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Identification of pp120, an endogenous substrate for the hepatocyte insulin receptor tyrosine kinase, as an integral membrane glycoprotein of the bile canalicular domain

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ABSTRACT **An endogenous membrane-bound substrate of the insulin receptor β-subunit tyrosine kinase in liver, pp120, has been identified as HA4, a 110-kDa membrane glycoprotein localized primarily to the bile canalicular domain of the hepatocyte. HA4 has been implicated in bile salt transport and cell adhesion. Monoclonal antibodies to HA4 were used to identify it as a substrate of the insulin receptor kinase. Anti-pp120 and anti-HA4 were found to cross-react, and phosphopeptide maps for each of the corresponding antigens were identical. The identification of pp120 as HA4 serves to link insulin action through the receptor tyrosine kinase activity to bile metabolism and raises questions pertaining to the intracellular site(s) of action of the insulin receptor.**

Several potential mechanisms of action have been proposed to explain the pleiotypic actions of insulin in tissues such as liver. These have included the generation of second messengers (1–3), interaction with guanine nucleotide-binding proteins (4), the activation of phosphoinositol turnover (5), and change in protein phosphorylation (1–3). The last has become an attractive hypothesis following the discovery that the 95-kDa β-subunit of the insulin receptor possessed tyrosine kinase activity (2) against itself (autophosphorylation) as well as exogenous and endogenous substrates (2, 6–11). Several membrane-bound and cytosolic substrates for insulin receptor β-subunit tyrosine kinase activity have been identified in a number of tissues (1). However, in most cases neither the functions of the endogenous proteins nor the effect of insulin-stimulated phosphorylation on them is known. An exception is pp15, a soluble substrate in adipocytes that is thought to act as the intermediary in insulin-stimulated glucose transport (11).

One of the endogenous substrates present in liver is a protein called pp120. It is found in a plasma membrane-enriched fraction, is detergent-soluble, and binds to wheat germ agglutinin (WGA), indicating that it is a glycoprotein with complex oligosaccharide chains. pp120 is also phosphorylated on tyrosine residues by physiological levels of insulin *in vitro* and intact hepatoma cells (6–9). However, the identity and precise cellular location of pp120 *in vivo* are not known. In this study, we have identified pp120 as HA4, an integral plasma membrane glycoprotein that is largely restricted to the bile canalicular membrane domain *in vivo*.

MATERIALS AND METHODS

Preparation of Antibodies. Antibodies to pp120 were raised in New Zealand White rabbits (6), and antiinsulin receptor antiserum B-d was obtained from a human patient with extreme insulin resistance (12). Mouse monoclonal antibodies to rat liver membrane proteins HA301, HA201, and HA4 were obtained as described by Hubbard *et al.* (13, 14) and coupled to Sepharose beads.

Membrane Protein Preparations. Plasma membrane sheets were prepared from livers of fasted rats (13) and the integral membrane proteins were extracted (15). pp120 and the insulin receptor were partially purified from solubilized rat liver plasma membranes by using lectin affinity chromatography (6–9, 16). Peak fractions eluted from the WGA columns were pooled and an aliquot was used to determine insulin binding.

Phosphorylation Reaction. Aliquots of WGA eluates were incubated with or without 100 nM insulin (crystalline porcine insulin, lot no. 615–2H2–300, Eli Lilly) for 30 min at room temperature in the presence of protease inhibitors (leupeptin, aprotinin, bacitracin, and pepstatin at 1 μg/ml; 1 mM phenylmethylsulfonyl fluoride). Fifty microcuries of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was added and the incubations were continued for a further 10 min at room temperature. Reactions were stopped by the addition of 4 mM EDTA/100 mM NaF/4 mM sodium pyrophosphate/2 mM sodium vanadate (final concentrations).

Immunoprecipitations. Plasma membrane extracts, WGA eluates, or aliquots of the phosphorylation mix were incubated with polyclonal antisera (anti-pp120 or antiinsulin receptor) or monoclonal antibodies coupled to Sepharose (anti-HA301, anti-HA201, anti-HA4) overnight at 4°C. Immunoprecipitations with polyclonal antibodies required the addition of *Staphylococcus aureus* cells (Pansorbin, Behring Diagnostics, San Diego, CA). All immune complexes were collected and washed as described (6, 13, 14, 17, 18). In some cases (Fig. 2), the unbound material (supernate) was concentrated by acid precipitation and prepared for $NaDodSO_4$/PAGE (see below). In other instances (Fig. 3), extracts were depleted of one antigen by preincubation with the corresponding antibody. After removal of immune complexes the supernates were incubated with a second antibody and immune complexes were precipitated.

Gel Electrophoresis. ^{32}P-labeled immune pellets or aliquots of the supernates were solubilized in Laemmli buffer (19) and electrophoresed in 7.5% $NaDodSO_4$ slab gels, and the gels were fixed, dried, and exposed to Kodak X-Omat AR2 film with intensifying screens at −70°C. HA4 was detected by immunoblotting after transfer of the polypeptides from $NaDodSO_4$ gels to nitrocellulose (20). The blots were incubated first with rabbit antiserum to HA4; this was followed by incubation with ^{125}I-labeled protein A (New England Nuclear) and then exposed to x-ray film, as described above.

Phosphopeptide Mapping. WGA eluate fractions, pretreated with 100 nM insulin, were phosphorylated with 1 mCi of [γ-^{32}P]ATP as described above and then incubated overnight at 4°C with antiinsulin receptor, anti-HA4, or anti-pp120.

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Abbreviation: WGA, wheat germ agglutinin.

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Immune pellets were processed for $NaDodSO_4$/PAGE as described above, and then the gels were fixed, dried, and exposed to x-ray film for 2 hr. Gel bands corresponding to individual labeled proteins were cut out, rehydrated, and eluted overnight at 4°C with WGA elution buffer (0.3 ml per band). After sedimentation of the gel bands, aliquots of the supernates were incubated for 10 min at room temperature with varying concentrations of *S. aureus* V8 protease (21). Samples were then electrophoresed on 15% $NaDodSO_4$/polyacrylamide gels and processed for autoradiography.

Isolated Liver Perfusion. The livers from overnight-fasted, adult male rats (150 g) (CD strain, Charles River Breeding Laboratories) were excised and perfused as described by Dunn *et al.* (22) using phosphate-free medium. Carrier free $^{32}P_i$ (5 mCi; 3000 Ci/mmol; New England Nuclear) was injected and allowed to circulate for 5 min. A biopsy (≈100 mg) was taken and rapidly frozen using aluminum clamps cooled to the temperature of liquid nitrogen (23). One hundred micromolar insulin (Eli Lilly) was then added and allowed to circulate for 3 min, and a second liver biopsy was taken. The frozen samples were homogenized using a Polytron (Brinkmann) in ice-cold 0.25 M sucrose/1 mM imidazole, pH 7.4, with the phosphatase inhibitors listed above. Small aliquots were removed for determination of protein concentrations (bicinchoninic acid, Pierce). Homogenates were diluted with 5× KBO buffer (0.5% Triton X-100/0.3 M NaCl/25 mM NaP_i, pH 7.4/20 mM octyl glucoside/0.02% sodium azide), dispersed occasionally in a Vortex for 30 min and centrifuged for 60 min at 45,000 rpm in a Ti 50 rotor (Beckman). The supernates were removed, and equivalent protein amounts were diluted in KBO buffer with 5 mg of bovine serum albumin per ml, added to 50 μl of anti-HA4-Sepharose, and incubated overnight at 4°C. The beads were processed as described (17). Samples were solubilized and electrophoresed and autoradiography was carried out as described above.

RESULTS

Based on the electrophoretic mobility of pp120 and its characterization as a detergent-soluble glycoprotein with affinity for WGA, we asked whether antibodies to rat hepatocyte membrane proteins having similar characteristics and electrophoretic mobilities in $NaDodSO_4$/PAGE (13, 14) would be phosphorylated in an insulin-dependent fashion. Three proteins were chosen, all of which have been shown to be concentrated in the apical or bile canalicular membrane *in situ* (13). These are dipeptidylpeptidase IV (monoclonal designation HA301), leucine aminopeptidase N (HA201), and a putative bile acid transporter (HA4). WGA eluates prepared from a plasma membrane-enriched fraction were incubated with or without 100 nM insulin, phosphorylated with [^{32}P]ATP, and then incubated with antiinsulin receptor, anti-pp120, anti-HA4, anti-HA201, or anti-HA301. As shown earlier (6–9) and presented in Fig. 1, the insulin receptor β-subunit and pp120 were phosphorylated in an insulin-dependent fashion. Neither HA301 (110 kDa) nor HA201 (150 kDa) could be detected as phosphoproteins in immune pellets. However, HA4, the third hepatocyte apical membrane protein with a published molecular mass of 105–115 kDa (13, 14), was phosphorylated and had an electrophoretic mobility similar to that of pp120.

Experiments were then performed to test whether pp120 and HA4 were immunologically related. Plasma membrane extracts were incubated with either HA4-Sepharose or anti-pp120 antiserum plus protein A-Sepharose; then the immune complexes and supernates from each immunoprecipitation were analyzed by $NaDodSO_4$/PAGE and immunoblotting with anti-pp120 and anti-HA4 antisera. As illustrated in Fig. 2, both antibodies recognized the same antigen, which had a

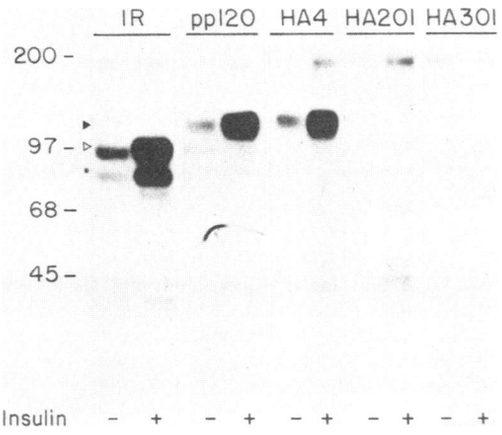

FIG. 1. WGA-agarose eluates from adult rat liver were preincubated with (+) or without (−) insulin (0 and 0.1 μM) and then phosphorylated. Aliquots were immunoprecipitated with monoclonal or polyclonal antibodies against five rat liver membrane glycoproteins: insulin receptor (IR), pp120, HA4, HA201, or HA301. Samples were prepared for $NaDodSO_4$/PAGE on 7.5% gels. Size markers are indicated in kDa. The positions of pp120 and HA4 (▶) and the β-subunit of the insulin receptor (▷) are marked. A common proteolytic fragment of the 95-kDa β-subunit can be seen (small asterisk).

characteristic doublet appearance, with a broad band migrating at 105 kDa and a narrower band of a slower mobility (115 kDa). This identity was further substantiated by sequential immunoprecipitations of WGA eluates that had been incubated with insulin and then phosphorylated. As shown in Fig. 3, polyclonal anti-pp120 removed virtually all of the ^{32}P-labeled HA4 in the sample, since subsequent incubation of the resulting supernate with anti-HA4 gave no evidence of a phosphoprotein. When the sequence of immunoprecipitation was reversed, significant levels of ^{32}P-labeled HA4 were present in the first immune pellet and substantially lower amounts were present after incubation with anti-pp120. Only the more slowly migrating band of the HA4 doublet was phosphorylated (data not shown). If immunoprecipitations were performed before phosphorylation of the remaining material, no ^{32}P-labeled proteins were found upon subsequent exposure to anti-HA4 or anti-pp120 . However, anti-insulin receptor antibody still immunoprecipitated a ^{32}P-labeled β-subunit (data not shown).

As a final determination of the identity of HA4 and pp120, the two ^{32}P-labeled polypeptides were eluted from rehydrated gels, incubated with *S. aureus* V8 protease, and reelectrophoresed (21). The resulting phosphopeptide maps gave identical patterns for pp120 and HA4 (Fig. 4). These peptide maps were in turn distinct from the pattern of the insulin

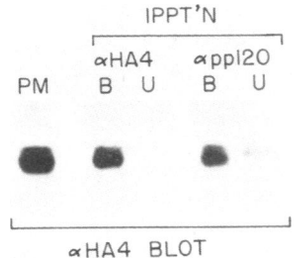

FIG. 2. Detergent extracts of isolated plasma membrane sheets (PM) were incubated with either anti-HA4-Sepharose (αHA4) or pp120 antiserum (αpp120) followed by protein A-Sepharose. After sedimentation the unbound (U) and bound (B) material in the supernates and pellets, respectively, was analyzed by $NaDodSO_4$/PAGE and immunoblotting with polyclonal anti-HA4 and ^{125}I-labeled protein A. IPPT'N, immunoprecipitation.

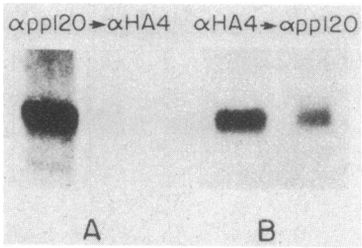

FIG. 3. WGA eluates were incubated with insulin (100 nM), ^{32}P-phosphorylated, and then exposed to anti-pp120 (*A*) or anti-HA4 (*B*). Immune complexes were obtained, the pellets were saved, and the supernates were then incubated with either anti-HA4 (*A*) or with anti-pp120 (*B*), and immune complexes were obtained. All pellets were analyzed by NaDodSO$_4$/PAGE and autoradiography.

receptor β-subunit, indicating that HA4 and pp120 were distinct from insulin receptor but were identical to each other.

Having established that HA4, a bile canalicular membrane protein, is an *in vitro* substrate for the insulin receptor tyrosine kinase, we next asked whether HA4 *in situ* could be phosphorylated in an insulin-dependent manner. As shown in Fig. 5, addition of 100 μM insulin to an isolated, perfused liver for only 3 min resulted in the enhanced incorporation of ^{32}P into HA4.

DISCUSSION

In this study we have identified pp120, an endogenous protein substrate of the insulin-stimulated insulin receptor β-subunit tyrosine kinase, as HA4, an integral glycoprotein that has been localized to the apical or bile canalicular plasma membrane domain of the hepatocyte *in situ*. Several lines of evidence indicate that pp120 and HA4 are the same protein. First, immunodepletion of one antigen from a WGA eluate or a detergent-solubilized extract of plasma membrane eliminates detection of the other antigen, as assayed by insulin-dependent phosphorylation and immunoprecipitation either before or after phosphorylation (Fig. 3) or by direct immunoblotting (Fig. 2). Second, peptide maps of the ^{32}P-labeled forms of pp120 and HA4 are identical and distinct from that

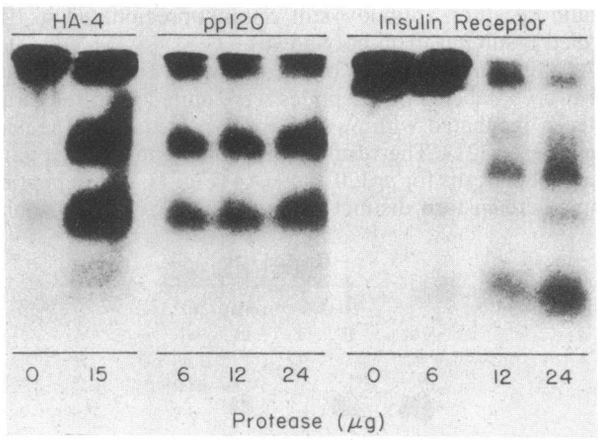

FIG. 4. WGA eluates were incubated with insulin, ^{32}P-phosphorylated, and then immunoprecipitated with either antiinsulin receptor, anti-pp120, or anti-HA4. Immune complexes were separated on 7.5% NaDodSO$_4$/polyacrylamide gels, which were then fixed, dried, and exposed to x-ray film for 2 hr. Labeled bands were cut from the dried gels, rehydrated, extracted (0.3 ml), and then incubated with indicated amounts of *S. aureus* V8 protease for 15 min at 30°C. The samples were boiled in Laemmli buffer and the ^{32}P-labeled peptides were separated on 15% NaDodSO$_4$ gels. Dried gels were exposed for 2–3 weeks.

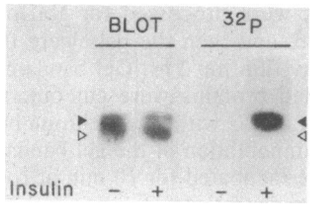

FIG. 5. Detergent extracts of biopsy specimen obtained from an isolated, perfused liver preparation perfused in the presence of 5 mCi of ^{32}P$_i$ were immunoprecipitated with anti-HA4-Sepharose, and the immune complexes were analyzed by NaDodSO$_4$/PAGE and autoradiography (^{32}P) following transfer to nitrocellulose. After the ^{32}P signal had decayed (>2 weeks), polyclonal anti-HA4 and ^{125}I-labeled protein A were applied, the blot was reexposed to x-ray film, and the two developed films (^{32}P and ^{125}I) were aligned. The positions of the upper (▶) and lower (▷) bands of HA4 are marked.

of the insulin receptor β-subunit (Fig. 4). Other possible candidates, such as leucine aminopeptidase N (HA201) or dipeptidylpeptidase IV (HA301), were ruled out (Fig. 1).

The function of HA4 at the bile front of hepatocytes is not yet known; however, two putative functions have been ascribed to molecules with similar biochemical characteristics and similar cellular location. Recently, Ruetz *et al.* (24), using a photolabile [^{3}H]taurocholate compound, identified a bile canalicular membrane protein of 100 kDa that acts as a bile acid transporter. This integral membrane glycoprotein binds to WGA and, when deglycosylated, migrates at an apparent molecular mass of 48 kDa. These reported properties are strikingly similar to those of HA4 (13), suggesting that HA4 may correspond to this bile acid transporter. However, Obrink and colleagues (25) have reported that antibody to a molecule of 105 kDa is able to block adhesion of collagenase-dissociated adult rat hepatocytes. We have found that Fab fragments of anti-HA4 will also block cell–cell adhesion (D. Cox, L. T. B. Braiterman, A.L.H., and S. R. Roseman, unpublished data).

A recent study of the role of insulin in functional differentiation of the hepatocyte during late gestation revealed that the insulin receptor with active β-subunit tyrosine kinase was present as early as day 14 of gestation (22-day term) (26). Although autophosphorylation of the receptor β-subunit was observed very early in liver development, phosphorylation of pp120 was not observed until day 17 of gestation. HA4 was first detected immunologically in fetal liver at day 17 of gestation as well (18), a time that corresponds to the onset of fetal rat liver bile salt (taurocholate) synthesis and secretion (27). This observation, together with the observations of Ruetz *et al.* (24), is at least consistent with a putative role for HA4 in some aspect of bile metabolism. Consequently, the identity of pp120 as HA4 raises questions concerning the role of insulin in bile metabolism.

All preceding studies of pp120 have been conducted *in vitro* or in unpolarized, cultured hepatoma cells, not with the polarized hepatocyte *in situ*. Our preliminary studies using the isolated, perfused liver system (22) indicate that HA4 *in situ* is phosphorylated in an insulin-dependent manner. This raises an important question: How and in what cellular compartment does the insulin receptor kinase phosphorylate HA4, given the apical disposition of the latter and the basolateral disposition of the former? Bartles *et al.* (17) have reported that five newly synthesized hepatocyte plasma membrane proteins are initially delivered to the basolateral membrane, with subsequent transcellular transport of those functioning at the apical domain by as yet unknown mechanisms. Perhaps the HA4 that is phosphorylated *in vivo* represents new molecules transiently present in the basolateral membrane. It is possible that phosphorylation expedites the transit of HA4 to the apical domain. An alternative

possibility is that internalized insulin receptor kinase phosphorylates HA4 *in situ* at the bile canaliculus or in some other intracellular membrane compartment. There is substantial evidence that the insulin receptor is internalized into the endosomal compartment(s) with active β-subunit kinase activity (28), where HA4 might also be in transit to the apical domain. Yet another alternative would be that the insulin receptor β-subunit tyrosine kinase initiates a cascade of tyrosine kinase phosphorylations that ultimately result in phosphorylation of HA4 by another kinase not directly attached to the receptor β-subunit.

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