

## Insulin-induced serine phosphorylation of IRS-2 via ERK1/2 and mTOR: studies on the function of Ser<sup>675</sup> and Ser<sup>907</sup>

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**Fritsche L, Neukamm SS, Lehmann R, Kremmer E, Hennige AM, Hunder-Gugel A, Schenk M, Häring H, Schleicher ED, Weigert C.** Insulin-induced serine phosphorylation of IRS-2 via ERK1/2 and mTOR: studies on the function of Ser<sup>675</sup> and Ser<sup>907</sup>. *Am J Physiol Endocrinol Metab* 300: E824–E836, 2011. First published November 23, 2010; doi:10.1152/ajpendo.00409.2010.—The identity of specific serine phosphorylation residues of insulin receptor substrate (IRS)-2 and their impact on insulin signal transduction are largely unknown. Ser<sup>675</sup> and Ser<sup>907</sup> of mouse IRS-2 are adjacent to PI 3-kinase or Grb2 binding domains, respectively. Using monoclonal phosphosite-specific antibodies, we demonstrated the phosphorylation of both serines after stimulation of FaO hepatoma cells with insulin, anisomycin, or phorbol esters. Phosphorylation of both sites was a late and prolonged event during insulin treatment and was also detected in liver tissue of insulin-treated as well as refed mice. Inhibition and siRNA-mediated knockdown of ERK1/2 indicated that the insulin-induced phosphorylation of Ser<sup>907</sup> was ERK dependent. Phosphorylation of Ser<sup>907</sup> did not prevent the insulin-induced association of IRS-2 with Grb2, but phosphorylation of the adjacent Tyr<sup>911</sup> was proved to be crucial in HEK 293 cells expressing IRS-2 Ala mutants. The insulin-induced phosphorylation of Ser<sup>675</sup> was prevented by inhibition and siRNA-mediated knockdown of mTOR but not of p70<sup>S6K1</sup>. Mutation of Ser<sup>675</sup> to Ala did not affect downstream insulin signaling but increased the half-life of the protein, suggesting an involvement of phospho-Ser<sup>675</sup> in an accelerated degradation of IRS-2. Moreover, the insulin-induced degradation of IRS-2 was blocked by inhibition of mTOR. We conclude that the two novel insulin-dependent serine phosphorylation sites of IRS-2 were not involved in the regulation of the adjacent PI 3-kinase and Grb2 binding domains but might be implicated in the ERK- and mTOR-mediated negative feedback control.

insulin receptor substrate 2; extracellular signal-regulated kinase 1/2; mammalian target of rapamycin

THE INSULIN RECEPTOR SUBSTRATE (IRS) proteins are a family of adaptor proteins that are crucial for the distribution, amplification, and termination of the insulin and IGF-I signal (49). The most prominent IRS isoforms are IRS-1 and IRS-2, and their importance has been demonstrated with different knock-out mouse models. The lack of IRS-1 results in impaired somatic growth with a mild insulin-resistant phenotype (3, 47). IRS-2 deficiency leads to severe insulin resistance and type 2 diabetes mellitus due to  $\beta$ -cell failure (28, 61). Both IRS-isoforms show distinct tissue distribution with IRS-1 being the

predominant IRS-protein in the muscle and adipose tissue, while IRS-2 is most important in the  $\beta$ -cells (60) and the detrimental whole body knockout of IRS-2, which results in overt diabetes, has been cured with transgenic islets expressing IRS-2 (20). The complete knockout of IRS-2 also suggested a prominent role of IRS-2 in hepatic metabolism (34, 50), and the liver-specific deletion of IRS-2 indicates that IRS-1 and IRS-2 have rather complementary functions rather than one or the other being the dominant isoform in the liver (14, 48). Recent data imply that the transient upregulation of IRS-2 expression during fasting and in the early postprandial state regulates the complementary function of IRS-1 and IRS-2 in hepatic insulin signaling (27).

The distribution of the insulin signal via the PI3K/PKB cascade and the ras/MAP kinase pathway toward the numerous downstream effectors is regulated by posttranslational modifications of IRS-1 and IRS-2. The phosphorylation on tyrosine residues (19) upon insulin receptor activation is the prerequisite for interaction with downstream signaling partners containing an src homology region 2 (SH2) domain, most importantly the p85 subunit of PI 3-kinase (PI3K) (5, 32, 45) and the adaptor protein Grb2 (43, 55). In addition, many kinases phosphorylate IRS-1 on a large number of different serine/threonine residues. These phosphorylations regulate the interaction of IRS-1 with the insulin receptor (1), with serine/threonine kinases such as PKC (56, 59) or their intracellular distribution and degradation (41, 46). Most of the phosphoserine/threonine sites of IRS-1 are implicated in the downregulation of the insulin signal leading to insulin resistance when IRS-1 serine/threonine kinases are chronically activated (7, 25). Some reports also demonstrate that this modification can enhance insulin signal transduction (11, 30, 57). Beyond the regulation of the intensity and duration of the insulin effects the serine/threonine phosphorylation of IRS proteins might determine the diversification of the insulin signal by modifying the docking sites for protein interactions (59).

IRS-1 and IRS-2 share 40% of sequence homology with a unique kinase regulatory loop binding domain (KRLB) in IRS-2 (39), which appears to be involved in limiting the tyrosine phosphorylation of this protein (62). The modification of IRS-1 by serine/threonine phosphorylation has been extensively studied, and the importance for the regulation of physiological and pathophysiological functions of IRS-1 is undoubtful. Considerably less is known about the serine/threonine phosphorylation of IRS-2. In vitro kinase assays using PKC $\zeta$  have shown that, despite the high homology of serine residues, substrate specificity of serine kinases toward IRS-1

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and IRS-2 might exist (29). Thr<sup>348</sup>, which is a functional homolog to Ser<sup>307</sup> of IRS-1, has been identified as an *in vitro* target of JNK (44), and the use of IRS-2 alanine mutants indicated the sequential phosphorylation on Ser<sup>484</sup> and Ser<sup>488</sup> by JNK and GSK-3 (42). The concomitant mutation of five putative PKC-dependent phosphoserine sites to alanine (serines 303, 343, 362, 381, 480) proved to be protective against cytokine-induced apoptosis in murine  $\beta$ -cells (18). To the best of our knowledge, no site-specific serine/threonine phosphorylation of IRS-2 has been demonstrated in cell culture models or *in vivo* using phospho-site-specific antibodies.

We developed monoclonal phosphospecific antibodies of IRS-2 based on a screening for potential serine/threonine phosphorylation sites with online available bioinformatic tools. After a prescreening for positive hybridoma clones, we focused on Ser<sup>675</sup> and Ser<sup>907</sup> (corresponding to mouse IRS-2 sequence), since both sites are in close proximity to functional tyrosine residues: Ser<sup>675</sup> lies within the IRS-2-specific KRLB domain and is adjacent to a PI3K binding motif (pY<sub>671</sub>MXM), and Ser<sup>907</sup> is adjacent to the Grb2 binding domain of IRS-2 (pY<sup>911</sup>INI) (45, 62). We studied the regulation of these phosphorylations in hepatoma cells and primary human hepatocytes and *in vivo* in mouse liver tissue and used serine-to-alanine (loss-of-function) mutants to investigate their insulin-regulated function.

## MATERIALS AND METHODS

**Materials.** Fao rat hepatoma cells and BHK cells were from The European Collection of Cell Cultures (ECACC), HEK 293 cells were from ATCC (Wesel, Germany). Cell culture media and supplements were from LONZA (Basel, Switzerland). Rapamycin, SP-600125, anisomycin, insulin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and cycloheximide were obtained from Sigma (Munich, Germany). G-418 was from Biochrom (Berlin, Germany). Wortmannin, PD-98059, and lactacystin were from Calbiochem (Schwabach, Germany). Human recombinant TNF $\alpha$  was from R&D Systems (Wiesbaden-Nordenstadt, Germany). Phosphatase inhibitor mix contained 10 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium orthovanadate, and 10 mM  $\beta$ -glycerophosphate from Sigma (Munich, Germany). Protease inhibitor cocktail (Complete) was obtained from Roche (Mannheim, Germany). Protein A-sepharose was from GE Healthcare Europe (Munich, Germany). Mouse IRS-2 coding sequence was kindly provided by M. F. White (Boston, MA).

IRS-2 protein antibody (06-506), IRS-1 protein antibody (06-248) and p85 antibody (06-195) were from Millipore (Schwalbach, Germany), the Akt/PKB protein and PKC $\delta$  antibody were from BD Transduction laboratories (Erembodegem, Belgium). Antibodies against phosphotyrosine, phospho-Akt/PKB Thr<sup>308</sup> and Ser<sup>473</sup>, phospho-p70<sup>S6K1</sup> Thr<sup>389</sup>, p70<sup>S6K1</sup>, phospho-p44/42 (ERK1/2) Thr<sup>202</sup>/Tyr<sup>204</sup>, p44/42 (ERK1/2), phospho-IRS-1 Ser<sup>1101</sup>, and  $\beta$ -actin were from Cell Signaling Technology (Frankfurt, Germany). The antibody against mTOR/FRAP1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against Grb2 was from Abcam (Cambridge, UK).

Rat monoclonal phosphospecific IRS-2 antibodies were generated by immunization of Lou/C rats with peptides corresponding to the mouse IRS-2 sequence surrounding Ser<sup>675</sup> and Ser<sup>907</sup> (Fig. 1) by use of standard procedures (33). IRS-10 5E4 (rat IgG1) and IRS-14 9C12 (rat IgG2a) are used in this study.

**Cell culture, transfection, and treatments.** Fao cells were kept in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. For experiments the cells were starved overnight in FCS-free medium and subsequently treated with various substances as described in RESULTS. Primary human hepato-

mouse	670	DYMPM\$ <u>S</u> P <sub>675</sub> TSVS	680
rat	671	DYMPM\$ <u>S</u> P <sub>675</sub> TSVS	681
human	674	DYMPM\$ <u>S</u> PASVS	684
mouse	902	PTEPK\$ <u>S</u> PGEYI	912
rat	905	PTEPK\$ <u>S</u> PGEYI	915
human	910	PPEPK\$ <u>S</u> PGEYI	920
mouse IRS-2	670	DYMPM\$ <u>S</u> P <sub>675</sub> TSVS	680
mouse IRS-1	627	DYMPM\$ <u>S</u> PKSVS	637
mouse IRS-2	902	PTEPK\$ <u>S</u> PGEYI	912
mouse IRS-1	882	PPEPK\$ <u>S</u> PGEYV	892

Fig. 1. Amino acid sequence surrounding Ser<sup>675</sup> and Ser<sup>907</sup>. *Top*: comparison of mouse, rat, and human IRS-2. *Bottom*: amino acid sequence of the peptides used to generate phosphospecific antibodies and corresponding amino acid sequence of mouse IRS-1. The unphosphorylated peptides are shown.

cytes were isolated from liver tissue obtained from therapeutic hepatectomies using a two-step collagenase perfusion protocol as described (40). The cells were plated onto collagen, cultured in Hepatocyte Maintenance Medium (Provitro, Berlin, Germany), 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 nonessential amino acids. All other cell lines were cultured in DMEM with 4.5 g/l glucose supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Cells were serum starved overnight in the same medium as the growth medium but without FCS. BHK cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Twenty-eight hours after transfection, cells were starved in serum-free medium overnight and treated with 100 nM insulin for various time points. HEK 293 cells were stably transfected to express wild-type and mutated IRS-2 proteins using Lipofectamine, and stable clones were selected with G418.

**Site-directed mutagenesis.** Mutation of serine sites of mouse IRS-2 to alanine were generated using the Stratagene QuikChange XL Site-Directed Mutagenesis method (Stratagene, La Jolla, CA). Positive clones were verified by sequencing.

**Cell lysis, immunoprecipitation, and Western blotting.** Cells were lysed in 175–300  $\mu$ l of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerin, 1% Triton X-100, containing protease and phosphatase inhibitors) per well. Total protein (100–250  $\mu$ g) was used for immunoprecipitation. Immunoprecipitated proteins or 100  $\mu$ g of protein of total extracts were separated by SDS-PAGE (7.5%), and Western blot analysis was performed as described elsewhere (58).

**Gene expression.** Total RNA was extracted from cells by use of an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (1  $\mu$ g) was used for reverse transcription-PCR with random hexamer primers using the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Expression of rat IRS-2 and rat  $\beta$ -actin was measured by real-time quantitative PCR on a Light Cycler 480 System (Roche) using FastStart DNA-MasterSYBR Green I (Roche) or the QuantiFast SYBR Green PCR Kit (Qiagen). For  $\beta$ -actin the following primer pair was used: sense, AGC CAT GTA CGT AGC

CAT CC; antisense, CTC TCA GCT GTG GTG AA. For IRS-2 the QuantiTect primer assay Rn\_IRS2\_1\_SG was used (Qiagen).

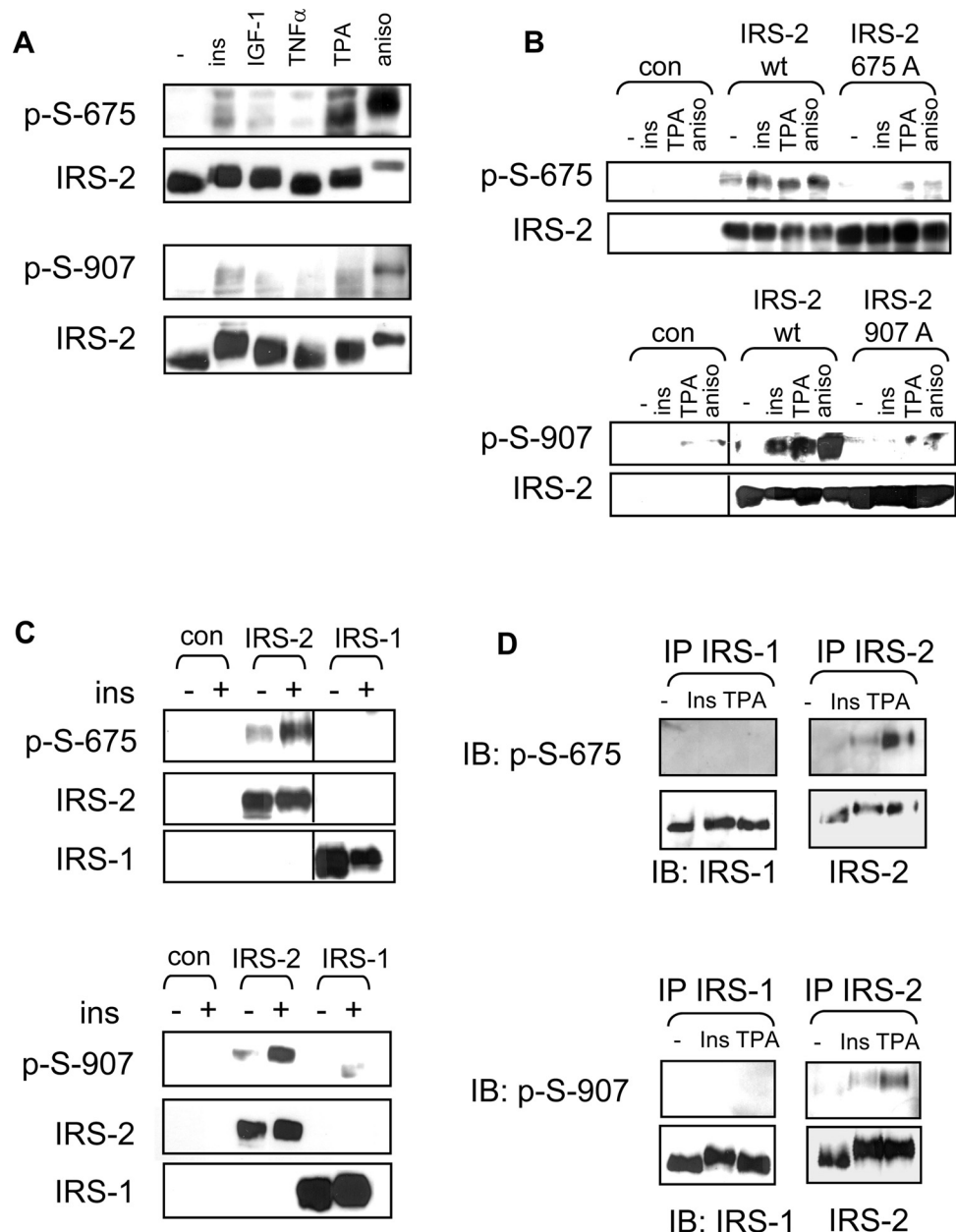
**Small interfering RNA.** Small interfering (si)RNA oligonucleotides targeting rat mTOR/FRAP1 (NM\_019906), rat p70 S6K1 (NM\_031985), rat ERK1/MAPK3 (NM\_017347), and rat ERK2/MAPK1 (NM\_053842) were designed, synthesized, and annealed at Dharmacon Research (Lafayette, CO). An unrelated siRNA targeting firefly luciferase was used as control in all experiments. Transfection was performed by electroporation using the Gene Pulser II with capacitance extender (Bio-Rad Laboratories, Hercules, CA) and 4-mm cuvettes. Fao cells ( $3.5 \times 10^6$ ) were electroporated with 100 nM siRNA in 100  $\mu$ l of RPMI with 1% FCS at 290 V and 450  $\mu$ F.

**Animal studies.** Four-week-old male C57Bl/6 wild-type mice were obtained from Charles River Laboratories. They were maintained on a normal 12:12-h light-dark cycle and kept on a standard chow diet for 8 wk. For in vivo stimulation, a bolus of human insulin (2 IU/mouse for 10 min) was injected into the inferior vena cava of overnight-fasted mice. Controls received a comparable

amount of diluent. In a fasting/refeeding experiment, 13-wk-old wild-type 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. Liver tissue was removed and homogenized at 4°C in lysis buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, containing protease inhibitor Complete (Roche) and phosphatase inhibitors]. Homogenates were allowed to solubilize for 30 min on ice and clarified by three subsequent centrifugation steps at 16,000 g for 10 min. For detection of IRS-2 phosphorylation, supernatants containing 1 mg of total protein were used for immunoprecipitation. All procedures were approved by the local Animal Care and Use committee.

**Statistical analysis.** Data are presented as means  $\pm$  SE from three to five independent experiments. Statistical analyses were performed with SPSS for Windows v. 15.0.1 (SPSS, Chicago, IL) using a Mann-Whitney *U*-test for not normally distributed variables and Student's *t*-test for normally distributed variables. A result was considered significant if  $P < 0.05$ .

Fig. 2. Phosphorylation of Ser<sup>675</sup> and Ser<sup>907</sup> in cell culture and in vivo. **A:** Fao cells were treated with 100 nM insulin (ins), 50 ng/ml IGF-I, 5 nM TNF $\alpha$ , 0.5  $\mu$ M TPA or 5  $\mu$ g/ml anisomycin (anso) for 30 min. After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with monoclonal phosphosite-specific Ser<sup>675</sup> (top) or Ser<sup>907</sup> (bottom) antibody. Blots were re-probed with a polyclonal IRS-2 antibody. **B:** BHK cells were transiently transfected with empty vector (con), IRS-2 wild-type (IRS-2 wt), IRS-2<sup>675Ala</sup>, or IRS-2<sup>907Ala</sup> and treated with 100 nM insulin, 0.5  $\mu$ M TPA, or 5  $\mu$ g/ml anisomycin for 30 min. Lanes separated by lines were from the same gel. **C:** HEK 293 cells were transiently transfected with empty vector (con), IRS-2, or IRS-1 and treated with 100 nM insulin for 30 min. Lanes separated by lines were from the same gel. **D:** Fao cells were treated with 100 nM insulin or 0.5  $\mu$ M TPA for 30 min. IRS-1 and IRS-2 were immunoprecipitated (IP) with polyclonal antibodies. **E:** primary human hepatocytes were stimulated with 100 nM insulin or 0.5  $\mu$ M TPA for 30 min. **F:** male C57Bl/6 mice were fasted overnight and injected intravenously with 2 IU insulin. Liver samples were obtained after 10 min of insulin treatment. IRS-2 was immunoprecipitated (IB) with phosphosite-specific antibodies. Shown are immunoblots of liver extracts obtained from one unstimulated and two insulin-treated mice. **G:** male C57Bl/6 mice were fasted overnight and refed for 5 h. Liver samples were obtained immediately after fasting or after the refeeding period. Shown are immunoblots of liver extracts obtained from two fasted and two refed mice. Phosphorylation intensity was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean  $\pm$  SE;  $n = 4$ . \* $P < 0.05$  vs. fasted mice).



**RESULTS**

*Identification of potential serine/threonine phosphorylation sites.* We studied first which serine kinase activators are able to induce the phosphorylation of Ser<sup>675</sup> and Ser<sup>907</sup> of IRS-2 in Fao rat hepatoma cells by using the generated monoclonal phosphospecific antibodies. Both sites were phosphorylated by stimulation with insulin, IGF-I, TPA, and anisomycin, whereas TNF $\alpha$  induced only a weak phosphorylation at Ser<sup>675</sup> and Ser<sup>907</sup> (Fig. 2A). Although 1 nM insulin was sufficient to induce the phosphorylation of both sites (data not shown), 100 nM was used in further studies to achieve a maximum activation of downstream insulin signaling.

To test the specificity of the antibodies, baby hamster kidney (BHK) cells were transiently transfected with mouse IRS-2 wild-type (IRS-2 wt), IRS-2 Ala<sup>675</sup>, and IRS-2 Ala<sup>907</sup> and stimulated with 100 nM insulin, TPA, and anisomycin for 30 min. The phospho-site-specific antibodies clearly recognized phosphorylated IRS-2 wt, whereas only a weak signal was detected in cells expressing the IRS-2 Ala mutants (Fig. 2B). Despite the high homology in the sequences

of IRS-2 and IRS-1 adjacent to both phosphorylation sites (Ser<sup>632</sup> and Ser<sup>887</sup>, respectively; Fig. 1), the antibodies showed no or only marginal reaction with ectopic expressed IRS-1 in HEK 293 cells (Fig. 2C). Moreover, the antibodies only recognized immunoprecipitated IRS-2 in the samples obtained from insulin- and TPA-treated Fao cells but not immunoprecipitated IRS-1 (Fig. 2D). The phosphorylation of Ser<sup>675</sup> was also detected in primary human hepatocytes after stimulation with insulin and TPA (Fig. 2E; phosphorylation of Ser<sup>907</sup> was not studied). After demonstrating that endogenous as well as transiently overexpressed IRS-2 is phosphorylated on Ser<sup>675</sup> and Ser<sup>907</sup> in different cell culture models, we investigated whether these phosphorylations occurred *in vivo* as well. Insulin-treated mice showed a marked increase in the phosphorylation of both sites in liver tissue (Fig. 2F). Moreover, the phosphorylation of both sites in the liver was significantly induced when fasted mice were refed for 5 h (Fig. 2G). Of note, the total IRS-2 protein amount was clearly reduced by refeeding in the liver, as has been described earlier (27).

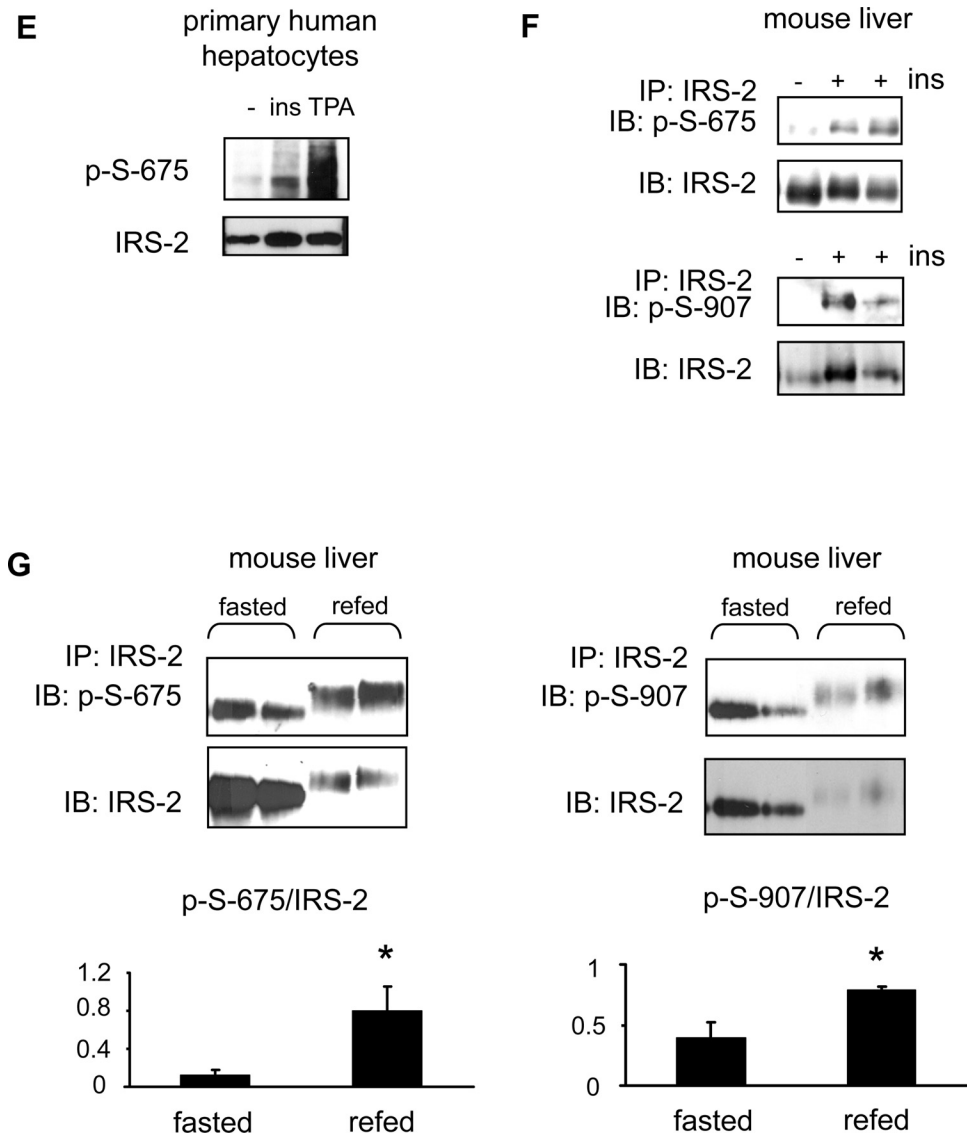


Fig. 2—Continued

Insulin-induced Ser<sup>675</sup> phosphorylation is mediated by mTOR. The insulin-induced phosphorylation kinetics of Ser<sup>675</sup> were studied in Fao cells. Insulin induced a strong phosphorylation that was maximal after 60 min and continued for at least 240 min (Fig. 3A). We also observed phosphorylation of this site after 8 h of insulin stimulation (data not shown).

To identify the kinase(s) responsible for phosphorylation of Ser<sup>675</sup>, we used a set of different pharmacological inhibitors in Fao cells. Inhibition of mTOR with rapamycin and inhibition of PI3K with wortmannin clearly reduced the insulin-depend

ent phosphorylation of Ser<sup>675</sup> (Fig. 3B). To investigate this further, we used siRNA to silence either mTOR (Fig. 3C) or its target kinase p70<sup>S6K1</sup> in Fao cells (Fig. 3D). The protein level of mTOR could be clearly reduced, leading to abolished insulin-induced p70<sup>S6K1</sup> phosphorylation (Fig. 3C). The knockdown of mTOR also resulted in a significantly reduced basal and insulin stimulated Ser<sup>675</sup> phosphorylation of endogenous IRS-2 (Fig. 3C). In contrast to the treatment with rapamycin (Fig. 3B), the electrophoretic mobility of IRS-2 was not markedly influenced, suggesting some remaining mTOR kinase activity. The knockdown of p70<sup>S6K1</sup> in Fao cells was highly efficient, resulting in undetectable protein bands in Western blot and leading to reduced Ser<sup>1101</sup> phosphorylation of IRS-1, which has been identified as a p70<sup>S6K1</sup>-dependent phosphorylation site (51) (Fig. 3D). However, the insulin-induced 675-phosphorylation of IRS-2 was not influenced (Fig. 3D). These data indicate that mTOR is involved in the phosphorylation of this site.

Phosphorylation of Ser<sup>675</sup> has no effect on downstream insulin signaling but influences IRS-2 degradation. To study the function of Ser<sup>675</sup>, we used HEK 293 cells stably expressing wild-type IRS-2 (IRS-2 wt) and the insulin receptor (IR) or HEK 293 cells stably expressing the IRS-2<sup>675</sup>Ala mutant (IRS-2<sup>675</sup>A) simulating the unphosphorylated state, and the IR. We could not detect differences between these cells in the insulin-induced activation of Akt/PKB or ERK1/2 signaling (Fig. 4A). Moreover, the<sup>675</sup>Ala mutation had no effect on the insulin-induced phosphorylation of IRS-2 tyrosine residues and on the association of IRS-2 and p85 as observed in IRS-2 or p85 coimmunoprecipitation experiments (Fig. 4, B and C). The Ser<sup>675</sup> phosphorylation was present in IRS-2 wt coprecipitated with p85 (Fig. 4C), demonstrating that the IRS-2 fraction that is relevant for insulin-induced effects is Ser<sup>675</sup> phosphorylated. A basal IRS-2-p85 association was detected using untreated IRS-2<sup>675</sup>A-expressing cells (Fig. 4C), but this effect did not carry over to downstream insulin signaling (Fig. 4A). The mTOR-mediated serine phosphorylation of IRS-2 might be implicated in the degradation of the protein. We studied the insulin-induced degradation of IRS-2 in Fao cells and observed a reduction of IRS-2 protein after 120 and 240 min of stimulation (Fig. 4D), which was completely prevented in the presence of rapamycin or the proteasomal inhibitor lactacystin. Of note, the insulin-induced pronounced retardation of the elec

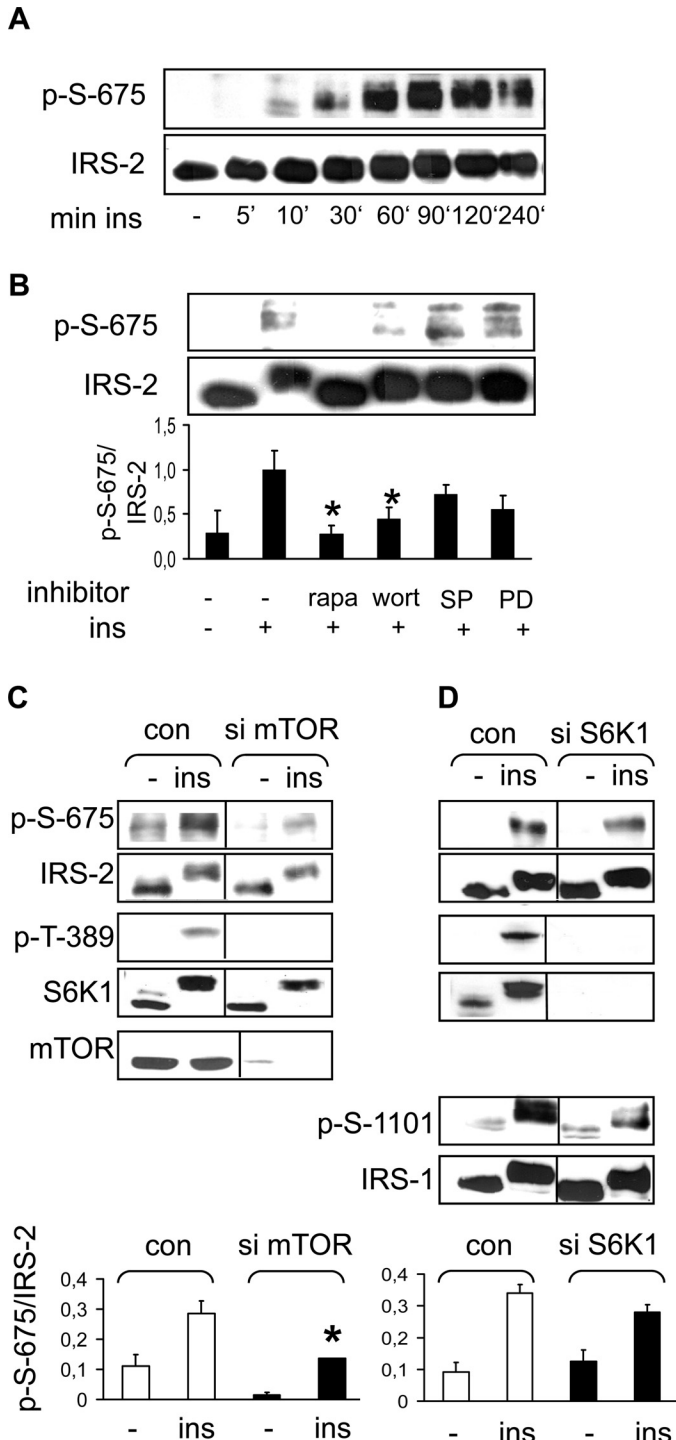


Fig. 3. Insulin-induced Ser<sup>675</sup> phosphorylation is mediated by mTOR. **A:** Fao cells were stimulated with 100 nM insulin (ins) for 0–240 min. After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE. Representative immunoblots with site-specific Ser<sup>675</sup> antibody and reprobe of the same blot with polyclonal IRS-2 antibody are shown. **B:** Fao cells were treated with 25 nM rapamycin (rapa), 100 nM wortmannin (wort), 10 μM SP-600125 (SP), or 50 μM PD-98059 (PD) for 30 min and subsequently stimulated with 100 nM insulin for 60 min. Phosphorylation intensity was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean ± SE; n = 5. \*P < 0.05 insulin stimulated cells vs. insulin-stimulated cells without inhibitor). **C and D:** Fao cells were electroporated with siRNA targeting mTOR (C) or S6K1 (D) or control siRNA (con). Forty-eight hours after transfection, cells were treated with 100 nM insulin for 30 min. Phosphorylation of IRS-2 (Ser<sup>675</sup>), S6K1 (Thr<sup>389</sup>) (C and D), and of IRS-1 (Ser<sup>1101</sup>) (D) with corresponding reblots and total mTOR protein (C) are shown. Phosphorylation intensity of Ser<sup>675</sup> was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein (mean ± SE; n = 4. \*P < 0.05 con ins vs. si mTOR ins). Lanes separated by lines were from the same gel.

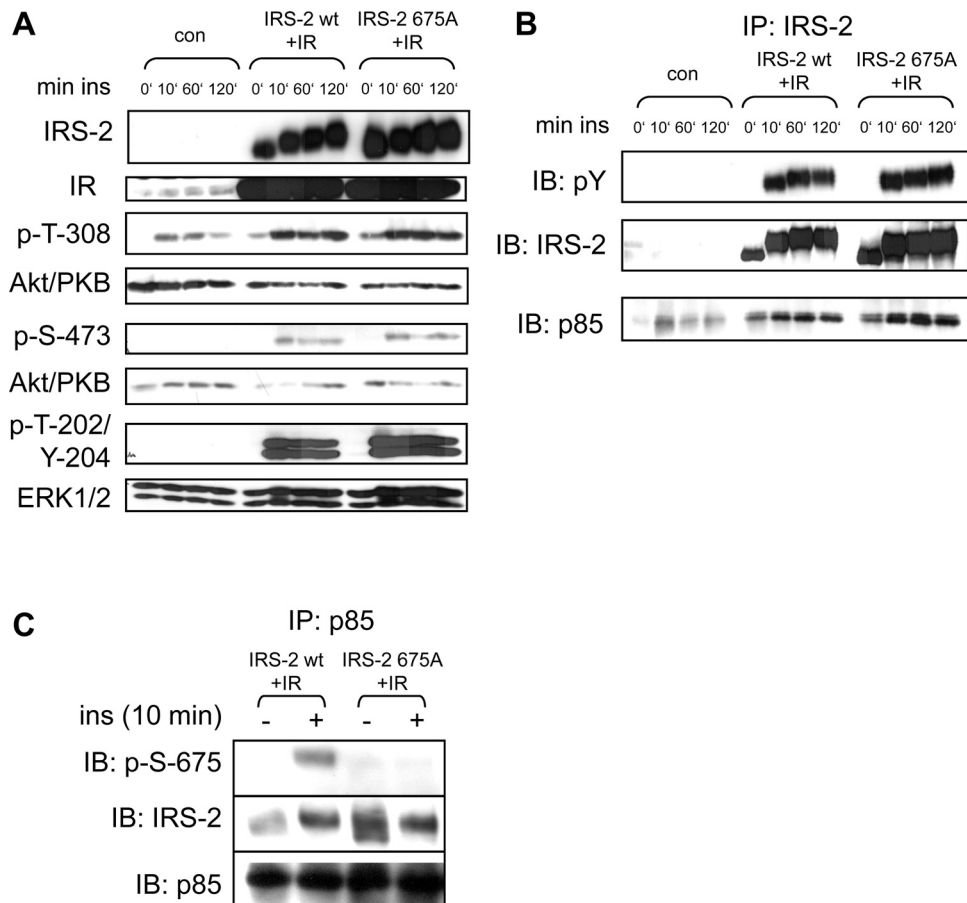


Fig. 4. Phosphorylation of Ser<sup>675</sup> has no effect on downstream insulin signaling but influences IRS-2 degradation. *A–C*: HEK 293 cells, stably transfected with either IRS-2 wild-type (wt) and insulin receptor (IR) or IRS-2<sup>675</sup>Ala (675A) and IR, were treated with 100 nM insulin (ins) for the indicated times. After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE. *A*: representative immunoblots for phospho-Akt/PKB Ser<sup>473</sup> and Thr<sup>308</sup> and phospho-ERK1/2 Thr<sup>202</sup>/Tyr<sup>204</sup> with corresponding reblots for total protein are shown. *B*: IRS-2 was immunoprecipitated with a polyclonal antibody, and the blot was probed for phosphotyrosine (*top*) and p85 (*bottom*). Blot was reprobed with IRS-2 (*middle*). *C*: p85 was immunoprecipitated with a polyclonal antibody, and the blot was probed with the site-specific phospho-Ser<sup>675</sup> antibody and for p85 and reprobed with a polyclonal IRS-2 antibody. *D* and *E*: Fao cells were treated with 25 nM rapamycin (rapa) or 10  $\mu$ M lactacystin (lacta) 30 min prior to stimulation with 100 nM insulin for 0–240 min. *D*: after stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE. Representative immunoblot for IRS-2 is shown. IRS-2 protein was quantified based on scanning densitometry of immunoblots normalized for  $\beta$ -actin (mean  $\pm$  SE;  $n = 4$ , \* $P = 0.05$ , † $P = 0.08$  vs. 0 min ins). *E*: mRNA expression of endogenous IRS-2 normalized to  $\beta$ -actin (mean  $\pm$  SE;  $n = 3$ ). *F* and *G*: HEK 293 cells, stably transfected with either IRS-2 wt and IR or IRS-2<sup>675</sup>A and IR, were treated with 100 nM insulin and 25  $\mu$ g/ml cycloheximide (CHX) for indicated times. After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE. Representative immunoblot for IRS-2 and  $\beta$ -actin is shown. *G*: IRS-2 protein was quantified based on scanning densitometry of immunoblots normalized for  $\beta$ -actin (mean  $\pm$  SE;  $n = 4$ , \* $P < 0.05$  wt ins vs. 675A ins).

trophoretic mobility of IRS-2, which was visible in Fao cells (Figs. 3A and 5A) as well as in liver lysates (Fig. 2F) and after refeeding (Fig. 2G), was also blocked by rapamycin and lactacystin. Insulin also reduced IRS-2 mRNA expression (Fig. 4E), which, however, was not prevented by lactacystin and rapamycin, indicating that the insulin-induced decrease of IRS-2 protein is caused by both proteasomal degradation and reduced gene expression. Furthermore, the data led to the assumption that an mTOR-dependent modification of IRS-2, namely phosphorylation of Ser<sup>675</sup>, could be involved in the insulin-induced degradation. In the HEK 293 cells stably expressing IRS-2 wt and IRS-2<sup>675</sup>A inhibition of protein synthesis with cycloheximide enabled the visualization of IRS-2 degradation, which otherwise is not possible in this cell model (Fig. 4F). In contrast to Fao cells, insulin treatment did not further reduce the half-life of the wild-type protein (Fig. 4F), but the disappearance of IRS-2 was clearly delayed when

Ser<sup>675</sup> was mutated to Ala with a comparable reduction after 6 h (Fig. 4G), supporting the hypothesis that phosphorylation of Ser<sup>675</sup> might be involved in an accelerated degradation of the protein.

*Insulin-induced Ser<sup>907</sup> phosphorylation is dependent on ERK.* To investigate the regulation of IRS-2 Ser<sup>907</sup>, we first studied the insulin-induced phosphorylation kinetics in Fao cells. Insulin treatment resulted in slowly increasing phosphorylation of Ser<sup>907</sup> with maximal phosphorylation after 60 min and continuing for at least 240 min (Fig. 5A), demonstrating that the phosphorylation of Ser<sup>907</sup> is a rather late event in insulin signaling.

Using different pharmacological inhibitors revealed that neither the inhibition of mTOR nor PI3K nor JNK showed any effect on insulin induced Ser<sup>907</sup> phosphorylation (Fig. 5B). Furthermore, 24-h pretreatment with 0.1  $\mu$ M TPA, which downregulates classical and novel PKC isoforms (e.g., PKC $\delta$ ),

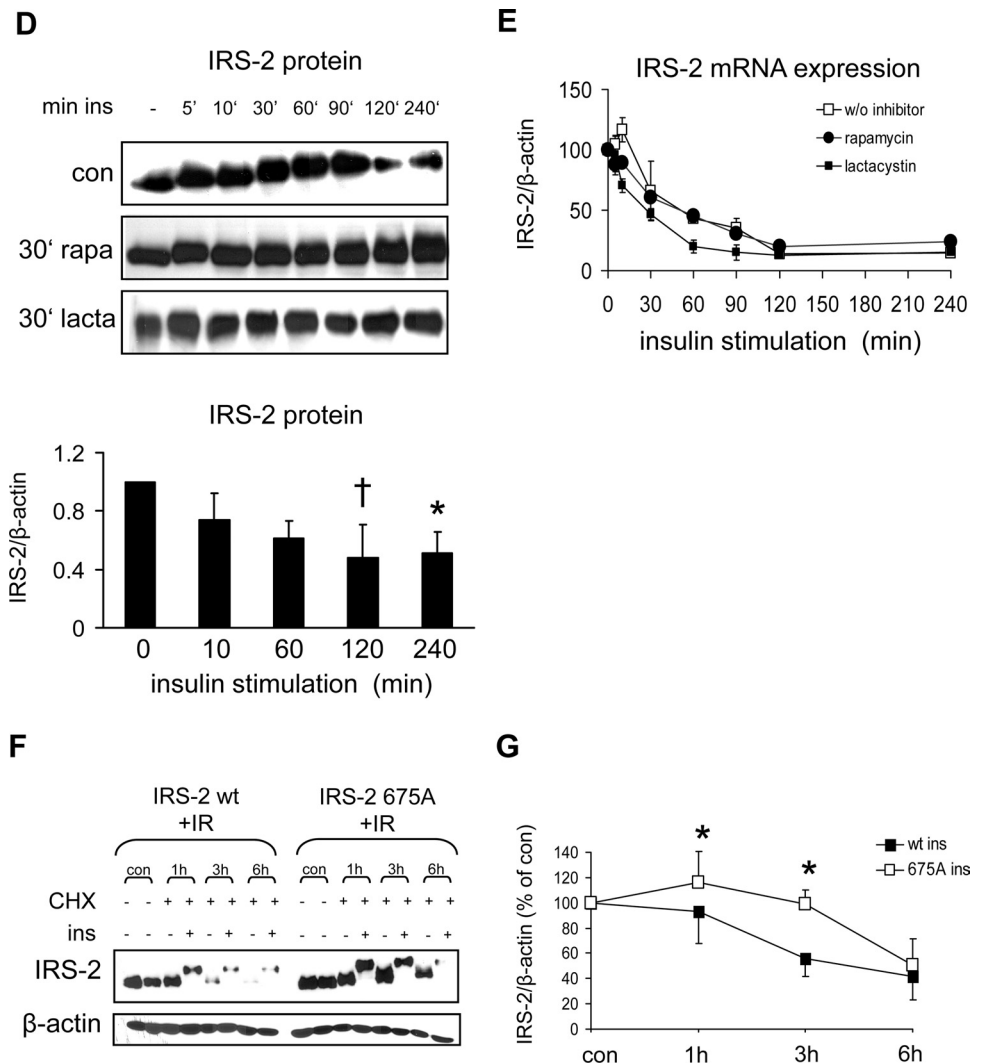


Fig. 4—Continued

but not atypical PKC isoforms, did not prevent the insulin-induced Ser<sup>907</sup> phosphorylation (Fig. 5C). However, treatment with the MEK1-specific inhibitor (PD-98059) to inhibit the activation of the MAP kinases ERK1/2 led to a strong reduction of the insulin-induced Ser<sup>907</sup> phosphorylation (Fig. 5C), and siRNA-mediated knockdown of these MAP kinases resulted in an impaired insulin-induced Ser<sup>907</sup> phosphorylation, despite the incomplete knockdown of ERK2 (Fig. 5D). These data imply that the MAP kinases ERK1 and -2 are involved in the insulin-induced phosphorylation of IRS-2 Ser<sup>907</sup>, which moreover lies within a MAP kinase consensus sequence (PXpS/pTP) (Fig. 1).

*Abrogation of Ser<sup>907</sup> phosphorylation does not influence the binding of Grb2 to IRS-2.* Ser<sup>907</sup> is located in close proximity to Tyr<sup>911</sup>, which is proposed to bind Grb2 after phosphorylation. Therefore, we tested whether the phosphorylation of Ser<sup>907</sup> influences the interaction of IRS-2 with Grb2 and thus the activation of ERK1/2 as well. HEK 293 clones were generated that stably express IRS-2<sup>907</sup>Ala + IR and in addition clones that stably express IRS-2 with an amino acid exchange of Tyr<sup>911</sup> to phenylalanine (<sup>911</sup>Phe). The insulin-induced total tyrosine phosphorylation of IRS-2, the association of IRS-2 with p85, and the downstream signaling toward

Akt/PKB and GSK-3 were not different in these cells (Fig. 6, A–C). The phosphorylation of Ser<sup>907</sup> was present in p85-coprecipitated IRS-2 wt after insulin treatment (Fig. 6B). The association to p85 was also detected in unstimulated IRS-2<sup>907</sup>Ala-expressing cells (Fig. 6, A and B), but no influence of this effect on insulin-induced signal transduction was observed. The association of IRS-2 with Grb2 was clearly and significantly induced by insulin treatment in IRS-2 wt-expressing cells, whereas it was greatly diminished in IRS-2<sup>911</sup>Phe-expressing cells (Fig. 6, D and E). No difference was observed between wt and <sup>907</sup>Ala-expressing cells. The phosphorylation of Ser<sup>907</sup> was present in IRS-2 coprecipitated with Grb2, even though the degree of phosphorylation was less than the phosphorylation of total IRS-2 when similar amounts of IRS-2 protein were compared, as is shown exemplarily in Fig. 6F. Despite the pronounced reduction of the Grb2 binding to IRS-2<sup>911</sup>Phe, the activation of ERK1/2 phosphorylation by insulin was not affected in these cells and indistinguishable from wt- and IRS-2<sup>907</sup>Ala-expressing cells (Fig. 6G). Further studies in HEK 293 cells stably expressing IR or IRS-2 alone or in combination demonstrated that elevated levels of IR protein alone were sufficient for the insulin-induced ERK1/2 phosphorylation, which was not further amplified in the presence of

IRS-2 wt or IRS-2<sup>907Ala</sup> (Fig. 6H). These results indicate that the phosphorylation of Tyr<sup>911</sup> is crucial for the insulin-induced binding of Grb2, but this interaction is dispensable in HEK 293 cells for downstream activation of ERK, and not prevented by phosphorylation of the adjacent Ser<sup>907</sup>.

**DISCUSSION**

In this study, we provide evidence that insulin induces the phosphorylation of two novel IRS-2 serine phosphorylation

sites, Ser<sup>675</sup> and Ser<sup>907</sup>. Both phosphorylation sites have been previously described using quantitative phosphoproteomics in HeLa cells (13). We demonstrate here the phosphorylation of both sites using monoclonal phosphospecific antibodies, which do not detect the homologous sites of IRS-1 but the phosphorylation in human, mouse, and rat IRS-2. Both serine residues were phosphorylated in different cell culture models and in vivo, indicating the physiological importance of these sites. Phosphorylation of Ser<sup>675</sup> and Ser<sup>907</sup> are late events detected after sustained insulin stimulation of cells (60–240 min) and found after 5 h of refeeding in mouse liver. The kinases responsible for the phosphorylation, mTOR and ERK1/2, have been implicated in the attenuation of the insulin signal. These data point to an involvement of both sites in the feedback control of insulin signal transduction.

Ser<sup>675</sup> is homologous to Ser<sup>632</sup> of IRS-1 (Ser<sup>636</sup> in human IRS-1), which has been shown to be a target of mTOR as well (16, 53), but activation of p70<sup>S6K1</sup> (41, 54) and ERK (8) could also induce the phosphorylation of Ser<sup>632</sup>. Unregulated hyperphosphorylation of Ser<sup>632</sup> has been associated with insulin resistance in obese mice (24, 52, 54). While p70<sup>S6K1</sup> or ERK1/2 were not responsible for the insulin-dependent phosphorylation Ser<sup>675</sup> of IRS-2 in Fao cells, these kinases could be involved in the phosphorylation of this site in vivo, for example, in the livers of refed mice.

The findings of our study suggest that Ser<sup>675</sup> 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 onset of refeeding has been described in mice (27), and it has been speculated that this is the prerequisite to shifting the insulin signaling toward IRS-1, which is suspected to be dominant for postprandial insulin signaling (17). In this context, the IRS-2 downregulation via degradation seems to be essential for hepatic nutrient homeostasis. Moreover, diminished protein levels of IRS proteins were found in liver and muscle in different animal models of obesity, insulin resistance, and diabetes, suggesting a function in the pathogenesis of these diseases (2, 23, 24). In the present study, Ser<sup>675</sup> is phosphorylated by mTOR, and the inhibition of this kinase abolished insulin-induced degradation of endogenous IRS-2, which has also been reported previously (37). Furthermore, in livers of refed mice, a robust Ser<sup>675</sup> phosphorylation was observed concomitantly with a pronounced IRS-2 protein decrease, possibly suggesting the relevance of this site

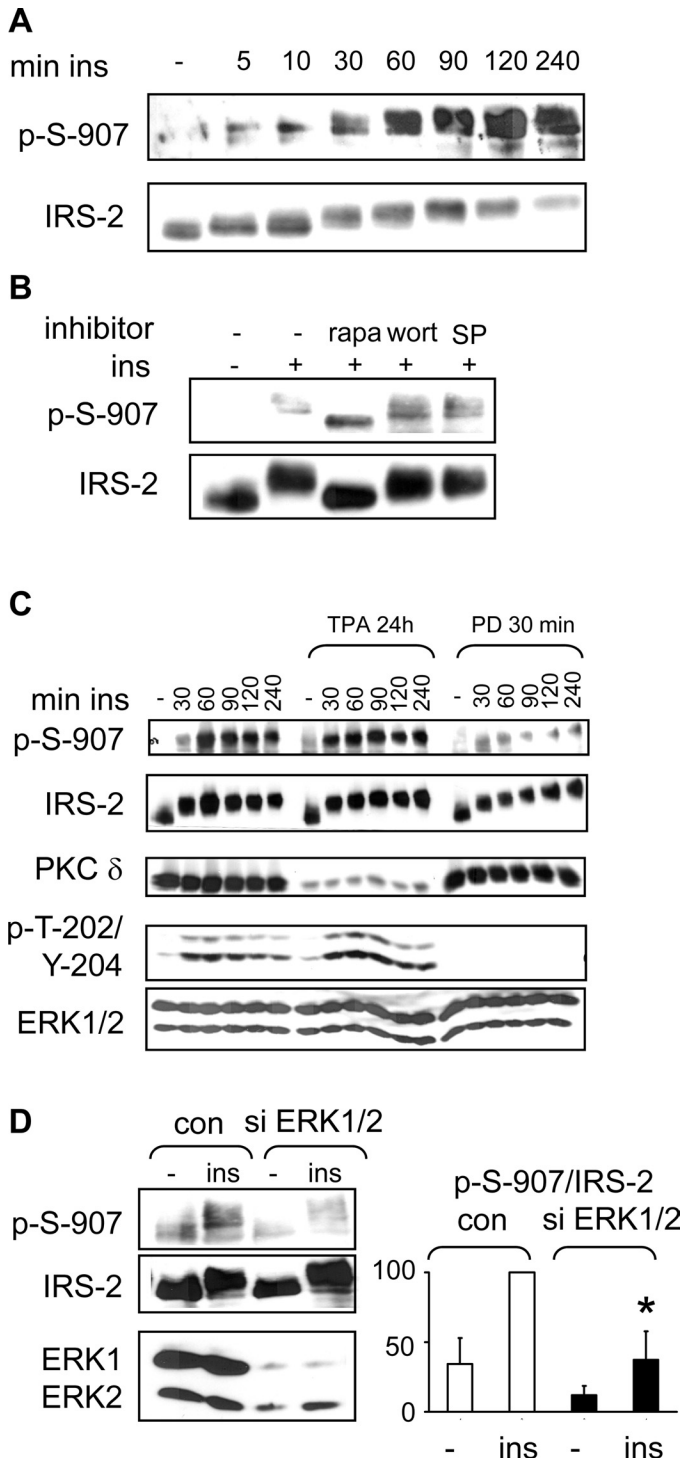


Fig. 5. Insulin-induced Ser<sup>907</sup> phosphorylation is dependent on ERK1/2. **A:** Fao cells were stimulated with 100 nM insulin (ins) for 0–240 min. After stimulation, cells were lysed and analyzed by 7.5% SDS-PAGE. Representative immunoblots with site-specific Ser<sup>907</sup> antibody and reprobe of the same blot with polyclonal IRS-2 antibody are shown. **B:** Fao cells were treated with 25 nM rapamycin (rapa), 100 nM wortmannin (wort), or 10 μM SP-600125 (SP) for 30 min and subsequently stimulated with 100 nM insulin for 60 min. **C:** Fao cells were pretreated with 0.1 μM TPA for 24 h or 20 μM PD-98059 (PD) for 30 min and subsequently stimulated with 100 nM insulin for 0–240 min. Representative immunoblots with site-specific Ser<sup>907</sup> antibody, reblot with IRS-2 antibody, PKCδ protein antibody, phospho-ERK1/2 Thr<sup>202</sup>/Tyr<sup>204</sup> antibody, and reblot with ERK1/2 antibody are shown. **D:** Fao cells were electroporated with siRNA targeting ERK1 and ERK2 and control siRNA (con). Forty-eight hours after transfection, cells were treated with 100 nM insulin for 30 min. Phosphorylation intensity was quantified based on scanning densitometry of immunoblots normalized for IRS-2 protein; con + ins was set as 100% (mean ± SE; n = 4). \*P < 0.05 con ins vs. si ERK1/2 ins.



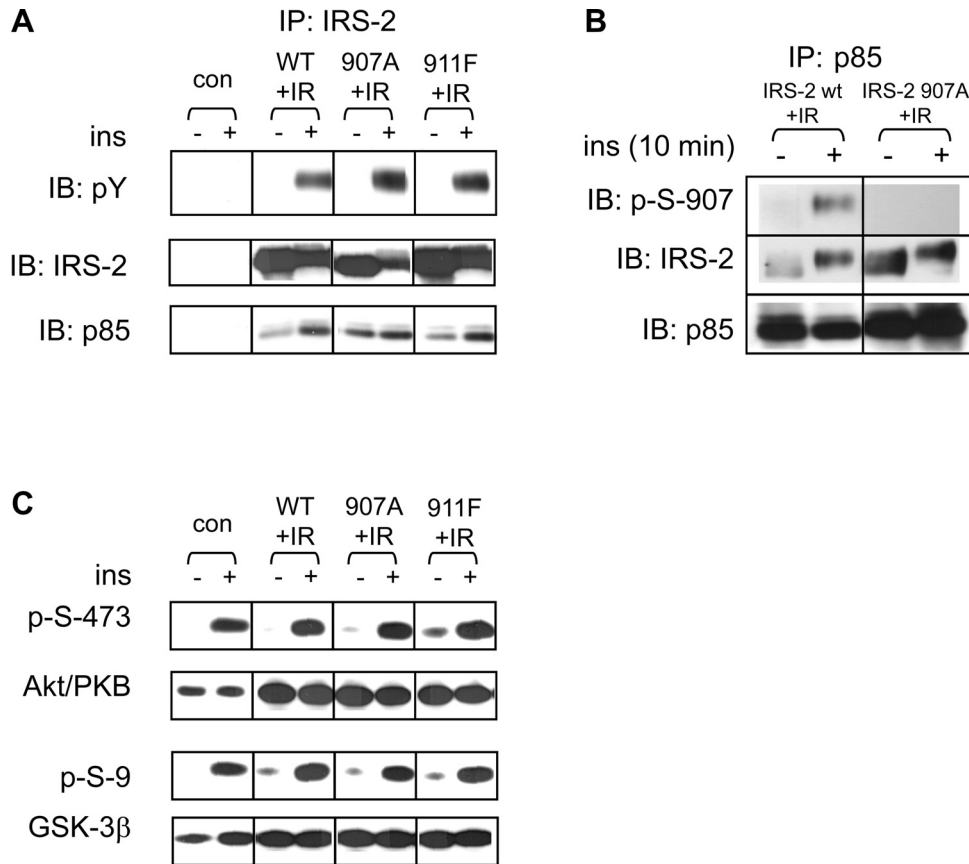


Fig. 6. Abrogation of Ser<sup>907</sup> phosphorylation does not influence the binding of Grb2 to IRS-2. HEK 293 cells were stably transfected with empty vector as control (con), IRS-2 wt, IR, IRS-2<sup>907</sup>Ala, IRS-2<sup>911</sup>Phe (F), or together with IR and treated with 100 nM insulin (ins) for 10 min. **A:** IRS-2 was immunoprecipitated with a polyclonal IRS-2 antibody, and the blot was probed for phosphotyrosine (pY) (top) and p85 (bottom) and reprobed for IRS-2 protein (middle). **B:** p85 was immunoprecipitated with a polyclonal antibody, and the blot was probed with site-specific phospho-Ser<sup>907</sup> antibody and for p85 and reprobed with a polyclonal IRS-2 antibody. Lanes separated by lines were from the same gel. **C:** representative immunoblots of lysates for phospho-Akt/PKB Ser<sup>473</sup> and phospho-GSK $\beta$  Ser<sup>9</sup> with corresponding reblot for total protein are shown. Lanes separated by lines were from the same gel. **D:** Grb2 was immunoprecipitated with a monoclonal Grb2 antibody, and the blot was probed for IRS-2 protein (top). Lysates from the same experiment were additionally probed for total IRS-2 protein (bottom). Lanes separated by lines were from the same gel. **E:** quantification of IRS-2 from Grb2 IP. Amount of coimmunoprecipitated IRS-2 was quantified based on scanning densitometry of immunoblots and normalized for IRS-2 protein from lysates (mean  $\pm$  SE;  $n = 6$ . \* $P < 0.05$  con vs. ins; # $P < 0.05$  wt ins vs. <sup>911</sup>F ins; NS, not significant). **F:** representative immunoblots of IRS-2 Ser<sup>907</sup> phosphorylation in lysates from insulin-treated cells with increasing amounts of total protein ( $\mu$ g) and of Grb2-coimmunoprecipitated IRS-2 (from 150  $\mu$ g total protein). Blot was probed with phosphosite-specific Ser<sup>907</sup> antibody and reprobed for IRS-2 with a polyclonal IRS-2 antibody. The ratio of IRS-2 phosphorylation was calculated based on scanning densitometry of immunoblots of 4 independent experiments (p-Ser<sup>907</sup>/IRS-2). Rectangles indicate comparable amounts of IRS-2 in lysate and IP and corresponding Ser<sup>907</sup> phosphorylation intensity. **G:** representative immunoblots of lysates for IRS-2 and IR protein, phospho-ERK1/2 Thr<sup>202</sup>/Tyr<sup>204</sup> with corresponding reblot for total ERK1/2 are shown. Lanes separated by lines were from the same gel. **H:** HEK 293 cells were transiently transfected with empty vector as control (con), IRS-2 wt, IR, IRS-2<sup>907</sup>Ala, or together with IR and treated with 100 nM insulin (ins) for 10 min. Representative immunoblots of lysates for IRS-2, IR, phospho-ERK1/2 Thr<sup>202</sup>/Tyr<sup>204</sup>, and reprobed ERK1/2 total protein are shown.

for degradation (Fig. 2G). To specifically address the function of phospho-Ser<sup>675</sup> for IRS-2 degradation, we used the nonphosphorylated IRS-2<sup>675</sup>Ala mutant in stably transfected HEK 293 cells. In this model, IRS-2 degradation could only be detected when protein translation was blocked with cycloheximide, but under these conditions a prolonged half-life of IRS-2<sup>675</sup>Ala was visible. The degradation of IRS proteins involves their ubiquitinylation (38). Two flanking lysines of Ser<sup>675</sup> (Lys<sup>667</sup> and Lys<sup>683</sup>) have a probability for this modification (35). It can be speculated that the phosphorylation of IRS-2 on Ser<sup>675</sup> is a prerequisite for the ubiquitination of either one or both of these lysine residues, thus facilitating the proteasomal degradation. Studies that aimed to prove the relevance of the phosphorylation of the homologous Ser<sup>632</sup> for IRS-1 degradation failed (53), whereas the importance of mTOR and mTOR-dependent,

but also mTOR-independent, serine phosphorylation could be demonstrated (15, 41, 46). With the pronounced effect of rapamycin on the electrophoretic mobility of IRS-2 taken into consideration, which indicates a major blockade of posttranslational modifications, it appears likely that further mTOR-dependent phosphorylations of other yet unknown residues of IRS-2 and other modifications are also important for the degradation of IRS-2 protein. The relevance of Ser<sup>675</sup> phosphorylation for the development of insulin resistance and diabetes will have to be investigated in suitable animal models, e.g., mice fed a high-fat diet.

Ser<sup>675</sup> is adjacent to a p85 binding motif (pY<sub>671</sub>MPM), and an inhibitory effect on the association with p85 has been described for the homologous Ser<sup>632</sup> and its neighboring Ser<sup>635</sup> of IRS-1 (53). Phosphorylation of Ser<sup>675</sup> appeared not to

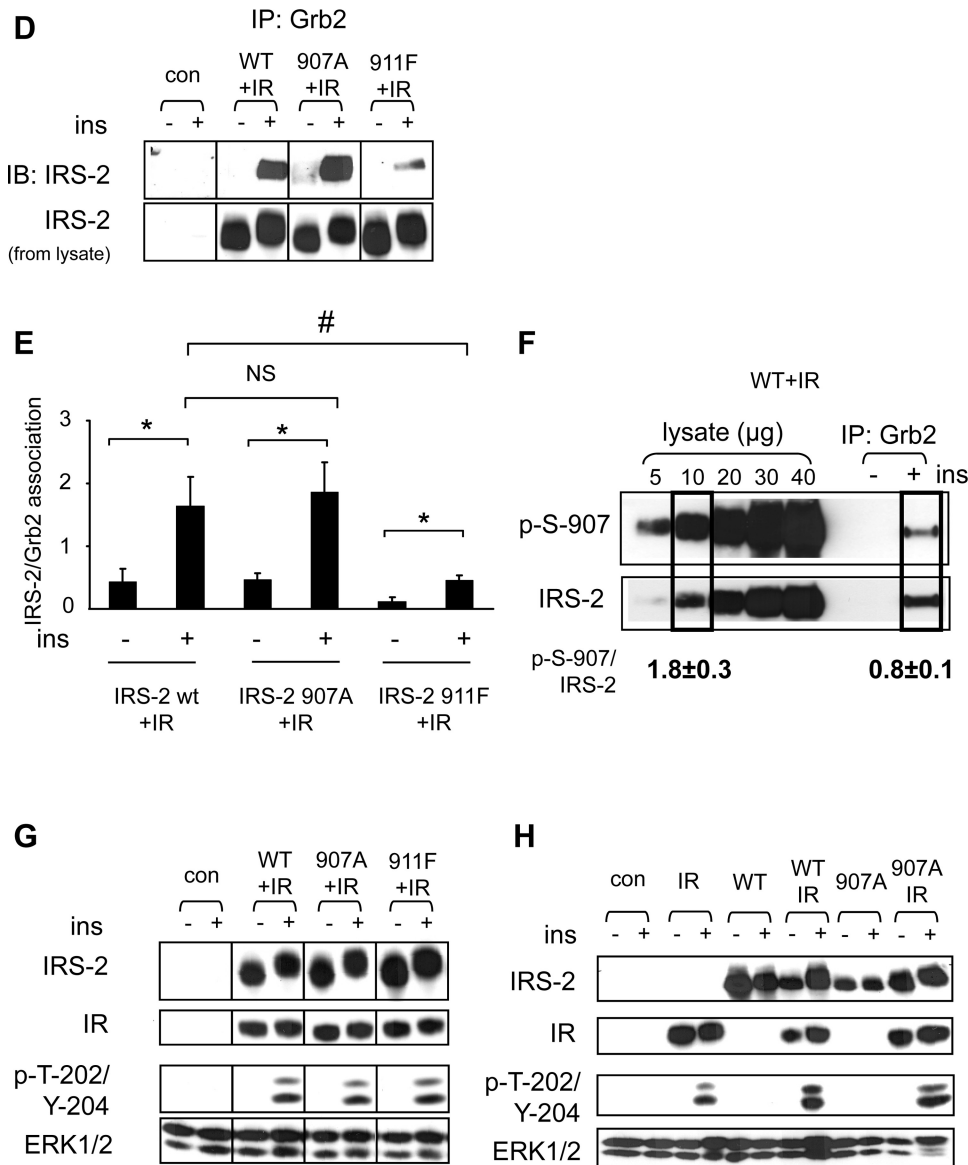


Fig. 6—Continued

influence the interaction of IRS-2 with the insulin receptor as determined by IRS-2 tyrosine phosphorylation (Fig. 4B, top), although Ser<sup>675</sup> is located within the KRLB domain. However, the region of the KRLB domain that binds directly to the insulin receptor (602–637) (62) is not in close proximity to Ser<sup>675</sup>, and phosphorylation of Ser<sup>675</sup> might therefore have no direct effect on IRS-2-insulin receptor interaction. Thus phosphorylation of Ser<sup>675</sup> does not appear to have direct effects on insulin signal transduction but plays a role in the degradation of the protein.

In contrast to Ser<sup>675</sup>, which has a well-described corresponding serine residue in IRS-1, the function of the IRS-1 homologous residue to Ser<sup>907</sup>, Ser<sup>887</sup> (corresponding to mouse IRS-1) (26, 64) is unknown. The serine kinase ERK1/2, which is responsible for insulin-induced phosphorylation of Ser<sup>907</sup>, has also been recognized as an IRS-1 kinase that is involved in the negative regulation of insulin signaling by phosphorylation of Ser<sup>612</sup> (12, 31) and Ser<sup>632</sup> (8). Therefore, it could be speculated that the ERK-mediated phosphorylation of IRS-2 might also be involved in the attenuation of insulin's effects.

On the other hand, IRS-2 has been shown to be crucial for the transduction of the insulin signal into the MAP kinase pathway in myotubes (9, 22) and β-cells (60). Tissue-specific knockout of IRS-2 in the liver of mice strongly reduced insulin-induced ERK phosphorylation (14). Although these studies showed that IRS-2 is an important mediator of the insulin/IGF-I signal into the MAP kinase pathway in different tissues, the molecular cascade transducing the insulin signal toward ERK via IRS-2 is less clear. It has been considered that the binding of Grb2 to the Grb2 consensus motif (pYI/VN) located at Tyr<sup>911</sup> is responsible for IRS-2-mediated ERK activation (45). Abrogation of the binding of Grb2 to IRS-1, which is dependent on the phosphorylation of the homologous tyrosine residue Tyr<sup>891</sup>, leads to reduced ERK activation (55). Thus, we hypothesized that phosphorylation of Tyr<sup>911</sup> is responsible for the insulin-induced binding of Grb2 to IRS-2 and the subsequent activation of the ERK pathway, and that insulin-induced, ERK-dependent phosphorylation of adjacent Ser<sup>907</sup> might interfere with the Grb2-IRS-2 association as a negative feedback regulation.

Expression of the unphosphorylated <sup>911</sup>Phe mutant in HEK 293 cells could demonstrate that the phosphorylation of Tyr<sup>911</sup> is necessary for the association of Grb2 to IRS-2. The unphosphorylated <sup>907</sup>Ala mutant did not influence the Grb2-IRS-2 interaction, and phosphorylation of Ser<sup>907</sup> did not block the interaction since the IRS-2 associated to Grb2 is phosphorylated on Ser<sup>907</sup>. A minor effect could not be excluded because the degree of Ser<sup>907</sup> phosphorylation of GRB-2 bound IRS-2 is less compared with total IRS-2. Moreover, IRS-2 has no apparent effect on the insulin-induced ERK activation, which appears to be completely dependent on the IR in this cell culture model. These results suggest that not IRS-2 but other adaptor proteins, such as Shc or Grb-associated binder-1 (Gab1), are the predominant interaction partners of the IR to transduce the mitogenic effects of insulin. It has been shown that Shc could compete with IRS-1 and probably IRS-2 as well for binding of the Grb2-Sos complex (63). Gab1 shares structural homology with the IRS proteins (21, 36) and could interact with SH2 domain-containing proteins, most notably SHP-2 (36), to control the activation of ERK1/2 (4, 10). Deletion of Gab1 in the liver of mice results in abolished insulin-induced ERK1/2 phosphorylation (6). Thus, the involvement of both, Gab1 or Shc, provides an explanation for the IR-dependent but IRS-2-independent activation of the MAP kinase pathway observed in stable HEK 293 clones. Our data also suggest that the expression levels of IR, IRS-2, and their interaction partners could be critical for the investigation of downstream effects. In HEK 293 cells, we could not verify that the ERK-mediated phosphorylation of Ser<sup>907</sup> of IRS-2 regulates the intensity of insulin signal transduction, but the late onset of this phosphorylation suggests an involvement in a negative feedback control.

To conclude, we were able to provide clear evidence for the phosphorylation of two novel IRS-2 serine residues in different cell systems and in vivo, and we were able to demonstrate their insulin-dependent kinetics and the responsible kinases and give first insights into their putative role in insulin signal transduction.

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#### DISCLOSURES

No conflicts of interest are reported by these authors.

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