Modulation der Insulinsignalübertragung durch Phosphorylierung von Serin-357 im Insulin-Rezeptor-Substrat-1

Modulation of Insulin Signal Transduction by Serine-357 Phosphorylation of Insulin Receptor Substrate-1

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   R. Sanaullah Waraich, C. Weigert, E. D. Schleicher, H. U. Häring, R. Lehmann;
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CONTENTS

ABBREVIATIONS

°C	Degree Celsius
Ala	Alanine
aPKC	Atypical protein kinace C
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Cb1	Cannabinoid receptor-1
cPKC	Conventional/classical protein kinase C
Da	Dalton
ddNTPs	Dideoxynucleotides
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
DTT	Dithiothreitol,
E.coli	Escherichia coli
ECL	"Enhanced Chemiluminescence-System"
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	"Extracellular signal-regulated kinase"
Gab1	"Grb2-associated binder 1"
Glu	Glutamic acid
Grb2	"Growth factor receptor binding protein 2"
GSK-3	Glycogen-synthase-kinase-3
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IGF	Insulin growth factor
IKKß	IκB(inhibitor of kappa B) kinase β
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin receptor substrate

JNK	C-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
nPKC	Novel protein kinase C
p	Phospho
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PKB	Protein kinase B
PDK	Phosphoinositide-dependent kinase
PH	Pleckstrin homology domain
PI3-K	Phosphoinositide 3-kinase
PI3,4P2	Phosphatidylinositol-3,4-bisphosphate
PI3,4,5P3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PTB	Phosphotyrosine-binding domain
Ser	Serine
SH2	Src homology 2 domain
SHP-2	SH2-homologe protein tyrosine phosphatase
SOS	Son of sevenless
TNFα	Tumor necrosis factor-alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
Tyr	Tyrosine
w/v	"Weight per volume"

SUMMARY

The activation of the protein kinase C (PKC) family of serine/threonine kinases contributes to the modulation of insulin signaling and the PKC-dependent phosphorylation of IRS-1 has been implicated in the development of insulin resistance. Here we demonstrate Ser-357 of rat IRS-1 as a novel PKC-δ-dependent phosphorylation site in skeletal muscle cells upon stimulation with insulin and phorbol ester using phospho-Ser-357 antibodies and active and kinase dead mutant of PKC-δ. Phosphorylation of this site was simulated using IRS-1 Glu357 and shown to reduce insulin-induced tyrosine phosphorylation of IRS-1, to decrease activation of Akt and subsequently to diminish phosphorylation of GSK-3. When the phosphorylation was prevented by mutation of Ser-357 to alanine, these effects of insulin were enhanced. When the adjacent Ser-358, present in mouse and rat IRS-1, was mutated to alanine, which is homologous to the human sequence, the insulin-induced phosphorylation of GSK-3 or tyrosine phosphorylation of IRS-1 was not increased. Moreover, both, active PKC-δ and phosphorylation of Ser-357 were shown to be necessary for the attenuation of insulinstimulated Akt phosphorylation. The phosphorylation of Ser-357 could lead to increased association of PKC-δ to IRS-1 upon insulin stimulation, which was demonstrated with IRS-1 Glu357. Together, these data suggest that phosphorylation of Ser-357 mediates at least in part the adverse effects of PKC-δ activation on insulin action.

1- INTRODUCTION

1.1 Diabetes

Diabetes mellitus (DM) is a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects of insulin secretion, insulin action, or a combination of both (1). The epidemic of type 2 diabetes mellitus (T2DM) and impaired glucose tolerance is one of the main causes of morbidity and mortality worldwide (2). In 2007, it is estimated that diabetes currently affects 246 million people worldwide and is expected to affect 380 million by 2025 with the most of this increase occurring in developing countries. The Western Pacific region and the European region have the highest number of people with diabetes, approximately 67 and 53 million respectively. The highest rate of diabetes prevalence is to be found in the North American region (9.2%) followed by the European region (8.4%) (3).

1.2 Insulin resistance

The pathogenesis of type 2 diabetes involves abnormalities in both, insulin action and secretion (4). At the molecular level, insulin resistance, the first detectable defect in type 2 diabetes, correlates with impaired insulin signaling in peripheral tissues (5). Clinically, the term insulin resistance implies that higher than normal concentrations of insulin are required to maintain normoglycemia. On a cellular level, this term defines an inadequate strength of insulin signaling from the insulin receptor downstream to the final substrates of insulin action involved in multiple metabolic and mitogenic aspects of cellular function (6). The earliest abnormality observed in insulin resistance is a decrease in insulin-induced glucose uptake in skeletal muscle and adipose tissue and a reduced ability of the hormone to suppress glucose production by the liver (2;7). At the early stage of the pathology, pancreatic ß cells first compensate for peripheral insulin resistance by increasing insulin secretion to maintain euglycemia. When chronic, this hyperinsulinemia exacerbates insulin resistance and contribute to ß cell failure leading to the development of impaired glucose tolerance and overt clinical type 2 diabetes.

Insulin resistance is also associated with a variety of physiological and patho-physiological states other then type 2 diabetes, such as hypertension, glucose intolerance, and obesity (7) However, understanding the molecular mechanisms that modulate insulin signaling under

these conditions, has proved a more difficult 'nut to crack'; even 50 years after the discovery of insulin, therefore a full understanding of insulin action is still evolving (8).

1.3 Insulin signaling - the basics

The discovery of insulin in 1921 was one of the key biological and medical advances of the twentieth century. However, it took the rest of the century to understand how this hormone regulates intracellular metabolism (8). The insulin signaling system plays a significant role in many physiological processes, including carbohydrate and fat metabolism, reproduction, cellular growth, and survival (9). Nonetheless, insulin is best known for its role in the regulation of blood glucose, as it suppresses hepatic gluconeogenesis and promotes glycogen synthesis and storage in liver and muscle, triglyceride synthesis in liver and storage in adipose tissue, and amino acid storage in muscle (10). Thus, understanding the cellular mechanisms of insulin action may contribute significantly to new treatments for type 2 diabetes mellitus, which grounds for a cohort of systemic disorders, such as dyslipidemia, hypertension, cardiovascular disease, stroke, blindness, kidney disease, female infertility, and neuro-degeneration (11).

1.3.1 Insulin receptor

In the 1970s, the insulin receptor was discovered, and 10 years later the demonstration of its tyrosine kinase activity pointed toward the mechanism of signal transduction (12;13). A 150-kb gene on chromosome 19 composed of 22 exons encodes the human pro-receptor. Dysregulation of insulin receptor gene splicing alters fetal growth patterns and contributes to insulin resistance in adults (14). The insulin receptor is the prototype for a family of homologous integral membrane proteins composed of two extracellular α-subunits and two transmembrane β-subunits that have protein tyrosine-kinase activity. There are at least seven tyrosine autophosporylation sites in three distinct regions of the β-subunits. The holoreceptor has a molecular weight of 350, 000 (15). The diverse biological actions of insulin and insulin-like growth factor I (IGF-I) are initiated by binding of the polypeptides to their respective cell surface tyrosine kinase receptors. Insulin binding activates an intramolecular autophosphorylation reaction in which one β-subunit phosphorylates the other at several sites. This reaction activates the tyrosine kinase activity of the receptor enabling it to recruit and phosphorylate cellular substrates to initiate signal transduction (2;16).

1.3.2 Binding partners of insulin receptor

Auto-phosphorylation of the A loop of β-subunit of IR creates binding sites for the other signaling proteins that modulate kinase activity including Grb10, Dock 1 and 2, Gab1 and 2, Cb1, APS and SH2B (17). Auto-phosphorylation in the juxtamembrane region of IR is essential for the recruitment of IRS proteins that propagate insulin signal. Autophosphorylation in the COOH region of IR (Tyr1314 Tyr1328) is poorly understood, however, it has been shown to regulate tyrosine kinase activity and receptor internalization (18). The activated IR subsequently phosphorylate these adaptor proteins that function as docking sites for effector proteins containing Src homology-2 (SH2) domains (19).

1.3.3 Insulin receptor substrates (IRS)

The first evidence for such an adaptor protein emerged when insulin was shown to stimulate the tyrosine phosphorylation of a 185-kDa cytosolic protein (20), now known as insulin receptor substrate-1 (IRS1). Insulin receptor substrate (IRS) molecules are key mediators in insulin signaling and play a central role in maintaining basic cellular functions such as growth, survival, and metabolism (21). They act as docking proteins between the insulin receptor and a complex network of intracellular signaling molecules containing Src homology 2 (SH2) domains. IRS proteins have no intrinsic catalytic activity but contain several domains that mediate interaction with the receptor and with IRS effectors.

1.3.4 Structural organization of IRS proteins

IRS proteins are composed of multiple interaction domains and phosphorylation motifs. An amino-terminal pleckstrin homology (PH) domain is adjacent to a phospho-tyrosine binding (PTB) domain. The nonconserved 46-residue linker between these two domains is disordered (22). The carboxy-terminal domain of IRS protein encodes numerous tyrosine and serine residues prone to phosphorylation (7;23). PH and PTB domain appear to facilitate recruitment of IRS proteins to activated insulin receptor. The PTB domain interacts with the tyrosine phosphorylated sequence NPXY in the insulin and insulin-like growth factor I (IGF-I) receptors. Deletion of both these domains in IRS-1 and IRS-2 almost completely prevent insulin stimulated tyrosine phosphorylation of the tail (24). No Src homology 2 or 3 (SH2 or SH3) domains have been identified in IRS proteins.

1.3.5 IRS isoforms

Four members (IRS-1, IRS-2, IRS-3, IRS-4) of IRS family have been identified that differ as to tissue distribution, subcellular localization, developmental expression, binding to the insulin receptor and interaction with SH2 domain containing proteins. Nevertheless, the IRS-protein isoforms display several structural similarities. Of note, mammalian IRS-1, -2, -3, -4 and drosophila ortholog Chico contain a NH2-terminal pleckstrin homology (PH) domain adjacent to a PTB domain. The structures of these domains are remarkably similar (25). Despite the structural similarities between these IRSs, analyses of the IRS knockout mice demonstrated that IRS proteins have different functions in development and metabolism (26;27). Moreover, analysis of the human genome sequence reveals at least two putative IRS proteins, IRS-5 and IRS-6 (7).

1.3.5.1 Insulin receptor substrate 1 (IRS- 1)

IRS-1 was the first substrate of insulin receptor, identified in hepatoma cells using an antiphosphotyrosine antibody and was subsequently identified in several tissues, including muscle, heart, liver, adipocyte and kidney (20;28). Discovery of IRS-1 provided the first clue that substrate phosphorylation together with autophosphorylation are important steps in signal transduction. The human IRS-1 gene is localized on chromosome 2q36–37 (29). The relevance of IRS-1 in insulin/IGF-I signaling was primarily suggested by *in vitro* studies in which IRS-1 was overexpressed or its levels were decreased by anti-sense ribozyme, which resulted in increase in insulin sensitivity and responsiveness for glucose transport and enhanced mitogenetic effects of insulin (30;31). IRS1 controls body growth and peripheral insulin action (32). Mice lacking IRS-1 are small and insulin resistant, but generally fail to develop diabetes owing to consistent compensatory hyperinsulinemia (26). Islets from knockout mice lacking IRS-1 exhibited marked defects in insulin content and the insulin secretory response to glucose (33). In agreement with these results, it has been shown in human adipocytes that IRS-1 is the main docking protein for the binding and activation of PI 3-kinase in response to insulin. Defects in IRS expression and function have been reported in target tissues of insulin action from insulin resistant subjects. In skeletal muscle strips from morbidly obese subjects, it was observed a significant reduction in IRS-1 content, insulin-stimulated IRS-1 phosphorylation, and PI 3-kinase activation that was paralleled by a decrease in insulin-stimulated glucose uptake (34).

Taken together, these data indicate that IRS-1 plays a key role in mediating both metabolic and mitogenic effects of insulin in peripheral tissues such as muscle and adipose tissue, and suggest a novel important role for IRS-1 in β cell function. Thus, insulin receptor substrate 1 is one of the important early site of divergence in insulin signaling, which seems to be a relevant target for modulation of the signal (35).

1.3.5.2 Insulin receptor substrate 2 (IRS-2)

IRS-2 was cloned from a myeloid cell line and was identified as the alternative IR substrate in liver and muscle of IRS1-null mice (36;37). The amino acid sequence identity between IRS-1 and IRS-2 is 43%, with some domains such as the PH and PTB domains exhibiting higher degrees of identity (65 and 75%, respectively) (38-40). Mice lacking IRS-2 develop diabetes with in 6-10 weeks of age owing to peripheral insulin resistance and ß-cell failure (26). Upregulation of insulin receptor substrate-2 in pancreatic beta cells prevents diabetes (41). IRS-2 regulates brain growth, body weight control, glucose homeostasis, and female fertility (42). It is clear that IRS1 and IRS2 are responsible for relaying insulin signals from the receptor to intracellular effectors, but, IRS1 and IRS2 differ significantly from each other (43). For example, it has been shown that IRS-1 and IRS-2 differ in their sub-cellular localization, IRS-2 is dephosphorylated more rapidly and activates PI3-kinase more transiently than IRS-1, thus indicating that differences in kinetics of activation may contribute to the diversity of the insulin signaling transduced by IRS-1 and IRS-2 (44). In fact, IRS-1 appears to have its major role in skeletal muscle whereas IRS-2 appears to regulate hepatic insulin action as well as pancreatic β cell development and survival (21).

1.3.5.3 Insulin receptor substrate 3 (IRS- 3)

Rodents express IRS-3 which is largely restricted to adipose tissue where it display activity similar to IRS-1 (27), whereas the gene might not be active in humans (45) . Although disruption of IRS-3 has small effect, mice with combined deficiency of IRS-1 and IRS-3 developed sever lipoautrophy associated with hyperglycemia, hyperinsulinemia and insulin resistance (27). In addition to metabolic signals, IRS-3 seems to be able to mediate mitogenic signals (46).

1.3.5.4 Insulin receptor substrate 4 (IRS- 4)

IRS-4 expression is limited to thymus, brain, kidney and ß-cell (47). Overall, IRS-4 displays only 27% and 29% sequence identity with IRS-1 and IRS-2, respectively. *In vitro* studies with HEK cells confirmed that IRS-4 binds PI3-kinase and Grb-2 (48). Overexpression of IRS-4 in rat adipocytes led to a marked increase in the number of GLUT4 molecules recruited to the cell surface (27). More recently, it has been shown that IRS-3 and IRS-4 may act as negative regulators of IGF-I signaling by suppressing the function of IRS-1 and IRS-2 at several steps (46).

1.3.5.5 Insulin receptor substrate 5 and 6 (IRS- 5, - 6)

IRS-5/DOK4 and IRS-6/DOK5 were recognized in the human genome owing to their NH-terminal tendum PH-PTB domain (49) however, they contain very short COOH tails with a few tyrosine phosphorylation sites, so their function remains unknown (7).

1.3.6 Binding partners of IRS-1

The tyrosine phosphorylation sites in the COOH-terminal of each IRS protein recruit and regulate various downstream signaling proteins. Over 20 tyrosine phosphorylation residues contribute to docking sites for downstream signaling proteins. However, only a few sites that bind p85, Grb2, or SHP2 have been formally identified (50). Many of the tyrosine residues cluster in the common motifs that recruit or activate enzymes (PI3-Kinase, SHP2, fyn) or adaptor molecules (Grb2, nck, crk, SH2B). Grb2 and possibly SHP2 couple Grb2/SOS to IRS proteins, which promotes ras $\longrightarrow$ raf cascade (51). All IRS proteins contain multiple p85 binding motifs that recruit the PI3-Kinase, which is the best studied insulin signaling pathway.

1.3.7 The PI3-Kinase cascade

PI3-kinase is ubiquitous and used by nearly all receptor signaling systems to promote cell division survival and growth. During insulin and IGF signaling, the PI3-kinase is accessed through tyrosine phosphorylation of IRS- proteins (17). IRS proteins couple insulin/IGF receptors to the PI3-kinase and extra cellular signal-regulated kinase (ERK) cascade. Other signal transduction proteins interact with IRS including Grb2, an adaptor protein that contains SH3 domains, which in turn associates with the guanine nucleotide exchange factor son-of

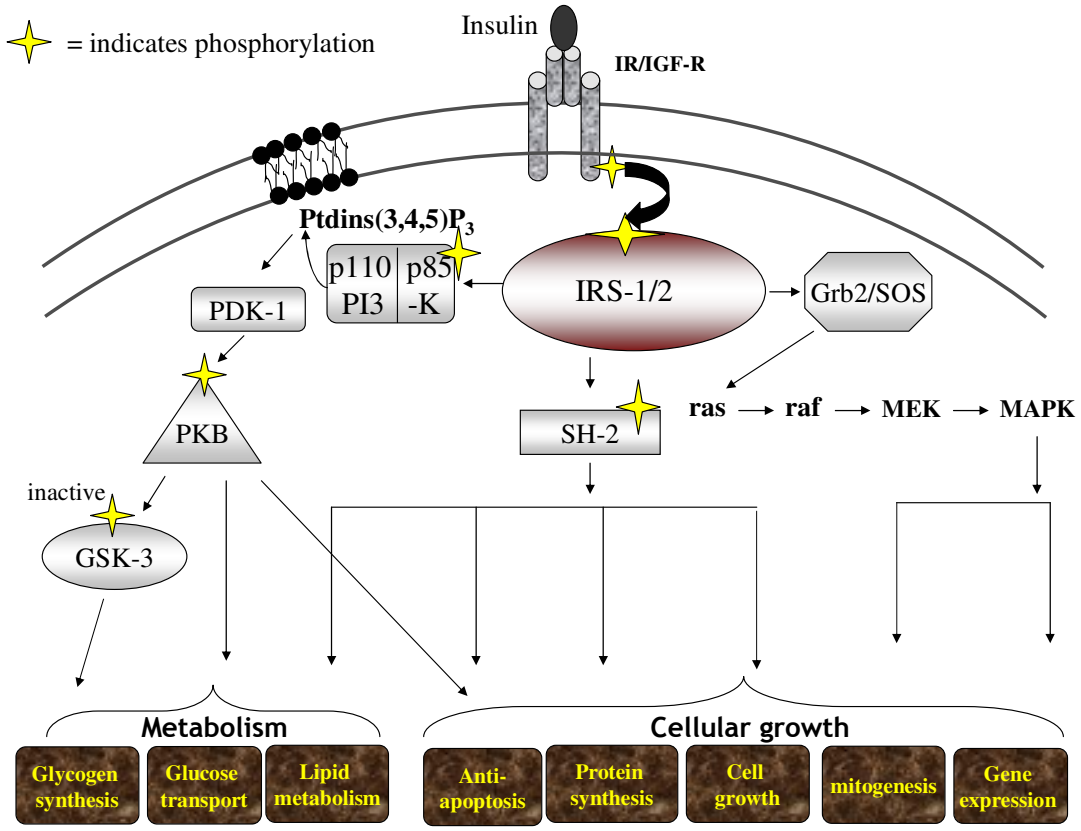


Fig. 1. IRS protein-dependent insulin/IGF-I-signaling cascade and various biological effects.

Activation of the receptors for insulin and IGF-I results in tyrosine phosphorylation of the IRS proteins. The IRS proteins thereby bind PI3-kinase, Grb2/son of sevenless (SOS). The Grb2/SOS complex mediates the activation of p21ras, thereby activating the ras/raf/mitogen-activated protein (MAP) kinase (MEK)/MAP kinase cascade. The activation of PI 3-kinase by IRS protein recruitment results in the generation of PI-3,4-diphosphate(PI3,4P2) and PI-3,4,5-triphosphate (PI3,4,5P3). In aggregate, PI3,4P2 and PI3,4,5P3 activate a variety of downstream signaling kinases, including PKB. PKB regulates glycogen synthase kinase 3 (GSK-3), which may regulate glycogen synthesis, and a variety of regulators of cell survival. PKB-mediated phosphorylation of the proapoptotic protein BAD inhibits apoptosis. The IRS proteins also interact with a variety of signaling molecules containing Src homology 2(SH2) domains, to regulate lipid and protein metabolism and cell growth.

SH-2	Src homology 2	PI3-Kinase	Phosphatidylinositol-3-kinase
Grb2/SOS	"Growth factor receptor bound protein" 2/ "son of sevenless"	PI3,4,5P3	Phosphatidylinositol-3,4,5-triphosphate
MEK	MAPK/Erk-kinase	PDK1/PDK 2	Phosphoinositide-dependent kinases-1/2

MAPK	"mitogen activated protein kinase"	mTor	"mammaliam target of rapamycin"
PKC	Protein kinase C	PKB/Akt	Protein kinase B
GSK3	Glycogen synthase kinase-3	IR	Insulin receptor
IGF-R	Insulin like Growth Factor receptor	Ras/raf	A membrane associated GTPases /MAP kinase kinase kinase

sevenless (sos) and elicits activation of the MAPK cascade leading to mitogenic responses (52) (Fig. 1). Activation of the PI 3-kinase cascade is an important insulin/IGF-regulated pathway. PI3-kinase is a dimer composed of a 110-kDa catalytic subunit that is associated non-covalently to a 55- or 85-kDa regulatory subunit. PI3-kinase is activated when the phosphorylated YMXM motifs in IRS proteins occupy both src homology-2 (Sh2) domains in the regulatory subunit (53). Products of PI3-kinase, including phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3, 4, 5-trisphosphate, attract serine kinases to the plasma membrane, including the phosphoinositide-dependent kinase (PDK1 and PDK2) and at least three protein kinase B (PKB) isoforms (Fig. 1). During co-localization at the plasma membrane, PDK1 phosphorylates and activates PKB-1, -2, or -3.

1.3.8 Insulin-activated AKT/PKB

Kinase Akt is one of the best characterized insulin-stimulated enzyme as it is broadly implicated in growth and metabolism (54;55). The activated protein kinase B (PKB or Akt) phosphorylates many substrates to control various biological signaling cascades, including glucose transport, protein synthesis, glycogen synthesis, cell proliferation, and cell survival, in various cells and tissues (Fig. 1)(37;56). AKT/protein kinase B (PKB) phosphorylates glycogen synthase kinase-3α (GSK3α) and GSK3β at serine residues located in Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr motifs (where Xaa is any amino acid) (57). This motif is crucial for the specificity of AKT/PKB (8). There is also increasing evidence that phosphorylation of the Rab GTPase-activating protein AS160 by AKT/PKB underlies the insulin-stimulated translocation of the glucose transporter GLUT4 to the plasma membranes of muscle cells and adipocytes, a crucial event in promoting the uptake of glucose from the blood (58).

1.3.9 Insulin-activated GSK-3

Glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase that consists of highly homologous α and β isoforms (59), phosphorylates and thereby inactivates glycogen synthase

(GS), resulting in reduced glycogenesis (57;60). Indeed, overexpression of GSK3 β in skeletal muscle of male mice is associated with a marked decrease in glucose tolerance (61). An association between skeletal muscle GSK3 and insulin resistance has been demonstrated in type 2 diabetes and animal models of insulin resistance (62;63). Additionally by inhibiting GSK-3, insulin stimulates the dephosphorylation and activation of eIF2B, contributing to an increased rate of protein synthesis (64).

1.4 Regulatory aspects of insulin signaling: cause of type 2 diabetes

1.4.1 Genetic aspects

Polymorphisms have been identified in human genes encoding proximal insulin signaling components that might contribute to metabolic disease. Although insulin receptor polymorphism provides important insight into receptor function they fail to uncover a general cause of type 2 diabetes (65). A few polymorphisms in the gene of IRS-1 have been found, some of which are more common in type 2 diabetic patients (66), of these, the Gly to Arg change at codon 972 (Arg^{972} IRS-1) is the most common, and has been studied most extensively (67;68). However, they don't reveal a simple genetic basis for insulin resistance (67). Thus genetic defects in the insulin signaling system validate the importance of the insulin signaling cascade, but they fail to explain the common cause of type2 diabetes.

1.4.2 Tyrosine phosphorylation of IRS-1 proteins

The COOH-terminal end of each IRS protein contains a set of tyrosine phosphorylation sites that act as on/off switches to recruit and regulate various downstream signaling proteins (7). Tyrosine phosphorylation of IRS-1 proteins constitutes the first event beyond activation of the IR tyrosine kinase that unleashes the intracellular transmission of insulin signals (69). Therefore, phosphorylation of IRS1 on tyrosine residues is mandatory for insulin-stimulated responses (70). The IRS proteins are phosphorylated on tyrosine residues by the activated insulin receptor kinase (71). It results in the generation of binding sites for SH2 domain containing proteins. Many of the tyrosine residues cluster into common motifs that bind and possibly activate specific effector proteins, including enzymes, the phosphotyrosine phosphatases and the Src-like kinase or adapter molecules (72). At the molecular level,

decrease in glucose uptake is linked to a reduced tyrosine phosphorylation of IRS1 in animal models of insulin resistance and in type 2 diabetic patients (21).

While the mechanisms involved in the decrease in IRS1 tyrosine phosphorylation have not been completely identified, the involvement of serine phosphorylation of IRS1 (70), protein phosphatases (2) and polymorphism in IRS gene (73) has been pointed out over the last decade.

1.4.3 Serine phosphorylation of IRS-1: Modulator of insulin signaling

In addition to tyrosine phosphorylation, both the insulin receptor and IRS proteins undergo serine phosphorylation, which may attenuate signaling by several mechanisms (74). IRS-1 and IRS2 each contain more than 100 potential serine phosphorylation sites. Current studies have demonstrated hyper-serine phosphorylation of IRS-1 on Ser-302, Ser-307, Ser-612, and Ser-632 in several insulin-resistant rodent models (75;76), as well as in lean insulin-resistant offspring of type 2 diabetic parents (77). Lately a study provide evidence for inhibitory role of serine phosphorylation of IRS-2 in hepatic insulin signaling by indicating phosphorylation of serines 484/488 of IRS-2 by JNK and GSK-3 (78). While the phosphorylation of IRS1 on tyrosine residue is required for insulin-stimulated responses, the phosphorylation of IRS1 on serine residues has a dual role, either to enhance or to terminate the insulin effects (70).

1.4.3.1 Serine phosphorylation as a feedback regulatory mechanism of insulin signaling

Control mechanisms are essential for cellular signaling. Tyrosine phosphorylated IRS proteins are key players in propagating insulin signaling and are the targets of such feedback regulatory systems. Regulation of IRS proteins involves proteasome-mediated degradation (79), phosphatase mediated dephosphorylation, (80) or Ser/Thr phosphorylation. The latter is an attractive regulatory mechanism because it enables multilevel control of the activation of IRS kinases and the specific targets among potential Ser/Thr phosphorylation sites in IRS proteins that might play regulatory roles during the insulin response (Figure 1). Ser/Thr phosphorylation reduces IRS-1 ability to undergo Tyr phosphorylation by the insulin receptor kinase and might serve as a physiological negative feedback control mechanism to turn off insulin signaling by uncoupling the IRS proteins from their upstream and downstream effectors (Fig. 2) (81-83).

1.4.3.2 Serine phosphorylation of IRS proteins as a negative modulator of insulin signaling

Recent studies reveal that agents that induce insulin resistance, exploit phosphorylation-based negative feedback control mechanisms otherwise utilized by insulin itself to uncouple the insulin receptor from its downstream effectors and thereby terminate insulin signal

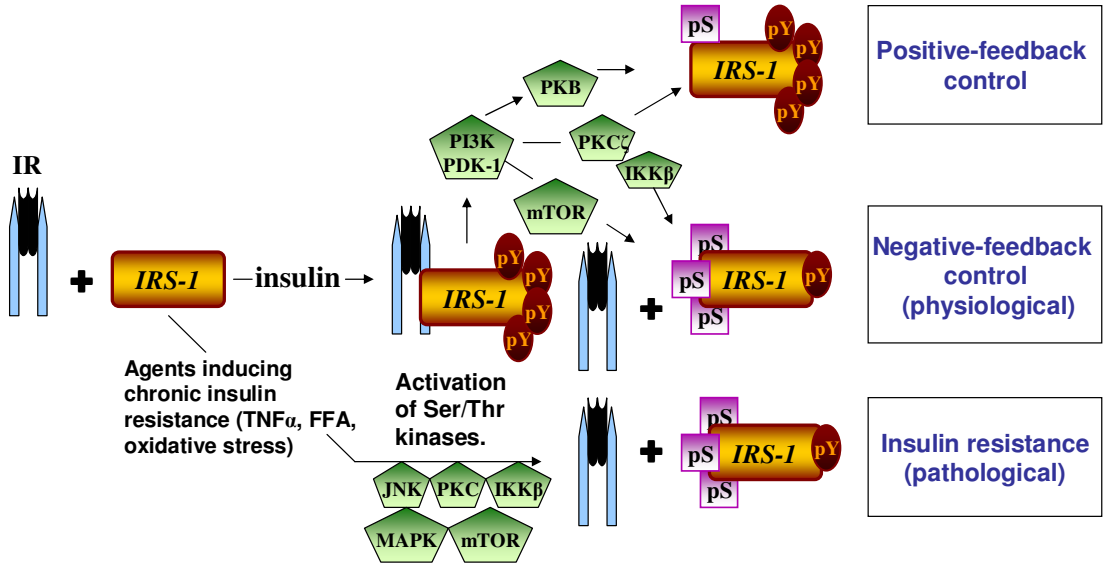


Fig .2. Serine phosphorylation of IRS-1 as modulator of insulin action and resistance. *Ser/Thr phosphorylation of IRS proteins has a dual role, either to enhance or to terminate signaling by insulin. Ser residues of the phosphotyrosin -binding (PTB) domain of IRS-1, located within consensus protein kinase B (PKB) phosphorylation sites, presumably function as positive effectors of insulin sigailing, thus, generating a positive-feedback loop for insulin action. Insulin also activates mTOR and PKC, which mediate phosphorylation of Ser/Thr residues within the IRS protein either directly or through activation of downstream effectors such as IκB kinase β (IKKβ). Phosphorylation of these sites is part of the negative-feedback control mechanism induced by insulin that results in the termination of insulin signaling. Agents that abrogate insulin action, such as free fatty acids (FFA) and tumor necrosis factor α (TNFα), take advantage of this negative feedback control mechanism by activating Ser/Thr kinases (e.g. JNK, PKCs, IKKβ, mTOR, MAPK) that, by mediating phosphorylation of IRS proteins, inhibit the function of IRS proteins, terminate insulin action and induce insulin resistance (84).*

transduction (Fig. 2) (85). The involvement of serine phosphorylation of IRS1 in the desensitization of insulin action has been pointed out over the last decade (21). Considerable data suggest that various pathological conditions associated with insulin resistance can promote serine phosphorylation of IRS-1 proteins. Stress-induced cytokine like TNF-α cause insulin resistance, at least in part, by serine phosphorylation of IRS-1. Regarding insulin resistance, studies have also suggested that agents, such as free fatty acids (FFA) (86;87), cellular stress (70), amino acids (88;89), endothelin-1 (90-92), angiotensin II (93) and hyperinsulinemia (94) which induce insulin resistance, can also activate serine/threonine kinases that phosphorylate IRS1 and inhibit its function (Fig. 2). The plasma concentrations of FFA (86), amino acids , angiotensin II (95;96), and endothelin-1 (97;98) are elevated in insulin resistant, obese and type 2 diabetic patients (99). IRS-1 Ser-312 and Ser-636 phosphorylation increased, in the insulin-resistant offspring of parents with type 2 diabetes compared to the control subjects (77). Many Ser/Thr kinases phosphorylate IRS-1 including Raf, MEK, MAPK, $P90^{rsk}$, JNK and PKC isoforms. Kinases downstream of the PI3-kinase cascade, PDK1, AKT, mTOR, $p70^{S6K}$, and GSK-3β are also involved (100-103). Consequently, the inhibition of IRS1 functions could represent a unifying mechanistic link between all factors involved in insulin resistance.

1.4.3.3 Mechanisms employed by Ser phosphorylation of IRS-1 to inhibit its function

Several mechanisms have been proposed to explain how serine phosphorylation can regulate insulin signaling. Ser-307 phosphorylation inhibit PTB domain function, which uncouple IRS-1 from insulin receptor (81). Some serine residues in IRS-1 bind 14-3-3 isoforms, which can target IRS-1 to sub-cellular compartments (Fig. 2) (104). Others sites might electrostatically block access to nearby tyrosine phosphorylation sites. Serine phosphorylation of IRS1 could induce conformational changes, steric hindrance, and cellular re-localization leading to decrease in tyrosine phosphorylation by IR, to reduce the recruitment of PI 3-kinase and to stimulate the IRS1 degradation pathway. Altogether, these events inhibit the IRS1 functions and promote insulin resistance (70).

1.4.3.4 Serine phosphorylation as a positive modulator of insulin signaling

The activation of PKB in response to insulin propagates insulin signaling and promotes the phosphorylation of IRS1 on serine residue in turn generating a positive-feedback loop for insulin action. Phosphorylation of Ser residues within the P-Tyr-binding (PTB) domain of IRS-1, by insulin-stimulated PKB, protects IRS proteins from the rapid action of PTPs, and enables the IRS proteins to maintain their Tyr-phosphorylated active conformation (105). Furthermore, activation of mTOR signaling induces Ser-302 phosphorylation and this positively influence signaling through the IR/IRS-1 axis (106).

1.4.3.5 Important serine phosphorylation sites of IRS-1

IRS-1 contains 232 Ser/Thr residues (23) many of which could be subjected to phosphorylation. Several candidate Ser residues were identified as potential targets for IRS-1 kinases. These include Ser-24 (107), 302 (108), 307 (109), 318 (110), 332 (103), 408 (83), 522 (111), 612 (91), 636 and 639 (112), 731 and 789 (113) (Numbering of Ser residues of IRS-1 is based on the rat sequence) (Fig. 3). It is becoming apparent that Ser/Thr phosphorylation of IRS proteins has a dual function, serving either a positive or a negative modulatory role in insulin signaling. Under pathological condition, inducers of insulin resistance presumably trigger the phosphorylation of only the negative sites with no effects on the positive sites, thus preventing the propagation of insulin signals mediated by IRS proteins and thus causing insulin resistance (114). Furthermore, data describing one specific serine residue of IRS-1 either as a positive (106;115) or as a negative (116;117) regulatory phosphorylation site suggest a time-and-stimulus dependent function of serine phosphorylation of IRS-1 (110).

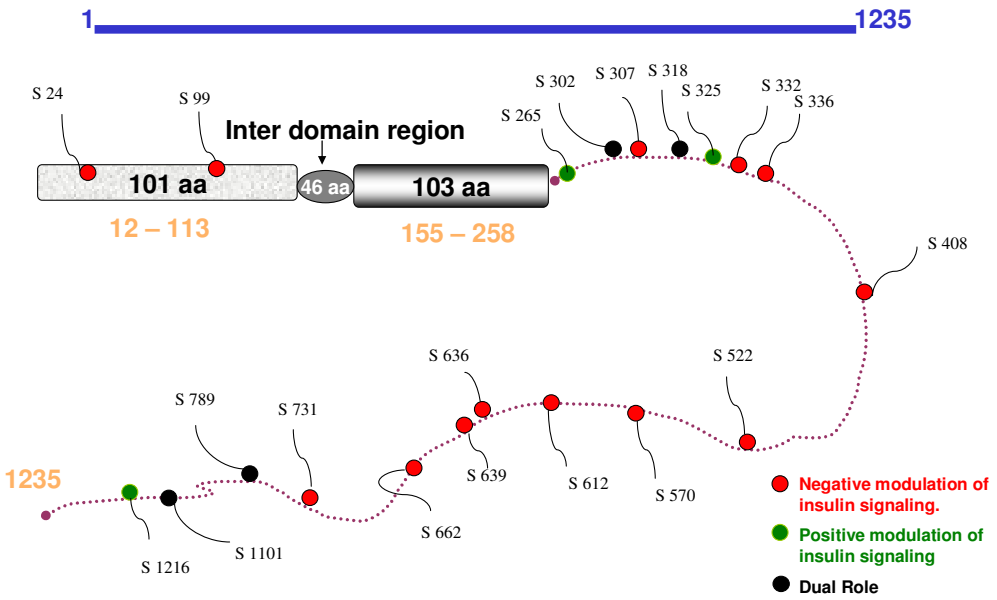


Fig. 3. Schematic diagram of rat IRS-1 serine phosphorylation sites.

Amino acid residues from 1 to 1235 are indicated. The N-terminal PH and PTB domains of IRS-1 are represented as boxes. IRS-1 serine phosphorylation sites with their relative location in IRS-1 are indicated. Positive, negative or dual role of the sites in insulin signaling is represented in different colours.

1.5 Protein kinase C as modulators of insulin signal transduction

Among IRS-1 kinases, members of the protein kinase C family of serine/threonine kinases have received considerable attention for their regulatory role in insulin signaling. The protein kinase C (PKC) family plays important roles in many intracellular signaling events, cell growth and differentiation (118;119). It is composed of a number of individual isoforms which belong to three distinct categories, conventional, novel and atypical, based upon their structurally distinct N-terminal regulatory domains. The major insulin-responsive tissues, skeletal muscle, liver and adipose tissue, express PKC isoforms from each of the categories, and the total number in each of these cells is in the range of 6–8 isoforms. These include conventional PKCs; α, βI and βII, novel PKCs; δ, ε and θ, and atypical PKCs; ζ or λ. Each of these isoforms has been shown to be activated by insulin stimulation or conditions important

for effective insulin stimulation (120;121). Most PKC isoforms have a dual action in stimulating insulin signaling and feeding back to induce insulin resistance in disease (122).

1.5.1 PKCs mediated upregulation of insulin signaling

There is increasing evidence that protein kinase C (PKC) isoforms modulate insulin-signaling pathways in both, positive and negative ways. Insulin stimulation or conditions important for effective insulin stimulation, activates PKCs α, βII, δ and ε in several cell types. In cultured mayotubes and intact muscles, it was shown that PKC βII is required for insulin-stimulated glucose uptake (123;124). Although PKC δ is primarily being reported as negative modulator of insulin signaling. But some of the reports suggest its positive effects on insulin signaling as well. Sampson et al (125) showed that insulin specifically induces PKC δ to associate with IR and that this IR-PKC δ association plays an important role in early IR signaling. In another study it was reported that insulin-induced GLUT4 translocation and glucose uptake was abrogated by inhibition of PKC δ, either pharmacologically or by overexpression of a kinase dead (dominant negative) PKC δ. Moreover, overexpression of WT PKC δ in the primary skeletal muscle cells increased GLUT4 translocation and glucose uptake in the absence of insulin stimulation (126).

A potential role of PKC ε as a transducer of insulin action was shown by its overexpression in NIH-3T3 fibroblasts, where the translocation of GLUT1 transporters to plasma membrane was greatly enhanced compared to (untransfected) cells where no translocation of GLUT1 was detected (127).The expression of PKC ε was shown to be depressed in muscle from Zucker obese insulin resistant rats (128). In one of the study it was reported that 4 weeks of physical exercise improved insulin-signaling responses in these animals and that this was associated with an increase in association of PKC ε with IR (145).

1.5.2 PKCs mediated downregulation of insulin signaling

While the activation of PKC isoforms is not necessarily a negative regulation of insulin action (85;129;130). But PKCs have been implicated in impaired insulin signaling for over one decade (131-133), including classical PKC, novel (134) and more recently also atypical PKC isoforms (135). Knockout of PKC-α in mice enhances insulin signaling and PKC-θ-deficient mice are protected from fat-induced insulin resistance (136;137). Protein kinase C α, β and ζ are increased in the liver of humans and rats with non-insulin-dependent diabetes mellitus

(138). It would thus appear that the previously described positive regulation by PKC δ of IR signaling might involve the intervention of another protein such as Src tyrosine kinase (Sampson 60). PKCα, expressed in cells co-expressing either IR or IRS1 (3T3ir, HEK293, COSIR) induced their phosphorylation, supporting the idea that PKCα might in fact play a role in development of insulin-resistance (134;139). As a modulator of insulin action, PKCε (140) has been shown to increase the inhibitory effect of TNF-α on insulin signaling (141). In skeletal muscle from obese, insulin resistant patients, the decrease in PKC θ expression was noted (142). In summary, each of PKC isoforms has been shown one way or another to modify insulin-stimulated effects in one or all of the insulin-responsive tissues (143).

1.5.2.1 Regulation of serine phosphorylation of IRS-1 by PKC isoforms

In vitro and *in vitro* studies have shown that increased Ser/Thr phosphorylation of IRS-1 by protein kinase C (PKC) impairs insulin signaling (144). PKCs phosphorylates IRS proteins on serine residues and acts as a negative feedback control regulator that turns off insulin signals by inducing the dissociation of IRS proteins from IR (81). Indeed, 12-O-tetradecanoylphorbol 13-acetate, a potent activator of various PKC isoforms, effectively inhibits both, IRS-1 interactions with the juxta-membrane region of the insulin receptor and insulin's ability to phosphorylate IRS proteins, thus implicating diacylglycerol-activated PKCs as potential regulators of IR-IRS interactions (81). Prolonged insulin stimulation of PKC-ζ can also participate in the negative regulation of insulin signaling by phosphorylating IRS-1 at serine residues (145). The phosphorylation of Ser-318 of IRS-1 by PKC-ζ has been suggested to be involved in the negative regulation of insulin signal transduction (81;110;146). Additionally, PKC-θ-dependent phosphorylation of Ser-1101 of IRS-1 has been reported in muscle cells (147).

1.5.2.2 PKC-δ-mediated downregulation of insulin signaling via serine phosphorylation of IRS-1

As a serine–threonine kinase, PKC-δ might be expected to function primarily as a negative regulator of IR signaling, particularly in view of the role of serine phosphorylation on activities of IR and IRS (148-150). In deed many studies have shown that PKC-δ decrease tyrosine phosphorylation of IRS-1 and may be involved in increase in serine phosphorylation of IR and IRS-1 (122;151), suggesting that the same kinases that mediate insulin signaling

might also play roles in negative feedback of it. PKC-δ, although presumably requiring co-expression of IRS-1, inhibits the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (152) . In addition, PKC-δ has been overexpressed in CHO cells and when activated by phorbol esters found to increase serine phosphorylation of IRS-1 (153;154). In the same report, it was shown in H4IIE hepatoma cells that expression of either constitutively active PKC-δ, or of wild type PKC-δ followed by phorbol ester stimulation, inhibited tyrosine phosphorylation of IRS-1 in response to insulin. Previously, PKC-δ was shown to phosphorylate several serine residues, including Ser-307, Ser-323 and Ser-574 (human sequence) that inhibit IRS-1 tyrosine phosphorylation (155). It has been suggested that the phosphorylation of IRS-1 on serine/threonine residues is the mechanism by which PKCs regulate insulin action (122). A few PKC-δ-dependent phosphorylation sites had been identified in recent times, among them, Ser-24 (155), Ser-318 (155) are important. These results support the notion that insulin-activated PKC-δ serves as a negative regulator of IR signaling.

1.6 Aims of the study

Albeit Ser/Thr phosphorylation has been increasingly recognized as a negative counterbalance to positive IRS signaling through tyrosine phosphorylation (84) and Ser/Thr phosphorylation could be a generalized mechanism for insulin resistance. But from a molecular perspective it has been difficult, however, to identify discrete sites that are both phosphorylated *in vivo* and when phosphorylated, have relevant functional consequences. Therefore attempts to reveal potential Serine phosphorylation sites in IRS-1 that regulate IRS-1 function during insulin simulation is worthwhile. Studies on regulation and function of novel serine phosphorylation sites of IRS-1 provide the rational mechanism to explain insulin resistance.

With this background, following aims were established for the project to study the role and function of Ser-357 of IRS-1 in insulin signal transduction;

1. To generate polyclonal phospho-site-specific Ser-357 antibody by immunizing the rabbits with synthetic peptide flanking phosphorylated-Ser-357.
2. To purify the antiserum raised against phospho-Ser-357. Additionally, to eliminate cross reactivity of the antibody with adjacent Ser-358 by immuno-purification.
3. To characterize specificity of phospho-site-specific Ser-357 in living cell.
4. To study the stimuli which induce phosphorylation of Ser-357 in skeletal muscle.

5. To generate following mutants of IRS-1 by PCR site-directed mutagenesis, to study the function of Ser-357.

    a. Loss of function mutants
        i. IRS-1 Ala-357
        ii. IRS-1 Ala-357358
    b. Gain of function mutants
        i. IRS-1 Glu-357
    c. To study the single effect of Ser-357 and Ser-358
        i. IRS-1 Ala-358
        ii. IRS-1 Glu-357 Ala-358
6. To study specific kinase involve in the phosphorylation of Ser-357.
7. To study the function/biological relevance of Ser-357 phosphorylation in insulin signaling by investigation of effect of Ser-357 phosphorylation on following key molecules of insulin signaling;
    a. Tyrosine phosphorylation of IRS-1
    b. PKB/AKT phosphorylation
    c. GSK-3 phosphorylation
8. To study the single function of Ser-357 and Ser-358 in insulin signaling
9. To study the phosphorylation of Ser-357 in tissues from mice stimulated with insulin.
10. To study the phosphorylation of Ser-357 phosphorylation in human mayotubes.

2- MATERIALS, SOFTWARE AND DEVICES

2.1 Enzymes, Proteins, Nucleic acids and substrates

λ- Protein phosphatase (λ-PPase)	New England BioLabs, Beverly MA, USA
λ-PPase reaction buffer	New England BioLabs, Beverly MA, USA
Ligase	Roche molecular Diagnostics, Mannheim, Germany
Ligase buffer	Roche molecular Diagnostics, Mannheim, Germany
Plasmid DNA IRS-1 (rat)	Lab Internal tool
Plasmid DNA PKC-δ (mouse)	Lab Internal tool
Plasmid PKC ζ, PKC-θ, PKC-λ	Lab Internal tool
pcDNA3	Lab Internal tool
Plasmid DNA kinase-negative (KN) PKC-δ	Lab Internal tool
Sequencing primers	Invitrogen, Karlsruhe, Germany
PWO polymerase	peqLab GmbH, Munich, Germany
10x Reaction buffer for Pwo polymerase	peqLab GmbH, Munich, Germany
DNTPs	peqLab GmbH, Munich, Germany
Restriction endonuclease	Roche, Mannheim, Germany
DNA-sequencing mix premix	Perkin Elmer, Überlingen, Germany
BamHI	Roche Mannheim, Germany
XhoI	Roche Mannheim, Germany

2.2 Antibodies

2.2.1 Antibodies against IRS-1

IRS-1, polyclonal, against C-terminus	Dr Rainer Lehmann
IRS-1, polyclonal, against C-terminus	Upstate, Lake Placid, USA
IRS1 phospho-Ser 318, polyclonal	Dr Rainer Lehmann

2.2.2 Antibodies against PKCs

Anti PKC δ, monoclonal	BD TransductionLaboratories, San Diego,

2.2.3 Antibodies against signal transduction molecules

Anti-phospho-PKB/Akt (Ser 473)	Cell Signaling, Frankfurt, Germany
Anti-PKB/Akt (mouse), polyclonal	Upstate Ltd, Wolverton, UK
Anti-phospho-GSK-3α/β (Ser 21/9), polyclonal	Cell Signaling, Heidelberg, Germany
Anti-GSK-3β (mouse) monoclonal	Santa Cruz Biotechnology, USA
Phospho-tyrosine, (P-tyr-100) (Mouse), monoclonal	Cell Signaling, Frankfurt, Germany

2.2.4 Secondary Antibodies

Anti rabbit IgG HRP, goat	Santa Cruz Biotechnology, USA
Anti mouse IgG HRP, goat	Santa Cruz Biotechnology, USA

2.3 Kits

QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
Plasmid Maxi Kit (25)	Qiagen, Hilden, Germany
Plasmid Mini Kit (25)	Qiagen, Hilden, Germany
PCR Purification Kit (250)	Qiagen, Hilden, Germany
Quick change site-directed mutagenesis kit	Stratagene, Germany
Bio-Rad-Kit	Biorad, München, Germany

2.4 Chemicals and solvents

Agarose, peqGold	peqLab GmbH, Munich,Germany
Ammonium persulfate (APS)	Sigma, Steinheim, Germany
β-Mercaptoethanol	Merck, Darmstadt, Germany
BSA, bovine serum albumin	Roche, Mannheim, Germany
Bromophenol Blue	Merck, Darmstadt, Germany
$CaCl_2$	Sigma, Deisenhofen, Germany

Chloroform	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma, Deisenhofen, Germany
DMSO (dimethyl sulfoxide)	Merck, Darmstadt, Germany
Dulbeccos-PBS	Bio Whittaker, Verviers, Belgium
EDTA	Sigma, Deisenhofen, Germany
EGTA	Sigma, Deisenhofen, Germany
Acetic acid	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidiumbromide	Sigma, Deisenhofen, Germany
Formaldehyde	Aldrich, Steinheim, Germany
FUGENE 6	Roche, Mannheim, Germany
Gelatine	Merck, Darmstadt, Germany
Glycine	Bio-Rad, Munich, Germany
Glycerol	Merck, Darmstadt, Germany
HEPES	Sigma, Deisenhofen, Germany
Insulin from bovine pancrease	Sigma, Deisenhofen, Germany
Isopropanol	Merck, Darmstadt, Germany
Rapid fixer for curix 60G354	Agfa, USA
Developer for curix 60A/BG153	Agfa, USA
Luminol (3-aminophtalhydrazide)	Aldrich, Steinheim, Germany
$MgCl_2 .6 H_2O$	Sigma, Deisenhofen, Germany
Sodium carbonate	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium fluoride	Merck, Darmstadt, Germany
Natriumorthovanadate	Sigma, Deisenhofen, Germany
Natriumpyrophosphate	Sigma, Deisenhofen, Germany
p-Iodophenol	Aldrich, Steinheim, Germany
Ponceau	Sigma, Deisenhofen, Germany
Phorbol-12-myristate-13-acetate, for cell culture	Sigma, Deisenhofen, Germany
Protein A-Sepharose (Suspension)	Amersham Biosciences, Freiburg, Germany
Hydrochloric acid (37 %)	Applichem, Darmstadt, Germany
SDS ultra pure	Roth, Karlsruhe, Germany

Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe, Germany
Tris-(hydroxymethyl)-aminomethan	Merck, Darmstadt, Germany
Tris-(hydroxymethyl) aminomethan, ultrapur -	ICN Biomedicals Inc, USA
TritonX-100 (t-octylphenoxypolyethoxyethanol)	Sigma, Deisenhofen, Germany
Tryptan blue	Biochrom, Berlin, Germany
Hydrogen peroxide, 30% solution	Aldrich, Steinheim, Germany
Xylenecyanol	Sigma, Deisenhofen, Germany

2.5 Molecular markers

"Low Molecular Weight" Marker	Amersham Biosciences, Freiburg, Germany
"High Molecular Weight" Marker	Amersham Biosciences, Freiburg, Germany
DNA-Molecular Weight Marker X (0.07-12.2 kbp)	Roche Molecular Diagnostics, Mannheim, Germany

2.6 Solutions and buffers

All buffer substances and solvents were p.a. or better quality. Water was either Milli Q or HPLC-Water.

Electrophoresis-running buffer: The Stock Buffer (10X) was prepared by dissolving 0.025 M (30.3 g) Tris, 0.19 M (144 g) glycine, 0.1% (10g) SDS in 1 litrer of water, pH was adjusted at 8.3.
Shortly before use stock was diluted by 1:10 with water to make 1X buffer.

Separation gel buffer: 1.5 M (90.8 g) Tris, 2 % (10 g) SDS were dissolved in 500 ml H_2O and pH was adjusted at 8.8.

Stacking gel buffer: 0.5 M (30.3 g) Tris; 2 % (2 g) SDS were dissolved in 500 ml H_2O. The pH was maintained with 25 % HCl at 6.8.

Sample buffer, Laemmli: Stock buffer (5X) was prepared by dissolving 1M Tris HCl (pH 6.8), 10% SDS; 1% bromphenolblue, 50% glycerol and β-mercaptoethanol, volume was raised to 10 ml with H_2O. Stock was diluted by 1:5 with protein sample.

Sample buffer, for agarose gel electrophoresis: 0.1 % bromphenolblue and 0.1% xylenecyanol, 60 % glycerol and 2 ml of 10x TAE buffer was dissolved in H_2O and volume was raised till 10 ml.

TAE(1X) (Tris/Acetat/EDTA): To prepare TAE buffer 40 mM Tris and 2 mM EDTA were dissolved in 1 liter of H_2O.The pH was adjusted with natrium acetate at 8.0.

KCM buffer: Stock solution (5X) of KCM buffer was prepared by dissolving 0.5M KCl, 0.25M $MgCl_2$ and 0.09M $CaCl_2$ in 1 liter of H_2O.

Blotting buffer: Stock blotting buffer (10X) was prepared by dissolving 480 mM Tris, 390 mM glycin and 0.4 % SDS.
100 ml of Stock blotting buffer was diluted with 200 ml Methanol and 700 ml H_2O.

10 x NET "G": Stock solution of NET "G" was prepared by dissolving 1.5 M NaCl, 50 mM EDTA, 500 mM Tris, 10% Triton X100 and 2.5 % gelatin. Shortly before use the stock solution was diluted by 1:10 with water and pH was adjusted at 7.4 with HCl.

ECL Solutions:
Luminol : 0.5 M Luminol was prepared by dissolving 0.885 g of Luminol in 10 ml of DMSO.
p-Iodophenol : 0.5M p-iodophenol was prepared by dissolving 1.1 g of p-iodophenol in 10 ml of DMSO.
Luminol and p-iodophenol solutions were stored away from light, at 4 ° C, and shortly before preparing ECL-Solution A and B, were liquified at room temperature (RT).

0.1 M Tris/HCl buffer; 12.11 g Tris was dissolved in H_2O and after adjusting the pH at 9.35 volume was raised to 1 L.

ECL-Solution A; 880 µl, 0.5 mol Luminol and 864 µl, 0.5 mol p-iodophenol were dissolved in 100 ml of 0.1 M Tris.

ECL-Solution B; 0.0075 % (25ul from 30%)of H_2O_2 was dissolved in 100 ml of 0.1 M Tris/HCl: 25 µl

The ECL-solutions A and B were stored at 4°C (maximum till 2 weeks) and were used in 1:1 ratio.

Stripping-buffer: Stripping buffer was prepared by dissolving 66 mM Tris, 0.5 % β-Mercaptoethanol and 2 % SDS in 500 ml H_2O, the pH was adjusted with around 10 ml, 25 % HCl at 6.8 and volume was raised with H_2O to 1 liter.

TBS/T buffer 10X : The stock(10X) buffer was prepared by dissolving 200 mM Tris and 1.37 M NaCl in 1 liter H_2O.

TBS/T buffer was prepared by dissolving TBS (10X) in water at 1:10 ratio and 0.1% Tween 20 was added.in the end addition of 5% BSA produced complete TBST/T buffer.

BBS: To prepare BBS buffer for transfection in mammalian cells, 50 mM BES, 280 mM NaCl-solution and 1.5 mM Na_2HPO_4 solution was mixed and volume was raised till 90 ml with H_2O. Then pH was adjusted exactly at 6.96 with NaOH and volume was raised till 100 ml with H_2O.

BBS was sterilized by passing through a 0.22 micron filter.

BBS buffer was first tested for a transfection test before use in the experiment.

Lysis buffer (A):

Lysis buffer (A) was prepared by dissolving 50mM HEPES, 150mM NaCl, 1.5mM $MgCl_2$, EGTA, 10% glycerole ,1% Triton-X-100, 100mM NaF and 10mM $Na_4P_2O_7$ together and the volume was raised with H_2O to100ml. The pH was adjusted with 37% HCl to 7.5.

Phosphatase inhibitor was freshly added to the lysis buffer shortly before use.

Lysis buffer (B): Lysis buffer (B) was prepared by dissolving 50 mM Tris,150 mM NaCl and 1 % Triton X-100 together and the volume was raised with H_2O to100ml. The pH was adjusted with 37% HCl to 7.6.

Phosphatase inhibitor (10 x): Stock solution (10X) of phosphatase inhibitor was prepared by dissolving 100 mM sodiumfluoride (NaF), 100 mM sodiumorthovanadate (Na_3VO_4), 100 mM β-glycerophosphate (glycerol-2-phosphate) ($C_3H_7O_6PNa_2$) and 50 mM sodium pyrophosphate ($Na_4P_2O_7$ .10 H_2O) in water and volume was raised to 100ml.
Solution was stored at -20°C in 1ml aliquots.

25 X solution of complete protease inhibitor:
One tablet complete protease inhibitor (Roche Germany) was dissolved in 2 ml H_2O to make a working solution of protease inhibitor. Protease inhibitor and phosphatase inhibitor were freshly added to the lysis buffer (B) shortly before use.

HNTG-Buffer: HNTG buffer was prepared by dissolving 20 mM HEPES, 10 mM NaF, 150 mM NaCl, 1% TritonX 100 and 10% Glycerol in 100ml $H_2O$and the pH was adjusted at 7.5.

2.7 Culture media and antibiotics

Glucose	Sigma, Deisenhofen, Germany
L-Glutamine	GIBCO, Invitrogen, Karlsruhe, Germany
Penicillin-streptomycin (PenStrep, 100.000 U)	GIBCO, Invitrogen, Karlsruhe, Germany
LB Broth,	Miller Sigma, Deisenhofen, Germany
LB Agar (Lennox L Agar)	Sigma, Deisenhofen, Germany
DMEM 1 g Glc/L, without glutamine	Bio Whittaker, Verviers, Belgium
DMEM 4.5 g Glc/L, without glutamine	Bio Whittaker, Verviers, Belgium
Ampicillin GIBCO,	Invitrogen, Karlsruhe, Germany

LB-Medium (Luria-Broth): 25 g LB was dissolved in 1 L H_2O and autoclaved.

LB/Amp-Medium: after autoclaving ampicilin was added in 100 µg/ml concentration in the LB-Medium.
LB/Amp-agar plates: after autoclaving and cooling the LB agar to 50°C, ampicillin was added in 100 µg/ml end concentration.15 ml LB-agar was added in each plate.

Standard medium for C2C12 and BHK-IR cells: Standard growth medium for cells was prepared by mixing 430 ml of DMEM with 4.5 g glucose/L (Bio Whittaker, Verviers, Belgium), 10% heat inactivated FCS, 100 U Penicillin and streptomycin, 2 mM glutamine, 1mM sodium pyruvate and 1x non-essential amino acids.

Starvation medium for C2C12 and BHK-IR cells: Standard starvation medium for cells was prepared by mixing 500 ml of DMEM with 1 g glucose/L (Bio Whittaker, Verviers, Belgium) and 2 mM glutamine.

2.8 Consumable material

Gel blotting paper	Schleicher & Schüll, Dassel, Germany
Hyper cassette for the detection of chemiluminescence of protein blots	Amersham Biosciences, Freiburg, Germany
Hyperfilm ECL 18 x 24 cm^2	Amersham Biosciences, Freiburg, Germany
Millipore Filter 0.22 µm pore size	Millipore, Bedford, USA
Pipette tipps	Eppendorf, Hamburg, Germany
Nitrocellulose membrane protein (BA 85 0.45µm)	Schleicher Schüll, Dassel, Germany

2.9 Cells and Bacterial strains

2.9.1 Cells

BHK (ATCC No. CCL 10): This cell line was established in 1961 from the kidney of five hamsters (Mesocricetus auratus), which was one day old. This cell line was kindly provided by R. Lammers, Tübingen.

BHKIR-cell line is a cell line derived from BHK-cells with a stably transfected insulin receptor (IR). This cell line was kindly provided by NP Møller, Novo Nordisk, Denmark.

C2C12: C2C12 cells were from ATCC (Wesel, Germany).

2.9.2 Bacterial Strains

Competent E. coli- XL1 Blue for transformation	Novagen, Madison, USA

2.10 Laboratory equipments

Genesys 5 spectrophotometer	Spectronic, Runcorn, Cheshire, England
Sterling Diagnostic Imaging apparatus	DuPont de Nemours, Bad Homburg, Germany
HP Scanjet 4600	HP, USA
Hera safe hood	Heraeus , Germany
Incubators	Heraeus, Germany
Thermocycler:	
• Progene	Techne, Dexford-Cambridge, UK
• Mastercycler 5330	Eppendorf, Hamburg, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortex-Genie 2	Scientific Industries, USA
Heating block	Scientific Industries, USA
Water bath	Julabo, Seelbach, Germany
Centrifuges:	
• Minicentrifuge	Qualtron Inc., Korea
• AvantiTM J-25 with Rotors JA-17and JLA-10.500	Beckman, Munich, Germany
• Biofuge fresco	Heraeus/Kendro, Hanau, Germany
• Megafuge 2.0 R	Heraeus/Kendro, Hanau, Germany
pH-Electrode SenTix 81	WTW, Weilheim, Germany
pH-Meter inoLab	WTW, Weilheim, Germany
Electrophoresis chamber for agarose	PolyMehr, Kassel, Germany
Electrophoresis chamber for SDS-PAGE ($20x20cm^2$)	PolyMehr, Kassel, Germany
Blotting apparatus	PolyMehr, kassel, Germany
Autoklav SANOclav	Wolf, Geislingen, Germany

2.11 Software

GelScan 5.02 BioSciTec,	Frankfurt am Main, Germany
Powerpoint 2000	Microsoft Corporation, USA
Reference Manager 10.0 ISI	ResearchSoft, USA
Literature –data base	http://www.ncbi.nlm.nih gov/entrez/query.fcgi
Protein data base	http://www.expasy.org/ http://www.expasy.org/prosite/ http://www.hprd.org /PhosphoMotif_finder

3. METHODS

3.1 Protein Biochemical Methods

3.3.1 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

To separate cell lysates and immunoprecipitated proteins, the discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used (156). SDS binds to hydrophobic domains of proteins and disrupts their folded structure allowing them to exist stable in solution. The resulting SDS-protein complex is proportional to the molecular weight of the protein. SDS-protein complexes have all a negative charge and can be size-separated; SDS-treatment masks individual charge differences of proteins. During separation, SDS-protein complexes are attracted to the anode and separated by enforcement through the porous acrylamide gel. Usually, proteins are first concentrated on a stacking gel and later separated on a separating gel.

In this work, a separating gel of 7.5% acrylamide was used. Protein samples were combined with 5x Laemmli-buffer and heated at 95 °C for 5 min.

3.3.2 Dephosphorylation assay

For dephosphorylation of phosphorylated proteins from crude extract of cells, Lambda Proteins Phosphatase (λ-PPase) was used in dephosphorylation assays. λ-PPase is a Mn^{2+} - dependent protein phosphatase with activity towards phosphorylated serine, threonine and tyrosine residues (157). Immunoprecipitated IRS-1 was incubated with 100 U of λ-phosphatase and reaction buffer for 30 min at 30°C. Protein was then separated by SDS-PAGE and analysed for dephosphorylation by western blot.

3.3.3 Quantification of proteins

Total protein concentrations were determined using the dye-based Bradford assay. This assay is also referred to as Bio-Rad assay after the company which sells the kit. Aliquots of samples (ranging from 1-5 µl) were diluted in water. 800 µl of sample and 200 µl of the Bio-Rad-Kit were added. Extinction of the contained Coomassie Brilliant Blue G-250 dye, in response to various concentrations of protein, was then measured after 20-30 min at 595 nm in a spectrophotometer.

A standard curve with samples of known protein concentration was prepared in parallel, to assess the unknown protein concentrations. Bovine serum albumin, BSA, was used as a standard and the following dilutions were usually used for the standard curve:

Table 1. Known protein concentrations used for the standard curve.

Standard	Protein /µg	µl BSA(200µg/ml)
1	0	0
2	4	20
3	8	40
4	12	60
5	16	80
6	20	100

Extinction was measured in plastic cuvettes and linear regression was used for the calculation of unknown protein concentration of the sample.

3.2 Immunological Methods

3.2.1 Generation and purification of phospho-site-specific-Ser-357 IRS-1 (p-Ser-357) antibodies

The antibody was generated as described in (158). Polyclonal phospho-site-specific-Ser357 antiserum was raised against a synthetic peptide (AHRHRGpSSRLHPPLNHSRSI) flanking Ser357 in IRS-1 (the numbering of IRS-1 refers to the rat sequence). The peptide was synthesized as a single peptide and as a multiple antigen peptide (MAP) (AHRHRGpSSRLHPPLNHSRSI)8 -(Lys)4-(Lys)2-Lys-Gly-OH using standard Fmoc/tBu chemistry as described above. After purification, the peptide was coupled to keyhole limpet hemocycanin using the glutardialdehyde method. The antiserum was obtained after repeated immunization of a rabbit with a 1:1 mixture of the peptide-keyhole limpet hemocycanin conjugate and the MAP. This antiserum was purified by multiple purification steps using immuno-affinity chromatography. In the first step of purification, the antiserum was purified using a non-phospho-peptide affinity column to eliminate the ability of the antibody to react with non-phosphorylated versions of the peptide. Since antiserum was showing cross reactivity with adjacent Ser358 as well, consequently, this cross reactivity was removed by performing an additional purification step, using phosho-Ser358 peptide, through immuno-affinity chromatography.

3.2.2 Enzyme-linked immunoSorbent assay (ELISA)

The wells of the microtiter plates (Nunc Brand Products, MaxiSorb surface, Wiesbaden, Germany) were coated with 5 µg of different peptides, listed in Table 1, in PBS in a final volume of 100 µl/well at 4°C overnight. The plates were washed three times with 200 µl of washing buffer (PBS/0.05% Tween 20, pH 7.0) and blocked with blocking buffer (PBS/0.05% Tween 20, pH 7.0, containing 2% BSA) for 2 hr at 37°C. After washing, the plates were treated for 1 h at 37°C with purified phospho-site-specific-Ser357 antibody (diluted in PBS/0.05% Tween 20, pH 7.0, containing 0.5% BSA) or the phospho-site-specific-Ser357 antiserum. After washing, the plates were incubated with HRP-conjugated goat anti-rabbit Ig (Dianova, Hamburg, Germany; 1:5000 diluted in PBS/0.05% Tween 20/0.5% BSA). ABTS (azinodiethyl-benzthiazoline-sulfonate) and H_2O_2 in substrate buffer (citrate buffer 100mM, pH 4.5) was added (100 µl/well) and the colour development analyzed at a wavelength of 405 nm (158).

3.2.3 Immunoprecipitation (IP)

For co-immunoprecipitation, cells were lysed in lysis buffer-1, supplemented with phosphatase inhibitors. Immunoprecipitation was carried out by incubating 250 µg of protein with 3 µl of specific antibody and 30 µl of protein A-Sepharose to capture the complexes at 4°C for 4 hours. Immunoprecipitates were washed twice with HNTG buffer supplemented with phosphatase inhibitors. After washing, protein was mixed with Laemmli (5X) and incubated at 95°C for 5 min. Sample was run on SDS-PAGE for Western blot analysis.

3.2.4 Protein transfer to nitrocellulose membranes (Western blot)

Western Blotting has been devised for the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets and their subsequent detection by antibodies (159). This technique can give information about the size of protein (with comparison to a size marker or ladder in kDa), or identify the phosphorylation expression level.

The separated protein of cellular extracts or IP from SDS-PAGE were transferred and immobilized to nitrocellulose membranes (0.45 µm pore size) by semi-dry Western blotting. Gel and membrane was placed between two blotting papers soaked in blotting buffer, to remove electrophoresis salts and detergents. Transfer was performed at 0.8 mA / cm^2 for 2 hours at room temperature.

3.2.5 Staining of proteins on nitrocellulose membranes with Ponceau S

The transfer efficiency of proteins from polyacrylamide gel to nitrocellulose membrane was checked by Ponceau staining of the nitrocellulose membranes. It is a ready–to–use, reversible staining solution designed for rapid (5 minute) staining of protein bands on nitrocellulose membranes. Ponceau S stain is easily reversed with water washes, facilitating subsequent immunological detection.

3.2.6 Detection of proteins by antibodies

In a western blot, it is important to block the non-reacted sites on the membrane to reduce the amount of non-specific binding of proteins during subsequent steps in the assay. Therefore, nitrocellulose membranes were subsequently soaked with blocking agent (NET-G) in a plastic container. This step was repeated 3x, each time for 15 min.

After the membrane was blocked, so that primary antibodies can not bind to the membrane non-specifically, a solution of the primary antibody or also called first antibody in NET-G was added and gently rocked for 2h at room temperature or overnight at 4 °C. Antibody solutions for Western blotting were typically diluted from 1/500 to 1/10,000.

Membranes were subsequently washed in NET-G 4x, each time for 15 min (first antibody wash). Second antibodies were HRP-conjugate, anti-mouse or anti-rabbit, depending on the animal in which the primary antibody was raised (the host species). A dilution (ranging from 1:2500 -1:5000) of the appropriate second antibody was added and incubated at room temperature for 30-60 min. The second antibody recognizes the Fc portion of the first antibody. Second antibody wash was performed four times, lasting 15 min, each.

Enhanced chemiluminescence system was used for the detection of proteins of interest on nitrocellulose membranes. This method has become the method of choice for Western blot analysis because it gives several advantages over other detection methods.The membranes were incubated with a 1:1 (v/v) ratio of solution A and B (as described in materials) for 3 min. Emitted light was detected by hyperfilm ECL films. Films were developed using a Sterling Diagnostic Imaging apparatus

3.2.7 Densitometry

Protein images on films were scanned using a HP Scanjet (4600 Series). Densitometry was carried out using Gelscan professional V5.1 software.

3.2.8 Stripping antibodies from nitrocellulose- membranes

Stripping is the term used to describe the removal of primary and secondary antibodies from the membrane. Stripping was used when more than one protein was investigated on the same blot or the same protein with different antibodies (for example when a phospho-specific antibody was probed, then the relative total amount of protein was reprobed).

The detection reagents from membrane was removed and membrane was washed for 15 min in NET"G". The blot was incubated with stripping buffer at 56°C for exactly 30 min in a water bath. The membrane was blocked with NET"G" for another 15 min and incubated with a new antibody solution overnight.

Membranes were stored after use in heat-sealed plastic bags at 4 °C.

3.3 Molecular methods

3.3.1 Cell cultivation

All works were carried out under sterile conditions in a "Sterile Hood".

Cryopreserved cells C2C12/BHK-IR were transferred in 15 ml DMEM (4.5g/L glucose) standard growth medium for C2C12/BHK-IR cells. Cells were incubated at 37 °C / 5% CO_2 either in 15 cm plates or 175 cm^2 cell culture flasks. After 4 hours, the medium was changed and after 24 hours cells were sub-cultivated in 1:10 split ratio.

3.3.2 Cell splitting/passaging

C2C12 cells were grown for 48 hours and BHK-IR for 72 hours. As they were 80% confluent, they were considered ready for splitting. For splitting, first medium was removed, and then cells were rinsed with 5 ml of PBS. After removal of PBS, 4 ml trypsin-EDTA was added in the plate and incubated at 37 °C / 5% CO_2 for 4 min. When cells were detached from the plate 5 ml DMEM growth media was added to stop trypsin activity and cells were centrifuged at 900 rpm for 4 min. The pellet was resuspended in 1ml DMEM growth media. 100 µl of cell suspension (1:10 split ratio) was added in a new plate containing 15 ml fresh DMEM growth media. Cells were incubated in 37 °C / 5% CO_2 for another 48 hours.

3.3.3 Cell counting

For cell culture experiments it is necessary to determine cell concentration to use particular number of cells in the experiment.

50µl of cell suspension was mixed with 450 µl of Trypan blue. After short vortexing, 20 µl of this mixture was added in the Neubauer cell counting chamber. Cells were counted in four large corner squares. The cell number was determined by multiplying the average of four squares with 10^5 (10^4 chamber factor x dilution factor).

3.3.4 Transfection

Transfection is the introduction of cloned eukaryotic DNA into cultured mammalian cells. The most widely used method is transfection mediated by calcium phosphate, although the mechanism remains ambiguous, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and it is transferred to the nucleus. To transfer wild type or mutated IRS-1 into the cells, pRK5 was used as a vector. For transient transfection, cells were seeded one day before and grown to 80% confluence in 6 well plates having $1x10^5$ cells per well in 2 ml DMEM growth medium. All transfections were performed with standard calcium phosphate precipitate protocol (160). In this method, cells were transfected with 4µg of rat IRS-1 cDNA in pRK5-vector or PKCs cDNA in pLXSN (161;162). Total DNA amount used for transfecting one well of 6 well plate was 4 µg, while performing co-transfection of two different DNAs, 2 µg of each plasmid was used for each well. DNA was mixed with 0.25 M $CaCl_2$ and 2x BBS buffer and incubated to develop fine precipitates. Empty vector, pcDNA3 was used as negative control of transfection.

200 µl of calcium-phosphate-DNA suspension was transferred into the medium above the cell monolayer in dropwise fashion. Cells were incubated at 37 °C / 5% CO_2 for 24 hours. At the end of this incubation the medium and precipitates were removed by aspiration and 2 ml of fresh growth medium was added.

3.3.5 Stimulation and lysis of the cells

After 48 hours of transfection, medium was changed with serum free (starvation medium) medium and cells were incubated with this medium for 3 hours. At the end of this incubation cells were stimulated with 10 nM insulin to investigate insulin signal transduction or with 0.5 µM 12-O-tetradecanoylphorbol-13-acetate (TPA) to activate novel and classical PKCs.

After stimulation medium was removed and cells were washed with PBS. To perform lysis of the cells, 200 µl of lysis buffer 1 was added in each well of six well plates and incubated at 4°C for 5 min. To remove cell debris, cells were harvested and centrifuged for 5 min at 13000 rpm and 4°C.The supernatant containing proteins was either processed for IP or mixed with

Laemmli (5X) and incubated at 95°C for 5 min. 40µg of protein was loaded on gel for SDS-PAGE.

3.3.6 Cryopreservation of mammalian cells

Mammalian cells are cryopreserved to avoid loss by contamination, to minimize genetic change in continuous cell lines, and to avoid aging and transformation in finite cell lines. Before cryopreservation, cells were characterized and checked for contamination. The media used to freeze cells included 10% DMSO and 90% FCS. Cells growing in log phase were detached, centrifuged, the supernatant was removed and the cell pellet was suspended in freezing medium to a concentration of $5x10^6$ to $1x\ 10^7$cells. For cell suspension, aliquots were prepared into cryogenic storage vials. First cells were placed on ice and then stored at -80°C in liquid nitrogen.

3.3.7 Statistical analysis

After quantifying the data in Gelscan software, it was exported to excel. All data are expressed as means ± SEM. The one-tailed student's unpaired test was used for comparison of mean values. Statistical significance was accepted at $p < 0.05$.

3.4 Standard DNA-methods

3.4.1 "Overlap" extension polymerase chain reaction (PCR)

The PCR is a rapid procedure for the *in vitro* amplification of specific segments of DNA. A segment of DNA is amplified using specific two single-stranded oligonucleotides (primers). Furthermore, a DNA polymerase, deoxyribonucleoside triphosphate (dNTPs) and a buffer system are needed. Denaturation (melting the DNA to single strands), annealing (flanking of single stranded DNA by primers) and synthesis (amplification of complementary strands to produce double-stranded DNA) are the three components of PCR amplification cycle. Usually 30 cycles are performed resulting in a 228-fold amplification of a discrete product.

Site-directed mutagenesis can be used to change particular base pairs in a piece of DNA (163). In this work overlap extension PCR was used to introduce site-directed mutagenesis in the IRS-1 wild type gene. For a typical, one site directed mutagenesis reaction, for instance mutation of Ser-357 of IRS-1 to Ala357, two mutagenic primers were designed containing the mutation and completely complementary to each other (Table 2). Each primer was used in a separate reaction (separate tubes, same conditions) with an outer flanking primer pRK5'PCR

or IRS-1-318/1 designed to one end of the IRS-1. These two reactions were given names as reaction for fragment A and reaction for fragment B (Table 3).The PCR program was set as described in Table 4. Two halves of the IRS-1, "A" and "B" were generated in this manner in two separate reactions. PCR products were purified by using a PCR purification kit. In the next step both those fragments were put together (reaction for fragment C ,Table 5), where they anneal in the 25-30 bp region of complementarity and prime off each other, to give the IRS-1 product with the mutation, Ala357, in IRS-1. This PCR product was separated on agarose gel and its concentration was determined. Several other mutants of IRS-1 was generated in the same manner.

Table 2. Primers used to generate different mutants of IRS-1by site directed mutagenesis.

Plasmid	**Mutagenic Upstream Primer**	**Sequence**
pRK5-IRS-1 WT	IRS-1-Ala357	cc cac gcc cat cgg cat cga ggc ***gcc*** tcc agg ttg cac ccc cca ctc aac cac
pRK5-IRS-1 WT	IRS-1-Glu357	cc cac gcc cat cgg cat cga ggc ***gag*** tcc agg ttg cac ccc cca ctc aac cac
pRK5-IRS-1 WT	IRS-1-Ala357/358	cc cac gcc cat cgg cat cga ggc ***gcc gcc*** agg ttg cac ccc cca ctc aac cac,
pRK5-IRS-1 WT	IRS-1-Ala 358	cc cac gcc cat cgg cat cga ggc agc ***gcc*** agg ttg cac ccc cca ctc aac cac
pRK5-IRS-1 WT	IRS-1-Glu357/ Ala 358	cc cac gcc cat cgg cat cga ggc ***gag gcc*** agg ttg cac ccc cca ctc aac cac
pRK5-IRS-1 WT	pRK5' PCR flanking primer	ttg cct ttc cca cag gtg
pRK5-IRS-1 WT	IRS-1 318 1 flanking primer	gct gct gct gct gtt gct ctc gta ccc ggg gta aag t

Table 3. Reaction conditions for the amplification of fragment A and B of IRS-1 to generate IRS -1 Ala-357 mutant.

Content	Reaction for Fragment A	Reaction for Fragment B	End concentration
	Volume [µl]		
H_2O	37.5	37.5	
10x Reaction buffer for Pwo polymerase (see 2.1)	2	2	1x
pRK5'PCR primer 100 µM (see table 2)	1	-	2 µM
IRS-1-318/1 primer 100 µM (see table 2)	-	1	2 µM
Ala 357 upstream primer 100 µM (see table 2)	1	-	2 µM
Ala 357 downstream primer 100 µM (see table 2)	-	1	2 µM
Template DNA 100 ng/µL	2	2	4 ng/µL
DNTPs 200 µM (see 2.1)	4	4	4 µM
Pwo polymerase 1U/µL (see 2.1)	2.5	2.5	0.05 U/µL

Table 4. PCR program for the amplification of fragment A and B of IRS-1 to generate IRS-1 Ala-357 mutant.

Segment	Duration (min)	Temperature	cycles
1 Denaturation	5	95°C	1x
2 Annealing	1	55°C	30x
Extension	6	72°C	
Denaturation	1	95°C	
3 Annealing	1	55°C	1x
Extension	5	72°C	

Table 5. Reaction conditions for the amplification of fragment C of IRS-1 to generate IRS-1 Ala-357 mutant.

Content	*Reaction for IRS-1 Ala 357 (Fragment C)*	End concentration
	Volume [µL]	
H_2O	35.5	
10x Reaction buffer for Pwo polymerase	2	1x
pRK5'PCR primer 100 µM	1	2 µM
IRS-1-318/1 primer 100 µM	1	2 µM
Fragment A ca. 100 ng/µL	2	4 ng/µL
Fragment B ca. 100 ng/µL	2	4 ng/µL
DNTPs 200 µM	4	4 µM
Pwo polymerase 1 U/µL	2.5	0.05 U/µL

3.4.2 DNA electrophoresis in agarose gel and isolation from agarose gel

The DNA from PCR reaction was diluted in DNA sample buffer and separated on horizontal agarose gels (1% agarose was prepared in 100 ml TAE buffer and 5 µl ethidium bromide (10 mg/ml) was added). Gel was run in TAE buffer at 5 V/cm. The detection was carried out with a UV lamp.

3.4.3 Isolation of DNA from agarose gel

For the isolation of DNA from agarose gel, QIAquick ® Gel Extraction Kit from Qiagen was used. The bands were excised from the gel as closely as possible. Gel slice were dissolved in buffers as specified by the manufacturer. The elution was conducted in 30 µl dH_2O.

3.4.4 Determination of DNA concentration

The DNA concentration was determined photometrically and calculated from the absorbance measured at 260 nm. Since a solution containing 50 µg per ml of double stranded DNA has an absorbance (optical density) of 1.0 at a wave length of 260 nm. Therefore, DNA concentration = absorbance (260 nm) x 50 µg/ml. DNA samples having a 260/280 ratio below 1.6 were discarded.

3.4.5 Restriction digestion of mutated PCR product

The PCR product containing the mutation at 357Ala of IRS-1 (fragment C) was excised from agarose gel and purified (3.4.3). The restriction digestion was carried out in order to separate the required insert containing 357 Ala mutations.

Digestion of DNA was performed by incubating 1µl of each BamHI and XhoI restriction enzymes, with 3 µl of digestion buffer and 25 µl of PCR product of fragment C, at 37°C for 1 hour. Restriction products were run on agarose gel and the 2500 bp band of interest was excised and purified.

3.4.6 Ligation of the digested mutated product into expression vector

The Purified restriction product was then ligated into the pRK5 IRS-1WT plasmid vector. Ligation reaction mixture consisted of 1 µl (10-50ng) of pRK5 IRS-1 WT (a BamHI and XhoI digested fragment of the vector), 7 µl digested and mutated fragment of IRS-1 containing the mutation of Ala 357, 1 µl 10x of the ligation buffer and 1 µl ligase enzyme (1U/ µl). Reaction was set overnight at 16°C.

3.4.7 Transformation of ligation product in Ecoli

The transformation of plasmid DNA into E. coli was made using heat shock method. 10 µl of the ligation product was mixed with 20 µl of KCM 5 x buffer (see 2.6) and 100 µl H_2O was added and incubated on ice for 5 min. 100µl of competent E-coli XL1 Blue was added in the KCM mixture and incubated on ice for 30 min. At the end of this incubation, temperature shock was given at 42°C for exactly 2 min. 1 ml of LB media (see 2.7)was added in the mixture and incubated at 37°C for 30 min. Centrifugation was done at 6500 rpm for 2 min. The pellet was resuspended and plated on LB/Amp agar plates (see 2.7) and incubated at 37°C for overnight.

3.4.8 Miniprep for isolation of plasmid DNA from Escherichia coli

Positive clones from LB/Amp agar plates were incubated in 2 ml LB medium with ampicillin overnight at 37°C under shaking at 350 rpm. Plasmid DNA was isolated from E.coli, using Plasmid Mini Kit from Qiagen, according to manufacturer's instructions.

3.4.9 Sequencing

PCR was set to verify the mutated sequence of 357A in IRS-1. The sequencing of the manufactured constructs was made by the chain termination method of Sanger (164). The Premix (BigDye ® Terminator v.1.1 Cycle Sequencing Kit) contains the dNTPs (dATP, dCTP, dGTP, dTTP), fluorescence labeled ddNTPs, thermostable AmpliTaq ® DNA polymerase, a thermostable pyrophosphatase and magnesium chloride in Tris-buffer pH 9.0. For 10µl reaction, 300 ng of plasmid DNA as template, 4 µl of sequencing mix, 1 pM of 318/1 Ala primer, 1x sequencing buffer and 3 µl of double distilled H_2O was used. After 1 min of initial denaturation, the PCR mix was incubated in the thermocycler for 30 of the following cycles: 10 sec denaturation at 96°C, 30 sec annealing at 50°C and 4 min elongation at 60°C.. The sequencing reaction products were purified by gel filtration and eluates were analyzed by capillary electrophoresis. The evaluation was done with the help of Abi Prism software "Sequencing analysis 3.7". The sequence comparison was performed on the Internet with BLAST, which is freely available at http://www.ncbi.nlm.nih.gov/blast.

3.4.10 Isolation of plasmid DNA- maxiprep

For the preparation of large amounts of plasmid DNA with great purity for the plasmid transfection into the mammalian cells, Maxi kit from Qiagen was used. A single bacterial colony from a selective agar plate was inoculated in 100 ml LB culture medium with

ampicillin. Culture was grown overnight on the shaker at 150 rpm / 37°C. The bacteria were then distributed into two 50 ml falcon tubes and centrifuged at 4000 rpm at 4°C for 10 min. The purification of DNA was performed according to manufacturer's instructions. After drying the pellet, the DNA was dissolved in 100 µl H_2O.The DNA concentration was determined as described in (3.4.4). The purity of the plasmid isolation was verified by restriction analysis (3.4.5) and subsequent agarose gel electrophoresis (3.4.2) or by sequencing (3.4.9).

Some mutants of IRS-1 were also generated by Quick Change Site-Directed Mutagenesis kit, Stratagene, following the manufacturer's instructions.

4. RESULTS

4.1 Characterization and specificity of phospho-Ser-357 Antibody

4.1.1 Initial characterisation of antiserum raised against p-Ser-357 showed cross reactivity with adjacent Ser-358

To identify novel Ser /Thr phosphorylation sites in IRS-1, *in vitro* phosphorylation assays were performed with the isolated N-terminal amino acid residues 2–304 and amino acid residues 265–522 of GST-IRS-1 protein fragments (146;165). Upon incubation of recombinant IRS-1 fragments with various PKC-isoforms, Ser-357 of rat IRS-1 (Ser-362 in human IRS-1) was identified as an *in vitro* PKC phosphorylation site (data not shown). Next, polyclonal phospho-site-specific antibodies were generated using a phosphopeptide sequence corresponding to the region of rat IRS-1 surrounding Ser-357 (Fig. 4).

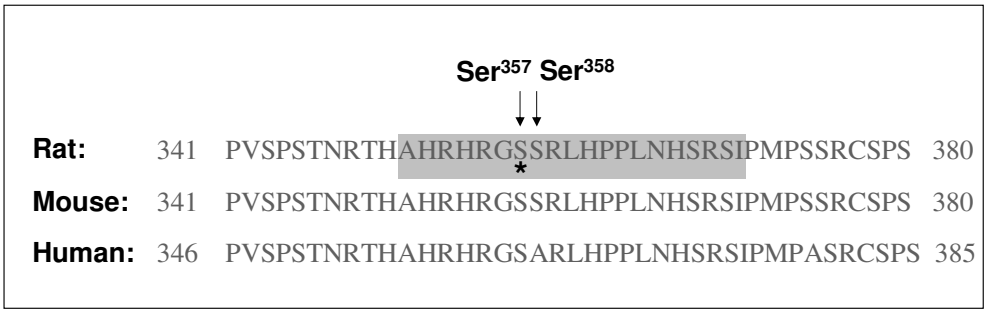


Fig. 4. Sequence alignment of the corresponding amino acid sequences around Ser-357 and Ser-358 of IRS-1 proteins from different species.

*Sequence alignment of the corresponding amino acid around Ser-357 for rat, mouse and human IRS-1 (amino acid numbers are indicated) is shown. Only the small region of rat IRS-1 containing the sequence AHRHRGpSSRLHPPLNHSRSI (antigenic peptide can be seen as shaded box, * shows phosphorylated residue) IRS-1 351–367 was used for generation of polyclonal phospho-site-specific Ser-357 antibody.*

The antiserum was used to test the specificity of the phospho-site-specific-Ser-357antibody. Mouse skeletal muscle cell line C2C12 overexpressing IRS-1 WT were stimulated with insulin or phorbol ester 12-O-tetradecanoyl 13-phorbolacetate (TPA), a pharmacological activator of classical and novel PKC isoforms for 30 min (Fig. 5). Before stimulation, the

antibody did not react with IRS-1, while insulin and TPA strongly stimulated phosphorylation of Ser-357, indicating that the antibody recognise phosphorylation of Ser-357.

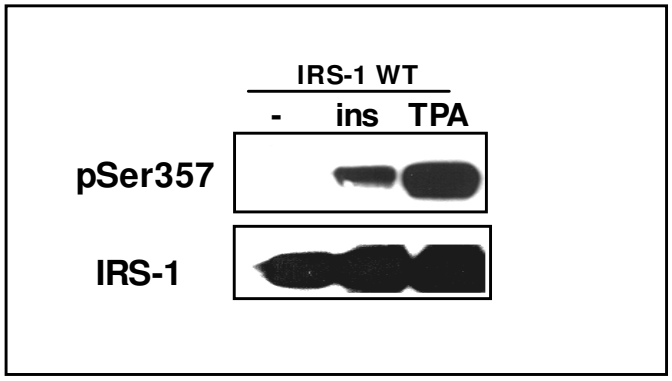


Fig. 5. Initial characterization of the antiserum raised against phospho-Ser-357.
Antibody characterization for phospho-Ser-357 was performed in C2C12 cells transiently transfected with IRS-1 wild type (WT) (4 µg) and stimulated with TPA (0.5 µM) and insulin for 30 min. The blot was reprobed to show the expressed IRS-1 protein levels.

To further verify that the antibody specifically recognises phosphorylation of Ser-357, an IRS-1Ala357 mutant was generated by PCR site-directed mutagenesis, to prevent phosphorylation at Ser-357. C2C12 cells were transiently transfected with either IRS-1 WT or IRS-1Ala357 mutant and stimulated with insulin or TPA for 30 min. Surprisingly,

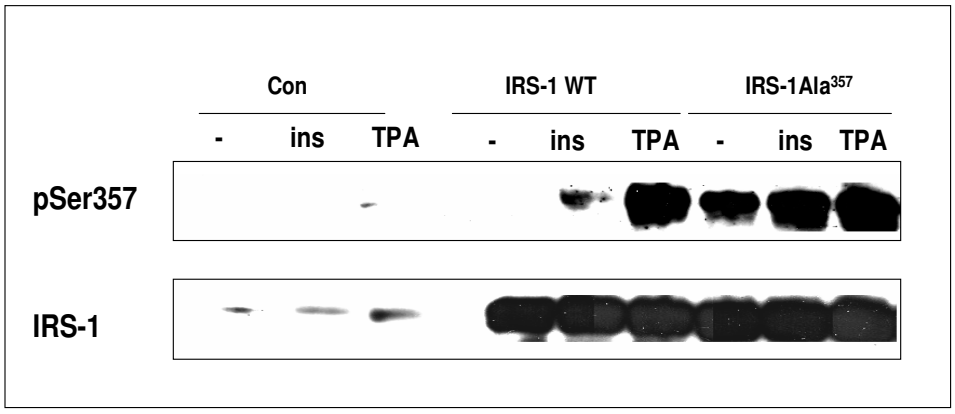


Fig. 6. Initial characterization of the antiserum raised against phospho-Ser-357.
C2C12 cells were transiently transfected with either IRS-1 wild type (WT) or IRS-1 Ala357 (4 µg each) and stimulated with insulin (10 nM) and TPA (0.5 µM) for 30 min. The blot was reprobed to show the expressed IRS-1 protein levels.

phosphorylation signal detected by antiserum was comparable between IRS-1Ala357 and IRS-1WT expressing cells (Fig. 6), indicating an unspecific binding of the antibody, possibly a cross reactivity with the adjacent Ser-358.

This was itself a novel phenomenon in the development of phospho-site specific antibodies for IRS-1. At this point we decided to eliminate cross reactivity of the antibody with Ser-358 to elucidate single effect of Ser-357 of IRS-1in insulin signaling , as prior studies has already described Ser-358 as a functional serine site of IRS-1 (166).

4.1.2 Synthesis and purification of synthetic peptides

In an attempt to immuno-purify antiserum and to determine specificity of purified- phospho-site-specific-Ser-357 antibody by indirect ELISA it was decided to generate chemically synthesized phosphopeptides as antigens. The peptides were synthesized using standard Fmoc/tBu chemistry (Table 6).

Table 6. Sequences of synthetic modified IRS-1 351-377 peptides.

Peptide	Sequence
a) IRS-1 351-377;p357	AHRHRG+SRLHPPLNHSRSI
b) IRS-1 351-377;p357(Ala at 358)	AHRHRG+ARLHPPLNHSRSI
c) IRS-1 351-377; p357p358	AHRHRG++RLHPPLNHSRSI
d) IRS-1 351-377;p358	AHRHRGS+RLHPPLNHSRSI
e) IRS-1 351-377;np357np358(Ala at 358)	AHRHRGSARLHPPLNHSRSI
f) IRS-1 351-377;np357np358	A HRHRGSSRLHPPLNHSRSI

Plus(+) sign indicates phosphorylated amino acids
p = phosphorylated
np = non phosphorylated

Crude peptides were purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC) Fig 7. The integrity of the peptides and phosphopeptides was verified by mass spectroscopy. Table 1 lists the sequences of the synthetic peptides. Each peptide was 20 amino acids long. The peptides were either unphosphorylated, contained single phosphate groups on Ser-357 or Ser-358, or phosphates on either serines or alanine at position 358.

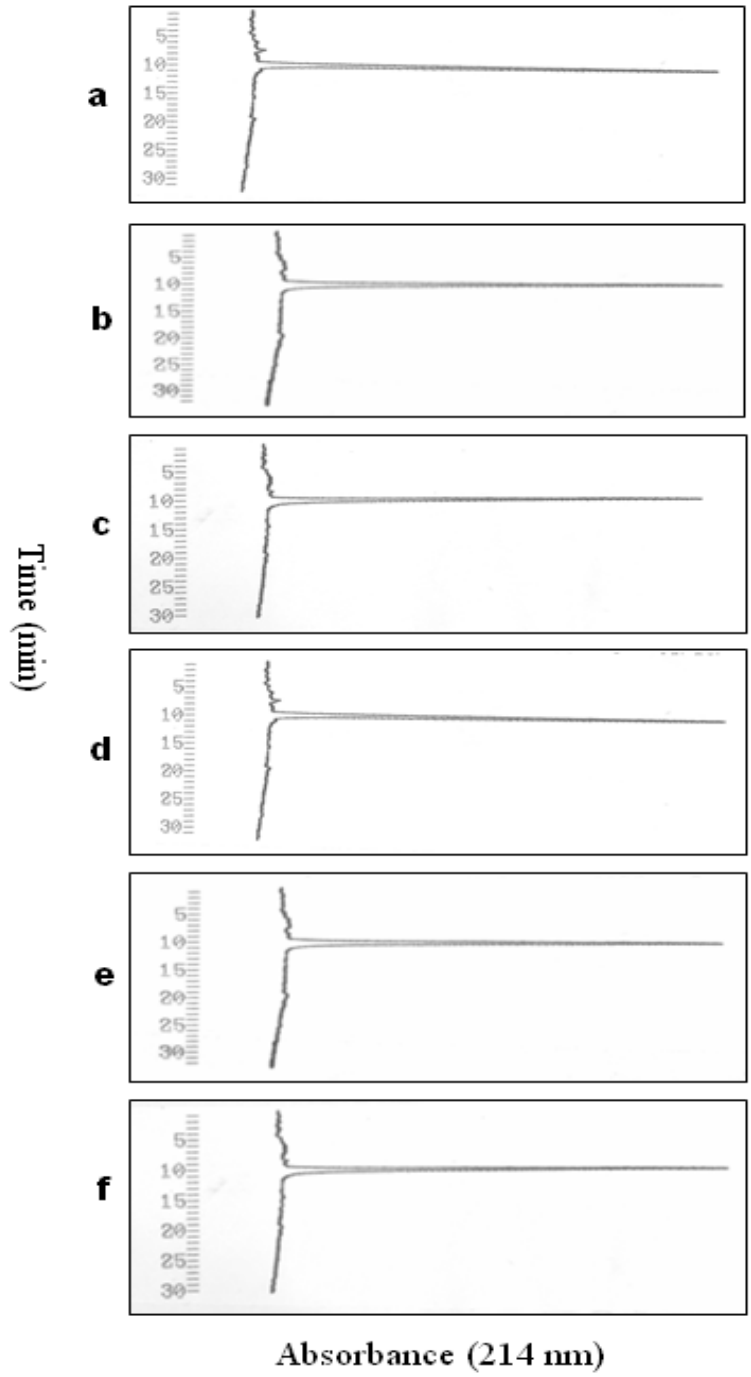


Fig. 7. RP-HPLC elution profile of synthetic modified IRS-1 351-377 peptides.

Purified peptides were separated via analytical RP-HPLC using a C8 column (150 × 2 mm, Reprosil 100,) with the following solvent system: (A) 0.055% (v/v) trifluoroacetic acid in H_2O, and (B) 0.05% (v/v) trifluoroacetic acid in ACN/H_2O (4:1, (v/v)). The column was eluted with a 10-90% gradient of solvent B for 30 min. UV detection was carried out at 214 nm (a,b,c,d,e and f refers to the peptide sequences in Table 6).

4.1.3 Immuno-purification of antiserum and determination of specificity of purified phospho-site-specific-Ser-357 antibody by indirect ELISA

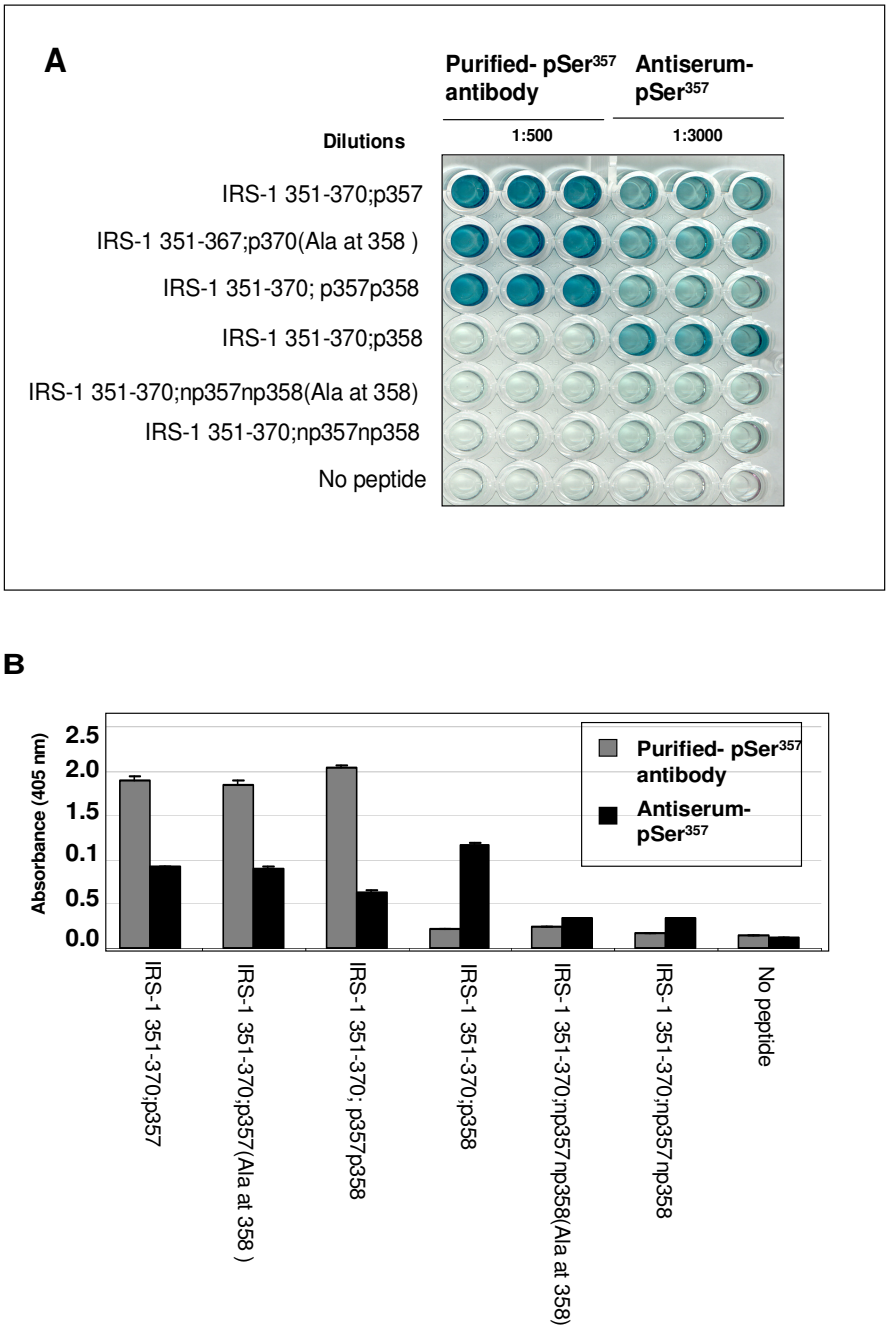


Fig. 8. Determination of specificity of polyclonal phospho-site-specific-Ser-357 antibody by indirect ELISA.*. The purified polyclonal phospho-site-specific-Ser-357 antibody specifically recognizes phospho-Ser-357 of IRS-1 peptide and gives a negative reaction towards different related synthetic peptides. 5 µg of different synthetic peptides were coated on an ELISA plate. Phospho-site-specific-Ser-357 antiserum and purified phospho-site-specific-Ser-357*

*antibodies were used for the detection at dilutions of 1: 3000 and 1: 500 respectively. (**B**) Quantification of relative binding of each polyclonal antibody to phospho-Ser-357 of IRS-1 protein-based synthetic peptides (listed in Table 5) using the PEPSCAN-ELISA method, values are mean +SEM, where n = 3.*

The generated phospho-site-specific-Ser-357 antibody was immuno-purified by multiple purification steps using non-phosphorylated Ser-357 and p-Ser-358 peptide. To determine and compare the specificity and cross-reactivity of the antiserum and purified-p-Ser-357 antibody, an indirect ELISA was performed using different non-phospho and phospho-synthetic peptides (Table 5). The results of the indirect ELISA (Fig. 8A) showed that the purified antibody binds specifically to the immunogenic phosphopeptide but not to the non-phosphorylated form of the peptide or to other phosphopeptides (Fig. 8B), suggesting that the antibody specifically recognized phosphorylation of Ser-357 and showed complete negative reaction towards phosphorylation of adjacent Ser-358.

4.1.4 Final determination of the specificity of purified phospho-site-specific-Ser-357 antibody in living cells

In order to establish the specificity of the antibody against phosphorylation of Ser-357 at the entire IRS-1 protein, two more mutants of IRS-1 were generated: IRS-1 Ala358 to prevent phosphorylation at Ser-358 and a double mutant IRS-1Ala357/358 to prevent phosphorylation at both, Ser-357 and Ser-358, simultaneously. Baby hamster kidney cells stably expressing the human insulin receptor (BHKIR-cells) were transiently transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358 and stimulated with insulin or TPA for 30 min (Fig. 9A).

The antiserum non-specifically recognized Ser-357 phosphorylation upon stimulation with TPA and insulin in the cells overexpressing IRS-1 Ala357 and IRS-1 Ala357/358 comparable to the cells overexpressing IRS-1WT and IRS-1 Ala358 (Fig. 6A, B).Whereas with the purified-p-Ser-357 antibody we detected almost no or a very low signal of phosphorylation was detected in IRS-1 Ala357 and IRS-1 Ala357/358-transfected cells, similar to the immunoblots obtained with control transfected cells (Fig. 9B).

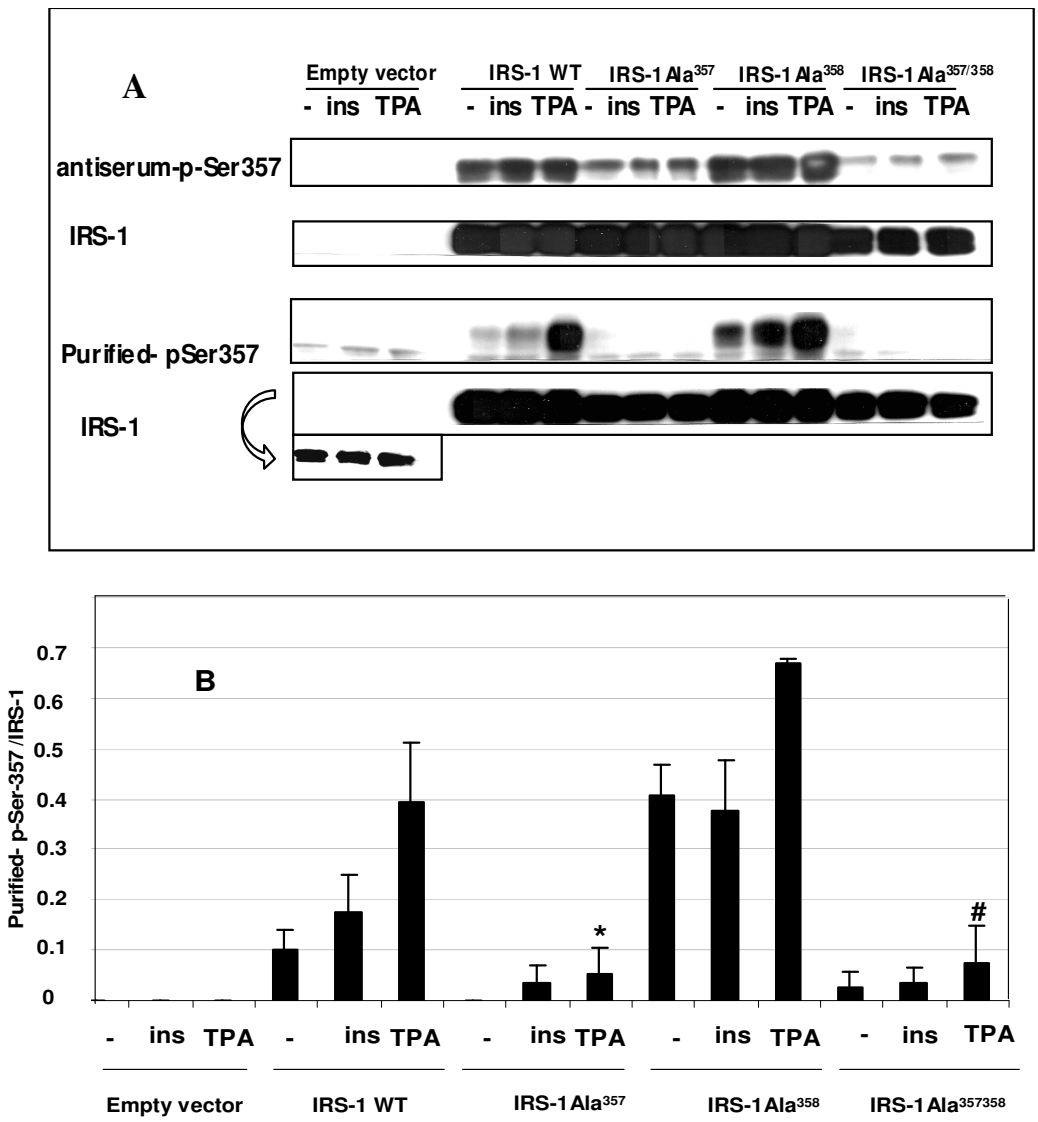


Fig. 9. Specificity of phospho-Ser-357 antibodies on the entire IRS-1 protein analyzed in cultured cells. *(**A**) Baby hamster kidney cells stably expressing the human insulin receptor (BHKIR-cells) were transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358, and IRS-1 Ala357/358 (4 μg each) and treated with insulin (10 nM, 30 min) or TPA (0.5 μM, 30 min). After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with phospho-site-specific-Ser-357 antiserum and purified-phosho-Ser-357 antibodies. The same blots were stripped and reprobed with a polyclonal IRS-1 antibody. Arrow indicates endogenous IRS-1 visible after long exposure.. Representative results from three independent experiments are shown. (B) Phosphorylation intensity of Ser-357 of IRS-1 was quantified based on scanning densitometry of immunoblots normalized for IRS-1 protein. (Mean +SEM, n = 3, *p < 0.05 IRS-1 Ala357 mutants vs. IRS-1 WT; # p < 0.05 IRS-1Ala357/358 mutant vs. IRS-1 WT).*

The very low signal depends most likely on the endogenous IRS-1 in the transfected cells, which can be phosphorylated at Ser-357. These data clearly show that the immunopurified antibody recognizes also *in vitro* IRS-1 solely, when it is phosphorylated on Ser-357 without any cross-reactivity with the adjacent putative phosphorylation residue Ser-358. Similar results have been obtained using C2C12 skeletal muscle cells.

4.1.5 Determination of specificity of purified phospho-site-specific-Ser-357 antibody in living cells with dephosphorylated IRS-1

To further confirm the specificity of the phospho-site-specific Ser-357 antibodies, C2C12 cells were transfected with IRS-1 WT and stimulated with TPA. Immunoprecipitated IRS-1 was incubated with λ-phosphatase at 37 °C for 30 min which led to a complete loss of the immunoreactivity of the antibody, indicating that dephosphorylated IRS-1 is not recognized (Fig. 10).

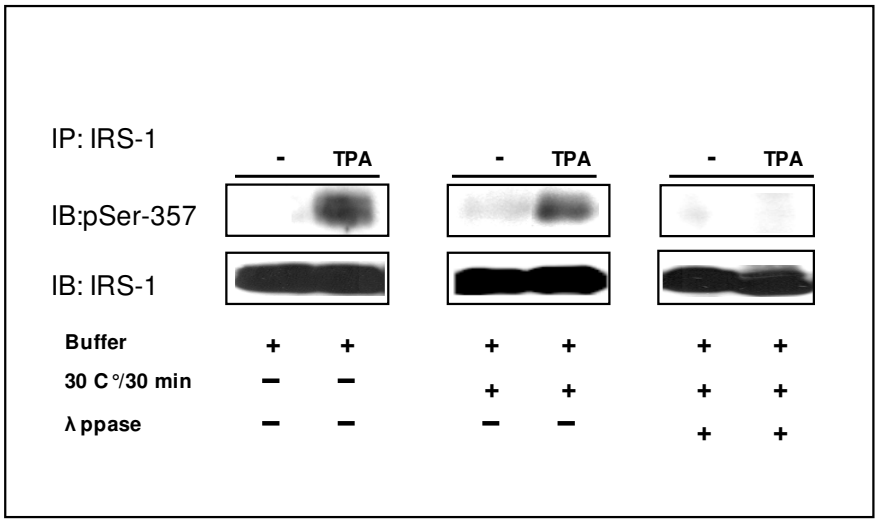


Fig. 10. Specificity of phospho-Ser-357-antibodies in cultured cells.

C2C12 cells were transfected with IRS-1 WT (4 μg) and stimulated for 30 min with TPA (0.5μM). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody and immunoprecipitates (IP) were incubated with buffer alone or with buffer and λ PPase (100 U) for 30 min at 30 °C and immunoblotted (IB) with the phospho-site-specific Ser-357 antibody. Addition of buffer without incubation at 30 °C served as another control. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody.

4.1.6 Determination of specificity of phospho-Ser-357-antibodies in cultured cells with endogenous IRS-1

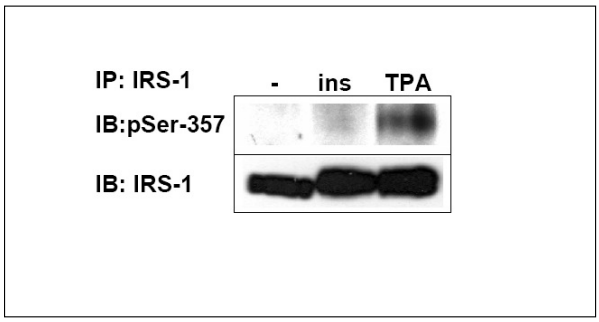


Fig. 11. Specificity of phospho-Ser-357-antibodies in cultured cells with endogenous IRS-1.
C2C12 cells were stimulated with insulin (10 nM, 30 min) or TPA (0.5 μM, 30 min). IRS-1 was immunoprecipitated and immunoblotted with site-specific Ser-357 antibody and reprobed with a polyclonal IRS-1 antibody.

In another set of experiments, we analyzed non-transfected C2C12 cells for phosphorylation of Ser-357. As shown in Fig. 11, phosphorylation of Ser-357 of endogenous IRS-1 was detected after stimulation with insulin or TPA.

4.2 Phosphorylation of Ser-357 of IRS-1 in insulin signaling

4.2.1 Insulin-stimulated phosphorylation of Ser-357 in C2C12 cells: Insulin dose kinetics

In order to examine the effect of insulin on the phosphorylation of Ser-357, time- and dose response curves of insulin-stimulated phosphorylation of Ser-357 were studied. C2C12 cells overexpressing IRS-1 WT were treated with various insulin concentrations for 30 min. A dose dependent increase in Ser-357 phosphorylation after 30 min of insulin stimulation was observed (Fig. 12).

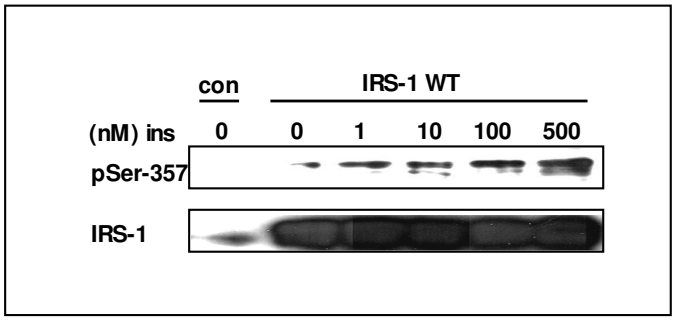


Fig. 12. Insulin dose kinetics of Ser-357 phosporylation in muscle cells.

C2C12 cells were transfected with IRS-1 wild type and treated with the indicated doses of insulin for 30 min and analyzed by immunoblotting with site-specific- Ser-357- antibody and reprobed with a polyclonal IRS-1 antibody.

4.2.2 Insulin-stimulated phosphorylation of Ser-357 in C2C12 cells: Insulin time kinetics

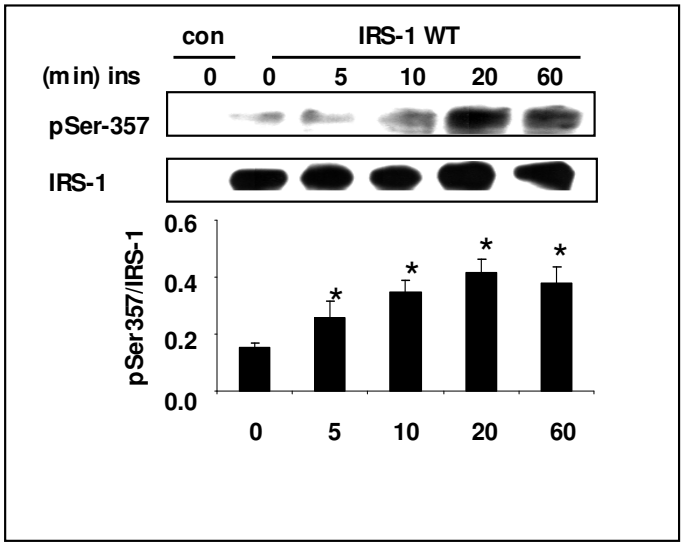


Fig. 13. Insulin-stimulated time kinetics of phosphorylation of Ser-357 in C2C12 cells.

*The time course of insulin action on the phosphorylation of Ser-357 was investigated by stimulation of C2C12 cells transfected with IRS-1 wild type (IRS-1 WT) (4 µg) with 10 nM insulin for indicated time points. "Con" indicates cells transfected with empty vector. Cell lysates were analyzed by 7.5% SDS-PAGE. A representative immunoblot of three independent experiments with the site-specific Ser-357 antibody and reprobe of the same blot with the polyclonal IRS-1 antibody are shown. Densitometric quantification of the increase of Ser-357 phosphorylation after insulin stimulation (mean +SEM, n = 3, *p < 0.05 vs. unstimulated cells.)*

In order to examine the effect of insulin on the phosphorylation of Ser-357 observed in figure 5, the time-response curve of insulin-stimulated phosphorylation of Ser-357 was studied. The phosphorylation kinetics of Ser-357 phosphorylation was performed using 10nM insulin for various time points. It was found that insulin induced significant phosphorylation of Ser-357 after 5 minutes of stimulation with a maximum increase after 20 minutes. The site remained strongly phosphorylated until 60 minutes of insulin stimulation (Fig. 13A and B) implicating a potential role in an early and late phase of insulin signaling.

4.2.3 PKC-δ mediates IRS-1 Ser-357 phosphorylation in C2C12 cells

Ser-357 is located at putative phosphorylation motif for PKC (RXS/ SXR/ RXSXR) a family of Ser kinases whose novel and atypical isoforms (e.g. PKC-δ and PKC-ζ) have been described as potential IRS-1 kinases that modulate IRS-1 function (85;162;167). Since the novel isoform PKC-δ was able to phosphorylate Ser-357 in the *in vitro* phosphorylation assays (data not shown) and treatment with TPA as an activator of classical and novel PKCs resulted in the pronounced phosphorylation of Ser-357 (Fig. 5) we focused on the role of PKC-δ in Ser-357 phosphorylation in muscle cells. C2C12 cells transiently co-transfected with PKC–δ and IRS-1 WT and stimulated by insulin and TPA, showed significant increase in the phosphorylation of Ser-357 in comparison to cells transfected with IRS-1WT alone (Fig. 14A). Phosphorylation of Ser-357 in unstimulated co-transfected cells was also observed, which might be due to basal activitiy of the cotransfected PKC-δ. Because TPA may have cellular targets other than PKC-δ, we co-transfected cells with IRS-1 and PKC-δ kinase negative mutant (KN). Phosphorylation of Ser-357 in unstimulated, insulin- and TPA-treated cells was significantly abrogated when PKC–δ-KN mutant was used (Fig. 14B). These findings suggest that the insulin and TPA-induced phosphorylation of Ser-357 required PKC-δ activity.

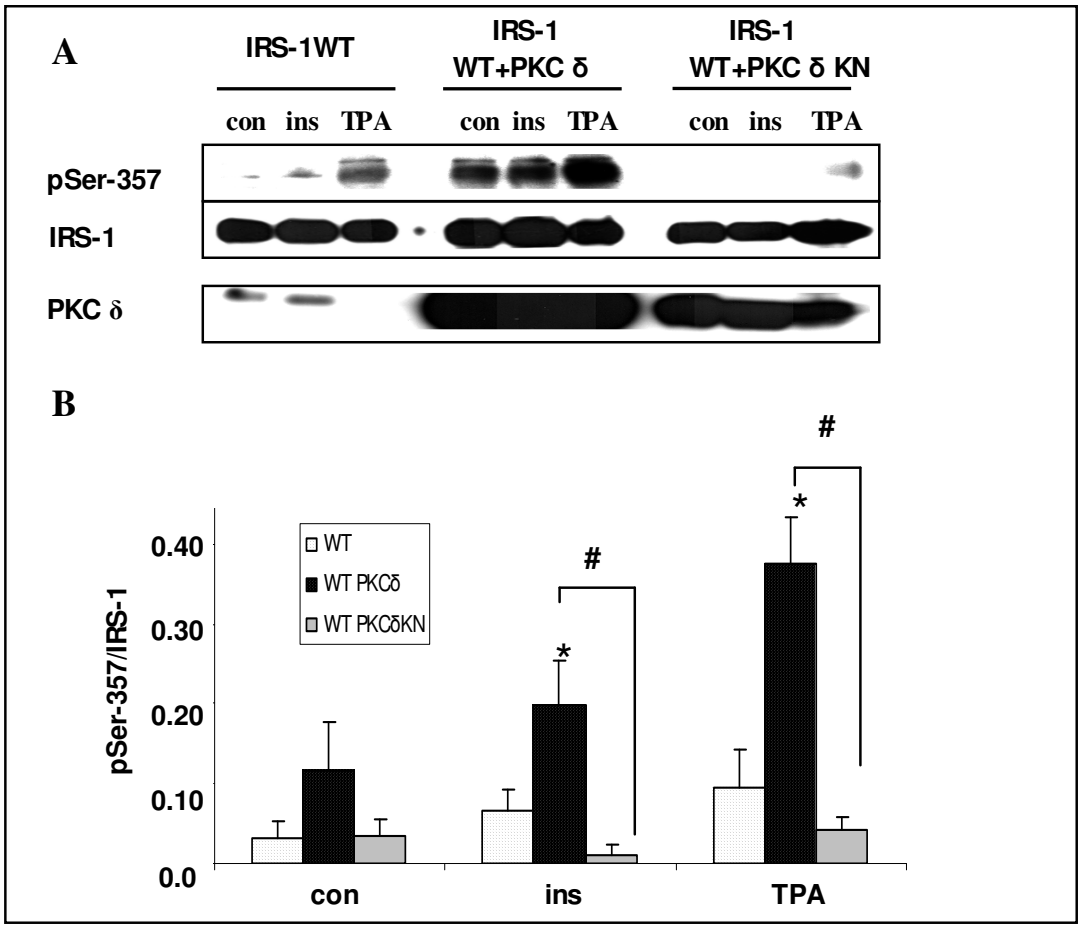


Fig. 14. PKC-δ-mediated IRS-1 Ser-357 phosphorylation in C2C12 cells. *(A) C2C12 cells were transfected with IRS-1 wild type (WT), co-transfected with PKC-δ and IRS-1 WT or kinase-dead mutant of PKC-δ (PKC-δ KN) and IRS-1 WT (2 µg each). Cells were stimulated with either 10 nM insulin or 0.5 µM TPA for 30 min. A representative immunoblot with the phospho-site-specific Ser-357 antibody and PKC–δ and reprobe of the same blot with the polyclonal IRS-1 antibody is shown. (B) Ser-357 phosphorylation intensity was quantified based on scanning densitometry of the immunoblots (means +SEM, n=3, * p < 0.05 PKC δ cotransfected cells vs. cells transfected with IRS-1 WT alone; # p < 0.05 PKC δ KN cotransfected cells vs. PKC δ cotransfected cells).*

4.2.4 Insulin induces the phosphorylation of Ser-357 in skeletal muscle of mice

The rapid phosphorylation of Ser-357 during acute insulin stimulation might contribute to an early modulation of the insulin response. To determine whether insulin-stimulated

phosphorylation of IRS-1 on Ser-357 occurs in the whole organism, skeletal muscle from male mice was isolated after 5 min of insulin stimulation (4 IU/mouse, intravenously). Immunoblotting with the Ser (P) 357-specific antibody revealed a very rapid (within 5min) insulin-stimulated phosphorylation of Ser-357 in skeletal muscle from these mice (Fig. 15), similar to the results obtained in cell culture. The data indicate that insulin also stimulates the phosphorylation of IRS-1 Ser-357 in skeletal muscle tissue within minutes.

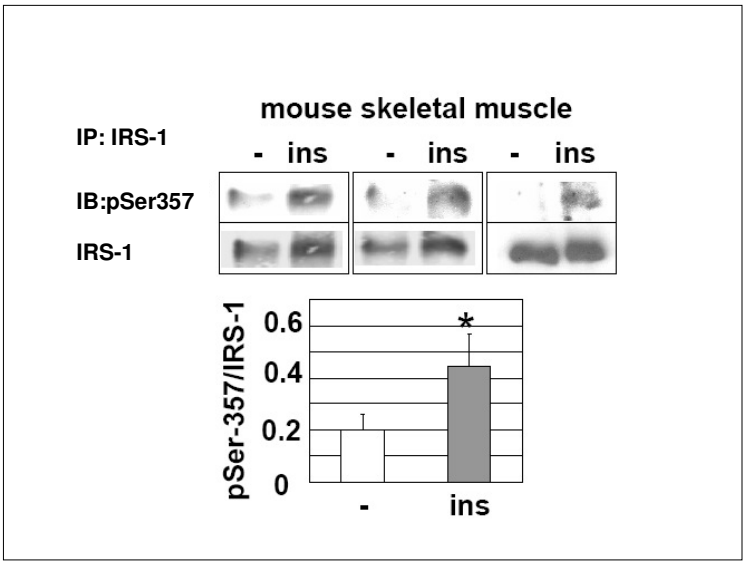


Fig. 15. Insulin-stimulated phosphorylation of Ser-357 in vitro.

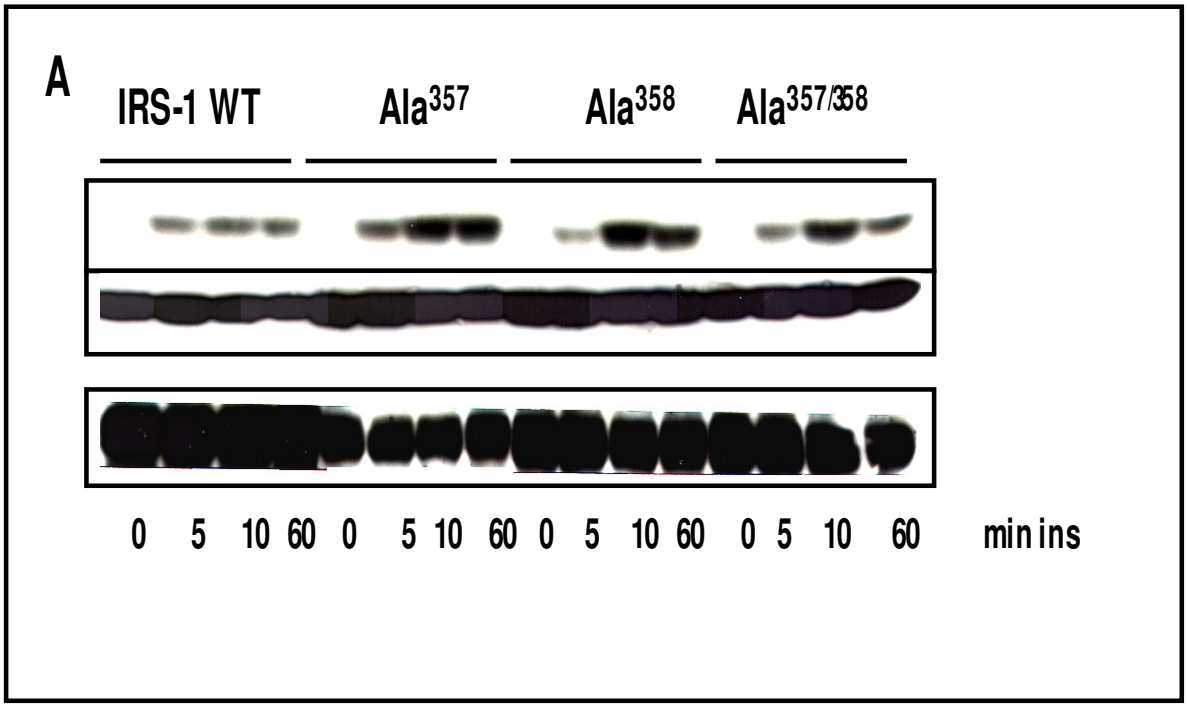
*Male CH3 mice were fasted overnight and subsequently intravenously injected with 4 IU of insulin into the inferior vena cava. Muscle samples were obtained after 5 min of insulin treatment. Shown are immunoblots of muscle tissue of 3 control and 3 insulin-treated mice. Ser357 phosphorylation intensity was quantified based on scanning densitometry of the immunoblots (means +SEM, n=7, * $p < 0.05$ insulin-treated mice vs. control).*

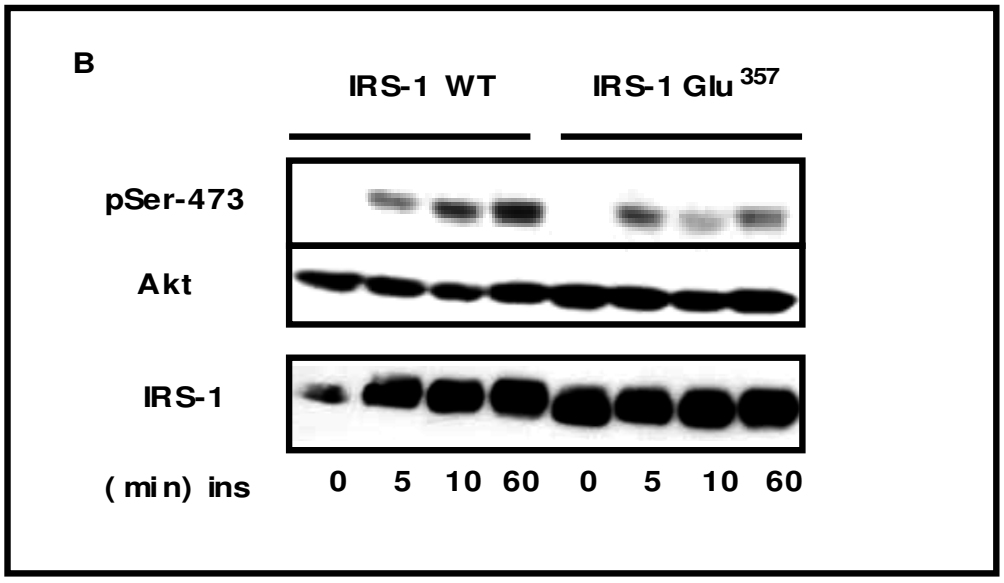
4.3 Functional role of phosphorylation of Ser-357 of IRS-1 in insulin-stimulated signal transduction.

4.3.1 The phosphorylation of Ser-357 of IRS-1 leads to reduced phosphorylation of Akt in skeletal muscle cells

Next, the functional role of phosphorylation of Ser-357 in insulin signal transduction was investigated. The kinase PKB/AKT is an important insulin signaling molecule, shown to transduce the metabolic actions of insulin, including increases in transport and storage of glucose in muscle, and its activation is largely controlled by IRS-1 during insulin action

(47;168). Therefore, we studied the influence of Ser-357 of IRS-1 on the insulin-induced time-dependent phosphorylation of Akt. To examine a possible influence of the adjacent Ser-358 residue, C2C12 cells were transiently transfected with IRS-1 WT, IRS-1 Ala357, and IRS-1 Ala358 and IRS-1 Ala357/358 and stimulated for various time points with 10 nM insulin (Fig. 16A).





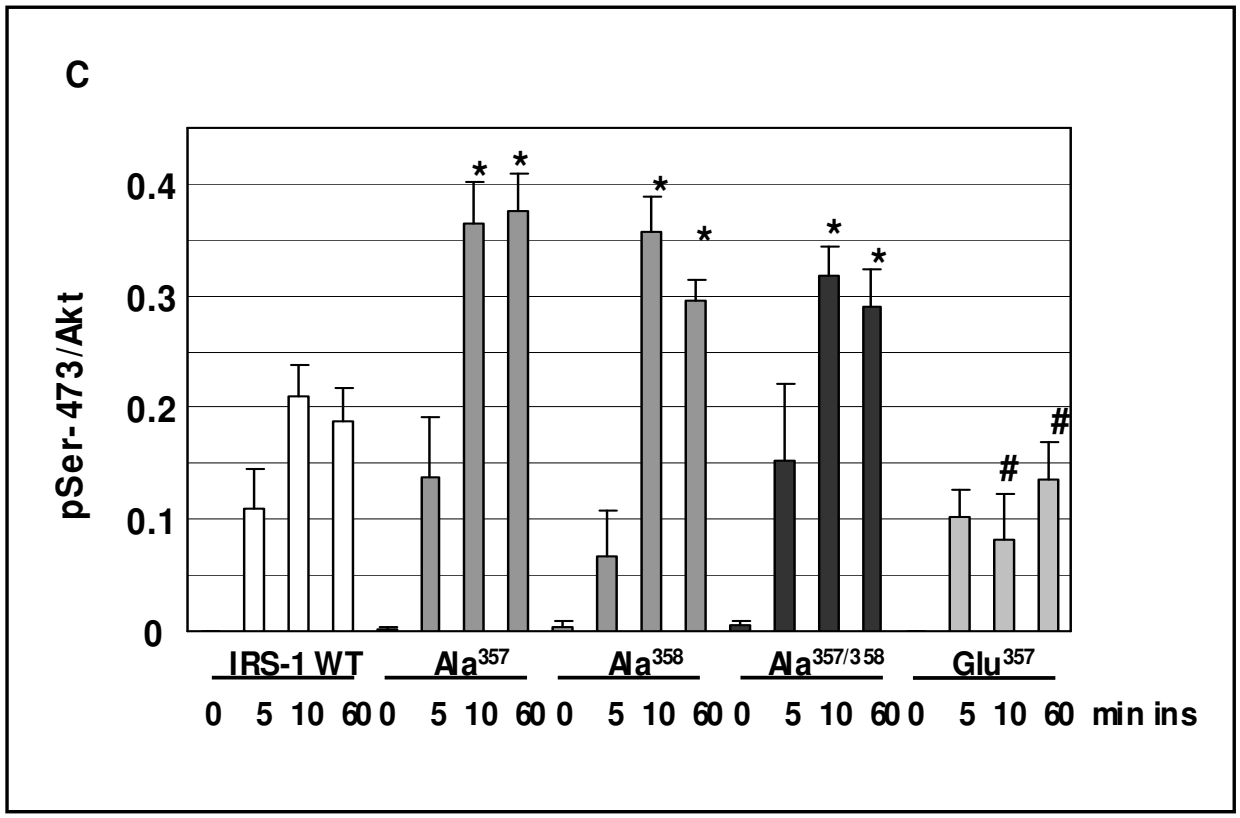


Fig. 16. The phosphorylation of Ser-357 of IRS-1 leads to reduced phosphorylation of Akt in skeletal muscle cells. *(A, B) C2C12 cells, transiently transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358, and IRS-1 Ala357/358 (A) or IRS-1 WT and IRS-1 Glu357(4 µg each) (B) were stimulated for 5, 10, and 60 min with 10 nM insulin. Cell lysates were analyzed with 7.5% SDS-PAGE, and after Western blotting the phosphorylation of Ser-473 of Akt was investigated. The same blot was stripped and reprobed with an Akt protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (C) Phosphorylation intensity of Ser-473 of PKB/Akt was quantified based on scanning densitometry of immunoblots normalized for PKB/Akt protein. (Mean +SEM, n = 3,*p < 0.05 IRS-1 Ala mutants vs. IRS-1 WT; # p < 0.05 IRS-1 Glu mutant vs. IRS-1 WT.)*

To mimick a permanent phosphorylation of Ser-357, cells also were transfected with IRS-1 Glu357 (Fig. 12B). The insulin-dependent increase in Ser-473 phosphorylation of Akt was markedly increased in IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358 expressing cells after 10 and 60 min of insulin stimulation compared with cells expressing wild-type IRS-1 (Fig. 12C). Of note, phosphorylation of Ser-357 and Ser-358 appeared to have similar negative effects on Akt phosphorylation with no additive effect when phosphorylation at both sites was prevented in the IRS-1 Ala357/358 mutant (Fig. 12C).

The possible negative role of Ser-357 phosphorylation in insulin action was further demonstrated in IRS-1 Glu357 expressing cells, which exhibited a significantly diminished Ser-473 phosphorylation of Akt after 10 and 60 min of insulin stimulation compared with wild-type expressing cells. Since we did not observe any difference of the two alanine mutants (Ala357 and Ala357/358) on insulin signal transduction we used in further experiments only IRS-1 Ala357/358.

4.3.2 Insulin-stimulated phosphorylation of GSK-3α in skeletal muscle cells is modulated by Ser-357 phosphorylation of IRS-1.

The sustained effect of Ser-357 phosphorylation on Akt suggests a physiological function of this site in downstream insulin signaling. Thus we asked whether the possible regulation of Akt activity was reflected by a modulation of its downstream effector GSK-3. C2C12 cells were transiently transfected with IRS-1 WT, IRS-1 Ala357/358 or IRS-1 Glu357 and stimulated for various time points with 10 nM insulin (Fig. 17A). The increase in Ser-21 phosphorylation of GSK-3α was more pronounced in IRS-1 Ala357/358 expressing cells after 10 and 60 min of insulin stimulation compared with cells expressing IRS-1 WT, while expression of IRS-1 Glu357 resulted in a clear decrease in this phosphorylation (Fig. 17B). Thus, the negative effect of Ser-357 phosphorylation on insulin signaling could also be demonstrated at the level of GSK-3. While both isoforms α and ß of GSK-3 were present in C2C12 cells (data not shown), we observed insulin-induced phosphorylation only at Ser^{21} of GSK-3α. These findings are in agreement with previous studies (169) which reported that the α-isoform of GSK-3 was more responsive to insulin as compared to GSK-3β and had a stronger association with parameters of insulin resistance.

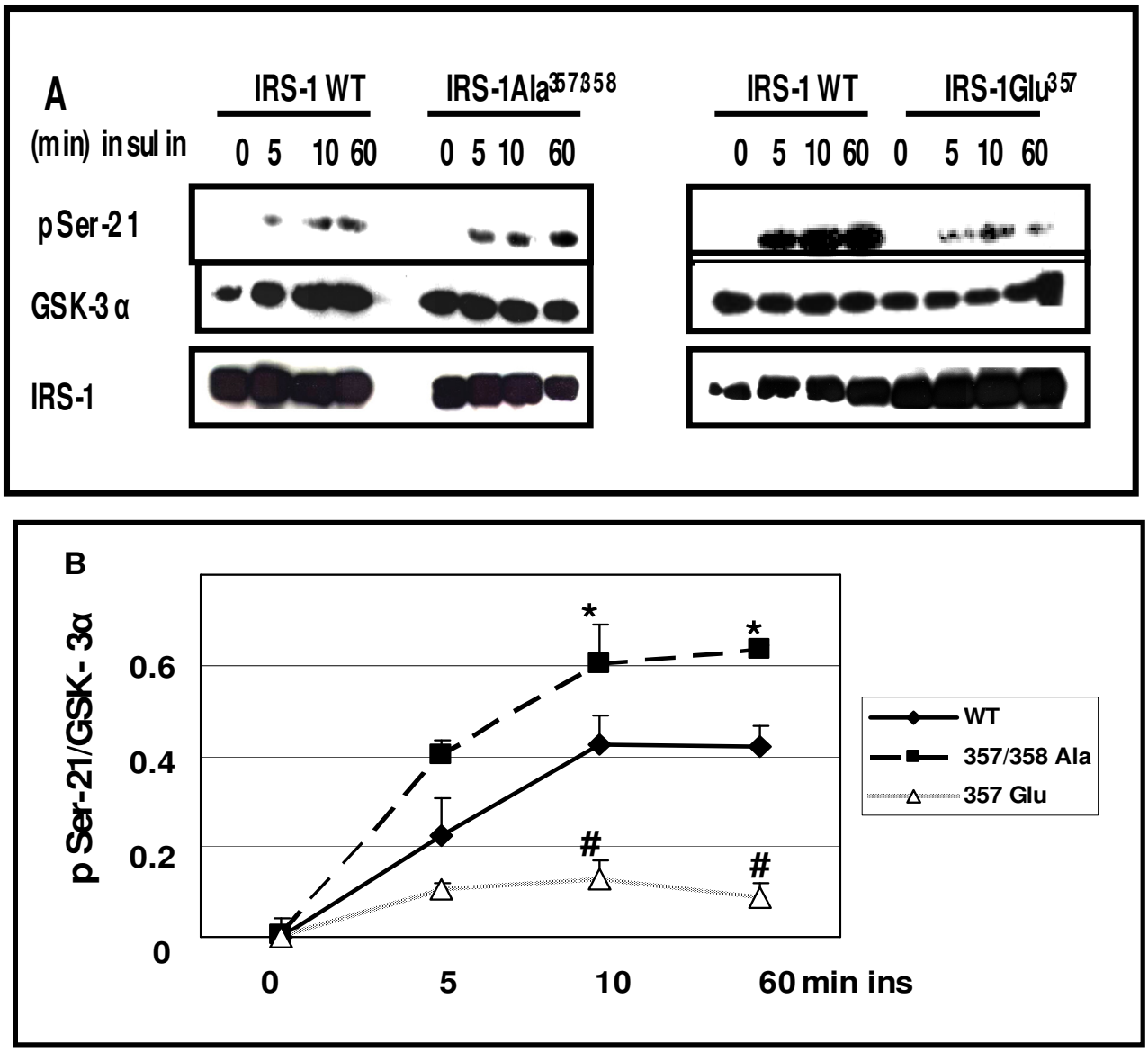


Fig. 17. Insulin-stimulated phosphorylation of GSK-3α in skeletal muscle cells is modulated by Ser-357 phosphorylation of IRS-1.

(A) C2C12 cells were transfected with IRS-1 WT, IRS-1 Ala357/358 or IRS-1 Glu357. Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). Cell lysates were resolved by 7.5% SDS-PAGE and immunoblotted with Ser 21 of GSK-3α. The same blot was stripped and reprobed with a GSK-3α protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (B) Phosphorylation intensity of Ser21 of GSK-3 was quantified based on scanning densitometry of immunoblots normalized for GSK-3α protein. (Mean +SEM, n = 3, $p < 0.05$ IRS-1 Ala357/358 vs. IRS-1 WT; # $p < 0.05$ IRS-1 Glu357 vs. IRS-1 WT.*

4.3.3 Effect of Ser-357 phosphorylation on insulin-stimulated Tyr phosphorylation of IRS-1.

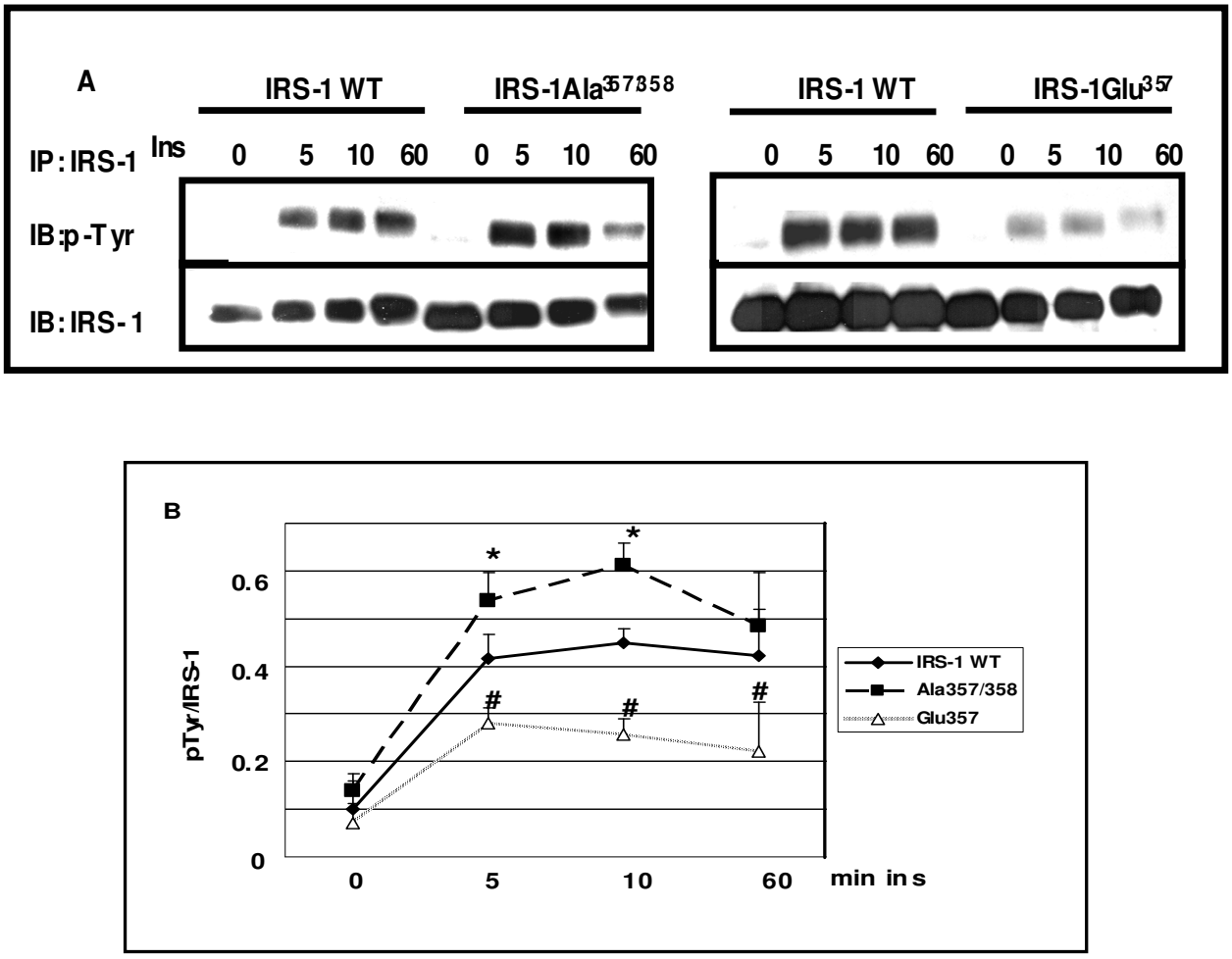


Fig.18. Effect of Ser-357 phosphorylation on insulin-stimulated Tyr phosphorylation of IRS-1.

*(A) C2C12 cells were transfected with IRS-1 WT, IRS-1 Ala357/358 or IRS-1 Glu357 (4 µg each). Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody (IP) and immunoblotted (IB) with the phospho-tyrosine antibody. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody. (B) Tyrosine phosphorylation of immunoprecipitated IRS-1 was quantified based on scanning densitometry of immunoblots normalized for IRS-1. (Mean ± SE, n = 3, *p < 0.05 IRS-1 Ala357/358 vs. IRS-1 WT; # p < 0.05 IRS-1 Glu357 vs. IRS-1 WT).*

Among several mechanisms by which serine phosphorylation of IRS-1 modulates insulin signaling altered tyrosine phosphorylation of IRS-1 is a very obvious one (70) and it also been implicated in the negative effects of PKC-δ on insulin signal transduction (170). Therefore we investigate whether phosphorylation of Ser-357 of IRS-1 could reduce the proximal insulin signaling by preventing or mimicking phosphorylation at Ser-357 in C2C12 cells expressing IRS-1 WT, IRS-1 Ala357/358 or IRS-1 Glu357 (Fig. 18).

Consistent with an inhibitory role of phosphorylation of Ser-357, insulin-stimulated tyrosine phosphorylation of IRS-1 was enhanced using IRS-1 Ala357/358 after 5 and 10 min of insulin stimulation, while it was reduced in IRS-1 Glu357 expressing cells after all time points of insulin stimulation studied (Fig. 18B). Together these experiments suggest that IRS-1 tyrosine phosphorylation was altered by Ser-357, and that phosphorylation of this residue can attenuate IRS-1 mediated insulin signaling cascade in muscle cells.

4.3.4 Inhibition of the PKC-δ-induced downregulation of PKB/Akt phosphorylation by IRS-1 Ala357/358

Activation of PKC isoforms leads to enhanced Ser/Thr phosphorylation of IRS-1 and thus is implicated in impaired insulin signal transduction (155). To illustrate this concept further, we focused on the inhibitory effect of PKC-δ in downstream insulin signaling i.e. on Akt phosphorylation and the potential role of Ser-357 hereby.

TPA-pretreatment of C2C12 cells expressing PKC-δ and IRS-1 WT led to clear downregulation of the insulin-induced phosphorylation of Ser-473 of Akt, an effect prevented by co-transfection of PKC-δ KN indicating the inhibitory effect of PKC-δ on insulin action (Fig. 19A). Moreover, co-transfection of IRS-1 Ala357/358 could clearly reduce the inhibitory action of PKC-δ on insulin-induced Akt phosphorylation (Fig. 19B). Thus we conclude that phosphorylation of Ser-357, by PKC-δ at least partially mediates the adverse effects of PKC-δ on insulin signaling.

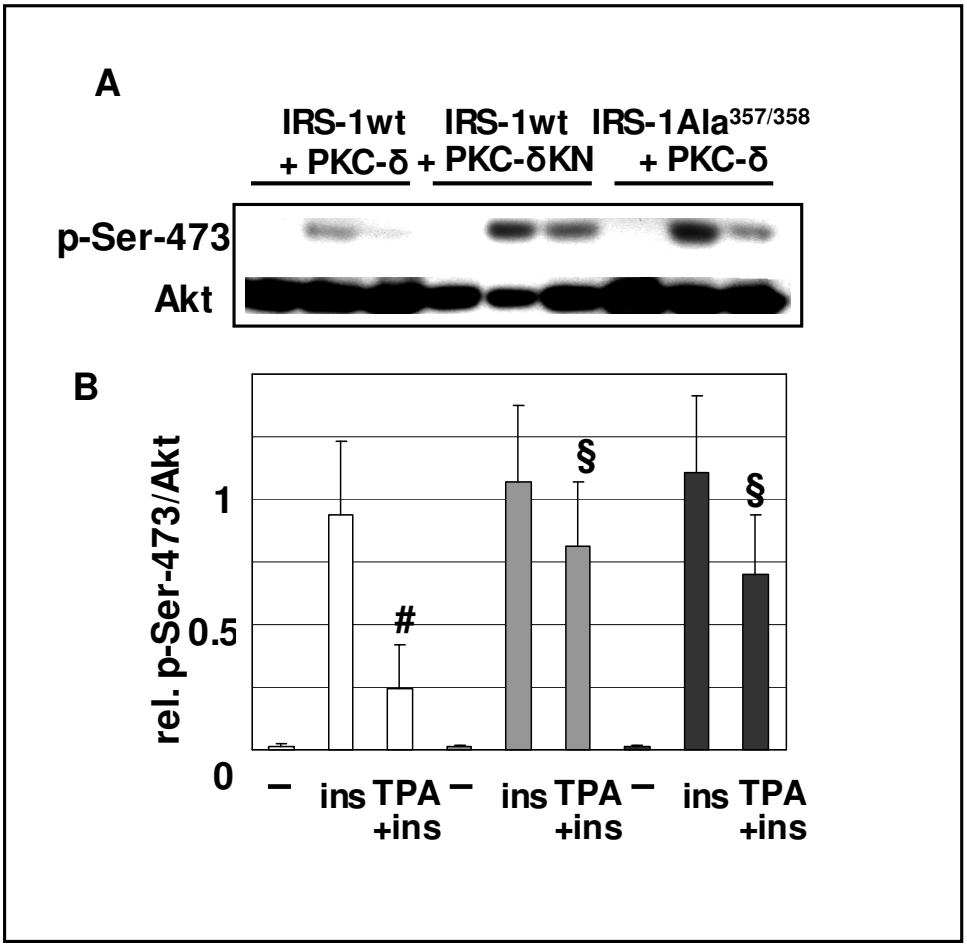


Fig. 19. PKC-δ and Ser-357 phosphorylation of IRS-1 mediated downregulation of insulin-induced phosphorylation of Akt. *(A) C2C12 cells were co-transfected with PKC δ and IRS-1 WT, kinase-dead mutant of PKC δ (PKC δ KN) and IRS-1 WT or PKC δ and IRS-1 WT357/358 Ala. Cells were stimulated with 10 nM insulin for 10 min (ins) or preincubated with 0.5 μM TPA for 20 min before insulin stimulation (TPA+ins). A representative immunoblot demonstrating the phosphorylation of Ser-473 of Akt and the reprobe of the same blot with an Akt protein antibody is shown. (B) Ser-473 phosphorylation intensity was quantified based on scanning densitometry of immunoblots ((means+SEM, n=3, # p < 0.05 TPA + insulin vs. insulin alone in cells cotransfected with IRS-1 WT andPKC-δ, § p< 0.05 vs. IRS-1 WT and PKC-δ transfected cells.)*

4.3.5 Influence of the phosphorylation of Ser-357 on the interaction of PKC-δ and IRS-1

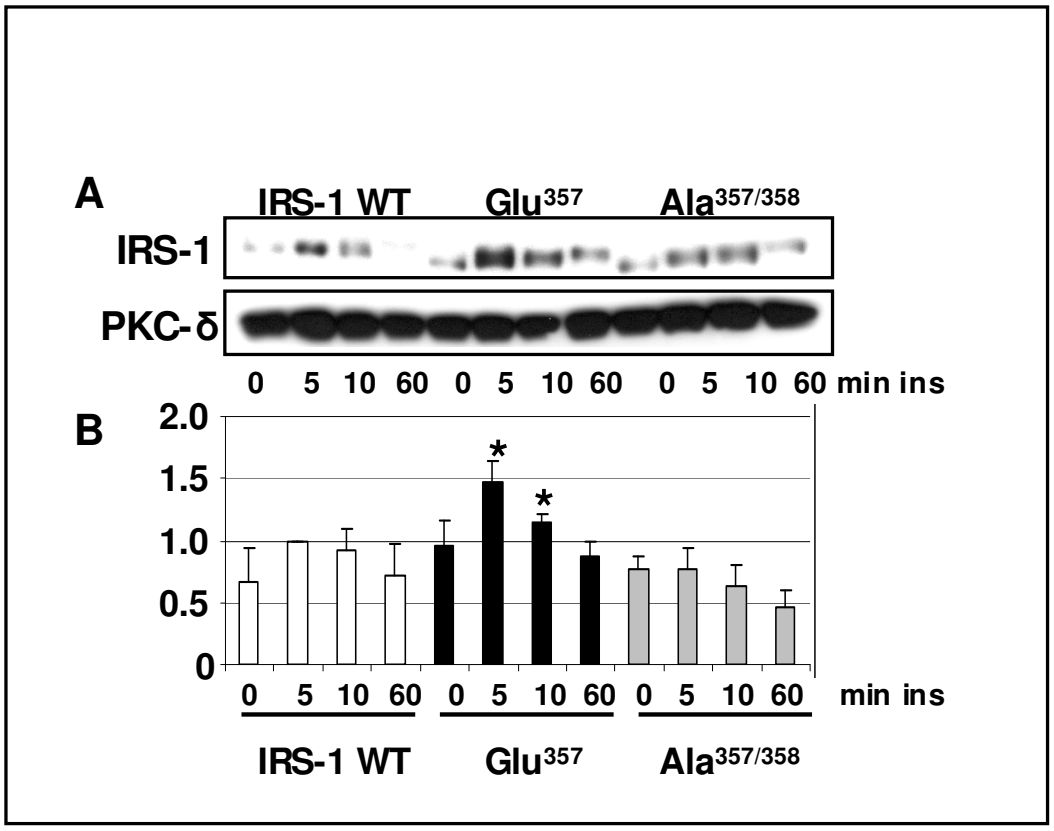


Fig. 20. Influence of the Phosphorylation of Ser-357 on the Interaction of PKC-δ and IRS-1. ***(A)*** *C2C12 cells were transfected with IRS-1 WT, IRS-1 Glu357 or IRS-1 Ala357/358 (4 µg each). Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). PKC-δ was immunoprecipitated and co-precipitated IRS-1 was detected by immunoblotting.* ***(B)*** *Quantification based on scanning densitometry is shown as histogram. (Mean + SEM, n =4,*p < 0.05 IRS-1 Glu357 vs. IRS-1 WT; coprecipitated IRS-1 WT after 5 min of insulin stimulation was set as 1).*

Also it was studied whether the phosphorylation of Ser-357 could regulate the association of IRS-1 and PKC-δ. In IRS-1 WT-expressing cells an insulin-stimulated increase in the association of both proteins after 5 and 10 min of stimulation was observed (Fig. 20), similar to the results obtained in primary mouse skeletal muscle cells (171). The insulin-dependent recruitment of PKC-δ to IRS-1 appeared to be enhanced in IRS-1 Glu357 expressing cells after 5 min of insulin stimulation, while the mutation of Ser-357 to alanine showed similar effect as found for IRS-1 WT (Fig. 20).These data suggest that the phosphorylation of Ser-357

could be involved in the insulin-mediated regulation of the interaction of this serine kinase and IRS-1.

4.3.6 The phosphorylation of Ser318 prevents the phosphorylation of Ser-357 of IRS-1 in the early phase of insulin action

The important physiological balance between activation and termination of insulin action can be altered by Ser-/Thr-phosphorylation of insulin receptor substrate (IRS)-1. Phosphorylation of Ser^{318} enhances insulin signal transduction in the early phase of insulin action; while later-on it is involved in the attenuation of the downstream insulin effects. We aimed to investigate the activation and interplay of Ser-318 and negative acting Ser-357. C2C12 cells were transiently transfected with IRS-1 WT or IRS-1 Ala318 and stimulated for various time points with 10 nM insulin (Fig. 21). The increase in Ser-357 phosphorylation was more pronounced in IRS-1 Ala318 expressing cells in basal condition and after 5 min of insulin stimulation compared with cells expressing IRS-1 WT. These results indicate that the phosphorylation of Ser-318 prevents in the early phase of insulin action the phosphorylation of Ser-357.

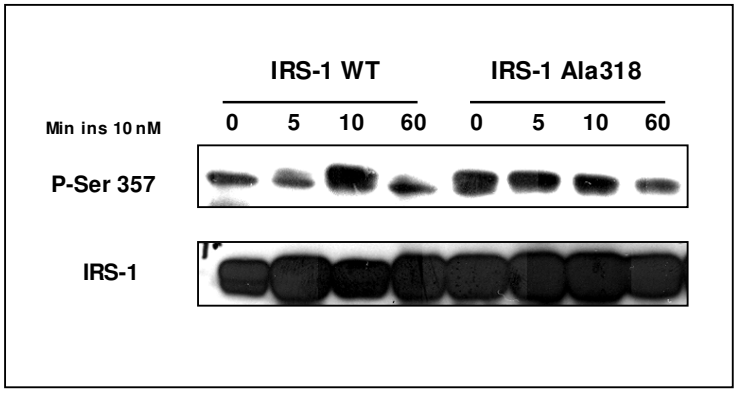


Fig. 21. The phosphorylation of Ser^{318} prevents the phosphorylation of Ser-357 of IRS-1 in the early phase of insulin action.

C2C12 cells transfected with either IRS-1 wild type (WT) or IRS-1 Ala318 (4 µg of each) were stimulated for 5, 10 and 60 min with 10 nM insulin and the phosphorylation of Ser-357 of IRS-1 was studied. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody.

4.3.7 Effects of novel, classical and atypical PKC isoforms on phosphorylation of Ser-357 in IRS-1

Based on the very intense phosphorylation of Ser-357 by TPA, an activator of classical and novel PKCs (Fig.5), as well as findings of the phosphorylation of Ser-357 by the novel PKC, in C2C12 cells (Fig 10), and because members of all PKC classes can be activated by insulin

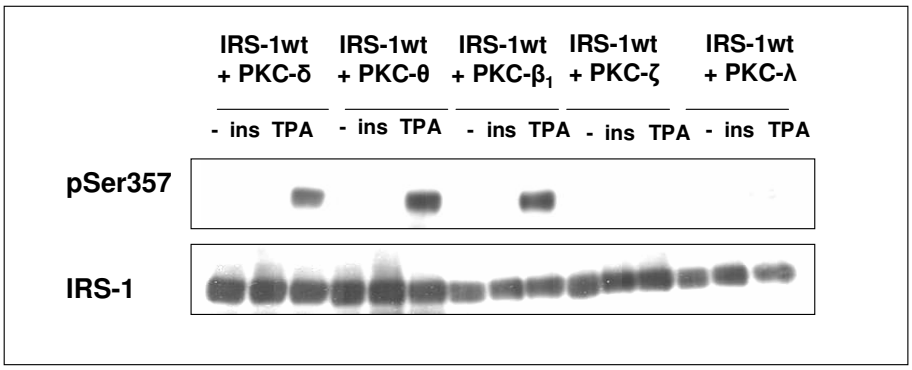


Fig. 22. Effects of novel, classical and atypical PKC isoforms on phosphorylation of Ser-357 in IRS-1.

C2C12 cells were transfected with IRS-1 wild type (WT), co-transfected with PKC–δ and IRS-1 WT, or PKC–θ and IRS-1 WT, or PKC–β_1 and IRS-1 WT, or PKC– ζ and IRS-1 WT, or PKC–λ and IRS-1 WT. Cells were stimulated with either 10 nM insulin or 0.5 μM TPA for 30 min. A representative immunoblot with the phospho-site-specific Ser-357 antibody and reprobe of the same blot with the polyclonal IRS-1 antibody is shown.

(165;172-174), PKC isoforms other then PKC-δ were studied to see their effect on phosphorylation of Ser-357. C2C12 cells were transiently co-transfected with PKC–δ and IRS-1 WT, or PKC–θ and IRS-1 WT, or PKC–β_1 and IRS-1 WT, or PKC– ζ and IRS-1 WT, or PKC–λ and IRS-1 WT and stimulated by insulin and TPA. Cells expressing PKC–δ and IRS-1 WT or PKC–θ and IRS-1 WT or PKC–β_1 and IRS-1 WT showed significant increase in the phosphorylation of Ser-357 in comparison to cells transfected with PKC– ζ and IRS-1 WT, or PKC–λ and IRS-1 WT (Fig. 22). Taken together, these results suggest that the phosphorylation of Ser-357 in skeletal muscle cells is mediated by novel and classical PKCs, whereas atypical PKCs are not involved.

4.8 Single effect of Ser 357, Ser 358 on insulin signal transduction

4.8.1 Single effect of Ser-357 and Ser-358 on tyrosine phosphorylation of IRS-1

In previous experiments, double Ala mutant (Ala 357/ Ala 358) was used to interpret the effect of Ser-357 phosphorylation on insulin signal transduction, these findings needed,

however further substantiation. Therefore, single mutants Ala357 and Ala358 of IRS-1 were used along with double mutant Ala357/358 to observe their single effect on insulin signal transduction.

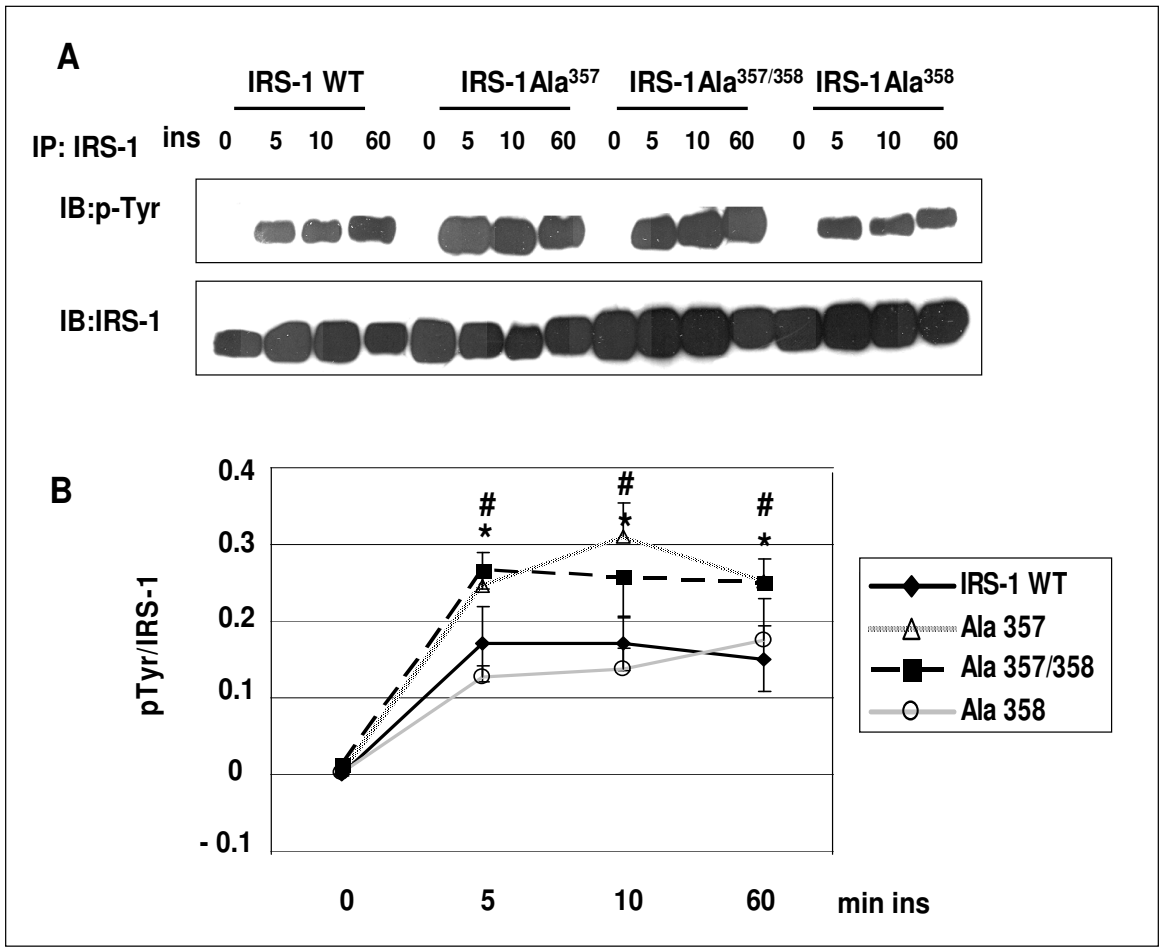


Fig. 23. Single effect of Ser-357 and Ser-358 phosphorylation on insulin-stimulated Tyr phosphorylation of IRS-1. *(A) C2C12 cells were transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358(4 µg each). Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody (IP) and immunoblotted (IB) with the phospho-tyrosine antibody. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody. (B) Tyrosine phosphorylation of immunoprecipitated IRS-1 was quantified based on scanning densitometry of immunoblots normalized for IRS-1. (Mean ± SE, n = 3,* p < 0.05 IRS-1 Ala357 vs. IRS-1 WT; # p < 0.05 IRS-1 Ala357/358 vs. IRS-1 WT).*

C2C12 cells were transfected with each construct and insulin-stimulated tyrosine phosphorylation was determined by immunoblotting. Substitution of Ser-357 IRS-1 by Ala357 IRS-1 increased short and long term insulin stimulated tyrosine phosphorylation of IRS-1 (Fig 23A).Unlike Ala357 IRS-1, Ala358 IRS-1 displayed normal insulin-stimulated tyrosine phosphorylation in C2C12 cells, whereas the double mutant also displayed increased insulin stimulated tyrosine phosphorylation, supporting the idea that Ser-357 plays important regulatory role (Fig 23B).

These experiments maintain the conclusion that phosphorylation of Ser 357 inhibit insulin stimulated tyrosine phosphorylation of IRS-1 while phosphorylation of Ser-358 apparently has no effect.

4.8.2 Single effect of Ser-357 and Ser-358 on Thr 308 phosphorylation of PKB

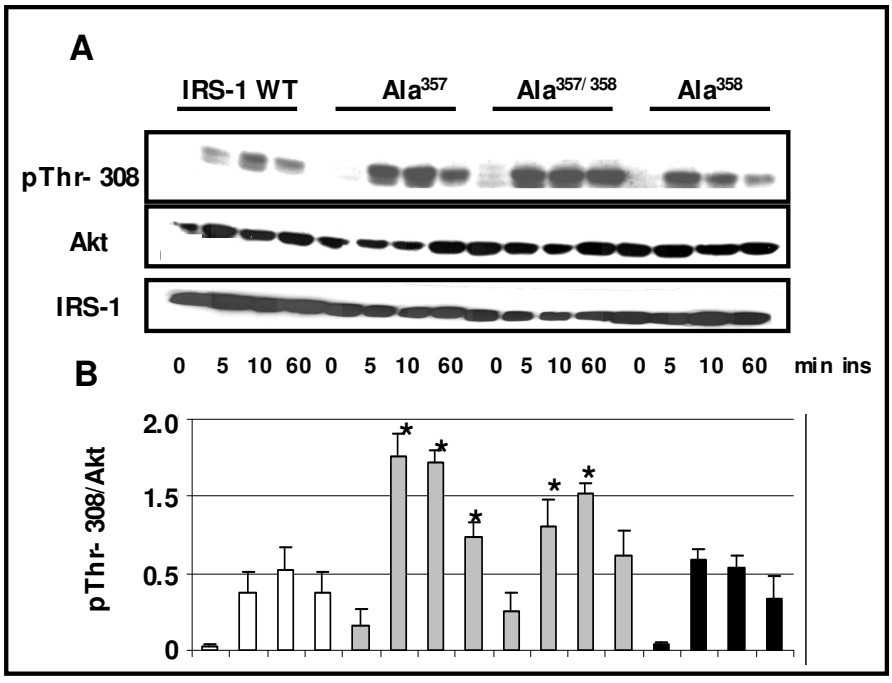


Fig. 24. The phosphorylation of Ser-357 of IRS-1 leads to reduced phosphorylation of Akt in skeletal muscle cells. *(A) C2C12 cells, transiently transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358, and IRS-1 Ala357/358 (4 µg each) were stimulated for 5, 10, and 60 min with 10 nM insulin. Cell lysates were analyzed with 7.5% SDS-PAGE, and after Western blotting the phosphorylation of Thr-308 of Akt was investigated. The same blot was stripped and reprobed with an Akt protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (B) Phosphorylation intensity of Thr-308 of PKB/Akt was quantified based on scanning densitometry of immunoblots normalized for PKB/Akt protein. (Mean +SEM, n = 3,*p < 0.05 IRS-1 Ala mutants vs. IRS-1 WT.)*

Previous results indicated a negative effect of Ser-358 on insulin-stimulated activation of PKB and since it was the only substantial effect of Ser-358 on insulin signaling molecules, therefore it needed further elucidation. Insulin-induced activation of AKT/PKB results from its phosphorylation at its two residues, Thr-308 and Ser-473 (8;175).Thus it was worthwhile to examine influence of Ser-357 and Ser-358 on PKB phosphorylation at Thr-308 as well. C2C12 cells were transiently transfected with IRS-1 WT, IRS-1 Ala357, and IRS-1 Ala358 and IRS-1 Ala357/358 and stimulated for various time points with 10 nM insulin (Fig. 24A). The insulin-dependent increase in Thr-308 phosphorylation of Akt was markedly increased in IRS-1 Ala357 and IRS-1 Ala357/358 expressing cells after 5 and 10 min of insulin stimulation compared with cells expressing wild-type IRS-1 (Fig. 24B). Interestingly, cells expressing IRS-1 Ala358 showed comparable phosphorylation of Thr-308 as in IRS-1 WT leading to the conclusion that Ser-358 does not have a steady and pronounced effect on insulin-mediated activation of PKB.

4.8.3 Single effect of Ser-357 and Ser-358 on GSK-3 phosphorylation

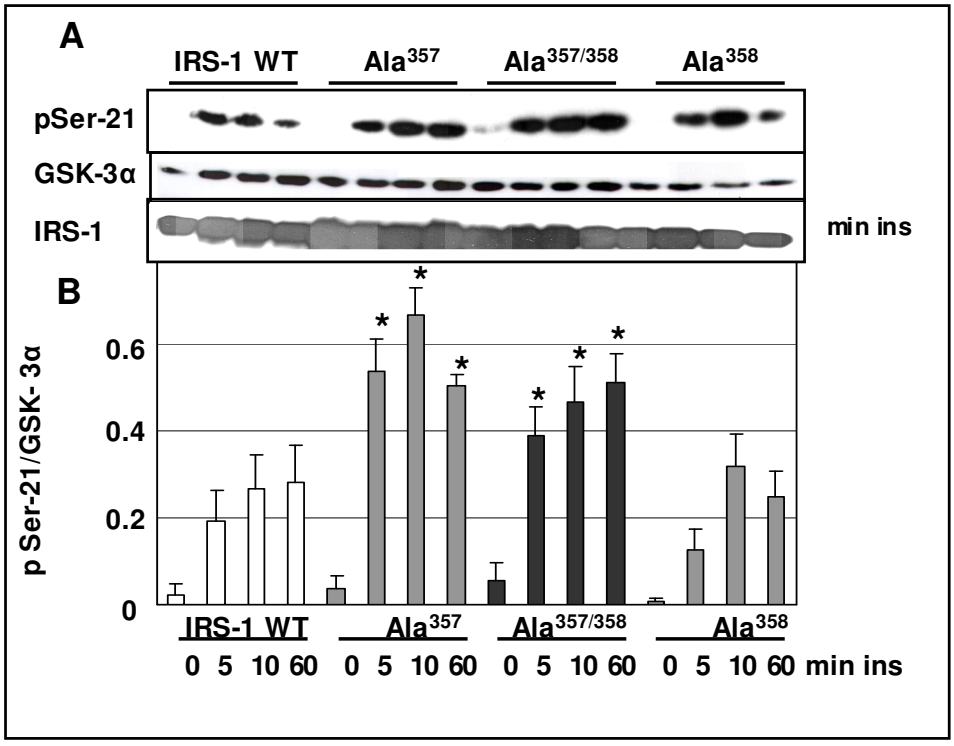


Fig.25. Single effect of Ser-357 and Ser-358 on GSK-3 phosphorylation of IRS-1.

(A) C2C12 cells were transfected with IRS-1 WT, IRS-1 Ala357/358 or IRS-1 Glu357. Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). Cell lysates

were resolved by 7.5% SDS-PAGE and immunoblotted with Ser 21 of GSK-3-3. The same blot was stripped and reprobed with a GSK-3α protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (B) Phosphorylation intensity of Ser21 of GSK-3α was quantified based on scanning densitometry of immunoblots normalized for GSK-3α protein. (Mean +SEM, n = 3, p < 0.05 IRS-1 Ala357 vs. IRS-1 WT; # p < 0.05 IRS-1 Ala357/358 vs. IRS-1 WT.*

The enhanced activation of PKB by Ser-357 phosphorylation was translated into higher phosphorylation of its downstream effectors. As shown in (Fig. 25A), GSK-3α underwent significantly higher phosphorylation in cells over expressing either IRS-1 Ala357 or IRS-1 Ala357/358 as compared to cells expressing wild type IRS-1. On the contrary cells transfected with IRS-1 Ala358 showed insulin stimulated phosphorylation of GSK-3 to the similar extent as in the cells expressing IRS-1 WT.

4.8.4 Phosphorylation of Ser-357 but not Ser-358 triggers deactivation of tyrosine phosphorylation of IRS-1, PKB and its downstream effectors ; relevance of phosphorylation of Ser-357 in human

The main query which remained obscure and need further elucidation was, if Ser-358 is mutated to Ala, Ser-357 phosphorylation has a functional effect? This is important because in human IRS-1, Ser-558 is replaced by Ala-358. Therefore, in order to establish the functional effect of Ser-357 in human IRS-1, an IRS-1 mutant was constructed in which Ser-357 was constitutively phosphorylated, while phosphorylation at Ser-358 was permanently blocked. Using PCR site directed mutagenesis such mutant, IRS-1 357Glu/358Ala, was generated and further used to see its effect in insulin signal transduction.

Phosphorylation of all three molecules of insulin signaling was reduced in the cells over-expressing either IRS-1 Glu357/Ala358 or IRS-1 Glu357 as compared to IRS-1WT expressing cells (Fig 26, 27, 28). These results clearly indicate that insulin stimulated Akt/GSK-3 phosphorylation and tyrosine phosphorylation of IRS-1 is regulated by Ser-357 phosphorylation alone and does not effected by phosphorylation of adjacent residue Ser-358.

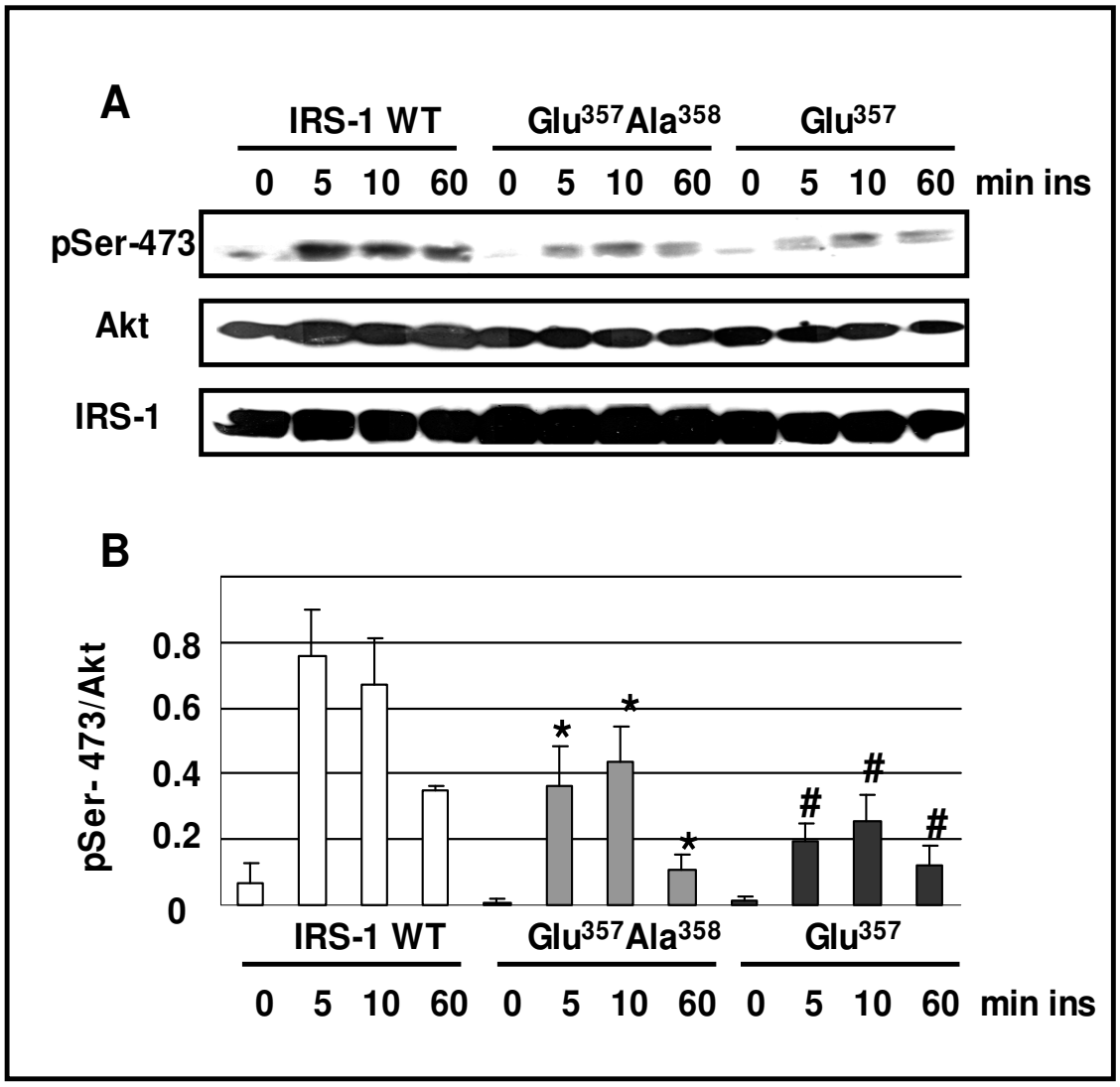


Fig. 26. Phosphorylation of Ser-357, but not Ser-358 triggers deactivation of PKB. *(A) C2C12 cells, transiently transfected with IRS-1 WT, IRS-1 Glu-357Ala-358 and IRS-1 Glu-357(4 µg each), were stimulated for 5, 10, and 60 min with 10 nM insulin. Cell lysates were analyzed with 7.5% SDS-PAGE, and after Western blotting the phosphorylation of Ser473 of Akt was investigated. The same blot was stripped and reprobed with an Akt protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (B) Phosphorylation intensity of Ser473 of PKB/Akt was quantified based on scanning densitometry of immunoblots normalized for PKB/Akt protein. (Mean +SEM, n = 3,*p < 0.05 IRS-1 Glu-357Ala-358 mutants vs. IRS-1 WT; # p < 0.05 IRS-1 Glu mutant vs. IRS-1 WT.)*

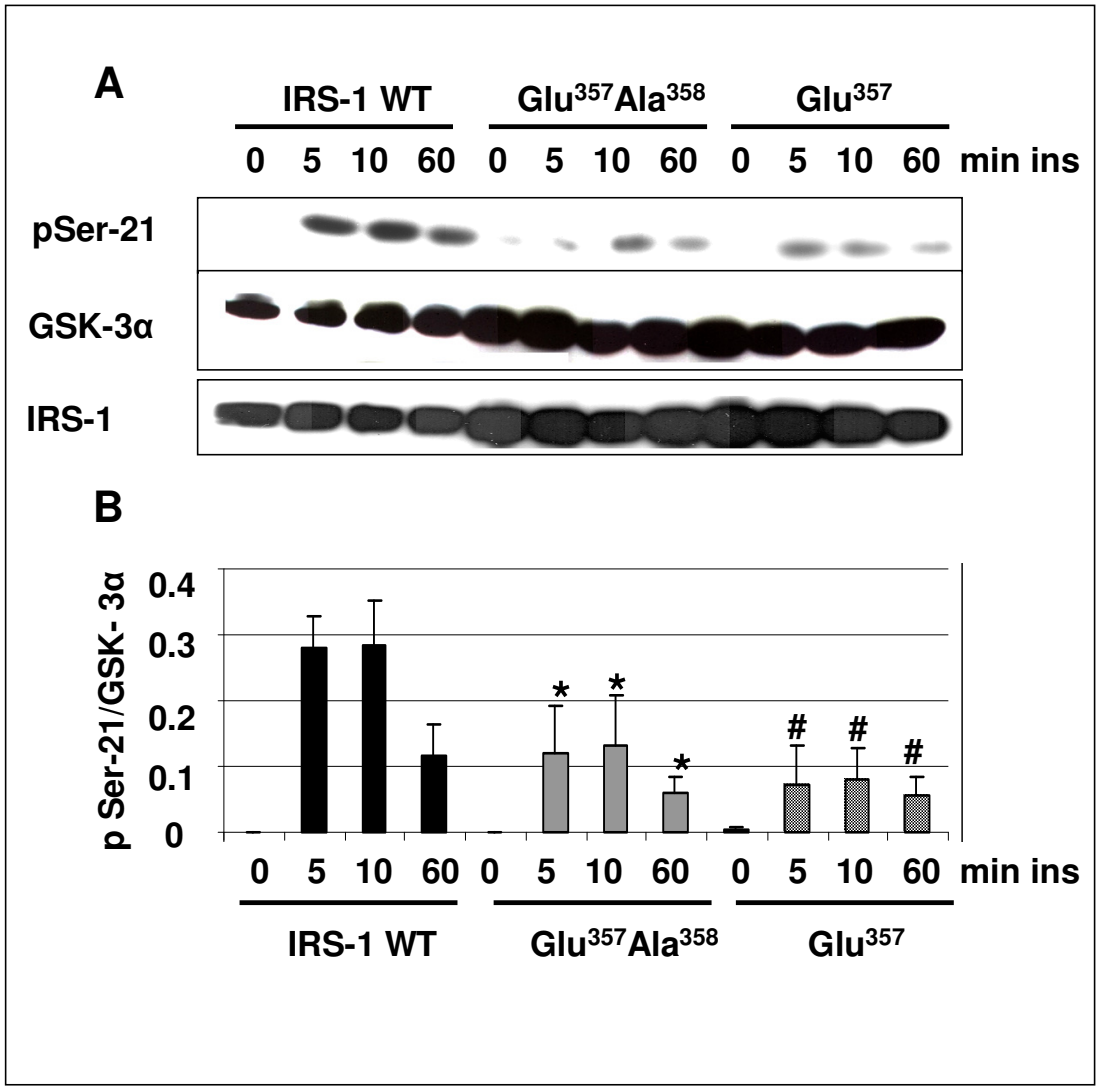


Fig. 27. Phosphorylation of Ser-357 but not Ser-358 down regulate phosphorylation of GSK-3. *(A) C2C12 cells, transiently transfected with IRS-1 WT, IRS-1 Glu-357Ala-358 and IRS-1 Glu-357(4 μg each), were stimulated for 5, 10, and 60 min with 10 nM insulin. Cell lysates were analyzed with 7.5% SDS-PAGE, and after Western blotting the phosphorylation of Ser-21 of GSK-3α was investigated. The same blot was stripped and reprobed with an GSK-3 protein antibody. The IRS-1 amount was analyzed using a polyclonal IRS-1 antibody. (C) Phosphorylation intensity of Ser-21 of GSK-3 was quantified based on scanning densitometry of immunoblots normalized for GSK-3 protein. (Mean +SEM, n = 3,*p < 0.05 IRS-1 Glu-357Ala-358 mutants vs. IRS-1 WT; # p < 0.05 IRS-1 Glu mutant vs. IRS-1 WT.)*

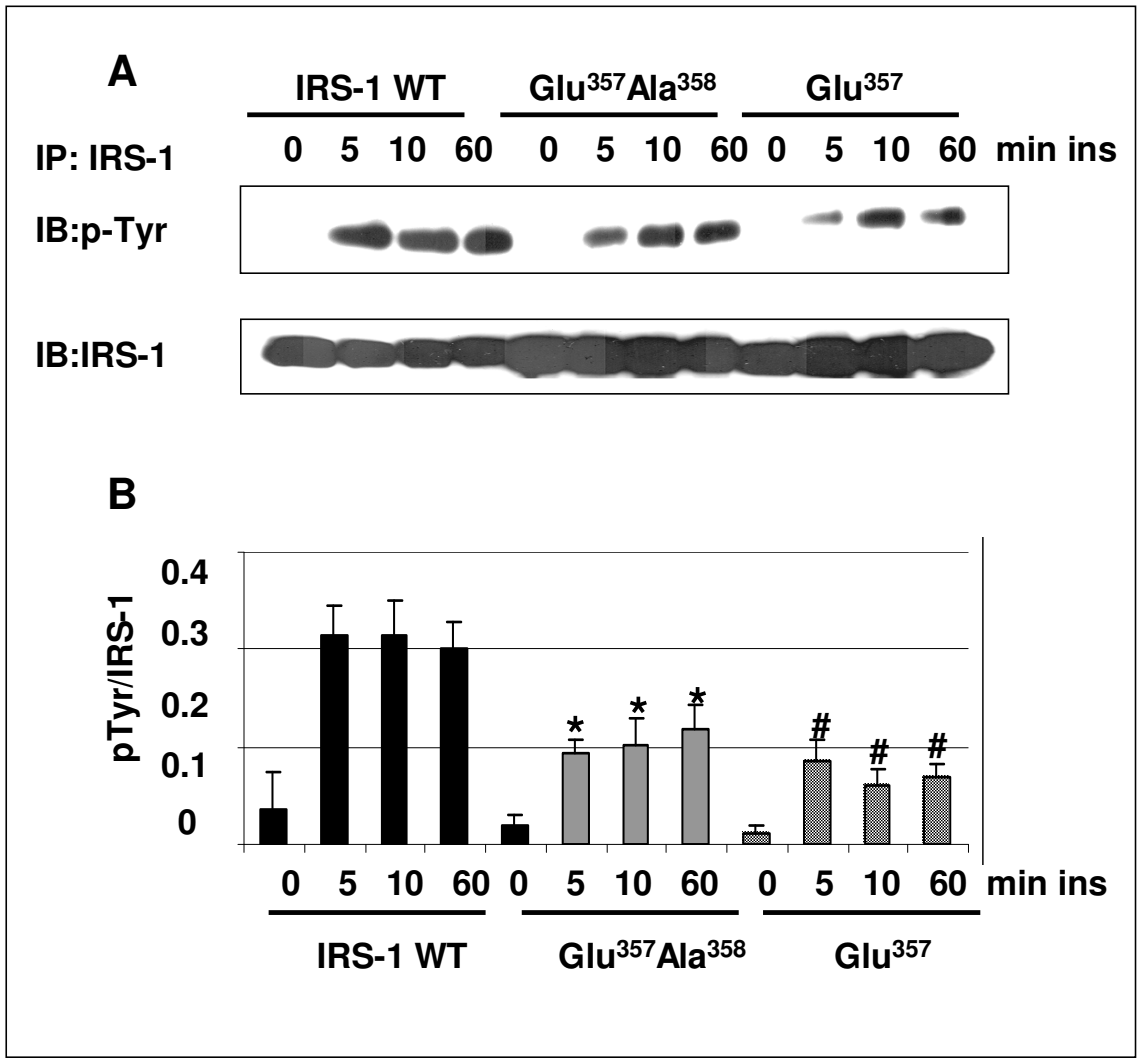


Fig. 28. Phosphorylation of Ser-357 but not Ser-358 down regulate tyrosine phosphorylation of IRS-1. *(A) C2C12 cells were transfected IRS-1 WT, IRS-1 Glu-357Ala-358 and IRS-1 Glu357. Cells were incubated with 10 nM insulin for the indicated times (0, 5, 10 and 60 min). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody (IP) and immunoblotted (IB) with the phospho-tyrosine antibody. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody. (B) Tyrosine phosphorylation of immunoprecipitated IRS-1 was quantified based on scanning densitometry of immunoblots normalized for IRS-1. (Mean ± SE, n = 3,*p < 0.05 IRS-1 Glu357Ala358 vs. IRS-1 WT; # p < 0.05 IRS-1 Glu357 vs. IRS-1 WT).*

4.8.5 Insulin-induced phosphorylation of Ser-357 in human myotubes

IRS-1 plays an essential role in peripheral insulin action and consequently in impaired insulin action (insulin resistance) in insulin sensitive tissues such as skeletal muscle. Therefore, investigation of the effects of physiologically relevant stimuli such as insulin were carried out on the phosphorylation of Ser-357 using human myotubes obtained from biopsies from quadriceps femoris. Insulin increased the phosphorylation of Ser-357 in human myotubes as compared to non-stimulated cells (Fig. 29). Additionally, since we observed in insulin or TPA-treated human myotubes enhanced phosphorylation of Ser357 of the endogenous human IRS-1 protein, which contain alanine instead of serine at position 358, it support the evidence that Ser-357 is functional site in human as well.

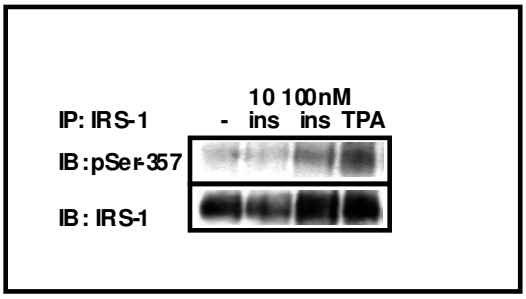


Fig. 29. Insulin-induced phosphorylation of Ser-357 in human myotubes.

Human myotubes were treated with10, 100 nM insulin and 0.5 µM TPA for 30 min.IRS-1 was immunoprecipitated (IP) with a polyclonal IRS-1 antibody and Immunoblotted (IB) with the phosphorylation site-specific Ser-357 antibody.The same blot was stripped and reprobed with a polyclonal IRS-1 antibody.

5- DISCUSSION

The results of a number of studies have indicated that Ser/Thr phosphorylation can affect the ability of IRS-1 to transmit insulin signal, most often in a negative fashion (83;101;103;111;165;170;176;177) ; therefore, the identification of IRS kinases and their target Ser phosphorylation sites is of physiological and pathological importance. Individual Ser-Thr phosphorylated sites of IRS-1 can make it a poor substrate for the IR kinase , resulting in the inhibition of downstream signaling molecules activation and insulin action (178).

The family of protein kinase C (PKC) isoforms represents important modulators of signaling molecules which regulate among other cellular functions the metabolic and mitogenic properties of insulin. All classes of PKCs have been involved in this regulation, and the effects described so far covers the transduction of the positive effect of insulin on glucose uptake and insulin secretion by atypical and novel PKCs (179), the participation in the self-induced attenuation of insulin action by atypical PKCs (173) , and the implication in lipid- and hyperglycemia-induced insulin resistance by classical and novel PKCs (142;177;180;181). These kinases are also activated by various inducers of insulin resistance, placing them at a point of convergence between physiological and pathological stimuli.

Data of the novel PKC isoform δ mirrored this positive and negative modulation of insulin signaling. PKC-δ is shown to be important for insulin stimulated glucose uptake (126), and it participates in the insulin-dependent activation of Akt (182). On the other hand, it has been shown that activation of PKC-δ by lipids and leptin is involved in the impairment of insulin signaling (165;183) and in the induction of apoptosis of insulin-secreting cells (161).

In addition, PKCs are involved in the subsequent downregulation of insulin signaling (155). PKCs may contribute to serine phosphorylation of IRS-1 in response to insulin, thereby involve in negative feed back of insulin signaling (170;184;185). Serine phosphorylation of IRS-1 appears to be a major mechanism for the adverse effects of PKC-δ on insulin action (155;165;170). While *in vitro* at least 18 PKC-δ-dependent phosphorylation sites in IRS-1 have been identified (155), so far only two sites could be demonstrated to be phosphorylated *in vitro* and to be functionally active, these are Ser-24 and Ser-318 (165;170;186). None of these sites were shown to be phosphorylated upon insulin stimulation by PKC-δ, although insulin could activate PKC-δ and induce its association to IRS- 1 (187).

5.1 Characterization and specificity of phospho-Ser-357 antibody

In the current study we could demonstrate Ser-357 of IRS-1 as a novel, genuine and PKC-δ-dependent phosphorylation site in cells, which is phosphorylated upon insulin stimulation. This conclusion is based upon the fact that its phosphorylation after insulin treatment can be detected using phospho-site-specific antibodies that selectively recognize the phosphorylated form of Ser-357.

This site was first identified by an *in vitro* kinase assay and verified in cell culture systems using phospho-specific antibodies. Cross reactivity of the phospho-Ser-357 antibody with the adjacent serine site was eliminated by immuno purification. The specificity of the antibody, after immuno-purification, was first determined by ELISA and then demonstrated after phosphatase-treatment and using IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358 mutants. It was necessary to include the Ala358 mutation since this amino acid adjacent to Ser-357 is also a serine residue in mouse and rat IRS-1 and has been reported as a putative phosphorylation site by Liu,Y.F (83).

Finally, to determine whether our observations in the C2C12 cells were applicable to a more physiological system, we examined tissues from mice for the presence of the IRS-1 Ser-357 phosphorylation. Extracts from muscle of insulin stimulated wild type mice exhibited phosphorylation of Ser-357 as compared to non stimulated mice. These results are therefore consistent with the hypothesis that Ser-357 is an insulin stimulated phosphorylation site of IRS-1.

5.2 PKC-δ mediated IRS-1 Ser-357 phosphorylation

In the present study we identified Ser-357 of IRS-1 as a PKC-δ mediated novel phosphorylation site. IRS-1 contains several PKC substrate motifs (RXS/SXR/RXSXR) and Ser-357 resides in one of these motifs (RGS/SSR/RGSSR). The results implicated PKC-δ as the responsible serine kinase for the phosphorylation of Ser-357 since it was the kinase used in the *in vitro* phosphorylation assays, it could be activated by phorbol ester and insulin. Here, we provide the evidence that our results are consistent with the hypothesis that Ser-357 of IRS-1 is phosphorylated by PKC- δ. Two lines of evidence support the notion that Ser-357 serve as an *in vitro* substrate of PKC-δ. First, overexpression of PKC-δ enhanced phosphorylation of Ser-357 as compared to control cells. Second, PKC-δ KN (kinase negative) failed to mimic the stimulatory effects of its wild-type counterpart, when transiently overexpressed in muscle cells. That is, phosphorylation of Ser-357 of IRS-1 in cells required

intact kinase activity of PKC-δ. Since overexpression strongly enhanced the phosphorylation of Ser-357 also in unstimulated cells and expression of a kinase dead PKC-δ blocked the phosphorylation almost completely, this observation supports the hypothesis that PKC-δ is involved in phosphorylation of Ser-357.

Our results also suggest the likelihood of involvement of classical PKCs and novel PKC isoforms other then PKC-δ in the phosphorylation of Ser-357 in skeletal muscle cells which support the hypothesis that Ser-357 is a PKC-dependent phosphorylation site.

Direct phosphorylation of Ser-357 is possible by PKC-δ, but we cannot rule out the role of other kinases as the phosphorylation motif is not completely selective for PKCs. This site is found in the substrate motif of PKB/Akt, and calmodulin-dependent protein kinase II; thus these can be possible kinases involved in phosphorylation of Ser-357. Albeit, some studies report that PKC-δ negatively regulates the insulin-induced JNK activation (188), but some studies indicate that novel PKCs can mediate serine phosphorylation of IRS-1 by activating MAP kinase and JNK in kidney and skeletal muscle cells (101;189) therefore, at present, we cannot completely rule out the possibility that PKC-δ can mediate indirect phosphorylation of Ser-357 by activating MAPKinase / JNK. While GSK-3 is highly improbable to mediate phosphorylation of Ser-357 because this site is not in the phosphorylation motif of GSK-3, besides, phosphorylation of GSK-3 is inhibited by Ser-357 phosphorylation and therefore it is unlikely that GSK3 can mediate phosphorylation of Ser-357. Hence, our study provides sufficient evidence that PKC-δ is at least partly responsible for cellular phosphorylation of Ser-357.

5.3 Functional role of phosphorylation of Ser-357 of IRS-1 in insulin-stimulated signal transduction.

5.3.1 Influence of Ser-357 and Ser-358 phosphorylation on PKB/AKT

To investigate the biological relevance of this phosphorylation site in insulin signaling, we compared effects of Ser-357 phosphorylation of IRS-1 on central molecules of insulin signaling. The key result of our study was that IRS-1 Ala357/358 can better propagate insulin signal as compared to IRS-1WT, which is manifested for instance by its ability to dramatically enhance PKB phosphorylation. This result indicates that Ser-357 and Ser-358 of IRS-1 are negative regulatory sites in insulin signal transduction. Additional findings support this conclusion, when Ser-357 was changed to Glu to mimic phosphorylation; insulin-stimulated PKB phosphorylation was blocked. Overexpressed IRS-1Ala357/358 enhanced

PKB phosphorylation to the similar extent as IRS-1 Ala357. This observation, further support the conclusion that mutation of both serine sites had no additive effect on insulin signal transduction. In order to further elucidate the single effect of these serine sites on insulin mediated PKB activity, regulation of phosphorylation of Thr-308 of PKB was tested with single mutants of IRS-1 (IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358), surprisingly it was observed that Ser-358 has no effect on Thr-308 phosphorylation while Ser-357 maintained its consistent negative role in insulin signaling by down regulation of Thr-308. Since different kinases are involved in phosphorylation of PKB, Ser-473 being phosphorylated by mammalian target of rapamycin complex-2 (mTORC2) while Thr-308 by PIP3-bound 3-phosphoinositide-dependent protein kinase-1 (PDK1) (8), it is likely that Ser-357 and Ser-358 albeit being adjacent sites, exploit different pathways for the regulation of insulin signaling and consequently contributed differently in down regulation of insulin signaling.

5.3.2 Influence of Ser-357 phosphorylation on GSK-3

Interestingly we were also able to translate this inhibitory effect of Ser-357 on the regulation of GSK3 activity, a downstream target of PKB. The double mutant IRS-1Ala 357/358 displayed increase insulin stimulated Ser-21 phosphorylation of GSK-3. Conversely, substitution of Ser-357 with glutamic acid blunted phosphorylation of Ser-21 of GSK3-α during insulin stimulation indicating a constant and stable negative effect of Ser-357 in insulin signal transduction.

5.3.3 Influence of Ser-357 phosphorylation on tyrosine phosphorylation of IRS-1

The additional strength of our study is that our experimental data support the conclusion that phosphorylation of Ser-357 of IRS-1 inhibits insulin-stimulated tyrosine phosphorylation of IRS-1. Substitution of IRS-1WT with IRS-1Ala357/358 increased insulin-stimulated IRS-1 tyrosine phosphorylation, whereas substitution of IRS-1Glu357 of IRS-1 decreased insulin-stimulated IRS-1 tyrosine phosphorylation, supporting the conclusion that Ser-357 has an essential regulatory function in insulin signaling. Though the mechanism by which Ser-357 blunted IRS-1 tyrosine phosphorylation of IRS-1 is not completely clear, but it might be possible that Ser-357 phosphorylation causes electrostatic blockade for access of IRS-1 to IR like other serine sites of IRS-1(44;179). Alternatively, Ser-357 phosphorylation might target IRS-1 to sub-cellular compartments inaccessible to the activated insulin receptor and thus inhibits subsequent IRS-1-mediated downstream insulin signaling.

5.4 Single effect of Ser 357 and Ser 358 on insulin signal transduction

Ser-358 has been reported to have both, positive and negative effects on insulin signal cascade (177;190), but these studies were carried out on IRS-1 mutated at the multiple serine residues (IRS-1- Ala 265/302/325/336/358/407/408 mutant) and none of these studies focused on single effect of Ser-358 on insulin signaling. Here a part of the studies was focused on IRS-1 mutated at only Ser-358.

5.4.1 Single effect of Ser-357 and Ser-358 on tyrosine phosphorylation of IRS-1

In order to confirm single effect of Ser-357 and Ser-358 on insulin signal transduction, single Ala mutants were used along with the double Ala to compare the effect of these serine residues on insulin signal transduction.In contrast to upregulation of tyrosine phosphorylation of IRS-1 by IRS-1 Ala357, IRS-1 Ala358 displayed normal insulin stimulated tyrosine phosphorylation, whereas the double Ala mutant also displayed increased insulin-stimulated tyrosine phosphorylation. These experiments support the conclusion that phosphorylation of Ser 357 inhibits insulin-stimulated proximal insulin signaling, while phosphorylation of Ser-358 apparently had no effect on it.

5.4.2 Single effect of Ser-357 and Ser-358 on GSK-3 phosphorylation of IRS-1

To test the effect of Ser-358 on GSK-3 phosphorylation it was found that there was significant enhanced GSK-3 phosphorylation when Ala-357 IRS-1 was used, while Ala 358 IRS-1 showed similar phosphorylation of GSK-3 as IRS-1WT.

Over all, our data showed no effect of Ser-358 phosphorylation on insulin signaling, except at the level of Ser-473 phosphorylation of PKB. However, there is no effect of Ser-358 phosphorylation on Thr-308 phosphorylation of PKB, tyrosine phosphorylation of IRS-1 and phosphorylation of GSK-3.

Although in rodents both serine residues contribute to the Ser-473 phosphorylation of Akt, as shown by our previous experiments with cells overexpressing single mutants (Fig 16), nevertheless Ala 358 had no effect on Thr-308 phosphorylation of PKB, tyrosine phosphorylationof IRS-1 and on GSK-3 phosphorylation. On the other hand, Ser-357 exhibited a consistent negative modulation of proximal and distal insulin signaling, as has been proven by using diverse loss of function and gain of function mutations at Ser-357 in IRS-1 molecule (IRS-1 Ala357, IRS-1 Ala358, IRS-1 Ala357/358, IRS-1 Glu357 and IRS-1

Glu357/Ala358). Therefore, we conclude that Ser-358 has a subordinate effect on overall insulin signal transduction as compared to Ser-357.

Moreover Ser-357 has been conserved in rodents and human, while Ser-358 is absent in human. In the present study enough evidence was provided to support the conclusion that Ser-357 but not Ser-358 has a conserved and putative function in insulin signal transduction.

5.4.3 Phosphorylation of Ser-357, but not Ser-358 triggers deactivation of tyrosine phosphorylation of IRS-1, PKB and its downstream effectors ; relevance of phosphorylation of Ser-357 in human

Finally to determine whether our observations of Ser-357 phosphorylation and its impact on insulin signaling were applicable to human, a new mutant was generated, known as IRS-1 357Glu/358Ala, because the human residue homologous to murine Ser-358 actually is alanine. Insulin-mediated phosphorylation of central molecules of insulin signaling was examined in C2C12 cells overexpressing IRS-1 WT or IRS-1 Glu357/Ala358 or IRS-1 Glu 357. Tyrosine phosphorylation of IRS-1, PKB phosphorylation at Ser-473 and GSK-3 phosphorylation at Ser-21 was reduced in the cells overexpressing either IRS-1 357Glu/358Ala or IRS-1 Glu 357 as compared to IRS-1 WT expressing cells. These results clearly indicate that in human, insulin stimulated phosphorylation of these signaling molecules, at least in part, is regulated by Ser-357. In addition, it was also observed that in human mayotubes this site is phosphorylated in response to insulin and TPA.

Therefore, Ser-357, but not Ser-358 is the primary phosphorylation site of IRS-1 both in rodents and human, which can modulate insulin signaling.

5.4.4 The phosphorylation of Ser-318 prevents the phosphorylation of Ser-357 of IRS-1 in the early phase of insulin action

To investigate the interplay of previously described phosphorylation site Ser-318 of IRS-1 (110) and the phosphorylation of Ser-357 was of interest. It was observed that in the early phase of insulin action, the phosphorylated state of Ser-318 prevents the phosphorylation of Ser-357. Since phosphorylation of Ser-318 enhances insulin signal transduction in the early phase of insulin action; while later-on it is involved in the attenuation of the downstream insulin effects, while Ser-357 has a negative effect in insulin signal transduction, this can be the part of complex regulation of IRS-1 serine phosphorylation to keep a tight balance between activation and inhibition of insulin signal transduction.

5.4.5 Inhibition of the PKC-δ-induced downregulation of Akt phosphorylation by IRS-1 Ala357/358; potential mechanism behind downregulation of insulin signaling by PKC-δ

Finally we addressed the hypothesis that insulin stimulated activation of PKC-δ may enhance serine phosphorylation of IRS-1 and attenuate the downstream effectors of insulin signaling. Prior studies have also attempted to explain the mechanism employed by PKC-δ to down - regulate insulin signaling, but these studies remained limited to the effect of PKC-δ on proximal insulin signaling/tyrosine phosphorylation of IRS-1 (155). We address the question about the effect of PKC-δ on distal insulin signaling. The adverse effect of PKC-δ activation on insulin action was further demonstrated, when we induced the negative effect of PKC-δ by pre-treatment of IRS-1 WT- and PKC-δ- overexpressing cells with phorbol ester. We found that insulin stimulated PKB phosphorylation was blunted by PKC-δ and that PKC-δ is not sufficient to cause this effect independent of Ser-357 phosphorylation, supporting the idea that PKC-δ could mediate the physiological negative feed back of insulin signaling through phosphorylation of Ser-357. Thus, both, active PKC-δ and IRS-1 phosphorylated on serine 357 are necessary for the observed adverse effects of phorbol ester-mediated PKC activation on insulin signaling. Taken together these results further strengthen the role of PKC-δ in attenuating insulin signaling via serine phosphorylation of IRS-1 and explain, at least in part, the inhibitory mechanism of PKC-δ in insulin signaling.

The phosphorylation of Ser-357 did not result in the dissociation of PKC-δ and IRS-1, as it was e.g. reported for PKC-ζ and IRS-1 after phosphorylation of Ser318 (110), but even appeared to result in an enhanced recruitment. This could facilitate other serine phosphorylation events on IRS-1 e.g. the previously published PKC-δ-dependent sites Ser-24 and Ser-318, or its receptor, leading to the described reduced tyrosine phosphorylation and attenuation of insulin signaling (170). Interestingly, the insulin-induced activation of PKC-δ has also been implicated in a positive modulation of insulin action. Overexpression of PKC-δ in mouse skeletal myotubes led to a very rapid increase in tyrosine phosphorylation of the IR without insulin stimulation, and overexpression of a dominant negative PKC-δ prevented the insulin-dependent tyrosine phosphorylation of the IR (125;187). Moreover, the insulin-dependent activation of PKC-δ was shown to be important for a maximum stimulation of Akt in skeletal muscle cells (182). Thus, insulin alone might not be sufficient to induce the adverse effects of PKC-δ on insulin action. These effects of PKC-δ had been reported after pharmacological activation using phorbol ester (170), stimulation with leptin (165) or lipid infusion (183). Following this aspect, it must be noted that we observed an insulin-dependent

phosphorylation of Ser-357, but this effect was not as strong as the phosphorylation intensity after stimulation with phorbol ester. It could be speculated that in the pathophysiological situation of metabolic disturbance with hyperinsulinemia, hyperglycemia, and hyperlipidemia a more pronounced and sustained activation of PKC-δ occurs which then leads to a significant phosphorylation of Ser-357. This phosphorylation could then mediate the desensitizing effect on insulin action in combination with other phosphorylation events.

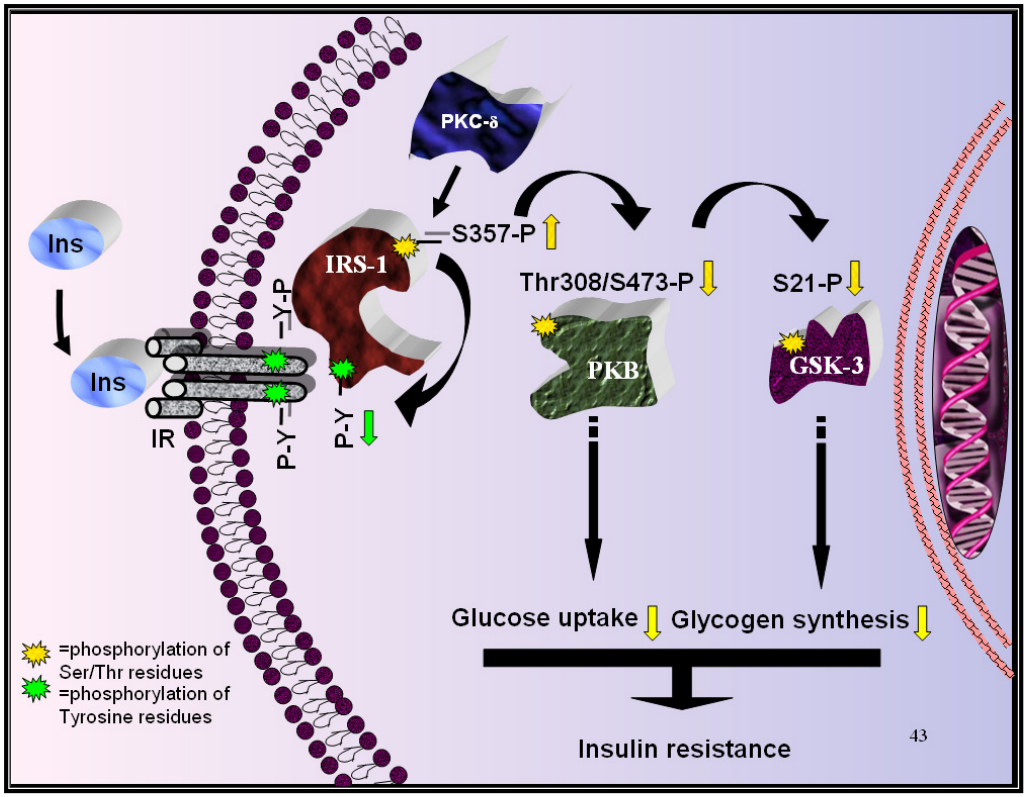


Fig. 30. Schematic diagram outlining the role of Ser-357 of IRS-1 in insulin action in skeletal muscle cells. *Arrow indicates change in phosphorylation.*

Insulin binding to IR results in recruitment of IRS-1 to the activated receptor and its tyrosine phosphorylation. Activated PKC-δ mediates Ser-357 phosphorylation of IRS-1. Phosphorylation of Ser-357 inhibits tyrosine phosphorylation of IRS-1 and appears to lead to the reduction of phosphorylation of PKB and GSK-3. This may eventually result in insulin resistance due to reduced glucose uptake and impaired glycogen synthesis.

Ins	Insulin	IRS-1	Insulin receptor substarte-1
IR	Insulin receptor	PKC	Protein kinase C
PKB	Protein kinase B	GSK-3	Glycogen-Synthase-Kinase 3
P-Y	Phosphorylated tyrosine	S	Serine

Number of distinct models may explain the observed data (Fig. 30). The simplest interpretation is that activation of PKC phosphorylates IRS-1 at Ser-357 and possibly other serine residues, and this phosphorylation inhibits the subsequent ability of the IRS-1 to serve as a substrate for the IR tyrosine kinase and results in reducd tyrosine phosphorylation of IRS-1. This event reduces IRS-1 ability to activate PKB and relay insulin signal further down stream i.e Phosphorylation of GSK-3. Deactivation of PKB and activation of GSK-3 may lead to reduce glucose uptake and reduced glycogen synthesis respectively which can be the basis of insulin resistance. Together, these data suggest that phosphorylation of Ser357 mediates at least in part the adverse effects of PKC-δ activation on insulin action.

In summary, we could clearly show that the phosphorylation of Ser-357 is functional and resulted in impaired insulin-stimulated tyrosine phosphorylation of IRS-1, reduced activation of Akt and subsequently reduced phosphorylation of GSK-3. These data suggest that Ser-357 is the primary site for the modulation of insulin signaling. Albeit, Ser-358 have an interchangeable and secondary function in mice and rats but in human only Ser-357 is functional. The substantial role of Ser-357 is underlined by the fact that in human IRS-1 Ser-358 (position 363 in human IRS-1) is replaced by alanine and our data support the conclusion that this site is phosphorylated in human myotubes and still modulate insulin signaling when 358 is Ala. In short, we identified Ser-357 as a new phosphorylation site in IRS-1 and showed that PKC-δ is the potential kinase involved in phosphorylation of Ser-357. Our results are consistent with the assumption that PKC-δ mediated phosphorylation of Ser-357 appears to be the part of physiological negative feedback inhibition of insulin signaling. In conclusion we demonstrated here a novel, functionally relevant serine residue of IRS-1, which could be involved upon PKC-δ-dependent phosphorylation in the attenuation of insulin signaling in the insulin resistant state.

In future, the *in vitro* role of Ser-357 phosphorylation needs to be resolved using tissues from animals or humans with insulin resistance and to follow changes in the phosphorylation status of Ser-357 after therapeutic interventions. Additional work is needed to identify other PKC-δ -mediated important regulatory serine sites of IRS-1; this should help to provide a more complete understanding of the molecular basis of insulin resistance.

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8- AUFLISTUNG DER AKADEMISCHEN LEHRER

W.Voelter	PhD research work
R.Lehmann	PhD research work
C.Weigert	PhD research work
E.Schleicher	PhD research work
H.U.Häring	PhD research work
F.Abbas	Research work
A.Saddiqui	Biochemistry
S.A.Qureshi	Biochemsitry/Molecular biology
Z.Hasan	Signal transduction
R.Hasan	Molecular immunology
S.Hussain	Cell biology
P.M.Frossard	Molecular biology
S.A.Saeed	Physiology
N.Ahmad	Molecular genetics
O.Y.Khan	Advance evolution
S.R.Farooqi	Human genetics
A.Wahidi	Plant genetics
M.Maqsood	Biochemsitry
M.Muneer	Microbiology
S.Qamar	Chemsitry
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9- LEBENSLAUF

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Titel: "Modulation der Insulinsignalübertragung durch Phosphorylierung von Serin-357 im Insulin-Rezeptor-Substrat-1"