Interaction of Insulin Receptor Substrate 3 with Insulin Receptor, Insulin Receptor-related Receptor, Insulin-like Growth Factor-1 Receptor, and Downstream Signaling Proteins*

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Insulin receptor substrates (IRS) mediate biological actions of insulin, growth factors, and cytokines. All four mammalian IRS proteins contain pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains at their N termini. However, the molecules diverge in their C-terminal sequences. IRS3 is considerably shorter than IRS1, IRS2, and IRS4, and is predicted to interact with a distinct group of downstream signaling molecules. In the present study, we investigated interactions of IRS3 with various signaling molecules. The PTB domain of mIRS3 is necessary and sufficient for binding to the juxtamembrane NPXY motif of the insulin receptor in the yeast two-hybrid system. This interaction is stronger if the PH domain or the C-terminal phosphorylation domain is retained in the construct. As determined in a modified yeast two-hybrid system, mIRS3 bound strongly to the p85 subunit of phosphatidylinositol 3-kinase. Although high affinity interaction required the presence of at least two of the four YXXM motifs in mIRS3, there was no requirement for specific YXXM motifs. mIRS3 also bound to SHP2, Grb2, Nck, and Shc, but less strongly than to p85. Studies in COS-7 cells demonstrated that deletion of either the PH or the PTB domain abolished insulin-stimulated phosphorylation of mIRS3. Insulin stimulation promoted the association of mIRS3 with p85, SHP2, Nck, and Shc. Despite weak association between mIRS3 and Grb2, this interaction was not increased by insulin, and may not be mediated by the SH2 domain of Grb2. Thus, in contrast to other IRS proteins, mIRS3 appears to have greater specificity for activation of the phosphatidylinositol 3-kinase pathway rather than the Grb2/Ras pathway.

Insulin binding to the extracellular domain of the insulin receptor (IR) results in the activation of the insulin receptor tyrosine kinase, which undergoes autophosphorylation of tyrosine residues in its cytoplasmic domain and subsequently phosphorylates several intracellular proteins including insulin receptor substrates (IRS), Shc (1), Gab1 (2), pp120/HA4 (3, 4), and p62 (5). Upon phosphorylation, IRS proteins serve as docking molecules for downstream signaling proteins that contain SH2 domain(s) and therefore activate signal transduction pathways.

To date four different IRS molecules have been cloned (6–10). Recently the roles of IRS1 and IRS2 in insulin signaling have been extensively investigated (11). However less is known about the role of IRS3 in insulin signal transduction (8, 9, 12–17). Sequence analysis reveals that IRS3 has the same general architecture as IRS1, IRS2 and IRS4: a pleckstrin homology (PH) domain at the N terminus, followed by a phosphotyrosine binding (PTB) domain, and a C-terminal domain containing multiple sites for tyrosine phosphorylation (designated as the phosphorylation domain) (8, 9). Both PH and PTB domains of IRS3 exhibit a high degree of similarity to those in IRS1, IRS2, and IRS4. Although the specific ligands for the PH domain of IRS proteins are still unknown, the PH domains of IRS1 has been shown to be required for its coupling to the activated insulin receptor (18–21). In studies using the yeast two-hybrid systems, the PTB domains of IRS1 and IRS2 have been demonstrated to bind to the NPXY motif in the juxtamembrane domain of the IR and IGF1R (22–26). The phosphorylation domain of IRS3 is only about half the size of, and shares little if any homology with, IRS1, IRS2, and IRS4. Nevertheless, it has similar tyrosine motifs that can be phosphorylated by the insulin receptor tyrosine kinase in response to insulin stimulation, and subsequently bind to SH2 domains of signaling molecules such as p85, SHP2, and Grb2 (8, 9).

In the present study, we used the yeast two-hybrid system (27, 28) to investigate the interaction of IRS3 with the insulin receptor, the insulin receptor-related receptor, and the insulin-like growth factor-1 receptor and also to downstream SH2 domain-containing signaling proteins. The minimal PTB domain of IRS3 binds relatively weakly to the IR cytoplasmic domain, but the binding was markedly enhanced by the presence of either the PH domain or the phosphorylation domain of IRS3. This interaction is dependent upon the intact NPXY motif within the juxtamembrane domain of the IR. Deletion of either the PH domain or the PTB domain abolished the in vivo tyrosine phosphorylation of IRS3 by the IR in COS-7 cells. In addition, by fusing the IR kinase domain to the phosphorylation domain of IRS3, we used the yeast two-hybrid system to study the interaction of IRS3 with downstream SH2 domain-containing signaling proteins such as p85 subunit of PI 3-kinase, SHP2, Grb2, Nck, and Shc. In this modified yeast two-hybrid system, IRS3 interacts strongly with the SH2 domains of p85, moderately with Grb2, Nck, and the SH2 domains of Shc and SHP2, but only weakly with the SH2 domains of PLCγ1. These interactions required intact kinase activity of the insulin receptor. Moreover, the high affinity binding of IRS3 with p85 required at least two intact YXXM motifs in IRS3 and two
tandem SH2 domains in p85. Furthermore, using an immunoprecipitation approach, we showed that insulin stimulated the association of IRS3 with p85, Nck, SHP2, Shc but not with PLCγ1 or Grb2 in COS-7 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The yeast MATCHMAKER LexA two-hybrid system (27, 28) reagents were purchased from CLONTECH. Yeast Saccharomyces cerevisiae strain EGY48 (MATa trpl 1, his3, ura3, 6LexA-Oep-LU2, LYS2) pretransformed with pbbop-LacZ was used as the host for the in vivo interaction studies.

cDNA Plasmid Constructs—All cDNA constructs for yeast transformation were generated by cloning PCR products into the yeast two-hybrid vectors pLexA or pB42AD. PCR reactions were carried out using high fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA) and primers with appropriate restriction sites incorporated as needed. The following cDNA clones were used as templates for PCR reactions: expressed sequence tag clones AA111517 (GenBank accession number, mouse IRS3), AA100052 (IGF1R), R18818 (Grb2), AA517594 (Nck), W539296 (SH2PA) and AA081539 (p66Shc), rat PLCγ1-cDNA (a gift from Dr. S. G. Rhee), and bovine P8-i kinase p85α (from Dr. K. Hara). Human pB42AD-IRS1 and mouse pB42AD-IRS2 constructs were generous gifts from Dr. Thomas A. Gustafson. To generate IRK-IRS3 chimera constructs, IRS3 YXXM mutants were digested with BgIII and SalI, and the inserts were cloned into the pLEX-IRκ construct such that the C-terminal sequence of IRS3 (amino acids 338–495) was fused in-frame to the IR kinase domain (amino acids 941–1271). The PCR products were cloned into the yeast two-hybrid vectors pLexA or pB42AD as listed in Table I. The IRS3 YXXM motif Tyr→Phe point mutation mutants, IRS3<sub>C1018A</sub>, IRS3<sub>L1018A</sub>, IR juxtamembrane NPXY motif mutant (N957A/V960A; Ulrich numbering; Ref. 29) and IR kinase-dead mutant (K1018A) were generated by site-directed mutagenesis. The detailed cloning and mutagenesis strategies for the above constructs are available upon request. All constructs were verified by sequencing using an Applied Biosystems Inc. Prism 377 DNA automatic sequencer.

**Yeast Transformation and Interaction Assays**—Yeast strain EGY48 was cotransformed with plasmid constructs by the polyethylene glycol/lithium acetate method according to CLONTECH’s protocol. Transformants were grown on appropriate SD glucose agar plates for 3 days at 30 °C. Five to 10 independent colonies were transferred to SD galactose/raffinose agar plates and grown overnight at 30 °C to induce the expression of B42 fusion proteins. For colony lift filter assay for β-galactosidase activity, the colonies were transferred to Whatman 3MM filter paper, washed in liquid nitrogen for 10–15 s, placed on a 300-μm filter that was presoaked with 5 ml of Z-buffer/X-gal/2-mercapto ethanol (60 mM Na<sub>H</sub>P<sub>O</sub>4 7H<sub>2</sub>O, 40 mM Na<sub>H</sub>PO<sub>4</sub>·H<sub>2</sub>O, 100 mM KCl, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.0, 0.27% (v/v) 2-mercaptoethanol and 0.334% (w/v) X-gal), and incubated at 30 °C. For the liquid β-galactosidase activity assay, at least six independent yeast colonies were picked and cultured in appropriate SD medium with galactose/raffinose (in which the glucose/galactose/raffinose was carbon source) before incubating with shaking at 30 °C. The culture was diluted in the same medium and regrown for additional 3 h at 30 °C. Yeast cells were harvested by low speed centrifugation, suspended in Z-buffer, and lysed by three cycles of freezing in liquid nitrogen, followed by thawing at 37 °C. The β-galactosidase activity was measured using p-nitrophenyl β-D-galactoside as substrate, and the results were expressed as Miller units (30) according to the CLONTECH protocol. The liquid β-galactosidase activity was defined arbitrarily as 100% for the interaction between IR and IRS3. The LacZ activity assay on Gal/Raf/-UWHL/X-gal plates, which is based defined arbitrarily as 100% for the interaction between IR and IRS3.

**Results**

Interactions of IRS3 with IR, IRR, and IGF1R—In this study, we used the LexA-based yeast two-hybrid system (28) to characterize interactions of IRS3 with IR, IRR, and IGF1R. One plasmid encoded proteins with the DNA-binding domain of LexA fused to cytoplasmic domains of each receptor (Table I, a). The other construct contained the transcriptional activation domain of B42 fused either to full-length IRS3 or various fragments of the molecule (Table I, b). Interactions between the two proteins were assayed semiquantitatively by observing blue color in a colony filter assay and quantitatively with β-galactosidase assays (see “Experimental Procedures”).

When yeast cells were cotransformed with expression vectors for both fusion proteins, (i.e., pLexA-IR and pB42AD-IRS3), a strong blue color developed rapidly (within minutes) in the filter assay. As shown in Fig. 1, in the quantitative liquid β-galactosidase assay, the interaction of IR with IRS3 was comparable to the interaction with IRS2, but approximately twice as strong as the interaction with IRS1. Relative to the interaction of IRS3 with IR, β-galactosidase activity was 110% and 47% in yeast transformed with pB42AD-IRS3 and either pLexA-IGF1R or pLexA-IRR, respectively. As negative control, when yeast cells were transformed with a single construct of either IR or IRS3, the β-galactosidase activity was negligible (about 2% of maximal activity in yeast co-transformed with pB42AD-IRS3 and pLexA-IR; Fig. 1). Similarly when yeast cells were co-transformed with a LexA or a B42 construct (with insert) paired with the appropriate empty expression vector, the β-galactosidase activity was barely detectable (data not shown).

The PTB domains of both IRS1 and IRS2 have been shown to bind the juxtamembrane domain of IR (22–26, 31–33). In addition, IRS2 (but not IRS1) has an additional insulin receptor binding domain named receptor binding domain 2 (25) or kinase regulatory loop binding domain (30) that is distinct from the PTB domain. To map the domain(s) of IRS3 required for binding to IR, we characterized deletion mutants of IRS3 (Fig. 2). As shown in Fig. 2, the minimal PTB domain of IRS3 bound weakly to IR. Neither the isolated PH domain nor the isolated phosphorylation domain of IRS3 interacted significantly with IR. Deletion of the PTB domain completely abolished binding to IR, while removal of either the PH domain or the phosphorylation domain had little effect. Furthermore, the receptor tyrosine kinase domain is required for the interaction inasmuch as
TABLE I

**cDNA constructs**

All cDNA fragments (except pB42AD-IRS1 and pB42AD-IRS2, which were gifts from Dr. T. A. Gustafson) were generated by PCR using *Pfu* polymerase, and cloned into either pLexA or pB42AD vector as described under “Experimental Procedures.” The constructs were verified by sequencing.

| Construct name | Description of insert |
|----------------|-----------------------|
| **a. cDNA constructs in pLexA vector used for the yeast two-hybrid experiments** | |
| pLexA-IR      | IR cytoplasmic domain (amino acids 941–1343) |
| pLexA-IGF1R   | IGF1R cytoplasmic domain (amino acids 929–1337) |
| pLexA-IR      | Juxtamembrane NPEY motif mutant (N957A/Y960A) of the IR cytoplasmic domain (amino acids 918–1271) |
| pLexA-IRKD    | Kinase dead mutant (K1018A) of the IR cytoplasmic domain (amino acids 941–1343) |
| pLexA-IRJM/IRS3cp | IR kinase domain (amino acids 941–1271) |
| pLexA-IRJM/IRS3cp | IRS3 C-terminal phosphorylation domain (amino acids 338–495) |
| pLexA-IRJM/IRS3cp | IIR kinase fused to IRS3cp |
| pLexA-IRJM/IRS3cp | IRS3 without the PH domain |
| pLexA-IRJM/IRS3cp | IRS3 Without the PTB domain |
| pLexA-IRJM/IRS3cp | IRS3 without the C-terminal phosphorylation domain |
| pLexA-IRJM/IRS3cp | Bovine PI 3-kinase p85 N-terminal SH2 domain (amino acids 333–428) |
| pLexA-IRJM/IRS3cp | Bovine PI 3-kinase p85 C-terminal SH2 domain (amino acids 624–718) |
| pLexA-IRJM/IRS3cp | Wild type human Grb2 |
| pLexA-IRJM/IRS3cp | Wild type mouse Nck |
| pLexA-IRJM/IRS3cp | Human SHP2 containing two tandem SH2 domains (amino acids 1–218) |
| pLexA-IRJM/IRS3cp | Human p66Shc containing the SH2 domain (amino acids 291–458) |
| pLexA-IRJM/IRS3cp | Rat PLCγ1 containing two tandem SH2 domains and a SH3 domain (amino acids 547–853) |
| **b. cDNA constructs in pB42AD vector used for the yeast two-hybrid experiments** | |
| pB42AD-IRS1   | Wild type IRS1 |
| pB42AD-IRS2   | Wild type IRS2 |
| pB42AD-IRS3   | Wild type IRS3 |
| pB42AD-IRS3PH | IRS3 PH domain (amino acids 32–131) |
| pB42AD-IRS3PTB| IRS3 PTB domain (amino acids 161–276) |
| pB42AD-IRS3cp | IRS3 C-terminal phosphorylation domain (amino acids 277–495) |
| pB42AD-IRS3ANTB | IRS3 without the PTB domain |
| pB42AD-IRS3cp | IRS3 without the C-terminal phosphorylation domain |
| pB42AD-p85mSH2 | Bovine PI 3-kinase p85 N-terminal SH2 domain (amino acids 333–428) |
| pB42AD-p85mSH2 | Bovine PI 3-kinase p85 C-terminal SH2 domain (amino acids 624–718) |
| pB42AD-Grb2   | Wild type human Grb2 |
| pB42AD-Nck    | Wild type mouse Nck |
| pB42AD-SHP21–218 | Human SHP2 containing two tandem SH2 domains (amino acids 1–218) |
| pB42AD-SHPh201–438 | Human p66Shc containing the SH2 domain (amino acids 291–458) |
| pB42AD-PLCγ1454–453 | Rat PLCγ1 containing two tandem SH2 domains and a SH3 domain (amino acids 547–853) |
| **c. cDNA constructs used for transfection in mammalian cells** | |
| pCDNA3.1-IRS3 | Wild type IRS3 |
| pCDNA3.1-IRS3PH | IRS3 without the PH domain |
| pCDNA3.1-IRS3PTB | IRS3 without the PTB domain |
| pBPV-IRS3-IRS3-neo | Wild type IRS3 |
the kinase inactive mutant of IR fails to bind to IRS3. Moreover, similar to what has been observed for PTB domains of IRS1 and IRS2 (22–26, 31, 33), mutations in the juxtamembrane domain NP<sup>N</sup>X<sup>Y</sup> motif of IR (N957A/Y960A) abolished the interaction of the insulin receptor with either the isolated PTB domain (data not shown) or the full-length IRS3 (Fig. 2). Thus, the PTB domain of IRS3 binds to the NP<sup>N</sup>X<sup>Y</sup> motif in the juxtamembrane domain of the IR, and the binding can be greatly enhanced by the presence of either the PH domain or the phosphorylation domain.

Phosphorylation of IRS3 in COS-7 Cells—Epitope-tagged IRS3 expressed in COS-7 cells contained an unexpectedly high phosphotyrosine content, even when cells were incubated in the absence of insulin (Fig. 3). Nevertheless, when the transfected cells were incubated in the presence of insulin (100 nM), this led to approximately 50% increase in tyrosine phosphorylation of IRS3. Since, the results from our yeast two-hybrid system showed that the PTB domain of IRS3 is required for binding to IR, we inquired whether deletion of the PTB domain would alter tyrosine phosphorylation of IRS3 in COS-7 cells. As shown in Fig. 3, wild type IRS3 and the mutants were expressed at a similar level in the COS-7 cells. As expected, deletion of the PTB domain abolished phosphorylation of IRS3 in both the presence and absence of insulin. Interestingly, deletion of the PH domain also abolished tyrosine phosphorylation of IRS3, both in the presence or absence of insulin.

Interaction of IRS3 with the SH2 Domains of the p85 Subunit of PI 3-Kinase—Both the PH domain and the PTB domain are highly conserved among IRS proteins, but there is little conservation of the phosphorylation domains. For instance, the IRS3 molecule is substantially shorter than the other three members of the family. Therefore, it is important to study structure-function relationships that determine the signaling specificity of IRS3. We used the yeast two-hybrid system to investigate interactions of phosphorylated IRS3 with downstream signaling molecules. As expected, when yeast cells were cotransformed with pLexA-IRS3 plus pB42AD-p85<sub>nnc</sub>SH2, we did not detect an interaction between IRS3 and p85 presumably due to the lack of IRS3 phosphorylation in this system. However, constitutive expression of c-Src in the yeast three hybrid system (34) did not permit detection of an interaction between IRS3 and p85 (data not shown). Presumably, these YXXM motifs in IRS3 molecules were poor substrates for phosphorylation by c-Src. Thus, we modified the yeast two-hybrid system so that the insulin receptor tyrosine kinase could phosphorylate tyrosine residues in IRS3 (Fig. 4). To accomplish this, we fused the IR kinase domain with the phosphorylation domain of IRS3, and the chimera was then fused to the LexA domain to yield pLexA-IR<sub>k</sub>-IRS3 construct (Table I). When yeast cells were cotransformed with pLexA-IR<sub>k</sub>-IRS3 and p85 SH2 domains (pB42AD-p85<sub>nnc</sub>SH2), we could detect strong in-
interaction. As judged by the β-galactosidase assay, the interaction was 60% as strong as the interaction between the IR cytoplasmic domain and full-length IRS3 (Fig. 4). In contrast, p85cSH2 did not interact directly with the isolated IR kinase domain when it was not fused to the IRS3 phosphorylation domain. Taken together, these observations demonstrate that the tandem SH2 domains of p85 bind to phosphotyrosine residues in the phosphorylation domain of IRS3.

Four Tyr residues of mIRS3 are located in YXXM motifs: Tyr^341, Tyr^350, Tyr^361, and Tyr^390 (9). We used our modified yeast two-hybrid system to investigate which of the phosphotyrosine residues bind to the SH2 domains of p85. When phenylalanine (Phe) was substituted for all four of these Tyr residues in the phosphorylation domain plus amino acid residues 338–495 of IRS3 (p85cSH2) (a plasmid encoding a fusion protein containing the IR kinase domain plus amino acid residues 338–495 of IRS3 and p42AD-p85cSH2) were substituted in the experiment, then 48 h were required for the development of visible blue color. Control experiments provided evidence for the specificity of the assay. We did not detect the development of a blue color, even after 48 h in yeast transformed singly with any of the following constructs in the absence of an interacting construct: pLexA-IR^k; pLexA-IR^k SH2, or pLexA-IR^k SH2). Finally, receptor tyrosine kinase activity is required for the interaction of IR^k-IRS3 with the tandem SH2 domains (p85cSH2) (Table II).

Interaction of IRS3 with Other Proteins Containing SH2 Domains—We used a similar approach to study the interactions of phosphorylated IRS3 with SH2 domains derived from SHP2, Grb2, Nck, Sce, or PLCγ1. In yeast co-expressing the IR^k-IRS3cp fusion protein together with fusion proteins containing SH2 domains derived from SHP2, Grb2, Nck, and Sce, we did detect evidence for interaction (Table III). Because yeast expressing the IR^k domain did not interact with any of the constructs, we conclude that the SH2 domains do not bind directly to phosphotyrosine residues in the insulin receptor kinase domain. Assuming that the length of time required to develop blue color is inversely related to the affinity of the interaction, we conclude that IRS3 binds strongly to the tandem SH2 domains of p85, and less strongly to SHP2^311–3120, Grb2, Nck, and Sce^101–345, but we did not obtain convincing evidence for an interaction with PLCγ1^547–853.

Specificity of the Modified Yeast Two-hybrid System—Be-
were carried out as described in Table IV. C-terminal SH2 or N-terminal SH2) is present. In addition, such interaction requires the active kinase activity of the IR. The interaction assays were carried out as described in Table IV.

proteins of B42 with SHP2 1–218, p85
with pLexA-IR
of SHP2 but not of p85 (35–39). Yeast cells were co-transformed
that conform to consensus binding motifs for the SH2 domains
Mammalian Cells—
Next, we carried out studies in COS-7 cells
IRS3 with p85, Shc, SHP2, Nck, and Grb2; in contrast, little if
cause IRS3 contains multiple potential sites of tyrosine phosphorylation, this raises the question of which phosphotyrosine residues interact with which SH2 domains. In the case of p85, our mutational studies support the conclusion that the tandem SH2 domains interact with phosphotyrosine residues in the context of YXXM motifs (Fig. 4). Like p85, SHP2 also contains two SH2 domains. The C terminus of IRS3 (amino acid residues 405–495) contains at least two motifs ((Y467VDL and Y490ASI) that conform to consensus binding motifs for the SH2 domains of SHP2 but not of p85 (35–39). Yeast cells were co-transformed
IRS3 interacts more strongly with tandem SH2 domains than with individual SH2 domains

Table II

| pLexA fusion | pB42AD fusion | LacZ activity (blue/white) |
|--------------|---------------|---------------------------|
| IRk          | Empty         |                           |
| IRk-IRS3cp   | Empty         |                           |
| LamC         | p85ncSH2      |                           |
| IRKD-IRS3cp  | p85ncSH2      |                           |
| IRS3         | p85ncSH2      |                           |
| IRk-IRS3cp   | p85ncSH2      |                           |
| IRk          | p85ncSH2      | +                         |
| IRk-IRS3cp   | p85ncSH2      | +                         |
| IRk          | p85ncSH2      | +                         |
| IRk-IRS3cp   | p85ncSH2      | +++                       |

Table III

The modified yeast two-hybrid system can be used to study the interaction of IRS3 with other SH2 domain-containing proteins
When the entire IRS3 phosphorylation domain is fused to the IR kinase domain, the modified yeast two-hybrid system can be used to analyze the interaction of IRS3 with SH2 domain containing proteins such as Grb2, Nck, SHP2, Shc, and PLCγ-1. The interaction assays were carried out as described in Table IV.

| pLexA fusion | pB42AD fusion | LacZ activity (blue/white) |
|--------------|---------------|---------------------------|
| IRk          | Grb2          |                           |
| IRk-IRS3cp   | Grb2          |                           |
| IRk          | Nck           |                           |
| IRk-IRS3cp   | Nck           |                           |
| IRk          | SHP21–218     |                           |
| IRk-IRS3cp   | SHP21–218     |                           |
| IRk          | Shc201–458    |                           |
| IRk-IRS3cp   | Shc201–458    |                           |
| IRk          | PLCγ1547–853  |                           |
| IRk-IRS3cp   | PLCγ1547–853  |                           |

Structure-Function Analysis of Mouse IRS3

DISCUSSION
The yeast two-hybrid system provides a powerful technique to study protein-protein interactions. This system has been applied previously to characterize structure-function relationships of both IRS1 and IRS2, specifically, their interactions with IR and IGF1R (22–26, 33). We have used a similar approach to define the domains of IRS3 that interact with the insulin receptor, the IGF-1 receptor, and the insulin receptor-
related receptor. In addition, we have modified the yeast two-hybrid approach to allow us to study interactions with downstream signaling molecules that interact via their SH2 domains with phosphotyrosine residues in IRS3. The majority of the predictions based upon observations obtained with the yeast two-hybrid system were confirmed by carrying out appropriate studies in mammalian cells.

**Interactions of IRS3 with Receptor Tyrosine Kinases**—Using the yeast two-hybrid system, we demonstrated that the PTB domain of IRS3 (like the PTB domains of IRS1 and IRS2) is essential for binding to the NPX(p)Y motif within the juxtamembrane domain of the insulin receptor in a phosphorylation-dependent manner. However, the isolated minimal PTB domain binds weakly to the IR, and this binding is markedly enhanced by the presence of either the PH domain or the phosphorylation domain. On the other hand, neither the isolated PH domain nor the isolated phosphorylation domain could interact directly with the IR cytoplasmic domain. Since the sequence downstream of the minimal PTB domain in IRS3 has no detectable homology with IRS1, IRS2, or IRS4, we suggest that the PTB domain of IRS3 is the only domain that binds directly to IR. Rather, the effects of both the PH and phosphorylation domains to promote the interaction of PTB domain with the IR may be explained by favorable effects upon either the folding of the fusion protein or the stability of the molecule. As predicted by the yeast two-hybrid system, deletion of the PTB domain prevented insulin-stimulated phosphorylation of IRS3 in COS-7 cells (Fig. 3). Furthermore, deletion of the PH domain also inhibited phosphorylation of IRS3. Similar results were reported previously in studies in which the PH domain of IRS1 was deleted or replaced with PH domains derived from proteins other than IRS family members (18–21). Thus, both the PH domain and the PTB domain or IRS3 are required for normal phosphorylation of IRS3 by the insulin receptor in mammalian cells.

Multiple factors may contribute to determining which IRS molecules are phosphorylated by specific receptor tyrosine kinases. Thus, we compared the strength of the interactions between IRS3 and various members of the insulin receptor family of tyrosine kinases. The interaction of IRS3 was approximately twice as strong with the IR or IGF1R as with IRR. In addition, we compared the strength of the interaction of the IR with IRS1, IRS2, and IRS3. In the yeast two-hybrid system, the interaction of IR with IRS2 or IRS3 was approximately twice as strong as with IRS1. These data are consistent with observations with endogenous IRS proteins in rat adipocytes. Rat adipocyte IRS3 binds more strongly than IRS1 to immobilized peptides containing phosphorylated NPX(p)Y motifs (15). Furthermore, IRS3 was phosphorylated more rapidly than IRS1 in rat adipocytes (15). In contrast, although the yeast two-hybrid system suggests that IR binds to IRS2 with greater affinity than to IRS1, IRS1 appears to be phosphorylated more heavily than IRS2 in rat adipocytes (15, 16, 40). It is likely that this discrepancy is accounted for by differences in the level of expression of the two substrates. However, it is possible that other factors (e.g. subcellular localization) might also contribute.

**Interaction of IRS3 with Downstream Effector Molecules**—It is necessary to modify the yeast two-hybrid system to analyze an interaction between proteins if the interaction requires one of the proteins to undergo post-translational modification (in this case, phosphorylation). For example, in the yeast three-hybrid system (34, 41–44), expression of a tyrosine kinase has enabled screening of libraries for tyrosine phosphorylation-dependent interactions between proteins (41, 44), and studying tyrosine phosphorylation-dependent interactions between two proteins (34). To our knowledge, the yeast three-hybrid system has not been applied to study the interaction of IRS proteins with downstream signaling proteins containing SH2 domains. In the present study, we utilized a modified yeast two-hybrid system. Rather than express a separate tyrosine kinase to phosphorylate the substrate, we fused the phosphorylation domain of IRS3 directly to the IR kinase domain. This chimera was fused to the C terminus of the transcription activation domain LexA to yield a chimeric LexA-IR<sub>Δ</sub>-IRS3<sub>Δ</sub> fusion. Both the LexAop-LacZ gene in the p8op-LacZ plasmid vector and the LexAop-LEU2 gene in the yeast EGY48 chromosome allow multiple LexA fusion proteins to bind to the LexA operators simultaneously. This binding results in activation of the IR kinase, possibly by phosphorylation in an intramolecular manner (22). The activated IR kinase subsequently phosphorylates these tyrosine motifs in IRS3 allowing their binding to the SH2 domain of B42 fusion proteins. Eventually, the transcription of
both the *LexAop-LEU2* and *LexAop-LacZ* genes is activated, allowing the yeast cells to grow in leucine-deficient plates and express \(\beta\)-galactosidase activity, respectively.

Using this modified yeast two-hybrid system, we demonstrated that tyrosine phosphorylated IRS3 binds to the SH2 domains of the p85 subunit of PI 3-kinase, SHP2, Nck, and Grb2, but not PLC-\(\gamma\). This is consistent with other data obtained in mammalian cells obtained in this and other studies (12–17, 45). Wild type IRS3 interacts strongly with the p85 SH2 domains via four tandem YXXM motifs in the phosphorylation domain of IRS3. By substituting Phe for Tyr residues within these YXXM motifs, we demonstrated that at least two intact tandem YXXM motifs in IRS3 were required for high affinity binding to the tandem SH2 domains of p85, and that the individual SH2 domains of p85 alone were not sufficient to bind strongly to wild type IRS3. This is in agreement with the conclusion from other groups that at least two tyrosine phosphorylation sites are necessary to confer high affinity binding of tandem SH2 domains from several signaling molecules such as p85, SHP2, ZAP-70, Syk, and PLC\(\gamma\)1 (38, 39, 46). Our data indicate that Tyr\(^{361}\) and Tyr\(^{350}\) in IRS3 confer higher binding affinity for SH2 domains of p85 than Tyr\(^{341}\) and Tyr\(^{350}\), as mutation of either Tyr\(^{361}\) or Tyr\(^{350}\) has a greater adverse impact than mutation of Tyr\(^{341}\) and Tyr\(^{350}\) upon the interaction of IRS3 with the SH2 domains of p85. It is noteworthy that Tyr\(^{341}\) is contained in the sequence YITM (lacking a Met residue at the +1 position). In contrast, Tyr\(^{360}\) Tyr\(^{361}\), and Tyr\(^{350}\) are contained in YXXM motifs that conform to the optimal motif (Y(M/V)M) to bind the SH2 domain of p85. In addition, Ottinger *et al.* demonstrated that the spacing between two YXXM motifs is not critical for high affinity binding of tandem SH2 domains in p85. For example, in comparing bisphosphoryl peptides containing two pYXXM motifs, the length of the sequence separating the two pTyr residues did not affect binding to the tandem SH2 domains in p85. Accordingly, we speculate that in the mammalian cells p85 could bind to any pair of YXXM motifs in IRS3 with high affinity, although the relative contribution of each YXXM motifs remains unknown. Given that a large portion of phosphorylated IRS3 binds to p85 in mammalian cells (12–17, 45), we postulate that at least two if not all four YXXM motifs are phosphorylated rapidly by the insulin receptor upon insulin stimulation. Subsequently, these phosphorylated YXXM motifs serve as high affinity sites for the simultaneous binding of tandem p85 SH2 domains.

It has been demonstrated that Grb2 can associate with IRS3 in rat adipose cells (14, 17) and rat hepatoma cells overexpressing insulin receptor (HTC-IR) (14). It has been suggested that Grb2 may bind to a putative binding motif (i.e. YIIV) in rat IRS3 (8). Unlike rat IRS3, mouse IRS3 does not possess an obvious motif for binding to Grb2 (9). The corresponding sequence in mouse IRS3 is YIIFPKF, which does not conform to the consensus sequence for Grb2 binding. Consistent with the absence of an obvious binding site, insulin did not enhance the interaction of recombinant mIRS3 with Grb2 in COS-7 cells. However, we did detect a weak interaction between Grb2 and IRS3 in cells incubated in the absence of insulin. Because of the presence of proline-rich sequences in IRS3, it seems likely that the insulin-independent interaction may involve the SH3 domain of Grb2. However, it is also possible that insulin-independent tyrosine kinases may have phosphorylated IRS3 at low stoichiometry, and this could explain the low level interaction of IRS3 and Grb2 observed in COS-7 cells in the absence of insulin.

SHP2 is a phosphotyrosine phosphatase containing two tandem SH2 domains. Its SH2 domains bind preferentially to phosphotyrosines in (pY-hydrophobic-X-hydrophobic motifs, such as Y-(V/I/L/P)-(V/I/L/P)) (35). In IRS1, the SHP2 binding motif was previously demonstrated to be Y\(^{1172}\)IDL (36, 47). More recently it has been demonstrated that both Y\(^{1172}\)IDL and Y\(^{222}\)ASI motifs in IRS1 are necessary for bivalent high affinity binding to the tandem SH2 domains of SHP2 (37–39). Originally the Y\(^{46}\)VDL motif in mouse IRS3 and the Y\(^{46}\)VDL motif in rat IRS3 were suggested to bind SHP2 (8, 9). Recently we noticed that both mouse and rat IRS3 have an additional potential SHP2 binding motif (i.e. YAS1) located in the C-terminal tail. Our results from the yeast two-hybrid system and immunoprecipitation clearly indicate that SHP2 binds to IRS3 with considerable affinity. Other groups have also demonstrated that SHP2 could associate with IRS3 in rat adipose cells (14, 17) and rat hepatoma cells overexpressing insulin receptor (HTC-IR) (14). We propose that the two tandem SH2 domains in SHP2 can simultaneous bind to the YVDL and YASI motifs in IRS3 when both tyrosine residues are phosphorylated. However we cannot exclude the possibility that other tyrosine motifs in IRS3 also participate in SHP2 binding.

**Role of IRS3 in Intracellular Signaling**—The existence of multiple IRS molecules raises the interesting question of whether each IRS molecule serves a distinct function. All four molecules (IRS1, IRS2, IRS3, and IRS4) possess homologous PH and PTB domains. These domains appear to be required for binding to the receptors, thereby permitting efficient phosphorylation of the substrate molecule. However, IRS proteins differ in several respects. First, each IRS protein has a characteristic pattern of expression in specific tissues and at specific times of development. Second, it has been suggested that there may be differences in the subcellular localization of individual IRS molecules (48, 49). Finally, IRS proteins differ in their C-terminal sequences. Several tyrosine phosphorylation sites seem to be conserved in all four IRS molecules (e.g. YXXM motifs that provide binding sites for PI 3-kinase). This shared structural motif correlates with the observation that all four IRS proteins are capable of mediating the metabolic actions of insulin in rat adipose cells (45). However, there appears to be some specificity with respect to interactions with other downstream proteins. For example, unlike other IRS proteins, murine IRS3 does not appear to possess a binding motif for the SH2 domain of Grb2. Accordingly, IRS3 might lack the ability to mediate the activation of Ras, and therefore might be predicted to be specialized in triggering the metabolic actions of insulin rather than mediating mitogenic activity.

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