Receptor-mediated Internalization of Insulin

POTENTIAL ROLE OF pp120/HA4, A SUBSTRATE OF THE INSULIN RECEPTOR KINASE*

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pp120/HA4 is a hepatocyte membrane glycoprotein phosphorylated by the insulin receptor tyrosine kinase. In this study, we have investigated the role of pp120/HA4 in insulin action. Transfection of antisense pp120/HA4 cDNA in H35 hepatoma cells resulted in inhibition of pp120/HA4 expression and was associated with a 2- to 3-fold decrease in the rate of insulin internalization. Furthermore, insulin internalization in NIH 3T3 fibroblasts co-transfected with insulin receptors and pp120/HA4 was increased 2-fold compared with cells expressing insulin receptors alone. In contrast, no effect on internalization was observed in cells overexpressing a naturally occurring splice variant of pp120/HA4 that lacks the phosphorylation sites in the intracellular domain. Insulin internalization was also unaffected in cells expressing three site-directed mutants of pp120/HA4 in which the sites of phosphorylation by the insulin receptor kinase had been eliminated (Y488F, Y488F/S503F, and S503A). Our data suggest that pp120/HA4 is part of a complex of proteins required for receptor-mediated internalization of insulin. It is possible that this function is regulated by insulin-induced phosphorylation of the intracellular domain of pp120/HA4.

Insulin binding to its receptor triggers the rapid endocytosis of the ligand-receptor complex (1, 2). Internalization of the insulin-insulin receptor complex constitutes the major mechanism of insulin degradation and down-regulation of cell surface receptors (3–5). The molecular events involved in the internalization process, however, are yet to be well defined. Specific sequences in the submembranous portion of the receptor are required for internalization to occur. Furthermore, activation of the receptor kinase is required for ligand-induced receptor internalization (6–15). Following insulin-induced receptor autophosphorylation, several intracellular substrates are phosphorylated, the best characterized of which is insulin receptor substrate-1 (IRS-1) (16). However, the role of substrate phosphorylation in receptor internalization is not established.

In the present study, we have investigated the role of pp120/HA4, a substrate of the insulin receptor kinase that is predominantly expressed in liver, in the internalization process. pp120/HA4 is a transmembrane glycoprotein that is phosphorylated by the insulin receptor tyrosine kinase in intact cells (17–20) and in cell-free systems (21). It is composed of a large extracellular domain containing 16 sites of potential N-linked glycosylation and a 71-amino acid cytoplasmic domain (22). Studies of site-directed mutants indicate that the intracellular domain contains a site of constitutive serine phosphorylation (SerS503) as well as one tyrosine residue that is phosphorylated in response to insulin (Tyr488) (20, 23). Alternative splicing of pp120/HA4 mRNA generates two isoforms, one of which lacks 61 amino acids at the C terminus of the cytoplasmic domain, including all the potential phosphorylation sites (24). The function of pp120/HA4 is still unknown. It has been proposed to serve as a Ca²⁺/Mg²⁺-dependent ecto-ATPase (22) and a bile acid transporter (25, 26).

We report that insulin-induced receptor internalization is inhibited 2–3-fold in H35 hepatoma cells transfected with an antisense pp120/HA4 cDNA. inhibition of internalization paralleled the reduction of expression of pp120/HA4. Moreover, expression of pp120/HA4 in NIH 3T3 cells transfected with insulin receptors increased the rate of internalization of the insulin-insulin receptor complex. In contrast, internalization rates were unaffected in NIH 3T3 cells expressing the short isoform of pp120/HA4 or by expression of mutant pp120/HA4 molecules in which the potential phosphorylation sites of the intracellular domain (Tyr488 and SerS503) had been replaced by site-directed mutagenesis with nonphosphorylatable amino acids.

Experimental Procedures

Antibodies—pp120/HA4 antibody (α-295) is a rabbit antipeptide polyclonal antiserum directed against the sequence VLLLAHNL-LOQEFQV (amino acids 51–64) of the extracellular domain of rat liver pp120 (32). Anti-insulin receptor antibody 50 was described in previous publications (20).

Cell Culture—H35 hepatoma cells were grown in RPMI medium (Biofluids Inc., Rockville, MD) supplemented with 10% fetal calf serum. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Biofluids Inc., Rockville, MD) containing 10% fetal calf serum (U1, Lake Placid, NY) and 2 mM glutamine (Biofluids, Inc). Transfected cells were routinely maintained at 37 °C in 5% CO₂.

Construction and Transfection of Antisense pp120/HA4 cDNA—cDNA corresponding to nucleotides 8 to 1547 of pp120/HA4 (20, 22) was ligated in a reverse orientation into a bovine papilloma virus-based expression vector (pBPV, Pharmacia Biotech Inc.) at the XhoI/Akt sites. Transfections of H33 cells at 70% confluence were carried out in 100-mm plates with 20, 50, or 100 μg of the expression vector (pBPV/ASpp120) and 1, 2.5, and 5 μg, respectively, of a plasmid containing the neomycin resistance gene (pRSVNe) in the presence of 90 μg of Lipoheitamine (Life Technologies, Inc.) according to the manufacturer's instructions. Single G418-resistant colonies were isolated and analyzed for pp120/HA4 expression by immunoblotting with α-295 antibody as described previously (20).

NIH 3T3 cells co-expressing human insulin receptors and wild-type (WT) pp120/HA4 (full-length and truncated isoforms) or phosphorylation-defective pp120/HA4 mutants (Y488F, Y513F, Y488F/S503F, and S503A) were described previously (20).

Insulin Binding, Internalization, and Degradation—Confluent monolayers of H35 hepatoma and NIH 3T3 cells were washed three times with phosphate-buffered saline and incubated at 4 °C for 4 h in...
binding buffer (100 mM HEPES, 120 mM NaCl, 1.2 mM MgSO₄, 1 mM EDTA, 15 mM CH₃COONa, 10 mM glucose, 1% bovine serum albumin, pH 7.4) containing 30 pm (125I) insulin. Unbound ligand was removed by washing with ice-cold binding buffer. Cells were either lysed with 1 N NaOH (to measure total insulin binding) or further incubated with the same buffer at 37°C for the indicated times (to measure insulin internalization). At each time point, cells were washed with ice-cold phosphate-buffered saline at pH 3.0 and incubated in the same buffer twice for 5 min to remove insulin that was still bound at the cell surface. Following an additional wash with ice-cold phosphate-buffered saline, pH 7.4, cells were solubilized in 1 N NaOH, and internalization rates were calculated as described by Lund et al. (27). Insulin degradation was determined in the incubation medium or in cell lysates (to measure intracellular degradation products) by precipitation with 10% trichloroacetic acid.

PDGF-1 Internalization—Untransfected NIH 3T3 cells or WTpp120 cells were incubated with 125I-PDGF (Amersham Corp.) as described by Mori (28). Thereafter, internalization of labeled ligand was measured using the same wash procedure as described above.

Insulin Receptor Down-regulation—Confluent monolayers were incubated at 37°C in serum-free culture media (RPMI for H35 cells and Dulbecco’s modified Eagle’s medium for NIH 3T3 cells), containing 1% bovine serum albumin and 10 mM HEPES, with or without 100 nM insulin. Surface-bound insulin was removed by the acid wash technique described above, and insulin binding was determined by further incubating cells with 125I-insulin for 4 h at 4°C, as described above.

Insulin Receptor Internalization—Cell monolayers were incubated with biotin (0.5 mg/ml) as described (29). Following incubation at 37°C for 20 min in the absence or presence of 100 nM insulin, cells were incubated with Pronase (2.5 mg/ml) for 1 h at 4°C and solubilized in a buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and a mixture of protease inhibitors. Cell lysates were immunoprecipitated with anti-insulin receptor antisera (Ab50) and analyzed by Western blotting as described (29). PhosphorImager analysis was used to quantitate insulin receptors.

Thymidine Incorporation—Subconfluent 12-well plates were incubated in serum-free medium for 24 h. Thereafter, insulin was added at the indicated concentrations for an additional 16 h. Subsequently, the incubation medium was replaced with medium supplemented with [3H] thymidine (500 nCi/ml), and cells were incubated for 1 h at 37°C. After being washed twice with phosphate-buffered saline pH 7.4, twice with 5% trichloroacetic acid, and twice with ethanol, cells were solubilized in 300 µl of 1 N NaOH for 1 h at 37°C. An equal volume of 1 N HCl was added to the solubilized extracts, and the radioactivity was measured by liquid scintillation counting.

RESULTS

Insulin Internalization and Degradation in H35 Cells Expressing Antisense pp120/HA4 RNA—H35 hepatoma cells were transfected with antisense pp120/HA4 cDNA and analyzed by immunoblotting with anti-pp120/HA4 polyclonal antisera (Fig. 1, upper panel). In six independent clones (AS1-AS6), the steady state levels of pp120/HA4 were decreased 4–6-fold compared with untransfected H35 cells and with mock-transfected cells (Fig. 1, neo1, neo2). Insulin binding and receptor autophosphorylation were similar in all clones studied and in control cells (data not shown). To measure internalization, cells were allowed to bind 125I-insulin at 4°C and then were transferred to 37°C for various lengths of time. In control cells (untransfected H35 and Neo-transfected cells), 50% of bound 125I-insulin was internalized after 1 min at 37°C (Fig. 2A). In contrast, cells expressing antisense pp120/HA4 RNA (AS) internalized only 35% of bound insulin after 1 min at 37°C (Fig. 2A).

Following receptor-mediated internalization, insulin molecules are released from the receptor by the acidic pH of the endocytic vesicles, and insulin is subjected to intracellular degradation. Insulin degradation was measured as trichloroacetic acid-soluble radioactivity in clones expressing antisense pp120/HA4 RNA (Fig. 2B). Consistent with the decreased internalization rate, insulin degradation was also decreased 2-fold in AS cells (15% of bound insulin, following 20 min incubation at 37°C) compared with both untransfected and Neo-transfected cells (35 and 42% of bound insulin, respectively). After 30 min at 37°C, 78% of bound insulin was degraded in H35, 82% in Neo cells, and 28% in AS cells, indicating that insulin degradation occurred at a slower rate in cells expressing antisense pp120/HA4 RNA. Similar results were also obtained when insulin degradation was measured in cell lysates (data not shown).

Insulin-induced receptor down-regulation was analyzed in AS and control cells following incubation with 100 nM insulin for 24 h at 37°C. At the end of the incubation period, the
corresponding to major bands were detected at approximately 130 and 95 kDa, 

In the absence of Pronase treatment, two

with anti-insulin receptor antibody (Ab50) and probed with

surface proteins, lysed with Triton X-100, immunoprecipitated

monolayers were treated with Pronase to remove residual cell

the absence or presence of 100 nM insulin (Fig. 5). Thereafter, PDGF internalization in NIH 3T3 cells (open squares) or cells expressing WTpp120 (filled squares) was measured by the acid wash technique as described. Experiments were performed in triplicate.

receptor number on the surface of control cells (H35 and Neo) was decreased by 15–20%. In contrast, AS cells did not down-regulate the number of cell surface receptors in response to insulin (data not shown).

Insulin Receptor Internalization in NIH 3T3 Cells Transfected with pp120/HA4—Next, we studied insulin receptor internalization as a measure of insulin endocytosis in NIH 3T3 cells co-expressing insulin receptors (hIR) and either full-length (WTpp120) or the truncated form (∆448) of pp120/HA4 that is expressed as a splice variant in liver. In cells expressing hIR, 37% of bound hormone was internalized after 10 min at 37 °C (Fig. 3). In cells co-transfected with hIR and full-length pp120/HA4 (WTpp120), incubation at 37 °C resulted in internalization of 70% of bound insulin after 10 min. In contrast, in cells co-transfected with truncated pp120/HA4 (∆448) and hIR, insulin internalization was comparable with that of cells expressing hIR alone. This effect appeared to be specific for the insulin receptor in that ligand-dependent internalization of the PDGF receptor, another member of the receptor tyrosine kinase family, was unaffected by transfection of pp120/HA4. In untransfected NIH 3T3 cells, 70% of bound 125I-PDGF was internalized after 20 min at 37 °C (Fig. 4). In WTpp120 cells, internalization of PDGF proceeded at a slightly faster rate and peaked at 15 min but was otherwise similar in extent to that observed in untransfected cells. Likewise, we failed to detect differences between hIR cells and WTpp120 cells when we measured internalization of transferrin by the transferrin receptor, a constitutively recycled, non-tyrosine kinase receptor (data not shown). Thus, it appears that the effect of pp120/HA4 to increase internalization is specific for insulin.

Insulin receptor internalization was also studied using biotin labeling of cell surface proteins followed by immunoprecipitation with anti-insulin receptor antibody and immunodetection with [125I]streptavidin. Cell surface proteins were biotinylated at 4 °C and then transferred to a 37 °C incubator for 15 min in the absence or presence of 100 nM insulin (Fig. 5). Thereafter, monolayers were treated with Pronase to remove residual cell surface proteins, lysed with Triton X-100, immunoprecipitated with anti-insulin receptor antibody (Ab50) and probed with [125I]streptavidin. In the absence of Pronase treatment, two major bands were detected at approximately 130 and 95 kDa, corresponding to α- and β-subunits of the insulin receptor (29). Pronase treatment resulted in a 90–95% decrease of cell surface receptors (Fig. 5, solid bars). Preincubation with insulin rendered a larger fraction of insulin receptors expressed in NIH 3T3 cells resistant to digestion with Pronase as a result of having undergone internalization (Fig. 5, hatched bars). Consistent with insulin internalization data, the amount of internalized receptors was 2-fold higher in WTpp120 cells (33 ± 5% of the total biotinylated receptors were internalized) than in hIR (16 ± 4%) and ∆448 cells (12 ± 3%).

Thus, expression of full-length pp120/HA4, but not of the truncated isoform, was associated with increased internalization of insulin-receptor complexes in NIH 3T3 cells.

Insulin Receptor Internalization in Cells Transfected with Phosphorylation-defective Mutants of pp120/HA4—We have recently shown that mutation of Tyr488 (Y488F) impaired phosphorylation of pp120/HA4 by the insulin receptor in vitro and in intact cells (20). Similarly, mutation of both Tyr488 and Tyr513 (Y488F/Y513F) also impaired insulin-induced phosphorylation. In contrast, mutation of Tyr513 did not affect the ability of pp120/HA4 to undergo insulin-stimulated phosphorylation. Finally, substitution of Ser503 with alanine (S503A) impaired basal as well as insulin-induced phosphorylation of pp120/HA4 (20).

In NIH 3T3 cells co-transfected with hIR and phosphorylation-defective mutants of pp120/HA4 (Y488F, Y488F/Y513F, or S503A), insulin internalization rates were similar to those observed in cells expressing hIR alone and cells co-expressing hIR and ∆448 (Fig. 6A). In contrast, insulin internalization rates in cells transfected with Y513F mutant were similar to those observed in cells expressing WTpp120 (Fig. 6A). Thus, we observed a correlation between increased rates of insulin endocytosis and expression of phosphorylation-competent forms of pp120/HA4.

Insulin Degradation and Insulin-induced Receptor Down-regulation in NIH 3T3 Transfected Cells—As shown in Fig. 6B,
insulin degradation products were detected in the medium of WTpp120 cells after 20 min at 37 °C (7 ± 2% of bound insulin). Over the same time period, insulin degradation in WTpp120 cells was 2-fold higher (15 ± 3%), whereas in Δ448 cells insulin degradation was similar to that measured in hiR cells (6 ± 3%). Further incubation at 37 °C resulted in increased degradation of insulin in all clones, so that at 60 min 52% of insulin was degraded in hiR cells, 59% in WTpp120 cells, and 50% in Δ448 cells (data not shown). In cells expressing phosphorylation-defective mutants of pp120/HA4 (Y488F, Y488F/Y513F, and S503A) degradation of insulin was similar to hiR and Δ448 cells (Fig. 6B). In contrast, Y513F cells internalized and degraded insulin at the same rate as cells transfected with WTpp120 (Fig. 6B). Thus, in all clones studied we observed a good correlation between insulin endocytosis and intracellular degradation.

Receptor down-regulation was evaluated in clones transfected with hiR and different forms of pp120/HA4. In hiR cells, insulin binding decreased by 37% after 24 h of insulin treatment. In contrast, at the same time point, the number of surface receptors in WTpp120 cells decreased by 50% (Fig. 7). In cells transfected with the truncated isoform of pp120/HA4 (Δ448), insulin receptor down-regulation was similar to hiR cells (34%). Insulin-induced receptor down-regulation of Y488F, Y488F/Y513F, and S503A cells was comparable with that observed in Δ448 cells. In cells expressing Y513F, receptor down-regulation displayed a pattern similar to that of WTpp120 cells, with 47% of receptors being removed from the cell surface by 24 h (Fig. 7).

Insulin-stimulated Thymidine Incorporation—We measured thymidine incorporation in cells transfected with sense and antisense cDNA encoding pp120/HA4 as an index of the mitogenic effect of insulin (Table I). Maximal thymidine incorporation in all clones was observed between 10⁻⁹ and 10⁻⁸ M insulin. However, cells expressing antisense pp120/HA4 RNA showed a leftward shift in the dose-response curve to insulin (ED₅₀ = (1.5 ± 0.3) × 10⁻⁹ M), compared with control H35 (ED₅₀ = (4.9 ± 0.2) × 10⁻⁹ M) and Neo cells (ED₅₀ = (5.1 ± 0.3) × 10⁻⁸ M). Conversely, cells transfected with WTpp120 showed a rightward shift of the dose-response curve for insulin (ED₅₀ = (3.0 ± 0.4) × 10⁻¹⁰ M) compared with hiR cells (ED₅₀ = (0.7 ± 0.1) × 10⁻¹⁰ M) and Δ448 cells (ED₅₀ = (0.6 ± 0.2) × 10⁻¹⁰ M). Therefore, cells overexpressing hiR alone are more sensitive to the mitogenic effects of insulin than cells expressing WTpp120/HA4. Cells co-transfected with hiR and Y513F showed a dose-response curve similar to WTpp120 cells (ED₅₀ = (2.4 ± 0.2) × 10⁻¹⁰ M), whereas cells co-transfected with hiR and one of the three phosphorylation-defective mutants displayed ED₅₀ = (0.8 ± 0.4) × 10⁻¹⁰ M (Y488F), (0.7 ± 0.2) × 10⁻¹⁰ M (Y488F/Y513F), and (0.8 ± 0.1) × 10⁻¹⁰ M (S503A).

**DISCUSSION**

pp120/HA4 is a transmembrane glycoprotein that is phosphorylated on tyrosine residues by the insulin receptor kinase (17-21). In the present study we have shown that inhibition of the expression of pp120/HA4 is associated with decreased endocytosis of insulin. We have also shown, using transfected cells, that overexpression of pp120/HA4 is associated with increased insulin endocytosis. Thus, manipulation of pp120/HA4 expression correlates with changes in insulin receptor-mediated internalization and degradation of insulin. Moreover, this effect of pp120/HA4 appears to require phosphorylation by the insulin receptor or other kinases, inasmuch as expression of phosphorylation-defective mutants of pp120/HA4 (20) is not associated with changes in internalization of the insulin-insulin receptor complex. The effect is specific for insulin endocytosis, because internalization of PDGF, a ligand that is also internalized via a receptor tyrosine kinase, is not affected by expression of pp120/HA4, nor is internalization of the unrelated molecule transferrin. Our data do not indicate whether there is a causal relation between expression of pp120/HA4 and endocytosis of insulin. It is, however, interesting to note that pp120/HA4 is predominantly expressed on the plasma mem-

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**FIG. 6. Insulin internalization and degradation in cells expressing pp120HA4 phosphorylation-defective mutants.** Insulin internalization (upper panel) and degradation (lower panel) were measured as described in the legend to Fig. 2. Insulin internalization rates (upper panel) were determined according to Lund et al. (27). Insulin degradation (lower panel) represents the fraction of trichloroacetic acid-soluble radioactivity detected in the extracellular medium after 20 min at 37 °C. All results are expressed as the mean ± S.D. of three triplicate experiments with at least two different clones of each cell type.

**FIG. 7. Insulin-induced receptor down-regulation in NIH 3T3 transfected cells.** Down-regulation was measured in hiR (open circles), WTpp120 (open squares), Δ448 (open triangles), Y488F (solid squares), Y513F (solid circles), Y488F/Y513F (solid triangles), and S503A (open diamond) cells as described under “Experimental Procedures.” Data are means ± S.D. of three triplicate experiments with at least two clones of each cell type.
brane of the hepatocyte, a major site of insulin clearance from the circulation. The mechanism by which pp120/HA4 might affect insulin receptor internalization is not clear. Our data suggest that phosphorylation of pp120/HA4 by the insulin receptor kinase may play a role in this process. The lack of a dominant negative effect by phosphorylation-defective mutants of pp120/HA4 on insulin internalization is consistent with the notion that there might exist pp120-dependent and pp120-independent internalization pathways. The tissue distribution of pp120/HA4, which is predominantly found in liver, is also consistent with the idea that pp120 may play a specific role in hepatic insulin clearance. pp120/HA4 may be part of a complex of proteins contributing to the interaction of insulin receptors with clathrin-coated pits (30–32). In support of the latter hypothesis, it is of interest to note that the amino acid sequence of pp120/HA4 shares homology with tyrosine-containing sequences thought to be important recognition elements for AP-2 adapters (33, 34). These sequences are found at positions 448–491 (Tyr-Ser-Val-Leu) and 513–516 (Tyr-Ser-Val-Val) of pp120/HA4. This finding is in agreement with the hypothesis that phosphorylation of pp120/HA4 by the insulin receptor kinase activates a second messenger mechanism by which this effect is elicited. Differential expression of the two isoforms of pp120/HA4 in liver may be important to modulate insulin clearance and to regulate receptor number at the cell surface.

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### Table I

| Cell type | $E_{50}$ (× 10$^{-10}$ M) |
|-----------|-------------------|
| hIR       | 0.7 ± 0.1         |
| WTpp120   | 3.0 ± 0.4         |
| ΔΔ46      | 0.6 ± 0.2         |
| Y488F     | 0.8 ± 0.4         |
| Y513F     | 2.4 ± 0.2         |
| Y488FY513F| 0.7 ± 0.2         |
| SS03A     | 0.8 ± 0.1         |

Phosphorylation of pp120/HA4 and Insulin Internalization