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ARTICLES

Disruption of a Putative SH3 Domain and the Proline-Rich Motifs in the 53-kDa Substrate of the Insulin Receptor Kinase Does Not Alter Its Subcellular Localization or Ability to Serve as a Substrate

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Abstract The recently identified 53-kDa substrate of the insulin receptor family was further characterized in several retroviral-generated stable cell lines overexpressing the wild type and various mutant forms of the protein. To facilitate the study of its subcellular localization in NIH3T3 cells overexpressing insulin receptor, a myc epitope-tag was added to the carboxy terminus of the 53-kDa protein. Like the endogenous protein in Chinese hamster ovary cells, the expressed myc-tagged 53-kDa protein was found partially in the particulate fraction and was tyrosine phosphorylated in insulin-stimulated cells. Immunofluorescence studies showed for the first time that a fraction of the 53-kDa protein was localized to the plasma membrane. Confocal microscopy of cells double-labeled with antibodies to the insulin receptor and the myc epitope showed the two proteins co-localize at the plasma membrane at the level of light microscopy. Further analyses of the protein sequence of the 53-kDa substrate revealed the presence of a putative SH3 domain and two proline-rich regions, putative binding sites for SH3 and WW domains. Disruption of these three motifs by the introduction of previously characterized point mutations did not affect the membrane localization of the 53-kDa protein, its ability to serve as substrate of the insulin receptor, or its colocalization with the insulin receptor, suggesting these domains are not important in the subcellular targeting of the protein and instead may function in the interaction with subsequent signaling proteins. J. Cell. Biochem. 68:139–150, 1998.

Key words: tyrosine phosphorylation; insulin signaling; tyrosine kinase; confocal microscopy

The insulin receptor (IR) tyrosine kinase is activated by the binding of insulin to its extracellular domain. The phosphorylation of cytosolic substrates such as IRS-1 (Insulin Receptor Substrate 1), IRS-2, and SHC results in the formation of signaling complexes via the binding of Src Homology 2 (SH2) domain-containing proteins to these tyrosine phosphorylated molecules [Draznin, 1996]. In addition, the autophosphorylated IR has also been shown to be tightly bound by other SH2 domain-containing proteins such as the Grb-IR/Grb 10 family of proteins [Liu and Roth, 1995; Dey et al., 1996; Hansen et al., 1996; Morrione et al., 1996; O'Neill et al., 1996]. Another set of substrates of the IR tyrosine kinase includes a number of distinct 60-kDa proteins, including one which binds to the phosphatidylinositol 3-kinase [Sung and Goldfine, 1992; Lavan and Lienhard, 1993; Milarski et al., 1995], another which binds to the GTPase activating protein of Ras [Hosomi et al., 1994], and a third that did not appear to be bound by any tested SH2 domain-containing protein [Yeh et al., 1996].

The substrate of the IR whose binding partners have not been identified appears as a 58- and 53-kDa doublet in Chinese hamster ovary cells [Yeh et al., 1996], possibly due to alternative splicing. Recently, a full-length cDNA was isolated which encodes for the 53-kDa substrate [Yeh et al., 1996], henceforth called

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IRSp53 for Insulin Receptor Substrate Protein of 53 kDa. IRSp53 is tyrosine phosphorylated by various members of the IR family including the insulin-like growth factor I receptor and the insulin receptor-related receptor [Yeh et al., 1996]. Like other IR substrates [Draznin, 1996], its tyrosine phosphorylation requires the presence of the juxtamembrane tyrosine in IR [Danielsen and Roth, 1996]. Most intriguing, unlike other endogenous substrates of the IR kinase, IRSp53 is partially found in the particulate fraction of cells [Yeh et al., 1996].

Three separate types of sequence analyses indicate that IRSp53 contains a putative Src homology 3 (SH3) domain. First of all, a standard BLAST sequence search [Altschul et al., 1990] with the entire sequence showed that the sequence with the highest homology to IRSp53 was that of the SH3 domain of a yeast protein called BOB1. The corresponding IRSp53 sequence was then used as the query sequence in a second BLAST search, resulting in the identification of many other SH3 domains including that of BOB1, as expected, and of other proteins such as Vav and Crk. Finally, a search with the entire cDNA against the PROSITE motif database [Bairoch et al., 1997] confirmed the presence of a putative SH3 domain consisting of residues 375–438.

Since the SH3 domain has been shown to be responsible for the targeting of several proteins to the cytoskeleton [Bar-Sagi et al., 1993], its presence in IRSp53 is consistent with the presence of this molecule in the particulate fraction. In this study, we have further characterized the subcellular localization of IRSp53 and analyzed the role of the SH3 domain as well as two putative SH3 and WW binding motifs in the subcellular targeting of IRSp53 as well as its ability to serve as a substrate of the IR.

METHODS

Construction of myc-Tagged IRSp53 and Mutants

A 1.8-kB BamHI fragment, containing the complete open reading frame and 200 bp of the 3′-UTR of IRSp53, was subcloned from the full-length cDNA [Yeh et al., 1996] into the BamHI site in the retroviral pWZL-hygro vector [Pear et al., 1993]. The myc tag was added by the substitution of a BstXI-BglII fragment with a shorter fragment containing 3′ coding sequence and the myc epitope EQKLISEEDL immediately before the stop codon (see Fig. 4). This fragment was obtained by PCR of the pWZL-IRSp53 plasmid using a 5′ internal IRSp53 primer and a 3′ primer consisting of IRSp53 and myc sequences.

Mutants were made by replacing a 370-bp XcmI-BstXI fragment (PxxP) or a 440-bp BstXI-BglII fragment (SH3-W, SH3-P, PPxY) with a similar fragment containing the appropriate point mutations (see Fig. 4). Mutagenized fragments were generated using a PCR-based strategy. Briefly, two initial PCR reactions were performed using pWZL/IRSp53-myc as template. One set of primers consisted of a 5′ flanking primer and a 3′-internal mutagenesis primer; the other set consisted of a 5′-internal mutagenesis primer and a flanking 3′ primer. Both PCR products were combined in a final PCR reaction with only the flanking 5′ and 3′ primers. This amplified fragment was then subcloned into the pWZL/IRSp53-myc plasmid and sequenced to confirm the point mutations and integrity of the remaining region.

Generation of Stable Cell Lines Using Retrovirus

The packaging cell line (Phoenix) and retroviral vector (pWZL-hygromycin resistance) were kindly provided by Dr. Garry Nolan [Pear et al., 1993]. Phoenix cells were plated at 4×10^6 cells per 60-mm plate. On the following day, they were transfected with the appropriate retroviral vector by the calcium phosphate method. The media was changed 8 h after transfection and, again, 24 h after transfection, at which time they were transferred to a 32°C/5% CO_2 incubator for 2 days in order to optimize retroviral titer. The supernatant, which contains the retroviral particles, was then cleared by centrifugation and added to NIH3T3/IR cells that had been plated at 7.5×10^4 cells/6-well the night before. Polybrene was added to the cells at a final concentration of 5 μg/ml. The 6-well plates were then spun at 2,500 rpm for 90 min in a tabletop centrifuge to increase infection efficiency. After an 18-h incubation in a 32°C incubator, the media was changed and the cells were transferred to a 37°C incubator. Selection with hygromycin (200 μg/ml) began the following day. The drug-resistant total cell population was used in all experiments. NIH3T3/IR (NIH3T3 cells overexpressing IR) were also generated by the retroviral method and selected by resistance to neomycin.

Detection of Proteins by Western Blotting

Cells were treated with insulin as described, and lysed in lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, pH 7.6, 5 mM EGTA, pH 7.6, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mg/ml bacitracin). H720 (anti-p58/p53), 9E10 (anti-myc, Babco, Berkeley, CA), and mouse IgG immunoprecipitates of the cell lysates were Western blotted with either RC20 (anti-phosphotyrosine, 1/5,000 dilution, Transduction Laboratories) or 9E10 (1/1,000 dilution, Babco). H720 was coupled to Affigel-10 agarose beads (Bio-rad, Richmond, CA) while 9E10 and MIgG were bound to Protein G-Sepharose beads (Pharmacia, Piscataway, NJ). Proteins were detected by incubation with a secondary antibody conjugated to alkaline phosphatase or horseradish peroxidase, followed by the appropriate substrates.

Subcellular Fractionation

Cells were washed once with PBS and lysed in Buffer 1 (20 mM HEPES, pH 7.5, 1 mM PMSF, 10 μg/ml aprotinin, 20 mM β-glycerolphosphate) using a glass Teflon homogenizer. The cell lysates were then centrifuged at 3,000 rpm (900*g*) in a microcentrifuge to pellet unlysed cells and nuclei. The supernantant was then transferred to an airfuge or an ultracentrifuge tube and spun at 100,000*g* for 1 h at 4°C. The resulting supernatant, designated the cytosolic fraction, was incubated overnight at 4°C with H720 (anti-p58/p53) coupled to agarose beads or 9E10 (anti-myc) antibody bound to sepharose beads. The pellet was resuspended in Buffer 2 (Buffer 1 containing 1% Triton X-100, 150 mM NaCl), placed on ice for 20 min, and spun at 100,000*g* for 1 h at 4°C. This supernatant, designated the particulate fraction, was also incubated overnight at 4°C with H720-agarose or 9E10-sepharose beads. The Triton-insoluble pellet was resuspended in SDS sample buffer, boiled, and analyzed, along with the immunoprecipitates, by Western blotting with 9E10 or anti-phosphotyrosine antibodies.

Immunofluorescence

Cells were plated on glass coverslips 1–2 days before immunolabeling. The coverslips were rinsed quickly in PBS and fixed in 1.5% formaldehyde/PBS for 20 min. After another rinse in PBS, the coverslips were treated with 50 mM NH_4Cl/PBS for 10 min. After washing in PBS, the cells were permeabilized in block solution (PBS with 0.2% gelatin, 0.2% Triton X-100, 0.1% BSA, 20% calf serum) for 30 min. The coverslips were then incubated with a purified 9E10 anti-myc antibody, (1/200 dilution, a gift of Dr. Carsten Möller, Stanford University) or affinity-purified polyclonal anti-IR (1/10 dilution). After a 90-min incubation with the primary antibody, the coverslips were washed 2×10 min in blocking solution and in PBS followed by an incubation with rhodamine-conjugated anti-mouse IgG (1/100 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC-conjugated anti-rabbit IgG (1/500 dilution, Cappel, Malvern, PA) for 1 h in the dark. After washing, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed with a Leitz Aristoplan fluorescence microscope using single or dual filters, and with a confocal microscope (Molecular Dynamics, Sunnyvale, CA). Double labeling was achieved by incubating coverslips in both primary and both secondary antibodies.

RESULTS

Generation of Stable Cell Lines Overexpressing IRSp53

To further characterize the IR substrate IRSp53, stable cell lines expressing the recombinant protein were developed. In order to differentiate between expressed IRSp53 and endogenous IRSp53, a myc-tagged IRSp53 was constructed using a PCR-based strategy to attach a 10-amino-acid epitope to the carboxy terminus of the protein. This expressed protein could then be detected by Western blot analysis using anti-myc antibodies, another advantage since the anti-p58/p53 (H720) antibody [Yeh et al., 1996] works poorly on Westerns. IRSp53 was expressed in mouse NIH3T3 cells overexpressing IR (NIH3T3/IR) using a retroviral system [Pear et al., 1993].

NIH3T3/IR cells infected with retroviruses pWZL, pWZL/IRSp53, or pWZL/IRSp53-myc were screened for the expression of IRSp53. Lysates from untreated and insulin-treated cells were incubated with either monoclonal anti-p58/p53 antibody (H720) coupled to agarose beads or monoclonal anti-myc antibody (9E10) coupled to sepharose beads. Immunoprecipitates were then Western blotted with anti-myc or anti-phosphotyrosine antibodies. As expected,

IRSp53-myc was immunoprecipitated by both H720 and 9E10, the latter being more efficient, while IRSp53 was immunoprecipitated with H720 (Fig. 1A and B). More important was the finding that IRSp53-myc, like IRSp53, was tyrosine phosphorylated in response to insulin (Fig. 1B). This observation is consistent with IRSp53 being a substrate of the IR kinase and demonstrates that the presence of the myc-epitope does not interfere in the interaction of the IR and this substrate. H720 immunoprecipitates from control NIH3T3/IR/pWZL cells showed no tyrosine phosphorylated IRSp53 (Fig. 1B), confirming the low expression of endogenous IRSp53 in these cells.

Subcellular Localization of IRSp53

Previous subcellular fractionation studies with metabolically labeled CHO.T (Chinese hamster ovary cells overexpressing IR) cells have shown that endogenous IRSp53 is mainly found in the particulate fraction [Yeh et al., 1996] suggesting that the protein is in close association with membranes or cytoskeletal el-

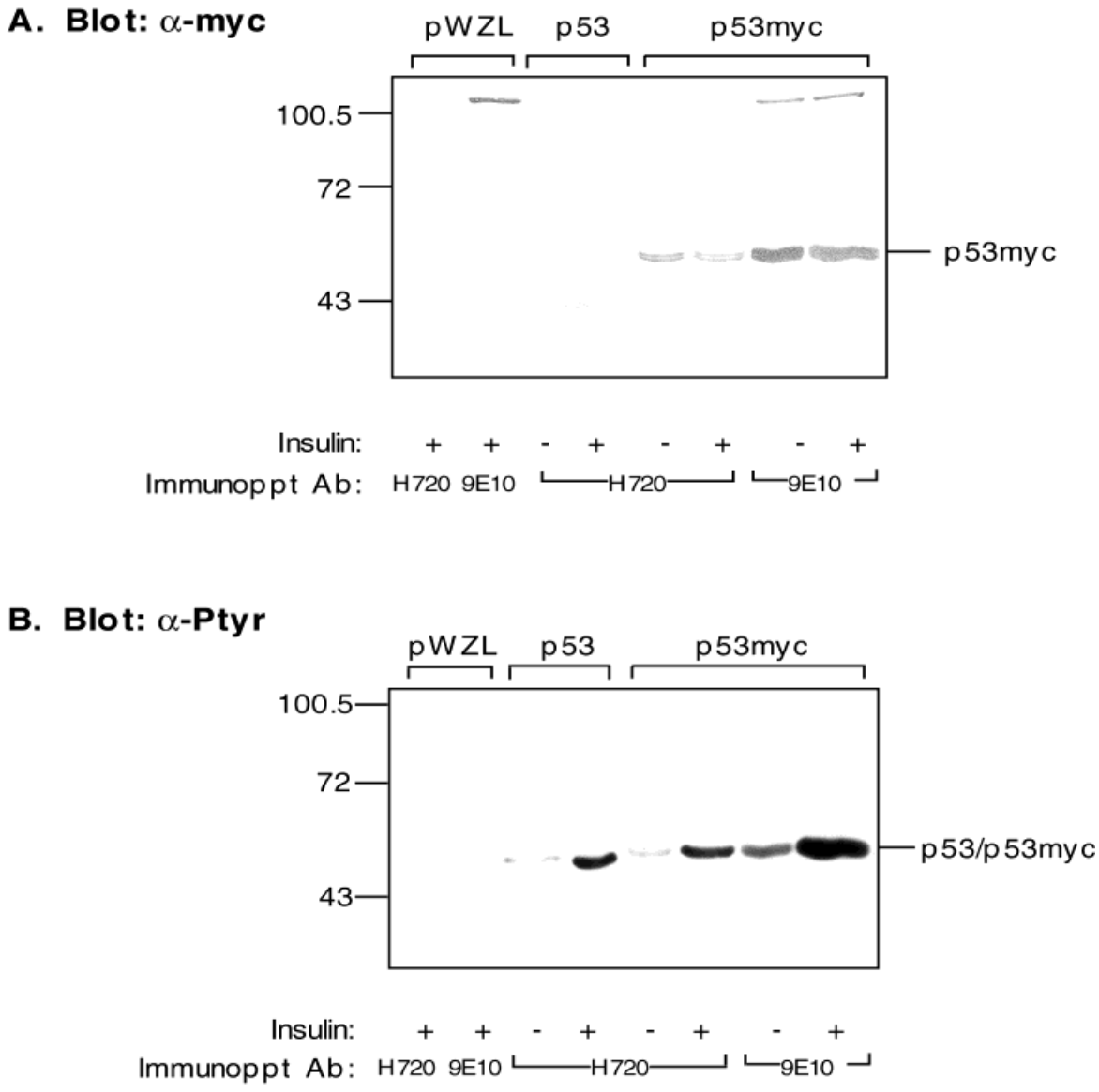

Fig. 1. Stable overexpression and tyrosine phosphorylation of IRSp53. NIH3T3/IR cells infected with the parental retrovirus pWZL (pWZL) or retrovirus encoding IRSp53 (p53) or IRSp53-myc (p53myc) were serum-starved for 5 h and treated with 1 μM insulin for 10 min as indicated. H720 (anti-p58/p53) and 9E10 (anti-myc) immunoprecipitates of cell lysates were analyzed by Western blotting with anti-myc antibody (9E10) **(A)** or anti-phosphotyrosine antibody **(B).** The position of standard marker proteins (in kDa) and the IRSp53 protein are shown.

ements. In addition, this localization was not altered upon insulin treatment. To determine the subcellular localization of overexpressed IRSp53, NIH3T3/IR/p53 and NIH3T3/IR/p53-myc cells were treated with or without insulin, lysed, and fractionated into cytosolic and particulate fractions. Anti-myc immunoprecipitates of these fractions were analyzed by Western blotting with anti-phosphotyrosine and anti-myc antibodies. The overexpressed IRSp53 and IRSp53-myc from both untreated and insulin-treated cells were found partially in the particulate fraction, in agreement with prior studies of the endogenous protein (Fig. 2A). The tyrosine phosphorylated form of the protein was also found in the same fraction (Fig. 2B). Another advantage of using the myc-tagged IRSp53 was the ability to analyze the presence of IRSp53 in the Triton-insoluble pellets, which were directly boiled in SDS sample buffer. Interestingly, there was still a significant amount of IRSp53-myc in this fraction (see Fig. 6A), fur-

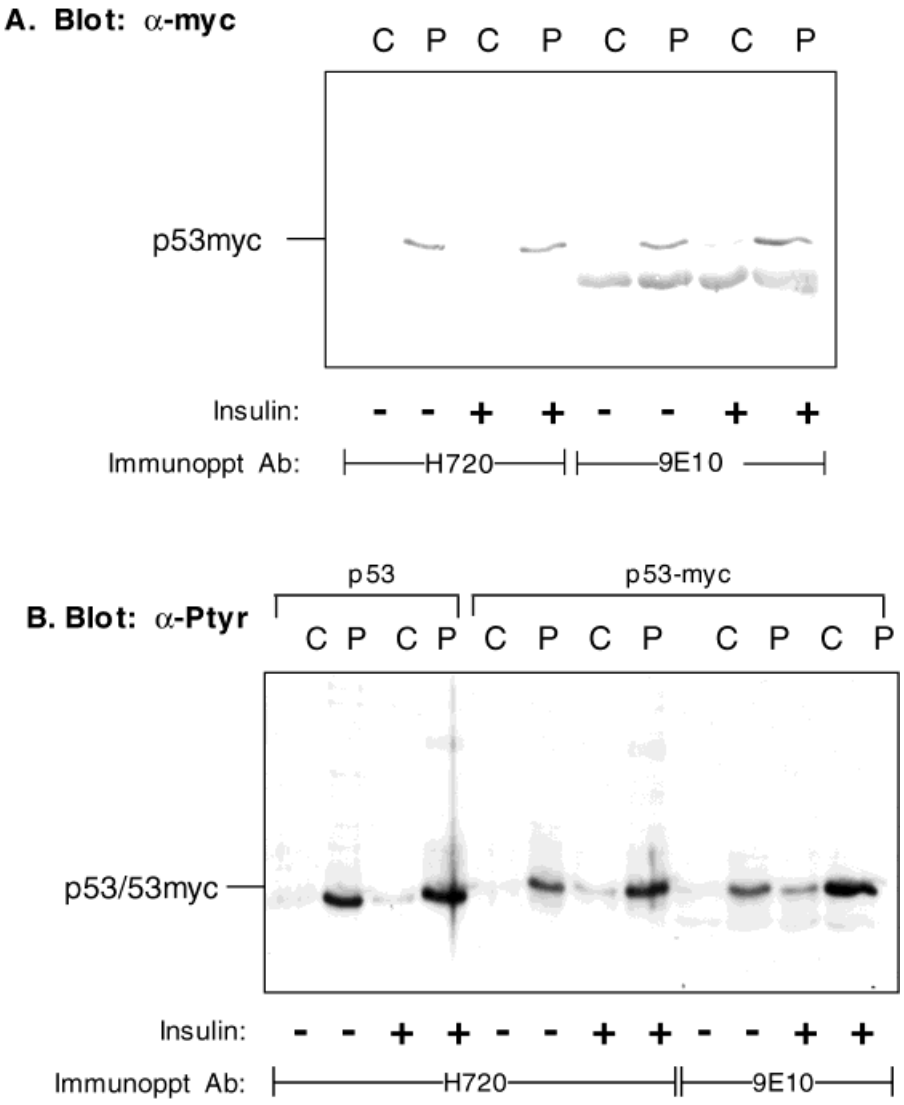

Fig. 2. Subcellular localization of IRSp53. NIH3T3/IR cells overexpressing IRSp53-myc were serum-starved for 1 h 30 min and treated with 1 μM insulin as indicated. The cells were lysed by homogenization and centrifuged at 100,000*g* for 1 h. The supernatant was designated as the cytosolic fraction (C). The pellet was resuspended in a buffer containing 1% Triton and 150 mM NaCl and centrifuged at 100,000*g* for 1 h. This second supernatant was designated as the Triton-soluble particulate fraction (P). H720 (anti-p58/p53) and 9E10 (anti-myc) immunoprecipitates of both cytosolic and particulate fractions were analyzed by Western blotting with anti-myc antibody (9E10) **(A)** or anti-phosphotyrosine antibody **(B).** H720 (anti-p58/53 antibody) immunoprecipitates from cells overexpressing IRSp53 are also included in the blot shown in B. The 50-kDa bands in the 9E10 immunoprecipitates shown in A are the immunoglobulin heavy chain bands.

ther suggesting a partial cytoskeletal localization.

Immunofluorescence studies were carried out to determine the subcellular localization in NIH3T3/IR/IRSp53-myc cells using the 9E10 antibody followed by a rhodamine-conjugated anti-mouse IgG antibody. The resulting immunofluorescence was consistent with a partial localization to the plasma membrane, as well as the cytosol. This was best seen using confocal microscopy techniques as shown in Figure 3A. The immunolabeling observed was specific for the IRSp53-myc protein since control NIH3T3/IR/pWZL cells showed low background staining (data not shown).

Since IRSp53 is partially localized to the plasma membrane and is a substrate of the IR, NIH3T3/IR/IRSp53-myc cells were double-labeled with 9E10 and a polyclonal antibody against the IR β subunit to determine if the two proteins colocalized. Figure 3 shows a confocal section of the same cell. The IRSp53-myc protein was visualized with a rhodamine-conjugated anti-mouse IgG antibody (Fig. 3A), while the IR was visualized with a FITC-conjugated anti-rabbit IgG antibody (Fig. 3B). There was no FITC-immunofluorescence at the plasma membrane in parental NIH3T3 cells or with only the secondary antibody (data not shown), confirming that the plasma membrane FITC staining seen in NIH3T3/IR/IRSp53-myc cells was specific for IR. Colocalization of IRSp53 and IR appears as a yellow staining of the plasma membrane (Fig. 3C).

Generation of IRSp53 Mutants

Sequence analyses of IRSp53 revealed the presence of several potential domains that have been proposed to be involved in protein-protein interactions. These include a putative SH3 domain, a putative SH3-binding domain, and a putative WW-binding domain. To determine whether one of these domains was responsible for the subcellular localization of the protein, we generated and characterized four IRSp53 mutants that carried point mutations that should, based on prior studies, lead to the disruption of each domain (Fig. 4). In the SH3-W mutant, tryptophan 414 was changed to alanine. In the SH3-P mutant, proline 429 was changed to leucine. The PxxP motif was changed to AxxA. Likewise, the PPxY motif was changed to PAxA.

All four IRSp53 mutants were created by introducing point mutations in the PWZL/IRSp53-myc construct using a PCR-based strategy. The point mutations were confirmed by sequencing the region of amplification in each construct. Stable NIH3T3/IR cells overexpressing wild type and mutated IRSp53 were then generated using the retroviral method. To determine the expression and insulin-stimulated tyrosine phosphorylation of the IRSp53 mutants, cells were serum-starved, treated with insulin as indicated, and lysed. H720 or 9E10 immunoprecipitates of cell lysates were Western blotted with anti-phosphotyrosine antibodies followed by anti-myc antibodies. All four IRSp53 mu-

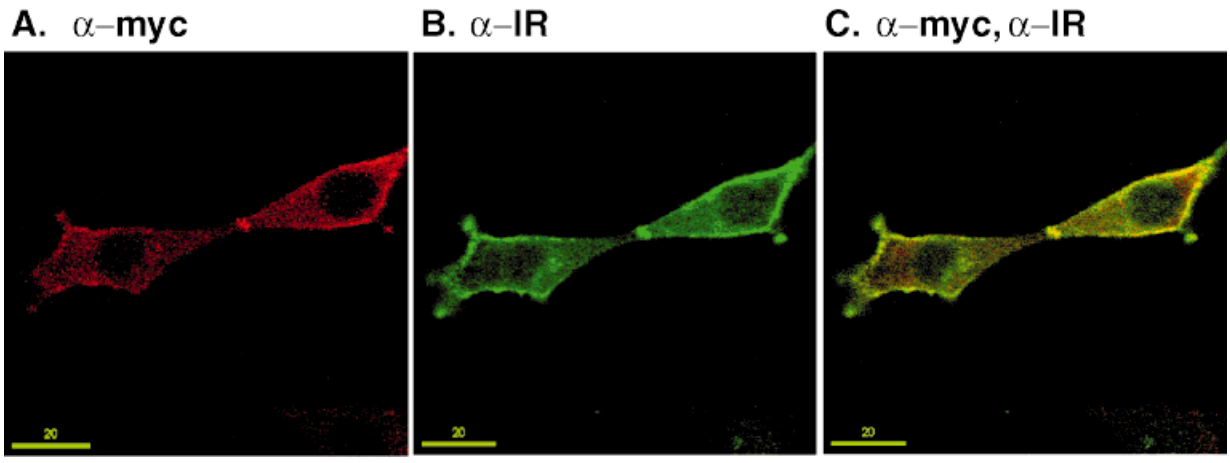

Fig. 3. Colocalization of IRSp53 and IR as analyzed by confocal microscopy. NIH3T3/IR cells overexpressing IRSp53-myc were incubated with purified 9E10 (anti-myc) and affinity-purified polyclonal anti-IR (β subunit) antibodies. The IRSp53-myc protein was visualized with a rhodamine-conjugated anti-mouse IgG antibody **(A).** The insulin receptor was visualized with a FITC-conjugated anti-rabbit IgG antibody **(B)**. **C:** Superimposition of images A and B, showing the colocalization of IRSp53 and IR at the plasma membrane, which appears in yellow.

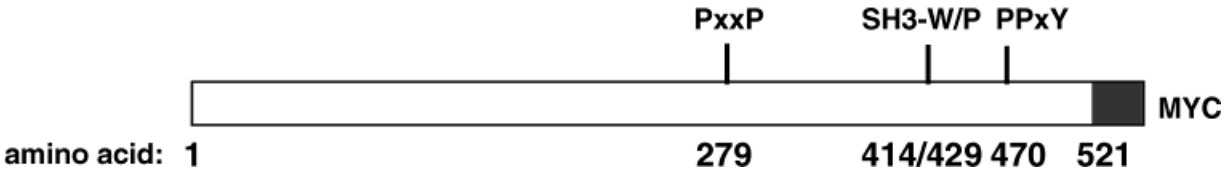

Fig. 4. Location of point mutations in IRSp53 mutants. Four separate IRSp53 mutants were generated, each containing point mutations in the SH3 domain (SH3-W changed to SH3-A, SH3-P changed to SH3-L), the proline-rich SH3-binding motif (PxxP changed to AxxA), or the proline-rich WW-binding motif (PPxY changed to PAxA).

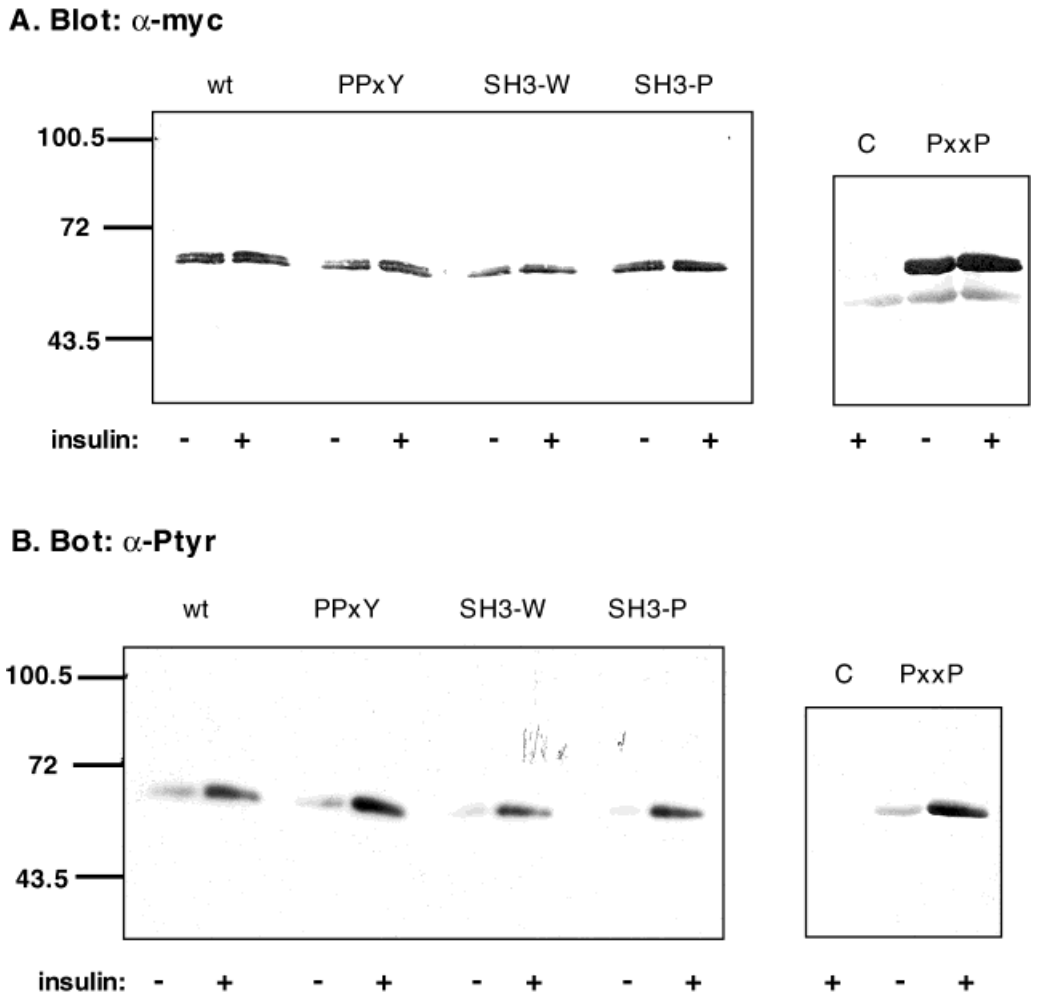

Fig. 5. Stable expression and tyrosine phosphorylation of IRSp53 mutants. Control NIH3T3/IR/pWZL cells (C) and cells overexpressing IRSp53-myc (wt) or IRSp53-myc with mutations in the WW-binding domain (PPxY), SH3 domain (SH3-W, SH3-P), or the SH3-binding domain (PxxP), were serum-starved for 2 h 30 min and treated with 1 μM insulin for 10 min as indicated. H720 (anti-p58/p53) immunoprecipitates (wt, PPxY, SH3-W, SH3-P) and 9E10 (anti-myc) immunoprecipitates (C, PxxP) were analyzed by Western blotting with anti-myc antibody (9E10) **(A)** or anti-phosphotyrosine antibody **(B).**

tants were readily expressed and recognized by both H720 and 9E10 (Fig. 5A). In addition, insulin treatment stimulated the tyrosine phosphorylation of all four IRSp53 mutants (Fig. 5B), suggesting that the point mutations did not lead to severe disruptions in overall protein structure since the mutants could still be recognized by both antibodies as well as the IR kinase.

Subcellular Localization of the IRSp53 Mutants

NIH3T3/IR cells expressing wild type and mutant IRSp53 proteins were fractionated into cytosolic and particulate fractions which were

then incubated with anti-myc antibodies. The immunoprecipitated proteins, along with the remaining proteins in the Triton-insoluble pellet, were analyzed by Western blotting with anti-myc antibodies. All four mutant IRSp53 proteins, like the wild type molecule, were found partially in the particulate and the Triton-insoluble fractions, indicating a partial membrane or cytoskeletal association (Fig. 6A and B). There appeared to be no differences between the wild type and any of the four mutant proteins in terms of their subcellular distribution patterns. The addition of 2% octylglucoside in the fractionation procedure, which can lead to the solubilization of some cytoskeletal proteins [Sargiacomo et al., 1993], did not have any effect on protein distribution (Fig. 6A).

Immunofluorescence was also used to analyze the effects of these mutations on the subcellular localization of IRSp53. Cells expressing wild type and mutant IRSp53 proteins were double-labeled with anti-myc and anti-IR antibodies, as described above. The results of these experiments showed no detectable differences in the subcellular localization of the wild type protein and any of the four mutants. All five proteins were partially localized to the plasma membrane and partially to the cytosol (data not

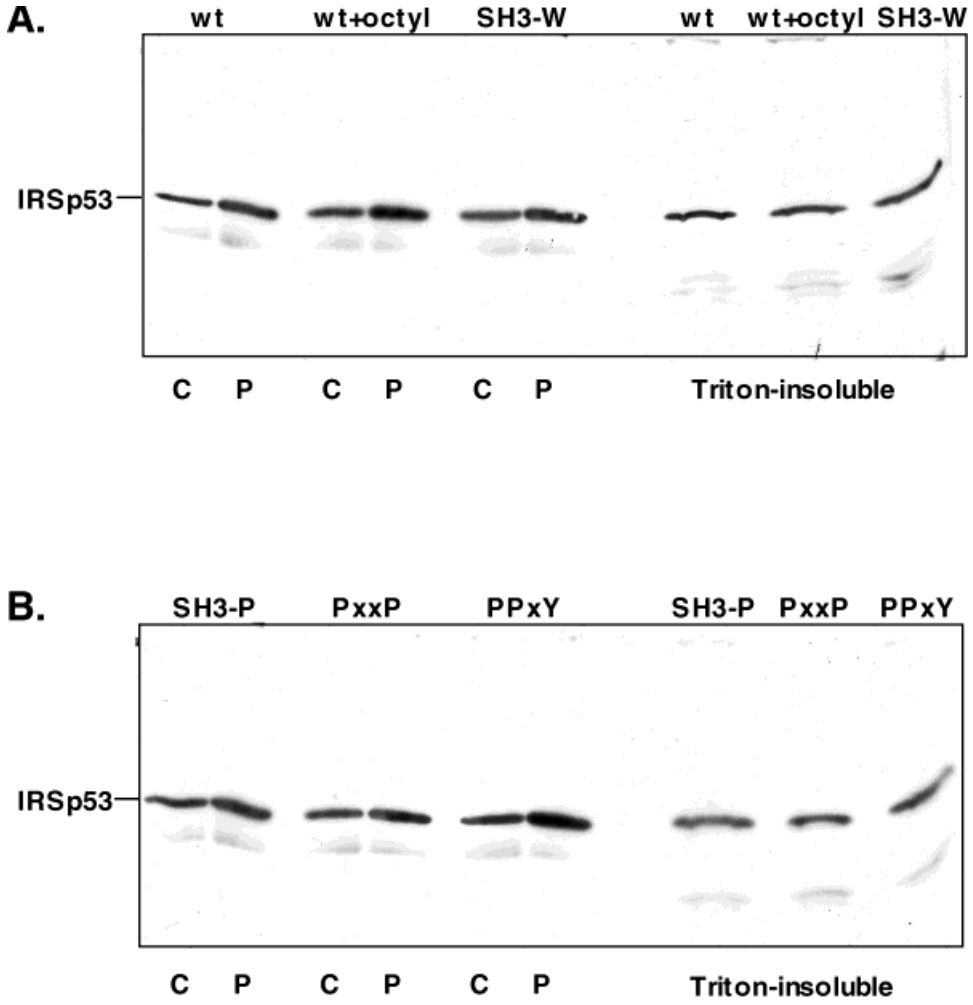

Fig. 6. Subcellular localization of IRSp53 mutants. NIH3T3/IR cells overexpressing wild type IRSp53 (wt) or IRSp53 with mutations in the SH3 domain (SH3-W, SH3-P), SH3-binding domain (PxxP), or the WW-binding domain (PPxY), were lysed in a non-detergent, non-salt containing buffer and centrifuged at 100,000*g* for 1 h. The supernatant was designated as the cytosolic fraction (C). The pellet was resuspended in lysis buffer containing 1% Triton and 150 mM NaCl and centrifuged at 100,000*g* for 1 h. This second supernatant was designated as the Triton-soluble particulate fraction (P). 9E10 (anti-myc) immunoprecipitates of both cytosolic and particulate fractions were analyzed by Western blotting with 9E10. The remaining Triton-insoluble pellets were boiled in sample buffer and also analyzed by Western blotting with 9E10. In one experiment (wt + octyl), 2% octylglucoside was added to the lysis buffer containing Triton and NaCl.

shown). The colocalization of IRSp53-myc and IR at the plasma membrane, shown in yellow with the use of a dual filter, was clearly evident in cells expressing wild type (Fig. 7B) and the two SH3 mutants (Fig. 7C,D). Similar observations were seen in cells expressing the two proline-rich mutants (data not shown). Control NIH3T3/IR/pWZL cells, as expected, only stained green (i.e., positive for anti-IR antibody immunostaining) through the double filter due to the absence of any myc-tagged proteins (Fig. 7A).

DISCUSSION

In this study, we sought to further characterize the subcellular localization of IRSp53. To this end, we expressed an epitope-tagged version of the protein in NIH3T3 cells overexpressing IR. This cell line was selected for these subcellular localization studies because of their flattened phenotype, which is optimal for immunofluorescence analysis. Also, the low expression of endogenous p58/p53 in these cells made it less likely that the overexpression of IRSp53 would saturate the system, leading to changes in localization. In addition, since these cells have a functional IR tyrosine kinase, we could also look at the localization of the tyrosine-phosphorylated form of IRSp53. Finally, since the infection efficiency is high (70–90%) in these cells, the entire population of stably transfected cells could be used in experiments, allowing us to avoid clonal artifacts.

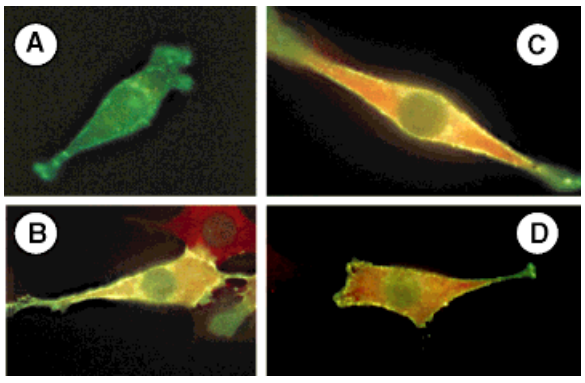

Fig. 7. Disruption of the SH3 domain in IRSp53 does not alter its colocalization with IR. Control NIH3T3/IR/pWZL cells **(A)** and cells overexpressing wildtype IRSp53 **(B)** or IRSp53 mutants SH3-P **(C)** and SH3-W **(D)** were immunolabeled for both IRSp53-myc and IR. The distribution of wild type IRSp53-myc and the two SH3 mutants is the same; a fraction of all forms of IRSp53 colocalizes with the IR at the plasma membrane as indicated in yellow. In control cells, IRSp53 is not detectable whereas IR can be clearly seen at the cell surface, shown in green.

Western analysis of H720 and 9E10 immunoprecipitates of NIH3T3/IR/IRSp53-myc cells using anti-myc or anti-phosphotyrosine antibodies showed that the addition of the myc tag did not affect expression of the protein, its ability to be recognized by the H720 antibody, or its ability to be tyrosine phosphorylated after insulin stimulation (Fig. 1). When the same cells were subjected to subcellular fractionation, IRSp53 protein was found partly in the particulate fraction, suggesting a partial membrane or cytoskeletal association (Fig. 2). There was no change in protein distribution due to the insulin-stimulated tyrosine phosphorylation of IRSp53. The fact that these observations are consistent with previous studies of the endogenous IRSp53 [Yeh et al., 1996] suggests that overexpression of IRSp53 as well as the addition of the myc-epitope did not alter the subcellular localization of IRSp53. In addition, since the protein was epitope-tagged, we were able to detect a significant amount of IRSp53 in the Triton-insoluble pellet, further implicating its association with the cytoskeleton (Fig. 6).

Immunofluorescence analysis of NIH3T3/IR/IRSp53-myc cells immunolabeled with 9E10 revealed that the IRSp53 protein was localized to the plasma membrane as well as to the cytosol, an observation that was consistent with the subcellular fractionation studies. The partial plasma membrane localization was confirmed by confocal microscopy (Fig. 3). Furthermore, when the same cells were double-labeled with 9E10 and anti-IR antibodies, the two proteins were shown to colocalize at the plasma membrane, at least at the level of light microscopy. This observation is again consistent with the role of IRSp53 as a direct substrate of the IR.

Since the immunofluorescence patterns were partially punctate and around the plasma membrane, we speculated that perhaps IRSp53 was localized to the caveolae, a membrane structure similar to clathrin-coated pits, which is believed to harbour signaling molecules [Parton, 1996]. However, studies involving anti-myc antibody blotting of sucrose density gradient fractions [Song et al., 1996] from these cells did not show colocalization of IRSp53 with caveolin, the main component of caveolae. Furthermore, the addition of 2% octylglucoside [Sargiacomo et al., 1993] to the lysis buffer did not release additional IRSp53 from the Triton-insoluble

fraction, an observation that could be seen with caveolin.

Since there is no signal peptide or any significant stretch of hydrophobic residues in IRSp53, it is likely that this protein is targeted to the vicinity of the plasma membrane by interacting with as yet unidentified proteins, which themselves are targeted to or near the plasma membrane. Furthermore, since IRSp53 is found partially in the particulate fraction, triton-soluble and triton-insoluble, this raises the possibility that perhaps IRSp53 is targeted through its interaction with the cytoskeleton near the plasma membrane. To this end, it is interesting to note that IRSp53 contains a putative SH3 domain as well as two proline-rich motifs that have been implicated in mediating protein-protein interactions, especially in the context of cytoskeleton [Musacchio et al., 1992; Bork and Sudol, 1994; Sudol et al., 1995]. Consequently, another goal of these studies was to determine the role of these domains in targeting IRSp53 to the particulate fraction, as well as to the plasma membrane region.

Since previous studies have shown that the SH3 domain is important for targeting PLC-γ to the actin cytoskeleton and Grb2 to the membrane ruffles [Bar-Sagi et al., 1993], it is possible that the putative SH3 domain in IRSp53 may be important for its subcellular targeting. To examine this possibility, the SH3 domain was disrupted by the introduction of two well-characterized point mutations, W414A and P429L (Fig. 4). The SH3 proline-to-leucine mutation was first identified during the characterization of several loss-of-function mutants in the *Caenorhabditis elegans* Grb2 counterpart, sem5 [Clark et al., 1992]. When the coresponding mutation was generated in the SH3 domain of Grb2, the protein localization shifted from the membrane ruffles to a more diffuse cytoplasmic distribution [Bar-Sagi et al., 1993]. Finally, the disruption of the SH3 domain in Src by proline-to-leucine or tryptophan-to-alanine point mutations, resulted in its loss-of-regulation by Csk as well as its loss of protein interactions with Sam68 and the p85 subunit of PI-3 kinase [Erpel et al., 1995].

In addition to the SH3 domain, further sequence analysis of IRSp53 revealed a potential SH3-binding proline-rich motif. The minimal consensus sequence for this motif is P-x-x-P. Several studies involving the screening of protein expression libraries or phage-displayed random peptide libraries with specific SH3 domains have shown the presence of at least 8 types of consensus sequences for proline-rich regions [Cheadle et al., 1994; Rickles et al., 1994; Sparks et al., 1996]. The one P-x-x-P motif in the IRSp53 sequence, KPLPVPP, is most related to the Src SH3 Class I motif, RPLPxxP, raising the possibility that a Src-like protein may interact with IRSp53 at its P-x-x-P motif, although Src itself has been shown not to interact with IRSp53 [Yeh et al., 1996]. The alteration of either proline in the P-x-x-P motif of the Nef protein leads to the loss of Hck and Lyn binding [Saksela et al., 1995]. Also peptide binding studies have shown that the alteration of the second proline leads to a decrease in SH3 binding [Cheadle et al., 1994]. To investigate whether IRSp53 is targeted to the particulate fraction by its interaction with a SH3 domain of another protein, we created a IRSp53 mutant where both prolines were replaced with alanine (Fig. 4).

The WW domain, similar to the SH3 domain in mediating protein-protein interactions, also binds to proline-rich regions but at regions containing the P-P-x-Y motif [Bork and Sudol, 1994; Sudol et al., 1995; Macias et al., 1996]. Mutational studies of the P-P-x-Y motif have revealed that the substitution of either proline or the tyrosine with alanine leads to the disruption of WW binding [Chen and Sudol, 1995; Macias et al., 1996; Staub et al., 1996]. To study the potential interactions of IRSp53 with the WW domains of other proteins, the one P-P-x-Y motif found in IRSp53 was changed to P-A-x-A (Fig. 4).

Western analysis of H720 and 9E10 immunoprecipitates of cell lysates from NIH3T3/IR cells expressing mutant IRSp53 proteins showed that all four mutants were readily expressed, recognized by both antibodies, and tyrosine phosphorylated in response to insulin stimulation, indicating that the point mutations did not lead to severe alterations in protein structure (Fig. 5). Subcellular fractionation studies showed that the same fraction of the four mutants was located in the particulate fractions as the wild type protein (Fig. 6). Therefore, the disruption of the putative SH3 domain and the putative SH3 binding and WW binding domains had no effect on protein distribution. This was further confirmed by immunofluorescence studies, which revealed no differences in subcellular localization between the wild type IRSp53 and

any of the mutants. A fraction of all four mutants continued to colocalize with the IR (Fig. 7), which was consistent with the observed insulin-stimulated tyrosine phosphorylation of all four mutants (Fig. 5). From these results, we conclude that the putative SH3 domain and the proline-rich P-x-x-P and P-P-x-Y motifs are not critical, at least individually, for the targeting of IRSp53 to the particulate fraction or the plasma membrane, nor for its ability to serve as a substrate for the IR.

Finally what, if any, are the roles of the putative SH3 domain and the two proline-rich motifs in IRSp53? Since IRSp53 is partially present around the plasma membrane both in the absence and presence of insulin stimulation, it is possible that once tyrosine phosphorylated, this protein can serve as a docking protein to recruit other signaling proteins to the plasma membrane through their SH2 domains. Furthermore, these protein-protein interactions could be further enhanced by the putative SH3, SH3-binding, or WW-binding domains in IRSp53. Thus the role of these domains could lie in their participation in the recruitment of other proteins to the plasma membrane, rather than the direct targeting of IRSp53 itself.

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