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Characterization of an Endogenous Substrate of the Insulin Receptor in Cultured Cells*

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Using antiphosphotyrosine antibodies, we have characterized the tyrosine phosphorylation of an endogenous substrate of the insulin receptor in Fao hepatoma cells and in Chinese hamster ovary cells transfected with a eukaryotic expression vector containing the human insulin receptor cDNA. In Fao cells, besides the β-subunit of the insulin receptor, a protein with a molecular mass between 170 and 210 kDa designated pp185, undergoes tyrosine phosphorylation immediately after insulin stimulation reaching a maximum level within 30 s. After 4 h of continuous insulin stimulation, the labeling of pp185 decreased to less than half of its original intensity, whereas the insulin receptor was unchanged. After 24 h of insulin stimulation, the phosphotyrosine-containing insulin receptor decreased by 75% owing to down-regulation, whereas the pp185 was completely undetectable. By several biochemical and physiological criteria, the pp185 is distinct from the insulin receptor. The pp185 and the β-subunit of the insulin receptor were strongly labeled with [^{32}P]orthophosphate, but in contrast to the insulin receptor, the pp185 was not labeled by cross-linking with ^{125}I-insulin or surface ^{125}I iodination. Unlike the insulin receptor, the pp185 was extracted from Fao cells without detergent, and tryptic phosphopeptide mapping of the pp185 and the insulin receptor yielded distinct patterns. Thus, the pp185 is not located at the external face of the plasma membrane and does not bind insulin. Treatment of Fao cells with the phorbol ester, phorbol 12-myristate 13-acetate, stimulated the phosphorylation of two proteins with molecular weights of 170 and 210 kDa which were immunoprecipitated with the anti-phosphotyrosine antibody. Subsequent insulin stimulation increased the phosphorylation of the 210 kDa protein, but the pp185 was not detected. Increasing the concentration of the human insulin receptor in the Chinese hamster ovary cells by transfection with a plasmid containing the human insulin receptor cDNA caused a higher level of tyrosine phosphorylation of the β-subunit and the pp185. These data support the notion that the insulin signal may be transmitted to a cellular substrate (pp185) which may initiate insulin action at intracellular sites.

One of the earliest detectable responses to insulin binding is activation of the insulin receptor kinase and tyrosine autophosphorylation of its β-subunit (1-3). Autophosphorylation appears to facilitate the interaction of other proteins with the β-subunit so that the receptor catalyzes tyrosine phosphorylation of protein substrates (4-6). The phosphorylation of cellular substrates may be the initial step which transmits the extracellular insulin signal beyond the plasma membrane receptor. Support for the substrate hypothesis of insulin action has come from two experimental approaches. Initially, *in vitro* experiments were carried out to identify proteins with known regulatory significance that undergo insulin-stimulated tyrosine phosphorylation during incubation with the purified insulin receptor (7-12). Several substrates were identified this way, but their physiological significance remains unclear largely because they have not been found in the intact cell (13).

We have used another approach to search for the physiologically significant substrates which employ polyclonal antiphosphotyrosine antibodies to immunoprecipitate phosphotyrosine-containing proteins that occur in the intact cell during insulin stimulation. Using this approach, we identified a protein of approximate molecular mass 185 kDa, which we designated pp185, that occurred during the initial response of Fao hepatoma cells to insulin (14). Tyrosine phosphorylation of pp185 is maximum within seconds after exposure of Fao cells to insulin and exhibits a similar dose response as receptor autophosphorylation. We and others have subsequently identified a phosphotyrosine-containing protein of similar molecular mass in several other cell lines such as Madin-Darby canine kidney cells and normal rat kidney cells (15), rat hepatocytes,[1] 3T3-L1 adipocytes (16), and L6 myocytes.[2]

In this report, we have characterized pp185 and present additional information supporting the notion that pp185 is a substrate for the insulin receptor kinase that is distinct from the receptor itself. The pp185 displays characteristics that are consistent with its involvement in insulin action. Its phosphorylation may provide a molecular link between the membrane-bound insulin receptor and the regulation of metabolic reactions in the cytoplasm of insulin-sensitive cells.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated sources: [^{32}P]orthophosphate, [^{125}I]iodine, and Triton X-100 were from New England Nuclear; [^{35}S]methionine and ^{125}I-insulin

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were from Amersham Corp.; HEPES,[3] aprotinin, phenylmethylsulfonyl fluoride, *N*-acetylglucosamine, bovine serum albumin, lactoperoxidase, and glucose oxidase were from Sigma. The reagents for SDS-PAGE and the reverse phase HPLC column (RP-318) were purchased from Bio-Rad, and the HPLC system was from Waters Associates. Crude Pansorbin was obtained from Behring Diagnostics and purified as previously described (17); Porcine insulin (Lot 1JM95AN) was from Elanco. Tissue culture medium, fetal bovine serum, and Geneticin (G418) were obtained from GIBCO. All tissue culture supplies were from Nunclon or Costar. Disuccinimidyl suberate (DSS) was obtained from Pierce Chemical Co.

Cell Culture—The experiments were performed with a differentiated and insulin-sensitive hepatoma cell (Fao) which possesses a high concentration of insulin receptors and many insulin-stimulated responses (18, 19), and Chinese hamster ovary (CHO) cells transfected with the human insulin receptor. All cell cultures were maintained at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2. The Fao cells were grown in plastic tissue culture dishes (15-cm diameter) containing 30 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. The CHO cells were grown in 10- or 15-cm dishes containing 20 or 30 ml of F12 medium supplemented with 10% fetal bovine serum, respectively. In each case, the medium was changed 12 h before each experiment to serum-free RPMI 1640 or F12 medium, respectively.

Transfection of the CHO Cells with the Wild-type Human Insulin Receptor—A eukaryotic expression vector (HIRc) containing the wild-type human insulin receptor was constructed as described previously (20, 21). This plasmid contains the origin of replication and the ampicillin-resistance gene of the *Escherichia coli* plasmid pBR322 and an insert containing the complete human insulin receptor coding sequence under the control of the SV40 early promoter (SVE). The plasmids were prepared as described by Maniatis *et al.* (22).

CHO cells (10^6/10-cm dish) were cotransfected with HIRC (10 μg) and a neomycin resistance gene under control of SVE in the plasmid pSVEneo (2 μg) by calcium phosphate precipitation (23). The plasmid DNA was diluted in 0.55 ml of 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 250 mM $CaCl_2$. The precipitate was formed by slowly adding with gentle agitation a solution containing 0.5 ml of 50 mM HEPES, pH 7.12, 280 mM NaCl, and 1.5 mM Na_2HPO_4. Within 45 min, a fine precipitate formed which was added to the cultured cells incubating in 10 ml of F12 medium. The cells were incubated with the DNA for 24 h before the medium was changed to fresh F12 medium. After a 24-h period of recovery, 800 μg/ml of the neomycin analog G418 was added to the medium to select cells which expressed the neomycin resistance gene contained in the pSVEneo plasmid. Ten to 14 days later, surviving colonies were harvested and cultured in the presence of G418 to amplify the cell line.

CHO cells that expressed high levels of surface insulin receptors were selected by fluorescence-activated cell sorting. Single cell suspensions of CHO cells were prepared from sparse colonies by brief incubation with a phosphate-buffered saline solution containing 0.05% trypsin and 0.5 mM EDTA. The cells were suspended in 0.5 ml of serum-containing F12 medium and incubated for 30 min at 4 °C with a monoclonal anti-insulin receptor antibody directed against the α-subunit of the human insulin receptor (αIR-1, S. Jacobs, Burroughs Wellcome) or a nonspecific mixture of mouse IgG. The cells were washed twice with 15 ml of serum-containing F12 medium, suspended in 1.0 ml of this medium, and incubated for an additional 30 min with fluorescein-conjugated anti-mouse IgG (5 μl). The cells were washed twice with 15 ml of serum-containing F12 medium, suspended in 1 ml of this medium, and analyzed by Coulter Epics V flow cytometer (24). The brightest cells were collected and amplified in tissue culture. The sorting procedure was repeated three times and yielded populations of CHO cells that expressed various levels of cell surface insulin receptors. The transfected CHO cell lines are designated HIRC-S0, HIRC-S1, and HIRC-S2, for no sort, one, and two sorts, respectively.

Cell-surface Insulin Binding—Confluent monolayers of Fao or CHO cells were grown in 24 well trays (Costar). The cells were incubated for 3 h at 15 °C in 0.25 ml of serum-free RPMI 1640 medium containing 0.1% bovine serum albumin, ^{125}I insulin (10^{-11} M, 20,000 cpm), and various concentrations of unlabeled insulin between 0.01 nM and 1 μM. The unbound insulin was removed from the monolayers by three washes (2 ml) with PBS at 4 °C using the apparatus previously described (25). The cells were solubilized with 1 ml of 0.1% SDS, and the bound radioactivity was measured in a γ counter. The binding curves were analyzed by a nonlinear least squares analysis (26) assuming the existence of two saturable and one nonsaturable component as described by the following equation:

$$\text{insulin bound} = \frac{B_1(\text{insulin})_f}{K_1 + (\text{insulin})_f} + \frac{B_2\,(\text{insulin})_f}{K_2 + (\text{insulin})_f} + P\,(\text{insulin})_f$$

B_1 and B_2 represent the total binding capacity of the high affinity and low affinity binding sites, respectively, and K_1 and K_2 are the corresponding affinity constants. P is the slope of the nonsaturable component on the curve of bound insulin *versus* free insulin ($(\text{insulin})_f$). The total number of specific insulin binding sites (R_0) is the sum of B_1 and B_2.

^{32}P Phosphorylation of the Intact Cells—Confluent cells in 15-cm dishes were incubated for 2 h with 10 ml of phosphate-free and serum-free RPMI 1640 medium (GIBCO) containing carrier-free [^{32}P]orthophosphate (0.5 mCi/ml) (2). Insulin was added, and the incubation was continued at 37 °C for the indicated time intervals. The experiments were stopped quickly by removing the incubation medium and freezing the cell monolayers with liquid nitrogen (2). The monolayers were thawed and homogenized immediately at 4 °C with 2 ml of a solution containing 50 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The cells were scraped from the dishes, and the insoluble material was sedimented by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor for 60 min. Triton X-100 was either included during homogenization of the cells to collect a mixture of cytoplasmic and membrane proteins or added only to the particulate pellet obtained after centrifugation of the detergent-free cell homogenate to separate membrane proteins from cytoplasmic proteins.

[^{35}S]Methionine Labeling of the Intact Fao Cells—Metabolic labeling of cells with [^{35}S]methionine was carried out in methionine-free RPMI 1640 medium (1). Monolayer cultures of Fao cells in a 10-cm dish were incubated for 15 h with 5 mCi of [^{35}S]methionine. Then the cells were incubated without or with 100 nM insulin for 1 min at 37 °C and prepared for immunoprecipitation by solubilization with 1% Triton X-100.

Surface ^{125}I Iodination of the Intact Fao Cells—Surface labeling of cultured cells was accomplished by lactoperoxidase-catalyzed iodination of extracellular proteins (27). The Fao cell cultures in 10-cm dishes were washed three times in PBS, pH 7.5, to remove serum proteins and then the cells were incubated at 10 °C with 5 ml of PBS containing 10 mM glucose, 2 units/ml lactoperoxidase, 1 unit/ml glucose oxidase, and 0.5 mCi/ml Na^{125}I. The reaction was allowed to proceed for 30 min. The cells were washed several times with PBS to stop the reaction. The labeled cells were kept at 4 °C until stimulation with insulin at which time they were quickly warmed to 37 °C and incubated without or with insulin (100 nM) for 1 min. Finally, the cell monolayers were prepared for immunoprecipitation as described above.

^{125}I-Insulin Affinity Labeling of the Insulin Receptor—Fao cells in 10-cm plastic dishes were incubated with ^{125}I-insulin (10 μCi) in the absence or presence of unlabeled insulin (100 nM) for 3 h at 15 °C (28). The unbound insulin was removed by washing the cells three times at 4 °C with PBS containing 0.1% bovine serum albumin. DSS (0.1 mM) dissolved in 10 ml of PBS was added to the cell cultures and incubated at 15 °C for 15 min. The solution was removed, and the cells were washed at 4 °C several times with PBS containing 25 mM Tris-HCl to inactivate remaining functional groups. Finally, the cells were incubated at 37 °C for 1 min and then they were prepared for immunoprecipitation.

Immunoprecipitation of Phosphotyrosine-containing Proteins—The whole cell extract was purified by immunoprecipitation with anti-phosphotyrosine (αpY) or anti-insulin receptor (αIR) antibodies as described previously (3, 17). The cell extracts (1–2 ml) were incubated with 2 μg of the αPY or 10 μg of αIR (B9) for at least 2 h at 4 °C. The antibody complex was precipitated with 50 μl of 10% Pansorbin during a 1-h incubation, and the precipitates were washed three times with a solution containing 50 mM HEPES, 150 nM NaCl, 100 mM NaF, 1.0% Triton X-100, and 0.1% SDS. The proteins were eluted from washed precipitates with Laemmli sample buffer, reduced with

[3] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGF, epidermal growth factor; IGF, insulin-like growth factor; DSS, disuccinimidyl suberate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; DTT, dithiothreitol; αPY, anti-phosphotyrosine antibody; αIR, anti-insulin receptor antibody; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate.

DTT, and separated by SDS-PAGE on 7.5% resolving polyacrylamide gels as previously described (17). The phosphoproteins were identified by autoradiography at −70 °C of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The radioactivity in the gel fragments was quantified by Cerenkov counting or scanning densitometry.

HPLC Separation of the Tryptic Phosphopeptides and Identification of Phosphoamino Acids—Tryptic phosphopeptides were obtained from the pp185 and the β-subunit separated in polyacrylamide gel fragments as previously described (29). The fixed and dried gel fragments were rehydrated and incubated in 1 ml of 50 mM NH_4HCO_3 containing 100 μg of L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin for 6 h at 37 °C. An additional 100 μg of trypsin was added, and the incubation was continued for 12 h. The phosphopeptides which eluted from the gel fragment (nearly 95%) were separated with a Waters high performance liquid chromatography system equipped with a wide-pore C_{18} column (Bio-Rad, RP-318). Phosphopeptides were applied to the column which was washed at a flow rate of 1 ml/min with 0.05% trifluoroacetic acid, 95% water, and 5% acetonitrile and eluted with an acetonitrile gradient increasing linearly to 25% during 85 min. Fractions (1 ml) were collected in 1.5-ml polypropylene microfuge tubes, and the radioactivity in each tube was measured as Cerenkov radiation using a Beckman scintillation counter with an efficiency of 40%. The percentage of acetonitrile in each sample had no effect on the efficiency of the Cerenkov radiation. All of the radioactivity in the trypsin digest was routinely recovered from the reverse phase HPLC column. The phosphoamino acids were identified in tryptic peptides by a modification (30) of the method of Hunter and Sefton (31). To identify the phosphotyrosine-containing peptides in the pp185, the peptides in fractions 20–70 were individually immunoprecipitated with 0.5 μg of the αPY to obtain a second elution profile of phosphotyrosine-containing tryptic peptides. The radioactivity in the washed precipitates was measured by Cerenkov counting.

RESULTS

Identification of the Insulin Receptor and the pp185 in Fao Cells by Metabolic Labeling, Surface ^{125}I-Iodination, and Cross-linking with ^{125}I-Insulin—Phosphotyrosyl residues occur rarely in cells (31), thus in practice, an antiphosphotyrosine antibody is a specific reagent to study tyrosine phosphorylation reactions that occur in the intact cell following insulin binding. In the absence of insulin or EGF stimulation, the αPY immunoprecipitated two proteins from [^{32}P]orthophosphate-labeled Fao cells which had relative molecular masses by SDS-PAGE of 120 and 70 kDa (Fig. 1, *lane a*). Insulin significantly stimulated tyrosine phosphorylation of two other proteins that were immunoprecipitated with the αPY. One had a molecular mass of 95 kDa and the other was approximately 185 kDa (Fig. 1, *lane b*). We reported these observations previously and identified the smaller insulin-stimulated protein as the β-subunit of the insulin receptor and proposed that the larger protein is a substrate for the insulin receptor kinase called pp185 (14). Since Fao cells do not contain receptors for epidermal growth factor, EGF had no effect on this pattern (Fig. 1, *lane c*), and neither insulin nor EGF had any reproducible effects on the phosphorylation of the 120- and 70-kDa proteins in the Fao cells.

To estimate the relative amount of the pp185, we labeled Fao cells with [^{35}S]methionine for 24 h and immunoprecipitated the phosphotyrosine-containing proteins with αPY before and after insulin stimulation for 1 min. In the absence of insulin, pp120 and pp70 were the major proteins detected (Fig. 1, *lane d*). After insulin stimulation, the α- and β-subunits of the insulin receptor and a faint band at M_r = 185,000 were also immunoprecipitated (Fig. 1, *lane e*). Scanning densitometry indicated that the labeling of the 135-kDa α-subunit of the insulin receptor was about 50% of that of the β-subunit which is consistent with the 1 to 2 ratio of methionine residues in the α *versus* β subunits (20). The intensity of the pp185 was less than 5% of that of the β-subunit of the insulin receptor suggesting that it is less abundant, contains few methionine residues, or has a long half-life and was poorly labeled during a 15-h incubation.

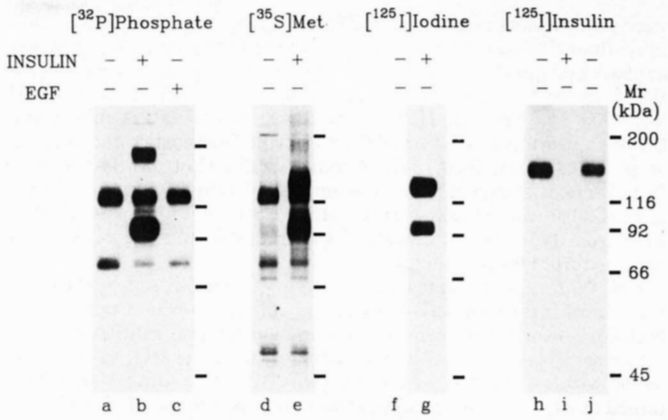

FIG. 1. **Identification of the insulin receptor and the pp185 in Fao cells.** Confluent monolayers of Fao cells were labeled with [^{32}P]orthophosphate (*lanes a–c*), [^{35}S]methionine (*lanes d* and *e*), [^{125}I]iodine (*lanes f* and *g*), or ^{125}I-insulin (*lanes h–i*). After incubation with 100 nM insulin (*lanes b*, *e*, and *g*) or EGF (*lane c*) for 1 min, or with 100 nM insulin for 3 h during insulin binding (*lane i*), the cells were prepared for immunoprecipitation as described under "Experimental Procedures." The αPY was used to immunoprecipitate the phosphotyrosine-containing proteins from the whole cell extracts. In one case (*lane j*), αIR was used to immunoprecipitate the insulin receptor precipitated with αPY. The autoradiograms represent exposures of 6 h (*lanes a–c*), 12 h (*lanes d* and *e*), 12 h (*lanes f* and *g*), and 4 days (*lanes h–j*).

To locate and distinguish pp185 with respect to the insulin receptor, Fao cells were surface-labeled with [^{125}I]iodine. After incubation of these cells with insulin (100 nM) for 1 min at 37 °C, the phosphotyrosine-containing proteins were immunoprecipitated with αPY. In the absence of insulin, no iodinated proteins were recovered (Fig. 1, *lane f*), suggesting that neither the pp120 nor the pp70 were exposed at the external face of the plasma membrane. After insulin stimulation, two proteins with molecular masses by SDS-PAGE of 135 and 95 kDa were immunoprecipitated by the αPY (Fig. 1, *lane g*). These proteins were subsequently immunoprecipitated completely by αIR indicating that they were the α- and β-subunits of the insulin receptor, respectively (data not shown). Although the α-subunit has never been found to be phosphorylated on tyrosine residues in the intact cell, it is immunoprecipitated by the αPY because it is covalently linked to the phosphorylated β-subunit by disulfide bonds (13). The pp185 was not detected in this experiment suggesting that it was not exposed on the cell surface. In addition, no other proteins were immunoprecipitated by the αPY from surface-iodinated cells after insulin stimulation, suggesting that no integral membrane proteins other than the insulin receptor were phosphorylated on tyrosine residues and recognized by the αPY during insulin stimulation.

To confirm that pp185 was not a form of the insulin receptor, we labeled the α-subunit of the insulin receptor with ^{125}I-insulin by cross-linking it with DSS (28). After cross-linking the ^{125}I-insulin at a concentration of 0.01 nM, the phosphotyrosine antibody immunoprecipitated a single labeled protein with a molecular mass by SDS-PAGE of 140 kDa (Fig. 1, *lane h*). Its labeling was completely inhibited by 100 nM unlabeled insulin (Fig. 1, *lane i*) and this protein was subsequently immunoprecipitated with αIR (Fig. 1, *lane j*) confirming that it was the α-subunit of the insulin receptor. These results indicated that the occupied insulin receptor is activated and its β-subunit undergoes tyrosine autophospho-

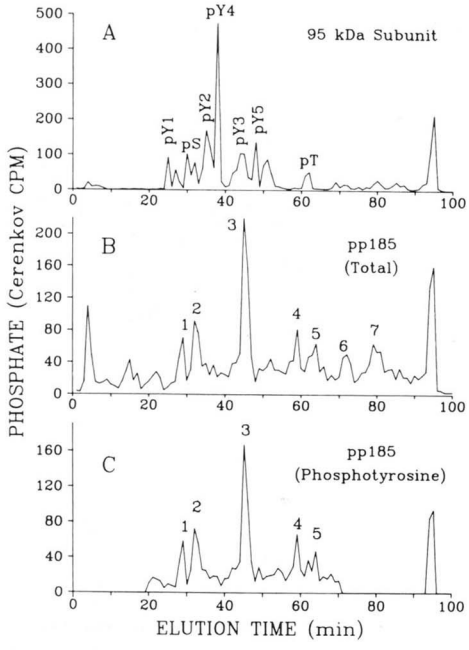

FIG. 2. **Separation of tryptic phosphopeptides of the β-subunit of the insulin receptor and the pp185.** The phosphorylated β-subunit (*panel A*) and the pp185 (*panel B*) were separated by SDS-PAGE and digested exhaustively with trypsin as described under "Experimental Procedures." The mixtures of peptides were then separated by reverse phase HPLC on an RP-318 column. *Panel C* shows the immunoprecipitation of the radioactivity in fractions 30–70 of *panel B* by the anti-phosphotyrosine antibody.

rylation at physiological insulin concentrations even after cross-linking. Furthermore, this experiment shows that the pp185 did not directly bind insulin. Therefore, the pp185 most likely undergoes tyrosine phosphorylation by an interaction with an insulin-stimulated tyrosine kinase, possibly the receptor itself, at the inner face of the plasma membrane.

Separation of Tryptic Phosphopeptides from the β-Subunit of the Insulin Receptor and the pp185—We characterized the pp185 by tryptic peptide mapping and compared its profile to that of the β-subunit of the insulin receptor. After insulin stimulation, the insulin receptor and pp185 were purified by immunoprecipitation with the αPY and SDS-PAGE. The phosphopeptides obtained after exhaustive trypsin digestion of the β-subunit were separated on a RP-318 reverse phase column (Fig. 2*A*). Five phosphotyrosine-containing peptides have been identified by phosphoamino acid analysis (data not shown) and are labeled as pY1–pY5. The tyrosine residues in some of these peptides have recently been deduced by partial enzymatic digestion (32) and by Edman degradation.[4] Using the sequence of Ullrich *et al.* (20), the pY2 and pY3 are derived from the C-terminal domain of the β-subunit and represent phosphorylation of tyrosine residues 1316 and 1322, whereas the pY4 and pY5 represent the phosphopeptide containing phosphotyrosine residues 1146 and 1150 or 1151. In addition, a phosphoserine-containing (*pS*) and a phosphothreonine-containing (*pT*) peptide have been observed previously (2), but their locations in the β-subunit are unknown.

The HPLC profile of the pp185 was distinct from that of the β-subunit of the insulin receptor suggesting that it is a unique protein (Fig. 2*B*). Immunoprecipitation of fractions 20–70 with the αPY indicated that the peptides 1–5 contained phosphotyrosine (Fig. 2*C*). Analysis of the total phosphoamino acid content has previously shown that pp185 also contains considerable phosphoserine and phosphothreonine (14).

Extraction of the pp185 in Detergent-free Solution—We determined the solubility of the pp185 in comparison to the insulin receptor and other phosphotyrosine-containing proteins that were immunoprecipitated by the αPY after a brief insulin-stimulation. Fao cells labeled with [^{32}P]orthophosphate and incubated with 100 nM insulin for 1 min were frozen with liquid nitrogen and homogenized in a detergent-free solution. The particulate fraction was separated by centrifugation at 100,000 × *g* for 90 min, and the proteins in the supernatant were immunoprecipitated with αPY (Fig. 3). In the absence of insulin, pp120 and pp70 were detected (Fig. 3, *lane a*). After insulin stimulation, pp185 was clearly detected in the detergent-free extract along with some increases in pp120 and pp70. However, the β-subunit of the insulin receptor was not immunoprecipitated from the detergent-free extract.

The membrane fraction that collected in the pellet during centrifugation of the cell homogenate was resuspended in the same buffer supplemented with 1% Triton X-100. The detergent-insoluble proteins were removed by centrifugation and the αPY was used to immunoprecipitate the solubilized membrane proteins which contain phosphotyrosine. In the absence of insulin, only trace amounts of a few proteins were observed (Fig. 3, *lane c*). After insulin stimulation, the β-subunit of the insulin receptor was the major phosphoprotein precipitated with αPY (Fig. 3, *lane d*). A small amount of pp185 and pp120 were also observed. These may represent contamination of

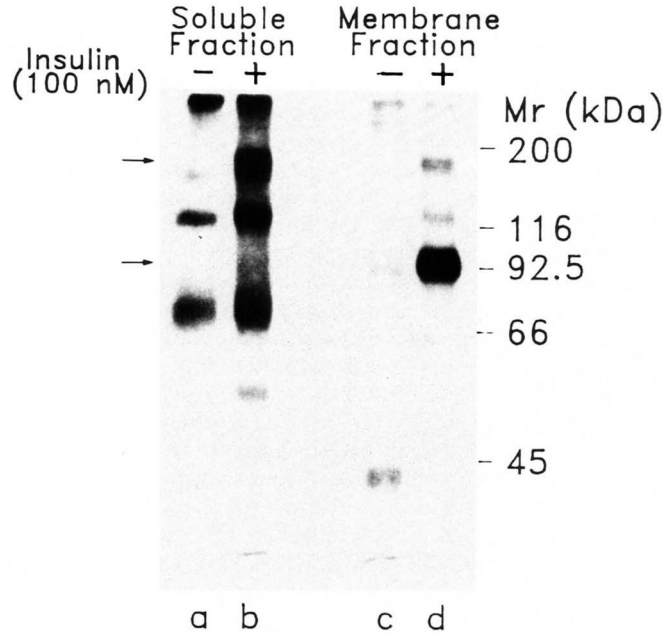

FIG. 3. **Separation of the pp185 and the insulin receptor by extraction of the cells with a detergent-free solution.** Fao cells were labeled with [^{32}P]phosphate for 2 h and then incubated without (*lanes a* and *c*) or with 100 nM insulin for 1 min (*lanes b* and *d*). The cells were frozen with liquid nitrogen and thawed into 2 ml of a detergent-free solution containing 50 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The insoluble material was sedimented by centrifugation and the phosphoproteins in the supernatant were immunoprecipitated by the αPY (*lanes a* and *b*). The pellet which contained the membrane fraction was suspended by sonication in the same solution containing 1% Triton X-100. The detergent insoluble material was removed by centrifugation and the phosphotyrosine-containing proteins in the supernatant were immunoprecipitated by the αPY (*lanes c* and *d*).

[4] White, M. F., Stegmann, E., Keutmann, H., Shoelson, S., and Kahn, C. R., (1987) *J. Biol. Chem.*, submitted for publication.

the membrane fraction with cytoplasm or indicate that a small fraction of the pp185 and pp120 bind to the membrane during insulin stimulation. These results further confirm that pp185 is distinct from the insulin receptor and may be a weakly membrane-bound or soluble substrate for the insulin receptor *in vivo*.

The Effect of Prolonged Incubation of the Fao Cells with Insulin on the Phosphorylation of pp185—The amount of pp185 immunoprecipitated from Fao cells by the αPY increased to a maximum level immediately after insulin stimulation (Fig. 4, *lanes a* and *b*) and then decreased by 50–60% during the continued exposure of the Fao cells to insulin for 1 h (14). After 4 h with insulin, the level of phosphorylation of the insulin receptor β-subunit was unchanged; however, the phosphorylation of pp185 measured by scanning densitometry and Cerenkov counting was decreased by 70% (Fig. 4, *lane c*). Furthermore, two bands with molecular masses of 210 and 170 kDa were seen at this time interval rather than the single band of pp185. The 210-kDa protein was phosphorylated more intensely than the 170-kDa band which allowed for their detection by SDS-PAGE. It is possible that the broad band observed on SDS-PAGE immediately after insulin stimulation at about 185 kDa is composed of two distinct phosphotyrosine-containing proteins which become apparent at later times, or alternatively, some of the pp185 undergoes a further covalent modification which alters its mobility.

After 24 h, the concentration of insulin receptors decreases 75% due to down regulation (27). Insulin stimulation of pp185 was nearly undetected in these cells due to a complete loss of the 210-kDa band and the presence of a basal level of the 170-kDa component. This cannot be attributed completely to an inactive insulin receptor kinase because the remaining

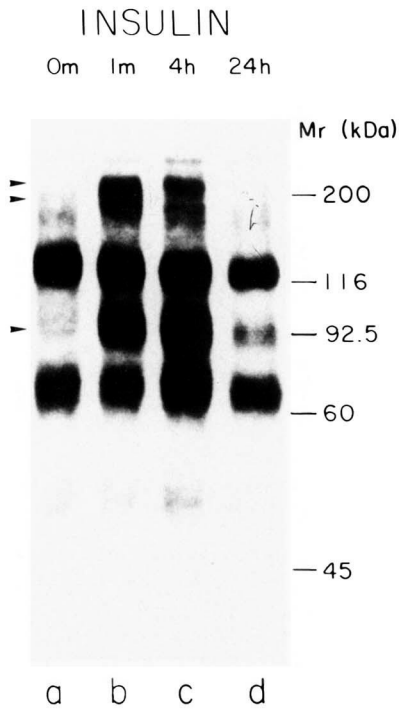

FIG. 4. **A time course of insulin-stimulated phosphorylation of the pp185 and the insulin receptor.** Fao cells were incubated without insulin or with 100 nM insulin (*lane d*) for 20 h. These cells were then labeled with [^{32}P]orthophosphate for 2 h without insulin (*lanes a–c*) or in the continued presence of 100 nM insulin (*lane d*). Then the cells were solubilized immediately (*lane a*) or incubated with 100 nM insulin for 1 min (*lane b*), 4 h (*lane c*), or 2 h (*lane d*, to give a total of 24 h). The solubilized phosphotyrosine-containing proteins were immunoprecipitated with the αPY, reduced with 100 mM DTT and separated by SDS-PAGE.

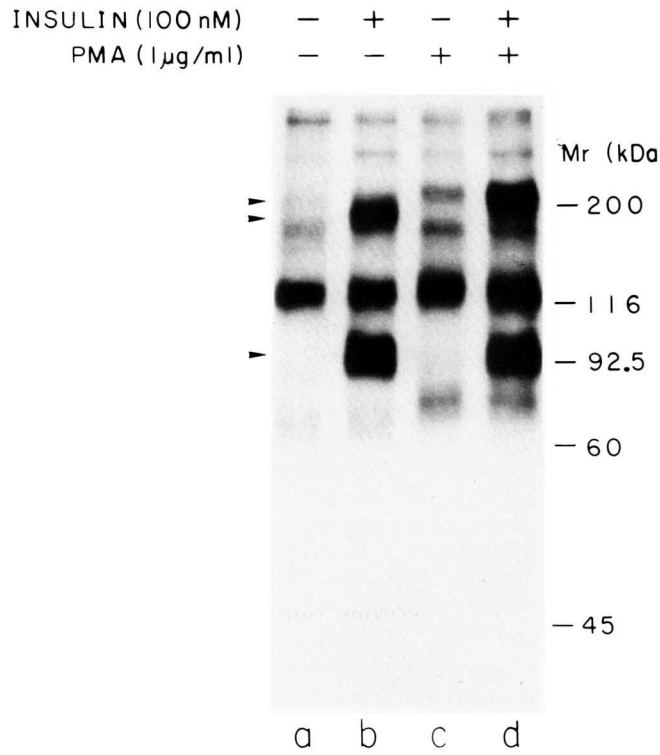

FIG. 5. **Insulin-stimulated phosphorylation of pp185 in PMA-treated Fao cells.** Fao cells were labeled with [^{32}P]orthophosphate for 2 h and then incubated without (*lanes a* and *b*) or with PMA (1 μg/ml) for 30 min (*lanes c* and *d*). Insulin was added to two dishes (*lanes b* and *d*) for 1 min and then all of the cells were solubilized and the phosphotyrosine-containing proteins were immunoprecipitated with the αPY. The proteins in the immunoprecipitate were reduced with DTT and separated by SDS-PAGE. The autoradiogram was obtained during a 12-h exposure.

receptors still undergo tyrosine autophosphorylation and were immunoprecipitated with the αPY suggesting that they were active (Fig. 4, *lane d*). The loss of pp185 is consistent with the total desensitization of the insulin response which occurs during the prolonged incubation of the Fao cell with insulin (27).

The Effect of PMA on the Phosphorylation of pp185—The phorbol ester PMA stimulates serine and threonine phosphorylation of the β-subunit of the insulin receptor possibly through the activation of the protein kinase C (33, 34).[5] Thus, we examined the effect of PMA on phosphorylation of pp185. Before incubation of the Fao cells with PMA, insulin stimulated within 1 min the phosphorylation of the β-subunit of the insulin receptor and the pp185 (Fig. 5, *lanes a* and *b*). Following a 30-min incubation with PMA alone, no phosphorylated β-subunit was immunoprecipitated with the αPY since PMA does not stimulate tyrosine phosphorylation of the insulin receptor (33). In contrast, PMA alone stimulated the phosphorylation of a 75-kDa protein and two proteins with molecular masses of 170 and 210 kDa which migrated similarly to those seen after 4 h of insulin stimulation only (compare Fig. 5, *lane c*, with Fig. 4, *lane c*). Subsequent insulin stimulation of the PMA-treated cells caused tyrosine phosphorylation of the β-subunit of the insulin receptor, although by Cerenkov counting, the total phosphate incorporated was 20% lower than with insulin alone. (Fig. 5, *lanes b* and *d*) (33). Insulin, in the presence of PMA, significantly stimulated the phosphorylation of the 210-kDa protein (Fig. 4, *lanes b*

[5] Takayama, S., White, M. F., and Kahn, C. R. (1987) *J. Biol. Chem.*, submitted for publication.

and *d*) with no detectable effect on the 170-kDa species. The net result was a shift in the molecular mass of the major insulin receptor substrate from the 185-kDa protein found during insulin stimulation in the absence of PMA to a 210-kDa phosphoprotein found in the presence of PMA.

Detection of pp185 in CHO Cells Expressing the Wild-type Human Insulin Receptor—Chinese hamster ovary cells contain about 0.1 pmol of insulin binding sites/mg of protein (data not shown). After transfection of the CHO cells with a plasmid containing the neomycin resistance gene (pSVEneo), the insulin binding capacity measured by Scatchard analysis was unchanged (Fig. 6). Consistent with this result, only small amounts of phosphorylated insulin receptor were immunoprecipitated with the αPY from [^{32}P]orthophosphate-labeled CHO cells after insulin stimulation (Fig. 7, *lanes a* and *b*).

The cell-surface concentration of insulin receptors in the CHO cells was increased by cotransfection with two expression plasmids containing the wild-type human insulin receptor (HIRC) and neomycin resistance (pSVEneo). Transfected CHO cells expressing high concentrations of the insulin receptor at their surface were selected by flow cytometry. Scatchard analysis indicated that the total insulin binding capacity of these transfected cells was 10- to 20-fold higher than the control cell population and only 3-fold less than Fao cells (Fig. 6). In parallel, the insulin-stimulated phosphorylation of the β-subunit of the insulin receptor also increased 10-fold (Fig. 7, *lanes e–h*).

In addition to the increase in the β-subunit phosphorylation in CHO cells expressing the human insulin receptor, a protein of molecular weight 175,000 was also detected during immunoprecipitation of the insulin-stimulated cells with the αPY (Fig. 7, *lanes f* and *h*). To rule out the possibility that this protein was the precursor of the insulin receptor, we compared the phosphopeptide maps of the β-subunit of the insulin receptor and the 175-kDa protein (Fig. 8). The resulting HPLC elution profiles were clearly different for each protein, suggesting that the 175-kDa protein found in the CHO cells was not structurally related to the β-subunit of the insulin receptor. A further comparison was possible between the pp185 of Fao cells and the 175-kDa protein in CHO cells. A similar elution profile was observed for both proteins although the intensities of the peaks were different (compare Fig. 2*B* with Fig. 8). We provisionally suggest that the 175-kDa protein in transfected CHO cells is pp185.

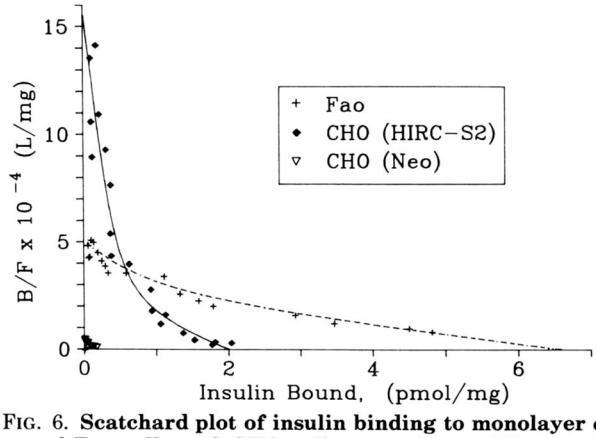

FIG. 6. **Scatchard plot of insulin binding to monolayer cultures of Fao cells and CHO cells expressing pSVneo which lacks the insulin receptor cDNA insert but contains neomycin resistance gene (*Neo*) or contains the insulin receptor cDNA insert (*HIRC-S2*).** The cells were grown to confluence in 24-well cluster trays. Each well was quickly washed with PBS and the cells were incubated at 15 °C with 10^{-11} M ^{125}I-insulin and various concentrations of nonradioactive insulin between 10^{-11} and 10^{-6} M. After 3 h, the free insulin was removed by three successive washes with PBS and the radioactivity in each well was quantified in a γ-counter. The values of *B/F* and *B* and the theoretical curves were calculated as described under "Experimental Procedures."

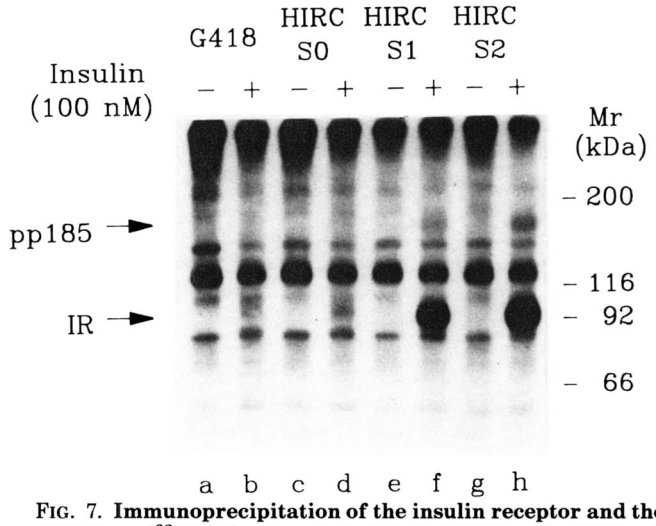

FIG. 7. **Immunoprecipitation of the insulin receptor and the pp185 from [^{32}P]orthophosphate-labeled CHO cells.** CHO cell lines which express various amounts of the human insulin receptor (total binding (R_0) = *HIRC-S0*, R_0 = 0.06 ± 0.01 pmol/mg protein; *HIRC-S1*, R_0 = 2.0 ± 0.3 pmol/mg protein; *HIRC-S2*, R_0 = 2.0 ± 0.02 pmol/mg protein) or only the endogenous CHO receptor (*G418*, R_0 = 0.1 ± 0.03 pmol/mg protein) were labeled for 2 h with [^{32}P]orthophosphate. The cells were incubated without (−) or with 100 nM insulin (+) for 1 min, frozen with liquid nitrogen, and solubilized as described under "Experimental Procedures." The phosphotyrosine-containing proteins were immunoprecipitated with the αPY, reduced with DTT, and separated by SDS-PAGE. The autoradiogram was obtained during a 6-h exposure. The relative intensity of phosphorylation after insulin stimulation of the β-subunit and the pp185 was measured by scanning densitometry for the β-subunit (*lane b* = 532, *lane d* = 534, *lane f* = 8750, *lane h* = 12072) and the pp185 (*lane b* = 44, *lane d* = 97, *lane f* = 418, and *lane h* = 819).

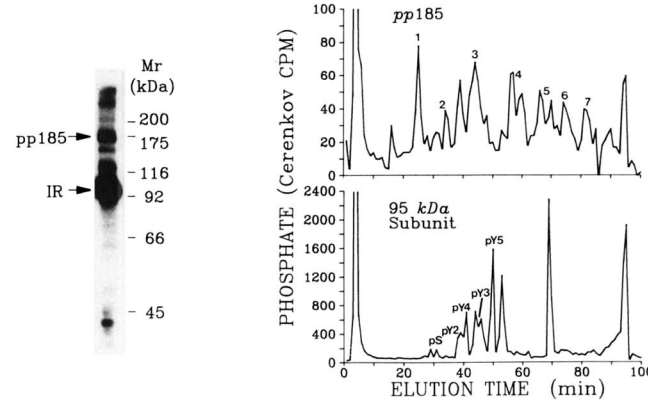

FIG. 8. **Separation of the tryptic phosphopeptide of the β-subunit of the insulin receptor and the pp185 immunoprecipitated from the HIRC-S2 cells by the αPY.** The cells were labeled and processed as described under "Experimental Procedures." The proteins identified in the autoradiogram were excised from the gel and digested exhaustively with trypsin. The mixtures of peptides were then separated by reverse phase HPLC on an RP-318 column.

DISCUSSION

Transmission of the insulin signal may occur in cells by tyrosine phosphorylation of cellular substrates of the insulin receptor kinase. Thus, we have extended our characterization of a 185-kDa phosphoprotein (pp185) that is immunoprecipitated by polyclonal antiphosphotyrosine antibodies from Fao

hepatoma cells and Chinese hamster ovary cells during insulin stimulation. A growing list of criteria suggest that this protein may be an important substrate for the insulin receptor kinase. Its phosphorylation occurs immediately during insulin stimulation, reaching a maximum level after 30 s of incubation with insulin. The level of phosphorylation of the pp185 is sensitive to the duration of insulin stimulation as it decreases to 50% of its initial level after 1 h (14), 30% after 4 h, and is undetectable after 24 h. Like other phosphotyrosine-containing proteins, the pp185 also contains phosphoserine and phosphothreonine (14). It is not an integral membrane protein as it cannot be labeled during surface iodination, and it is probably a cytoplasmic protein because it can be extracted from Fao cells by low-salt and detergent-free solutions. It is distinct from the insulin receptor as indicated by its cytoplasmic location, a unique tryptic phosphopeptide map, and failure to bind to anti-insulin receptor antibodies or wheat germ agglutinin agarose (14). The phorbol ester PMA influences the phosphorylation of both the insulin receptor and the pp185 by decreasing the phosphotyrosine content of the former (33) and either completely inhibiting the phosphorylation of the latter or changing its mobility to give a M_r 210,000 protein. Finally, the steady-state phosphorylation of the pp185 increases in CHO cells which express high levels of the insulin receptor, suggesting that phosphorylation of pp185 is closely related to the kinase activity of the insulin receptor and is probably a direct substrate. Together, these results suggest that the pp185 is a potential molecular link between the membrane-bound insulin receptor and metabolic pathways in the cytoplasm.

Like the receptors for epidermal growth factor (35, 36), platelet-derived growth factor (37, 38), insulin-like growth factor I (39, 40), and several oncogene products (41), the insulin receptor is a tyrosine-specific protein kinase (20, 42, 43). The mechanism by which these kinases regulate intracellular events is unknown; however, there appears to be little doubt that the tyrosine kinase activity is required for signal transmission (44).

A cascade of tyrosine phosphorylation provides an attractive mechanism to regulate the metabolism and growth of normal and transformed cells. Thus, considerable effort has been expended to identify the cellular substrates of the tyrosine kinases. Viral protein-tyrosine kinases have generally been shown to phosphorylate certain glycolytic enzymes (enolase, phosphoglycerate mutase, lactate dehydrogenase) and cytoskeletal proteins (p81, vinculin, calpactin) (41, 45). The receptors for EGF and platelet-derived growth factor appear to phosphorylate a different set of protein with molecular mass of 35 kDa, 40–45 kDa, and 81 kDa (41), but in most cases, the receptor itself is the major phosphotyrosine-containing protein detected during ligand stimulation (46, 47).

Similarly, the insulin receptor is the major phosphotyrosine-containing protein detected in insulin-stimulated cells by immunoprecipitation with the αPY, and pp185 is the only substrate detectable with the αPY in Fao and H35 hepatoma cells (14, 15), CHO cells transfected with the human insulin receptor, and 3T3-L1 cells (16). A 175–180-kDa protein that is tyrosine phosphorylated in response to insulin is also found in freshly isolated hepatocytes and L6 myoblasts.[6] In the latter two cell types, the substrate can only be extracted with SDS, however, suggesting that it is either distinct from the pp185 described in this report or that the pp185 in these cell types is tightly associated with insoluble cellular structures.

In addition to this set of 185-kDa proteins, two other substrates for the insulin receptor have been identified in intact cells labeled with [^{32}P]orthophosphate. One of them is a glycoprotein of molecular mass 120 kDa that has been found in H35 hepatoma cells (48). This protein binds to wheat germ agglutinin agarose and contains phosphotyrosine, but does not bind to αPY, suggesting that it is distinct from the 120-kDa insulin-insensitive protein described in this report. In the freshly isolated rat adipocytes, pp185 was not detected; however, a phosphotyrosine-containing protein of M_r 45,000 was immunoprecipitated with the αPY after insulin stimulation (49).

Recently, using the αPY, Izumi *et al.* (15) identified a protein of 185 kDa in normal rat kidney and Madin-Darby canine kidney cells that was stimulated in its tyrosine phosphorylation. These cells contain few or no insulin receptors, thus, pp185 appears to be a substrate for the type I IGF receptor. This receptor has been known for many years to be structurally similar to the insulin receptor (50), and the highest amino acid sequence homology (84%) between the type I IGF receptor and the insulin receptor is found in the cytoplasmic region of the β-subunit which defines the catalytic domain of the tyrosine-specific protein kinase (40). The finding that pp185 is phosphorylated by both the insulin and type I IGF receptor suggests that the substrate specificity of these receptors are similar which could provide a molecular link to explain the similar biological responses observed during IGF-I and insulin binding (51).

Prolonged exposure of cells to insulin causes down-regulation of the insulin receptors (52), increases the insulin binding affinity (18), changes the structure of the receptor (27), and reduces the biological responses to the hormone (18, 27, 53). Incubation of the well-differentiated Fao hepatoma cells with insulin for 24 h causes a 75% decrease of insulin receptors and a total desensitization of the cells to the effect of insulin on glycogen synthase, tyrosine aminotransferase, and amino acid transport (18). Similar results have been described for HTC hepatoma cells (53) and rat adipocytes (54). The mechanism of insulin-induced desensitization is unknown but may involve post-receptor changes in the signaling mechanism since the partial loss of insulin receptors cannot fully explain the complete absence of biological effects (53).

The duration of the insulin incubation has two effects on the phosphorylation of pp185 that could be partially responsible for the changes in the biological response: it alters the migration of the protein on SDS-PAGE and it decreases its phosphorylation. After 4 h of insulin stimulation, the pp185 migrates as two phosphotyrosine-containing proteins with molecular masses of 170 and 210 kDa. The detection of these two proteins is possible because the 170-kDa protein becomes dephosphorylated faster than the 210-kDa protein. This effect can be seen as early as 1 h after insulin stimulation (14). After a 24-h incubation of the Fao cells with insulin, the phosphorylation of pp185 is undetectable due to the complete loss of the 210-kDa component and a decrease of the 170-kDa components to an apparent basal level.

We have previously shown that PMA treatment of intact Fao cells stimulates serine phosphorylation of the insulin receptor β-subunit (33). Similar observations have been reported in IM-9 lymphocytes and HepG2 cells (55) and in bovine endothelial cells.[7] In Fao cells, the serine phosphorylation is associated with a decrease in insulin stimulation of glycogen synthase and a decrease in the induction of tyrosine amino transferase; these changes occur without an effect of PMA on insulin binding (33). PMA inhibits the kinase activity of the insulin receptor,[6] but the maximum decrease is only

[6] F. Beguinot, and R. Smith, personal communication.

[7] Hachiya, H., Takayama, S., White, M. F., and King, G. L. (1987) *J. Biol. Chem.* **262,** 6417–6424.

50% which does not fully explain the nearly complete loss of the insulin response.

In this study, we found that PMA alters the phosphorylation of pp185 which may be linked to the loss of the insulin response. Before insulin stimulation, PMA increased the phosphorylation of two proteins with molecular masses of 170 and 210 kDa that were immunoprecipitated with the αPY. Only phosphorylation of the 210-kDa component was stimulated by insulin. Thus, pp185 was not detected after a 1-min insulin stimulation of PMA-treated cells. Instead, the autoradiogram looked similar to that obtained after 4 h of insulin stimulation alone, suggesting a possible relationship between the insulin signal obtained in PMA-treated cells and that obtained after prolonged incubation with insulin. We do not understand the mechanism of this effect, but two possibilities should be mentioned: i) PMA-treatment or prolonged insulin stimulation may alter the substrate specificity of the catalytic domain of the β-subunit or other tyrosine kinase so that it no longer recognizes the pp185 but phosphorylates a new protein of 210 kDa; ii) PMA and prolonged insulin stimulation may alter the migration of the pp185 by causing phosphorylation of its serine or threonine residues or by altering its apparent size in some other way. However, the exact role of this changes and its effect on insulin action will require the identification of the catalytic function of the pp185.

Expression of the wild-type human insulin receptor in CHO cells leads to an increased sensitivity of the insulin response measured by stimulation of 2-deoxyglucose uptake (23). We have confirmed this result using glycogen synthase as the measured physiologic response in our CHO cells transfected with the wild-type human insulin receptor.[8] This has been attributed to the increased number of active insulin receptors in the plasma membrane of these cells. This increase in sensitivity also correlates with an increased phosphorylation of pp185. We do not know if the pp185 is the substrate that transmits the insulin signal beyond the plasma membrane to regulate the biological responses; however, we can provisionally conclude from our results that insulin stimulation of substrate phosphorylation in the intact cell increases with the concentration of the insulin receptor. Thus, the increased ability to phosphorylate the pp185 or some other protein may be the mechanism by which the insulin sensitivity of the transfected cells increases.

Unfortunately, the function of the pp185 is unknown at present, but it is possible to speculate about its role in insulin action. As it is a soluble protein, pp185 is probably not a structural protein, but may be a component of a multienzyme complex. Since many enzyme systems are regulated by serine and threonine phosphorylation (44), pp185 could be a serine/threonine kinase. Alternatively, many insulin bioeffects are mediated by dephosphorylation (56), thus, pp185 could be a component of a serine or threonine phosphatase. In addition, it could play a role in the generation of low molecular weight mediators which regulate the activity of other cellular enzymes (57, 58).

In conclusion, the pp185 or a protein of similar molecular mass is found in a number of cell types (14–16). In these systems, it is the only phosphoprotein in addition to the β-subunit of the insulin receptor that is recognized by the αPY during the initial stages of the insulin response. Although other protein substrates may exist, their concentration may be too low for detection or their phosphorylated tyrosine residue may not be available to interact with the αPY. Although the function of the pp185 is not known, many of its characteristics described here and previously (14, 15) are consistent with its having a role in insulin action.

[8] M. F. White, V. Lauris, and C. R. Kahn, unpublished results.

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