# Multi-peptide-based Vaccines for Personalized Cancer Therapy

## – Analytical fundamentals translated into clinical applications

# Multi-Peptid-Impfstoffe zur individuellen Behandlung von Tumorpatienten

## – Von den analytischen Grundlagen zur klinischen Anwendung

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**Toni Weinschenk**

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Dekan:      Prof. Dr. H. Probst

1. Berichterstatter:      Prof. Dr. H.-G. Rammensee

2. Berichterstatter:      PD Dr. S. Stevanović

3. Berichterstatter:      Dr. phil. Dr. med. habil. F. Lottspeich

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# Preface

The results of this thesis are presented in three parts:

**Part 1** introduces the concept for the identification of multi-peptide-based vaccines for personalized cancer therapy by integrating genome and proteome analysis. The results achieved during establishing the approach up to its clinical usage in personalized medicine are given. Genome-wide gene expression analysis by DNA-chip-technology is presented only in this part of the work, which is the central one of this thesis.

The results presented in **Part 2** provide new insight in antigen processing. The rules for the events making parts of antigens presented as short peptides on the cell surface are fundamental for peptide-based vaccines. Whereas Part 1 represents applied clinical investigations, Part 2 shows results related to basic research.

**Part 3** shows the inset of mass spectrometry in the field of proteome analysis as analytical basics for Part 1: Mass spectrometrical sequencing of peptides only present in limited amounts as well as protein identification by mass spectrometry.

At the beginning of each chapter it is indicated which experiments were contributed by the author of this thesis. Some chapters are parts of publications. The authors of these publications are mentioned at the ends of these chapters.

Die Ergebnisse der vorliegenden Dissertation sind in drei Teile gegliedert:

**Teil 1** stellt das Konzept der Identifizierung von Multi-Peptid-Impfstoffen zur individuellen Behandlung von Tumorpatienten durch die Integration von Genom- und Proteomanalyse dar. Die Ergebnisse der Etablierungsphase bis hin zum klinischen Einsatz des Verfahrens in der personalisierten Medizin werden gezeigt. Die genomweite Genexpressionsanalyse durch DNA-Chip-Technologie wird ausschließlich in diesem Kapitel behandelt. Ergebnisse dieses Teils stehen im Zentrum der Dissertation.

**Teil 2** behandelt neue Erkenntnisse in der Antigenprozessierung. Die Regeln für den Weg vom Antigen zum präsentierten Peptid bilden das Fundament für Peptid-basierte Impfstoffe. Während also in Teil 1 eher angewandte, klinische Arbeiten im Vordergrund stehen, besteht Teil 2 aus Ergebnissen der Grundlagenforschung.

**Teil 3** zeigt den Einsatz der Massenspektrometrie in der Proteomanalyse als analytische Grundlage für Teil 1: Die massenspektrometrische Sequenzierung von Peptiden bei geringen Analytmengen sowie die Identifizierung von Proteinen über die Massenspektrometrie.

Generell ist der Anteil des Autors dieser Dissertation an den Ergebnissen der einzelnen Kapitel jeweils zu Beginn der Kapitel beschrieben. Manche Kapitel sind Teil von Veröffentlichungen. Die Autoren der Veröffentlichungen sind an den jeweiligen Kapitelenden aufgeführt.

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# 1    Introduction

## 1.1    The immune system

The adaptive immune system is a typical feature of vertebrates. It has developed during evolution to prevent higher organisms from severe damage by viruses, bacteria, fungi and other parasites. But also tumor cells can be destroyed. The different parts of the immune system arise from haematopoietic stem cells in the bone marrow. The lymphoid organs are called central – bone marrow and thymus – and peripheral – lymph nodes, lymphatic vessels and the spleen. Immune responses are mediated by specialized cells or proteins. Both have fast responding but rigid innate components and parts which are very flexible and able to perfectly adapt to the problem: the antigen. Moreover, the immune system does not forget anything. Encountering the same antigen for the second time, the immune system remembers how to handle the problem and solves it much faster. And it knows when to slow down again after the job is done.

Despite of its huge diversity, theoretically based on ~$10^{18}$ different T cell receptors and ~$10^{14}$ B cell receptors, nevertheless, each receptor is highly specific. This specificity in combination with lessons learned during development prevents from self-destruction. As usual, there are failures also in this system, which attract interest from scientists as well as from suffering patients. But more often, the immune system saves lives and fascinates many immunologists.

## 1.2    MHC-molecules and T cells

T cells are very diverse in their phenotype, their function and in the target structures they recognize. Early in development, the progenitors differentiate in T cells expressing γδ-T cell receptors (γδ-TCR) or αβ-T cell receptors. γδ-T cells recognize non-peptidic antigens such as sphingolipids bound to non-classical MHC molecules like CD1 and, additionally, might have regulatory effects. αβ-T cells can be NK T cells, CD8+ T cells or CD4+ T cells. NK T cells express NK1.1,

CD4 or no coreceptor and show a limited TCR diversity. CD4 CD8 positive T cells are positively selected by self MHC molecules during their development in the thymus. A T cell expressing a T cell receptor which binds to an MHC class I molecule loses its CD4 expression and becomes CD8 positive. The opposite is true for T cells with appropriate receptors for MHC class II molecules. They become CD4 positive T cells. T cells with TCR binding to neither self MHC class I nor II molecules die. To prevent self-destruction, T cells recognizing self peptides, which are presented on healthy cells, are also removed during the following negative selection step. Some antigens are not expressed in the thymus but in other tissues or organs. Self-reactive T cells recognizing peptides from those antigens become anergic because they lack important additional signals during the first antigen encounter. Thus, T cells are able to distinguish between self and foreign or malignant.

T cells which successfully passed all selection criteria circle through the body via the blood stream as so-called naïve T cells. When they encounter a professional antigen presenting cell (APC) such as dendritic cells, B cells or macrophages, they can be activated. Activation requires engagement of the TCR by a peptide/MHC complex plus delivery of a second signal from the APC. In the case of CD8 positive T cells, the resulting armed effector cells now are able to kill specifically infected or malignant transformed target cells. Activated CD4 T cells differentiate into cells with a $T_H1$ or $T_H2$ phenotype, determined by the secreted cytokine profile. $T_H1$ cells have a rather direct inflammatory effect due to secretion of IFN-γ and lead to cellular responses whereas $T_H2$ cells support a humoral response.

A further modulation of T cell response is achieved by the recently identified regulatory T cells ($T_{reg}$). They are CD4 positive and constitutively express CD25. In the presence of $T_{reg}$, T cell resonses are impaired for reasons which are not completely understood until now.

Together with TCR, MHC molecules are in the centre of cellular immunity. MHC class I molecules – called human leukocyte antigen (HLA) in human – are membrane bound glycoproteins found on the surface of all nucleated cells. They are heterodimers built by a heavy α-chain which is non-covalently linked to β2-microglobulin (β2m). The α-chain has a short cytoplasmic C-terminal part, a transmembrane domain and three extracellular immunoglobulin-like domains $\alpha_1$-$\alpha_3$. The two N-terminal domains $\alpha_1$ and $\alpha_2$ form a peptide binding groove – two

parallel α-helical domains on a β-sheeted floor – which is closed at both ends. This peptide binding groove harbours the presented peptide with a length of 8-10 amino acids. Non-covalent hydrophobic and ionic interactions between the peptide and the α-chain mainly take place between so called pockets in the $\alpha_1$-domain and the anchor amino acids of the ligand and involve the N- and C-termini of the peptide.

A remarkable feature of the major histocompatibility complex is its polymorphism. On the human chromosome 6, there are three gene loci for the MHC class I α-chain: HLA-A, -B and –C. Each of these loci is highly polymorphic. Table 1.2.1 shows the number of named HLA class I alleles known in April 2003.

Table 1.2.1 *Number of known HLA alleles\**

| Gene locus | Number of alleles |
|---|---|
| HLA-A | 275 |
| HLA-B | 521 |
| HLA-C | 134 |

*data from the Anthony Nolan Trust, HLA informatics group (http://www.anthonynolan.com/HIG)

Major differences between the alleles are found at the positions responsible for the peptide binding, namely the $\alpha_1$-helix. Every allele has its more or less unique set of two pockets. Each of them allows binding of only a restricted set of amino acids with common special chemical features at defined positions within the peptide sequence. All features a peptide should exploit for the capacity to bind to a given HLA-allele are called "peptide binding motif" of this allele [1]. The motif for HLA-A*24 [2], shown in Table 1.2.2, gives an example.

**Table 1.2.2 *Peptide binding motif of HLA-A\*24***

| Position | 1 | **2** | 3 | 4 | 5 | 6 | 7 | 8 | **9** |
|---|---|---|---|---|---|---|---|---|---|
| Anchor amino acids | | **Y** | | | | | | | **L** |
| | | **F** | | | | | | | **F** |
| | | | | | | | | | **I** |

The three different gene loci together with codominant heterozygous gene expression result in the expression of up to six different HLA-alleles in an individual. This allows each individuum to present mostly more than only one peptide from hardly any protein in the context of different HLA-alleles and thus lowers the likelihood of immune evasion of pathogens. Moreover, looking at the whole population of a species, survival of some individuals is likely in the case of an epidemy.

MHC class II molecules are found on professional antigen presenting cells and occasionally also on tumor cells. They are heterodimers built up by α- and β-chains. These genes are also highly polymorphic. The overall structure is similar but in contrast to MHC class I molecules, the peptide binding groove is open at both ends. This allows binding of longer peptides with 10 to 15 amino acids. Due to the different antigen processing pathway, MHC class II-bound peptides are mainly derived from extracellular proteins, but also peptides from cytosolic proteins are frequently found. Another characteristic feature is the presentation of length variants from one protein covering the same core sequence, the N- and C-termini are not that clearcut as in MHC class I-ligands.

## 1.3   Antigen processing

### 1.3.1   Processing of MHC class I epitopes

MHC class I-bound peptides are derived from cytosolic proteins. Exo- and endoproteolytic events during the physiological protein turnover lead to the generation of peptides of different length. But, probably, even more important, misfolded proteins and premature chains released during translation feed the pool of epitope precursors [3-5]. They are called defective ribosomal products (DRiPs) and presumably contribute the most significant part of substrates for epitope production, allowing a cell to quickly display changes from inside to the outside. The proteasome, a large multienzyme complex with the shape of a barrel, is mainly responsible for the generation of the C-termini of T cell epitopes. In some cases it directly excices the epitope, in many cases it provides N-terminal elongated precursors, which are further trimmed by other proteases as already suggested in 1990 [6]. The 20S core proteasome with its three constitutive

proteolytic subunits (δ/Y, Z and X) can be converted into an immuno proteasome by IFNγ. In this proteasome species, δ/Y, Z and X are replaced by low molecular weight protein 2 (LMP2), MECL-1 and low molecular weight protein 7 (LMP7). On both sides of the barrel, the 20S proteasome to some extent is singly or doubly capped, either by the 19S regulatory particle or by the IFNγ-inducible activator PA28, both of which regulate substrate access to the core particle. For a long time it was assumed that substrates are channelled sequentially into the proteasome and cut in an exopeptidase-like way, but recently it has been shown that the proteasome can also act as an endoprotease [7].

Several cytosolic proteases besides the proteasome have been described being involved in trimming events: leucine aminopeptidase (LAP) [8], tripeptidyl-peptidase II (TPPII) [9], bleomycin hydrolase (BH), and puromycin-sensitive aminopeptidase (PSH) [10].

Epitopes and their precursors enter the endoplasmatic reticulum via the transporter associated with antigen processing (TAP). To be transported by human TAP, peptides have to fullfill some requirements. Their length must be between 8 and 16 amino acids and the C-terminus has to be hydrophobic or basic [11, 12]. After some discussion in the last years about the relevance of trimming proteases in the ER [13-19], several publications in the last months could show the impact of an aminopeptidase called ERAAP1 present in the ER [20-22].

Several ER resident proteins (calreticulin, calnexin, tapasin, Erp57) provide help for the loading of empty MHC class I molecules with processed peptides matching the respective motifs. The complete complex of MHC class I α-chain, β2m and peptide is exported to the cell membrane by the classical secretion pathway. Recently, it was shown that peptides are rapidly degraded if not loaded on MHC molecules and that a large part is rescued from degradation by entering the nucleus, where peptides bind to chromatin [23].

## 1.3.2  Processing of MHC class II epitopes

Peptides presented by MHC class II molecules are mainly derived from extracellular or membrane-bound proteins. They are taken up by phagocytosis, pinocytosis or receptor-mediated endocytosis, are degraded in vesicles fused to lysosomes and end up in the "so-called" MHC class II loading compartment MIIC.

Several different proteases mainly from the cathepsin family are involved in antigen processing. The MHC class II α- and β-chains are synthesized into the ER and transported in association with the trimeric invariant chain (Ii) to the MHC class II loading compartment. There, the class II-associated invariant-chain peptide (CLIP), a part of the invariant chain which occupies the peptide binding groove of the MHC class II-molecules, is replaced by the later presented peptides. This exchange is catalyzed and regulated by HLA-DM and HLA-DO [24, 25].

### 1.3.3 Cross presentation

Presentation of peptides from extracellular proteins on MHC class I-molecules is called cross presentation. This phenomenon was observed in several cases and breaks the general separation of the two processing pathways. It is necessary for the generation of CD8^{+} T cell-responses against viruses which do not infect professional antigen presenting cells. In this situation, the infected cells are not capable to provide the necessary costimulatory signals for T cell priming in addition to the TCR-MHC class I-interaction. And without cross presentation, the APCs would not present peptides from this virus on MHC class I. Still it is not clear how the peptides channel from the endocytotic pathway to the cytosol. But also for the generation of most naturally occurring CTL responses against tumor cells, cross presentation is a prerequisite, because most tumor cells do not have MHC class II molecules neither do they have costimulatory molecules. But necrotic tumor material can be taken up by surrounding stroma cells, which then cross present the digested tumor-derived proteins on MHC class I [26].

## 1.4  Immunotherapy in cancer

### 1.4.1  Classes of tumor associated antigens

Nearly all possibilities for tumor associated antigens one can think about have been found (Table 1.4.1).

**Table 1.4.1** *Classes of tumor antigens\**

| class | examples | recognized by | |
|---|---|---|---|
| | | T cells | antibodies |
| point mutation of normal gene | CDK4 [27] | x | - |
| frame shift mutation of normal gene | TGFβRII [28] | x | - |
| anti sense transcript of normal gene | RU2AS [29] | x | - |
| expressed intron of normal gene | N-acetylglucosaminyltransferase V [30] | x | - |
| fusion protein caused by translocation | BCR-ABL [31] | x | - |
| altered postranslational modifications | tyrosinase [32] | x | - |
| cancer/embryonic antigen | CEA [33, 34] | x | x |
| overexpressed antigens – protein | Her2/neu, MUC1 [35, 36] | x | x |
| overexpressed antigens – non-protein | gangliosid GD3 [37] | - | x |
| cancer testis antigen | NY-ESO-1 [38] | x | x |
| | MAGE family [39] | x | x |
| oncogenes | ras [40-42] | x | x |
| tumor suppressor genes | p53 [43, 44] | x | x |
| differenciation antigen | tyrosinase [45, 46] | x | x |
| | gp100 [47, 48] | x | x |
| viral proteins | HPV [49, 50] | x | x |

\* adapted from Rammensee et al. [51]

This is not a big surprise considering the estimated number of all changes in a tumor. However, there are major differences in the quality of the named classes. For vaccination purposes, antigens which play a central role in the transformation event itself such as the observed point mutation in CDK4 are favorable, because to escape the selection pressure of an immune response these genes cannot be easily avoided by the tumor. On the other hand, this criterium might be neglectible, if many targets are addressed at the same time. Another interesting point is the frequency of the antigen among many patients or even among several tumors. This topic is especially relevant for the question, how many percent of all patients fit to the vaccine. E.g. a mutated gene leading to a mutated epitope, which accidently arose in a single patient compared with the overexpression of p53, which is found in many tumor entities and in frequencies of more than 50% of all patients in some entities. For sure, antigens not exclusively expressed in the tumor when compared to other healthy tissues and cells are problematic because of existing tolerances towards these antigens. And vaccination against them might

induce autoimmunity. Many antigens – most of them in melanoma – have been identified yet, but still there is need for more.

## 1.4.2  Vaccination trials

Although W. C. Coley's first pioneering attempt to fight cancer by immunotherapy in the 1890s is most likely uneffective, it was the beginning of a new area in the field of oncology. He injected Coley toxins – bacterial cultures – into cancer patients, believing the bacterial infection helps to stimulate the immune system, causing it to fight off cancer cells. This approach from ancient times is not too far away from some vaccines recently still used: tumors taken out of patients, mixed, loaded on dendritic cells – the best known adjuvant today – and reinjected again [52]. The number of different types of vaccines used in clinical trials until now is immense: irradiated allogenic [53] or autologous tumor cells [54], modified in several ways to become more immunogenic, hybrid cells made by fusion of tumor cells and professional antigen presenting cells [55], DNA coding for tumor rejection genes delivered by sophisticated methods such as viral transporters [56], heat shock proteins isolated from the autologous tumor [57], DCs transfected with total RNA [58] or with RNA coding for defined tumor antigens [59] or DCs loaded with MHC class I peptides [52], proteins alone [60] and, last but not least, peptides, peptides plus adjuvants such as GM-CSF [61], IL-2 [62], Montanide ISA-51 [63], keyhole-limpet hemocyanin (KLH) [61], tumor associated [64] or artificial MHC class II ligands [65]. The many different vaccination protocols – injection sites, time schedule concerning the number of injections and the frequency, amount of vaccine used for injection – make comparisons between trials from different groups impossible.

All vaccines made from autologous tumors have the advantage that the number of antigens is as high as the molecular changes within this tumor. In principle, this should lead to an immune response against many targets, thus the risk of immune evasion due to tumoral changes under the selection pressure of the immune response is minimized [66]. But these advantages are accompanied by some side effects. In some cases, the amount of the vaccine is related to the tumor size and, therefore, limited with respect to the number of possible repeated vaccinations. In general, these tumor derived vaccines are not defined on the molecular level, and

monitoring as well as the final immunological readout as a surrogate marker for the decision about the success of a vaccine depends on the knowledge about T cell epitopes. Furthermore, the relevant antigens in this category of vaccines are diluted in a large excess of irrelevant structures. E.g. gp96 isolated from the autologous tumor, where most of the bound peptides are probably self-peptides. Dendritic cells loaded with tumor lysates give another example. Here, the lysate mainly consists of housekeeping proteins and the same is true for DCs transfected with total RNA.

Immunological responses against molecular defined vaccines such as peptides can be monitored with several sensitive up-to-date methods. They can be synthesized in machines in any amounts, allowing repeated vaccinations over years. But the big limitation so far is the small number of known T cell epitopes from tumor rejection antigens. This fact prevented studies with many epitopes at the same time, but now there is evidence from first trials designed in this way for superior clinical outcome the more peptides were included in the vaccine [67]. Thus, after the first use of a peptide derived from a tumor antigen in a vaccination trial in the mid 1990s [68], several studies showed clinical responses [52, 69-80] and even the correlation to immunological response could be shown [67].

## 1.4.3 Identification of T cell epitopes derived from tumor associated antigens

### 1.4.3.1 The classical approach: Starting with T cells recognizing tumor cells

The very first gene encoding an antigen recognized by CTL on a human tumor has been identified by expression cloning in 1991 [81]. Cytotoxic T cells were used to screen expression libraries prepared from the recognized tumor cell. The gene was called MAGE-1. The gene segment containing the epitope was further narrowed down to a 0.3 kb region by expressing fragments of MAGE-1 and analysis of the transfectants using again the specific CTL line. The last step to the epitope was done by "epitope mapping", a frequently used method since these days. The primary sequence is represented by many adjacent small peptides of about 15 amino acids in length and each of the peptides is analyzed for its ability to mediated CTL response. When a 15-mer peptide is recognized, all possible

included nonamer peptides are tested again. Thus, the HLA-A*01-restricted epitope (EADPTGHSY) was identified [39].

Another approach also starting with T cells was carried out for the first time by the group of Slingluff Jr. [82]. Using the method described by Falk et al. [1], they isolated MHC-bound peptides from a melanoma cell line, which was recognized by melanoma-specific CTL lines from five different melanoma patients. After separation by HPLC, fractions were tested for their ability to reconstitute epitopes for two of the five CTLs after loading on T2 cells. Three peptides coeluting with the cytotoxic activity were sequenced by tandem mass spectrometry, and finally one of them, (YLEPGPVTA), has been shown to be recognized by all the five melanoma-specific CTLs.

### 1.4.3.2 Reverse Immunology: T cell epitope prediction and verification – Combining computer algorithms with experimental approaches allows for rapid and accurate identification of T cell epitopes from defined antigens

This chapter is published in the Journal of Immunological Methods by Markus Schirle, Toni Weinschenk and Stefan Stevanović

*Summary*

The identification of T cell epitopes from immunologically relevant antigens remains a critical step towards the development of vaccines and methods for the monitoring of T cell responses. This review presents an overview over strategies that employ computer algorithms for the selection of candidate peptides from defined proteins and subsequent verification of their *in vivo* relevance by experimental approaches.

Several computer algorithms are currently being used for epitope prediction for various MHC class I and II molecules, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides. Moreover, the analysis of proteasomal digests of peptides and whole proteins has led to the development of algorithms for the prediction of proteasomal cleavages. In order to verify the generation of the predicted peptides during antigen processing *in vivo* as well as their immunogenic potential, several experimental approaches have been pursued

in the recent past. Mass spectrometry-based bioanalytical approaches have been used to specifically detect predicted peptides among isolated natural ligands. Other strategies employ various methods for the stimulation of primary T cell responses against the predicted peptides and subsequent testing of the recognition pattern towards target cells that express the antigen.

## *Introduction*

MHC molecules are highly polymorphic cell surface molecules that present peptidic ligands to cells of the T cell compartment of the immune system. MHC class I ligands have a typical length of 8-12 amino acids and are derived from endogenously expressed proteins that are degraded by cytosolic proteases, most notably the proteasome. The proteolytic fragments are transported into the endoplasmatic reticulum in an ATP-dependent fashion by the transporter associated with antigen processing (TAP), where they bind to newly synthesized empty MHC class I molecules. The MHC-peptide complex is subsequently transported to the cell surface and can be recognized by the T cell receptor (TCR) of $CD8^+$ cytotoxic T cells (CTL). MHC class II ligands have a more variable length of 9-25 amino acids and are derived mainly from exogenous or transmembrane, but also from cytosolic proteins that are degraded by various proteases which originate from the lysosomal compartment. After binding to MHC class II-molecules in the late endosomal/lysosomal compartment, the MHC-peptide complex is also transported to the cell surface, where it can be recognized by the TCR of $CD4^+$ T cells.

Recognition of a peptide derived from a disease-associated protein, e g. a viral or a tumor-specific protein, in presence of a costimulatory signal leads to T cell activation and triggers a T cell-mediated immune response. Therefore, the question which peptide fragments of immunologically relevant antigens are available in context of a certain MHC-molecule for recognition by T cells is crucial for the development of peptide-based or other defined antigen-based vaccines. Moreover, tools for monitoring specific T cell responses such as MHC-peptide tetramers, intracellular cytokine staining or ELISPOT assay depend on the identification of the relevant T cell epitope.

A major breakthrough for the identification of T cell epitopes was the discovery that ligands of a certain MHC-molecule carry chemically related amino acids in

certain positions of their primary sequence, which allows the definition of a "peptide motif" for every MHC allele [1]. This information was rapidly used to predict potential epitopes from protein sequences [83, 84] and was the start signal for the so called "reverse immunology" approach [85], which has since then become the most successful strategy for the identification of T cell epitopes.

The first step of such studies is usually the computer-based prediction of potential MHC ligands from a protein of interest which is followed by experiments to verify the natural processing of the predicted peptides and their recognition by T cells. The classical and most straightforward approach relies on the stimulation of primary T cell responses against the predicted peptides *in vitro* or in (transgenic) animals and subsequent testing of the recognition pattern of the generated T cells towards target cells that endogenously express the antigen. Alternatively, patient-derived T cells, which might have been already primed in vivo, are used. This "pure" reverse immunology-approach has been used extensively in the recent past for the identification of CTL and to a lesser extent helper T cell epitopes from a wide variety of immunologically relevant proteins. However, a major drawback of this labor- and resource-intensive approach is the high failure rate, even if the peptide in question binds well to the MHC molecule and the generated T cells recognize target cells loaded with the synthetic peptide. The reason for this is either that the peptide is not produced by the processing machinery of the cell, or the T cells raised by primary peptide stimulation *in vitro* are of low affinity, or a combination of both.

In consequence, various groups have made attempts to decrease the number of candidate peptides by applying additional criteria to epitope prediction. For example, the increasing knowledge about specificities of other cellular components of the antigen processing machinery, most notably the proteasome and TAP, has made it possible to apply these specificities as additional filters to the selection of candidate peptides. Moreover, experimental selection criteria such as MHC–peptide binding assays and the bioanalytical verification of presentation of predicted MHC-ligands have been applied successfully.

This review tries to give a comprehensive overview on available computer-based prediction algorithms and strategies that combine the predictive power of these theoretical approaches with experimental approaches for a reliable and rapid identification of T cell epitopes.

Since it was written from an immunologist's point of view, this review focuses mostly on prediction programs that have already been applied to the identification of novel epitopes. Furthermore, it should be noted that there are tremendous differences between different prediction softwares in terms of availability. Some programs are easily accessible for free in the WWW, others are available from authors of the corresponding publications, still others are available on a commercial basis only.

### *Computational predictions*

*Epitope prediction*

The recognition of MHC-peptide complexes by the T cell receptor (TCR) is the first step in a series of cellular processes that lead to the initiation of T cell-mediated immunity. It is therefore not surprising, that as soon as the peptidic nature of MHC ligands was discovered, but even before the rules for the binding of peptides to MHC molecules were elucidated, first attempts for the predicition of T cell antigenic structures from protein sequences were published. These predictions were based solely on comparisons of precursor peptide sequences known to contain T cell epitopes. Suggested characteristics of T cell epitopes were predicted secondary structures (amphipathic helices) [86] or short primary sequence motifs [87, 88]. It was the discovery of allele-specific motifs shared by eluted natural MHC ligands [1], that finally allowed the exact prediction of peptides from a given protein sequence presented on MHC class I molecules [83, 84].

For MHC class II ligands the elucidation of peptide motifs turned out to be more difficult due to the variable length of the ligands as well as the more degenerate anchor positions, which are sometimes not used at all. Several methods for the determination of MHC class II peptide motifs have been based on sequence information of natural ligands, including the alignment of natural ligands, pool sequence analysis, or substitution variants of known ligands [89-93]. In 1992, the first HLA-DR-motif based on binding assays using a phage peptide display library was published [94].

While in the beginning of the 1990s, the selection of candidate peptides was done manually or using simple pattern search programs by scanning protein sequences for main anchor positions only, the constant refinement of peptide motifs and knowledge about secondary anchor amino acids, disfavored amino acids and

other chemical interactions important for binding to the MHC molecule quickly led to the development of more complex computational predictions.

Depending on the focus of research, the determination of peptide motifs has been approached from two sides:

On the one hand, the binding properties of a certain MHC molecule have been analyzed by determining the binding of synthetic peptides from protein antigens using MHC-peptide binding assays [95-98]. The most refined descriptions of such "peptide binding motifs" have been achieved by using positional scanning with random synthetic peptide libraries [99-101].

On the other hand, the analysis of natural ligands by individual ligand analysis as characterized by Edman degradation [1, 92, 102, 103] and tandem mass spectrometry [91] as well as Edman pool sequencing [1] leads to so-called "natural ligand motifs", that give information about the natural peptide repertoire presented by a certain MHC molecule. These motifs are therefore not only a result of the binding properties of this MHC molecule, but reflect also – to a certain extent – features of the antigen processing machinery, e. g. cleavage specificities of the proteasome or sequence requirements for transport into the ER by TAP. Indeed, MHC binding motifs derived from random peptide libraries show significant differences, especially for the C-terminal amino acids, compared to motifs derived from natural ligands [104]. This raises the question of the *in vivo* relevance of motifs based on binding properties of synthetic peptides.

*Epitope prediction: MHC class I*

Historically, MHC class I epitope prediction had a headstart over its MHC class II counterpart. The reasons for this include the defined length of MHC class I ligands of mostly 9 amino acids and the more clearcut rules of MHC class I motifs, that in turn have been more clearly characterized. Currently there are two predictive algorithms available in the world wide web, that allow unrestricted predictions free of charge: "BIMAS", developed by K. C. Parker et al. [97] (http://www-bimas.dcrt.nih.gov/molbio/hla_bind/) and "SYFPEITHI", developed by our group [105] (http://www.syfpeithi.de). SYFPEITHI uses motif matrices deduced from refined motifs based on the pool sequencing and single peptide analysis exclusively of natural ligands. Potential binders for various human, mouse and rat

MHC class I molecules are ranked according to the presence of primary and secondary anchor amino acids as well as favored and disfavored amino acids. BIMAS ranks potential HLA-binders according to predicted half-time dissociation of MHC-peptide complexes. While the motif matrix for HLA-A*02 is based solely on binding studies, other matrices have been deduced from reported motifs, regardless of the method used for their identification. This might explain the highly similar results that were obtained with both algorithms in a recent study that identified HLA-B*07-presented epitopes from the tumor-associated antigen (TAA) carcinoembryonic antigen (CEA) [106], since for HLA-B*07 the reported motifs are based on natural ligands. Both algorithms have been used successfully during the last years for identification of CTL epitopes from a wide range of antigens, for example from the human TAAs telomerase (BIMAS) [107] and TRP2 (SYFPEITHI) [108].

Other prediction programs that have been applied to the identification of MHC class I-presented epitopes are distributed on disk (e. g., "MOTIFS" [109]), part of commercial websites (e. g., "EPIMATRIX" [110] (http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html)) or are currently not publicly available [111].

The motif-based algorithms so far described are still relatively simple programs that consider every amino acid position in a peptide individually. For every position, a coefficient is assigned to each amino acid and the overall score or binding value of the peptide are determined by addition or multiplication of amino acid coefficients. Besides, there are several reports employing artificial neural networks (ANN) [112-115] or the polynomial method [114]. These approaches open up the possibility to consider amino acid preferences that depend on the properties of amino acids in other positions of the peptide.

Still another approach is based on structural information from crystallographically solved peptide-MHC complexes [116, 117].

**Table 1.4.2** *Comparison of MHC class I prediction programs*

| Sequence | Source (position) | HLA molecule | program used for identification | reference | SYFPEITHI rank/score | BIMAS rank/value | PAPROC* |
|---|---|---|---|---|---|---|---|
| ILAKFLHWL | Human TERT (540-548) | A*0201 | BIMAS | [107] | 1/30 | 1/ 1745.714 | No |
| KVLEYVIKV | Human MAGE-A1 (278-286) | A*0201 | SYFPEITHI | [118] | 3/26 | 1/743.189 | Yes |
| ILHNGAYSL | Human Her-2/neu (435-443) | A*0201 | Sette et al. | [119] | 3/27 | 16/36.316 | Yes |
| HLSTAFARV | Human G250 (254-262) | A*0201 | MOTIFS | [120] | 19/22 | 38/4.493 | No |

* prediction of proteasomal generation of C-terminus by human proteasome wild type III

Comparison of MHC class I predictions. Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge on interactive websites: the epitope prediction programs BIMAS and SYFPEITHI and a program for prediction of proteasomal cleavage sites PAPROC.

Table 1.4.2 demonstrates the similarities and differences of some of the currently used MHC class I prediction programs. It shows selected CTL epitopes that were identified using four different computer programs and also lists the corresponding results from SYFPEITHI, BIMAS and a prediction algorithm for proteasomal cleavages, PAPROC (see below), i. e. programs that are available at interactive websites at no cost. However, it should be noted that some studies do not give the results of the original prediction procedure, such as ranks or values, but only lists of peptides, that were selected after performing additional tests.

*Epitope prediction: MHC class II*

The main obstacle for the prediction of MHC class II-ligands has been the varying degree of degeneration of motifs. While some alleles, e. g. HLA-DRB1*0405 show a strong preference for certain related amino acids in the anchor positions that is comparable to MHC class I motifs (in this case D, E in P9) [105], other alleles make the definition of primary anchor amino acids virtually impossible (e. g. for HLA-DRB1*0401 all amino acids except G, P, F, W have been assigned anchor amino acids in P9) [105]. To date, the focus has been on predictions of HLA-DR ligands, with only one report on predictions of HLA-DQ-restricted epitopes [121]. The first matrix-based prediction algorithms for HLA-DRB1*0401 were based on the results of side chain scanning experiments using simplified, polyalanine-based peptide libraries [98, 122]. The work by Hammer and colleagues resulted in the

commercial program TEPITOPE, which focuses on promiscuous HLA-DR binding peptides, but also allows allele-specific predictions. It has been used in several studies, for example in the identification of an HLA-DR11-presented epitope from MAGE-A3 [123].

Another matrix-based HLA-DRB1*0401-prediction algorithm, also based on binding studies using synthetic peptides [124] has been used for the retrospective calculation of an epitope from human gp100 [125] that had been identified before [126]. This program is available for free on request from the Surgery Branch of the NCI.

Still another customized computer algorithm also based on published HLA-DR binding motifs has been used for the identification of HLA-DR-presented promiscuous T cell epitopes from *Plasmodium falciparum* [127].

The first program for prediction of MHC class II based on natural ligands of two HLA-DR13 alleles was the PAP program [128]. This program, which can be obtained for free from the author, uses corrected amino acid yields from Edman pool sequencing directly as coefficients in motif matrices. One of the following publications also reports for the first time predictions for HLA-DQ2 and –DQ8 and the identification of a novel HLA-DQ2-presented epitope from gliadin, the antigen of celiac disease [121].

Currently, SYFPEITHI is the only the interactive website offering unrestricted MHC class II predictions free of charge. As for MHC class I, the available predictions for HLA-DR and several mouse MHC class II alleles are based on motif matrices deduced from natural ligands by single ligand analysis and Edman pool sequencing.

For HLA-DRB1*0401, predictions by artificial neural networks have already been applied successfully to the identification of helper T cell epitopes. Zarour and colleagues have used an ANN, based on the work by Honeyman, Brusic and colleagues [129, 130], for the identification of epitopes from the TAAs Melan-A/MART-1 [131] and NY-ESO-1 [132]. These prediction programs are currently not publicly available.

Finally, several publications have explored the possibilities of other more complex computational predictions. E. g., Mallios has described an iterative algorithm to align and optimize an MHC class II-binding matrix based on sequences of known

class II ligands alone or in combination with suggested motifs [133]. Up to now, none of these predictions have been applied to the identification of T cell epitopes. Table 1.4.3 shows examples of helper T cell epitopes, that were identified by four different computer programs and also lists the corresponding results from SYFPEITHI and the HLA-DRB1*0401 prediction program from the NCI. However, in contrast to MHC class I, comparisons of MHC class II predictions can be even more difficult for several reasons.

**Table 1.4.3 *Helper T cell epitopes identified by prediction***

| Epitope sequence | Source (position) | HLA molecule | reference | program used for identification | SYFPEITHI rank/score | NCI rank/value |
|---|---|---|---|---|---|---|
| WNRQLYPEWTEAQRLD | Human gp100 (44-59) | DRB1*0401 | [125] | NCI | 2/26 | 1/0.0195 |
| RNGYRALMDKSLHVGTQCALTRR | Human MART-1 (51-73) | DRB1*0401 | [132] | ANN | 1/22 | -/- |
| TSYVKVLHHMVKISG | Human MAGE-A3 (281-295) | DRB1*1101 | [123] | TEPITOPE | 8/22** | n. a. |

putative core sequences are underlined; **best ranked peptides 284-298 (score 28) and 280-294 (score 24) also contain other putative core sequences; n. a. not available

Comparison of MHC class II predictions. Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge either on an interactive website (SYFPEITHI) or by email (NCI).

First, due to the variable length of MHC class II ligands, the identified epitopes may harbor more than one putative core sequence. Furthermore, some studies focus on promiscuous MHC class II-binding peptides, while others use allele-specific predictions.

*Predictions of antigen processing*

Much of the work combining computational predictions and applied immunology has focused on MHC-peptide binding only. However, the increasing knowledge about specificities of other cellular components involved in antigen processing, such as the proteasome and TAP, has been used to create comparable prediction algorithms with the future prospect of fusing them to epitope prediction programs. The proteasome is a cytosolic multisubunit protease that is, among other functions, involved in the generation of peptide ligands for MHC class I molecules and responsible for the generation of the correct C-terminus of CTL epitopes [134,

135]. The proteolytically active core complex (20S proteasome) can associate with the 19S cap complex to form the 26S proteasome, which is responsible for the degradation of ubiquitin-marked proteins. The three different active sites show different proteolytic specificities, cleaving after large, hydrophobic amino acids, basic amino acids and acidic amino acids, respectively. Upon exposure of cells to interferon $\gamma$ (IFN$\gamma$), the catalytically active subunits are replaced by three IFN$\gamma$-inducible subunits. This results in a shift in the overall cleavage specificity of this immunoproteasome towards hydrophobic amino acids, while cleavages after acidic amino acids are disfavored. This corresponds to the fact that almost all reported peptide motifs of vertebrate MHC molecules demand hydrophobic or basic C-terminal anchor amino acids.

An experimental approach was chosen by Kessler at al., for the identification of HLA-A*0201-presented CTL epitopes from the TAA PRAME by an extended reverse immunology strategy that incorporates proteasomal digest analysis [136]. Predicted HLA-A*0201-binding peptides were tested in binding and stability assays, followed by analysis of in vitro proteasome-mediated digestions of 27mer precursor peptides encompassing candidate epitopes. For only 4 out of 19 high affinity binders efficient generation of the correct C-terminus was observed and all 4 peptides were found to be efficient CTL epitopes.

The first published prediction algorithm for cleavages by 20S proteasomes, "FRAGPREDICT", is based on published peptide cleavage data [137] (http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html). The "PAPROC"-algorithm [138] (http://www.paproc.de), developed by our group, is based on an evolutionary algorithm trained on cleavage data of 20S proteasomal digests of a whole protein substrate (enolase [139]), and additional peptide digest data from the literature. While the currently available prediction programs are based only on semi-quantitative data from human or yeast constitutive 20S proteasomal digests, more accurate predictions based on fully quantified protein digestion data as well as immunoproteasomal digestion data [140] are currently being developed. Another program, "NETCHOP" [141] (http://www.cbs.dtu.dk/services/NetChop/) is an ANN that was trained on data from the analysis of residues at the termini and flanking regions of known natural MHC class I ligands. All these programs for the prediction of proteasomal cleavages are available free of charge on interactive websites.

The overall goal of combining proteasomal prediction algorithms with epitope prediction is already possible using combinations of FRAGPREDICT and PAPROC with SYFPEITHI and BIMAS at the website of the Max-Planck-Institute for Infection Biology (www.mpiib-berlin.mpg.de/MAPPP/). It should be noted that this program also predicts C-terminally extended precursor peptides.

An ANN-based device has also been reported for prediction of human TAP peptide-binding affinities using a polyalanine-based peptide library [142]. The program predicted a higher affinity to TAP for eluted natural ligands compared to randomly selected peptides with similar binding affinities to the same HLA molecule, indicating that such predictions might be – at least for some HLA-alleles – a valuable addition for epitope prediction tools. However, this would probably require a more profound investigation of peptide translocation by TAP, not only binding of peptides. The reported preferences of TAP for peptide translocations [12] have not yet led to a prediction program.

## *Experimental verification of predicted epitopes*

### *Analysis of binding properties*

In most cases epitope prediction is followed by binding studies with synthetic peptides in order to reduce the number of potential candidate peptides by discarding non-binding peptides for the final analysis of recognition by peptide-specific T cells.

Binding of synthetic peptides to MHC molecules is mainly studied by assays based on competition [111, 143, 144], reconstitution of MHC-peptide complexes on the cell surface [145-147], or stabilization of MHC-peptide complexes on TAP-deficient cells [148].

In competition assays, the studied peptides compete with a labeled reference peptide of intermediate affinity for binding to MHC molecules. Such assays are performed either using whole cells or purified MHC molecules in solution. In both cases the amount of labeled MHC-bound peptide is measured. While for MHC class II binding assays usually fluorescence dyes like fluorescein or 7-amino-4-methylcoumarin-3-acetic acid (AMCA) are used for labeling the N-terminal amino acid of the MHC ligand, for MHC class I binding studies amino acid side chains have to be labeled by fluorescent dyes or by radioactive isoltopes such as [125]I

without interfering with the more restricted binding properties of MHC class I molecules. Performing in-solution assays using purified MHC molecules, MHC-peptide complexes have to be separated from unbound free peptide by gel filtration [111], spun columns [149] or high performance size exclusion chromatography (HPSEC) [144]. In addition, ELISA-based assays [150, 151] or the more sensitive time-resolved fluorimetry in an europium fluoroimmunoassay [152] have been used as the readout. Competition based binding assays result in $IC_{50}$ values, which do not represent an absolute dissociation constant since they are dependent on the reference peptide. However, they allow a better comparison of binding affinities of different peptides than the results obtained by reconstitution or stabilization assays. Competition assays using purified MHC molecules employ soluble MHC molecules, which might have a slightly different structure compared to native molecules embedded in the cell membrane. Whole cell assays overcome these problems by using MHC molecules on the surface of intact cells. As an additional feature, mild acid treatment of the cells can be used to remove peptides from the MHC binding groove. Cells are then incubated with a fluorescence-labeled reference peptide together with different concentrations of the peptides of interest [153]. The amount of bound reference peptide can be measured by FACS analysis. As peptide-depleted MHC molecules have a short half time and $\beta_2$-microglobulin dissociates rapidly, only membrane-anchored MHC-$\alpha$-chains are left and thus this competition assay might also represent a reconstitution assay.

In classical reconstitution assays, cells expressing the appropriate HLA alleles are stripped by incubation at pH 3.2 for a short time, then the peptide of interest and a conformation dependent monoclonal antibody are added. The difference in fluorescence intensity determined by FACS analysis between cells incubated with and without peptide after staining with fluorescence-labeled secondary antibody is used to determine peptide binding [147].

The stabilization assay was first described by Stuber et al. in 1992 [148]. Cell lines with defective peptide loading mechanisms like the TAP-deficient murine cell line RMA-S [154] or the human cell lines T2 [155] or ST-EMO [156] display a low level of mostly empty MHC class I molecules on their surface, due to the rapid turnover of empty MHC molecules. Addition of exogenous binding peptide to the cells, however, leads to stabilization and accumulation of the MHC molecules, which can be determined by using monoclonal antibodies and FACS analysis. Due to its

simplicity, this assay can serve for screening large numbers of peptides. Moreover, transfection of any human cell with cDNA of the immediate early protein ICP47 of the Herpes simplex virus generates a TAP deficient phenotype and thus allows to expand the stabilization assay to virtually any HLA allele [157].

*Analysis of presentation*

Since the first identification of a natural MHC class I-ligand by tandem mass spectrometry (MSMS), this technique has become the bioanalytical method of choice for high sensitivity sequencing of natural MHC ligands. This has lead to the development of approaches that combine epitope prediction and HPLC-MS and – MS/MS for specific detection and identification of disease-associated MHC class I-ligands. Brockman and colleagues used a simple main-anchor-based epitope prediction to identify masses of candidate H2-K$^b$-ligands from *Trypanosoma cruzi* [158]. The corresponding mass chromatograms showed in two cases significant peaks and subsequent HPLC-MS/MS analysis revealed indeed two naturally processed peptides. A variant is the "Predict, Calibrate, Detect" (PCD) -approach [159], that allows a more rapid and sensitive screening of tumor samples by calibrating the HPLC-MS-system with synthetic analogs of predicted potential HLA-A*02 ligands from tumor-associated proteins. HLA-A*02 ligands from TAAs p53, CEA and MAGE-A1 have been identified using PCD [118, 159]; the MAGE-A1-derived peptide has been verified as a CTL epitope using an HLA-A*02 transgenic mouse model [118].

These approaches do not substitute for testing peptide-specific recognition of tumor cells by CTL, but they allow a further focussing of these efforts to those peptides that are not only binding to the MHC molecule, but are actually processed and presented.

*Analysis of T cell recognition*

The final experimental verification of a predicted epitope is achieved by the identification of T cells, which specifically recognize the naturally processed epitope in an HLA-restricted fashion. One major source for T cells are peripheral blood mononuclear cells (PBMC) from patients or healthy donors, which either are already primed *in vivo* or which can be primed *in vitro*, respectively. In addition, immunization of various HLA class I and class II transgenic mice (reviewed in

[160] and [161]) can be used to generate specific T cells. T cell responses after stimulation can be measured by means of lytic activity, proliferation and cytokine production. Lytic activity of cytotoxic T lymphocytes has been analyzed since 1968 using the $^{51}$Cr-release assay [162, 163], but this assay requires the cumbersome *in vitro* expansion of specific T cells from PBMCs. Nevertheless, it is still widely used. As an alternative, T cell proliferation has been investigated by radioactive $^{3}$H-thymidine incorporation. To determine peptide specific T cell response, a sensitive proliferation assay was established in 1996. This assay avoids radioactivity and uses BrdU incorporation instead [164]. Comparison with the $^{51}$Cr-release assay revealed a much higher sensitivity of the proliferation assay in this report. The first quantitative assay for IL-2 production by activated T cells described in 1978 is still used today [165]. This sensitive method is based upon an IL-2 dependent mouse T cell line, CTLL, as target cell population, which proliferates and incorporates $^{3}$H-thymidine in response to IL-2 in culture medium. After IL-2 and IFNγ specific antibodies have been available, enzyme-linked immunosorbent assays (ELISA) have been used to measure T cell responses [166-168]. Advantages of ELISA assays are their simple setup and the capacity to proceed with many samples in parallel.

All methods discussed so far cannot detect cytokine production by T cells on a single cell level. The first application to address this question was the ELISPOT assay reported in 1988 for enumerating IFNγ-secreting lymphocytes [169, 170], originally developed for detection of single specific antibody-secreting cells [171]. In this assay activated T cells are plated in anti-IFNγ antibody coated wells; single IFNγ-secreting cells are detected as spots after staining trapped IFNγ with an insoluble dye generated by an enzyme coupled to the second anti-IFNγ antibody. Peptide-specific T cells can be assessed this way with higher sensitivity compared to the $^{51}$Cr-release assay [172], even without need for prior *in vitro* expansion, which may bias the result. ELISPOT has been used to measure TNFα secretion [173] or IFNγ secretion [174, 175] as markers for T cell activation. The tedious visual counting of spots was no longer necessary after introduction of a computer-assisted image analysis system, which has made the data analysis convenient, objective and suitable for handling large sample pools [176].

Another way to assess cytokine production on a single cell level is achieved by intracellular cytokine staining and flow cytometry analysis. After stimulation in the

presence of monensin T cells are fixed by paraformaldehyde and permeabilized by saponin to allow antibodies to penetrate through the cell membrane, the cytosol and the membranes of the endoplasmatic reticulum and Golgi organelle. Monensin interrupts intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex and thus an increase of the signal/noise ratio leads to a higher sensitivity [177, 178]. Intracellular cytokine staining allows high throughput of samples and multiparameter characterization of cytokine production as well as parallel detection of cell surface markers on a single cell basis without the need for prolonged in vitro culture and cloning. However, the sensitivity is limited by the fluorescence-activated cell-sorting analysis.

Visualization of antigen-specific T cells is possible by staining T cell receptors with MHC/peptide tetrameric complexes [179-182]. These tetramers are made by *in vitro* folding MHC heavy chain in the presence of $\beta_2$-microglobulin and a specific peptide ligand. The heavy chain is engineered in a way that the purified MHC-peptide complex can be biotinylated and than tetramerized via fluorochrome dye-coupled avidin. Tetramerization enhances the affinity of the $\alpha\beta$TCRs to soluble peptide-loaded MHC molecules. By this method, a quantitation of T cells displaying TCRs specific for the analyzed peptide is possible, but nothing can be said about functionality of these T cells. The tetramer approach is well established for MHC class I molecules, but detection of antigen-specific CD4^{+} T cells with soluble MHC class II tetramers is about to begin [183-189]. Application of MHC class I tetramers in the field of virus immunology is reviewed by Doherty and Christensen [190].

Probably the most sensitive method to detect T cell responses is quantitation of induced cytokine mRNA after activation by quantitative real time RT-PCR. This quantitative method is based on the 5` nuclease assay in which during PCR the 5`$\rightarrow$ 3` endonuclease activity of the Taq DNA polymerase cleaves a hybridized sequence-specific fluorogenic internal oligonucleotide probe. Relative increase in fluorescence emission from the reporter dye is detected online in an analytical thermal cycler [191]. This assay has been used to quantify cytokine mRNA expression (IL-2, IL-4, IL-10, TNF$\alpha$, IFN$\gamma$) in previously frozen whole blood samples [192]. More recently, this very sensitive and highly reproducible method was adapted by normalizing copy numbers of cytokine mRNAs to CD8 mRNA copy numbers in order to analyze specific T cell responses in fresh PBMCs

without any further stimulation [193, 194]. This way it was possible to monitor the specific T cell response after peptide vaccination in melanoma patients. Though this is no single cell assay, it is very sensitive, highly reproducible and allows high sample throughput.

***Outlook***

While computational predictions have already become a familiar tool for many immunologists searching for T cell epitopes from immunologically relevant proteins, there is the constant need of using new data to update and extend the available programs. Reliable epitope prediction is still only available for a limited number of organisms and alleles because no or not enough information is available about the corresponding peptide specificities. However, several studies have shown both for MHC class I and II, that the knowledge, which amino acids occupy those positions of the MHC polypeptide chain that are responsible for the interaction with peptide ligands, may in some cases allow the prediction of peptide specificities for MHC molecules with yet unknown motifs [195, 196].

Furthermore, the predictions for other components of the antigen processing such as proteasomal cleavage specificities are still in their infancy, with programs that are based on quantified cleavage data from immuno- and constitutive proteasomes being not yet publicly available. On the other hand, the development of prediction programs for MHC class II that are able to take antigen processing into consideration are still in the distant future.

Since the increasing usage of screening techniques such as DNA microarrays [197] and SEREX (Serological analysis of recombinant cDNA expression libraries) [198] provide a rapidly growing pool of candidate protein antigens, interactive combinations of computational epitope predictions with these approaches, such as the recently described combination of DNA microarray analysis and TEPITOPE [196], are expected to provide a rapidly growing number of epitopes to be used in immunotherapy of a wide range of diseases.

However, while the described strategies combining computational prediction and experimental methods are likely to provide a rapidly increasing number of T cell epitopes as potential tools for therapeutic and diagnostic purposes, the identified epitopes still have to pass the ultimate test: they have to prove to be useful in the *in vivo* situation.

**Abbreviations**

ANN           artificial neural network

CTL           cytotoxic T lymphocyte

ELISA         enzyme linked immuno-sorbent assay

FACS          fluorescence-activated cell sorting

HLA           human leukocyte antigen

MHC           major histocompatibility complex

MSMS          tandem mass spectrometry

RT-PCR        reverse transcriptase-polymerase chain reaction

TAA           tumor-associated antigen

TAP           transporter associated with antigen processing

TCR           T cell receptor

WWW           world wide web


**Participating Reviewers**

Markus Schirle,Toni Weinschenk and Stefan Stevanović

Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany,

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### 1.4.3.3 Exploring the MHC-Ligandome

Parts of this chapter are parts of a publication in Immunological Reviews by Hans-Georg Rammensee, Toni Weinschenk, Cecile Gouttefangeas, and Stefan Stevanović.

Ideal would be the identification of all presented MHC ligands of a given tumor. In principle this aim was tackled in this thesis. It is shown here that identification of

more than 100 peptides is feasible, but still this is probably less than 10 % of the complete MHC-ligandome [199]. But as the big majority of all MHC-bound peptides are derived from self antigens and thus are not useful for immunotherapy, immediately the question about the selection of the appropriate antigens and peptides arises.

*Selection of tumor antigens for individual patients*

To get a selection of tumor antigens well suited to be targeted in a particular cancer patient, first an analysis of differences between this tumor and normal cells is necessary on the molecular level. The amount of starting material ranges between whole organs such as a kidney in the case of renal cell carcinoma on the one hand and small biopsies on the other hand. In the first case malignant and normal material is easily available for analysis, in the latter case amounts are limited and it can be difficult especially to get material from normal tissues. Moreover, accurate analysis requires homogenous tumor and normal cell populations, which sometimes only can be obtained after enrichment procedures. Enrichment can be achieved by several methods, e.g. laser capture microdissection, cell sorting by magnetic beads or fluorescence-assisted cell sorting, but cell numbers available for analysis are then further decreased. Expression analysis of single tumor cells by chip technology has been described [200]; however, a critical issue here is the fidelity of quantitative representation of the original individual mRNA species after PCR-based cDNA amplification.

Large scale screening for differences between tumor and normal cells can be done on the level of DNA, protein, mRNA or MHC ligands. Array-based comparative genomic hybridization (array-CGH) [201] can be used for genome-wide analysis of DNA copy-number changes, but to identify target genes appropriate for immunotherapy, they are of less interest compared to changes in gene expression. Another interesting feature detectable in principle on the DNA level are point mutations and frameshifts due to microsatellite instability [28, 202], but methods for rapid parallel large scale mutation analysis as needed for patient individual analysis are lacking. Commercially available is a microarray for analysis of mutations in the tumor suppressor gene p53, a gene frequently mutated in tumors, but parallel screening for the most frequently occuring mutations distributed among several genes is not possible. An array covering the hot spots

of these genes such as ras [203] would be acclaimed. The classical proteomics approach uses two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for high-resolution protein separation followed by identification via mass spectrometry. Here, not only proteins overexpressed in the tumor versus the normal tissue are detectable, but additional TAA-classes are accessible, such as frameshift-derived antigens, antigens translated from the antisense strand, fusion-derived antigens and antigens showing different posttranslational modifications (PTM) in the tumor. For the aim to immunize with peptides, MHC ligand-prediction can be performed on most of these TAA-classes, but differences due to PTM cannot easily be transferred to MHC ligands.

Analysis based on electrophoresis is laborious, time-consuming, and of low sensitivity, despite still having the highest separation resolution. Thus, new simplified methods were developed: a method well suited to identify proteins only present in one of two samples, the surface-enhanced laser desorption/ionization (SELDI) technique [204, 205], and an approach to quantitative proteome analysis, using isotope-coded affinity tags (ICAT) [206, 207]. In the last years, development of protein based microarrays – arrays of antibodies, recombinant proteins or whole proteomes – made its contribution to tumor immunology [208-210].

For most cellular requirements, it is the proteins which do the job, suggesting the protein-level to be more relevant than the mRNA-level. But there is increasing evidence that the majority of MHC ligands are not derived from native proteins, but instead are processed from defective ribosomal products (DRiP) by proteasomal degradation [3-5, 211, 212]. This suggests that the number of peptides displayed by MHC molecules on the surface of a cell is closer related to the expression of the encoding mRNA species compared to the respective expression of the protein measured in the steady state.

Comparative expression profiling of a tumor and the corresponding autologous normal tissue enabled by DNA microarray technology [197, 213] is an excellent method for identifying large numbers of candidate TAA from individual tumor samples [214-216]. Using chip technology, genome wide expression analysis of all genes is possible within few days, and customized arrays can be used for high throughput screenings [217]. One disadvantage is that some important TAA-classes are not considered such as products of frameshift mutations, proteins translated from the antisense strand or fusion proteins. A big advantage is the

possibility to get expression levels for all known TAA in an individual patient on call. This means one could screen an MHC class I-ligand database (e.g. SYFPEITHI at http://www.syfpeithi.de) for all reported cancer-related epitopes restricted to the HLA alleles expressed by an individual patient, check the expression of the respective encoding genes in the very same patient and use the appropriate epitopes for vaccination on short notice. In addition, expression data for each gene in almost all normal human tissues and organs are available from already existing databases (see below) and databases that are in development. This is especially important for the choice as to which antigens are suitable for vaccination with respect to autoimmunity and T cell tolerance.

Ideal would be a differential "MHC-ligandome" analysis of the tumor and the corresponding normal cells because in this setting also differences in antigen processing between tumor and normal tissues are detected. Epitopes might exist which only are presented on the tumor cells although the source protein is present in equal amounts in both cell types. As an example a HLA-B*40-restricted T cell epitope of MAGE-3 was only generated in tumor cells expressing the immunoproteasome [218]. There is increasing evidence that the peptide pool generated by the immunoproteasome differs from that produced by the standard proteasome [140, 219, 220], an interesting aspect in the context of tumors exposed to local release of IFN-$\gamma$. But differential MHC-ligandome analysis is hard to achieve because of the low amounts of individual peptides obtained after MHC immunoprecipitation and elution. Comparison can be performed by off-line mass spectrometrical analysis of corresponding fractions obtained after reverse-phase HPLC separation. A more sensitive approach is the comparison of liquid-capillary mass spectrometry (LC-MS) runs of peptide mixtures eluted from the tumor and from the normal cells, a method already reported ten years ago for comparative analysis of different cell lines [91]. For quantitative conclusions, the challenge in this case is how to normalize.

*Data-mining*

How to select peptides out of the pool eluted from an individual patient's tumor for their suitability as vaccine components? Apart from the obvious considerations – overexpression in tumor, limited expression in other tissues – other criteria, such as known cancer association, involvement in the oncogenic process or

immunogenicity of the gene product, can be made. The analysis of existing databases should provide assistance: Serological identification of antigens by recombinant expression cloning (SEREX) [46, 221] gives a list of TAA recognized by antibodies. Some antigens identified by this method were shown to be recognized concomitantly by CD8^{+} T cells [38]. Despite the fact that there is no direct linkage between detection of antibodies in cancer patient's sera against a particular TAA and the presence of CD8^{+} T cells recognizing the very same TAA, at least one could state that antigens identified by SEREX are immunogenic, in general. These antigens are suitable enough for the immune system to be recognized by antibodies and CD4^{+} T cells, so probably likewise by CTL. For these reasons, data-mining after MHC ligand analysis of an individual tumor should include comparison of the peptide-encoding source proteins with the SEREX database (http://www.licr.org/SEREX.html).

Other useful tools are databases for gene expression. Compared to data sets obtained from the individual patient, they have only a small impact regarding the overexpression of genes, but they can help to decide if a gene is a TAA shared among several tumors or not. Moreover, they are important for limitation of risk concerning autoimmunity because more and more data about tissue distribution of a candidate gene will be available. Some examples for databases that allow unrestricted access are: SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE) [222], a database compiled from data obtained by serial analysis of gene expression [223, 224], allows to display virtual northern blots. Gene Expression Omnibus (GEO) [225] (http://www.ncbi.nlm.nih.gov/geo) is a public repository for high-throughput gene expression and genomic hybridization data derived from SAGE, hybridization on filter, microarray and high-density oligonucleotide array experiments. HugeIndex [226] is a database especially for data from normal human tissues (http://www.hugeindex.org). Certainly, in-house gene expression databases are preferable, because data sets are more coherent due to exactly identical sample preparations, but they are rather expensive to build up. One general drawback of expression databases lies in the diversity of platforms. Even within one technology platform such as high-density oligonucleotide arrays, data generated from new chips covering more genes and with another design are not comparable to data obtained from out-of-date chip generations. As an example, data from HugeIndex are based on Affymetrix GeneChip[®] Hu6800 arrays, therefore only a small

number of genes is represented and comparison with data from recently released arrays is not possible.

Further useful quick information, especially gene annotations, for the selection of peptides out of the pool eluted from an individual patient can be obtained from databases about gene ontology. A nice tool for this purpose is the Gene Ontology database (http://www.geneontology.org) [227] also linked to LokusLink (http://www.ncbi.nlm.nih.gov/LocusLink), a central platform for many informations about genes [228].

## 1.5   References – Introduction

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## 1.6    Aims of this thesis

Therapeutic tumor-immunotherapy in human beings in general as well as vaccination with peptides have been more or less established at the beginning of this thesis. Even personalized vaccines – vaccines especially adapted to the needs of every patient – have been studied in a variety of applications. On the one hand, personalization was achieved using processed autologous material from each patient. This autologous material was either whole tumor lysate, total RNA isolated from the tumor or gp96 isolated from autologous tumor cells. On the other hand, all studies using MHC class I-restricted peptides have been sort of personalized because almost all of them studied HLA-A*02-restricted peptides and it's obvious that patients had to be selected according to their HLA-type. Expression of the respective antigen was not analyzed in most of the latter cases.

Thus, the aim of this thesis was to establish an approach for the identification of personalized tumor vaccines consisting of MHC class I-bound peptides. This approach should take into account:

- the expressed antigens in the tumor
- the expressed HLA alleles
- antigen processing in the tumor

Further demands were:

- many different targets should be addressed
- peptides should be presented preferably on many HLA-alleles

Several up-to-date technologies were used for this aim:

- capillary LC-MS (already established in the laboratory)
- oligonucleotide microarray expression profiling (established by the author of this thesis in the lab already in 2000)
- real-time RT-PCR (established by the author of this thesis in the lab)
- LCM (established by the author of this thesis in the lab)

Especially LC-MS was also applied to the second and related topic of this thesis, antigen processing. Here, the focus was set on processing enzymes beside the proteasome. During establishment and optimisation of the above mentioned technologies, some side projects, mainly protein identifications by tryptic in-gel digestions followed by mass spectrometric analysis (MALDI and ESI), were performed.

# 2 Results, Part 1: Tumor Immunology

## 2.1 Integrated functional genomics approach for the design of patient-individual antitumor vaccines

This chapter is published in Cancer Research. The author of this thesis performed all experiments shown in this chapter with the exception of Figure 2.1.3, Figure 2.1.4, and Figure 2.1.5. Figure 2.1.3 resulted from the work of Oliver Schoor, Markus Schirle did the LC-MS run shown in Figure 2.1.4 and Steffen Walter did the tetramer stainings in Figure 2.1.5.

### 2.1.1 Summary

Our aim is to identify as many candidates as possible for tumor-associated T cell epitopes in individual patients. First, we performed expression profiling of tumor and normal tissue to identify genes exclusively expressed or overexpressed in the tumor sample. Then, using mass spectrometry, we characterized up to 77 different MHC ligands from the same tumor sample. Several of the MHC ligands were derived from overexpressed gene products, one from a proto-oncogene, and another from a frameshift mutation. At least one was identified as an actual T cell epitope. Thus, we could show that by combining these two analytic tools, it is possible to propose several candidates for peptide-based immunotherapy. We envision the use of this novel integrated functional genomics approach for the design of antitumor vaccines tailored to suit the needs of each patient.

### 2.1.2 Introduction

Treatment of cancer by T cell-based immunotherapy can induce antigen-specific T cell responses *in vivo* and lead to clinical benefit, as shown in several clinical trials [1-6]. Induction of a defined specific CD8^{+} CTL response directed against the tumor is dependent on identification of MHC class I-ligands derived from TAA.

TAA can be exclusively present in malignant cells, such as the products of mutated genes. Another important class of TAA are tissue-specific structures such as the cancer-testis antigens and a third class of TAA are proteins overexpressed in tumors. Classically, the identification of tumor-associated T cell epitopes involved patient-derived T cells and either a gene expression approach [7] or MS-assisted sequencing of the recognized peptides [8]. A more recent approach is "reverse immunology", which uses the prediction of MHC class I-ligands from a selected TAA followed by their verification as T cell epitopes [9]. Major drawbacks of T cell-based strategies are the time-consuming culture techniques, and, more importantly, their limitation by the frequency of pre-existing T cells. Recently, we developed a T cell-independent approach, combining epitope prediction and screening for the predicted peptides in complex peptide mixtures eluted from tumors by highly sensitive capillary LC-MS [10].

Comparative expression profiling of a tumor and the corresponding autologous normal tissue enabled by DNA microarray technology [11, 12] is an excellent method for identifying large numbers of candidate TAA from individual tumor samples [13-15]. HLA-presented peptides from overexpressed or selectively expressed proteins should provide targets for specific CTL recognition of tumors. The feasibility of combining expression analysis with epitope prediction for a successful vaccine design has been demonstrated in a mouse model [16]. However, epitope prediction even for only a few target genes results in a vast number of candidate peptides, the majority of which are actually not presented by MHC molecules. Ideal would be a combination of epitope prediction with biochemical verification.

Here we describe an integrated functional genomics approach that provides the basis for making an individual selection of peptides that can be used for peptide-based multi-epitope immunotherapy tailored to suit the needs of each patient (Figure 2.1.1).
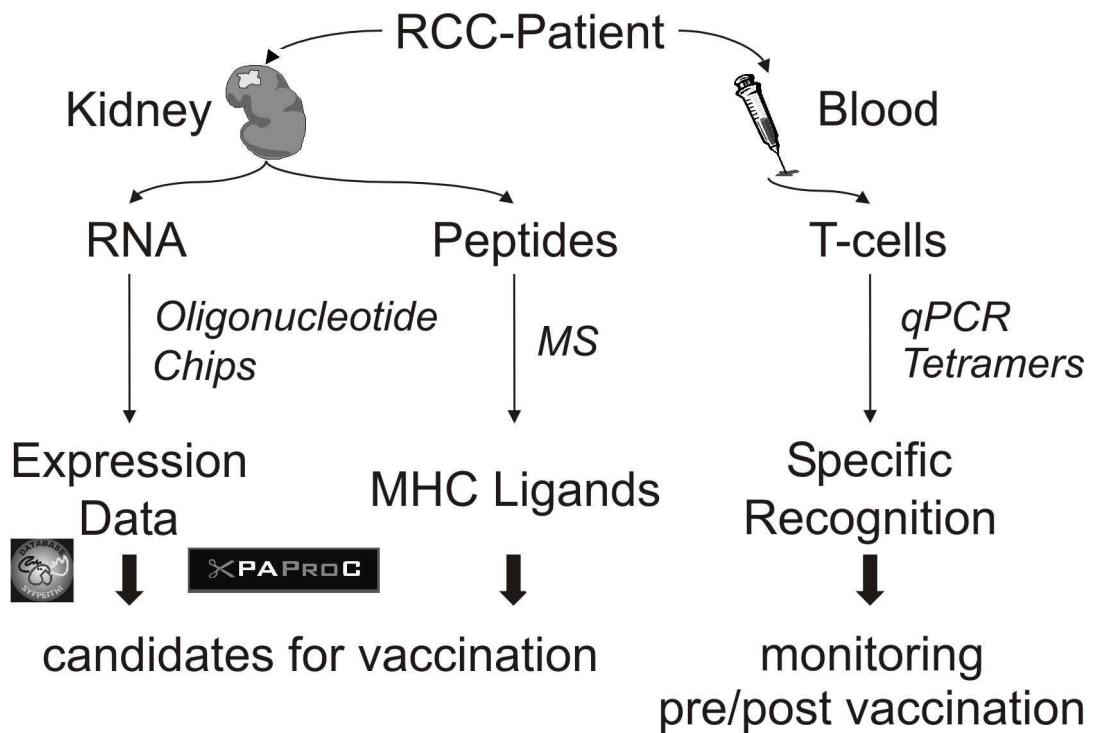
**Figure 2.1.1** *Integrated functional genomics approach for an optimized identification of tumor-associated epitopes.*

MHC class I-ligands presented on solid tumors are identified by MS. Expression data for the source proteins are immediately available from microarrays. T cell reactivity against new potential tumor-associated epitopes before and after vaccination can be monitored by MHC-tetramer technique and a quantitative real-time PCR-based T cell assay. SYFPEITHI (http://www.syfpeithi.de) and PAPROC (http://www.paproc.de) allow epitope and proteasomal cleavage prediction for identified target genes, respectively.

We analyzed samples from surgically removed malignant and normal tissue and blood from RCC patients in three different ways.

First, we performed gene expression profiling using high density oligonucleotide array-technology to identify genes selectively expressed or overexpressed in the malignant tissue. Second, we identified MHC class I-ligands from the malignant material by mass spectrometry. Newly identified MHC ligands encoded by selectively expressed or overexpressed genes as detected in step one should be suitable candidates for an individual, multi-epitope-based vaccine. Furthermore, all known ligands from TAA reported already can be included immediately in the

vaccine if they match the patient's HLA type and if the TAA are expressed in the individual tumor. Third, peripheral CD8^{+} T cells of tumor patients and healthy individuals were tested for reactivity against several of the tumor-associated MHC class I-ligands by a qPCR-based T cell assay [17] and by MHC peptide-tetramers [18].

## 2.1.3   Materials and Methods

### 2.1.3.1  Patient samples

Patient samples were obtained from the Department of Urology, University of Tübingen. The local ethical committee approved this study and informed consent was obtained from the patients. Both patients had histologically confirmed RCC, clear cell subtype, and had not received preoperative therapy. RCC01 was staged pT3bNxMx (G2), RCC13 was pT2NxMx (G3). HLA-typing for patient RCC01 was HLA-A*02 A*68 B*18 B*44 and for patient RCC13 HLA-A*02 A*24 B*07 B*40.

### 2.1.3.2  Isolation of MHC Class I-Bound Peptides

Shock-frozen tumor samples were essentially processed as described [10]. Peptides were isolated according to standard protocols [19] using the mAb W6/32 [20] specific for HLA class I or HLA-A*02-specific mAb BB7.2 [21].

### 2.1.3.3  Mass spectrometry.

Peptides from tumor RCC01 were separated by reversed phase HPLC (SMART system, μRPC C2/C18 SC 2.1/10, Amersham Pharmacia Biotech, Freiburg, Germany) and fractions analyzed by nanoESI MS on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-TOF, Micromass, Manchester, United Kingdom) as described [10]. Peptides from tumor RCC13 were identified by on-line capillary LC-MS as described [10] with minor modifications: Sample volumes of about 100 μl were loaded, desalted, and preconcentrated on a 300 μm * 5 mm C18 μ-Precolumn (LC Packings, San Francisco, CA). A syringe pump (PHD 2000, Harvard Apparatus, Inc., Holliston, MA), equipped with a gastight 100 μl syringe (1710 RNR, Hamilton, Bonaduz,

Switzerland), delivered solvent and sample at 2 µl/min. For peptide separation, the preconcentration column was switched in line with a 75 µm * 250 mm C-18 column (LC Packings, San Francisco, CA). A binary gradient of 25%-60% B within 70 min was performed, applying a 12 µl/min flow rate reduced to approximately 300 nl/min with a pre-column split using a TEE-piece (ZT1C, Valco, Schenkon, Switzerland) and a 300 µm * 150 mm C-18 column as a backpressure device. A blank run was always included to ensure that the system was free of residual peptide. On-line fragmentation was performed as described [10]. Fragment spectra were analyzed manually and database searches (NCBInr, EST) were made using MASCOT[1] [22].

### 2.1.3.4  Preparation of RNA

Fragments of normal and malignant renal tissue were dissected, shock-frozen, ground by mortar and pestle under liquid nitrogen and homogenized with a rotary homogenizer (Heidolph Instruments, Schwabach, Germany) in TRIZOL (Life Technologies, Inc., Karlsruhe, Germany). Total RNA was prepared according to the manufacturer's protocol followed by a clean-up with RNeasy (QIAGEN, Hilden, Germany). Total RNA from human tissues were obtained commercially (Human total RNA Master Panel II, Clontech, Heidelberg, Germany).

### 2.1.3.5  High-Density Oligonucleotide Microarray Analysis

Double-stranded DNA was synthesized from 40 µg of total RNA using SuperScript RTII (Life Technologies, Inc., Karlsruhe, Germany) and the primer (Eurogentec, Seraing, Belgium) as given by the Affymetrix manual. *In vitro* transcription using the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY), fragmentation, hybridization on Affymetrix HuGeneFL GeneChips (Affymetrix, Santa Clara, CA), and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, The Netherlands) followed the manufacturer's protocols (Affymetrix). The Affymetrix GeneArray Scanner was used and data were analyzed with the Microarray Analysis Suite 4.0 software. After scaling, exclusive expression was

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[1] Internet address: http://www.matrixscience.com

determined by the absolute call algorithms. Only genes showing an increase according to the difference call algorithm were considered to be upregulated by the factor as given by the fold change, calculated from average difference changes.

### 2.1.3.6 Real-time RT-PCR

The cDNA generated for microarray analysis was used for qPCR analysis. Tissue expression of adipophilin and keratin 18 was analyzed using single-stranded cDNA synthesized from 1 µg total RNA by SuperScript RTII (Life Technologies, Inc., Karlsruhe, Germany) and a random hexamer primer. Each gene was run in duplicates (40 cycles, 95°C x 15 s, 60°C x 1 min) using SYBRGreen chemistry on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Samples were independently analyzed 2 to 3 times. Primers (MWG-Biotech, Ebersberg, Germany) were selected to flank an intron and PCR efficiencies were tested for all primer pairs and found to be close to 1. Primer sequences (forward; reverse) were for ADFP: 5′-CACTGT GCTGAGCAATTTGAG-3′; 5′-TTGGCTTGATCTTGGATGTTC-3′. KIAA0367: 5′-AATGCCTCAGTAGTTTGTTCCC-3′; 5′-TTTATTCTGAGCAATCCAATGC-3′. LGALS2: 5′-AAGATCACAGGCAGCATCG-3′; 5′-GACAATGGTGGATTCGCTG-3′. CCND1: 5′-CACGATTTCATTGAACACTTCC-3′; 5′-TGAACTTCACATCTGTG GCAC-3′. MET: 5′-ACATTGAAATGCACAGTTGGTC-3′; 5′-ACAGGATCCACAT AGGAGAATG-3′. ETS1: 5′-AAAGTGCCAACTTCCCCTG-3′; 5′-GGAAATCCGA CTTTCTTCCC-3′. KRT18: 5′-GAGCCTGGAGACCGAGAAC-3′; 5′-TTGCGAAG ATCTGAGCCC-3′. LMP2: 5′-TGGGATAGAACTGGAGGAACC-3′; 5′-CATATAC CTGACCTCCTTCACG-3′. LMP7: 5′-CTATCTGCGAAATGGAGAACG-3′; 5′-CCT TCTTATCCCAGCCACAG-3′. 18S rRNA: 5′-CGGCTACCACATCCAAGGAA-3′; 5′-GCTGGAATTACCGCGGCT-3′. PCR products were analyzed on 3% agarose gels for purity and sequence-verified after cloning into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands). Data analysis involved the delta $C_T$ method for relative quantification.

### 2.1.3.7  Laser Capture Microdissection

Embedded frozen-tissue specimens were cut at 6 $\mu$m thickness and transferred in 70% ethanol for less than 15 min. Slides were incubated 90 s in Mayer's hematoxylin (Merck, Darmstadt, Germany), rinsed in water, incubated 1 min in 70% ethanol, 1 min in 95% ethanol, 30 s in 1% alcoholic eosin Y (Sigma, Munich, Germany), 2 x 2 min in 95% ethanol, 2 x 2 min in 100% ethanol and finally 2 x 5 min in xylene. After air-drying for 15 min, slides were stored under dry conditions. Nonmalignant epithelial tubular cells and carcinoma cells were isolated by LCM using the PixCell II LCM System (Arcturus Engineering, Harpenden, United Kingdom). Total RNA was extracted in 400 $\mu$l TRIZOL.

### 2.1.3.8  PBMC, Tetramer Production and Flow Cytometry

Buffy coats from HD were provided by the Bloodbank, Tübingen. HD1 and HD2 were serologically typed as CMV-positive, HD4 and HD6 as CMV-negative. PBMC were isolated by gradient centrifugation (FicoLite H, Wertheim-Bettingen, Germany) and frozen. HLA-A*0201 tetrameric complexes were produced as previously described [18, 23], with minor modifications. The HLA-A*02 binding peptides used for the refolding were (ALLNIKVKL) from keratin 18 and the epitope (NLVPMVATV) from pp65 HCMVA. Tetramers were assembled by mixing biotinylated monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 ratio. PBMC were thawed and 2-3 x $10^6$ cells were incubated 30 min at 4 °C with both tetramers (10 $\mu$g/ml for each monomer in PBS, 0.01% $NaN_3$, 2 mM EDTA, 50% fetal calf serum; optimal concentration determined after titration experiments), then anti-CD4-FITC (Coulter-Immunotech, Hamburg, Germany) and anti-CD8-PerCP (Becton Dickinson, Heidelberg, Germany) mAbs were added for 20 min. After 3 washes, samples were fixed in FACS buffer, 1% formaldehyde. Four-color analysis was performed on a FACSCalibur cytometer (Becton Dickinson). Under these conditions, the average background of nonspecific staining with tetramers was found to be less than 0.01% on CD8$^+$ cells.

## 2.1.4  Results

### 2.1.4.1 Candidates for Target Antigens in Individual Cancer Patients are Revealed by Expression Profiling

We analyzed the expression of approximately 7000 genes in tumors and corresponding normal tissues of two RCC. Between 400 and 500 genes were found to be overexpressed or selectively expressed in the tumors. In RCC01, we found 268 overexpressed and 129 exclusively detected genes (partially shown in Table 2.1.1). Most of the overexpressed genes might be cancer-related, i.e. they are either oncogenes, tumor suppressor genes or genes already described as overexpressed in cancer, such as cyclin D1 (CCND1, increased by factor 4.9, data not shown) [24], carbonic anhydrase IX (CA9) [25], cerebroside sulfotransferase (CST) [26], and parathyroid hormone-like hormone [27] (Table 2.1.1). The cancer-associated adipose differentiation-related protein (ADFP), or adipophilin, showed the second highest degree of overexpression. We compared the list of overexpressed genes with the SEREX database[1] [28] and found KIAA0367 to be included (clone ID NGO-St-87). Both tumors showed increased expression levels of IFN-$\gamma$-inducible genes: TAP1, MHC class I heavy chain, and the immunoproteasomal subunits LMP2 and LMP7 (not shown).

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[1] Internet address: http://www.licr.org/SEREX.html

**Table 2.1.1 *Genes overexpressed in RCC01 (partial list)*[a]**

| Gene Name[b] | Acc. No. | SEREX database[c] | cancer related[d] | Fold Change[e] |
|---|---|---|---|---|
| CST (cerebroside sulfotransferase) | D88667 | - | + | EE[f] |
| VEGF (vascular endothelial growth factor) | M27281 | - | + | EE |
| INHBB (inhibin) | M31682 | - | + | EE |
| LGALS2 (galectin 2) | M87860 | - | - | EE |
| FOLR1 (folate receptor 1) | U20391 | - | + | EE |
| CA9 (carbonic anhydrase IX) | X66839 | - | + | EE |
| EGFR (epidermal growth factor receptor) | X00588 | - | + | EE |
| ANG (angiogenin) | M11567 | - | + | EE |
| TYMS (thymidylate synthetase) | D00596 | - | + | EE |
| BTN3A2 (butyrophilin, subfamily 3, member A2) | U90546 | - | - | EE |
| TGFA (TGFα) | X70340 | - | + | EE |
| MMP1 (matrix metalloproteinase 1) | X54925 | - | + | EE |
| PLD1 (phospholipase D1) | U38545 | - | + | EE |
| ABP1 (amiloride binding protein 1) | U11862 | - | - | 34.6 |
| ADFP (adipose differentiation-related protein) | X97324 | - | + | 29.1 |
| GSTA2 (glutathione S-transferase A2) | M16594 | - | - | 23.3 |
| HSF4 (heat shock transcription factor 4) | D87673 | - | - | 19.6 |
| ASM3A (acid sphingomyelinase-like phosphodiesterase) | Y08136 | - | - | 17.6 |
| ESM1 (endothelial cell-specific molecule 1) | X89426 | - | + | 14.9 |
| ASPA (aspartoacylase) | S67156 | - | - | 12.8 |
| CP (ceruloplasmin) | M13699 | - | + | 12.4 |
| PTHLH (parathyroid hormone-like hormone) | M24349 | - | + | 11.6 |
| KIAA0367 | AB002365 | + | - | 11.3 |
| ENPP2 (autotaxin) | L35594 | - | + | 10.7 |
| P4HA1 (proline 4-hydroxylase) | M24486 | - | - | 9.6 |
| SHMT1 (serine hydroxymethyltransferase 1) | L23928 | - | + | 9.4 |

[a] to constrain the size of Table 2.1.1, genes overexpressed more than 9.0 fold are shown and ranked by fold change; in addition arbitrarily selected genes exclusively expressed in the tumor are shown;

[b] gene symbols and names refer to GeneCards (http://bioinformatics.weizmann.ac.il/cards); whenever possible symbols approved by the HUGO Gene Nomenclature Committee were used.

[c] http://www.licr.org/SEREX.html.

[d] oncogenes, tumor suppressor genes or genes overexpressed in cancer.

[e] expression in tumor relative to corresponding normal tissue.

[f] exclusively expressed in tumor, not in normal tissue.

To verify data obtained by microarray analysis, we analyzed the expression of selected genes – genes from which ligands were identified (see below) and which are interesting because of either reported overexpression or tumor-association – by qPCR (Figure 2.1.2 A).
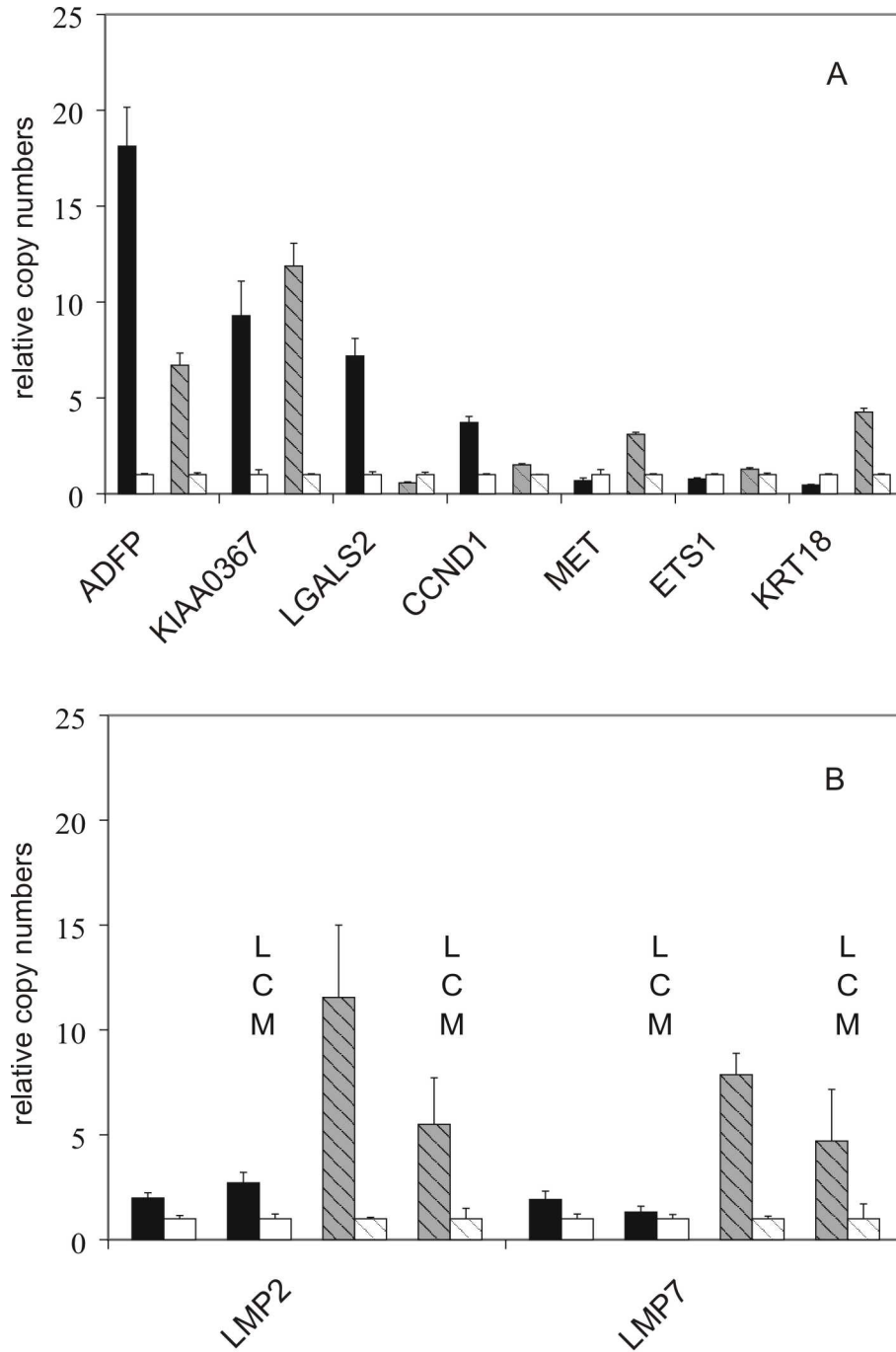
**Figure 2.1.2** *Expression analysis by quantitative RT-PCR for selected genes.*

Expression was analyzed by qPCR using the same cDNA as generated for microarray analysis or cDNA generated from laser-capture microdissected (LCM) pure cell populations. Copy numbers are relative to 18S rRNA and normalized to the normal tissue of each patient, which is set at 1 (■ RCC01 tumor, □ RCC01 normal tissue, ▨ RCC13 tumor, □ RCC13 normal tissue). *A*, Expression of potential target genes. Symbols for gene names are adipophilin (ADFP), galectin 2 (LGALS2), cyclin D1 (CCND1), met proto-oncogene/hepatocyte growth factor receptor (MET), ets-1 (ETS1), and keratin 18 (KRT18). *B*, Expression of immunoproteasome subunits LMP2 and LMP7; *bars*, ±SE.

Overexpression of adipophilin (ADFP) and cyclin D1 (CCND1) and equal expression of ets-1 (ETS1) was confirmed. Relative expression levels detected by both techniques were roughly comparable: for example adipophilin was overexpressed in RCC01 by a factor of 29.1 by microarray, compared to 18.1 by qPCR. The corresponding numbers in RCC13 are 11.4 and 6.7 (Figure 2.1.2 A). Galectin 2 (LGALS2) was overexpressed in RCC01 and keratin 18 (KRT18) in RCC13, but not vice versa. An exception to the congruence between microarray and qPCR was the overexpression of KIAA0367 and met proto-oncogene (MET) in RCC13. Analysis of pure tumor and normal cell populations obtained by LCM revealed the expression of adipophilin and galectin 2 in the tumors of both patients but not in normal tissue (data not shown). To confirm that LMP2 and LMP7 are indeed upregulated in tumor cells, we also performed qPCR on LCM-derived cells (Figure 2.1.2 B).

### 2.1.4.2  77 MHC Class I Ligands are Identified from One Individual RCC

HLA class I-associated peptides were isolated from tumor RCC01. Sequence analysis by nanoESI tandem mass spectrometry allowed the identification of 77 ligands of either HLA-A*02, HLA-A*68, HLA-B*18, or HLA-B*44, according to the HLA-typing of patient RCC01 and the respective peptide motifs (Table 2.1.2). To our knowledge, this is the largest number of ligands identified from a single solid tumor.

**Table 2.1.2 *Identified MHC class I-ligands***

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| **Solid renal cell carcinoma from patient RCC01** | | | | | |
| HLA-A*02 | | | | | |
| YVDPVITSI | MET (met proto-oncogene) | J02958 | 654-662 | - | nc |
| **SVASTITGV** | ADFP (adipose differentiation-related protein) | X97324 | 129-137 | - | **29.1** |
| ALLNIKVKL | KRT18 (keratin 18) | M26326 | 365-373 | - | nc |
| **ALFDGDPHL** | KIAA0367 | AB002365 | 1-9 | + | **11.3** |
| RLLDYVVNI | FLJ20004 (hypothetical protein FLJ20004) | AB040951 | 679-687 | - | |
| ALANGIEEV | APOL3 (apolipoprotein L, 3) | AY014906 | 101-109 | - | |
| **QLIDKVWQL** | SEC14L1 (SEC14 (S. cerevisiae)-like 1) | D67029 | 593-601 | - | **1.9** |
| ALSDLEITL | MIG2 (mitogen inducible 2 ) | Z24725 | 389-397 | - | nc |

**Table 2.1.2 - *continued***

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| ILDTGTIQL | CML1( kidney- and liver-specific gene ) | AB013094 | 174-182 | - | |
| SLLGGDVVSV | DSIPI (delta sleep inducing peptide, immunoreactor) | AF153603 | 27-36 | - | nc |
| FLDGNELTL | CLIC1 (chloride intracellular channel 1) | U93205 | 167-175 | - | nc |
| NLLPKLHIV | CLIC1 (chloride intracellular channel 1) | U93205 | 179-187 | - | nc |
| ALASHLIEA | EHD2 (EH-domain containing 2) | AF181263 | 507-515 | - | |
| SLYGGTITI | FLJ11189 (hypothetical protein FLJ11189) | AK000697 | 296-304 | + | |
| FLLDKKIGV | CCT2 (chaperonin containing TCP1, subunit 2 (beta)) | AF026166 | 218-226 | - | |
| FLDGNEMTL | CLIC4 (chloride intracellular channel 4) | AF097330 | 178-186 | - | |
| AIVDKVPSV | LOC51137 (coat protein gamma-cop) | AF100756 | 147-155 | - | |
| DVASVIVTKL | SRP54 (signal recognition particle 54kD) | U51920 | 241-250 | - | nc |
| LASVSTVL | HBA2 (hemoglobin, alpha 2) | AF230076 | 130-137 | - | nc |
| VMAPRTLVL | HLA-A | | 3-11 | | |
| LLFDRPMHV | HNRPM (hnRNP M) | L03532 | 267-275 | - | nc |
| SLAGGIIGV[e] | HNRPK (hnRNP K) | BC000355 | 154-162 | - | nc |
| **TLWVDPYEV**[e] | BTG1 (B-cell translocation gene 1, anti-proliferative) | X61123 | 103-111 | - | **1.8** |
| **ALSDHHIYL**[e] | ALDOA (aldolase A, fructose-bisphosphate) | X12447 | 216-224 | + | **1.8** |
| LLDVPTAAV[e] | IFI30 (interferon, gamma-inducible protein 30) | J03909 | 27-35 | - | nc |

HLA-A*68

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| **MTSALPIIQK** | ADFP (adipose differentiation-related protein) | X97324 | 62-71 | - | **29.1** |
| **MAGDIYSVFR** | ADFP (adipose differentiation-related protein) | X97324 | 349-358 | - | **29.1** |
| **ETIPLTAEKL** | CCND1 (cyclin D1/PRAD1) | X59798 | 115-124 | - | **4.9** |
| DVMVGPFKLR | AKAP2 (A kinase (PRKA) anchor protein 2) | AJ303079 | 934-943 | - | |
| TIIDILTKR | ANXA1 (annexin A1) | X05908 | 64-72 | - | -2.6 |
| TIVNILTNR | ANXA2 (annexin A2) | BC001388 | 55-63 | + | -2.0 |
| TIIDIITHR | ANXA6 (annexin A6) | J03578 | 385-393 | - | nc |
| SIFDGRVVAK | LOC54499 (putative membrane protein) | AB020980 | 107-116 | - | |
| STIEYVIQR | SEC23B (Sec23 (S. cerevisiae) homolog B) | BC005032 | 115-123 | - | nc |
| ELIKPPTILR | AP3M1 (adaptor-related protein complex 3) | AF092092 | 132-141 | - | |
| **EIAMATVTALR** | ALDOA (aldolase A, fructose-bisphosphate) | X12447 | 248-258 | + | **1.8** |
| EVAQLIQGGR[e] | LOC51660 (brain protein 44-like) | AF125101 | 88-97 | - | |
| DTIEIITDR[e] | HNRPA2B1 (hnRNP A2/B1) | M29065 | 127-135 | - | nc |
| ETIGEILKK | HNRPK (hnRNP K) | BC000355 | 95-103 | - | nc |
| DVFRDPALK[e] | RPL27 (ribosomal protein L27) | L19527 | 99-107 | - | nc |
| SLADIMAKR | RPL24 (ribosomal protein L24) | BC000690 | 86-94 | - | |
| EVILIDPFHK[e] | RPL15 (ribosomal protein L15) | L25899 | 131-140 | + | |

HLA-B*44 or HLA-B*18

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| EEIAFLKKL | VIM (vimentin) | M14144 | 229-237 | - | nc |
| DEAAFLERL | CALD1 (caldesmon 1) | M64110 | 92-100 | - | nc |
| DEMKVLVL | SPTBN1 (spectrin, beta, non-erythrocytic 1) | M96803 | 545-552 | - | nc |
| **DEVKFLTV** | ANXA4 (annexin A4) | M82809 | 191-198 | - | **3.1** |

**Table 2.1.2 - *continued***

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| NENSLFKSL | CLTC (clathrin, heavy polypeptide (Hc)) | D21260 | 935-943 | - | nc |
| DEFKVVVV | LOC51137 (coat protein gamma-cop) | AF100756 | 373-380 | - | |
| EEVKLIKKM | FTL (ferritin, light polypeptide) | M11147 | 137-145 | - | nc |
| DEVKLPAKL | PTRF (polymerase I and transcript release factor) | AF312393 | 158-166 | - | |
| KESTLHLVL[e] | UBB (ubiquitin B) | X04803 | 63-71 | - | nc |
| TERELKVAY | FLJ20004 (hypothetical protein FLJ20004) | AB040951 | 637-645 | - | |
| NEFSLKGVDF | ETS1 (ets-1) | J04101 | 86-95 | - | nc |
| **NEQDLGIQY** | CTNNA1 (catenin alpha 1) | D13866 | 169-177 | + | **1.8** |
| EERIVELF | STAT3 (signal transducer and activator of transcription 3 ) | BC000627 | 306-313 | - | nc |
| EEIREAFRVF | CALM3 (calmodulin 3) | J04046 | 84-93 | - | nc |
| DEYIYRHFF | CPR8 (cell cycle progression 8 protein) | AF011794 | 344-352 | - | |
| DELELHQRF | BS69 (adenovirus 5 E1A binding protein) | X86098 | 308-316 | + | nc |
| **SEVKFTVTF** | LGALS2 (galectin 2) | M87842 | 80-88 | - | **EET** |
| IETIINTF | S100A9 (calgranulin B) | M26311 | 12-19 | - | nc |
| KENPLQFKF | VIL2/RDX (villin 2 (ezrin)/(radixin) | J05021/L02320[f] | 61-69/72-80 | +/- | 3.2/-2.3 |
| DEVRTLTY | HRMT1L2 (hnRNP methyltransferase, S. cerevisiae-like 2) | Y10807 | 41-48 | - | -1.6 |
| **GEAVVNRVF** | PSMB9 (large multifunctional protease 2, LMP2) | Z14977 | 43-51 | - | **2.6** |
| EEVLIPDQKY | FBXL3A (F-box and leucine-rich repeat protein 3A) | AF126028 | 385-394 | - | |
| DEGRLVLEF | SOAT1 (sterol O-acyltransferase 1) | L21934 | 163-171 | - | nc |
| DEVELIHF | FACTP140 (chromatin-specific transcription elongation factor) | AF152961 | 838-845 | - | |
| VEVLLNYAY | NS1-BP (NS1-binding protein ) | AF205218 | 83-91 | - | |
| TENDIRVMF | CUGBP1 (CUG triplet repeat, RNA-binding protein 1) | AF267534 | 120-128 | - | nc |
| LEGLTVVY | LOC51644 (coatomer protein complex, subunit zeta 1) | AF151878 | 62-69 | - | |
| NELPTVAF | FLJ10613 (hypothetical protein) | AK001475 | 192-199 | - | |
| EEFGQAFSF | HLA-DPA1 (MHC, class II, DP alpha 1) | X03100 | 77-85 | - | nc |
| VEAIFSKY | HNRPC (hnRNP C (C1/C2)) | M29063 | 33-40 | - | nc |
| DERTFHIFY | MYH10 (myosin, heavy polypeptide 10, non-muscle) | M69181 | 277-285 | + | -1.8 |
| TEKVLAAVY | ALDOB (aldolase B, fructose-bisphosphate) | K01177 | 206-214 | - | EEN |
| VESPLSVSF | FLJ22318 (hypothetical protein FLJ22318 ) | AK025971 | 159-167 | - | |
| SEAGSHTLQW | MHC-I | | | | |
| DEGKVIRF | EST (rf -1) | BF431469 | 56-63 | + | |

**Solid renal cell carcinoma from patient RCC13**

HLA-A*02

| Sequence | Gene Name | Acc. No. | Position | SEREX | Fold Change |
|---|---|---|---|---|---|
| YVDPVITSI | MET (met proto-oncogene) | J02958 | 654-662 | - | nc |
| **ALLNIKVKL** | KRT18 (keratin 18) | M26326 | 365-373 | - | **3.7** |
| ALAAVVTEV | frameshift, DDX3 reading frame +2 | AF061337 | | - | nc[g] |
| TLIEDILGV | DKFZP727M231(transient receptor protein 4 associated protein) | AL132825 | 209-217 | - | |
| ALFGALFLA | PLTP (phospholipid transfer protein) | L26232 | 2-10 | - | |
| VLATLVLLL | EST | AA483794 | 72-80 | - | |
| TLDDLIAAV | FLJ10042 (hypothetical protein FLJ10042) | AK000904 | 325-333 | - | |
| YLDNGVVFV | DDB1 (damage-specific DNA binding protein 1 (127kD)) | U18299 | 316-324 | - | nc |

**Table 2.1.2 - *continued***

| Sequence[a] | Gene Name[b] | Acc. No. | Position | SEREX database[c] | Fold Change[d] |
|---|---|---|---|---|---|
| SVFAGVVGV | GUCY1A3 (guanylate cyclase 1, soluble, alpha 3) | U58855 | 581-589 | - | nc |
| SLINVGLISV | SSP29 (acidic protein rich in leucines) | BC000476 | 48-57 | - | nc |
| ALADGVQKV | APOL1 (apolipoprotein L, 1)) | AF323540 | 176-184 | - | |
| FLGENISNFL[e] | APOL1 (apolipoprotein L, 1)) | AF323540 | 273-282 | - | |
| GLVPFLVSV[e] | KPNA2 (karyopherin alpha 2 (RAG cohort 1, importin alpha 1)) | BC005978 | 377-385 | - | nc |
| **LLDVPTAAV**[e] | IFI30 (interferon, gamma-inducible protein 30) | J03909 | 27-35 | - | **3.5** |
| **MVDGTLLLL**[e] | HLA-E (major histocompatibility complex, class I, E) | M21533 | 1-9 | - | **2.2** |
| YLLPAIVHI[e] | DDX5 (DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5) | AF015812 | 148-156 | + | nc |
| SLLPAIVEL[e] | PP2R1A (protein phosphatase 2, regulatory subunit A, alpha) | J02902 | 403-411 | - | nc |
| **ALSDHHIYL**[e] | ALDOA (aldolase A, fructose-bisphosphate) | X12447 | 216-224 | + | **1.9** |

**HLA-A*24**

| | | | | | |
|---|---|---|---|---|---|
| TYGEIFEKF | NDUFC2 (NADH dehydrogenase (ubiquinone) 1, ( B14.5b)) | AF070652 | 107-115 | - | |
| **YYMIGEQKF** | NNMT (nicotinamide N-methyltransferase) | U08021 | 203-211 | - | **EET** |

**HLA-B*40**

| | | | | | |
|---|---|---|---|---|---|
| KESTLHLVL[e] | UBB (ubiquitin B) | X04803 | 63-71 | - | nc |

[a] peptides printed in bold letters are encoded by overexpressed genes.

[b] gene symbols and names refer to GeneCards (http://bioinformatics.weizmann.ac.il/cards); whenever possible symbols approved by the HUGO Gene Nomenclature Committee were used.

[c] Internet address: http://www.licr.org/SEREX.html.

[d] expression in tumor relative to corresponding normal tissue; fold change is only given for genes included on the array; nc no change; EET exclusively expressed in tumor, not in normal tissue; EEN exclusively expressed in normal tissue.

[e] ligands already published, see SYFPEITHI database (http://www.syfpeithi.de/) for references.

[f] peptide derived from sequence homologous in VIL2 and RDX.

[g] no change for DDX3.

Peptides assigned to HLA-A*02 reflected the allele-specific peptide motif (L/V/I/A/M in position 2, L/V/I/A at the C-terminus) (Table 2.1.2). 21 of 25 identified HLA-A*02 restricted ligands were new, 4 have been reported before. Most ligands were derived from abundantly expressed housekeeping proteins, but we could also detect ligands from proteins with reported tumor association such as (YVDPVITSI), derived from met proto-oncogene, (ALLNIKVKL) from keratin 18, and (SVASTITGV) from adipophilin. HLA-A*68 ligands were recognized by their anchor amino acids T/I/V/A/L in position 2 and C-terminal R/K, which also indicated that the subtype was most probably HLA-A*6801. Two other ligands from adipophilin, (MTSALPIIQK) and (MAGDIYSVFR), were found among HLA-

A*68-presented peptides, as well as (ETIPLTAEKL), derived from tumor-associated cyclin D1. Annexin II, from which the peptide (TIVNILTNR) was identified, has been shown to be immunogenic in the context of MHC class II in melanoma patients [29].

All other 35 ligands carried E in position 2, an anchor residue of HLA-B*44. The peptide motif of HLA-B*18 is still unknown; therefore, a distinction between ligands of these two HLA-B-molecules was not possible. Fragments of vimentin (EEIAFLKKL) and caldesmon (DEAAFLERL), both overexpressed in RCC [13], were identified as well as ligands derived from ets-1 (NEFSLKGVDF), alpha-catenin (NEQDLGIQY) and galectin 2 (SEVKFTVTF).

Comparison with microarray data indicated 10 overexpressed genes as sources of MHC ligands: adipophilin, KIAA0367, SEC14-like 1, B-cell translocation gene 1, aldolase A, cyclin D1, annexin A4, catenin alpha 1, galectin 2, and LMP2. Three of them were also included in the SEREX database: KIAA0367, aldolase A, and catenin alpha 1.

### 2.1.4.3 Highest Expression Levels of Adipophilin In Tumors Compared to the Most Essential Human Organs and Tissues

If the difference in expression of a particular antigen between tumor cells and all normal cells is high, this antigen should also have a greater potential as a possible vaccine candidate. To this aim we analyzed the expression of adipophilin and keratin 18 in 21 human tissues and organs in addition to the kidney. Adipophilin, selectively overexpressed in the two tumors tested for comparison, was only marginally expressed in all other organs. Kidney was the organ with the highest expression level among all analyzed normal tissues (Figure 2.1.3 A). In contrast to adipophilin, keratin 18 showed a more heterogeneous tissue distribution, in particular with high expression levels in colon and placenta (Figure 2.1.3 B).
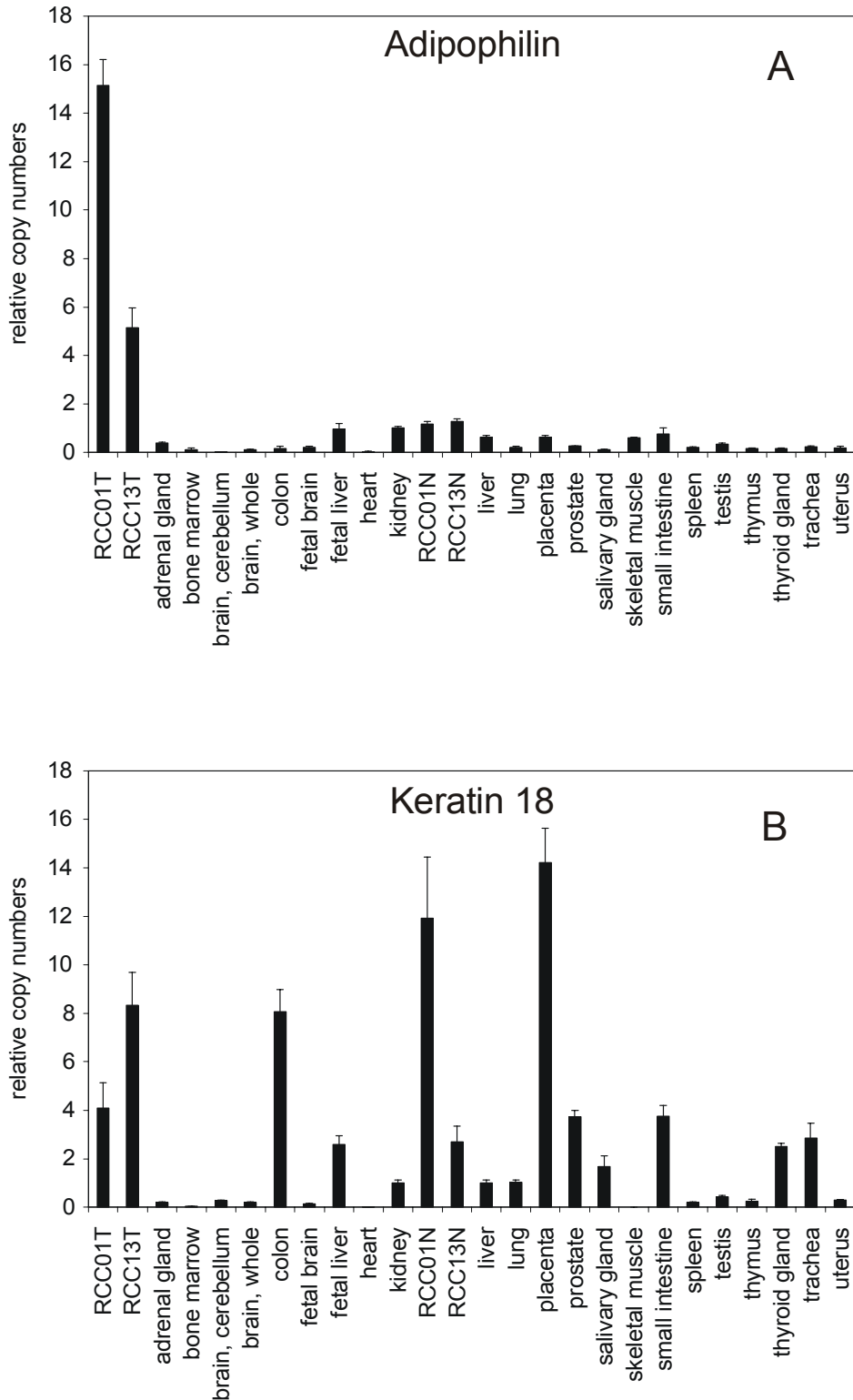
**Figure 2.1.3** *Tissue expression of adipophilin and keratin 18.*

Adipophilin was strongly expressed in tumors but only weakly in healthy organs and tissues (*A*) whereas keratin 18 was widely distributed among a variety of normal tissues (*B*). Expression was analyzed by qPCR using cDNA generated from a commercial total RNA tissue panel. Copy numbers are relative to 18S rRNA and normalized to kidney, which is set at 1; *bars*, ±SE.

### 2.1.4.4 Some of the New MHC Class I-Ligands Are Shared Between Tumors

MHC class I-ligands can be analyzed with utmost sensitivity by on-line capillary LC-MS using the "predict, calibrate, detect" approach [10]. After eluting MHC class I ligands from RCC13 we were again able to detect the ligands derived from met proto-oncogene (Figure 2.1.4) and keratin 18 (not shown). Interestingly, the keratin 18-derived ligand was also presented on a solid colon carcinoma (data not shown). In addition, several other ligands were identified from RCC13 (Table 2.1.2). We found one ligand derived from nicotinamide N-methyltransferase (NNMT), a gene which is overexpressed in more than 95% of all RCC [14]. Some other ligands overlap with the peptide repertoire of RCC01.
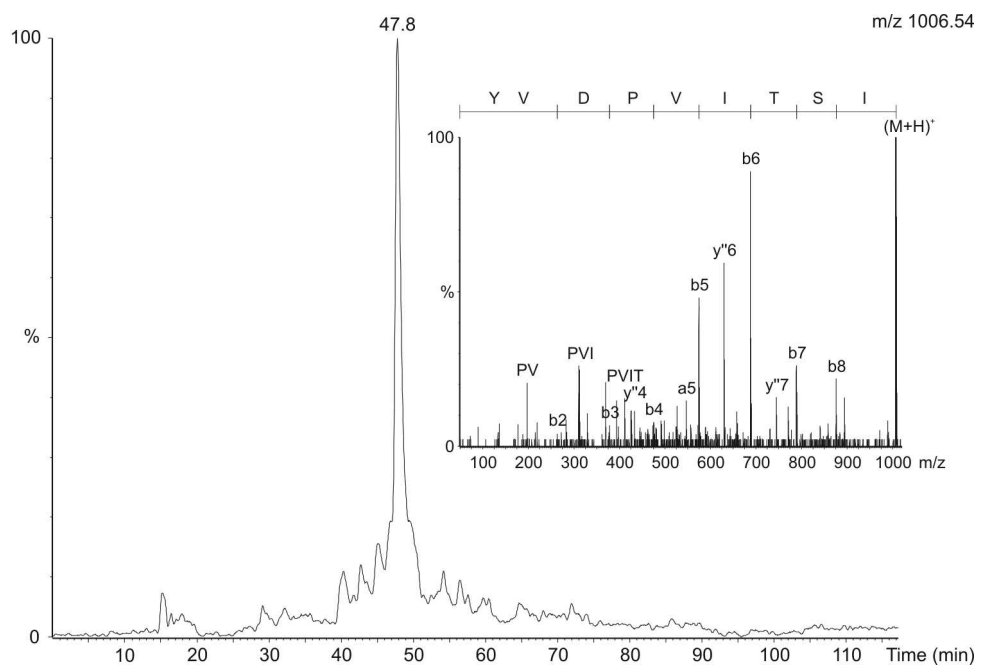


**Figure 2.1.4** *Presentation of ligand (YVDPVITSI) from MET on RCC13.*

Nanocapillary HPLC ESI MS was done on peptides eluted from RCC13. The mass chromatogram for 1006.54 $\pm$ 0.15 Da shows a peak at retention time 47.8 min. Collisionally induced decay mass spectrum from m/z 1006.54, recorded in a second LC-MS run at the given retention time and shown in the insert, confirmed the presence of (YVDPVITSI).

## 2.1.4.5 A New Frameshift-Derived MHC Class I-Ligand may Represent a Target Unique for one Patient

The most interesting ligand from RCC13 is (ALAAVVTEV), encoded by a reading frame shifted by one nucleotide compared to the frame coding for DEAD/H box polypeptide 3 (DDX3). (ALAAVVTEV) is encoded by nucleotides 317-343 of the coding strand of DDX3, whereas nucleotides 316-342 code for GIGSRGDRS of the DDX3 protein. T cell reactivity against frameshift-derived epitopes in anti-tumor response has been reported [30].

## 2.1.4.6 Keratin 18-Specific T Cells are Present in the Normal CD8$^+$ T Cell Repertoire

PBMC from patients RCC01 and RCC13 were not available, which prevented us from testing T-cell reactivity against the frameshift-derived peptide identified above. Nevertheless, we tested 6 HLA-A*02 positive RCC patients for reaction against 4 of the relevant peptides (HLA-A*02 restricted ligands from adipophilin, keratin 18, KIAA0367 and met proto-oncogene, see Table 2.1.2). We used a very sensitive qPCR assay for IFN-γ mRNA production by CD8$^+$ T cells [17], following a 7-day *in vitro* sensitization with peptide. In all patients, high production of IFN-γ mRNA was observed after stimulation with recall viral-derived peptides, showing that the cells were functional after isolation and stimulation. In contrast, responses against KIAA0367 were not detected, and sporadic but marginal responses were seen after stimulation with met proto-oncogene, keratin 18 or adipophilin peptides (data not shown). We also stained PBMC of few patients and healthy individuals with HLA-A*0201 tetramers folded either with the adipophilin, keratin 18 or met proto-oncogene peptides. A significant population of CD8$^+$ T lymphocytes specific for keratin 18 (between 0.02% and 0.2% of CD8$^+$ T cells) was found in 4 out of 22 healthy individuals tested (Figure 2.1.5 shows the staining of two positive and two negative donors, 0.02% of CD8$^+$ T cells for HD1 and 0.17% for HD6 bind to the keratin 18 tetramer). These results were reproduced in at least three independent experiments using two or more blood samples obtained at different time points and involving different batches of independently folded keratin 18 tetramers. Moreover, in a colabeling experiment it was shown that the binding of keratin 18 tetramer was specific since this population did not stain with a CMV tetramer

(Figure 2.1.5, bottom row). We conclude that for the keratin 18 peptide at least, specific CD8$^+$ T lymphocytes are contained in the human T cell repertoire. However, the 4 peptides identified on RCC are generally not recognized by spontaneously arising T cells detectable in the blood of patients using tetramers *ex vivo* or even qPCR after one *in vitro* stimulation with peptides. Vaccination, however, might lead to activation and expansion of specific CD8$^+$ T cells against these peptides, which then could be detected by accurate T cell monitoring.
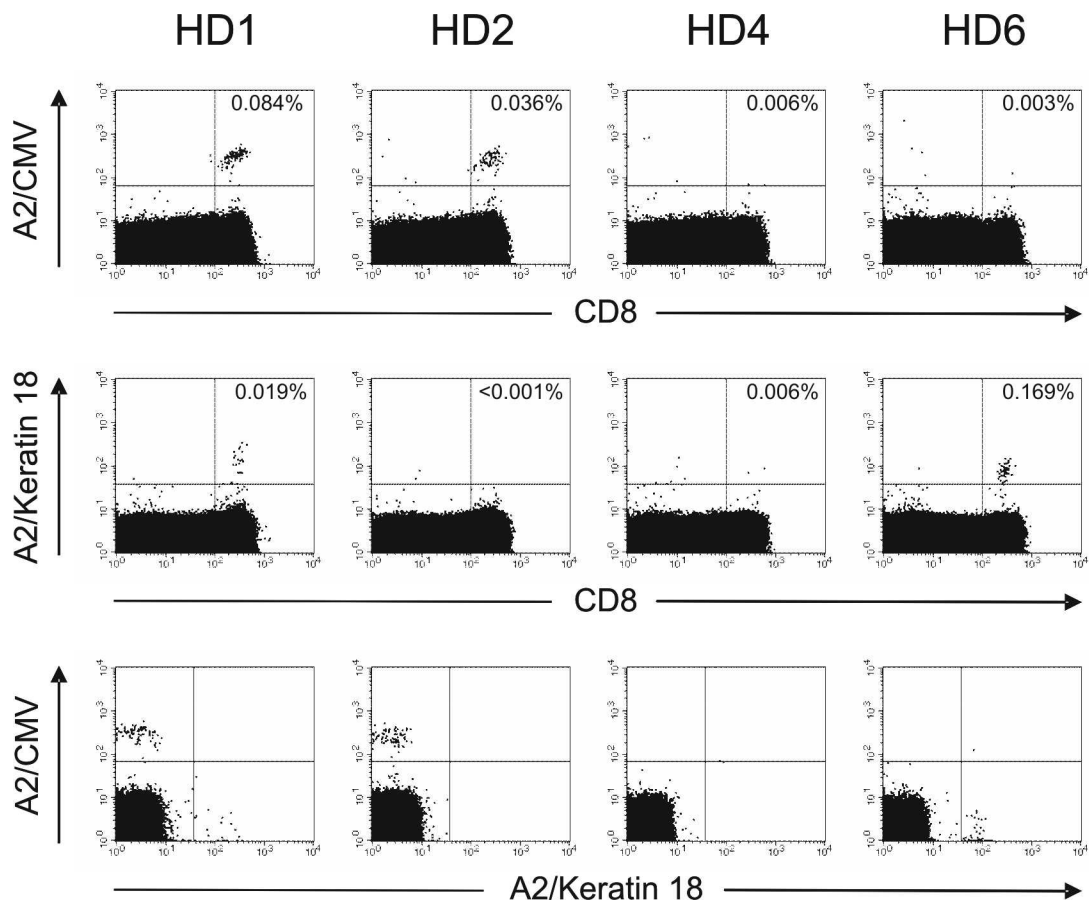


**Figure 2.1.5** *Detection of keratin 18-specific CD8+ T lymphocytes.*
PBMC from four healthy HLA-A*02$^+$ donors (HD1, 2, 4, 6) were stained simultaneously with HLA-A2/keratin 18-PE tetramers, HLA-A2/CMV-APC tetramers, CD8-PerCP and CD4-FITC. Dot plots show results from 1 of 3 independent experiments for 1 x 10$^6$ counted PBMC gated on CD4$^-$ lymphocytes (top and middle row) or gated on CD4$^-$CD8$^+$ lymphocytes (bottom row). Percentage of tetramer$^+$ cells within the CD8$^+$CD4$^-$ population are indicated.

## 2.1.5  Discussion

Gene expression analysis of the individual renal cell carcinomas revealed approximately 400 genes exclusively detected or overexpressed in tumor versus normal tissue. From tumor RCC01, 77 HLA class I-ligands were identified, 10 derived from overexpressed genes. Some of the latter have a broad tissue distribution, whereas others are more restricted. Which of the HLA ligands detected would now be candidates for vaccination? According to previous experience in tumor immunology, gene products overexpressed in the tumor but otherwise only expressed in a few, possibly nonvital cell types, can be targets for tumor-directed T cells.

**Table 2.1.3 *Candidates for peptide based immunotherapy of RCC01***

| Gene Name | Sequence | HLA Restriction | remarks | expression in normal tissues | candidate for vaccine |
|---|---|---|---|---|---|
| **New ligands** | | | | | |
| Adipophilin | SVASTITGV | A*02 | highly overexpressed in tumor | low[a] | +++ |
| Adipophilin | MTSALPIIQK | A*68 | highly overexpressed in tumor | low | +++ |
| Adipophilin | MAGDIYSVFR | A*68 | highly overexpressed in tumor | low | +++ |
| KIAA0367 | ALFDGDPHL | A*02 | limited tissue distribution | brain, prostate[b] | +++ |
| Galectin-2 | SEVKFTVTF | B*44/B*18 | exclusively expressed in tumor | n.t[c] | ++ |
| Cyclin D1 | ETIPLTAEKL | A*68 | cell cycle involved | low[b] | ++ |
| **Known epitopes[d]** | | | | | |
| CA9 | HLSTAFARV | A*02 | known TAA, overexpressed in RCC01T | n.t. | +++ |
| Cyclin D1 | LLGATCMFV | A*02 | cell cycle involved | low[b] | ++ |
| Cyclin D1 | RLTRFLSRV | A*02 | cell cycle involved | low[b] | ++ |

[a] see Figure 2.1.3 A

[b] based on qPCR analysis as shown in Figure 2.1.3, data not shown

[c] not tested

[d] reported T cell epitopes. These peptides were not detected in RCC01, but should be included because of overexpression of the encoding genes in RCC01 as revealed by the microarray data

Apart from these more obvious points, additional considerations can be made, such as known cancer association, involvement in the oncogenic process or immunogenicity of the gene product. The analysis of existing databases should support this endeavour. For example, the SEREX database indicates that antibodies against alpha catenin have been found in renal cancer patients, antibodies against KIAA0367 in stomach cancer patients and antibodies against

vimentin in pancreas cancer patients, suggesting a spontaneous immune response.

Based on the following detailed discussion of each new ligand, we would propose an individual combination of peptides for vaccination of patients RCC01 (Table 2.1.3) and RCC13 (Table 2.1.4).

**Table 2.1.4 *Candidates for peptide based immunotherapy of RCC13***

| Gene Name | Sequence | HLA Restriction | remarks | expression in normal tissues | candidate for vaccine |
|---|---|---|---|---|---|
| **New ligands** | | | | | |
| NNMT | YYMIGEQKF | A*24 | limited tissue distribution | liver[a,b] | +++ |
| MET | YVDPVITSI | A*02 | proto-oncogene | low[a]. | ++ |
| DDX3 rf +2[c] | ALAAVVTEV | A*02 | frameshift-derived | n.t.[d] | ++ |
| Adipophilin | SVASTITGV | A*02 | highly overexpressed in tumor | low[e] | +++ |
| KIAA0367 | ALFDGDPHL | A*02 | limited tissue distribution | brain, prostate[a] | +++ |
| **Known epitopes[f]** | | | | | |
| Cyclin D1 | LLGATCMFV | A*02 | cell cycle involved | low[a] | ++ |
| Cyclin D1 | RLTRFLSRV | A*02 | cell cycle involved | low[a] | ++ |
| PRAME | VLDGLDVLL | A*02 | cancer testis antigen | testis | +++ |
| PRAME | SLYSFPEPEA | A*02 | cancer testis antigen | testis | +++ |
| PRAME | ALYVDSLFFL | A*02 | cancer testis antigen | testis | +++ |
| PRAME | SLLQHLIGL | A*02 | cancer testis antigen | testis | +++ |
| PRAME | LYVDSLFFL | A*24 | cancer testis antigen | testis | +++ |
| CA9 | HLSTAFARV | A*02 | known TAA, overexpressed in RCC13T | n.t. | +++ |

[a] based on qPCR analysis as shown in Figure 2.1.3, data not shown

[b] expression in liver was about 3x lower compared to RCC13, expression in all other tissues tested was more than 16x lower

[c] reading frame +2

[d] not tested

[e] see Figure 2.1.3 A

[f] reported T cell epitopes. These peptides were not detected in RCC13, but should be included because of overexpression of the encoding genes in RCC13 as revealed by the microarray data

Adipophilin is an interesting candidate for a vaccine component for several reasons: First, it is only expressed in a few cell types [31] such as adipocytes, lactating mammary epithelial cells, adrenal cortex cells, Sertoli and Leydig cells, and pathological hepatocytes in alcoholic liver cirrhosis. Second, this gene was reported to be overexpressed in cancer [13, 32]. Third, we demonstrated that adipophilin is selectively expressed in the tumor cells of RCC01 and RCC13 but not in LCM-derived pure normal kidney cell populations. We also found that

expression in all analyzed normal tissues is much lower than in tumor cells (Figure 2.1.3 A). Finally, we identified 3 MHC class I ligands from this protein. KIAA0367 was overexpressed in patient RCC01 and according to the SAGEmap[1], a public database for gene expression [33], KIAA0367 is mainly expressed in prostate carcinoma and in the brain. Expression analysis by qPCR also showed that the highest levels were to be found in brain and in prostate (data not shown). Since the brain expresses low MHC and is partially immunoprivileged, and since patient RCC01 was female thus lacking prostate, the KIAA0367 peptide (ALFDGDPHL) would seem to be a first-class choice of vaccine component in this patient. According to analysis of LCM-derived samples, galectin 2, the source protein of (SEVKFTVTF), was selectively expressed in both tumors and not in normal kidney cells. Other members of the galectin gene family, including galectin-1, galectin-3 and galectin-12, are overexpressed in malignancies [13, 34]. Cyclin D1 is also overexpressed in several malignancies, including renal cell carcinoma [24]. We found an overexpression of cyclin D1 in RCC01, from which (ETIPLTAEKL) has been eluted, as well as in RCC13. The expression of cyclin D1 in all analyzed healthy organs and tissues was lower compared to RCC01 and RCC13 (data not shown).

For RCC13, three new ligands were identified which could have been used for vaccination: The ligand from met proto-oncogene was found in both tumor samples analyzed, whereas the gene was only overexpressed in RCC13 (Figure 2.1.2 A). Thus, this gene is an interesting vaccine candidate for RCC13, especially because of its oncogenic property. Expression analysis in healthy organs and tissues showed the highest level in normal kidney followed by brain and trachea (data not shown). Nicotinamide N-methyltransferase (NNMT) was exclusively expressed in RCC13 compared to the corresponding normal tissue and expression in other tissues was limited to liver, which still expressed lower amounts compared to the tumor (data not shown). It is highly probable that the frameshift-derived ligand was presented exclusively on tumor cells and for this reason should be included in the vaccine.

We identified ligands from keratin 18 and from vimentin, (ALLNIKVKL) and (EEIAFLKKL), respectively. Both genes were overexpressed in RCC13 but not in

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[1] Internet address: http://www.ncbi.nlm.nih.gov/SAGE

RCC01. Keratin 18 has been shown to be overexpressed in carcinomas, and the coexpression of keratin 18 and 8 together with vimentin augments tumor cell motility [35, 36]. But because of the rather high expression of keratin 18 in colon, we would not recommend the keratin 18-derived ligand for vaccination. We also found MHC class I-ligands from 3 members of the annexin gene family, (TIIDILTKR) derived from annexin I, (TIVNILTNR) from annexin II and (DEVKFLTV) from annexin IV. Annexin II-specific CD4$^+$ T cells were reported to specifically recognize melanoma cells overexpressing annexin II [29], but we did not find overexpression in RCC01 from which this ligand has been eluted. In RCC01, annexin IV was overexpressed and a ligand from this protein was eluted. But since annexin IV is mainly expressed in all epithelial cells [37], this ligand is excluded from the proposed vaccine. All other new ligands derived from overexpressed genes and not discussed in detail (SEC14L1, BTG1, ALDOA, CTNNA1, PSMB9, IFI30, HLA-E) were not considered to be suitable for vaccination because they either displayed a broad tissue expression pattern or the level of overexpression was too low.

As a second source of vaccine components, known epitopes from described TAA can be included without hesitation in the vaccine provided that they match the patient's HLA type and the TAA are expressed in the individual tumor (Table 2.1.3 and Table 2.1.4). This can be checked by referring to the expression data. Thus, in our microarray data we looked for expression of known TAA such as NY-ESO-1, LAGE-1, Hom-Mel-40, SSX2, SSX4, CT7, MAGE-1, SCP1, MUC-1, RAGE, GAGE, survivin, hTERT, RU1, Her-2/neu, CEA, WT1, SART3, gp75, PRAME, and CA9. In RCC01, CA9 was expressed exclusively in the tumor (Table 2.1.1); the vaccine for this particular patient could thus contain the CA9-derived epitope (HLSTAFARV) [25]. Similarly, the two known epitopes of cyclin D1 [38] could also be applied. These three epitopes would have been relevant for RCC13 as well. In addition, the exclusive expression of PRAME in RCC13 became clear. Thus, the four epitopes of PRAME restricted to HLA-A*02 [39] and the HLA-A*24-restricted epitope [40] can be classed as suitable for this patient. Finally, our new HLA-A*02 restricted ligands from adipophilin and from KIAA0367 eluted from RCC01 should be included in the selection because of the overexpression of these genes in RCC13.

In addition to identified HLA ligands from overexpressed gene products, epitope prediction[1] for the relevant HLA alleles combined with proteasomal cleavage prediction[2] [41] can be applied to overexpressed genes (Figure 2.1.1). This provides more potential candidates for vaccination, which should be screened for presentation on the solid tumor as carried out for selected peptides in the tumor RCC13 by online LC-MS. The combination of expression profiling and epitope prediction has been suggested [42, 43] and reported before, but only one target gene alone has been used to predict mouse epitopes, and neither the presentation of the predicted epitopes on the cell surface nor the type of anti-tumor activity has been shown [16].

There is increasing evidence that the peptide pool generated by immunoproteasome differs from that produced by the standard proteasome [44-46]. As we could show upregulation of immunoproteasome subunits in tumor cells, one might expect differences among the MHC-ligands presented on tumor versus normal cells.

All of our identified ligands except for the frameshift-derived peptide could be self peptides and as such also be presented on the surface of normal cells. If specific T cells against these peptides exist, they might induce autoimmunity once activated by vaccination. In all human vaccination studies published so far, no indication of autoimmunity has been reported. For example, TAAs such as carcinoembryonic antigen (CEA) or Her2/neu, which are broadly expressed by epithelial cells, are being targeted without any sign of autoimmunity in ongoing clinical trials. One exception might be T cell-mediated vitiligo after antigen-specific therapy of melanoma [47]. However, even in cases in which either whole tumor lysate was loaded onto DC for vaccination or autologous cancer-derived heat shock proteins were used, no evidence for autoreactivity towards normal tissue was observed [2, 48]. Therefore, we believe that the risk of inducing autoimmunity using our vaccine candidates is no greater than in these previous instances.

Being aware of the fact that self-protein derived peptides might be subject to T-cell tolerance, we selected 4 of our interesting peptides to be screened for specific peripheral CD8^{+} T cells in patients and healthy donors: only in the case of keratin

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[1] Internet address: http://www.syfpeithi.de

[2] Internet address: http://www.paproc.de

18 was a significant tetramer-positive population seen in 4/22 healthy donors tested. It is likely that such T cells can also be found in cancer patients, and more renal cell carcinoma patients must be screened to evaluate the frequency of this keratin 18-specific CD8$^+$ population. It is not clear if these T cells specific for a self antigen are tolerized *in vivo*, and experiments are being carried out to characterize them functionally. For this reason, and considering the high expression of keratin 18 in normal colon, at the present time this antigen does not constitute a first choice candidate target for immunotherapy. For those antigens which are shared among tumors from several patients, their immunogenicity should be tested, for example in classical *in vitro* priming experiments [9, 25]. Using tetramer staining or qPCR for IFN-$\gamma$, we could generally detect no spontaneous expansion of specific T cells against the peptides tested after one *in vitro* stimulation, suggesting either that these T cells are not activated *in vivo*, or that their frequency in the blood is too low to be detected in our assays. Here again, the number of RCC patients screened must be increased. However, we do not believe that the spontaneous *in vivo* response to these peptides in cancer patients is a prerequisite for their use as a vaccine. Indeed, previous research has shown that induction of tumor peptide-specific CD8$^+$ T cells (not detectable before vaccination) can correlate with clinical benefit [5, 49, 50]. Using qPCR, we also observed the induction of MUC1-specific CD8$^+$ T cells in a vaccinated patient simultaneously to metastatic regression (C.G. unpublished data, and [5]).

Moreover, most immunization protocols tested until now have used antigens defined by spontaneous immune responses in patients. One could argue that such spontaneous T or B cell responses, in most cases, are not efficient enough to destroy the tumor. We hypothesize that redirecting immunity to tumor-associated structures not obviously attacked might result in more efficient responses.

The combination of gene expression analysis and MHC ligand identification allows the selection of several peptide candidates for immunotherapy. This can be performed in an appropriate timeframe after surgical intervention to allow rapid subsequent vaccination. Analysis of the T-cell response before and during vaccination, together with clinical monitoring, will help to determine those peptides which induce a beneficial immune response for the patient. Thus, we have demonstrated that it is possible to extract information from a single excised tumor

specimen that leads to an optimized design of a multi-epitope, peptide-based vaccine directed against the tumor of an individual patient and considering all expressed HLA-alleles.

## 2.1.6  References

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## 2.1.7  Abbreviations

CTL          cytotoxic T lymphocyte

TAA          tumor-associated antigen

MS           mass-spectrometry

LC-MS        liquid chromatography mass-spectrometry

RCC          renal cell carcinoma

qPCR         quantitative PCR

mAb          monoclonal antibody

LCM          laser capture microdissection

HD           healthy donor

PBMC peripheral blood mononuclear cells

## 2.1.8  Participating Researchers

Toni Weinschenk, Cécile Gouttefangeas, Markus Schirle, Florian Obermayr, Steffen Walter, Oliver Schoor, Raffael Kurek, Wolfgang Loeser, Karl-Horst Bichler, Dorothee Wernet, Stefan Stevanović  & Hans-Georg Rammensee

Department of Immunology, Institute for Cell Biology [C.G., M.S., F.O., S.W., O.S., S.S., HG.R.] and Department of Obstetrics and Gynecology [R.K.], and Department of Urology [W.L., KH.B.], and Department of Transfusion Medicine [D.W.], University of Tübingen, D-72076 Tübingen, Germany

## 2.1.9  Acknowledgments

## 2.2    Identification of c-MET oncogene as a broadly expressed tumor associated antigen recognized by cytotoxic T-lymphocytes

The author of this dissertation, Toni Weinschenk, identified the MHC ligand analyzed in this chapter.

### 2.2.1  Summary

C-Met protooncogene is a transmembrane heterodimeric receptor tyrosine kinase that mediates the potentially oncogenic activities of the hepatocyte growth factor/scatter factor. In chapter 2.1, identification of an MHC class I-ligand restricted to HLA-A*02 and encoded by c-Met protooncogene was shown. We used this novel HLA-A*02 binding peptide for the induction of c-Met specific cytotoxic T-lymphocytes (CTL) *in vitro* in order to analyze the presentation of this epitope by human tumor cells. After several restimulations the *in vitro* induced CTL efficiently lysed target cells pulsed with the cognate peptide while they spared cells presenting an irrelevant peptide. Furthermore, c-Met specific CTL recognized allogeneic HLA matched tumor cell lines including melanoma, breast, colon and renal cell carcinomas as well as multiple myeloma cells in an antigen specific and HLA-restricted manner. To further analyze the effector function of the generated CTL in an autologous system we used DC from the same donor as target cells in a $^{51}$Cr-release assay. The *in vitro* induced c-Met peptide specific CTL lysed autologous DC pulsed with the antigenic peptide or transfected with whole tumor mRNA purified from a c-Met expressing cell lines. In our study we demonstrate that c-Met oncogene is a tumor rejection antigen recognized by CTL and expressed on a broad variety of epithelial and hematopoetic malignancies.

## 2.2.2  Introduction

C-Met encodes a heterodimeric transmembranous receptor with tyrosine kinase activity that is composed of an $\alpha$-chain that is disulfide-linked to a $\beta$ subunit [1, 2]. Both subunits are expressed on the surface, the heavy $\beta$ subunit is responsible for the binding of the ligand, hepatocyte growth factor (HGF), the $\alpha$ subunit contains an intracellular domain that mediates the activation of different signal transduction pathways. Met signaling is involved in organ regeneration, as demonstrated for liver and kidney, embryogenesis, hematopoesis, muscle development, and in the regulation of migration and adhesion of normally activated B-cells and monocytes. Furthermore, numerous studies indicated the involvement of c-Met overexpression in malignant transformation and invasiveness of malignant cells [3-12].

Using an integrated functional genomics approach that combines gene expression profiling with analysis of MHC ligands by mass spectrometry to identify genes and corresponding MHC ligands that are selectively expressed or overexpressed in malignant tissues a HLA-A*02 binding peptide derived from the c-Met protooncogene could be identified [13, see chapter 2.1]. In our study we analyzed the possible function of this peptide as a T-cell epitope and its presentation by malignant cells using antigen specific cytotoxic T-lymphocytes (CTL) that were generated by *in vitro* priming with monocyte derived dendritic cells (DC) as antigen presenting cells. We show here that the CTL generated from several healthy donors elicited an antigen specific and HLA-A*02 restricted cytolytic activity against tumor cells endogenously expressing the c-Met protein including renal cell carcinomas, breast cancer, colon cancer, melanoma and multiple myeloma cells. Furthermore, they lysed autologous DC pulsed with the antigenic peptide or electroporated with RNA isolated from c-Met expressing tumor cell lines indicating that this peptide is also expressed upon transfection of DC. Our results demonstrate that c-Met oncogene is a tumor rejection antigen recognized by CTL and expressed on a broad variety of epithelial and hematopoetic malignancies.

## 2.2.3 Materials and Methods

### 2.2.3.1 Tumor cell lines

Tumor cell lines used in the experiments were grown in RP10 medium (RPMI 1640 supplemented with 10% heat inactivated FCS and antibiotics). The following c-Met expressing tumor cell lines were used in experiments: MCF-7 (breast cancer, HLA-A*02+, purchased from ATCC), A 498 (RCC, HLA-A*02+) and MZ 1257 (RCC, HLA-A*02+, kindly provided by Prof. A. Knuth, Frankfurt, Germany), U 266 (multiple myeloma, HLA-A*02+), HCT 116 (colon cancer, HLA-A*02+), Mel 1479 (malignant melanoma, HLA-A*02+, kindly provided by Prof. G. Pawelec, Tübingen, Germany), SK-OV-3 (ovarian cell line, HLA-A*03+, kindly provided by O. J. Finn, Pittsburgh, USA). The c-Met negative cell lines T2 (HLA-A*02+, TAP-deficient) and Croft (EBV-immortalized B-cell line, kindly donated by O. J. Finn, Pittsburgh, USA, HLA-A*02+) were used as controls. K 562 cells were utilized to determine the NK-cell activity.

### 2.2.3.2 Cell isolation and generation of dendritic cells (DC) from adherent peripheral blood mononuclear cells

Generation of DC from peripheral blood monocytes was performed as described previously [14]. In brief, peripheral blood mononuclear cells (PBMNC) were isolated by Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of heparinized blood obtained from buffy coat preparations of healthy volunteers from the blood bank of the University of Tübingen. Cells were seeded ($1 \times 10^7$ cells/3 ml per well) into 6 well plates (Falcon, Heidelberg, Germany) in RP10 media. After 2 h of incubation at 37°C, nonadherent cells were removed and the adherent blood monocytes were cultured in RP10 medium supplemented with the following cytokines: human recombinant GM-CSF (Leukomax, Novartis, 100 ng/ml), IL-4 (R&D Systems, Wiesbaden, Germany, 1000 IU/ml), and TNF-$\alpha$ (R&D Systems, Wiesbaden, Germany, 10 ng/ml).

### 2.2.3.3 RT-PCR

RT-PCR was performed with some modifications as recently described [14]. Total RNA was isolated from cell lysates using QIAGEN RNeasy "Mini" anion-exchange spin columns (QIAGEN GmbH, Hilden, Germany) according to the instructions of the manufacturer and was subjected to a 20 µl cDNA synthesis reaction (Invitrogen, Karlsruhe, Germany). Oligo(dT) was used as primer. 1 µl of cDNA was used for PCR amplification. To control the integrity of the RNA and the efficiency of the cDNA synthesis, 1 µl of cDNA was amplified by an intron-spanning primer pair for the ß2-microglobulin gene. The PCR temperature profiles were as follows: 2 minutes pre-treatment at 94°C and 30 cycles at 94°C for 30 seconds, annealing at 59°C for 30 seconds and 72°C for 60 seconds for the c-Met and ß2-microglobulin cDNA. Primer sequences were deduced from published cDNA sequences: ß2-microglobulin: 5' GGGTTTCATCCATCCGACAT 3' and 5' GATGCTGCTTACATGTCTCGA 3', c-Met: 5' AGTCAAGGTTGCTGATTTTGGT 3' and 5' ATGTGCTCCCCAATGAAAGTAGA 3'. 10 µl of the RT-PCR reactions were electrophoresed through a 2.5% agarose gel and stained with ethidium bromide for visualization under UV light.

### 2.2.3.4 Induction of antigen specific CTL response using HLA-A*02 restricted synthetic peptides

The HLA-A*02 binding peptides derived from c-Met (YVDPVITSI, amino acids 654-662) [13], survivin (ELTLGEFLKL) [15], and HIV (ILKEPVHGV, pol HIV-1 reverse transcriptase peptide, amino acids 476-484) were synthesized using standard F-moc chemistry on a peptide synthesizer (432A, Applied Biosystems, Weiterstadt, Germany) and analyzed by reversed-phase HPLC and mass spectrometry.

For CTL induction, $5 \times 10^5$ DC were pulsed with 50 µg/ml of the synthetic c-Met peptide for 2 h, washed, and incubated with $2,5 \times 10^6$ autologous PBMNC in RP10 medium. After 7 days of culture, cells were restimulated with autologous peptide pulsed PBMNC and 1 ng/ml human recombinant IL-2 (R&D Systems) was added on days 1, 3 and 5. The cytolytic activity of induced CTL was analyzed on day 5 after the last restimulation in a standard ^{51}Cr-release assay.

### 2.2.3.5  CTL assay

The standard $^{51}$Cr-release assay was performed as described [14]. Target cells were pulsed with 50 μg/ml peptide for 2 h and labelled with [$^{51}$Cr]-sodium chromate in RP10 for 1 h at 37°C. $10^4$ cells were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTL were added to give a final volume of 200 μl and incubated for 4 h at 37° C. At the end of the assay supernatants (50 μl/well) were harvested and counted in a beta-plate counter. The percent specific lysis was calculated as: 100 x (experimental release - spontaneous release / maximal release-spontaneous release). Spontaneous and maximal release were determined in the presence of either medium or 2% Triton X-100, respectively.

Antigen specificity of tumor cell lysis was further determined in a cold target inhibition assay [14] by analyzing the capacity of peptide pulsed unlabeled T2 cells to block lysis of tumor cells at a ratio of 20:1 (inhibitor to target ratio).

### 2.2.3.6  PAGE and Western Blotting

Protein concentration of cell lysates were determined using a BCA assay (Pierce, Rockford, IL). 30 μg of total protein were separated on 7.5% polyacrylamide gel, blotted on nitrocellulose membrane, and probed with a c-Met specific polyclonal antibody (h-Met, clone 28, sc-161, polyclonal rabbit, Santa Cruz Biotechnology, Heidelberg, Germany). Bands were visualized by ECL staining (Amersham Pharmacia, Freiburg, Germany).

### 2.2.3.7  Electroporation of DC with whole tumor derived RNA

Total RNA was isolated from tumor cell lysates using RNeasy$^{TM}$ Maxi anion-exchange spin columns (Qiagen GmbH, Germany) according to the protocol for isolation of total RNA from animal cells provided by the manufacturer. Quantity and purity of RNA were determined by UV spectrophotometry. Prior to electroporation on day 6, immature DC (grown in RP10 medium in the presence of IL-4 and GM-CSF) were washed twice with serum-free X-VIVO 20 medium (BioWhittaker,Apen. Germany) and resuspended to a final concentration of 2 x $10^7$ cells/ml. Subsequently, 200 μl of the cell suspension were mixed with 10 μg of

total RNA and electroporated in a 4 mm cuvette using an Easyject Plus™ unit (Peqlab, Erlangen, Germany). The physical parameters were: voltage of 300 V, capacitance of 150 µF, resistance of 1540 $\Omega$ and pulse time of 231 ms [16]. After electroporation the cells were immediately transferred into RP10 medium and returned to the incubator.

## 2.2.4  Results

### 2.2.4.1 Induction of c-Met specific cytotoxic T-lymphocytes (CTL) using peptide pulsed DC

C-Met protooncogene was shown to be overexpressed in a variety of malignant cells of epithelial and hematopoetic origin and to be involved in the malignant phenotype and invasiveness of these cells [3, 17-19]. As demonstrated in Figure 2.2.1, expression of c-Met mRNA and protein could be detected in several tested human tumor cell lines including malignant melanoma, renal cell carcinoma (RCC) and multiple myeloma.
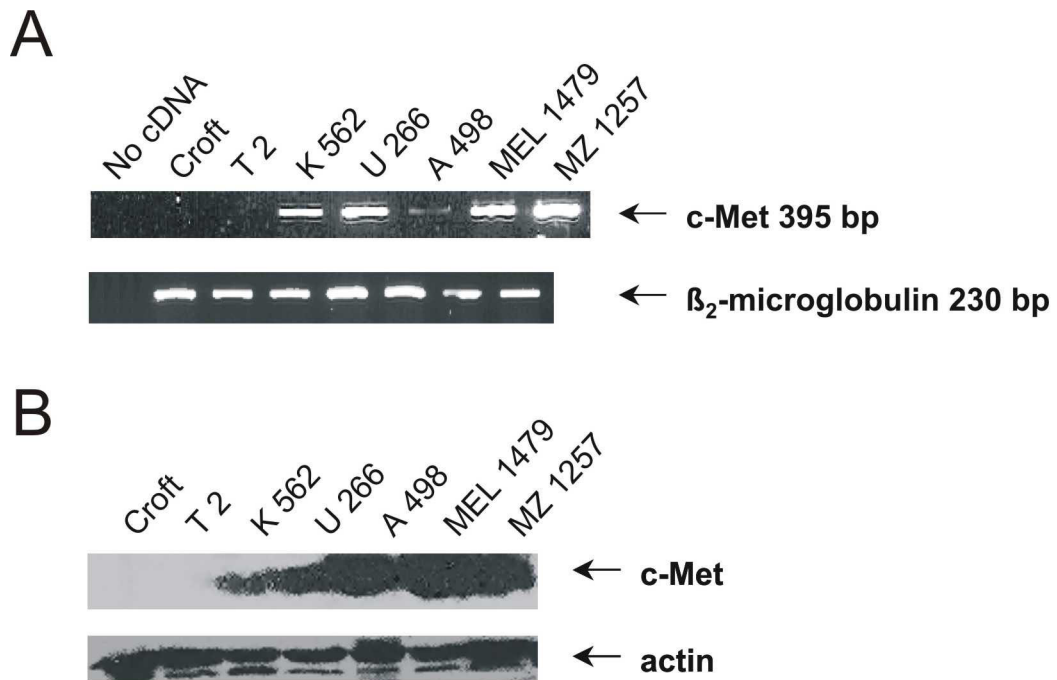
**Figure 2.2.1** *C-Met expression in human tumor cell lines and peripheral blood B- and T-lymphocytes.*

The expression of c-Met mRNA was analyzed in human tumor cell lines by RT-PCR (A). Total RNA was subjected to cDNA synthesis. PCR products were run on a 2.5 % agarose gel and visualized by ethidium bromide staining. Western blot analysis using a c-Met specific polyclonal antibody was performed to determine the protein expression (B).

Comparative analysis of gene expression profiling of a tumor sample of a patient with renal cell carcinoma and the corresponding autologous renal tissue using DNA microarray technology followed by the characterization of MHC ligands present in the tumor by mass spectrometry resulted in the identification of a HLA-A*02 binding peptide derived from the c-Met protein (YVDPVITSI, aminoacids 654-662) [13, see chapter 2.1].

To analyze the presentation of this epitope by tumor cells and its recognition by CTL we induced c-Met specific CTL *in vitro* using DC derived from adherent PBMNC of HLA-A*02 positive healthy donors. These monocyte derived DC were pulsed with the HLA-A*02 binding c-Met peptide and utilized as antigen presenting cells for *in vitro* priming. The cytotoxicity of the induced CTL was analyzed in a standard $^{51}$Cr-release assay using peptide loaded T2 cells and autologous DC as targets. The CTL line obtained after two weekly restimulations demonstrated antigen specific killing. The T-cells only recognized T2 cells or DC coated with the

cognate peptide while they did not lyse target cells pulsed with irrelevant HLA-A*02 binding peptides derived from survivin protein or HIV-1 reverse transcriptase confirming the specificity of the cytolytic activity (data not shown).

### 2.2.4.2 C-Met peptide specific CTL efficiently recognize tumor cells endogeneously expressing the c-Met protooncogene

We next analyzed the ability of the *in vitro* induced CTL to lyse tumor cells that express the c-Met protein. We used the HLA-A*02 positive cell lines HCT 116 (colon cancer), A498, MZ 1257 (renal cell carcinoma, RCC), MCF-7 (breast cancer), Mel 1479 (malignant melanoma) and U266 (multiple myeloma) that express c-Met as targets in a standard $^{51}$Cr-release assay. The EBV-transformed B-cell line Croft (HLA-A*02+/c-Met-) and the ovarian cancer cell line SK-OV-3 (HLA-A*03+/c-Met+) were included to determine the specificity and HLA-restriction of the CTL. As demonstrated in Figure 2.2.2 A-D, the c-Met peptide specific CTL were able to efficiently lyse malignant cells expressing both HLA-A*02 and c-Met. There was no recognition of the ovarian cancer cells SK-OV-3 or Croft cells demonstrating that the presentation of c-Met peptide in context of HLA-A*02 molecules on the tumor cells is required for the efficient lysis of target cells and confirm the antigen specificity and MHC restriction of the CTL. The *in vitro* induced T-cells did not recognize the K 562 cells indicating that the cytotoxic activity was not NK-cell mediated.
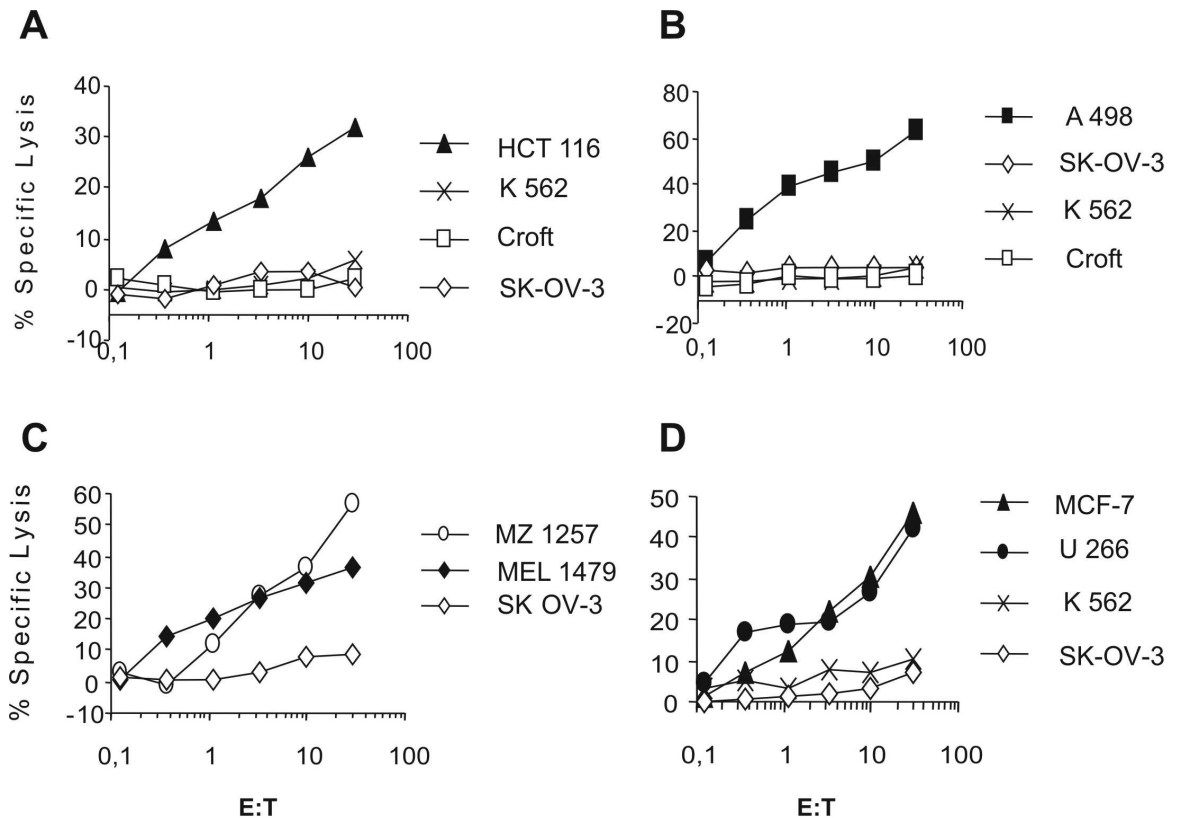
**Figure 2.2.2** *Antigen specific lysis of human tumor cell lines endogenously expressing c-Met by c-Met specific CTL.*

Human HLA-A*02+/c-Met+ colon cell carcinoma cells HCT 116, renal cell carcinoma (MZ 1257, A498), melanoma (Mel 1479), breast cancer cell line MCF-7, multiple myeloma (U266) cell line and the EBV-immortalized Croft cells (HLA-A*02+/c-Met-) as well as the ovarian cancer cell line SK-OV-3 (HLA-A*02-/c-Met+) were used as targets in a stardard $^{51}$Cr-release assay. K 562 cells were included to determine the NK-cell activity.

To further verify the antigen specificity and MHC restriction of the *in vitro* induced CTL lines we performed cold target inhibition assays. The lysis of the target cells (U 266 and A 498) could be blocked in cold target inhibition assays. The addition of cold (not labeled with $^{51}$Cr) T2-cells pulsed with the cognate peptide reduced the lysis of tumor cells whereas T2-cells pulsed with an irrelevant peptide showed no effect (data not shown).

### 2.2.4.3 C-Met specific CTL can lyse autologous DC transfected with whole tumor RNA

To analyze the cytotoxic activity of the CTL in an autologous setting and test the presentation of c-Met specific T-cell epitopes upon transfection of whole tumor

RNA we used autologous DC, generated from the same PBMNC that were utilized for CTL induction, as target cells. As shown in Figure 2.2.3, CTL efficiently lysed autologous DC electroporated with the whole tumor RNA isolated from the c-Met expressing A498 or MCF-7 tumor cell lines indicating that the identified c-Met peptide is processed and presented after transfection of DC with RNA derived from c-Met positive tumor cells.
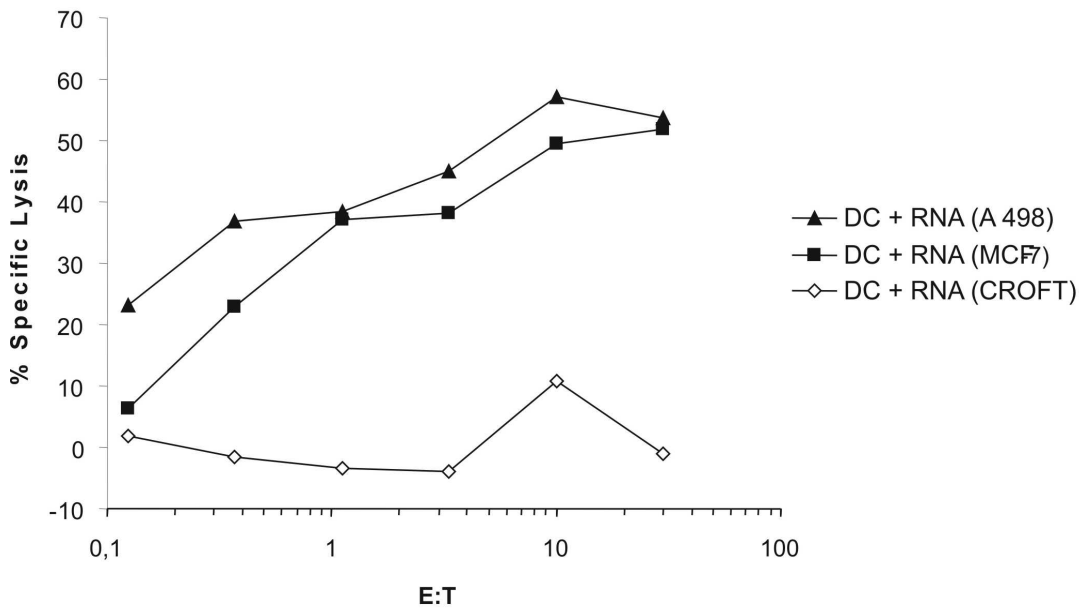


**Figure 2.2.3** *C-Met specific CTL recognize autologous dendritic cells transfected with the total RNA isolated from A 498 or MCF-7 tumor cells.*

Autologous DC from a healthy HLA-A*02+ donor generated from peripheral blood monocytes with GM-CSF and IL-4 were electroporated with the total tumor RNA isolated from the c-Met expressing MCF-7 or A498 cells were utilized as target cells in a standard $^{51}$Cr-release assay (closed symbols). DC electroporated with RNA derived from the c-Met negative Croft cell line were used as controls in the assay (open symbols).

## 2.2.5  Discussion

Therapeutic vaccinations of patients with malignant diseases aim at stimulation of anti-tumor directed immune responses, mainly cytotoxic T-lymphocytes, capable to recognize and eliminate malignant cells. During the last years considerable efforts have been made to identify antigens specifically recognized by CTL using reverse immunology, expression cloning or SEREX technology. However, with the exception of some tumor associated antigens most of the identified T-cell epitopes are restricted to a limited set of malignancies [20-22].

Comparative expression profiling of a tumor and its corresponding autologous healthy tissue by DNA microarray technology allows the identification of antigens selectively expressed or overexpressed in malignant cells making these proteins suitable targets for immunotherapeutic approaches. Combination of this genetic analysis with mass spectrometry enables the characterization of antigenic peptides encoded by these antigens. Using this integrated functional genomics approach a HLA-A*02 binding peptide derived from the c-Met protooncogene was identified [13].

C-Met is a heterodimeric tyrosine kinase receptor that mediates the multifunctional and potentially oncogenic activities of the hepatocyte growth factor/scatter factor including promotion of cell growth, motility, survival, extracellular matrix dissolution and angiogenesis [1-3]. Binding of HGF to the receptor induces autophosphorylation of c-Met and activates downstream signaling events including the ras, PI3 kinase, PLC-$\gamma$ and MAPK related pathways [4,5, 23-26]. The c-Met gene is predominantly expressed in epithelial cells and is overexpressed in several malignant tissues and cell lines [27-35]. An increasing number of reports have shown that nonepithelial cells such as hematopoetic, neural and skeletal cells respond to HGF and hematological malignancies like multiple myeloma, Hodgkin disease, leukemias and lymphomas express the c-Met protein [36-40]. Deregulated control of the invasive growth phenotype by oncogenically activated c-Met provoked by c-Met activating mutations, c-Met amplification/overexpression, and the acquisition of HGF/c-Met autocrine loops confers invasive and metastatic properties to malignant cells. Notably, constitutive activation of c-Met in HGF-overexpressing transgenic mice promotes broad tumorigenesis [41, 42]. Therefore, targeting of c-Met and/or c-Met oncogenic transduction pathways could represent a promising therapeutic option.

In our study we show that c-Met specific CTL recognizing tumor cells in an antigen specific and MHC restricted manner can be induced *in vitro* suggesting that c-Met protooncogene is a novel tumor rejection antigen. We used the previously identified c-Met derived HLA-A*02 binding peptide for CTL induction and were able to demonstrate that this c-Met epitope is expressed on a broad spectrum of

epithelial and hematological malignancies including renal cell carcinomas, breast cancer, colon cancer, malignant melanoma and multiple myeloma indicating that this c-Met peptide is an interesting candidate for the development of a broadly applicable vaccination therapy. The specificity of the elicited CTL responses was confirmed in cold target inhibition assays. Furthermore, we performed the experiments in an autologous setting and utilized autologous DC that were either pulsed with the cognate peptide or electroporated with the RNA isolated from c-Met expressing tumor cell lines as targets. The *in vitro* induced c-Met peptide specific CTL efficiently lysed peptide pulsed autologous DC and DC transfected with whole tumor RNA, thus demonstrating that the peptide used for CTL induction is also processed and presented upon transfection of DC with whole tumor RNA.

Previous studies have shown that under normal conditions c-Met gene can be found in many epithelial tissues and its expression can be induced by treatment with phorbol esters, serum, and in a paracrine fashion by mesenchymally derived HGF. HGF/SF-Met signaling is required for normal development of liver, skeletal muscle and placenta [36]. In the hematopoetic system, HGF is produced by stromal bone marrow cells and induces together with other cytokines and growth factors proliferation and differentiation of a subset of c-Met positive progenitor cells. The c-Met/HGF interaction plays an important role in the lymphoid microenvironment and induces adhesion as well as migration of normally activated B-cells and monocytes [3-12].

These observations indicate that the c-Met/HGF network promotes pleiotropic effects in normal cells and is not a cancer-specific antigen and caution is required when targeting this protein in clinical vaccination trials.

In several clinical vaccination trials using dendritic cells presenting tumor associated antigens or adoptive transfer of tumor reactive CTL generated ex vivo, it was shown that these approaches can induce antitumor immunity in patients with malignant diseases [21, 22, 43-47]. However, with the exception of some reports in malignant melanoma trials where induction of vitiligo was observed after vaccinations with DC even when antigens that are expressed in normal tissues like MUC1 or Her-2/neu were applied there has been so far no evidence for the

development of autoimmune reactions in these patients [48]. In conclusion, in our study we describe the identification of a novel broadly expressed T-cell epitope derived from a protooncogene that is an interesting candidate to be applied in immunotherapies of a broad variety of human malignancies.

## 2.2.6  References

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## 2.2.7  Abbreviations

| | |
|---|---|
| CTL | cytotoxic T lymphocyte |
| HLA | human leukocyte antigen |
| DC | dendritic cell |
| HGF | hepatocyte growth factor |
| GM-CSF | granulocyte-makrophage colony stimulating factor |
| IL | interleukin |
| TNF | tumor necrosis factor |
| RT-PCR | reverse-Transcriptase Polymerase chain reaction |
| PBMNC | peripheral blood mononuclear cells |
| PAGE | polyacrylamide gel electrophoresis |
| MHC | major histocompatibility complex |
| EBV | Epstein-Barr virus |
| NK-cell | natural killer cell |
| SEREX | serological identification of antigens by recombinant expression cloning |

## 2.2.8  Participating Researchers

These results are submitted for publication by

Kerstin Schag, Susanne M. Schmidt, Martin R. Müller, Toni Weinschenk[1], Silke Appel, Markus M. Weck, Frank Grünebach, Stefan Stevanović[1], Hans-Georg Rammensee[1], Peter Brossart.

University of Tübingen, Department of Hematology, Oncology and Immunology, and [1]Institute for Cell Biology, Department of Immunology, Tübingen, Germany.

# 2.3 Induction of adipophilin specific cytotoxic T lymphocytes using a novel HLA-A*02 binding peptide that mediates tumor cell lysis

The author of this dissertation, Toni Weinschenk, identified the MHC ligand this chapter is dealing with.

## 2.3.1 Summary

Identification of tumor associated antigens (TAA) and recent advances in tumor immunology resulted in the development of vaccination strategies to treat patients with malignant diseases. However, most of the so far identified T-cell epitopes are restricted to certain malignancies or HLA alleles. Using a novel approach that combines high-density oligonucleotide microarray analysis of tumor samples with isolation and sequencing of peptides from the surface of tumor cells, a peptide derived from the adipophilin protein was recently identified (see chapter 2.1). In the present study we sought to develop specific cytotoxic T-lymphocyte (CTL) responses directed against adipophilin using this novel HLA-A*0201 binding peptide. The *in vitro* induced CTL efficiently lysed cells pulsed with the adipophilin peptide and allogeneic HLA-matched tumor cell lines, including melanoma, breast, and renal cell carcinomas as well as multiple myeloma cells in an antigen specific and HLA-restricted manner. Finally, the *in vitro* induced CTL recognized autologous DC pulsed with the antigenic peptide or transfected with whole tumor mRNA purified from an adipophilin expressing tumor cell line. To further analyze the possible use of this peptide in immunotherapies of malignancies, we induced adipophilin specific CTL using PBMNC and DC from a patient with chronic lymphatic leukemia (CLL). The *in vitro* generated T-cells efficiently recognized autologous malignant CLL cells while they spared autologous purified non-malignant B-cells or DC. Our results demonstrate that this peptide might represent a novel important candidate for the development of cancer vaccines designed to target adipophilin derived epitopes in a wide range of malignancies.

## 2.3.2  Introduction

The discovery of tumor associated antigens (TAA) and recent advances in the identification of T-cell epitopes using expression cloning, SEREX technology or the "reverse immunology" approach opened new perspectives to treat malignant diseases. Several Phase I/II studies have demonstrated that cancer vaccines designed to target epitopes derived from these TAA by cytotoxic T-lymphocytes (CTL) can be successful in several types of malignancies such as renal cell carcinoma (RCC), melanoma, prostate and breast cancer or Non-Hodgkin lymphoma resulting in immunological and clinical responses. However, the application of these immunotherapeutical approaches is restricted by the limited number of known tumor antigens and epitopes, by patients HLA-type or availability of tumor tissue.

DNA microarray analysis of tumor samples in comparison to the corresponding non-malignant autologous tissue provides a promising new technology to identify large numbers of possible TAA. Using gene expression profiling with analysis of MHC ligands by mass spectrometry, an HLA-A*0201-presented peptide derived from the adipophilin protein was recently described (see chapter 2.1). In the same study it was demonstrated that in line with other previous reports, adipophilin, a protein involved in lipid homeostasis of adipocytes and macrophages, is overexpressed in renal cell carcinomas while only marginally found in non-malignant tissues.

We analyzed the possible function of this peptide as a T-cell epitope and its presentation by malignant cells using antigen specific cytotoxic T-lymphocytes (CTL) that were generated by *in vitro* priming with monocyte derived dendritic cells (DC) as antigen presenting cells. We show here that the CTL generated from several healthy donors elicited an antigen specific and HLA-A*0201 restricted cytolytic activity against tumor cells endogenously expressing the adipophilin protein including renal cell carcinomas, breast cancer, melanoma, multiple myeloma cells and primary autologous chronic lymphatic leukemia cells. Furthermore, they lysed autologous DC electroporated with RNA isolated from adipophilin expressing tumor cell lines. This indicates that this peptide is also presented upon transfection of DC. Our results demonstrate that adipophilin is a

tumor rejection antigen recognized by CTL and expressed on a broad variety of epithelial and hematopoetic malignancies.

### 2.3.3 Materials and Methods

#### 2.3.3.1 Tumor cell lines

Tumor cells were essentially the same as in chapter 2.2.3.1. MZ 1774 (kindly provided by Prof. A. Knuth, Frankfurt, Germany) was a renal cell carcinoma cell line which was positive for adipophilin and HLA-A*0201. B-cells from a patient with chronic lymphatic leukemia (CLL) were grown in RP10 medium for 24 hours before they were used as target cells in a standard $^{51}$Cr-release assay.

#### 2.3.3.2 Cell isolation and generation of dendritic cells (DC) from adherent peripheral blood mononuclear cells

All steps have already been described in chapter 2.2.3.2.

#### 2.3.3.3 RT-PCR

See chapter 2.2.3.3.

#### 2.3.3.4 Induction of antigen specific CTL response using HLA-A*02 restricted synthetic peptides.

The HLA-A*0201 binding peptides derived from adipophilin (SVASTITGV) and from survivin (ELTLGEFLKL) and pol HIV-1 reverse transcriptase (ILKEPVHGV), both used as irrelvant controls, were synthesized using standard F-moc chemistry on a peptide synthesizer (432A, Applied Biosystems, Weiterstadt, Germany) and analyzed by reversed-phase HPLC and mass spectrometry. For CTL induction, $5 \times 10^5$ DC were pulsed with 50 $\mu$g/ml synthetic adipophilin peptide for 2 h, washed, and incubated with $3 \times 10^6$ autologous PBMNC in RP10 medium. After 7 days of culture, cells were restimulated with autologous peptide pulsed PBMNC and 1 ng/ml human recombinant IL-2 (R&D Systems) was added on days 1, 3 and 5. The cytolytic activity of induced CTL was analyzed on day 5 after the last restimulation in a standard $^{51}$Cr-release assay.

**2.3.3.5  CTL assay**

This assay has been described in chapter 2.2.3.5. For antibody blocking experiments, cells were incubated for 30 minutes with 10 µg/ml of monoclonal antibody BB7.2 (IgG2b, kindly provided by Stefan Stefanović, Tübingen, Germany) recognizing HLA-A*02 or isotype control antibody (ChromPure Mouse IgG, Dianova, Hamburg, Germany, 0,2µg/ml) before seeding in 96-well plates.

**2.3.3.6  PAGE and Western Blotting**

PAGE and western blot was performed as already described in chapter 2.2.3.6. Briefly, 30 µg of total protein were separated on 12% polyacrylamide gel, blotted on nitrocellulose membrane (Schleicher & Schuell), and probed with an adipophilin specific antibody (Mouse monoclonal, AP 125, Hybridoma culture supernatant, Research Diagnostics, Flanders, USA).

**2.3.3.7  Electroporation of DC with EGFP *in vitro* transcript (IVT) or whole tumor derived RNA.**

EGFP-IVT was synthesized from the plasmid pSP64 Poly(A) EGFPII (generously provided by V.F.I. Van Tendeloo, Antwerp, Belgium). Total RNA was isolated from tumor cell lysates using QIAGEN RNeasy "Mini" anion-exchange spin columns (QIAGEN, Hilden, Germany) according to the protocol for isolation of total RNA from animal cells provided by the manufacturer. Quantity and purity of RNA was determined by UV spectrophotometry. RNA samples were routinely checked by formaldehyde/agarose gel electrophoresis for size and integrity and stored at -80°C in small aliquots.

Prior to electroporation on day 6, immature DC were washed twice with serum-free X-VIVO 20 medium (BioWhittaker, Walkersville, MD) and resuspended to a final concentration of $2 \times 10^7$ cells/ml. Subsequently, 200 µl of the cell suspension were mixed with 10 µg of total RNA and electroporated in a 4 mm cuvette using an Easyject Plus™ unit (Peqlab, Erlangen, Germany). The physical parameters were: voltage of 300 V, capacitance of 150 µF, resistance of 1540 W and pulse time of 231 ms. After electroporation the cells were immediately transferred into RP10

medium and returned to the incubator. More than 80% of the cells were still alive after electroporation.

## 2.3.4  Results

### 2.3.4.1 Generation of adipophilin specific cytotoxic T-lymphocytes (CTL) using peptide pulsed DC

As demonstrated in Figure 2.3.1, expression of adipophilin mRNA and protein could be detected in all tested human tumor cell lines.
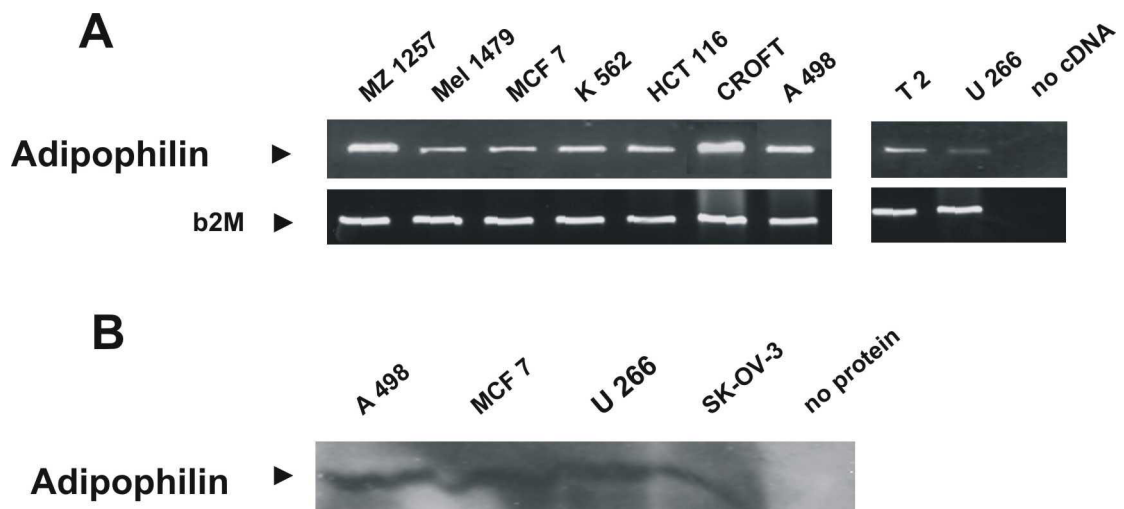


**Figure 2.3.1** *Expression of adipophilin in human tumor cell lines.*
RT-PCR (A) and Western blot analysis (B) using adipophilin specific primers and an adipophilin recognizing monoclonal antibody were performed to analyze the adipophilin expression.

To analyze the presentation of the adipophilin derived HLA-A*0201 restricted peptide by human tumor cells, we induced specific CTL *in vitro* using dendritic cells (DC) derived from adherent PBMNC of HLA-A*0201 positive healthy donors as antigen presenting cells. The cytotoxicity of the *in vitro* induced CTL was assessed after several restimulations in a standard $^{51}$Cr-release assay. As shown in Figure 2.3.2, the CTL line obtained after several weekly restimulations demonstrated peptide specific killing. T-cells only recognized T2-cells or autologous DC coated with the cognate adipophilin peptide while they did not lyse target cells pulsed with an irrelevant peptide confirming the specificity of the cytolytic activity.
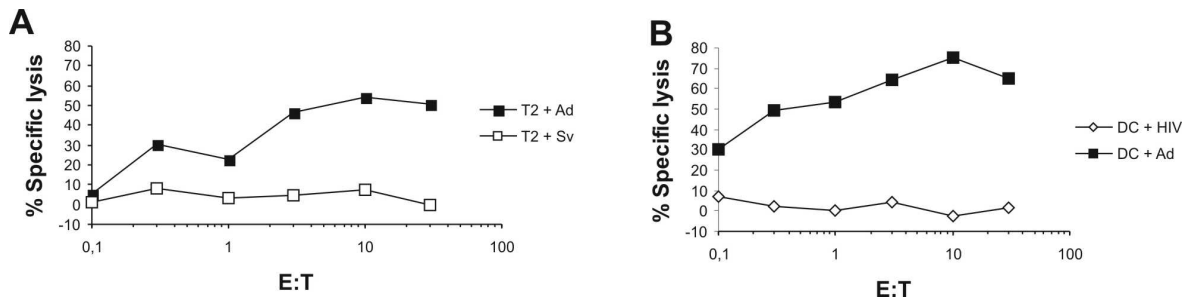
**Figure 2.3.2** *Induction of adipophilin specific CTL responses in vitro.*

DC generated from adherent PBMNC in the presence of GM-CSF, IL-4 and TNF-α were pulsed with the synthetic adipophilin peptide and used to generate a CTL response *in vitro*. Lytic activity of induced CTL was assessed in a standard $^{51}$Cr-release assay using T2-cells or autologous DC pulsed with the cognate peptide (Ad, closed symbols) or an irrelevant peptide (Sv, survivin derived, or HIV peptide, open symbols) as targets.

## 2.3.4.2 The identified adipophilin peptide is endogenously processed and presented in tumor cells and mediates tumor cell lysis

We next assessed presentation of the endogenously synthesized adipophilin peptide in the context of the HLA-A*0201 molecule. To this end, *in vitro* induced CTL specific for the adipophilin peptide were tested for their ability to lyse human tumor cells expressing adipophilin. The adipophilin positive and HLA-A*0201 expressing cell lines A498, MZ 1257 and MZ 1774 (renal cell carcinoma, RCC), MCF-7 (breast cancer), Mel 1479 (malignant melanoma) and U266 (multiple myeloma) were used as target cells in a standard $^{51}$Cr-release assay. As demonstrated in Figure 2.3.3 A-B the peptide induced adipophilin specific CTL were able to efficiently recognize malignant cells expressing adipophilin in the context of HLA-A*0201. There was no killing of the ovarian cancer cells SK-OV-3 (HLA-A*03+, adipophilin+) or the K562 cells indicating that the cytotoxic activity was not NK-cell mediated.
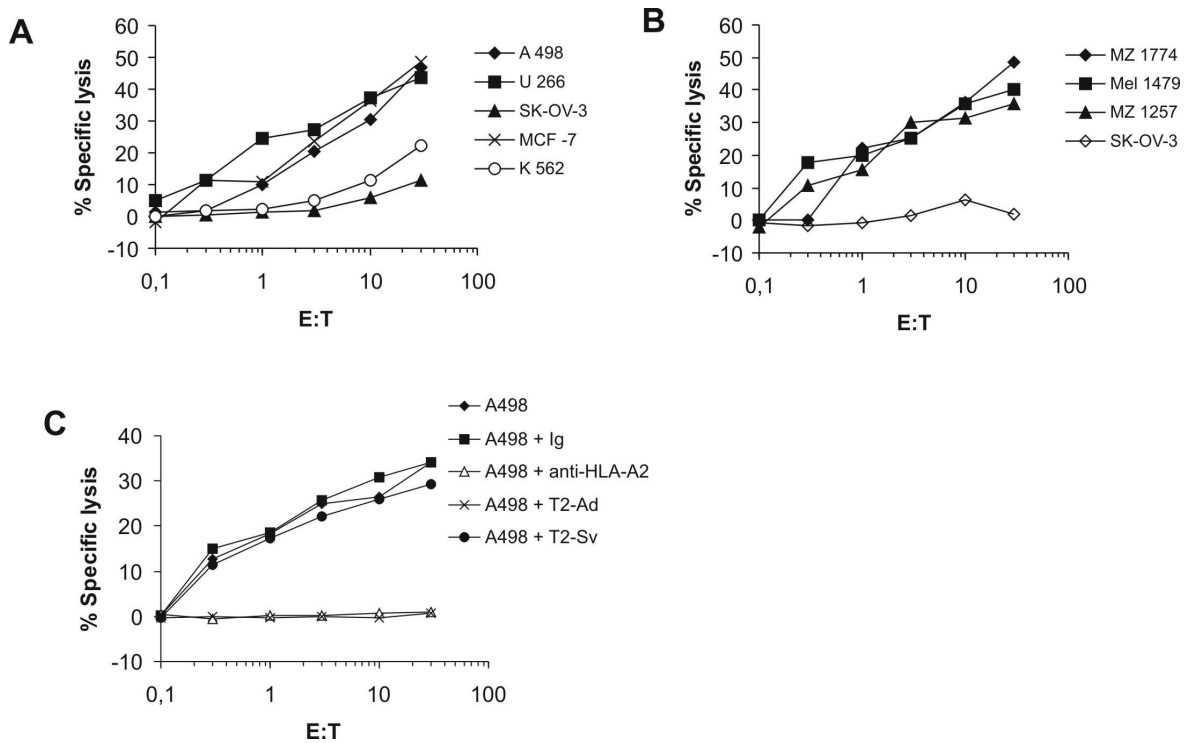
**Figure 2.3.3** *Adipophilin peptide induced CTL are able to lyse human tumor cell lines endogenously expressing the antigen.*

Human renal cell carcinoma (MZ 1774, MZ 1257, A498), malignant melanoma (Mel 1479), breast cancer carcinoma (MCF-7) and multiple myeloma (U 266) cell lines expressing adipophilin and HLA-A*0201 as well as the ovarian cancer cell line SK-OV-3 (HLA-A*02-/ adipophilin+) were used as targets in a stardard $^{51}$Cr-release assay (A-B). K 562 cells were included to determine the NK-cell activity. The antigen specific and HLA-A*02 restricted lysis of the A498 tumor cells by the CTL lines was confirmed using a blocking HLA-A*02 specific monoclonal antibody and in cold target inhibition assays (C) using unlabeled T2-cells pulsed with the cognate adipophilin (Ad) or the irrelevant survivin (Sv) peptide at an inhibitor:target ratio of 20:1.

The antigen specificity and MHC restriction of the cytotoxic activity mediated by the peptide induced CTL lines was further confirmed by using an HLA-A*02 specific monoclonal antibody and in cold target inhibition assays (Figure 2.3.3 C). The lysis of the A498 tumor cells could be blocked by incubation of target cells with the antibody specific for the HLA-A*02 molecule or the addition of cold (not labeled with $^{51}$Cr) T2-cells pulsed with the cognate peptide (Ad). T2 cells pulsed with an irrelevant survivin derived peptide showed no effect.

## 2.3.4.3 Adipophilin specific CTL can lyse autologous DC transfected with whole tumor RNA purified from an adipophilin positive A498 tumor cell line

Next, we analyzed the extent to which the CTL are able to recognize target cells in an autologous setting and investigated the presentation of the identified adipophilin peptide upon transfection of autologous HLA-A*0201+ DC with whole tumor RNA. As shown in Figure 2.3.4, CTL efficiently lysed autologous DC pulsed with the cognate adipophilin peptide or electroporated with the whole tumor RNA isolated from the survivin expressing A498 tumor cell line indicating that the adipophilin peptide is processed and presented after transfection of DC with RNA.
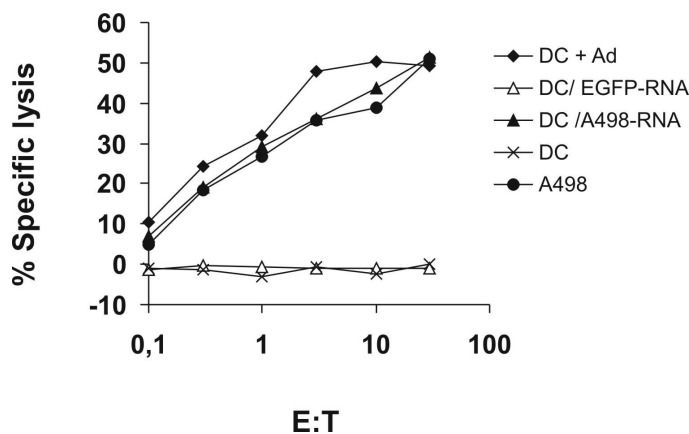


**Figure 2.3.4** *Peptide induced CTL recognize autologous dendritic cells (DC) transfected with the total RNA isolated from A498 tumor cells.*
Autologous DC from a healthy HLA-A*0201 donor generated from peripheral blood were pulsed with the cognate peptide (Ad) or electroporated with the total tumor RNA isolated from the adipophilin positive A498 tumor cell line. Then they were utilized as target cells in a stardard ^{51}Cr-release assay (closed symbols). DC electroporated with *in vitro* transcribed EGFP-RNA were used as controls in the assay (open symbols).

## 2.3.4.4  Induction of adipophilin specific CTL in a patient with CLL

Using RT-PCR analysis we found that primary malignant B-cells from a patient with chronic lymphocytic leukemia express adipophilin (data not schown). We therefore generated adipophilin specific CTL from PBMNC of a HLA-A*0201 positive patient with CLL who was in remission after treatment with fludarabine and utilized autologous primary CLL cells as targets in a standard ^{51}Cr-release assay.
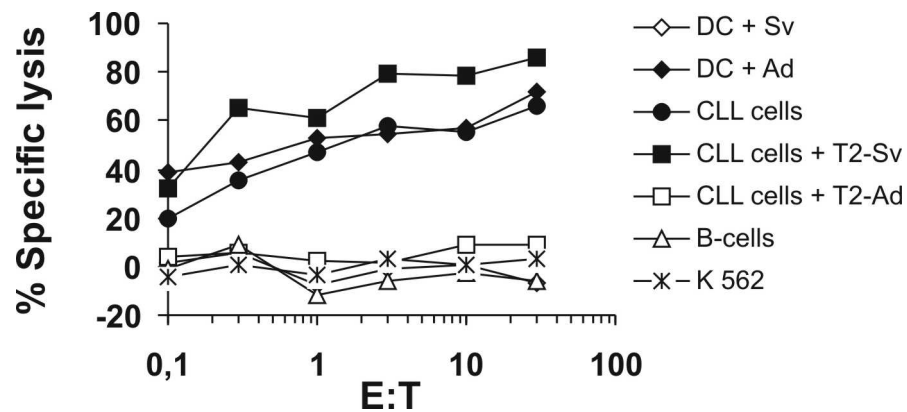
**Figure 2.3.5** *In vitro generated adipophilin specific CTL recognize autologous malignant chronic lymphocytic leukemia cells.*

Malignant autologous CLL cells from a HLA-A*0201+ patient and autologous DC pulsed with the cognate adipophilin peptide (Ad) or an irrelevant survivin peptide (Sv) were used as targets. Autologous non-malignant B-cells and the K 562 cells were included as controls. The antigen specificity of the CTL lines was analyzed in cold target inhibition assays using unlabeled T2-cells pulsed with the adipophilin (Ad) peptide or an irrelevant survivin (Sv) at an inhibitor:target ratio of 20:1.

As demonstrated in Figure 2.3.5, the *in vitro* peptide induced CTL efficiently lysed autologous DC from this patient that were pulsed with the cognate peptide as well as the autologous CLL cells while they spared the non-malignant B-cells or the K562 cell line. The specificity of the mediated cytotoxic T-cell responses was further confirmed in a cold target inhibition assay using T2-cells pulsed with the cognate or an irrelevant peptide.

## 2.3.5  Discussion

Adipophilin, officially called adipose differentiation-related protein, was originally thought to be selectively expressed only in adipocytes, but it can be found at the surface of lipid droplets in several other cell types such as monocytes and macrophages or human tumor cells. Comparative expression analysis of normal kidney and renal cell carcinomas (RCC) by the differential display approach, quantitative RT-PCR or DNA microarray technology revealed that adipophilin is highly overexpressed in malignant RCC samples. However, only low levels of adipophilin expression were detected in normal tissues, therefore peptides from

this protein might be interesting candidates as components of cancer vaccines aimed to target this antigen.

Recently, an HLA-A*0201 binding peptide derived from the adipophilin protein was identified in tumors of RCC patients by applying an integrated functional genomics approach (see chapter 2.1). This procedure combines comparative expression profiling of a tumor sample with the corresponding autologous normal tissue using high-density oligonucleotide microarray technology to find genes overexpressed in the malignant cells with mass spectrometry to identify MHC class I ligands derived from these selectively expressed or overexpressed antigens.

In our study we analyzed the possible use of this peptide as a T-cell epitope that can induce antigen specific CTL and mediate tumor cell lysis. To accomplish this, we used PBMNC from HLA-A*0201 positive donors in an *in vitro* immunization protocol. CTL induction was carried out by using monocyte derived DC pulsed with the identified adipophilin peptide as antigen presenting cells. After several rounds of restimulations, cultures were tested for their lytic activity against target cells pulsed with the cognate peptide or human tumor cell lines. Using RT-PCR and western blot analysis expression of adipophilin was found in different human tumor cell lines, indicating that this protein is expressed in a broad variety of human malignancies. The *in vitro* induced peptide specific CTL were able not only to lyse target cells pulsed with the antigenic peptide but also recognized tumor cells endogenously expressing the adipophilin protein in an antigen specific and HLA-A*02 restricted manner including RCC, malignant melanoma, breast cancer and multiple myeloma cells. The specificity of the lytic activity was confirmed by the addition of monoclonal antibodies blocking the HLA-A*02 molecules to the assay or by performing cold target inhibition assays.

To further analyze the specificity of the elicited CTL responses we utilized autologous DC that were either pulsed with the antigenic peptide or electroporated with the RNA isolated from an adipophilin expressing tumor cell line. The *in vitro* generated CTL efficiently lysed peptide pulsed autologous DC and DC transfected with whole tumor RNA, demonstrating that the peptide used for CTL induction is also processed and presented upon transfection of DC with whole tumor RNA.

Finally, we tested the ability of the identified adipophilin peptide to elicit CTL responses in patients with malignant diseases. CTL lines were generated from the

PBMNC of an HLA-A*0201 positive patient with chronic lymphatic leukemia by a first round of *in vitro* immunization with peptide pulsed autologous DC followed by two rounds of restimulations. The peptide specific CTL were used as effectors against the malignant CLL cells. The expression of adipophilin in malignant B-cells that represented more than 90% of the peripheral blood lymphocytes and were cryopreserved before treatment with fludarabine was confirmed by RT-PCR (data not shown). The *in vitro* induced CTL efficiently lysed in an MHC restricted and antigen specific manner the malignant leukemia cells but spared purified B-cells or autologous DC from this patient thus demonstrating that adipophilin specific CTL are able to recognize primary autologous tumor cells.

In conclusion, we have characterized a novel HLA-A*0201 restricted T-cell epitope derived from adipophilin, a protein involved in accumulation and storage of lipid. Adipophilin occurs in a wide range of cultured tumor cell lines, is overexpressed in some malignant tissues such as RCC while only marginally expressed in non malignant organs. Our results demonstrate that adipophilin is a tumor rejection antigen expressed in a wide range of solid and hematopoetic malignancies and extend the number of possible CTL epitopes that can be used for the design of cancer vaccines.

## 2.3.6  Abbreviations

| | |
|---|---|
| TAA | tumor associated antigens |
| CTL | cytotoxic T-lymphocyte |
| DC | Dendritic cell |
| HLA | Human leukocyte antigen |
| CLL | chronic lymphatic leukemia |
| SEREX | serological identification of antigens by recombinant expression cloning |

## 2.3.7  Participating Researchers

These results are submitted for publication by

Susanne M. Schmidt, Kerstin Schag, Martin R. Müller, Toni Weinschenk[1], Silke Appel, Markus M. Weck, Frank Grünebach, Lothar Kanz, Stefan Stevanović[1], Hans-Georg Rammensee[1], Peter Brossart.

University of Tübingen, Department of Hematology, Oncology and Immunology, and [1]Institute for Cell Biology, Department of Immunology, Tübingen, Germany.

## 2.4 RCC frequently overexpress genes involved in the presentation of MHC ligands

Reports about tumor escape mechanisms often include down-regulation of MHC class I-molecules or genes involved in antigen processing. Using the oligonucleotide microarray data generated for some renal cell carcinomas and few colon carcinomas, we analyzed the expression of genes involved in antigen processing and MHC class I-ligand presentation (Table 2.4.1). In five of six renal cell carcinomas, we found increased expression of MHC class I α-chain together with stronger expression of β2m. This is in accordance with the observation that also many other genes known to be induced by γ-IFN were upregulated. Interestingly, some of the RCCs might also express MHC class II-molecules. It was known before that melanomas in some cases do express MHC class II-molecules. Immunoproteasomal subunits LMP2 and LMP7 were frequently upregulated as already shown even in samples collected by laser capture microdissection (Figure 2.1.2), whereas the constitutive subunits were either unchanged, decreased or only in two cases increased. TAP1 was increased in all cases but two.

**Table 2.4.1 *Expression of genes involved in MHC ligand presentation*[1]**

| | RCC[2] 01 | RCC 20 | RCC 13 | RCC 19 | RCC 40 | RCC 44 | CCA 130 LCM | CCA 132 bulk | CCA 132 LCM |
|---|---|---|---|---|---|---|---|---|---|
| MHC class I | I | I | I | NC/D | I | I | NC | D | D |
| MHC class II | NC | I | I | I | | NC | | NC | NC |
| B2M | I | I | I | D | I | I | D | NC | D |
| LMP2 | I | I | I | NC | I | D | I | I | D |
| LMP7 | I | I | I | NC | I | I | I | MD | D |
| MECL-1 | NC | I | I | NC | I | NC | D | NC | D |
| Delta | NC | NC | D | NC | I | D | I | I | I |
| MB1 | NC | NC | NC | I | I | D | I | I | I |
| Z | NC | NC | D | I | NC | D | I | I | I |
| TAP1 | I | I | I | NC | I | D | I | I | MD |
| TAP2 | NC | NC | NC | NC | NC | NC | NC | NC | NC |

[1] Expression in tumor compared to autologous normal tissue: I increased, D decreased, NC no change

[2] RCC renal cell carcinoma, CCA colon carcinoma, LCM laser capture microdissection

Only two colon carcinomas were analyzed, one of them by using LCM samples. No MHC class II-expression was detected and class I was either not changed or decreased, even in the LCM samples. LMPs and TAP were increased in CCA130 but decreased in CCA132. LMP2, which finally was found to be decreased in CCA132, was considered to be increased, when bulk tissue was analyzed. In comparison to renal cell carcinoma, where the cell populations in bulk tissue samples are quite homogenous, it is important to use purified cell populations in colon carcinoma. The expression levels of genes involved in MHC class I-presentation reflect the probability to isolate reasonable amounts of peptides, which is higher in RCC compared to CCA.

# 2.5  Integrated functional genomics approach: renal cell carcinoma RCC44

## 2.5.1  Materials and Methods

Please refer to chapter 2.1.3.

## 2.5.2  Results

Patient RCC44 was typed positive for HLA-A*11, B*2705, B*40, and HLA-C*0202 by cloning and sequencing the α-chains. Peptides were eluted as described in 2.1.3.2, separated off-line and analyzed by nanoESI mass spectrometry. Table 2.5.1 shows the identified peptides together with the relative expression differences between the tumor and the corresponding autologous normal kidney tissue analyzed by a high-density oligonucleotide chip U133A from Affymetrix (see chapter 2.1.3.5).

Some of the identified MHC class I-ligands were interesting because of the strong overexpression of the respective genes: (SSLPTQLFK) from insulin-like growth factor 1 (IGF1), (FRFENVNGY) from asparagine synthetase (ASNS), (QRYGFSAVGF) from Rh type C glycoprotein (RHCG), (ARLSLTYERL) from ATPase lysosomal interacting protein 1 (ATP6IP1), and (GRYQVSWSL) from signal sequence receptor delta (SSR4).

**Table 2.5.1** *MHC class I-ligands from RCC44*

| Sequence[a] | Gene Symbol[b] | Gene Name[b] | LocusID[c] | Fold Change[d] |
|---|---|---|---|---|
| **HLA-A*11** | | | | |
| | | | | |
| **SSLPTQLFK** | IGF1 | insulin-like growth factor 1 (somatomedin C) | 3479 | **32.0** |
| **ATFPDTLTY** | ITGA6 | integrin, alpha 6 | 3655 | **1.6** |
| SIFDGRVVAK | LOC54499 | putative membrane protein | 54499 | nc |
| | | | | |
| **HLA-B*27** | | | | |
| | | | | |
| **FRFENVNGY** | ASNS | asparagine synthetase | 440 | **17.1** |
| **QRYGFSAVGF** | RHCG | Rh type C glycoprotein | 51458 | **9.0** |
| **ARLSLTYERL** | ATP6IP1 | ATPase, H+ transporting, lysosomal interacting protein 1 | 537 | **8.6** |
| **GRYQVSWSL** | SSR4 | signal sequence receptor, delta | 6748 | **6.5** |
| **QRKKAYADF**[e] | COX6C | cytochrome c oxidase subunit VIc | 1345 | **2.2** |
| **KRFDDKYTL** | KIAA0102 | KIAA0102 | 9789 | **1.7** |
| **TRWNKIVLK** | UBL5 | ubiquitin-like 5 | 59286 | **1.4** |
| **LRFDGALNV** | TUBA2 | tubulin, alpha 2 | 7278 | **1.3** |
| **ARFSGNLLV** | SEC61A1 | protein transport protein SEC61 alpha subunit isoform 1 | 29927 | **1.2** |
| **NRIKFVIKR** | GTF2I | general transcription factor II, i | 2969 | **1.1** |
| GRVFIIKSY | HGRG8 | high-glucose-regulated protein 8 = NY-REN-2 | 51441 | nc |
| GRFDVKIEV[e] | ATP1B1 | ATPase, Na+/K+ transporting, beta 1 polypeptide | 481 | nc |
| SRFGNAFHL | PRPF8 | PRP8 pre-mRNA processing factor 8 homolog (yeast) | 10594 | nc |
| GRTGGSWFK | ATP1B1 | ATPase, Na+/K+ transporting, beta 1 polypeptide | 481 | nc |

[a] peptides printed in bold letters are encoded by overexpressed genes.

[b] gene symbols and names refer to LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink); whenever possible symbols approved by the HUGO Gene Nomenclature Committee were used.

[c] Internet address: http://www.ncbi.nlm.nih.gov/LocusLink.

[d] expression in tumor relative to corresponding normal tissue; nc no change;.

[e] ligands already published, see SYFPEITHI database (http://www.syfpeithi.de/) for references.

## 2.5.3  Discussion

The reports about insulin-like growth factor 1 (IGF1) and its receptor in cancer have a long history [1]. The IGF1/IGF1R system is strongly involved in the control of proliferation, differentiation and transformation in several cancer entities. The potential usage of the ligand derived from IGF1 and all other ligands from RCC44 for vaccination is discussed in chapter 2.5.

Asparagine synthetase is frequently discussed in the context of L-asparaginase-treatment in acute lymphocytic leukemia (ALL) [2, 3]. Up-regulation of ASNS

happens in cells as a result of limited access to asparagine, as induced by L-asparaginase-therapy.

Rh type C glycoprotein (RHCG) is a nonerythroid Rh glycoprotein homologue, which acts as an ammonia transporter [4, 5]. Recently it was shown that RHCG within the kidney is dominantly expressed in the connecting tubules and the cortical and outer medullary collecting ducts [6]. Therefore, if the tumor has developed from one of these particular regions, it might be the case that the dissected normal bulk tissue was low in the content of these structures and, therefore, the overexpression was biased.

The ATPase lysosomal interacting protein 1 (ATP6IP1)-derived ligand deserves special attention because this gene was shown being a SEREX antigen in melanoma patients. Moreover, antibody-levels against ATP6IP1 correlate with tumor destruction [7]. Hodi and collegues vaccinated melanoma patients with irradiated autologous melanoma cells engineered to secrete GM-CSF. They observed dense infiltration of CD8+ and CD4+ T cells in metastatic lesions after vaccination in 11 out of 16 patients. They applied the SEREX approach to a long term responder and identified antibodies against ATP6S1. Longitudinal analysis of one patient showed increases in serum antibody-levels against ATP6IP1 in parallel to tumor destruction. This correlation was also shown in 3 additional melanoma patients, in 3 metastatic non-small cell lung carcinoma patients and in a CML patient with a complete remission after a CD4 donor lymphocyte infusion. This was seen also in a murine tumor model: C57BL/6 mice vaccinated with irradiated GM-CSF-secreting B16 melanoma cells but not naïve mice generated high-titer IgG antibodies to ATP6S1. In conclusion, it seems that ATP6S1 is at least able to induce CD4+ T cell responses, but the relevance for CD8+ T cell responses is still not clear. Anyway, for many reported SEREX antigens, also CD8+ T cell responses could be induced.

Signal sequence receptor delta (SSR4) is one subunit of a tetrameric complex called translocon-associated protein (TRAP), formerly called signal sequence receptor [8, 9]. Its function within this complex is not clear so far.

## 2.5.4  References

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# 2.6  A gene expression database: 22.000 genes in 21 human healthy tissues

This database was built by the author of this thesis together with Oliver Schoor.

## 2.6.1  Materials and Methods

### RNA sources

Total RNA from human tissues were obtained commercially (Ambion, Huntingdon, UK; Clontech, Heidelberg, Germany; Stratagene, Amsterdam, The Netherlands). Total RNA from several individuals (Table 2.6.1) was mixed in a way that RNA from each individual was equally weighted. Quality and quantity was confirmed on the Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) using the RNA 6000 Nano LabChip Kit (Agilent).

**Table 2.6.1** *Total RNA used for expression analysis*

| Tissue | Number of Individuals | total RNA [µg] | RNA source |
|---|---|---|---|
| Adrenal gland | 62 | 5 | Clontech |
| Bladder | 2 | 5 | Stratagene |
| Bone marrow | 7 | 5 | Clontech |
| Whole brain | 3 | 5 | Clontech |
| Colon | 4 | 5 | Clontech, Stratagene |
| Heart | 2 | 5 | Clontech, Ambion |
| Kidney | 6 | 5 | Clontech |
| Liver | 2 | 5 | Clontech, Ambion |
| Lung | 8 | 5 | Clontech, Stratagene |
| Placenta | 21 | 5 | Clontech |
| Prostate | 32 | 5 | Clontech |
| Salivary gland | 24 | 5 | Clontech |
| Small intestine | 5 | 5 | Clontech |
| Skeletal muscle | 9 | 5 | Clontech |
| Spleen | 20 | 5 | Clontech |
| Stomach | 33 | 5 | Stratagene |
| Testis | 45 | 5 | Clontech |

**Table 2.6.1** *- continued*

| Tissue | Number of Individuals | total RNA [μg] | RNA source |
|--------|-----------------------|----------------|------------|
| Thymus | 12 | 5 | Clontech |
| Thyroid | 65 | 5 | Clontech |
| Trachea | 86 | 5 | Clontech |
| Uterus | 11 | 5 | Clontech |

### *High-Density Oligonucleotide Microarray Analysis*

Double-stranded DNA was synthesized from 5 μg of total RNA using SuperScript RTII (Life Technologies, Inc., Karlsruhe, Germany) and the primer (Eurogentec, Seraing, Belgium) as given by the Affymetrix manual. *In vitro* transcription using the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY), fragmentation, hybridization on Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA), and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, The Netherlands) followed the manufacturer's protocols (Affymetrix). The Affymetrix GeneArray Scanner was used and data were analyzed with the Microarray Analysis Suite 5.0 software. For normalization, 100 housekeeping genes provided by Affymetrix were used[1]. Pairwise comparisons were calculated using the expression values in kidney as baseline. Accordingly, all expression values calculated from signal log ratios are relative to kidney, which was set at 1. Significance of differential expression was judged by the "change" values given by the statistical algorithms implemented in the Microarray Analysis Suite 5.0 software. For absolute detection of expression, data were analyzed again using the statistical algorithms. Presence or absence of gene expression was determined by the absolute call algorithms. Error bars were given as 95% confidence intervals computed using the one-step Tukey's Biweight method by taking a mean of the signal log ratios of probe pair intensities across the two arrays.

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[1] http://www.affymetrix.com/support/technical/mask_files.affx

## 2.6.2 Example: Tissue expression profiles of candidate target genes in RCC44

Knowledge about the expression of a given gene in any kind of organ or tissue is very important for the decision about the quality of a candidate gene as a target in cancer immunotherapy. The first criterium for the selection of candidate peptides useful for vaccination is the overexpression of the coding genes in the tumor compared to the autologous normal renal tissue. After this first filter, of course it is necessary to make sure that the selected genes are not stronger expressed in other vital organs because of the risk of induction of autoreactivity. The best way to get this information at short notice is the presented gene expression database covering about 22.000 genes. It allows to call for the tissue expression pattern of any gene included on the chip within a second. This database is not closed but rather should be enlarged with data from additional tissues and organs.

In the following, the usage of this database within the presented integrated functional genomics approach is demonstrated. For the decision which of the identified MHC class I-ligands shown in chapter 2.5.2 would have been useful for vaccination of patient RCC44, the expression profiles of the genes coding for these peptides were displayed.

**209541_at, IGF1, LocusLink 3479**
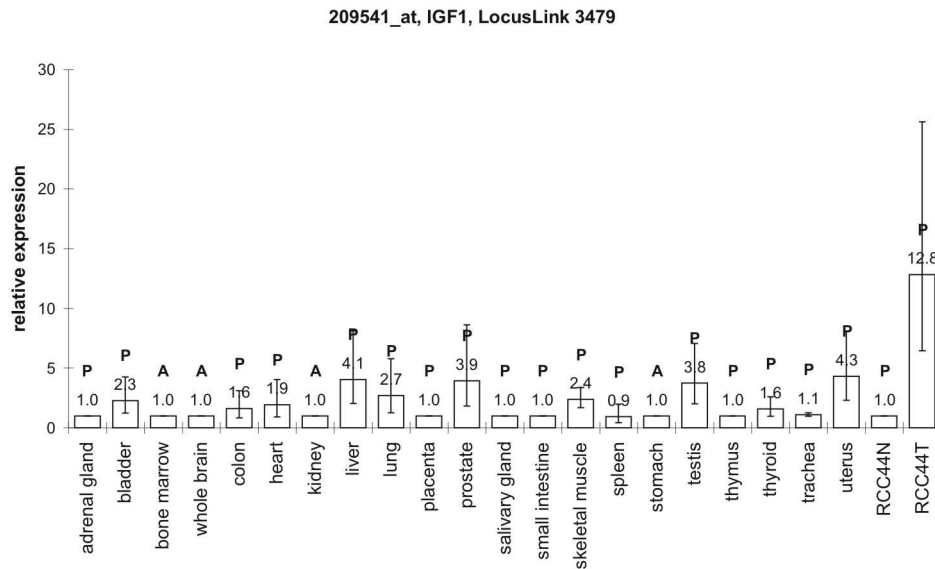
**Figure 2.6.1 Tissue expression of insulin-like growth factor 1 (IGF1).**

Expression was analyzed by oligonucleotide microarrays. Copy numbers are relative to kidney, which is set at 1.0. P means that the gene is present, A absent and M marginal according to the statistical absolute call algorithms. When the expression in a tissue is computed as being not significantly changed in the pairwise comparison with the kidney, the value is set at 1.0. For significant changes (increases or decreases), the expression value relative to kidney is calculated from the signal log ratio and displayed on top of the bars. In the case that the gene is considered not to be expressed in the kidney (A above the bar for kidney), all other expression values are given relative to the background signal in the kidney. Nevertheless, in this case expression differences between two other organs remain untouched. Error bars show upper and lower 95% confidential intervals.

IGF1 was expressed 3 fold higher in the tumor of RCC44 compared to the organs with the highest expression, uterus, liver, and prostate (Figure 2.6.1). Therefore, the HLA-A*11 restricted ligand from IGF1 would have been a candidate for vaccination.

**205047_s_at, ASNS, LocusLink 440**
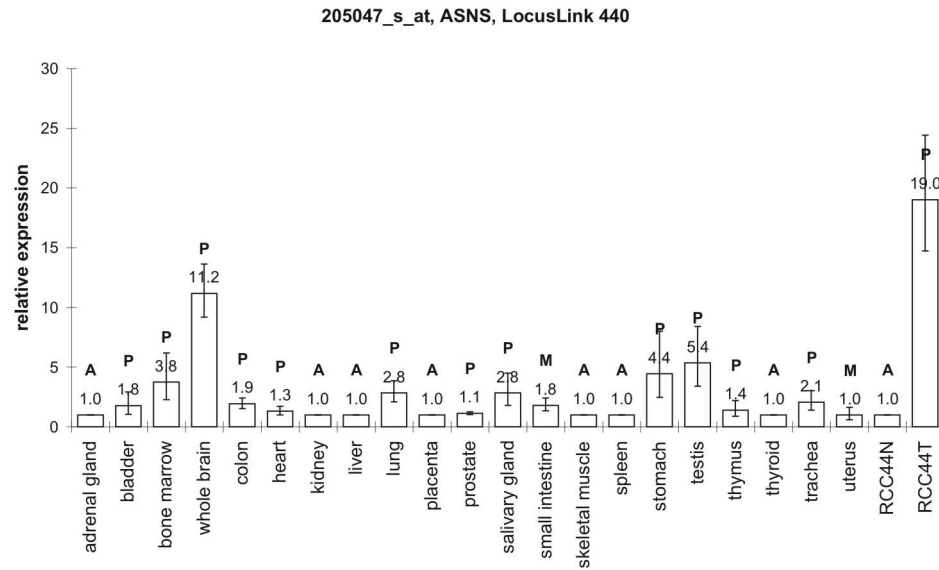


**Figure 2.6.2 Tissue expression of asparagine synthetase (ASNS).**

Please refer to legend of Figure 2.6.1

Expression of ASNS in the tumor is twice as high as in the organ with the second highest expression level, whole brain (Figure 2.6.2). As brain is an immune privileged region and ASNS is either expressed at much lower levels in all other analyzed tissues or not at all, it would have been possible to include the ligand derived from ASNS in a vaccine tailored to meet the needs of patient RCC44.

**219554_at, RHCG, LocusLink 51458**
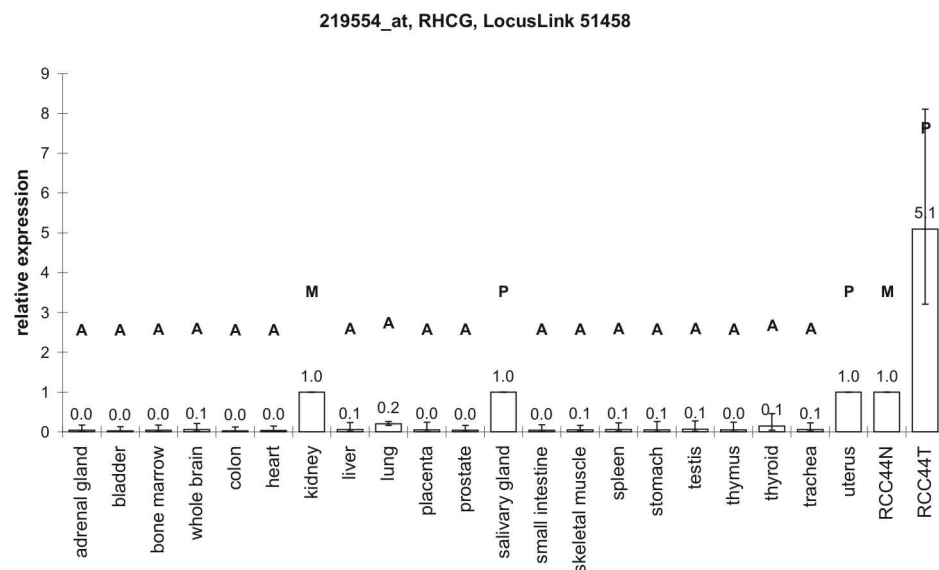


**Figure 2.6.3 Tissue expression of Rh type C glycoprotein (RHCG).**

Please refer to legend of Figure 2.6.1

RHCG was only expressed in few tissues and high in the tumor (Figure 2.6.3). As already mentioned above, within the kidney, RHCG is dominantly expressed in the connecting tubules and the cortical and outer medullary collecting ducts. Therefore, if the tumor has developed from one of these particular regions, it might be the case that the dissected normal bulk tissue was low in the content of these structures and, therefore, the expression value for kidney is just a mean value from part of the cells not expressing RHCG and some cells expressing RHCG at the same level as the tumor does. This possibility cannot be ruled out easily as one does not know from which single cell the tumor developed.

**207809_s_at, ATP6IP1, LocusLink 537**
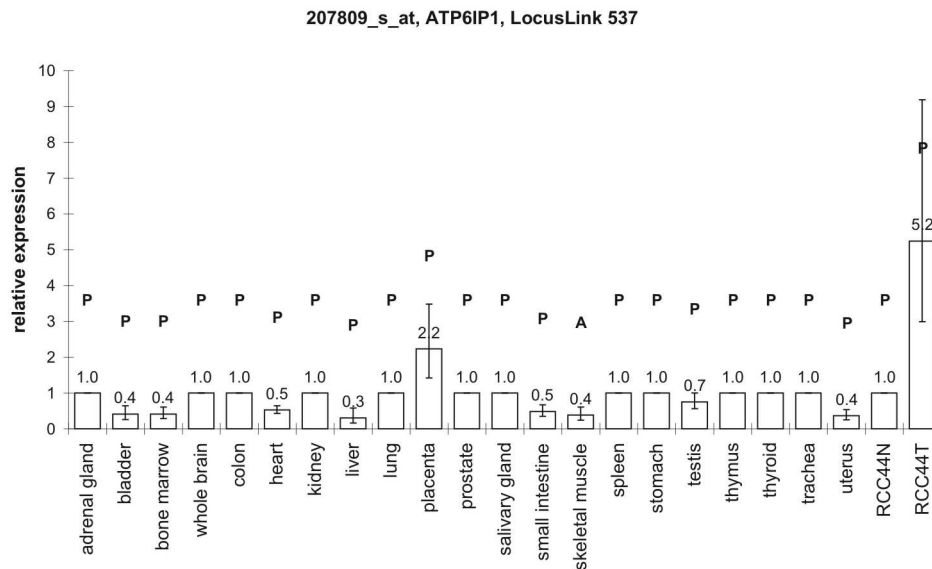


**Figure 2.6.4 Tissue expression of ATPase lysosomal interacting protein 1 (ATP6IP1).**
Please refer to legend of Figure 2.6.1

ATP6IP1 is already discussed as a promising vaccine candidate in chapter 2.5.3. The tissue expression profile shows expression in most of the tissues, but a 5 fold higher expression in the tumor (Figure 2.6.4). Placenta, where the expression level is about half of the tumor, is an immune privileged organ. Thus, (ARLSLTYERL) from ATP6IP1 would have been a good vaccine candidate.

**201004_at, SSR4, LocusLink 6748**
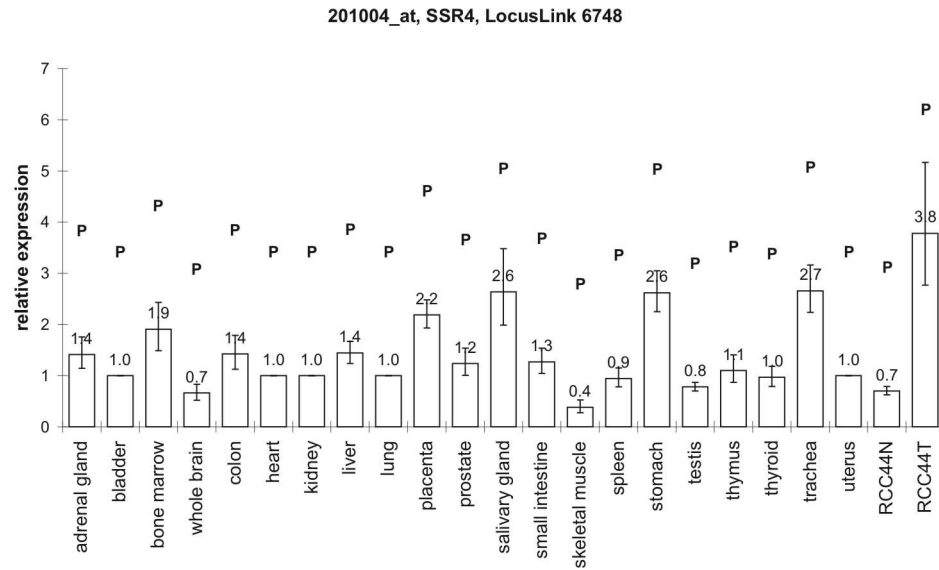


**Figure 2.6.5 Tissue expression of signal sequence receptor delta (SSR4).**
Please refer to legend of Figure 2.6.1

SSR4 is broadly expressed and the expression differences between the tumor and other organs like stomach or trachea are rather small. Therefore this ligand should probably be omitted.

# 2.7 Integrated functional genomics approach: a personalized cancer vaccine clinical trial

This trial is conducted by Dr. Brossart from the Department of Hematology, Oncology and Immunology, University of Tübingen. Patients were from the Department of Urology, University of Tübingen, headed by Prof. Stenzl. Sequencing of MHC class I-ligands was done (beside the author of this thesis) by Claudia Lemmel, Jörn Dengjel, and Oliver Schoor. MHC class I precipitation was done by Christian Reichle. Oliver Schoor also performed the expression profiling experiments.

## 2.7.1 Results

A monocentric clinical phase I/II trial using the integrated functional genomics approach presented in detail in chapter 2.1 was initiated. Advanced metastatic renal cell carcinoma patients are currently recruited. Until now, four patients were analyzed, RCC68, RCC70, RCC73, and RCC75. Peptide and expression analysis were performed as described in chapter 2.1.3. Three patients were vaccinated using autologous dendritic cells loaded with peptides at days 0, 14, 28, 42.

### 2.7.1.1 Patient RCC68

HLA typing for RCC68 was HLA-A*02, A*29, B*15, and B*45. Tumor weight was about 20 g, the patient was male. More than 100 MHC class I-ligands could be identified from this patient. Because the gene expression database was not finished when this patient was analyzed, newly identified peptides derived from overexpressed genes could not be included due to the missing tissue expression profiles. The peptides shown in Table 2.7.1 were chosen because they were already known before and the genes coding for these peptides were found to be overexpressed in the tumor of this particular patient.

**Table 2.7.1** *Peptides included in the vaccine for patient RCC68*

| HLA-Allele | Gene | Position | Sequence | Length |
|---|---|---|---|---|
| A*0201 | BIRC5 | 94-103 | ELTLGEFLKL | 10 |
| | MET | 654-662 | YVDPVITSI | 9 |
| | CA9 | 254-262 | HLSTAFARV | 9 |
| | ADFP | 129-137 | SVASTITGV | 9 |
| | CCND1 | 101-109 | LLGATCMFV | 9 |
| | CCND1 | 228-236 | RLTRFLSRV | 9 |
| | MUC1 | 950-958 | STAPPVHNV | 9 |
| | MUC1 | 12-20 | LLLLTVLTV | 10 |
| | PRAME | 425-433 | SLLQHLIGL | 9 |
| DR | (PADRE)[1] | - | aKXVAAWTLKAAa | 13 |

[1] this peptide is not derived from a gene; PADRE (Pan DR epitope) has promiscuous HLA-DR binding capacity.

## 2.7.1.2 Patient RCC70

HLA typing for RCC70 was HLA-A*01, A*02, B*07, B*0806, and B*42. Tumor weight was about 8.5 g, the patient was male. Unfortunately, no peptides could be identified from this tumor. Nevertheless, known peptides were chosen for vaccination according to the gene expression data obtained from this patient (Table 2.7.2).

**Table 2.7.2** *Peptides included in the vaccine for patient RCC70*

| HLA-Allele | Gene | Position | Sequence | Length |
|---|---|---|---|---|
| A*0201 | MET | 654-662 | YVDPVITSI | 9 |
| | CA9 | 254-262 | HLSTAFARV | 9 |
| | ADFP | 129-137 | SVASTITGV | 9 |
| | CCND1 | 101-109 | LLGATCMFV | 9 |
| | CCND1 | 228-236 | RLTRFLSRV | 9 |
| | MUC1 | 950-958 | STAPPVHNV | 9 |
| | MUC1 | 12-20 | LLLLTVLTV | 10 |
| | PRAME | 425-433 | SLLQHLIGL | 9 |
| DR | (PADRE)[1] | - | aKXVAAWTLKAAa | 13 |

[1] this peptide is not derived from a gene; PADRE (Pan DR epitope) has promiscuous HLA-DR binding capacity.

## 2.7.1.3 Patient RCC73

HLA typing for RCC73 was HLA-A*02, A*03, B*07, B*57. Tumor weight was about 10 g, the patient was male. Selection of the peptides shown in Table 2.7.3 was

done considering the gene expression data from this patient, because peptide identification failed in this case.

**Table 2.7.3** *Peptides suggested for patient RCC73*

| HLA-Allele | Gene | Position | Sequence | Length |
|---|---|---|---|---|
| A*0201 | ADFP | 129-137 | SVASTITGV | 9 |
| | MET | 654-662 | YVDPVITSI | 9 |
| | CA9 | 254-262 | HLSTAFARV | 9 |
| | CCND1 | 101-109 | LLGATCMFV | 9 |
| | CCND1 | 228-236 | RLTRFLSRV | 9 |
| | MUC1 | 950-958 | STAPPVHNV | 9 |
| | MUC1 | 12-20 | LLLLTVLTV | 9 |
| DR | (PADRE)[1] | - | aKXVAAWTLKAAa | 13 |

[1] this peptide is not derived from a gene; PADRE (Pan DR epitope) has promiscuous HLA-DR binding capacity.

This patient changed his mind at a later point of time, after the analysis had already been done, and preferred not to be immunized.

### 2.7.1.4 Patient RCC75

HLA typing for RCC75 was HLA- A*03, B*07, B*40. Tumor weight was about 16 g, the patient was male. More than 100 MHC class I-ligands could be identified from this patient. Peptides listed in Table 2.7.4 were chosen for the vaccine.

**Table 2.7.4** *Peptides included in the vaccine for patient RCC75*

| HLA-Allele | Gene | Position | Sequence | Length |
|---|---|---|---|---|
| A*0301 | EF1e | 114-122 | TLADILLYY | 9 |
| | FACL4 | 81-90 | KLFDHAVSKF | 10 |
| | NSAP1 | 269-277 | GLTDVILYH | 9 |
| | unknown | - | SVASISLTK | 9 |
| B*0702 | FACL4 | 617-626 | VPNQKRLTLL | 10 |
| DR | (PADRE)[1] | - | aKXVAAWTLKAAa | 13 |

[1] this peptide is not derived from a gene; PADRE (Pan DR epitope) has promiscuous HLA-DR binding capacity.

Until now, none of the patients showed a clinical response. Monitoring of T cell responses against the vaccinated peptides as a surrogate marker was not analyzed yet.

# Results, Part 2: Antigen Processing

## 2.8    A role for a novel luminal endoplasmic reticulum aminopeptidase in final trimming of 26S proteasome-generated major histocompatability complex class I antigenic peptides

The author of this dissertation, Toni Weinschenk, contributed the relative quantification of SIINFEKL containing peptides by liquid-capillary mass spectrometry analysis shown in Table 2.8.1 to this work.

### 2.8.1   Summary

Peptides presented to cytotoxic T lymphocytes by class I major histocompatability complex are 8-11 residues long. While proteasomal activity generates the precise C-termini of antigenic epitopes, the mechanism(s) involved in generation of the precise N-termini is largely unknown. To investigate the mechanism of N-terminal peptide processing, we utilized a cell-free system in which two recombinant ornithine decarboxylase constructs, one expressing the native H2-K$^b$-restricted ovalbumin-derived epitope SIINFEKL (ODC-ova), whereas the other the extended epitope, LESIINFEKL (ODC-LEova), were targeted to degradation by 26S proteasomes followed by import into microsomes. We found that the cleavage specificity of the 26S proteasome was influenced by the N-terminal flanking amino acids leading to significantly different yields of the final epitope SIINFEKL. Following incubation in the presence of purified 26S proteasome, ODC-LEova generated largely ESIINFEKL that was efficiently converted to the final epitope SIINFEKL following translocation into microsomes. The conversion of ESIINFEKL to SIINFEKL was strictly dependent on the presence of H2-K$^b$, and was completely inhibited by the metalloaminopeptidase inhibitor 1,10-phenanthroline. Importantly, the converting activity was resistant to a stringent salt/EDTA wash of

the microsomes and was only apparent when TAP transport was facilitated. These results strongly suggest a crucial role for a lumenal ER-resident metallo-aminopeptidase in the N-terminal trimming of major histocompatibility complex class I-associated peptides.

## 2.8.2  Introduction

MHC class I molecules associate with peptides of 8-11 amino acids derived from the proteolytic degradation of intracellular protein antigens and present them to CD8+ T cells on the cell surface [1-3]. While the vast majority of class I ligands are translocated from the cytosol into the endoplasmic reticulum (ER) by the ATP-dependent transporter associated with antigen processing, TAP [3], alternative pathways have been described including the liberation of minigene-encoded peptides from ER signal sequences [4-6], TAP-independent processing of signal sequences [7, 8], and processing of membrane-associated or soluble proteins in the secretory pathway [9-12]. Peptides that are not retained in the ER by binding to class I or glycosylation are released back into the cytosol through the Sec61 channel [13-15]. Studies using membrane-permeable inhibitors of proteasomes have indicated that the proteasome is the major proteolytic activity responsible for the generation of antigenic peptides [16-21] although the incomplete inhibition of antigen processing by proteasome inhibitors has also documented the involvement of nonproteasomal cytosolic proteases [22-28]. The proteasome is an abundant cytosolic multisubunit protease consisting of the proteolytic 20S core particle that associates with PA700 regulatory complexes to form 26S proteasomes, or with PA28 complexes [29]. The 26S proteasome usually degrades ubiquitinylated proteins [30]. Ornithine decarboxylase1 (ODC) constitutes an exception as it is targeted to 26 proteasomes by antizyme in an ubiquitin-independent fashion [31-33]. The 26S proteasome seems to mediate the degradation of most antigenic proteins in living cells [34-36] and, therefore, deserves particular interest. The *in vitro* cleavage specificities of purified 20S proteasomes and the relevance of proteasomal processing for the generation of immunodominant epitopes have been investigated in great detail [1, 37]. While 20S proteasomes have been shown to precisely cleave a number of known class I ligands out of model polypeptide substrates, a minor part of cleavage products is

longer than the canonical length of class I-binding peptides [38-41]. Limited information is, however, so far available about protein processing by 26S proteasomes. A direct comparison of 26S and 20S proteasomes revealed overlapping but substantially different cleavage patterns for the protein substrate β-casein [41]. On average, the 26S proteasomal cleavage products were found to be slightly shorter than peptides generated by 20S proteasomes containing greater proportions of peptides that are too short for efficient TAP-mediated translocation as well as for class I binding [40, 41]. Proteasomes seem to be the dominant, if not the only, protease that generates the correct C-terminus of class I ligands, whereas the N-terminal trimming of proteasomal products is insensitive to proteasome inhibitors [20, 42, 43]. Leucine aminopeptidase, puromycin-sensitive aminopeptidase, and bleomycin hydrolase have been implicated in the cytoplasmic trimming of proteasomal products [44, 45]. In addition, tri-peptidyl peptidase II has been suggested to play a role in the generation of class I ligands or precursors [46]. Different lines of evidence suggest that precursors of class I ligands can be trimmed in the ER lumen to their final lengths. Processing of peptide imported into microsomes was directly shown by biochemical methods [14, 47]. The broad but not random substrate specificity of TAP precludes that 9-mer peptides containing a proline residue at position 2 or 3 are efficiently bound and transported by TAP [48- 50], which can be rescued by extending such peptides with N- and/or C-terminal flanking residues [49]. Nevertheless, several MHC class I alloforms in man and mouse prefer a proline residue at position 2 or 3 in the associated 9-mer peptide [51]. Also for some peptides not containing proline, addition of flanking residues to minimal epitopes strongly improved TAP affinities [50, 52, 53] implicating that precursors become trimmed in the ER. The analysis of extended minimal epitopes or tandem arrays of epitopes that were directed into the ER of TAP-deficient T2 cells by a leader sequence have clearly indicated the predominance of an aminopeptidase activity [10, 47, 53-55], whereas the carboxypeptidase activity in the ER lumen seems to be very poor [5, 11, 53-55]. N-terminal trimming of a precursor peptide was recently suggested to depend on the presence of the correct class I restriction element [47]. However, the identity and biochemical properties of the aminopeptidase(s) in charge remains elusive. To investigate the relative contribution of the proteasome, cytosolic and ER peptidases to the generation of the definite class I epitope, we

have developed an *in vitro* system in which recombinant ODC containing ovalbumin (ova) peptides are targeted to degradation by 26S proteasomes followed by import into purified microsomes. We find that the yield of a finally processed H2-K$^b$-binding epitope was strongly influenced by N-terminal flanking amino acids in the protein sequence. We show that a slightly extended ova epitope precursor was efficiently trimmed following TAP-mediated import into microsomes. The conversion into the definite epitope could be blocked by the aminopeptidase inhibitor 1,10-phenanthroline (PNT) and was strictly dependent on the presence of MHC class I molecules known to associate with the epitope.

## 2.8.3 Materials and Methods

### 2.8.3.1 Peptides

The peptide SIINFEKL was synthesized by Anaspec (San Jose, CA). The peptide ESIINFEKL was synthesized by Dr. M. Fridkin at the Department of Organic Chemistry, The Weizmann Institute, Rehovot, Israel. The peptides HESIINFEKL, HLESIINFEKL, QSHESIINFEKL and QSHLESIINFEKL were synthesized at the peptide synthesis core facility of the DKFZ (Heidelberg, Germany). All peptides were purified to ~95% homogeneity by HPLC and their identity confirmed by mass spectrometry. To accurately determine the concentration of peptides used in this study each peptide solution in double-distilled water was analyzed by Edman degradation.

### 2.8.3.2 Chemicals

Bestatin and lactacystin were from Calbiochem (San Diego, CA). Complete$^{TM}$ EDTA-free protease inhibitors (referred to as protease inhibitors) were from Roche Molecular Biochemicals (Mannheim, Germany). 1,10-phenanthroline, all standard reagents and reagents for cell culture were from Sigma (St. Louis, MO)

### 2.8.3.3 Cell lines and mice

The B3Z T-cell hybridoma and the antigen presenting cell line K$^b$-L have been described by Karttunen et al. [56] and were granted by Dr. Chris Norbury (NIH,

Bethesda, MD). C57BL/6 (B6) and BALB/c mice were from local breeding in the animal facility of the Sackler Faculty of Medicine, Tel Aviv University (Tel Aviv, Israel). H2-D$^{b-/-}$ (B6.K$^b$) and H2-K$^{b-/-}$ (B6.D$^b$) knockout mice [57] were a kind gift of Dr. Lea Eisenbach, (Weizmann Institute, Rehovot, Israel). TAP1$^{-/-}$ mice [58] were courtesy of Dr. Natalio Garcia-Garbi (DKFZ, Heidelberg, Germany).

### 2.8.3.4 Preparation of 26S proteasome, maltose-binding protein antizyme-fusion protein (MBP-Az) and recombinant ODC-ova derivatives

The 26S proteasome was purified from B6 livers, and the recombinant proteins, MBP-Az and the ODC-ova derivatives were expressed in bacteria and purified by affinity chromatography as previously described [59]. In order to generate the ODC-LEova expression vector, an adaptor encoding the peptide LESIINFEKL with *Bst*XI-compatible ends was generated by annealing the two synthetic oligonucleotides 5'-ATCTGGAAAGTATAATCAACTTCGAAAAACTGAGCC-3' and 5'- CAGTTTTTCGAAGTTGATTATACTTTCCAGATGGCT-3'. The adaptor was then inserted into the ODC sequence as previously described for ODC-ova [59]. Production of $^{35}$S-labeled ODC-ova and ODC-LEova was in the methionine auxotroph strain B834(DE3) (Novagen Inc., Madison, WI) and purification of the $^{35}$S-labeled proteins was carried out as previously described [59].

### 2.8.3.5 Preparation of sub-cellular fractions

*Lactacystin-treated cytosol:*
Preparation of cytosol (100,000 x g supernatant) from RMA cells was carried out as previously described [60]. To inhibit proteasome activity, a 2 ml aliquot of the cytosol (20 mg of protein) was incubated with 35 µM lactacystin for 30 min at 37°C. The lactacystin-treated cytosol was then dialyzed for 16 hours at 4°C against 500 ml of 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT and stored in aliquots at -70°C. Inhibition of proteasome activity in the cytosol was greater than 90% as determined by the inhibition of fluorogenic peptide suc-LLVY-MCA hydrolyzing activity [61].

*Microsomes:*

Microsomes were prepared according to the protocol described by Shepherd et al. [58]. Briefly, mice (8-10 weeks old) were injected intraperitoneally with 0.2 mg/mouse of poly-inosine-cytosine 24 hours prior to sacrifice. The livers and spleens were thoroughly washed with phosphate buffered saline and then homogenized in a motor-driven Potter-Elvehjem Teflon tissue grinder. Homogenization was in 3 ml/liver of buffer A containing: 50 mM triethanolamine-Ac (pH 7.5), 1 mM DTT, 5 mM Mg(Ac)$_2$, 50 mM KAc, 250 mM sucrose and 1:25 (w/v) solution of protease inhibitors. The extract was then subjected to fractional centrifugation at 1000 x g and then at 10,000 x g. The supernatant from the 10,000 x g spin was then placed on top of a sucrose cushion containing 1.3 M in buffer A at a ratio of 2:1 (sample:cushion) and then centrifuged at 140,000 x g for 2.5 hours. The resulting pellet was then re-suspended in buffer C (50 mM 7.5, 1 mM DTT and 250 mM sucrose) to a concentration of 200 OD$_{280nm}$ units/ml. The microsome suspension was then stored in aliquotes at -70°C.

### 2.8.3.6 Stripping off microsomes-associated proteins

The microsome suspension was incubated on ice for 15 min in 10 volumes of buffer D containing: 50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 0.5 M KCl and 15 mM EDTA. The membrane suspension was then centrifuged at 10,000 x g in a microfuge. The membrane pellet was then re-suspended in 10 volumes of buffer D without EDTA and immediately centrifuged as described above. The final membrane pellet was resuspended in the processing reaction mixture. The protein content of the stripped microsomes was less than 40% of that of untreated membranes as determined by measurement of the optical density at 280nm.

### 2.8.3.7 Assay of TAP-mediated peptide transport

TAP-mediated peptide transport was determined by measuring the ATP-dependent transport of the radio-iodinated peptide TNKTRIDGQY into isolated microsomes as previously described [63]. Purified microsomes (1 unit) were incubated in a reaction mixture containing the following components in a final volume of 100µl: 50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 5 mM MgCl$_2$, 10 mM creatine phosphate, 2.5 units creatine phosphokinase and ^{125}I-labeled peptide (40

ng, 106 cpm). In reactions without ATP, 2- deoxyglucose (20 mM) and hexokinase (3 µg) were added instead of ATP and the ATP regenerating system. Following incubation for 10 min at 37°C the microsomes were pelleted by centrifugation at 10,000 x g in a microfuge. The supernatant was removed and the membrane pellet was re-suspended in 1 ml lysis buffer (20 mM Hepes-KOH (pH 7.5), 5 mM MgCl$_2$ and 1% NP-40) and then sonicated for 30 sec at 50% output power in a water bath sonicator (Ultrasonic Processor, Heat Systems Inc., Farmingdale, NY). The detergent extract was centrifuged for 10 min at 10,000 x g in a microfuge. The resulting supernatant was then mixed with 25 µl concanavalin A (ConA)-Sepharose beads (Amersham-Pharmacia Biotech AB, Uppsala, Sweden) and the mixture was then mixed gently for 16 hours at 4oC. The beads were then pelleted by centrifugation at 1000 x g in a microfuge and then washed twice with 1 ml portions of lysis buffer. The ATP-dependent transport of peptide was then determined by measuring the amount of radioactivity associated with the beads in the presence of ATP after subtraction of the ConA-associated radioactivity obtained in a parallel reaction carried out in the absence of ATP.

### 2.8.3.8  Antigen processing assays

Antigen processing reaction mixtures contained the following components in a final volume of 75 µl: 50 mM Hepes-KOH (pH 7.5), 5 mM MgCl$_2$, 0.5 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 1.8 units creatine phosphokinase, 10 µg of recombinant ODC, 15 µg MBP-Az and 10 units of 26S proteasome (for the definition of proteasome units see ref. 59). Where indicated purified microsomes (1 OD$_{280}$ unit) and lactacystin-treated RMA lysate (60 µg of protein) were also supplemented. Following incubation for 10 min at 37°C, the reaction was extracted in 400 µl ice-cold lysis solution (0.5% trifluoroacetic acid, 1% NP-40). The mixture was then sonicated for 60 sec at 50% output power in an ice-cooled bath sonicator. In processing reactions containing microsomes the membranes were first pelleted by centrifugation at 4°C for 15 min at 10,000 x g in a microfuge and then extracted in lysis buffer as described above. The acid extract was then filtered through a 10kDa cut-off filter (Microcon 10 concentrator, Millipore Corp. Bedford, MA) and the filtrate was collected and lyophilized. The lyophilized material was then chromatographed by reverse phase-HPLC and the fractions

corresponding the elution position of synthetic SIINFEKL were pooled and lyophilized exactly as previously described [59].

### 2.8.3.9  Peptide processing assays

Reactions were carried out in a final volume of 100 µl containing the following components: 50 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 2.5 units of creatine phosphokinase, microsomes (1 unit) and 50 fmoles of synthetic ESIINFEKL or HLESIINFEKL. Where indicated, 1,10-phenanthroline (2 mM) or bestatin (250 µM) were added. In reaction without ATP, hexokinase (3 µg) (Roche Molecular Biochemicals) and 2-deoxyglucose (20 mM) were added instead of ATP and the ATP regenerating system. The reaction was pre-incubated for 4 min at 37°C and then ESIINFEKL was added for a further incubation for 8 min at 37°C. The reaction was then stopped by addition of 450 µl lysis buffer and then processed as described above. We detected low SIINFEKL activity even in the absence of microsomes. This activity was probably due to residual SIINFEKL present in the synthetic ESIINFEKL preparation that we estimate ~5%. Therefore, the conversion of ESIINFEKL to SIINFEKL was calculated after subtraction of the value obtained in a parallel experiment without microsomes.

### 2.8.3.10     Quantification of SIINFEKL

Except for Figure 2.8.1 B where the amount of SIINFEKL was quantified by flow cytometry using the H2-$K^b$-SIINFEKL-specific monoclonal antibody 25.D1-16 [62], SIINFEKL was quantified by the B3Z Tcell hybridoma activation assay [56]. Briefly, B3Z cells ($5 \times 10^4$) were co-cultured overnight in 100 µl phenol red-free RPMI-1640 at 37°C with $K^bL$ cells ($3 \times 10^4$) and in the presence of either synthetic peptides or HPLC-purified processed peptides. After activation, cells were lysed by addition of 50 µl to each well of stop buffer (50 mM $Na_2HPO_4$, 34 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 0.125% NP-40, 150 mM β-mercaptoethanol) containing 0.45 mM CPRG (chlorophenol red β-galactopyranoside, Calbiochem, San Diego CA.). The cells were then further incubated for 4 hours at 37°C and the absorbance at 595 nm in each well was subsequently determined using a 96-well ELISA reader. The amount of SIINFEKL

was calculated based on the activity of known amounts of synthetic peptide that were tested in parallel.

### 2.8.3.11    HPLC mass spectrometry

Synthetic and proteasomal digest-derived peptide mixtures were analyzed by a reversed phase HPLC system (ABI 140D, Applied Biosystems) coupled to a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-TOF, Micromass, Manchester, England) equipped with an ESI source. Solvent A was 4 mM ammonium acetate adjusted to pH 3.0 with formic acid. Solvent B was 2 mM ammonium acetate in 70% acetonitrile/water adjusted to pH 3.0 with formic acid. Loading and desalting of typical sample volumes of 100 µl was achieved by preconcentration on a 300 µm x 5 mm C18 µ-Precolumn (LC Packings, San Francisco, CA). A syringe pump (PHD 2000, Harvard Apparatus, Inc., Holliston, MA), equipped with a gastight 100 µl syringe (1710 RNR, Hamilton, Bonaduz, Switzerland), was used to deliver solvent and sample at a flow rate of 2 µl/min. For peptide separation, the preconcentration column was switched in line with a 75 µm x 250 mm C-18 column (LC Packings, San Francisco, CA). A binary gradient of 25%-60% B within 70 min was performed, applying a flow rate of 27 µl/min reduced to approximately 300 nl/min with a pre-column split using a TEE-piece (ZT1C, Valco, Schenkon, Switzerland) and a 300 µm x 150 mm C-18 column as a backpressure device. A gold-coated glass capillary (PicoTip, New Objective, 11 Cambridge, MA) was used as the needle in the ESI source. A blank run was performed prior to any subsequent HPLC MS run in order to ensure that the system was free of any residual peptide.

For on-line nanocapillary HPLC tandem mass spectrometry experiments, fragmentation of the parent ion was achieved at the given retention time by collision with argon atoms. Q1 was set to the mass of interest +/- 0.5 Da and collision energy was applied for 1-2 min.

Quantitative analysis of different compounds in HPLC mass spectrometry is possible by calibrating with synthetic substances of known amounts. For quantifying relative amounts of different SIINFEKL-containing peptides in one digest, relative signal intensities have been evaluated by calibration with different amounts of the corresponding synthetic peptides.

For comparison of the amounts of SIINFEKL and ESIINFEKL in the digests of the two different substrates, the efficiencies of the digests were normalized using three proteasomal cleavage products from various parts of ODC, which for this reason should be generated in equal amounts in all digests.

## 2.8.4  Results

### 2.8.4.1 The efficiency of generation of the H2-K$^b$ ova-derived epitope is dependent upon its N-terminal flanking residues

We have generated two recombinant ODC derivatives that express the optimal H2-K$^b$ epitope SIINFEKL (ODC-ova), and the naturally extended ovalbumin-derived peptide LESIINFEKL (ODC-LEova). We have previously shown that ODC-ova is processed very efficiently to generate almost exclusively the optimal epitope SIINFEKL [59]. When we compared the proteolytic processing of ODC-ova with ODC-LEova by purified 26S proteasome under linear kinetic conditions we noticed that the amount of SIINFEKL produced from ODC-ova was consistently 3-4 fold higher than that produced from ODC-LEova (Figure 2.8.1 A). The difference was not due to decreased proteolysis of ODC-LEova since the rate of degradation of the two derivatives was similar (Figure 2.8.1 B). Consequently, mass spectrometry analysis indicated that the distribution of the SIINFEKL-containing peptides produced by the two derivatives was markedly different in respect to the proportion of the optimal epitope relative to the other SIINFEKL containing peptides. Indeed, the proportion of SIINFEKL relative to the overall SIINFEKL-containing peptides from ODC-ova and ODC-LEova was 90% and 25% respectively. Instead, the predominant peptide produced from ODC-LEova was ESIINFEKL (62%).

Consistent with previous observations that the proteasome is the sole proteolytic activity that generates the correct C-terminus of MHC class I-restricted epitopes, no SIINFEKL-containing peptides with C-terminal extensions could be detected (Table 2.8.1). The finding that ESIINFEKL became the dominant peptide generated from ODC-LEova is probably due to the introduction of the leucine residue, a preferential proteasomal cleavage site [39], upstream of the epitope in ODC-LEova.
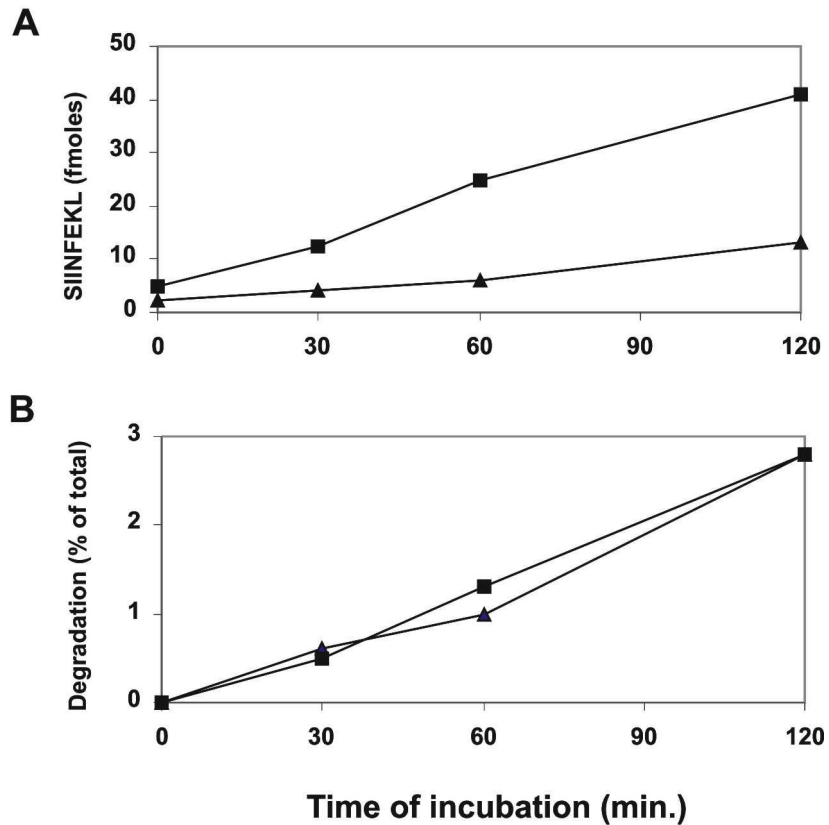
**Figure 2.8.1** *Proteolytic processing of 35S-labeled ODC-ova and ODC-LEova by purified 26S proteasome.*

[35]S-Labeled recombinant ODC-ova (squares) and ODC-LEova (triangles) were incubated for the indicated time periods with purified 26S proteasome and MBP-Az. To quantify the amount of SIINFEKL (A), 4 µg of each of the ^{35}S-labeled proteins was incubated in a final volume of 100 µl in a standard reaction mixture. The peptides were then isolated as described under "Experimental procedures" and then tested for recognition by mAb 25-D1.16 as previously described [59]. To determine the percentage of degradation (B), ^{35}S-labeled recombinant ODC derivatives (1 µg of protein being 11,000 cpm for ODC-ova and 6,000 cpm for ODC-LEova) were incubated in a volume of 25 µl in a standard reaction mixture. The percentage of degradation of ^{35}S labeled ODC derivative was then determined as previously described [59] by measuring the amount of soluble radioactivity after addition of trichloroacetic acid and after subtraction of the soluble radioactivity obtained at time zero. The degradation results are the mean of duplicate incubations.

**Table 2.8.1** *Mass spectrometric analysis*

*of SIINFEKL containing peptides generated by the 26S proteasome*

| Antigen | Processed peptide | Relative amount (%) |
|---------|-------------------|---------------------|
| ODC-ova | SIINFEKL | 90 |
|  | HSIINFEKL | 5.5 |
|  | QSHSIINFEKL | 4.5 |
| ODC-LEova | SIINFEKL | 25 |
|  | ESIINFEKL | 62 |
|  | HLESIINFEKL | 6 |
|  | QSHLESIINFEKL | 7 |

Mass spectrometric analysis and quantification of processed peptides was carried out as described in 2.8.3.11.

### 2.8.4.2 Processing of ODC-ova and ODC-LEova in the presence of cytosol and isolated microsomes

It has been previously reported that antigenic peptide precursors with N-terminal extensions are further processed either in the cytoplasm or in the ER (see Introduction). To investigate the role of cytoplasmic and ER peptidases in the generation of the final antigenic epitope, we subjected the recombinant antigens to proteolytic processing by the 26S proteasome in the presence of a cytosolic fraction and isolated microsomes. Following incubation of the antigens in the cell-free system, the microsomes were pelleted and the yield of SIINFEKL measured by the ability to activate the $K^b$/SIINFEKL-specific T cell hybridoma B3Z.

 When ODC-ova was incubated in the presence of 26S proteasome and B6.$K^b$ microsomes, 54% of the SIINFEKL, initially produced by the proteasome alone was recovered (6 out of 11.2 fmoles) (Figure 2.8.2 A). However, when ODC-LEova was processed under the same conditions, the yield of SIINFEKL was 175% (6.3 compared with 3.6 fmoles) (Figure 2.8.2 B). These results proposed that the N-terminally extended SIINFEKL precursors generated from ODC-LEova and specifically ESIINFEKL were further trimmed by the microsomes to produce the optimal epitope. To exclude the possibility that the augmentation in SIINFEKL yield was due to exceedingly higher affinity of ESIINFEKL to TAP resulting in preferential transport followed by further trimming in the ER, we tested the

affinities of the extended peptides to TAP. Using a radio-iodinated and glycosylatable reporter peptide [63], we determined the $IC_{50}$ values of SIINFEKL, ESIINFEKL, HLESIINFEKL, and QSHLESIINFEKL to be 4.5 µM, 3.0 µM, 2.3 µM, and >1 mM, respectively. Thus, we found that the affinity of all the N-terminally extended ova peptides generated from ODC-LEova was similar to that of SIINFEKL (except for the 13-mer) suggesting that the increment in the recovery of SIINFEKL was mainly owing to processing of the predominant proteasomal product, ESIINFEKL. The increment in SIINFEKL activity when ODC-LEova was processed could also not be explained by recognition of $K^b$-bound ESIINFEKL since the B3Z T-cell hybridoma recognized this peptide at least ten-fold less efficiently than SIINFEKL (Figure 2.8.2 D). The addition of cytosol to the processing reaction caused a considerable reduction in the yield of SIINFEKL from both substrates (compare + and - cytosol in Figs. 2A and B). These results indicated that the proteasome-processed peptides were subjected to non-specific degradation in the cytosol. Nevertheless, the significant recovery of SIINFEKL in microsomes indicated that the transport of peptides into the ER lumen rescues a significant amount of peptides from complete cytosolic degradation. When processing was performed with $B6.D^b$ microsomes no SIINFEKL could be recovered (Figure 2.8.2 A, B). Furthermore, SIINFEKL could not be detected when ODC-LEova was processed in the presence of BALB/c microsomes (Figure 2.8.2 C). These results clearly indicate that the correct restriction element was essential for precursor peptide processing and retention of the definite epitope. These results are also consistent with previous reports that, unless the peptide is retained in the ER by binding to the appropriate MHC class I molecule it is degraded either in the microsomal lumen or after release to the cytosol [14].

No SIINFEKL could be recovered when processing of ODC-LEova was carried out in the presence of TAP1-deficient microsomes instead of $B6.K^b$ microsomes, indicating that entry of the peptides into the microsomal lumen was necessary for further processing (Figure 2.8.2 C).
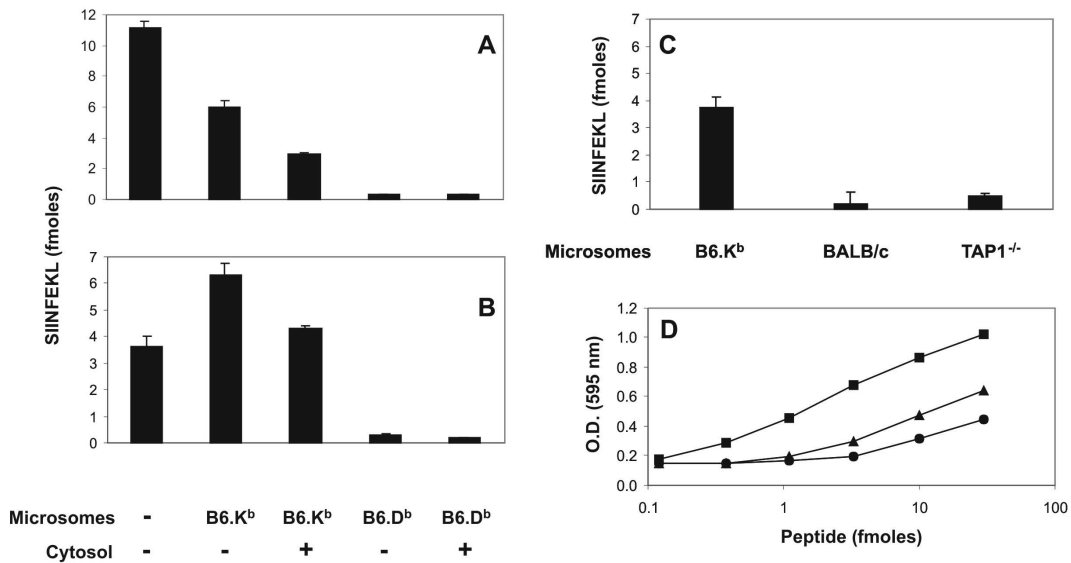
**Figure 2.8.2** *Processing of ODC-ova and ODC-LEova by the 26S proteasome in the presence of cytosol and microsomes.*

ODC-ova (A) and ODC-LEova (B and C) were incubated in a standard antigen processing reaction mixture with the indicated additions as described in 2.8.3.8. Following incubation at 37°C for 10 min, microsomes were isolated and extracted. The peptides were then purified by RP-HPLC and the amount of processed SIINFEKL was then quantified based on the ability to activate the B3Z T-cell hybridoma as described in 2.8.3.10. Results of A-C were calculated as the mean value of duplicate incubations. (D) Recognition of synthetic ODC-ova peptides by B3Z. Various amounts of synthetic peptides were incubated with $K^bL$ cells and then tested for recognition by the B3Z. Squares, SIINFEKL; triangles, ESIINFEKL; circles, HLESIINFEKL.

## 2.8.4.3  Processing of synthetic ESIINFEKL by isolated microsomes

In order to investigate the peptidase activity responsible for the N-terminal trimming, we studied the conversion of synthetic ESIINFEKL to SIINFEKL by isolated microsomes. As observed in the antigen processing experiments, incubation of synthetic ESIINFEKL with isolated B6.$K^b$ microsomes resulted in a remarkable enhancement of SIINFEKL recovery (Figure 2.8.3).
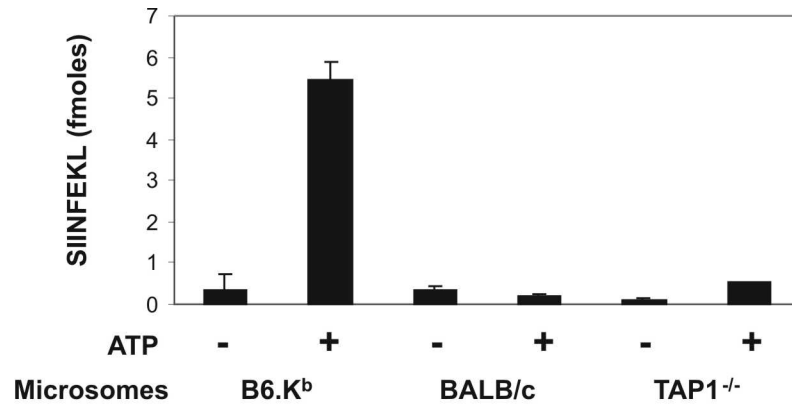
**Figure 2.8.3** *Processing of synthetic ESIINFEKL by isolated microsomes.*

Synthetic ESIINFEKL was incubated with 1 $OD_{280}$ unit of the indicated microsomes either in the presence or absence of ATP. Microsomes were then extracted and the peptides were isolated as described in "Experimental procedures" and in the legend to Figure 2.8.2. The amount of SIINFEKL was then determined by the B3Z activation assay as described under "Experimental procedures". Results were calculated as the mean value of duplicate incubations.

In agreement with the results of the ODC-LEova processing experiment, TAP1-/-, BALB/c as well as $B6.D^b$ microsomes could not generate SIINFEKL (Figure 2.8.3 and data not shown). To further characterize the specific trimming activity associated with the microsomes, we tested the sensitivity of the putative peptidase to the metallo-aminopeptidase inhibitor bestatin [64] as well as to the ion chelator PNT that was previously shown to inhibit various metallopeptidases [65]. We found that when both inhibitors were present, the ESIINFEKL to SIINFEKL conversion was completely blocked (Figure 2.8.4).
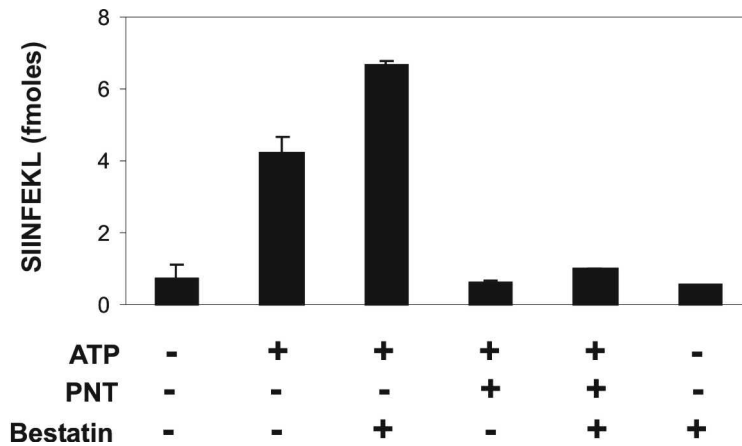
**Figure 2.8.4 *Effect of peptidase inhibitors on processing of synthetic ESIINFEKL by isolated microsomes.***

Synthetic ESIINFEKL was incubated with with 1 $OD_{280}$ unit of B6.$K^b$ microsomes with the indicated additions. Microsomes were then extracted and the amount of SIINFEKL was determined by the B3Z activation assay. Results were calculated as the mean value obtained in two independent experiments.

PNT alone was sufficient to sustain the block. Surprisingly however, in the presence of bestatin alone, there was a significant augmentation in the yield of SIINFEKL. These results propose that at least two types of peptidases influence the fate of ESIINFEKL: a bestatin-sensitive peptidase that had a deleterious effect and a PNTsensitive "trimmase" that converted ESIINFEKL to SIINFEKL. The PNT-mediated inhibition was not due to inhibition of peptide transport into the ER because the compound even slightly stimulated TAP-mediated peptide transport (data not shown). Although the processing of ESIINFEKL was dependent on TAP transport and was class I-specific it was still possible that peptidases operating at the cytoplasmic leaflet of the microsomes initially converted ESIINFEKL to SIINFEKL and that only subsequent transport into the ER lumen and binding to the correct class I molecule prevented further degradation of the peptide epitope. While the ultimate proof for lumenal localization of the trimming aminopeptidase would have been resistance to proteolysis, this approach could not have been applied, as treatment of microsomes with trypsin, for example, would have also digested TAP and prevented peptide transport. Thus, to establish the localization of the ESIINFEKL to SIINFEKL converting enzyme, we attempted to remove the external peptidases. To this end the microsomes were washed with 15 mM EDTA and 500 mM KCl. This washing procedure removed over 60% of the proteins

associated with the microsomes (data not shown) and should have removed almost entirely any peptidase activity associated with the outer face of the microsomes.
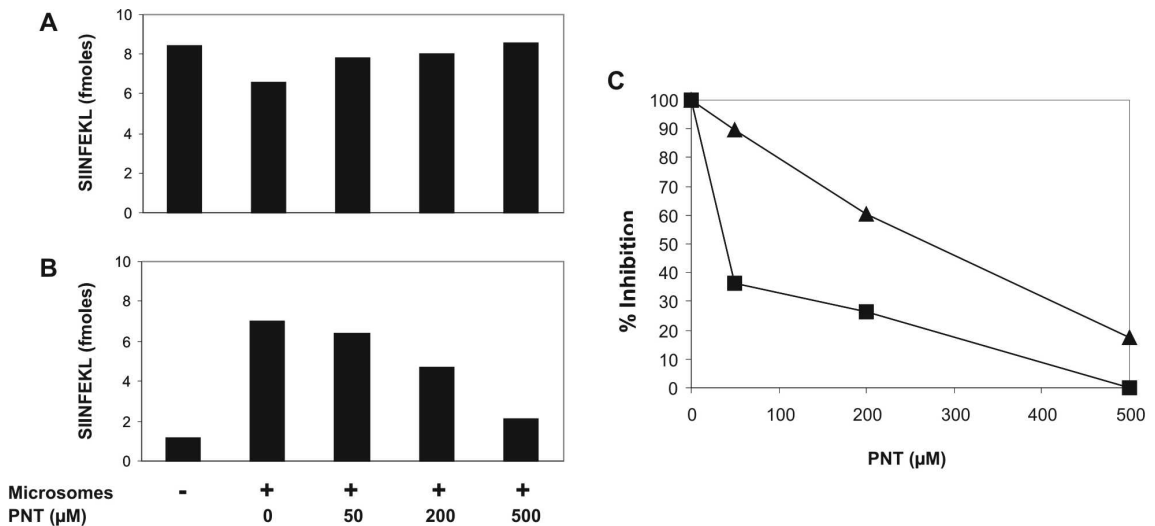


**Figure 2.8.5** *Inhibition of peptidase activity by 1, 10-phenanthroline (PNT).*
(A) Synthetic SIINFEKL was incubated in the absence of ATP, and (B) synthetic ESIINFEKL was incubated in the presence of ATP with B6.K$^b$ microsomes and with the indicated concentrations of PNT. Microsomes were then extracted and the amount of SIINFEKL was determined by the B3Z activation assay. (C) Quantification of the inhibition of peptidase activity. The percent inhibition of the external peptidase activity (squares) was calculated according to the following formula:

$$\frac{[(SIINFEKL\ without\ microsomes)-(SIINFEKL\ with\ microsomes\ plus\ PNT)]\times100}{[(SIINFEKL\ without\ microsomes)-(SIINFEKL\ with\ microsomes)]}$$

The percent inhibition of ESIINFEKL processing (triangles) was calculated as the ratio between the activity observed in the absence of PNT relative to that observed in the presence of the inhibitor after subtraction of the activity measured in the absence of microsomes in each case. The results were calculated as the mean of the values obtained in two independent experiments.

As shown in Figure 2.8.5, when synthetic SIINFEKL was incubated with the washed microsomes in the absence of ATP (i.e. when TAP transport was blocked) there was only a small reduction in the amount of SIINFEKL relative to the input level obtained in the control incubation with buffer alone (Figure 2.8.5 left and next to the left columns). Low and intermediate PNT concentrations (50-200 μM) were sufficient to restore the input level of SIINFEKL indicating that the remaining peptidase activity on the cytosolic face was completely inhibited (Figure 2.8.5 A and C). However, when ESIINFEKL was incubated with the washed microsomes in the presence of ATP, the conversion of ESIINFEKL to SIINFEKL was

significantly inhibited only at higher PNT concentrations (Figure 2.8.5 B and C). These results demonstrate that under conditions where the activity of the peptidase on the external surface of the microsomes was completely inhibited, there was an additional PNT-sensitive activity that became only apparent in the presence of ATP (see also Figure 2.8.4). We conclude that an ER-lumenal metallopeptidase contributes to the conversion of ESIINFEKL to SIINFEKL.

## 2.8.5 Discussion

In this work we have reconstituted *in vitro* the entire antigen processing pathway, from proteolysis of the antigen by the 26S proteasome to the binding of the final epitope to the specific MHC class I molecule in the ER. This cell-free system that is comprised of the 26S proteasome, a protein antigen, cytosol and isolated microsomes enables the analysis of the relative contribution of the proteasome, cytosolic and microsomal peptidases to the generation of the definite epitope. The major impediment for the analysis of the mechanism of the 26S proteasome in antigen processing lies in the difficulty to produce large enough ubiquitinated protein antigen for *in vitro* processing experiments to allow mass spectrometrical analysis of the cleavage products. We have overcome this crucial problem by targeting the protein antigen to the 26S proteasome through the ubiquitin-independent mechanism employed by ODC [31, 59]. By varying the amino acid residues immediately N-terminal to SIINFEKL we were able to generate an ODC-ova derivative ODC-LEova that was degraded at a similar rate by the 26S proteasome, however, the proteolytic processing resulted in a significant reduction in the abundance of SIINFEKL (see Figure 2.8.1 and Table 2.8.1). The fact that ESIINFEKL, the dominant extended SIINFEKL, was a poor activator of B3Z T cells allowed us to follow for the first time post-proteasomal processing activity in a physiologically relevant fully reconstituted cell-free system. Consistent with our previous results [59], we found by mass-spectrometrical quantification that the 26S proteasome efficiently liberates the SIINFEKL peptide from the ODC sequence context (...WQLMKQIQSH-SIINFEKL-SHGFPPEVEE...) while the N-terminal extended peptides HSIINFEKL and QSHSIINFEKL were only minor products. When introducing the extended epitope LESIINFEKL into the same ODC sequence context, the predominant cleavage now occurred between the N-

terminal Leu and Glu residues which is in full agreement with the cleavage specificities of both the 20S and the 26S human proteasome assayed on the model proteins enolase and β-casein, respectively [39, 41]. The 26S-processed SIINFEKL is efficiently delivered into the ER lumen despite losses that are due to the activity of peptidases co-purifying with ER membranes. The cytosolic origin of the SIINFEKL degrading peptidases is suggested by the finding that addition of cytosol led to further degradation (see Figure 2.8.2 A). Nevertheless, the significant proportion of SIINFEKL that was retained in the microsomes suggested that the TAP-mediated translocation of peptide occurred at a higher rate. Three cytosolic peptidases have thus far been implicated in the trimming of N-terminally extended peptide epitope precursors to their final size. Rock and co-workers have shown that a bestatin-sensitive leucine aminopeptidase can cleave SIINFEKL precursors to generate the final 8-mer [43, 44]. In a recent study, puromycin-sensitive aminopeptidase and bleomycine hydrolase have been implicated in the N-terminal trimming of a VSV nucleoprotein-derived epitope precursor [45]. These observations indicate that individual peptidases contribute to limited N-terminal trimming of epitope precursors. Nevertheless, the cumulative effect of cytosolic peptidases on SIINFEKL and its precursors seems to be deleterious. This can be concluded from our finding that addition of cytosol to the antigen processing reaction caused a major reduction in the amount of SIINFEKL retained in the ER (see Figure 2.8.2). In other experiments not presented in this study we found that cytosol completely destroyed 26S proteasome-processed peptides in the absence of microsomes, and that this degradation could not be inhibited by bestatin. The stimulatory effect of bestatin on the yield of SIINFEKL in the absence of cytosol (see Figure 2.8.4) likely resulted from inhibition of a particular membrane-associated peptidase(s). This also suggested that the bestatin-sensitive peptidase acted prior to TAP transport and thus limited the availability of peptide substrates. López and co-workers have recently provided evidence for a PNT-sensitive cytosolic aminopeptidase activity [28]. When the metallopeptidase inhibitor PNT was administered to cells infected with recombinant vaccinia virus expressing an HIV env epitope either in the context of the natural HIV envelope protein or of a recombinant HBV core protein, the authors noticed efficient blockade of antigen presentation. However, the processing of another recombinant HBc construct or a long epitope precursor targeted to the ER in a TAP-independent manner was not

affected by PNT suggesting that a PNT-sensitive peptidase was operative prior to TAP transport. We have also identified a PNT-sensitive peptidase associated with the external leaflet of the microsomal membranes. However, in addition we show that a distinct PNT-sensitive peptidase is operative in the lumen of the ER and that this lumenal peptidase is involved in the final processing of 26S proteasome processed peptides subsequent to introduction into the ER by TAP. This follows from our observation that the final epitope SIINFEKL was generated by EDTA/salt-stripped microsomes from ESIINFEKL when the activity of the external peptidase was inhibited and when peptide translocation was facilitated (see Figure 2.8.5). The presence of H2-K$^b$ was strictly required to convert ESIINFEKL to SIINFEKL whereas only background levels could be recovered from H2-D$^b$- or H2-K$^d$/D$^d$-expressing microsomes (Figure 2.8.2 B, C and Figure 2.8.3). This would invoke a model according to which N-terminal trimming is initiated by binding of the precursor peptide to the appropriate class I receptor followed by recruitment of the aminopeptidase to the class I/peptide complex as originally proposed by Rammensee and co-workers [51]. In this model, the class I molecules would prevent N-terminal trimming beyond the optimal peptide length. Earlier work has provided evidence that selected peptides could only be extracted from tissues expressing class I molecules able to associate with these peptides [66]. Furthermore, it has been shown that antigenic peptides that are not retained in the ER undergo retrotranslocation to the cytosol for degradation [13- 15, 58]. The herein presented findings that SIINFEKL processed from ODC-ova was undetectable in reactions containing H2-D$^b$- but not H2-K$^b$-expressing B6 microsomes (Figure 2.8.2 A), or H2-K$^d$-expressing BALB/c microsomes (unpublished result) are fully consistent with the idea that class I molecules retain and protect TAP-translocated peptides. Recently, Shastri and colleagues demonstrated the efficient conversion of an N-terminally extended SIINFEKL derivative in H2-K$^b$- but not H2-K$^d$-expressing cells [47]. We have shown that in a cell-free system the presence of H2-K$^b$ is essential for the recovery of SIINFEKL following processing of ESIINFEKL by a microsomal metallo-aminopeptidase. However, we cannot conclusively decide whether binding of the precursor peptide to the correct restriction element is strictly required for the initiation of the processing, or whether class I molecules merely capture partially digested peptides released from slowly acting luminal aminopeptidases in a random

process and protect them from further degradation. Additional work is required to distinguish between these two potential mechanisms. Furthermore, the general role of this aminopeptidase will have to be dissected using additional protein antigens.

## 2.8.6 References

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### 2.8.7 Abbreviations

PNT     1,10-phenanthrolin

Az     Antizyme

ODC     ornithine decarboxylase

### 2.8.8 Participating Researchers

These results are published by

Arthur Komlosh[*], Frank Momburg[§], Toni Weinschenk[¶], Hansjörg Schild[¶], Niels Emmerich[¶], Eran Nadav[*], Isabella Shaked[*], and Yuval Reiss[*]

[*]Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

[§]Division of Molecular Immunology, German Research Research Center (DKFZ), 69120 Heidelberg, Germany

[¶]Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany

### 2.8.9 Acknowledgments

## 2.9 An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope

The author of this dissertation, Toni Weinschenk, contributed the analysis of the major cleavage products generated by 26S proteasomes from the Nef-ODC-OVA fusion protein by liquid-capillary mass spectrometry, shown in Figure 2.9.1b, to this work.

### 2.9.1 Summary

Most of the peptides presented by major histocompatibility complex (MHC) class I molecules require processing by proteasomes. Tripeptidyl peptidase II (TPPII), an aminopeptidase with endoproteolytic activity, may also have a role in antigen processing. Here, we analyzed the processing and presentation of the immunodominant human immunodeficiency virus epitope HIV-Nef (73-82) in human dendritic cells. We found that inhibition of proteasome activity did not impair Nef (73-82) epitope presentation. In contrast, specific inhibition of TPPII led to a reduction of Nef (73-82) epitope presentation. We propose that TPPII can act in combination with or independent of the proteasome system and can generate epitopes that evade generation by the proteasome-system.

### 2.9.2 Introduction

The ability of an organism to eliminate viral infections relies largely on its capacity to generate peptides from viral antigens, which, in the context of major histocompatibility complex (MHC) class I molecules, can be presented on the cell surface to CD8^{+} cytotoxic T lymphocytes (CTLs). To allow binding to MHC molecules, intracellular proteins must be processed to smaller fragments, which are translocated by TAP (transporter associated with antigen processing) into the endoplasmic reticulum (ER). Finally, peptides of 8-11 residues in length, called epitopes, containing an appropriate binding motif bind to MHC class I molecules for transportation to the cell surface[1-5]. Most peptides that bind to MHC class I molecules are generated from cellular proteins by the 26*S* ubiquitin-proteasome

system, the major proteolytic machinery in the cytosol[5]. Its proteolytic activity is exerted by the 20S core proteasome[1,3] by three of the seven β-subunits in the two inner rings of the four-ring particle. The 20*S* core proteasomes are usually found associated with a 19*S* regulatory complex that binds to the outer α-rings of the 20*S* core to form the 26*S* proteasome. This complex is responsible for the binding of ubiquitin-tagged substrates and transportation of the substrates into the 20*S* core complex for processing. Stimulation of cells with interferon (IFN)-γ results in an exchange of the catalytic subunits, the formation of so-called immunoproteasomes and an adaptation of the proteasome to the specific requirements of an enhanced cellular immune response[1,3,5]. Apart from its ability to generate peptides of the appropriate length, the central role of the proteasome in the antiviral CD8^{+} T cell-dependent immune response is largely based on its intrinsic ability to efficiently generate the C-terminal anchor residue of an epitope, which allows its proper binding to the peptide binding groove of the MHC class I protein[1,3,5]. Many epitopes are generated as precursor peptides that carry the correct C terminus and an N-terminal extension of several residues[3,6-8]. These epitope precursor peptides require N-terminal trimming by aminopeptidases either in the ER[9-12] or in the cytosol[13,14]. MHC class I-associated epitope generation from the Nef protein encoded by human immunodeficiency virus 1 (HIV-1 Nef) has been extensively studied, as this protein may be a good target for vaccination against AIDS. The Nef protein is expressed early and its processed MHC class I epitopes are recognized by CD8^{+} T lymphocytes on the cell surface before structural proteins are synthesized. Thus, infected cells could potentially be lysed and viral replication inhibited before virus release. The proteasome is known to produce a substantial number of MHC class I-restricted epitopes from HIV-1 Nef in the context of different HLA haplotypes[15,16]. However, production of the HIV Nef epitope from amino acids 73-81 (HIV Nef (73-82)) that is restricted to both HLA-A*03 and HLA-A*11 (HLA-A*03/A*11) appeared to be insensitive to proteasome inhibition[16]. In agreement with this, it had been suggested that epitopes carrying a lysine residue at its C terminus, as is the case for HIV Nef (73-82), may be generated by proteasomes with strongly reduced efficiency[17]. These data indicated that there may be additional proteolytic pathways that are involved in the cytosolic processing of MHC class I epitopes. A protease that was previously suggested to also have a role in MHC class I antigen processing is the cytosolic

subtilisin-like tripeptidyl peptidase II (TPPII)[18,19]. Due to its aminopeptidase activity, TPPII was suggested to function as a post-proteasomal trimpeptidase for epitope precursor molecules[20]. In addition to its exo-peptidase activity, TPPII also exhibits endo-proteolytic cleavage properties and it is able to cleave after lysine residues[19]. Thus, TPPII may be a candidate protease for the generation of those epitopes that cannot be produced by the proteasome. We undertook these studies to identify the proteases that were essential for the generation of the HLA-A*03/A*11-restricted HIV Nef (73-82) epitope (QVPLRPMTYK) in human dendritic cells (DCs) expressing the full-length Nef protein. DCs are probable ports of entry for HIV during mucosal infection, which is the most frequent transmission mode, and they are the only antigen-presenting cells (APCs) that can stimulate naïve T lymphocytes (that is, at the onset of infection as well as for vaccination). Using biochemical and immunological approaches, we found that purified 20$S$ or 26$S$ proteasomes were unable to generate the HIV Nef (73-82) epitope *in vitro* from larger synthetic polypeptides or from an ODC-Nef fusion protein, and that specific inhibition of proteasomes in human DCs did not affect epitope presentation. We found that purified high-molecular weight TPPII generates the HIV Nef (73-82) epitope *in vitro* from a synthetic polypeptide, and that inhibition of TPPII by AAF-CMK or by TPPII-specific small interfering RNA (siRNA) resulted in abrogation of epitope presentation *in vivo*. We propose that TPPII can work in combination with the proteasome or independent of the proteasome system to generate a subset of those epitopes that evade generation by the proteasome.

### 2.9.3 Materials and methods

*Cells*

DCs, Josk-M, T2 (TAP-deficient), T2-217 (T2 + immunoproteasome subunits[33]) and EBV-transformed lymphoblastoid cells were maintained in RPMI-1640 with 10% fetal calf serum (FCS). Nef (73-82)-specific CD8^{+} T cell lines were generated from HIV^{+} individuals from cohort studies established with approval of Cochin Hospital's ethics committee as described[34]. DCs were differentiated from elutriated monocytes cultured for 7 d in the presence of GM-CSF and IL-4[34].

*Antigen presentation assays*

HLA-*02[+] and HLA-A*03[+] DCs were infected with recombinant Copenhagen Vaccinia viruses encoding HIV-1 Lai nef or pol (VV.TG.1147 or 3167, Transgène, 5 plaque forming units (PFU) per cell, 5% FCS), or incubated with peptides overnight, then washed and assessed for viability. DCs were incubated with the CD8^{+} T cell lines and assayed for intracellular IFN-γ production in CD3^{+}CD8^{+} lymphocytes by flow cytometry as described[34].

*Peptides, Nef-ODC fusion protein and inhibitors*

The peptides EEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGL (HIV Lai Nef amino acids 64-100), PVTPQVPLRPMTYKAAVDL (Nef amino acids 69-87) and QVPLRPMTYK (Nef amino acids 73-82) were synthesized using standard Fmoc methodology on an Applied Biosystems (Norwalk, CT) 433A automated synthesizer at >90% purity by the peptide synthesis group of the Institute of Biochemistry-Charité. The peptide ILKEPVHGV (RT 476-484) was obtained from Neosystem (Strasbourg, France). Expression and purification of Nef-ODC-OVA and purification of the maltose binding protein-antizyme (MBP-AZ) were performed as described[22]. Protease inhibitors were epoxomicin (added at 10 μM 30 min before infection, then diluted to 2 μM; Alexis, Grünberg, Germany), and MG 132, bestatin, E64, AAFCMK (Sigma, Taufkirchen, Germany), ALLM, LLnL (Calbiochem, Schwalbach, Germany) and butabindide[26] (added 30 min after vaccinia infection). The fluorogenic calpain substrate II (Calbiochem) and the L-leucine *p*-nitroanilide substrate (Sigma) were used as controls to check for enzyme inhibitor activities in human DCs.

*Proteasome isolation*

The 20*S* and 26*S* proteasomes were essentially purified as described[22,35]. The 20*S* constitutive proteasomes were isolated from Josk-M and T2 (TAPdeficient) cells, and 20*S* immunoproteasomes were isolated from T2-217 (T2 + immunoproteasome subunits[33]) cells and from Josk-M cells stimulated for 72 h with 200 U/ml IFN-γ. The 26*S* proteasomes were purified from human red blood cells.

*TPPII purification*

TPPII was purified from human erythrocytes at 4°C. The 100,000$g$ supernatant obtained from washed and lysed erythrocytes was mixed with 100 g DEAE-Cellulose SERVACEL (SERVA, Heidelberg, Germany) in TEAD buffer (20 mM Tris/HCL (pH 7. 5), 1 mM EDTA, 1 mM NaN$_3$, 1 mM Dithioerythrit). After washing with TEAD, bound proteins were eluted with 500 mM NaCl in TEAD, then further fractionated by ammonium sulfate. Proteins precipitating between 35% and 70% saturation were pelleted at 15,000$g$, resuspended, dialyzed against TEAD and applied to a DEAE-Sephacel-column in 50 mM NaCl, TEAD. Proteins were eluted with a linear gradient of 50-500 mM NaCl, TEAD. Fractions containing H-AAF-MCA hydrolyzing activity were pooled. Residual proteasomes were removed by affinity chromatography with monoclonal antibody mcp2136. Unbound TPPII exhibiting H-AAFMCA hydrolyzing activity inhibited by H-AAF-CMK was further purified by successive chromatography on MonoQ, arginine-Sepharose 4B and Superose 6B. All columns were equilibrated in 20 mM HEPES (pH 7.2), 15% glycerol and 1 mM ATP. TPPII was eluted from Mono Q and arginine-Sepharose columns with linear increasing gradients (0-400 mM NaCl in HEPES, ATP, glycerol). The purity of TPPII was checked by SDS-PAGE combined with immunoblot analysis using polyclonal chicken anti-(human TPPII) Ig (Immunsystem, Uppsala, Sweden). In some preparations minor amounts of spectrin (major erythrocyte component) and α-actin copurified as judged by mass spectrometric sequencing (MS/MS) analysis. TPPII activity was confirmed by digestion of fluorogenic peptide substrate and its complete inhibition by butabindide. TPPII activity was insensitive to the proteasome inhibitor *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL).

*Peptide digestion and mass spectrometry*

Nef (64-100) polypeptide (20 µg) and 20*S* proteasomes (2 µg) were incubated in 300 µl assay buffer (20 mM HEPES/KOH, pH 7.8, 2 mM MgAc$_2$, 1 mM dithiothreitol) at 37°C for different times. Nef-ODC-OVA fusion protein was incubated with 26*S* proteasome[22]. Nef (64-100) and Nef (69-87) (10 µg) were incubated with 100 ng or 1 µg TPPII in 50 µl assay buffer and incubated for 3, 24 and 48 h at 37°C in the presence of the proteasome inhibitor LLnL. TPPII-dependent processing was sensitive to butabindide. Reversed-phase chromatography and mass spectrometric (MS) analyses performed online with an ion trap mass spectrometer (LCQ, Thermo-Finnigan, Engelsbach, Germany) equipped with an electrospray ion source were performed as described[35]. Peptides were identified by tandem mass spectrometry experiments. Cleavage products from the Nef- ODC-OVA fusion protein were analyzed as described[28].

*siRNAs and electroporation*

We synthesized 21-nucleotide interfering RNA duplexes with two 3' end overhang dT nucleotides in the antisense strand. The sequences of the antisense strands of the siRNAs targeting TPPII were 5'- GUGGCGAUGUGAAUACUGCdTdT-3', and the control scrambled oligoribonucleotide scrambled-siRNA was 5'-UGUAUAGGUGUG GGCACACdTdT-3' (Eurogentec, Seraing, Belgium). TPPII expression was decreased in transfected HeLa cells in fluorogenic substrate digestion assays and in immunoblots. Transfection used an ECM 830 square wave electroporation system (BTX, San Diego, CA). Briefly, 4 × 10$^5$ EBV-transformed lymphoblastic cells were washed twice in PBS and placed in 4-mm gap cuvettes in the presence of oligonucleotides, subjected to 5 cycles of 20 V for 10 ms separated by 500-ms gaps in electroporation buffer (120 mM KCl, 0.15 mM CaCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl$_2$, 50 mM glutathione, 2 mM ATP, pH 7.6). Cells were washed again and transferred to culture medium for 48 h before incubating with Vaccinia viruses and performing antigen presentation assays.

## 2.9.4  Results

### 2.9.4.1  HIV-1 Nef (73-83) is not generated by proteasomes

The HIV-1 Nef (73-82) epitope is an immunodominant CTL epitope recognized in 60% of infected patients in the context of both HLA-A*03 and HLA-A*11 MHC molecules[16,21]. To study the proteasome-mediated generation of this peptide, we digested a synthetic polypeptide derived from the Nef central immunodominant region (Nef amino acids 64-100) with 20S proteasomes *in vitro*. Independent of whether standard proteasomes or immunoproteasomes were used, we failed to generate the correct C terminus of the HLA-A*03-restricted Nef (73-82) epitope. In agreement with previous observations[16], we identified a dominant cleavage at residue Nef-Y81 that destroyed this major HLA-A*03 epitope. Dominant cleavages were also observed at residues Nef-F68, Nef-A84 and Nef-L87 within the two flanking regions of the epitope (Figure 2.9.1).
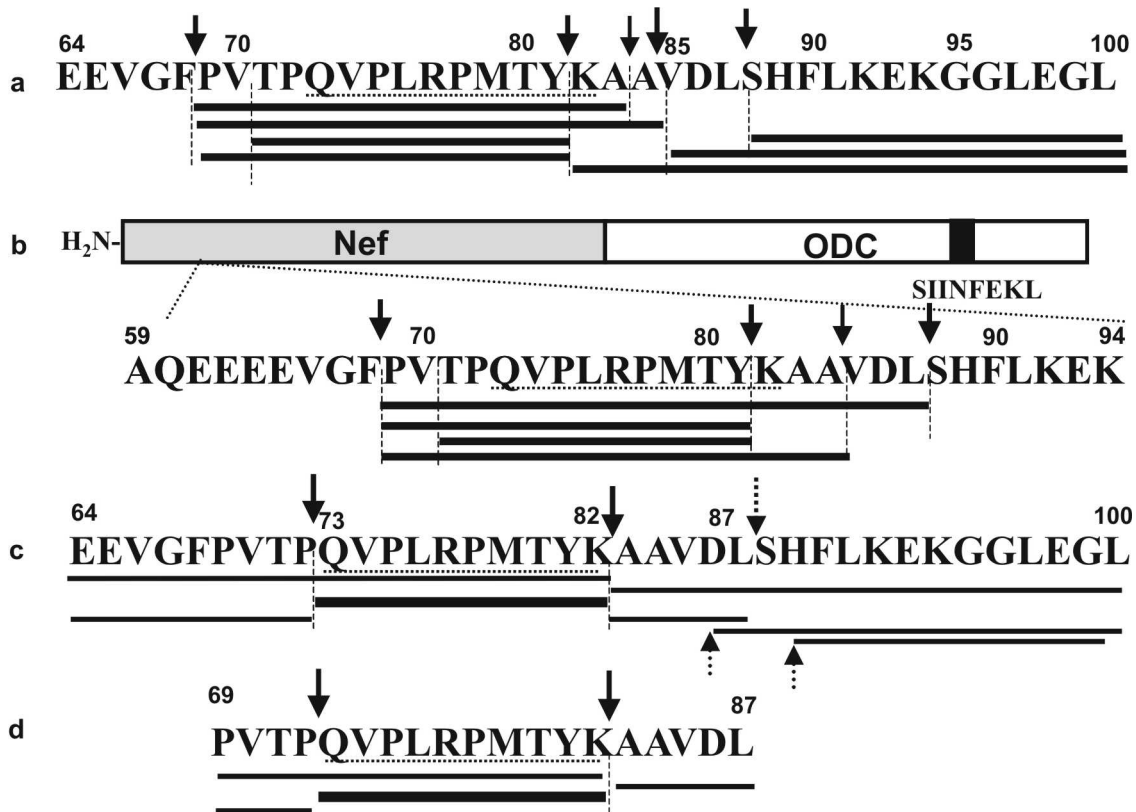
**Figure 2.9.1** *Proteasomes do not generate the HLA-A\*03/A\*11-restricted HIV-1 Nef (73-82) epitope, but TPPII does.*

(**a**) Major cleavage products generated by 20*S* proteasomes from the Nef (64-100) polypeptide. (**b**) Major cleavage products generated by 26*S* proteasomes from the Nef-ODC-OVA fusion protein. (**c**) Major fragments generated by TPPII from the Nef (64-100) polypeptide. (**d**) Fragments generated by TPPII from a synthetic Nef (69-87) polypeptide. In both experiments, fragments directly flanking the epitope were identified. Dotted arrows mark fragments that are the result of a tripeptidyl trimming reaction. Solid arrows mark major cleavage sites. Dotted lines underline the location of the Nef (73-82) epitope.

To exclude that the inability to generate the epitope was due to the use of 20*S* proteasome that is not the enzyme involved in antigen processing *in vivo*, we took advantage of the ornithine decarboxylase (ODC) system, which allows the ubiquitin-independent degradation of an ODC-fusion protein by 26*S* proteasomes in the presence of a chaperoning protein called antizyme[22]. Therefore, we constructed an ODC-Nef fusion protein containing the ovalbumin (OVA) epitope SIINFEKL as an internal control for antigen processing activity (Nef-ODC-OVA)[22]. Recombinant Nef-ODC-OVA was degraded *in vitro* by the 26*S* proteasome in the presence of recombinant antizyme, allowing the analysis of the degradation products of full-length Nef in a physiologically relevant system[22]. Although 26*S*

proteasomes produced the OVA epitope, they failed to produce the Nef (73-82) epitope or a peptide carrying the correct C-terminal residue (Figure 2.9.1). The 26$S$ proteasome degradation products also included the longer peptides Nef (69-84) and Nef (69-87), suggesting that these longer peptides were authentic Nef processing products. Thus, two different experimental approaches, which used either the 20$S$ core complex in combination with a Nef (64-100) polypeptide or the 26$S$ proteasomes in the context of an ODC-Nef fusion protein, resulted in the generation of an almost identical peptide pattern for the Nef region analyzed, but did not produce the desired epitope.

### 2.9.4.2  Nef (73-82) presentation was insensitive to epoxomicin

To test Nef (73-82) presentation by professional APCs, we infected HLA-A*03 human monocyte-derived DCs with the recombinant Vaccinia virus Vac-Nef, or as a control with Vac-RT, to induce the synthesis of HIV-1 Nef and reverse transcriptase, respectively. After overnight culture, Nef (73-82)-specific CD8$^+$ T cell lines derived from HIV-infected patients were added to the DCs and tested for intracellular production of IFN-γ. Flow cytometric measurement of the expression of IFN-γ in CD8$^+$ cells allows the assay of an effector response actually mediated by CD8$^+$ T cells. In accordance with the *in vitro* processing data that showed the inability of proteasomes to generate the Nef epitope, HIV-Nef (73-82) presentation was not inhibited by the 20$S$ and 26$S$ proteasome inhibitors epoxomicin or MG132 (Figure 2.9.2 a, b).
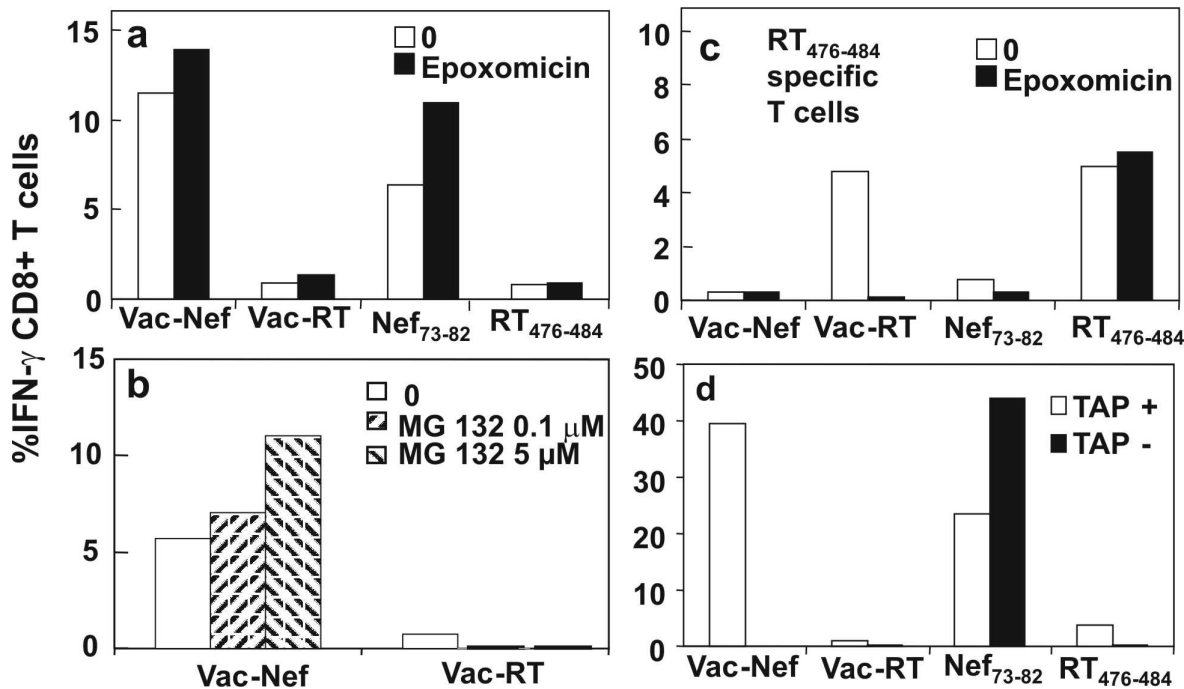
**Figure 2.9.2** *Proteasome-independent presentation of the HLA-A\*03/A\*11-restricted HIV-1 Nef (73-82) epitope in human DCs.*

DCs infected with recombinant Vaccinia viruses or incubated with peptides were cultured overnight, then cocultured with CD8^{+} T cells that were tested for intracellular IFN-γ secretion; epoxomicin was added at 10 µM for 30 min before Vaccinia or peptide incubation, then diluted to 2 µM. (**a**, **b**) Nef (73-82)-specific T cells. (**c**) RT (476-484)-specific CD8^{+} T cells, to test the same DCs as in (**a**) in parallel. (**d**) TAP-negative (ST-EMO37) or TAP-positive (EBV-1) HLA-A\*03 B lymphoblastoid cells were infected or incubated with peptides overnight, then tested with Nef (73-82)-specific T cells. All experiments are representative of at least two experiments, except epoxomicin was tested at least six times at similar concentrations for Nef-specific responses. The average response percentages in the presence of epoxomicin compared with the noninhibited control were, for anti-Nef (73-82) responses, 115 ± 38% for Vac-Nef *versus* 115 ± 46% for Nef (73-82) (not significant).

In contrast, recognition of the RT (476-484) epitope on Vac-RT infected, HLA-A\*02[+], HLA-A\*03[+] DCs by specific T cells was completely inhibited (Figure 2.9.2 c), as expected from its known proteasome-dependent generation[23]. These results raised the question of whether, in fact, HIV Nef (73-82) was generated by a cytosolic protease and therefore required transport from the cytosol into the ER by TAP for its presentation. We tested whether Nef (73-82) presentation after Vac-Nef infection was dependent on the presence of TAP. Surface presentation of Nef (73-82) was only observed in TAP-positive, EBV-transformed lymphoblastoid cells demonstrating the requirement for cytosolic Nef processing (Figure 2.9.2 d). The observed TAP dependence of Nef (73-82) presentation indicated that processing

of the peptide took place in the cytosol. This result, combined with the proteasome inhibitor independence of Nef (73-82) presentation, suggested that the proteasome system is either not rate-limiting or not responsible for the generation of this epitope. In an attempt to identify the proteases involved in Nef-epitope generation, we tested various inhibitors of cytosolic proteases that were previously discussed to have a role in antigen presentation[2,4,13,14]. Treatment of DCs with ALLM (inhibits calpains)[2], bestatin (inhibits various aminopeptidase including leucine amino peptidase and puromycin sensitive aminopeptidase)[14] or E64 (inhibits cysteine proteases, calpains and the exo- and endoproteolytic activities of bleomycin hydrolase)[14] revealed no effect on Nef (73-82) presentation (Fig. 3a, b).
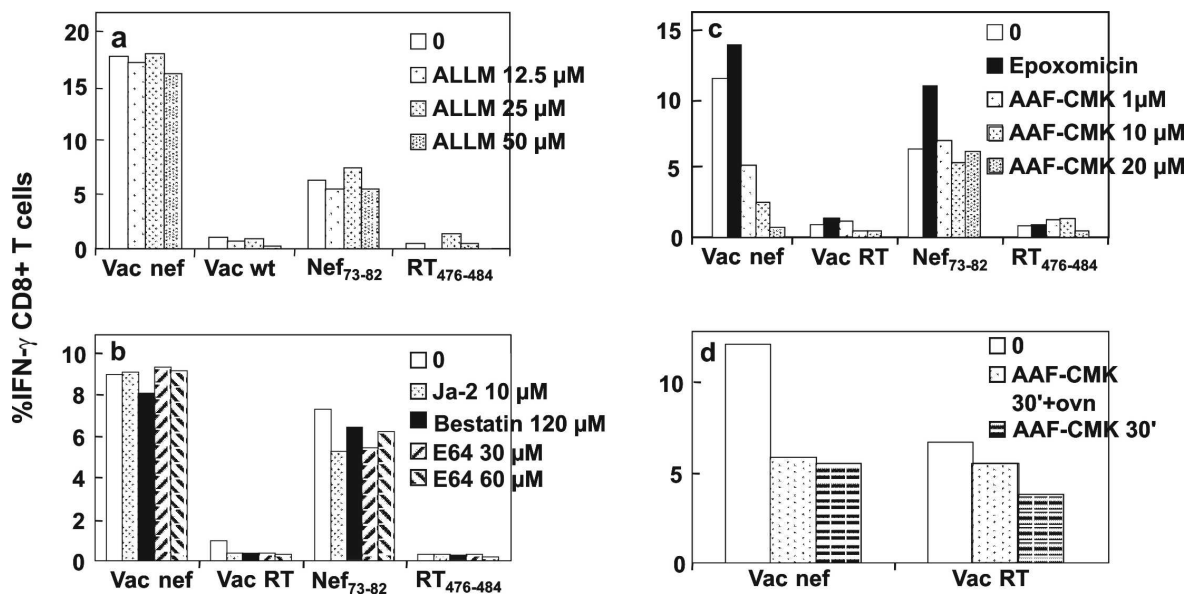


**Figure 2.9.3** *Irreversible inhibition of HLA-A\*03- Nef (73-82) presentation by the TPPII inhibitor AAF-CMK.*

(**a**, **b**, **c**) DCs were tested as in Figure 2.9.2 a. (**d**) AAF-CMK (2 μM) was added for 30 min, washed and then added again (or not) for overnight culture. All experiments are representative of at least two experiments, except ALLM dose response curve and bestatin were tested only once, and AAF-CMK was tested six times at similar concentrations. The average response percentage in the presence of AAF-CMK compared with the noninhibited control was decreased for Vac-Nef compared with Nef (73-82): 32 ± 17% *versus* 94 ± 12% (*P* = 0. 01).

At the concentrations used in these experiments, the inhibitors were active in human DCs, as ALLM and E64 inhibited fluorogenic calpain substrate II digestion, and bestatin inhibited L-leucine *p*-nitroanilide substrate digestion *in vitro* with cell extracts of inhibitor-treated DCs (data not shown). Thus, the involvement of

calpains[2], bleomycin hydrolase[14] or other cysteine proteases and leucine aminopeptidase[13] was unlikely.

### 2.9.4.3 Inhibition of HIV Nef (73-82) epitope presentation

The results of the experiments described above led us to speculate that the cytosolic protease TPPII[19,24] may be responsible for Nef (73-82) epitope generation. Thus, DCs were treated with the known TPPII inhibitor Ala-Ala-Phe-chloromethylketone (AAF-CMK). Low concentrations (1 μM) of AAF-CMK inhibited the presentation of Nef (73-82) by 60%, and 20 μM of AAF-CMK abolished epitope presentation (Figure 2.9.3 c). AAF-CMK also inhibited Nef-epitope presentation in EBV-transformed lymphoblastoid cells, indicating that the role of TPPII was not restricted to DCs (data not shown). To further substantiate the role of TPPII and exclude that of lysosomal TPPI, which is reversibly inhibited by AAF-CMK[25], AAF-CMK was added to Vac-Nef-infected human DCs for 30 min and then washed away. Even this short period of AAF-CMK treatment irreversibly inhibited the presentation of the Nef (73-82) epitope (Figure 2.9.3 d). Thus, our data suggested that TPPII might be the rate-limiting cytosolic protease involved in generation of the Nef (73-82) epitope. We hypothesized that if TPPII was responsible for Nef (73-82) epitope generation, then its endoproteolytic function would essentially be required for liberation of the Nef (73-82) epitope from a Nef (73-82)- containing polypeptide substrate.

### 2.9.4.4 Generation of the Nef (73-82) epitope by TPPII

To test the hypothesis that TPPII is responsible for Nef (73-82) epitope processing, we purified high-molecular-weight TPPII complexes to homogeneity from human erythrocytes. The identity of TPPII was verified by mass spectrometry, immunoblotting with TPPII antibody and its H-Ala-Ala-Phe-MCA hydrolyzing activity, which was completely inhibited by the TPPII-specific inhibitor butabindide[26]. The purity of the preparations was tested by SDS-PAGE (data not shown). To assay the antigen processing capacity of TPPII in *in vitro* processing experiments, we used the synthetic Nef (64-100) polypeptide containing the Nef (73-82) epitope as substrate (Figure 2.9.1 a). The analysis of the TPPII processing products showed that the enzyme generated the correct Nef (73-82) epitope with

high efficiency (Figure 2.9.1 c). This TPPII-dependent epitope generation seemed to be the consequence of two endoproteolytic cleavages behind residues Nef-P72 and Nef-K82. Thus, in contrast to the 20$S$ and 26$S$ proteasomes, TPPII used the Nef K82-A83 peptide bond at the C terminus of the Nef epitope as a preferential endoproteolytic cleavage site. In addition, and allowing the liberation of the correct epitope from the polypeptide substrate, TPPII also generated the correct N terminus of the epitope by cleavage after residue Nef-P72 (Figure 2.9.1 c). To study whether the length of the substrate influenced the efficiency of TPPII-dependent epitope production, we used a shorter polypeptide (Nef amino acids P69 through L87) that was shown to be a processing intermediate of the 20$S$ and 26$S$ proteasomes as substrate for TPPII processing (Figure 2.9.1 a, b). Again, TPPII used the C-terminal K82-A83 peptide bond as the major cleavage site (Figure 2.9.1 d). As evidenced by the identification of the N-terminal leaving fragment 69PVTP72, the generation of the correct N terminus was again the result of an endoproteolytic cleavage behind the Nef-P72 residue. The generation of the Nef (73-82) epitope by purified TPPII was impaired by butabindide, a specific inhibitor of TPPII, which, unlike AAF-CMK, did not permeate the cells.

### 2.9.4.5  TPPII siRNAs inhibit Nef (73-82) presentation

Both the inhibitor data and our *in vitro* processing data suggested that TPPII is most likely the rate-limiting cytosolic protease involved in generation of the Nef (73-82) epitope. To demonstrate that TPPII was indeed responsible for the generation of the Nef (73-82) epitope, Epstein Barr virus (EBV)-transformed lymphoblastoid cells were transfected with siRNAs for 48 h, then infected with Vac-Nef. Epitope recognition was inhibited up to 60% in a dose-dependent manner using the TPPII-specific siRNAs. In contrast, the scrambled control siRNAs had no effect on epitope presentation (Figure 2.9.4).
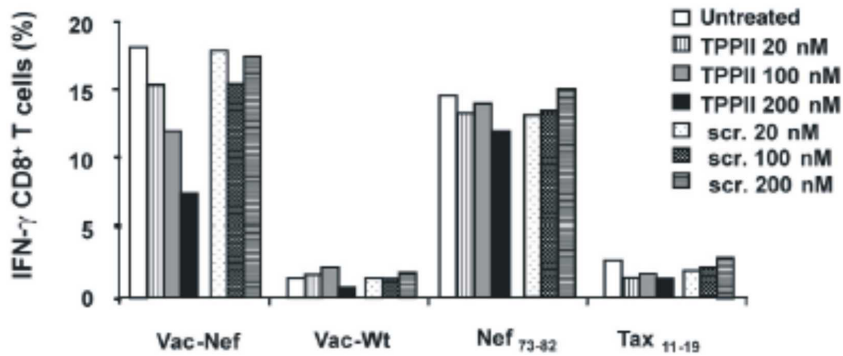
**Figure 2.9.4** *TPPII-specific siRNAs inhibit Nef (73-82) epitope presentation.*
EBV-1 cells were transfected with either TPPII-specific or scrambled siRNAs (scr.) for 48 h, infected or incubated with peptides overnight, then tested with Nef (73-82)-specific T cells. The experiment shown is representative of two.

Our combined *in vivo* and *in vitro* data presented evidence that TPPII can be the rate-limiting enzyme for the generation of a MHC class I epitope with the correct C terminus, which could not be supplied by the proteasome.

## 2.9.5  Discussion

Based on the findings of proteasome cleavage characteristics, inhibitor studies, and *in vitro* and *in vivo* antigen processing experiments[1-5], the proteasome seemed to be the only cellular enzyme with the capacity to generate the correct C termini and anchor residues of MHC class I ligands with the required efficiency. The involvement of other proteases in MHC class I antigen processing seemed to be restricted to the TAP-independent pathway[6,27] or the trimming of N-terminally extended epitope precursor peptides[11,12,14,28]. Previously it was found that loading of HLA-A*03 binding peptides or maturation of HLA-A*03, -A*11 and –B*35 molecules was insensitive to proteasome inhibition[17]. As lysine is not a preferred cleavage site for proteasomes, it was proposed that the frequent presence of lysine residues at the C terminus of HLA-A*03 epitopes may interfere with proteasome function[17,29]. Here we showed that cytosolic TPPII is essential for the efficient generation of the immunodominant HLA-A*03 and –A*11-restricted HIV-1 Nef (73-82) epitope, which possesses a lysine as the C-terminal anchor residue. From previous work in which proteasome activity was impaired by prolonged treatment of cells with the proteasome inhibitor lactacystin, it was proposed that

TPPII may be able to perform basic proteasome functions such as, for example, the removal of misfolded proteins and may support cell survival[19,30]. The analysis of an OVA-derived polypeptide showed that TPPII possessed endo-proteolytic activity in addition to its aminopeptidase activity and that it degraded larger polypeptides[19]. In agreement with this, we found that TPPII generated an immunodominant HLA-A*03 restricted CTL epitope by endo-proteolytic cleavages, indicating that TPPII can complement or even substitute proteasome function – also in the context of MHC class I antigen processing. It was suggested that the >2,000-kDa TPPII complex, which is composed of multiple 138-kDa subunits, may function as a compartmentalized enzyme like the proteasome[31]. Although TPPII is a relatively abundant protein, there is very little known so far about its physiological function. Our analysis of HIV-Nef protein processing supports the concept that, under physiological conditions, TPPII functions downstream of an active ubiquitin-proteasome system, because TPPII was able to generate the HLA-A*03 HIV Nef (73-82) epitope from proteasomal Nef-protein processing intermediates. An efficient peptide supply is essential for MHC class I antigen presentation. Therefore, a cooperative action between the proteasome system and TPPII would guarantee that the infected cell is able to generate epitopes that cannot be efficiently generated by the proteasome system. Our experiments, however, also demonstrated that inhibition of the proteasome system had no effect on TPPII-dependent production of the HLA-A*03 HIV-Nef (73-82) epitope. This suggests that TPPII can also work in parallel to the proteasome system and can contribute to the MHC class I peptide pool independent of the proteasome. Thus, HIV Nef (73-82) is most likely not the only epitope requiring TPPII activity. Such an independent role of TPPII may be of importance under conditions in which proteasome activity is impaired as a result of viral infections or metabolic stress. Thus, inhibition of proteasome activity seems to up-regulate TPPII activity[30], and it has also been shown that viral proteins like HIV-TAT can directly interfere with proteasome function[32]. These observations suggest that within the MHC class I antigen processing pathway, TPPII possesses a 'housekeeping' function under normal physiological conditions by taking up proteasomal processing intermediates, but that under conditions of physiological stress TPPII can also work independent of the proteasome and process protein with the help of chaperones or other yet to be defined protein factors.

## 2.9.6  References

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## 2.9.7  Participating Researchers

These results are published by

Ulrike Seifert[1], Concepción Marañón[2*], Ayelet Shmueli[3*], Jean-François Desoutter[2*], Lisa Wesoloski[1], Katharina Janek[1], Peter Henklein[1], Susanne Diescher[4], Muriel Andrieu[2], Henri de la Salle[5], Toni Weinschenk[6], Hansjörg Schild[6], Diego Laderach[7], Anne Galy[7], Gaby Haas[4*], Peter-M. Kloetzel[1], Yuval Reiss[3*†] and Anne Hosmalin[2*]

[1]Institut für Biochemie-Charité, Medical Faculty of the Humboldt-University Berlin, Monbijoustr. 2, 10117 Berlin, Germany. [2]Institut Cochin, Département d´Immunologie, U Inserm 567, CNRS UMR 8104, IFR 116, 27 rue du Fg St Jacques, 75014 Paris, France. [3]Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, 69978, Israel. [4]Max Planck Institut für Infektionsbiologie, Department of Molecular Biology, Schumannstrasse 20/21, 10117 Berlin, Germany. [5]INSERM EP99-08, EFS-Alsace, 10 rue Spielmann, 676065 Strasbourg, France. [6]Institute for Cell Biology, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany. [7]INSERM U362, Institut Gustave Roussy, Villejuif and Généthon, BP 60, 91002, Evry, France. †Present address: Proteologics Ltd., 2 Holzman str., Rehovot 76124, Israel. *These authors contributed equally to this work.

## 2.9.8  Acknowledgments

# Results, Part 3: Protein Identification

## 2.10  A Conserved Sequence in the Mouse Variable T Cell Receptor α Recombination Signal Sequence 23 bp Spacer Can Inhibit Recombination

The author of this dissertation contributed the mass spectrometrical protein identification to this work.

### 2.10.1 Summary

Although the variable gene segments coding for the TCR α and δ chains are mixed together in the α/δ locus and are recombined by the same machinery (RAG1 and RAG2) through recognition of the same signal sequences (Recombination Signal Sequences, RSS, with a 23 bp spacer), some gene fragments are rearranged only with TCR Jα gene segments (classified then as TRAV gene segments), some only with TCR Dδ gene segments (TRDV gene segments) and some with both (TRADV gene segments). No molecular signal is known which can characterize these three different types of gene segments. Studying the RSS of TCR variable gene segments we observed that 80% of the mouse TRAV gene segments contain a palindrome sequence (CTGCAG) or its related variant CTGTAG in their 23 bp spacer. Using gel shift assays we could show that these sequences are specifically recognized by some nuclear proteins expressed by fresh thymocytes, fresh lymphocytes and tumor cells. Two distinct complexes recognize this conserved sequence: one is specific for palindrome structures and one is specific for the CTGPyAG sequence. Spacers from TRDV or TRADV gene segments do normally not contain this sequence (only 1 in a total of 14 contains the CTGPyAG sequence) and are not recognized by the CTGPyAG-binding complexes. Using a plasmid substrate and the RAG1, RAG2 expressing

transformed pre-B cell 18.8, we could show that RSS containing the CTGCAG sequence are less efficiently used for recombination than RSS having no such sequence in their 23 bp spacer. We could identify three proteins which associate *in vitro* to a DNA sequence containing the CTGCAG motif: G3BP1, a nucleic acid binding protein with a proposed helicase activity and two proteins from the HMG family: HMGB2 (already known to participate in RAG-mediated recombination) and HMGB3. We hypothesize that these proteins might prevent recombination of the TRAV gene segments at the thymocyte CD4CD8 double negative stage when only TRDV and TRADV genes can recombine although the TCR α/δ locus is accessible to RAG proteins.

## 2.10.2 Materials and methods

### 2.10.2.1    Protein Identification

In gel tryptic digestion was performed as described [1] and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega) at a concentration of 67 ng/µl in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1-2 µl were left and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method [2] was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a $N_2$ 337 nm laser, gridless pulsed ion extraction and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, England) equipped with a nanoflow electrospray ionization source. Gold-coated glass

capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [3].

## 2.10.3 Results

We observed, that 80% of the mouse TRAV gene segments contain a palindrome sequence (CTGCAG) or its related variant CTGTAG in their 23 bp spacer. Using gel shift assays we could show that these sequences are specifically recognized by some nuclear proteins. Point mutations in but not outside the CTGPyAG sequence abrogated its recognition by nuclear factors. Two distinct complexes recognized this conserved sequence: one was specific for palindrome structures and one was specific for the CTGPyAG sequence. The CTGPyAG binding complex was present in all four thymocyte sub-populations. Using a plasmid substrate and the RAG1, RAG2 expressing transformed pre-B cell 18.8, we could show that RSS containing the CTGCAG sequence are less efficiently used for recombination than RSS having no such sequence in their 23 bp spacer (data for all the findings up to this point are not shown in this thesis). We made attempts to identify the proteins contained in the two CTGPyAG-binding complexes seen on gel shift assays. Using a two step approach where nuclear extracts were first enriched with CTGCAG-binding activity through the utilization of immobilized double stranded oligonucleotides containing this sequence and a subsequent FPLC separation of the oligo-attached proteins, we could unambiguously identify at least three proteins contained in the non-palindrome-specific complex (the lower band on the gel shifts). Figure 2.10.1 details the two steps of this purification process: the upper panel shows the profile of elution of proteins associated to streptavidin-agarose via biotinylated double stranded TRAV7D-3 oligonucleotides (in the presence of an excess of free double stranded oligonuleotide not containing the CTGCAG sequence: the TRAV7D-3 Mut 2 double stranded oligonucleotide). Each fraction from this chromatography was tested in EMSA where the radioactive TRAV7D-3 23 bp spacer was used as a probe and incubated with the FPLC fractions in the presence of an excess of an oligonucleotide containing a CTGCAG sequence (marked A on Figure 2.10.1 A) or

an excess of a TRD-V-derived RSS 23 bp spacer that does not contain a CTGCAG sequence (double stranded oligonucleotide representing the 23bp spacer from TRDV6-2 marked D on Figure 2.10.1 A). In fractions C9 to C14 the binding to TRAV7D-3 is competed away with a CTGCAG-containing oligonucleotide pair but not with an irrelevant oligonucleotide pair. This shows that in these fractions we detected the CTGCAG-specific complex. All fractions containing the protein from the lower complex were pooled (pool 1) and all fractions containing the palindrome-specific complex were mixed in pool 2. Each pool contains many different proteins (data not shown) and was consequently further fractionated by an FPLC system using a monoQ column. The fractions eluted from this column were tested again in EMSA using a radioactive TRAV7D-3 23bp spacer double stranded oligonucleotide. All the palindrome specific activity from pool 2 was lost after FPLC (data not shown). On the contrary, two FPLC fractions from pool 1 contain the CTGCAG-binding activity. These two fractions were separated on a acrylamide gel (Figure 2.10.1 C) together with flanking fractions and we could clearly visualize four proteins on the gel especially in the fraction C1 which shows the strongest CTGCAG-binding activity in EMSA (Figure 2.10.1 B). Mass spectrometry analysis of these 4 bands showed that they correspond to three proteins: G3BP1 (Ras-GTPase-activating protein SH3-domain-binding protein) present as full length (protein P1 on Figure 2.10.1 C) and as a sub-fragment (protein P2 on Figure 2.10.1 C) has nucleic acid-binding activity and a proposed helicase function, High Mobility Group protein 2 (HMGB2 previously called HMG2, protein P4 on Figure 2.10.1 C) already known to participate in RAG-mediated recombination and HMGB3 (previously called HMG2a or HMG4, protein P3 on Figure 2.10.1 C) . While G3BP1 and HMGB2 are ubiquitously expressed, HMGB3 is supposed to be expressed mainly in the embryo but still, ESTs corresponding to HMGB3 can be found in cDNA libraries from adult tissues like skeletal muscle, thymus, testis and liver [4].
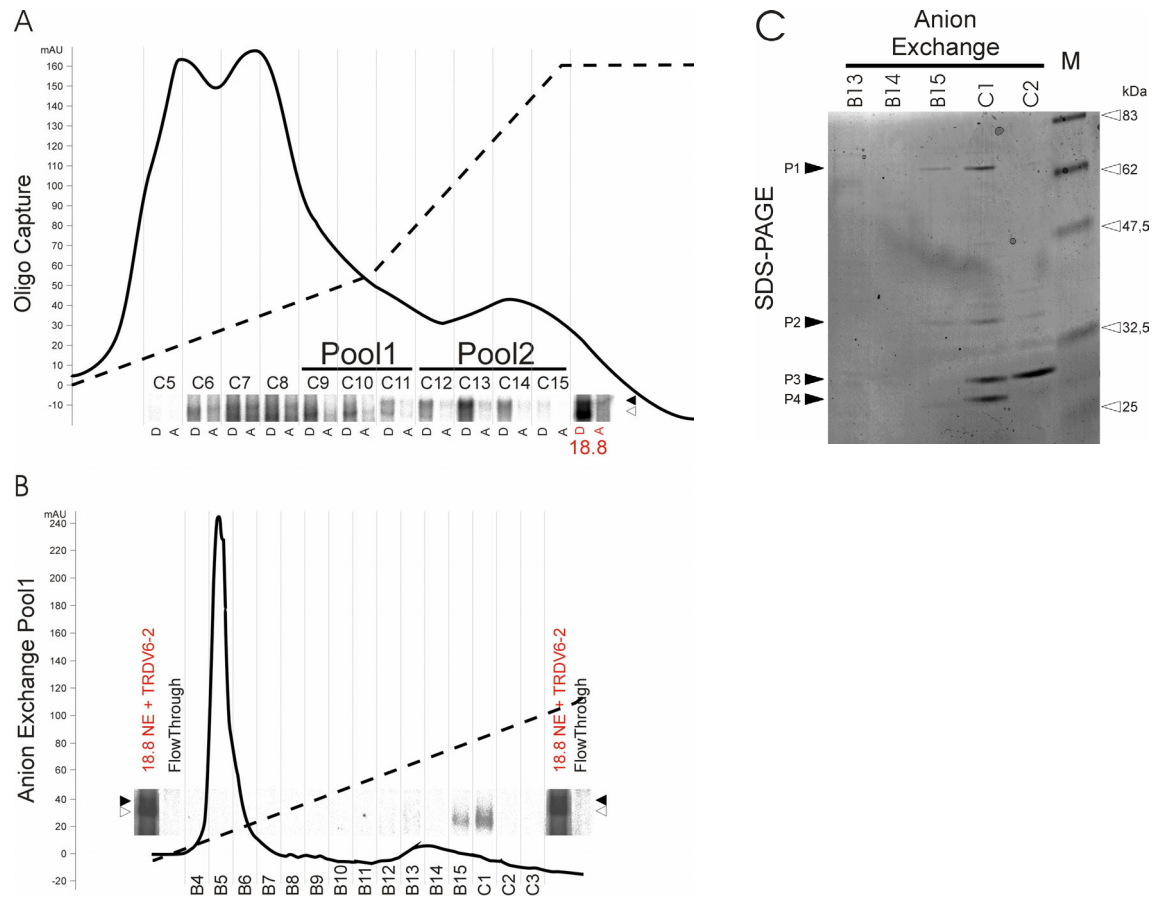
**Figure 2.10.1** *Identification of the CTGPyAG-specific nuclear proteins.*

To identify the proteins that bind specifically to the CTGPyAG sequence contained in the TRAV RSS 23 bp spacer, a 18.8 nuclear extract was mixed with a biotinylated double stranded oligonucleotide that contains a CTGCAG sequence in the presence of an excess of non-specific competitors: double stranded oligonucleotide that is mutated for the palindrome (TRAV7D-3 Mut 2) and poly dI:dC DNA. Biotinylated oligonucleotides were immobilized on a streptavidin column and co-precipitated proteins were eluted from the column usin increasing salt concentration. Fractions eluted from the column were tested by EMSA for the presence of the CTGCAG-binding complex (upper panel) when an excess of CTGCAG containing (A) or non-containing (D) non-labelled oligonucleotide was added. Two pools were made by mixing together consecutive fractions which showed a similar profile in EMSA. Pool 2 was further fractionated using anion exchange and again each fraction was tested for the presence of CTGCAG-binding proteins by EMSA. The fractions that show binding as well as the immediate neighboring fractions were concentrated and run on a preparative SDS PAGE. The 4 dominant bands visualized after staining were cut out and analyzed by mass spectrometry.

## 2.10.4 Discussion

From all the results in the present work, only the identified proteins are discussed here in this thesis. The larger (68 kDa) of the three proteins involved in CTGPyAG recognition, G3BP1 was first identified in human cells as a protein interacting with GAP (ras-GTPase-Activating Protein) which is a negative regulator of Ras[5]. Although no spliced variant was found for G3BP1, the related G3BP2 gene can be alternatively spliced in mouse cells [6]. Such a yet un-identified splicing event in G3BP1 pre-mRNA might generate the two G3BP1 proteins that we observed with an apparent mass of 68 kDa for the full length protein and of 34 kDa for the protein potentially encoded by the uncharacterized short splice variant. As it is the case for the CTGPyAG-binding complex, G3BP1 is found to be ubiquitously expressed but a closer analysis of mouse spleens indicated a cell specific staining [7]. The identity of the cells expressing or not G3BP1 in lymphoid tissues has not yet been determined. The two known roles of G3BP1 are its RNase activity (cleavage in-between C and A residues in AU rich regions at the 3' UTR of unstable RNA like the c-myc RNA [8]) and its helicase capacity [9]. Besides, G3BP1 contains an RNA recognition motif (RRM) that also exists in other proteins like SSAP (Stage-Specific Activator Protein). SSAP is a sequence-specific DNA-binding protein that recognizes DNA through its RRM domain. Interestingly, the DNA sequence recognized by SSAP is a specific palindrome: TTTAAA. Since G3BP1 contains a RRM domain, it can also be expected to bind DNA in a sequence specific manner. The localization and the activity of G3BP1 are regulated by phosphorylation of serine residues [10]: hypophosphorylated in dividing cells, it localizes mainly in the cytosol and interacts with GAP, hyperphosphorylated in quiescent cells, it is translocated in the nucleus due to its nuclear transport 2 (NTF2)-like domain [11] and is active as an RNA-specific endonuclease [10, 8]. Nevertheless, G3BP1 can be detected and purified from the nuclei of dividing HeLa cells [9] in which it was shown to have a helicase activity. The ubiquitous expression of G3BP1, its regulated localization and activity, its homology to the sequence specific DNA-binding SSAP as well as its DNA helicase activity fit with the experimental and expected characteristics of the proteins contained in the CTGPyAG-binding complex. Further experiments will be required to precisely characterize the role of G3BP1 in TCR-alpha recombination and the

engineering of G3BP1 knock out mice might allow us to get more insights into the role of the CTGPyAG sequence in TCR recombination. The two proteins that are found in the CTGPyAG-binding complex on top of G3BP1 are from the High mobililty group proteins B (HMGB): HMGB2 and HMGB3. Proteins of the HMGB family [12] have acidic tails that allow them to bind DNA in the minor groove and in a structure- rather than sequence-dependent manner [13]. In humans, HMGB3 (previously called HMG4 or HMG2a) is expressed during embryonic development and is found to be expressed in adult placenta and skeletal muscles [14] (thymus was not examined in that study). Since ESTs coding for HMGB3 are found in a library made from adult thymus (as well as from cDNA libraries made from adult testis and liver), the protein must be expressed also in this organ [4]. The fine tuning of the HMGB3 protein expression suggests that it may be a key player in the regulatory role of the CTGPyAG-binding complex on TRAV gene segments recombination. The smallest identified band corresponds to HMGB2 (previously called HMG2). Numerous evidences have indicated that this abundant and ubiquitous protein participates in RAG-mediated recombination together with HMGB1 (previously called HMG1). These two proteins improve RAG-mediated cleavages of DNA especially at a 23bp RSS through both enhancement of the bending of the DNA and stabilization of the RAG1-RAG2-RSS complex [15, 16, 17]. HMGB2-deficient mice are viable but have a defect in sperm development [18]. They have a normal thymus and normal numbers of T and B cells. A detailed study of the TRAV genes expressed by peripheral T cells would have to be done in order to eventually identify deregulation in the identity of TRAV, TRDV or TRADV gene segments that would fit with our identification of HMG2B as part of the CTGPyAG-binding complex. As hypothesized for the RAG machinery, HMG proteins might stabilize the DNA-specific recognition of the CTGPyAG sequence by G3BP1. Besides, as suggested above, the regulation of HMG2B expression may account for stage specific activity of the CTGPyAG-binding complex. A fine study of G3BP1 and HMG2B gene expression in purified thymocytes subpopulations using real time quantitative PCR should also help in the characterization of the involvement of these proteins in the TRAV genes rearrangement.

Besides G3BP1, HMGB2 and HMGB3 that are contained in the CTGPyAG-specific binding complex, some other proteins contained in the palindrome specific

complex (pool 2 in Figure 2.10.1) might be involved in the regulation of TRAV recombination through recognition of the 23bp spacer. Using one step purification of the CTGCAG-binding protein by fishing with oligonucleotides, many proteins could be identified on a SDS PAGE gel (not shown). Some might be non-specific DNA binding proteins (although we used an excess of non specific DNA oligonucleotide that does not contain the CTGCAG sequence during the purification) but some might be part of the palindrome-specific complex that is lost during anion exchange HPLC. Interestingly, with such one step preparation we could visualize on a preparative SDS PAGE a strong band at a size of 95 kDa which contained both NP95, a potential DNA binding protein, and nucleolin which is a DNA Helicase [9]. More investigations are necessary to confirm the presence of these proteins in the palindrome-specific complex.

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## 2.10.6 Abbreviations

| | |
|---|---|
| TCR | T cell receptor |
| RSS | recombination signal sequences |
| TRAV | variable gene segments coding for the TCR α and δ chains which are rearranged only with TCR Jα gene segments |
| TRDV | variable gene segments coding for the TCR α and δ chains which are rearranged only with TCR Dδ gene segments |
| TRADV | variable gene segments coding for the TCR α and δ chains which are rearranged with both, TCR Jα and TCR Dδ gene segments |
| FPLC | fast performance liquid chromatography |
| EMSA | electrophoretic mobility shift assay |

## 2.10.7 Participating Researchers

These results are submitted for publication by

Jochen Probst[1], Sibylle G. Blumenthal[2], Stefan Tenzer[1], Toni Weinschenk[1], Jürgen Dittmer[2], Adrien Six[3], Hans-Georg Rammensee[1] and Steve Pascolo[1]

[1]Institute for Cell Biology, Department of Immunology; Auf der Morgenstelle 15; D-72076 Tuebingen, Germany

[2]Institute for Cell Biology, Department of Molecular Biology, Auf der Morgenstelle 15, D-72076 Tuebingen, Germany

[3]Institut Pasteur, Departement d'Immunology, 28 rue du Dr. Roux, 75724 Paris Cedex 15

## 2.11   Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels: selective targeting of endothelial regulatory protein pigpen

The author of this dissertation contributed to this work by identifying the protein binding to the aptamer.

### 2.11.1 Summary

Tumor microvessels differ in structure and metabolic function from normal vasculature, and neoangiogenesis is associated with quantitative and qualitative changes in expression of endothelial proteins. Such molecules could serve as molecular addresses differentiating the tumor vasculature from those of the normal brain. We have applied Systematic Evolution of Ligands by EXponential enrichment (SELEX) against transformed endothelial cells as a complex target to select single-stranded DNA-ligands (aptamers) that function as histological markers to detect microvessels of rat experimental glioma, a fatal brain tumor that is highly vascularized. Both the SELEX selection procedure as well as subsequent deconvolution-SELEX were analyzed by fluorescence based methods (flow cytometry and fluorescence microscopy). Of 25 aptamers analyzed, one aptamer was selected that selectively bound microvessels of rat brain glioblastoma but not the vasculature of the normal rat brain including peritumoral areas. The molecular target protein of aptamer III.1 was isolated from endothelial cells by ligand-mediated magnetic DNA affinity purification. This protein was identified by mass spectrometry as rat homologue of mouse pigpen, a not widely known endothelial protein the expression of which parallels the transition from quiescent to angiogenic phenotypes *in vitro*. Because neoangiogenesis, the formation of new blood vessels, is a key feature of tumor development, the presented aptamer can be used as a probe to analyze pathological angiogenesis of glioblastoma. The presented data show that pigpen is highly expressed in tumor microvessels of experimental rat brain glioblastoma and may play an important role in warranting blood supply, thus growth of brain tumors.

## 2.11.2 Introduction

Systematic Evolution of Ligands by EXponential enrichment (SELEX) is a nucleic acid based combinatorial chemistry procedure that has been used to isolate relatively short high affinity ssDNA or RNA ligands, termed aptamers, to a wide variety of protein or low molecular weight compounds [14, 15]. Single-stranded nucleic acids, which can fold into very small and complex three-dimensional shapes with a great diversity of binding specificities, are isolated from a large pool of random sequence molecules ($10^{14}$-$10^{15}$ sequences) by reiterative rounds of selection and amplification [reviewed in 16]. Here we describe a fluorescence-based SELEX procedure. Using transformed EC as a complex target allowed systematic evolution of fluorescence-labeled oligonucleotides and subsequent *in situ* deconvolution-SELEX by flow cytometry and fluorescence microscopy on cryostat tissue sections (Figure 2.11.1). Finally aptamers were generated that function as a histological marker to selectively stain microvessels of experimental rat glioblastoma, a brain tumor that is highly vascularized [17]. We present aptamer III.1, a ssDNA-ligand binding to pigpen, a not widely known endothelial protein of the Ewing's sarcoma family that parallels the transition from quiescent to angiogenic phenotypes *in vitro* [18].
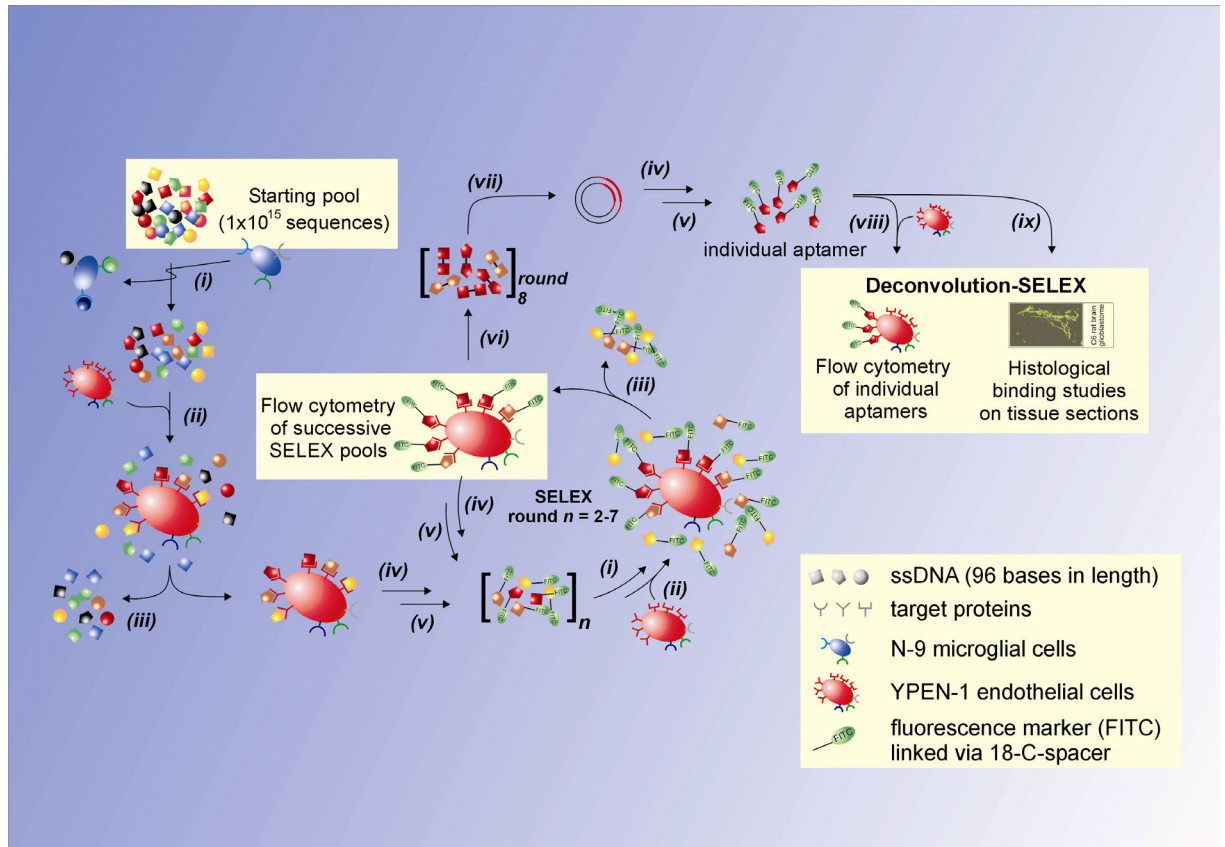
**Figure 2.11.1** *Selection procedure for the generation of DNA aptamers binding to angiogenic microvessels of rat experimental glioma.*

*(i)*, counterselection against N9 microglial cells (to reduce co-selection of aptamers binding to other cell types). *(ii)*, incubation of successive SELEX pools with YPEN-1 endothelial cells. *(iii)*, removal of unbound sequences. *(iv)*, PCR amplification of cell-bound aptamers using modified primers. *(v)*, FITC-ssDNA generation. *(vi)*, PCR amplification using unmodified primers for *(vii)* sorting round 8 pool aptamers into individual clones. *(viii)*, deconvolution-SELEX (step 1) of cloned aptamers as a preselection to evaluate aptamers binding to endothelial cells. *(ix),* deconvolution-SELEX (step 2) to evaluate preselected aptamers selectivity against tumor microvessels embedded in their natural surrounding on tissue sections of rat brain glioblastoma.

## 2.11.3 Materials and Methods

### 2.11.3.1    Ligand Mediated Protein Purification

1 mg (100 ml) of magnetic streptavidin beads were coated with 200 pmol of trB aptamer III.1 (MWG-Biotech AG) by incubation in 1 ml selection buffer (30 min, room temperature). As a control, 100 ml of magnetic beads were coated with 200 pmol of unselected FITC-ssDNA (trB-96-nt). $1.5 \times 10^8$ Ypen-1 endothelial cells were lysed in the presence of protease inhibitors as described [27]. After

centrifugation, the protein pellet was resuspended in 400 ml of selection buffer, treated by ultra-sonication (0°C, 20 s), and incubated with aptamer III.1-coated magnetic beads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer (total volume 1.5 ml, 0°C, 15 min). The protein-aptamer-magnetic bead-complex was removed in a magnet stand and washed four times (first wash: 1 ml of selection buffer with 150 mM NaCl; second through fifth wash: 200 ml of selection buffer with 100 mM NaCl with 2 nmol tRNA). Protein is removed from aptamer-coated beads by incubation in 30 ml of 1 M NaCl (0°C, 30 min) and analyzed by polyacrylamide gel electrophoresis after staining with Coomassie Blue.

### 2.11.3.2    Protein Identification

In gel tryptic digestion was performed as described [28] and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega) at a concentration of 67 ng/ml in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by two changes of 50% trifluoroacetic acid/50% water followed by two changes of 50% trifluoroacetic acid/50% acetonitrile. The combined extracts were vacuum-dried. The dried peptides were redissolved in 0.1% trifluoroacetic acid and purified using a ready-to-go pipette tip filled with C18 spherical silica reverse phase material (ZipTipC18™, Millipore). Peptides were eluted with 10 ml of 50% methanol/1% formic acid, and sequencing was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, England) equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). The needle was filled with 3 μl of the sample and subsequently opened by breaking the tapered end of the tip under a microscope. A stable spray was observed applying a needle voltage of 1200-1400 V, a back pressure of 2 p.s.i., and a source temperature of 40°C. The estimated flow rate was 20-50 nl/min. For nanoflow electrospray ionization tandem mass

spectrometry experiments, fragmentation was achieved by collision with argon atoms. Q1 was set to the mass of interest, and an optimized collision energy was applied. The integration time for the time of flight analyzer was 1 s with an interscan delay of 0.1 s. Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [29].

## 2.11.4 Results

### 2.11.4.1    Selection of ssDNA Aptamers that Bind Transformed Endothelial Cells

SELEX was used essentially as described [30] to generate ssDNA aptamers against Adenovirus-12 SV40-transformed YPEN-1 rat endothelial cells. The selection was started with a SELEX library of ~1 x $10^{15}$ sequences, each containing a central randomized region of 60 nt flanked by two primer hybridization sites [22].

Aptamers cloned from the eighth round of selection were evaluated by a two step deconvolution-SELEX procedure: (*i*) each sequence affinity against the transformed YPEN-1 EC cell line was preevaluated by flow cytometry, and (*ii*) the selective binding of aptamers to microvessels of C6 brain tumor was analyzed by fluorescence microscopy on cryostat tissue sections of rat brain glioblastoma. Of 25 sequences (FITC-18C-96-nt) tested by flow cytometry, by comparison of endothelial cells stained with unselected ssDNA (FITC-18C-96-nt), 23 showed binding to YPEN-1 endothelial cells. Of these, 16 bound to the pathological microvasculature of C6 glioblastoma, and 7 showed no binding. Aptamer III.1 displayed the most intensive staining of the vasculature exclusively in areas of solid tumor growth and was therefore chosen as the candidate to be further characterized as outlined below.

Fluorescence-labeled aptamer III.1-staining showed increased fluorescence intensity of YPEN-1 EC by comparison with unselected ssDNA (FITC-18C-96-nt) as a negative control in fluorescence-activated cell sorter histograms (not shown). EC-specific binding was confirmed by staining against N9 microglial cells, which was negative (not shown). Histological analyses of aptamer III.1. revealed staining of the complex architecture of the pathological microvasculature within the cell-rich

tumor region but not of vasculature in peritumoral areas. Thus, our histological data gave strong indication that aptamer III.1 binds an endothelial molecular target that is involved in the process of EC proliferation, a key step of angiogenesis.

### 2.11.4.2    Aptamer III.1-Mediated Target Identification

The molecular target has been isolated by magnetic DNA affinity purification. Solubilized EC proteins were incubated with trB aptamer III.1 beforehand coupled to magnetic streptavidin beads. After thorough washing and subsequent release of aptamer-bound protein in high salt solution, proteins were analyzed by SDS/polyacrylamide gel electrophoresis (Figure 2.11.2). Polyacrylamide gel analyses revealed a distinct band migrating at ~70 kDa in the lane of aptamer III.1, which is absent in the lane containing a product of the control reaction (performed with unselected ssDNA) which revealed one single band of ~50 kDa. Peptide mass fingerprinting and sequencing of three of the tryptic peptide fragments of the protein by mass spectrometry and tandem mass spectrometry, respectively, were used to identify the aptamer-specific protein as the rat homologue of mouse pigpen protein (67 kDa), a so far, widely unknown endothelial protein that has been considered to parallel the transition from quiescent to angiogenic phenotypes *in vitro* [31] (Figure 2.11.3).
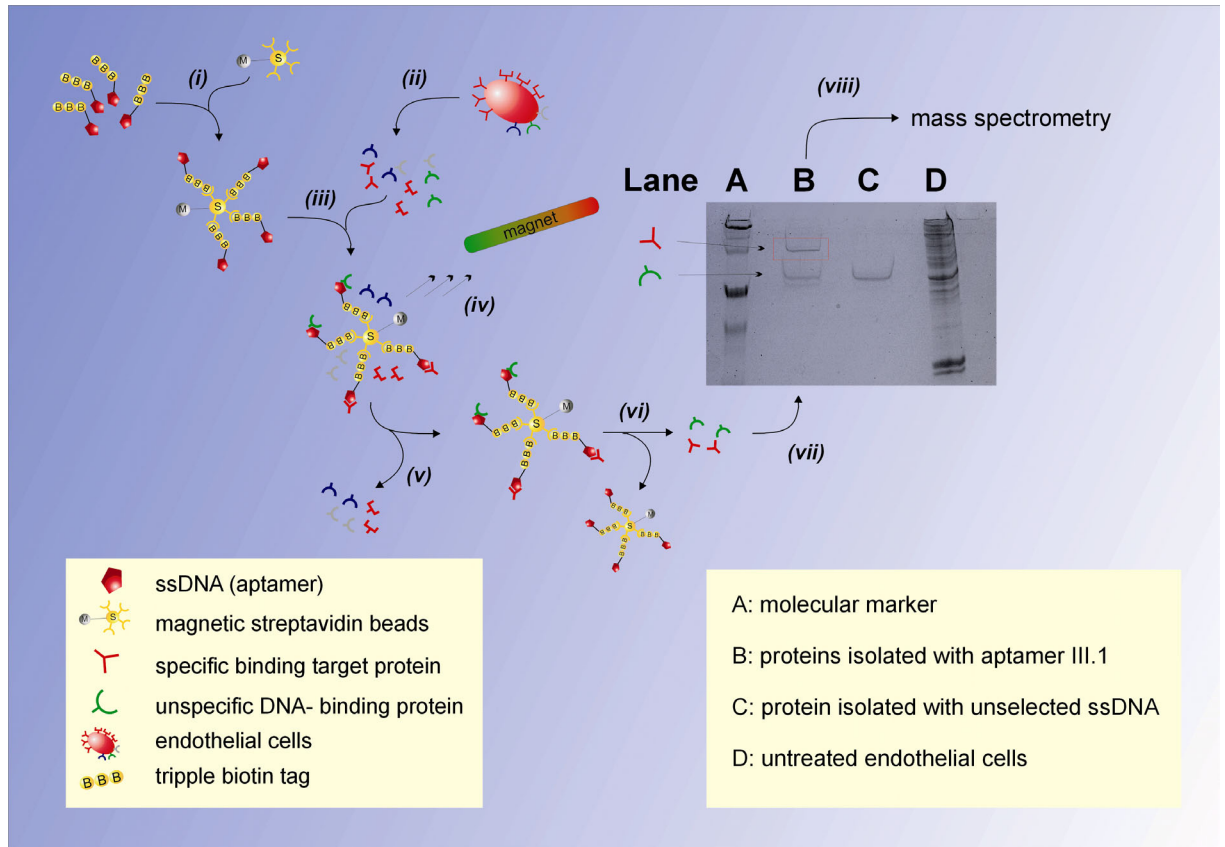
**Figure 2.11.2** *Coomassie Blue-stained polyacrylamide gel used to analyze the ligand-mediated target purification.*

*Lane A*, molecular marker. *Lane B*, purification with aptamer III.1. *Lane C*, purification with unselected ssDNA (trB-96-nt). *Lane D*, untreated endothelial cells. The target band isolated in the reaction containing the aptamer III.1 is indicated by an *asterisk* and was identified as the 67-kDa protein pigpen.

MASNDYTQQATQSYGAYPTQPGQGYSQQSSQPYGQQSYSGYGQSADTSGYGQSSYGSSYGQTQNTGYGTQSAP
QGYGSTGGYGSSQSSQSSYGQQSSYPGYGQQPAPSSTSGSYGGSSQSSSYGQPQSGGYGQQSGYGGQQQSYG
QQQSSYNPPQGYGQQNQYNSSSGGGGGGGGGGNYGQDQSSMSGGGGGGGGYGNQDQSGGGGGGGYGGGQQDR
GGRGRGGGGGYNRSSGGYEPRGRGGGRGGRGGMGGSDRGGFNKFGGPRDQGSRHDSEQDNSDNNTIFVQGLG
ENVTIESVADYFKQIGIIKTNKK**TGQPMINLYTDR**ETGK**LKGEATVSFDDPPSAKAAIDWFDGKEFSGNPIKVSFATR**R
ADFNRGGGNGRGGRGRGGPMGRGGYGGGGSGGGGRGGFPSGGGGGGGGQQRAGDWKCPNPTCENMNFSWRN
ECNQCKAPKPDGPGGGPGGSHMGGNYGDDRRGRGGYDRGGYRGRGGDRGGFRGGRGGGDRGGFGPGKMDSR
GEHRQDRRERPY

**Figure 2.11.3** *Sequence of murine pigpen and identified tryptic fragments (highlighted in grey).*

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## 2.11.6 Abbreviations

EC              endothelial cell(s)

SsDNA           singlestranded DNA

SELEX           Systematic Evolution of Ligands by Exponential enrichment

nt(s)           nucleotide(s)

FITC            fluorescein isothiocyanate

18C             18-carbon ethylene glycol spacer

trB             triple biotin

PCR             polymerase chain reaction

BSA             bovine serum albumin

DAPI            49,6 diamidino-2-phenylindole

## 2.11.7 Participating Researchers

These results are part of a publication by

Michael Blank[‡], Toni Weinschenk[¶], Martin Priemer[i], and Hermann Schluesener[‡]

From the [‡]Institute of Brain Research, University of Tuebingen, Calwer Strasse 3, D-72076 Tuebingen, Germany, the [¶]Institute for Cell Biology, Department of Immunology, University of Tuebingen, Auf der Morgenstelle 15, D-72076 Tuebingen, Germany, and the [i]Institute for Cell Biology, Department of Molecular Biology, University of Tuebingen, Auf der Morgenstelle 15, D-72076 Tuebingen, Germany

# 2.12 Broadband detection electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry to reveal enzymatically and chemically induced deamidation reactions within peptides

These results are part of a publication. Only data from the author of this dissertation are shown in the following chapters.

## 2.12.1 Introduction

Coeliac disease is a complex inflammatory disorder, influenced by both, environmental factors and genetic disposition. Main characteristics are intolerance to ingested wheat gluten, leading to chronic inflammation in the small intestine. Coeliac disease can be efficiently treated by gluten-free diet. Gluten consists of a complex mixture of many gliadin and glutenin polypeptides. They are rich in proline and glutamine residues and poor in glutamic acid and aspartic acid. Serum antibodies specific for gluten and the autoantigen tissue transglutaminase are found in celiac disease patients. Primary HLA association in most celiac-disease patients is with HLA-DQ2, and in a minority of patients with DQ8. There is strong evidence that these MHC molecules present disease-related peptides derived from gluten to CD4$^+$ T cells in the small intestine. Due to the preference of DQ2 and DQ8 for negatively charged residues at the anchor amino acids and the low frequency of glutamic and aspartic acid in gluten proteins, it was found that lesion-derived T cells predominantly recognize deamidated gluten peptides and that this deamidation can be mediated *in situ* by tissue transglutaminase. Current data suggest, that during an inflammation tissue transglutaminase is upregulated in the small intestine. In the presence of gluten from the diet, this leads to production of deamidated peptides, which can bind to DQ2 or DQ8 and which are recognized by CD4$^+$ T cells. Tolerance might only exist towards unmodified peptides [1].

Because enzymatic specificity of tissue transglutaminase is probably involved in selection of gluten T cell epitopes [2], it was analyzed which glutamine residue in the peptide LPFPQQPQQPFPQPQQ derived from γ-gliadin (85-100) is deamidated by tissue transglutaminase. The results of the following chapter support the observations described in the publication of Fleckenstein et al. [2] but are not shown therein.

## 2.12.2 Results

The peptide LPFPQQPQQPFPQPQQ derived from γ-gliadin (85-100), which was identified as a B-cell epitope in patients with coeliac disease [3], is enzymatically deamidated by tissue transglutaminase (tTGase), the autoantigen in celiac disease. It has a molecular mass of 1903.96 Da. The peptide was incubated with the commercial enzyme guinea pig (gp) tTGase for 4 h. After enzymatic treatment (done by Florian von der Mülbe), the sample was measured by nanoESI-MS and sequence analysis was done by CID. The overview spectra of the incubated peptide obtained after 0 h and 4 h is shown in Figure 2.12.1. After 4 h the molecular mass was increased by 1 Da due to the deamidation of Q (128 Da) to E (129 Da). Only small amounts were left unchanged (see small peak at 1904.3 Da after 4 h).
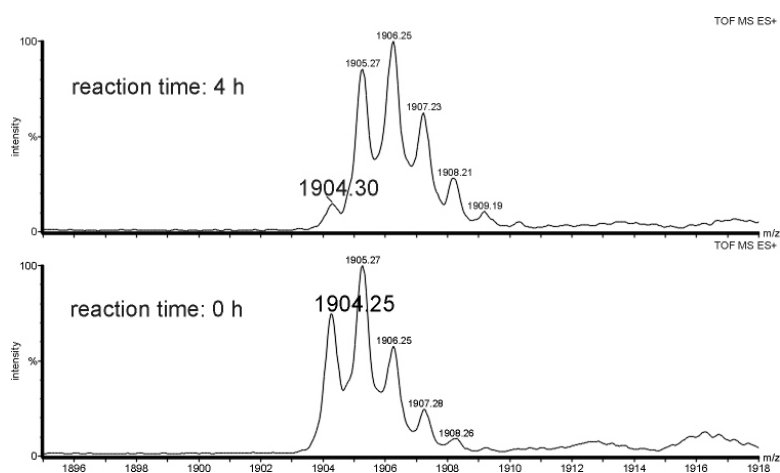


**Figure 2.12.1** *Overview spectra of peptide LPFPQQPQQPFPQPQQ before and after 4 h treatment with tTGase.*

To figure out which of the 7 glutamine residues has been converted to E, fragmentation was done by CID. Figure 2.12.2 shows masses of b- and y"-series fragments resulting from selected possible Q→E conversions.

```
A              583.3
                 711.3
b   1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
    L   P   F   P   Q   Q   P   Q   Q   P   F   P   Q   P   Q   Q
y"  16  15  14  13  12  11  10   9   8   7   6   5   4   3   2   1
                         1097.5
                             969.4
B                584.3
                 712.3
b   1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
    L   P   F   P   E   Q   P   Q   Q   P   F   P   Q   P   Q   Q
y"  16  15  14  13  12  11  10   9   8   7   6   5   4   3   2   1
                         1097.5
                             969.4
C                583.3
                 712.3
b   1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
    L   P   F   P   Q   E   P   Q   Q   P   F   P   Q   P   Q   Q
y"  16  15  14  13  12  11  10   9   8   7   6   5   4   3   2   1
                         1097.5
                             969.4
```
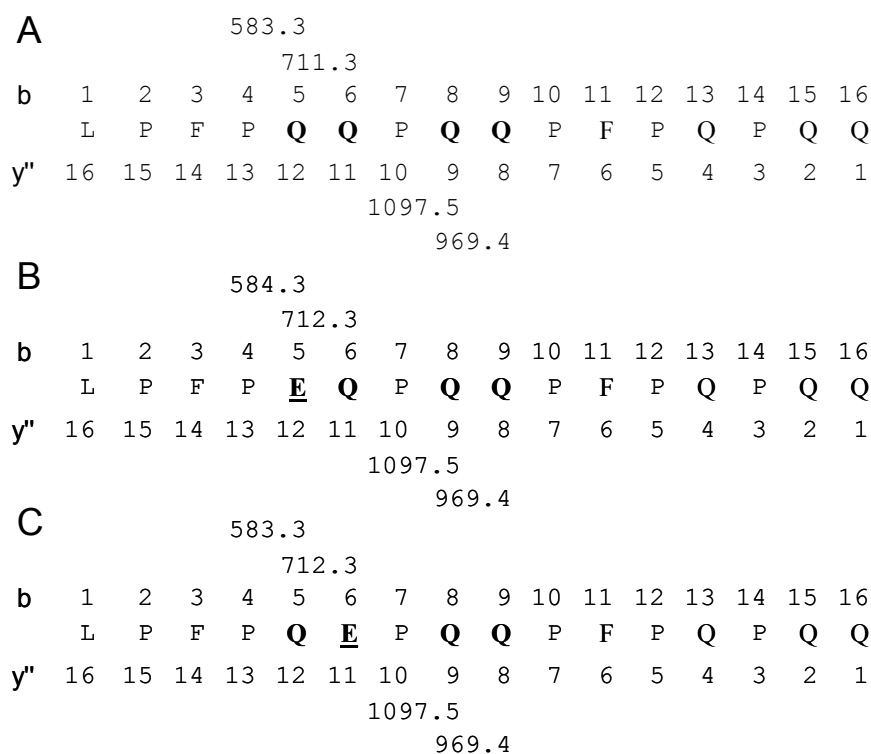
**Figure 2.12.2** *Possible deamidation products of peptide LPFPQQPQQPFPQPQQ and masses of corresponding b- (above) and y"-(below) series ions.* A original sequence, B Q5→E5 deamidation, C Q6→E6 deamidation.

A peak at m/z = 969.4 could still be detected after 4 h incubation, suggesting that Q9, Q13, Q15 and Q16 of this peptide were unchanged (not shown). After 4 h incubation with tTGase, the peak of 711.55 Da was almost completely shifted by 1 Da to 712.56 Da. This indicated that mainly Q5 or Q6 was deamidated, and that alternatively, to a smaller extent, Q8 was deamidated.
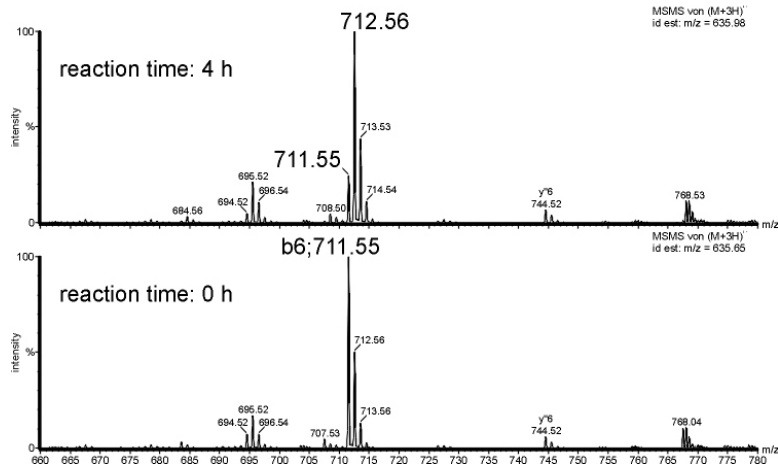
**Figure 2.12.3** *Fragment spectra of peptide LPFPQQPQQPFPQPQQ before and after 4 h treatment with tTGase.* Significant but not complete shift of 711.55 Da towards 712.56 Da indicates, that mainly Q5 or Q6 was deamidated.

The b5-ion allows to distinguish between deamidation Q5 or Q6. Because additional signals of other fragments (not b-series ions) were detected in the region of interest (583.5 Da or 584.5 Da, not shown), acetylation was done to increase all fragments containing the N-terminus by 42 Da. After this, a peak of 626.56 Da (584.5 Da + 42 Da) was seen, which has not been present before in the non-acetylated samples, showing that it was Q5 being deamidated to E after incubation with tTGase.
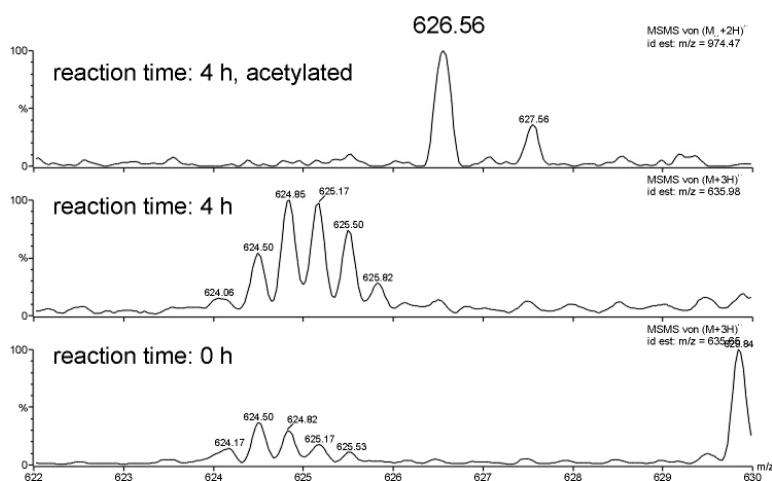


**Figure 2.12.4** *Glutamine at position 5 is deamidated to glutamic acid.* The peak of 626.56 Da corresponds to acetylated b-series fragment Ac-LPFPE.

Thus, it was shown that the peptide LPFPQQPQQPFPQPQQ from γ-gliadin (85-100) was mainly deamidated to LPFPEQPQQPFPQPQQ and to a lower extent to LPFPQQPEQPFPQPQQ by tTGase. This was in accordance to the results shown by Fleckenstein et al. and others [4]: human tissue transglutaminase preferred mostly Q in the motif QxP but not in QP or QxxP.

### 2.12.3 References

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3. Sollid,L.M. (2000). Molecular basis of celiac disease. Annu. Rev. Immunol. 18, 53-81.
4. Vader,L.W., de Ru,A., van der,W.Y., Kooy,Y.M., Benckhuijsen,W., Mearin,M.L., Drijfhout,J.W., van Veelen,P., and Koning,F. (2002). Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. J. Exp. Med. 195, 643-649.

### 2.12.4 Abbreviations

TTGase       tissue transglutaminase
Ac-           acetyl-

### 2.12.5 Participating Researchers

These results are part of a publication by
Dietmar G. Schmid,[†] Florian von der Mülbe,[†] Burkhard Fleckenstein,[‡] Toni Weinschenk[¶], and Günther Jung[†]

[†] Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany

[¶] Institute for Cell Biology, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany

[‡] Present address: Institute of Immunology, University of Oslo, Rikshospitalet, N-0027, Oslo, Norway

## 2.13 Release of regulators of angiogenesis following Hypocrellin-A and -B photodynamic therapy of human brain tumor cells

The author of this dissertation contributed the mass spectrometrical protein identification shown in Figure 2.13.3 to this work.

### 2.13.1 Summary

Photodynamic therapy (PDT) is an innovative strategy for the treatment of solid neoplasms of the brain. Aside from inducing cell death in tumor cells, PDT induces endothelial cell death and promotes formation of blood clots, however, exact mechanisms that trigger these phenomena remain largely unknown. We now used Western Blotting to analyze secretion of regulators of angiogenesis to the supernatants of one glioma, one macrophage and one endothelial cell line following Hypocrellin-A and -B photodynamic therapy. We observed induction of proangiogenic VEGF (vascular endothelial growth factor), and of antiangiogenic sFlt-1, angiostatin, p43, allograft inflammatory factor-1 and connective tissue growth factor. Release of thrombospondin-1 was diminished. Endostatin release was induced in glioma cells and reduced in macrophages and endothelial cells. These data show that a wide range of antiangiogenic factors is secreted by brain tumor cells following Hypocrellin photochemotherapy. However, VEGF release is also induced thus suggesting both favorable and deleterious effects on tumor outgrowth.

### 2.13.2 Introduction

During the course of maturation, brain tumor cells activate and recruit endothelial cells, thereby starting the fatal process of tumor tissue expansion that can only rarely be intercepted by conventional therapy strategies. Photodynamic therapy (PDT) has been extensively investigated *in vitro* and in clinical trials for the treatment of a variety of brain tumors, particularly gliomas [1]. The main advantage of PDT is considered to selectively target infiltrating tumor cells that are

predominantly responsible for local tumor recurrence and rapid formation of intracranial metastases remote from the primary tumor [2]. Subsequent light activation induces photooxidation, followed by selective tumor destruction via vascular and direct cellular mechanisms [3,4]. Aside from inducing cell death in tumor cells, PDT induces endothelial cell death and promotes formation of blood clots. Following sensitizer accumulation and irradiation, damage to sensitive sites within the microvasculature, namely to endothelial cells and the vascular basement membrane, is induced and leads to the establishment of thrombogenic sites within the vessel lumen. This initiates a physiological cascade of responses including platelet aggregation, the release of vasoactive molecules, leukocyte adhesion, increases in vascular permeability and vessel constriction. This results in tumor destruction by vascular collapse, blood flow stasis and tissue hemorrhages. However, exact mechanisms that trigger these phenomena remain largely unknown. Malignant brain tumors carry a lethal prognosis with a median survival of 15 months despite surgery, radiotherapy and chemotherapy [5,6]. PDT is, therefore, a logical therapeutic concept for brain tumors infiltrating into normal brain. However, no information is available about release of regulators of angiogenesis following PDT.

Angiogenesis plays an essential role not only in the physiological formation and maintenance of vessels, but also in pathological conditions that range from tumor growth to wound healing [7]. Several steps are involved in the successful formation of new blood vessels including the stimulation of endothelial cells by growth factors, subsequent degradation of the extracellular matrix by proteolytic enzymes followed by invasion of the extracellular matrix, migration and proliferation of endothelial cells and, finally, the formation of new capillary tubes [8]. The initiation of angiogenesis, the angiogenic switch, is dependent on a dynamic regulation between proangiogenic and antiangiogenic factors in the immediate environment of endothelial cells [9]. A positive balance in favor of angiogenic factors leads to new vessel formation, whereas the prevalence of antiangiogenic factors shifts the equilibrium to vessel quiescence or, even to vessel regression [10].

In order to give a comprehensive overview of the release of angiogenic factors in brain tumor cells after photodynamic therapy, we now used Western Blotting to analyze proangiogenic VEGF (vascular endothelial growth factor), its receptor

VEGFR-1 (vascular endothelial growth factor receptor-1, Flt-1), and antiangiogenic thrombospondin-1, angiostatin, AIF-1 (allograft inflammatory factor-1), connective tissue growth factor (CTGF), endothelial-monocyte-activating polypeptide II/p43 and endostatin/Collagen XVIII in the supernatants of LN229 glioma, U937 macrophage and SVHCEC brain endothelial cell lines following Hypocrellin-A and -B photodynamic therapy.

## 2.13.3 Materials and Methods

### 2.13.3.1    Cell culture

U937 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SVHCEC endothelial cell line was derived of brain endothelial cells and was a kind gift from Professor A. Muruganandam and has been described before [11]. The LN229 glioma cell line was a kind gift of Professor Michael Weller, Department of Neurology, Tübingen and has been described before [12]. Cells were raised in RPMI 1640 medium with Glutamax II (Gibco BRL, Paisley, Great Britain) containing 10% fetal calf serum (FCS, Gibco) and 100 units/ml penicillin and 100 μg/ml streptomycin (Fluka, Buchs, Switzerland) at 37°C and 5% $CO_2$.

### 2.13.3.2    Photodynamic therapy

Hypocrellin-A and -B were used as photosensitizers because they both share a broad absorption spectrum in the visible light range (not shown). Preliminary experiments were used to determine illumination times and Hypocrellin concentrations. Normal growth media were supplemented with a concentration of 5 μmol Hypocrellin A or Hypocrellin B (both Molecular Probes Europe BV, Leiden, The Netherlands) for 24 h in the dark. Cells were then irradiated using a 11-W Dulux lamp (Osram, Germany, not shown) placed 1 inch above the cell surface for 15 min at a light dose of approximately 4 mW/cm$^2$ as assessed using a luxmeter. All irradiations were performed at room temperature. Controls included cell preparations without illumination and Hypocrellin A- and -B supplementation.

### 2.13.3.3    Protein preparation and Western Blotting

Following stimulation, cell lines were washed twice with phosphate buffered saline, pH 7.5 and then resubstituted with serum-free medium. After 24 h incubation time, supernatants were collected by centrifugation and proteins were precipitated by acetone incubation for 15 min at -20°C. Cells were collected following trypsinization. The resulting pellet was washed twice with PBS, resuspended in running buffer containing 125 mM Tris base, 20 % glycerol, 2 % SDS, 1 % bromophenol blue and 2 % 2-ME, sonicated for 1 min and boiled for 10 min in a water bath. Protein concentration was determined using a Bradford assay with albumin as a standard. 50 µg of total protein was loaded, electrophoresed on a 10 % SDS-polyacrylamide gel and transferred to PVDF membranes (Biorad, Munich, Germany) by semi-dry blotting. Membranes were blocked with FCS and the primary monoclonal antibodies directed against vascular endothelial growth factor (VEGF, rabbit polyclonal, Ab-2, Oncogene Research Products, San Diego, CA, USA, PC37), vascular endothelial growth factor recepor-1 (Flt-1, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-9029), thrombospondin-1 (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-12312), angiostatin (rabbit polyclonal, AB-1, Oncogene Research Products, San Diego, CA, USA, PC371), allograft inflammatory factor-1 (AIF-1) [13], endothelial monocyte activating polypeptide (EMAP II/p43) [14], connective tissue growth factor (CTGF) [15] and endostatin/Collagen XVIII [16] (all BMA Biomedicals, Augst, Switzerland) were visualized using HRP conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies and consecutive ECL visualization.

### 2.13.3.4    AIF-1 protein confirmation

In one experiment that was conducted to analyze the nature of the 50 kDa bands, we added 8 M urea, a chaotropic agent that is well known to disrupt oligomer formation of proteins [17] and/or ß-mercaptoethanol to the sample buffer, incubated the samples for the indicated time spans and consequently performed either 12% SDS-PAGE or 12% SDS 6M urea PAGE followed by Western analyses. Protein concentration was determined using the Bradford technique with bovine albumin as a standard. Indicated amounts of total protein were loaded per lane, electrophoresed and transferred to PVDF membranes (Biorad, Munich,

Germany) by semi-dry blotting. Then, membranes were blocked with FCS and primary monoclonal antibodies directed against AIF-1 were visualized using HRP conjugated avidin-biotin complex and ECL visualization.

In gel tryptic digestion was performed as described [18] and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega) at a concentration of 67 ng/µl in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1-2 µl were left and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method [19] was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a $N_2$ 337 nm laser, gridless pulsed ion extraction and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionisation mode at 23 kV.

Sequence verification of some fragments were performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, England) equipped with a nanoflow electrospray ionisation source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [20].

## 2.13.4 Results

For photodynamic therapy, we chose Hypocrellin-A and -B because of their broad absorption spectrum in the visible light range (not shown). For illumination, a common table top fluorescent tube with an emission spectrum that matches the absorption spectra of Hypocrellin-A and -B was chosen. Another advantage is its

shape that exactly matches the shape of the cell culture flasks thus allowing an even illumination of the cells.

Hypocrellin A and -B photodynamic therapy induced visible morphological changes in LN229 glioma, U937 macrophage and SVHCEC endothelial cells. After 6 hours, LN229 glioma cells showed multiple intracellular bodies (not shown). In addition, the cells showed a rounder morphology. Occasionally, protrusions of the cellular membrane were observed. However, no clear signs of cell death could be detected. U937 cells also showed multiple inclusion bodies and occasionally protrusions of the cellular membrane (not shown). SVHCEC cells were characterized by the formation of inclusion bodies. However, their shape mainly remained unaltered (not shown). 24 hours after illumination (not shown), all cells analyzed were characterized by morphological alterations that did not differ from the ones at 6 hours post illumination. No further deterioration was detected.
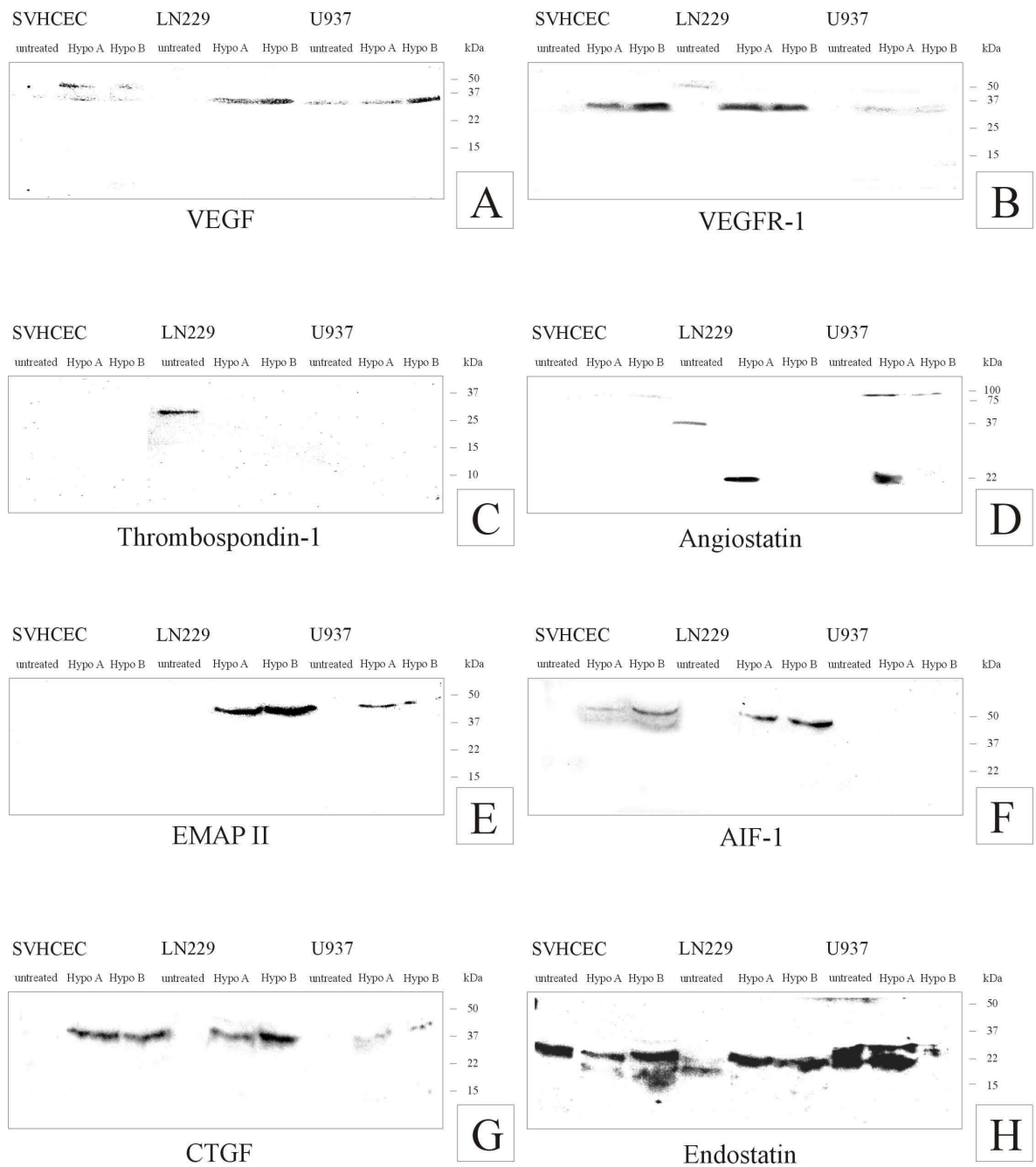
**Figure 2.13.1** *Western Blotting of SVHCEC, LN229 and U937 cells demonstrates accentuated release of VEGF (A) and VEGFR-1/Flt-1 (B).*

A singular thrombospondin-1 band was observed in unstimulated LN229 supernatants that disappeared after Hypocrellin A and -B PDT (C). Induction of different molecular weight angiostatin proteins was detected in all cells analyzed (D). A 43 kDa EMAP II band was induced in LN229 and U937 supernatants after Hypocrellin A and -B PDT (E). A 51 kDa AIF-1 band was induced in SVHCEC and LN229 cells by Hypocrellin A and -B (F). CTGF was released to the supernatants of all analyzed cells after PDT (G). Endostatin release to the supernatants (H).

Western Blotting revealed increased induction of proangiogenic VEGF in all analyzed cell lines following both Hypocrellin A and -B stimulation (Figure 2.13.1 A). Surprisingly, we observed induction of an approximately 30 kDa Flt-1 immunoreactive band that most likely represents the soluble form of Flt-1 (Figure 2.13.1 B). Thrombospondin-1 was detected only in unstimulated LN229 cells but neither in unstimulated SVHCEC or U937 cells nor in PDT stimulated LN229 cells suggesting diminution by Hypocrellin A and -B photochemotherapy (Figure 2.13.1 C). The 38 kDa angiostatin was only detected in supernatants of unstimulated LN229 but not in SVHCEC or U937 cells (Figure 2.13.1 D). After PDT, we observed induction of approximately 90 kDa bands in SVHCEC and U937 cells and of approximately 20 kDa bands in Hypocrellin A stimulated LN229 and U937 cells. No EMAP II/p43 immunoreactive bands were detected in unstimulated and Hypocrellin A and -B stimulated SVHCEC supernatants. In LN229 and U937 cells, we observed the induction of an approximately 43 kDa band following both Hypocrellin A and -B stimulation (Figure 2.13.1 E). No AIF-1 was observed in the supernatants of unstimulated cells (Figure 2.13.1 F). In contrast, we observed release of an approximately 51 kDa band to Hypocrellin A and -B stimulated SVHCEC and LN229 but not U937 cell supernatants (Figure 2.13.1 F). No CTGF was detected in unstimulated SVHCEC, LN229 and U937 cells (Figure 2.13.1 G). After Hypocrellin A and -B photodynamic therapy, prominent induction of CTGF was readily observed in all analyzed supernatants (Figure 2.13.1 G). Endostatin was observed in all analyzed unstimulated cell supernatants (Figure 2.13.1 H). After Hypocrellin A and -B PDT, more prominent bands were observed in the supernatants of LN229 cells and weaker bands in SVHCEC and U937 supernatants (Figure 2.13.1 H).
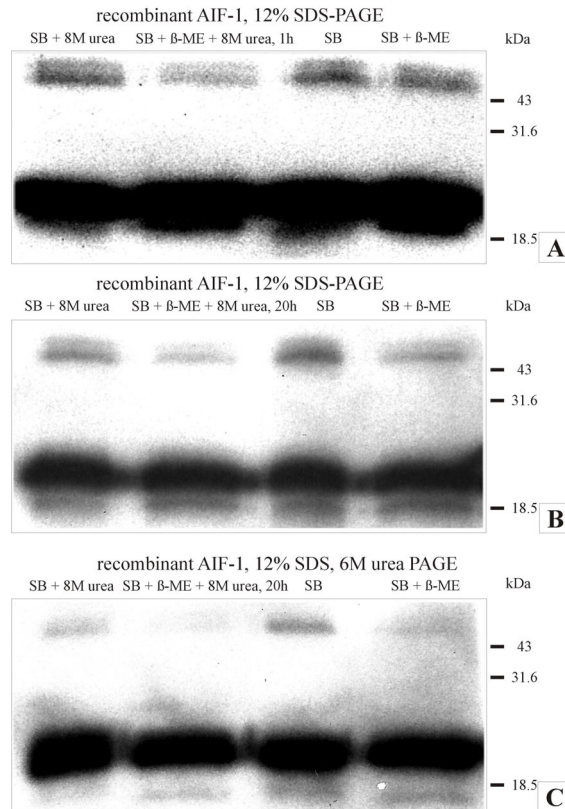
**Figure 2.13.2 Western Blot analyses of recombinant AIF-1.** Western Blot analyses of 5 µg of the recombinant AIF-1 protein per lane using 12 % SDS-PAGE shows reduction of the observed 50 kDa bands predominantly following incubation of the recombinant protein for 1 h in 8 M urea under reducing conditions (ß-ME = ß-mercaptoethanol) (A). After a 20 h incubation period at 25°C of the recombinant AIF-1 in the 8 M urea ß-ME buffer, the otherwise observed 50 kDa bands were further reduced (B). Using the same samples, 6M urea SDS-PAGE resulted in the complete diminution of the 50 kDa bands in the ß-ME 8 M urea sample (C).

In order to determine the origin of the observed aproximately 50 kDa AIF-1 bands, we performed 12% SDS PAGE with preincubation of the recombinant AIF-1 protein with either non-reducing sample buffer (SB), reducing sample buffer (ß-ME) or chaotropic 8 M urea for 1 h at 25°C (Figure 2.13.2 A). The 50 kDa bands in the reducing sample were slightly weaker than the ones in the non-reducing sample, while non-reducing 8 M urea preincubation resulted in no alteration. After preincubation of the sample under chaotropic (urea) and reducing (ß-ME) conditions, we observed a strong diminution of the 50 kDa bands. These results were confirmed following preincubation of the same samples for 20 h at 25°C (Figure 2.13.2 B). To further increase stringency, we used the same samples and performed 6 M urea SDS PAGE followed by Western Blotting (Figure 2.13.2 C).

Here, the bands generally appeared much weaker and the reduced and urea-incubated sample completely lost the 50 kDa bands.

To provide more evidence for these data, we performed in gel tryptic digestion of the three observed AIF-1 bands. The tryptic digest of the 17 kDa-band of recombinant AIF-1 revealed 4 tryptic fragments (AIF-1$_{89\text{-}108}$ MH$^+$: 2161.00, AIF-1$_{54\text{-}71}$ oxidized MH$^+$: 2106.91, AIF-1$_{120\text{-}127}$ oxidized MH$^+$: 1088.50, and AIF-1$_{82\text{-}87}$ MH$^+$: 740.41) as determined by MALDI MS, signal m/z = 2161.00 being the highest one (Figure 2.13.3 A). Most likely because of the weak intensity of the highest band, in this digest only the previously highest signal with m/z = 2161.00 again could be detected (Figure 2.13.3 B). Sequencing of the peptide with the mass 2160.00 Da by tandem ESI MS confirmed identity to AIF-1$_{89\text{-}108}$ in the digest of the 17 kDa band as well as in the highest band (Figure 2.13.3 C). From these results, together with the above named data, we conclude that AIF-1 forms trimers *in vivo*, and in part, *in vitro*.
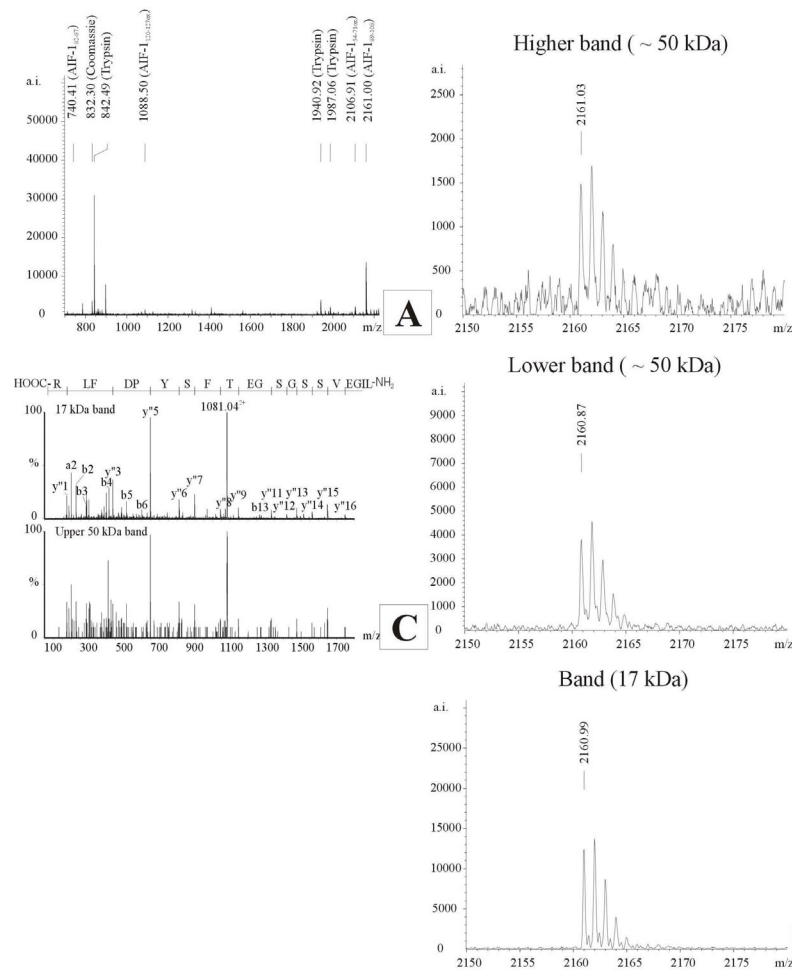
**Figure 2.13.3** *Peptide mass fingerprint of the tryptic digest of the recombinant 17 kDa AIF-1 band.* Four tryptic fragments of AIF-1 were detected, the peak of AIF-1$_{89-108}$ being the most dominant (A). AIF-1$_{89-108}$ was detected in tryptic digests of all three bands of recombinant AIF-1 (B). Fragment spectra of the peptide with the mass 2160.00 confirmed that the peptide was indeed AIF-1$_{89-108}$. Upper spectrum shows fragmentation of doubly charged parent ion ((M+2H)$^{2+}$: 1081.04) in the digest of the 17 kDa band, lower spectrum was recorded from digest of the upper 50 kDa band (C).

## 2.13.5 Discussion

Approximately 17 000 glioblastoma brain tumors are diagnosed and 12000 deaths from glioblastoma occur within the European Community annually. Despite surgery, radiotherapy and chemotherapy, the 5 year survival rate in patients with glioblastoma is below 1 %. Radical resection is oftentimes not possible because of infiltrating tumor growth to the normal brain parenchyma. Photosensitizers are known to accumulate in tumor cells to a greater extent than in normal glial or

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neuronal cells and have therefore become an alternative to battle solid neoplasms of the brain [21,22]. Interestingly, photodynamic therapy has profound effects on the vasculature of solid neoplasms [23]. PDT induces hypoxia by reducing tumor perfusion [24], a concept that recently gave rise to specific PDT protocols to selectively target the tumor vasculature [25]. A staged process is thought to account for the overall observed reduction of blood flow following PDT, with endothelial cell death being the final consequence [26]. However, increased permeability in endothelial cells that results in the release of clotting factors and in turn vessel constriction and occlusion, vascular collapse and disruption, blood flow stasis and tissue hemorrhage are consequences prior to endothelial cell death [27,28,29]. Only few individual factors that mediate this process have been identified including thromboxane and prostacyclin [30].

Using hypocrellin A and -B photodynamic therapy, we now observed release of both pro- and antiangiogenic factors not only from brain tumor cells but also macrophages and endothelial cells. This is the first description of hypocrellin function on brain tumor cells and of the cell-type specific induction of release of regulators of angiogenesis following photodynamic therapy in general.

VEGF is probably the most prominent inducer of vessel formation. VEGF was initially defined to induce vascular leak and promote vascular endothelial cell proliferation [31,32]. It is required for both vasculogenesis, where mesoderm-derived angioblasts form tubes, and for angiogenesis, where capillaries form by sprouting or intussusception from existing vessels [7]. Other members of the VEGF family were identified on their homology to VEGF [33] with overlapping abilities to interact with a set of cell-surface receptors that trigger response to these factors, VEGF-B/VRF [34,35], VEGF-C/VRP [36,37] and VEGF-D/FIGF [38,39]. VEGF-C and VEGF-D bind both VEGFR-3/Flt-4 and VEGFR-2 [36,40]. The corresponding receptor(s) for VEGF-B has not been reported. VEGF-B exists as two alternatively spliced forms, VEGF-B167 and VEGF-B186, which differ in their affinity for heparin and thus release and bioavailability, and it forms heterodimers with VEGF [34,41], a property likely to alter its receptor specificity, biological effects and appearance in Western Blotting experiments.

VEGF exerts its functions through binding to two receptor tyrosine kinases, VEGFR-1/Flt-1 and VEGFR-2/KDR [8]. These receptors are expressed almost exclusively on endothelial cells, although VEGFR-1 is also found in monocytes,

where it mediates migration [42,43]. Interestingly, a mRNA for a soluble truncated form of Flt-1 (sFlt-1) was generated by alternative splicing in endothelial cells that functions as a competitive inhibitor of VEGF [44]. Accordingly, transfection experiments revealed prominent antitumor activity of sFlt-1 [45]. Recombinant sFlt-1 revealed that this antitumor activity is due to dominant-negative inhibition of VEGF-induced angiogenesis [46].

CTGF is a 349 amino-acid protein that has been originally described as a mitogen produced by human umbilical cord endothelial cells [47]. Until recently, CTGF has been thought to mediate predominantly fibrotic diseases [48] by modulating fibroblast reactivity [49]. However, CTGF has been recently identified as a regulator of angiogenesis [50] by binding to integrins $alpha_v \text{ß}_3$ that has been implicated in tumor neoangiogenesis and metastasis [51]. Thus, CTGF functions, at least in part, through integrin-dependent pathways with a direct role in the adhesion, migration and survival of endothelial cells during blood vessel growth. Close interactions between VEGF, Flt-1 and CTGF have been described. VEGF induces expression of CTGF via KDR, Flt-1, and phosphatidylinositol 3-kinase-akt-dependent pathways in vascular cells [52]. VEGF then is capable to bind CTGF to inhibit its proangiogenic function [53], a process that can be reversed by matrix metalloproteinases [54]. In this context, it is of note that we observed release of VEGF, sFLT-1 and CTGF after Hypocrellin A and -B stimulation in a similar fashion in all cells analyzed. In addition, we observed a reduction of thrombospondin-1 release by LN229 cells. Thrombospondin-1 is a potent natural inhibitor of angiogenesis that induces endothelial cell apoptosis *in vitro* and downregulates neovascularization *in vivo* [55]. Interestingly, thrombospondin-1 attenuates VEGF-mediated Bcl-2 expression in endothelial cells *in vitro* [56], suppresses VEGF mobilization [57] and therefore acts, at least in part, as VEGF counterpart. These data are supported by opposing up- or downregulation of VEGF and Thrombospondin by p73 [58] and Smad4/DPC4 [59].

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, and extracellular administration inhibits endothelial proliferation and migration *in vitro* and angiogenesis and tumor growth *in vivo* by inducing apoptosis in endothelial cells [60,61]. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth [62] by inhibiting the binding of VEGF to endothelial cells and the

extracellular domain of KDR/Flk-1 [63] and has therefore become a promising inhibitor of neovascularization in solid neoplasms.

In addition, we analyzed three angiogenic molecules that have yet not been described to interact with VEGF: angiostatin, p43/EMAP II and AIF-1. Angiostatin, a proteolytic fragment of plasminogen, contains either the first three or first four kringle domains of plasminogen and is a potent inhibitor of tumor-induced angiogenesis in animal models [64,65]. Angiostatin has been reported to increase endothelial cell apoptosis and the activity of focal adhesion kinase [66] and block the migration of endothelial cells [67]. Smaller fragments of angiostatin have been described that display differential effects on the suppression of endothelial cell growth [68].

EMAP II is a 23 kDa protein that is proteolytically cleaved from the C-terminal region of the p43 protein, which in turn constitutes a noncatalytic subunit of the aminoacyl tRNA synthetase complex. p43 is one of the three auxiliary components invariably associated with nine aminoacyl-tRNA synthetases as a multienzyme complex ubiquitous to all eukaryotic cells from flies to humans [69]. *In vivo*, EMAP II induces hemorrhage of blood vessels of certain experimental tumors, like fibrosarcoma, melanoma and adenocarcinoma and EMAP II administration has therefore become a novel and promising antiangiogenic strategy to battle neoplasia [70-72]. Release of the 43 kDa antiangiogenic endothelial-monocyte-activating polypeptide II/p43 (EMAP II/p43) band from the glioma and the macrophage cell line is of note, because EMAP II can be derived by proteolytical cleavage from the C-terminal region of the p43 precursor molecule, which in turn constitutes a noncatalytic subunit of the aminoacyl tRNA synthetase complex. Interestingly, a recent publication showed evidence that p43 itself is specifically secreted from intact mammalian cells while EMAP II is released only when the cells were disrupted. Secretion of p43 was also observed when its expression was increased. These results suggest that p43 itself should be a real cytokine secreted by an active mechanism. THP-1 macrophages treated with the full-length p43 protein showed higher cytokine activity than EMAP II, further supporting p43 as an active cytokine. p43 was also shown to activate MAPKs and NFkB, and to induce cytokines and chemokines such as TNF, IL-8, MCP-1, MIP-1a, MIP-1b, MIP-2a, IL-1b, and RANTES [73].

AIF-1 is a 17 kd IFN-$\gamma$ inducible $Ca^{2+}$- binding EF-hand protein that is encoded within the HLA class III genomic region [74] and has originally been cloned from activated macrophages in human and rat atherosclerotic allogenic heart grafts undergoing chronic transplant rejection [75]. It was initially demonstrated that AIF-1 is a modulator of the immune response during macrophage activation [74-75]. Three proteins are probably identical with AIF-1 termed Iba1 (ionized $Ca^{2+}$-binding adaptor), MRF-1 (microglia response factor) and daintain. Considerable but not complete sequence identity with AIF-1 has been described for IRT-1 (interferon responsive transcript), BART-1 (balloon angioplasty responsive transcript), and other, yet unassigned alternatively spliced variants [76]. Some evidence points to a proangiogenic function of AIF-1. Transfection and constitutive expression of AIF-1 in a primary and a rat vascular smooth muscle cell line resulted in enhanced growth proportional to the amount of AIF-1 expressed. Constitutive expression of AIF-1 results in a shorter cell cycle and AIF-1 overexpression also permits growth in serum-reduced media [77,78]. Other data show evidence that AIF-1 has antiangiogenic function by augmenting the production of interleukin-6, -10 and -12 by a mouse macrophage line [79], cytokines that differentially inhibit angiogenesis [80-83].

In conclusion, Hypocrellin A and B photodynamic therapy of cells of human gliomas not only induce apoptosis but moreover elicits the release of distinct angiogenic proteins that are capable to crucially determine neoplastic outgrowth and vascularization, a fact that needs to be taken into account during photodynamic therapy.

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## 2.13.7 Abbreviations

PDT          photodynamic therapy
SB           sample buffer

## 2.13.8 Participating Researchers

These results are part of a publication by

Martin H. Deininger, Toni Weinschenk[1], Matthias H. Morgalla,[2] Richard Meyermann and Hermann J. Schluesener

Institute of Brain Research, University of Tuebingen, Calwer Str. 3, D-72076 Tuebingen, Germany

[1] Institute for Cell Biology, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany

[2] Department of Neurosurgery, University of Tuebingen, Tuebingen, Germany

# 3    Summary

Genomics (oligonucleotide microarray based expression profiling) and MHC class I-ligandome analysis (mass spectrometry) were combined to analyse tumors of renal cell carcinoma patients. This can be performed in an appropriate timeframe after surgical intervention to allow rapid subsequent vaccination. Thus, it was demonstrated in seven cases that it is possible to extract information from a single excised tumor specimen that leads to an optimized design of a multi-epitope, peptide-based vaccine directed against the tumor of an individual patient and considering all expressed HLA-alleles.

New tumor associated antigens together with MHC class I-ligands encoded by them can be identified using this approach. *In vitro* T cell analyses carried out for two of the newly discovered ligands have proven that, first, they were T cell epitopes, and, second, T cells specific for these epitopes mediated lysis of tumor cells but not control cells.

As part of the complete approach, a database based on oligonucleotide microarrays was built covering 22.000 gene expression values of twenty one human healthy tissues and organs.

Finally, a clinical study using this approach was started and four RCC patients were already analyzed and three patients vaccinated.

The results of this thesis presented in part two provided new insights into the antigen processing mechanisms. First, there was evidence for a novel luminal endoplasmic reticulum aminopeptidase involved in epitope trimming events, which in the meantime has been identified as being ERAP1.
Second, it could be shown that tripeptidyl peptidase plays an essential role in the generation of an MHC class I epitope.

Small projects, mainly performed to improve technical skills in protein and peptide analysis, were related to protein identification, another dealt with detection of glutamine deamidations.

# 4    Acknowledgments

# 5    Abbreviations

| | | | | |
|---|---|---|---|---|
| APC | Antigen presenting cell | | SDS | Sodium Dodecyl Sulfate |
| AA | Amino acid | | TAP | Transporter Associated with Antigen Processing |
| BCR | B cell receptor | | | |
| BPI | Base peak ion | | TFA | Trifluoro Acetic Acid |
| CD | Cluster of differentiation | | TIC | Total Ion Current |
| CID | Collision induced decay | | TIL | Tumor Infiltrating Lymphocyte |
| CTL | Cytotoxic T lymphocyte | | TOF | Time of Flight |
| ER | Endoplasmic reticulum | | | |
| ESI | Electrospray ionization | | | |
| EST | Expressed sequence tag | | One letter code for amino acids | |
| FACS | Fluorescence activated cell sorter | | | |
| HLA | Human leucocyte antigen | | A | Alanine |
| HSP | Heat shock protein | | C | Cysteine |
| IFN | Interferon | | D | Aspartic acid |
| Ig | Immunoglobulin | | E | Glutamic acid |
| IL | Interleukin | | F | Phenylalanine |
| LCM | Laser capture microdissection | | G | Glycine |
| LMP | Low molecular weight polypeptide | | H | Histidine |
| | | | I | Isoleucine |
| | | | K | Lysine |
| MALDI | Matrix assisted laser desorption ionization | | L | Leucine |
| | | | M | Methionine |
| MHC | Major histocompatibility complex | | N | Asparagine |
| MS | Mass spectrometry | | P | Proline |
| MS/MS | Tandem mass spectrometry | | Q | Glutamine |
| MW | Molecular weight | | R | Arginine |
| NK-cell | Natural killer cell | | S | Serine |
| PAGE | Polyacrylamide gel electrophoresis | | T | Threonine |
| | | | V | Valine |
| PCR | Polymerase chain reaction | | W | Tryptophane |
| PH | Potentia hydrogenii | | Y | Tyrosine |
| RP-HPLC | Reversed Phase-High Performance Liquid Chromatography | | | |

# 6 Academic Teachers

Academic teachers from the University of Tübingen, *from the Max-Planck-Institute for Biochemistry/Neurobiology in Martinsried, Munich, or **from the Klinikum rechts der Isar, Munich.

Prof. Albert, Prof. Bayer, Prof. Bisswanger, Prof. Bock, Prof. Bohley, Prof. Breyer-Pfaff, Prof. Eisele, *Dr. Essen, PD Dr. Fröhlich, **Prof. Gänsbacher, Prof. Gauglitz, Prof. Günzl, Prof. Hagenmaier, Prof. Hamprecht, Prof. Hanack, Dr. Hildt, *Prof. Hofschneider, *Dr. Holak, Prof. Jung, Prof. Kaiser, PD Dr. Kalbacher, Prof. Krug, Prof. Lindner, Prof. Mayer, Prof. Mecke, Prof. Müller, Prof. Nakel, Prof. Ninnemann, Prof. Oberhammer, *Prof. Oesterhelt, PD Dr. Pawelec, Prof. Pfaff, Prof. Pfeiffer, Prof. Pommer, Prof. Probst, Prof. Rammensee, Dr. Reinecke, Prof. Reutter, PD Dr. Schild, Prof. Schöffl, *Prof. Stamm, Prof. Stegmann, PD Dr. Stevanovic, PD Dr. Stoeva, Prof. Strähle, Prof. Voelter, Prof. Wegmann, Prof. Weser, PD Dr. Wiesinger, Prof. Wohlleben

# 7     CV and Publications

| | |
|---|---|
| Name: | Toni Weinschenk |
| Geburtstag: | 5.1.1973 |
| Geburtsort: | Esslingen am Neckar |
| | |
| 1979-1983 | Grundschule |
| | |
| 1983-1992 | Theodor-Heuss-Gymnasium, Esslingen a.N. |
| | |
| 5/1992 | Abitur |
| | |
| 1992-1993 | Zivildienst |
| | |
| 10/1993-7/1999 | Studium der Biochemie an der Eberhard-Karls-Universität Tübingen |
| | |
| 5/1996 | Vordiplom in Biochemie |
| | |
| 4/1997-4/1998 | Studienaufenthalt an den Max-Planck-Instituten für Biochemie und Neurobiologie in Martinsried bei München (in den Abteilungen von Prof. Dr. Dieter Oesterhelt, Prof. Dr. Dr. Peter Hans Hofschneider, Prof. Dr. Robert Huber) sowie am Klinikum Rechts der Isar (Prof. Dr. Bernd Gänsbacher) |
| | |
| 6/1998-11/1998 | Diplomprüfungen |
| | |
| 11/1998-7/1999 | Diplomarbeit bei Prof. Dr. H.-G. Rammensee am Institut für Zellbiologie, Abt. Immunologie, Universität |

Tübingen: „HLA-Klasse-I- präsentierte Peptide auf Tumoren des Gastrointestinaltrakts"

20/07/1999          Diplom in Biochemie

8/1999-1/2004       Doktorarbeit bei Prof. Dr. H.-G. Rammensee am Institut für Zellbiologie, Abt. Immunologie, Universität Tübingen:
„Multi-Peptid-Impfstoffe zur individuellen Behandlung von Tumorpatienten – Von den analytischen Grundlagen zur klinischen Anwendung"

| Name: | Toni Weinschenk |
|---|---|
| Date of birth: | 5.1.1973 |
| Place of birth: | Esslingen am Neckar |

| 1979-1983 | Primary school |
|---|---|

| 1983-1992 | Theodor-Heuss-Gymnasium, Esslingen a.N. (Grammar School) |
|---|---|

| 5/1992 | Abitur (general qualification for university entrance) |
|---|---|

| 1992-1993 | Community Service |
|---|---|

| 10/1993-7/1999 | Biochemistry studies at the Eberhard-Karls-University in Tübingen |
|---|---|

| 5/1996 | Intermediate diploma in Biochemistry |
|---|---|

| 4/1997-4/1998 | Practical training at the Max-Planck-Institute for Biochemistry and Neurobiology in Martinsried near Munich (Departments of Prof. Dr. Dieter Oesterhelt, Prof. Dr. Dr. Peter Hans Hofschneider, Prof. Dr. Robert Huber) and at the Klinikum Rechts der Isar (Prof. Dr. Bernd Gänsbacher) |
|---|---|

| 11/1998-7/1999 | Diploma thesis at the Institute for Cell biology, Dept. of Immunology, University of Tübingen supervised by PD Dr. S. Stevanović and Prof. Dr. H.-G. Rammensee, title: „HLA-class-I-presented peptides on gastrointestinal tumors" |
|---|---|

| 20/07/1999 | Diploma in Biochemistry (graduation) |
|---|---|

| | |
|---|---|
| 8/1999-1/2004 | PhD thesis at the Institute for Cell Biology, Dept. of Immunology, University of Tübingen supervised by PD Dr. S. Stevanović and Prof. Dr. H.-G. Rammensee (doctoral thesis supervisor), title: |
| | „Multi-peptide-based Vaccines for Personalized Cancer Therapy – Analytical fundamentals translated into clinical applications" |

## Publications Toni Weinschenk

1. Appel S, Rupf A, Weck MM, Grünebach F, Schoor O, Brümmendorf TL, Weinschenk T and Brossart P. c-Abl tyrosine kinase is critically involved in the differentiation and function of monocyte derived dendritic cells. *submitted*

2. Probst J, Blumenthal SG, Tenzer S, Weinschenk T, Dittmer J, Six A, Rammensee HG and Pascolo S. A conserved sequence in the mouse variable T cell receptor α recombination signal sequence 23 bp spacer can inhibit recombination. *submitted*

3. Schag K, Schmidt SM, Müller MR, Weinschenk T, Appel S, Weck MM, Grünebach F, Stevanović S, Rammensee HG, Brossart P. Identification of c-MET oncogene as a broadly expressed tumor associated antigen recognized by cytotoxic T-lymphocytes. *Submitted*

4. Schmidt SM, Schag K, Müller MR, Weinschenk T, Appel S, Weck MM, Grünebach F, Kanz L, Stevanović S, Rammensee HG, Brossart P. 2004. Induction of adipophilin specific cytotoxic T-lymphocytes using a novel HLA-A2 binding peptide that mediates tumor cell lysis. *Cancer Research* 64(3)

5. Schoor O, Weinschenk T, Hennenlotter J, Corvin S, Stenzl A, Rammensee HG, Stevanovic S. 2003. Moderate degradation does not preclude microarray analysis of small amounts of RNA. *Biotechniques* 35(6):1198-201.

6. Deininger MH, Weinschenk T, Meyermann R, Schluesener HJ. 2003. The allograft inflammatory factor-1 in Creutzfeldt-Jakob disease brains. *Neuropathol Appl Neurobiol* 29(4):389-99.

7. Kayser S, Watermann I, Rentzsch C, Weinschenk T, Wallwiener D, Gückel B. 2003. Tumor-associated antigen profiling in breast and ovarian cancer: mRNA, protein or T cell recognition? *J Cancer Res Clin Oncol* 129(7):397-409.

8. Seifert U, Marañón C, Shmueli A, Desoutter JF, Wesoloski L, Janek K, Henklein P, Diescher S, Andrieu M, de la Salle H, Weinschenk T, Schild H, Laderach D, Galy A, Haas G, Kloetzel PM, Reiss Y and Hosmalin A. 2003. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nature Immunol* 4(4):375-9.

9. Rammensee HG, Weinschenk T, Gouttefangeas C, Stevanović S. 2002. Towards patient-specific tumor antigen selection for vaccination. *Immunol Rev* 188(1):164-76. Review.

10. Deininger MH, Weinschenk T, Morgalla MH, Meyermann R, Schluesener HJ. 2002. Release of regulators of angiogenesis following Hypocrellin-A and -B photodynamic therapy of human brain tumor cells. *Biochem Biophys Res Commun* 298(4):520-30.

11. Weinschenk T, Gouttefangeas C, Schirle M, Obermayr F, Walter S, Schoor O, Kurek R, Loeser W, Bichler KH, Wernet D, Stevanović S, Rammensee HG. 2002. Integrated functional genomics approach for the design of patient-individual antitumor vaccines. *Cancer Res* 62(20):5818-27.

12. Schmid DG, von der Mulbe FD, Fleckenstein B, Weinschenk T, Jung G. 2001. Broadband detection electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry to reveal enzymatically and chemically induced deamidation reactions within peptides. *Anal Chem* 73(24):6008-13.

13. Schirle M, Weinschenk T, Stevanović S. 2001. Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens. *J Immunol Methods* 257(1-2):1-16. Review.

14. Komlosh A, Momburg F, Weinschenk T, Emmerich N, Schild H, Nadav E, Shaked I, Reiss Y. 2001. A role for a novel luminal endoplasmic reticulum aminopeptidase in final trimming of 26S proteasome-generated major histocompatability complex class I antigenic peptides. *J Biol Chem* 276(32):30050-6.

15. Blank M, Weinschenk T, Priemer M, Schluesener H. 2001. Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels: selective targeting of endothelial regulatory protein pigpen. *J Biol Chem* 276(19):16464-8.