Identification and functional studies of two novel serine phosphorylation sites of insulin receptor substrate (IRS)-2: Ser 675 and Ser 907

Dissertation zur Erlangung des Doktorgrades

der Naturwissenschaften (Dr. rer. nat.)

Fakultät Naturwissenschaften

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Abteilung für Innere Medizin IV-

Pathobiochemie und klinische Chemie

vorgelegt von

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2010

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Eingereicht am: 15.06.2010

Mündliche Prüfung am: 12.10.2010

Die vorliegende Arbeit wurde am 29.07.2010 von der Fakultät Naturwissenschaften der Universität Hohenheim als „Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften" angenommen.

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1.1.1 Insulin

Insulin is the most important anabolic hormone acting on multiple target tissues. It controls the uptake of glucose and amino acids into muscle and adipose tissue, regulates synthesis of glycogen and inhibits gluconeogenesis in the liver (1). It promotes fatty acid synthesis in the liver and inhibits lipolysis in adipose tissue (2). Furthermore, it regulates proliferation (3) and apoptosis, it controls food uptake (4) and insulin synthesis (5). The absolute (type I diabetes) or relative (type II diabetes) lack of insulin results in dramatic metabolic consequences and leads, when treated insufficiently, ultimately to pathological lesions such as retinopathy, neuropathy and nephropathy. The untreated type I diabetes leads to death.

Insulin is released from the pancreatic β-cells in response to increased blood glucose concentrations and this is amplified by free fatty acids (6), amino acids (7) and incretins, such as glucagon-like peptide (GLP-) 1 and glucose-dependent insulinotropic polypeptide (GIP) (8). The hormone acts on its target tissues via the insulin receptor and glucagon is its major physiological antagonist.

1.1.2 The insulin receptor (IR)

The insulin signaling cascade is initiated by binding of insulin to its receptor. The complete insulin receptor (IR) is a heterotetramer comprised of two α and two β-subunits, linked by disulfide-bonds (Figure 1). Both IR subunits are encoded by a 22-exon gene, spanning 120 kb located on chromosome 19. The extracellular α-unit exists in two isoforms: IR-A and IR-B (reviewed in (9)). The isoforms differ by the presence (IR-B) or absence (IR-A) of exon 11 (12 amino acids) and they are generated via differential splicing (10). Differences regarding their expression patterns (11), receptor activation, binding of insulin and IGF-I and -II (9) and differences in proliferative and metabolic signaling have been observed (12). Briefly, both IR

isoforms predominantly bind insulin but in addition, IR-A has a higher affinity for IGF-I and -II as compared to IR-B and an aberrant expression in tumorous tissues has been reported. IR-B is predominantly involved in metabolic signaling.

The binding of insulin induces a conformational change of the receptor leading to autophosphorylation of at least 7 tyrosine residues in the membrane spanning β-subunit of the IR (13). These residues are located in the juxtamembrane (JM) domain (Tyr 953, Tyr 960, corresponding to human IR amino acid sequence) as well as the tyrosine kinase activation loop (A-loop) (Tyr 1146, Tyr 1151, Tyr 1152) and the C-terminal domain (Tyr 1316, Tyr 1322). Autophosphorylation in the A-loop activates the kinase activity of the IR (14) and facilitates the binding of docking molecules containing a src homology 2 (SH2)-domain (see below). Phospho-tyrosine residues in the JM domain (located in *NPXpY* motifs) have distinct functions: the motif *NPEpY$_{960}$* enables the binding and IR mediated phosphorylation of docking proteins containing a phosphotyrosine binding (PTB) domain, thus initiating signal transduction (15) (Figure 1). Furthermore the JM domain contains the motif for insulin stimulated IR internalization (16). The C-terminal phospho-tyrosines are involved in regulation of IR kinase activity (17).

The IR is expressed not only in the classical insulin target tissues liver, skeletal muscle and adipose tissue but also in the brain, vasculature and endocrine pancreas. The importance of the IR expression in these tissues for glucose homeostasis has been demonstrated with tissue specific IR-knock out mice (reviewed in (18;19)). Other tissues that express the IR in low concentrations are lungs, kidney, heart, spleen, monocytes and erythrocytes (20;21).

1.1.3 Insulin signal transduction

The activated IR recruits a number of docking proteins which act as scaffolds directing the insulin signal into different signaling pathways, most importantly the phosphatidylinositol-3 (PI-3) kinase and the mitogen activated protein (MAP) kinase pathway and the activation of the insulin dependent glucose transporter (GLUT) 4 translocation in muscle and adipose tissue (22) (Figure 1). This signal diversification is accomplished by IR-mediated phosphorylation of tyrosine residues of these docking proteins, which are then bound by other signaling proteins via SH2-domains, a conserved region with a high affinity for phosphorylated tyrosine residues. Depending on the motif in which the phosphorylated tyrosine is located (23), proteins with different classes of SH2 domains can bind to the docking proteins.

Several adapter proteins are substrates of the activated IR (Figure 1): Src homology s/α-collagen-related protein (Shc) (24) and Grb associated binder 1 (Gab1) (25) both of which

direct the insulin signal into the mitogenic MAP kinase pathway; Gab1 can also bind p85, the regulatory subunit of the PI-3 kinase. Another adapter is SH2-B which has been known as a substrate of the IR for long (26) but only recently the function of SH2-B1 as endogenous insulin sensitizer (27) and regulator of metabolism, body weight (28), longevity and oxidative stress (29) has been described. The IR also phosphorylates the adapter protein with a PH and SH2 domain (APS, also known as SH2-B2) (30) and this protein might be involved in negative regulation of the insulin signal (31), but has also been shown to promote mitogenic signaling (32). APS forms a complex with Casitas b-lineage lymphoma (Cbl) and Cbl associated protein (CAP) (33) and the IR-dependent phosphorylation of Cbl leads to the recruitment of this complex to lipid rafts (34). This eventually activates the small G-protein TC10 which provides the second signal for the translocation of GLUT4 vesicles and may function in parallel with the activation of the PI-3 kinase pathway, which is the other main effector of GLUT4 translocation (35). All the mentioned adapter proteins, except Shc, bind the IR via their SH2-domains.

The most important substrates of the activated IR are the insulin receptor substrates (IRS) 1 – 6. Like Shc they bind to phospho-Tyr 960 of the IR via a PTB domain (36) (refer also to section 1.2.1). The IR-induced tyrosine phosphorylation of the IRS proteins creates multiple binding site for different downstream signaling proteins. Many of the phospho-tyrosine residues in IRS proteins are bound by the regulatory p85 subunit of the PI-3 kinase (Figure 4) leading to the activation of the kinase (37). They also associate with the small docking protein growth receptor bound 2 (Grb2) thus activating the MAP kinase pathway (38). Other adaptor proteins, like Nck and Crk have been shown to interact with IRS proteins as well (39-41). Furthermore enzymes, such as the src like kinase Fyn and the tyrosine phosphatase SHP-2 are activated (42;43). SHP-2 is not only important for deactivation of the insulin signal by dephosphorylation of IRS-1 (44) but also acts as another scaffolding protein associating with Gab1 (45) and acting as a positive effector of insulin-activated MAP kinase pathway and mitogenesis (46-49) (Figure 1).

The binding of so many different effector molecules and the activation of various signaling pathways is a unique feature of the IRS proteins which enables the broad spectrum of effects that insulin has on metabolism, proliferation and survival.
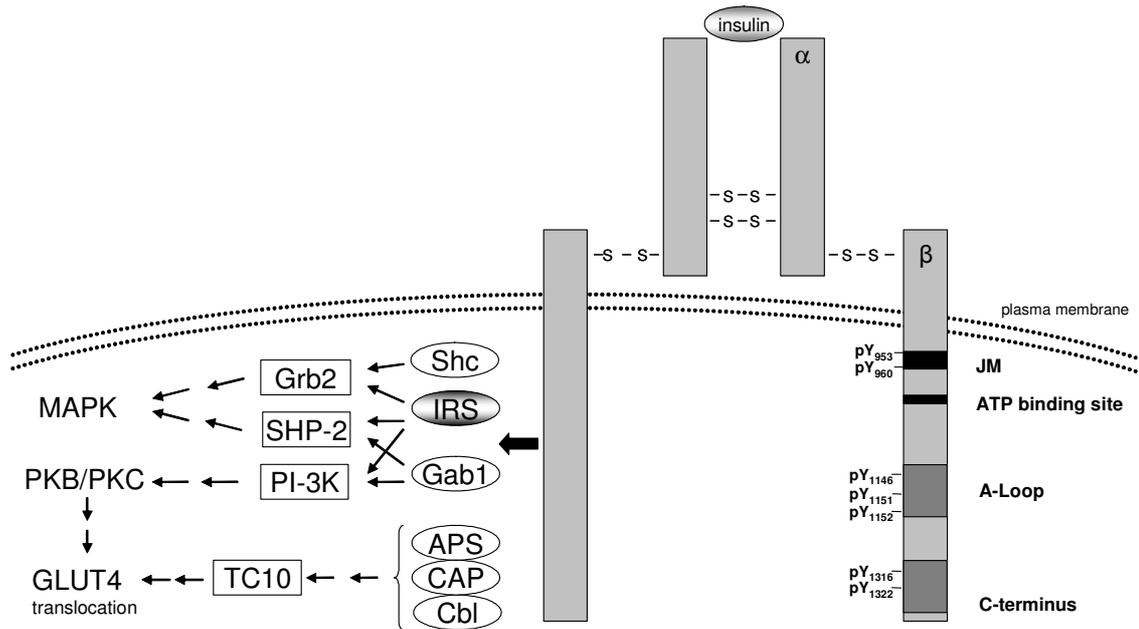
Figure 1 Structure and binding partners of the activated human insulin receptor (IR). Upon insulin binding the IR undergoes autophosphorylation thus creating docking sites in the juxtamembrane domain (JM) for a number of different proteins, which become phosphorylated by the IR. The phosphorylated substrates of the IR can bind different downstream signaling proteins which enables the activation of the PI-3 kinase- and MAP kinase pathway as well as initiation of GLUT4 translocation. Abbreviations: A-loop, activation loop; APS, adapter protein with a PH and SH2 domain; CAP, Cbl associated protein; Cbl, Casitas b-lineage lymphoma; Gab-1, Grb associated binder-1; GLUT4, glucose transporter 4; Grb2, growth receptor bound 2; JM, juxtamembrane domain; MAPK, mitogen activated protein kinase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; pY, phosphotyrosine residues; Shc, Src homology s/α-collagen-related protein; SHP-2, Src homology phosphatase-2; -S-S, disulfide bonds.

1.1.4 PI-3 kinase pathway and PKB/Akt dependent signaling

Binding of p85 to IRS proteins directs the catalytical p110 subunit of the PI-3 kinase class IA towards the plasma membrane where it phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) thus leading to the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Recently it has been shown that mostly p110α, not p110β is responsible for the insulin dependent generation of PIP3 (50). This second messenger is needed to recruit the kinases PDK1 and PKB/Akt via their PH domains towards the membrane where PDK1 phosphorylates PKB/Akt on threonine 308 (51). Another kinase, PDK2, which has been identified as mammalian target of rapamycin complex (mTORC) 2 (52) phosphorylates PKB/Akt in its hydrophobic motif on serine 473 leading to its full activation. Of note, mTORC2

consists of the kinase mammalian target of rapamycin (mTOR), Sin1, GβL and rictor and is insensitive to rapamycin. After activation PKB/Akt detaches from the membrane and phosphorylates a number of downstream kinases and transcription factors (Figure 2).

PKB/Akt phosphorylates glycogen synthase kinase (GSK)-3, which enables the activation of glycogen synthase (GS), the key enzyme responsible for the formation of glycogen. Simultaneously glycogenolysis is stopped by the action of PKB/Akt, which reduces the activity of glycogen phosphorylase (53).

PKB/Akt also phosphorylates the forkhead box (Fox) family of transcription factors, with the most prominent members FoxO1 and FoxA2 (54). These transcription factors are regulated in a complex manner with insulin dependent and independent phosphorylations and acetylations (55;56). FoxO1 for instance is phosphorylated by PKB/Akt on Thr 24, Ser 256 and Ser 319 (57) and this enables the binding of 14-3-3 proteins, which mask the nuclear import sequence and this leads to its exclusion from the nucleus (58). Other Fox proteins are regulated similarly. The Fox proteins are important transcriptions factors of the gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) in the liver and genes involved in hepatic fatty acid oxidation and ketogenesis (59;60). Another member of the FoxO family, the proapoptotic FoxO3a is phosphorylated and regulated by PKB/Akt in a similar manner as FoxO1 (61). PKB/Akt also inactivates other proapoptotic signaling proteins such as Bad (62) and Bax (63), thereby regulating survival signals and mediating the antiapoptotic effects of insulin (Figure 2).

Another target of PKB/Akt is the tuberous sclerosis complex 1/2 (TSC1/TSC2) which acts as GTPase activating protein (GAP) towards the small G-Protein Rheb leading to the transition from the active GTP-bound state to the inactive GDP-bound state (64). The phosphorylation of TSC1/TSC2 inhibits its function and leads to the accumulation of Rheb GTP and this activates by yet undefined mechanisms the rapamycin sensitive mTORC1, a nutrient sensitive complex (65) that contains the mTOR kinase and the proteins GβL and raptor. Active mTORC1 phosphorylates the p70 S6 kinase and the transcription factor 4E-BP1 (66), which is the prerequisite for the initiation of protein synthesis (Figure 2). A recent study showed, that mTORC1 is also involved in the insulin dependent induction of sterol regulatory element binding protein (SREBP) -1c (67), a transcription factor responsible for the regulation of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) -1. The induction of SREBP-1c is apparently also dependent on the insulin-induced generation of PIP3 by the PI-3 kinase as this second messenger activates atypical protein kinase C (PKC) isoforms which are important for the induction of this transcription factor (68). PKB/Akt therefore regulates a broad range of metabolic and antiapoptotic functions (Figure 2).
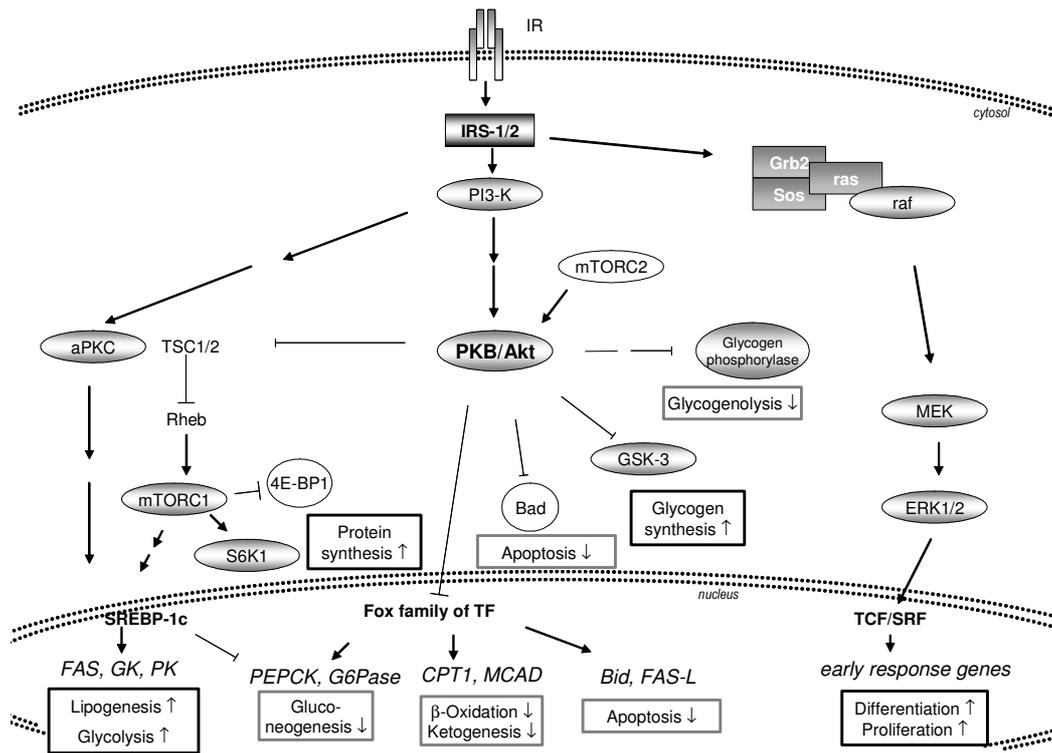
Figure 2 Insulin signaling via insulin receptor substrates (IRS). Abbreviations: aPKC, atypical protein kinase C; CPT1, carnitine palmitoyltransferase 1; ERK, extracellular signal regulated protein kinase; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; Grb2, growth receptor bound 2; GSK-3, glycogen synthase kinase-3; GK, glucokinase; IRS, insulin receptor substrate; MEK, MAPkinase-ERK kinase; MCAD, medium-chain acyl-CoA dehydrogenase; mTORC, mammalian target of rapamycin complex; PEPCK, phosphoenolpyruvate carboxykinase; PI3-K, phosphatidylinositol-3 kinase; PK, pyuvate kinase; PKB, protein kinase B; Sos, son of sevenless; SRE, serum response factor; SREBP-1c, sterol regulatory element binding protein 1c; TCF, ternary complex factor; TF, transcription factors; TSC, tuberous sclerosis complex; ↑, upregulation; ↓, downregulation.

1.1.5 MAP kinase pathway

Insulin controls via the PI-3 kinase/PKB pathway a broad range of metabolic functions. However, it also has a role in differentiation and proliferation as the MAP kinase pathway is activated via the binding of the small adaptor protein Grb2 to the IRS proteins and Shc. Grb2 associates with the ras nucleotide exchange factor mSos (son of sevenless) via its SH3 domains and thereby recruits it to the plasma membrane where it promotes the activation of a small G-protein p21ras, which binds and activates the serine/threonine kinase raf. A phosphorylation cascade from raf to the dual specific kinase MAP kinase-ERK kinase (MEK)1 to extracellular signal-regulated protein kinase (ERK)1/2 leads to the translocation of ERK1/2 into the nucleus. Here ERK1/2 phosphorylates different transcription factors and induces

thereby several early response genes, such as FBJ osteosarcoma oncogene (c-fos) and activating transcription factor (ATF)3 (69) (Figure 2). Alternatively the activated IR binds and phosphorylates Gab1 which associates with SHP2 and also leads to activation of MAP kinase signaling (70).

1.2   IRS proteins

IRS proteins are important mediators of the insulin signaling, despite their lack of enzymatic activity. They are central for the transduction, amplification, modulation and termination of the insulin signal. As described in the previous sections IRS proteins function as branching point for different signaling pathways by binding to various downstream signaling proteins, such as the PI-3 kinase, Grb2 and SHP-2. So far, there are six different isoforms identified with different tissue specific distributions (reviewed in (71). At least five of them are expressed in human tissues: IRS-1, -2, -4, -5 and -6, whereas IRS-3 only appears in rodent adipose tissue and brain. The most prominent IRS isoforms are IRS-1 and IRS-2, which are highly conserved proteins in human, mouse and rat. Their functional importance has been demonstrated with different knock out mouse models (72). Studies with a global knock out of IRS-1 demonstrated that this IRS protein is mainly involved in insulin-like growth factor (IGF)-1 signaling. IRS-1 knock out mice show growth retardation but only mild insulin resistance which never progresses to diabetes (73;74). Abe et al. demonstrated that IRS-1 knock out mice have a metabolic syndrome-like phenotype with insulin resistance in fat and muscle tissue, increased blood pressure and elevated plasma triglycerides as well as impaired endothelial vascular relaxation (75). All groups also reported a compensatory up-regulation of IRS-2, which maintains an almost normal PI-3 kinase activity in the liver (76;77). Importantly, the restoration of hepatic IRS-1 expression via adenoviral infection is sufficient to normalize insulin sensitivity in the IRS-1 knock out mice (78).

In contrast, the general IRS-2 knock out mouse is characterized by reduced β-cell mass and insulin resistance in liver and muscle without compensatory up-regulation of IRS-1, leading ultimately to type 2 diabetes mellitus (79;80). Male IRS-2 knock out mice die from dehydration and hyperosmolar coma after 12 to 16 weeks of life (79). Transgenic islets expressing IRS-2 in the IRS-2 knock out mice cured diabetes, demonstrating an essential function of IRS-2 for normal β-cell function (81). Hence, based on the studies mentioned above IRS-1 and IRS-2 are not only alternative substrates for the IR (77) but are differentially regulated and exert different functions.

For the liver there is currently some controversy whether IRS-1 or IRS-2 is the dominant mediator of hepatic insulin action. Studies with complete IRS-1 or IRS-2 knock out mice indicated a prominent role of IRS-2 in the liver (82-86) but studies with liver specific knock down and knock out models showed that IRS-1 and IRS-2 have rather complementary functions than one or the other being the dominant isoform (87-89). Recent data imply that IRS-2 is important during fasting and early postprandial insulin signaling (90;91) and that it has a function in the regulation of lipid metabolism since the liver-specific knock down of IRS-2 with shRNA resulted in hepatic steatosis (88).

1.2.1 Protein structure of IRS-1 and -2

Both IRS isoforms appear to have a similar general architecture (71;92;93) (Figure 3). They are composed of a N-terminal pleckstrin homology (PH) domain which enables their interaction with phospholipids in the plasma membrane. The PH-domain is followed by a PTB-domain that mediates the interaction with the tyrosine phosphorylated IR (92;93). These two domains are highly conserved by 75% homology among IRS-1 and IRS-2 (94) and they could be crystallized for IRS-1 in 1999 (93). In contrast, the C-terminal part of the IRS proteins is poorly conserved with only 35% of matching amino acids (95). A crystallization of this apparently unstructured region was not achieved up to now.

The C-terminal part contains a large number of tyrosine phosphorylation motifs. In their phosphorylated state these sites represent binding motifs for many downstream signaling proteins (Figure 4), such as adaptor proteins involved in ras signaling (Grb-2 (24), Nck (39), Crk (41)), the SH2 domain-containing protein-tyrosine phosphatase (SHP)-2, which is involved in attenuation of the insulin signal by dephosphorylation of IRS-1 (44) as well as the lipid phosphatase SHIP2, the suppressor of ras signaling ras-GAP and the p85 regulatory subunit of the lipid kinase PI-3 kinase (96) which is of great importance for the metabolic actions of insulin. Only IRS-2 has a unique kinase regulatory loop binding domain (KRLB), ranging from amino acid residue 591 to 733 (Figure 3). This domain interacts with the tyrosine phosphorylated kinase activation loop of the IR (97-99) and it appears that its function is to limit IRS-2 tyrosine phosphorylation hereby regulating the extent of IRS-2 activation (100).

mouse IRS-1
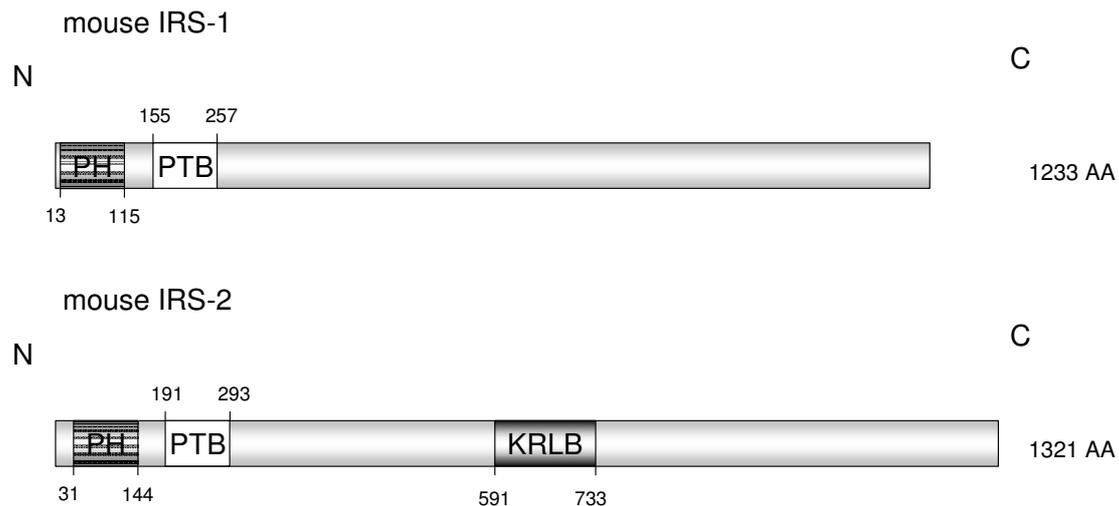
mouse IRS-2

Figure 3 Insulin receptor substrate (IRS) protein structure. Schematic diagram of IRS-1 and IRS-2 domain structure. Amino acid numbering corresponding to mouse IRS-1/2. Abbreviations: C, C-terminus; KRLB, kinase regulatory loop binding domain; N, N-terminus; PH, pleckstrin homology domain; PTB; phosphotyrosine binding domain.

Despite their common features IRS-1 and -2 show a number of differences which renders them as point of diversification of the insulin signal. This is accomplished via several mechanisms: both molecules appear in different subcellular compartments (101) with unequal frequency, IRS-2 being higher concentrated in the cytosol than in other intracellular compartments (102). The IRS proteins also show different activation kinetics (103;104), probably due to structural differences such as the KRLB domain. Recently, it was found that the phosphotyrosine residues of IRS-1 and -2 bind different interaction partners. IRS-2 binds exclusively PLCγ, the adaptor protein Shc and the tyrosine kinase Fyn (105) whereas only IRS-1 binds the kinase Csk (105). Of note, despite the common interaction partners of IRS-1 and -2 both proteins have different numbers of binding sites for each partner. IRS-1 has eight p85 binding sites, whereas IRS-2 has up to eleven and IRS-1 can bind up to five SH2-containing inositol phosphatase (SHIP)2 molecules while IRS-2 has only two potential binding sites (105). These differences between both IRS proteins are likely to enable their distinct roles in insulin and IGF-1 signaling.
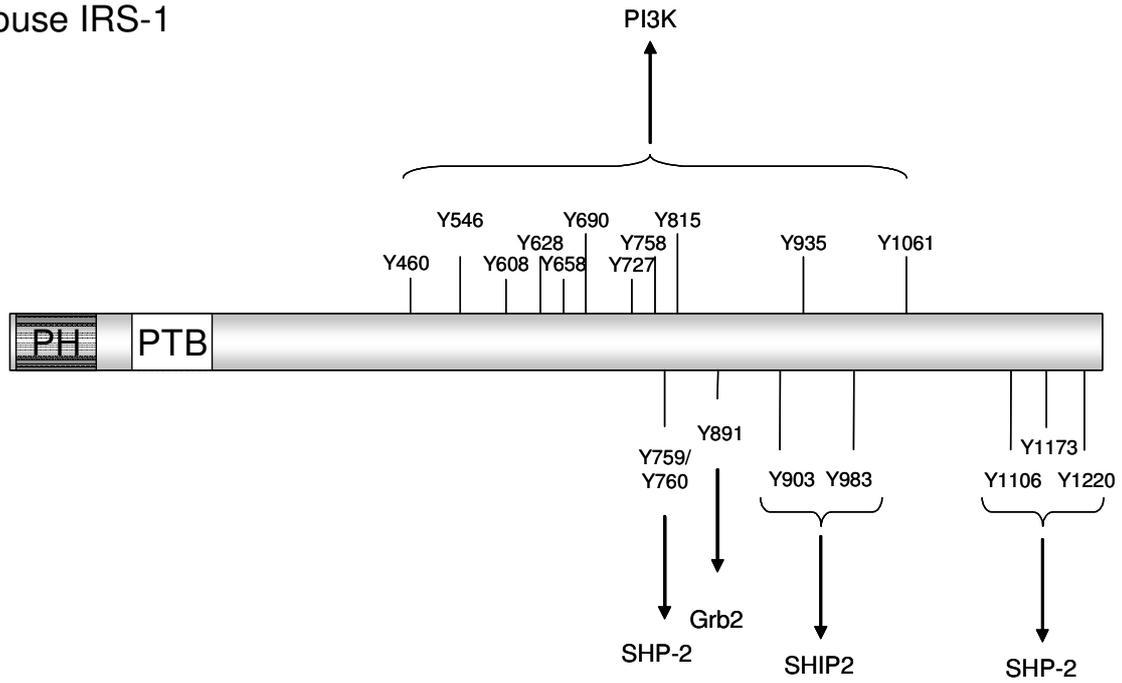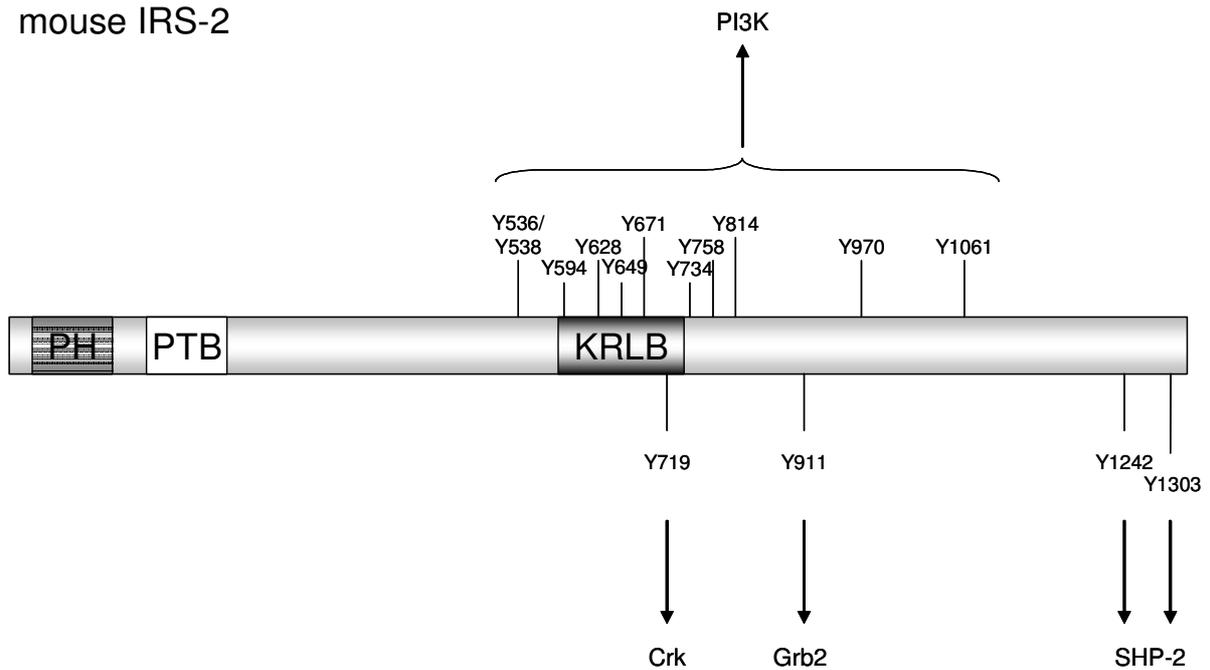
Figure 4 IRS-1 and IRS-2 phospho-tyrosine residues and binding partners. Abbreviations: Grb2, Growth receptor bound 2; PI3K, phosphoinositide-3 kinase; SHP-2, SH2 domain containing protein tyrosine phosphatase 2.

1.2.2 Regulation of IRS proteins

The regulation of both IRS-1 and IRS-2 isoforms occurs on different levels: gene expression, degradation, change of subcellular localization, and posttranslational modification such as tyrosine phosphorylation and serine/threonine phosphorylation, as well as S-nitrosation, O-linked β-N-acetylglucosamine-modification and acetylation. Although IRS-proteins are expressed in several tissues the focus of the following sections will be hold on IRS regulation in the liver because the phosphorylation of hepatic IRS-2 and its functional consequences were investigated in this thesis.

1.2.2.1   Expression

The major regulation of IRS-1 action in the liver appears not to be on the transcriptional level, however, some information is available: a) short term insulin stimulation of rat hepatoma cells is reported to result in an upregulation of IRS-1 protein (106) and refeeding increases the hepatic IRS-1 levels in mice (106), b) stimulation with dexamethasone increases the amount of IRS-1 in Fao rat hepatoma cells (107) and also in the liver of rats (108), c) short term insulin injection in humans resulted in increased muscular IRS-1 levels (106). Contrary to that, fasting had only minimally increasing effects on the hepatic IRS-1 amount (109).

Unlike IRS-1, IRS-2 is highly regulated at the transcriptional level: fasting strongly induces hepatic IRS-2 mRNA (90;110) and protein level (111). IRS-2 protein was 3.5-fold increased after a 16 hour-fast in C57Bl6 wildtype (wt) mice (112). Furthermore signaling molecules known to be activated during fasting induce IRS-2 mRNA and protein expression in different cell culture models. IRS-2 expression is induced by cAMP and the glucocorticoid dexamethasone in HeLa cells (113) and in Fao rat hepatoma cells (L. Fritsche, unpublished data). Dexamethasone alone induces IRS-2 protein in adult rat hepatocytes (114) and primary rat adipocytes (115) and it leads to an increased IRS-2 promotor activity as determined in a luciferase reporter assay (110). The group of Montminy could demonstrate that forskolin-induced increase of cAMP induces IRS-2 expression via activation of cAMP response element binding protein (CREBP) in murine β-cells (116) and Canettieri et al. showed that IRS-2 expression in the liver is stimulated by the CREBP-Torc2 (transducer of regulated CREBP activity 2) pathway (111). Glucagon, the hormone which controls the hepatic glucose metabolism during fasting has been shown to induce IRS-2 mRNA expression in primary rat hepatocytes (83). This induction of IRS-2 during fasting appears critical for glucose homeostasis and serves as a feedback response that limits glucose output from the liver.

Studies investigating the promoter region of the IRS-2 gene revealed several response elements. It contains an insulin response element (IRE) (117) which is recognized by forkhead transcription factors like FoxO1 (110). Guo and co-workers showed that FoxO1 and IRS-2 are regulated reciprocally, with FoxO1 increasing the expression of IRS-2, while insulin signaling via IRS-2 results in FoxO1 down-regulation (118). Liver specific FoxO1 knockout mice display a 50% decrease of IRS-2 gene expression (119) and the expression of a constitutively nuclear FoxO1 in mouse liver induces IRS-2 significantly (120). Transcription factor E3 (TFE3), a leucine zipper–containing basic helix loop helix protein, has also been show to promote IRS-2 transcription (121). Interestingly the IRS-2 promotor also contains a region which binds SREBP (named sterol regulatory element, SRE), which partially overlaps with the IRE. SREBP is induced by insulin and it negatively regulates the expression of IRS-2, by replacing FoxO1 from the promotor (110). Furthermore, ERK has been shown to induce IRS-2 expression upon oxidative stress in HepG2 cells via the transcription factors NF1 and SP1 (122).

1.2.2.2   Degradation

Targeted degradation has been verified as a regulator of IRS-1 protein levels: *in vitro* studies demonstrated that long term (up to 24 h) insulin stimulation of cultured Fao hepatoma cells results in proteasomal degradation of the IRS-1 protein without a change of IRS-1 mRNA levels (123-126). Other agents such as tumor necrosis factor (TNF)α, interferon (INF)γ, platelet derived growth factor (PDGF) and phorbol esters also reduce the IRS-1 protein levels in different cell culture models (127;128). Suppressor of cytokine signaling (SOCS)-1 and -3 are reported to bind via their SH2 domains to IRS-1 and promote its ubiquitination and degradation (129). Since several cytokines are inducers of SOCS expression this provides a mechanism for the interaction of cytokines with the insulin signaling pathway. Insulin-induced degradation of IRS proteins is thought to be mainly dependent on mTOR. Serine phosphorylation of IRS-1 via the mTOR-p70 S6K1 pathway is reported to release IRS-1 from intracellular complexes thereby enabling its degradation. The nutrient sensitive mTOR mediates the phosphorylation of Ser 636/639 in human IRS-1 (corresponding to Ser 632/635 in rodent IRS-1) in muscle and adipose tissue (130;131) and this site is hyperphoshorylated in the liver during prolonged stimulation with insulin (132), palmitate (133) and in diet-induced obesity (134). Rapamycin, the widely used mTOR inhibitor, prevents insulin-induced IRS-1 degradation in CHO cells (135) and 3T3-L1 adipocytes (125;136), stressing the importance of this kinase for the degradation of IRS-1. The mTOR-activated p70 S6K1 has recently been shown to be essential for the cullin 7 (CUL7) E3 ligase dependent ubiquitination of IRS-1 *in vitro* (137).

For IRS-2 similar degradation mechanisms are described. High insulin concentrations which emerge during the fed state lead to reduced IRS-2 concentrations in the liver (83). This is accomplished through inhibition of transcription (117) and subsequent downregulation of IRS-2 mRNA (123) and increased proteasomal degradation of the IRS-2 protein. Rui and colleagues demonstrated that insulin mediates the ubiquitination and subsequent proteasomal degradation of IRS-2 via the PI-3 kinase – PKB/Akt – mTOR pathway (138). The role of insulin as a suppressor of IRS-2 protein levels is eminent in liver insulin receptor knock out (LIRKO) mice. The complete lack of insulin signaling in the liver of these mice results in 5-fold increased IRS-2 but not IRS-1 protein concentrations (139). It has been speculated that the fast degradation of IRS-2 protein in the liver after onset of refeeding (90) is the prerequisite to shift the insulin signaling towards IRS-1, which is suspected to be dominant for postprandial insulin signaling (91). In this context, the degradation of IRS-2 seems to be essential for the physiological regulation of hepatic nutrient homeostasis.

However, the proteasomal degradation of IRS-2 is also induced under pathophysiological conditions, such as insulin resistance. SOCS-1 and -3, negative regulators of cytokine signaling, are not only reported to promote ubiquitination and degradation of IRS-1, but also of IRS-2 in mice (129). SOCS-1 and -3 can inhibit IRS-2 function in an additional manner. Both SOCS isoforms can bind to different regions in the IR and inhibit thereby the interaction and activation of the IRS proteins (140). Furthermore, SOCS-1 has been also implicated as negative regulator of IRS-2 expression (141): SOCS-1 knock out mice are reported to have increased IRS-2 protein levels and increased tyrosine phosphorylation of IRS-2 and subsequently enhanced PKB/Akt activation.

In conclusion, accelerated degradation of IRS proteins stimulated by hyperinsulinemia and hyperlipidemia as it occurs in obesity is one of the mechanisms discussed in the development of insulin resistance (142).

1.2.2.3   Posttranslational modification

1.2.2.3.1 Phosphorylation

Apart from degradation and expression, the IRS-proteins are extensively regulated via multiple reversible posttranslational modifications, most importantly phosphorylations. The amino acid sequence of IRS-1 and -2 provides a multitude of tyrosine, serine and threonine residues as potential phosphorylation sites. The phosphotyrosine interactome of both IRS isoforms has recently been analyzed demonstrating at least 21 (IRS-1) and 16 (IRS-2) functional tyrosine

residues that are mandatory for the transduction of the insulin signal (105) (Figure 4). In addition, even more potential and verified serine/threonine phosphorylation sites exist: With Netphos2.0 (143) 124 potential serine/threonine phosphorylation sites for IRS-1 can be calculated and up to now more than 20 of these sites have been studied in great detail. For IRS-2 a similar number of potential serine/threonine phosphorylation sites is expected, however, only 2 sites have been studied so far.

The vast amount of possible posttranslational modifications of the IRS is a unique feature of these proteins and the significance of such modifications, especially the serine/threonine phosphorylations of IRS-1 are thoroughly studied. In general, the function of serine/threonine phosphorylations is to modulate and precisely regulate the insulin (and IGF) signal in a positive and negative fashion (144;145), with many phospho-sites involved in the termination of the signal. Furthermore, other extra- and intracellular stimuli are integrated into the insulin signal via the phosphorylation of IRS proteins and the pathological hyperphosphorylation of certain serine/threonine residues is implicated in the development of insulin resistance and type II diabetes (142).

The mechanisms involved in the regulation of insulin signal transduction by serine/threonine phosphorylation are the association of IRS-1 with other signaling molecules (146;147), the subcellular localization of IRS-1 (102) and regulation of its degradation (136). The sites implicated in a negative regulation (corresponding to rat IRS-1 sequence) are Ser 24 (148), Ser 267 (149), Ser 270 (150), Ser 307 (151;152), Ser 332 (153), Ser 357 (154), Ser 522 (155), Ser 612 (156-158), Ser 632/635 (159-161), Ser 662 (157;158), and Ser 1099/1100 (162).

The IRS-1 kinases responsible for phosphorylation of IRS-1 serine residues and for attenuation of insulin signaling are the nutrient sensitive kinase mTOR (131;163-165) (166) and its downstream kinase p70 ribosomal S6 kinase (p70 S6K) 1 (150;161). The mTOR/p70 S6K pathway is over-activated in states emerging from overnutrition characterized by hyperglycemia, hyperinsulinemia and increased concentrations of free fatty acids (FFA) (133;134;167).

Other important serine/threonine kinases like c-jun-N-terminal kinase (JNK) (151), protein kinase C (PKC)-θ (168;169), PKC-δ (154), inhibitor of κB (IκB) kinase (148;170), glycogen synthase kinase (GSK)-3 (153) and MAP kinases (160) also phosphorylate IRS-1. These kinases help to turn off the insulin signal when activated under physiological conditions but when stimulated permanently they are implicated in the pathogenesis of insulin resistance. Chronically elevated concentration of insulin, FFA (171;172) and TNFα (151;173) are all reported to activate the above mentioned kinases resulting in decreased hepatic insulin sensitivity and glucose tolerance.

One of the most intensely studied sites is Ser 307 (Ser 312 in the human IRS-1 homolog). This site is responsible for the PI-3 kinase dependent downregulation of the insulin signal

because it interferes with the IRS-1-IR interaction (174). There are many kinases that phosphorylate Ser 307, with JNK being the first one reported (151). The introduction of a dominant negative JNK isoform into the liver of obese diabetic mice led to an improved insulin sensitivity and also to a decreased hepatic glucose output due to decreased expression of gluconeogenic enzymes (144;175;176). But in contrast to the cell culture based results the transgenic mouse expressing a non-phosphorylatable IRS-1 307 Ala mutation was not protected from high fat diet-induced insulin resistance and developed, compared to the control animals, even a more pronounced insulin resistant phenotype (177). Thus, it is necessary to verify which of the phosphorylated serine residues of IRS-1 in the insulin resistant state are markers of chronic activation of serine/threonine kinases and which are causally involved in the impairment of insulin action.
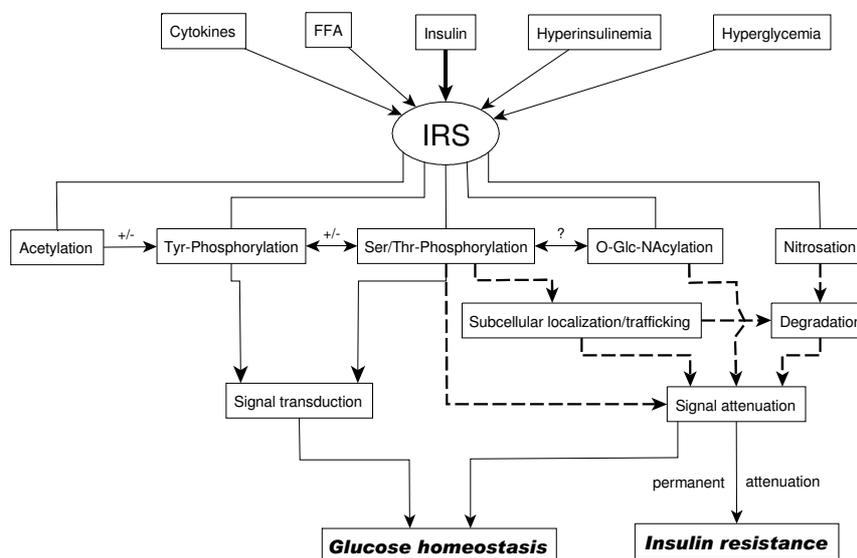
Figure 5 Posttranslational modification of IRS proteins and their interplay. IRS-proteins are extensively posttranslationally modified. Insulin induces tyrosine phosphorylation, which is the prerequisite for further signal transduction and serine/threonine phosphorylation, leading to both activation and attenuation of the signaling. Other stimuli like acutely and chronically elevated cytokines, free fatty acids, glucose and insulin not only induce serine/threonine phosphorylation but also acetylation, *O*-Glc-NAcylation and nitrosation. The roles of these modifications are less clear than of the phosphorylations, but they have been implicates in positive as well as negative regulation of signal transduction. Bold lines represent the activation of the system, dashed lines indicate an attenuation. Abbreviations: IRS, insulin receptor substrates; FFA, free fatty acids.

Other serine phosphorylation sites of IRS-1 are involved in positive regulation of the insulin signal transduction. The phosphorylation of Ser 302 (178;179), Ser 318 (180), Ser 325 (181), Ser 629 of human IRS-1 (182), Ser 789 (183) and Ser 1216 (168) has been associated with improved insulin signaling (Figure 5), although the data are not consistent presumably due to the stimulus, kinetics and the cell type. Phosphorylation on one residue can also have different effects depending on the time course of insulin action. The early phosphorylation of Ser

318 is involved in enhanced insulin action, but phospho-Ser 318 is also necessary for the attenuation in the late phase (184). It has been hypothesized that the net result of serine phosphorylations of IRS-1 depends on the time course and the interdependency of phosphorylated serine sites rather than the phosphorylation of single residues (185).

Surprisingly little is known about the regulation of IRS-2 by serine/threonine phosphorylations To date only two sites are described as possible targets of JNK (186;187). Based on *in vitro* studies Solinas and colleagues proposed that Thr 348 in IRS-2 is a functional homolog to Ser 307 in IRS-1, which is involved in negative regulation of the insulin signal (186). Another group could show that JNK phosphorylates IRS-2 on Ser 488 and that this is a prerequisite for the GSK-3β-dependent phosphorylation of Ser 484. This sequential phosphorylation led to an inhibition of the insulin signal in hepatocytes and it could be speculated that it contributes to the development of insulin resistance (187).

Interestingly there appears to be some substrate specificity of serine kinases to IRS proteins: PKC-ζ phosphorylates IRS-1, -3 and -4 but not IRS-2 in *in vitro* kinase assays (188).

1.2.2.3.2 Other posttranslational modifications

Beside phosphorylations several other posttranslational modifications are present in IRS proteins.

The posttranslational modification on serine/threonine residues of IRS-1 with *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) has been demonstrated in muscle and adipose tissue (189). This modification, first identified in the 1980s in rat liver subcellular organelles (190;191), is enhanced by the increased activity of the hexosamine biosynthetic pathway, which generates UDP N-acetylglucosamine, the substrate for the addition of *O*-GlcNAc-moieties by *O*-GlcNAc-transferase (192). Hyperglycemia and hyperlipidemia have been shown to increase the flux through this pathway (193), thus *O*-GlcNAc-modification is enhanced during insulin resistant states and has also been related to impaired insulin action (194;195). In some proteins *O*-GlcNAc-modification occurs on the same sites as phosphorylations thereby inhibiting the proper phosphorylation. Ball et al. showed that in IRS-1 Ser 1036 is the major site of *O*-GlcNAc-modification and under conditions that model the diabetic state (high glucose, chronic insulin stimulation) the level of *O*-GlcNAc-modification was increased in human embryonic kidney (HEK293) cells at this site (196). A number of serine residues (Ser 984, Ser 985, Ser 1011 in human IRS-1) which are located adjacent to functional tyrosine residues can be modified with *O*-GlcNAc and it has been speculated that this affects the interaction of IRS-1 with

SH2-domain containing proteins (197). However, the effect of the *O*-GlcNAc-modification on the interaction of IRS-1 with downstream signaling partners needs further clarification.

For IRS-2 only little information is available regarding the *O*-GlcNAc-modification. One study could demonstrate the existence of this modification in IRS-2 derived from skeletal muscle of glucosamine plus insulin infused rats (195).

A further posttranslational modification of IRS-1 described in muscle tissue is the S-nitrosation via nitric oxide which has been implicated in the down-regulation of insulin action (198;199). It appears that this modification induces the proteasomal down-regulation of IRS-1 in cultured skeletal muscle cells (200) as well as in rat liver (201). The relevance of this modification for IRS-proteins needs to be clarified further.

Furthermore, IRS-1 can also be modified by acetylation, i.e. the transfer of an acetyl group to a lysine residue. This modification is found on histones and it is important for chromatin regulation (202) but it is known that the histone acetyltransferases (HATs) and histone deacetylases (HDACs) also have non-histone substrates (203), among them many proteins involved in the pathogenesis of diabetes (204). It was demonstrated that IRS-1 is acetylated on lysine residues and this modification led to improved insulin signaling, whereas the activity of a specific deacetylase (HDAC2) was associated with insulin resistance (205). In contrast, the acetylation of IRS-2 seems to have opposing effects on insulin signaling: a study of Zhang demonstrated that IRS-2 is acetylated at the basal state and for sufficient insulin signal transduction the removal of acetyl residues by SirT1 protein deacetylase is needed (206). Acetylation of IRS-2 was furthermore shown in a cell free system (207).

1.3   Insulin resistance and type II diabetes mellitus

Type II diabetes mellitus, insulin resistance and the metabolic syndrome are diseases with epidemic proportions. For 2010 worldwide 285 million patients will be affected with diabetes (208). The prevalence of diabetes in the United States is estimated with 12.3 %, in Europe 8.6 %. Germany has a higher prevalence than the average of european countries with 12% (208). Leading causes are overnutrition, obesity and a sedentary lifestyle, which is common in westernized civilizations.

Type II diabetes is preceded by the development of insulin resistance. This is a metabolic disorder of multifactorial genesis defined as the inability of insulin to exert its effects on target tissues. Rare monogenetic forms of diabetes have been described (209) and genome-wide association studies are conducted to identify genes responsible for the development of im-

paired insulin secretion, insulin resistance and diabetes (210). But in general the development of diabetes is believed to be caused by complex genetic and epigenetic factors to be defined yet (211). Impaired cellular insulin action is found on the level of IR and IRS-proteins: reduced expression/increased degradation and impaired function due to pathologically increased post-translational modifications, which have been described in detail in section 1.2.2.3.

Insulin resistance leads to transient hyperglycemia due to unsuppressed hepatic gluconeo-genesis and impaired glucose uptake into muscle and adipose tissue. Simultaneously, lipoly-sis is not inhibited and therefore free fatty acids (FFA) are released from the adipose tissue into the circulation. Paradoxically, the hepatic activation of SREBP-1c, although being con-trolled by insulin, is increased in the insulin resistant state, resulting in enhanced lipogenesis. This is called selective insulin resistance (212). To compensate the impaired insulin re-sponse, the β-cells secrete more insulin, causing hyperinsulinemia and this hypersecretion could eventually lead to β-cell failure. Untreated, insulin resistance is a *circulus vitiosus* be-cause hyperinsulinemia, hyperglycemia and increased FFA concentrations results in glucoli-potoxicity which not only impairs insulin signaling in all insulin responsive tissues but which also promotes β-cell apoptosis, thereby exacerbating insulin resistance further. The enlarged fat mass, which occurs during obesity, contributes to insulin resistance due to secretion of FFA (213;214) and proinflammatory cytokines (adipokines), e. g. TNFα (215) and IL-6 (216;217).

Insulin resistance can lead to type II diabetes mellitus which is characterized by a combina-tion of impaired or even absent insulin secretion, insulin resistance, increased fasting plasma glucose and impaired glucose tolerance. Patients often need oral antidiabetic medication or have to administer exogenous insulin. A badly controlled diabetes is the major cause for macroangiopathy, retinopathy, nephropathy and neuropathy.

Since dysregulated posttranslational modifications of IRS proteins are a hallmark in the de-velopment of insulin resistance and diabetes, the study of these is expected to deepen the understanding of the development insulin resistance. The final goal in the future would be to identify pharmacological agents that modulate certain phosphorylations to overcome insulin resistance.

1.4   Aims of the thesis

As outlined in the previous sections, IRS-2 has been recognized as a crucial mediator of he-patic and pancreatic insulin signaling and complementary rather than interchangeable func-

tions with IRS-1 are described. The study of posttranslational modifications, particularly of serine/threonine phosphorylations, is needed to deepen the understanding of IRS-2 regulation.

In vitro kinase assays with PKC isoforms and subsequent mass spectroscopic analysis were conducted in the Division of Pathobiochemistry and Clinical Chemistry of the Department of Internal Medicine, University of Tübingen to identify phosphorylated serine/threonine residues of IRS-2. Based on these data, together with the bioinformatical evaluation (143;218;219) of the IRS-2 amino acid sequence 20 potential serine/threonine phosphorylation sites were selected and phospho-site specific monoclonal antibodies were generated in cooperation with Dr. E. Kremmer, Helmholtz Zentrum München, Germany.

The first aim of this thesis was to screen these monoclonal antibodies for their specificity and sensitivity to detect site specific serine/threonine phosphorylations of IRS-2. The antibodies were generated against peptides of 10 amino acids surrounding the potential phosphorylation site, exemplarily shown in Figure 6. Antibodies for 5 out of 20 potential phospho-sites were positive in the initial screening and the corresponding clones chosen and propagated. Two previously unknown phosphorylation sites, Ser 675 and Ser 907, where chosen for further analysis, since both serine residues are located in close proximity to two functional tyrosine residues: Ser 675 lies within the IRS-2 specific kinase regulatory loop binding (KRLB) domain and is adjacent to a PI-3 kinase binding motif (**pY$_{671}$MPM**), Ser 907 is adjacent to the Grb2 binding domain of IRS-2 (**pY$_{911}$INI**) (100;220). In a further testing the antibodies were evaluated for their phospho-site specificity using alanine mutants, for their specificity towards IRS-2 compared to IRS-1 and recognition of IRS-2 from different species.

The second part of the thesis was aimed to identify the kinases responsible for phosphorylating Ser 675 and Ser 907 and to study the function and biological relevance of these IRS-2 phosphorylation sites. Finally, the IRS-2 serine phosphorylations were investigated *in vivo* in mice.

^{670} DYMPM**S**PTSVS ^{680}

^{902} PTEPK**S**PGEYI ^{912}

Figure 6 Phosphopeptides (corresponding to mouse IRS-2 amino acid sequence) used for the generation of phospho-site specific monoclonal antibodies. The bold and underlined serine indicates the phosphorylated residue.

2 Materials

2.1 Chemicals

12-O-tetradecanoylphorbol 13-acetate (TPA)	Merck, Darmstadt, Germany
Acetic acid, 100%, waterfree	Sigma, München, Germany
Acrylamid 30 (37.5:1)	Roth, Karlsruhe, Germany
Agarose, peqgold Universal	Peqlab, Erlangen, Germany
Ammonium persulfate (APS)	Sigma, München, Germany
Anisomycin	Sigma, München, Germany
BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid)	Sigma, München, Germany
Biorad Protein Assay	Biorad, München, Germany
β-Glycerophosphate	Sigma, München, Germany
β-Mercaptoethanol	Sigma, München, Germany
Bromophenolblue	Sigma, München, Germany
BSA (bovine serum albumin)	Roche, Mannheim, Germany
$CaCl_2$	Sigma, München, Germany
Cycloheximide	Sigma, München, Germany
Developer	Agfa Healthcare GmbH, Berlin, Germany
Complete	Roche, Mannheim, Germany
DMSO (dimethylsulfoxide)	Roth, Karlsruhe, Germany
EDTA	Sigma, München, Germany
EGTA	Sigma, München, Germany
Ethanol p.A.	Merck, Darmstadt, Germany
Ethidium bromide	Sigma, München, Germany
Gelatine	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Roth, Karlsruhe, Germany
H_2O, HPLC-grade	Merck, Darmstadt, Germany
HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid)	Roth, Karlsruhe, Germany
H_2O_2	Merck, Darmstadt, Germany
HCl	AppliChem, Darmstadt, Germany
Insulin, human recombinant	Sigma, München, Germany

Ketamin-ratiopharm injectable solution	Ratiopharm GmbH, Ulm, Germany
KCl	Sigma, München, Germany
Lactacystin	Calbiochem, Schwabach, Germany
Lipofectamine 2000	Invitrogen, Karlsruhe, Germany
Luminol (3-Aminophthalhydrazide)	Sigma, München, Germany
LY294002	Sigma, München, Germany
NaCl	Merck, Darmstadt, Germany
NaF	Sigma, München, Germany
$Na_4P_2O_7$	Sigma, München, Germany
Na-Pyrophosphate	Sigma, München, Germany
Na-Orthovanadate	Sigma, München, Germany
Methanol Normapur	VWR, Darmstadt, Germany
$MgCl_2$	Sigma, München, Germany
$MgSO_4$	Sigma, München, Germany
PEG 3350	Sigma, München, Germany
PD98059	Calbiochem, Schwabach, Germany
p-Iodophenole	Sigma, München, Germany
Ponceau S-Solution	AppliChem, Darmstadt, Germany
Propidium iodide	Sigma, München, Germany
Protein-A-Sepharose (suspension)	GE Healthcare, München, Germany
Rapamycin	Sigma, München, Germany
Rapid fixer	Agfa Healthcare GmbH, Berlin, Germany
Ribonuclease A	Serva Electrophoresis, Heidelberg, Germany
SDS (sodium dodecyl sulfate)	Biorad, München, Germany
Sepharose G-50 Superfine	GE Healthcare, München, Germany
Sequencing Buffer 5X	Applied Biosystems, Foster City, CA, USA
SP600125	Sigma, München, Germany
SuRE/Cut Buffer B for restriciton enzymes	Roche, Mannheim, Germany
TE-buffer, sterile, pH 8.0	Sigma, München, Germany
TEMED (*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine)	Roth, Karlsruhe, Germany
TNFα, human recombinant	R&D Systems, Wiesbaden-Nordenstadt, Germany
Triton-X-100	Sigma, München, Germany
TRIS, Ultra Pure (for ECL)	MP Biomedicals Inc., Solon, OH, USA
Trizma base (TRIS)	Sigma, München, Germany
Wortmannin	Calbiochem, Schwabach, Germany

2.2   Buffers and solutions

All buffers and solutions were prepared with aqua$_{dest}$.

Cell lysis buffer (pH 7.5) (store at 4°C)	HEPES	50 mM
	NaCl	150 mM
	$MgCl_2$	1.5 mM
	EGTA	1 mM
	glycerol	10%
	Triton-X-100	1%
	NaF	100 mM
	$Na_4P_2O_7$	10 mM

Shortly before use the cell lysis buffer was mixed with 10 X phosphatase inhibitors (see below).

Tissue lysis buffer (pH 7.6) (store at 4°C)	TRIS	50 mM
	NaCl	150 mM
	Triton-X-100	1%

Shortly before use the tissue lysis buffer was mixed with 10 X phosphatase inhibitors (see below) and 25 X Complete protease inhibitor.

Cell lysis buffer for λPP treatment (store at 4°C)	TRIS	50 mM
	NaCl	150 mM
	Triton-X-100	1%
	glycerol	10%

HNTG-buffer (pH 7.5) (store at 4°C)	HEPES	20 mM
	NaF	10 mM
	NaCl	150 mM
	Triton-X-100	0.1%
	glycerol	10%

10 X Phosphatase inhibitors (store at -20 °C)	NaF	10 mM
	Na-pyrophosphate	5 mM
	Na-orthovanadate	10 mM
	β-glycerophosphate	10 mM

Bradford assay solution

To obtain the working solution of the Biorad Protein Assay the solution was diluted 1:5 with water and filtered through filter papers. The solution was kept at room temperature, protected from light and it was stable for 1 week.

5 X Laemmli sample buffer (pH 6.8) (store at 4 °C)	TRIS	60 mM
	glycerol	25%
	SDS	2%
	β-mercaptoethanol	5%
	bromophenolblue	0,1%

Stacking gel buffer (pH 6.8) (store at room temperature)	TRIS	0.5 M
	SDS	2%

Separation gel buffer (pH 8.8) (store at room temperature)	TRIS	1.5 M
	SDS	2%

10 X Electrophoresis buffer (store at 4 °C)	TRIS	250 mM
	glycine	2 M
	SDS	1%

10 X Blotting buffer (store at room temperature)	TRIS	480 mM
	glycine	390 mM
	SDS	0.4%

1 X Blotting buffer (store at room temperature)	10 X Blotting buffer	10%
	methanol	20%
	H_2O	70%

Stripping buffer (pH 6.8) (store at room temperature)	TRIS	66 mM
	β-mercaptoethanol	0.5%
	SDS	2%

10 X NET-G (store at 4°C)	gelatine	2.5%
	NaCl	1.5 M
	EDTA	50 mM
	TRIS	500 mM
	Triton-X-100	0.5%

10 X NET-G was diluted 1:10 with water and the pH was adjusted to 7.4 using 37% HCl to obtain 1 X NET-G.

Enhanced chemiluminiscence (ECL) solutions

Solution A (pH 9.35) (store at 4°C)	TRIS, Ultra Pure	0.1 M
	luminol	4.4 mM (in DMSO)
	p-jodophenol	4.4 mM (in DMSO)

Solution B (pH 9.35) (store at 4°C)	TRIS, Ultra Pure	0.1 M
	H_2O_2 (30%)	0.4%

Shortly before use solution A and B were mixed 1:1.

50 X TAE buffer (pH 8) (store at room temperature)	TRIS	1.25 M
	acetic acid	625 mM
	EDTA	50 mM

10 X Sample buffer for DNA (store at 4°C)	bromophenolblue	0.1%
	xylenecyanole	0.1%
	glycerol	60%
	50 X TAE	20%

5 X KCM (store at -20°C)	KCl	0.5 M
	$CaCl_2$	0.09 M
	$MgCl_2$	0.25 M

TSB (freshly prepared)	LB-medium	50%
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	PEG 3350	10%
	H_2O	40%

| Cryomedium for cells (freshly prepared) | FCS | 90% |
| | DMSO | 10% |

2 X BBS (pH 6.96) (store at -20 °C)	BES	50 mM
	NaCl	280 mM
	Na_2HPO_4	1.5 mM

2.3   Gels

Stacking gel (11.08 mL)	H_2O	7.05 mL
	stacking gel buffer	2.55 mL
	acrylamid 30 (37.5:1)	1.35 mL
	TEMED	15 µL
	APS (10%)	112.5 µL

Separation gel 7.5% (40.4 mL)	H_2O	20 mL
	separation gel buffer	10 mL
	acrylamid 30 (37.5:1)	10 mL
	TEMED	66 µL
	APS (10%)	270 µL

Separation gel 5% (40.4 mL)	H_2O	23.4 mL
	separation gel buffer	10 mL
	acrylamid 30 (37.5:1)	6.66 mL
	TEMED	66 µL
	APS (10%)	270 µL

Gradient gel 5 -->15%

The gradient gel was made by pouring the gel into the gelchamber while mixing the heavy (15%) and the light (5%) gel in a gradient maker.

Separation gel 5% (18.5 mL)	H_2O	10.5 mL
	separation gel buffer	5 mL
	acrylamid 30 (37.5:1)	2.8 mL
	TEMED	33 µL
	APS (10%)	135 µL

Separation gel 15% (18.5 mL)	H_2O	3.1 mL
	separation gel buffer	5 mL
	glycerol	2 mL
	acrylamid 30 (37.5:1)	8.1 mL
	TEMED	33 µL
	APS (10%)	135 µL

Agarose gel (1%, 2%)

Agarose gels were prepared by dissolving agarose in 1 X TAE buffer to obtain 1% or 2% gels.

2.4   Culture media and supplements

RPMI 1640 with 2 g/L glucose	Lonza, Basel, Switzerland
DMEM with 1 g/L glucose	Lonza, Basel, Switzerland
DMEM with 4.5 g/L glucose	Lonza, Basel, Switzerland
Hepatocyte maintenance medium	Provitro, Berlin, Germany
OptiMEM	Invitrogen, Karlsruhe, Germany
L-Glutamine	Lonza, Basel, Switzerland
Sodium-pyruvate	Lonza, Basel, Switzerland
Non essential amino acids	Lonza, Basel, Switzerland
Penicillin/ Streptomycin	Lonza, Basel, Switzerland
G418 BC Sulfate	Biochrom AG, Berlin, Germany
Fetal Calf Serum (FCS)	Invitrogen, Karlsruhe, Germany
DPBS	Lonza, Basel, Switzerland

Trypsin/EDTA	Lonza, Basel, Switzerland
Collagen CSP	Pentapharm, Basel, Switzerland
Luria Broth, Millers LB broth	Sigma, München, Germany
LB Agar	Sigma, München, Germany
Ampicillin	Sigma, München, Germany

2.5   Kits

Big Dye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, CA, USA
QIA Prep Spin Miniprep kit	Qiagen, Hilden, Germany
Qiagen Plasmid Maxi Kit	Qiagen, Hilden, Germany
QIA Quick PCR Purification	Qiagen, Hilden, Germany
MinElute PCR Purification Kit	Qiagen, Hilden, Germany
QuickChange XL Site Directed Mutagenesis Kit	Stratagene, La Jolla, CA, USA
Immobilon Western HRP Substrate Luminol Reagent	Millipore, Schwalbach, Germany

2.6   Enzymes and molecular markers

Hind III (10 U/μL)	Roche, Mannheim, Germany
Xba I (10 U/μL)	Roche, Mannheim, Germany
SDS-PAGE molecular weight standard, high range	Biorad, München, Germany
Precision Plus Protein standard	Biorad, München, Germany
Quickload 1 kb DNA ladder	New England BioLabs, Beverly MA, USA

2.7   Consumables

Gel blotting paper	VWR, Darmstadt, Germany
Nitrocellulose Transfer Membrane, BA 85, 0.45 µm	VWR, Darmstadt, Germany

Amersham Hyperfilm ECL	GE Healthcare, München, Germany
Filterpapers MN 615 ¼	Machery-Nagel GmbH Co KG Germany
Transparency film	Lyreco, Impega, Barsinghausen, Germany
Costar 150 mm TC-treated culture dish	Corning B.V. Life Sciences, Amsterdam, Netherlands
Costar TC-treated 6-well plates	Corning B.V. Life Sciences, Amsterdam, Netherlands
Tissue Culture Dishes 87 mm and 137 mm	TPP, Trasadingen, Switzerland
Tissue Cluture Test Plates (6-well, 12-well)	TPP, Trasadingen, Switzerland
Cryocups	Greiner Bio-One GmbH, Frickenhausen, Germany
96-well ELISA Microplate,PS, flat bottom	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell Scraper	Corning B.V. Life Sciences, Amsterdam, Netherlands
Centri Sep Spin Columns	Applied Biosystems, Foster City, CA, USA
4 mm cuvettes for electroporation	Peqlab, Erlangen, Germany
Dounce homogenizer for 2 mL	Sartorius, Göttingen, Germany
Plunger for dounce homogenizer, size S	Sartorius, Göttingen, Germany
Inoculation loop	Sarstedt, Nümbrecht, Germany
Drigalski applicator	Neolab, Heidelberg, Germany

2.8   Laboratory equipment

Electrophoresis chamber for SDS-PAGE	Selfmade
Semi dry blotting chamber	Hölzel, Wörth, Germany
Table shaker	Hecht Assistent, Sondheim, Germany
Shaker incubator	Edmund Bühler GmbH, Hechingen, Germany
Heating block Thermostat Plus	Eppendorf, Hamburg, Germany
Magnetic stirrer IKAMAG RCT	IKA Labortechnik, Staufen, Germany
Hera Safe Hood	Thermo Fisher Scientific, Schwerte, Germany
Precision scale ALJI60-4NM	Gottl. Kern & Sohn GmbH, Balingen, Germany
Laboratory balance BL1500	Sartorius, Göttingen, Germany
Waterbath	Memmert, Schwabach, Germany
Incubator for cells Cytoperm	Heraeus, Hanau, Germany

Incubator for bacteria	Heraeus, Hanau, Germany
ELISA reader Model 680	Biorad, München, Germany
Biophotometer	Eppendorf, Hamburg, Germany
Vortex Genie 2	Scientific industries, USA
Microscope Axiovert 40	Zeiss, Oberkochen, Germany
Neubauer chamber	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Centrifuge Heraeus Pico 17	Thermo Fisher Scientific, Schwerte, Germany
Centrifuge Biofuge Fresco	Heraeus, Hanau, Germany
Centrifuge Hettich Rotana RPC	Andreas Hettich GmbH & Co KG, Tuttlingen, Germany
Powersupply Consort E 802	Consort nv, Turnhout, Belgium
Agarosegel chamber Midi	Harnischmacher Labortechnik, Kassel, Germany
UV-Transilluminator 254 nm	LTF Labortechnik gmbH & Co KG, Wasserburg, Germany
Camera Powershot A710IS	Canon, Krefeld, Germany
Printer Selphy CP510	Canon, Krefeld, Germany
Developer machine Agfa Curix 60	Agfa Healthcare GmbH, Berlin, Germany
Gradient maker	Pharmacia LKB Biotechnology AB, Bromma, Sweden
Lightcycler 480 system	Roche, Mannheim, Germany
Thermocycler Progene	Techne, Dexford-Cambridge, UK
Thermocycler Mastercycler 5330	Eppendorf, Hamburg, Germany
Autoclave Systec DX-65	Systec, Wettenberg, Germany
Gene Pulser II with capacitance extender	Biorad, München, Germany
Rotator	Grünewald GmbH & Co. KG PSI Medizintechnik, Laudenbach, Germany
HP scanjet 4670	Hewlett-Packard GmbH, Berlin, Germany

2.9   Software

Gelscan Professional V5.1	BioSciTec GmbH, Frankfurt, Germany
SPSS for Windows V15.0.1	SPSS GmbH Software, München, Germany
Lightcycler 480 Software V1.5.0.39	Roche, Mannheim, Germany
Netphos2	http://www.cbs.dtu.dk/services/NetPhos/
Scansite	http://scansite.mit.edu/
Human Protein Reference Database	http://www.hprd.org/PhosphoMotif_finder

2.10 Primers and siRNA oligonucleotides

2.10.1 Primers for real time PCR

rat β -actin   sense   AGC CAT GTA CGT AGC CAT CC
                                 antisense   CTC TCA GCT GTG GTG GTG AA
                                                TIB Molbiol, Berlin, Germany
rat IRS-2   Rn_IRS2_1_SG   QuantiTect Primer assay Qiagen, Hilden,
                                                Germany

2.10.2 Primers for PCR-mutagenesis

All primers were from Invitrogen, Karlsruhe, Germany.

Mutated triplets are presented bold and underlined.

Ser 675 --> Ala 675

sense   5' CAA GAG CGA TGA CTA CAT GCC CAT **GGC** CCC CAC AAG CGT GTC TGC TC 3'

antisense 5' GA GCA GAC ACG CTT GTG GGG **GCC** ATG GGC ATG TAG TCA TCG CTC TTG 3'

Ser 675 --> Glu 675

sense   5' C AAG AGC GAT GAC TAC ATG CCC ATG **GAA** CCC ACA AGC GTG TCT GCT C 3'

antisense 5' G AGC AGA CAC GCT TGT GGG **TTC** CAT GGG CAT GTA GTC ATC GCT CTT G 3'

Ser 907 --> Ala 907

sense   5' CCT CTA CCC ACA GAG CCC AAG **GCC** CCT GGC GAG TAC ATC AAC ATT GAC 3'

antisense 5' GTC AAT GTT GAT GTA CTC GCC AGG **GGC** CTT GGG CTC TGT GGG TAG AGG 3'

Ser 907 --> Glu 907

sense   5' CCT CTA CCC ACA GAG CCC AAG **GAA** CCT GGC GAG TAC ATC AAC ATT GAC 3'

antisense 5' GTC AAT GTT GAT GTA CTC GCC AGG **TTC** CTT GGG CTC TGT GGG TAG AGG 3'

2.10.3 Sequencing primers

For sequencing the following primers were used. Primers were obtained from TIB Molbiol, Berlin, Germany.

Position 675: 5' CTT ACC CAG AGG ACT ATG GAG 3'

Position 907: 5' GCC TTC CTC CAT GCG GCC GAG 3'

2.10.4 siRNA oligonucleotides

All siRNA oligonucleotides were designed, synthesized and annealed at Dharmacon Research (Lafayette, CO, USA).

Gene	target sequences
rat mTOR/FRAP1 (NM_019906)	CAA GAA UGG UGC CGA AAG U
	GCG GAU GGC UCC UGA CUA U
	GAA GAA GAC CCU UUG AUU U
	GGC AUA UGG UCG AGA UUU A
rat RPS6KB1 (NM_031985)	GAA CAG UCA CGC ACA CAU UUU
	CGG AGA ACA UCA UGC UUA AUU
	CCG AUC GCC UCG AAG AUU UUU
	GCG CCU GAC UUC CGA CAC AUU
rat MAPK3 (ERK1) (NM_017347)	CAA CCA CAU UCU AGG UAU A
	UAC AGU CUC UGC CCU CUA A
	CCC AAG AGG ACC UAA AUU G
	CAU GAA UUC CCU AAA CUA C
rat MAPK1 (ERK2) (NM_053842)	ACA CUA AUC UCU CGU ACA U
	AAA AUA AGG UGC CGU GGA A
	UAU ACC AAG UCC AUU GAU A
	UCG AGU UGC UAU CAA GAA A

2.11 Antibodies

2.11.1 Primary antibodies

Antibody	Dilution	Manufacturer
Grb2 for IP	-	Abcam, Cambrigde, UK
IRS-1	1:500	Millipore, Schwalbach, Germany
IRS-2 (also for IP)	1:1000	Millipore, Schwalbach, Germany
phospho-IRS-2 S-675 Clone 5E4	1:20	Dr. E. Kremmer, Helmholtz center, München, Germany
phospho-IRS-2 S-907 Clone 9B12	1:20	Dr. E. Kremmer, Helmholtz center, München, Germany
phospho-IRS-1 S-1101	1:1000	Cell Signaling Technology, Frankfurt, Germany
Akt/PKB	1:1000	BD Transduction laboratories Erembodegem, Belgium
phospho Akt/PKB T-308	1:1000	Cell Signaling Technology, Frankfurt, Germany
phospho Akt/PKB S-473	1:1000	Cell Signaling Technology, Frankfurt, Germany
β-actin	1:1000	Cell Signaling Technology, Frankfurt, Germany
GSK-3β	1:1000	Cell Signaling Technology, Frankfurt, Germany
phospho-GSK-3α/β S-9/ 21	1:1000	Cell Signaling Technology, Frankfurt, Germany
Insulin receptor β	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
IGF-1 receptor	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
JNK	1:1000	BD Transduction laboratories Erembodegem, Belgium
phospho-JNK	1:1000	Cell Signaling Technology, Frankfurt, Germany
mTOR/FRAP1	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
p44/42 (ERK1/2)	1:1000	Cell Signaling Technology, Frankfurt, Germany

phospho-p44/42 (ERK1/2)	1:1000	Cell Signaling Technology, Frankfurt, Germany
p85	1:1000	Millipore, Schwalbach, Germany
PKC δ	1:1000	BD Transduction laboratories, Erembodegem, Belgium
p70 S6K	1:1000	Cell Signaling Technology, Frankfurt, Germany
phospho-p70 S6K T-389	1:1000	Cell Signaling Technology, Frankfurt, Germany
phospho-tyrosine	1:1000	Cell Signaling Technology, Frankfurt, Germany
Ubiquitin	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

2.11.2 Secondary antibodies

Antibody	Dilution	Manufacturer
goat anti mouse IgG-HRP	1:3000 to 1:10,000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
goat anti rabbit IgG-HRP	1:3000 to 1:10,000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
goat anti rat HRP	1:1500	Dianova, Hamburg, Germany

2.12 Plasmids

Plasmid pRK5 IRS-2 DNA (mouse)	lab internal tool
Plasmid pRK5 IRS-1 DNA (rat)	lab internal tool
Plasmid pRK5 IRS-2 DNA (human)	Prof. Calum Sutherland, Ninewells Hospital, Dundee, Scotland
Plasmid pRK5	lab interal tool
Plasmid pRK5 insulin receptor B (human)	lab internal tool

2.13 Cells, bacterial strains and animals

2.13.1 Cells

Fao rat hepatoma cells   ECACC, Salisbury, UK

Baby hamster kidney (BHK) cells   ECACC, Salisbury, UK

Human embryonic kidney (HEK293) cells   ATCC, Wesel, Germany

Human hepatoma (Huh-7)   Riken Cell Bank, Tsukuba, Japan

Primary human hepatocytes   Dr. Martin Schenk, University Hospital Tübingen, Germany

2.13.2 Bacteria Strains

Competent E. coli - XL1 Blue for transformation (store at -80 °C)   Novagen, Madison, USA

2.13.3 Animals

Male C57/Bl6 mice, age 4 weeks   Charles River Laboratories, Sulzfeld, Germany

3   Methods

3.1   Cell culture

3.1.1   Cultivation, passaging and seeding for experiments

All cells were maintained in an incubator at a temperature of 37℃ and in an atmosphere of 95% humidity and 5% CO_2. Every work step with cells was carried out under sterile conditions using a cell culture bench (sterile hood). To dissociate cells from the cell culture dish (diameter 150 mm), cells were washed once with 10 mL DPBS and treated with 5 mL trypsin EDTA for 5 to 10 minutes in the cell incubator. The trypsination was stopped with 10 mL growth medium containing 10% FCS. The cells were then centrifuged at 100 X g for 4 minutes at room temperature and the supernatant was removed. The cell pellet was resuspended in 1 mL growth medium and the volume was increased to 10 mL with growth medium. The cell count was determined using a Neubauer chamber.

Fao cells were grown in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin. Confluent cells were passaged weekly and seeded at a density of 10,000 cells per cm^2 for the next passage. For experiments $1x10^6$ cells were seeded in 2 mL growth medium in a 6-well plate and grown for 36 h. The cells were then serum-starved in RPMI 1640 without supplements for 16 h. Immediately before any experiment the starvation medium was changed and the cells were treated with various substances, chemicals, kinase activators and kinase inhibitors as described in the results part.

HEK293 cells, BHK and Huh-7 cells were kept in DMEM with 4.5 g/L glucose supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM non essential amino acids. Confluent cells were passaged weekly and seeded at a density of 1000 cells per cm^2 for the next passage. For transfection both cell lines were seeded to $4x10^6$ cells per well of a 6-well plate in 2 mL growth medium. After 24 h the cells were either transfected transiently or stably (see section transfection) in a growth medium without antibiotics. For experiments cells were serum-starved in DMEM with 1 g/L glucose, supplemented with 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM non essen-

tial amino acids for 16 h. The cells were treated in freshly added starvation medium as described in results.

Primary human hepatocytes were isolated in the group of Dr. M. Schenk. Briefly, the cells were isolated from liver tissue obtained from therapeutic hepatectomies using a two-step collagenase perfusion protocol as described in (221). The cells were plated onto collagen CSP, cultured in Hepatocyte Maintenance Medium and stimulated in serum-free DMEM with 4.5 g/L glucose supplemented with 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM non essential amino acids.

3.1.2 Cryopreservation of cell lines

To cryopreserve cells confluent 15 cm dishes were trypsinated. After centrifugation the cell pellet was resuspended in 1 mL cryomedium and another 4 mL of cryomedium was added. This suspension was immediately aliquoted into 5 cryocups and frozen at -140°C.

3.1.3 Electroporation of Fao cells with siRNA

Confluent cells were trypsinated and counted as described above. After a second centrifugation step 3.5×10^6 cells were resuspended in 200 µL RPMI 1640 supplemented with 1% FCS and 100 nM siRNA oligonucleotides were added. The mix was transferred into 4 mm cuvettes and the cells were electroporated in a Gene Pulser II with capacitance extender at 290 mV and 450 µF according to the manufacturer's instructions. The electroporated cells were transferred into 2 mL of Fao growth medium without antibiotics. After 24 h medium was changed to normal growth medium. After 36 h cells were serum starved for 16 h and treated in fresh starvation medium as described in the results part.

3.1.4 Transient transfection

3.1.4.1   Lipofection of BHK cells

BHK cells were seeded at a density of 4×10^6 cells per well of a 6-well plate. 24 h after seeding the cells were transfected with either the empty pRK5 vector as control or the plasmids according to the specific experiment. At first, the medium was changed towards transfection medium (HEK293/BHK growth medium without antibiotics). Next, the transfection mix was prepared:

<div align="center">

tube 1: 48 µL OptiMEM + 2 µg DNA (c= 1µg/µL)

tube 2: 46 µL OptiMEM + 4 µL Lipofectamine 2000

</div>

Both tubes were incubated at room temperature for 5 minutes and then combined, mixed by tapping the tube gently and incubated again at room temperature for 20 minutes. The complete transfection mix was transferred onto one well of a 6-well plate.

36 h after transfection cells were serum-starved for 16 h and treated in fresh starvation medium as described in the results part.

3.1.4.2   Calcium phosphate transfection of HEK293 and Huh-7 cells

HEK293 cells were transfected using a standard calcium phosphate transfection protocol (222). The cells were seeded at a density of 4×10^6 cells per well of a 6-well plate. 24 h after seeding the cells were transfected with either the empty pRK5 vector as control or the plasmids according to the specific experiment. After medium change to transfection medium (see 3.1.4.1), the transfection mix was prepared:

<div align="center">

96 µL	0.25 M CaCl$_2$
4 µL	DNA (c= 1 µg/µL)
100 µL	2 X BBS

</div>

The transfection mix was vigorously mixed by tapping the tube, followed by incubation at room temperature for 20 minutes. The complete transfection mix was transferred onto one well of a 6-well plate.

36 h after transfection cells were serum-starved for 16 h and treated in fresh starvation medium as described in the results part.

3.1.5 Generation of HEK293 and Huh-7 cells stably expressing IRS-2 mutants

HEK293 cells were stably transfected to express IRS-2 wildtype (wt) + IR or the IRS-2 675 Ala mutant + IR or the IRS-2 907 Ala mutant + IR. Huh-7 cells were stably transfected to express the IRS-2 wt only. To achieve resistance against the selecting agent G418 (geneticin) the cells were also co-transfected with a plasmid coding for the neomycin resistance gene, pSVneo.

HEK293 and Huh-7 cells were seeded at a density of 4×10^6 cells per well of a 6-well plate. 24 h after seeding the medium was exchanged towards transfection medium and the following transfection mixes were prepared and pipetted onto the cells:

HEK293 Control

96 μL	0.25 M $CaCl_2$
3.2 μL	pRK5 (c=1 μg/μL)
0.8 μL	pSV neo (c=1μg/μL)
100 μL	2 X BBS

HEK 293 IRS-2 wt + IR

96 μL	0.25 M $CaCl_2$
1.6 μL	IRS-2 wt (c=1μg/μL)
1.6 μL	IR (c=1μg/μL)
0.8 μL	pSV neo (c=1μg/μL)
100 μL	2 X BBS

HEK293 IRS-2 675 Ala + IR

96 μL	0.25 M $CaCl_2$
1.6 μL	IRS-2 675 Ala (c=1μg/μL)
1.6 μL	IR (c=1μg/μL)
0.8 μL	pSV neo (c=1μg/μL)
100 μL	2 X BBS

HEK293 IRS-2 907 Ala + IR

96 μL	0.25 M $CaCl_2$
1.6 μL	IRS-2 907 Ala (c=1μg/μL)
1.6 μL	IR (c=1μg/μL)
0.8 μL	pSV neo (c=1μg/μL)
100 μL	2 X BBS

Huh-7 IRS-2

96 µL	0.25 M CaCl$_2$
3.2 µL	IRS-2 wt (c=1 µg/µL)
0.8 µL	pSV neo (c=1µg/µL)
100 µL	2 X BBS

24 h after transfection all cells of one 6-well were seeded onto 12 wells of a 12-well plate in transfection medium. On the next day the selection process was initiated by adding 1 % of G418 to the transfection medium. Over the next 14 days the medium was changed every 2 to 3 days against fresh transfection medium containing 1 % G418. Selected clones were finally transferred into new 6-well plates and propagated in normal growth medium. The stable clones were tested for IRS-2 and IR expression.

3.2   Animal studies

Four-week old male C57Bl/6 wt mice were maintained on a normal 12 hour light/dark cycle and kept on a standard chow diet for 8 weeks. For *in vivo* stimulation, the mice were fasted overnight. After induction of anesthesia with Ketamin (150 mg/kg bodyweight), the abdomen was opened and a bolus of human insulin (2 IU/mouse for 10 minutes) was injected into the vena cava inferior. Controls received a comparable amount of diluent. The mice were then sacrificed by decapitation and the liver was immediately extracted and processed as described below in section 3.3.2.

For fasting experiments, 11-14-week-old wt mice were either fasted over 16 h or had free access to standard lab animal chow. In a fasting/refeeding experiment 13-week old wt mice were fasted over 16 h and afterwards had access to chow for 5 h. The control animals were killed immediately after the fasting period.

3.3   Protein biochemical methods

3.3.1 Cell lysis

After completion of each experiment cells were washed once with cold DPBS and then lysed at 4°C on a table shaker for 15 minutes (BHK, HEK293) or 30 minutes (Fao) with either 175 µL or 300 µL (for immunoprecipitation) cell lysis buffer which contained 1 X phosphatase inhibitors. All following steps were carried out on ice. The lysed cells were collected from the plate using a cell scraper and transferred into 1.5 mL tubes. The lysate was centrifuged at 4°C for 5 minutes at 16,000 X g and the supernatant containing the proteins was pipetted into a new tube. The lysates were mixed with 5 X Laemmli buffer (223) and incubated at 95°C for 5 minutes. The denatured lysates were loaded onto a SDS-PAGE gel.

3.3.2 Liver tissue lysis

After sacrification of mice, the liver was quickly removed and a piece of approximately 50 mg was transferred into a precooled cylinder of a 2 mL dounce homogenizer containing 1 mL of tissue lysis buffer with phosphatase inhibitor and Complete protease inhibitor. The liver tissue was gently homogenized using a plunger (size S) with approximately 10 strokes. The homogenates were transferred into a 1.5 mL tube and incubated on ice for 30 minutes. The homogenates were then clarified by three subsequent centrifugation steps at 4°C for 10 minutes at 16000 X g. The protein concentration of the supernatant was measured and the lysates were immediately used for immunoprecipitation (3.3.4) or stored at -80°C for further analysis.

3.3.3 Protein assay for determination of protein concentration (Bradford assay)

The protein concentration of cell and liver tissue lysates was determined using the standard procedure from Bradford (224). Cell and liver tissue lysates were diluted with HPLC-grade water: cell lysates 1:20 and liver tissue lysates 1:100. A calibration curve was made with BSA (c=1 mg/mL) according to the following table:

Table 1 Calibration curve for Bradford assay

No	BSA (c=mg/mL) (µL)	H$_2$O (µL)	final concentration (mg/mL)
1	0	40	0
2	2	38	0,05
3	4	36	0,1
4	8	32	0,2
5	12	28	0,3
6	16	24	0,4
7	20	20	0,5

10 µL of diluted sample and standards were pipetted directly into one well of a 96-well microplate and 200 µL of diluted Bradford solution was added. The samples and standards were measured in triplicate. After 5 minutes of incubation at room temperature the extinction was measured at 595 nm in an ELISA reader.

3.3.4 Immunoprecipitation (IP)

Co-IP was used to demonstrate the interaction of IRS-2 with Grb2 and p85. Furthermore, IRS-2 was immunoprecipitated to specifically demonstrate the phosphorylation of IRS-2. 150 µg of protein from cell or liver tissue lysates were mixed with 30 µL protein A sepharose and 2 µL of antibody. If necessary, the total volume was adjusted to 400 µL with HTNG-buffer containing phosphatase inhibitor. The (co-) IP mix was continuously mixed for 4 h at 4 °C using a rotating wheel with a rotation of 15 rpm. Afterwards the tubes were centrifuged at 4 °C for 30 seconds at 4000 x g and the pellet was washed with 250 µL HNTG-buffer containing phosphatase inhibitors. Centrifugation and washing was repeated 2 times. The last centrifugation step was performed at 2000 x g for 1 minute at 4 °C and the supernatant was completely removed by aspiration. 25 µL of 5 X Laemmli-buffer were added to the pellet and incubated for 5 minutes at 95 °C. After a quick spin the complete supernatant was loaded onto a SDS-PAGE gel.

3.3.5 SDS-PAGE

The SDS-PAGE technique (223) was used for the separation of proteins according to their size. After assembly of the glass casket, the separation gel was poured until approximately 80% of the casket were filled. The separation gel was then covered with a layer of water. After polymerization the water was decanted and the stacking gel was poured on top of the separation gel and the comb for the formation of the gel pockets was inserted. After polymerization the comb was removed and the glass casket was fixed into the gel electrophoresis chamber. Two separate reservoirs on top and bottom of the gel were filled with 1 X running buffer and the samples were loaded onto the gel. One gel pocket was used for the molecular marker. The electrophoresis was carried out overnight at 50 mV.

3.3.6 Western blotting

The SDS-PAGE gel, the gel blotting paper and the nitrocellulose membrane were soaked with 1 X blotting buffer and the proteins were transferred with 0.8 mA / cm^2 for 2 h onto the nitrocellulose membrane (225) using a semidry blotting system. The membrane was stained with Ponceau S solution to examine the quality of the blot and to mark the standard bands.

3.3.7 Immunodetection

For immunodetection the membranes with the blotted proteins were blocked with 1 X NET-G (3 times for 15 minutes) and subsequently incubated with the specific primary antibodies overnight on a table shaker at 4°C. On the next day the membranes were washed with three changes of 1 X NET-G and then incubated with the secondary antibodies that were coupled to horseraddish peroxidase (HRP) for 45 minutes at room temperature. Finally the membranes were washed again in 1 X NET-G and then transferred into the complete ECL-solution (see 2.2) for 3 minutes. The membranes were placed between two sheets of transparency film and autoradiographs on Hyperfilm ECL films were taken. The films were developed in an Agfa Curix 60 developer machine.

3.3.8 Stripping of membranes

After the detection of phosphorylated proteins with phospho-specific antibodies it was necessary to reprobe the membrane with the corresponding protein antibody. For this purpose the membrane was stripped off the initial antibody by incubating it in stripping buffer at 56 °C (waterbath) for 30 minutes. After the stripping the membranes had to be blocked again with 1 X NET-G.

3.4 Generation of monoclonal phospho-specific antibodies

The generation of phospho-specific antibodies was conducted in the group of Dr. E. Kremmer (Helmholtz Zentrum München, Germany). Phosphopeptides corresponding to amino acid residues 670 - 680 and 902 - 912 (Figure 6) of mouse IRS-2 with the phosphorylation of serine residues Ser 675 and Ser 907 were synthesized (NMI, Reutlingen, Germany) and coupled to bovine serum albumin and ovalbumin by cystein linkage at the N-terminus. The non-phosphorylated peptides were synthesized as controls and coupled to ovalbumin. Lou/c rats were immunized with 50 µg of phosphopeptide, IFA (Freund's adjuvant, incomplete), and CpG 2006 as adjuvant and received a boost with another 50 µg of coupled phosphopeptides and CpG after 6 weeks (Dr. E. Kremmer, Helmholtz Zentrum München, Germany). Three days later, hyperimmune spleen cells were fused with the mouse myeloma cell line P3X63Ag8.653 to generate hybridoma cell lines using standard procedures (226). Supernatants were first screened in a differential ELISA with the phospho- and the corresponding unphosphorylated peptide to select phospho-specific monoclonal antibodies. Bound monoclonal antibodies were detected using a cocktail of biotinylated mouse monoclonal antibodies against rat IgG heavy chains (a-IgG1, a-IgG2a, a-IgG2b and a-IgG2c) thus avoiding detection of monoclonal antibodies of the IgM class. The biotinylated antibodies were visualized with peroxidase-labeled avidin (Alexis, Grünberg, Germany) and o-phenylenediamine as chromogen in the peroxidase reaction. Positive cell lines were frozen and the monoclonal antibodies were tested in a first prescreening step as described in section 4.1.

3.5   Molecular methods

All work was carried out with PCR-grade water.

3.5.1   PCR-mutagenesis and DpnI digestion

PCR-mutagenesis was performed to generate IRS-2 mutants which represent the unphosphorylated (alanine residue) and the constantly phosphorylated state (glutamate residue). The mutagenesis was performed with the QuickChange XL mutagenesis kit and self designed primers (see section 2.10.2). The following PCR mix in a total volume of 25 µL was prepared:

2.5 µL	10 X reaction buffer
5 ng	IRS-2 plasmid
3.6 µL	sense primer (c=1 pmol/L)
3.6 µL	antisense primer (c=1 pmol/L)
0.5 µL	dNTP-mix
1.5 µL	quicksolution
12.7 µL	H_2O
0.5 µL	Pfu ultra polymerase (2.5 U/µL)

The PCR reaction was carried out in the Techne-thermocycler from Progene, which performed the program outlined in Table 2:

Table 2 PCR mutagenesis thermocycler program

Step	Temperature	Time	Iteration
denaturation	95 °C	1 min	1x
denaturation	95 °C	50 sec	
annealing	65 °C	50 sec	18x
elongation	68 °C	10 min	
proofreading	68 °C	7 min	1x
cool	4 °C	hold	

For the elimination of methylated and hemimethylated DNA the following mix was prepared:

20 µL   PCR product

0.5 µL   DpnI (10 U/ µL)

The mix was incubated for 1 hour at 37°C.

As control 5 µL of the undigested PCR product was mixed with 15 µL H_2O. The digested and the undigested PCR products were then purified using the QIA Quick PCR Purification kit according to the manufacturer's instructions. The purified DNA was eluted with 50 µL TE-buffer.

3.5.2 Transformation

Plasmid-DNA was transformed into competent E. coli - XL1 bacteria using the KCM-method. For transformation of purified PCR product the following mix with a total volume of 100 µL was prepared:

50 µL   purified PCR product

20 µL   5 X KCM

30 µL H_2O

For transformation of plasmid DNA for a Maxi preparation 1 µg of DNA was used and the volume of water was adjusted accordingly. This mix was vortexed and incubated on ice for 5 minutes. 100 µL of E. coli bacteria was thawed and the mix was pipetted into the bacteria-tube and vortexed for 3 seconds. The tube was placed on ice for 15 minutes followed by the temperature shock at 42°C (waterbath) for 2 minutes. The mix was then pipetted into 1 mL LB-medium, incubated at 37°C for 30 minutes and subsequently centrifuged for 2 minutes at 2000 x g. The supernatant was discarded and the pellet was resuspended in 50 µL LB medium and plated with a Drigalski applicator onto prewarmed LB-agar petri dishes containing 0.1 mg/mL ampicillin. The petri dishes were incubated at 37°C overnight and formation of single colonies was monitored.

3.5.3 Miniprep/Maxiprep for the isolation of plasmid-DNA from transformed E. coli

Single colonies were picked with an inoculation loop and grown in 2 mL LB-medium with 0.1 mg/mL ampicillin either overnight (Miniprep) or 6 h in 2 mL LB-medium with 0.1 mg/mL ampicillin with subsequent transfer into 100 mL LB-medium with 0.1 mg/mL ampicillin and incubation overnight (Maxiprep) in a shaker incubator at 37°C at 125 rpm. On the following day the Miniprep and Maxiprep was carried out according to the manufacturer's instructions.

3.5.4 Sequencing of plasmid-DNA

The sequencing of plasmid-DNA was carried out using a commercially available kit for sequencing-PCR. The following reaction mix in a total volume of 10 µL was pipetted:

0.5 µL	Miniprep- or Maxiprep-eluate
1 µL	sequencing primer (c=10 pmol/µL)
2 µL	sequencing mix
1 µL	5 X sequencing buffer
5.5 µL	H_2O

The PCR reaction was carried out in the Mastercycler-thermocycler from Eppendorf, which performed the program outlined in Table 3 :

Table 3 Sequencing PCR thermocycler program

Step	Temperature	Time	Iteration
denaturation	96°C	1 min	1x
denaturation	96°C	10 sec	
annealing	50°C	10 sec	24x
elongation	60°C	4 min	
cool	15°C	hold	

The PCR products were subsequently purified. Spin columns were prepared by pipetting 750 µL 5% well swelled sephadex (room temperature) into mini spin columns and centrifuging them at room temperature for 3 minutes at 2400 x g. 10 µL of water were added to the PCR mix and it was transferred onto the spin columns. The columns were centrifuged again at room temperature for 3 minutes at 2400 x g. The eluates were sequenced by the Genotyping Facility Tübingen (University Hospital Tübingen, Department of Internal Medicine IV).

3.5.5 Digestion with restriction enzymes and separation of DNA fragments with agarose gel

To verify that the correct plasmid had been amplified with Miniprep or Maxiprep a control digestion with restriction enzymes was performed. The following reaction mixes with a total volume of 10 µL were pipetted:

8 µL	Miniprep-eluate
0.5 µL	Hind III (10 U/µL)
0.5 µL	Xba I (10 U/µL)
1 µL	SuRE/Cut buffer B

1 µL	original IRS-2 plasmid (c=1µg/µL)
0.5 µL	Hind III (10 U/µL)
0.5 µL	Xba I (10 U/µL)
1 µL	SuRE/Cut buffer B
7 µL	H_2O

The mixes were incubated for 1 hour at 37°C and then mixed with DNA sample buffer and loaded onto a 1% agarose gel. Electrophoresis was conducted for approximately 45 minutes at 100 mV. Figure 7 shows exemplarily the pattern of the digested IRS-2 plasmids.
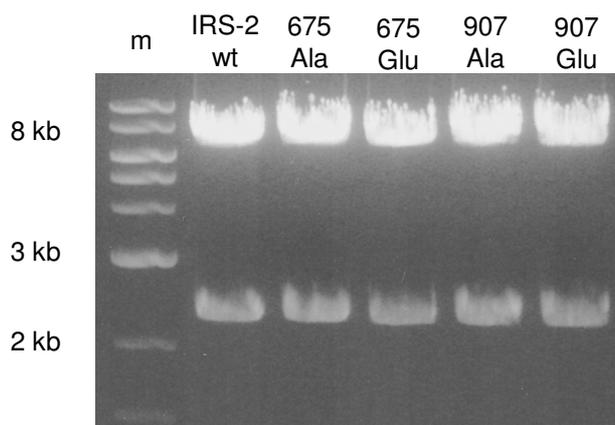
Figure 7 Digestion of IRS-2 wildtype (wt) and IRS-2 mutant plasmids with restriction enzymes HindIII and XbaI.

3.5.6 Generation of competent E. coli

100 μL of original competent E. coli bacteria were incubated in 2 mL LB medium overnight at 37°C in a shaker incubator. 150 mL LB-medium were inoculated with 1 mL of the bacterial suspension and again incubated at 37°C for 2.5 to 3.5 h until the optical density at 600 nm (OD_{600}) was 0.6. The bacteria were centrifuged at 2150 x g for 10 minutes at room temperature and the pellet was resuspended in 15 mL TSB with 5 % DMSO on ice. The suspension was incubated on ice for 10 minutes and then aliquoted and stored at -80°C.

3.5.7 RNA extraction from cultured cells

RNA was extracted using the RNeasy Mini kit according to the manufacturer's instructions. Briefly, cells were lysed in 350 μL RLT-buffer containing 10 μL β-mercaptoethanol per mL. The emerging slurry was transferred into QIA shredder mini spin columns and centrifuged at 17,000 x g for 2 minutes at room temperature. The eluate was mixed with 350 μL 70% ethanol and transferred onto the mini spin columns provided in the RNeasy Mini kit and centrifuged for 15 seconds at 17,000 x g at room temperature. All following washing steps were carried out at these centrifuging conditions. The RNA which adhered to the column was washed once with 350 μL RW1 buffer and then incubated for 20 minutes with RNAse-Free DNAse. Another washing step with 350 μL RW1 buffer was conducted and then the mini spin column was washed twice with 500 μL RPE-buffer. The last centrifugation step was carried

out for 2 minutes. The RNA was finally eluted with 50 µL RNAse-free water and the concentration was measured in the Biophotometer from Eppendorf.

3.5.8 Reverse transcriptase (RT)-reaction

RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit according to the manufacturer's instructions. First, the concentration of RNA was adjusted to 1 µg/µL with water. 11 µL of adjusted RNA were mixed with 2 µL random hexamer primer (600 pmol/µL) and the template-primer mixture was denatured at 65 °C for 10 minutes in the Mastercycler-thermocycler from Eppendorf. Then the following components were added to the mix:

4 µL	5 X reaction buffer
2 µL	dNTP mix (c=10 mM each nucleotide)
0.5 µL	RNase inhibitor (40 U/µL)
0.5 µL	reverse transcritase (20 U/µL)

The program outlined in Table 4 was performed in the Mastercycler:

Table 4 RT reaction thermocycler program

Step	Temperature	Time
annealing	25 °C	10 min
transcription	55 °C	30 min
inactivation of reverse transcriptase	85 °C	5 min
cool	4 °C	hold

Finally, the cDNA was aliquoted into to 2 µL aliquots and stored at -20 °C

3.5.9 Real time quantitative PCR on Lightcycler 480

For the quantitative analysis of mRNA-expression the real time PCR technique was used which facilitates the detection of the PCR product via the intercalating agent SYBR Green in real time in contrast to endpoint detection which is used in the older northern blot technique. SYBR Green is a fluorescent dye which is excited with light at 494 nm and whose emission maximum is 521 nm. For this work two different kit systems were used. Rat IRS-2 was measured using QuantiFast SYBR Green PCR kit from Qiagen and rat β-actin was measured with FastStart DNA Master SYBR Green I from Roche. The following PCR mixes were prepared:

Qiagen protocol

2 µL	cDNA
2 µL	QuantiTect Primer Assay
10 µL	2 X QuantiFast SYBR Green
6 µL	H_2O

Roche protocol

2 µL	cDNA
0.5 µL	primer sense (c=20 µM)
0.5 µL	primer antisense (c=20 µM)
1.6 µL	$MgCl_2$ (c=50 mM)
13.6 µL	H_2O
2 µL	FastStart DNA Master SYBR Green I

The complete mixes was transferred onto a LC480 Multiwell plate and the following programs were performed in the Lightcycler 480 System from Roche:

Table 5 Qiagen protocol LC 480 program for rat IRS-2

Step	Temperature	Time	Iteration
denaturation	95 °C	3 min	1 X
denaturation	95 °C	3 sec	
combined annealing and elongation	60 °C	30 sec	40 X
denaturation	95 °C	5 sec	1 X
melting curve	62 °C --> 98 °C	continuous	1 X
cool	40 °C	10 sec	

Table 6 Roche protocol LC 480 program for rat β-actin

Step	Temperature	Time	Iteration
denaturation	95 °C	10 min	1 X
denaturation	95 °C	15 sec	
annealing	67 °C	10 sec	40 X
elongation	72 °C	11 sec	
denaturation	95 °C	5 sec	1 X
melting curve	69 °C --> 98 °C	continuous	1 X
cool	40 °C	30 sec	

To quantify the expression of mRNA external standard curves were run for each gene and in each experiment. For the establishment of a standard curve the PCR product from a reaction conducted under the above described conditions was purified using the MinElute PCR purification kit from Qiagen according to the manufacturer's instructions. The eluted DNA was measured with the Biophotometer from Eppendorf and the concentration was adjusted with water to 5 ng/µL and aliquots of 5 µL were stored at -20 °C. For each experiment the following dilution series was pipetted by diluting each dilution 1:10 to achieve the following dilution step.

Table 7 Dilution series calibration
curve for real time quantitative PCR

Dilution	Concentration
standard stock	5 ng / µl
1	500 pg / µl
2	50 pg / µl
3	5 pg / µl
4	500 fg / µl
5	50 fg / µl
6	5 fg / µl
7	500 ag / µl
8	50 ag / µl
9	5 ag / µl

2 µL of dilutions 3 to 9 were used for the calibration curve and subsequently treated like samples.

The mRNA expression was quantified with the Lightcycler 480 software by determination of the crossing point (C_T) of standards and samples. The crossing point or threshold cycle is defined as the cycle at which the amplification plot (Figure 8A, B) crosses a threshold at which there is a significant increase in fluorescence. This C_T is determined automatically by the software. The standard curve is plotted from the C_T of the standards against the log of amount of standard (Figure 8C) and the samples are determined on the basis of the standard curve.

A Amplification curves of standards

B Amplification curves of standards and samples

C Standard curve

Error: 0,00608
Efficiency: 1,991
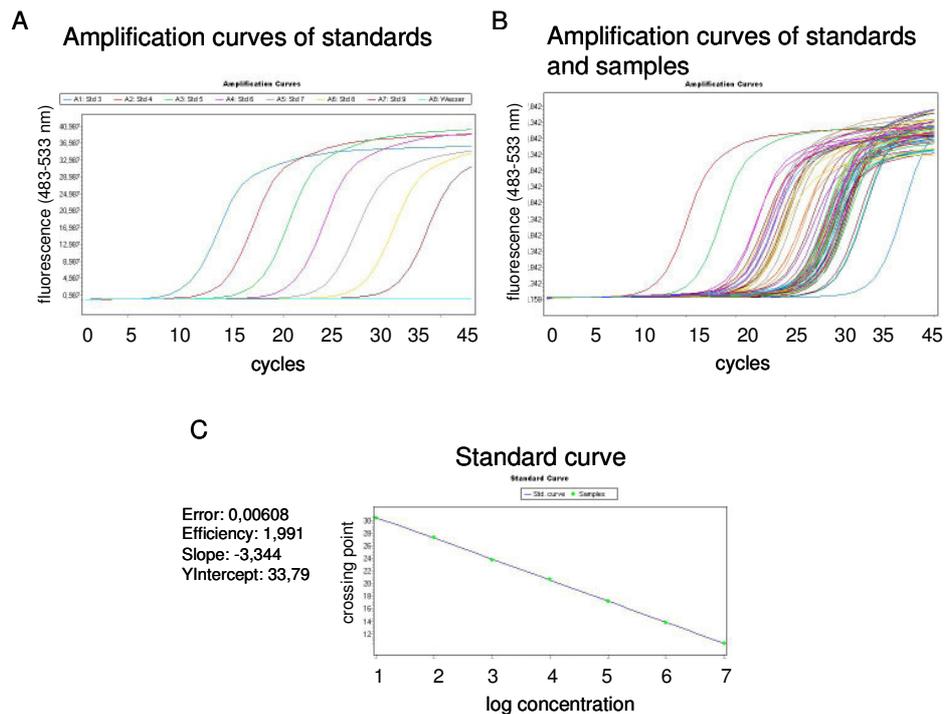Slope: -3,344
YIntercept: 33,79

Figure 8 Quantification of Roche Lightcycler 480 real time PCR. (A) Amplification curves of standards, (B) amplification curves of standards and sample and (C) standard curve calculated from CT of standards against the log of amount of standard.

3.6 Data analysis

3.6.1 Quantification of immunoblots by scanning densitometry

Films with phospho-protein bands and protein bands were scanned using a HP scanjet 4670. The densitometric analyses were carried out using Gelscan Professional V5.1.

3.6.2 Statistics

Data are presented as mean ± SEM from 3 to 5 independent experiments. Phospho-protein values were normalized by the value of the corresponding protein and protein values were normalized using β-actin. mRNA expression data of IRS-2 was normalized by β-actin expression. Statistical analyses were performed with SPSS for Windows (version 15.0.1) using Mann-Whitney-U test for not normally distributed variables and the Students t-test for normally distributed variables. A result was considered significant if p<0.05.

4 Results

4.1 Testing of antibodies

4.1.1 Prescreening in Fao rat hepatoma cells

A prescreening of the monoclonal antibodies generated to detect site specific serine/threonine phosphorylations of IRS-2 was performed in Fao rat hepatoma cells. These cells express large amounts of endogenous IR and IRS-2 protein. Fao cells were treated with 0.5 µM phorbol ester (TPA) for 30 minutes that activate PKC isoforms and further serine/threonine kinases and cell extracts were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with the monoclonal antibodies. Antibodies that gave positive results (data not shown) were retested using untreated Fao cells or cells after treatment with 100 nM insulin or TPA. Antibodies were chosen for their ability to recognize IRS-2 after treatment with serine/threonine kinase activators insulin and TPA and to give only weak signals in untreated cells and the corresponding clones were subcloned and propagated. Antibodies against two previously unknown phosphorylation sites were used in this thesis: phospho-Ser 675 and phospho-Ser 907. Both serine residues are particularly interesting since they are located in close proximity to two functional tyrosine residues: Ser 675 lies within the IRS-2 specific kinase regulatory loop binding (KRLB) domain and is adjacent to a PI-3 kinase binding motif ($pY_{671}MPM$), Ser 907 is adjacent to the Grb2 binding domain of IRS-2 ($pY_{911}INI$) (100;220).

For phospho-Ser 675 three monoclonal antibodies were chosen for further experiments: hybridoma clones 5E4, 8A8 and 1C4 (Figure 9A). The antibody from clone 5E4 gave the strongest signal and was therefore used in the majority of the experiments. In the initial immunization round only one clone (2F4) was positive for the detection of phospho-Ser 907 (Figure 9B), however, this clone could not be stably subcloned and therefore new hybridoma cell lines were established. Finally the hybridoma clone 9C12 (Figure 9B) was stable and sufficient amounts of antibody were produced.
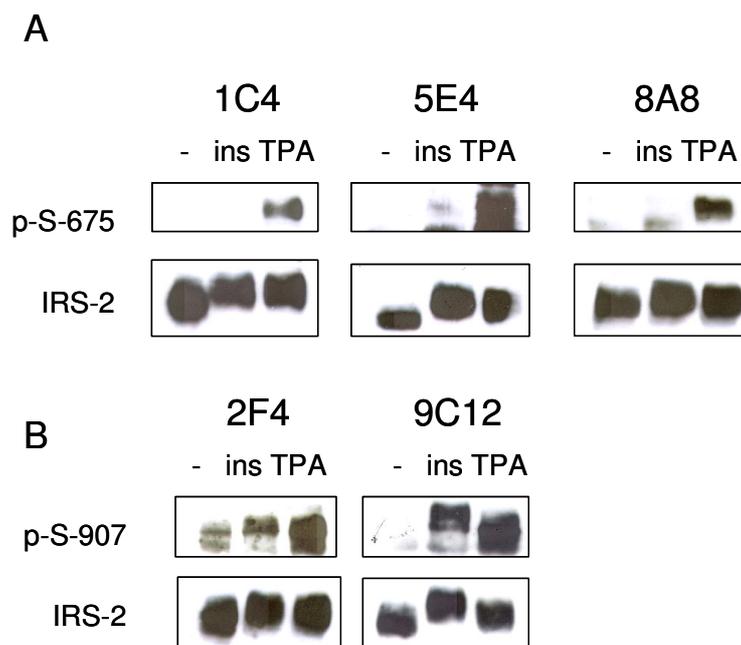
Figure 9 Detection of IRS-2 using phospho-Ser 675 (A) or phospho-Ser 907 antibodies (B) in Fao cells. Cells were serum starved overnight and treated with 100 nM insulin or 0.5 µM TPA for 30 minutes. After stimulation cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with different monoclonal phospho-site specific Ser 675 (A) or Ser 907 (B) antibodies. The blots were reprobed with a polyclonal IRS-2 protein antibody .

4.1.2 Antibody specificity

4.1.2.1   Phospho-site specificity in BHK cells transiently expressing Ala mutants

The specificity of the antibodies towards the phosphorylated serine residues was tested in baby hamster kidney (BHK) cells transiently expressing IRS-2 wildtype (IRS-2 wt), IRS-2 675 Ala (675 Ala) or IRS-2 907 Ala (907 Ala), which could not be phosphorylated on these residues. The cells were treated with insulin, TPA or anisomycin for 30 minutes and the lysates were separated by 7.5% SDS-PAGE. Both antibodies clearly recognized IRS-2 wt in stimulated cells, but only a weak signal was detected in the cells expressing the IRS-2 Ala mutants (Figure 10). This demonstrated that the antibodies specifically recognized the phosphorylated state of the IRS-2 protein.
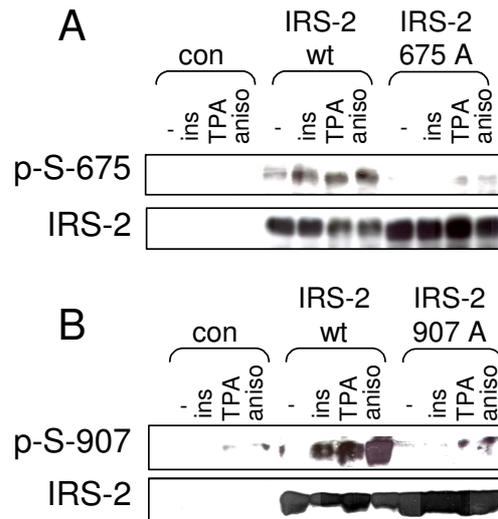
Figure 10 Antibody specificity in BHK cells transiently overexpressing IRS-2 and IRS-2 675 Ala and 907 Ala. BHK cells were transiently transfected with empty vector (con), IRS-2, IRS-2 675 Ala or IRS-2 907 Ala and treated with 100 nM insulin (ins), 0.5 µM TPA or 5 µg/mL anisomycin (aniso) for 30 minutes. The blots were probed with phospho-site specific antibodies and reprobed with polyclonal antibodies against IRS-2.

4.1.2.2   IRS-1/2 specificity in Fao cells

IRS-1 and 2 share 36% of overall sequence homology and the amino acid sequences of the peptides that were used to raise the antibodies are almost identical for IRS-1 (Figure 11). It was therefore necessary to test whether the antibodies would also detect the corresponding IRS-1 serine, if phosphorylated. To test the IRS-2 phospho-protein specificity Fao cells were left untreated or treated with insulin or TPA and from the cell lysates either IRS-1 or IRS-2 was immunoprecipitated with polyclonal antibodies and immunoblotted with the phospho-site specific antibodies. In this setting only the endogenous IRS-2 but not the endogenous IRS-1 was detected by the antibodies (Figure 12).

mouse IRS-2 ^{670} DYMPM**S**PTSVS ^{680}

mouse IRS-1 ^{627} DYMPM**S**PKSVS ^{637}

mouse IRS-2 ^{902} PTEPK**S**PGEYI ^{912}

mouse IRS-1 ^{882}PPEPK**S**PGEYV ^{892}

Figure 11 Sequence homology of IRS-1 and IRS-2 surrounding the phosphorylated serine residues 675 (top) and 907 (bottom).

A

IP IRS-1   IP IRS-2

IB: p-S-675

IB: IRS-1   IRS-2
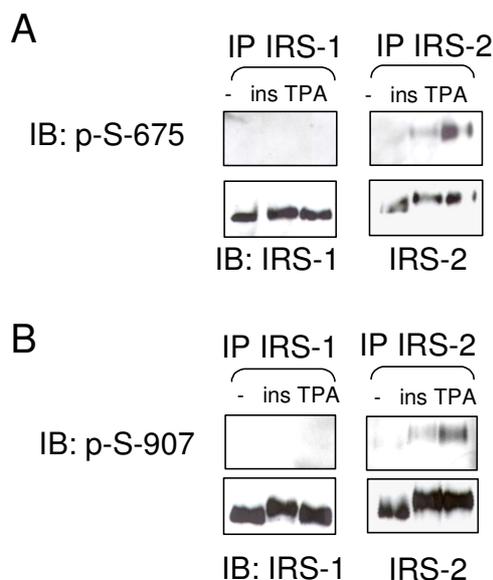
B

IP IRS-1   IP IRS-2

IB: p-S-907

IB: IRS-1   IRS-2

Figure 12 Antibody specificity towards IRS-2. Fao cells were treated with 100 nM insulin or 0.5 µM TPA for 30 minutes and IRS-1 and -2 were immunoprecipitated (IP) with polyclonal antibodies and immunoblotted (IB) with phospho-site specific monoclonal antibodies. The blots were reprobed for IRS-1 and IRS-2.

4.1.2.3   IRS-1/2 specificity in HEK293 cells transiently expressing rat IRS-1, mouse IRS-2 or human IRS-2

The specificity of the monoclonal antibodies towards IRS-2 was further tested in human embryonic kidney (HEK293) cells transiently overexpressing the IR and either mouse IRS-2, rat IRS-1 or human IRS-2 (Figure 13). The antibodies clearly detected the mouse and the human IRS-2 in insulin stimulated HEK293 cells and the phospho-Ser 675 antibody did not recognize IRS-1. The phospho-Ser 907 antibody detected a faint signal in insulin stimulated cells expressing IRS-1 (Figure 13B). This signal was very weak when compared to the cells expressing the respective IRS-2 protein and this phenomenon was only observed in an IRS-1-overexpressing system, but nevertheless, it showed that the antibody had some cross-reactivity towards IRS-1.

4.1.3 IRS-2 phosphorylation in primary human hepatocytes

The antibody against phospho-Ser 675 not only detected overexpressed human IRS-2 in HEK293 cells but also phosphorylated endogenous IRS-2 in primary human hepatocytes after

treatment with insulin and TPA (Figure 14). Of note, the pronounced IRS-2 shift due to al-
tered electrophoretic mobility after insulin and TPA stimulation which is detectable in Fao cells
(see also 4.2.1) and overexpressing cell systems, could not be demonstrated in these cells.
Unfortunately due to the instability of the original phospho-Ser 907 antibody (clone 2F4) it
was not possible to test the phosphorylation on Ser 907 in primary human hepatocytes at the
time when these cells were available.
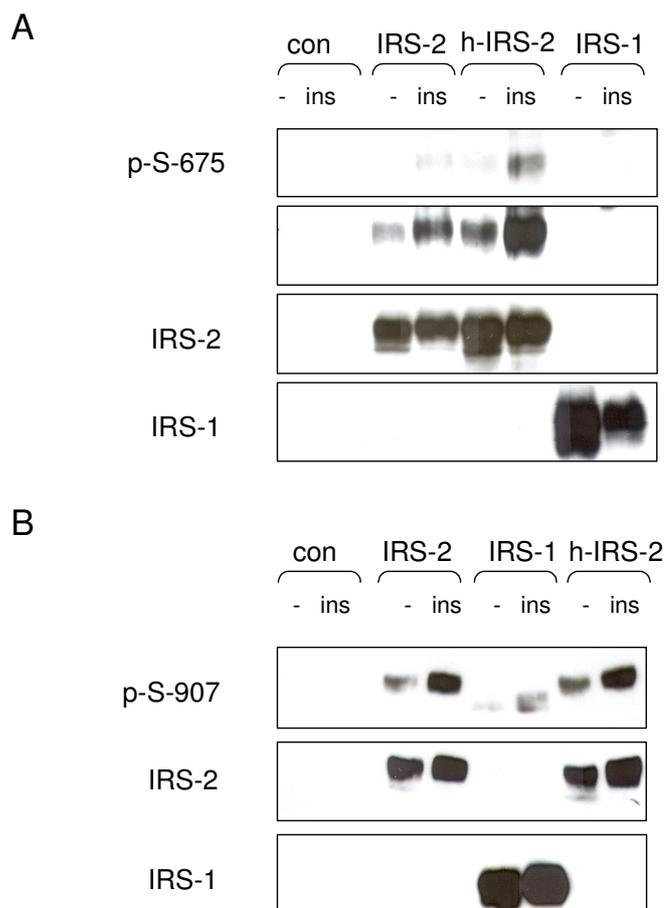
Figure 13 Antibody specificity in HEK293 cells transiently overexpressing rat IRS-1, mouse IRS-2 or
human IRS-2. (A, B) HEK293 cells were transiently transfected with empty vector (con), IRS-2, IRS-1
or human IRS-2 and treated with 100 nM insulin for 30 minutes. The blots were probed with phospho-
site specific antibodies and reprobed with polyclonal antibodies against IRS-1 and IRS-2. The arrow
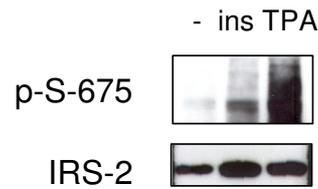indicates phospho-Ser 675 after longer exposure time.

- ins TPA

p-S-675

IRS-2

Figure 14 Ser 675 phosphorylation of endogenous IRS-2 in primary human hepatocytes.

4.1.4 IRS-2 phosphorylation *in vivo*

After demonstrating that endogenous as well as transiently overexpressed IRS-2 is phosphorylated on Ser 675 and Ser 907 in different cell culture models it was studied whether these phosphorylations occurred *in vivo* as well. C57Bl/6 wt mice were treated with insulin intravenously for 10 minutes and the liver tissue lysates were immunoprecipitated with IRS-2 antibody and immunoblotted for phospho-Ser 675 (Figure 15A) and phospho-Ser 907 (Figure 15B). A clear signal was detected on both phosphorylation sites.

Furthermore the phosphorylation of both sites was studied in the livers of mice that had been fasted overnight and then refed for 5 h (Figure 15C and D). Refeeding induced a significant phosphorylation on both sites, demonstrating that not only the rather unphysiological administration of insulin i.v. but also the physiological process of feeding leads to the phosphorylation of serine residues of IRS-2. Of note, feeding also induced a pronounced decrease of IRS-2 protein which has been demonstrated in several studies (90;112) and a clearly reduced electrophoretic mobility of the protein.
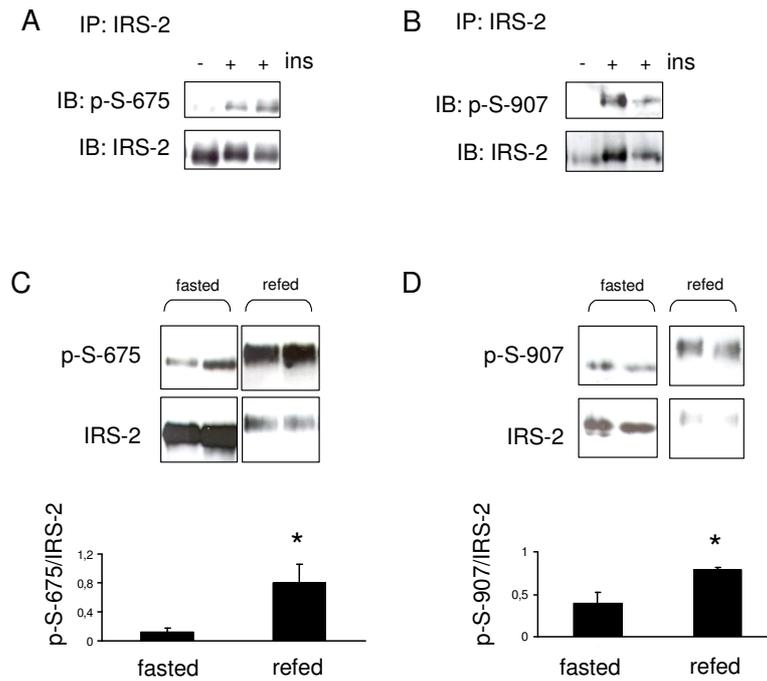
Figure 15 IRS-2 serine phosphorylation *in vivo*. (A) and (B) Male C57/Bl6 mice were fasted overnight and injected intravenously with 2 IU insulin. Liver samples were obtained after 10 minutes of insulin treatment. Immunoblots of liver extracts obtained from one untreated and two insulin-treated mice are shown. (C) and (D) Male C57/Bl6 mice were fasted overnight and refed for 5 h. Liver samples were obtained immediately after the refeeding period. Immunoblots for 2 mice of each group are shown. Phosphorylation intensity was quantified based on scanning densitometry of immunoblots normalized for IRS-2 protein (mean + SEM, n=4; *p<0.05 fasted vs. refed)

4.2   Characterization of IRS-2 phospho-serine sites

4.2.1   Effect of different stimuli on serine phosphorylation in Fao cells

A set of different stimuli and kinase activators was tested for their ability to induce a phosphorylation on Ser 675 and Ser 907. For this purpose Fao cells were stimulated for 30 minutes with either insulin, IGF-1, TNFα, TPA or anisomycin. TNFα was used because it is associated with the induction of insulin resistance and serine-phosphorylation of IRS-1 (227). Anisomycin was used to stimulate stress activated protein kinases in general. IGF-1 stimulation was examined because IRS-2 is also a substrate of the IGF-1 receptor, which predominantly signals into mitogenic pathways. Both IRS-2 serine residues were phosphorylated upon insulin, TPA and anisomycin stimulation (Figure 16) whereas TNFα induced only a weak phosphorylation but it neither activated JNK suggesting a missing effect of this cytokine in Fao

cells. This phenomenon was not examined further. IGF-1 had a surprisingly small effect on the phosphorylation of both serine sites but in Fao cells only weak expression signals of the IGF-1 receptor compared to the high protein levels of the IR were detected (Figure 16A). This difference of IR and IGF-1 receptor protein levels has also been described earlier in Fao cells (228). The small effect of IGF-1 is also reflected by the relatively low activation of PKB/Akt (p-S-473) in the IGF-1 treated cells (Figure 16A).

Of note, particularly the stimulation with insulin, IGF-1, TPA and anisomycin resulted in the pronounced retardation of the electrophoretic mobility of the IRS-2 protein in SDS-PAGE („shift"). This shift can occur upon tyrosine, serine and threonine phosphorylation and probably other kinds of posttranslational modification. In all experiments the strongest effect on the electrophoretic mobility was produced by treatment of Fao cells with anisomycin (Figure 16) resulting in a condensed and retained band of IRS-2 protein on the western blot. Other stimuli like insulin and TPA also produced a shift but in these cases the IRS-2 band appeared more square-shaped.
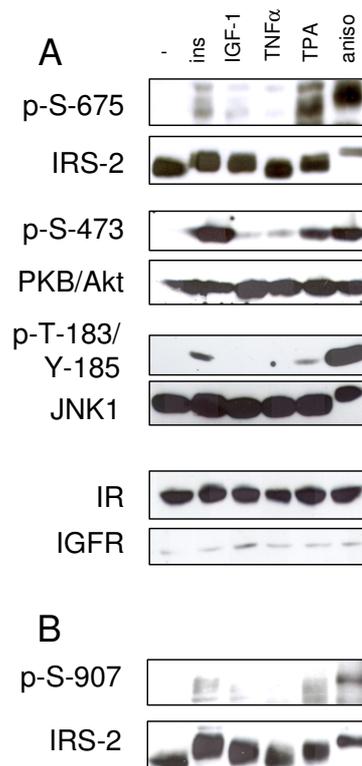
Figure 16 Effect of different stimuli on IRS-2 serine phosphorylation. Fao cells were treated with either 100 nM insulin (ins), 50 ng/mL IGF-1, 5 nM TNFα, 0.5 μM TPA or 5 μg/mL anisomycin (aniso) for 30 minutes. After stimulation cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with monoclonal IRS-2 phospho-site specific Ser 675 (A) or Ser 907 (B) antibody. The blots were reprobed with a polyclonal IRS-2 antibody. Furthermore phospho-PKB/Akt Ser 473, phospho-JNK Thr 183/Tyr 185 and protein reblot as well as insulin receptor (IR) and insulin like growth factor (IGF)-1 blots are shown (A).

4.2.2 Dose response of IRS-2 phosphorylation with different insulin concentrations

In Fao cells a dose response of IRS-2 phosphorylation on Ser 675 and Ser 907 to increasing levels of insulin was assessed (Figure 17A). In addition different components of the insulin signaling pathway were studied as well (Figure 17B). Insulin concentrations as low as 0.1 nM were sufficient to trigger a phosphorylation of Ser 675, demonstrating that physiological insulin concentrations are able to induce the phosphorylation at this site. Ser 907 phosphorylation on the other hand appeared to increase with higher insulin concentrations (Figure 17A). The reduction of electrophoretic mobility of the IRS-2 protein was visible already at 0.1 nM insulin and became more pronounced with increasing insulin concentrations.

The activation of downstream signaling proteins of the insulin signaling pathway as well as the tyrosine phosphorylation of IRS-2 was increased with rising insulin concentrations (Figure 17B) and strongest tyrosine phosphorylation of IRS-2 and phosphorylation of PKB/Akt was achieved with 10 to 100 nM insulin. The MAP kinase ERK1/2 was only phosphorylated over basal levels with 100 nM insulin. Therefore in all experiments the maximal insulin concentration of 100 nM was used to induce the strongest possible activation of downstream kinases.
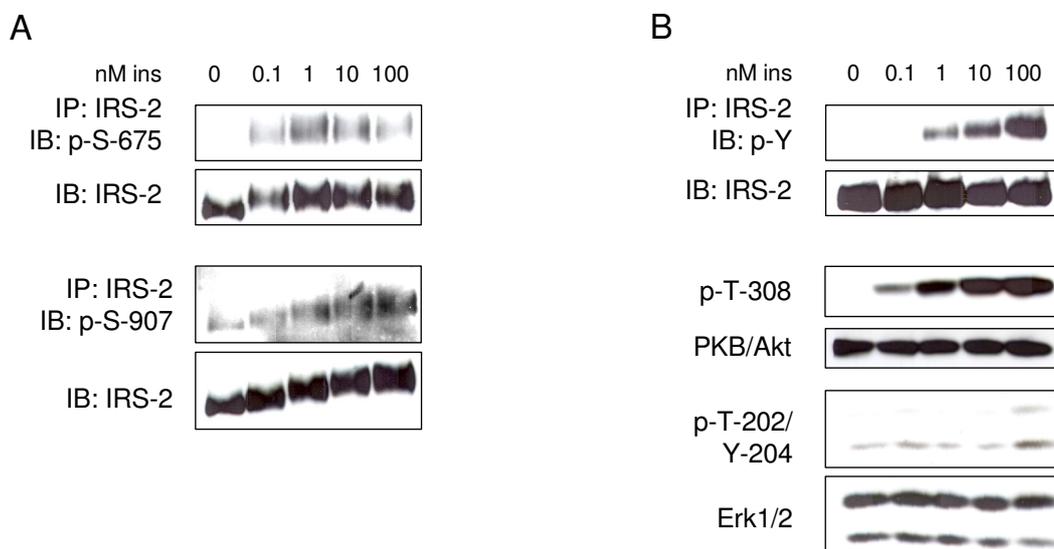
Figure 17 Insulin dose response in Fao cells. Fao cells were treated with different insulin concentrations ranging from 0.1 to 100 nM for 30 minutes. (A) and (B) top panel, endogenous IRS-2 was immunoprecipitated (IP) using a polyclonal antibody and immunoblotted (IB) with phospho-site specific IRS-2 antibodies and a general phospho-tyrosine antibody (B, top panel). Normal lysates were separated with 7.5 % SDS-PAGE and immunoblotted with phospho-PKB/Akt and phospho-ERK1/2 and reprobed with the respective protein antibody.

4.2.3 Phosphorylation kinetics

To record the time course of insulin-induced phosphorylation of Ser 675 and Ser 907 Fao cells were treated with insulin for 0 to 240 minutes. Both phospho-sites were phosphorylated after 30 to 60 minutes of insulin stimulation and the phosphorylation continued for the rest of the experiment (Figure 18). The phosphorylation of Ser 675 was also observed after 8 h of insulin treatment (data not shown). This indicates that Ser 675 and Ser 907 are rather late events of insulin signaling as opposed to very rapid phosphorylations of IRS-proteins, e.g. tyrosine phosphorylations which occur immediately after insulin stimulation (229).
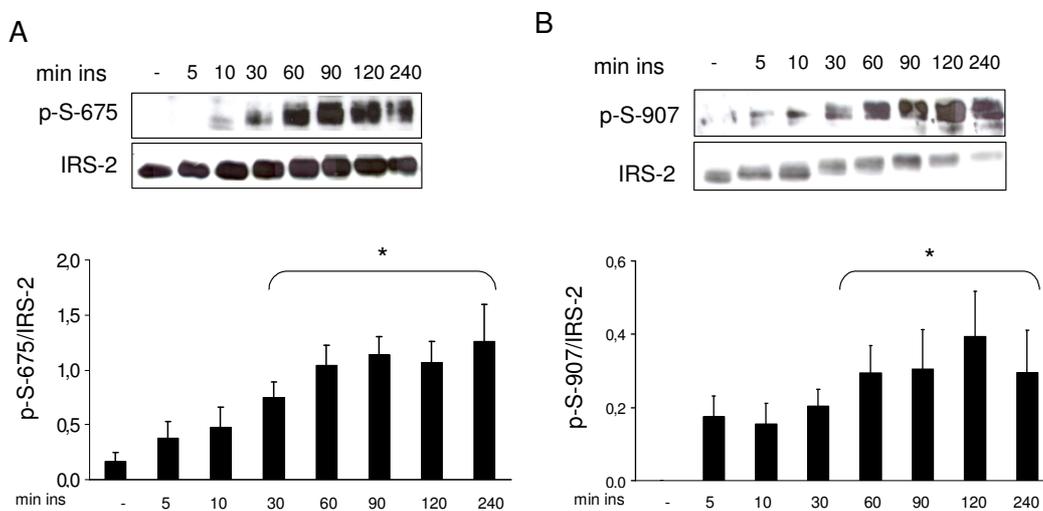
Figure 18 Phosphorylation kinetics of IRS-2 Ser 675 (A) and Ser 907 (B). Fao cells were treated with 100 nM insulin for 0-240 minutes. Cells were lysed and analyzed with 7.5% SDS-PAGE and im-munoblotted with monoclonal phospho-site specific antibodies. The blots were reprobed for IRS-2 with a polyclonal antibody. Quantification was based on scanning densitometry of immunoblots normalized for IRS-2 protein (mean + SEM, n=3, * $p<0.05$ ins vs. control (-)).

4.3   Biological regulation and relevance of Serine 675 phosphorylation

4.3.1 Characterization of kinases

4.3.1.1   *In silico* amino acid sequence analysis and comparison with IRS-1

Ser 675 is located in a functional relevant region of the IRS-2 protein. It lies within the IRS-2 specific KRLB domain next to a recruitment site (**pY$_{671}$MPM**) for the regulatory subunit p85 of the PI-3 kinase (Figure 19A). Position 676 is occupied by a proline residue and the motif **pS/pTP** has been recognized as a mTOR phosphorylation motif (66). Furthermore, the bioinformatical tool Human Protein Reference Database (219) suggested that ERK1 and 2 as well as GSK3 and cyclin-dependent kinase (CDK)5 could phosphorylate Ser 675.

The amino acid sequence surrounding Ser 675 is highly conserved in mouse, rat and human IRS-2 (Figure 19B) and as shown above the phospho-site specific antibody specifically recognizes phosphorylated IRS-2 in all three species (Figure 12A, Figure 13A, Figure 14, Figure 15A, C).

IRS-2 Ser 675 has a homologous serine residue in IRS-1: Ser 632 (mouse IRS-2 amino acid sequence) (Figure 19C) which however is not detected by our phospho-site specific antibodies (Figure 13A) although the sequences in that region are almost identical in IRS-1 and -2. They differ only by one amino acid, Thr 677 instead of Lys 634 (Figure 19C). This site along with Ser 635 is well studied and it has been shown that mTOR/p70 S6K1 as well as ERK1/2 phosphorylate Ser 632/635 (134;159). Furthermore, the phosphorylation of Ser 632/635 has been shown to reduce the insulin-induced association of IRS-1 with p85 (131).

A

p85 binding via
SH2 domain

^{670} D *Y M P M* **S** P T S V S ^{680} ^{675}

phosphorylation
motif of mTOR

B

mouse	^{670} DYMPM**S**PTSVS ^{680}
rat	^{671} DYMPM**S**PTSVS ^{681}
human	^{674} DYMPM**S**PASVS ^{684}

C

IRS-2	^{670} DYMPM**S**PTSVS ^{680}
IRS-1	^{627} DYMPM**S**PKSVS ^{637}
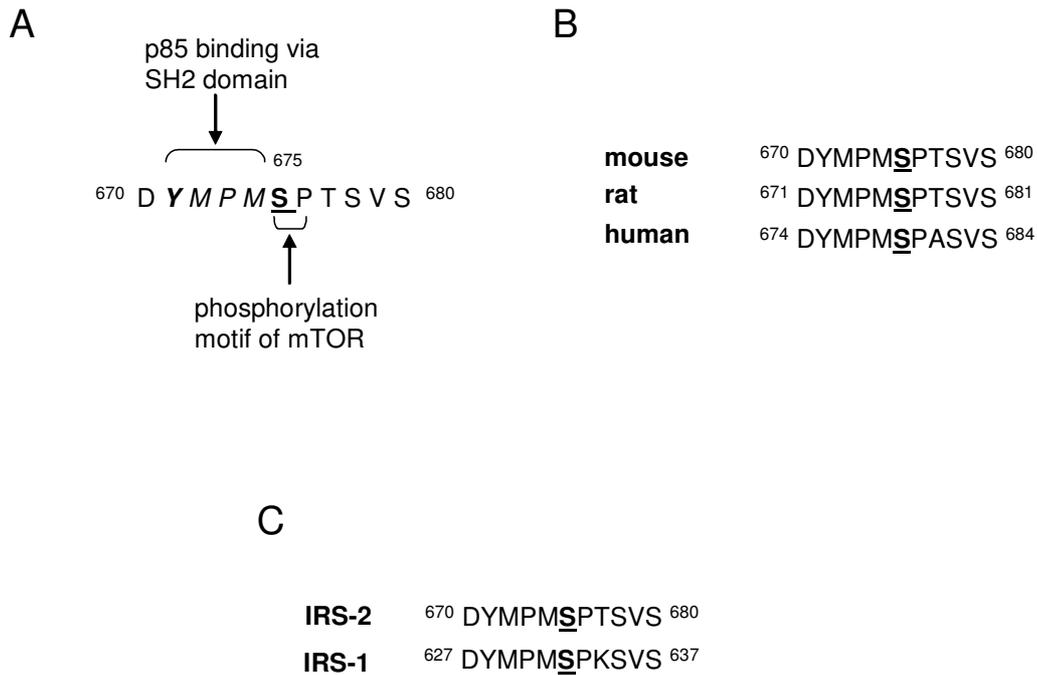
Figure 19 Amino acid sequence of mouse IRS-2 surrounding Ser 675. (A) Phosphorylation and protein binding motifs in mouse IRS-2. (B) Comparison of mouse, rat and human IRS-2. (C) Comparison of mouse IRS-2 and IRS-1.

4.3.1.2   Inhibition of insulin-induced phosphorylation by pharmacological inhibitors

To identify the kinase(s) responsible for phosphorylating IRS-2 on Ser 675 first a number of inhibitors was used to block specific kinases in insulin treated Fao cells. The inhibition of the PI-3 kinase or mTOR by wortmannin or rapamycin respectively resulted in a significantly reduced insulin dependent phosphorylation of Ser 675 (Figure 20). In contrast, the inhibition of stress inducible JNK by SP600125 and the ERK1/2 activating dual specific MAP kinase ERK kinase (MEK)1 by PD98059 had no significant effect on the insulin-induced phosphorylation of Ser 675.

Figure 20 Inhibition of insulin-induced Ser 675 phosphorylation in Fao cells. Fao cells were treated with either 25 nM rapamycin (rapa), 100 nM wortmannin (wort), 10 μM SP600125 (SP) or 50 μM PD98059 (PD) for 30 minutes and subsequently stimulated with 100 nM insulin (ins) for 60 minutes. Phosphorylation intensity was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean + SEM, n=5, * p<0.05 insulin stimulated cells with inhibitor vs. insulin stimulated cells without inhibitor)
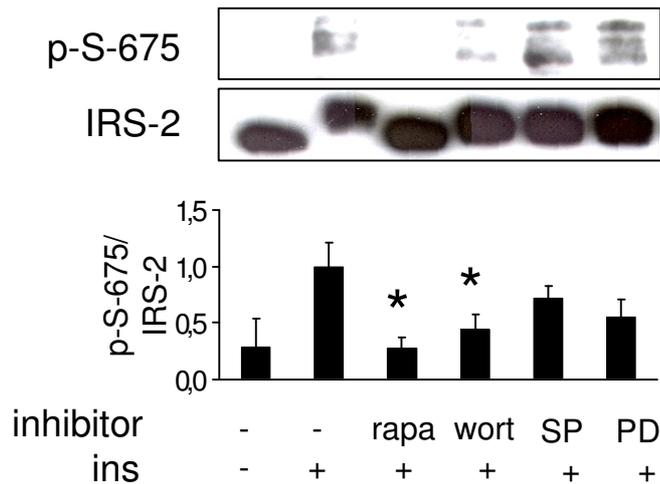
4.3.1.3   Inhibition of insulin-induced Ser 675 phosphorylation by siRNA targeting mTOR

The clear effects of rapamycin on the inhibition of the insulin-induced phosphorylation of Ser 675 led to the assumption that mTOR or a mTOR-dependent kinase could phosphorylate this site. To test this hypothesis Fao cells were electroporated with siRNA oligonucleotides against mTOR and stimulated with 100 nM insulin for 60 minutes. This approach led to a pronounced reduction of mTOR protein (Figure 21A) and it resulted in abolished insulin-induced phosphorylation of Thr 389 of p70 S6K1, the downstream target of mTOR. The knockdown of mTOR resulted in significantly reduced basal and insulin-induced phosphorylation of Ser 675 on IRS-2 (Figure 21A). Since the knockdown of mTOR also inhibited the activation of p70 S6K1 this kinase was knocked down in the same cell system as well (Figure 21B). The silencing of p70 S6K1 was highly efficient resulting in undetectable protein bands and leading to a reduced phosphorylation of Ser 1101 on IRS-1, which has been identified as a p70 S6K1-dependent phosphorylation site (167). However, the knockdown of p70 S6K1 had no significant effect on the insulin-induced Ser 675 phosphorylation of IRS-2 (Figure 21B). These results led to the conclusion that mTOR is most likely the kinase responsible for phosphorylating Ser 675.
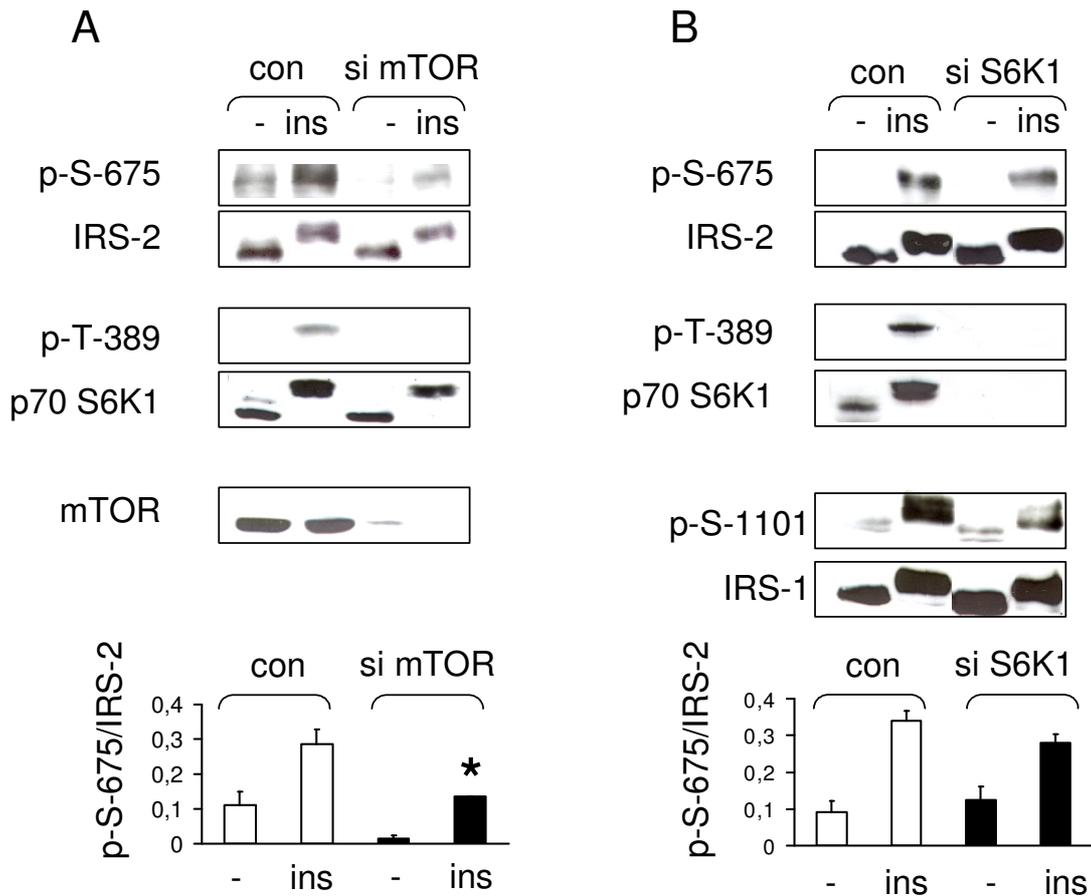
Figure 21 Phosphorylation of IRS-2 Ser 675 after knockdown of mTOR (A) and p70 S6K1 (B) in Fao cells. Fao cells were electroporated with siRNA targeting either mTOR (A) or p70 S6K1 (B) and control siRNA (con). 48 h after transfection cells were treated with 100 nM insulin (ins) for 30 minutes. Phosphorylation of IRS-2 (Ser 675), p70 S6K1 (Thr 389) (A, B) and of IRS-1 (Ser 1011) (B) with the corresponding reblots and total mTOR (A) protein are shown. Phosphorylation intensity of Ser 675 was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean + SEM, n=4, * $p < 0.05$ con ins vs. si mTOR ins).

The interaction of mTOR with IRS-2 was further confirmed with a co-immunoprecipitation experiment in Fao cells: IRS-2 was immunoprecipitated and at approximately 280 kDa a band was detected which was expected to be mTOR (Figure 22A, left panel). The association of IRS-2 and mTOR was apparently present at the basal state and was not further influenced by insulin or rapamycin. However, rapamycin treatment together with insulin appeared to reduce the association of both molecules (Figure 22A, B). The inverse co-immunoprecipitation experiment with the immunoprecipitation of mTOR and subsequent detection of IRS-2 produced only a weak IRS-2 signal (data not shown).

A

IP: αIRS-2   lysate

rapa   rapa

ins   -   +   -   +   -   +   -   +

IB: mTOR

IB: IRS-2

B

IP: αIRS-2

rapa

min ins   0   30   60 120 60

IB: mTOR

IB: IRS-2
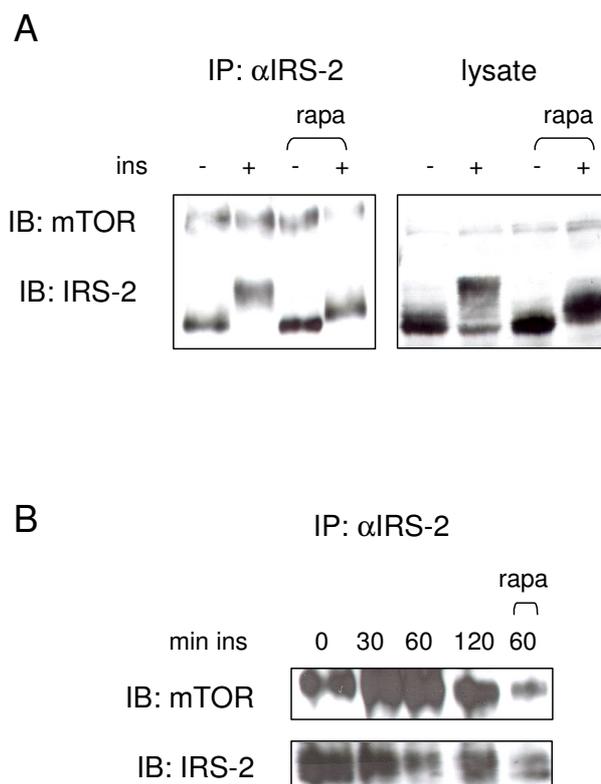
Figure 22 Co-immunoprecipitation of IRS-2 and mTOR in Fao. (A) Cells were treated with 25 nM rapamycin (rapa) for 30 minutes and subsequently stimulated with 100 nM insulin (ins) for 60 minutes. Cells were lysed and IRS-2 was immunoprecipitated (IP) with a polyclonal IRS-2 antibody. IP and lysates were separated by 7.5% SDS-PAGE and immunoblotted for mTOR and IRS-2. (B) Cells were pretreated with 25 nM rapamycin for 60 minutes and stimulated with 100 nM insulin for additional 60 minutes or were treated with insulin for 0 to 120 minutes without rapamycin. Cells were lysed and IRS-2 was immunoprecipitated (IP) with a polyclonal IRS-2 antibody. The blots were probed for mTOR and IRS-2.

Interestingly, the inhibition of mTOR with rapamycin completely prevented the insulin-induced phosphorylation on Ser 675 and also partially the TPA-induced phosphorylation, but it had no effect on the anisomycin-induced phosphorylation (Figure 23), indicating that other mTOR-independent kinase(s) could also phosphorylate IRS-2 on Ser 675. Except for anisomycin treated cells, rapamycin treatment led to a significant increase of IRS-2 mobility even in the basal state, suggesting a reduced overall phosphorylation of IRS-2. Furthermore, a reduced electrophoretic mobility of p70 S6K1 was observed after stimulation with either insulin, TPA or anisomycin and this mobility was greatly increased when the cells were treated with the inhibitor and could not further be influenced by any stimulation (Figure 23).
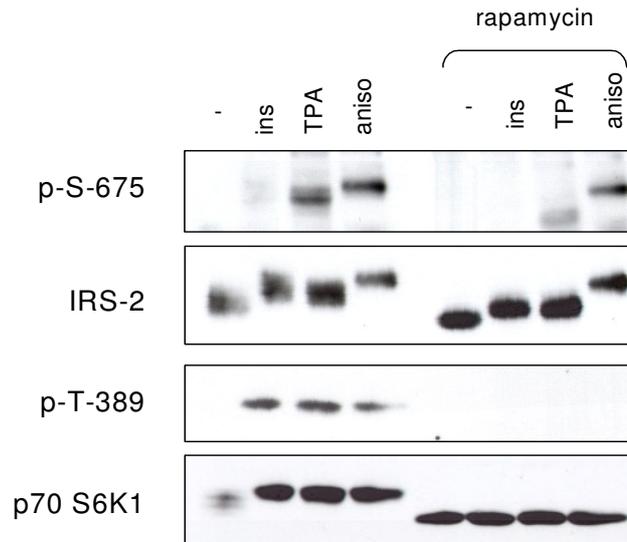
Figure 23 Rapamycin inhibits the insulin-induced but not the TPA- and anisomycin-induced phosphory-lation of IRS-2 Ser 675. Fao cells were treated with 25 nM rapamycin for 30 minutes and subsequently stimulated with 100 nM insulin (ins), 0.5 µM TPA or 5 µg/mL anisomycin (aniso) for 30 minutes. Lysates were separated by 7.5% SDS-PAGE and immunoblotted with phospho-site specific monoclonal Ser 675 antibody and phospho p70 S6K1 (Thr 389) antibody. The membranes were reprobed with protein antibodies for IRS-2 and p70 S6K1.

4.3.2 Function of Ser 675

To elucidate the functional role of the Ser 675 phosphorylation for insulin signaling the effects of the non-phosphorylated 675 Ala mutant and also the 675 Glu mutant, which simulates the phosphorylated state, were investigated in different cell culture models. Fao cells were not used for two reasons: first, these cells already express high amounts of endogenous IRS-2 and second, Fao cells can only be efficiently transfected with retroviral methods which however is not established in the lab and needs a S2-laboratory. It was attempted to transfect Fao cells with standard methods (lipofection, calcium phosphate transfection, electroporation) but only very low transfection rates were achieved. Therefore, other cell culture models were used in this thesis.

4.3.2.1   Transient transfection of BHK cells

BHK cells were transiently transfected with the empty pRK5 vector as control, with IRS-2 wt, 675 Ala and 675 Glu mutants and stimulated with insulin for 0 to 120 minutes. Proximal and distal insulin signaling was studied by immunoblotting. Insulin-induced a strong tyrosine phosphorylation of all IRS-2 proteins, with slightly different kinetics for the Ala and the Glu mutant (Figure 24A). The IRS-2 wt protein was strongly phosphorylated after 10 minutes. This phosphorylation decreased significantly after 60 minutes of insulin stimulation and increased again after 120 minutes similarly as it has been shown for IRS-1 (184). The 675 Ala mutant was constantly tyrosine phosphorylated upon insulin treatment whereas in the 675 Glu mutant the tyrosine phosphorylation tended to decrease. However, effect did not reach statistical significance (Figure 24A). Insulin also activated the PI-3 kinase and the MAP kinase signaling cascades as monitored by the phosphorylation of PKB/Akt, GSK3α and ERK1/2 (Figure 24B). The phosphorylation states of all these members of the signaling cascades were not different between the control, the IRS-2 wt expressing cells and the two 675 mutants. These results suggest that phosphorylation of Ser 675 does not influence insulin signal transduction in this cell culture model after transient transfection, but also that the introduction of IRS-2 into the cells had no effect on downstream insulin signaling events.

4.3.2.2   Stable transfection of HEK293 cells

To avoid the problem of acutely induced cellular stress by the ectopic overexpression of a distinct protein and to circumvent the necessity of repeated transfection experiments with variable transfection efficiencies, clones of HEK293 cells were made that stably express either the pRK5 vector as control or IRS-2 wt + IR or the IRS-2 675 Ala mutant + IR. The IRS-2 wt and the 675 Ala expressing cells had about similar amounts of IRS-2 and IR (Figure 25A and B). These cells were stimulated with insulin for 0 to 120 minutes and several steps of the insulin signaling cascade were investigated. Insulin-induced a strong tyrosine phosphorylation of IRS-2 wt and 675 Ala alike and promoted the association of both IRS-2 variants with p85, the regulatory subunit of the PI-3 kinase (Figure 25A). The activation of the PKB/Akt and of the MAP kinase pathway, evidenced by the phosphorylation of PKB/Akt and ERK1/2 was increased in both cell lines equally (Figure 25B) indicating that there was no effect of Ser 675 phosphorylation neither on the proximal nor distal insulin signaling.
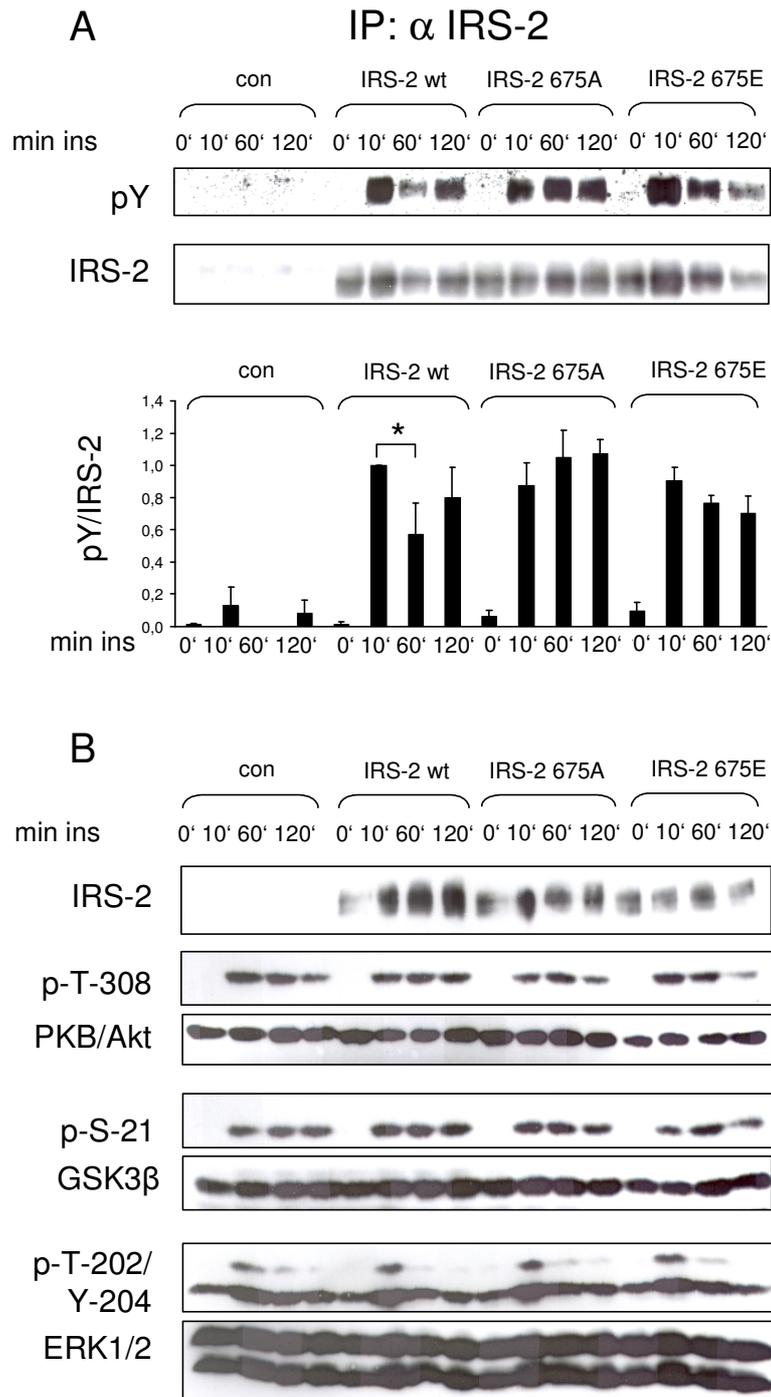
Figure 24 Function of IRS-2 Ser 675 in BHK cells. (A, B) Cells were transiently transfected with empty vector as control (con), IRS-2 wildtype (wt), IRS-2 675 Ala or IRS-2 675 Glu (E) and treated with 100 nM insulin (ins) for 0-120 minutes. Lysates were analyzed by 7.5% SDS-Page. (A) IRS-2 was immuno-precipitated (IP) with a polyclonal IRS-2 antibody and the blot was probed for phospho-tyrosine (pY) and reprobed for IRS-2 protein. Quantification of IRS-2 tyrosine phosphorylation was based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean + SEM, n=5). (B) Representative immunoblots for IRS-2 protein, phospho-PKB/Akt Thr 308, phospho-GSKα Ser 21 and phospho-ERK1/2 Thr 202/ Tyr 204 with the corresponding reblots for total protein are shown. * $p < 0.05$ wt 10' vs. wt 60'
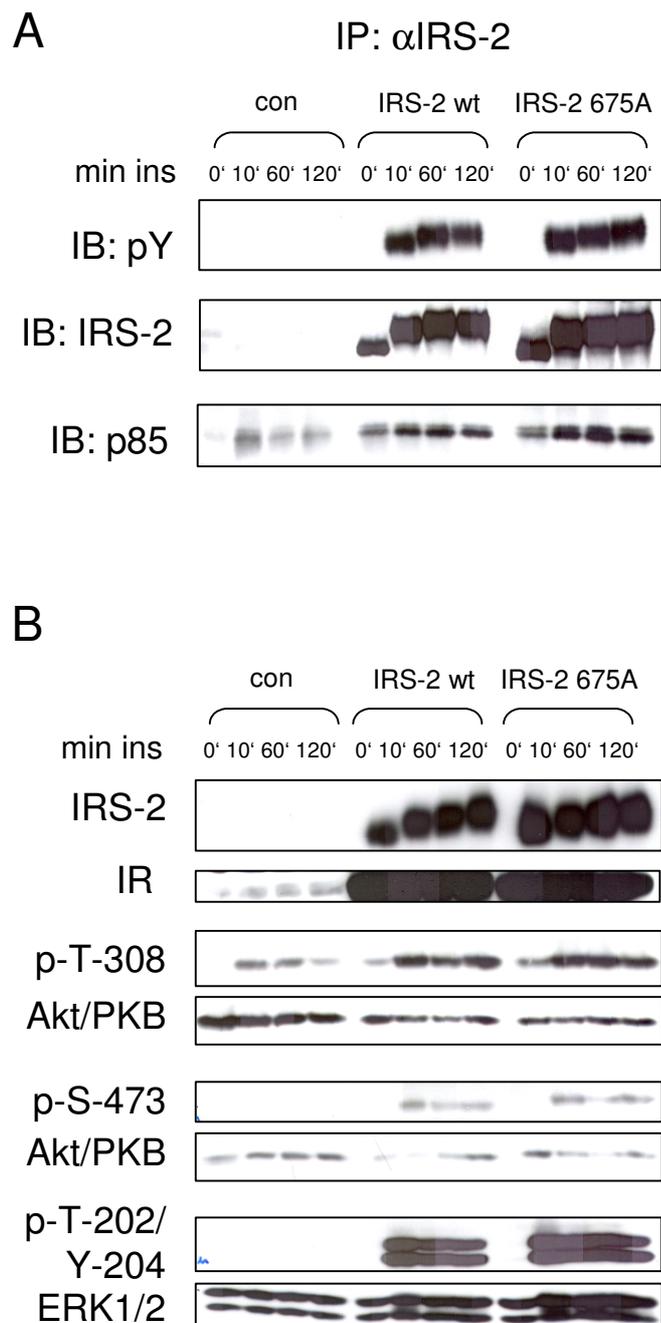
Figure 25 Effect of Ser 675 phosphorylation on proximal and distal insulin signaling in HEK293 cells. (A, B) Cells were stably transfected with either empty vector as control (con), IRS-2 wildtype (wt) and insulin receptor (IR) or IRS-2 675 Ala + IR and treated with 100 nM insulin (ins) for 0-120 minutes. Lysates were analyzed by 7.5% SDS-Page. (A) IRS-2 was immunoprecipitated (IP) with a polyclonal IRS-2 antibody and the blot was probed for phospho-tyrosine (pY) (upper panel) and p85 (lower panel) and reprobed for IRS-2 protein (middle panel). (B) Representative immunoblots for IRS-2 and IR protein, phospho-PKB/Akt Thr 308 and Ser 473 and phospho-ERK1/2 Thr 202/ Tyr 204 with the corresponding reblots for total protein are shown.

4.3.3 Degradation

It has been shown, that IRS-1 can be translocated and degraded upon insulin stimulation. In both processes, mTOR seems to have an important function since the inhibition of this kinase leads to impaired degradation of IRS-1. It has been hypothesized that mTOR's role for IRS-1 regulation is mainly to phosphorylate IRS-1 on different Ser residues leading to increased ubiquitination and degradation or altered subcellular localization (125;164). Since the preceding experiments showed that Ser 675 is phosphorylated by mTOR the question arose whether the phosphorylation on this site also influences the degradation of IRS-2.

4.3.3.1 Degradation of hepatic IRS-2 *in vivo*

Degradation is an important feature of IRS-2 and constitutes an important regulatory mechanism. Hepatic IRS-1 and -2 are differentially regulated during fasting and feeding, a state in which high insulin levels occur. As shown in section 4.1.4 IRS-2 is degraded *in vivo* after the onset of refeeding (Figure 15). In line with these data, IRS-2 levels in livers of fed and fasted wt mice differed extremely while the protein amount of IRS-1 remained unchanged (Figure 26).
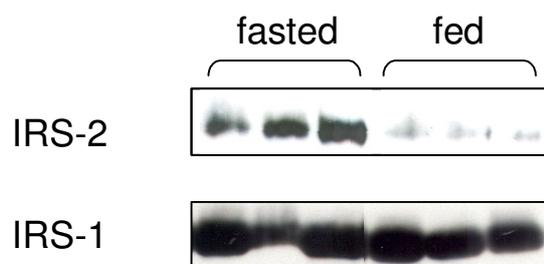

Figure 26 Degradation of hepatic IRS-2 and IRS-1 in fed wt mice. C57 Bl/6 wt mice were either fasted for 16 h or had free access to chow. After sacrification the livers were removed and homogenized immediately. Liver lysates of 6 animals were separated with 7.5% SDS-PAGE and immunoblotted for IRS-2 and IRS-1 protein.

4.3.3.2   Degradation of endogenous IRS-2

The degradation of IRS-2 was further studied in Fao cells. Insulin-induced a visible decline of endogenous IRS-2 protein after 120 minutes of treatment (Figure 27A) which was paralleled by a decrease of IRS-2 mRNA (Figure 27B). The insulin-induced decrease of IRS-2 protein could be prevented by lactacystin, a specific inhibitor of the proteasome and also by rapamycin, thus by inhibition of mTOR (Figure 27A). Both treatments however, had no effect on the insulin-induced decrease of IRS-2 mRNA (Figure 27B) indicating that the decrease of protein and the decrease of mRNA are distinct processes. Since inhibition of the proteasome resulted in abolished insulin-induced protein decrease it was assumed that the IRS-2 protein was decreased via degradation. The apparent difference of IRS-2 protein levels in untreated cells and that observed after 10 minutes of insulin treatment was a quite consistent phenomenon throughout all experiments in Fao cells. This could be due to a different subcellular distribution and thus accessibility to cell lysis treatment, but experiments designed to clarify that observation failed.

An additional observation was made on the electrophoretic mobility of the IRS-2 protein which was reduced with prolonged insulin stimulation as shown numerous times before, while in the rapamycin treated cells the IRS-2-shift was almost completely abrogated. On the other hand lactacystin treated IRS-2 was not discernible from control regarding the electrophoretic mobility.
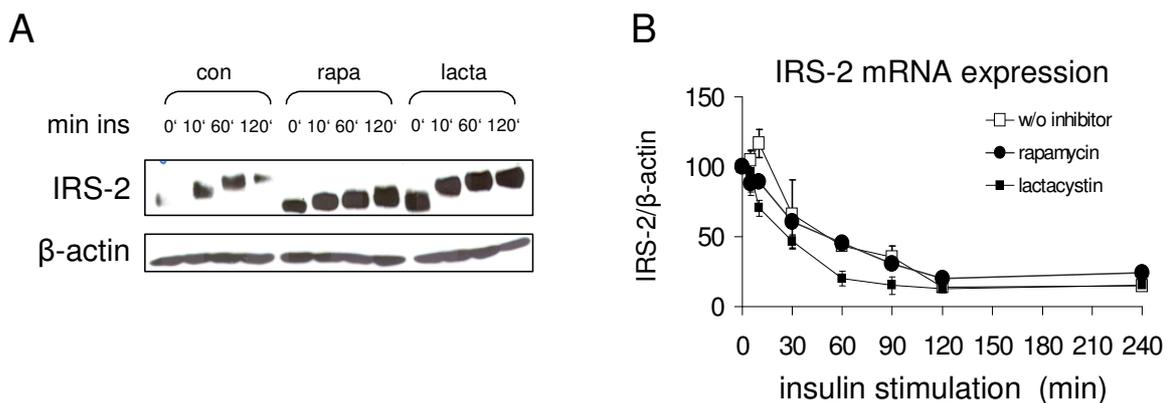
Figure 27 Degradation of endogenous IRS-2 in Fao cells. Cells were treated with either 25 nM rapamycin (rapa) or 10 µM lactacystin (lacta) 30 minutes prior to stimulation with 100 nM insulin (ins) for 0 to 120 (A) or 0 to 240 minutes (B). (A) After stimulation cells were lysed and analyzed with 7.5% SDS-PAGE. A representative immunoblot for IRS-2 is shown. (B) mRNA expression of endogenous IRS-2 normalized to β-actin (mean ± SEM, n=3). Expression in untreated cells was set as 100%.

4.3.3.3 Degradation of ectopically expressed IRS-2

To study the possible involvement of Ser 675 in the insulin-induced IRS-2 protein degradation it was necessary to establish an adequate cell culture model. Since Fao cells could not be used it was attempted to visualize IRS-2 degradation in HEK293 cells stably expressing IRS-2 and the IR. These cells were treated with insulin for 0 to 240 minutes but the IRS-2 protein amount was not reduced (Figure 28A). To exclude that the IRS-2 degradation might be liver tissue dependent the human hepatoma cell line Huh-7 was used. Initially, IRS-2 was transiently transfected into Huh-7 cells and the insulin-induced degradation was studied. As shown in Figure 28B IRS-2 was well expressed, but insulin treatment had no effect on the stability of the protein. To exclude the possibility that the transient transfection was masking certain insulin effects, clones of Huh-7 cells which stably express IRS-2 wt were generated and studied but similar to the other IRS-2-overexpressing cell culture models insulin could not induce IRS-2 protein degradation (Figure 28C).
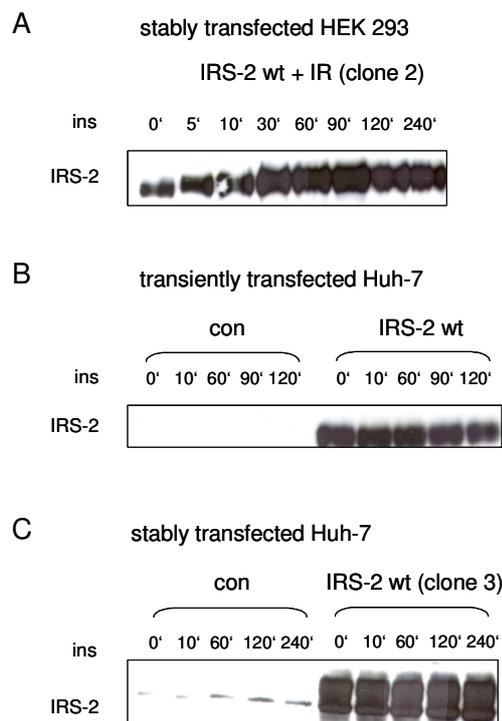
Figure 28 Degradation of ectopically expressed IRS-2 protein. (A) HEK293 cells were stably transfected to express IRS-2 wt and the insulin receptor (IR) and stimulated with 100 nM insulin for 0 to 240 minutes. The lysates were separated by 7.5% SDS-PAGE and immunoblotted for IRS-2 protein. (B) Huh-7 cells were transiently transfected with empty vector control (con) or IRS-2 wt and stimulated with 100 nM insulin (ins) for 0 to 120 minutes. (C) Huh-7 cells were stably transfected to express IRS-2 wt and treated with 100 nM insulin for 0 to 240 minutes.

Based on the results of these experiments it was assumed, that protein degradation of ectopically expressed IRS-2 cannot be visualized due to unregulated overexpression of the protein which is under control of the potent CMV promotor in the expression vectors used in this thesis. To circumvent this problem, cycloheximide (CHX) was used to inhibit protein synthesis and this approach enabled the visualization of IRS-2 degradation. In contrast to Fao cells insulin treatment of HEK293 cells stably expressing IRS-2 + IR or IRS-2 675Ala + IR did not further reduce the half-life of the wt protein (Figure 29A), but the disappearance of IRS-2 was clearly delayed when Ser 675 was mutated to Ala with a comparable reduction after 360 minutes (Figure 29B), supporting the hypothesis that phosphorylation of Ser 675 might be involved in the degradation of the protein.
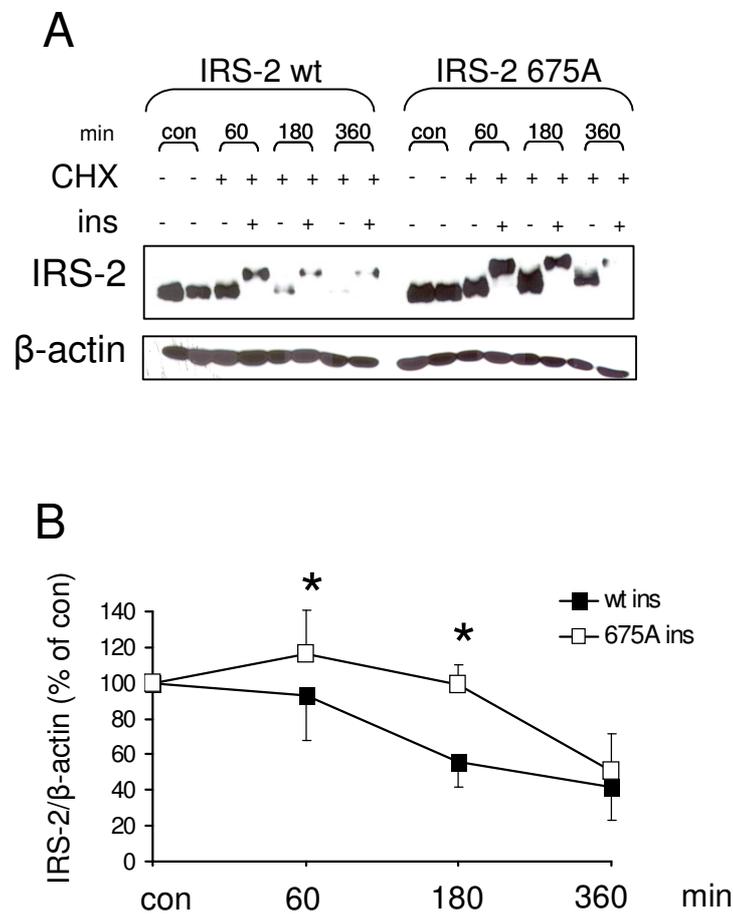
Figure 29 The phosphorylation of Ser 675 influences the degradation of IRS-2. HEK293 cells were stably transfected to express either IRS-2 wildtype (wt) and the insulin receptor (IR) or IRS-2 675 Ala + IR. The cells were treated with 100 nM insulin (ins) and 25 µg/mL cycloheximide (CHX) for the indicated times. (A) After stimulation cells were lysed and analyzed by 7.5% SDS-PAGE. A representative immunoblot for IRS-2 and β-actin is shown. (B) IRS-2 protein was quantified based on scanning densitometry of immunoblots normalized for β-actin (mean ± SEM, n=4, * p<0.05 wt ins vs. 675A ins).

4.3.4 Ubiquitination of IRS-2

Ubiquitination is an important posttranslational modification of proteins which are thereby labeled for proteasomal degradation. As described above insulin induced the proteasomal degradation of endogenous IRS-2 in Fao cells (section 4.3.3.2) and it was therefore attempted to demonstrate an increased ubiquitination of IRS-2. Fao cells were treated 30 minutes with lactacystin followed by insulin stimulation for different time periods and cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against ubiquitin (Figure 30A). Insulin alone did not increase the ubiquitination status of cellular proteins. An increase of ubiquitination was only visible after inhibition of the proteasome by lactacystin and in contrast to previously published data (138) insulin treatment did not increase the ubiquitination status of Fao cells further. To specifically study the ubiquitination of IRS-2 the protein was immunoprecipitated from Fao cells pretreated with lactacystin and stimulated with insulin for 60 minutes. The immunoprecipitates were separated by 7.5% SDS-PAGE and immunoblotted for ubiquitin. Lactacystin clearly enhanced the signal of ubiquitinated IRS-2, but insulin had no additional effect (Figure 30B).

Finally, it was attempted to demonstrate the ubiquitination of ectopically expressed IRS-2 and IRS-2 675 Ala in HEK293 cells to study the effect of Ser 675 phosphorylation on the ubiquitination status of IRS-2. However, this approach failed (data not shown).
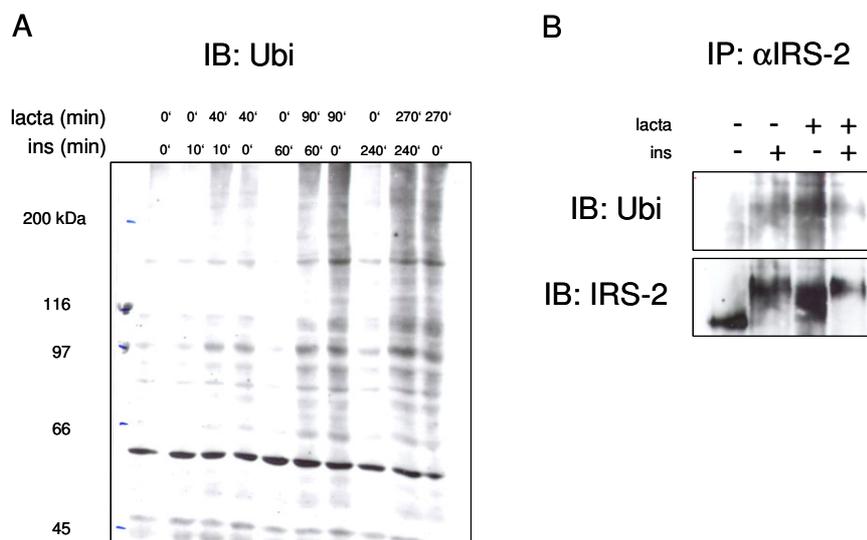
Figure 30 Ubiquitination of IRS-2. (A) Fao cells were pretreated with 10 µM lactacystin (lacta) for 30 minutes and then stimulated with 100 nM insulin (ins) for 0 to 240 minutes. Cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted for ubiquitin (Ubi). (B) Fao cells were treated with 10 µM lactacystin for 30 minutes and 100 nM insulin for 60 minutes. Lysates were immunoprecipitated with a polyclonal IRS-2 antibody and immunoblotted for ubiquitin. The blot was reprobed for IRS-2.

4.4 Biological regulation and relevance of Serine 907 phosphorylation

4.4.1 Characterization of kinases

4.4.1.1 *In silico* amino acid sequence analysis

Ser 907 is located in the C-terminal part of the IRS-2 protein, 4 amino acid residues apart from the only Grb2-binding site of IRS-2 Tyr 911 (Figure 31A). Ser 907 is positioned in a consensus MAP kinase phosphorylation site (*PXpS/pTP*) (230) and the analysis with human protein reference database indicated that this serine residue could be indeed a target of ERK1/2, but also of GSK-3 or of CDK5.

Ser 907 is a conserved site in human, mouse and rat IRS-2 and in accordance to that the phospho-site specific antibody detected phospho-Ser 907 in IRS-2 of all three species (Figure 9B, Figure 13B).

IRS-2 Ser 907 has a homologous serine residue in IRS-1, Ser 887 (mouse IRS-1 amino acid sequence), which has been identified using mass spectrometric analysis of insulin treated HEK293 cells (197) and human muscle biopsies (231) but is otherwise not further characterized. Probably due to the high homology of IRS-1 and -2 in this region, the phospho-site specific Ser 907 antibody also weakly recognized ectopically overexpressed IRS-1 (Figure 13B).

A

Grb2 binding via
SH2 domain

907

^{902} P T E P K **S** P G E *Y* I N I ^{914}

phosphorylation
motif of Erk1/2

B

mouse	^{902} PTEPK**S**PGEYI ^{912}
rat	^{905} PTEPK**S**PGEYI ^{915}
human	^{910} PPEPK**S**PGEYI ^{920}

C

mouse IRS-2	^{902} PTEPK**S**PGEYI ^{912}
mouse IRS-1	^{882} PPEPK**S**PGEYV ^{892}
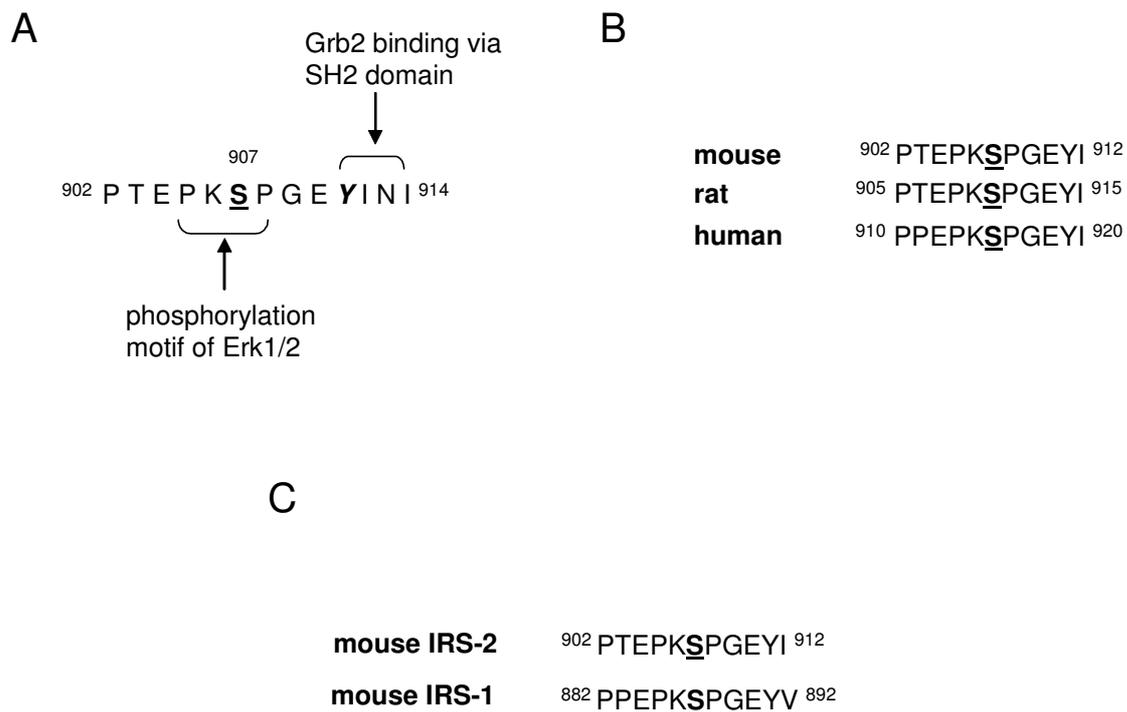
Figure 31 Amino acid sequence of mouse IRS-2 surrounding Ser 907. (A) Phosphorylation and protein binding motifs in mouse IRS-2. (B) Comparison of mouse, rat and human IRS-2. (C) Comparison of mouse IRS-2 and IRS-1.

4.4.1.2   Inhibition of insulin-induced phosphorylation of Ser 907 by pharmacological inhibitors

In order to identify the Ser 907-kinase different pharmacological kinase inhibitors were used. Figure 32A shows the effects of rapamycin, wortmannin and SP600125 on the insulin-induced phosphorylation of Ser 907: neither the inhibition of mTOR, of PI-3 kinase nor JNK had any significant effect on the phosphorylation.

Since short term treatment with TPA produced a Ser 907 phosphorylation similar to insulin (Figure 16B) it was assumed that classical or novel PKC isoforms, which are activated by TPA, could also be involved in the insulin-induced phosphorylation of this site. To test this, Fao cells were treated with 0.1 µM TPA for 24 h to deplete these PKC isoforms (232) and subsequently stimulated with insulin for 0 to 240 minutes. Figure 32B shows exemplarily the protein amount of PKC δ (a novel PKC isoform) which was clearly diminished after the long-term TPA treatment, however, this had no effect on the insulin-induced Ser 907 phosphorylation, arguing against the involvement of PKCs.

Since the sequence analysis (4.4.1.1) revealed that the MAP kinases ERK1 and 2 could potentially phosphorylate Ser 907 the MEK1 specific inhibitor PD98059 was used to prevent ERK1/2 activation. The inhibition of MEK1 resulted in abolished insulin-induced ERK1/2 activation (Figure 32B) and reduced the phosphorylation of Ser 907 clearly.

A

B
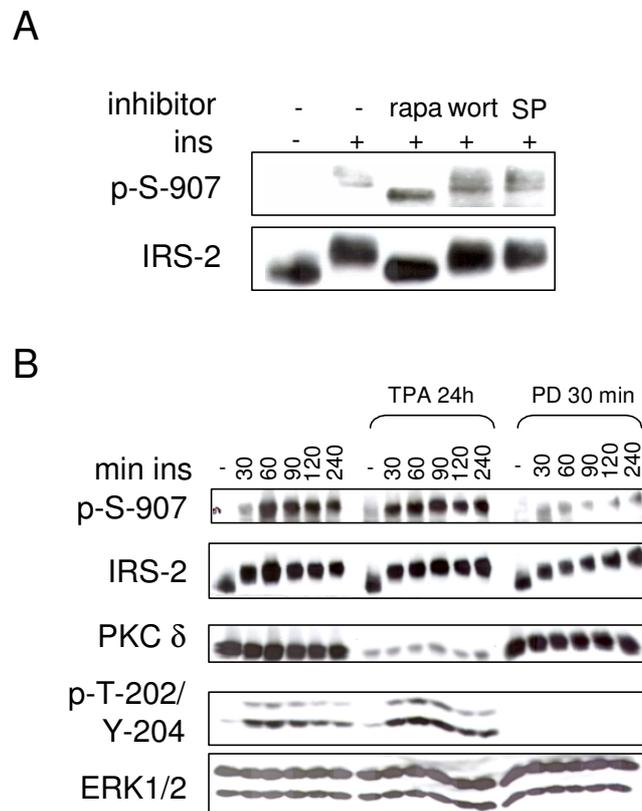
Figure 32 Inhibition of insulin-induced Ser 907 phosphorylation in Fao cells. (A) Cells were treated with either 25 nM rapamycin (rapa), 100 nM wortmannin (wort) or 10 μM SP600125 (SP) for 30 minutes and subsequently stimulated with 100 nM insulin (ins) for 60 minutes. Cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with phospho-site specific Ser 907 antibody and reprobed with IRS-2 protein antibody. (B) Fao cells were pretreated with either 0.1 μM TPA for 24 h or 20 μM PD98059 (PD) for 30 minutes and subsequently stimulated with 100 nM insulin (ins) fro 0 to 240 minutes. Representative immunoblots with site specific Ser 907 antibody, reblot with IRS-2 antibody, PKCδ protein antibody, phospho-ERK 1/2 Thr 202/Tyr 204 antibody and reblot with ERK 1/2 antibody are shown.

4.4.1.3   Inhibition of insulin-induced phosphorylation of Ser 907 by targeting ERK1/2 with siRNA

To investigate the role of ERK1 and 2 more closely, both kinases were knocked down in Fao cells using siRNA oligonucleotides. ERK1 expression could be efficiently reduced but ERK2 protein levels were only decreased by approximately 50% as determined by western blotting

(Figure 33). The knockdown of both kinases resulted in a statistically significant impaired in-sulin-induced Ser 907 phosphorylation, despite the incomplete knockdown of ERK2. These results indicate that ERK1 and 2 are Ser 907 kinases.
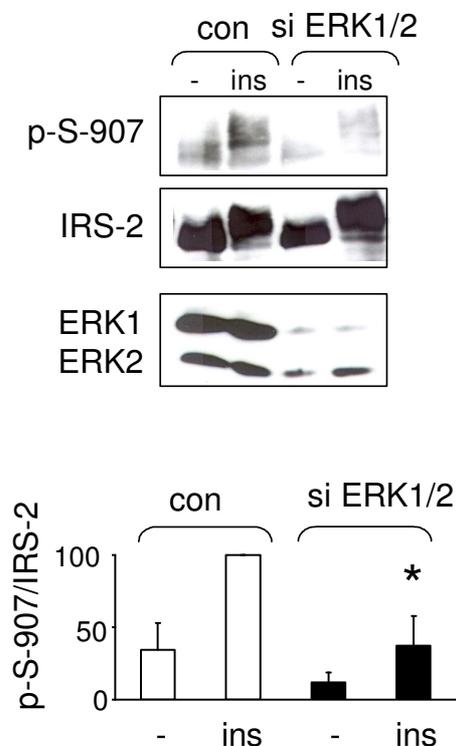
Figure 33 Phosphorylation of IRS-2 Ser 907 after knockdown of ERK1/2 in Fao cells. Cells were elec-troporated with siRNA targeting either ERK1, ERK2 or both and control siRNA (con). 48 h after trans-fection cells were treated with 100 nM insulin (ins) for 30 minutes. Phosphorylation of IRS-2 (Ser 907) with the IRS-2 reblot and total ERK1/2 are shown. Phosphorylation intensity of Ser 907 was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean + SEM, n=4, * $p < 0.05$ con ins vs. si ERK1/2 ins).

4.4.2 Function of Ser 907

To study the function of phospho-serine 907 IRS-2 mutants with an amino acid exchange on position 907 generating either a Ser to Ala or a Ser to Glu mutation were made. In different sets of experiments these mutants were transiently or stably transfected in different cell lines.

4.4.2.1   Transient transfection of BHK cells

In a first step, BHK cells were used to investigate the effects of the IRS-2 907 mutants on the proximal and distal insulin signaling. BHK cells were transiently transfected with IRS-2 wt, IRS-2 907 Ala or IRS-2 907 Glu mutants and stimulated with insulin for 10, 60 and 120 minutes (Figure 34). The tyrosine phosphorylation of IRS-2 was not affected by the 907 Ala mutant, but the Glu mutant, simulating the phosphorylated state, diminished the insulin-induced tyrosine phosphorylation. This effect was statistically significant for time point 10 and 120. However, both mutants had no effect on the distal insulin signaling evidenced by similar phosphorylation patterns of PKB/Akt, GSK-3α and ERK1/2. Only 907 Ala appeared to slightly increase PKB/Akt phosphorylation but this effect never reached statistical significance. Of note, downstream insulin signaling was not affected by the introduction of IRS-2 when compared to the empty vector transfected controls (Figure 34B). This general lack of an IRS-2 mediated effect, as it has already been demonstrated in experiments with Ser 675 (Figure 24), indicated that this cell model was not appropriate to study the function of Ser 907 and therefore stably transfected HEK293 cells were used for further experiments.
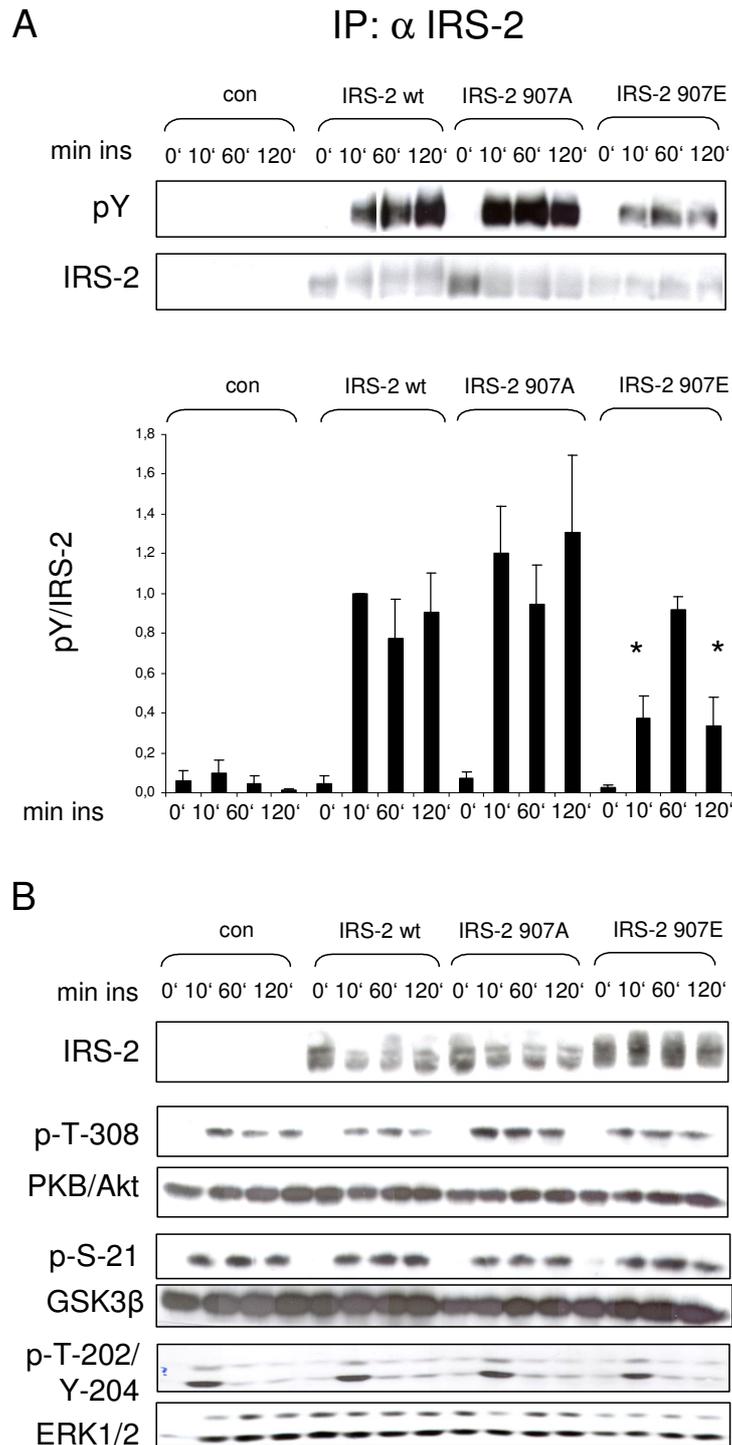
Figure 34 Function of IRS-2 Ser 907 in BHK cells. (A, B) Cells were transiently transfected with empty vector as control (con), IRS-2 wildtype (wt), IRS-2 907 Ala or IRS-2 907 Glu (E) and treated with 100 nM insulin (ins) for 0 to 120 minutes. Lysates were analyzed by 7.5% SDS-Page. (A) IRS-2 was immunoprecipitated (IP) with a polyclonal IRS-2 antibody and the blot was probed for phospho-tyrosine (pY) and reprobed for IRS-2 protein. Quantification of IRS-2 tyrosine phosphorylation was based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean + SEM, n=5). (B) Representative immunoblots for IRS-2 protein, phospho-PKB/Akt Thr 308, phospho-GSKα Ser 21 and phospho-ERK1/2 Thr 202/ Tyr 204 with the corresponding reblots for total protein are shown.

4.4.2.2   Effect of Ser 907 on insulin signaling in HEK293 cells stably overexpressing IRS-2 wildtype and 907 Ala mutant

Since Ser 907 is located in close proximity to Tyr 911, which binds Grb2 after phosphorylation, it was hypothesized that the phosphorylation of Ser 907 could influence the interaction of IRS-2 with Grb2 and probably the activation of ERK1/2 as well. To study the effect of Ser 907 on MAP kinase signaling HEK293 clones were generated which stably express the empty pRK5 vector as control, IRS-2 wt + IR and IRS-2 907 Ala + IR. In addition HEK293 cells which stably express IRS-2 with an amino acid exchange of 911 tyrosine to phenylalanine (911 Phe), which cannot become phosphorylated, were investigated. For the experiments 2 different clones of each cell line expressing IRS-2 wt and IRS-2 mutants were used.

To test whether Ser 907 is influencing the interaction of Grb2 with IRS-2 stable HEK293 clones were treated with insulin and the lysates were immunoprecipitated with a Grb2 antibody and immunoblotted for IRS-2. The association of IRS-2 with Grb2 was clearly and significantly induced by insulin treatment in wt and 907 Ala expressing cells but this interaction was greatly diminished in IRS-2 911 Phe expressing cells (Figure 35A,B). The insulin-induced Grb2 binding to IRS-2 was not different between wt and 907 Ala expressing cells. Of note, the reduced Grb2 binding in 907 Ala clone 3B15 was caused by smaller IRS-2 protein content (Figure 35A).

In the same cells the activation of the MAP kinase pathway was studied. Except for the control cells insulin induced in all cells an ERK1/2 phosphorylation but surprisingly the 911 Phe mutant had no effect on the activation (Figure 35B) despite the clearly reduced IRS-2/Grb2 interaction (Figure 35A). Similarly in 907 Ala expressing cells the ERK1/2 phosphorylation was unaffected indicating that the activation of the MAP kinase pathway was independent of IRS-2. Since in all cells, apart from the controls, the IR was also stably overexpressed it was speculated, that the insulin-induced activation of ERK1/2 was solely dependent on the IR.

Furthermore the insulin-induced IRS-2 tyrosine phosphorylation and association with p85 was investigated. IRS-2 wt and all mutants were tyrosine-phosphorylated to the same extent and the binding of p85 was not different (Figure 36). Of note, the abrogation of one single tyrosine phosphorylation site (Tyr 911) by mutation to phenylalanine had no effect on the overall tyrosine phosphorylation status of the IRS-2 protein (Figure 36). The phosphorylation of PKB/Akt and of its downstream target GSK-3 as well as the activation of p70 S6K1 were also investigated in these HEK293 clones but the results did not indicate a significant effect of the 907 Ala or 911 Phe mutation on these pathways (data not shown).
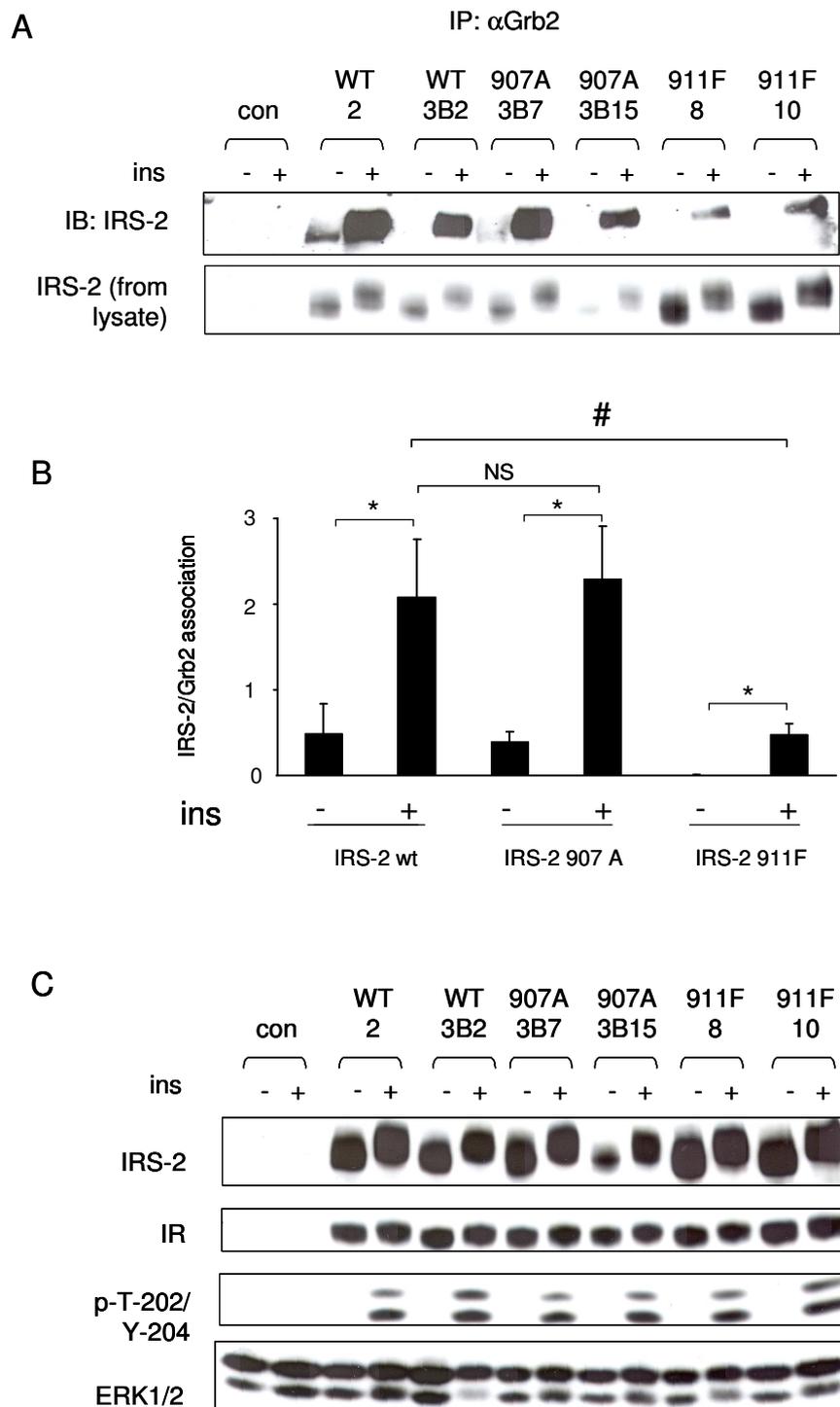
Figure 35 Effect of Ser 907 phosphorylation on insulin-induced activation of the MAP kinase pathway in HEK293 cells. Cells were stably transfected with either empty vector as control (con), IRS-2 wildtype (wt) and insulin receptor (IR), IRS-2 907 Ala + IR or IRS-2 911 Phe (F) + IR and treated with 100 nM insulin (ins) for 10 minutes. (A) Grb2 was immunoprecipitated (IP) with a monoclonal Grb2 antibody and the blot was probed for IRS-2 protein (upper panel). Lysates from the same experiment were additionally separated by 7.5% SDS-PAGE and probed for total IRS-2 protein (lower panel). (B) Quantification of IRS-2 from Grb2 IP with 2 different clones for each cell line. Amount of co-immunoprecipitated IRS-2 was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein from lysates (mean + SEM, n=4, * p<0.05 con vs. ins, # p<0.05 wt ins vs. 911F ins, NS not significant). (C) Lysates were analyzed by 7.5% SDS-Page and representative immunoblots for IRS-2 and IR protein, phospho-ERK1/2 Thr 202/ Tyr 204 with the corresponding reblot for total ERK1/2 are shown.

IP: αIRS-2

	con	WT 2	WT 3B2	907A 3B7	907A 3B15	911F 8	911F 10
ins	- +	- +	- +	- +	- +	- +	- +
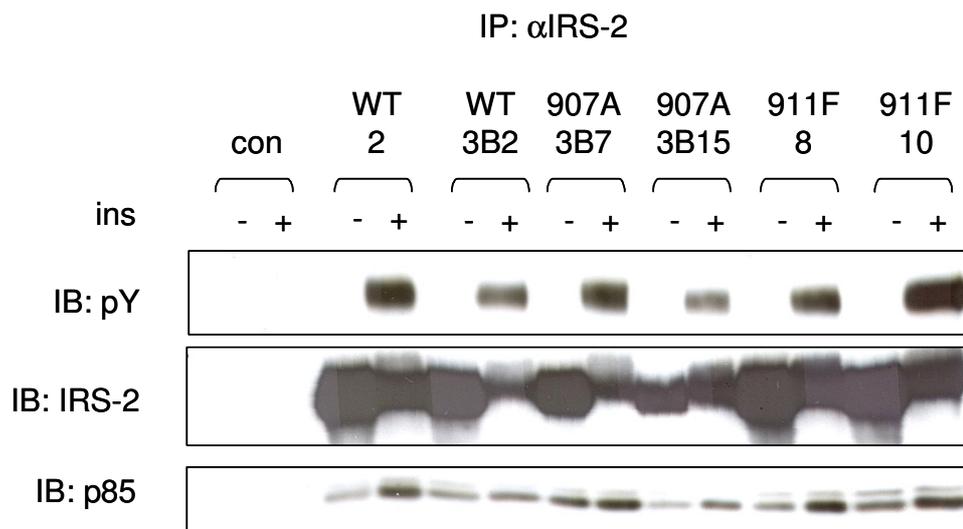
Figure 36 Effect of Ser 907 phosphorylation on insulin-induced tyrosine phosphorylation of IRS-2 in HEK293 cells. Cells were stably transfected with either empty vector as control (con), IRS-2 wildtype (wt) and insulin receptor (IR), IRS-2 907 Ala + IR or IRS-2 911 Phe (F) + IR and treated with 100 nM insulin (ins) for 10 minutes. IRS-2 was immunoprecipitated with a polyclonal IRS-2 antibody and the blot was probed for phospho-tyrosine (pY) (upper panel) and p85 (lower panel) and reprobed for IRS-2 protein (middle panel).

The finding that ERK1/2 activation is apparently independent of IRS-2 protein expression but probably dependent on the IR expression was supported by a number of HEK293 cell lines, which stably overexpress the IR alone or together with IRS-2 wt or the 907 Ala mutant. In these cell lines IRS-2 wt, 907 Ala and IR were stably transfected with different levels of efficiency (Figure 37A, two top panels) leading to pronounced differences in the expression level. Because of this apparent problem these cell lines were not used for further studies. Of note, the problem of uneven expression levels was not encountered with the 675 Ala clone, which had the same expression level of IRS-2 and IR as the wt (Figure 25B).

In these HEK293 cells, the insulin-induced activation of ERK1/2 was completely dependent on the expression of the IR (Figure 37A). Furthermore, ERK1/2 activation was independent of IRS-2 co-expression as in cells that only expressed the IR a strong insulin-induced ERK1/2 phosphorylation was observed (Figure 37A, clone IR11). Of note, HEK293 cells express low levels of IR, but due to short exposure time of the ECL-developed membranes to films, the endogenous IR is not visible.

The reduced electrophoretic mobility, constantly observed in IRS-2 after insulin treatment, was absent in cells without ectopically expressed IR (Figure 37A, clone 3B15), indicating a reduced or even missing posttranslational modification of the protein. The insulin-induced tyrosine phosphorylation of IRS-2 was only detectable in cells that expressed IRS-2 together with the IR (data not shown).

The IR-effect on ERK1/2-activation was further studied in HEK293 cells transiently transfected with different amounts of plasmid-DNA (Figure 37B). 0.25 µg and 2 µg IR plasmid DNA were transfected and the stimulation with insulin led to a strong ERK1/2 phosphorylation comparable to results obtained in stable HEK293 clones. Transfection efficiency was very high and even the small amount of transfected IR plasmid DNA was sufficient to produce high levels of IR protein (Figure 37B, compare lanes 2, 3, 4 and 5). Transfecting the cells with IRS-2 alone had no effect on ERK1/2 activation, only in combination with the IR a robust phosphorylation was observed (Figure 37B, last 4 lanes).

To summarize these data, Grb2 associates with IRS-2 via phospho-tyrosine 911 upon insulin stimulation but this does not affect the activation of the MAP kinase pathway, which is regulated IR-dependently but IRS-2-independently in HEK293 cells.
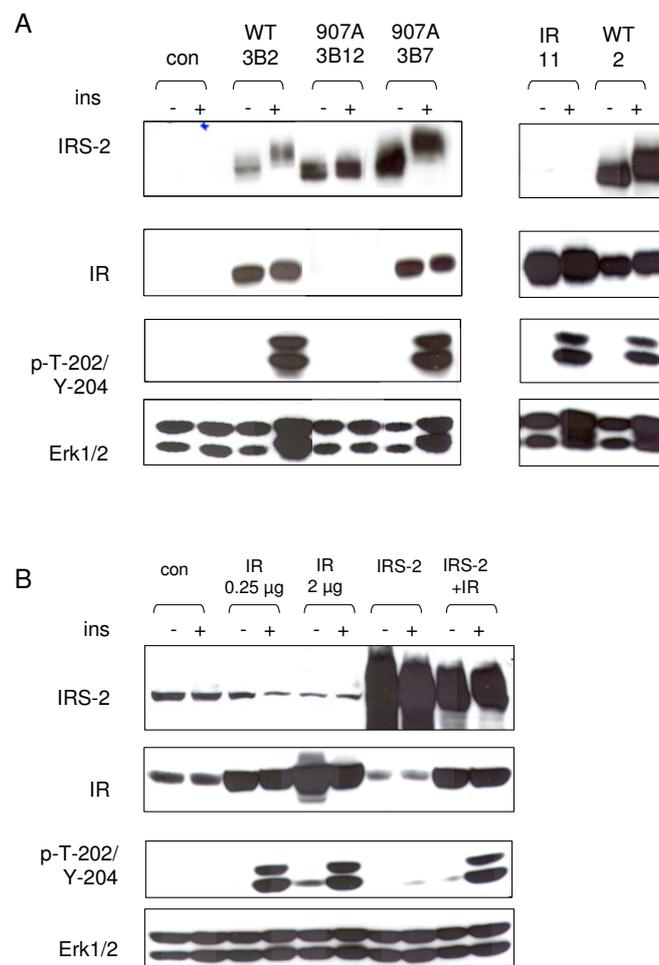
Figure 37 Effect of stable (A) and transient (B) transfection of insulin receptor (IR) on ERK1/2 activation in HEK293 cells. (A) Cells were stably transfected with either empty vector as control (con), IR, IRS-2 wildtype (wt) and/or IRS-2 907 Ala generating several clones and treated with 100 nM insulin (ins) for 10 minutes. Lysates were analyzed by 7.5% SDS-Page and the blot was probed for IRS-2, IR, phospho-ERK1/2 Thr 202/ Tyr 204 and reprobed for ERK1/2 total protein (B) Cells were transiently transfected with IR and or IRS-2 and treated with 100 nM insulin for 10 minutes.

5   Discussion

Numerous studies have shown that IRS-1 serine/threonine phosphorylation is one major regulatory mechanism of insulin and IGF-1 signal transduction. The pathophysiological dysregulation of these phosphorylation events and its connection to the development of insulin resistance and type II diabetes mellitus is well accepted (142). In contrast to IRS-1, only a few serine phosphorylation sites have been identified and characterized in IRS-2 (186;187). In this thesis two novel serine phosphorylation sites were described and their function was studied. To accomplish this task, phospho-site specific monoclonal antibodies have been generated and tested for their specificity. The regulation of these insulin-induced phosphorylations was determined in several cell culture models and *in vivo*. Finally, the potential functions of Ser 675 and Ser 907 were studied in detail.

5.1   Suitability of monoclonal phospho-site specific antibodies

In order to study novel serine/threonine phosphorylation sites of IRS-2 phospho-site specific antibodies had to be generated and tested. The antibodies used in this thesis for the detection of novel phosphorylation sites Ser 675 and Ser 907 were highly suitable because they could only recognize the phosphorylated but not the unphosphorylated respective serine of IRS-2 as demonstrated with the corresponding 675 and 907 alanine mutants (Figure 10). Furthermore, it was shown that the antibodies detect rat (Figure 12), mouse (Figure 15) and human IRS-2 (Figure 14). The p-S-675 antibody showed no cross reactivity with endogenous IRS-1 in Fao cells, despite a high sequence homology in the surrounding region of Ser 675 (Figure 12). And although the antibody for p-S-907 weakly recognized IRS-1 in insulin treated cells, this occurred only in an IRS-1 overexpressing cell model (Figure 13). In contrast, endogenous IRS-1 was not detected by the p-S-907 antibody. From these data it was concluded that the antibodies used in this thesis are highly suitable for the investigation of IRS-2 serine phosphorylation.

Monoclonal antibodies were used in this thesis because they have a number of advantages over polyclonal antibodies. Once the monoclonal antibody-producing hybridoma cell line is established and the properties of the antibody is characterized the antibody can be produced unlimited and with constant quality. In contrast, polyclonal antibodies are produced once and

after the host animal is sacrificed the amount of antibody is limited. Furthermore monoclonal antibodies are specific for one epitope whereas the antisera of immunized host animal contain a mixture of different antibodies which recognize various antigens. This has been demonstrated with the p-S-675 and p-S-907 antibodies used in this thesis, which would not recognize IRS-1 despite a very high sequence homology (Figure 19C and Figure 31C) differing only in one amino acid residue. However, the production of monoclonal antibodies is expensive, time consuming and possibly more laborious than that of polyclonal antibodies but the high and constant quality of monoclonal antibodies render them superior over polyclonal antibodies.

5.2   Serine 675

5.2.1 Phosphorylation of Ser 675

The present data show that IRS-2 is phosphorylated on Ser 675 by a number of different stimuli i.e. insulin, TPA and anisomycin. Insulin is known to activate a large number of serine/threonine kinases including members of the PI-3 kinase/PKB/GSK pathway, the MAP kinase pathway and the mTOR/p70 S6K1 pathway. In addition several PKC isoforms are activated by insulin. TPA is best known as an activator of classical and novel PKC isoforms (233) and also other kinases, e.g. ERK1/2 (234). Anisomycin induces stress activated kinases (SAPK/JNK1-3) (235). All these kinases are known to be involved in the phosphorylation of different serine/threonine residues of IRS-1 (144) and a role for all of these kinases for IRS-2 phosphorylation is very likely.

The phosphorylation of Ser 675 was observed in Fao and BHK cells transiently transfected with mouse and human IRS-2. In addition, the insulin- and TPA-induced phosphorylation was demonstrated in primary human hepatocytes which are an excellent model for *in vivo* conditions since they don't have the constraints of an immortalized cell line. Ser 675 was also strongly phosphorylated in the livers of insulin treated mice, demonstrating the existence of this phosphorylation *in vivo*. Ser 675 phosphorylation was observed in different species (rat, mouse and human) and the insulin-induced phosphorylation of Ser 675 was maximal with 1 nM of insulin, well within the physiological range of insulin concentration suggesting a functional role of this phosphorylation site *in vivo*, which was further demonstrated with feeding-induced phosphorylation of hepatic IRS-2 in wt mice (Figure 15B). The consistent occurrence

of Ser 675 phosphorylation across different species and models suggests a physiological relevance of this site.

To identify the kinase responsible for phosphorylating Ser 675 under physiological conditions the focus was set on insulin as a physiologically relevant stimulus. The inhibition of the PI-3 kinase by wortmannin and of mTOR by rapamycin resulted in a pronounced decrease of insulin-induced Ser 675 phosphorylation indicating a role of the PI-3 kinase/mTOR pathway. The nutrient sensitive kinase mTOR is known as an essential controller of cell growth which integrates insulin/growth factor signals (236) and nutrient signaling (237). mTOR protein is downregulated in livers of fasted mice (238) and the activity of the mTOR/p70 S6K1 pathway is increased in livers of obese rats (134). Via PKB/Akt insulin induces a phosphorylation of mTOR on Ser 2448 which is associated with increased kinase activity (239). Only mTORC1, which is a multiprotein complex consisting of mTOR, raptor, GβL and possibly other members, is sensitive to the inhibition with rapamycin. The mechanism by which rapamycin acts on mTORC1 is not completely understood but it has been proposed that it precludes the complex of mTOR and raptor thus uncoupling mTORC1 from its substrates, without affecting mTOR's intrinsic catalytical activity (240;241). Rapamycin has no effect on the function of mTORC2, a complex comprised of mTOR, Gβl, rictor and Sin1. This second mTOR complex is involved in the regulation of actin remodeling and has been identified as PDK2, which phosphorylates PKB/Akt on Ser 473 (52). mTORC1 phosphorylates and activates p70 S6K1 and inhibits 4E-binding protein 1 (4E-BP1), the repressor of eukaryotic translation initiation factor 4E. The phosphorylation of both mTOR targets results in the initiation of mRNA translation (237) and by that mechanism insulin stimulates cell growth and proliferation.

The strong effect of rapamycin on the insulin-induced phosphorylation of Ser 675 indicates a role for mTORC1 or a mTORC1-dependent kinase for the phosphorylation of this site. Furthermore, Ser 675 is located in a mTOR phosphorylation motif (*pS/pTP*) (66). The involvement of mTOR was further proved by the knock down of mTOR in Fao cells which resulted in impaired insulin-induced phosphorylation of Ser 675. Of note, the silencing of mTOR should have affected the activity of the mTORC2 complex as well, but since the phosphorylation of Ser 675 was highly sensitive to rapamycin treatment, which only affects mTORC1 (242), it could be concluded that mTOR in the mTORC1 complex but not mTORC2 is responsible for the phosphorylation this site.

Furthermore, an actual co-localization of IRS-2 and mTOR in Fao cells could be demonstrated with co-immunoprecipitation experiments (Figure 22). It was demonstrated that mTOR does not directly associate with IRS-2 but is directed to its substrate via raptor which binds a region upstream of Ser 675, termed Shc and IRS-1 NPXY binding (SAIN) domain, which is actually the PTB domain (243;244). The association of mTOR with IRS-2 was constitutive and

the same has been demonstrated for the association of mTOR with IRS-1 in 3T3-L1 adipocytes (160) and myotubes (130). The concomitant stimulation of Fao cells with insulin and rapamycin seemed to decrease the association of IRS-2 and mTOR (Figure 22) probably due to inhibition of the association of raptor and mTOR leading to dissociation of mTOR from IRS-2. However, this does not explain the association of mTOR and IRS-2 still present in rapamycin treated cells particularly in the absence of insulin. It could be speculated that insulin induces other regulatory mechanisms that lead to the reduced association of mTOR and IRS-2 in rapamycin treated cells, maybe via mTOR phosphorylation on Ser 2448 or other phosphorylation sites but this hypothesis needs further clarification.

Insulin activates mTOR and this leads to protein synthesis and cell growth but this kinase also exerts a physiological feedback regulation on the level of the IRS-proteins by increasing the phosphorylation of serine/threonine residues and increasing the degradation of these proteins thus turning off the insulin signal (245). In hyperinsulinemic, insulin resistant states however the pathological increased activation of mTOR/p70 S6K1 is frequently associated with insulin resistance by inhibition of IRS protein function via serine phosphorylation (132;160;163;246), translocation (247) and degradation (123;125;164;165). Especially Ser 632 which is the homologous Ser residue of IRS-1 to Ser 675 of IRS-2, has been shown to be a target of mTOR (130;131;160;244) and the chronic inhibition of mTOR with rapamycin in rats results in reduced hepatic IRS-1 Ser 632 phosphorylation after refeeding (248).

Ser 632 of IRS-1 is not only a target of mTOR but could also be phosphorylated by the activation of p70 S6K1 (161;164) and of the MAP kinases (159). However, the involvement of p70 S6K1 in Ser 675 phosphorylation is not likely, since the knock down of this kinase in Fao cells had no effect on the insulin-induced phosphorylation of this site and Ser 675 is not located in a p70 S6K1 consensus phosphorylation motif (*RXRXXpS)* (249). Nevertheless, experiments with anisomycin in conjunction with inhibition of mTOR by rapamycin showed, that IRS-2 Ser 675 is also phosphorylated independently of mTOR, indicating a role of other kinases (Figure 23). This finding was further demonstrated by the inability of rapamycin to affect the electrophoretic mobility of IRS-2 in anisomycin treated cells proving that pathways other than the mTOR/p70 S6K1 pathway lead to phosphorylation of Ser 675. A likely candidate for another Ser 675 kinase are the JNK isoforms, which are activated by TNFα and anisomycin. Despite the lack of a TNFα effect on Ser 675 phosphorylation in Fao cells, which for unknown reasons did also not activate JNK, it could be speculated, that under circumstances that activate these kinases a phosphorylation of Ser 675 might be observed. This is particularly important as TNFα is connected to insulin resistance by serine/threonine hyperphosphorylation of IRS-1 (151;250) and the strong anisomycin effect on Ser 675 phosphorylation could indicate that in insulin resistant states Ser 675 might also be phosphorylated. The effect of TNFα should be investigated in a suitable model in the future.

Of note, other stimuli like hyperglycemia (131) and high concentrations of amino acids in combination with hyperinsulinemia (251) have been shown to be involved in mTOR-dependent phosphorylation of mouse IRS-1 Ser 632. Nutrient excess and obesity are connected to insulin resistance and type II diabetes mellitus (252) and indeed, the phosphorylation of human IRS-1 at Ser 636 is upregulated in primary culture of skeletal muscle cells from type II diabetic patients (159) and in livers of mice fed a high fat diet (161). Furthermore, the overactivation of the mTOR/p70 S6K1 pathway in liver and muscle of high fat diet fed rats has been linked with increased Ser 636 phosphorylation (134). The role of nutrient excess for the phosphorylation of IRS-2 Ser 675 has not been addressed in the course of this thesis but could be investigated *in vivo* in the future, for instance in a model of high fat diet-fed mice.

The phosphorylation of Ser 675 was shown to be a late event of insulin signaling occurring after 30 to 60 minutes of Fao cell stimulation (Figure 18A). This kind of phosphorylation kinetic has been described for several serine sites of IRS-1: Ser 636 of human IRS-1 is phosphorylated maximally after 30 minutes of insulin stimulation in CHO cells overexpressing the IR and IRS-1 (182). The phosphorylation of IRS-1 Ser 307 was maximal after 15 to 30 minutes in 32D cells expressing the IR and IRS-1 protein (174), in 3T3-L1 adipocytes (160) and in L6 myotubes (185). These phosphorylation kinetics are in contrast to the rapid phosphorylation of IRS-1 and -2 on tyrosine residues, which occurs within 2 minutes after insulin stimulation (37;77;104;229;253) and which is the prerequisite for downstream signal transduction. Ser 302, a phosphorylation site associated with positive regulation of insulin signaling, is already strongly phosphorylated 5 minutes after insulin treatment (185). In general, early IRS-phosphorylation events might therefore be associated with positive regulation while a late phosphorylation of serine residues might rather indicate negative regulation. Accordingly, the late phosphorylation of Ser 675 could imply this site as a negative regulator of insulin signaling.

5.2.2 Degradation of IRS-2 and potential involvement of Ser 675

The findings in this thesis suggest that Ser 675 phosphorylation might be involved in IRS-2 degradation, which is an important feature of IRS-2 regulation. The fast degradation of IRS-2 protein in the liver after the onset of refeeding has been described in mice (90) and it has been speculated that this is the prerequisite to shift the insulin signaling towards IRS-1, which is suspected to be dominant for the hepatic nutrient homeostasis in the postprandial state (91) and in this context IRS-2 downregulation via degradation seems to be essential. Moreover, the IRS proteins show diminished protein expression in liver and muscle in different

animal models of obesity, insulin resistance and diabetes (134;254-256). It is therefore of great interest to understand and possibly modulate the mechanisms responsible for the insulin-induced degradation of the IRS proteins.

The present data show that Ser 675 is phosphorylated by mTOR and the inhibition of this kinase led to abolished insulin-induced proteasomal degradation of endogenous IRS-2 in Fao cells (Figure 27) and (138)). Furthermore, in livers of refed mice a robust Ser 675 phosphorylation was observed concomitantly with a pronounced IRS-2 protein decrease possibly suggesting the relevance of this site for degradation. To investigate the precise function of Ser 675 in IRS-2 degradation the insulin-induced degradation of wt IRS-2 and the non phosphorylatable 675 Ala mutant was investigated in transiently and stably transfected cell models. This proved to be difficult due to the strong and unregulated expression of ectopic IRS-2, which was under the control of the CMV promotor. To circumvent this problem cycloheximide was used to monitor the half life and insulin-dependent IRS-2 degradation. Wildtype IRS-2 was reduced by about 50% after 180 minutes, similar to data obtained for IRS-1 (137), but an additional effect of insulin was not visible. However, under these conditions the IRS-2 675 Ala mutant had a prolonged half life (Figure 29), indicating a role of Ser 675 for the stability of IRS-2.

In order to degrade proteins by the proteasome they need to be labeled with ubiquitin, a small 8.5 kDa protein (257;258). Ubiquitination is a three step process: The ubiquitin-activating enzyme E1 activates the C-terminal glycine of the ubiquitin molecule. Following activation, an enzyme from the E2 ubiquitin-conjugating enzyme family transfers the ubiquitin molecule to a member of the ubiquitin-protein ligase family (E3) which specifically binds the substrate protein. E3 catalyzes the covalent attachment of ubiquitin to its substrate. Ubiquitin is bound to the ε-NH2 group of a lysine residue of the target protein to form an isopeptide bond. The 26-S proteasome recognizes poly-ubiquitinated proteins for degradation, therefore the attachment of more ubiquitin molecules to lysine 48 of the previously conjugated ubiquitin molecule is catalyzed. Suppressor of cytokine signaling (SOCS) 1 and 3, which are involved in negative feedback regulation of Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signaling, have been shown to facilitate the binding of IRS-1 and -2 to elongin BC ubiquitin ligase, a member of the large family of E3 ubiquitin ligases and are thereby involved in the degradation of IRS-1 and IRS-2 (129). Another E3-ubiquitin ligase is Cul7, which specifically targets serine phosphorylated IRS-1 (137) for proteasomal degradation. The involvement of the mTOR/p70 S6K1 pathway for this IRS-1 phosphorylation was demonstrated, in particular the combined phosphorylation of serine residues 307, 312, 527 and 636/639 (corresponding to human IRS-1) (137).

A sequence scan of IRS-2 with UBPred, a software for the prediction of ubiquitination sites (259), indicated that two lysines (Lys 667 and Lys 683) flanking Ser 675 have a probability for ubiquitination. It can be speculated that the phosphorylation of IRS-2 on Ser 675 is a prerequisite for the ubiquitination of either or both of these lysine residues thus facilitating the proteasomal degradation. The prerequisite of phosphorylation for subsequent ubiquitination has been suggested for IRS-1 (125;260) (261) and reported for other proteins (262;263). An attempt to demonstrate a differential ubiquitination pattern of wt IRS-2 and 675 Ala in stably transfected HEK293 cells failed, however, a literature screening showed, that ubiquitination is usually demonstrated by co-expression of the protein of interest together with ubiquitin (129) and therefore, it might be necessary to co-express ubiquitin to demonstrate the regulation of IRS-2-ubiquitination.

Studies that aimed to prove the relevance of the phosphorylation of the homologous Ser 632 (mouse numbering) for IRS-1 degradation failed (131;137), while the importance of mTOR and mTOR-dependent, but also mTOR-independent serine phosphorylation could be demonstrated (125;164;248;264;265), namely the involvement of Ser 307 (266). Therefore, it can be suggested that the mTOR-dependent phosphorylation of other yet unknown residues of IRS-2 and further mTOR dependent effects despite serine phosphorylation of IRS-2 are also important for the degradation of IRS-2 protein. This hypothesis is supported by the drastic effects of rapamycin on the electrophoretic mobility of IRS-2 (Figure 27A): mTOR inhibition not only inhibits proteasomal degradation but almost completely prevents the IRS-2 shift usually seen with insulin treatment, thus mTOR inhibition leads to reduced posttranslational modification of IRS-2, most likely serine/threonine phosphorylation of several other sites, which remain yet to be identified.

Of note, the investigation of IRS-2 phosphorylations in other insulin responsive tissues was beyond the scope of this thesis but a relevance of Ser 675 in other tissues is very likely. IRS-2 is of great importance for the survival of β-cells, shown by the diabetic whole body IRS-2 knock out mouse in which β-cell hyperplasia in response to insulin resistance is abrogated (79). The diabetes of these IRS-2 knock out mice could be cured by transgenic islets expressing IRS-2 (81). Agents that improve the expression or stability of IRS-2 should therefore be beneficial for the survival of β-cells in diabetic patients and inhibition of Ser 675 phosphorylation could aid in the stability of IRS-2 protein.

5.2.3   Effect of Ser 675 on the IRS-2-association with IR and PI-3 kinase

The serine/threonine phosphorylations of IRS-1 have been shown to exert hindrance effects on the association of IRS-1 and the IR and of IRS-1 and p85 respectively. The phosphorylation of Ser 24 in the PH domain of IRS-1 was shown to influence the association with the IR negatively (267). And the phosphorylations on Ser 302/307/612 have been shown to negatively influence the association of IRS-1 with p85 (268). Since Ser 675 is adjacent to a p85 binding motif (**pY$_{671}$MPM**), an interaction with the binding of p85 appears likely. For the homologous Ser 632 and the neighboring Ser 635 of IRS-1 the reduced binding of p85 has been previously described (131). However, the association of p85 and IRS-2 was not affected in IRS-2 675 Ala expressing cells (Figure 25A) despite the close proximity of Ser 675 to Tyr 671. Moreover, the phosphorylation of Ser 675 appeared not to influence the association of IRS-2 with the IR as determined by IRS-2 tyrosine phosphorylation, despite the localization of Ser 675 in the KRLB domain. However, the region of the KRLB domain which binds directly to the IR (residues 602-637) (100) is not in close proximity to Ser 675 and phosphorylation of Ser 675 might therefore have no direct effect on the IRS-2-IR interaction.

In conclusion, the phosphorylation of Ser 675 does not appear to have direct effects on insulin signal transduction, but might play a role in the degradation of the protein.

5.3   Serine 907

5.3.1   Phosphorylation of Ser 907

The phosphorylation of Ser 907 was induced in cell culture by insulin, TPA and anisomycin indicating a potential involvement of kinases of the PI-3 kinase- and the MAP kinase pathway but also of PKC isoforms and stress activated kinases. The intensity of the Ser 907 phosphorylation was dependent on the insulin concentration and 100 nM of insulin induced a maximal response in Fao cells. This is in contrast to Ser 675, which was phosphorylated already at low nM insulin concentrations. Nevertheless, Ser 907 was also phosphorylated *in vivo* after insulin treatment and, more importantly, after refeeding as well. The phosphorylation after 5 hours of refeeding was not as pronounced as that of Ser 675 but statistically significant (Figure 15), indicating a physiological relevance of the Ser 907 phosphorylation.

As for Ser 675, the insulin-induced phosphorylation proved to be a late event of insulin signaling, occurring after 60 minutes of insulin treatment of Fao cells. As discussed for Ser 675 in section 5.2.1 this late phosphorylation could indicate a role in negative regulation of insulin signaling.

To identify the kinase which phosphorylates Ser 907 upon insulin treatment several kinase inhibitors were used. The phosphorylation was abrogated in cells treated with the MEK1 inhibitor PD98059, suggesting ERK1/2 (or an ERK1/2-dependent kinase) as a likely candidate. Despite an incomplete knock down of ERK2 using siRNA oligonucleotides, the insulin-dependent Ser 907 phosphorylation was clearly impaired after the silencing of both ERK-isoforms. Moreover, since Ser 907 is located in an ERK1/2 consensus phosphorylation motif (*PXpS/pTP* (230)), it was concluded that this is indeed the kinase responsible for the phosphorylation Ser 907 after insulin treatment.

The possible involvement of other insulin-activated kinases could not be demonstrated with the pharmacological inhibitors used in this thesis. GSK-3 was proposed by the human protein reference database (219) but the involvement of this kinase is not very likely, since it phosphorylates the motif *pSXXXpS* (269) in which the serine in position +5 needs prior phosphorylation by a priming kinase. However, Ser 907 is not located in such a motif.

Anisomycin treatment strongly phosphorylated Ser 907 suggesting a role for SAPK/JNK. However, these kinases were only weakly activated in response to insulin and the inhibition of JNK with SP600125 did not interfere with the insulin dependent phosphorylation of Ser 907. The focus of this thesis was to identify the kinase responsible for phosphorylation of Ser 907 after insulin stimulation, which is the physiological stimulus, therefore the possible involvement of JNK, which is frequently activated in pathological states like insulin resistance (151) was not studied further.

The serine kinase ERK1/2 has been recognized as an IRS-1 kinase as it phosphorylates Ser 612 (160;270) and has been shown to phosphorylate Ser 632 in primary human skeletal muscle cells of type 2 diabetic patients (159). The ERK1/2-dependent IRS-1 phosphorylation has been involved in negative regulation of insulin signaling and in the context of insulin resistance the phosphorylation of Ser 612 is increased in skeletal muscle of mice fed a high fat diet compared to control animals (268). The activation of the MAP kinase pathway has been shown to facilitate the negative feedback regulation of insulin signaling on the level of IRS-1 serine phosphorylation (156;270-272).

5.3.2 Function of Ser 907

IRS-2 has been shown to be crucial for the transduction of the insulin signal into the MAP kinase pathway in different tissues. In L6 myotubes (273) and human myotubes (274) insulin signals predominantly through IRS-2 into the MAP kinase pathway as the silencing of IRS-2 in these cells resulted in ablated insulin-induced ERK1/2 activation. In the β-cells IRS-2 has been shown to be crucial for development and proliferation (84) and IGF-1 signaling into the MAP kinase pathway was restored in IRS-2 overexpressing β-cells from IRS-2 knock out mice (81). In the liver a tissue specific knock out of IRS-2 in mice strongly reduced insulin-induced ERK1/2 phosphorylation (87), while a liver specific knock down of IRS-2 by only 70% had no effect on the MAP kinase signaling (88) suggesting that the remaining IRS-2 protein levels are sufficient to maintain downstream signaling. These studies showed that IRS-2 is an important mediator of the insulin/IGF-1 signal into the MAP kinase pathway in different tissues.

Tyr 911 of IRS-2 is part of a consensus Grb2 binding site (*pYl/VN* (24)) and in contrast to the numerous possible p85 binding sites of IRS-2 it is the only one for Grb2. Although there are no reports on the actual role of Tyr 911 of IRS-2 on the association with Grb2 the binding of Grb2 to IRS-1 is facilitated solely via Tyr 891 (mouse IRS-1 amino acid sequence), which is the homologous tyrosine residue in IRS-1 (275). Since Ser 907 is located in close proximity to Tyr 911 it was hypothesized that phosphorylation of this serine residue influences the interaction of IRS-2 with Grb2 and/or the phosphorylation of Tyr 911. ERK1/2 dependent phosphorylation of activators of the MAP kinase pathway has been shown to exert negative feedback control: Grb2 associated binder 2 (Gab2) phosphorylation downregulates its interaction with SHP-2, which is a component of the MAP kinase activating pathway induced by IL-2 in T-lymphocytes (276) and ERK1/2 is also involved in the negative regulation of Grb2 and Sos association (277). Furthermore, the close proximity of phospho-serines to SH2-domain binding motifs has been shown to negatively modulate the interaction of SH2 domain-containing proteins with phospho-tyrosine residues (131;168;270). Therefore it would be possible that phosphorylation of Ser 907 reduces the binding of Grb2 to the phosphorylated Tyr 911 motif of IRS-2 thus leading to an attenuation of the insulin-induced activation of the MAP kinase pathway.

The functional role of Ser 907 was studied in stably transfected HEK293 cells. Experiments with different clones revealed that the co-expression of the IR and IRS-2 was necessary for the insulin-induced tyrosine phosphorylation of IRS-2 (Figure 36). Surprisingly, the strong insulin-induced activation of the MAP kinase pathway was only dependent on the expression of IR (either transiently or stably (Figure 37)) but not on the presence of IRS-2 which had no

effect whatsoever on this pathway, suggesting that the IR is the limiting factor for the activation of the MAP kinase pathway. This finding could be explained by studies showing that Shc is the predominant adapter protein for the IR --> MAP kinase signal transduction in rat fibroblasts (278). Shc competes with IRS proteins for binding of the Grb2-Sos complex (279). While IRS-2 possesses only one Grb2-binding site (*pY$_{911}$INI*), Shc has at least two (*pY$_{317}$VNV, pYpY$_{239/240}$NDF*) with Tyr 317 being of major importance for the interaction with Grb2 (280). Shc has therefore more possibilities to associate with Grb2 molecules from a limited pool than IRS-2. Furthermore, Shc and IRS-2 not only compete for Grb2 but also for the activated IR, since they both bind to the same *NPEpY$_{960}$* motif in the juxtamembrane region of the IR (Figure 38) (281;282). In the experimental setting used in this thesis however ectopic IRS-2 was greatly overexpressed and the number of IRS-2 molecules should exceed the number of Shc molecules by far and a displacement of Shc by IRS-2 would be expected. This was apparently not the case as IRS-2 did not influence MAP kinase activation. An explanation of this finding could be that the HEK293 cells are of human origin as is the ectopically co-expressed IR, but the co-expressed IRS-2 was of murine origin. The IR might therefore preferably bind human Shc than IRS-2 of mouse origin. However arguing against this hypothesis is the high homology of human and murine IR and the strong IRS-2 tyrosine phosphorylation observed after insulin treatment (Figure 36). The decision whether the insulin signal is transduced into the MAP kinase pathway via IR --> Shc or IR --> IRS might also be tissue dependent.

Another possible explanation for the apparently IRS-2-independent signaling into the MAP kinase pathway is provided by another substrate of the IR: Gab1 (Figure 38). This molecule shares structural homology with the IRS-proteins, especially in the PH domain (25), which is essential for its localization to the plasma membrane where it becomes phosphorylated on several tyrosine residues in the C-terminal part by the activated IR (45). Gab1 does not possess a PTB domain, therefore a direct binding to the IR is not likely, at least not via IR Tyr 960. A number of phosphorylated tyrosine residues facilitate the binding to SH2-domain containing proteins, most notably SHP-2 (45). Several studies demonstrated that Gab1 acts via SHP-2 to control the activation of ERK1/2 (283;284), however, the precise molecular events that lead to the ERK1/2 activation exerted by the phosphatase/adapter protein SHP-2 are not completely dissolved (49). Nevertheless, the IR-->Gab1-->SHP-2 pathway provides an alternative to the IR-->Shc--> or the IR-->IRS pathway for the activation of ERK1/2 (Figure 38). The importance of Gab1 for the activation of ERK1/2 was shown by the liver specific knock out of this adapter protein in mice causing an abolished insulin-induced ERK1/2 phosphorylation (271). The involvement of Gab1 provides an explanation for the IR-dependent but IRS-2-independent activation of the MAP kinase pathway observed in stable HEK293 clones.
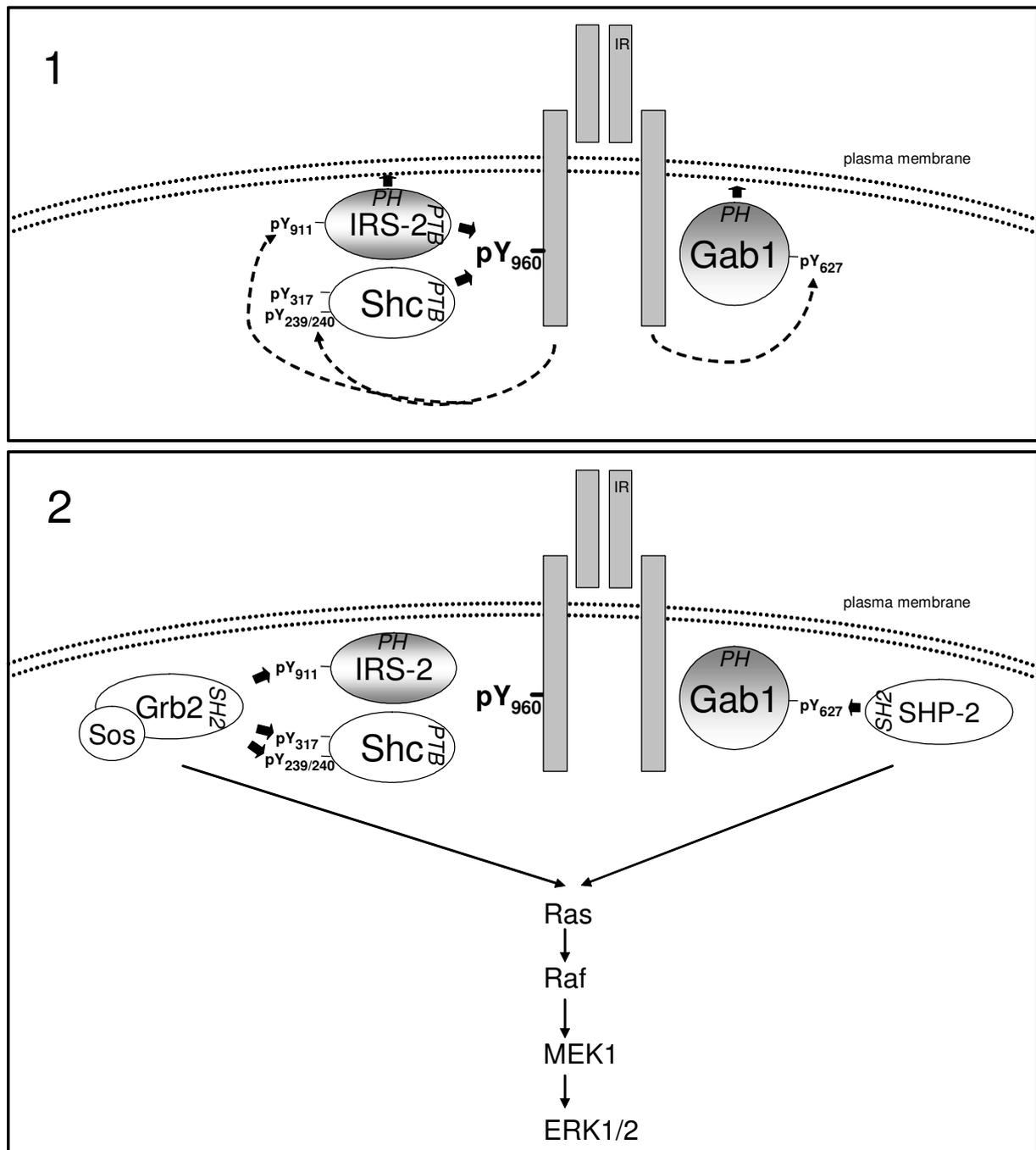
Figure 38 IR-dependent activation of the MAP kinase pathway. **1** Phospho-Tyr 960 of the activated IR is bound by IRS-2 and Shc via their PTB domains. IRS-2 and Gab1 associate with their PH domains on the plasma membrane in close proximity of the IR. After appropriate localization and binding of adapter molecules the IR phosphorylated several Tyr residues of IRS-2, Shc and Gab1. **2** These phospho-Tyr residues are now recognized and bound by Grb2 or SHP-2 via SH2 domains. This interaction leads subsequently to the activation of the Ras→ Raf→ MEK1→ERK1/2 signaling cascade. Abbreviations: Gab1, Grb2 associated binder 1; Grb2, growth factor receptor bound 2; PH, pleckstrin homology; PTB, phosphotyrosine binding; pY, phospho-tyrosine; SH2, src homology 2; bold arrows indicate binding of domains; dashed arrows indicate phosphorylation; thin arrows indicate activation.

Nevertheless, IRS-2 does associate with Grb2 in cell culture (Figure 35A) and *in vivo* (77) and the interaction was clearly abrogated in IRS-2 911 Phe expressing HEK293 cells (Figure 35A, B). However the introduction of the 907 Ala mutant had no effect on the Grb2-IRS-2 interaction demonstrating no role of this site for the interaction of IRS-2 with Grb2 in the cell culture model used in this thesis. It was also attempted to specifically study the effect of the Ser 907 phosphorylation on the phosphorylation of Tyr 911, but the commercial antibody against the homologous phospho-Tyr 891 of IRS-1 was not specific for phospho-Tyr 911 of IRS-2 as tested with IRS-2 911 Phe mutant despite the high homology of these two sites (data not shown).

From the data obtained with IRS-2 907 Ala + IR transfected cells it is evident that the phosphorylation of Ser 907 has no effect on the association of IRS-2 with Grb2. The role of IRS-2 for the insulin-induced activation of the MAP kinase pathway appears to be dependent on several determinants such as the cell culture model and the expression levels of the participating signaling proteins.

5.4   Limitations of cell culture models used in this thesis

In this thesis the phosphorylation of IRS-2 on serine residues 675 and 907 has been clearly demonstrated in different cell culture models and primary hepatocytes. The phosphorylations occurred in livers of refed as well as insulin treated mice and both phosphorylations were observed in different species. In order to elucidate the responsible IRS-2 kinases and the function of both sites by alanine mutants studies had to be performed in cell culture. Unfortunately each of the used model systems had certain limitations.

The BHK cells were easily transfectable and showed appropriate responses to insulin stimulation, however an IRS-2 effect on downstream insulin signaling was not observed. Only a weak effect of both phospho-sites on IRS-2 tyrosine phosphorylation was noted, which however did not confer to downstream signaling events.

A very good cell culture model of liver metabolism is the Fao rat hepatoma cell line. These cells express large amounts of IR and IRS-1 and -2 (Figure 16) and therefore respond well to physiological concentrations of insulin (107). Furthermore Fao cells could be used for metabolic studies, for instance regulation of gluconeogenesis (112;285-287) and lipid metabolism (288;289). However, the large endogenous IRS-2 protein amount hinders the study of ectopically expressed mutant IRS-2 proteins as they would likely derange insulin signaling and a discrimination between effects of wt and mutant IRS-2 might not be possible. In addition, Fao

cells express little amounts of IGF-1 receptor thus precluding the study of this effector of IRS protein dependent signaling.

The Huh-7 cell line was used as an alternative for the Fao cell line. These cells were easily transfectable but due to the problem of unregulated IRS-2 overexpression and failure to demonstrate IRS-2 degradation the work with these cells was discontinued.

HEK293 cells are a widely used cell line since they can be transfected with high efficiency and the establishment of clones stably overexpressing the protein of interest is easy. Therefore HEK293 cells were used to study the effects of IRS-2 mutants on proximal and distal insulin signaling. In the course of this thesis it became evident that the effect of IRS-2 on the MAP kinase pathway could not be studied due to solely IR-dependent activation of this pathway. However, the insulin-induced degradation of IRS-2 was demonstrated clearly by use of cycloheximide.

All models presented limitations making it difficult to draw absolute conclusions from the obtained data. The findings of this thesis should be ideally confirmed with adequate *in vivo* models, namely by use of transgenic mice bearing IRS-2 mutants with alanine substitutions of Ser 675 and Ser 907. The usage of such transgenic mouse models expressing IRS-1 mutants have been recently described (177;268). The transgenic muscle-specific overexpression of skeletal muscle IRS-1 with mutations of serine residues 302, 307 and 612 to alanine resulted in partial protection against high fat diet induced muscular insulin resistance by improved IRS-1 tyrosine phosphorylation (268). The effect was not attributed to the overexpression of IRS-1 which was shown by control animals with muscular IRS-1 wt overexpression. From this study it was concluded that the phosphorylation of these three serine residues per se mediate muscular insulin resistance and that these phosphorylation are not a mere side-effect of this pathological condition. However, this model is somewhat problematic as it used the alanine mutant of Ser 302, a site which is actually connected to positive regulation of insulin signaling (179) but the effect of Ser 302 might have been superimposed by the other two phosphosites. Another transgenic mouse model investigated the effect of Ser 307 on whole body insulin signaling. These mice expressed IRS-1 with a mutation of Ser 307 to alanine instead of wt IRS-1 and the animals exhibited a very surprising phenotype. In contrast to data frequently obtained in cell culture (151;152;170;174) which place Ser 307 as a negative feedback regulator of insulin signaling and connect it to insulin resistance due to its phosphorylation by JNK, a kinase strongly upregulated in insulin resistant states, these mice were not protected against high fat diet-induced insulin resistance and showed defective insulin signaling in muscle and cultured primary hepatocytes. The Ala 307 expressing mice developed an even more severe insulin resistance, indicating that Ser 307 in mice is a positive regulatory site. This surprising result shows that it is crucial to confirm all *in vitro* data with appropriate *in vivo* models.

Creating such a transgenic mouse is difficult and time consuming. An attractive alternative to a transgenic *in vivo* model could be the use of primary mouse hepatocytes from the existing IRS-2 knock out mouse (79) and the transfection with the IRS-2 alanine mutants as it has been shown with the reconstitution of IRS-2 in such an primary hepatocyte culture (290). This would give valueable information about the effect of the serine phosphorylation sites on hepatic metabolism and insulin signaling. An even more elegant approach is the transfection of the IRS-2 knock out mouse with the desired IRS-2 mutants. This is possible via tail vein injection of "naked DNA", which leads to expression in the liver (291) or even liver tissue-specific adenoviruses carrying the plasmids of interest (292).

Furthermore, it should be kept in mind that phosphorylation of single serine residues is not a unique event, but activation of insulin-regulated kinases rather result in an array of multiple serine/threonine phosphorylations, which may even be phosphorylated interdependently (185). It is possible that Ser 907 has a function together with other yet undefined serine/threonine residues for instance other sites that are phosphorylated in the late phase of insulin signaling as Ser 907 itself. The investigations of such "phosphorylation-arrays" is challenging, particularly *in vivo* and to date only one report studied the *in vivo* phosphorylation pattern of IRS-1 after insulin infusion using mass spectrometric analysis methods (231). With this method the detection of many more serine/threonine site of IRS-2 is very likely and might provide the basis for understanding and treatment of insulin resistance. To elucidate the regulation of insulin signal transduction by these phosphorylation patterns however is even more challenging and appears not to be possible *in vivo* in the near future.

5.5   Conclusion

The understanding of insulin signal regulation and its dysregulation in pathological states is crucial for the development of therapeutical strategies to treat insulin resistance and type 2 diabetes. Hyperphosphorylation of serine/threonine residues of IRS-1 has been recognized as possible drug target (293) and the same could be expected for IRS-2. Therefore the thorough investigation of novel serine/threonine phosphorylation sites like the ones presented in this thesis is of great importance. The modulation of certain IRS-Ser/Thr kinases and the inhibition of IRS phosphatases (294) has been investigated in regard to the improvement of insulin signaling. The use of salicylates has been shown to protect IRS-1 from Ser/Thr phosphorylation in TNFα treated cells (149) and the downregulation of JNK has been shown to improve hepatic glucose tolerance in obese *db/db* mice (295). However, the mere inhibition of single kinases *in vivo* might have undesirable effects like it has been recently demonstrated with

chronic inhibition of mTOR by rapamycin in rats which resulted in insulin resistance despite an improved activation of the IRS/PI-3 kinase/PKB axis (248). This was due to previously unknown inhibitory effects of mTOR on the hepatic gluconeogenesis which were abrogated under rapamycin treatment. In conclusion, an exact understanding of IRS-1 and -2 serine/threonine phosphorylation and the regulation and function of the responsible kinases but also of the complex interplay of these phosphorylation sites is needed to obtain new treatment options for insulin resistance and type 2 diabetes mellitus. For this purpose the present work, providing a thorough study on the phosphorylation of two novel serine phospho-sites of IRS-2, is a next step in this direction indicating similarities but also distinct regulatory mechanisms for IRS-1 and IRS-2. The basic understanding of phosphorylation of Ser 675 and Ser 907 is the prerequisite for further investigations regarding the function of both sites *in vivo* for normal and pathologically impaired insulin signaling.

6   Summary

Insulin receptor substrate (IRS) proteins are major transducers of the insulin and IGF-1 signal into the PI-3 kinase/PKB and the MAP kinase pathway. In addition to tyrosine phosphorylation, a large number of serine/threonine phosphorylation sites enable the IRS proteins to integrate different extra- and intracellular stimuli resulting in positive and negative modulation of the insulin and IGF-1 signal. Chronic hyperphosphorylation of serine/threonine sites of IRS-1 is involved in the development of insulin resistance. IRS-2 is of great importance for β-cell survival and for the regulation of hepatic metabolism. The study of serine/threonine phosphorylations is required to understand the physiological and pathophysiological regulation of this important mediator of insulin signaling. In this thesis two novel IRS-2 serine phosphorylation sites have been identified and characterized (mouse amino acid numbering): Ser 675, which is located in the kinase regulatory loop binding (KRLB) domain unique to IRS-2 and Ser 907, which is adjacent to the Grb2 binding site Tyr 911. Using phospho-site specific antibodies both sites were demonstrated to be phosphorylated upon insulin, phorbol ester and anisomycin treatment in Fao rat hepatoma cells. The phosphorylation was also detected in primary human hepatocytes and in liver tissue of insulin treated or refed mice.

The insulin-induced phosphorylation of Ser 907 was mediated by the MAP kinase ERK1/2. Simulation of a permanent phosphorylation of this site in BHK cells expressing IRS-2 Glu 907 led to a slight decrease of IRS-2 tyrosine phosphorylation with no apparent effect on insulin downstream signaling. The insulin-induced association of IRS-2 with Grb2 in HEK293 cells was abrogated by mutation of the adjacent Tyr 911 to Phe, but not influenced by mutation of Ser 907 to Ala. Of note, the activation of MAP kinase signaling was not impaired in HEK293 cells expressing IRS-2 Phe 911 and not regulated by the expression level of IRS-2 wildtype, but completely dependent on IR expression, indicating the importance of an alternative, IRS-2-Grb2-independent pathway for the activation of MAP kinase signaling in these cells.

The insulin-induced phosphorylation of Ser 675 was dependent on mTOR, but not on the downstream kinase p70 S6K1. Prevention of this phosphorylation in BHK cells or HEK293 cells expressing IRS-2 Ala 675 had no effect on proximal or distal insulin signal transduction. But compared with IRS-2 wildtype, the mutated IRS-2 protein Ala 675 showed increased half life in cycloheximide-treated HEK293 cells. Thus, phosphorylation of Ser 675 could have a similar function as its homologous site Ser 632 in IRS-1 and could be involved in the regulation of mTOR-dependent IRS-2 proteasom-mediated protein degradation.

7 Zusammenfassung

Die Insulin Rezeptor Substrate (IRS) sind bedeutende Vermittler des Insulin- und des IGF-1-Signales in den PI-3 Kinase- und den MAP Kinase-Signaltransduktionsweg. Zusätzlich zu Tyrosinphosphorylierungen ermöglichen eine große Anzahl an Serine/Threonin-Phosphorylierungsstellen der IRS-Proteine die Integration von verschiedenen extra- und intrazellulären Stimuli was zu einer positiven und negativen Modulation des Insulin- und IGF-1 Signales führt. Chronische Hyperphosphorylierungen der Serin/Threonin-Reste des IRS-1 sind an der Entstehung von Insulinresistenz beteiligt. IRS-2 hat eine besondere Bedeutung für das Überleben der β-Zellen sowie für die Regulation des hepatischen Metabolismus. Die Untersuchung der Serin/Threonin-Phosphorylierungen ist die Voraussetzung um die physiologische und pathophysiologische Regulation dieses wichtigen Vermittlers des Insulinsignales zu verstehen. In dieser Doktorarbeit wurden zwei neue IRS-2 Phosphorylierungsstellen identifiziert und charakterisiert (Maus IRS-2 Aminosäure Nummerierung): Ser 675, das in der IRS-2-spezifischen kinase regulatory loop binding (KRLB) Domäne liegt und Ser 907, das in der Nähe eines Grb2-Bindungsmotives (Tyr 911) liegt. Mit phospho-site-spezifischen Antikörpern wurden beide Phosphorylierungen in Fao Ratten Hepatoma-Zellen nach Insulin-, Phorbolester- und Anisomycinstimulation nachgewiesen. Die Phosphorylierungen wurden auch in primären humanen Hepatocyten sowie in Lebergewebe von insulinbehandelten oder gefütterten Mäusen detektiert.

Die insulininduzierte Phosphorylierung von Ser 907 wurde durch die MAP Kinase ERK1/2 vermittelt. Die Simulation einer permanenten Phosphorylierung dieses Serinrestes in BHK-Zellen, die IRS-2 Glu 907 transient exprimierten, führte zu einer leichten Abnahme der IRS-2-Tyrosinphosphorylierung hatte aber keinen offensichtlichen Einfluss auf die nachgeordneten Insulin-Signaltransduktionskaskaden. Die insulininduzierte Bindung von Grb2 an IRS-2 war in HEK293 Zellen durch Mutation des benachbarten Tyrosin 911 zu Phenylalanin aufgehoben, wurde jedoch durch eine Mutation von Serin 907 zu Alanin nicht beeinflusst. Die Aktivierung des MAP Kinase Signalweges in IRS-2 Phe 911-überexprimierenden HEK293 Zellen war jedoch nicht beeinträchtigt und war auch nicht induziert durch die Überexpression des Wildtyp IRS-2. Allerdings war die Aktivierung dieses Signalweges vollständig abhängig von der Höhe der Insulinrezeptor-Expression, was auf einen alternativen, IRS-2-Grb2-unabhängigen Signalweg bei der Aktivierung der MAP Kinase hindeutet.

Die insulininduzierte Phosphorylierung von Ser 675 war abhängig von mTOR, jedoch nicht von seiner nachgeschalteten Kinase p70 S6K1. Die nicht-phosphorylierbare Ser 675 Ala

Mutante, exprimiert in BHK und HEK293 Zellen, hatte keinen Einfluss auf die proximale oder distale Insulin-Signalweiterleitung. Allerdings hatte das mutierte IRS-2 675 Ala Protein eine verlängerte Halbwertszeit in Cycloheximid-behandelten HEK293 Zellen. Die Phosphorylierung von Ser 675 könnte daher eine ähnlich Funktion haben wie das homologe Ser 632 im IRS-1 und an der mTOR-abhängigen proteasomalen IRS-2 Proteindegradation beteiligt sein.

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9   Supplement

9.1   Abbreviations

A	alanine
Ala	alanine
APS	adapter protein with a PH and SH2 domain
ATF3	activating transcription factor 3
BHK	baby hamster kidney cells
BSA	bovine serum albumin
cAMP	cyclic adenosine-monophosphate
CAP	Cbl associated protein
Cbl	Casitas b-lineage lymphoma
cFOS	FBJ murine osteosarcoma viral oncogene homolog
CHX	cycloheximide
CREBP	cAMP response element binding protein
C_T	crossing point
CMV	cytomegalie virus
DNA	desoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
E	glutamine
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ERK1/2	extracellular signal-regulated protein kinase 1/2
F	Phenylalanine
FAS	fatty acid synthase
FCS	fetal calf serum

FFA	free fatty acids
FoxO1/A2	forkhead box O1/ A2
G6Pase	glucose-6-phosphatase
Gab1	Grb associated binder-1
GAP	GTPase activating protein
GDP	guanosine-diphosphate
GIP	glucose dependent insulinotropic polypeptide
Glu	glutamate
GLP	glucagon-like peptide
GLUT4	glucose transporter 4
Grb2	growth receptor bound 2
GS	glycogen synthase
GSK	glycogen synthase kinase
GTP	guanosine-triphosphate
h	hour(s)
HEK293	human embryonal kidney cells
HPLC	high pressure liquid chromatography
HRP	horseraddish peroxidase
IGF	insulin-like growth factor
INFγ	interferon γ
IP	immunoprecipitation
IR	insulin receptor
IRE	insulin response element
IRS	insulin receptor substrate
i.v.	intravenous
JM	juxtamembrane
JNK	c-jun-N-terminal kinase
KRLB	kinase regulatory loop binding
Lys	lysine
MAP kinase	mitogen activated protein kinase
MEK1	MAP kinase-ERK kinase 1
mTOR	mammalian target of rapamycin

mTORC1/2	mTOR complex 1/2
NFκB	nuclear factor κB
O-GlcNAc	*O*-linked β-N-acetylglucosamine
p	phospho
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDK	Phosphoinositide-dependent protein kinase
PEG	polyethylene glycol
PEPCK	phosphoenolpyruvate carboxykinase
PH	pleckstrin homology
Phe	phenylalanine
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PI-3 kinase	phosphatidylinositol-3 kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospho-lipase C
PTB	phosphotyrosine binding
RT	reverse transcriptase
S	serine
S6K 1	S6 ribosomal protein kinase 1
SAPK	stress activated protein kinase
SCD	stearoyl-CoA desaturase
SDS-PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
Ser	serine
SH2	Src homology 2
Shc	Src homology s/α-collagen-related protein
SHIP2	SH2-containing inositol phosphatase 2
SOCS	suppressor of cytokine signaling
SREBP	sterol regulatory element binding protein
T	threonine
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine

TF	transcription factor
TFE3	transcription factor E3
Thr	threonine
TNFα	tumor necrosis factor α
TSC	tuberous sclerosis complex
Tyr	tyrosine
TPA	12-O-tetradecanoylphorbol 13-acetate
Y	tyrosine

9.2 Declaration/Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig durchgeführt habe und bei der Verfassung der Arbeit keine anderen Quellen und Hilfsmittel als die angegebenen verwendet habe, sowie wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Ich habe bisher an keinem in- und ausländischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende Arbeit oder eine andere Dissertation vorgelegt.

(Louise Fritsche)

Tübingen, 15.06.2010

9.3   Acknowledgement

9.4   Curriculum vitae

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08/1992 – 07/1999	Humboldt-Gymnasium Potsdam, 1999 Abitur (Note 1,4)
10/2000 – 12/2005	Studium der Ernährungswissenschaft an der Universität Hohenheim
06/2005 – 12/2005	Diplomarbeit: „Detection and improvement of fat soluble vitamin status in patients with short bowel syndrome using two different supplement formulations"
2005	Diplom-Ernährungswissenschaflterin (Note 1,0)
08/2006 – 07/2009	Promotionsstipendium des Internationalen Graduiertenkollegs 1302 (DFG): The PI3K Pathway in Tumor Growth and Diabetes
08/2006 – 08/2010	Doktorarbeit: "Identification and functional studies of two novel serine phosphorylation sites of insulin receptor substrate (IRS)-2: Ser 675 and Ser 907"

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05/2004 – 11/2005	Universität Hohenheim, Institut für Biologische Chemie und Ernährung, Wissenschaftlische Hilfkraft, Labor
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9.5   List of publications

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