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The Pleckstrin Homology Domain Is the Principle Link between the Insulin Receptor and IRS-1*

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Interaction domains located in the NH_2 terminus of IRS-1 mediate its recognition by the insulin receptor. Alignment of IRS-1 and IRS-2 reveals two homology regions: the $IH1^{PH}$ contains a pleckstrin homology (PH) domain, and the $IH2^{PTB}$ contains a phosphotyrosine binding (PTB) domain. A third region in IRS-1 called SAIN was proposed to contain another functional PTB domain. Peptide competition experiments demonstrated that the $IH2^{PTB}$ in IRS-2, like the corresponding domain in IRS-1, binds directly to peptides containing NP*X*Y motifs. In contrast, these peptides do not bind to $IH1^{PH}$ or the SAIN regions. In 32D cells the $IH1^{PH}$ was essential for insulin-stimulated tyrosine phosphorylation of IRS-1 and insulin-stimulated phosphatidylinositol 3-kinase activity and $p70^{s6k}$ phosphorylation. In contrast, the $IH2^{PTB}$ and the SAIN regions were not required for these insulin actions; however, the $IH2^{PTB}$ improved the coupling between IRS-1 and the insulin receptor. Overexpression of the insulin receptor in $32D^{IR}$ cells increased IRS-1 tyrosine phosphorylation and mediated insulin-stimulated DNA synthesis. The sensitivity of these responses was partially reduced by deletion of either the $IH1^{PH}$ or the $IH2^{PTB}$ and significantly reduced when both regions were deleted together. Thus, the PH and PTB domains equally couple IRS-1 to high levels of insulin receptor normally expressed in most cells, whereas at low levels of insulin receptors the PTB domain is inefficient and the PH domain is essential for a productive interaction.

During stimulation of cells with growth factors or cytokines, specific cell surface receptors are activated by dimerization, which stimulates the phosphorylation of tyrosine residues on the intracellular domain of the receptor itself or in tightly associated subunits. In many cases, signaling proteins with Src homology-2 domains (SH2 proteins)[1] bind directly to these phosphotyrosine-containing motifs (1). However, several plasma membrane receptor complexes do not associate directly with SH2 proteins. A growing number of these receptors utilize cytoplasmic docking proteins to provide an interface between the activated receptor and various SH2 proteins (2–6). One of the first docking molecules described was IRS-1, a major substrate of the insulin receptor tyrosine kinase (7). Two other such molecules have been described, including IRS-2 and Gab-1 (5, 6).

The IRS proteins and Gab-1 provide a scaffolding to assemble multiple SH2 proteins into a signaling complex, which is transiently linked to an activated receptor (8). The transient association of the IRS proteins with the activated receptors provides a mechanism for signal amplification and diversity. Thus, in a single cell, one activated insulin receptor may engage multiple copies of IRS-1, IRS-2, and Gab-1 to initiate a broad range of downstream signals. Similarly, multiple receptors can engage a single docking molecule. Although first described as a substrate for the insulin receptor, IRS-1 is now known to be phosphorylated by multiple systems, including the receptors for insulin and IGF-1, several interleukins (IL-2, IL-4, IL-9, IL-13 and IL-15), growth hormone, and interferons (interferon-α/β and interferon-γ) (3, 4, 9, 10).

Tyrosine phosphorylation of IRS proteins requires a sensitive mechanism for its selective interaction with an activated receptor. Analysis of IRS-1 using the yeast two-hybrid system reveals the importance of its NH_2 terminus for recognition by the insulin receptor (11, 12).

The cloning of IRS-2 and its alignment with IRS-1 revealed two strongly conserved regions in the NH_2 terminus of the IRS proteins (5). The first is called $IH1^{PH}$ because of its similarity to a pleckstrin homology (PH) domain. PH domains have been identified in several signaling proteins and may serve to localize them to the plasma membrane (13, 14). The second conserved region was called the $IH2^{PTB}$, reflecting a weak similarity to putative phosphotyrosine-binding (PTB) domains in Shc and other proteins (5). PTB domains recognize phosphotyrosine within NP*X*Y motifs, providing a potential mechanism to couple IRS-1 to Tyr^{960} in the juxtamembrane region of the insulin receptor (11, 12, 15). The $IH1^{PH}$ was shown previously to mediate the interaction of the insulin receptor with IRS-1, whereas peptide binding experiments revealed that the $IH2^{PTB}$ contains a functional PTB domain (16, 17). These regions are distinct from the so-called SAIN region, which contains a weak sequence similarity to the PTB domain of Shc and was also proposed to function as a PTB domain.

In this study, we compared the contribution of the $IH1^{PH}$, $IH2^{PTB}$, and SAIN to the interaction between the insulin receptor and IRS proteins in 32D cells. We concluded that the only functional PTB domain that binds to phosphorylated NP*X*Y motifs is located in the $IH2^{PTB}$ region. Surprisingly, in

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[1] The abbreviations used are: SH2 protein, signaling protein with Src homology-2 domain; IL, interleukin; PH, pleckstrin homology; PTB, phosphotyrosine binding; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; s6k, ribosomal S6 kinase.

32D cells, the IH1^{PH} is the principle element coupling the insulin receptor to IRS-1, although the IH2^{PTB} increases the sensitivity of this coupling.

MATERIALS AND METHODS

Construction of Fusion Proteins—Histidine-tagged fusion proteins were constructed using PCR products corresponding to the IH2^{PTB} regions of IRS-1 (amino acids 131–331) and IRS-2 (amino acids 166–376) and the SAIN domain of IRS-1 (amino acids 313–462). These regions were generated using specific oligonucleotide primers containing the appropriate restriction sites as described previously (16). The PCR products were digested with *Eco*RI and *Sal*I, subcloned into the pET30a (Novagen), and confirmed by sequencing. Glutathione *S*-transferase fusion proteins of the IH2^{PTB} regions in IRS-1 (amino acids 130–331) and IRS-2 (amino acids 166–376) and the IH1^{PH} region of IRS-1 (amino acids 1–134) were constructed as described above, except that the PCR products were subcloned into the pGEX2T vector (Pharmacia Biotech Inc.).

Peptide Binding and Competition—Synthetic peptides were generated using the F-moc synthesis method (Harvard Biopolymers Lab, Boston, MA) and were labeled with [^{125}I]Bolton-Hunter reagent (DuPont NEN) (18). For competition experiments, 20 μM of the appropriate fusion protein (His-tagged or glutathione *S*-transferase fusion) were incubated with 10 μl of a 1:1 slurry of Ni^{2+}- or glutathione *S*-transferase-agarose beads (Quiagen, Pharmacia) for 1 h at 4 °C in 50 mM Tris (pH 8.0) containing 200 mM NaCl. ^{125}I-Peptides (40,000 cpm) and the indicated amount of unlabeled competitor peptides were incubated with the recombinant regions for 3.5 h. Unbound peptides were removed by vacuum filtration on GF/C filters (VWR). The filters were then washed twice with cold binding buffer and dried, and radioactivity was determined by γ-counter.

Construction of IRS-1 Deletion Mutants—The construction of the IRS1$^{\Delta PH}$ mutant has been described (16). The IRS1$^{\Delta IH2}$, IRS1$^{\Delta SAIN}$, and IRS1$^{\Delta IH1/2}$ mutants were constructed as described (19). Briefly, specific oligonucleotide primers were used to generate PCR fragments, which resulted in the deletion of amino acids 155–309 (IRS1$^{\Delta IH2}$), residues 309–555 (IRS1$^{\Delta SAIN}$), or residues 6–309 (IRS1$^{\Delta PH/PTB}$). The PCR products were subcloned into rat IRS-1 in pBluescript (7) using *Bsp*EI and *Pfl*M1 and confirmed by sequencing. The full-length mutant cDNAs were subcloned into pCMVhis for expression using *Sac*I and *Hin*dIII (19).

Cell Lines—32D cells were used for these experiments because they contain a very low level of endogenous insulin receptor and no detectable IRS-1 or IRS-2. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% WEHI-3-conditioned medium (a source of IL-3). 32D cells (approximately 500 receptors/cell) and 32D^{IR} cells (approximately 16,000 receptors/cell) overexpressing IRS-1 or IRS1$^{\Delta IH1}$ have been described (16, 20). To introduce the new mutant cDNAs, 32D and 32D^{IR} cells were transfected by electroporation with the cDNA for IRS1$^{\Delta IH2}$, IRS1$^{\Delta IH1/2}$, or IRS1$^{\Delta SAIN}$. Transfected cells were selected with 5 mM histidinol (19, 20), and cell lines expressing similar levels of proteins were identified by immunoblotting with α-IRS-1 antibodies; constant expression of the human insulin receptor was confirmed by immunoblotting cell lines with antibody against the insulin receptor. Before each experiment, 32D cells were made quiescent by a 4-h incubation in serum-free and WEHI-free Dulbecco's modified Eagle's medium.

Antibodies and Growth Factors—Polyclonal antibodies against the COOH terminus of IRS-1 were used at a 1:300 dilution to immunoblot and 1:100 to immunoprecipitate, as described previously (21). This antibody recognizes IRS-1 and each deletion mutant equivalently as it is directed against a peptide in the COOH terminus of IRS-1. Antiphosphotyrosine (α-pY) antibodies were affinity-purified rabbit polyclonal antibodies (22); they were used at a 1:500 dilution for immunoblotting. p70 s6k antibodies were used at a dilution of 1:300 for Western blotting as described (23). Insulin was from Calbiochem.

Immunoblotting—Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol, resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Schleicher & Schuell) in Towbin buffer containing 0.02% SDS and 20% methanol (24). Membranes were blocked, probed, and developed as described previously and visualized using ^{125}I-protein A (Amersham Corp.) (19, 25). Blots were exposed to Kodak X-AR film or imaged on a Molecular Dynamics PhosphorImager.

Phosphatidylinositol 3-Kinase Activity—32D cell lines were grown, stimulated, lysed, and immunoprecipitated as for immunoprecipitations (above). Immune complexes were precipitated from the supernatant with protein A-Sepharose (Pharmacia) and washed successively in phosphate-buffered saline containing 1% Nonidet P-40 and 2 mM Na_3VO_4 (3 times), 100 mM Tris-HCl (pH 7.5) containing 500 mM LiCl and 2 mM Na_3VO_4 (3 times), and 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 2 mM Na_3VO_4 (2 times). The pellets were resuspended in 50 μl of 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA and combined with 10 μl of 100 mM $MnCl_2$ and 10 μl of 2 μg/μl phosphatidylinositol (Avanti) sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EGTA. The phosphorylation reaction was started by adding 10 μl of 440 μM ATP containing 30 μCi of [γ-^{32}P]ATP. After 10 min at 22 °C, the reaction was stopped with 20 μl of 8 N HCl and 160 μl $CHCl_3$:methanol (1:1). The samples were 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. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, visualized, and quantitated on a Molecular Dynamics PhosphorImager.

p70^{s6k} Gel Shift Assay—Quiescent cells were stimulated with 100 nM insulin for 30 min and collected as described above. Cells were lysed in ice-cold 10 mM potassium phosphate, 1 mM EDTA (pH 7.05) containing 0.5% Nonidet P-40, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mg/ml each of aprotinin and leupeptin. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Samples were denatured by boiling, and phosphorylated substrates were separated by 10% SDS-PAGE and analyzed by Western blotting, as described above.

Incorporation of [^{3}H]Thymidine into DNA in 32D Cells—Insulin-stimulated thymidine incorporation was assayed as described previously (20). Briefly, cells in log phase growth were washed, and 2×10^5 cells were seeded into 1 ml of medium in each of 24 wells containing RPMI with 10% fetal bovine serum alone or containing various concentrations of insulin or IL-3-containing conditioned medium (WEHI). Cells were grown for 48 h at 37 °C. [^{3}H]thymidine (ICN) was added to a final concentration of 0.5 mCi/ml, and incubation was continued for 2 h. Cells were collected onto glass microfiber filters, cells were lysed, and unincorporated nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid for 1 min.

RESULTS

Identification of the Functional PTB Domain in IRS-1 and IRS-2—The relative positions the IH1^{PH}, IH2^{PTB} and SAIN regions in IRS-1 are shown in Fig. 1. To confirm which region binds to phosphorylated NP*X*Y peptides, recombinant domains expressed as glutathione *S*-transferase or polyhistidine fusion proteins were incubated with an [^{125}I]peptide. The sequence of this peptide, GGLYASSNPApYSASD, is based on the sequence around Tyr^{960} in the insulin receptor juxtamembrane, except that an alanine residue was substituted at the Tyr^{-1} position to increase its affinity as described previously (17). Recombinant IH2^{PTB} from IRS-1 and IRS-2 was incubated with the ^{125}I-NPApY peptide and various concentrations of the unlabeled competitor peptides: NPApY peptide, the wild-type sequence GGLYASSNPEpYSASD (designated NPEpY peptide), or the unphosphorylated NPEY peptide. As previously reported, about half of the ^{125}I-NPApY peptide was displaced from the IH2^{PTB} of IRS-1 with 5 μM of the unlabeled NPApY peptide; the native NPEpY peptide was 6-fold less sensitive (Fig. 2*A*). As expected, displacement of the ^{125}I-NPApY peptide was dependent on tyrosine phosphorylation, since the unphosphorylated NPEY peptide did not compete in these assays (Fig. 2*A*). Similar results were observed with the unphosphorylated NPAY peptide (data not shown).

The relative affinities of the IH2^{PTB} region in IRS-1 and IRS-2 were compared in binding competition experiments using the iodinated and uniodinated NPApY peptide. In each case, half of the bound ^{125}I-NPApY peptide was displaced with 5 μM of the unlabeled peptide (Fig. 2*B*). In contrast, ^{125}I-NPApY peptide did not bind to the recombinant SAIN region, suggesting that this region is not a functional PTB domain. Similarly, the ^{125}I-NPApY peptide did not bind to the IH1^{PH} domain of IRS-1 (Fig. 2*C*). Thus, the IH2^{PTB} region contains the

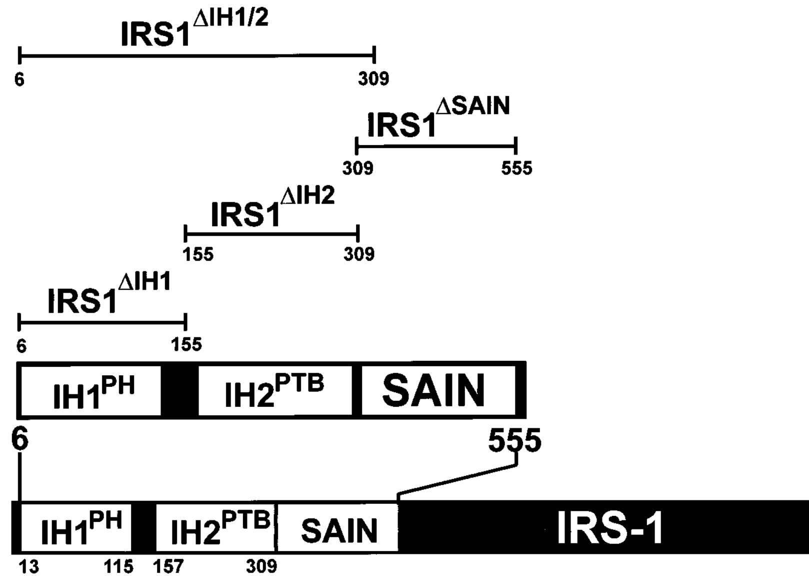

FIG. 1. **Structure of IRS proteins.** Schematic representations of the various deletion mutants of IRS-1 are shown. The locations of the IH1^PH^, IH2^PTB^, and SAIN domains of IRS-1 are indicated.

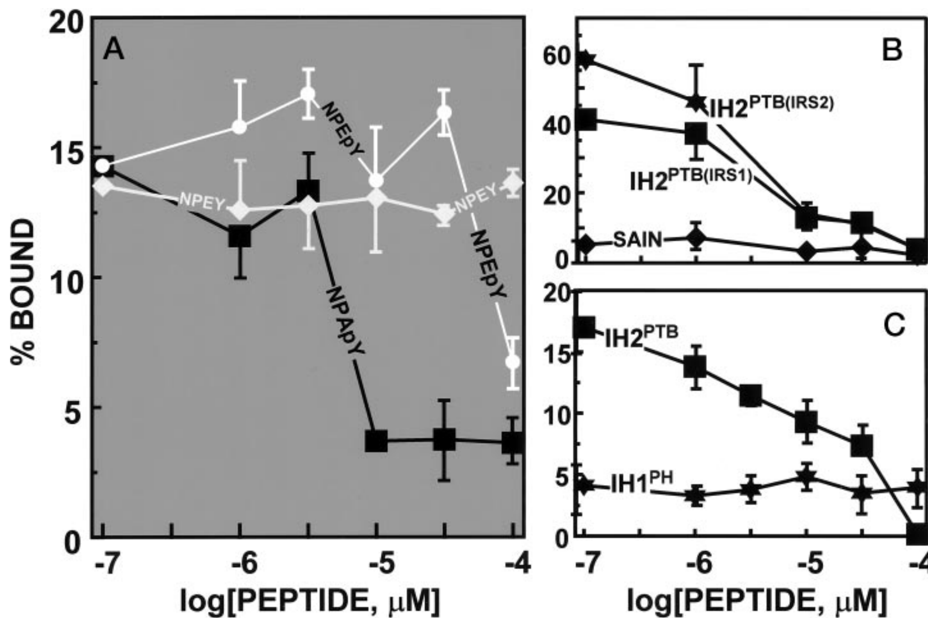

FIG. 2. **Peptide competition.** *A*, ^{125}I-NPApY-containing peptide was bound to a His-tagged IRS-1^{PTB} fusion protein as described under "Materials and Methods." Unlabeled NPEpY-, NPApY-, and unphosphorylated NPEY-containing peptides were used to displace the labeled peptide at the indicated concentrations. Data are triplicate determinations, and *error bars* represent standard error for each point. *B*, ^{125}I Bolton-Hunter-labeled NPApY-containing peptide was bound to His-tagged IRS-1^{PTB}, IRS-2^{PTB}, and IRS-1^{SAIN} fusion proteins and displaced by the indicated amounts of unlabeled NPApY peptide. Data represent the average of three separate experiments, and *error bars* represent the standard error for each point. *C*, glutathione *S*-transferase-IRS-1^{PTB} and glutathione *S*-transferase-IRS1^{PH} fusion proteins were analyzed as in *B*. Data are triplicate determinations, and *error bars* represent the standard error for each point.

only functional PTB domain, and it displays similar binding characteristics in IRS-1 and IRS-2.

The IH1^{PH} and IH2^{PTB} Mediate the Tyrosine Phosphorylation of IRS-1 in 32D Cells—We analyzed the contributions of the NH_2-terminal regions of IRS-1 for insulin signaling *in vivo* by modifying the cDNA of rat IRS-1 to delete the IH1^{PH}, IH2^{PTB}, or SAIN regions. These cDNA constructs were expressed in parental 32D cells, which contain no IRS proteins and low levels of the murine insulin receptor (approximately 500 receptors/cell) (20, 26). The expression of wild-type and mutant IRS-1 molecules in several isolated clones was measured by immunoblotting with antibodies directed against the COOH terminus. A set of 32D cell clones expressing similar levels of IRS-1 molecules was selected for further analysis (Fig. 3*A*).

Insulin-stimulated tyrosine phosphorylation of IRS-1 in specific immunoprecipitates was examined by immunoblotting with α-pY antibodies. In agreement with previous results, deletion of the IH1^{PH} region abrogated insulin-stimulated tyrosine phosphorylation of IRS1$^{\Delta IH1}$, even at maximum insulin concentrations (Fig. 3*B*). In contrast, deletion of the IH2^{PTB} region reduced tyrosine phosphorylation of IRS1$^{\Delta IH2}$ at low to moderate insulin doses (Fig. 3*B*). After normalizing for the expression of IRS-1, the deletion of the IH2^{PTB} region reduced the sensitivity of the phosphorylation, but the maximal response at 100 nM insulin was obtained (Fig. 3*C*). Thus, the IH1^{PH} region provided sufficient coupling in the absence of the IH2^{PTB} region, but the latter region was insufficient alone.

To confirm that the IH1^{PH} region contributes significantly to this residual phosphorylation, both the IH1^{PH} and IH2^{PTB} regions were deleted (amino acids 6–309). Although the IRS1$^{\Delta IH1/2}$ was expressed stably in 32D cells, its tyrosine phosphorylation was completely impaired (Fig. 3, *B* and *C*). These results further suggest that the IH1^{PH} contributes significantly to the interaction between IRS-1 and the insulin receptor. Previous studies suggest that the SAIN domain, located immediately COOH-terminal to the IH2^{PTB}, was important for the recognition between the insulin receptor and IRS-1 (11, 12). However, these results suggest that the SAIN region alone cannot promote the interaction with the insulin receptor. Consistent with these results, an IRS-1 molecule lacking the SAIN region (amino acids 309–555 in IRS-1) but retaining the IH1^{PH} and IH2^{PTB} displayed nearly normal insulin-stimulated tyrosine phosphorylation in 32D cells (Fig. 3, *B* and *C*). Moreover, another mutant IRS-1 molecule, designated IRS1$^{\Delta N2}$, which

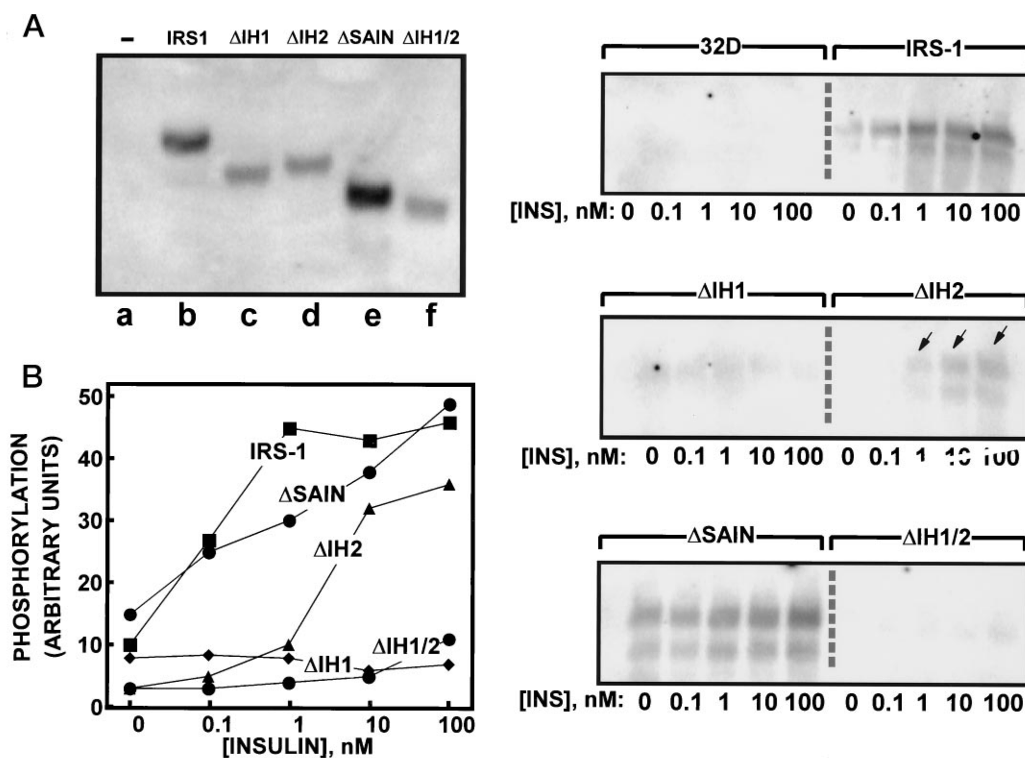

FIG. 3. **Expression and tyrosine phosphorylation of mutant IRS-1 molecules expressed in 32D cells.** *A*, clonal 32D cell lines were lysed, and proteins were separated by SDS-PAGE, transferred, and Western blotted with anti-IRS-1 antibodies. *B*, the same cell lines were starved, stimulated with the indicated amount of insulin, immunoprecipitated with anti-IRS-1 antibodies, and probed with antiphosphotyrosine antibodies. Similar results were observed in three separate experiments using different clonal cell lines of each mutant. *Arrows* indicate the position of the $IRS1^{\Delta IH2}$ deletion mutant. *C*, the *graph* depicts the quantitation of the tyrosine phosphorylation dose-response experiment. Data are normalized for the amount of IRS-1 expressed in each cell line.

lacked both the $IH2^{PTB}$ and SAIN but retained the $IH1^{PH}$ underwent insulin-stimulated tyrosine phosphorylation similar to $IRS1^{\Delta PTB}$.[2] Thus, deletion of the SAIN region does not impair IRS-1 phosphorylation, whereas the presence of the $IH1^{PH}$ is essential for interaction with the insulin receptor in 32D cells.

PI 3-Kinase Activity and $p70^{s6k}$ Phosphorylation in 32D Cells—We next examined two downstream IRS-1-dependent signaling events in the 32D cells expressing equivalent amounts of the various IRS-1 deletion mutants. IRS-1 immunoprecipitates were prepared, and PI 3-kinase activity was determined in an *in vitro* kinase assay. Deletion of the $IH1^{PH}$ domain markedly decreased the amount of PI 3-kinase activity associated with IRS-1 at maximum doses of insulin (100 nM) (Fig. 4*A*). In contrast, deletion of either the $IH2^{PTB}$ or the SAIN domain did not affect the amount of IRS-1-associated PI 3-kinase activity at maximum doses of insulin. Furthermore, cell lines expressing $IRS1^{\Delta IH1/2}$ showed no insulin-stimulated PI 3-kinase activity, since this molecule was not efficiently tyrosine-phosphorylated even at maximal doses of insulin. These results are consistent with the tyrosine phosphorylation data described above.

The serine/threonine kinase p70 s6k, is downstream of PI 3-kinase, and in 32D cells it requires IRS-1 for insulin-stimulated activation (16). The increase in phosphorylation associated with the activation of $p70^{s6k}$ was monitored by the decrease in mobility during SDS-PAGE analysis. Similar to the tyrosine phosphorylation and PI 3-kinase results, insulin-stimulated (100 nM) phosphorylation of $p70^{s6k}$ is abrogated in 32D cell lines expressing the $IH1^{PH}$ deletion mutant, whereas cell lines expressing deletion mutants of either the $IH2^{PTB}$ or SAIN domain mediate this signaling event (Fig. 4*B*). Moreover, insulin failed to stimulate the phosphorylation of $p70^{s6k}$ in 32D/$IRS1^{\Delta IH1/2}$ cells.

Phosphorylation of IRS-1 in 32D Cells Overexpressing the Insulin Receptor—Based on previous experiments, we hypothesized that the $IH1^{PH}$ and the $IH2^{PTB}$ regions together mediate the sensitive coupling of IRS-1 and the insulin receptor. However, our previous work suggested that one of these regions may be sufficient at high levels of receptor expression (16). $32D^{IR}$ cells prepared previously and shown to express stable levels of the insulin receptor (approximately 16,000 receptors/cell) were transfected with wild-type or mutant IRS-1 molecules, as shown by immunoblotting (Fig. 5*A*). Unlike our results with 32D cells, $IRS1^{\Delta IH1}$, $IRS1^{\Delta IH2}$, and $IRS1^{\Delta SAIN}$ were tyrosine-phosphorylated during insulin stimulation of the $32D^{IR}$ cells (Fig. 5*B*). However, insulin-stimulated tyrosine phosphorylation of $IRS1^{\Delta IH1/2}$ was markedly impaired even at maximal doses of insulin (100 nM). Thus, at high insulin receptor levels, both $IH1^{PH}$ and the $IH2^{PTB}$ independently mediate insulin-stimulated tyrosine phosphorylation of IRS-1; however, the results in 32D cells with low levels of insulin receptor suggest that the $IH2^{PTB}$ domain is less efficient than the $IH1^{PH}$ domain.

IRS-1-mediated DNA Synthesis in $32D^{IR}$ Cells—Expression of both the insulin receptor and an IRS protein is required for insulin-stimulated mitogenesis in 32D cells (5, 20). Mitogenic activity was determined by assaying the amount of insulin-stimulated [^{3}H]thymidine incorporation in $32D^{IR}$ cells matched for expression of both insulin receptor and IRS-1 (Fig. 6). As expected, 32D cells expressing only the insulin receptor showed no insulin-stimulated mitogenesis, whereas co-expression with IRS-1 resulted in strong insulin-stimulated mitogenesis (Fig. 6). In agreement with the tyrosine phosphorylation data,

[2] M. G. Myers and M. F. White, unpublished observations.

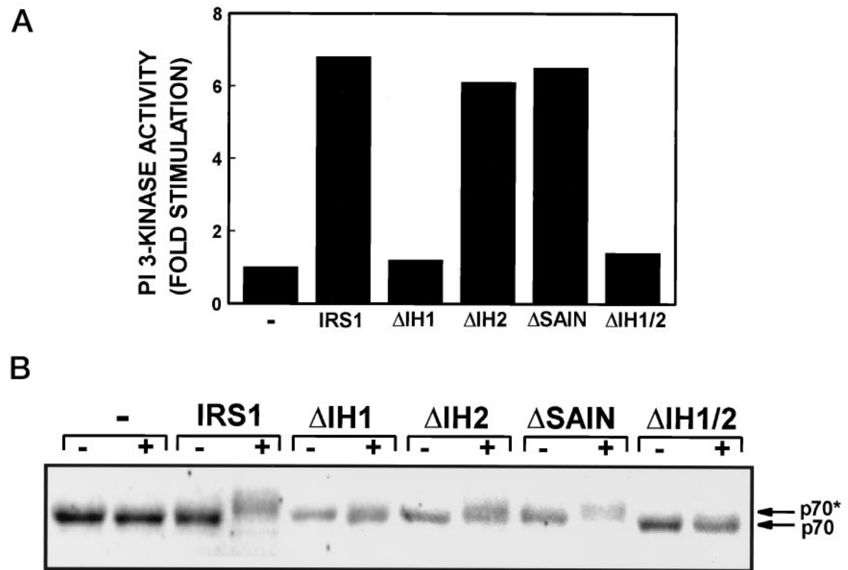

FIG. 4. **PI 3-kinase and p70^{s6k} activity in 32D cells.** *A*, 32D cell lines expressing equivalent amounts of the various IRS-1 deletion mutants were starved in unsupplemented medium for 4 h and stimulated for 5 min with 100 nM insulin. IRS-1 immunoprecipitates were prepared, and *in vitro* PI 3-kinase assays were performed as described under "Materials and Methods." Data represent the average of duplicate determinations and are expressed as -fold stimulation. *B*, 32D cell lines were starved in unsupplemented media for 4 h and stimulated with 100 nM insulin for 30 min. Lysates were prepared and separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-p70^{s6k} antibodies.

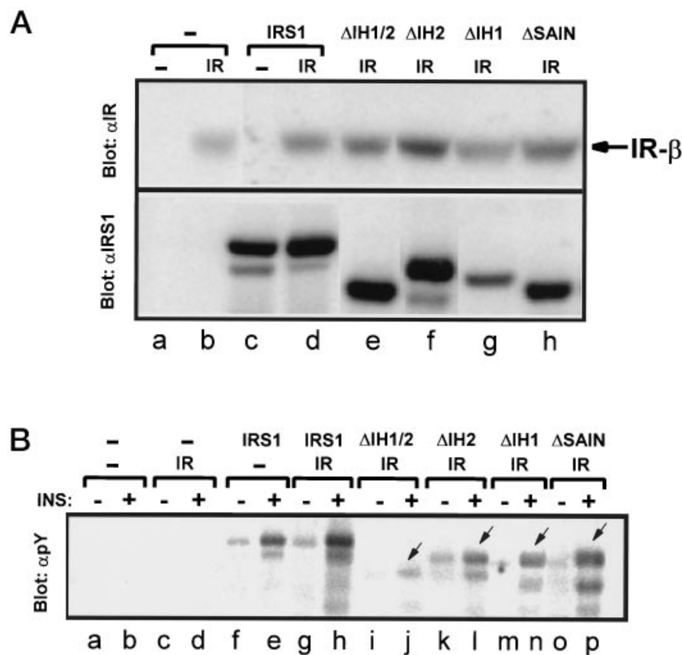

FIG. 5. **Phosphorylation of wild-type and mutant IRS-1 proteins in 32D^{IR} cells.** Various 32D cell lines expressing equal amounts of IR and IRS-1 are shown (*A*). *B*, these cell lines were made quiescent in unsupplemented Dulbecco's modified Eagle's medium, stimulated with 100 nM insulin, and lysed. Cell lysates were immunoprecipitated with anti-IRS-1 antibodies, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibodies. *Arrows* indicate the position of the IRS-1 deletion mutants. Similar results were observed in three separate experiments.

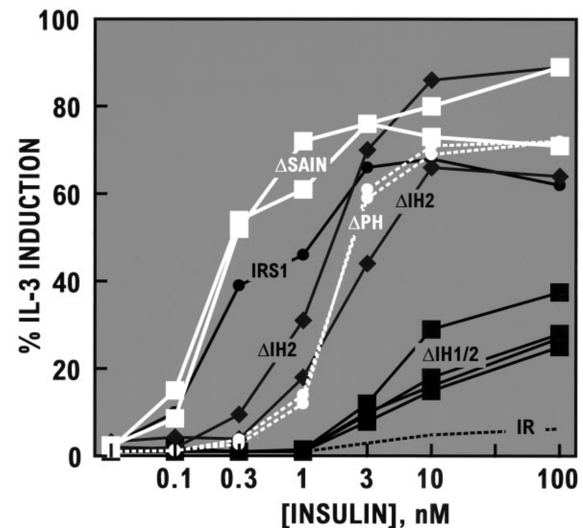

FIG. 6. **Insulin-stimulated [^{3}H]thymidine incorporation in 32D^{IR} cells.** 32D cell lines expressing equal amounts of IRS-1 and insulin receptor were analyzed for insulin-stimulated mitogenic activity as described under "Materials and Methods." Each *line* represents individual clonal cell lines. The *graph* depicts a representative experiment, and similar results were obtained with multiple clonal cell lines expressing each deletion mutant ($n = 6$).

IRS1$^{\Delta SAIN}$ also mediated DNA synthesis in 32D^{IR} (Fig. 6). Thus, the SAIN region is unlikely to mediate the interaction between IRS-1 and the insulin receptor and is not essential for this downstream response.

Consistent with the phosphorylation studies, the 32D^{IR}/ΔIH1 cells were slightly less sensitive to insulin but reached the same maximum response as cells expressing wild type IRS-1 (Fig. 6). Moreover, the 32D^{IR}/ΔIH2 cells exhibited a similar decrease in sensitivity (Fig. 6). These results are consistent with the dose response of insulin-stimulated IRS-1 tyrosine phosphorylation observed in these cells (data not shown). However, the IRS1$^{\Delta IH1/2}$ poorly mediated DNA synthesis during insulin stimulation of 32D^{IR} cells. The response to insulin was very insensitive, and the impaired responses persisted even at high insulin concentrations. The weak signaling by IRS1$^{\Delta IH1/2}$ was consistent with its impaired tyrosine phosphorylation in 32D and 32D^{IR} cells (Fig. 6).

DISCUSSION

Several years ago we proposed that Tyr^{960} in the intracellular juxtamembrane region of the insulin receptor was essential for IRS-1 phosphorylation and normal biological responses during insulin stimulation (15). Tyr^{960} is one of the first residues phosphorylated in the β-subunit of the receptor (27). This residue is located in an NP*X*Y motif, and insulin receptors with mutations in this region are catalytically active but poorly phosphorylate IRS-1 (previously called pp185) (15). Similarly, Shc utilizes a similar mechanism, since it is not phosphorylated by the mutant receptor (28). Based on these results, we proposed that the phosphorylated NP*X*Y motif mediates substrate recognition, which is essential for the insulin response (7, 15). The recent identification of the PTB domain in Shc and the

recognition of a functionally similar domain in the IH2^{PTB} region of the IRS proteins provide a provisional explanation for the role of Tyr^{960} during insulin receptor signaling (5, 29–31).

Tyr^{960} is not absolutely required for the phosphorylation of IRS-1, especially when IRS-1 is expressed at high levels, and the present study demonstrates that the PTB domains is not essential for IRS-1 coupling at high or low receptor levels (32). Therefore, other mechanisms to couple the insulin receptor to IRS-1 must exist. Our results point to the important contribution of the PH domain in the IH1^{PH} region of the IRS proteins as an essential and sensitive element in the recognition of IRS proteins by the insulin receptor. Consequently, tyrosine phosphorylation of IRS-1 and the activation of downstream signals such as PI 3-kinase and p70^{s6k} phosphorylation are markedly decreased during insulin stimulation of 32D cells expressing IRS1$^{\Delta IH1}$. Clearly, the PH domain in the IH1^{PH} region provides an important mechanism to sensitively couple IRS proteins to the insulin receptor. Under these conditions, the PTB domain contributes an additional site of interaction, which increases the sensitivity of insulin receptor coupling, but it is not essential. Moreover, when the insulin receptor is expressed at high levels, either domain is sufficient to couple IRS-1 to the insulin receptor. Thus, the PH domain in the IH1^{PH} region provides the most sensitive coupling to the insulin receptor, whereas the PTB domain in the IH2^{PTB} region provides less sensitive coupling. Under physiological conditions, it is likely that the contributions of both domains are necessary to facilitate a productive receptor/substrate interaction.

Recently, the first two-thirds of the IH2^{PTB} region was found by deletional analysis to contain the functional PTB domain, and this domain was successfully crystallized and found to be similar to the PTB domain of Shc (17). The NP*X*Y phosphopeptide adopts a β-turn and forms an antiparallel β-strand with a β-sheet of the PTB domain. This binding is stabilized by charge interactions between the phosphotyrosine residue in the peptide and arginine residues in the PTB domain and by hydrophobic interactions between residues in the NH_2-terminal portion of the peptide and the PTB domain (33). Interestingly, the PTB is structurally similar to PH domains, which are composed of a β-sandwich capped by an α-helix (34). From a structural perspective, the PTB domains in the IRS proteins and Shc can be classified as a type of PH domain that binds phosphorylated NP*X*Y motifs (35). From this point of view, the IRS proteins contain two PH domains in the IH1^{PH} and IH2^{PTB} regions; the relevant ligand for the PH domain in the IH1^{PH} region is unknown.

The physiologically relevant ligands for PH domain in various proteins have been investigated. In several cases, PH domains have been found to bind phospholipids, which leads to the generalization that PH domains mediate the interaction of signaling proteins with the plasma membrane (1, 36). By contrast, the PH domain in β-ARK binds to both the $\beta\gamma$-subunits of heterotrimeric G-proteins and phospholipids (37). These results have led to the general conclusion that PH domains may mediate the association of signaling proteins with membrane components. For IRS-1, both the IH1^{PH} and the IH2^{PTB} domains bring IRS-1 to the plasma membrane, the IH2^{PTB} providing a direct link to the insulin receptor and the IH1^{PH} providing another specific interaction. Since the IH1^{PH} region is essential for the coupling between low levels of insulin receptor and IRS-1, the ligand is expected to be specific and have high affinity. However, this ligand does not appear to exist in the insulin receptor, since several attempts to observe interactions between the PH domains and the insulin receptor have failed (11, 38). Possible candidates for specific PH domain ligands include various inositol phosphates, phosphorylated membrane components, and specific protein sequences containing phosphorylated tyrosine, serine, threonine, or histidine residues (35).

IRS proteins are the principle substrates for the insulin receptor, and their phosphorylation is observed in all insulin-sensitive cells. This may arise because IRS proteins contain two coupling mechanisms to ensure an efficient interaction with the receptor. However, it is well known that all insulin responses are not equally sensitive to insulin (39). One mechanism to adjust coupling efficiency between the insulin receptor and various substrates may be to include only one coupling domain. The recent discovery of Gab-1 as an insulin receptor substrate supports this hypothesis (6). Gab-1 is a 77-kDa protein that migrates at 115–120 kDa during SDS-PAGE. Gab-1 contains a PH domain with 44% similarity to IRS-1. Like IRS-1, the COOH-terminal region contains several putative tyrosine phosphorylation motifs with a potential to interact with various SH2 proteins. However, without an IH2^{PTB} region, Gab-1 may rely entirely on its PH domain for interaction with the insulin receptor. By analogy to our current results, Gab-1 is likely to be a less efficient substrate than the wild-type IRS proteins. It is likely that other insulin receptor substrates will be identified with similar PH domains to mediate receptor coupling.

Shc is an insulin receptor substrate that appears to be coupled solely through a PTB domain. This is consistent with the inhibition of Shc phosphorylation by mutations of Tyr^{960} in the insulin receptor (28). It is unknown whether overexpression of Shc would restore its phosphorylation by this mutant receptor; however, without an alternative mechanism for coupling it appears doubtful. Although alone the IH2^{PTB} region of IRS-1 is not sufficient to mediate IRS-1 phosphorylation, the PTB domain in Shc may succeed because it has a 6-fold higher affinity for the NPEY motif in the insulin receptor (17). Nevertheless, Shc is a rather poor substrate of the insulin receptor. Using 32D cells with low endogenous insulin receptor levels, insulin has no effect on the phosphorylation of Shc, whereas overexpression of the receptor mediates Shc phosphorylation during insulin stimulation (19). Thus, under ordinary conditions, Shc may compete weakly with IRS-1 for tyrosine phosphorylation by the insulin receptor kinase.

Although the IH1^{PH} and the IH2^{PTB} regions are very similar in IRS-1 and IRS-2, other regions in the IRS proteins may contribute to the coupling with the insulin receptor. IRS-2 contains an interaction domain between residues 591–786, which was detected in a yeast two-hybrid screen (38). Interestingly, the corresponding region in IRS-1 does not display this function, although the amino acid sequences are similar, and whether this region is important for coupling in cells is currently unknown. IRS-1 contains a region between residues 309 and 555 called SAIN, which appears to mediate the interaction between the insulin receptor and IRS-1 in the yeast two-hybrid screen (11). However, the SAIN region has no binding affinity for phosphorylated NP*X*Y peptides, and it is not essential for coupling between the insulin receptor and IRS-1 in 32D cells.

IRS proteins are also involved in signaling events initiated by several classes of receptors, including growth factor, cytokine, and interferon receptors (3–5, 40, 41). Many of these receptors are structurally disparate and utilize distinct signaling strategies; however, they all engage IRS proteins. The receptors for IGF-1 and IL-4 both contain analogous NP*X*Y motifs in their intracellular subunits, suggesting that PTB domain interactions at least partially mediate the association of these receptors; however, the receptors for growth hormone and interferon α and β do not contain this motif, and the way in which they recognize IRS proteins as substrates is presently

undefined. One commonality of these receptors is the engagement of Janus family kinases (JAK) (42). Therefore, it is possible that these kinases somehow bind to IRS proteins and recruit them to the receptor signaling complex. Consistent with the hypothesis, we recently observed that recombinant $IH1^{PH}$ associates with Tyk2 in Daudi cells (9). Future experiments are necessary to investigate this possibility and to further define the molecular basis of IRS protein substrate recognition in these receptor systems.

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