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IRS Pleckstrin Homology Domains Bind to Acidic Motifs in Proteins*

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Using a yeast two-hybrid system, we identified several proteins that interact with the PH domains in IRS-1 and IRS-2, including Lon protease, myeloblast protein, and nucleolin. Although the roles of these molecules in insulin action are not yet known, each protein contained an acidic motif that interacted with the PH domain of IRS-2. However, only the acidic motif in nucleolin bound to IRS-1, suggesting that the PH domain in IRS-1 and IRS-2 are not identical. Moreover, synthetic peptides based on the acidic motif in Lon protease and myeloblast protein inhibited the binding of nucleolin to the PH domain of IRS-2 but not to the PH domain of IRS-1, confirming the selectivity of these PH domains. The ability to bind acidic motifs may be a specific function of the PH domain in IRS proteins, because the PH domains in βARK, phospholipase Cγ, or spectrin did not bind nucleolin. In 32D cells, nucleolin bound to both IRS-1 and IRS-2, and expression of the acidic motif of nucleolin inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2. These results suggest that the binding of acidic motifs to the PH domain of IRS-1 and IRS-2 disrupts coupling to the activated insulin receptor. Our results are consistent with the hypothesis that the PH domain in the IRS proteins may ordinarily bind acidic peptide motifs in membrane proteins or other acidic membrane elements that couple IRS proteins to activated membrane receptors.

Early steps in cellular signaling by growth factors and cytokines are mediated by molecular interactions that are coordinated by common protein modules. In many cases the molecular basis of these protein-protein interactions are known: Src homology 2 domains and phosphotyrosine binding (PTB)[1] domains bind to tyrosine phosphorylated motifs in activated receptors or in transiently associated docking proteins; and Src homology 3 domains and WW domains bind to proline-rich motifs in receptors, enzymes, and the cytoskeleton (1). Pleckstrin homology (PH) domains are also frequent participants in the signaling cascades. Most molecules that contain a PH domain interact with membrane components, suggesting that PH domains mediate membrane targeting. However, PH domains display a broad range of binding specificity, which has hindered the identification of ligands for the various PH domain isoforms.

PH domains consist of approximately 100 amino acids, but amino acid sequence alignments reveal few overall identities. A single tryptophan residue in the COOH-terminal α-helix is the only residue conserved in all PH domains (2). Secondary structure is the best predictor of the PH domain. X-ray crystallographic and NMR analysis of several PH domains reveal a common structure with two orthogonal β-sheets assembled from seven β-strands closed at one end with a COOH-terminal α-helix (3–8). PH domains contain a positively charged binding pocket, and several reports suggest that these regions bind phospholipids, including the PH domain in PLCδ that binds to phosphatidylinositol diphosphate and inositol 1,4,5-trisphosphate (9, 10). Because inositol 1,4,5-trisphosphate is a product of PLC catalysis, inositol 1,4,5-trisphosphate may dissociate PLCδ from membranes containing its substrate phosphatidylinositol diphosphate (10). Recent studies reveal that the lipid product of phosphatidylinositol 3-kinase binds to the PH domains of Btk, SOS, Tiam-I, and PKB (11, 12). In the latter case, the interaction of PKB with phosphatidylinositol 3,4,5-triphosphate or phosphatidylinositol 3,4,-diphosphate, mediates the translocation of PKB to membranes, which places its regulation under the influence of phosphatidylinositol 3-kinase (13–15). Although the interaction of PH domains with phosphatidylinositol diphosphate and phosphatidylinositol triphosphate provides a common theme to understand their function, not all PH domains bind to these phospholipids (16). Some PH domains bind protein ligands, as illustrated by the association of the βark PH domain with βγ-subunits of heterotrimeric G-proteins (17).

Members of the insulin receptor substrate family (IRS proteins) contain an NH_2-terminal PH domain followed immediately by a PTB domain. The PTB domain binds to the phosphorylated NPEY motif in the juxtamembrane region of the activated insulin receptor (18). Although the structures of the PH and PTB domains are very similar, the ligands for the PH domains in IRS proteins are not yet defined (19). A direct interaction between the insulin receptor and the PH domain of IRS-1 has not been detected (20–22). Nevertheless, the PH domain is essential for efficient tyrosine phosphorylation of IRS-1 during insulin stimulation, because deletion of the PH domain significantly reduces insulin-stimulated IRS-1 tyrosine phosphorylation (18, 23). The PH domain in the IRS proteins may have a common function that mediates receptor coupling, because chimeric IRS-1 proteins bearing the PH domains from

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[1] The abbreviations used are: PTB, phosphotyrosine binding; PH, pleckstrin homology; PLCγ, phospholipase Cγ; β-gal, β-galactosidase; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary.

TABLE I
Characterization of PH ligands

A β-gal assay was used to evaluate the specificity of the interactions between the four groups of cDNA clones and the PH domains of IRS-1 and IRS-2. Additionally, the clones were introduced into yeast strains carrying the control baits which included the PTB domains of IRS-1 and IRS-2, the entire intracellular domain of the β-subunit of the insulin receptor, the catalytic domain of the IGF-I receptor, and the bait plasmid pGBT. Positive interacting colonies were scored positive based on the development of blue color according to a standard assay as described under "Experimental Procedures." Colonies that displayed blue color within 15 min were scored +++, and those that were positive after 30 min were assigned ++. The absence of color demonstrates the lack of an interaction and was determined after a 24-h incubation. The scoring presented here was compiled from at least two independent β-gal assays.

	Myeloblast protein	Lon protease	Nucleolin	Poly-Glu
Independent Clones	10	4	8	25
Coding region isolated	1065–1185	685–775	239–341	????
IRS1-PH	−	−	+++	−
IRS2-PH	+++	++	+++	+++
IRS1-PTB	−	−	−	−
IRS2 PTB	−	−	−	−
IR	−	−	−	−
IGF1 R	−	−	−	−
pGBT	−	−	−	−

IRS-2 or Gab-1 are phosphorylated normally by the insulin receptor (24). By contrast, chimeric IRS-1 proteins constructed with PH domains from βARK, PLCγ, or spectrin fail to couple to the insulin receptor, suggesting that these heterologous PH domains are incapable of mediating the correct protein coupling. Therefore, to identify potential protein ligands for the PH domains in the IRS proteins, we used the PH domains of IRS-1 and IRS-2 as baits in a yeast two-hybrid screen. Our analysis has revealed that acidic motifs in proteins define provisionally the ligand specificity for the PH domain in IRS proteins.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and β-Galactosidase Assay—PH domains of IRS-1 (amino acids 13–114) and IRS-2 (amino acids 31–143) were amplified by polymerase chain reaction and cloned into the yeast two-hybrid plasmid pGBT9 (25). Similarly, the PTB domains of IRS-1 (amino acids 131–331) and IRS-2 (amino acids 161–376) were prepared in the pGBT9 plasmid. These bait-containing plasmids were then used to screen a 9.5–10.5 day post-conception mouse embryo library prepared in plasmid pVP16 (26, 27). Positive library clones were isolated from His prototroph and β-galactosidase (β-gal) yeast assays (27). Plasmids were transfected into *Escherichia coli*, sequenced, and then re-introduced into *Saccharomyces cerevisiae* strain L40. The specificity of these interactions was then assessed by mating with the AMR70 strain carrying various test baits. These bait-prey interactions were revealed by the β-gal color assay (26). Construction of bait control plasmids has been described previously (27).

Preparation of GST Fusion Proteins—pVP16 clones containing the positive inserts identified by the yeast two-hybrid screen were excised with *Not*I and inserted into the *Not*I cloning sites of pGEX-4T-1 (Amersham Pharmacia Biotech). GST fusion proteins were expressed in *E. coli* strain BL-21 and purified using glutathione-agarose (Amersham Pharmacia Biotech).

Generation of Antibodies—Rabbit polyclonal antibodies directed against IRS-1 or IRS-2 have been described previously (23). Anti-lon protease antibodies were a gift of Michael Maurizi (National Institutes of Health) (28). Anti-nucleolin antibodies were provided by the laboratory of Dr. Nancy Maizels (Yale University) (29). Antibodies against sequences encoded by yeast two-hybrid clones (myeloblast protein, lon protease, and nucleolin) were generated by immunization of rabbits with GST fusion proteins of these partial cDNA clones (HRP, Denver, PA).

Preparation of Chimeric IRS-1 Proteins—The native PH domain in IRS-1 was replaced with the PH domains of IRS-2, Gab1, βARK, PLCγ, or spectrin as described previously (24). cDNAs for the PH domains of rat PLCγ (Asp^{863}–Thr^{972}), human β-spectrin (Pro^{1061}–Lys^{1274}) and bovine βARK (Pro^{469}–Gly^{688}) were graciously provided by Dr. Robert Lefkowitz (Duke University) (30). These PH domains were excised from pGEX-2T, adapted by polymerase chain reaction with initiation codons, *Sac*I restriction sites, and cloned in-frame into the $pCMV^{his}$ expression vector containing the cDNA for $IRS1^{\Delta PH}$ (18). The PH domains of IRS-2 (Val^{23}–Leu^{130}) and Gab-1 (Lys^{13}–Gly^{116}) were generated by polymerase chain reaction and ligated into the vector containing the cDNA for $IRS1^{\Delta PH}$ (18). All constructs were then excised from the $pCMV^{his}$ vector with *Sna*bI and *Sal*I and ligated into the pBABE expression vector. Constructs were then stably transfected into 32D cells. Lysates were prepared for GST pull-down experiments.

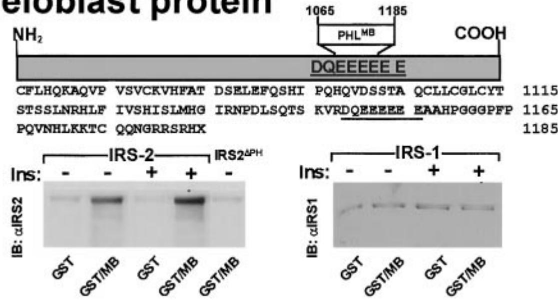
Transient Expression of PH Domain Interacting Clones—For expression in mammalian cells, inserts from the library clones were ligated into the expression vector pcDNAI (Invitrogen). The laboratory of Nancy Maizels, Yale University, provided the full-length nucleolin cDNA (29). Subconfluent cultures of CHO or CHO cells overexpressing the insulin receptor (CHO/IR) were transfected transiently using DEAE-dextran or calcium phosphate (Amersham Pharmacia Biotech). 24 h post-transfection cells were serum starved for 16 h and then stimulated with 100 nM insulin for 10 min. Cells were then lysed and analyzed for tyrosine phosphorylation of IRS proteins by immunoblotting. Expression of clones encoding potential PH domain ligands was verified using either a sequence-specific antibody or anti-hemagglutinin because recombinant proteins bear this tagged epitope.

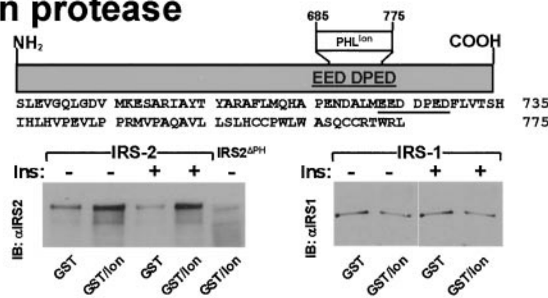
Immunoprecipitation and Western Blot Analysis—For GST pull-down experiments and immunoprecipitations, cells were lysed in 1% Triton X-100, 50 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Na_3VO_4, and protease inhibitors as described previously (23). For experiments testing insulin action, cells were starved either overnight (CHO cells) or 4 h (32D cells) and then stimulated with 100 nM insulin for 10 min. The preparation of IRS proteins containing heterologous PH domains has been described previously (24). One ml of cleared cell lysates (0.5 mg of protein) was incubated for 2 h with either antiserum or 25 μl (packed volume) of glutathione-agarose containing approximately 0.5 μg of GST fusion protein. Immune complexes were collected with protein A-Sepharose. After washing, complexes were subjected to SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose for Western analysis. Immunoblots were developed by ECL (Amersham Pharmacia Biotech).

Peptide Competition Experiments—Peptides derived from the amino acid sequence of the clone encoding nucleolin were synthesized by the Harvard Microchemistry Facility. Cell lysates and GST fusion proteins were prepared as described above. Various concentrations of peptides were incubated with one ml of cell lysates for one h prior to the addition of 25 μl of Sepharose containing 0.5 μg of nucleolin GST fusion protein. After a two-hour incubation with this affinity resin, the Sepharose pellet was washed and eluted in SDS-polyacrylamide gel electrophoresis sample buffer. Bound complexes were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS

Isolation and Characterization of PH Domain Ligands (PH Ligands)—We used the yeast two-hybrid system to identify proteins that bind to the PH domains of IRS-1 and IRS-2 in a 10.5-day mouse embryo cDNA library. Bait constructs were prepared with each PH domain, and the initial screen revealed 47 cDNA clones. The binding specificity of each clone was analyzed in yeast using the PH or PTB domains of IRS-1 or IRS-2 and different control constructs, including the intracellular domain of the insulin or the IGF-1 receptor, PKC_{δ}, or GAL4 (Table I). All 47 clones interacted with the PH domain of





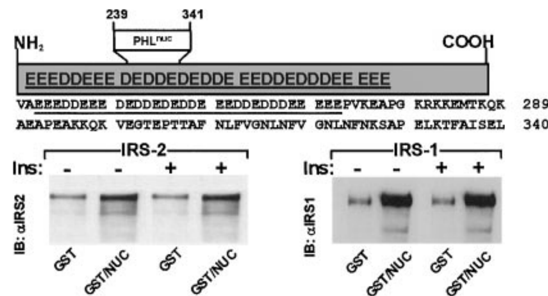


FIG. 1. **Characterization of IRS PH ligand clones.** A yeast two-hybrid cDNA library (10.5-day mouse embryo) was screened with bait plasmids containing the PH domain of either IRS-1 or IRS-2. Positive clones were purified and sequenced using primers based on the library plasmid pVP16. BLAST searches of data bases revealed that three of the four cDNA clones encode a known protein. GST fusion proteins were prepared from each of the clones, and pull-down experiments were performed using lysates of 32D cells overexpressing IRS proteins, including IRS proteins in which the PH domain was deleted, ΔPH. Lysates from unstimulated and insulin-stimulated (100 nM) cells were prepared as described. GST alone was used as a control in all pull-down experiments. *A*, clone 4 encodes a COOH-terminal (amino acids 1065–1185) region of the mouse homologue of the human myeloblast protein KIAA0211 (GenBank™ accession number D86966). *B*, clone 8 encodes a fragment of the mouse homologue of the human lon protease (EMBL accession number 74215). *C*, clone 23 encodes mouse nucleolin (EMBL accession number X07699). Regions encoded by the yeast cDNA clones are indicated with *boxed inserts*. The acidic peptide motifs in each PH ligand are *underlined*. The GST pull-downs are representative of triplicate experiments. *IB*, immunoblot.

IRS-2, whereas only 4 clones interacted with the PH domain of IRS-1. None of the clones interacted with the other test baits.

Many of the initial clones represented duplicates of the same cDNA, and restriction mapping classified the 47 cDNA clones into four unique groups. DNA sequence analysis revealed that these sets of cDNA molecules encoded partial murine isoforms of nucleolin, Lon protease, or myeloblast protein (Fig. 1). A fourth set of clones encoded a repetitive open reading frame that contained a Glu-Arg polymer; however, this clone was not studied further. In a yeast β-gal assay, the nucleolin fragment bound to the PH domain of IRS-1 and IRS-2, whereas the cloned fragments of lon protease or myeloblast protein bound

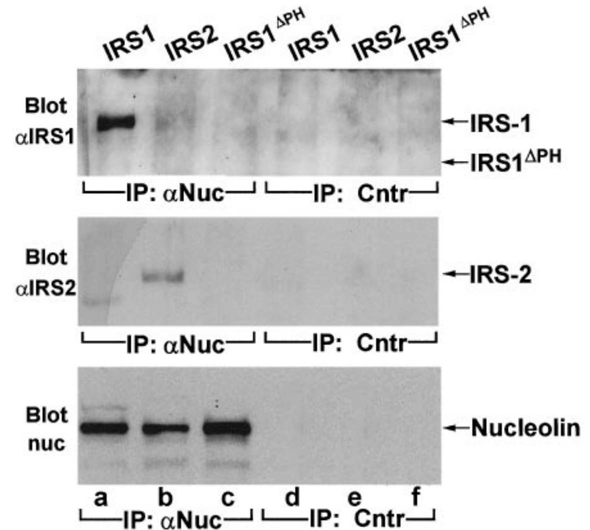


FIG. 2. **Nucleolin co-immunoprecipitates with IRS-1 and IRS-2.** Lysates of CHO cells overexpressing the insulin receptor and either IRS-1, IRS-1 without the PH domain ($IRS1^{\Delta PH}$), or IRS-2 were immunoprecipitated (*IP*) with anti-nucleolin antiserum (*αNuc*), or preimmune serum (*Cntr*). Immune complexes were probed with antibodies against IRS-1 and IRS-2. Subsequently, blots were re-probed with anti-nucleolin antibodies. Western blots are representative of duplicate experiments.

only to the PH domain of IRS-2 (Table I). Because the cloned fragments of nucleolin, Lon protease, and myeloblast protein bind to the PH domains of IRS-1 or IRS-2, they are PH ligands (PHL): PHL^{nuc}, PHL^{lon}, and PHL^{MB}, respectively. Nucleolin is a ubiquitously expressed nucleolar protein that becomes phosphorylated in response to insulin and may have a role in RNA export in cells (31). In bacteria Lon proteases function to degrade abnormal proteins; however, the function of these enzymes in mammalian cells is unknown (32). The human myeloblast protein was cloned from human KG-1 cells, but no information regarding its function is currently available.

Binding Selectivity of the PH Ligands in Vitro—The selectivity of the PH ligands was verified *in vitro* by measuring their binding to recombinant IRS-1 or IRS-2 in transfected 32D cells. GST fusion proteins expressing PHL^{nuc}, PHL^{lon}, or PHL^{MB} were prepared and attached to glutathione-Sepharose. Crude cell extracts containing IRS-1 or IRS-2 were incubated with each GST fusion protein. GST alone did not bind to IRS-1 or IRS-2. Consistent with the yeast screen, the GST fusion proteins containing PHL^{lon} or PHL^{MB} associated with IRS-2 but not with IRS-1 (Fig. 1). As expected, $IRS2^{\Delta PH}$, a mutant of IRS-2 that lacks the PH domain, did not associate with PHL^{lon} or PHL^{MB}, which is consistent with the conclusion that the PH domain mediated the binding (Fig. 1). By contrast, PHL^{nuc} associated with both IRS-1 and IRS-2 in the 32D cell extracts (Fig. 1*C*). Insulin stimulation of 32D cells failed to change the association of the PH ligands with IRS-1 or IRS-2, suggesting that these interactions in this cellular context were not regulated by insulin.

PH Domains of IRS-1 and IRS-2 Mediate Nucleolin Binding in Vivo—Based on the binding of PHL^{nuc} with IRS-1 and IRS-2, the interaction of nucleolin with IRS-1 and IRS-2 was investigated in CHO^{IR} cell extracts. Lysates from CHO^{IR} cells expressing IRS-1 or IRS-2 or a mutant of IRS-1 lacking the PH domain ($IRS1^{\Delta PH}$) were incubated with antibodies against nucleolin. Both IRS-1 and IRS-2 were co-immunoprecipitated with nucleolin antibodies from these cell lysates, whereas immunoprecipitations performed with preimmune serum did not contain nucleolin or IRS proteins (Fig. 2). Moreover, $IRS1^{\Delta PH}$ did not interact with nucleolin, demonstrating that *in vivo* the

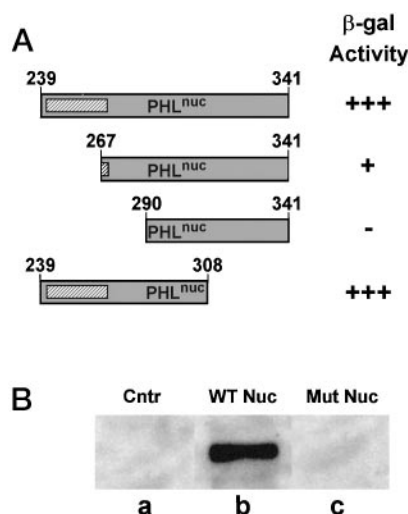


FIG. 3. **Identification of the PH domain binding motif in nucleolin.** *A*, the indicated deletions of the nucleolin clone were constructed using polymerase chain reaction. Fragments were subcloned into the yeast plasmid pVP16 and tested for interaction with the IRS-1 PH domain in a β-gal assay as described under "Experimental Procedures." The acidic domain (amino acids 241–271) is indicated by the *shaded box*. The quantitation of these interactions was based on two independent β-gal assays. *B*, residues 239–290 were deleted from the full-length nucleolin cDNA. Both wild-type nucleolin (*WT Nuc*) and the mutated cDNA lacking the PH ligand domain (*Mut Nuc*) were transiently transfected into CHO/IR/IRS-1 cells. Lysates were immunoprecipitated with preimmune serum (*Cntr*, control) or anti-nucleolin antibodies, and immune complexes were subsequently probed with anti-IRS-1 antibodies.

interaction between nucleolin and IRS proteins also occurred through the PH domain.

The PH Domain Binds Acidic Motifs in the PH Ligands—Alignment of PHL^{nuc}, PHL^{lon}, and PHL^{MP} did not reveal a common motif. However, each protein contained a stretch of acidic residues. Moreover, a fourth set of cDNA clones isolated by the yeast two-hybrid screen encoded an open reading frame containing a repetitive sequence of Glu residues, suggesting that acidic motifs in proteins may bind to the PH domain (Fig. 1). To test this possibility, PHL^{nuc} was truncated to partially or completely remove its acidic motif, and the interaction of the wild-type or mutant PHL^{nuc} with IRS-1 was evaluated in yeast (Fig. 3). As previously observed, PHL^{nuc} interacted strongly with the PH domain of IRS-1 in yeast color assay. Deletion of the first 28 NH_2-terminal residues partially reduced this association, whereas deletion of the next 23 residues, including the remaining acidic amino acids, completely abolished the interaction of PHL^{nuc} with the PH domain (Fig. 3). Deletion of the last 50 COOH-terminal amino acid residues of PHL^{nuc} had no effect on the interaction with the IRS-1 PH domain.

To verify that the PH domain of IRS-1 binds to the acidic motif in nucleolin, wild-type nucleolin containing a hemagglutinin tag at the COOH terminus was expressed in CHO^{IR} cells (29). Cell lysates were immunoprecipitated with an antibody against the hemagglutinin tag, and the presence of IRS-1 was demonstrated by immunoblotting (Fig. 3). A mutant nucleolin lacking nucleotides 687–887, which encode the acidic motif, was also expressed in CHO^{IR} cells. However, this truncated nucleolin protein did not co-immunoprecipitate IRS-1 (Fig. 3). These results suggest that the PH domain of IRS-1 binds to the acidic motif between residues 239–280 in nucleolin.

Nucleolin Interaction Is Specific for the PH Domain in IRS Proteins—Our previous results suggest that PH domains in homologous IRS proteins, including IRS-1, IRS-2, and Gab-1 have similar functions, because each PH domain couples IRS proteins to the insulin receptor (24). Chimeric IRS-1 proteins containing an homologous PH domain from IRS-2 or Gab-1 were phosphorylated by the insulin receptor, whereas chimeric proteins containing a heterologous PH domain from spectrin, βARK, or PLCγ were not phosphorylated (24). To examine whether the PH ligands isolated from our yeast two-hybrid screen specifically interact with PH domains from IRS proteins, we investigated the binding of these chimeric IRS1 proteins to PHL^{nuc}. These proteins were expressed in 32D cells as described previously and used in GST pull-down experiments. Consistent with their functional specificity, chimeric IRS-1 containing the PH domain from IRS-2 or Gab-1 associated with PHL^{nuc}; however, binding to the Gab-1 PH domain was significantly weaker. Interestingly, the IRS-1 chimeras containing heterologous PH domains from spectrin, βARK, or PLCδ did not associate with PHL^{nuc} (Fig. 4). These results suggest that the acidic motif in nucleolin interacts specifically with the PH domains of IRS proteins.

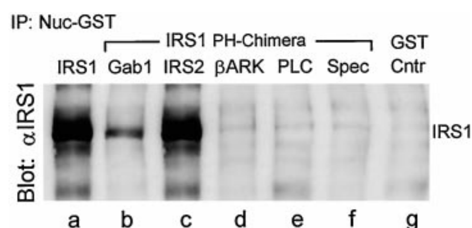
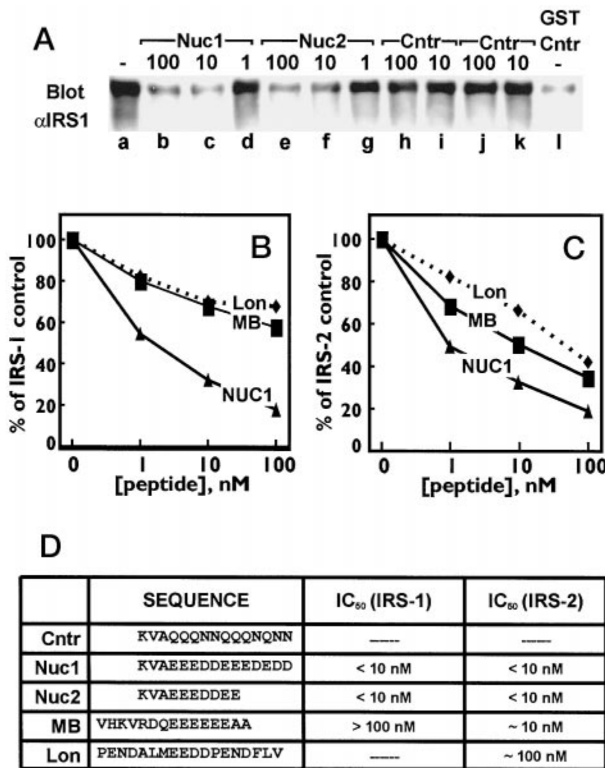


FIG. 4. **Nucleolin does not bind heterologous PH domains.** Chimeric IRS-1 proteins were prepared by replacing the IRS-1 PH domain with the PH domain from IRS-2, Gab1, βARK, spectrin (*Spec*), or PLCγ. Constructs were then expressed stably in 32D cells. For these GST pull-down experiments, lysates were prepared from cell lines expressing comparable amounts of these recombinant proteins and incubated with GST-nucleolin. Western blotting for IRS-1 revealed association with nucleolin. *IP*, immunoprecipitate; *GTS Cntr*, GST alone.

Peptides Containing Acidic Motifs Disrupt the Interaction between the PH Domain of IRS Proteins and PHL^{nuc}—Short peptides based on the acid motifs in nucleolin, Lon protease, or myeloblast proteins were tested for their ability to disrupt the interaction between PHL^{nuc} and recombinant IRS-1 or IRS-2. Lysates of CHO^{IR} cells expressing IRS-1 or IRS-2 were incubated for 30 min with immobilized PHL^{nuc} and various concentrations of Nuc1 (^{238}KVAEEEDDEEEDEDD^{252}) or Nuc2 (238KVAEEEDDEE^{247}) (Fig. 5). At concentrations of 10 and 100 nM, Nuc1 and Nuc2 blocked association of IRS-1 and IRS-2 with PHL^{nuc}, whereas a control peptide containing Asp → Asn and Glu → Gln mutations was not inhibitory (Fig. 5*A*). Based on these experiments, binding affinity of nucleolin-based peptides for IRS-1 and IRS-2 PH domains was less than 10 nM. In similar competition experiments, peptides derived from Lon protease and myeloblast protein were used to inhibit the binding of PHL^{nuc} to IRS-2 or IRS-1. Both peptides inhibited the interaction between PHL^{nuc} and IRS-2, although with less affinity; however, these peptides weakly inhibited the interaction with IRS-1 (Fig. 5, *B* and *C*). Thus, the PH domains of IRS-1 and IRS-2 may distinguish differences between acidic motifs.

PH Ligands Disrupt Tyrosine Phosphorylation of IRS-1—Previous experiments show that the PH domain provides important coupling between IRS-1 and the activated insulin receptor, although the mechanism of this coupling is unknown (18). To test whether the interaction of the PH domain with acidic motifs is involved in this interaction, the PHL^{nuc}, PHL^{lon}, or PHL^{MB} were expressed in 32D cells to determine whether they inhibited insulin-stimulated tyrosine phosphorylation. cDNAs encoding the PH ligands were subcloned into the pCDNAI and transfected into 32D cells expressing IRS-1 or IRS-2. Consistent with our *in vitro* experiments, expression of PHL^{nuc} inhibited insulin-stimulated tyrosine phosphorylation



	SEQUENCE	IC_{50} (IRS-1)	IC_{50} (IRS-2)
Cntr	KVAQQQNNQQQNQNN	-----	-----
Nuc1	KVAEEEDDEEEDEDD	< 10 nM	< 10 nM
Nuc2	KVAEEEDDEE	< 10 nM	< 10 nM
MB	VHKVRDQEEEEEEAA	> 100 nM	~ 10 nM
Lon	PENDALMEEDDPENDFLV	-----	~ 100 nM

FIG. 5. **Acidic peptides derived from nucleolin inhibit the association of IRS proteins with nucleolin.** *A*, two peptides derived from the PH-binding region of nucleolin were tested in competition experiments. Nuc1 (KVAEEEDDEEEDEDD), Nuc2 (KVAEEEDDEE), or a control peptide (KVAQQQNNQQQNQNN) at concentrations of 1, 10, and 100 nM were pre-incubated with nucleolin-GST for 30 min at 4 °C. Lysates from CHO cells overexpressing IRS-1 were then added and rotated with the affinity resin for 2 h. The Sepharose pellet was then washed, and bound complexes were analyzed for the presence of IRS-1 by immunoblotting. No peptide was added to the incubation in *lane a*, the IRS-1 positive control (*Cntr*) for nucleolin interaction. In *lane l*, IRS-1 lysates were incubated with GST alone to control for nonspecific binding. *B*, *C*, and *D*, acidic peptides derived from the other yeast clones, myeloblast protein (*MB*) and lon protease (*Lon*), were incubated with GST-nucleolin as described above. Lysates from either CHO/IRS-1 or CHO/IRS-2 cells were then added to the Sepharose beads and incubated for 2 h. The affinity resin was washed and then analyzed by Western blotting for IRS-1 (*B*) or IRS-2 (*C*). Blots were then quantitated by densitometric analysis. Graphs of these results are presented as the percentage of IRS controls, which were incubated with nucleolin-GST in the absence of peptides. This densitometric analysis was also used to approximate the IC_{50} for these peptides as presented in *D*. The Western blot and quantitation of the peptide competition are representative of three independent experiments.

of IRS-1 and IRS-2; however, PHL^{lon} and PHL^{MB} only inhibited IRS-2 tyrosine phosphorylation (Fig. 6, *A* and *B*, and data not shown). Direct blotting of these cells lysates demonstrated that samples in this experiment contain equal amounts of the IRS protein (*lower panel*, Fig. 6*B*). Quantitation of the anti-phosphotyrosine blots by densitometric analysis suggests that nucleolin overexpression in 32D/IR/IRS1 cells reduced the insulin-stimulated phosphorylation of IRS-1 by 80%; in 32D/IR/IRS2 cells transfected with either PHL^{MB} or PHL^{nuc}, tyrosine phosphorylation of IRS-2 was decreased by approximately 50%. These results imply the possibility that these PH ligands inhibit the active site of the PH domain that mediates coupling to the activated insulin receptor.

We analyzed the effect of overexpressing full-length nucleolin on tyrosine phosphorylation in CHO^{IR} and CHO^{IR}/IRS-1 cells. Cells were stimulated with insulin, and lysates were immunoblotted to assess tyrosine phosphorylation of endogenous or recombinant IRS-1. Overexpression of nucleolin significantly reduced tyrosine phosphorylation of IRS-1 in both cell lines (Fig. 7*A*). However, the inhibitory effect of nucleolin was more pronounced on the endogenous IRS-1 in CHO^{IR} cells. When the acidic motifs of nucleolin were deleted (amino acids 239–290), nucleolin failed to produce an inhibitory effect on IRS-1 phosphorylation, suggesting that this region was required to inhibit the interaction between IRS-1 and the insulin receptor (Fig. 7*B*).

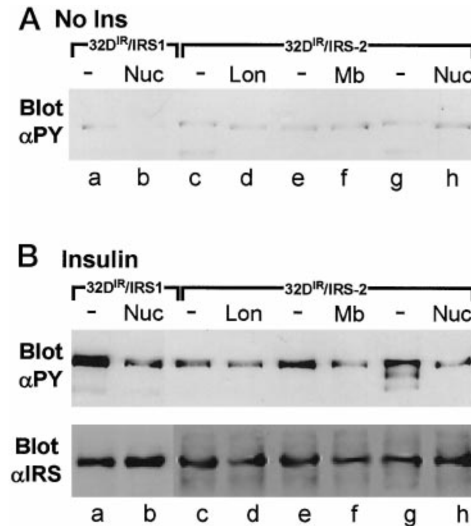


FIG. 6. **Overexpression of PHL proteins reduces tyrosine phosphorylation of IRS proteins in 32D/IR cells.** The cDNAs isolated from the yeast two-hybrid screen were subcloned into the pCDNAI vector and transfected transiently into 32D cells expressing either IRS-1 (*lanes a* and *b*) or IRS-2 (*lanes c–h*). Cells were then stimulated with 100 nM insulin, and lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and analyzed for tyrosine phosphorylation of IRS by anti-phosphotyrosine immunoblotting. The *upper panel* presents an anti-phosphotyrosine blot of unstimulated cell lysates. *Lanes a*, *c*, *e*, and *g* are control cells which were not transfected with vector alone. Insulin-stimulated tyrosine phosphorylation of IRS-1 or IRS-2 is revealed in the *lower panel*. These immunoblots are representative of results obtained in two separate transfection experiments. *Mb*, myeloblast protein; *αPY*, anti-phosphotyrosine; *Ins*, insulin.

To exclude the possibility that overexpression of nucleolin generally disrupts phosphorylation events, insulin-stimulated tyrosine phosphorylation of the insulin receptor and Shc were also analyzed in CHO^{IR} cells in the absence (Fig. 7*C*, *lanes a* and *b*) and presence of overexpressed nucleolin (Fig. 7*C*, *lanes c* and *d*). Anti-phosphotyrosine immunoblots revealed that only IRS-1 phosphorylation was reduced by overexpression of nucleolin, whereas the insulin receptor and Shc were not inhibited (Fig. 7*C*, *left panels*). Based on densitometric analysis of these immunoblots, tyrosine phosphorylation of IRS-1 was reduced more than 60% in the presence of overexpressed nucleolin, whereas the tyrosine phosphorylation of the insulin receptor and Shc were enhanced by approximately 10%. Re-probing of these blots with appropriate antibodies indicated that the samples contained equal levels of IRS-1, IR, or Shc (Fig. 7*C*, *lanes e–h*). These results are consistent with the hypothesis that the PH domain-mediated association between IRS-1 and overexpressed nucleolin disrupts the interactions required for productive coupling of the IRS proteins to the activated insulin receptor.

DISCUSSION

Although multiple mechanisms exist to couple IRS proteins to activated receptors, several observations suggest that the PH domain is essential for insulin-stimulated tyrosine phosphorylation of IRS-1 (18, 23, 33). However, the cellular elements that interact with the PH domains in IRS proteins are

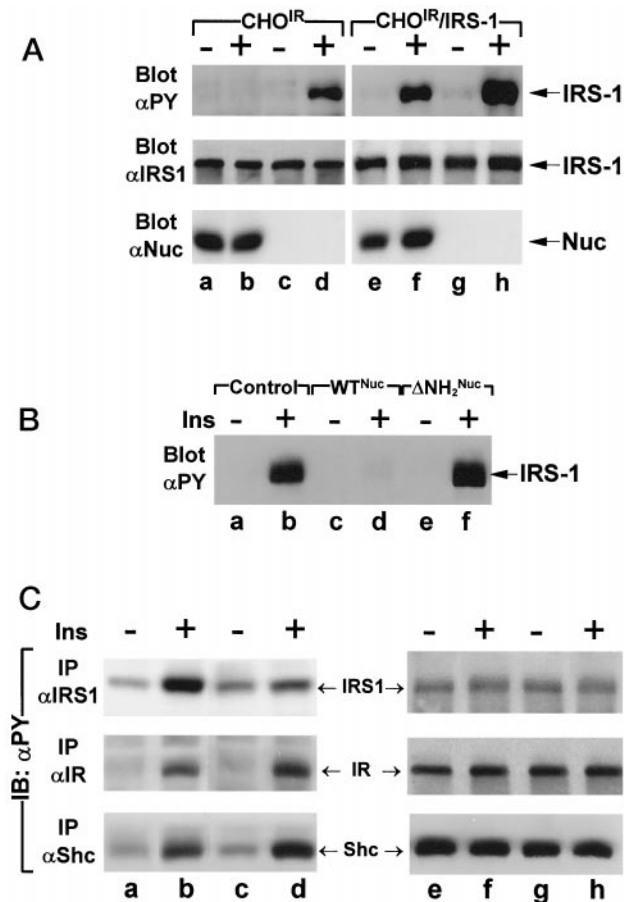


FIG. 7. **Overexpressed nucleolin exerts a dominant negative effect on IRS-1 tyrosine phosphorylation but does not alter the phosphorylation of the insulin receptor nor Shc.** *A*, CHO/IR (*lanes a–d*) and CHO/IR/IRS-1 (*lanes e–h*) cells were transfected with full-length nucleolin containing a hemagglutinin tag (*lanes a*, *b*, *e*, and *f*) or a plasmid control (*lanes c*, *d*, *g*, and *h*). Cells were stimulated with 100 nM insulin for 10 min and lysed as described under "Experimental Procedures." Lysates were immunoprecipitated (*IP*) with either anti-IRS-1 antibodies or an anti-hemagglutinin tag antibody. Immune complexes were then blotted and probed with anti-phosphotyrosine (*αPY*) antibodies, anti-IRS-1, or anti-nucleolin. *B*, CHO/IR cells were transfected with vector alone (*lanes a* and *b*), wild-type nucleolin (*lanes c* and *d*), or nucleolin lacking the PH domain binding region located in the NH_2 terminus. Cells were stimulated with insulin, lysed, and immunoprecipitated with anti-IRS antibodies. Blots were probed with anti-phosphotyrosine antibodies. *C*, lysates of insulin-stimulated CHO/IR cells transfected with vector (*lanes a* and *b*) or wild-type nucleolin (*lanes c* and *d*) were immunoprecipitated for IRS-1, the insulin receptor and Shc. Immune complexes were then probed with anti-PY (*left panels*). To demonstrate comparable expression of the relevant proteins, these blots were subsequently stripped and re-probed with anti-IRS-1, anti-insulin receptor, or anti-Shc antibodies (*right panels*). These blots are representative of results obtained from two independent transfection experiments. *IB*, immunoblot.

unknown. Because the insulin receptor does not interact directly with the PH domain, other membrane-associated elements may act as the interface between IRS proteins and activated receptors (20–22). By analogy with other PH domains, membrane phospholipids have been proposed as ligands for the PH domains in IRS proteins, but no published reports support this hypothesis. Our yeast two-hybrid screen identified nucleolin, Lon protease, and myeloblast protein as potential ligands for the IRS-2 PH domain. Although these proteins are unrelated, each contains an acidic motif that interacts with the PH domain of IRS-2. The acidic motif in nucleolin has the highest affinity for this PH domain, and unlike the other potential ligands, it binds equally well to the PH domain in IRS-1. This defines a novel and selective interaction that may be functionally important.

Based on information available from solved PH domain structures, certain PH domains may bind to a short peptide sequence that is not incorporated into larger folded domains of interacting proteins (3–10). Although such ligands could be variable in sequence, consistent with the PH domain divergence, the positive charge clustered in the putative binding pocket in solved PH domain structures suggests an interaction with a negatively charged ligand such as the acidic motifs contained in nucleolin or the myeloblast protein identified in our screen (3–8). Because the PH domains of IRS-1 and IRS-2 are only 60% identical, certain binding specificity might be expected, which may explain the selective binding observed in our experiments. The acidic motif in nucleolin that binds to the PH domain of IRS-1 and IRS-2 includes a stretch of 31 aspartic acid and glutamic acid residues. Shorter peptides based on this motif, including KVAEEEDDEE, also bind, suggesting that the recognition motif is somewhat shorter. The acidic motifs in lon protease (EEQDPED) and myeloblast protein (DQEEEEEE) include an asparagine residue, which may provide the apparent specificity for the IRS-2 PH domain. However, further work using a series of peptides will be necessary to fully define the selectivity of the IRS-1 and IRS-2 PH domain and extend the analysis to the PH domain of the other IRS proteins.

The different recognition of nucleolin, Lon protease, and myeloblast protein by the PH domain of IRS-2 and IRS-1 reveals a potential mechanism for differential recruitment or regulation of the IRS proteins. This provisional hypothesis is supported by the finding that overexpression of nucleolin inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2, whereas only IRS-2 is inhibited by lon protease or myeloblast protein. Recent work suggests that IRS-1 and IRS-2 mediate distinct signals during insulin stimulation. Without IRS-1, mice are small and insulin-resistant, but they do not develop diabetes unless additional insulin resistance is imposed by a second molecular or physiological defect (34). By contrast, disruption of IRS-2 causes progressive development of insulin resistance leading to diabetes owing to inadequate β-cell compensation (35). The distinct biological properties of IRS-1 and IRS-2 may arise partially from the unique specificity of the PH domain revealed by our experiments. However, other unique properties may also be involved, including the presence of different phosphorylation motifs or the KRLB domain in IRS-2 (36). Future experiments based on the hypothetical role of acidic motifs will address these questions.

Ligands for the PH domain of the IRS proteins could mediate several biological effects required for normal signaling. Membrane proteins with acidic motifs may be juxtaposed with the insulin receptor to recruit IRS proteins into a signaling complex. These proteins may be associated with activated membrane receptors to provide an efficient mechanism to recruit the substrate for tyrosine phosphorylation. Alternatively, cytoplasmic proteins with acidic motifs may disrupt the interaction between IRS proteins and membrane elements, thus facilitating the relocation of IRS proteins to other sites in the cell. Finally, other enzymes with acidic motifs, including phosphatases or serine kinases, may be recruited to the IRS protein complex through binding to the PH domain. Many kinases contain acid motifs that may cause them to interact with the IRS protein complex.

With the exception of nucleolin, which also interacts with the PH domain of both IRS-1 and IRS-2, there is as of yet no evidence that the proteins identified in our yeast two-hybrid screen are physiologically relevant for insulin signaling or contribute to the normal function of the IRS proteins. Nucleolin, a

ubiquitously expressed nucleolar protein, has many cellular activities including transcription of b-cell-specific complexes and processing of ribosomal RNA (29, 37). This protein is a substrate of casein kinase 2 (38), cyclin-D kinase (39), and PKCζ (40). Interestingly, for the present study, insulin stimulates the phosphorylation and nuclear translocation of nucleolin in 3T3–442A cells (31). Therefore, the interaction of this protein with the PH domains of IRS-1 and IRS-2 may reveal a novel mechanism to build the IRS signal transduction complex and may provide a means for IRS proteins to regulate such cellular events as RNA efflux.

Identification of potential ligands for the PH domains of IRS proteins will now facilitate studies aimed at defining the function of this structural module in IRS-mediated signaling. Although the physiological relevance of the interactions between IRS proteins and these PH ligands requires further study, the presence of a negatively charged motif provides a tool for designing structural studies of the IRS PH domain. It will be of interest to determine precisely using mutational analysis the amino acid residues in the IRS PH domain that mediate recognition of the negatively charged motif.

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