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Insulin-Stimulated Phosphorylation of Recombinant pp120/HA4, an Endogenous Substrate of the Insulin Receptor Tyrosine Kinase

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ABSTRACT: Insulin binding to the α-subunit of its receptor stimulates the receptor tyrosine kinase to phosphorylate the β-subunit and several endogenous protein substrates, including pp120/HA4, a liver-specific plasma membrane glycoprotein of M_r 120 000. Analysis of the deduced amino acid sequence of rat liver pp120/HA4 revealed two potential sites for tyrosine phosphorylation in the cytoplasmic domain (Tyr^{488} and Tyr^{513}), as well as a potential cAMP-dependent protein kinase phosphorylation site (Ser^{503}). To determine which of these sites is phosphorylated in response to insulin, each of these amino acid residues was altered by site-directed mutagenesis. Mutant cDNAs were then expressed by stable transfection in NIH 3T3 cells. Two mutations (Phe^{488} and Ala^{503}) impaired insulin-induced phosphorylation of pp120/HA4, suggesting that pp120/HA4 undergoes multisite phosphorylation. It seems likely that Tyr^{488} is phosphorylated by the insulin receptor kinase, and phosphorylation of Ser^{513} may contribute to the regulation of tyrosine phosphorylation. Since pp120/HA4 is believed to be associated with a Ca^{2+}/Mg^{2+}-dependent ecto-ATPase activity, we determined the effects of insulin-induced phosphorylation on this enzymatic activity. In NIH 3T3 cells co-expressing the insulin receptor and pp120/HA4, insulin caused a 2-fold increase in ecto-ATPase activity. Moreover, elimination of the phosphorylation sites of pp120/HA4 impaired the ability of insulin to stimulate the ecto-ATPase activity. These data suggest that tyrosine phosphorylation of pp120/HA4 may regulate Ca^{2+}/Mg^{2+}-dependent ecto-ATPase activity.

The tyrosine kinase of the insulin receptor plays an important role in mediating insulin action. Activation of the receptor kinase by insulin leads to phosphorylation of several intracellular protein substrates (Taylor *et al.*, 1991; Roth *et al.*, 1992; White & Kahn, 1994) including pp120/HA4, a transmembrane glycoprotein of M_r 120 000 (Rees-Jones & Taylor, 1985; Perrotti *et al.*, 1987). When the partial amino acid sequence of pp120/HA4 was determined (Margolis *et al.*, 1990), it was found to be identical to the deduced amino acid sequence of a membrane glycoprotein encoded by a cDNA previously cloned by Lin and Guidotti (1989).

The putative cytoplasmic domain of pp120/HA4 contains two potential sites for tyrosine phosphorylation (Tyr^{488} and Tyr^{513}), as well as a potential cAMP-dependent protein kinase phosphorylation site (Lys-Arg-Pro-Thr-Ser^{503}) (Lin & Guidotti, 1989; Sippel *et al.*, 1994b). To determine which of these sites is a target for insulin-dependent phosphorylation, we removed the potential phosphorylation sites by site-directed mutagenesis. Mutant cDNAs were then expressed by stable transfection in NIH 3T3 mouse fibroblasts, co-expressing human insulin receptor cDNA. Mutation of either Tyr^{488} or Ser^{503} abolished insulin-stimulated phosphorylation. This observation suggests that pp120/HA4 is a target for multisite phosphorylation by the insulin receptor kinase and perhaps other protein kinases as well. Furthermore, it is likely that there are important regulatory interactions among the multiple phosphorylation sites.

Although the function of the molecule remains controversial, pp120/HA4 has been reported to function as a bile acid transport protein (Sippel *et al.*, 1993) and as a Ca^{2+}/Mg^{2+}-dependent ecto-ATPase (Lin & Guidotti, 1989). In this study, we assayed ecto-ATPase activity in cells expressing pp120/HA4 and also investigated the effect of insulin upon ecto-ATPase activity. Expression of pp120/HA4 led to a relatively small increase in ecto-ATPase activity in NIH-3T3 cells. However, when cells were cotransfected with cDNAs encoding both pp120/HA4 and human insulin receptors, the cells acquired the ability to respond to insulin with an increase in ecto-ATPase activity. While these observations are consistent with the hypothesis that pp120/HA4 is related to hepatic Ca^{2+}/Mg^{2+}-dependent ecto-ATPase (Lin & Guidotti, 1989), they do not constitute proof that the protein possesses catalytic activity. For example, it remains possible that pp120/HA4 is a regulatory subunit of Ca^{2+}/Mg^{2+}-dependent ecto-ATPase.

EXPERIMENTAL PROCEDURES

Materials. The monoclonal antibody used to immunoprecipitate pp120/HA4 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). This monoclonal was originally raised against epitopes on the extracellular domain of HA4 (Hubbard *et al*, 1985), a bile canalicular glycoprotein from rat hepatocytes believed to be identical to pp120/HA4 (Margolis *et al.*, 1988). Coupling of the antibody to CNBr-activated Sepharose (Pharmacia) was performed according to the manufacturer's instructions. The pp120/HA4 antibody that was used in immunoblotting (α-295) is an antipeptide

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polyclonal antibody that was raised in rabbits against the peptide 51-VLLLAHNLPQEFQV-64 in the extracellular domain of the rat liver Ca^{2+}/Mg^{2+}-dependent ecto-ATPase based on the deduced amino acid that was reported by Lin and Guidotti (1989). Ab-50 was raised in rabbits against the C-terminus of the human insulin receptor (hIR) (Cama *et al*, 1988). Protease inhibitors, restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). ATP, CTP, *N*-acetyl-D-glucosamine, Triton X-100, and other reagents used in the phosphorylation assays were purchased from Sigma (St. Louis, MO). All reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). All reagents for the glutathione *S*-transferase (GST) fusion protein system were purchased from Pharmacia, Inc. (Piscataway, NJ). Human insulin was purchased from Lilly, Indianapolis, IN, and insulin-free BSA from Intergen Co. (Des Plaines, IL).

Construction of the Expression Vectors. The cDNA molecules encoding the full-length or the truncated isoforms of pp120/HA4 (Najjar *et al.*, 1993) were ligated into a bovine papilloma virus-based expression vector (pBPV, Pharmacia) at the *Xho*I/*Not*I sites. Site-directed mutagenesis was accomplished by amplification of a fragment of pp120/HA4 cDNA (nt 1079−1574) in a PCR reaction using the wild-type cDNA as a template and primers encoding substitution of Ala for Ser^{503} or Phe for either Tyr^{488} and/or Tyr^{513}. The PCR products were then subcloned into the pGEM-4Z plasmid containing pp120/HA4 cDNA by replacing a fragment of wild-type cDNA (nt 1079−1574) at the *Pst*I/*Not*I sites. The resulting mutant cDNAs were excised from the pGEM-4Z plasmid and subsequently ligated into the pBPV vector at the *Xho*I/*Not*I sites.

Cell Culture and Transfection. NIH 3T3 cells were maintained in Dulbecco-modified Eagle's medium (DMEM, Biofluids Inc., Rockville, MD) containing 10% fetal calf serum (UBI, Lake Placid, NY), 100 units/mL penicillin (Sigma), and 10 μg/mL streptomycin (Sigma) at 37 °C/5% CO_2. Approximately 10^7 cells were trypsinized, washed, and transferred to electroporation cuvettes (Bio-Rad) in 500 μL of phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ (100 mg/L of each) (Advanced Biotechnologies Inc., Columbia, MD). pBPV-hIR and pBPV-pp120/HA4 (15 μg of each) were then transfected by electroporation (Bio-Rad) into the cells in the presence of 1.5 μg of RSV-Neo (0.22 kV, 960 μF). After 48 h in regular medium, the cells were cultured for 2−3 weeks in medium containing G-418 (600 μg/mL, geneticin, Gibco, Gaithersburg, MD). Individual clones were picked and expanded in 12-well plates. Confluent cells were washed on ice in PBS buffer, frozen and thawed on liquid nitrogen, and lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Hepes, pH 7.6, 1 mM PMSF, and 10 μg/mL of each of the following protease inhibitors: antipain dihydrochloride, pepstatin A, leupeptin, aprotinin, bacitracin). Following centrifugation at 12000*g* for 30 min at 4 °C, the supernatants were collected, the protein concentration was measured by the Bio-Rad assay (Bio-Rad), and proteins (10 μg) were electrophoresed through 7.5% SDS−polyacrylamide gels after boiling in SDS solubilization buffer for 3 min. Following transfer to nitrocellulose paper (Schleicher and Schuell, Keene, NH), the proteins were immunoblotted with either α-295 (an anti-pp120/HA4 polyclonal antibody) or Ab-50 (an anti-hIR polyclonal antibody), and the antigen−antibody complex was then detected by a horseradish peroxidase-labeled anti-rabbit antibody raised in sheep (Amersham Life Science, Arlington Heights, IL) followed by the Enhanced Chemicoluminescence reaction (ECL, Amersham). In addition, insulin binding in intact cells was measured to screen for the expression of the insulin receptor in these cells.

In Vitro Phosphorylation. NIH 3T3 cells expressing pp120/HA4 cDNA were expanded in T^-75 flasks and lysed in Triton X-100. The glycoproteins were partially purified by affinity chromatography over wheat germ agglutinin (WGA)−agarose and eluted in 0.3 M *N*-acetylglucosamine, 0.1% Triton X-100, 150 mM NaCl, 50 mM Hepes, pH 7.6, and 10 μg/mL protease inhibitors (Hedo *et al.*, 1981). Lectin-purified glycoproteins (20 μg) were allowed to bind to insulin (10^{-7} M) in the presence of 15 μg of WGA-purified hIR (from NIH 3T3 cells overexpressing hIR) in a total volume of 200 μL for 1 h at room temperature. Phosphorylation was initiated by adding 75 μL of a solution containing $MgCl_2$ (75 mM), CTP (1 mM), ATP (50 μM), and 100 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, NEN-Dupont, Wilmington, DE). After 15 min at room temperature, the reaction was stopped at 4 °C in the presence of 70 μL of a phosphatase inhibitor cocktail (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). PMSF (1 mM) was then added, and immunoprecipitation was carried out immediately (Zick *et al.*, 1983). Aliquots (170 μL) of the proteins in the phosphorylated mixture were then separately immunoprecipitated with either Ab-50 (anti-hIR) or HA4 antibody (anti-pp120/HA4). The resulting immunoprecipitates were subjected to 7.5% SDS−PAGE, blotted onto a nitrocellulose paper for autoradiography and subsequent immunoblotting with antibodies as specifically mentioned in the figure legends. Experiments were carried out with at least three independent clones for each construct derived from the same transfection.

Insulin-Induced Phosphorylation of pp120/HA4 in Intact Cells. NIH 3T3 cells co-expressing hIR and various isoforms of pp120/HA4 were expanded to confluence in 10 cm plates. Following overnight incubation in serum-free DMEM containing 0.1% insulin-free BSA and 25 mM Hepes (pH 7.4), the cells were incubated in 4 mL of phosphate- and serum-free DMEM medium for 90 min. [^{33}P]Orthophosphate (800 μCi; Dupont-NEN) was added to label the cells for 90 min at 37 °C. Thereafter, cells were incubated in the presence or absence of insulin (10^{-7} M) for 3 min (unless otherwise noted), and the phosphorylation reaction was stopped by quickly aspirating the medium, washing the ice-cold PBS, freezing on liquid nitrogen, and thawing on ice in the presence of 1 mL of Triton X-100 lysis buffer containing protease and phosphatase inhibitors as described above. Following partial purification on WGA−agarose columns, equal amounts of solubilized proteins were specifically immunoprecipitated by either the anti-IR polyclonal (Ab-50) or the anti-pp120/HA4 monoclonal antibody (HA4). The immunoprecipitate was boiled in SDS solubilization buffer and subjected to electrophoresis on SDS−PAGE, followed by transfer onto nitrocellulose paper for autoradiography and immunoblotting. When sequential reimmunoblotting with another antibody was carried out, the previously employed antibody was removed by washing the blot in 100 mM DTT,

2% SDS, and 62.5 mM Tris (pH 6.7) buffer for 30 min at 47 °C, the nonspecific binding was blocked with 2% gelatin, and reimmunoblotting was performed with a new antibody. These experiments were carried out with at least three independent clones for each construct derived from the same transfection.

Ecto-ATPase Assay. Transfected and untransfected NIH 3T3 cells were grown in triplicate to confluence (2×10^5 cells) in DMEM (Biofluids, Inc., MD). Following overnight incubation in serum-free DMEM−25 mM Hepes (pH 7.4) (Advanced Biotechnologies Inc.) supplemented with 0.1% insulin-free BSA, the serum was replaced by 1.0 mL of fresh serum-free medium in order to remove the floating dead cells. Five hours later, 100 μL of insulin (10^{-7} M) was added in the presence of 50 μM ATP, 0.02 μCi of [γ-^{32}P]ATP, and 5 mM sodium azide to block interference from intracellular ATPases. Following incubation for 15 min at 37 °C, 200 μL was then removed from the medium and placed on 500 μL of ice-cold 20% (w/v) HCl-activated charcoal (Sigma) for 15 min (Filippini *et al.*, 1990). Unhydrolyzed [γ-^{32}P]-ATP was trapped on charcoal and sedimented by centrifugation (12000*g*, 15 min, 4 °C). Aliquots (100 μL) of the supernatant containing hydrolyzed [^{32}P]P_i were then removed and counted in a Beckman scintillation counter. In some experiments, the 100 μL aliquot was absorbed on a 1.5 × 1.5 cm Whatman 3 MM Chr filter paper soaked in 5.7% trichloroacetic acid, 2.9% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 29 mM triethylamine hydrochloride (Sigma) to precipitate hydrolyzed [^{32}P]P_i and minimize possible contamination by residual floating charcoal-trapped unhydrolyzed nucleotides. The pellet was then washed in 1.5% trichloroacetic acid, 0.74% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 7.4 mM triethylamine hydrochloride (Reimann *et al.*, 1978). No significant difference was detected between direct counting and the TCA/molybdate precipitation method. Duplicate wells were seeded with cells either for the determination of protein concentration (Bio-Rad) or for biotinylation with ImmunoPure NHS-LC-Biotin (Pierce), followed by immunoprecipitation of labeled pp120/HA4 with the monoclonal anti-pp120/HA4 antibody and immunoblotting with streptavidin-conjugated secondary antibody (Levy-Toledano *et al.*, 1993) to determine the level of pp120/HA4 expression on the cell surface. All experiments were carried out in triplicate with four independent clones for each construct.

Expression of the Intracellular Domain of pp120/HA4 in the GST Fusion Protein System. The cDNA fragment (nt 1354−1574) encoding the majority of the cytoplasmic tail of the pp120/HA4 (aa 452−519) was amplified in a PCR reaction by using the wild-type cDNA recombinant carried in the pGEM-4Z vector (see above) as a template and sense (s-1354, tcctaaggatccACTGGCGGGGGAAGT-1368) and antisense (α-1574, acaggaattcCAGGACAGACAATGTTAC-1557) primers. The sense oligonucleotide contained a *Bam*HI and the antisense primer an *Eco*RI restriction site to allow for the in-frame subcloning of the cDNA product into the glutathione *S*-transferase (GST) gene fusion vector (pGEX-1λT, Pharmacia). The added restriction enzyme sites are shown in lower case letters.

Purification of the GST Fusion Proteins. Competent *Escherichia coli* HB101 cells (Gibco BRL, Life Technologies) were transformed with the GST fusion pGEX-1λT vector as described in Smith and Johnson (1988) with some modifications. *E. coli* cells were grown in 100 mL of LB broth in the presence of 100 μg/mL ampicillin at 37 °C, subsequently transferred to 900 mL of fresh LB-ampicillin, and allowed to grow for an hour before induction with 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were grown to mid-log phase (0.5 OD_{600}), centrifuged at 10000*g* for 15 min at 4 °C, and resuspended in 100 mL of buffer A [50 mM Tris-HCl, 0.5 mM EDTA, 300 mM NaCl, pH 7.5, 10 μg/mL of each of the protease inhibitor cocktail (see above), and 1 mM PMSF]. Following centrifugation at 8000*g* for 15 min at 4 °C, the cells were then resuspended in 10 mL of buffer A supplemented with 1 mM DTT and 1 mg/mL lysozyme and placed on ice for 10 min prior to freezing on liquid nitrogen and thawing on ice. Five milliliters of buffer B (buffer A supplemented with 1 mM DTT, 1.5% Triton X-100, 30 mM $MgCl_2$, and 0.2 mg/mL DNase I) was added, and the mixture was placed on ice for 10 min before centrifugation at 35000*g* at 4 °C for 30 min. The supernatant was then collected and allowed to bind to 2.5 mL of 50% reduced glutathione Sepharose-4B beads in buffer C (20 mM Hepes, pH 7.4, 25 mM NaCl, 0.05% Triton X-100, 10% glycerol) supplemented with 10 mM DTT at 4 °C for at least 90 min.

Elution of the GST-pp120/HA4 Fusion Protein. Ten milliliters of slurry was packed into a 1 cm × 10 cm column in buffer C. Following washing to remove nonspecifically bound proteins in 3−4 volumes of buffer C, the GST fusion proteins were eluted in 100 mM of reduced glutathione prepared in EDTA-free buffer C, by collecting 20 fractions of 0.5 mL each.

Phosphorylation of the GST-pp120/HA4 Fusion Protein. Recombinant hIR (3 μg of protein; partially purified from NIH 3T3 cells overexpressing hIR by WGA−agarose affinity chromatography) was incubated in the presence or absence of insulin (10^{-7} M) and assayed for the ability to phosphorylate GST fusion proteins (1 or 10 μg) containing fragments of wild-type or mutant pp120/HA4. The phosphorylation reaction was immediately initiated by the addition of 25 μM ATP, 100 μCi of [γ-^{32}P]ATP, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM CTP in 0.1% Triton X-100, 150 mM NaCl, and 50 mM Hepes, pH 7.6, for 20 min at room temperature. The reaction was terminated on ice by the addition of phosphatase and protease inhibitors (see above) and immediately boiling in SDS solubilization buffer prior to analysis by 12% acrylamide SDS−PAGE. These experiments were repeated at least three times.

Measurement of Radioactive Label Incorporation. The Phosphorlmager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the level of incorporation of ^{32}P (in vitro phosphorylation) or ^{33}P (in situ phosphorylation in intact cells) in the pp120/HA4 bands. The relative effect of insulin stimulation was then measured as a percentage of the basal state in the absence of insulin.

RESULTS

In Vitro Phosphorylation of pp120/HA4. Partially purified recombinant human insulin receptors (from NIH 3T3 cells overexpressing hIR) were allowed to phosphorylate partially purified wild-type or mutant pp120/HA4 (from NIH 3T3 cells overexpressing pp120/HA4) in the presence or absence of insulin (10^{-7} M) for an hour at room temperature in the presence of [γ-^{32}P]ATP. The samples were immunoprecipitated with antibody directed against either pp120/HA4

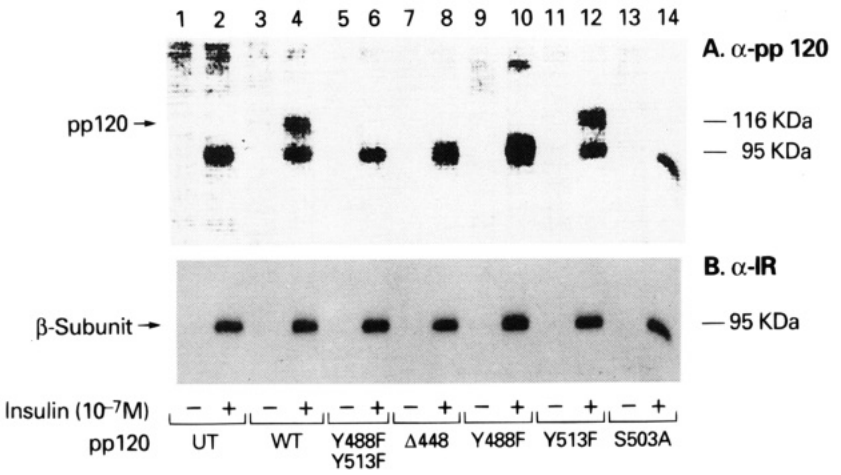

FIGURE 1: In vitro phosphorylation of pp120/HA4. Cell extracts were prepared from untransfected (UT) NIH 3T3 cells (lanes 1 and 2), from cells transfected with wild-type pp120/HA4 (WT; lanes 3 and 4) or mutant forms of pp120/HA4 (lanes 5−14), and from NIH 3T3 cells transfected with a human insulin receptor cDNA. Following affinity purification of glycoproteins on a lectin column, equal amounts of insulin receptors were added to extracts containing wild-type and mutant pp120 isoforms. Phosphorylation assays were carried out by incubating the extracts with [γ-^{32}P]ATP in the absence (odd numbered lanes) or presence of 10^{-7} M insulin (even numbered lanes). Following addition of phosphatase inhibitors, phosphorylated proteins were immunoprecipitated with either a monoclonal antibody directed against pp120/HA4 (panel A) or a polyclonal antipeptide antiserum raised against a carboxy-terminal peptide of the human insulin receptor sequence (panel B). Molecular mass markers are shown at the right-hand side of each panel. As discussed in the text, the identity of the M_r ~95 000 band in panel A is not known, but appears not to be the β-subunit of the insulin receptor. These experiments were carried out with at least three independent clones for each construct derived from the same transfection.

(Figure 1A) or human insulin receptor (Figure 1B), and the immunoprecipitates were analyzed by SDS−PAGE. As expected, insulin stimulated autophosphorylation of the insulin receptor (Figure 1B). In addition, insulin increased the phosphorylation of a M_r ~ 120 000 band corresponding to recombinant wild-type pp120/HA4 (Figure 1A, lane 4 vs 3) by approximately 10-fold relative to untreated cells. Deletion of the intracellular domain (Δ448) abolished the insulin-induced phosphorylation of pp120/HA4 (Δ448; Figure 1A, lanes 7 and 8). Thus, even in a cell-free system, the major phosphorylation sites are located in the intracellular domain of pp120/HA4. Substitution of Phe for Tyr^{488} completely inhibited insulin-stimulated phosphorylation of pp120/HA4 (Y488F; Figure 1A, lane 10 vs 9). This suggests that Tyr^{488} is a major site for phosphorylation by the insulin receptor. In contrast, substitution of Phe for Tyr^{513} (Y513F) did not impair the ability of insulin to stimulate phosphorylation of pp120/HA4 (Figure 1A, lane 12 vs 11), suggesting that Tyr^{513} does not constitute a major phosphorylation site in pp120/HA4. As expected, substitution of Phe for both Tyr^{488} and Tyr^{513} in a double mutant completely inhibited insulin-stimulated phosphorylation of pp120/HA4 (Y488F/Y513F; Figure 1A, lane 6 vs 5). These differences were not due to differences in the quantity of insulin receptors as evidenced by the observation of identical levels of phosphorylated hIR in all samples (Figure 1B, even lanes). Substitution of alanine for Ser^{503} (S503A) completely inhibited insulin-stimulated phosphorylation of pp120/HA4 despite the presence of potential phosphorylation sites at Tyr^{488} and Tyr^{513} (Figure 1A, lane 14 vs 13). Equal amounts of immunoprecipitable material from each pp120/HA4 isoform were applied to the phosphorylation assay in the absence and presence of insulin (data not shown). Identical results were obtained with multiple clones derived from independent transfections for each construct.

Moreover, insulin stimulated the phosphorylation of an additional species with M_r ~95 000 that was immunoprecipitated by monoclonal antibody directed against pp120/HA4 (Figure 1A, even numbered lanes). Because the M_r ~95 000 band was detected in cells that had not been transfected with pp120/HA4 (Figure 1A, lanes 1 and 2), it is unlikely that the presence of the band in the immunoprecipitate can be explained by binding to pp120/HA4. We have not established the identity of this M_r ~95 000 protein immunoprecipitated by anti-pp120/HA4 antibody. Nevertheless, when the immunoblots were probed with antibody directed against the hIR β-subunit, this M_r ~95 000 band was not detected (data not shown). Thus, although it has the same apparent molecular mass as the β-subunit of the insulin receptor, this M_r ~95 000 band is not the insulin receptor.

Phosphorylation of the GST-pp120/HA4 Fusion Protein Encoding the Intracellular Domain of pp120/HA. The majority of the intracellular domain of pp120/HA4 (amino acid residues 452−519) was expressed as a glutathione *S*-transferase fusion protein in *E. coli* (data not shown). We investigated whether partially purified hIR could phosphorylate this GST fusion protein in the absence or presence of 10^{-7} M insulin (Figure 2). Under these conditions, insulin led to a 2-fold increase in phosphorylation of the β-subunit of the insulin receptor (M_r ~95 000) (Figure 2A, lanes 2 vs 1 and lanes 4 vs 3; Figure 2C, lane 6 vs 5). Similarly, insulin led to a 70−130% increase in the phosphorylation of a M_r ~32 000 species, corresponding to the GST-pp120/HA4 fusion peptide (Figure 2B). In contrast, the GST protein lacking sequences from pp120/HA4 did not serve as a substrate for phosphorylation by the insulin receptor tyrosine kinase (Figure 2C, lane 6 vs 5). Thus, the extracellular domain of pp120/HA4 was not required for the cytoplasmic domain to undergo phosphorylation by the insulin receptor.

Phosphorylation of Recombinant pp120/HA4 in Intact NIH 3T3 Cells. NIH 3T3 cells co-expressing recombinant hIR and pp120/HA4 were incubated in the absence (Figure 3, lanes 1 and 2) or presence (Figure 3, lanes 3−8) of insulin (10^{-7} M). The phosphorylation reaction was allowed to proceed for 0−30 min at 37 °C. The cells were then

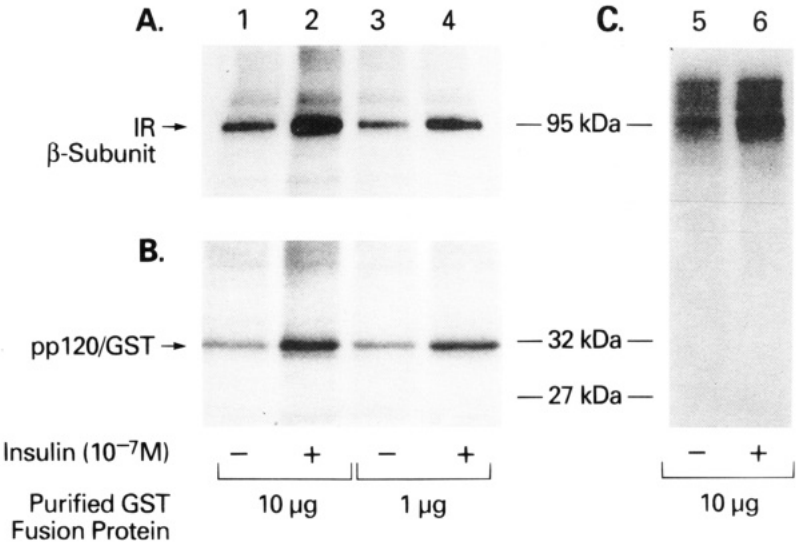

FIGURE 2: Phosphorylation of the GST-pp120/HA4 fusion protein encoding the intracellular domain of pp120/HA4. The intracellular domain of pp120/HA4 (aa 452−519) was expressed as a glutathione *S*-transferase fusion protein in *E. coli* and purified on glutathione Sepharose-4B. Partially purified insulin receptor (3 μg) was then added to either 10 μg (lanes 1 and 2) or 1 μg (lanes 3 and 4) of the purified GST fusion protein, or 10 μg of GST plasmid control (panel C, lanes 5 and 6). In vitro phosphorylation was then initiated by the addition of [γ-^{32}P]ATP in the absence (odd numbered lanes) or presence (even numbered lanes) of 10^{-7} M insulin. Phosphorylated proteins were then subjected to analysis on 12% SDS−PAGE. Panels A and B are from the same SDS−polyacrylamide gel, differing in the exposure time of the autoradiogram for optimal visualization of both bands [panel A (30 min), panel B (3 h)]. These experiments were repeated at least three times.

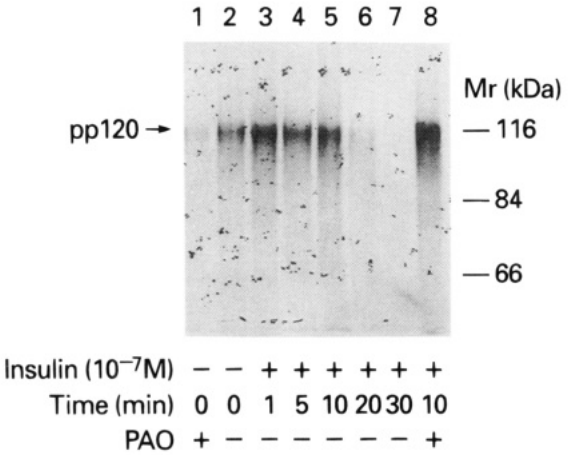

FIGURE 3: Time course of insulin-stimulated phosphorylation of pp120/HA4 in intact NIH 3T3 cells. NIH 3T3 cells co-expressing the insulin receptor and wild-type pp120 were incubated in phosphate-free medium for 90 min and were subsequently incubated in the presence of [^{33}P]orthophosphoric acid for 90 min. Insulin (10^{-7} M) was added (lanes 3−8), and the incubation was extended for variable periods of time (0−30 min) as indicated at the bottom of the figure. In some experiments, phenylarsine oxide (PAO) was added for 5 min prior to the addition of insulin (lanes 1 and 8). Detergent extracts were immunoprecipitated with anti-HA4 monoclonal antibody and analyzed by 7.5% SDS−PAGE followed by autoradiography. These experiments are representatives of two independent determinations on clone F10 and one determination on clone F8, two independent clones co-expressing wild-type pp120/HA4 and insulin receptors.

solubilized in Triton X-100, and the extract was affinity purified on WGA−agarose. The eluate was immunoprecipitated with the monoclonal anti-pp120/HA4 antibody, and the immunoprecipitate was analyzed by SDS−PAGE followed by autoradiography. Within 1 min, insulin increased the phosphorylation of pp120/HA4 (Figure 3, lane 3) approximately 2.5-fold; this increase was sustained for up to 10 min (Figure 3, lanes 4 and 5). Thereafter, the level of phosphorylation decreased even below the basal level, possibly due to activation of cellular phosphotyrosine phosphatases. Some cells were pretreated for 5 min with 25 μM phenylarsine oxide (PAO), a tyrosine phosphatase inhibitor (Pronk *et al.*, 1992), prior to stimulation with insulin (Figure 3, lanes 1 and 8). Under these conditions, PAO enhanced the effect of insulin to stimulate incorporation of [^{33}P]-phosphate (Figure 3, lane 8 vs 5). In contrast, PAO decreased the ^{33}P content of pp120/HA4 in cells incubated in the absence of insulin. Because basal phosphorylation of pp120/HA4 represents primarily serine phosphorylation (Perrotti *et al.*, 1987; Sippel *et al.*, 1994b), the effect of PAO upon basal phosphorylation probably represents a decrease in serine phosphorylation.

To investigate the sites of insulin-stimulated phosphorylation of pp120/HA4 in intact cells, NIH 3T3 cells were cotransfected with hIR cDNA and site-directed mutants of pp120/HA4. After transfected cells were loaded with [^{33}P]-orthophosphate, the cells were incubated in the presence or absence of insulin (10^{-7} M) for 5 min at 37 °C. Cell lysates were partially purified by WGA affinity chromatography, and the eluates were immunoprecipitated with anti-pp120/HA4 antibody. We did not detect expression of pp120/HA4 in nontransfected cells either by ^{33}P-labeling (Figure 4A, lanes 11 and 12; Figure 5, lanes 1 and 2) or by immunoblotting (Figure 4B). However, in intact cells expressing recombinant pp120/HA4, insulin increased ^{33}P content of wild-type pp120/HA4 by 42% in the experiment (Figure 4A, lanes 9 and 10) or 48% in the experiment (Figure 5, lanes 3 and 4). Substitution of Phe for Tyr^{488} (Y488F) almost completely inhibited both basal and insulin-stimulated phosphorylation of pp120/HA4 (Figure 4A, lane 5 vs 6), suggesting that Tyr^{488} is a major site of phosphorylation. In contrast, insulin induced a 49% increase over basal in the phosphorylation of the Y513F mutant (Figure 4A, lane 3 vs 4). The observation that Y513F mutant pp120/HA4 retains the ability to undergo insulin-stimulated phosphorylation suggests that Tyr^{513} is not a significant phosphorylation site

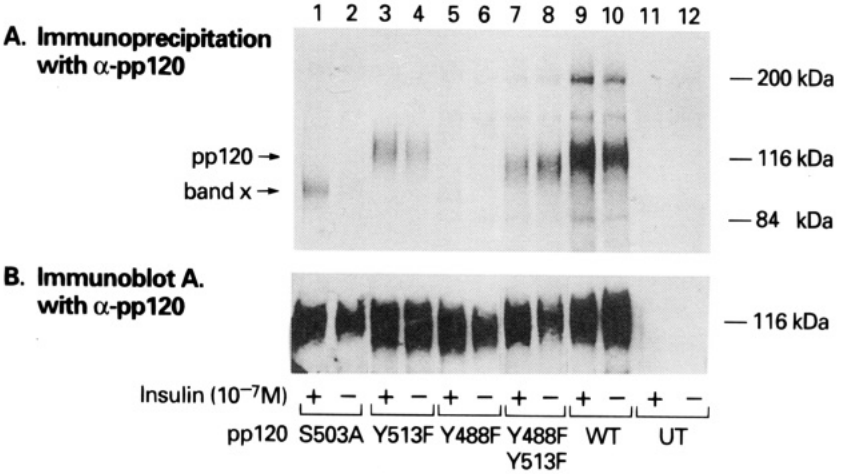

FIGURE 4: Phosphorylation of recombinant pp120/HA4 in intact NIH 3T3 cells. Stably transfected NIH 3T3 cells were phosphorylated as described in the legend to Figure 3 with the exception that cells were incubated in the presence or absence of insulin (10^{-7} M) for 5 min. Following SDS−PAGE electrophoresis, the proteins were transferred on nitrocellulose paper, subjected to autoradiography (panel A). Thereafter, the blots were probed with anti-pp120/HA4 polyclonal antibody (panel B). The ^{33}P content associated with the pp120/HA4 bands were quantitated with a Phosphorlmager. The relative effect of insulin was calculated by dividing the difference between ^{33}P content in the presence and absence of insulin by the ^{33}P content in absence of insulin. Except for the untransfected NIH 3T3 cells (UT, lanes 11 and 12), all cell lines were stably transfected with human insulin receptor plus either wild-type (WT, lanes 9 and 10) or various mutant forms of pp120/HA4 (S503A, lanes 1 and 2; Y513F, lanes 3 and 4; Y488F, lanes 5 and 6; Y488F/Y513F, lanes 7 and 8). The immunoblot in panel B shows the level of expression of pp120/HA4. The band migrating at M_r ~95 000 kDa in lane 1 (labeled band x) was also detected on the same nitrocellulose sheet using an antibody against the β-subunit of the insulin receptor (data not shown), suggesting that band x is the insulin receptor. The fact that band x was observed only in lane 1 suggests that the S503A mutation leads to an increased association of pp120/HA4 with the insulin receptor. Moreover, the decreased efficiency of immunoprecipitation of the Y488F pp120 mutant (lane 6, −insulin) was not consistent among the three independent clones that were examined in these experiments. At least three independent clones for each construct derived from the same transfection were employed in these experiments.

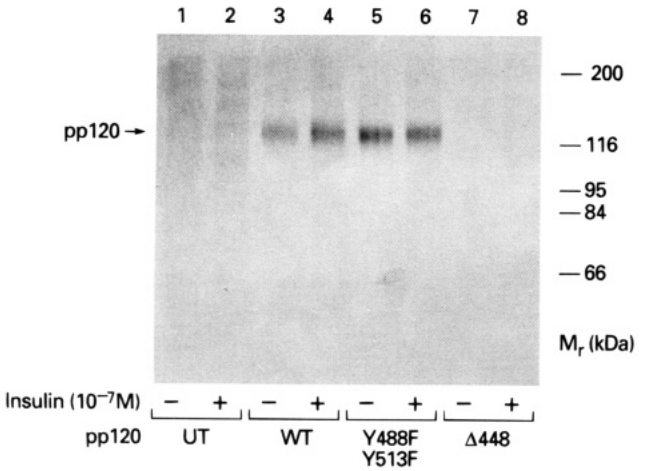

FIGURE 5: The truncated isoform of pp120/HA4 is not phosphorylated in intact NIH 3T3 cells. Cells that had been prelabeled with [^{33}P]orthophosphate were incubated in the presence or absence of insulin (10^{-7} M) as described in the legends to Figures 3 and 4. Phosphorylated proteins were immunoprecipitated with anti-HA4 monoclonal antibody and analyzed by SDS−PAGE followed by autoradiography. Except for the untransfected NIH 3T3 cells (UT, lanes 1 and 2), all cell lines were stably transfected with human insulin receptor plus either wild-type (WT, lanes 3 and 4), Y488/Y513F mutant (lanes 5 and 6), or truncated Δ448 pp120/HA4 isoform (lanes 7 and 8). This experiment is representative of three independent determinations. Additionally, clones overexpressing either the wild-type or the Y488F/Y513F double mutant form of pp120/HA4 were independent of those employed in the experiments described in Figure 4.

in response to insulin. The Y488F/Y513F double mutant form of pp120/HA4 underwent dephosphorylation in response to insulin. Thus, insulin decreased ^{33}P incorporation in pp120/HA4 by 34% (Figure 4A, lanes 7 and 8) and 30% (Figure 5, lanes 5 and 6). This raises the possibility that insulin leads to a decrease in the level of phosphorylation of Ser^{503}. Moreover, it appears that elimination of the phosphorylation site at Tyr^{488} increases the electrophoretic mobility of pp120/HA4 (Figure 4A, lanes 7 and 8 vs lanes 9 and 10, and vs lanes 3 and 4). Lastly, the S503A mutation completely inhibited both basal and insulin-stimulated phosphorylation (Figure 4A, lane 1 vs 2). This suggests that Ser^{503} is a major phosphorylation site of pp120/HA4 and raises the possibility that phosphorylation of Ser^{503} may be required for phosphorylation of Tyr^{488} residue. Finally, deletion of the intracellular domain of pp120/HA4 in the truncated isoform (Δ448) inhibited insulin-stimulated phosphorylation of pp120/HA4 (Figure 5, lanes 7 and 8). This observation supports the conclusion that pp120/HA4 is phosphorylated on sites in the intracellular domain in intact cells.

Ecto-ATPase Assay. To test whether insulin-induced phosphorylation of the intracellular domain of pp120/HA4 regulates cell-associated ecto-ATPase activity, we measured the ecto-ATPase activity of intact NIH 3T3 cells expressing high levels of recombinant pp120/HA4 in the presence and absence of insulin (10^{-7} M). Transfection of wild-type pp120/HA4 cDNA did not cause a statistically significant change in ecto-ATPase activity (Figure 6A; cells expressing wild-type pp120/HA4: 0.47 ± 0.18 vs untransfected cells: 0.23 ± 0.03 milliunit/mg; $p = 0.32$). Similarly, in cells coexpressing recombinant pp120/HA4 plus recombinant hIR, ecto-ATPase activity was not statistically significantly different from cells expressing only pp120/HA4 (Figure 6A; WTpp120-hIR: 0.77 ± 0.06 vs WTpp120: 0.47 ± 0.18 milliunit/mg; $p = 0.08$). However, as estimated by analysis of immunoblots with the Phosphorlmager, the levels of pp120/HA4 were 5-fold lower in cotransfected cells (expressing both insulin receptors and pp120/HA4) than in the singly transfected cells (expressing only pp120/HA4). Thus,

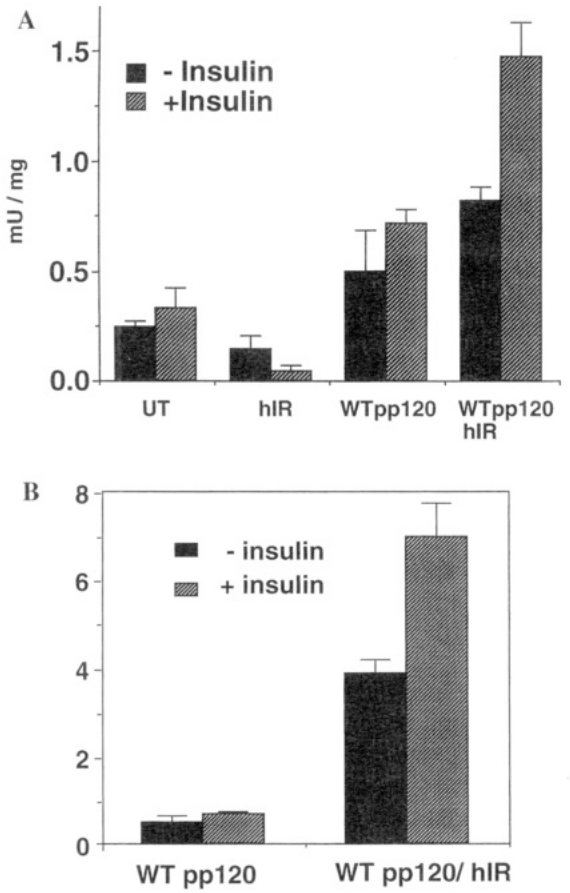

FIGURE 6: Ecto-ATPase assay in NIH 3T3 cells expressing pp120/HA4. Intact NIH 3T3 cells were incubated for 15 min at 37 °C in the presence of [γ-^{32}P]ATP. The following cell lines were studied: untransfected (UT) NIH 3T3 cells, cells expressing human insulin receptor (hIR), cells expressing wild-type pp120/HA4 (WTpp120), and cells co-expressing wild-type pp120/HA4 plus hIR (WTpp120-hIR). The ecto-ATPase reaction was stopped by placing the cells on ice and adding acid-activated charcoal. ATP hydrolysis was plotted as milliunits (1 milliunit of ecto-ATPase activity is defined as the hydrolysis of 1 nmol of ATP/min) per milligram of protein in the assay tube (panel A). Panel B presents the ratio of ecto-ATPase activity (panel A) divided by the content of pp120/HA4 content in each transfected cell line (quantitated by PhosphorImager analysis of immunoblotting with anti-pp120/HA4 polyclonal antibody). The calculated specific ecto-ATPase activities (per molecule of pp120/HA4) are not corrected for the contribution due to endogenous ecto-ATPases present in untransfected cells. Data are the means ± SEM of triplicate experiments. Measurements were repeated on four independent clones for each construct.

if one calculates a specific activity ecto-ATPase activity relative to the pp120/HA4 content per cell (omitting any contribution of endogenous ecto-ATPase), then basal ecto-ATPase activity in the cotransfected cells (Figure 6B; WTpp120-hIR: 3.87 ± 0.30) was approximately 8-fold higher than that of the cells expressing pp120/HA4 alone (Figure 6B, WTpp120: 0.47 ± 0.18). Furthermore, ecto-ATPase activity was increased approximately 2-fold when doubly transfected cells were exposed to insulin [Figure 6B; WTpp120-hIR (with insulin): 6.98 ± 0.75 vs WTpp120-hIR (no insulin): 3.87 ± 0.30, $p = 0.04$]. In contrast, insulin did not significantly increase ecto-ATPase activity in cells singly transfected with pp120/HA4 cDNA. These observations suggest that phosphorylation of pp120/HA4 contributes in the regulation of cell-associated ecto-ATPase activity. To further investigate the role of phosphorylation of pp120/HA4 in regulating ecto-ATPase activity, we determined the effect of insulin upon ecto-ATPase activity in cells co-expressing hIR and pp120/HA4 cDNAs carrying point mutations in the phosphorylation sites. As depicted in Table 1, insulin induces a 104.81 ± 7.23% increase in ecto-ATPase activity of cells co-expressing wild-type pp120/HA4 and insulin receptors (WT). This effect was not significantly different ($p > 0.05$ compared to WT) from that found in cells expressing the Y513F mutant form of pp120/HA4 (Y513F: 84.40 ± 5.11%). However, deletion of the intracellular domain in the truncated isoform (Δ448) completely abolished the ability of insulin to increase ecto-ATPase activity (−18.60 ± 2.22%). When amino acid residues Tyr^{488} or Ser^{503} were mutated, the insulin-induced increase in ecto-ATPase activity ($p < 0.05$ compared to WT) was intermediate in magnitude: a 24.50 ± 3.21% increase in cells expressing Y488F mutant pp120/HA4; 44.21 ± 3.32% in the Y488F/Y513F mutant; and 40.60 ± 5.02% in the S503A mutant. However, the elevated level of basal ecto-ATPase activity in some of these cell lines (i.e., Y488F, S503A, Δ448, and Y488F/Y513F) complicates the interpretation of these experiments.

DISCUSSION

When insulin binds to its receptor, this activates the receptor tyrosine kinase (Kasuga *et al.*, 1982). A large body of evidence supports the hypothesis that the receptor tyrosine kinase is required for the ability of the insulin receptor to mediate insulin action (Taylor *et al.*, 1991; Roth *et al.*, 1992). Activation of the insulin receptor kinase leads to phosphorylation of several intracellular protein substrates. In recent years, several of these endogenous substrates have been characterized (White & Kahn, 1994). Our laboratory has previously identified a M_r ~120 000 glycoprotein (pp120/HA4) in hepatocyte plasma membranes that is phosphorylated by the insulin receptor kinase in cell-free systems and in intact cells (Rees-Jones & Taylor, 1984; Accili *et al.*, 1986; Perrotti *et al.*, 1987; Margolis *et al.*, 1988). Sequence analysis of tryptic peptides derived from pp120/HA4 (Margolis *et al.*, 1990) demonstrated that the protein is identical to the deduced amino acid sequence of a plasma membrane glycoprotein encoded by a cDNA previously cloned from rat liver (Lin & Guidotti, 1989). In this report, we have directly demonstrated that the insulin receptor can phosphorylate the cytoplasmic domain of recombinant pp120/HA4 expressed by stable transfection in NIH 3T3 cells.

Site-Directed Mutagenesis of Phosphorylation Sites. pp120/HA4 can be expressed as two alternatively spliced isoforms, one of which lacks 61 of the 71 amino acids of the intracellular domain (Najjar *et al.*, 1993). In both intact cells and in the cell-free system, we demonstrated that only the long isoform of pp120/HA4 undergoes insulin-stimulated phosphorylation. This is consistent with the identification of three potential phosphorylation sites in the cytoplasmic domain (Figure 7). There are two tyrosine residues that are located in proximity to acidic amino acids: Tyr^{488} is located downstream from two aspartic acid residues (Asp-Asp-Val-Ser-Tyr), and Tyr^{513} is located downstream from a glutamic acid residue (Glu-Thr-Val-Tyr) (Lin & Guidotti, 1989). Location in proximity to acidic amino acids is the main feature that characterizes tyrosine residues that undergo phosphorylation (Pearson & Kemp, 1991). In addition, Ser^{503} is located in a sequence (Lys-Arg-Pro-Thr-Ser^{503}) that conforms to the motif for phosphorylation by the cyclic

Table 1: Effects of Mutations in pp120/HA4 upon the Ability of Insulin To Activate Ecto-ATPase in Transfected NIH 3T3 Cells[a]

	ecto-ATPase (milliunits/mg of protein)		pp120 (arb units)	no. of insulin receptors ($\times 10^{-6}$)
	−insulin	+insulin		
WT	1.22 ± 0.22	2.49 ± 0.23	1	1.12
Y488F	4.89 ± 0.27	6.12 ± 0.25	0.3	1.25
Y513F	2.24 ± 0.29	4.13 ± 0.32	1.2	0.62
Y488F/Y513F	4.35 ± 0.28	6.24 ± 0.25	0.5	1.25
S503A	5.69 ± 0.36	8.07 ± 0.25	0.4	0.95
Δ448	8.29 ± 0.21	6.75 ± 0.11	0.6	0.80

[a] Ecto-ATPase activity (expressed as mU/mg of protein) of intact NIH 3T3 cells was measured as described in the legend to Figure 6. Expression of pp120 was mesaured as arbitrary units of biotin-labeled pp120 detected by blotting with horseradish peroxidase-conjugated streptavidin. The number of insulin receptors was estimated from the *y*-intercept of Scatchard plots of insulin binding to intact cells. Data are the means ± SEM of triplicate experiments.

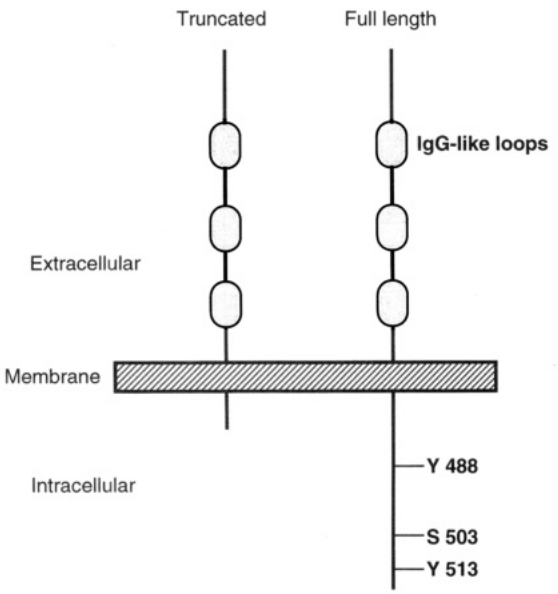

FIGURE 7: Two isoforms of pp120/HA4. pp120/HA4 contains immunoglobulin (IgG)-like loops in its extracellular domain. It is expressed as two alternatively spliced isoforms, one lacking 61 of the 71 amino acids of the intracellular domain. The long isoform contains two tyrosine residues (Y488 and Y513) that are located in proximity to acidic amino acids, suggesting that they are potential sites of phosphorylation by tyrosine kinases. In addition, there are 15 serine residues in the intracellular domain, one of which (S503) is placed in a consensus sequence for phosphorylation by cAMP-dependent protein kinase.

AMP-dependent protein kinase (Lin & Guidotti, 1989; Sippel *et al.*, 1993).

We have employed site-directed mutagenesis of pp120/HA4 in an effort to identify the sites of tyrosine phosphorylation by the insulin receptor tyrosine kinase. In both intact cells and the cell-free system, substitution of Phe for Tyr^{488} abolished insulin-stimulated phosphorylation of pp120/HA4. In contrast, mutation of Tyr^{513} had little if any effect upon insulin-stimulated phosphorylation of pp120/HA4. These data suggest that Tyr^{488} is likely to be the major site phosphorylated by the insulin receptor tyrosine kinase. Of course, it remains possible that Tyr^{513} is a minor phosphorylation site for the insulin receptor, or a major phosphorylation site for a different tyrosine kinase.

Subsequent to the completion of this work, a paper appeared describing the basal phosphorylation of an identical protein which is reported also to function as a bile acid transporter in rat liver (Sippel *et al.*, 1994b). In that study, similar mutations were introduced and similar conclusions were reached regarding the sites of phosphorylation. In particular, their phosphoamino acid analysis supports the conclusion that Tyr^{488} and Ser^{503} are the major sites of tyrosine and serine phosphorylation, respectively, in the basal state. Furthermore, our study complements the previous study (Sippel *et al.*, 1994b) because we have focused primarily upon insulin-stimulated phosphorylation of pp120/HA4.

Several lines of evidence suggest that Ser^{503} may be a substrate for phosphorylation by another protein kinase. Indeed, because the Y488F/Y513F mutant lacks both Tyr^{488} and Tyr^{513}, it is likely that Ser^{503} is the site of phosphorylation of this double mutant in intact cells. Furthermore, insulin decreases the ^{33}P content of the Y488F/Y513F mutant form of pp120/HA4—presumably because insulin either inhibits the relevant serine protein kinase or activates a phosphoserine phosphatase. These observations with recombinant pp120/HA4 are reminiscent of two previous observations in cultured H35 hepatoma cells: first, that phosphoserine is the major phosphoamino acid in pp120/HA4 in H35 cells incubated in the absence of insulin (Perrotti *et al.*, 1987); and second, that insulin decreased the phosphoserine content of pp120/HA4 in H35 cells (Perrotti *et al.*, 1987).

Functional Significance of pp120/HA4 Phosphorylation. Tyrosine phosphorylation has been shown to have several functions in various proteins. For example, autophosphorylation of the insulin receptor activates the receptor tyrosine kinase to phosphorylate other intracellular proteins (White & Kahn, 1994). In contrast, phosphorylation of Tyr^{527} in $pp60^{src}$ has an inhibitory effect on the transforming activity of that protein (Cobb & Parsons, 1993). However, the most common function of phosphotyrosine residues is to bind to SH2 domains in proteins that function in intracellular signaling pathways (Koch *et al.*, 1991; Pawson & Gish, 1992). It is reasonable to speculate that SH2 domains with the appropriate specificity to bind phosphotyrosine residues in pp120/HA4 may exist. In fact, the sequences flanking Tyr^{488} (Tyr-Ser-Val-Leu) and Tyr^{513} (Tyr-Ser-Val-Val) conform to motifs that have been proposed to bind the SH2 motifs (pTyr-Val,Ile,Thr-X-Val, Leu, Ile) of SHPTP-2 (Syp) (Case *et al.*, 1994). Furthermore, this sequence closely resembles SHC binding sites of SH2 domains from several proteins with important functions in the immune system: human CD3 gamma and delta chain (Tyr-Ser-His-Leu), the human CD3 zeta chain (Tyr-Asp-Val-Leu), the mouse CD3 epsilon chain (Tyr-Ser-Gly-Leu), and the mouse Ig epsilon receptor b (Tyr-Ser-Glu-Leu) (Songyang *et al.*, 1994). Thus, it is also possible that SHC binds to phosphorylated pp120/HA4.

pp120/HA4 Is Associated with an Ecto-ATPase Activity. In cells co-expressing human insulin receptors and full-length

recombinant rat pp120/HA4, insulin has the ability to increase ecto-ATPase activity. However, this effect of insulin is not observed in cells co-expressing human insulin receptors and the short isoform of recombinant rat pp120/HA4 (due to alternative splicing of mRNA, resulting in deletion of 61 amino acid residues from the C-terminus of the protein). These observations suggest that phosphorylation of pp120/HA4 may provide a mechanism whereby insulin stimulates ecto-ATPase activity. This hypothesis is entirely consistent with the view that pp120/HA4 possesses catalytic activity as a Ca^{2+}/Mg^{2+}-dependent ecto-ATPase as suggested in the original publication of Lin and Guidotti (1989) and in subsequent studies by Sippel *et al.* (1993, 1994a,b). However, even if pp120/HA4 does not possess ecto-ATPase activity as has been suggested by others (Obrink, 1991; Dr. Aileen F. Knowles, personal communication; Dr. Liselotte Plesner, personal communication), it is nevertheless possible that pp120/HA4 may be a regulatory subunit of an ecto-ATPase enzyme. This would account for the copurification of the pp120/HA4 glycoprotein together with ecto-ATPase activity. In addition, it would account for the fact that expression of pp120/HA4 is required in order to observe the activity of insulin to activate ecto-ATPase. Further studies are needed to elucidate the physiological role of this regulation on the function of pp120/HA4 and its possible relevance to insulin action in hepatocytes.

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