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The COOH-terminal Tyrosine Phosphorylation Sites on IRS-1 Bind SHP-2 and Negatively Regulate Insulin Signaling*

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Activation of tyrosine kinases by numerous growth factor and cytokine receptors leads to tyrosine phosphorylation of the insulin receptor substrate (IRS)-proteins. Tyrosine-phosphorylated motifs on the IRS proteins bind to the SH2 domains in proteins that mediate downstream signals, including phosphatidylinositol 3′-kinase, GRB-2, and SHP-2. We investigated the function of the two SHP-2 binding COOH-terminal tyrosines of IRS-1 by replacing them with phenylalanine (IRS-1^{FCT}). IRS-1^{FCT} failed to bind SHP-2 or mediate its tyrosine phosphorylation during insulin stimulation. Although several reports suggest a critical role for SHP-2 in insulin stimulated mitogen-activated protein kinase activation and cell proliferation, IRS-1^{FCT} mediated these effects normally in 32D cells. Indeed, IRS-1^{FCT} exhibited increased tyrosine phosphorylation, phosphatidylinositol 3′-kinase binding and activation of protein synthesis in response to insulin. These results suggest that SHP-2 attentuates the phosphorylation and downstream signal transmission of IRS-1 and that the interaction of IRS-1 and SHP-2 is an important regulatory event which attenuates insulin metabolic responses.

Insulin transmits cellular signals by binding the cell surface insulin receptor and activating the insulin receptor tyrosine kinase (1). The activated tyrosine kinase transmits downstream signals largely through the action of a class of intermediary docking proteins, including GAB-1, Shc, and a set of signaling molecules known as the insulin receptor substrate (IRS)[1] proteins, IRS-1, IRS-2, IRS-3, and IRS-4 (2–6). The NH_2-terminal region of the IRS proteins contains extended domains that cooperate in receptor recognition (1, 2, 7), while the COOH-terminal region possesses numerous tyrosine-containing motifs that become phosphorylated and bind specific Src homology 2 (SH2) domains in signaling proteins during insulin stimulation. While each IRS protein contains unique tyrosine phosphorylation sites, three classes of sites are well conserved across the IRS proteins, multiple YM*X*M motifs responsible for binding regulatory subunits of phosphatidylinositol (PI) 3′-kinase, a Y*XNX* motif that binds GRB-2, and a pair of COOH-terminal motifs (YIDL and YASI) responsible for binding the SHP-2 tyrosine phosphatase. The role of the PI 3′-kinase and GRB-2 binding motifs on IRS-1 have been extensively studied, but the role of the SHP-2 binding sites in IRS-1 signaling is unclear (1).

SHP-2 is a cytoplasmic protein tyrosine phosphatase that contains two SH2 domains (8). It is ubiquitously expressed in mammalian cells and appears to be the homolog of the *Drosophila corkscrew* gene responsible for activation of $p21^{ras}$ downstream of a variety of tyrosine kinases (9, 10). Coordinate binding of the two SH2 domains of SHP-2 to double tyrosine motifs in target proteins such as IRS-1 increases the affinity of the association and activates the phosphatase by moving the NH_2-terminal SH2 domain away from the phosphatase active site (11–14).

Homozygous disruption of SHP-2 in mice is early embryonic lethal, suggesting that SHP-2 plays a critical role in cell growth or differentiation. Consistent with this result, microinjection or overexpression of a catalytically inactive SHP-2, the SHP-2 SH2 domains, or αSHP-2 antibodies inhibits insulin-stimulated DNA synthesis and MAP kinase activation in a number of cell types (15–18). These approaches, however, interfere with all SHP-2-mediated signaling, rather than the IRS-1/SHP-2 signaling pathway specifically.

In this report, we examine signaling by a mutant IRS-1 molecule that lacks the COOH-terminal tyrosine phosphorylation sites and does not bind SHP-2. During insulin stimulation, this mutant is more highly phosphorylated and more strongly activates the PI 3′-kinase pathway, including protein synthesis, than does wild-type IRS-1. In contrast, MAP kinase activation and DNA synthesis occur normally. Thus, although SHP-2 may serve multiple roles, its association with IRS-1 appears to attenuate certain insulin signals important for metabolic responses.

MATERIALS AND METHODS

Cell Lines—Chinese hamster ovary cells overexpressing the human insulin receptor (CHO^{IR}) or the human insulin receptor and rat IRS-1 (CHO^{IR}/IRS-1) have been described (19). CHO^{IR} cells were maintained in Ham's F-12 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Sigma) and were made quiescent for experiments by incubation in F-12 medium containing 0.5% bovine serum albumin for 12–18 h. The mutant cDNA for IRS-1^{FCT} was co-transfected into CHO^{IR} cells with pHyg (containing the hygromycin B phosphotransferase gene) by calcium phosphate precipitation (20). The transfected cells were selected in medium containing 300 μg/ml hygromycin (ICN, Aurora, Ohio).

32D cell lines expressing the human insulin receptor ($32D^{IR}$) and/or rat IRS-1 have been described (21, 22); these cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum and 5% WEHI-3 conditioned medium (a source of IL-3). 32D cell lines were made quiescent by incubation in unsupplemented Dul-

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[1] The abbreviations used are: IRS, insulin receptor substrate; PI, phosphatidylinositol; MAP, mitogen-activated protein; CHO, Chinese hamster ovary; IL, interleukin; PAGE, polyacrylamide gel electrophoresis.

becco's modified Eagle's medium for 3–4 h before experiments. 32D cell lines were transfected with the cDNAs for isoforms of IRS-1 by electroporation (21, 22) and were selected and maintained in the presence of 10 mM histidinol (Bachem).

Antibodies and Growth Factors—Bovine insulin was from Calbiochem. Antibodies directed against SHP-2 were rabbit polyclonal antisera raised against human SHP-2 expressed as a glutathione *S*-transferase fusion protein (8). Antiphosphotyrosine antibodies (αPY) were rabbit polyclonal antibodies affinity-purified on phosphotyrosine (23) or monoclonal antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY). αIRS-1 antibodies were rabbit polyclonal antisera raised against the amino-terminal 135 amino acids of rat IRS-1 expressed as a glutathione *S*-transferase fusion protein (24) or against synthetic peptides comprising amino acids 1–15 of rat IRS-1 coupled to keyhole limpet hemocyanin (25). αPhosphoMAP kinase antibody was from New England Biolabs.

Generation of IRS-1^{FCT} cDNA—The cDNA for rat IRS-1 in pBluescript (3) was used as a template for polymerase chain reaction-mediated oligonucleotide-directed mutagenesis (26). The mutagenic primer 5′-AAG/AGT/CTT/AAC/TTT/ATA/GAC/TTG/GAT-3′ and its complement were used with outside primers flanking the *Eco*RI and *Aat*II sites in the rat IRS-1 cDNA to mutate Tyr^{1172}. This product was subjected to another round of mutagenesis using the mutagenic primers 5′-GAT/TTA/AGC/ACC/TTT/GCC/AGC/ATC/ACC-3′ and its complement and the same outside primers. The rat IRS-1 cDNA in pBluescript and mutant polymerase chain reaction products were digested with *Eco*RI and *Aat*II, and the mutant polymerase chain reaction fragments were inserted in place of the wild-type sequence. The presence of the desired mutations and the absence of adventitious mutations was confirmed by DNA sequencing of the mutant cDNA constructs. All mutant cDNAs were sucloned into the pCMVhis expression vector for expression in mammalian cells using the restriction enzymes *Sac*I and *Hin*dIII.

Immunoprecipitation—Quiescent confluent CHO^{IR} cells growing on 15-cm dishes were incubated in the absence or presence of insulin as indicated, the medium was aspirated, and cells were immediately washed with ice-cold PBS. Quiescent 32D^{IR} cells were stimulated as indicated and collected by centrifugation after the addition of ice-cold-phosphate-buffered saline. Cells were lysed in 137 mM NaCl, 20 mM Tris, 1 mM $NaCl_2$, 1 mM $CaCl_2$, pH 7.5, supplemented with 10% glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, and 0.1 mM sodium orthovanadate. Nuclei and insoluble materials were removed by centrifugation at 10,000 × *g* for 15 min. Supernatants were incubated at 4 °C with antibodies for several hours to overnight before being collected with protein A-Sepharose 6 MB (Amersham Pharmacia Biotech). Finally, immunoprecipitates were washed three times with lysis buffer, denatured by boiling in Laemmli sample buffer for 5 min, and separated by SDS-PAGE.

Phosphatidylinositol 3′-Kinase Activity—*In vitro* phosphorylation of PI was carried out in immune complexes as described previously (27). Cells were made quiescent, stimulated, lysed, and immunoprecipitated as above. Immune complexes were precipitated from the supernatant with protein A-Sepharose (Amersham Pharmacia Biotech) and washed (27). Immune complexes were incubated with phosphatidylinositol (Avanti) and [γ-^{32}P]ATP for 10 min at 22 °C. The reactions were stopped with 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1) and centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck) that had been coated with 1% potassium oxalate (27). TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized and quantitated on a Molecular Dynamics PhosphorImager.

Immunoblotting—Proteins in cell lysates prepared as for PI 3′-kinase assays or from immunoprecipitates similarly prepared were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by SDS-PAGE. Gels were transferred to nitrocellulose membranes (Schleicher & Schuell) in Towbin buffer containing 0.02% SDS and 20% methanol (28). Membranes were blocked, probed, and developed as described previously (29, 30). Blots were exposed to Kodak XAR film or imaged on a Molecular Dynamics PhosphorImager.

Incorporation of [^{3}H]Thymidine into DNA in 32D Cells—Insulin-stimulated thymidine incorporation was assayed as described previously (22, 29). Briefly, cells in log phase growth were washed and seeded into RPMI with 10% fetal bovine serum alone, 5% IL-3-containing WEHI-3-conditioned medium, or with various concentrations of insulin. Cells were grown for 48 h at 37 °C, [^{3}H]thymidine (ICN) was added to 0.5 mCi/ml, and incubation was continued for 3 h. Cells were collected onto glass microfiber filters and lysed, and unincorporated nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

[^{35}S]Methionine Incorporation into Protein in 32D Cells—32D cell lines were maintained at concentrations of 5 × 10^5 cells/ml and starved in RPMI 1640 medium supplemented with 10% fetal bovine serum for 6 h. Following starvation, 1-ml aliquots of cells were taken and incubated in the presence of IL-3 or various concentrations of insulin. Cells were incubated with [^{35}S]methionine (10 μCi/aliquot) in methionine-free RPMI 1640 for 60 min, washed once in phosphate-buffered saline containing 10 mM methionine, and lysed in 0.5 M NaOH for 30 min at 37 °C. Protein was precipitated with 12% ice-cold trichloroacetic acid containing 10 mM methionine, collected on glass filters, and washed with 5% trichloroacetic acid and ethanol. Filters were air-dried, and incorporated radionuclide was quantitated by liquid scintigraphy in an aqueous fluor.

RESULTS

The COOH-terminal Tyrosine Phosphorylation Sites in IRS-1 Mediate Interactions with SHP-2 and the Tyrosine Phosphorylation of SHP-2—In order to investigate the role of IRS-1/SHP-2 interactions in insulin signaling, we generated IRS-1^{FCT}, in which the two predicted SHP-2-binding residues (Tyr^{1172} and Tyr^{1222}) on IRS-1 were replaced by phenylalanine, and we expressed IRS-1^{FCT} in CHO^{IR} and 32D^{IR} cells at similar levels as wild-type IRS-1 (Fig. 1). We assayed insulin-stimulated tyrosine phosphorylation of IRS-1 isoforms in the CHO^{IR} cells by αPY immunoblotting immunoprecipitated IRS-1. While tyrosine phsophorylation of endogenous hamster IRS-1 was weakly detected in the CHO^{IR} cells, robust insulin-stimulated tyrosine phosphorylation of overexpressed IRS-1 and IRS-1^{FCT} was readily detected (Fig. 2*A*).

Since Tyr^{1172} and Tyr^{1222} on IRS-1 associate with the SH2 domains of SHP-2 (11, 12, 31), we assayed the association of SHP-2 with IRS-1 and IRS-1^{FCT} in αIRS-1 immunoprecipitates from lysates of insulin-stimulated CHO^{IR} cell lines (Fig. 2*A*). SHP-2 was detected in the αIRS-1 immunoprecipitates from insulin-stimulated CHO^{IR}/IRS-1 cells, but not the other cell lines. Thus, IRS-1^{FCT} failed to associate with SHP-2 in intact cells.

Insulin-stimulated Tyrosine Phosphorylation of SHP-2—αPY immunoblots of αSHP-2 immunoprecipitates from CHO^{IR} cells revealed that tyrosine phosphorylated IRS-1 associated with SHP-2 during insulin stimulation, consistent with previous results (32, 33) (Fig. 2*B*). In addition, overexpression of wild-type IRS-1 greatly increased the amount of SHP-2-associated tyrosine-phosphorylated IRS-1. Furthermore, SHP-2 became tyrosine-phosphorylated during insulin stimulation of CHO^{IR} cells, and the overexpression of wild-type IRS-1 similarly increased the tyrosine phosphorylation of SHP-2 during insulin stimulation. The identity of the approximately 60-kDa tyrosyl phosphoprotein in αSHP-2 immunoprecipitates was confirmed to be SHP-2 by reblotting with αSHP-2 antibodies and demonstrating that the bands overlay (data not shown). In contrast, IRS-1^{FCT} did not associate with SHP-2; nor did IRS-1^{FCT} increase the tyrosine phosphorylation of SHP-2. Thus, the COOH-terminal tyrosine phosphorylation sites in IRS-1 mediated the binding of SHP-2 and IRS-1 in these cells, and this interaction mediated the tyrosine phosphorylation of SHP-2 during insulin stimulation. A 95–100-kDa phosphoprotein (p100) also associated with αSHP-2 immunoprecipitates during insulin stimulation; its association similarly depended upon the presence of the COOH-terminal tyrosines on IRS-1

Insulin-stimulated Tyrosine Phosphorylation and PI 3′-Kinase Binding of IRS-1 Isoforms—In order to examine the function of IRS-1^{FCT} during insulin signaling, we used the 32D^{IR} cells, which contain no endogenous IRS-proteins (21, 22). We first examined tyrosine phosphorylation of IRS-1 and IRS-1^{FCT} during insulin stimulation in the 32D^{IR} cell lines (Fig. 3). At all doses of insulin the tyrosine phosphorylation of IRS-1^{FCT} was

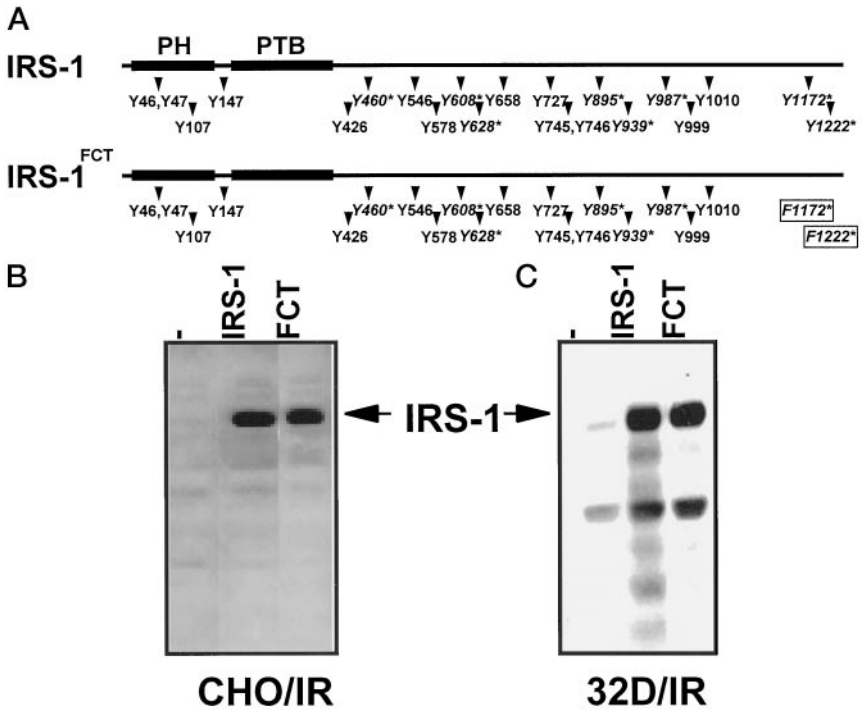

FIG. 1. **Construction and expression of COOH-terminal tyrosine mutants of IRS-1.** *A*, Schematic diagram of IRS-1. Shown is a linear diagram of IRS-1, including the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains required for upstream kinase interactions and 18 potential tyrosine phosphorylation sites (* = known tyrosine phosphorylation site). IRS-1^{F*CT} is shown below IRS-1, with the two mutated COOH-terminal tyrosine residues in *boxes*. Expression of IRS-1 and IRS-1 mutants in *B*, CHO^{IR} and *C*, $32D^{IR}$ cells. Lysates of control (-) cells and cells expressing IRS-1 (*IRS-1*) or IRS-1^{FCT} (*FCT*) were resolved by SDS-PAGE and immunoblotted with αIRS-1 before being exposed to autoradiography. There are two nonspecific bands in *panel C* (90 and 170 kDa) due to the cross-reactivity of the antisera when used in 32D cells.

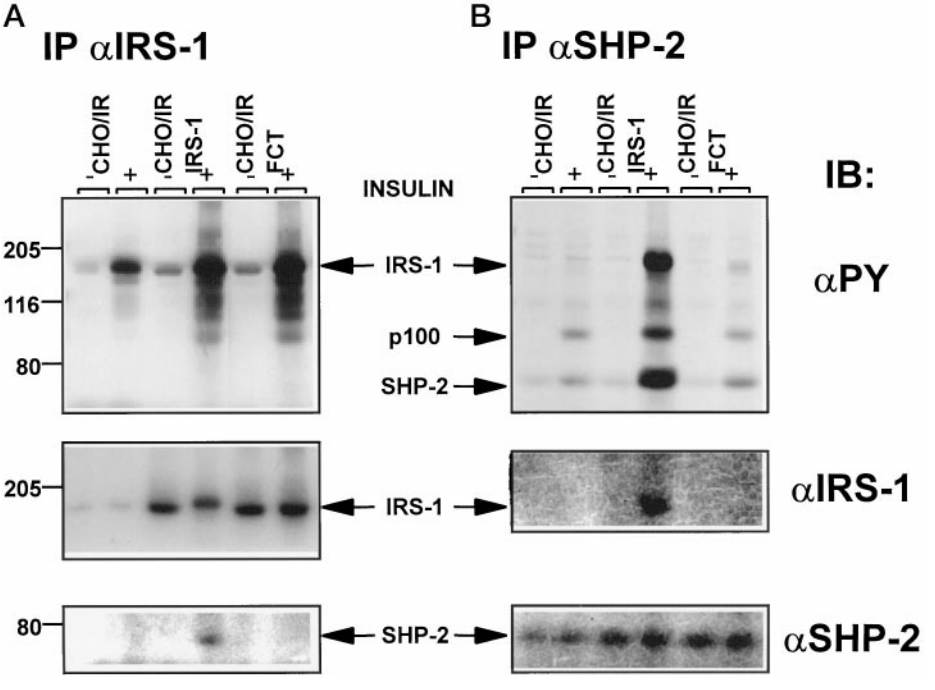

FIG. 2. **Insulin-stimulated tyrosine phosphorylation and SHP-2 association of IRS-1 isoforms in CHO^{IR} cells.** Quiescent CHO^{IR} cell lines expressing the indicated IRS-1 isoforms were incubated for 5 min in the absence (−) or presence (+) of 100 nM insulin before being lysed. Lysates were clarified and immunoprecipitated with either *A*, αIRS-1 or *B*, αSHP-2 antisera. Immunoprecipitates were collected, washed, and resolved by SDS-PAGE before being analyzed by immunoblotting with the indicated antisera and being exposed to autoradiography. Migration of molecular weight standards is shown on the *left* and the position of SHP-2, IRS-1, and p100 are indicated on the *right*.

greater than that of wild-type IRS-1 (Fig. 3); after normalizing for variations in expression of IRS-1 and IRS-1^{FCT}, the phosphotyrosine content of IRS-1^{FCT} was approximately 2-fold greater than for wild-type IRS-1 during stimulation with a range of insulin concentrations (Fig. 3, *graph*).

We investigated whether the increased insulin-stimulated tyrosine phosphorylation of IRS-1^{FCT} enhanced IRS-1 downstream signals by assaying PI 3′-kinase activity in αIRS-1 immunoprecipitates: Indeed, PI 3′-kinase activity associated with IRS-1^{FCT} was increased compared with wild-type IRS-1, and to a similar extent as tyrosine phosphorylation of IRS-1^{FCT} was increased (Fig. 4*A*).

We furthermore assessed downstream signaling by IRS-1^{FCT} by assaying generalized insulin-stimulated protein synthesis, which requires IRS-1-mediated PI 3′-kinase activity in the 32D cells (34, 35) (Fig. 4*B*). As previously shown, expression of the insulin receptor alone in $32D^{IR}$ cells was insufficient for the stimulation of protein synthesis by insulin, whereas co-expression of IRS-1 in these cells mediated insulin-stimulated protein synthesis. As with tyrosine phosphorylation and PI 3′-kinase binding, IRS-1^{FCT} enhanced insulin-stimulated protein synthesis compared with wild-type IRS-1. Therefore, the inhibitory effect of Tyr^{1172} and Tyr^{1222} on tyrosine phosphorylation and PI 3′-kinase activity carries over to the stimulation of protein synthesis.

MAP Kinase and Proliferative Signaling by IRS-1^{FCT}—The insulin receptor partially activates MAP kinase via the Shc/GRB-2 pathway in $32D^{IR}$ cells and expression of IRS-1 enhances this signal by recruiting GRB-2 to Tyr^{895} in IRS-1 (29). Since reagents which interfere with SHP-2 block insulin-stim-

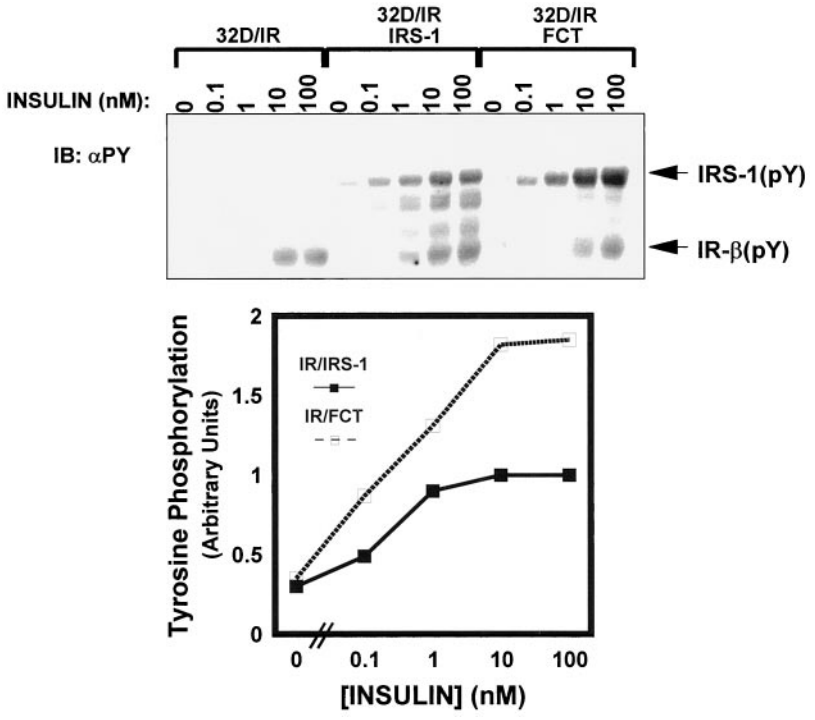

FIG. 3. **Tyrosine phosphorylation of IRS-1 and IRS-1^{FCT}.** Quiescent 32D^{IR} cells and 32D^{IR} cells expressing IRS-1 or IRS-1^{FCT} were incubated with the indicated concentration of insulin for 5 min before being collected and lysed. Lysates were resolved and immunoblotted with αPY (*upper panel*). Tyrosine phosphorylation of IRS-1 isoforms was quantitated on a PhosphorImager, normalized for IRS-1 expression, and plotted (*lower panel*). The data in the graph represent the average of two independent experiments.

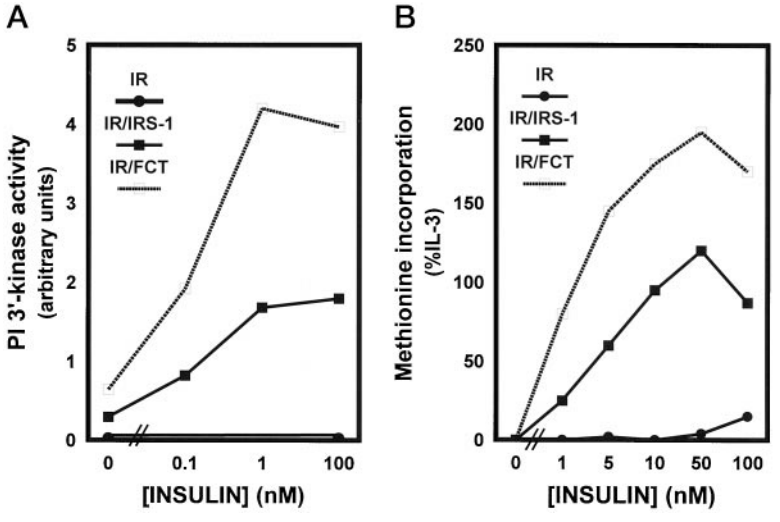

FIG. 4. **Stimulation of PI 3′-kinase and general protein synthesis by insulin in 32D^{IR} cell lines.** *A*, IRS-1-associated PI 3′-kinase activity. Quiescent 32D^{IR} cell lines expressing the indicated IRS-1 isoforms were incubated with various concentrations of insulin for 5 min before being lysed. Lysates were immunoprecipitated with αIRS-1. Immunoprecipitates were washed and assayed for associated PI 3′-kinase activity. Lipid kinase activity was quantitated on a PhosphorImager and is expressed in arbitrary units. *B*, insulin-stimulated [^{35}S]methionine incorporation. Quiescent 32D^{IR} cell lines expressing the indicated IRS-1 isforms were incubated with various concentrations of insulin for 6 h before being pulsed with [^{35}S]methionine for 1 h. Cells were collected and proteins precipitated with trichloroacetic acid and quantitated by radiation scintigraphy. Results are shown as percent of IL-3 effect. These data are representative of multiple similar experiments.

ulated MAP kinase signaling, we assayed the insulin-stimulated activation of MAP kinase in 32D^{IR} cells expressing the SHP-2 binding-defective IRS-1^{FCT} (Fig. 5*A*). Insulin stimulated the activation of MAP kinase in 32D^{IR} cells, and IRS-1 expression increased the sensitivity and intensity of the response. Furthermore, insulin stimulated MAP kinase activity similarly in 32D^{IR}/IRS-1^{FCT} and 32D^{IR}/IRS-1 cells, demonstrating that IRS-1 and IRS-1^{FCT} signal similarly to MAP kinase in 32D^{IR} cells. Therefore, the SHP-2-binding COOH-terminal tyrosine phosphorylation sites in IRS-1 are not involved in the insulin-stimulated MAP kinase signaling pathway in the 32D^{IR} cells.

Although insulin receptor expression alone mediates some activation of MAP kinase in the 32D cells, insulin-stimulated DNA synthesis, like protein synthesis, requires IRS-1 expression (Fig. 5*B*). In contrast to the effects observed in signaling to protein synthesis, however, IRS-1^{FCT} did not increase the proliferative response to insulin compared with wild-type IRS-1. Thus, the COOH-terminal tyrosines on IRS-1 mediate neither positive nor negative proliferative signals in 32D^{IR} cells.

DISCUSSION

We have examined the role of the COOH-terminal tyrosines (Tyr^{1172} and Tyr^{1222}) of IRS-1 for interactions with SHP-2 during insulin signaling. When phosphorylated by the insulin receptor, the motif containing Tyr^{1172} binds to the NH_2-terminal SH2 domain and the motif containing Tyr^{1222} binds to the COOH-terminal SH2 domain of SHP-2 (11). The binding of these motifs to the SH2 domains of SHP-2 activates the tyro-

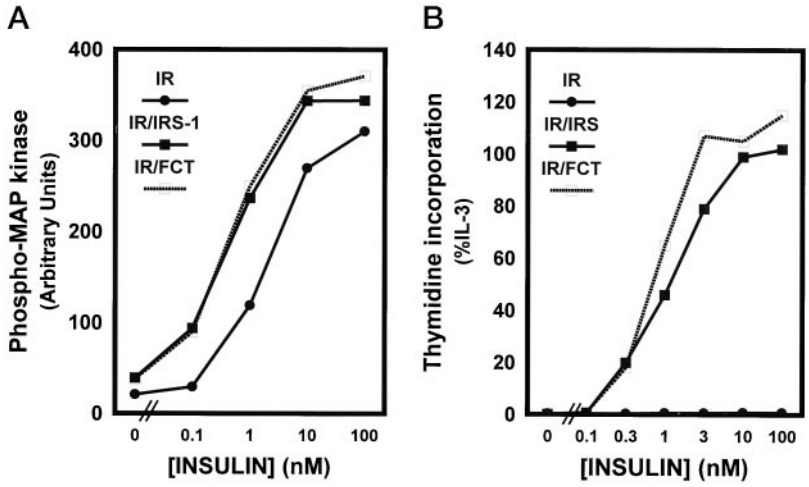

FIG. 5. **Insulin-stimulated MAP kinase activation and thymidine incorporation by IRS-1^{FCT}.** *A*, activation of MAP kinase. Quiescent 32D^{IR} cell lines were incubated with the indicated concentrations of insulin for 5 min before being lysed. Lysates were resolved by SDS-PAGE and phosphorylated MAP kinase was detected by immunoblotting with αPhospho-MAP kinase and quantitated on a PhosphorImager. Data for IR/IRS-1 and IR/FCT cells represent the average of two independently derived cell lines and are representative of multiple similar experiments. Results are expressed in arbitrary units. *B*, insulin-stimulated [^{3}H]thymidine incorporation. 32D^{IR} cell lines were incubated in medium containing the indicated concentrations of insulin for 48 h and pulsed with [^{3}H]thymidine for 3 h. DNA was collected by vacuum filtration. [^{3}H]Thymidine incorporation was determined by scintigraphy with aqueous fluor. Results are expressed as a percent of the IL-3-stimulated response. Results were obtained in duplicate or triplicate and are representative of numerous similar experiments.

sine phosphatase activity of the SHP-2 molecule by altering the conformation of the NH_2-terminal SH2 domain such that the active site of SHP-2 becomes available (12–14, 31–33). Thus, SHP-2 likely regulates insulin signaling by dephosphorylating tyrosine residues on IRS-1 or associated molecules.

Previous work on IRS-1 and SHP-2 has focused largely on interactions between purified proteins. Here we confirm in intact cells that the association of IRS-1 and SHP-2 depends upon Tyr^{1172} and Tyr^{1222}. Using the yeast two-hybrid system or purified proteins *in vitro*, others have suggested that SHP-2 and the insulin receptor can interact directly, without IRS-1 (36, 37). Our data in CHO^{IR} cells suggest that IRS-1 is necessary to link SHP-2 to the insulin receptor; insulin stimulates the tyrosine phosphorylation of SHP-2 when it is associated with IRS-1. The simplest interpretation of this data is that SHP-2 is phosphorylated when recruited to the insulin receptor-IRS-1 complex by the phosphorylated Tyr^{1172} and Tyr^{1222} of IRS-1. Furthermore, although previous reports suggest that SHP-2 enhances the coupling between IRS-1 and the insulin receptor, IRS-1^{FCT}, which does not bind SHP-2, mediates signaling as well or better than wild-type IRS-1. Thus SHP-2 is not required for insulin receptor-IRS-1 coupling in this system.

Numerous studies have suggested that SHP-2 activity is required for the insulin-stimulated activation of MAP kinases and proliferation. We examined the function of the SHP-2-binding defective IRS-1^{FCT} in insulin signal transmission in 32D^{IR} cells, as these cells contain no endogenous IRS proteins and facilitate the analysis of IRS-1 mutants (21, 22); IRS-1^{FCT} mediates MAP kinase activation and proliferation similarly to wild-type IRS-1 in the 32D^{IR} cells. Thus, at least in this system, IRS-1/SHP-2 interactions are not required for these two insulin-mediated events. In contrast, mutation of the SHP-2 binding site in IRS-1^{FCT} results in enhanced insulin-stimulated tyrosine phosphorylation of IRS-1; this increases the association of PI 3′-kinase with IRS-1^{FCT}, which in turn enhances insulin-stimulated protein synthesis. These data suggest a negative regulatory role for the COOH-terminal tyrosine phosphorylation sites on IRS-1 and, by extension, for the IRS-1/SHP-2 interaction.

A reasonable model for this effect is that tyrosine phosphorylation of the COOH-terminal sites on IRS-1 recruits and activates the SHP-2 phosphatase in complex with IRS-1. In this complex, SHP-2 dephosphorylates IRS-1 and/or the insulin receptor, reducing tyrosine phosphorylation of PI 3′-kinase binding YM*X*M motifs in IRS-1 (Fig. 6); indeed, others have shown that dephosphorylation of IRS-1 by SHP-2 *in vitro* is mediated by the IRS-1/SHP-2 interaction (38). Hence, our model of SHP-2/IRS-1 interactions is consistent with the emerging model of SHP-2/gp130 interaction; mutation of the SHP-2 binding sites in gp130 (the shared subunit of many IL-6-type cytokine receptors) prolongs activation of the associated JAK tyrosine kinase and the downstream STAT signaling proteins during oligomerization of gp130 or during CNTF signaling (39–41).

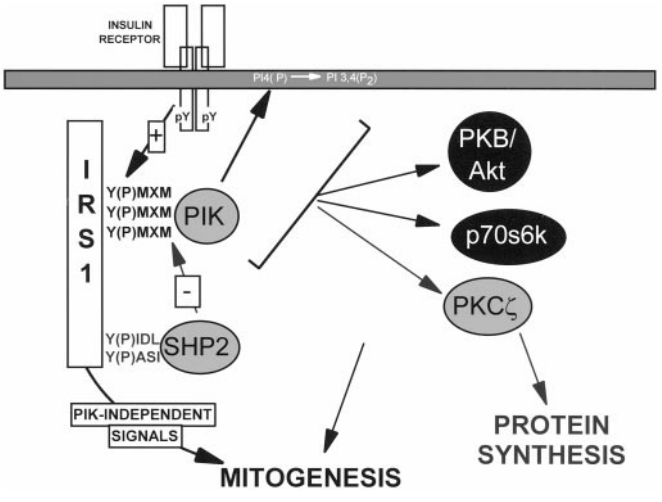

FIG. 6. **Model of SHP-2 function in IRS-1 signaling.** Activation of the insulin receptor causes tyrosine phosphorylation of multiple sites on IRS-1, including PI 3′-kinase and SHP-2 binding sites. PI 3′-kinase binding causes activation of downstream Ser/Thr kinases such as protein kinase B (*PKB*), p70s6k, and protein kinase Cζ (*PKC*ζ). Activation of protein kinase Cζ controls insulin-stimulated general protein synthesis. SHP-2 binding to IRS-1 causes dephosphorylation of some PI 3′-kinase-binding YM*X*M motifs, reducing the strength of the protein synthesis signal. Unlike protein synthesis, mitogenesis is not controlled only by PI 3′-kinase, but likely requires only a certain threshold level of PI 3′-kinase activation in conjunction with other, undefined signals mediated by IRS-1.

SHP-2 also binds and dephosphorylates other classes of tyrosyl phosphoproteins, including the SIRP/SHPS proteins and an approximately 95–100-kDa GAB-1-like tyrosyl-phosphorylated docking protein (p95–100) (42–51). Although, like IRS-1, these SHP-2 binding proteins are dephosphorylated by SHP-2, dephosphorylation in this case correlates with increased MAP kinase activation and proliferation. Thus, while SHP-2 dephosphorylates a variety of bound tyrosyl phosphoproteins, this dephosphorylation may either inhibit downstream signals (as in the case of IRS-1 and gp130) or mediate positive signals as in the case of SIRP/SHPS and p95–100.

While our data show no link between IRS-1/SHP-2 interactions and MAP kinase and proliferative signaling, a large body of data suggests that SHP-2 is required for insulin-stimulated activation of the $p21^{ras} \rightarrow$ MAP kinase $\rightarrow$ c-Fos pathway and proliferation (15–18, 52). These studies, however, have investigated SHP-2 signaling by overexpressing or microinjecting reagents (such as catalytically inactive SHP-2 mutants, SHP-2 SH2 domains, or αSHP-2 antibodies) which interfere with SHP-2 signaling. This approach blocks not only the SHP-2/IRS-1 interaction, but the interaction of SHP-2 with other proteins, such as SIRP/SHPS and p95–100. In contrast, we have examined the SHP-2/IRS-1 interaction specifically. Thus, we are able to draw conclusions specifically about the role of the SHP-2/IRS-1 interaction without disrupting signaling by other important SHP-2 interacting molecules which may mediate positive signals.

The function of $IRS\text{-}1^{FCT}$ also highlights differences between the signaling pathways connecting IRS-1 to protein synthesis and proliferation (Fig. 6). Although both pathways require PI 3′-kinase, the increased PI 3′-kinase activity generated by the $IRS\text{-}1^{FCT}$ enhances insulin-stimulated protein synthesis without altering insulin-stimulated proliferation. Similarly, an IRS-1 mutant whose only tyrosines are three YM*X*M motifs (and thus lacks the other 15 tyrosines in wild-type IRS-1, including six other YM*X*M motifs and both COOH-terminal tyrosine) mediates enhanced activation of PI 3′-kinase and protein synthesis, but impaired proliferation compared with wild-type IRS-1 (34, 53). Furthermore, insulin receptor variants which bind and activate PI 3′-kinase directly mediate protein synthesis but not proliferation in response to insulin (54).[2] Thus, activation of PI 3′-kinase and its downstream pathways suffices to stimulate general protein synthesis, while signals in addition to PI 3′-kinase (and which are not modified by the COOH-terminal tyrosine phosphorylation sites) are required for proliferation. A certain level of PI 3′-kinase may be required for proliferation, but PI 3′-kinase independent pathways mediated by IRS-1 must be limiting for this effect.

PI 3′-kinase and its downstream mediators control insulin stimulated protein synthesis directly, and may suffice to control other aspects of energy storage, such as glucose transport, as well (1, 35, 55, 56). The COOH-terminal tyrosine phosphorylation sites on IRS-1 may therefore offer potential therapeutic targets for the treatment of insulin resistance, since they appear to regulate PI 3′-kinase-mediated metabolic signals without affecting proliferative signaling.

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