Effect of pp120 on Receptor-mediated Insulin Endocytosis Is Regulated by the Juxtamembrane Domain of the Insulin Receptor*

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pp120, a substrate of the insulin receptor tyrosine kinase, does not undergo ligand-stimulated phosphorylation by the insulin-like growth factor-1 (IGF-1) receptor. However, replacement of the C-terminal domain of the IGF-1 receptor β-subunit with the corresponding segment of the insulin receptor restored pp120 phosphorylation by the chimeric receptor. Since pp120 stimulates receptor-mediated insulin endocytosis when it is phosphorylated, we examined whether pp120 regulates IGF-1 receptor endocytosis in transfected NIH 3T3 cells. pp120 failed to alter IGF-1 receptor endocytosis via either wild-type or chimeric IGF-1 receptors. Thus, the effect of pp120 on hormone endocytosis is specific to insulin, and the C-terminal domain of the β-subunit of the insulin receptor does not regulate the effect of pp120 on insulin endocytosis. Mutation of Tyr960 in the juxtamembrane domain of the insulin receptor abolished the effect of pp120 to stimulate receptor endocytosis, without affecting pp120 phosphorylation by the insulin receptor. These findings suggest that pp120 interacts with two separate domains of the insulin receptor as follows: a C-terminal domain required for pp120 phosphorylation and a juxtamembrane domain required for internalization. We propose that the interaction of pp120 with the juxtamembrane domain is indirect and requires one or more substrates that bind to Tyr960 in the insulin receptor.

The insulin receptor is essential to mediate the multiple effects of insulin on target cells (1, 2). Insulin binding to the α-subunit of its receptor activates the tyrosine kinase of the receptor in the cytoplasmic tail of the β-subunit to phosphorylate the receptor (3) and other endogenous substrates, including pp120 (4, 5), insulin receptor substrate proteins (IRS-1, -2, -3, and -4) (6–10), Shc (11, 12), and others (reviewed in Ref. 10).

Phosphorylated substrates engage in turn the formation of signaling complexes via phosphotyrosine-containing binding motifs with src homology-2 (SH2) domains (13) in order to propagate the signals of insulin in the cell. pp120 is a plasma membrane glycoprotein expressed in the liver as two spliced variants that differ by the inclusion (full-length) or exclusion (truncated) of 61 out of 71 amino acids of the cytoplasmic domain (14). In contrast to the truncated isoform, full-length pp120 undergoes insulin-stimulated phosphorylation (5). Site-directed mutagenesis revealed that phosphorylation on Ser503 by cAMP-dependent serine kinase occurs in the absence of insulin and that phosphorylation at this site is required for insulin-stimulated tyrosine phosphorylation on Tyr960, the major pp120 phosphorylation site by the insulin receptor kinase (5).

In marked contrast to insulin receptors, insulin-like growth factor-1 (IGF-1) receptors failed to mediate pp120 phosphorylation in response to IGF-1 (15). This is consistent with the predominant expression of pp120 and insulin receptors in the liver, an organ with low levels of IGF-1 receptors (16). Moreover, pp120 phosphorylation by the IGF-1 receptors was restored when the C-terminal domain of the β-subunit of the IGF-1 receptor was replaced by that of the insulin receptor, suggesting that pp120 phosphorylation by the insulin receptor is regulated by this domain. These features distinguish pp120 from other substrates, phosphorylation of which by both the insulin and the IGF-1 receptors is regulated by the juxtamembrane domain of the receptors. Thus, pp120 may mediate different physiologic functions of the two receptors, with the insulin receptor regulating metabolism (1) and the IGF-1 receptor mediating growth and differentiation (17, 18).

An important mechanism to regulate plasma insulin levels is receptor-mediated rapid vesicular endocytosis of insulin (19), followed by degradation (20). Whereas activation of the receptor kinase is required for this endocytosis (21–23), the molecular events involved in this process are not yet well defined. The juxtamembrane domain of the insulin receptor contains two tyrosine-centered sequences (Gly-Pro-Leu-Tyr963 and Asn-Pro-Glu-Tyr966) in tight β-turn structures that conform to the internalization signaling motif (24). However, the role of these sequences in insulin receptor endocytosis is controversial despite the general agreement that the juxtamembrane domain plays a significant role (22, 25). Although most reports agree that the Gly-Pro-Leu-Tyr963 sequence is required for insulin-stimulated receptor endocytosis, Berhanu et al. (26) observed that this sequence is not necessary for insulin-stimulated endocytosis of insulin receptor isoform B (exon 11+). The sequence around Tyr966 matches the internalization motif of the low density lipoprotein receptor (Asn-Pro-X-Tyr) (27) and is conserved in the IGF-1 receptor. This sequence is required for ligand-induced IGF-1 receptor endocytosis (28), but it is not required for insulin-induced insulin receptor endocytosis (25).
Instead, it is required for substrates binding such as IRS-1 and Shc (29–31). Since Tyr360 mediates binding of substrates to the insulin receptor, it is possible that these substrates play a role in insulin-mediated receptor endocytosis. However, phosphorylation of IRS-1 did not regulate insulin-receptor endocytosis in transfected Chinese hamster ovary cells (32). Shc, a substrate of the insulin and the epidermal growth factor (EGF) receptors, has been shown to bind to adaptor proteins in the clathrin coat of the endocytotic vesicles to participate in ligand-stimulated endocytosis of EGF receptors (33). Even though it may play a similar role in insulin receptor endocytosis, this hypothesis has not yet been tested.

We have observed that pp120 co-expression with insulin receptors enhanced receptor-mediated insulin endocytosis and degradation in transfected cells and that this effect required pp120 phosphorylation by the insulin receptor tyrosine kinase (34, 35). More recently, we have observed that pp120 stimulated insulin endocytosis without being directly associated with the insulin receptor. This effect required insulin-stimulated phosphorylation of pp120 by the receptor tyrosine kinase. Since pp120 phosphorylation by the insulin receptor is regulated by the C-terminal domain of the β-subunit of the receptor (15), we have examined in these studies whether this domain regulates the effect of pp120 on insulin-induced receptor endocytosis in transfected NIH 3T3 cells. We have observed that the C terminus of the β-subunit of the insulin receptor was not directly involved in regulating the stimulatory effect of pp120 on insulin endocytosis. Instead, phosphorylation on Tyr360 in the juxtamembrane domain of the receptor was required for the effect of pp120 on insulin endocytosis. This is consistent with the hypothesis that the juxtamembrane domain of the receptor is involved in insulin endocytosis, perhaps by mediating complex formation between pp120 and the insulin receptor via other signaling molecules.

**EXPERIMENTAL PROCEDURES**

**Materials—**LipofectAMINE reagent, G418 (Geneticin), and protein A-agarose were purchased from Life Technologies, Inc. Hygromycin B was purchased from Calbiochem. 125I-Inulin and 125I-IGF-1 (2000 Ci/mmol, radioimmunoassay grade), the sheep horseradish peroxidase-labeled anti-rabbit antibody, and the enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. Protease inhibitors were purchased from Boehringer Mannheim. All reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad. Human insulin was purchased from Lilly and used in insulin-free bovine serum albumin from Intergen Co. (Des Plaines, IL). Recombinant human IGF-1, monoclonal anti-phosphotyrosine (α-Tyr(P)) antibodies, polyclonal anti-IRS-1 (α-IRS-1) antibody, and fetal calf serum were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). pp120 antibodies used in these studies were previously described (5). Briefly, the monoclonal antibody used to immunoprecipitate pp120 (α-hPP120) is an identical protein to pp120 which was purified from ascites fluid from HA4 c19 cells purchased from the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City). The polyclonal antibody used to immunoblot pp120 (α-295) was raised in rabbit against a peptide (amino acids 51–64) in the extracellular domain of rat liver pp120. Ab-53, a polyclonal antibody raised in rabbit against a peptide (amino acids 1245–1343) was raised in rabbit against a peptide (amino acids 1230–1337) of the corresponding tail of the insulin receptor (amino acids 1245–1343) was described previously (38). The cDNA encoding the Y960F hIR mutant was synthesized by Kaburagi et al. (25), and resubcloned into a modified pGEM7Z plasmid that contained a glycerolphosphate kinase 1 promoter and poly(A) signals by Accili et al. (3).

**Cell Culture—**NIH 3T3 mouse skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Biofluids Inc., Rockville, MD) containing 10% fetal calf serum and 2 mM glutamine (Biofluids, Inc). Cells expressing insulin and IGF-1 receptors either alone or in addition to full-length pp120 were grown in medium supplemented with G418 (600 μg/ml, Life Technologies, Inc.) at 37 °C in 5% CO2. Cells co-expressing Y960F insulin receptors and pp120 were routinely maintained in medium supplemented with G418 (600 μg/ml) and hygromycin B (200 μg/ml).

**Transfection—**Stable transfection of NIH 3T3 cells with cDNAs encoding hIGF-1 receptors (WT and CHI) alone or in addition to full-length pp120 were described previously (15). Stable transfection of NIH 3T3 cells with cDNAs encoding Y960F hIR was achieved by the LipofectAMINE method (Life Technologies, Inc.) in the presence of 1.5 μg of the pBSV-Neo’ neo’ neomycin-resistant gene as we have previously described (34). Stable transfection of NIH 3T3 cells expressing full-length pp120 was also achieved by the LipofectAMINE method in the presence of 1.5 μg of PREP4-Hygro’ hygromycin-resistant gene. Individual clones were picked and expanded, and confluent cells were lysed in 1% Triton X-100 for analysis on 7.5% SDS-polyacrylamide (SDS-PAGE) gels and screening for pp120 expression by immunoblotting with a pp120 polypeptide antibody (α-295). As previously indicated, IGF-1 and insulin binding assays were performed on ~80% confluent cells grown in 6-well plates to screen for insulin and IGF-1 receptor expression (15, 35). Clones used in these studies typically expressed ~2.5–3.5 × 105 hIR and ~1.0–2.0 × 105 hIGF-1R per cell.

**Ligand Binding and Internalization—**As described previously (35), confluent monolayer of cells were maintained in 6-well plates in triplicate and allowed to grow to ~80% confluency. Cells were incubated overnight at 4 °C in binding buffer (100 mM Hepes, pH 7.4, 120 mM NaCl, 1.2 mM MgSO4, 1 mM EDTA, 15 mM CH3COONa, 10 mM glucose, and 1% BSA) containing 20 μl (50,000 cpm/ml) 125I-insulin or 125I-IGF-1 and incubated with prewarmed binding buffer at 37 °C for 0–90 min following removal of unbound ligand with ice-cold PBS, pH 7.4. At the end of each incubation period, cells were washed 3 × with ice-cold PBS, pH 7.4, and incubated in 0.1% BSA-supplemented PBS, pH 3.5, for 10 min. The acid wash was then collected to count acid-sensitive radioactivity that corresponds to noninternalized ligand. Cells were then washed 3 × with ice-cold PBS, pH 7.4, solubilized in 1.0 ml of ice-cold 0.4 N NaOH, 0.1% BSA for 30 min, and collected to count acid-resistant radioactivity that corresponds to internalized ligand. Specifically bound ligand was calculated as the sum of acid-sensitive plus acid-resistant ligand. Internalized insulin was calculated as percent acid-resistant per specifically bound ligand. Experiments were performed in triplicate and repeated three times on at least two different clones of each cell type. Since NIH 3T3 cells predominantly express IGF-1 binding protein-6 and some IGF-1 binding protein-1 (39) which do not associate with the cell surface and/or extracellular matrix of cultured cells, we did not expect IGF-1 binding proteins to interfere with the IGF-1 binding experiments described above.

**Statistical Analysis—**Curves were compared by a multivariate analysis of variance, and individual points were compared by paired t tests. p values of less than 0.05 were considered statistically significant.

**Biotin Labeling of Surface Membrane Proteins—**Following incubation in the absence or presence of 100 mM insulin 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), pH 7.4, supplemented with 0.1 mM CaCl2, 1 mM MgCl2, and 0.1% BSA as we have described previously (34). 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 (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml of each of the following protease inhibitors: antipain, dihydrochloride, pepstatin, leupeptin, aprotinin, leupeptin, and bactin), the samples were incubated with pp120/H4A monoclonal antibody (5), proteins were electrophoresed through 7.5% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), and immunoblotted with horseradish peroxidase (HRP)-labeled streptavidin followed by detection with enhanced chemiluminescence (ECL) as we previously described (34). The difference in the amount of biotin-labeled pp120 before and after insulin

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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. Experiments were repeated three times for each cell type to allow statistical analysis.

Phosphorylation of pp120 in Intact Cells—NIH 3T3 cells co-expressing full-length WT pp120 and insulin receptors (WT and Y960F mutant) were expanded to confluence in 100-mm plates. Following overnight incubation in serum-free Dulbecco's modified Eagle's medium containing 0.1% insulin-free BSA and 25 mM Hepes, pH 7.4, cells were treated with either buffer alone or insulin (10^(-7) M) for 5 min prior to lysis in 1% Triton X-100 in the presence of phosphatase (EDTA, 4 mM; NaF, 100 mM; sodium pyrophosphate, 10 mM; sodium phosphate, 10 mM; ATP, 2 mM; sodium orthovanadate, 20 mM; N-ethylmaleimide, 5 mM; and Hepes, 40 mM, pH 7.6), and protease inhibitors. Cell lysates were subjected to immunoprecipitation with a polyclonal antibody against IRS-1 (α-IRS-1) or a polyclonal antibody against the insulin receptor (α-IR). For pp120 immunoprecipitation, glycoproteins in the cell lysates were partially purified on wheat germ agglutinin affinity chromatography (5) prior to being subjected to immunoprecipitation with a polyclonal insulin receptor antibody and a monoclonal pp120/HA4 antibody since these two proteins do not co-immunoprecipitate.2 Following analysis on 7.5% SDS-PAGE, proteins were transferred on nitrocellulose membranes and immunoblotted with HRP-coupled α-Tyr(P) antibody to detect phosphorylated proteins by the ECL detection system (5). Contrary to previous experiments (40), the α-Tyr(P) antibody detected pp120 under these conditions. These experiments were carried out with two independent clones for each construct derived from the same transfection. Quantitation of Proteins—Autoradiograms were initially scanned on an imaging densitometer (Bio-Rad model GS-670), and the proteins were quantitated on the Image NIH version 1.59 Macintosh software program.

RESULTS

Effect of pp120 on IGF-1 Internalization (Endocytosis)—Co-expression of full-length pp120 with insulin receptors increased insulin endocytosis in NIH 3T3 cells compared with cells expressing insulin receptors alone (34, 35). To examine whether pp120 similarly regulates IGF-1 internalization, we measured internalized 125I-IGF-1 in NIH 3T3 cells expressing comparable amounts of WT IGF-1 receptors per cell with or without pp120. As Fig. 1A reveals, pp120 expression did not alter the amount of internalized 125I-IGF-1 in cells co-expressing WT hIGF-1 receptors by comparison to cells expressing WT hIGF-1 receptors alone (Fig. 1A, WT hIGF-1R/pp120 (1) versus WT hIGF-1R). Since replacement of the C-terminal domain of the β-subunit of the IGF-1 receptor with the corresponding segment of the insulin receptor restored pp120 phosphorylation by the chimeric receptor, we examined whether replacement of this domain restored the effect of pp120 on IGF-1 internalization. When stable clones expressing comparable amounts of chimeric receptors per cell with or without pp120 were examined, no significant effect of pp120 on internalized 125I-IGF-1 in cells co-expressing chimeric receptors was observed relative to cells expressing chimeric receptors alone (Fig. 1B, CHI hIGF-1R/pp120 (59) versus CHI hIGF-1R). Failure of pp120 to alter receptor-mediated IGF-1 endocytosis suggests that the effect of pp120 is specific to the insulin-insulin receptor complex. Moreover, failure to restore the effect of pp120 on IGF-1 endocytosis by replacing the C-terminal domain of the β-subunit of the IGF-1 receptor with that of the insulin receptor suggests that this domain does not regulate the effect of pp120 on receptor-mediated insulin endocytosis.

Surface Expression of pp120 in Response to IGF-1.—We have recently observed that the effect of pp120 on receptor-mediated insulin endocytosis depends on its ability to be endocytosed along with the insulin receptor.2 To test whether pp120 participated in IGF-1 receptor endocytosis, the effect of IGF-1 on surface expression of pp120 was examined in cells co-expressing IGF-1 receptors (WT and CHI). To this end, cells were treated with either insulin or IGF-1 prior to biotin labeling followed by Pronase treatment. Cells were lysed, and the proteins were immunoprecipitated with pp120/HA4 monoclonal antibody, electrophoresed, and immunoblotted with HRP-labeled streptavidin. The difference in the amount of biotin-labeled pp120 at the cell surface before and after ligand treatment was calculated as percent biotin-labeled pp120 in the absence of ligand and used as measure for the amount of pp120 internalized in response to ligand (Fig. 2, right). Consistent with our previous findings,2 the amount of biotin incorporated in the extracellular domain of full-length pp120 in cells co-expressing WT insulin receptors was substantially decreased by 65.84 ± 2.85% in response to insulin (Fig. 2, WT hIR/pp120 (10), lane 3 versus 1). In contrast, IGF-1 treatment failed to decrease the amount of biotin-labeled pp120 in cells co-transfected with WT IGF-1 receptors (Fig. 2, WT hIGF-1R/pp120 (1), lane 3 versus 1, -6.65 ± 1.69%). IGF-1 treatment did not lead to a significant decrease in surface expression of pp120 in cells co-transfected with chimeric IGF-1 receptors, as evidenced by the modest 15.16 ± 1.88% decrease in biotin-labeled pp120 in

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these cells (Fig. 2, CHI hIGF-1R/pp120 (59), lane 3 versus 1). This suggests that in contrast to insulin receptors, pp120 does not take part in the endocytosis of IGF-1 receptors.

The Effect of pp120 on Receptor-mediated Insulin Endocytosis Is Regulated by the Juxtamembrane Domain of the Insulin Receptor—Since the C-terminal domain of the β-subunit of the insulin receptor did not directly regulate the effect of pp120 on receptor-mediated insulin endocytosis (Fig. 1), the hypothesis that the juxtamembrane domain mediates the stimulatory effect of pp120 on insulin endocytosis was tested. Phosphorylation on Tyr960 in the juxtamembrane is not required for insulin-induced insulin receptor endocytosis (25). Therefore, mutating Tyr960 in the insulin receptor was not expected to alter its endocytosis in response to insulin binding. We investigated whether replacing Tyr960 by a non-phosphorylatable phenylalanine would interfere with the ability of pp120 to increase receptor endocytosis. To this end, internalized 125I-insulin was measured in NIH 3T3 cells expressing comparable amounts of Y960F insulin receptors per cell with or without pp120. Cells expressing WT insulin receptors with or without pp120 were included as controls. Consistent with our previous findings (34, 35), full-length pp120 increased internalized 125I-insulin in NIH 3T3 cells co-expressing WT insulin receptors by comparison to cells expressing WT insulin receptors alone (Fig. 3A, WT hIR/pp120(10) versus WT hIR(3006)). In contrast, co-expressing pp120 with Y960F insulin receptors did not increase the amount of internalized 125I-insulin by comparison to cells expressing Y960F insulin receptors alone (Fig. 3B, Y960F hIR/pp120(73–16) versus Y960F hIR(53)). Since the amount of internalized insulin is only slightly higher in cells expressing Y960F by comparison to cells expressing wild-type receptors, it is unlikely that the lack of effect of pp120 on insulin internalization by the Y960F receptor mutant is attributed to saturation of insulin internalization in cells expressing this mutant. This suggests that phosphorylation of the insulin receptor on Tyr960 is required for pp120 regulation of receptor-mediated insulin endocytosis.

Internalization of pp120 in Response to Insulin—We tested whether endocytosis of pp120 depends on phosphorylation of Tyr960 in the juxtamembrane domain of the receptor. To this end, the effect of insulin on surface expression of pp120 was examined in cells co-expressing Y960F insulin receptors and pp120 as described above. Consistent with our previous findings, the amount of biotin incorporated in the extracellular domain of full-length pp120 was substantially decreased upon treating cells co-expressing WT insulin receptors with insulin (Fig. 4, WT hIR/pp120(10), lane 3 versus 1). In contrast, insulin treatment failed to decrease the amount of biotin-labeled pp120 in cells co-transfected with Y960F insulin receptors (Fig. 4, Y960F hIR/pp120(73–31), lane 3 versus 1). This suggests that substituting phenylalanine for Tyr960 abolished the ability of insulin to induce a decrease in the surface expression of pp120. Thus, pp120 endocytosis is regulated by phosphorylation on Tyr960 in the juxtamembrane domain of the receptor.

Phosphorylation of pp120 by Y960F Insulin Receptors in Intact Cells—We then investigated whether pp120 phosphorylation depends on phosphorylation of Tyr960 in the juxtamembrane domain of the receptor. To this end, cells co-expressing full-length pp120 and insulin receptors (WT and Y960F) were treated with insulin, solubilized, and purified on wheat germ agglutinin-agarose affinity chromatography. The glycoprotein fraction was then subjected to immunoprecipitation with pp120 and insulin receptor antibodies, followed by electrophoresis and immunoblotting with an anti-phosphotyrosine antibody (Fig. 5A). The immunoblot was reprobed with a pp120 polyclonal antibody to account for the amount of immunoprecipitated pp120 (Fig. 5B). Corrected for the amount of insulin receptors and pp120, pp120 phosphorylation by WT insulin receptors was comparable to that by Y960F insulin receptors. This suggests that pp120 phosphorylation does not depend on the phosphorylation of Tyr960 in the juxtamembrane domain of the insulin receptor. This finding was not surprising since it had been observed that pp120 phosphorylation was regulated by the C-terminal domain of the β-subunit of the insulin receptor (15).

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**FIG. 2. Effect of IGF-1 on surface expression of pp120.** NIH 3T3 cells were stably transfected with rat liver pp120 cDNA and either wild-type or chimeric human IGF-1 receptors (WT hIGF-1R/pp120(1), CHI hIGF-1R/pp120(59), respectively). As control, NIH 3T3 cells were stably transfected with pp120 and wild-type human insulin receptors (WT hIR/pp120(10)). Numbers in parentheses denote different clone numbers. Following incubation of cells for 20 min at 37 °C in the absence (−, lanes 1 and 2) or presence (+, lanes 3 and 4) of 100 nM IGF-1 for cells expressing IGF-1 receptors or insulin for cells expressing insulin receptors, 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) at 4 °C. Cell lysates were then immunoprecipitated with a pp120/H4A monoclonal antibody, analyzed by 7.5% SDS-PAGE, and immunoprobed with HRP-labeled streptavidin. The difference in the amount of biotin-labeled pp120 before and after hormone treatment was calculated as percent biotin-labeled pp120 in the absence of ligand and used as measure for the amount of pp120 internalized in response to ligand (graph). Experiments were repeated three times for each cell type to allow statistical analysis.

**FIG. 3. Time course of insulin internalization in NIH 3T3 cells co-expressing pp120 and insulin receptors.** 125I-insulin internalization in NIH 3T3 cells expressing wild-type insulin receptors alone (WT hIR(3006), open squares) or with pp120 (WT hIR/pp120(10), closed squares), Y960F insulin receptors alone (Y960F hIR(53), open circles) or with pp120 (Y960F hIR/pp120(73–16), closed circles) was measured as described under “Experimental Procedures” and in the legend to Fig. 1. Numbers in parentheses following abbreviation of cell types denote different clone numbers. The data represent mean ± S.D. from triplicate experiments performed on at least two different clones of each cell type.
FIG. 4. Effect of insulin on surface expression of pp120. NIH 3T3 cells were stably transfected with rat liver pp120 CDNA and either wild-type or Y960F insulin receptors (WT hIR/pp120(10), Y960F hIR/pp120(73–31), respectively). Numbers in parentheses denote different clone numbers. Following incubation of cells in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 nM insulin for 20 min at 37 °C, cells were biotin-labeled and Pronase-treated as described in the legend to Fig. 2. Cell lysates were then immunoprecipitated with α-pp120/HA4 monoclonal antibody, analyzed by 7.5% SDS-PAGE, and immunoprobed with HRP-labeled streptavidin. Decrease in the amount of biotin-labeled pp120 following insulin treatment was used as measure of pp120 internalization inside the cell. Experiments were performed on at least two clones from each cell type.

FIG. 5. Phosphorylation of recombinant pp120 in intact NIH 3T3 cells. Stably transfected NIH 3T3 cells with cDNAs encoding pp120 and either wild-type or Y960F insulin receptors (WT hIR/pp120(10), Y960F hIR/pp120(73–31), respectively) were serum-starved overnight prior to incubation in the absence (+ lanes) or absence (− lanes) of insulin (10 −7 M) for 5 min. Following partial purification on affinity chromatography, proteins were immunoprecipitated with a monoclonal antibody against pp120/HA4 and a polyclonal antibody against insulin receptors, analyzed by SDS-PAGE electrophoresis, and immunoblotted with HRP-coupled anti-phosphotyrosine monoclonal antibody (α-pTyr) (A). The immunoblot was reprobed with anti-pp120 polyclonal antibody (B) to assess the level of expression of pp120. Molecular weight markers are indicated on the left-hand side of the gel. The M, ~95,000 band in A corresponds to the β-subunit of the receptors.

As control, we examined phosphorylation of endogenous IRS-1 in these transfected cells. To this end, insulin-treated serum-starved cultured cells were lysed and immunoprecipitated with antibodies against either IRS-1 (α-IRS-1) or insulin receptors (α-IR). Following electrophoresis and transfer to nitrocellulose membranes, proteins were probed with HRP-coupled anti-phosphotyrosine antibody (α-pTyr(P)). Insulin treatment caused phosphorylation of a M, ~95,000 band corresponding to the β-subunit of the insulin receptor in cells transfected with pp120 and either WT or Y960F insulin receptors (Fig. 6, IRβ, −versus − lane). Additionally, a M, ~185,000 band corresponding to IRS-1 was heavily phosphorylated in response to insulin in cells expressing WT insulin receptors. However, phosphorylation of this band was significantly decreased (by ~85%) in cells expressing Y960F insulin receptors, especially after being corrected for the amount of phosphorylated receptors in these transfected cell lines. These data are consistent with original reports on the regulation of IRS-1 phosphorylation by an intact Tyr960 in the juxtamembrane domain of the insulin receptor (29).

Fig. 6. Phosphorylation of IRS-1 in intact NIH 3T3 cells. Stably transfected NIH 3T3 cells with cDNAs encoding pp120 and either wild-type or Y960F insulin receptors (WT hIR/pp120(10), Y960F hIR/pp120(73–31), respectively) were serum-starved overnight prior to incubation in the absence (+ lanes) or absence (− lanes) of insulin (10 −7 M) for 5 min. Cell lysates were subjected to immunoprecipitation with antibodies against either IRS-1 (α-IRS-1) or insulin receptors (α-IR). Following analysis by SDS-PAGE, proteins were transferrered on nitrocellulose membrane for immunoblotting with HRP-coupled α-pTyr(P) antibody, and detection by the ECL system. Molecular mass markers are indicated between panels.

DISCUSSION

pp120, a substrate of the insulin receptor tyrosine kinase in the hepatocyte, stimulates receptor-mediated insulin endocytosis in transfected cells. Consistent with our previous observations that pp120 failed to regulate receptor-mediated endocytosis of platelet-derived growth factors (34), we have observed in the current studies that the effect of pp120 is specific to the insulin receptor insofar as pp120 failed to regulate IGF-1 receptor endocytosis in response to IGF-1. Since IGF-1 is not cleared in the liver as insulin is, the specific effect of pp120 on insulin endocytosis and degradation proposes a specific physiologic role for pp120 in regulating the cell sensitivity to insulin.

Since IGF-1 receptor does not phosphorylate pp120 in response to IGF-1 (15), failure of pp120 to stimulate receptor-mediated IGF-1 endocytosis supports our previous observations that the effect of pp120 on insulin endocytosis requires pp120 phosphorylation by the insulin receptor tyrosine kinase (34). However, restoration of pp120 phosphorylation by IGF-1 receptors upon replacing the C-terminal domain of the α-subunit of the receptor with that of the corresponding segment of the insulin receptor did not confer on pp120 the ability to stimulate endocytosis of IGF-1 via this chimeric receptor. Thus, pp120 phosphorylation by the receptor is required but not sufficient to mediate its effect on hormone endocytosis. This is consistent with our recent report that pp120 differentially increased insulin endocytosis via the high rather than the low affinity insulin receptor isoform despite being equally phosphorylated by both isoforms (35). Thus, an additional molecular mechanism must underlie the effect of pp120 on insulin endocytosis.

In this report, we have observed that Tyr960 in the juxtamembrane of the insulin receptor regulated the effect of pp120 on insulin endocytosis but not its phosphorylation by the insulin receptor. Since association between pp120 and the insulin receptor appears to be mediated by other signaling molecules,2 we propose a model in which insulin receptors phosphorylate pp120 through the C-terminal domain of the β-subunit. The phosphorylated pp120, in turn, engages one or more molecules that associate with the insulin receptor via Tyr960 in the juxtamembrane domain of the insulin receptor. This complex is required for insulin-mediated receptor endocytosis. The role of pp120 is probably to stabilize this complex and possibly to mediate its association with structural components of the clathrin-coated pits, such as adaptor proteins-2, since it
contains tyrosine-centered sequences (Tyr 488-Ser-Val-Leu and Tyr 513-Ser-Val-Val) known to target proteins to adaptor protein-2 (24). Insulin receptor substrates that bind to this Tyr 560 residue include IRS-1, Shc, GTPase-activating protein-1 (41), and Grb2-associated binding proteins-1 (42). A role for IRS-1 in receptor-mediated insulin endocytosis has been ruled out (32), and a role for GTPase-activating protein-1 and Grb2-associated binding protein-1 in ligand-induced endocytosis of receptors has not yet been identified. Even though a role for Shc in insulin-induced endocytosis of insulin receptors is not yet known, its role in ligand-induced endocytosis of EGF receptors has been documented (33). More studies are required to identify which of these molecules, if any, is involved with pp120 in the formation of the protein complex required for insulin receptor endocytosis.

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