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Coordinate Phosphorylation of Insulin-Receptor Kinase and Its 175,000-M_r Endogenous Substrate in Rat Hepatocytes

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To investigate the early events in insulin signal transmission in liver, isolated rat hepatocytes were labeled with ^{32}P, and proteins phosphorylated in response to insulin were detected by immunoprecipitation with anti-phosphotyrosine and anti-receptor antibodies and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and autoradiography. In these cells, insulin rapidly stimulated tyrosine phosphorylation of the 95,000-*M*, β-subunit of the insulin receptor and a 175,000-*M*, phosphoprotein (pp175). Both proteins were precipitated by anti-phosphotyrosine antibody, whereas only the insulin receptor was recognized with anti-insulin-receptor antibody. In the insulin-stimulated state, both pp175 and the receptor β-subunit were found to be phosphorylated on tyrosine and serine residues. Based on precipitation by the two antibodies, receptor phosphorylation was biphasic with an initial increase in tyrosine phosphorylation followed by a more gradual increase in serine phosphorylation over the first 30 min of stimulation. The time course of phosphorylation of pp175 was rapid and paralleled that of the β-subunit of the insulin receptor. The pp175 was clearly distinguished from the insulin receptor, because it was detected only when boiling SDS was used to extract cellular phosphoproteins, whereas the insulin receptor was extracted with either Triton X-100 or SDS. In addition, the tryptic peptide maps of the two proteins were distinct. The dose-response curve for insulin stimulation was shifted slightly to the left of the insulin receptor, suggesting some signal amplification at this step. These data suggest that pp175 is a major endogenous substrate of the insulin receptor in liver and may be a cytoskeletal-associated protein. Both serine and tyrosine sites are involved in insulin-stimulated phosphorylation in hepatocytes. *Diabetes* 40:66–72, 1991

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The current model of insulin signal transduction across the membrane is based on the nature of the insulin receptor as a tyrosine kinase (1,2). According to this model, insulin binds to the extracellular α-subunit of the receptor, activating the tyrosine kinase of the transmembrane β-subunit. The activated tyrosine kinase leads to phosphorylation of the receptor itself and intracellular proteins, which in their phosphorylated form may serve as messengers for the insulin signal. This model has been supported by studies with in vitro mutants of the receptor that lack kinase activity and by studies with monoclonal antibodies to the β-subunit antibodies that inhibit tyrosine kinase activity and lead to loss of insulin action (3,4).

In the pursuit of substrates that undergo phosphorylation by the insulin receptor in intact cells, several proteins have been identified in transformed and nontransformed cells (5–9). One of the first such proteins was a 185,000-*M*, protein we observed in Fao hepatoma cells (5). A protein of similar molecular weight has been observed in several transformed cell lines after insulin stimulation (6–8). In this study, we utilized anti-phosphotyrosine and anti-insulin-receptor antibodies to study insulin-stimulated phosphorylation in nontransformed freshly isolated rat hepatocytes. We found that in hepatocytes, insulin stimulates the rapid tyrosine phosphorylation of the β-subunit of the insulin receptor, which is followed by a slower rise in serine-threonine phosphorylation of the receptor. During the initial response to insulin, an endogenous substrate of a 175,000-*M*, phosphoprotein (pp175) is also phosphorylated on tyrosine residues in an insulin-dependent manner. This protein has similarities to pp185 but also has some unique characteristics in its solubility and immunoprecipitability compared with its counterpart in hepatoma cells.

RESEARCH DESIGN AND METHODS

Sprague-Dawley rats were purchased from Charles River UK (Margate). Type IV collagenase was obtained from Cooper (Malvern, PA), [^{32}P]orthophosphate and Triton X-100 from Du Pont-NEN (Boston, MA), phosphoamino acids from Sigma (St. Louis, MO), pork insulin from Elanco (Indianapolis, IN), reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad (Richmond, CA), Pansorbin from Calbiochem (La Jolla, CA), cellulose thin-layer plates from Analtech (Newark, DE), and RPMI-1640 tissue culture medium from Gibco (Grand Island, NY). Polyclonal anti-phosphotyrosine antibody was prepared in rabbits as previously described and affinity purified on phosphotyramine Sepharose (10). Anti-insulin-receptor antibody was from the serum of patient B-9 (11).

Hepatocytes were isolated from male Sprague-Dawley rats weighing 160–200 g fed ad libitum via a modification (12) of the method of Berry and Friend (13). Cells were washed with 137 mM NaCl supplemented with 2.7 mM KCl and 20 mM HEPES (pH 7.4) and resuspended in phosphate-free RPMI-1640 to give a final cell concentration of 2×10^6/ml. Cell viability was 80–90% as judged by trypan blue exclusion. Labeling of the hepatocytes with [^{32}P]-orthophosphate was accomplished by incubating 0.5-ml aliquots of the cell suspension for 90 min with 1 mCi of [^{32}P]orthophosphate at 37°C in a humidified atmosphere composed of 95% O_2/5% CO_2.

After stimulation by insulin for 1 min (except as otherwise indicated), the reaction was stopped with one of two methods. For Triton X-100 extracts, the reaction was stopped by adding 0.5 ml of ice-cold stopping solution composed of 50 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 2 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin. The mixture was vigorously vortexed, cooled on dry ice–methanol until minimal ice was detected in the bottom of the tube, vortexed again, and kept on ice for 30 min. For SDS extraction, 0.5 ml of boiling stopping buffer was added to give the same concentrations as described above except that 1% SDS replaced the 1% Triton X-100 and phenylmethylsulfonyl fluoride and aprotinin were omitted. After boiling for 15 min, the sample was cooled in an ice bath for 60 min. After both extraction procedures, the samples were centrifuged at 200,000 × *g* for 45 min at 0–4°C, and the supernatant was used for immunoprecipitation.

Immunoprecipitation with anti-insulin-receptor antibody B-9 and anti-phosphotyrosine antibody were performed at dilutions of 1:200 and 1:100, respectively (14). Immunoprecipitated proteins were solubilized in Laemmli buffer with 100 mM dithiothreitol and were separated in 7.5% polyacrylamide gels by electrophoresis. The gels were stained with Coomassie blue in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-OMAT film. Molecular weights of proteins were calculated with standard proteins (Bio-Rad). Incorporation of ^{32}P into individual bands was quantitated by scanning densitometry of the film.

Tryptic phosphopeptides were obtained from the protein bands in polyacrylamide gel fragments as previously described (15). The positions of the phosphorylated proteins were determined by autoradiography; the gel fragments containing the proteins were excised from the gel, washed for 12 h at 37°C in 20% methanol, dried at 80°C for 2 h, and digested with 2 ml 50 mM NH_2HCO_3 containing 100 μg trypsin (pH 8). After a 6-h incubation at 37°C, another 100 μg trypsin was added, and the digestion was continued for an additional 16 h. The supernatant was lyophilized, and the phosphopeptides were dissolved in 100 μl 6 N HCl and hydrolyzed for 2 h at 110°C. The phosphoamino acids were separated in one dimension by high-voltage electrophoresis on thin-layer plates (250 μm; Avicel, Analtech) with a solution of H_2O/acetic acid/pyridine (89:10:1 vol/vol). Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards (1 μl) were added to all samples and identified by reaction with ninhydrin, and the radioactivity was located by autoradiography.

The phosphopeptides were eluted from the gel fragments as described above with 95% efficiency and analyzed in two ways. Two-dimensional peptide mapping was performed as described by Ellis et al. (16). Peptide mapping was also performed with a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA) equipped with a wide-pore C-18 column (RP-0318, Bio-Rad) as previously described (15,17). Phosphopeptides were applied to the column, which was washed with 5% acetonitrile and eluted with an acetonitrile gradient increasing linearly to 25% during 85 min.

RESULTS

^{32}P-labeled hepatocytes were stimulated with or without 1 μg/ml insulin for different periods and extracted with 1% Triton X-100 as described in METHODS. Extract supernatants were immunoprecipitated with either anti-phosphotyrosine antibodies or anti-insulin-receptor antibodies and analyzed by SDS-PAGE (Fig. 1). With anti-phosphotyrosine antibody, in the basal state, phosphotyrosine-containing proteins of 200,000, 120,000, and 55,000 M_r were observed, but there was no tyrosine phosphorylation of the insulin receptor. After insulin stimulation, there was appearance of a 95,000-M_r band corresponding to the insulin-receptor β-subunit, and labeling of pp120 increased slightly, whereas phosphorylation of pp200 and pp55 was not affected (9,15). Both of the latter proteins could be absorbed by precipitation with protein A alone, indicating the nonspecific nature of their immunoprecipitation. The increase in tyrosine phosphorylation of the β-subunit of the insulin receptor reached a maximum after 1 min, plateaued for at least 20 min, and then gradually declined.

Via anti-insulin-receptor antibody, phosphorylation of the β-subunit of the insulin receptor could be detected in the basal state consistent with the presence of serine and threonine phosphate before insulin stimulation (1; Fig. 1*B*). Total phosphate incorporation was increased within 1 min after insulin stimulation due to the increase in tyrosine phosphorylation. However, with anti-receptor antibody, the level of phosphorylation of the β-subunit gradually increased throughout the 30-min stimulation. The different results with the two antibodies suggest that during the 1st min after insulin stimulation, the β-subunit of the insulin receptor becomes phosphorylated mainly on tyrosine residues, and this

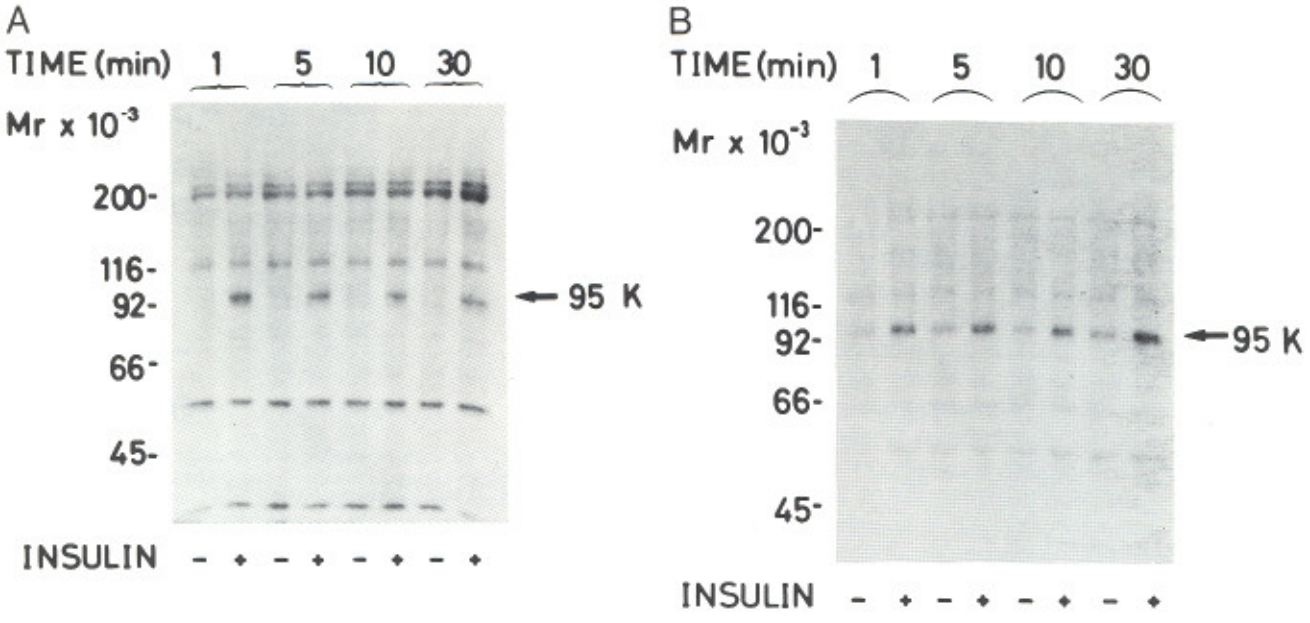

FIG. 1. Time course of insulin-stimulated autophosphorylation of insulin receptor. *A*: isolated rat hepatocytes were labeled with [^{32}P]orthophosphate and treated with 1 μg/ml insulin for indicated time intervals. Cells were extracted with 1% Triton X-100, immunoprecipitated with anti-phosphotyrosine antibody, reduced with dithiothreitol, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 7.5% resolving gel. *B*: procedure and conditions are identical as in *A*, except that antibody used for immunoprecipitation was anti-insulin-receptor antibody B-9.

is followed by a more gradual increase in phosphorylation on serine and threonine residues.

To further study the relationship between serine and tyrosine phosphorylation on the insulin receptor, the band representing the receptor immunoprecipitated by anti-insulin-receptor antibody (B-9) was eluted from the gel by trypsinization and subjected to phosphoamino acid analysis (Fig. 2). Without insulin, the receptor contained primarily phosphoserine and a small amount of phosphothreonine but no phosphotyrosine. After 1 min of insulin stimulation, there was no significant change in phosphoserine or phosphothreonine, but phosphotyrosine appeared. These results are consistent with previous studies demonstrating that the insulin receptor in the basal state contains mainly phosphoserine and that phosphotyrosine appears only after insulin stimulation (1,10).

Although autophosphorylation of the insulin-receptor β-subunit was easily detected in Triton X-100 extracts of intact rat hepatocytes with anti-phosphotyrosine antibodies, no other insulin-stimulated phosphotyrosine-containing proteins were detected. To further pursue potential substrates, SDS extracts of the hepatocytes were prepared as described in METHODS. Figure 3 compares the phosphoproteins solubilized from hepatocytes with Triton X-100 or SDS and precipitated with anti-phosphotyrosine antibody. As shown in the previous figures, only the β-subunit of the insulin receptor was immunoprecipitated from Triton X-100 extracts as an insulin-stimulated phosphotyrosyl protein. However, when cells were extracted with 1% boiling SDS and immunoprecipitated with anti-phosphotyrosine antibody, a new protein of 175,000 *M*, was observed in the basal state that was not present in the Triton X-100 extract. After insulin stimulation, ^{32}P incorporation into pp175 was increased tenfold, resulting in a labeled band that was more prominent than the β-subunit. No other phosphotyrosyl proteins were detected by SDS-PAGE with 15 and 5% gels (data not shown).

Phosphoamino acid analysis was performed on pp175 before and after insulin stimulation (Fig. 4). In the basal state, the major phosphoamino acid in pp175 was phosphoserine with a small amount of phosphotyrosine. After insulin stimulation, both phosphoamino acids increased. Whether the

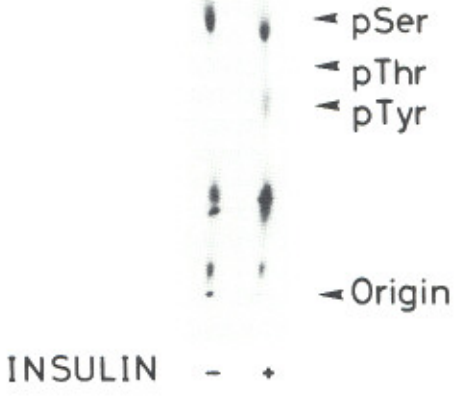

FIG. 2. Phosphoamino acid analysis of insulin receptor. Band corresponding to β-subunit of receptor was excised from sodium dodecyl sulfate gel of Triton X-100 extracts of hepatocytes precipitated with anti-insulin-receptor antibody (Fig. 1*B*). Fragment was then trypsinized to elute labeled peptides and partially hydrolyzed with 6 N HCl for 60 min. Phosphoamino acids were separated by high-voltage electrophoresis on a cellulose thin-layer plate and identified with unlabeled phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) added as standards. An autoradiogram of plate is shown.

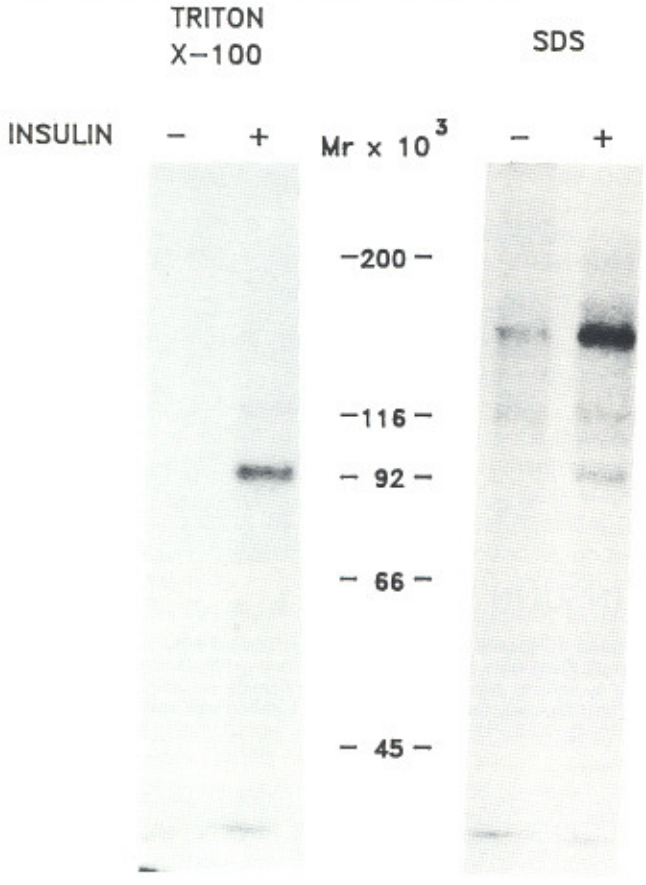

FIG. 3. Comparison of Triton X-100 and sodium dodecyl sulfate (SDS) extracts of phosphorylated proteins in rat hepatocytes. Isolated rat hepatocytes were labeled with [^{32}P]orthophosphate and stimulated with 1 μg/ml insulin for 1 min. Cells were then solubilized with 1% Triton X-100 (*left*) or 1% SDS (*right*). Samples were then immunoprecipitated by anti-phosphotyrosine antibody and analyzed by SDS–polyacrylamide gel electrophoresis as described in METHODS.

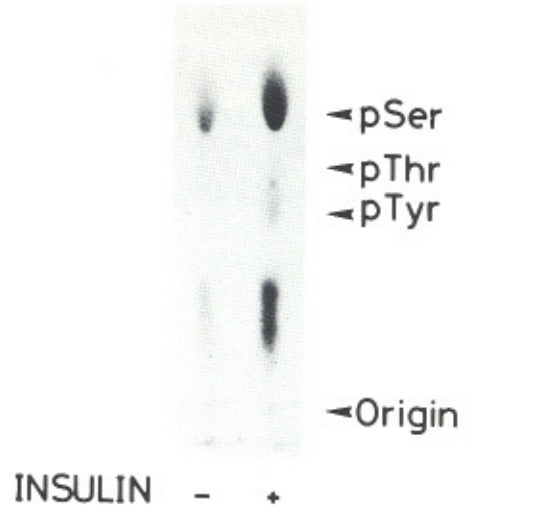

FIG. 4. Phosphoamino acid analysis of 175,000-*M*, phosphoprotein (pp175). pp175 in 1% sodium dodecyl sulfate cell extracts was identified as described in METHODS and Fig. 3. pp175 was extracted from gel by hydrolysis with trypsin, and phosphoamino acids were analyzed as described in Fig. 2.

increase in phosphoserine arises from de novo phosphorylation of this protein stimulated by insulin or is only apparent due to increased recovery of the protein during immunoprecipitation with the anti-phosphotyrosine antibody is unknown.

The time course of phosphorylation of pp175 closely paralleled that of the insulin-receptor β-subunit (Fig. 5). Autophosphorylation of the β-subunit of the receptor and phosphorylation of pp175 were almost maximal within 1 min, remained elevated for 10 min, and decreased at 30 min. As noted above, the phosphorylation of pp120 exhibited little or no stimulation by insulin.

The dose-response curves for insulin stimulation of pp175 and for the insulin-receptor β-subunit are shown in Fig. 6. Some increase in ^{32}P incorporation into these proteins was observed with an insulin concentration of 5 nM after only 1 min of stimulation, and the effect was maximal at 100 nM. The dose-response curves for stimulation of both proteins were similar, although the ED_{50} for pp175 was slightly to the left of that of the β-subunit of the insulin receptor, suggesting some signal amplification at this step. Both curves were about one order of magnitude less sensitive than insulin stimulation of amino acid transport in isolated hepatocytes but similar to the curve for insulin binding to its receptor.

Note that the phosphorylation data were obtained only 1 min after insulin addition, and at the lower insulin concentrations, insulin binding to its receptor probably had not come to equilibrium. This accounts, in part, for the apparent insensitivity of the cells to insulin.

The insulin receptor is synthesized via a proreceptor of ~180,000 *M*,. Thus, pp175 could be the precursor of the insulin receptor. This seemed unlikely, however, because pp175 was not detected in Triton X-100 extracts of cells or precipitated by anti-receptor antibody, and it contained trace amounts of phosphotyrosine in the basal state (both characteristics different from those of insulin receptor). To confirm their different origin, pp175 and the 95,000-*M*, β-subunit of the insulin receptor were subjected to two-dimensional phos-

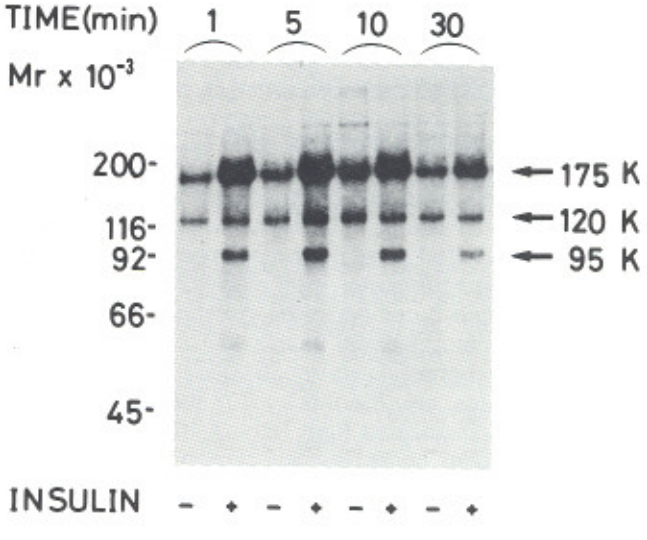

FIG. 5. Autoradiogram showing time course of phosphorylation on 175,000-*M*, phosphoprotein and β-subunit of insulin receptor. Procedures were identical to those in Fig. 1*A*, except that phosphorylated proteins were extracted by 1% sodium dodecyl sulfate as described in METHODS.

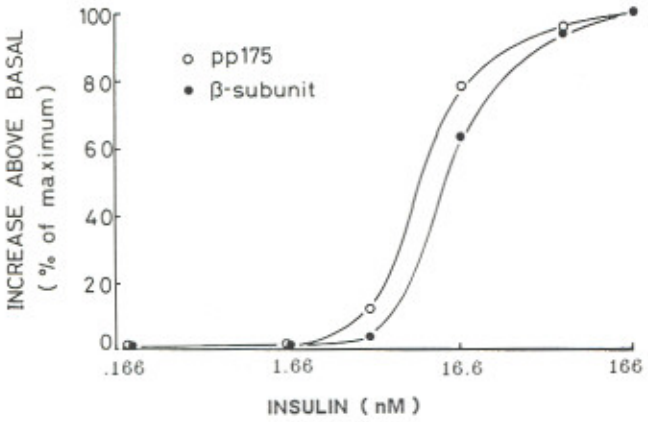

FIG. 6. Dose-response curve of phosphorylation on 175,000-*M*, phosphoprotein (pp175) and β-subunit of insulin receptor. After labeling isolated rat hepatocytes with [^{32}P]orthophosphate, cells were treated with indicated concentrations of insulin for 1 min. Phosphorylated proteins in sodium dodecyl sulfate (SDS) extracts were analyzed as described in Fig. 1*A*, and autoradiogram of SDS gel was subjected to densitometry scanning to quantitate amount of ^{32}P incorporated in proteins. Data are presented as percent increase above basal.

phopeptide mapping (Fig. 7). The peptide map of the β-subunit of the insulin receptor and the peptide map from pp175 showed completely different patterns. This different peptide map was also confirmed with reverse-phase HPLC (data not shown). Thus, pp175 is distinct from the insulin-receptor β-subunit and not likely to represent the precursor of the insulin receptor.

DISCUSSION

The insulin receptor is a tyrosine-specific protein kinase that is activated on insulin binding (1). This has led to a model of insulin transmembrane signaling in which insulin stimulates tyrosine phosphorylation of an intracellular substrate, which in its phosphorylated form serves as an intracellular messenger for the insulin signal (2). The first such substrate was described by us in Fao hepatoma cells and is a 185,000-*M*, cytoplasmic protein (5).

Since then, several proteins with different molecular weights that undergo insulin-stimulated tyrosine phosphorylation in intact cells have been described (6–9,18). In adipocytes, phosphorylation of calmodulin (19) and proteins of 160,000, 46,000, and 15,000 *M*, (6,9,18) was demonstrated. In liver, a 120,000-*M*, protein, which is retained on wheat-germ agglutinin together with the insulin receptor, is tyrosine phosphorylated in response to insulin (20). This protein has been identified as an integral membrane glycoprotein of the bile canalicular domain and is an unlikely messenger for the insulin signal (21). Exposure of hepatoma cells to vanadate and peroxide, in addition to insulin, has revealed additional phosphoproteins of 150,000, 114,000, 100,000, 85,000, 68,000, and 56,000 *M*, which undergo tyrosine phosphorylation in response to stimulation (22).

In this study, we identified a unique protein of ~175,000 *M*, that is extracted only with SDS and appears as a naturally occurring major endogenous substrate for the insulin-receptor kinase in intact hepatocytes. After incubation of labeled hepatocytes for 1 min with 1 μg/ml insulin and extraction with boiling SDS, two major proteins of 175,000 and 95,000 *M*, were stimulated in their tyrosine phosphorylation. The 95,000-*M*, band was immunoprecipitated by anti-insulin-receptor antibody, confirming its identity as the β-subunit of the insulin receptor. The 95,000-*M*, band was also detected with antibody against the COOH-terminal region of the insulin receptor with Western blotting after SDS extraction and boiling, whereas pp175 was not detected by this antibody, indicating that it is immunologically distinct from the insulin receptor (data not shown). This pp175 could be either a substrate for the insulin receptor or the biosynthetic precursor of the receptor, which has an approximate molecular weight of 180,000. However, the latter seems very unlikely, because the pp175 observed in this study has a phosphopeptide map after trypsinization that is distinct from that of the receptor, has different solubility properties, and is not precipitated by an antireceptor serum that is known to precipitate the precursor of the insulin receptor.

Further support for the possible role of pp175 as an endogenous substrate for the insulin receptor kinase is found in the parallel time course and dose response of insulin-stimulated tyrosine phosphorylation of both pp175 and the β-subunit of the receptor. The fact that the dose-response curve in these cells is slightly less sensitive than that in other cells may stem from the short time of exposure to insulin or from the presence of insulinases released by hepatocytes, leading to a lower effective insulin concentration. As for the stoichiometry of phosphorylation of pp175 in SDS extracts,

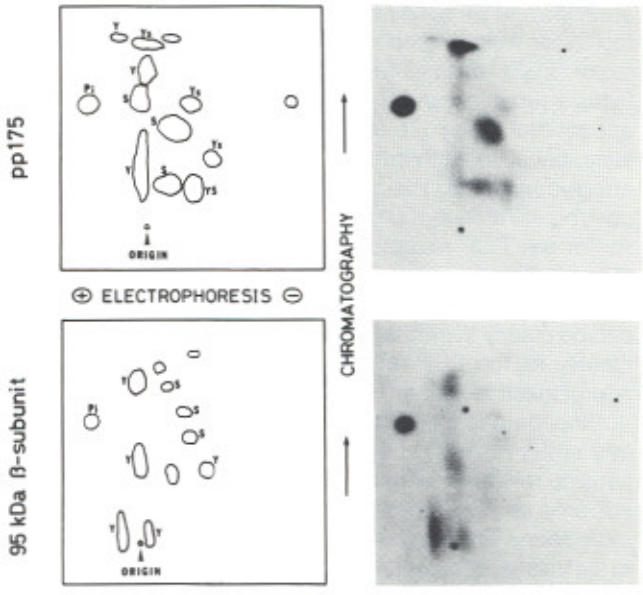

FIG. 7. Two-dimensional phosphopeptide mapping of 175,000-*M*, phosphoprotein (pp175) and β-subunit of insulin receptor. After stimulation by 1 μg/ml insulin, tyrosine-phosphorylated and immunoprecipitated proteins in hepatocytes were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gel fragments containing proteins were incubated with trypsin, and eluted phosphopeptides were separated on cellulose thin-layer plates by electrophoresis (pH 1.9) and ascending chromatography (pH 3.5) as described in METHODS. *Right*, autoradiograms of plate; *left*, corresponding schematic diagrams of phosphorylated spots. Phosphoamino acid (*Y*, phosphotyrosine; *S*, phosphoserine) in individual peptides was also determined. When both amino acids were present, size of letters represents relative amount of each phosphoamino acid in spot.

pp175 was more highly labeled than the β-subunit of the insulin receptor. Because we do not have any direct estimate of the amount of pp175, we cannot be certain whether its prominence is due to a higher stoichiometry of phosphorylation than the receptor or a greater abundance of the protein.

The 175,000- to 185,000-*M*, proteins that undergo insulin and insulinlike growth factor I–stimulated phosphorylation in different cells can be divided into two groups. The first are cytoplasmic proteins found in hepatoma (5), neuroblastoma (8), and NRK (23) cells. The second are proteins extracted with boiling SDS and found in L6 skeletal muscle cells (24), thyroid-derived FRTL-5 cells (25), and in this study, rat liver cells. The need for SDS for their extraction suggests that they are proteins associated with the cytoskeleton or nucleus. In the FRTL-5 cells, as in hepatocytes, there is very little tyrosine-phosphorylated pp175 in the Triton X-100 extracts. Subsequent solubilization of the Triton X-100–insoluble fraction of FRTL-5 cells with boiling SDS allowed identification of a larger fraction of tyrosine-phosphorylated pp175. This experiment is consistent with a cytoskeletal or nuclear origin for pp175 and weakens the alternate explanation that demonstration of this protein in the SDS extract is simply due to a more efficient termination of protease and phosphatase activity by the use of SDS (25). Thus, we believe that the 175,000-*M*, protein that has been recognized in muscle, liver, and FRTL-5 cells may be different from the cytoplasmic pp185, although purification and cloning will provide the only definitive answer.

Hepatocytes contain or may contain several proteins that are known to be tyrosine phosphorylated and are of similar molecular weight to pp175, including the epidermal growth factor (EGF) receptor (26), macrophage colony-stimulating factor I receptor, and the *neu* proto-oncogene. It does not appear that the pp175 described by us is any of these proteins, because all three are solubilized by Triton X-100 and are glycoproteins. The pp175 of hepatocytes is neither solubilized in Triton X-100 nor a glycoprotein (data not shown). As for the EGF receptor, we have shown that after EGF stimulation of hepatocytes, 185,000- and 160,000-*M*, bands appear (i.e., slightly above and below pp175). These species can be clearly separated on SDS gels from pp175, and their identity as the EGF receptor can be confirmed by immunoprecipitation with anti-EGF-receptor antibody (27).

Although phosphorylation of the insulin receptor in a cell-free system results in almost exclusive phosphorylation on tyrosine residues, in intact cells, insulin is known to activate not only its own receptor tyrosine kinase but also one or more serine kinases (28). In hepatocytes, both the β-subunit of the insulin receptor and pp175 contain phosphoserine and phosphotyrosine. In the case of the β-subunit, we were able to demonstrate an increase in serine phosphorylation in response to insulin with anti-insulin-receptor antibodies. Not having a specific antibody to pp175, we cannot determine whether the increase in phosphoserine after exposure to insulin reflects phosphorylation by a second activated serine kinase or simply an improved recovery due to tyrosine phosphorylation. The observation that insulin-induced phosphorylation on serine residues occurs later than on tyrosine residues may suggest a different role for each phosphorylation in transmission of the different effects of insulin that occur in liver. If indeed this 175,000-*M*, protein is an intracellular messenger for the insulin signal, multisite phosphorylation by tyrosine and serine kinases could modulate its activity, as is the case for the β-subunit of the insulin receptor (29).

Although all proteins undergoing tyrosine phosphorylation in response to insulin may be potential physiological substrates for the insulin-receptor kinase, the most consistently observed is the family of proteins that migrates with a 175,000–185,000 *M*, (5,8,22–24). Support for a role of pp185 phosphorylation in insulin signal transmission was found with CHO cells expressing a mutant of the insulin receptor whose tyrosyl residue 960 was substituted with phenylalanine. Cells expressing the nondiabetic human insulin receptor were insulin sensitive, and both the receptor and pp185 underwent insulin-stimulated autophosphorylation. In cells expressing the mutated receptor, the receptor underwent autophosphorylation but not phosphorylation of pp185, and no insulin-induced biological effects were found (30). We are currently pursuing the purification and sequence determination of this class of proteins in an attempt to better define their role in insulin action.

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