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) 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 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 necessary for its effect on insulin endocytosis.

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‡ 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; PGE, 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 &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 polyclonal immunoblotting were from Amersham Pharmacia 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 HA4c19 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 mutants of pp120 (Tyr-1574 (acaggaattcCAGGACAGAAAGCTTTG) and Tyr-1574 (acaggaattcCAGGACAGAAAGCTTTG) by electron transfer (ET)) were performed with PCR amplification from pcDNA3 (a eukaryotic vector) by using primers specific for the cloning site (Not I) and the 5′-end of the cDNA (see above). The PCR reaction products were subcloned into a vector (pAT34) containing a bovine papilloma virus-based expression vector (pBPV, Amersham Pharmacia Biotech) at the Xhol/NotI 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 in 5% CO2. Transfection of approximately 107 cells was performed with 0.4% Triton X-100 in buffer E (PBS, pH 7.4, 150 mM NaCl, 2 mM MgCl2) for 5 min at 20 °C and overnight incubation at 4 °C with or without primary antibodies in buffer E, 1% serum, 0.4% Triton. Washed cells were then incubated overnight at 4 °C with secondary antibodies in buffer E, 1% 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 IX 70 inverted microscope equipped with 100W oil immersion (1.4) to an oil immersion phase contrast lens. To label α-adaptin, we used the antibody against the extracellular domain, following detection with labeled streptavidin followed by detection with fluorescein isothiocyanate-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 of Intact Cells—Following overnight starvation, NIH 3T3 cells expressing human insulin receptor-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; 2 mM sodium orthovanadate, 20 mM; N-ethylmaleimide, 5 mM; and 40 μg/ml, pH 7.6). To this end, cells were collected in 1 ml of NaHCO3, 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 proteases (see above) and phosphatase inhibitors (EDTA, 4 mM; NaF, 100 mM; sodium pyrophosphate, 10 mM; sodium phosphate, 10 mM; 2 mM sodium orthovanadate, 20 mM; N-ethylmaleimide, 5 mM; and 40 μg/ml, pH 7.6). To this end, cells were collected in 1 ml of NaHCO3, 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 (nt 1354–1574) of the major variant 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 5′-ATCGTGGCGGAGGAATCGAAGG-3′ and 5′-ATCGTGGCGGAGGAATCGAAGG-3′ as sense and antisense primers, respectively. The sense oligonucleotide contained a BamHI and the antisense primer an
EcoRI restriction site (shown in lowercase letters) to allow for in-frame subcloning of the cDNA product into the GST gene fusion vector (pGEX-1AT, Amersham Pharmacia Biotech). Similarly, the 68-aa peptides carrying mutations at either Tyr488 or Tyr513 residues were synthesized, using pGEM4Z/pip20 mutant cDNAs as templates (Y488F, Y513F) (see above), and the wild-type oligomers containing BamHI (nucleotide 1335–1368) and EcoRI (nucleotide 1557–1574) as sense and antisense primers, respectively (2). Competent Escherichia coli HB101 (Life Technologies, Inc.) were transformed with the GST fusion pGEX-1AT 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 25 mM NaCl, pH 7.4, 0.5% Triton X-100, 10% glycerol, and 10 mM dithiothreitol as described previously (2).

Phosphorylation of the GST-pip20 Fusion Peptide by the Bacterial-purified Insulin Receptor Tyrosine Kinase—Per manufacturer’s instructions, the β-insulin receptor kinase (10 units) was initially auto-phosphorylated for 5 min at room temperature in the presence of 50 μM ATP, 75 mM MgCl₂, 1 mg/ml BSA, and 70 μCi of [γ-³²P]ATP (6000 Ci/mmol; NEN Life Science Products). Equal aliquots of the insulin receptor kinase reaction or of buffer alone were added to 2 μg of Sepharose-coupled GST containing fragments from wild-type and mutant (Y488F and Y513F) pip20, 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-pip20 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 pip20. The phosphorylation reaction was carried out for 10 min at room temperature in the presence of 25 μM ATP, 100 μCi of [γ-³²P]ATP, 5 mM MgCl₂ and MnCl₂, 1 mM DTT, 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 electrophoresed 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 pip20 as described previously (2). In some experiments, proteins were immunoblotted with a monoclonal antibody against phosphotyrosines per manufacturer’s instructions.

RESULTS

Internalization of pip20—To investigate the effect of insulin on surface expression of pip20, transfected NIH 3T3 cells were stimulated with insulin prior to biotin labeling and Pronase digestion. Following lysis, proteins were immunoprecipitated with pip20/HAA antibody, electrophoresed, and immunoblotted with HRP-labeled streptavidin. The difference in the amount of biotin-labeled pip20 before and after insulin treatment was calculated as percent biotin-labeled pip20 in the absence of insulin and used as measure for the amount of pip20 internalized in response to insulin (Fig. 1B, graph). As revealed in Fig. 1A, insulin treatment did not decrease the amount of biotin-labeled pip20 (full-length (FL) or truncated (Δ448)) in cells expressing pip20 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 pip20 was substantially decreased by 62±8% in response to insulin (Fig. 1B, FL pip20/hIR, lane 3 versus lane 1). Similarly, the amount of biotin-labeled pip20 was decreased by 50.6±9.1% in response to insulin when the phosphorylatable Y513F pip20 isoform was co-expressed (Fig. 1B, Y513F pip20/hIR, lane 3 versus lane 1). In contrast, the amount of biotin-labeled pip20 was not significantly decreased in cells co-transfected with phosphorylation-defective pip20 isoforms (Fig. 1B, lane 3 versus lane 1, truncated (Δ448): 11.1±3.4%, and site-directed mutants: Y488F (15.5±8.4%), Y488FY513F (−13.2±1.3%), S503A (−36.8±4.5%). Instead, most of the phosphorylation-defective pip20 remained at the surface membrane, as evidenced by the comparable decrease in the amount of biotin-labeled pip20 after the removal of the extracellular domains with Pronase (Fig. 1B, lane 2 versus lane 1 and lane 4 versus lane 3). Because the effect of pip20 on insulin endocytosis was only detected in cells co-expressing the phosphorylatable (full-length and Y513F) isoforms of pip20 (11), these data suggest that pip20 exerted its effect on receptor-mediated insulin endocytosis by co-internalizing with the insulin receptor and that this required insulin-induced pip20 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 pip20 as detected by immunoprecipitation with a polyclonal antibody against the 130-kDa α-subunit of the insulin receptor (data not shown).

To investigate whether pip20 undergoes constitutive endocytosis in the absence of insulin, we examined its co-localization with α-adaptin in cells expressing pip20 alone or with insulin receptors. To this end, we employed indirect immunofluorescence in which pip20 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 (Δ448 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 Cy3 (pp120; isothiocyanate (FITC) labeling (Fig. 2, A–D, and pp120 did not co-localize with α-adaptin (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 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 (Δ448) pp120 did not co-localize with α-adaptin when cells co-expressing insulin receptors (Δ448 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**—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 baculovirus-purified insulin receptor kinase. The β-insulin receptor kinase was initially autophosphorylated (Fig. 3, lane 10) in the presence of [γ-32P]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-
sequences (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 Mr ~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 Tyr488 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 Tyr513 with phenylalanine (Y513F) did not alter phosphorylation of GST-pp120 by the receptor (lane 8 versus lane 7), supporting our previous finding that Tyr488 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 (Mr ~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 Mr ~190,000 band that corresponds to the insulin receptor precursor, and the Mr ~90,000 band that was nonspecific, as evidenced by immunoprecipitation with normal rabbit globulin (data not shown), a Mr ~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 Mr ~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 Mr ~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-32P]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. 5A, lane 2 versus lane 1). However, when the GST-pp120 fusion wild-type peptide was used, insulin led to a 10-fold increase in the phosphorylation of a Mr ~95,000 protein (Fig. 5A, 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 Mr ~32,000 species that corresponds to the pp120 fusion wild-type peptide (Fig. 5A, 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. 5A, lane 2 versus lane 1). Moreover, insulin led to a significant increase in the co-precipitation of several proteins of Mr ~160,000, ~135,000, ~68,000, and ~58,000 (p160, p135, p68, p58). As evidenced by immunoblotting with phosphotyrosine antibody (α-pTyr) (Fig. 5C), p68 and p58, in addition to the β-subunit of the insulin receptor and three other proteins (Mr ~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 (Tyr488 and Tyr513) to nonphosphorylatable phenylalanine (Phe) on the complex formation with the insulin receptor using the same GST fusion binding assay described above (Fig. 6). Mutating Tyr488 to Phe abolished insulin-stimulated pp120 phosphorylation and its association with the receptor, as evi-
enanced by the failure to detect the \( \beta \)-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 \( \beta \)-subunit of the insulin receptor was associated with the Y513F pp120 fusion peptide (\( \beta IR, \) 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 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.

**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 underlies 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 \( \beta \)-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\textsuperscript{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\textsuperscript{488} and Tyr\textsuperscript{513} in pp120 are placed in tight \( \beta \)-turns (19) with flanking amino acid sequences (Tyr\textsuperscript{488}-Ser-Val-Leu; Tyr\textsuperscript{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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