Insulin Receptor Substrate-1 Pleckstrin Homology and Phosphotyrosine-binding Domains Are Both Involved in Plasma Membrane Targeting*

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The localization of insulin receptor substrate (IRS) molecules may be responsible for the differential biological activities of insulin and other peptides such as platelet-derived growth factor. The subcellular localization of IRS-1 is controversial, with some reports suggesting association with the cytoskeleton and other studies reporting membrane localization. In this study, we used immunofluorescence microscopy to define the localization of IRS-1. In the basal state, recombinant IRS-1 was localized predominantly in the cytoplasm. In response to insulin, recombinant IRS-1 translocated to the plasma membrane. We have also studied the localization of green fluorescent protein (GFP) fusion proteins. Unlike native IRS-1, a fusion protein containing GFP plus full-length IRS-1 appeared to localize in inclusion bodies. In contrast, when GFP was fused to the N terminus of IRS-1 (i.e. the pleckstrin homology and phosphotyrosine-binding domains), this fusion protein was targeted to the plasma membrane. Mutations of phosphoinositide-binding sites in both the pleckstrin homology and phosphotyrosine-binding domains significantly reduced the ability of Myc-tagged IRS-1 to translocate to the plasma membrane following insulin stimulation. However, these mutations did not cause a statistically significant impairment of tyrosine phosphorylation in response to insulin. This raises the possibility that IRS-1 tyrosine phosphorylation may occur prior to plasma membrane translocation.

Insulin receptor substrate (IRS)* molecules mediate multiple insulin signaling pathways downstream of the insulin receptor (IR). Ligand binding to the extracellular domain of the IR activates its intrinsic tyrosine kinase activity, resulting in autophosphorylation and phosphorylation of other intracellular proteins, including IRS molecules and SHC (1). Tyrosine-phosphorylated IRS molecules then activate various SH2 domain-containing molecules such as phosphatidylinositol 3-kinase (PI3K), Grb2, SHP2, and Nck (1). Thus, IRS molecules initiate a PI3K-mediated cascade leading to GLUT4 translocation.

Although both insulin and platelet-derived growth factor receptors are able to activate PI3K, only insulin-stimulated activation of PI3K leads to the translocation of the glucose transporter GLUT4 from intracellular vesicles to the cell surface in both 3T3-L1 cells and adipocytes (2). It has been hypothesized that IRS molecules and the platelet-derived growth factor receptor recruit PI3K to distinct locations (3). Thus, differential recruitment of PI3K may be responsible for the ability of insulin, but not platelet-derived growth factor, to stimulate GLUT4 translocation.

All IRS molecules contain both a pleckstrin homology (PH) domain and a phosphotyrosine-binding (PTB) domain. PH domains have been shown to be important both for plasma membrane targeting (4) and for ligand-induced tyrosine phosphorylation by the IR (5). PTB domains specifically bind to tyrosine-phosphorylated proteins at NPxY motifs. The PTB/NPxY interaction is required for the binding of IRS molecules to the IR following insulin-stimulated IR activation. Interestingly, the β-strands of the IRS-1 PTB domain superimpose on the framework of a PH domain, suggesting that the IRS-1 PTB domain may be a type of PH domain (6). In support of this, Takeuchi et al. (7) have demonstrated the ability of the IRS-1 PTB domain to bind phosphorylated inositol derivatives.

Many PH domain-containing molecules have been shown to translocate to the plasma membrane in response to insulin stimulation, e.g. protein kinase B, a serine/threonine-specific protein kinase downstream of IRS-1 in the insulin signaling pathway; phosphoinositide-dependent protein kinase-1, which regulates protein kinase B; and Gab1, a Grb2-associated protein that functions downstream of multiple growth factor receptors (8–11). Binding assays have shown that the IRS-1 PH domain binds to phosphatidylinositol phosphates (12). This binding occurs primarily in response to growth factor stimulation either by causing a conformational change in the protein or by changing the composition of phosphoinositides in the membrane bilayer (13). Thus, it has been hypothesized that IRS-1 might translocate to the plasma membrane following insulin stimulation.

In this study, we have investigated the role of the PH and PTB domains both in the subcellular localization of IRS-1 and in insulin signaling. By mutating predicted phosphoinositide-binding sites in the IRS-1 N terminus, we demonstrated the contribution of these domains to the plasma membrane targeting of full-length IRS-1. We found that mutation of either the PH or PTB domain significantly reduced the ability of IRS-1 to translocate to the plasma membrane. However, these same...
mutations did not significantly affect the ability of IRS-1 to be tyrosine-phosphorylated in response to insulin. This raises the possibility that association of IRS-1 with the plasma membrane may not be required for tyrosine phosphorylation. Indeed, it is possible that tyrosine phosphorylation may occur prior to plasma membrane translocation.

EXPERIMENTAL PROCEDURES

IRS-1 Constructs—cDNA constructs were generated by polymerase chain reaction amplification of either full-length IRS-1 or IRS-1 regions (PH domain, amino acids 1–113; PTB domain, amino acids 107–335; and N terminus, amino acids 1–262) using human IRS-1 cDNA (14) as a template. Polymerase chain reaction was carried out using high fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA), and primers with appropriate restriction sites were incorporated as needed. Polymerase chain reaction products were cloned into pcDNA3.1Myc, pEGFPN2, or pEGFPN3 (CLONTECH, Palo Alto, CA) with the appropriate restriction enzymes. All cDNA constructs were sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (PE Applied Biosystems, Foster City, CA) with the appropriate restriction enzymes. The details cloning strategies for these constructs are available upon request.

Site-directed Mutagenesis of IRS-1—The IRS-1 R28C mutation was made using a QuikChange™ site-directed mutagenesis kit (Stratagene). Mutagenesis was carried out on the human full-length IRS-1 cDNA. Introduction of a Myc epitope tag into IRS-1 R28C was accomplished by polymerase chain reaction amplification of IRS-1 cDNA, followed by TA cloning and subcloning into pcDNA3.1Myc, pEGFPN2, or pEGFPN3 (CLONTECH, Palo Alto, CA) with the appropriate restriction enzymes. All cDNA constructs were sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (PE Applied Biosystems, Foster City, CA) and an Applied Biosystems Prism 377 DNA automatic sequencer.

Antibodies—The primary antibodies used included anti-IRS-1 (C20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-e-Myc (9E10; Santa Cruz Biotechnology), and anti-phosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, NY). The secondary antibodies used included fluorescein-, rhodamine-, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Indirect Immunofluorescence Studies—COS-7 cells were seeded on two-chamber glass slides (Lab-Tek II chamber slide, Nalge Nunc International, Naperville, IL) at a density of 5 × 10^4 cells/ml and transfected using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, 3 μl of Fugene 6 and 1 μg of plasmid DNA were mixed and incubated in 100 μl of Opti-MEM serum-free medium (Life Technologies, Inc.) for 15 min before being added to cells in antibiotic-free medium. After 48 h, cells were fixed in 2% Formalin in Dulbecco's phosphate-buffered saline (DPBS) for 15 min, washed twice with DPBS, and incubated with DPBS and 10% fetal bovine serum for 5 min. Slides were then placed in a humidified 15-cm dish with water-soaked 3-mm paper and incubated with primary antibody as indicated at a dilution of 1:200 in DPBS, 10% fetal bovine serum, and 0.075% saponin for 1 h. Following two 5-min washes with DPBS and 10% fetal bovine serum, the slides were incubated with the appropriate secondary antibody at a 1:200 dilution in DPBS, 10% fetal bovine serum, and 0.075% saponin for 30 min. Following two 5-min washes with DPBS, the slides were mounted using Vectashield mounting medium (Vector Labs, Inc., Burlingame, CA) and glass coverslips (Corning Inc., Corning, NY). For experiments with green fluorescent protein (GFP) fusion proteins, the antibody incubations were omitted.

Confocal images of IRS-1-GFP were obtained with a Nikon Optiphot 2 fluorescence microscope equipped with a Bio-Rad 1024 confocal laser scanning imaging system. This system uses a mixed argon/krypton laser (λ = 488 nm, blue line for fluorescein isothiocyanate (FITC); λ = 586 nm, yellow line for rhodamine) and COSMOS/Lasersharp image analysis software (Bio-Rad). All other slides were viewed with a Zeiss Axiophot inverted microscope. Images were captured with a PentaMAX camera (Princeton Instruments Inc., Trenton, NJ) and IP Labs software (Analytical Imaging, Inc., Fairfax, VA) and processed with Adobe Photoshop.

Quantitation of plasma membrane association was conducted by blind scoring of 150 cells from three independent experiments, with each sample performed in duplicate. We used the following scoring system. Cells with little plasma membrane staining received a score of zero. Cells in this category exhibit a diffuse cytoplasmic and/or nuclear pattern (resembling GFP alone) or are primarily cytoplasmic with a faint plasma membrane outline. Cells with significant plasma membrane association received a score of 1. Cells in this category have fluorescence either exclusively on plasma membrane ruffles or in distinct plasma membrane ruffles with some cytoplasmic staining, or in a bright outline at the cell periphery with minimal cytoplasmic staining. Translocation score was calculated as the percentage of cells scored as 1. This score is a semiquantitative index and is not necessarily linearly related to the number of molecules undergoing translocation. Statistical analysis of plasma membrane scoring was accomplished using SigmaPlot Version 5.0.

Tyrosine Phosphorylation of IRS-1 R28C, IRS-1 3KQ, and IRS-1 R28C+3KQ—COS-7 cells were transfected in 10-cm dishes using LipofectAMINE Plus (Life Technologies, Inc.). The next day, cells were starved overnight (in Dulbecco’s modified Eagle’s medium containing 1% insulin-free bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine) and stimulated with 100 nM insulin for 5 min; and lysed in buffer containing 0.5% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.5) 0.3 mM NaCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, and a Complete proteinase inhibitor tablet (Roche Molecular Biochemicals). Samples were boiled in Laemmli buffer. Cell extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using the indicated primary antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and quantified using the NIH Image program.

RESULTS

Translocation of IRS-1 in Response to Insulin—We constructed IRS-1-GFP fusion expression plasmids to monitor the effect of insulin upon the subcellular localization of IRS-1 in live cells (Fig. 1). However, full-length IRS-1-GFP was localized to inclusion bodies seen as bright dots in the cytosol (Fig. 2E). Additional experiments demonstrated that IRS-1-GFP was not
efficiently phosphorylated in response to insulin (data not shown), consistent with the conclusion that IRS-1-GFP was localized in a non-physiological location. Therefore, we used either untagged or Myc-tagged protein in subsequent studies of full-length IRS-1.

Using indirect immunofluorescence microscopy, we investigated the effect of insulin on the subcellular localization of recombinant IRS-1. COS-7 cells were transiently transfected with either IRS-1 or IRS-1-Myc expression vectors. Both recombinant IRS-1 and IRS-1-Myc were primarily cytoplasmic when expressed in cells incubated in the absence of insulin (Fig. 2, A and C). In contrast, transfection of cells with expression vectors for IRSs and incubation in the presence of insulin promoted translocation of IRS-1 to distinct plasma membrane ruffles (Fig. 2, B and D). In preliminary studies, we did not detect an effect of insulin upon the localization of IRS-1 when cells were cotransfected with IR expression vectors (data not shown).

**The IRS-1 N Terminus Is Sufficient for Plasma Membrane Targeting**—We were interested in defining a region that was capable of targeting IRS-1 to the plasma membrane in response to insulin. Therefore, we analyzed fragments of IRS-1 for their ability to target GFP to the plasma membrane (Fig. 1). In contrast to recombinant full-length IRS-1, the IRS-1 N terminus (IRS-1-GFP) fused to GFP was associated with the plasma membrane in both the absence and presence of insulin (Fig. 3, A and B). Identical results were obtained using IRS-1-(1–262) with a C-terminal Myc tag (data not shown). These findings suggest that the N terminus of IRS-1 is sufficient for plasma membrane association. Furthermore, taken together with data on full-length IRS-1, these observations suggest that the C terminus of IRS-1 may inhibit the ability of the IRS-1 N terminus to be targeted to the plasma membrane.

**Subcellular Localization of Isolated PH and PTB Domains**—The IRS-1 N terminus consists of at least two potential targeting regions, the PH and PTB domains. Thus, we explored the ability of these individual domains to target GFP to the plasma membrane. When the PH domain was fused to GFP, the resulting protein was recruited to plasma membrane ruffles in both unstimulated (Fig. 4A) and insulin-stimulated (data not shown) cells. We confirmed that the recombinant Myc-tagged PH domain was also targeted to the plasma membrane, suggesting that the GFP moiety was not distorting the subcellular localization (Fig. 4B). In contrast to the PH domain, the isolated PTB domain tagged with either GFP or Myc did not target to the plasma membrane in either the absence (Fig. 4, C and D) or presence (data not shown) of insulin.

**Effect of Mutations of the PH and PTB Domains on Translocation of Full-length IRS-1**—To assess the contribution of the PH and PTB domains to the plasma membrane targeting ability of IRS-1, we individually mutated sites in these domains that are predicted to reduce their affinity for phosphoinositides. Like the PH domains from other molecules such as Bruton’s tyrosine kinase, Gab1, and protein kinase B, the PH domain of IRS-1 contains a conserved arginine residue at position 28 in the β2-strand. Mutation of this arginine to cysteine has been demonstrated to significantly reduce the affinity of PH domain-containing molecules for phosphatidylinositol 3,4,5-trisphosphate (4). In contrast, the IRS-1 PTB domain was found to bind to phosphoinositides and inositol polyphosphates, specifically inositol 1,3,4,5,6-pentakisphosphate and inositol P₆, via three critical lysine residues in the PTB domain (positions 169, 171, and 177) (7). Replacement of each individual lysine residue with glutamine also was shown to result in a 50% reduction in phosphoinositide binding (7).

Therefore, to investigate the membrane targeting ability of the PH and PTB domains, we mutated arginine 28 to cysteine (R28C) in the PH domain and/or lysines 169, 171, and 177 to glutamine (3KQ) in the PTB domain, which are believed to be critical for phosphoinositide and inositol phosphate binding. We then assessed the ability of these specific point mutations to affect the plasma membrane targeting ability of both full-length IRS-1-Myc and an IRS-1-(1–262)-GFP fusion protein containing the PH and PTB domains. The Myc-tagged IRS-1 protein containing mutations in the PH and PTB domains was used because the full-length IRS-1-GFP fusion protein predominantly accumulated in inclusion bodies. Following incubation in serum-free medium and/or insulin stimulation, cells with low levels of plasma membrane staining were scored as zero, whereas those with significant levels of plasma membrane staining were scored as 1 (Fig. 5) (see “Experimental Procedures”).

As we demonstrated earlier, full-length IRS-1-Myc was primarily cytoplasmic in the unstimulated state, and it translocated to plasma membrane ruffles following insulin stimulation (Fig. 6, upper panel, A and B). Quantitation of our observations revealed that wild-type IRS-1-Myc visibly translocated to the plasma membrane in response to insulin in ~40% of the cells (Fig. 6, lower panel). Mutation of either the PH or PTB domain reduced the ability of IRS-1 to associate with the plasma membrane in the insulin-stimulated state (Fig. 6, upper panel, C–F). The R28C mutation reduced the translocation score by ~75%, whereas the 3KQ triple mutation led to a 50% reduction. When both the PH and PTB domains...
Fig. 3. The IRS-1 N terminus is targeted to the plasma membrane. COS-7 cells were transiently transfected with IRS-1-(1–262)-GFP expression plasmid with either the IR (B) or empty vector (A). Cells were treated in the presence (B) or absence (A) of insulin for 5 min. Localization was observed by direct fluorescence microscopy. Arrows indicate plasma membrane ruffles. Direct fluorescence of a representative cell (n = 150) is shown. Bar = 20 μm.

Fig. 4. Plasma membrane targeting of the IRS-1 PH domain, but not the PTB domain. COS-7 cells were transiently transfected with IRS-1 PH domain-GFP (A), IRS-1 PH domain-Myc (B), IRS-1 PTB domain-GFP (C), or IRS-1 PTB domain-Myc (D) expression plasmid. Cells expressing GFP fusion proteins were observed by direct fluorescence, whereas those expressing Myc-tagged proteins were detected using an anti-Myc primary antibody followed by an FITC-conjugated secondary antibody. Representative cells (n = 150) are shown. Bar = 10 μm (A and B) and 20 μm (C and D).

GFP Fusions  Score  IMF
A  B  E  F  0
C  D  G  H  1

Fig. 5. Scoring cells for plasma membrane association. COS-7 cells transfected with GFP fusion proteins were observed by direct fluorescence microscopy (A–D), whereas cells transfected with Myc-tagged proteins were visualized by immunofluorescence microscopy (IMF) (E–H). Cells were scored as either zero (A and B and E and F) or 1 (C and D and G and H). See “Experimental Procedures” for details.

where mutated, translocation was inhibited to the same extent as with the R28C mutation alone.

Mutational Analysis of the IRS-1 N Terminus—To study the effects of our mutations on the isolated IRS-1 N terminus (IRS-1-(1–262)-GFP), we employed the same method to score cells for significant plasma membrane association (see “Experimental Procedures”). Mutation of either the PH or PTB domain reduced the plasma membrane association of the IRS-1-(1–262)-GFP fusion protein (Fig. 7, upper panel). Consistent with what we observed using IRS-1-Myc, the R28C mutation reduced the plasma membrane targeting ability of the IRS-1-(1–262)-GFP fusion protein by 90% in the unstimulated state and by 67% in the stimulated state (Fig. 7, lower panel). Additionally, when both the PH and PTB domains were mutated, the reduction in plasma membrane association was not detectably different from the effect of the R28C mutation alone. Interestingly, the 3KQ mutation significantly reduced the translocation score of the IRS-1-(1–262)-GFP fusion protein by 67% in the unstimulated state, but this reduction was less severe in the insulin-stimulated state (30%) and did not reach statistical significance.

Next, we confirmed the ability of the R28C mutation to reduce the plasma membrane targeting ability of the isolated PH domain-GFP fusion. The wild-type IRS-1 PH domain-GFP and IRS-1 R28C-GFP fusion proteins were expressed separately in COS-7 cells. After insulin stimulation, cells were scored for significant plasma membrane association. In contrast to the findings of Razzini et al. (4), our wild-type IRS-1 PH domain-GFP fusion protein was targeted to the plasma membrane in both the unstimulated and insulin-stimulated states (Fig. 8, upper panel). Although the percentage of cells displaying distinct plasma membrane localization increased from 45 to 65% (Fig. 8, lower panel) following insulin stimulation, this difference was not statistically significant (as determined by Student’s t test). Additionally, the R28C mutation reduced the plasma membrane association of IRS-1 PH domain-GFP by 67% in both the unstimulated and insulin-stimulated states.

Ability of IRS-1 Targeting Mutants to Be Tyrosine-phosphorylated in Response to Insulin—It has been suggested that plasma membrane targeting is a prerequisite for insulin-stimulated tyrosine phosphorylation of IRS molecules. The R28C and 3KQ mutations reduce the ability of IRS-1 to translocate to the plasma membrane in response to insulin. Therefore, we investigated the ability of these IRS-1 mutants to become tyrosine-phosphorylated. Since recombinant IRS-1 was not efficiently phosphorylated by the endogenous IR (data not shown), it was necessary to cotransfect COS-7 cells with recombinant IR.

COS-7 cells were transiently transfected with the Myc-tagged IRS-1 mutants and the IR. Following insulin stimulation, cell extracts were used for immunoblotting. Probing with an anti-Myc antibody showed that both the wild-type and mutant IRS-1 proteins were expressed at comparable levels (Fig. 9B). Interestingly, both the wild-type and mutant IRS-1 proteins were found to be tyrosine-phosphorylated to a similar extent (Fig. 9A, lanes 2, 4, 6, and 8). Thus, we were concerned
that the anti-phosphotyrosine antibody was recognizing endogenous IRS-1 molecules in addition to the recombinant IRS-1 molecules. However, when COS-7 cells were transfected with the IR alone, insulin-stimulated endogenous IRS-1 was not detectable by immunoblotting with an anti-phosphotyrosine antibody (data not shown). We also considered the possibility that overexpression of the IR might lead to maximal IRS-1 tyrosine phosphorylation, thereby masking the differences between the wild-type and mutant IRS-1 molecules. To address this issue, we conducted control experiments in which IRS-1 plasmid DNA levels were held constant while IR plasmid levels were varied (data not shown). We found that when IR expression levels were sufficient, further increases did not lead to additional insulin-stimulated tyrosine phosphorylation of IRS-1.

As judged by densitometric quantitation of three separate experiments (Fig. 9C), the ratio of phosphotyrosine to total recombinant IRS-1 in the mutants appeared slightly decreased compared with wild-type IRS-1. However, this difference was
Role of IRS-1 Domains in Plasma Membrane Targeting

**FIG. 8.** The R28C mutation disrupts plasma membrane targeting of the IRS-1 PH domain. Upper panel, COS-7 cells were transiently transfected with IRS-1 PH domain-GFP (A and B) or IRS-1 R28C-GFP (C and D) expression plasmid or with a plasmid encoding GFP alone (not shown). Cells were either transfected with IR expression plasmid and incubated in the presence of 100 nM insulin for 5 min (B and D) or transfected with empty vector and incubated in the absence of insulin (A and C). Direct fluorescence microscopy of representative cells is shown. Arrows indicate plasma membrane ruffles. Bar = 10 μm. Lower panel, over 150 cells per condition were scored for significant plasma membrane association (see "Experimental Procedures"); mean ± S.E.; *, p < 0.01. WT, wild-type IRS-1.

**DISCUSSION**

Subcellular Localization of IRS-1—IRS-1 was initially believed to be membrane-associated, but more recent studies suggest that the majority is associated with the cytoskeleton. In early subcellular fractionation experiments, it was reported that IRS-1 co-fractionated with either the low density microsomal fraction (16) or intracellular membranes (17). However, by more extensive fractionation, Clark et al. (3) separated IRS-1 from membranes, but not cytoskeletal elements. Additionally, IRS-1 was resistant to extraction with ionic detergent, suggesting that the majority of IRS-1 was associated with the cytoskeleton (18). Even though all IRS molecules contain a PH domain, presumably to target them to the plasma membrane, only IRS-3 is found predominantly in the plasma membrane fraction (19). In contrast, IRS-1 and IRS-2 primarily co-fractionate with the low density microsomes (19). Although the majority of IRS-1 is associated with intracellular membranes or the cytoskeleton, it remains possible that translocation of a percentage of IRS-1 molecules to the plasma membrane might be required to mediate insulin signaling.

It has been hypothesized that IRS-1 translocates to the plasma membrane upon insulin stimulation via its PH domain. Indeed, the translocation of individual IRS PH domains has been reported in NIH-3T3 fibroblasts (4). However, other groups have reported translocation from intracellular membranes to the cytosol (16, 17) or even no change at all in the subcellular distribution of IRS-1 in either Chinese hamster ovary cells or adipocytes (20, 21).

**Interaction of IRS-1 with Other Proteins**—The interaction of IRS-1 with other proteins provides a regulatory mechanism for both the subcellular localization and activity of IRS-1. IRS-1 can associate with other proteins via its PH and PTB domains in addition to its multiple Ser/Thr and Tyr phosphorylation sites. PH domains generally interact with phosphoinositides (12) and thus target proteins to the plasma membrane (8–11). PTB domains specifically bind to tyrosine-phosphorylated proteins at NXPY motifs. This interaction is required for the binding of IRS molecules to the IR following insulin stimulation (1). In addition to binding to phosphotyrosine residues, the PTB domain of IRS-1 reportedly binds specifically to phosphoinositides (7). Consistent with this finding, the PTB domain of SHC binds to phospholipids and associates with the plasma membrane (22). Thus, both the PH and PTB domains of IRS-1 are potential plasma membrane targeting regions. Moreover, other proteins may serve as receptors regulating the subcellular localization of IRS-1. It has been reported that the p38α subunit of the AP-3 adaptor protein complex serves as a receptor dictating the localization of IRS-1 to low density membranes (23). Additionally, the cytosolic protein 14-3-3 down-regulates insulin signaling by sequestration of phosphoserine-containing IRS-1, thereby inhibiting its association with the IR (24). Thus, the subcellular localization of IRS-1 may be regulated by its interactions with multiple proteins.

3. L. Zhou, S. I. Taylor, and S. W. Cushman, unpublished results.
Translocation of IRS-1 to the Plasma Membrane—Our studies were designed to investigate the subcellular localization of IRS-1 both before and after incubation with insulin. To this end, we utilized both GFP fusion proteins and immunofluorescence microscopy. The use of GFP fusions is advantageous because detection is independent of antibody specificity. However, in the case of the full-length IRS-1-GFP fusion protein, the presence of the GFP domain resulted in non-physiological targeting to inclusion bodies. We mapped plasma membrane targeting regions by studying both isolated PH and PTB domains and by mutational analysis of phosphoinositide-binding sites within these domains. Although isolated domains provide an opportunity to study a specific region, this reductionist approach ignores complex interactions that may occur between distinct domains in the full-length IRS-1 protein. Furthermore, it is possible that a targeting motif might be composed by noncontiguous amino acid residues dispersed throughout the full-length molecule. Thus, our studies were complemented by mutational analysis in the full-length molecule.

Recombinant IRS-1 localized to the cytoplasm in the unstimulated state, and insulin stimulation promoted its translocation to the plasma membrane. Although the translocation of IRS PH domains has been reported (4), to our knowledge, this is the first study in which the translocation of the full-length IRS-1 molecule was detected using immunofluorescence. Interestingly, mutation of phosphoinositide-binding sites in either the PH or PTB domain significantly reduced IRS-1 translocation to plasma membrane ruffles. We observed that the mutation reduced the translocation index by 75%, which is comparable in magnitude to other publications. For example, the R28C mutation was reported to decrease either translocation of the PH domain or particulate association of the IRS-1 N terminus (4, 25). The R28C mutation had a more dramatic effect on plasma membrane targeting than the 3KQ mutation, thereby suggesting that the PH domain may have a more significant role than the PTB domain. However, we cannot rule out the possibility that the PTB domain contains additional phosphoinositide-binding sites.

In our studies, a fusion protein containing GFP and the PH domain of IRS-1 was targeted to the plasma membrane even in the absence of insulin stimulation. Furthermore, a small, but statistically insignificant increase in plasma membrane association was observed following insulin stimulation. The IRS-1 PH domain likely interacts with the plasma membrane in unstimulated cells primarily via binding to phosphatidylinositol 4,5-bisphosphate, which is the most abundant phosphoinositide and is constitutively present in the membrane bilayer (4, 12). However, additional binding of the IRS-1 PH domain to the plasma membrane in the insulin-stimulated state likely occurs as a consequence of an increase in the local concentration of phosphatidylinositol 3,4,5-trisphosphate. Our data conflict with earlier findings that the PH domain fused to GFP was cytoplasmic and translocated to the plasma membrane only following insulin stimulation (4). However, these differences may result from the use of different cell types (NIH-3T3 versus COS-7) or different boundaries for the PH domain (residues 3–134 versus 1–113). We confirmed the plasma membrane targeting ability of the PH domain by mutation of the phosphoinositide-binding site Arg28 to Cys. This mutation significantly reduced the plasma membrane targeting of the PH domain in both the basal and insulin-stimulated states.

We did not detect plasma membrane targeting of a GFP fusion protein containing the isolated PTB domain. Similarly, Dhe-Paganon et al. (12) were unable to demonstrate binding of the IRS-1 PTB domain alone to phosphoinositides. However, mutation of lysines 169, 171, and 177 to glutamine in the PTB domain of either an N-terminal fragment of IRS-1 or the full-length molecule significantly reduced plasma membrane targeting. Since the PTB domain is normally found in the middle of IRS-1, it may misfold when expressed as an isolated fragment lacking the PH domain. Indeed, in previous studies using the yeast two-hybrid system, the isolated PTB domain of IRS-3 bound only weakly to the IR, but this binding was significantly enhanced by the presence of either the PH domain or the C terminus (26). Additionally, in binding assays, PH + PTB domains bound more strongly than the PH domain alone, indicating that the PTB domain may facilitate the binding of the PH domain to phosphoinositides (12). Indeed, crystallographic studies have found the PH and PTB domains to be closely associated, suggesting that they function cooperatively (12).

Although the full-length IRS-1 molecule translocates only in response to insulin, the IRS-1 N terminus is sufficient to target GFP to the plasma membrane even in the absence of insulin stimulation. Therefore, our data suggest that the C terminus of IRS-1 inhibits its plasma membrane targeting ability in the absence of insulin. Insulin stimulation appears to decrease this inhibition, allowing the translocation of IRS-1 to the plasma membrane.

Significance of Plasma Membrane Targeting of IRS-1 in Insulin Signaling—The IRS-1 targeting-deficient mutants were only slightly impaired in their ability to be tyrosine-phosphorylated in response to insulin. Although there was no statistical difference between the wild-type and mutant IRS-1 molecules, the R28C mutation in the PH domain had the smallest inhibitory effect on tyrosine phosphorylation. This differs from previous studies in which deletion of the PH domain nearly abolished the ability of IRS-1 to be tyrosine-phosphorylated in response to insulin (27–29). Interestingly, when IRS-1APH was expressed in 32DIR cells as opposed to 32D cells, its ability to be tyrosine-phosphorylated was not reduced (29). However, overexpression of the IR in COS-7 cells did not interfere with our ability to detect differences between wild-type and mutant IRS-1 molecules. Furthermore, deletion of the PH domain would be expected to have more severe consequences than point mutations particularly on protein stability.

We have conducted experiments in which there was significant impairment of IRS-1 translocation without detectable impairment of IRS-1 tyrosine phosphorylation. During the preparation of this manuscript, similar results were presented by Vainshtein et al. (25), who studied the same mutation. Although the phosphotyrosine content of IRS-1 was not directly measured, the R28C mutation caused disruption of IRS-1 subcellular localization without blocking activation of PI3K. Nevertheless, they demonstrated the ability of the R28C mutation to impair Akt activity (25). Taken together, these data suggest that the role of subcellular localization may be more relevant to events downstream of PI3K.

On the other hand, Goalstone et al. (30) presented data demonstrating that the overexpression of an isolated IRS-1 PH domain appears to inhibit IRS-1 tyrosine phosphorylation. The simplest explanation for this is that IRS-1 PH domain overexpression blocked IRS-1 targeting and subsequent phosphorylation. However, it may also block an intramolecular interaction between the IRS-1 PH and PTB domains that is necessary for IRS-1 targeting. Additionally, it may also interfere with the subcellular localization of other PH domain-containing proteins, which may be required for IRS-1 tyrosine phosphorylation. Thus, there are a number of different mechanisms by which overexpression of the PH domain could block IRS-1 phosphorylation.

Since our mutations did not completely abolish IRS-1 translocation, we cannot conclude from our data whether IRS-1
tyrosine phosphorylation occurs prior to translocation. However, if this were the case, then IRS-1 phosphorylation would be unaffected by impaired plasma membrane targeting, even if downstream signaling might be impaired. It remains possible that the interaction between IRS-1 and the IR may also occur at intracellular sites other than the plasma membrane. In support of this interpretation, Clark et al. (18) demonstrated the majority of tyrosine-phosphorylated IRS-1 to be associated with the cytoskeleton rather than the cytosol or plasma membrane. This suggests that the functional pool of IRS-1 is not entirely associated with the plasma membrane. Thus, when Kriauciunas et al. (31) forced IRS-1 to the plasma membrane using a CAAX motif, its tyrosine phosphorylation was slightly decreased. Surprisingly, PI3K binding and activation by IRS-1-CAAX were decreased by 75% compared with wild-type IRS-1 (31). This suggests that the proper subcellular distribution of IRS-1 is important for insulin signaling.

In this study, we have demonstrated that the majority of IRS-1 is found in the cytoplasm, likely associated with the cytoskeleton, as suggested by Clark et al. (18). However, there exists a pool of IRS-1 molecules that is targeted to the plasma membrane in response to insulin. By mutational analysis, we have implicated both the PH and PTB domains in this insulin-stimulated translocation of IRS-1. Although our mutations in IRS-1 significantly impaired translocation, we were unable to detect a corresponding impairment of tyrosine phosphorylation. However, we cannot rule out an effect on subsequent downstream signaling.

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