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Insulin Stimulates pp120 Endocytosis in Cells Co-expressing Insulin Receptors*

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pp120, a substrate of the insulin receptor tyrosine kinase, is a plasma membrane glycoprotein that is expressed in the hepatocyte as two spliced isoforms differing by the presence (full-length) or absence (truncated) of most of the intracellular domain including all phosphorylation sites. Co-expression of full-length pp120, but not its phosphorylation-defective isoforms, increased receptor-mediated insulin endocytosis and degradation in NIH 3T3 fibroblasts. We, herein, examined whether internalization of pp120 is required to mediate its effect on insulin endocytosis. The amount of full-length pp120 expressed at the cell surface membrane, as measured by biotin labeling, markedly decreased in response to insulin only when insulin receptors were co-expressed. In contrast, when phosphorylation-defective pp120 mutants were co-expressed, the amount of pp120 expressed at the cell surface did not decrease in response to insulin. Indirect immunofluorescence analysis revealed that upon insulin treatment of cells co-expressing insulin receptors, full-length, but not truncated, pp120 co-localized with α-adaptin in the adaptor protein complex that anchors endocytosed proteins to clathrin-coated pits. This suggests that full-length pp120 is part of a complex of proteins required for receptor-mediated insulin endocytosis and that formation of this complex is regulated by insulin-induced pp120 phosphorylation by the receptor tyrosine kinase. *In vitro* GST binding assays and co-immunoprecipitation experiments in intact cells further revealed that pp120 did not bind directly to the insulin receptor and that its association with the receptor may be mediated by other cellular proteins.

Insulin binding to the extracellular α-subunits of its receptor triggers activation of the tyrosine kinase in the intracellular domain of the β-subunits. Activation of the tyrosine kinase causes phosphorylation of the receptor and of endogenous substrates, including pp120, a plasma membrane glycoprotein of M_r ~120,000 that is expressed in the liver as two spliced variants differing by the inclusion (full-length) or exclusion (truncated) of a 61 amino acid (aa)[1] segment in the C terminus of its cytoplasmic domain (1). The truncated isoform lacks all phosphorylation sites. Site-directed mutagenesis studies in NIH 3T3 mouse skin fibroblasts revealed that full-length pp120 is constitutively phosphorylated by cAMP-dependent serine kinase on Ser^{503} and that this phosphorylation is required for its phosphorylation on Tyr^{488} residue by the insulin receptor tyrosine kinase (2). Tyr^{513}, the other tyrosine residue in the cytoplasmic domain of pp120, is not involved in insulin-mediated pp120 phosphorylation (2).

Binding of insulin to its receptor triggers the entry of the insulin-receptor complex into clathrin-coated pits, which pinch off the surface membrane to form clathrin-coated vesicles (3). The clathrin coat is formed upon receptor activation by the sequestration of the cytoplasmic adaptor proteins 2 (AP2) to the inner surface of the membrane where they bind to the clathrin tripods (4). Vesicular trafficking delivers the insulin-receptor complex to endosomes where it undergoes dissociation in the acidic environment. Insulin is then targeted for degradation, whereas the receptor recycles back to the cell surface (5, 6). Insulin-induced internalization and recycling of the receptor is believed to regulate sensitivity of the cell to insulin (7) and to constitute the basic mechanism of insulin clearance from the blood, an event that mainly occurs in the liver under normal physiological conditions.

The molecular mechanism for insulin endocytosis is not well defined. Insulin-stimulated receptor autophosphorylation is involved in this internalization process (8, 9). However, requirement for substrate phosphorylation is disputed (3, 10). Phosphorylation of insulin receptor substrate-1 and activation of phosphatidylinositol 3′-kinase did not regulate insulin endocytosis in transfected Chinese hamster ovary cells (3). In contrast, inhibiting pp120 expression in H4-II-E hepatoma cells by antisense mRNA transfection was associated with a decrease in the internalization rate of the insulin-receptor complex (11). Moreover, receptor-mediated insulin endocytosis and degradation were 2–3-fold higher in NIH 3T3 cells co-expressing full-length pp120 than in cells expressing insulin receptors alone. More recently, we have reported that full-length pp120 stimulated insulin endocytosis via high affinity (receptor-A) rather than low affinity (receptor-B) insulin receptors, despite being equally phosphorylated by both isoforms (12). This suggests that pp120-induced increase in insulin endocytosis and degradation does not depend on its phosphorylation state. However, elimination of phosphorylation sites in the truncated splice variant, and alteration of these sites by site-directed mutagenesis, abolished the effect of pp120 on insulin endocytosis and degradation (11), suggesting that pp120 phosphorylation is

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[1] The abbreviations used are: aa, amino acids; IR, insulin receptor; hIR, human insulin receptor; hIR-A, human insulin receptor isoform A; hIR-B, human insulin receptor isoform B; pp120, pp120/HA4/C-CAM; pBPV, bovine papilloma virus-based expression vector; NIH 3T3, NIH 3T3 mouse skin fibroblasts; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; BSA, bovine serum albumin.

required but not sufficient to mediate its effect on receptor-mediated insulin endocytosis and degradation.

Because the amino acid sequences in proximity to Tyr^{488} (Tyr-Ser-Val-Leu) and Tyr^{513} (Tyr-Ser-Val-Val) in tight β-turns share high homology with tyrosine-centered motifs known to interact with AP2 molecules that anchor endocytosed proteins to the clathrin coat of vesicles (13, 14), it is possible that full-length pp120 exerts its effect by targeting the receptor into clathrin-coated pits. To evaluate this hypothesis, we carried out these initial studies examining whether pp120 internalization is required to mediate its effect on insulin endocytosis. To this end, we used NIH 3T3 cells overexpressing either pp120 alone or with insulin receptors. Indirect immunofluorescence analysis revealed that pp120 did not significantly internalize in the absence of insulin. Biotin labeling of plasma membrane proteins revealed that full-length pp120 underwent endocytosis in response to insulin in cells co-expressing insulin receptors. In contrast, phosphorylation-defective isoforms of pp120 failed to endocytose in response to insulin. Because full-length, but not phosphorylation-defective, pp120 isoforms increased the rate of receptor-mediated insulin endocytosis and degradation (11), our data suggest that internalization of pp120 requires its phosphorylation by the insulin receptor tyrosine kinase in response to insulin. *In vitro* GST binding assays and co-immunoprecipitation experiments in intact cells revealed that pp120 did not directly bind to the insulin receptor. Instead, it may be indirectly associated with the receptor via other cellular proteins. Thus, it appears that pp120 exerts its effect on endocytosis by participating in a complex of proteins required for insulin receptor endocytosis.

EXPERIMENTAL PROCEDURES

Materials—ImmunoPure NHS-LC-biotin-sulfosuccinimidyl was purchased from Pierce. All reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad, and all reagents for immunoblotting were from Amersham Phamacia Biotech. All reagents for the GST system were purchased from Amersham Pharmacia Biotech. The baculovirus-purified β-insulin receptor kinase (aa 941–1343) of the cytoplasmic tail of β-subunit of the insulin receptor was from Stratagene (La Jolla, CA). The monoclonal antibody used to immunoprecipitate pp120 (α-HA4, an identical protein to pp120) was purified from ascites fluid from HA4 c19 cells purchased from the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City, IA). α-295 and α-778 polyclonal antibodies were raised in rabbit against aa 51–64 in the extracellular domain and aa 480–495 in the intracellular domain of rat liver pp120, respectively. The polyclonal antibody against aa 657–670 in the α-subunit of the human insulin receptor and the monoclonal antibody against phosphotyrosines were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-α-AP2 adaptin monoclonal antibody from the 100/2 hybridoma clone was purchased from Sigma. Ab-53, a polyclonal antibody raised in rabbit against the tyrosine kinase domain of the insulin receptor, was described previously (2).

Construction of Expression Vectors—Amplification and subcloning of the cDNA molecules encoding full-length, truncated, and site-directed pp120 mutants (Y488F, Y488F/Y513F, Y513F, and S503A) into a bovine papilloma virus-based expression vector (pBPV, Amersham Pharmacia Biotech) at the *Xho*I/*Not*I sites were described previously (2).

Cell Culture and Transfections—NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc., Herndon, VA) containing 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C/5% CO_2. Transfection of approximately 10^7 cells with 15 μg of each of pBPV-human insulin receptors A (pBPV-hIR) and pBPV-pp120 (full-length, truncated, and site-directed mutants) in the presence of 1.5 μg of RSV-Neo^r by the electroporation method (Bio-Rad) was originally described (2). Isolated clones were expanded and maintained in medium containing Geneticin (G-418, 600 μg/ml) (Life Technologies, Inc.). Cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Hepes, pH 7.6, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each of the following protease inhibitors: antipain dihydrochloride, pepstatin A, leupeptin, aprotinin, bacitracin) for analysis by 7.5% SDS-polyacrylamide (SDS-PAGE) gels and screening for pp120 expression by immunoblotting with a pp120 polypeptide antibody (α-295) and for insulin receptor expression by insulin binding in intact cells (2). Clones used in these studies typically expressed ~4.0×10^5 hIR-A per cell.

Biotin Labeling of Surface Membrane Proteins—Following incubation in the absence or presence of 100 nM insulin (Lilly) for 20 min at 37 °C, cells were incubated for 30 min at 4 °C with biotin (1 mg/ml) in phosphate-buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) supplemented with 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% BSA, as we have described previously (11). Cells were then incubated at 4 °C for 1 h with buffer alone or with Pronase (2.5 mg/ml). Following lysis in 1% Triton X-100 in the presence of protease inhibitors (see above), and immunoprecipitation with pp120/HA4 monoclonal antibody (2), proteins were electrophoresed through 7.5% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell), and immunoblotted with horseradish peroxidase (HRP)-labeled streptavidin followed by detection with enhanced chemiluminescence (ECL) as we described previously (11). The autoradiogram was scanned on an imaging densitometer (Bio-Rad model GS-670) and the proteins quantitated on the Image NIH v1.59 Macintosh software program. The difference in the amount of biotin-labeled pp120 before and after insulin treatment was calculated as percent biotin-labeled pp120 in the absence of insulin and used as measure for the amount of pp120 internalized in response to insulin. These experiments were repeated three times for each cell type.

In control experiments, biotin-labeled proteins were immunoprecipitated with a polyclonal antibody against the α-subunit of the insulin receptor prior to electrophoresis and immunoblotting with HRP-coupled streptavidin as described above.

Indirect Immunofluorescence—Transfected NIH 3T3 cells were grown to ~50% confluence in chamber slide culture wells (Lab-Tek, Naperville, IL), incubated overnight in serum-free medium supplemented with 0.1% BSA and 25 mM Hepes, pH 7.6, and treated with either 100 nM insulin or with buffer alone for 30 min at 37 °C as we described previously (2). As described in Ref. 15, with some modifications, cells were fixed in 2% (v/v) paraformaldehyde for 20 min at 4 °C followed by incubation for 15 min at 25 °C prior to permeabilization with 0.4% Triton X-100 in buffer E (PBS, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$) for 5 min at −20 °C and overnight incubation at 4 °C with or without primary antibodies in buffer E, 10% serum, 0.4% Triton. Washed cells were then incubated overnight at 4 °C with secondary antibodies in buffer E, 10% serum prior to washing and labeling the nucleus with 4,6-diamidino-2-phenylindole blue for 15 min at room temperature. Coverslips were then mounted on top of labeled cells with vectashield mounting medium and cells examined by an Olympus AX-70 microscope with an UplanApo 100× oil lens. To label pp120, we used the antibody against the extracellular domain, followed by detection with red $Cy^{TM}3$-conjugated AffiniPure goat anti-Rabbit IgG. To label α-adaptin, we used an anti-α-AP2 adaptin monoclonal antibody, followed by detection with fluorescein isothiocyanate-conjugated AffiniPure goat anti-mouse IgG.

Co-immunoprecipitation in Intact Cells—Following overnight starvation of serum, NIH 3T3 cells co-expressing human insulin receptors-A and full-length pp120 were treated with insulin (100 nM) for 3 min prior to subfractionation of the membrane pellet in the presence of protease (see above) and phosphatase inhibitors (EDTA, 4 mM; NaF, 100 mM; sodium pyrophosphate, 10 mM; sodium phosphate, 10 mM; ATP, 2 mM; sodium orthovanadate, 20 mM; *N*-ethylmalemide, 5 mM; and Hepes, 40 mM, pH 7.6). To this end, cells were collected in 1 mM $NaHCO_3$, pH 8.0, centrifuged at 1000 × *g* for 10 min at 4 °C, and the membrane fraction of the resulting supernatant was recovered through centrifugation at 20,000 × *g* for 30 min at 4 °C. Following lysis of the membrane pellet in 1% Triton X-100 in the presence of protease inhibitors and centrifugation at 100,000 × *g* for 1 h at 4 °C, cellular lysates were subjected to immunoprecipitation with antibodies against either pp120 or the α-subunit of the insulin receptor. Following three washes with PBS, pH 7.4, the immunopellets were electrophoresed through 7.5% SDS-PAGE and the proteins immunoblotted with the insulin receptor antibody followed by HRP labeling and ECL detection as described previously (2).

Expression of the Intracellular Domain of pp120 in the GST Fusion Protein System—As described previously (2), the cDNA fragment (nucleotide 1354–1574) encoding the majority of the cytoplasmic tail of wild-type pp120 (aa 452–519) was amplified in a polymerase chain reaction reaction using the wild-type recombinant cDNA (in the pGEM-4Z vector) as template and oligonucleotides s-1354 (tcctaaggatcACTGGCGGGGGAAGT-1368) and α-1574 (acaggaattcCAGGACAGACAATGTTAC-1557) as sense and antisense primers, respectively. The sense oligonucleotide contained a *Bam*HI and the antisense primer an

*Eco*RI restriction site (shown in lowercase letters) to allow for in-frame subcloning of the cDNA product into the GST gene fusion vector (pGEX-1λT, Amersham Pharmacia Biotech). Similarly, the 68-aa peptides carrying mutations at either Tyr^{488} or Tyr^{513} residues were synthesized, using pGEM4Z/pp120 mutant cDNAs as templates (Y488F, Y513F) (see above), and the wild-type oligomers containing *Bam*HI (nucleotide 1354–1368) and *Eco*RI (nucleotide 1557–1574) as sense and antisense primers, respectively (2). Competent *Escherichia coli* HB101 cells (Life Technologies, Inc.) were transformed with the GST fusion pGEX-1λT vectors, and the resulting GST peptides were allowed to bind at 4 °C for at least 90 min to 50% reduced glutathione-Sepharose 4B beads in the presence of 20 mM Hepes, pH 7.4, 25 mM NaCl, 0.05% Triton X-100, 10% glycerol, and 10 mM dithiothreitol as described previously (2).

Phosphorylation of the GST-pp120 Fusion Peptide by the Baculovirus-purified Insulin Receptor Tyrosine Kinase—Per manufacturer's instructions, the β-insulin receptor kinase (10 units) was initially autophosphorylated for 5 min at room temperature in the presence of 50 μM ATP, 75 mM $MgCl_2$, 1 mg/ml BSA, and 70 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; NEN Life Science Products). Equal aliquots of the insulin receptor reaction or of buffer alone were added to 2 μg of Sepharose-coupled GST containing fragments from wild-type and mutant (Y488F and Y513F) pp120, and the phosphorylation reaction was allowed to proceed for 10 min at room temperature. The reaction was terminated by adding phosphatase inhibitors on ice. Following mixing for 90 min at 4 °C, the Sepharose pellet was washed in HNTG buffer (150 mM Hepes, pH 7.6, 50 mM NaCl, 0.1% Triton X-100, 10% glycerol) and analyzed by 10% SDS-PAGE and autoradiography.

Phosphorylation of the GST-pp120 Fusion Peptide by Partially Purified Insulin Receptors—Glycoproteins derived from NIH 3T3 cells overexpressing human insulin receptors-A were partially purified by wheat germ agglutinin affinity chromatography as described previously (2). An aliquot of the partially purified fractions (5 μg) was then allowed to bind to insulin (100 nM) or to buffer alone for 30 min at room temperature prior to being assayed for its ability to phosphorylate GST fusion peptides (2 μg) containing fragments of wild-type pp120. The phosphorylation reaction was carried out for 10 min at room temperature in the presence of 25 μM ATP, 100 μCi of [γ-^{32}P]ATP, 5 mM $MgCl_2$ and $MnCl_2$, 1 mM CTP in 0.1% Triton X-100, 150 mM NaCl, 50 mM Hepes, pH 7.6. The reaction was terminated on ice by adding phosphatase inhibitors (2). The phosphorylation reaction was then allowed to mix for 90 min at 4 °C prior to washing the Sepharose pellet with HNTG buffer (see above) and electrophoresis through 10% SDS-PAGE. Following transfer to nitrocellulose membranes, proteins were detected by autoradiography and immunoblotted with a polyclonal antibody against the intracellular domain of pp120 as described previously (2). In some experiments, proteins were immunoblotted with a monoclonal antibody against phosphotyrosines per manufacturer's instructions.

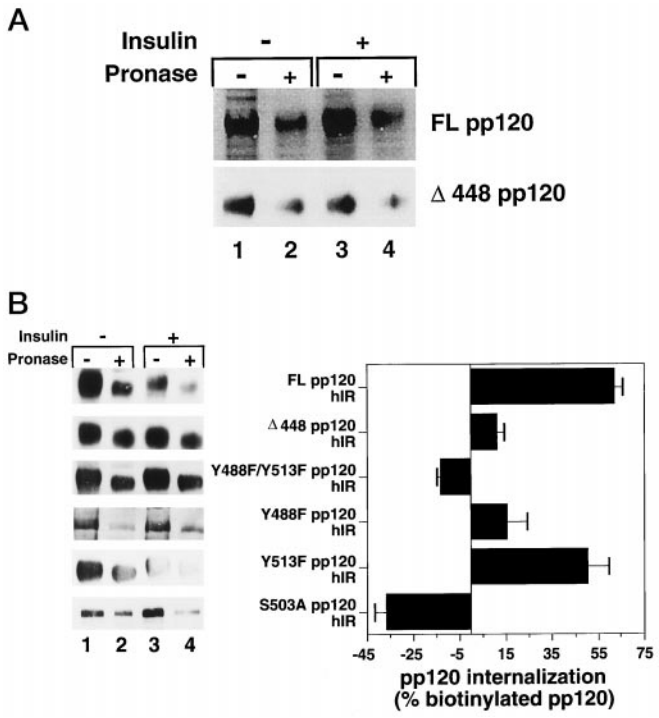

FIG. 1. **Biotin labeling, internalization of pp120.** NIH 3T3 cells were stably transfected with rat liver pp120 cDNA alone (*A*) or with human insulin receptors-A (*B*). pp120 cDNAs include wild-type (full-length (*FL*) and truncated (Δ*448*)) and site-directed mutant isoforms (Y488F,Y513F; Y488F; Y513F; and S503A). Following incubation of cells in the absence (−, *lanes 1* and *2*) or presence (+, *lanes 3* and *4*) of 100 nM insulin, the extracellular domains of proteins expressed at the surface membrane were labeled with biotin and treated with buffer alone (−, *odd-numbered lanes*) or with Pronase (+, *even-numbered lanes*). Cell lysates were then immunoprecipitated with α-pp120/HA4 monoclonal antibody and the proteins analyzed by SDS-PAGE and immunoblotted with HRP-labeled streptavidin, followed by the ECL detection system. The amount of pp120 internalized in response to insulin was calculated as the difference in the amount of biotin-labeled pp120 before and after insulin treatment relative to the amount of biotin-labeled pp120 in the absence of insulin (*B*, graph). Experiments were repeated three times for each cell type co-expressing pp120 and insulin receptors.

RESULTS

Internalization of pp120—To investigate the effect of insulin on surface expression of pp120, transfected NIH 3T3 cells were stimulated with insulin prior to biotin labeling and Pronase digestion. Following lysis, proteins were immunoprecipitated with pp120/HA4 antibody, electrophoresed, and immunoblotted with HRP-labeled streptavidin. The difference in the amount of biotin-labeled pp120 before and after insulin treatment was calculated as percent biotin-labeled pp120 in the absence of insulin and used as measure for the amount of pp120 internalized in response to insulin (Fig. 1*B*, graph). As revealed in Fig. 1*A*, insulin treatment did not decrease the amount of biotin-labeled pp120 (full-length (*FL*) or truncated (Δ*448*)) in cells expressing pp120 alone (*lane 3 versus lane 1*). However, when insulin receptors were co-expressed with human insulin receptors-A (*hIR*), the amount of biotin incorporated in the extracellular domain of full-length pp120 was substantially decreased by 62.2 ± 3.8% in response to insulin (Fig. 1*B*, *FL pp120/hIR*, *lane 3 versus lane 1*). Similarly, the amount of biotin-labeled pp120 was decreased by 50.6 ± 9.1% in response to insulin when the phosphorylatable Y513F pp120 isoform was co-expressed (Fig. 1*B*, *Y513F pp120/hIR*, *lane 3 versus lane 1*). In contrast, the amount of biotin-labeled pp120 was not significantly decreased in cells co-transfected with phosphorylation-defective pp120 isoforms (Fig. 1*B*, *lane 3 versus lane 1*, truncated (Δ448): 11.1 ± 3.4%, and site-directed mutants: Y488F (15.5 ± 8.4%), Y488F/Y513F (−13.2 ± 1.3%), S503A (−36.8 ± 4.5%)]. Instead, most of the phosphorylation-defective pp120 remained at the surface membrane, as evidenced by the comparable decrease in the amount of biotin-labeled pp120 after the removal of the extracellular domains with Pronase (Fig. 1*B*, *lane 2 versus lane 1* and *lane 4 versus lane 3*). Because the effect of pp120 on insulin endocytosis was only detected in cells co-expressing the phosphorylatable (full-length and Y513F) isoforms of pp120 (11), these data suggest that pp120 exerted its effect on receptor-mediated insulin endocytosis by co-internalizing with the insulin receptor and that this required insulin-induced pp120 phosphorylation by the insulin receptor tyrosine kinase. In fact, expression of the insulin receptor at the cell surface was also decreased in response to insulin in cells co-expressing full-length pp120 as detected by immunoprecipitation with a polyclonal antibody against the 130-kDa α-subunit of the insulin receptor (data not shown).

To investigate whether pp120 undergoes constitutive endocytosis in the absence of insulin, we examined its co-localization with α-adaptin in cells expressing pp120 alone or with insulin receptors. To this end, we employed indirect immunofluorescence in which pp120 was detected by a polyclonal antibody against its extracellular domain followed by red Cy™3 labeling (Fig. 2, *A*, *C*, *E*, *G*, *I*, and *K red*), and α-adaptin was detected by a monoclonal antibody followed by fluorescein iso-

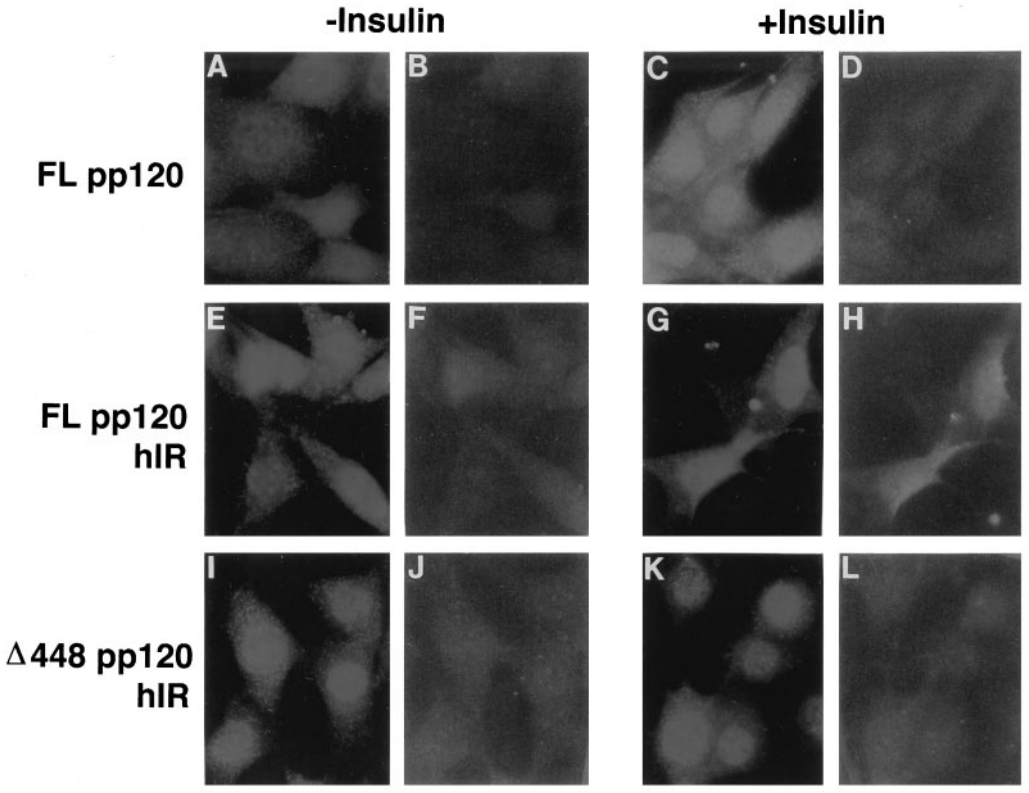

FIG. 2. **Indirect immunofluorescence, pp120 does not undergo endocytosis in the absence of insulin.** NIH 3T3 cells expressing full-length pp120 alone (*FL pp120*, *A–D*) or co-expressing human insulin receptors-A plus either full-length (*FL pp120/hIR*, *E–H*) or truncated pp120 (Δ*448 pp120/hIR*, *I–L*) were treated with either 100 nM insulin (+*Insulin*) or with buffer alone (−*Insulin*) prior to being fixed in paraformaldehyde, incubated with primary antibodies, and immunostained with red Cy™3 (pp120; *A*, *C*, *E*, *G*, *I*, and *K*) and fluorescein isothiocyanate (α-adaptin; *B*, *D*, *F*, *H*, *J*, and *L* panels). Cells were examined by a 100× oil lens.

thiocyanate labeling (Fig. 2, *B*, *D*, *F*, *H*, *J*, and *L* green). Absence of red or green stain of cells when primary antibodies were omitted indicated the absence of nonspecific binding of antibodies against pp120 and α-adaptin to these cells (data not shown). The punctate distribution of label in *A* revealed that in the absence of insulin, full-length (*FL*) pp120 was largely distributed on the surface membrane of cells expressing pp120 alone (*FL pp120*). In fact, pp120 distribution in these cells did not match the typical cytoplasmic distribution of α-adaptin (16) as shown in *B*. Insulin treatment did not noticeably alter this distribution (*C* and *D*), suggesting that full-length pp120 did not undergo significant constitutive endocytosis when expressed alone. When full-length pp120 was co-transfected with insulin receptors (*FL pp120/hIR*), pp120 and α-adaptin remained largely separated in the absence of insulin (*E* and *F*). However, when these cells were treated with insulin, most of pp120 co-localized with α-adaptin (*G* and *H*). This suggests that pp120 underwent insulin-stimulated endocytosis in cells co-expressing insulin receptors. In contrast, truncated (Δ*448*) pp120 did not co-localize with α-adaptin when cells co-expressing insulin receptors (Δ*448 pp120/hIR*) were treated with either buffer alone (*I* and *J*) or with insulin (*K* and *L*), indicating that truncated pp120 did not undergo insulin-stimulated internalization.

Phosphorylation of the GST Fusion Peptide from the Intracellular Domain of pp120 by a Baculovirus-purified Insulin Receptor Kinase—Because pp120 phosphorylation by the insulin receptor tyrosine kinase appears to regulate its effect on receptor-mediated insulin endocytosis (11), we examined whether pp120 is a direct substrate of the insulin receptor kinase. To this end, we examined phosphorylation of a Sepharose-coupled GST-pp120 fusion peptide from the entire C-terminal tail of wild-type, Y488F, and Y513F pp120 mutants by a

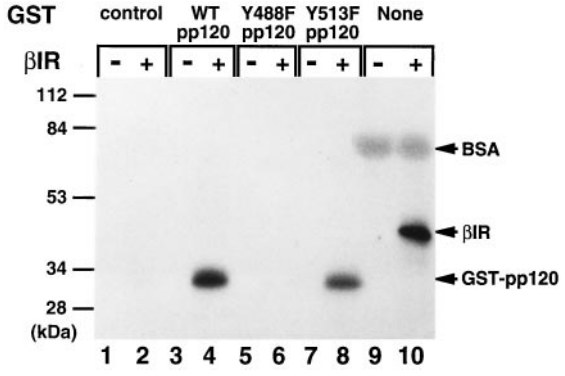

FIG. 3. **The intracellular domain of pp120, a direct substrate of the insulin receptor kinase, does not directly bind to the intracellular domain of the receptor *in vitro*.** Baculovirus-purified insulin receptor kinase was autophosphorylated in the presence of [γ-^{32}P]ATP and BSA. In control experiments, the kinase was replaced by buffer. Equal aliquots from the buffer (*odd-numbered lanes*) or the βIR kinase (*even-numbered lanes*) autophosphorylation reaction was added to Sepharose-coupled GST plasmid control (*lanes 1* and *2*) or GST fusion proteins containing fragments (aa 452–519) from wild-type (*WT*) or mutant (Y488F, Y513F) pp120 intracellular domain (*lanes 3–8*). Upon termination of the phosphorylation reaction on ice in the presence of phosphatase inhibitors, proteins were allowed to mix for 90 min prior to analysis on SDS-PAGE and autoradiography. One-fifth of the added buffer and the autophosphorylated βIR was also analyzed (*lanes 9* and *10*, respectively) to assess the autophosphorylation of the βIR kinase. Molecular mass markers are shown at the *left-hand side* of the gel.

baculovirus-purified insulin receptor kinase. The β-insulin receptor kinase was initially autophosphorylated (Fig. 3, *lane 10*) in the presence of [γ-^{32}P]ATP prior to being allowed to phosphorylate GST-pp120 fusion peptides (Fig. 3, *lanes 4, 6*, and *8*) or Sepharose-coupled GST proteins alone lacking pp120 se-

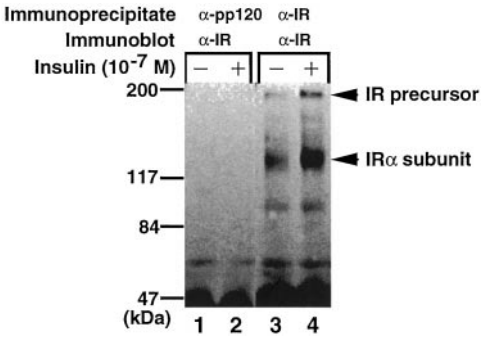

FIG. 4. **pp120 does not co-immunoprecipitate with the insulin receptor in intact cells.** Following overnight starvation of serum, NIH 3T3 cells co-expressing insulin receptors and full-length pp120 were treated with insulin (100 nM) prior to subfractionation of the membrane pellet in the presence of protease and phosphatase inhibitors. Detergent extracts of the membrane fraction were subjected to immunoprecipitation with either a polyclonal antibody against the α-subunit of the insulin receptor (*α-IR*) or with pp120/HA4 monoclonal antibody (*α-pp120*). Proteins were then electrophoresed through SDS-PAGE and immunoblotted with α-IR antibody. Molecular mass markers are shown at the *left-hand side* of the gel.

quences (Fig. 3, *lane 2*). In control experiments, the β-insulin receptor kinase was omitted from the phosphorylation mixture (Fig. 3, *odd-numbered lanes*). Phosphatase inhibitors were then added, and the mixture was kept at 4 °C to allow binding between proteins to occur. At the end of the incubation period, proteins were analyzed by electrophoresis and autoradiography (Fig. 3). GST peptide lacking sequences from pp120 did not serve as a substrate for phosphorylation by the insulin receptor tyrosine kinase (*lane 2 versus lane 1*). In contrast, the activated insulin receptor caused phosphorylation of a M_r ~32,000 species that corresponds to the GST-pp120 fusion wild-type peptide (*lane 4 versus lane 3*), indicating that pp120 is a direct substrate of the insulin receptor and that its extracellular domain is not required for its cytoplasmic domain to undergo phosphorylation by the insulin receptor. Moreover, mutating Tyr^{488} to nonphosphorylatable phenylalanine (Y488F) abolished phosphorylation of GST-pp120 fusion peptide by the tyrosine kinase of the insulin receptor (*lane 6 versus lane 5*). In contrast, replacing Tyr^{513} with phenylalanine (Y513F) did not alter phosphorylation of GST-pp120 by the receptor (*lane 8 versus lane 7*), supporting our previous finding that Tyr^{488} is the main phosphorylation site of pp120 by the insulin receptor tyrosine kinase (2). Interestingly, no band corresponding to the β-subunit of the insulin receptor (M_r ~48,000) was detected with the GST-pp120 pellet (*lanes 3–8*). Consistent with these findings, we have observed in collaboration with T. A. Gustafson that the intracellular domain of pp120 did not bind to that of the insulin receptor in the yeast two-hybrid system (data not shown). Thus, it appears that the intracellular domain of pp120 did not directly bind to the intracellular domain of the insulin receptor in these two *in vitro* systems.

Co-immunoprecipitation in Intact Cells—It remains possible that insulin binding to the extracellular domain of the insulin receptor induces conformational changes that would lead to a direct association between the intracellular domains of pp120 and the insulin receptor. To examine this hypothesis, we treated NIH 3T3 cells co-expressing recombinant human insulin receptors-A and full-length pp120 with either buffer alone (Fig. 4, *odd-numbered lanes*) or with 10^{-7} M insulin (Fig. 4, *even-numbered lanes*) prior to subcellular fractionation. Following lysis of the membrane fraction, proteins were immunoprecipitated with either HA4/pp120 monoclonal antibody (Fig. 4, *lanes 1* and *2*, α-pp120) or with a polyclonal antibody against the α-subunit of the insulin receptor (Fig. 4, *lanes 3* and *4*, *α-IR*), and the immunopellets were analyzed by SDS-PAGE and immunoblotted with the insulin receptor antibody (Fig. 4, all lanes). In addition to the M_r ~190,000 band that corresponds to the insulin receptor precursor, and the M_r ~90,000 band that was nonspecific, as evidenced by immunoprecipitation with normal rabbit globulin (data not shown), a M_r ~135,000 band corresponding to the α-subunit of the insulin receptor was detected in the immunopellets of the insulin receptor antibody (Fig. 4, *lanes 3–4*). However, when the pp120 antibody immunopellets were probed with the α-subunit of the insulin receptor, this M_r ~135,000 band was not detected (Fig. 4, *lanes 1* and *2*). Similarly, when the immunoblot was probed with a polyclonal antibody against pp120, the M_r ~120,000 band that corresponds to pp120 was not detected in the insulin receptor immunopellet (data not shown). Thus, the insulin receptor and pp120 failed to co-immunoprecipitate *in vivo* under our experimental conditions.

Co-precipitation of Partially Purified Insulin Receptors and GST-pp120 Fusion Peptides—It remains possible that pp120 associates with the insulin receptor via other proteins. To test this hypothesis, we employed the same phosphorylation/GST binding experiment described above using partially purified insulin receptors derived from NIH 3T3 cells overexpressing human insulin receptors-A. Because we have shown that partially purified insulin receptors could phosphorylate purified pp120 fusion peptides in the presence of 10^{-7} M insulin (2), we allowed insulin to initially bind to partially purified insulin receptors prior to introducing either Sepharose-coupled GST fusion wild-type pp120 peptides (*WT pp120*) or GST proteins lacking fusion peptides (*Control*) in the presence of [γ-^{32}P]ATP. Under these conditions, the GST protein lacking sequences from pp120 did not undergo phosphorylation by the insulin receptor tyrosine kinase nor did it allow detection of any binding protein (Fig. 5*A*, *lanes 3* and *4*). However, when the GST-pp120 fusion wild-type peptide was used, insulin led to a 10-fold increase in the phosphorylation of a M_r ~95,000 protein (Fig. 5*A*, *lane 2 versus lane 1*) that corresponds to the β-subunit of the insulin receptor, as evidenced by immunoblotting with Ab-53 antireceptor antibody (Fig. 5D, *α-IR*). Additionally, insulin led to a 5-fold increase in the phosphorylation of a M_r ~32,000 species that corresponds to the pp120 fusion wild-type peptide (Fig. 5*A*, *GST-WT pp120*, *lane 2 versus lane 1*). Interestingly, phosphorylated insulin receptors co-precipitated with Sepharose-coupled GST-pp120 fusion peptide in the presence of insulin under these conditions (Fig. 5*A*, *lane 2 versus lane 1*). Moreover, insulin led to a significant increase in the co-precipitation of several proteins of M_r ~160,000, ~135,000, ~68,000, and ~58,000 (p160, p135, p68, p58). As evidenced by immunoblotting with phosphotyrosine antibody (*α-pTyr*) (Fig. 5*C*), p68 and p58, in addition to the β-subunit of the insulin receptor and three other proteins (M_r ~80,000, ~72,000, and ~40,000), underwent a marked increase in tyrosine phosphorylation in the presence of insulin. Because the baculovirus-purified cytoplasmic domain of the insulin receptor did not co-precipitate with GST-pp120 fusion peptide under similar conditions (Fig. 4), it is reasonable to predict that co-precipitation of partially purified insulin receptors with pp120 in experiments shown in Fig. 5 may have been mediated by these unidentified proteins. These studies constitute the first evidence for an indirect association between pp120 with the insulin receptor.

We then examined the effect of mutating tyrosine residues in pp120 (Tyr^{488} and Tyr^{513}) to nonphosphorylatable phenylalanine (Phe) on the complex formation with the insulin receptor using the same GST fusion binding assay described above (Fig. 6). Mutating Tyr^{488} to Phe abolished insulin-stimulated pp120 phosphorylation and its association with the receptor, as evi-

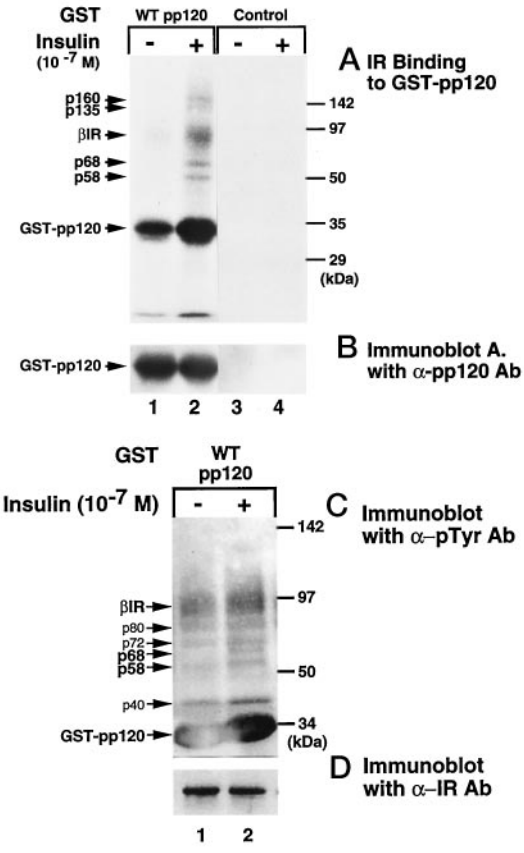

FIG. 5. **The intracellular domain of pp120 indirectly associates with the insulin receptor *in vitro*.** The intracellular domain of wild-type pp120 (aa 452–519) was expressed as a glutathione *S*-transferase fusion protein in *E. coli* and coupled to Sepharose. Lectin chromatography partially purified insulin receptors (from NIH 3T3 cells overexpressing insulin receptors-A) were then added to GST-pp120 fusion wild-type peptide (*lanes 1* and *2* of *A* and *C*) or GST plasmid control lacking pp120 sequences (*lanes 3* and *4*). *In vitro* phosphorylation was then initiated by adding [γ-^{32}P]ATP in the absence (−, *odd-numbered lanes*) or presence (+, *even-numbered lanes*) of insulin (10^{-7} M). Sepharose-coupled GST fusion proteins were analyzed by SDS-PAGE. Following autoradiography (*A*), proteins were immunoblotted with a polyclonal antibody against the intracellular domain of pp120 (*B*) or with a monoclonal antibody against phosphorylated tyrosines (*C*). Thereafter, proteins were immunoprobed with a polyclonal antibody (α-*IR*) against the β-subunit of the insulin receptor (*D*) to identify the M_r ~95,000 band. Phosphorylated proteins that were also detected by autoradiography are labeled in *bold* (***βIR***, ***p68***, and ***p58***). Additional tyrosyl phosphorylated proteins are labeled in roman type and in smaller text size (*p80*, *p72*, and *p40*). Molecular mass markers are shown at the *right-hand side* of the gel.

denced by the failure to detect the β-subunit of the insulin receptor with the GST-Y488F pp120 fusion peptide (*lane 2*). In contrast, mutating Tyr^{513} to Phe did not alter the phosphorylation state of pp120 (*lane 4 versus lane 6*). Similarly to wild-type, the β-subunit of the insulin receptor was associated with the Y513F pp120 fusion peptide (*βIR*, *lane 4 versus lane 6*). Interestingly, the p58 and p68 proteins persisted in the Phe^{513} fusion pellet but were virtually undetectable with the Phe^{488} complex. This suggests that tyrosine-phosphorylated p58 and p68 may mediate the indirect association between the insulin receptor and pp120 at Tyr^{488}. In these experiments, the higher molecular mass proteins (p135 and p160) did not separate on the 10% acrylamide SDS-PAGE. Nonetheless, they persist in the fusion pellet independently of the phosphorylation state of pp120, suggesting that they may not be significant components of the internalization complex.

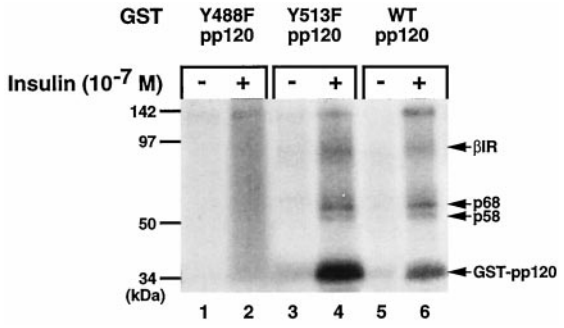

FIG. 6. **Effect of pp120 phosphorylation on its association with the insulin receptor *in vitro*.** Following *in vitro* phosphorylation by partially purified insulin receptors in the absence (−, *odd-numbered lanes*) or presence (+, *even-numbered lanes*) of insulin (10^{-7} M), GST binding experiments similar to those described in Fig. 5 were performed using GST-pp120 fusion wild-type (*lanes 5* and *6*), Y513F (*lanes 3* and *4*), and Y488F mutant peptides (*lanes 1* and *2*). Proteins bound to Sepharose-coupled GST-pp120 were detected by autoradiography. *βIR* denotes the β-subunit of the insulin receptor. Molecular mass markers are shown at the *left-hand side* of the gel.

DISCUSSION

We have reported previously that co-expression of full-length, not truncated, pp120 in NIH 3T3 cells increased receptor-mediated insulin endocytosis and degradation compared with cells expressing insulin receptors alone (11, 12). In the current studies, we observed that pp120 regulated receptor-mediated insulin endocytosis by taking part in a complex of proteins required for insulin endocytosis. Indirect immunofluorescence analysis revealed that a large portion of pp120 did not undergo constitutive endocytosis in the absence of insulin. However, in response to insulin, pp120 was endocytosed in cells co-expressing insulin receptors, as evidenced by biotin labeling of plasma membrane proteins. This suggests that insulin did not directly induce endocytosis of pp120. Because insulin binding to its receptor induces receptor internalization, our data suggest that the effect of pp120 on insulin endocytosis was mediated by its effect on the receptor. Moreover, insulin did not induce endocytosis of phosphorylation-defective pp120 isoforms that failed to regulate insulin receptor endocytosis in NIH 3T3 cells (11). This suggests that insulin-stimulated pp120 phosphorylation by the insulin receptor underlied its ability to endocytose as part of a complex of proteins required for insulin endocytosis.

In vitro assays (GST binding and the yeast two-hybrid system) and *in vivo* co-immunoprecipitation experiments revealed that the intracellular domain of pp120 did not directly bind to that of the insulin receptor. Instead, pp120 appeared to be associated with the insulin receptor via one or more unidentified proteins. We have shown that mutating Tyr^{488} to nonphosphorylatable phenylalanine altered pp120 endocytosis (11). In this report, we have shown that the reduced effect of pp120 on endocytosis was correlated with decreased association of pp120 with insulin receptor and with at least two other tyrosine-phosphorylated proteins (p68 and p58). Because one of the main functions of phosphorylated tyrosines in tight β-turns, such as Tyr^{488} and Tyr^{513} in pp120, is to expose these residues for associations with signaling molecules, it is reasonable to speculate that p58 and p68 play a role in insulin signaling. Mutating Tyr^{513} did not alter the phosphorylation state of pp120, nor did it modify its effect on insulin-induced receptor endocytosis (2, 11). This suggests that this residue is not significantly involved in the association between pp120 and other signaling proteins that play a role in insulin endocytosis. Thus, it is reasonable to conclude that an association between full-length pp120 and other signaling proteins is mediated by phos-

phorylated Tyr^{488} in its intracellular domain. Identification of these proteins would greatly advance our understanding of the basic mechanism of insulin endocytosis.

Receptor-mediated insulin endocytosis occurs via two distinct vesicular pathways, clathrin-coated pits and noncoated caveolae (17). When targeted for degradation, insulin is largely internalized through the clathrin-coated pathway (18). Because insulin is mainly degraded in the liver, its internalization in hepatocytes predominantly occurs through clathrin-coated vesicles. Consistent with these observations, clathrin-coated pits constitute a larger fraction of plasma membrane compartments in hepatocytes than caveolae (18). Tyr^{488} and Tyr^{513} in pp120 are placed in tight β-turns (19) with flanking amino acid sequences (Tyr^{488}-Ser-Val-Leu; Tyr^{513}-Ser-Val-Val) conforming to the motif that signals sorting of plasma membrane proteins to endosomes via clathrin-coated vesicles (aromatic-Xp-Xp (where Xp indicates any polar amino acid)-aromatic or large hydrophobic amino acid) (13, 14, 20). Thus, it is possible that pp120 exerts its effect on insulin endocytosis and degradation by targeting the insulin receptor protein complex to clathrin-coated pits.

pp120 is the first substrate of the insulin receptor tyrosine kinase that has been implicated in insulin receptor endocytosis and degradation (11, 12). In this report we have presented evidence that pp120 exerts its up-regulatory effect on receptor-mediated insulin endocytosis by engaging in complex formation with the insulin receptor when phosphorylated in response to insulin. This may direct the complex to the endocytotic vesicles and/or stabilize the insulin-receptor endocytosis complex. In this manner, pp120 would play a substantial role in accelerating insulin uptake and its degradation in the cell. Because pp120 is predominantly expressed in the liver, a major site for insulin degradation, insulin-induced pp120 endocytosis may constitute a basic mechanism for insulin degradation and clearance from the blood.

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Insulin Stimulates pp120 Endocytosis in Cells Co-expressing Insulin Receptors

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