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Comparison of the Intracellular Trafficking of Two Alternatively Spliced Isoforms of pp120, a Substrate of the Insulin Receptor Tyrosine Kinase

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Abstract pp120, a substrate of the insulin receptor tyrosine kinase, is a plasma membrane glycoprotein in the hepatocyte. It is expressed as two spliced isoforms differing by the presence (full length) or absence (truncated) of most of the intracellular domain including all phosphorylation sites. Because the two isoforms differ by their ability to regulate receptor-mediated insulin endocytosis and degradation, we aimed to investigate the cellular basis for this functional difference by comparing their intracellular trafficking. During its intracellular assembly, pp120 is transported from the *trans*-Golgi network to the sinusoidal domain of the plasma membrane before its final transcytosis to the bile canalicular domain. Because both isoforms are expressed in hepatocytes, we examined their intracellular trafficking in NIH 3T3 fibroblasts individually transfected with each isoform. Pulse-chase experiments demonstrated that most of the newly synthesized full-length isoform reached complete maturation at about 60 min of chase. By contrast, only about 40% of the newly synthesized truncated isoform underwent complete maturation, even at more prolonged chase. Moreover, a significant portion of the truncated isoform appeared to be targeted to lysosomes. Abolishing basal phosphorylation on Ser^{503} by cAMP-dependent serine kinase by mutating this residue to alanine was correlated with incomplete maturation of full length pp120 in NIH 3T3 cells and hepatocytes. This finding suggests that the intracellular domain of pp120 contains information that regulates its vectorial sorting from the *trans*-Golgi network to the plasma membrane. J. Cell. Biochem. 76:133–142, 1999.

Key words: kinetics; pulse-chase; intracellular synthesis; assembly; receptor-mediated endocytosis

The tyrosine kinase of the insulin receptor plays an important role in mediating insulin action. Activating the kinase upon insulin binding to its receptor leads to phosphorylation of several intracellular protein substrates [Yenush and White, 1997], including pp120, a transmembrane glycoprotein of M_r ~120 000 [Perrotti et al., 1987; Rees-Jones and Taylor, 1985].

Alternative splicing of a single gene generates two spliced variants of pp120 in the hepatocyte. The two isoforms differ by the presence (full-length) or absence (truncated) of 61 out of the 71 amino acid-cytoplasmic tail [Najjar et al., 1993], including all phosphorylation sites [Najjar et al., 1995]. Only the long isoform undergoes phosphorylation. Site-directed mutagenesis in NIH 3T3 cells showed that pp120 is basally phosphorylated on Ser^{503} by cyclic adenosine monophosphate (cAMP)-dependent serine kinase in the absence of insulin [Najjar et al., 1995] and that this phosphorylation is required for its phosphorylation on Tyr^{488} by the insulin receptor tyrosine kinase [Najjar et al., 1995]. Tyr^{513}, the other tyrosine in the cytoplasmic domain of pp120, does not appear to be

Abbreviations used: pp120, pp120/HA 4/C-CAM; S503A, a mutant isoform in which $Serine^{503}$ was mutated to alanine; TGN, *trans*-Golgi network; pBPV, bovine papilloma virus-based expression vector; NIH 3T3, NIH 3T3 mouse skin fibroblasts; $IR^{-/-}$ hepatocytes, SV-40 transformed hepatocytes derived from the insulin receptor knockout mouse; Endo F, endoglycosidase F/N-glycosidase F; Endo H, endoglycosidase H; ECL, enhanced chemiluminescence; E64, *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane; L-MME, L-methionine methyl ester hydrochloride.

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phosphorylated by the insulin receptor tyrosine kinase [Najjar et al., 1995].

The function of pp120 remains elusive. It may function as a tumor suppressor in colon, liver, and prostate [Lin et al., 1995] and as a downregulator of the mitogenic effects of insulin [Formisano et al., 1995]. pp120 may upregulate the transport of bile acids [Sippel et al., 1993] and insulin [Formisano et al., 1995] in the hepatocyte, as suggested by studies in transfected cells. Supportive evidence for a role of pp120 in cell adhesion has also emerged [Edlund and Öbrink, 1993]. The basic mechanism of pp120 functions is not completely understood. However, pp120 phosphorylation was found to be required for its function in insulin endocytosis and action [Formisano et al., 1995], in bile acid transport [Sippel et al., 1994], and in tumor suppression [Hsieh et al., 1995; Luo et al., 1998]. Dependence on an intact intracellular domain for the cell adhesion property of pp120 has also been reported [Cheung et al., 1993]. Because of the multiple functions ascribed to pp120, it has been referred to as pp120, C-CAM, and CBATP. On the basis of cDNA sequence analysis, pp120 has also been identified as biliary glycoprotein (BGP) [Nedellec et al., 1994] and Ca^{2+}/Mg^{2+} ecto-ATPase [Lin and Guidotti, 1989; Margolis et al., 1990].

Evidence obtained using a monoclonal antibody against the extracellular domain of HA 4, an integral plasma membrane glycoprotein primarily expressed in the bile canalicular domain of the hepatocyte, confirmed the identity of pp120 as HA 4 [Margolis et al., 1988]. HA 4 is a heavily glycosylated protein containing 14–28 N-linked with no classical O-linked glycans [Bartles et al., 1985]. After processing of its carbohydrate moieties in Golgi, HA 4, packaged in vesicles that bud out of the *trans*-Golgi network (TGN), is transported to the sinusoidal domain, where it temporarily resides before its final sorting to the bile canalicular domain [Bartles et al., 1985]. This rapid vectorial delivery from TGN to the sinusoidal membrane is shared by other hepatocyte glycoproteins, regardless of their final destination in the sinusoidal (CE9 and the asialoglycoprotein), or in the bile canalicular domain of the plasma membrane [polymeric IgA receptor (dipeptidyl peptidase IV), and aminopeptidase N] [Bartles et al., 1985, 1987].

Polarized expression at the plasma membrane domains of epithelial cells has been described for many proteins [Monlauzeur et al., 1995; Le Gall et al., 1997; Rutledge et al., 1998]. Immunofluorescence analysis demonstrated that C-CAM (a cell adhesion molecule identical to pp120) is expressed chiefly in the lateral membrane of NBT II rat bladder carcinoma cells, but it is also expressed, albeit to much lower extent, at the basal and apical domains of these epithelial cells [Hunter et al., 1994]. Thus, it appears that the relative distribution of the multiple pp120 isoforms varies among the different surface domains of epithelial cells. We aimed at comparing the intracellular trafficking of the two pp120 isoforms.

Because both pp120 isoforms are expressed in epithelial polar cells (liver, intestines, and kidneys) and because they electrophorese at a close apparent molecular mass, it would be technically difficult to distinguish between their immature and mature species in rat tissues. Thus, we employed transfection analysis as a tool to begin to compare the postsynthesis intracellular trafficking of pp120 isoforms. Because hepatocyte glycoproteins appear to reach the sinusoidal domain with similar kinetics [Bartles et al., 1987], we transfected mouse NIH 3T3 fibroblasts individually with each of the rat pp120 isoforms to compare post-translational modification, intracellular sorting, and initial delivery of the two pp120 isoforms with that of the plasma membrane. We have observed that, in contrast to full length, a significant portion of truncated pp120 did not undergo maturation. Instead, it was targeted to lysosomes, suggesting that the intracellular domain contains significant information that regulates vectorial transport from TGN to the plasma membrane. Abolishing basal phosphorylation on Ser^{503} in the cytoplasmic domain was associated with reduced maturation of a significant portion of full-length pp120 in transfected mouse fibroblasts and hepatocytes, suggesting that basal phosphorylation uniformly regulates the intracellular trafficking of pp120.

MATERIALS AND METHODS

Materials

ImmunoPure NHS-LC-Biotin-Sulfosuccinimidyl was purchased from Pierce (Rockford, IL). L-Methionine methyl ester hydrochloride was from Sigma Chemical Co. (St. Louis, MO), and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) was from Fluka Chemical Co. (Milwaukee, WI). All reagents for polyacryl-

amide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA), and all reagents for immunoblotting were from Amersham Life Science (Arlington Heights, IL). The monoclonal antibody used to immunoprecipitate pp120 (α-HA 4, an identical protein to pp120) was purified from ascites fluid from HA 4 c19 cells purchased from the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City, IA). α-295 polyclonal antibody was raised in rabbit against amino acids 51–64 in the extracellular domain of rat liver pp120.

Construction of Expression Vectors

Amplification and subcloning of the cDNA encoding full-length (wild-type and S503A) or truncated pp120 at the *Xho*I/*Not*I sites of a bovine papilloma virus-based expression vector (pBPV; Pharmacia) carrying the metallothionein 1 promoter were previously described [Najjar et al., 1995].

Cell Culture and Transfection

Mouse NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 10 µg/ml streptomycin at 37°C/5% CO_2. Transfection of approximately 10^7 cells with pBPV-cDNAs encoding pp120 (full-length or truncated) and wild-type human insulin receptors in the presence of 1.5 µg of RSV-Neo^r by the electroporation method (Bio-Rad) was originally described [Najjar et al., 1995]. The SV-40-transformed hepatocytes, derived from insulin receptor knockout mice ($IR^{-/-}$), were routinely maintained at 33°C/5% CO_2 in α-modified Eagle's medium (α-MEM) containing 4% FCS, 1% glutamine, 200 nM dexamethasone, 100 U/ml penicillin, and 10 µg/ml streptomycin. They were stably transfected with pBPV-cDNAs encoding wild-type insulin receptors and the S503A full-length pp120 mutant in the presence of 1.5 µg of the pREP4-Hygro^r hygromycin-resistant gene, as previously described [Najjar and Lewis, 1999]. Isolated clones were expanded and maintained in medium containing Geneticin (G-418, 600 µg/ml) (Gibco-BRL, Gaithersburg, MD) or hygromycin B (Calbiochem-Novabiochem, San Diego, CA). Cells were 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) for analysis by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and screening for pp120 expression by immunoblotting with a pp120 polypeptide antibody (α-295) [Najjar et al., 1995].

Biotin Labeling of Surface Membrane Proteins

As we have previously described [Najjar et al., 1998; Choice et al., 1998], transfected NIH 3T3 cells were incubated for 30 min at 4°C with biotin (1 mg/ml) in phosphate-buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) supplemented with 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% BSA. After lysis in 1% Triton-X-100 in the presence of protease inhibitors (see above), and immunoprecipitation with pp120/HA 4 monoclonal antibody [Najjar et al., 1995], proteins were treated with endoglycosidases (see below), electrophoresed through 7.5% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), and immunoblotted with horseradish peroxidase (HRP)-labeled streptavidin, followed by detection with enhanced chemiluminescence (ECL), as previously described [Najjar et al., 1998]. These experiments were repeated three times for each cell type.

Treatment With Endoglycosidase H and Endoglycosidase F/N-Glycosidase F

After labeling of transfected NIH 3T3 cells with biotin, pp120 was immunoprecipitated with pp120/HA 4 monoclonal antibody and solubilized in 1% SDS-10 mM Tris, pH 6.8. As described by Jui et al. [1994], solubilized pp120 was diluted 1:5 with buffer B (100 mM potassium phosphate buffer, pH 6.8, 5 mM EDTA, 2% n-octyl glucoside, and 1% 2-mercaptoethanol). Thereafter, 0.4 U/ml endoglycosidase F/N-glycosidase F (Endo F) (Boehringer Mannheim, Indianapolis, IN), 5 U endoglycosidase H (Endo H) (Boehringer Mannheim), or buffer alone, was added to individual aliquots, and the digestion was allowed to proceed for 18 h at 37°C.

Metabolic Labeling

Confluent monolayers of cells were incubated overnight in complete medium supplemented with dialyzed serum. Thereafter, cells were incubated with methionine- and cysteine-free me-

dium for 90 min before being pulsed with 0.2 mCi/ml of TRAN ^{35}S-Label (ICN Pharmaceuticals, Irvine, CA) for 20 min at 37°C. Cells were then chased with complete medium supplemented with 1 mM methionine and 1 mM cysteine for the designated time before lysis and analysis on SDS-PAGE. After transfer to nitrocellulose membranes, proteins were detected by autoradiography and analyzed by immunoprobing with a pp120 polypeptide antibody (α-295) and ECL, as described previously [Najjar et al., 1995]. To reach steady state, cells were sometimes pulsed for 15 h. In some experiments, the chase medium was supplemented with MME lysosomal inhibitor cocktail [20 mM L-methionine methyl ester hydrochloride-100 μg/ml leupeptin-100 μg/ml pepstatin A-100 μg/ml *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64)], as described by Bosshart et al. [1994].

Protein Quantitation

As previously described [Najjar et al., 1998; Choice et al., 1998], autoradiograms were scanned on an imaging densitometer (Bio-Rad model GS-670), and the proteins were quantitated on the Image NIH v1.60 Macintosh software program.

RESULTS

The Truncated, but Not the Full-Length Isoform, of pp120 Retains Some Immature N-Linked Glycans

The carbohydrate constituents of truncated and full-length pp120 at the surface membrane were compared in stably transfected NIH 3T3 cells individually expressing various amounts of full-length and truncated pp120. Figure 1 represents experiments performed in clones with higher levels of truncated than of full length pp120 (lane 2 vs 1). After labeling of the extracellular domain of proteins with biotin, pp120 was immunoprecipitated and treated with glycosidases before being analyzed on SDS-PAGE (Fig. 1). Because pp120 does not contain O-linked glycans, removal of all N-linked chains with Endo F yielded M_r ~66 000 and ~60 000 species corresponding to the apoproteins of the full-length and the truncated isoforms, respectively, as shown in Figure 1, lanes 3 and 6, pp120 (f_s). Treatment with Endo H, which cleaves immature, but not complex, N-linked glycans, did not significantly affect full-length

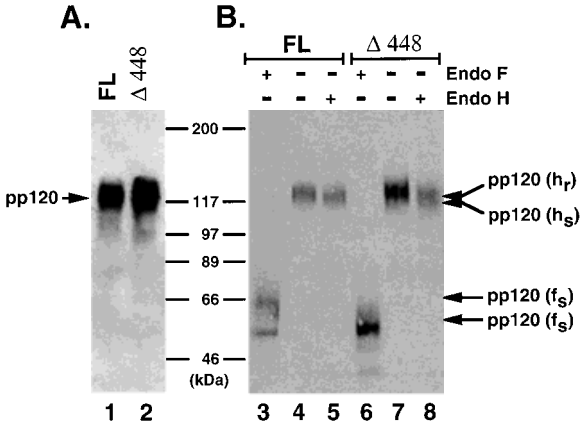

Fig. 1. Carbohydrate structure of recombinant pp120 at the surface membrane. The expression level of each of full-length (FL) and truncated (Δ448) pp120 in stably transfected NIH 3T3 cells was determined by immunoblotting proteins derived from cell lysates with a polyclonal antibody against pp120 (**A, lanes 1, 2**). In another set of experiments, the extracellular domains of proteins at the surface membrane of these cells were labeled with biotin (**B**). Cell lysates were then immunoprecipitated with α-pp120/HA 4 monoclonal antibody, and the proteins treated with buffer alone (**lanes 4, 7**), Endo F (**lanes 3, 6**), or Endo H (**lanes 5, 8**) before analysis on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting with horseradish peroxidase (HRP)-labeled streptavidin, and detection by the enhanced chemoluminescence (ECL) system. f_s, h_s, Endo F- and Endo H-sensitive N-linked glycans, respectively; h_r, Endo H-resistant N-linked glycans. Molecular mass markers are shown at the left-hand side of the gel. Experiments were repeated at least three times and on two different clones for each cell type.

pp120 (Fig. 1, lane 5 vs 4), suggesting that this pp120 isoform undergoes complete maturation of its carbohydrate chains. By contrast, Endo H treatment of truncated pp120 yielded two species, one Endo H-resistant (lane 8, pp120, h_r) and another Endo H-sensitive (lane 8, pp120, h_s), differing by ~4.8 kDa that correspond to 1–2 N-linked carbohydrate chains. This suggests that, in contrast to full-length pp120, the truncated isoform retains 1–2 N-linked glycans that are of the immature type and that this does not adversely affect the sorting of the truncated isoform to the cell surface membrane.

Comparison of the Intracellular Trafficking of Truncated and Full-Length pp120

The difference in the carbohydrate constituence of truncated and full-length pp120 at the cell surface prompted us to investigate the intracellular assembly of the two isoforms. In rat hepatocytes, HA 4 reaches maturation at about 45 min of chase [Bartles et al., 1987]. Hence, we

initially examined whether the protein undergoes similar kinetics when expressed in NIH 3T3 cells. To this end, we chased cells expressing the full length isoform with methionine- and cysteine-rich medium for 0.5 to 20 h after their chase with radioactive amino acids for 20 min (Fig. 2). As in the rat hepatocyte, conversion of the immature precursor (P_i) of M_r ~87 000 to the mature (P_m) species of M_r ~120 000 occurs rapidly, beginning at about 30 min of chase. Moreover, 94% of radioactivity remained incorporated in P_m, replicating the in vivo state of HA 4 in the rat hepatocyte [Bartles et al., 1987].

We then compared the intracellular trafficking of the two pp120 isoforms in NIH 3T3 cells expressing a comparable amount of pp120. After autoradiography, proteins were immunoprobed with a polyclonal antibody against pp120, to confirm the identity of the M_r ~120 000 protein as the mature species of pp120 (Fig. 3B). The amount of radioactivity in the precursor at various time of chase (0–180 min) was calculated as percent radioactivity in the precursor in the absence of chase (time = 0 min) and used as measure for the amount of intracellularly assembled pp120 (Fig. 3A, graphs). The immature precursor of full-length pp120 began to convert to the mature species at about 30 min of chase and reached complete conversion at 180 min of chase, as evidenced by the symmetrical recovery of the lost radioactivity from the immature in the mature species (Fig. 3A, graph). By contrast, ~40% of the immature precursor of the truncated isoform failed to reach maturation after 3 (Fig. 3A, graph) and even after 6 h of chase (data not shown). More strikingly, the amount of incorporated [^{35}S] amino acids at time 0 of chase in the truncated precursor was lower than that of its full-length counterpart (Fig. 3A), despite the comparable levels of pp120 expression in these cells (Fig. 3B). This observation was reproduced regardless of the duration of pulse (5, 15, 20, 30, 60, and 90 min and 24 h; data not shown), suggesting that truncated pp120 was synthesized at a lower rate than its full-length counterpart.

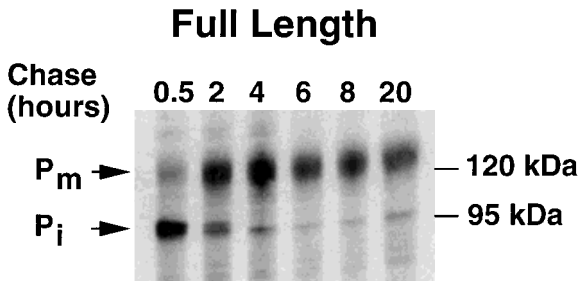

Fig. 2. Metabolic labeling of recombinant pp120. NIH 3T3 cells expressing full-length pp120 were pulsed with [^{35}S]methionine-cysteine for 20 min before being chased with methionine- and cysteine-rich medium for 0.5 to 20 h. After lysis, proteins were immunoprecipitated with α-pp120/HA 4 monoclonal antibody before analysis on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. P_i and P_m, immature and mature intracellular precursors, respectively. Molecular mass markers are shown at the right-hand side of the gel.

To compare these isoforms at steady state, transfected NIH 3T3 cells were pulsed for 15 h before undergoing a 0–40 chase with methionine- and cysteine-rich medium. As shown in Figure 4, the turnover rate of truncated P_m was indistinguishable from that of the full-length precursor at steady state, as evidenced by the comparable rate of decrease in the amount of radioactivity incorporated in the precursor at each chase time relative to steady-state levels at time 0 of chase (13%, 43%, 77% lost in full-length vs 13%, 31%, 91% lost in truncated at 8, 20, and 40 h of chase, respectively).

Sorting of the Truncated Isoform to Lysosomes

On the basis of previous experiments on HA 4 in rat hepatocytes [Bartles et al., 1987], the mature portion of the truncated precursor was presumably sorted from TGN to the plasma membrane. To investigate whether the portion of the truncated precursor that does not undergo maturation was targeted to lysosomes, we supplemented the chase medium of transfected NIH 3T3 cells with a lysosomal inhibitor cocktail (MME) and measured the amount of assembled mature and immature species as described above (Fig. 5). Under these conditions, ~83% of the truncated precursor underwent maturation in cells treated with MME (Fig. 5B; P_m + MME), compared with ~40% in untreated cells (Fig. 5A; P_m). Moreover, the symmetrical pattern of converting the immature to the mature species was restored upon inhibiting lysosomal enzymes in cells transfected with the truncated isoform. This finding suggests that a significant portion of the truncated precursor is normally targeted to lysosomes. As expected, treatment of cells expressing the full-length isoform with MME did not alter the pattern of assembly of this isoform (data not shown), suggesting that full-length

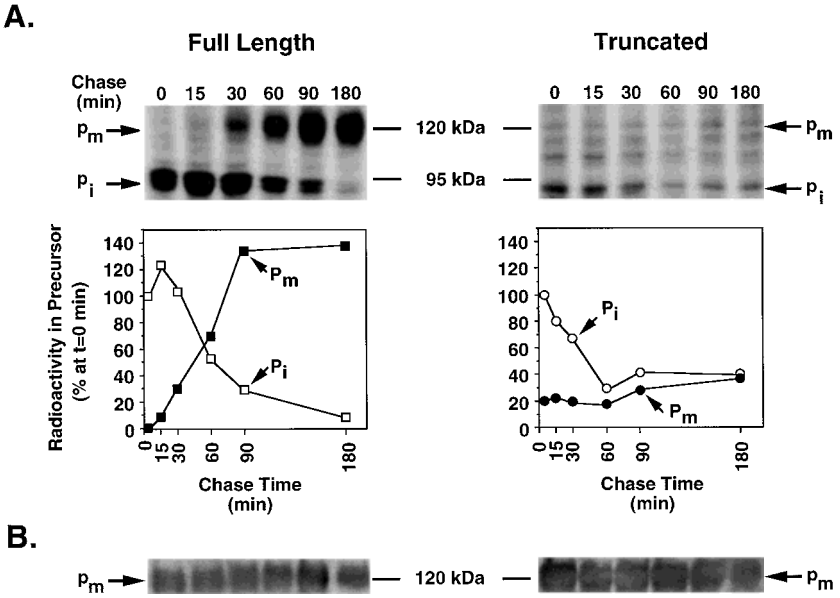

Fig. 3. Comparison of the intracellular synthesis and trafficking of recombinant truncated and full-length pp120. NIH 3T3 cells expressing either full-length or truncated pp120 were pulsed with [^{35}S]methionine-cysteine for 20 min before being chased with methionine- and cysteine-rich medium for 0–180 min. After lysis, proteins were immunoprecipitated with α-pp120/HA 4 monoclonal antibody before analysis on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography and densitometry (**A**) and by immunoblotting with a polyclonal antibody against pp120 and detection by the enhanced chemoluminescence (ECL) system (**B**). The amount of radioactivity incorporated in the immature (P_i) and the mature (P_m) precursor at each time of chase was calculated as a percentage of the newly synthesized pp120 (nonchased P_i at t = 0 min) and used as measure of the amount of intracellularly assembled pp120 (graphs). Molecular mass markers are shown between gels.

pp120 is not significantly targeted to lysosomes.

The Intracellular Domain of pp120 Regulates Its Intracellular Maturation in Transfected NIH 3T3 Cells

Because the truncated pp120 isoform differs from its full-length counterpart by the absence of most of the cytoplasmic domain, including phosphorylation sites, we investigated whether this domain contains the necessary information for the efficient intracellular synthesis and trafficking of the protein. Ser^{503} constitutes the basal phosphorylation site of full-length pp120 by cAMP-dependent serine kinase in the absence of insulin [Najjar et al., 1995]. To investigate whether this basal phosphorylation is required for the vectorial sorting of pp120 precursor from TGN to the plasma membrane, we examined the effect of mutating Ser^{503} to nonphosphorylatable alanine on the intracellular synthesis and trafficking of pp120. To this end, we used NIH 3T3 cells expressing S503A pp120 mutant at a comparable level to the wild-type full-length isoform. As shown in Figure 6, mutating Ser^{503} to alanine bestowed on full-length pp120 the intracellular assembly pattern characteristic of the truncated isoform: slower synthesis rate and incomplete maturation. This finding suggests that basal phosphorylation of pp120 on Ser^{503} regulates complete maturation of pp120 and its sorting from TGN to the plasma membrane.

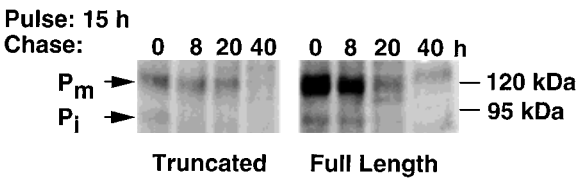

Fig. 4. Recombinant pp120 at steady state. NIH 3T3 cells expressing either full-length or truncated pp120 were pulsed with [^{35}S]methionine-cysteine for 15 h before being chased with methionine- and cysteine-rich medium for 0–40 h. After lysis, proteins were immunoprecipitated with α-pp120/HA 4 monoclonal antibody before analysis on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. P_i and P_m, immature and mature intracellular precursors, respectively. Molecular mass markers are shown at the right-hand side of the gel.

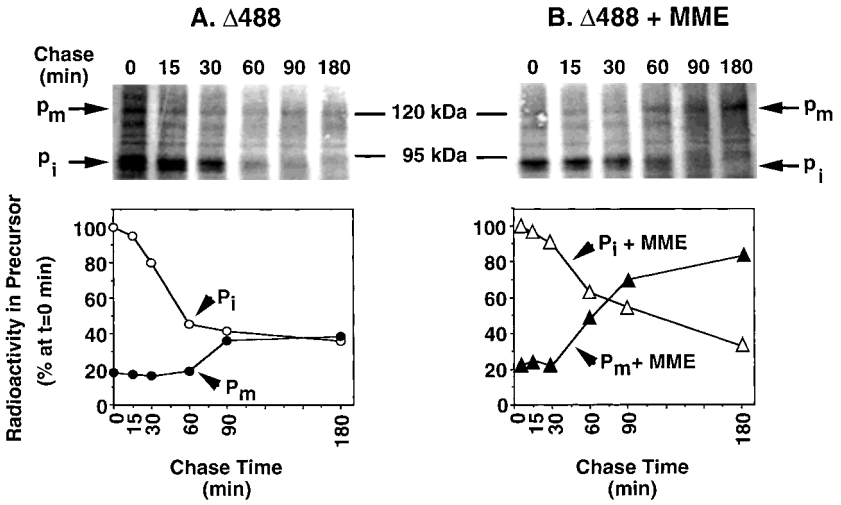

Fig. 5. Truncated pp120 is targeted to lysosomes. NIH 3T3 cells expressing truncated (Δ448) pp120 were pulsed-chased and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry as described in the legend to Fig. 3, except for the addition (**B**) or omission (**A**) of lysosomal inhibitors, MME, to the chase medium. P_i and P_m, immature and mature intracellular precursors, respectively. Molecular mass markers are shown between panels.

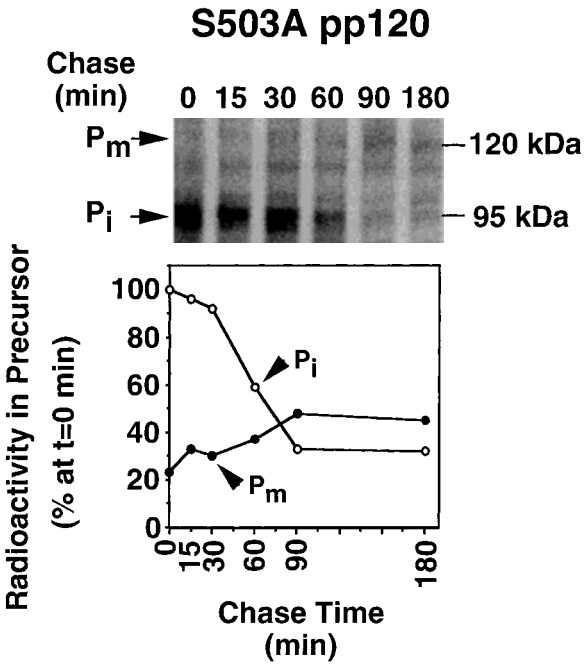

Fig. 6. Effect of basal phosphorylation on the intracellular trafficking of pp120 in NIH 3T3 cells. NIH 3T3 cells expressing the rat S503A pp120 mutant in which the site of basal phosphorylation by cAMP-dependent serine kinase, Ser^{503} (S), was mutated to alanine (A) were pulsed-chased and analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography and densitometry as described in the legend to Fig. 3. P_i and P_m, immature and mature intracellular precursors, respectively. Molecular mass markers are shown at the right-hand side of the gel.

The Intracellular Domain of pp120 Regulates Its Intracellular Maturationin Transfected Mouse Hepatocytes

Because the intracellular sorting pathways of proteins may differ in a cell-specific manner [Matter and Mellman, 1994], we aimed at examining pp120 sorting in an epithelial cell where it is physiologically expressed. Because the pp120/HA4 monoclonal antibody used in our immunoprecipitation studies recognizes the rat protein, but not the mouse protein, we transfected mouse hepatocytes with cDNA encoding either wild-type or S503A full-length pp120 with insulin receptors, and investigated the effect of mutating Ser^{503} to alanine on pp120 sorting in this epithelial cell line (Fig. 7). The S503A mutant clones used in these experiments expressed ~10-fold higher amount of pp120 than those expressing the wild-type isoform. As in NIH 3T3 cells, wild-type full-length pp120 in hepatocytes underwent almost complete maturation (~82%) at 3 h of chase (Fig. 7, left). Moreover, mutating Ser^{503} to alanine resulted in a marked decrease in pp120 maturation, as evidenced by the low fraction, ~38%, of the newly synthesized protein that reached maturation even after 3 h of chase (Fig. 7, right). Because mutating Ser^{503} to alanine was associated with decreased pp120 maturation in both cell types, our data suggest that basal pp120 phosphorylation on Ser^{503} is uniformly required for its complete maturation in transfected cells.

DISCUSSION

Hepatocytes and other epithelial polar cells express pp120, with hepatocytes and intestines expressing high amounts of pp120/C-CAM and kidneys expressing much lower amounts [Cheung et al., 1993]. The relative distribution of each isoform differs in a tissue-specific manner. In hepatocytes and kidneys, the level of the

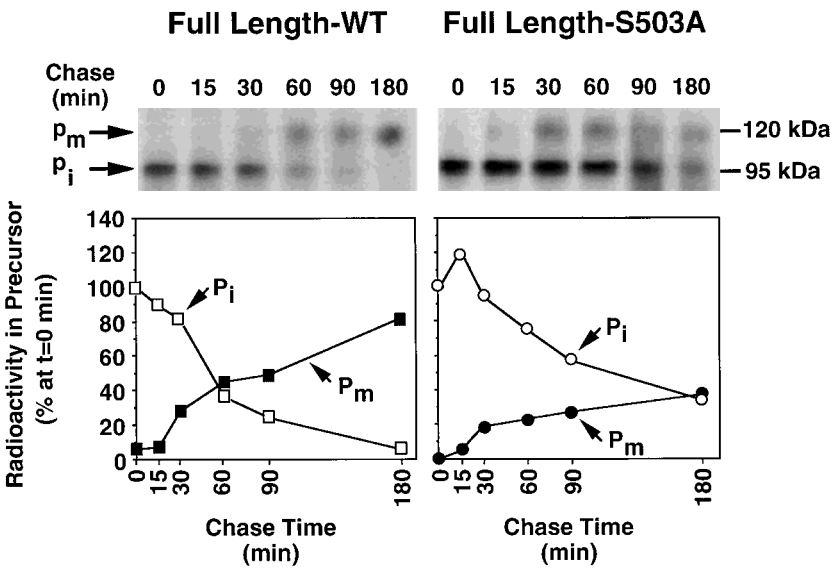

Fig. 7. Effect of basal phosphorylation on pp120 maturation in hepatocytes. SV40-transformed hepatocytes overexpressing wild-type (WT, left) or S503A (right) full-length pp120 were pulsed-chased and analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography and densitometry as described in the legend to Fig. 3. P_i and P_m, immature and mature intracellular precursors, respectively. Molecular mass markers are shown at the right-hand side of the gel.

truncated isoform is about two- to threefold higher than that of the full length [Najjar et al., 1993; Cheung et al., 1993], whereas the ratio is reversed in the intestines [Cheung et al., 1993]. To understand the regulatory mechanism of the relative distribution of pp120 isoforms in the cell, we aimed to begin by comparing the initial intracellular trafficking of these pp120 isoforms in transfected cells grown as monolayers.

Because the intracellular domain of pp120 does not contain methionine or cysteine residues, the presence of lower levels of radioactivity incorporated in the immature species of truncated per expressed pp120 suggests that this isoform is synthesized at a lower rate by comparison to the full length. Lower synthesis level may be due to transcriptional and/or post-transcriptional regulatory factors, such as mRNA stability. Experiments are under way to investigate which, if any, of these factors is responsible for the apparent difference in the rate of synthesis of the two pp120 isoforms.

In contrast to the immature precursor of the full-length isoform that underwent complete conversion to the mature species, only ~40% of the truncated precursor underwent maturation (Fig. 3). The remaining portion appeared to be targeted to lysosomes (Fig. 5). Similar to its full-length counterpart, the mature truncated precursor was sorted to the plasma membrane, as evidenced by biotin labeling of surface proteins (Fig. 1). At steady state, the turnover rate of the mature species of truncated pp120 appeared to be identical to that of the full-length isoform (Fig. 4). Thus, it appears that the two isoforms share the same long half-life previously reported for HA 4 in the rat hepatocyte (>30 h) [Bartles et al., 1987]. Because the mechanism of pp120/HA 4 degradation has not been delineated [Scott and Hubbard, 1992], it is hard to identify at the present time the specific mechanism of the relative distribution of the two pp120 isoforms in the hepatocyte. Nevertheless, it is reasonable to conclude from our present studies that the decreased rate of synthesis and partial lysosomal targeting of the truncated isoform constitute post-translational intracellular mechanisms that regulate its ultimate level at the surface membrane of the hepatocyte.

In the truncated isoform, the N-linked chains of the mature species remained partially Endo H-sensitive, reflecting failure of ~9–11% of the chains to achieve full complex structure in the Golgi (Fig. 1). This finding contrasts with the mature species of the full-length isoform, in which there is barely a suggestion of retained Endo H sensitivity (Fig. 1). Persistence of 1–2 immature high-mannose N-linked glycan chains in HA 4 has been previously noted [Bartles et al., 1985]. In the current studies, we have presented evidence that it is the truncated, not the full-length isoform, that retains the immature N-linked glycans. Despite the presence of these immature N-linked constituents, truncated pp120 was efficiently transported to the plasma membrane, suggesting that full maturation of N-linked glycans is not necessary for optimal vectorial transport to the cell surface. Absence of an absolute requirement for N-linked glyco-

sylation for transfer of insulin and insulin-like growth factor-1 receptors to the surface membrane of cultured cells has also been demonstrated [Duronio et al., 1986]. Thus, another mechanism must underlie the intracellular trafficking and the vectorial transport of pp120 from TGN to the plasma membrane.

Omission of most of the intracellular domain led to sorting of a significant portion of the newly synthesized truncated protein to lysosomes in NIH 3T3 cells. Mutating Ser^{503} in the intracellular domain of pp120 to nonphosphorylatable alanine was associated with altered synthesis and trafficking reminiscent of that of the truncated isoform in NIH 3T3 cells (Fig. 6) and hepatocytes (Fig. 7). Because this mutation abolished constitutive pp120 phosphorylation in transfected NIH 3T3 cells [Najjar et al., 1995], it appears that phosphorylation of pp120 on Ser^{503} by the cAMP-dependent serine kinase is generally required for its optimum intracellular trafficking and vectorial targeting from TGN to the plasma membrane of cells. Dependence on serine phosphorylation has also been shown to be required for the intracellular trafficking of other proteins. For instance, mutating Ser^{664} in the polymeric immunoglobulin receptor to alanine resulted in deficient transcytosis of this protein from the basolateral to the apical surface [Casanova et al., 1990]. Proper vesicular release of newly synthesized influenza hemagglutinin from TGN and its targeting to the apical domain of the plasma membrane of polarized Madin-Darby canine kidney cells appeared to require activation of protein kinase A and C (PKA, PKC) [Pimplikar and Simons, 1994]. It is not possible to predict from our studies in which cells were grown as monolayers, whether constitutive pp120 phosphorylation is required for its polarized targeting, if any, at the hepatocyte membrane. It remains possible that the phosphorylation-defective isoforms of pp120 would be targeted predominantly to the apical (bile canalicular) domain, had cells been grown polarized. Future experiments in epithelial cells grown on permeable filter supports are necessary to test this possibility.

Because mutating Ser^{503} to alanine was associated with a marked decrease in pp120 maturation in fibroblasts and hepatocytes, we may conclude that the intracellular domain of pp120 contains complex information necessary for proper intracellular targeting of pp120 in many cell types. It remains possible that the intracellular domain contains elements that inhibit the effects of Ser^{503} in hepatocytes, leading to comparable sorting of the truncated and full-length isoforms in these cells, as opposed to their differential trafficking in NIH 3T3 cells. Further experiments are required to test this hypothesis.

Because the truncated isoform does not increase receptor-mediated insulin endocytosis and degradation in transfected NIH 3T3 cells, as has been proposed for its full-length counterpart, regulation of the intracellular trafficking of the two isoforms in NIH 3T3 cells may constitute an intrinsic cellular control mechanism of the rate of insulin endocytosis and degradation in these cells. It would be of interest to study whether the differential intracellular trafficking of the pp120 isoforms correlates with their differential functions under in vivo conditions. These studies would advance our understanding of pp120 function in tumor suppression and in the transport of insulin and bile acids in hepatocytes.

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