Heterologous Pleckstrin Homology Domains Do NotCoupleIRS-1 to the Insulin Receptor*

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Pleckstrin homology (PH) domains occur in many signaling proteins, including substrates for the insulin receptor tyrosine kinase (IRS proteins). Based on the hypothesis that PH domains may have a common function such as membrane targeting we tested the ability of PH domains from other signaling molecules to link IRS-1 to the insulin receptor. Chimeric IRS-1 proteins containing a homologous PH domain derived from other IRS proteins (IRS-2 or Gab-1) were tyrosine phosphorylated normally in response to insulin. In contrast, heterologous PH domains from the β-adrenergic receptor kinase, phospholipase Cγ, or spectrin failed to mediate tyrosine phosphorylation of chimeric IRS-1 proteins, even in cells expressing high levels of insulin receptor. Moreover, IRS-1 proteins containing heterologous PH domains did not bind phosphorylated NPEY motifs derived from the insulin receptor, suggesting that the presence of these structures interfered with the function of the adjacent PTB binding domain. Thus, tyrosine phosphorylation of IRS-1 by the insulin receptor specifically requires a PH domain derived from IRS proteins.

Pleckstrin homology (PH)domains occur in more than 90 different proteins, many of which play a role in cellular signaling or cytoskeletal organization which require association with cell membranes. Although the amino acid sequences of PH domains are diverse, their general structure consists of seven β-sheets and an α-helix. Many PH domains interact with membrane elements to regulate the assembly or activity of signal transduction complexes. Potential PH domain ligands include various inositol polyphosphates, the γ subunits of heterotrimeric G proteins, and membrane components, as well as specific protein sequences containing phosphorylated tyrosine, serine, threonine, or histidine residues. Although PH domains have a common structure, variable loops between the β-strands, β₁/β₂, β₂/β₃ and β₁/β₁₀ may determine specific ligand binding sites. Interestingly, phosphorysine binding (PTB) domains found in Shc and IRS-1 adopt a PH domain structure, suggesting that PTB domains are a class of PH domains that bind to phosphorylated tyrosine residues in NPXY motifs. Thus, each class of PH domains may engage specific ligands that are required for efficient assembly of relevant membrane-associated complexes.

The activated insulin receptor kinase phosphorylates IRS proteins at multiple tyrosine residues that bind Src homology-2 domains in various signaling proteins. IRS-1 was the first member of the IRS protein family, which now includes IRS-2, p60(RHS, p62, and Gab-1 (15–17). The NH₂-terminally PH domain of IRS proteins is highly conserved; given the sequence variability among other PH domains, this degree of conservation suggests that it is critical for IRS protein function. Indeed, tyrosine phosphorylation of IRS-1 is mediated by the PH domain and the adjacent PTB domain (18–20). The PTB domain binds directly to the activated insulin receptor at a phosphorylated NPEY motif in the juxtamembrane region (19, 21–23). However, yeast two-hybrid screens and biochemical approaches fail to demonstrate a direct interaction between the insulin receptor and the PH domain (22, 24–26). Nevertheless, without the PH domain, IRS-1 is poorly tyrosine phosphorylated during insulin stimulation, especially in cells expressing few insulin receptors.

To determine if the PH domain of IRS-1 is specifically required for coupling to the activated insulin receptor, it was replaced by a PH domain from several related or unrelated proteins (Fig. 1). Chimeric IRS-1 proteins reveal that heterologous PH domains (βark, phospholipase Cγ, or spectrin) weakly couple IRS-1 and the insulin receptor, whereas the homologous domains (IRS-2, Gab-1) participate with the PTB domain to mediate the interaction of chimeric IRS-1 with the activated insulin receptor. These results are consistent with a conserved and specific adapter function for PH domains in the IRS protein family.

MATERIALS AND METHODS

Construction of IRS-1 PH Domain Chimeras—cDNAs for the PH domains of rat PLCγ (Asp62-Thr727), human β-spectrin (Pro861-Lys1273), and bovine βark (Pro469-Gly686) were graciously provided by Dr. Robert Lefkowitz, Duke University (10). These PH domains were excised from pGEX-2T, adapted by polymerase chain reaction with initiation codons, ScaI restriction sites, and cloned in-frame into the pcMVPH vector containing the cDNA for IRS-1 (18). Preparation of the IRS1PH and IRS-1PH (19). Deletion domains have been described previously (18, 19). The PH domains of IRS-2 (Val82-Leu100) and Gab-1 (Lys13-Gly116) were generated by polymerase chain reaction and ligated into the cDNA for IRS-1 (19). All constructs were excised from the pcMVPH vector with Snibl and SalI and ligated into pBABE.

Expression of IRS-1 PH Domain Chimeras—For stable expression, 32D cells and 32D cells overexpressing the human insulin receptor (32D/IR) were transfected as described (18). Positive clones selected in histidinol were assessed for expression of chimeric IRS-1 proteins by immunoblotting with antibodies against the COOH terminus of IRS-1 (α-IRS1 (27)). Transient expression of pBABE constructs in COS-7 cells
was accomplished by the calcium phosphate method. Clones of transfected cells were selected based on comparable levels of expressed IRS-1.

**Immunoprecipitations and Western Analysis—** Cell extracts were prepared in lysis buffer (137 mM NaCl, 20 mM Tris, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 100 μM vanadate, and protease inhibitors). After centrifugation to remove insoluble material, lysates were incubated with the appropriate antibody for 1 h at 4 °C. Protein-A Sepharose was added for an additional hour, and complexes were washed three times with phosphate-buffered saline. Immune complexes were resolved by SDS-polyacrylamide gel electrophoresis as described previously (19). For detection of IRS-1, membranes were blocked in 5% nonfat dried milk, incubated with α-IRS1CT (27), and developed by enhanced chemiluminescence (Amersham). Immunoblots were also performed with PY20 (Transduction Laboratories) or an antibody against p85 (α-p85), as described previously (28).

**PI-3 Kinase Assays—** Lysates were prepared from unstimulated or insulin-stimulated COS-7 cells expressing IRS-1 or various chimeric IRS-1 proteins. After immunoprecipitation with αIRS-1CT, in vitro phosphorylation of phosphatidylinositol was performed on immune complexes as described, separated by thin layer chromatography, and identified by autoradiography (19).

**NPEY Binding Studies—** For preparation of the NPEY affinity matrix, a polymerase chain reaction fragment encoding four tandem NPEY repeats (derived from amino acids 944–983 of the insulin receptor) was cloned into pGEX-2T and expressed as a glutathione S-transferase fusion protein. After coupling of the expressed NPEY-containing protein to glutathione-Sepharose, this motif was phosphorylated with the activated insulin receptor as described previously (19). Briefly, 200 μl of NPEY-bound glutathione-Sepharose beads (approximately 25 μg of NPEY fusion protein) was incubated with wheat germ agglutinin-purified insulin receptor in 50 mM Hepes (pH 7.4), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 100 μM vanadate. The kinase reaction proceeded at 22 °C and was terminated 1 h later by washing the Sepharose beads three times with 150 mM NaCl and 50 mM Hepes at pH 7.4. As a control for these binding experiments, a nonphosphorylated NPEY peptide column was generated as described above except that no insulin receptor was included in the preparation. Lysates from cells overexpressing wild type or chimeric IRS proteins were prepared by three freeze/thaw cycles in 0.15M sucrose containing 50 mM Hepes, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 100 μM vanadate. Cleared lysates from 5 × 106 cells in 200 μl was added to 20 μl of packed NPEY peptide beads and incubated for 4 h at 4 °C. After a brief wash with 100 μl of lysis buffer, proteins bound to immobilized NPEY peptides were eluted by an equal volume of 2 × SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and detected by immunoblotting with α-IRS1CT.

**RESULTS**

The capacity of chimeric IRS-1 proteins to serve as substrates of the insulin receptor was tested in stably transfected 32D myeloid progenitor cells. 32D cells are ideal for studying mutant IRS proteins because they contain few insulin receptors (approximately 500/cell) and no detectable endogenous IRS-1 or IRS-2 (13, 18). Cell lines expressing equivalent levels of wild type IRS-1 or various chimeric IRS-1 proteins were selected by immunoblotting with αIRS1CT (27), and developed by enhanced chemiluminescence (Amersham). Immunoblots were also performed with PY20 (Transduction Laboratories) or an antibody against p85 (α-p85), as described previously (28).

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domain from IRS-2 or Gab-1 restored insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 2A). In contrast, insulin did not stimulate tyrosine phosphorylation of chimeric IRS-1 proteins containing heterologous PH domains derived from βark, PLC, or spectrin (Fig. 2A). Therefore, the homologous PH domains coupled the chimeric IRS-1 proteins to the activated insulin receptor, whereas the heterologous PH domains did not.

As revealed by prior studies, overexpression of the human insulin receptor in 32D cells (32DIR) restored tyrosine phosphorylation of IRS-1 and IRS-2 PH domains to normal levels (Fig. 2B) (19). As expected, chimeric IRS-1 proteins containing a homologous PH domain from IRS-2 or Gab-1 were tyrosine phosphorylated comparably to wild type IRS-1 in 32DIR cells (Fig. 2B). In contrast, chimeric IRS-1 proteins with a PH domain from βark or spectrin were insensitive to insulin and poorly tyrosine phosphorylated; the PLCγ PH domain mediated more sensitiv-
levels of recombinant proteins. These same 32D cell lysates were incubated with immobilized phosphorylated or unphosphorylated NPEY peptides. After a 4-h incubation, bound proteins were eluted and then immunoblotted with α-IRS1\textsuperscript{CT}.

The incapability of chimeric IRS-1 proteins containing the PH domains of βark, PLCγ, or spectrin to undergo insulin-stimulated tyrosine phosphorylation in 32D cells was surprising and suggested that a heterologous PH domain interferes with the interaction between IRS-1 and the insulin receptor. Heterologous PH domains may incorrectly target the chimeric IRS-1 protein or disrupt the function of the adjacent PTB domain; the latter effect seems likely because the PTB domain alone is ordinarily sufficient to mediate phosphorylation of IRS1\textsuperscript{PTB} in 32D cells expressing the insulin receptor. To evaluate this possibility, the binding of chimeric IRS-1 proteins to immobilized, phosphorylated NPEY motifs was tested. Lysates from 32D cells expressing similar levels of wild type IRS-1 or IRS-1 chimeras were incubated with Sepharose beads containing non-phosphorylated or tyrosine-phosphorylated NPEY peptide (Fig. 3A). Wild type IRS-1 bound to the phosphorylated NPEY peptide but not to the nonphosphorylated control (Fig. 3, B and C). As expected, deletion of the PTB domain completely abrogated interaction with the phosphorylated NPEY motifs, whereas removal of the IRS-1 PH domain had no effect on binding. Chimeric IRS-1 proteins containing the PH domain from Gab-1 or IRS-2 bound normally, whereas the PH domain from βark, PLCγ, or spectrin blocked the interaction between chimeric IRS-1 proteins and the phosphorylated NPEY peptide (Fig. 3). Thus, the presence of a heterologous PH domain in IRS-1 impaired binding of the PTB domain to the phosphorylated NPEY motif, providing at least one explanation for the inability of these chimeric proteins to undergo insulin-stimulated tyrosine phosphorylation in 32D cells.

The capacity of chimeric IRS-1 proteins to engage p85 and activate PI-3 kinase was tested in COS-7 cells transiently expressing recombinant proteins. Consistent with the results of 32D cells, the heterologous PH domains of βark, PLCγ, or spectrin did not mediate tyrosine phosphorylation of the chimeric IRS-1 proteins nor their association with the PI-3 kinase (Fig. 4). The insulin-stimulated PI-3 kinase activity associated with chimeric IRS-1 proteins bearing the PH domains of Gab-1 or IRS-2 was comparable to that detected in immunoprecipitates of wild type IRS-1. Thus, homologous PH domains mediate IRS-1-specific signaling in at least two cell backgrounds.

**Discussion**

PH domains are diverse modules with a common structural fold which direct proteins to membranes or other cellular compartments. Our results suggest that the PH domains in IRS proteins are functionally similar and are required for coupling to the activated insulin receptor. Because the insulin receptor does not appear to bind these PH domains directly, our work supports the hypothesis that specific membrane elements or adapter proteins engage the PH domain to facilitate interaction between IRS proteins and the insulin receptor.

Many isoforms of PH domains bind phospholipids, which may target proteins to membrane surfaces: the PH-domain of spectrin contains a site for membrane binding (4); the PLC\textsuperscript{δ1} PH-domain binds inositol trisphosphate and phosphatidylinositol bisphosphate (7); the βark PH domain binds phosphatidylinositol bisphosphate and Gβγ subunits (10, 11, 29). But these PH domains do not function in IRS-1, and they actually impair tyrosine phosphorylation of IRS-1 in 32D cells. Thus, the ability to bind these membrane components may not be the essential feature for coupling IRS proteins to the activated insulin receptor.

We have demonstrated that disruption of PTB domain function is one explanation for the inability of heterologous PH domains to promote tyrosine phosphorylation of chimeric IRS-1 molecules. Recent results suggest that the PH and PTB domains in intact IRS proteins may function cooperatively to mediate coupling with the insulin receptor. The presence of a heterologous PH domain in IRS-1 may interfere with the proper folding of the adjacent PTB domain, thereby impairing recognition of the phosphorylated NPEY motif in the insulin receptor. However, in 32D cells a PTB domain is not required for insulin-stimulated phosphorylation of IRS-1, so disruption of its function cannot be the only explanation for this inhibition. Alternatively, the PTB domain may inhibit the function of the heterologous PH domains. These possibilities will be tested in the future when specific ligands are available for analysis.

Heterologous PH domains may target the chimeric IRS-1 proteins incorrectly, decreasing their interaction with the activated insulin receptors. If PH domains are needed simply to bind phospholipids and tether proteins to membranes, then the PH domains of βark and PLCγ which both bind membrane lipids should function in IRS-1 (10, 11). Their failure to do so suggests that either phospholipid binding is not sufficient to mediate productive interaction between IRS-1 and the insulin receptor or that heterologous PH domains bind certain phospholipids that are incompatible with coupling of IRS-1 and the insulin receptor. Recent studies of the PH domain provide additional support for the specificity of PH domain interactions; expression of chimeric pleckstrin variants containing either the βark or dynamin PH domain in COS cells failed to produce morphological characteristics associated with wild type pleckstrin (30). Similarly, the PH domains of PLCγ and pleckstrin were ineffective at blocking dynamin-mediated rapid exocytosis, whereas the native dynamin PH domain produces a dominant negative effect on endocytosis in adrenal chromaffin cells (31).

Although the PH domains of IRS-2 and Gab-1 were the only homologous structures tested in the present study, we conclude provisionally that similar domains from IRS-3 and p62\textsuperscript{dok} will also function in chimeric IRS-1 proteins since they are insulin receptor substrates. However, alignment of the PH domains from the five known IRS proteins does not clearly reveal potentially critical elements required for IRS-1 function because few identical regions occur. The β/βγ-loop is positively charged.
in all of the IRS proteins but absent from the heterologous PH domains; this loop could contribute the specificity for engaging a negatively charged membrane element. The $\beta_1/\beta_2$-loop may also convey binding specificity, but this region of the PH domain is rather variable even among the IRS proteins. A lethal mutation in the PH domain of Btk occurs in the second $\beta$-strand, suggesting that this subdomain could be important for ligand binding (32–34). Further analysis of the common regions in IRS PH domains may reveal the structural requirements for ligand recognition and provide a means to identify relevant binding partners.

The nature of the specific ligands for the PH domain in IRS proteins is unknown. Unlike the PTB domain, which binds directly to the phosphorylated NPEY motif in the juxtamembrane regions of the insulin receptor, the PH domain does not appear to interact directly with the insulin receptor; yeast two-hybrid screens and biochemical approaches repeatedly fail to demonstrate a direct interaction between the insulin receptor and this structural module of IRS-1 (22, 24–26). However, the specificity and sensitivity provided by the PH domain, especially in the absence of the PTB domain, strongly suggest that it binds to a ligand that is in close association with the insulin receptor. Perhaps the receptor coordinates a favorable phospholipid environment that binds to the PH domain. Alternatively, the insulin receptor may engage an adapter protein that recruits the PH domain into the activated insulin receptor complex. Elucidation of the mechanism used by the PH domain to couple IRS proteins to the insulin receptor may provide important insights into the molecular basis of insulin resistance and enable the design of new drugs to restore the insulin response in non-insulin-dependent diabetes mellitus patients or disrupt IRS protein function in cancer.

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