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Purification and Partial Sequence Analysis of pp185, the Major Cellular Substrate of the Insulin Receptor Tyrosine Kinase*

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Insulin stimulates the tyrosine phosphorylation of a 185-kDa putative cytosolic substrate protein (pp185) in diverse cell types. After intravenous insulin infusion into the live intact rat, pp185 and the 95-kDa insulin receptor β-subunit were the major proteins that tyrosine phosphorylated in liver, skeletal muscle, and adipose tissue. Both proteins were maximally phosphorylated within 30 s, and both increased in phosphotyrosine content in parallel with increasing insulin dose. However, pp185 tyrosine phosphorylation was transient, with almost complete dephosphorylation within 2–3 min despite continued insulin stimulation. To identify pp185 directly, we purified pp185 from insulin-stimulated rat liver, using a denaturation-based extraction procedure that blocks endogenous protein phosphatases and thus allows a high yield, single step isolation of phosphotyrosyl proteins by anti-phosphotyrosine antibody immunoaffinity absorption. From 50 rat livers, 50–100 pmol of pp185 was isolated. Edman degradation of seven internal tryptic peptide fragments of pp185 yielded novel amino acid sequences, indicating that pp185 is a new protein. Antipeptide antibodies were raised which specifically recognize a single, 185-kDa insulin-stimulated phosphotyrosyl protein in liver, skeletal muscle, adipose tissue, and several cultured cell lines. These results indicate that pp185 is expressed in a variety of insulin-responsive tissues, is the major protein rapidly tyrosine phosphorylated under physiological conditions in the intact animal, and also provide a route for cloning the pp185 gene and elucidating the function of pp185 in insulin signal transduction.

Insulin initiates its metabolic and growth-promoting effects upon binding to its tetrameric receptor (1–3), thereby activating a kinase in the β-subunit to catalyze the intramolecular autophosphorylation of specific tyrosine residues of its own β-subunits (4, 5, 17). Autophosphorylation enhances receptor tyrosine kinase activity toward other protein substrates (6–8). Considerable evidence demonstrates that insulin receptor tyrosine kinase activity is essential for many if not all of the biological effects of insulin (9–16). However, the exact biochemical mechanisms linking receptor kinase-mediated tyrosine phosphorylation to the regulation of cellular metabolic pathways are undefined.

Tyrosine phosphorylation of several cellular proteins and enzymes has been observed during the initial cellular response to some receptor tyrosine kinase-linked polypeptide growth factors, *e.g.* platelet-derived growth factor-induced phosphorylation of phospholipase C (18), 3′-phosphatidylinositol kinase (19), the *raf-1* kinase (20), and the GTPase-activating protein GAP (21). However, the nature of the physiologically relevant cellular protein substrates of the insulin receptor kinase has remained elusive. Although many purified proteins and synthetic peptides can be phosphorylated *in vitro* by isolated insulin receptors (reviewed in 22), these reactions do not occur *in vivo*, and thus their physiological significance is moot. When anti-phosphotyrosine antibodies are used to immunoprecipitate phosphotyrosine-containing proteins that appear in intact cultured cells during insulin stimulation, a protein of 185 kDa, designated pp185, appears in extracts of several cell types (41, 47–51). Additional phosphotyrosyl proteins of lower molecular mass have also been described in some cell lines (22, 65, 68, 80, 85, 87). The majority of these putative substrates are unidentified, and for all such putative insulin receptor kinase substrates no clear role in insulin signaling has yet been assigned.

The present studies were undertaken to characterize the nature, occurrence, and physiological relevance of insulin-stimulated tyrosine phosphorylations in the major insulin target tissues of the intact live animal, conditions most germane to normal insulin responses. To accomplish this we developed a new and generally applicable method both to assay and to isolate denatured phosphotyrosyl proteins. This technique enabled us to purify and partially sequence pp185, the major endogenous cellular substrate of the insulin receptor tyrosine kinase.

EXPERIMENTAL PROCEDURES

Materials—PMSF,[1] leupeptin, aprotinin, *p*-nitrophenyl phosphate, ovalbumin, DTT, dimethyl pimelimidate and Nonidet P-40 were purchased from Sigma. Sodium amobarbital (Amytal) and porcine insulin and human recombinant insulin (Humulin R) were from Lilly. Bovine serum albumin (fraction V) was from Armour. SDS (protein

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[1] The abbreviations used are: PMSF, phenylmethanesulfonic acid; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethypiperazine-*N*′-2-ethanesulfonic acid, sodium salt; PAGE, polyacrylamide gel electrophoresis; pNPP, *p*-nitrophenyl phosphate; aPY Ab, anti-phosphotyrosine antibody; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid.

chemistry special grade), Tris-HCl and HEPES (ultrapure grade) were from Boehringer Mannheim. ^{125}I-Protein A was from ICN Biomedicals, Costa Mesa, CA. Immobilized protein A beads (Trisacryl) and Triton X-100 (purified grade) were obtained from Pierce Chemical Co. Trichloroacetic acid and diethyl ether (anhydrous) were from Fisher. Wheat germ agglutinin-agarose was from Vector Laboratories. Male Sprague-Dawley rats were from Charles River Breeding Laboratories, Inc., Wilmington, MA. Nitrocellulose (BA85, 0.2 μm) was from Schleicher & Schuell. PVDF membranes were from Millipore. Reagents for SDS-PAGE, including molecular weight standards, were from Bio-Rad. Silver stain reagent kit was from Sigma, and colloidal gold stain from Janssen. Sequencing grade bovine trypsin was obtained from Boehringer Mannheim. HPLC grade trifluoroacetic acid was obtained from Applied Biosystems, Inc.; HPLC grade acetonitrile and water, from Burdick and Jackson; and Vydac HPLC columns, from The Nest Group. Automated sequenator and analyzer reagents were provided by the manufacturer. All other reagents were of at least analytical grade purity. Polyclonal anti-phosphotyrosine antibodies were raised in rabbits and affinity purified on phosphotyramine columns as described by Pang *et al.* (26).

Methods—Male rats (100–250 g) were fed *ad libitum* with Purina Laboratory Rodent Chow, except where indicated. Rats were injected with sodium amobarbital (150 mg/kg of body weight, intraperitoneally) and were used in experiments 10–15 min later as soon as anesthesia was assured by loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein or inferior vena cava exposed, and normal saline (0.9% NaCl) with or without hormone was infused through a 27-gauge needle connected to a mechanical syringe pump driven at 1 ml/min. After infusion, the liver or other tissues were excised rapidly, minced coarsely, and disrupted immediately for 45 s in 35 ml of solubilization buffer maintained at 100 °C in a water bath with a Polytron PTA 20S generator (Brinkmann Instruments model PT10/35) operated at maximum speed (setting 10). The solubilization buffer was composed of 2% SDS, 100 mM HEPES (pH 7.8 at 22 °C), 100 mM NaCl, 10 mM EDTA and 50 mM DTT. The homogenate was heated further to boiling with gentle stirring for 2 min and then left to cool to 22 °C. After centrifugation at 35,000 rpm for 2 h at 18 °C in a Beckman type 35 rotor (143,000 × *g* at r_{max}), the supernatant was acidified with 100% trichloroacetic acid, added slowly dropwise at 22 °C with vigorous stirring to a final trichloroacetic acid concentration of 10%. The mixture was then cooled on ice for 30 min. Under these conditions protein and nucleic acids form a copious, flocculent, pink precipitate while SDS remains largely soluble. Preliminary tests indicated that trichloroacetic acid concentrations of 5 and 15% gave identical results. The precipitate was collected by centrifugation at 5,000 rpm in a Sorvall SS-34 rotor at 4 °C for 5 min. The precipitate was washed once with 25 volumes of 10% trichloroacetic acid at 4 °C, and the trichloroacetic acid was then extracted by three washes, each with 25 volumes of ethanol:diethyl ether (1:1, v/v) at 4 °C. The precipitate was dried *in vacuo* for 4–18 h. The final yield of dry precipitate was about 0.05 g/g of liver (wet weight). The precipitate was pulverized thoroughly to a fine powder in a porcelain mortar. In this form the extracted proteins can be stored for at least 1 year at −70 °C without apparent degradation or significant loss of phosphotyrosine content.

Alternate methods of removing the SDS from the initial tissue extracts were evaluated. These include precipitation of the insoluble potassium salt of SDS by KCl addition (27, 28), dilution of the SDS with an excess of Triton X-100 (29), selective precipitation of proteins with cold organic solvents (30), and ion pair extraction of the SDS with triethylamine (31). These treatments were unsatisfactory because of much lower yields of phosphotyrosyl proteins and/or formation of intractable protein precipitates which could not be redissolved. Use of different lots of SDS obtained from different manufacturers altered the recovery of phosphotyrosyl proteins, perhaps because of variable contamination with hexadecyl sulfates, which have higher protein binding affinity and are less readily removed (32).

For the immunoprecipitation of phosphotyrosyl proteins, 0.1 g of dry tissue powder was dissolved in 0.1 N NaOH (0.05 g of powder/ml) with vigorous stirring at 22 °C for 3 min. The resulting solution was then neutralized rapidly to pH 8 with 2 volumes of 100 mM Tris-HCl. EDTA (1 mM), NaN_3 (0.1%), and the protease inhibitors PMSF (1 mM), leupeptin (1 μg/ml), and aprotinin (1 μg/ml) were added, and the slightly turbid solution was clarified with a 0.45-μm pore diameter cellulose/PVC filter (Millex-HA, Millipore Corp.). Control experiments demonstrated that once resolubilized, the phosphotyrosine content of the extracted proteins was stable for at least 3 days at 4 °C. In later experiments omission of the protease inhibitors was without effect. Protein concentrations were determined with the Bradford dye binding assay (33) using dye reagent and immunoglobulin protein standards from Bio-Rad or alternatively by optical density as described by Whitaker and Granum (34). Anti-phosphotyrosine antibodies were added to a final concentration of 3–4 μg of IgG/ml and incubated at 4 °C for 4 h (or overnight with equivalent results). Anti-phosphotyrosine antibody was then adsorbed to protein A beads (25 μl of a 50% bead slurry/ml of extract) for 2 h at 4 °C with gentle agitation. The immunocomplexes were washed twice by resuspension and brief centrifugation in 1 ml of wash buffer (1% Triton X-100, 0.1% SDS, 100 mM NaCl, 50 mM Tris, pH 7.3, at 22 °C) and once further in the same buffer lacking NaCl. After aspirating excess wash buffer, the immunoprecipitated proteins were solubilized in 50 μl of SDS-PAGE sample buffer (Laemmli, Ref. 35) with 50 mM DTT at 100 °C for 3 min. For some experiments, the immunoprecipitated phosphotyrosyl proteins were eluted competitively from the antibody bead pellet by incubating with 100 mM pNPP, 50 mM Tris, pH 7.4, 0.05% SDS for 1 h at 22 °C. The eluate was desalted by centrifugal passage (86) over a microcolumn of Sephadex G-25, pre-equilibrated with Laemmli sample buffer.

Electrophoresis and Immunoblotting—Immunoprecipitated proteins were separated on 0.5-mm thick, one-dimensional SDS-PAGE (5% T acrylamide) using the formulations of Laemmli (35) in a Bio-Rad miniature slab gel apparatus (Mini-Protean) at 175 V (constant). Standard molecular mass protein markers were: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (42.7 kDa). Electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 100 V (constant) at 5–15 °C in the Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin *et al.* (36) but with 0.05% SDS added to the transfer buffer to enhance elution of high molecular mass proteins. Preliminary experiments using alternate transfer buffers (37) or transfer times varying from 0.5 to 4 h gave qualitatively identical results. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4 °C in blocking buffer (5% bovine serum albumin, 1% ovalbumin in TNA (10 mM Tris, pH 7.2, 0.9% NaCl, 0.02% NaN_3)). The nitrocellulose blot was incubated with anti-phosphotyrosine antibodies diluted in blocking buffer (2 μg/ml) for 2 h at 22 °C and then washed twice for 10 min in TNA, once for 10 min in TNA containing 0.05% Nonidet P-40, and twice further for 10 min each in TNA. The blots were then incubated with 50 μCi of ^{125}I-protein A (6–30 μCi/μg) in 10 ml of blocking buffer for 1 h at 22 °C and then washed again as described above. Bound anti-phosphotyrosine antibodies were detected by autoradiography using preflashed (38) Kodak XAR film with Cronex Lightning Plus intensifying screens at −70 °C for 12–72 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS300) of the developed autoradiogram or by direct γ-scintillation spectrometry of bands excised from the nitrocellulose blots.

Preparation of Anti-phosphotyrosine Antibody Affinity Matrix—Thirty-eight milligrams of affinity-purified rabbit anti-phosphotyrosine antibody (aPY Ab) was adsorbed to 12 ml (settled gel volume) of protein A-Trisacryl by slow mixing at 4 °C overnite in 150 mM NaCl, 50 mM HEPES, pH 7.8. The gel matrix was washed three times with 100 ml of 0.2 M sodium borate, pH 9.0, at 22 °C and resuspended in 45 ml of 0.2 M sodium borate, pH 9.0, also containing 2 mM pNPP (to bind and protect the antibody combining site) for 2 h at 22 °C. Dimethyl pimelimidate was then added (20 mM final concentration) and the matrix gently mixed at 22 °C for 30 min to link the antibody to protein A covalently (57). The antibody matrix was then washed with excess 0.2 M ethylamine, pH 8.0, at 22 °C and incubated for 2 h further in the same buffer to quench unreacted dimethyl pimelimidate. The cross-linked matrix was washed extensively and stored at 4 °C in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN_3. Use of a Trisacryl matrix for this affinity column was essential, as the relatively hydrophilic nature of this material (25) minimized nonspecific adsorption of denatured proteins.

Preparative Purification of pp185—SDS-denatured protein extracts were prepared from whole livers of 3-day fasted male rats (200–300 g initial body weight) after an intraportal infusion of insulin (10^{-6} M) or 0.9% NaCl vehicle for 30 s, as described above. Sixty grams of dry protein precipitate (from a total of 50 livers) was dissolved quickly in 1,200 ml of 0.1 N NaOH with vigorous agitation for 5 min at 22 °C and the base neutralized by addition of 4800 ml of 100 mM Tris-HCl to a final pH of 7.4. The following additions were made to this solution: EDTA, 1 mM; NaN_3, 0.02%; leupeptin and aprotinin, 1 μg/

ml each; PMSF, 0.1 mM. After centrifugation (143,000 × *g* at r_{max}) for 1 h at 18 °C in a Beckman type 35 rotor the clear supernatant was filtered (0.45 μM cellulose Millex-HA) and then passed over a 15 × 1-cm column containing 12 ml of immobilized anti-phosphotyrosine antibody-protein A-Trisacryl matrix at 0.8 ml/min at 4 °C. The column was washed sequentially at 1 ml/min with 30 bed volumes of 1% Triton X-100, 0.1% SDS, 100 mM NaCl, 50 mM Tris, pH 7.3, at 22 °C, then with 30 bed volumes of the same buffer lacking NaCl, and finally at 22 °C with 1 bed volume of 50 mM Tris, pH 7.2. The adsorbed proteins were eluted at 22 °C for 2 h with 4 bed volumes of 100 mM pNPP in 0.025% SDS, 50 mM Tris, pH 7.2. The eluate was made 5 mM in DTT, and then simultaneously dialyzed (against 0.05% SDS, 5 mM DTT, 50 mM Tris, pH 7.2) and concentrated 125-fold *in vacuo* at 22 °C in a Micro-ProDiCon apparatus using PA-15 membranes (Bio-Molecular Dynamics, Beaverton, OR). The concentrated sample was made 10% in sucrose, 50 mM DTT and heated at 100 °C for 3 min. Since only about half of the phosphotyrosyl protein content of the original liver extract was removed by a single pass over the aPY Ab column under the conditions just described, the liver extract was recycled through the column, and the adsorption, column washing, hapten elution, dialysis, and concentration procedure was repeated, and the final sample combined with the first, and stored at −70 °C.

Amino Acid Analysis—To estimate the yield of purified phosphotyrosyl proteins, the samples were resolved on 5% SDS-PAGE, 0.5-mm thick gels, and then electrotransferred to PVDF membranes at 95 V for 2 h at 35 °C in 10 mM CAPS, pH 11.0, 10% methanol, as described by Matsudaira (58). The PVDF membranes were incubated in 0.1% Coomassie Blue R-250, 50% methanol, for 5 min, and after destaining (in 50% methanol, 10% acetic acid), the visible protein bands were excised individually. Proteins blotted onto PVDF membranes were placed in 6 × 50-mm tubes baked previously at 540 °C for 16 h. The tube(s) were placed in a Waters hydrolysis vial, 200 μl of constant boiling HCl added, and the vial evacuated and flushed with argon. After a final exposure to vacuum, the vial was sealed and heated at 110 °C for 22 h. After hydrolysis, the samples were dried *in vacuo* and the resultant amino acids analyzed as follows. The PVDF membrane was wet with 10 μl of MeOH and then extracted twice with 100 μl of 0.1 M HCl, 20% MeOH. This extract was taken to dryness, dissolved in 4 mM EDTA, and loaded onto an Applied Biosystems 420A derivatizer/analyzer for amino acid analysis. Phosphotyrosyl protein yields were also estimated by direct silver staining of SDS-PAGE gels (59) or, alternatively, by colloidal gold staining of nitrocellulose electroblots (23, 24) with comparison of band intensities with those of standard reference proteins included in the same gel or blot.

Enzymatic Cleavage of pp185—Anti-phosphotyrosine affinity-purified liver proteins, concentrated by vacuum dialysis into a solution containing 3% SDS, 50 mM Tris, pH 7.2, 50 mM DTT, 10% sucrose, were made 5% in SDS and then preparatively separated by reducing one-dimensional SDS-PAGE (5.5% T, 0.8% C) in a Bio-Rad miniature slab gel apparatus, using 1.2-mm-thick gels, run at 150 V, with electrophoresis buffers as described by Laemmli (35). After electrophoresis, the proteins were electrotransferred to BA85 nitrocellulose in transfer buffer (10 mM Tris, pH 8.0, 192 mM glycine, 20% methanol, 0.02% SDS) for 2 h at 4 °C and then for an additional 15 min in transfer buffer lacking SDS. To locate the protein bands the nitrocellulose was stained for 2 min in 0.1% Ponceau S, 1% acetic acid, and destained for 4 min in 1% acetic acid. The lightly stained bands were excised with a scalpel, washed three times with HPLC grade water, and stored moist at −20 °C.

Peptide fragments of the electrophoretically separated proteins were generated by *in situ* proteolytic digestion of the nitrocellulose-bound proteins with trypsin as described by Aebersold (69) but omitting the NaOH wash to minimize the loss of protein. After digestion the solution was immediately stored at −20 °C until separation of the resultant peptides by narrow bore reverse phase HPLC.

Reverse Phase HPLC Separation of Peptides—Peptides were separated by narrow bore reverse phase HPLC on a Hewlett-Packard 1090 HPLC equipped with a 1040 diode array detector, using a Vydac 2.1 × 150-mm C18 column. The gradient employed was a modification of that described by Stone *et al.* (70). Briefly, where buffer A was 0.06% trifluoroacetic acid/H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile, a gradient of 5% B at 0 min, 33% B at 63 min, 60% B at 95 min and 80% B at 105 min with a flow rate of 0.15 ml/min was used. Chromatographic data at 210 and 277 nm and ultraviolet spectra from 209 to 321 nm of each peak were obtained. While monitoring absorbance at 210 nm, fractions were manually collected by peak into microcentrifuge tubes and stored immediately without drying at −20 °C in preparation for sequence analysis.

Amino-terminal Peptide Sequence Analysis—Samples for amino-terminal sequence analysis were applied directly to a Polybrene precycled glass fiber filter and placed in the reaction cartridge of an Applied Biosystems model 477A protein Sequencer. The samples were subjected to automated Edman degradation using the program NORMAL-1, which was modified using the manufacturer's recommendations for faster cycle time (36 min) by decreasing dry-down times and increasing the reaction cartridge temperature to 53 °C during coupling. The resultant phenylthiohydantoin derivative fractions were identified subsequently using an on-line Applied Biosystems model 120A HPLC and Shimadzu CR4A integrator. Computerized protein and gene sequence database searches were performed using the Intelligenetics FASTDB program.

Preparation of Anti-pp185 Antibodies—Polyclonal antibodies to peptide segments of pp185 were raised in young adult New Zealand White rabbits. Synthetic peptides were prepared by the Protein Chemistry Core Facility of the University of Pennsylvania on the solid phase Milligen 9050 peptide synthesizer, and purified by reverse phase C18 HPLC. Peptides were coupled to rabbit serum albumin carrier by the bisdiazobenzidine method (71) and by glutaraldehyde (72), and a mixture of these conjugates was used to immunize three rabbits. Immunoglobulin fractions of the sera were prepared by ammonium sulfate precipitation and DEAE-Sephacel chromatography (73). Antibodies were affinity purified further on a column prepared by coupling synthetic peptide to Affi-Gel 10 (according to the manufacturer's directions), with antibody elution using 100 mM glycine, pH 2.5, and rapid neutralization. To assess antipeptide immunoreactivity with nondenatured pp185, whole rat liver cytosolic extracts were prepared by homogenizing 1 g of liver in 25 ml of homogenization buffer (0.25 M sucrose, 5 mM EDTA, 5 mM EGTA, 10 mM $Na_4P_2O_7$, 20 mM NaF, 50 mM HEPES, pH 7.5, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mg/ml bacitracin, 0.1 mg/ml benzamidine) at 0 °C and clarified by centrifugation at 100,000 × *g*) for 1 h.

Cell Culture—FaO hepatoma cells (39) were cultured in Falcon 150-mm-diameter plasticware dishes in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and penicillin/streptomycin (40) at 37 °C in a 5% CO_2 incubator. For experiments, 90% confluent cultures (10^7 cells) were serum deprived for 16–18 h prior to hormone stimulation, extraction, and analysis. In some experiments, cultures were labeled metabolically with 1 mCi of [^{32}P]orthophosphate in P_i-free medium for 4 h prior to analysis by methods described elsewhere (41). Solutions containing sodium orthovanadate were prepared at neutral pH to avoid loss of phosphatase inhibitory activity, as described previously (42).

RESULTS

Validation of Methods—Phosphotyrosyl proteins are susceptible to rapid phosphatase-mediated dephosphorylation both *in vivo* (43) and during cell extraction procedures (44). To assay hormone-stimulated tyrosine phosphorylation in intact organs of the live animal under conditions that block dephosphorylation, tissues were homogenized rapidly at 100 °C in a 2% SDS solution also containing 50 mM DTT reductant. The SDS denaturant was then removed by precipitation of proteins with trichloroacetic acid under conditions in which SDS remains soluble (45). Trichloroacetic acid was removed by organic extraction, the protein precipitate redissolved in 0.1 N NaOH (conditions in which phosphotyrosine is stable, (46)), and after neutralization the phosphotyrosyl proteins were precipitated quantitatively with anti-phosphotyrosine antibodies. The immunoprecipitated proteins were then resolved by one-dimensional SDS-PAGE, electroblotted to nitrocellulose, and detected with additional anti-phosphotyrosine antibody and ^{125}I-protein A.

We tested the validity of this method by examining the insulin response of FaO hepatoma, a cell line (39) with well characterized insulin receptor tyrosine autophosphorylation and endogenous protein phosphorylation (40, 41). Fig. 1 compares the recovery of phosphotyrosyl proteins from control and insulin-stimulated FaO cells using either the boiling SDS

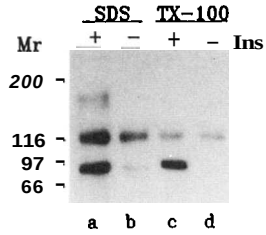

FIG. 1. Comparison of cell extraction methods for phosphotyrosyl protein analysis by anti-PY antibody immunoprecipitation and immunoblotting in FaO hepatoma cells. Confluent cultures (10^7 cells/dish, 150-mm diameter) were preincuhated in serum-free medium for **16** h. Insulin (10^{-6} M) was added for **1** min at 37 °C *(lane a* and *c)* or vehicle alone (phosphate-buffered saline) *(lanes b* and *d)* and the extraction performed using SDS at 100°C *(lanes a* and *b)* or $N_{2(liq)}$ quick-freezing and Triton X-100-based extraction at *0* °C *(lanes c* and *d)* as described under "Experimental Procedures."

denaturation/trichloroacetic acid precipitation method or using the generally employed, nondenaturing detergent (Triton X-100) extraction method at 0 °C with phosphatase inhibitors (10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_2VO_4, 10 mM EDTA) (41). Under nondenaturing extraction conditions, in the absence of insulin, FaO cells contain a major phosphotyrosyl protein at 120 kDa *(lane d)*. Upon insulin stimulation (10^{-6} M) for 1 min, only one new phosphotyrosyl protein appears *(lane* c) at 95 kDa, consistent with the autophosphorylated @-subunitof the insulin receptor. Use of denaturing SDS extraction demonstrates the same 120-kDa phosphotyrosyl protein *(lanes b* and *a)* and upon insulin stimulation the appearance of receptor @-subunitphosphorylation *(lanea)*. In addition, SDS extraction permits detection of a distinct insulin-stimulated phosphotyrosyl protein at 185 kDa *(lane a)*. This band has been designated previously as pp185 (41, 47, 48–51) and is likely an endogenous cellular substrate of the insulin receptor tyrosine kinase (52). The attenuated band intensities and the apparent lack of pp185 in the Triton **X**-100 extracts *(lanes* c and *d)* compared with the SDS extracts *(lanes a* and *b)* are attributed to incomplete inhibition of phosphotyrosyl phosphatase activity under nondenaturing conditions, despite inclusion of phosphatase inhibitors. When nondenaturing Triton X-100 buffers were used to solubilize animal tissues in preliminary experiments, virtually no phosphotyrosyl proteins could be observed (results not shown). In contrast, the SDS denaturation/trichloroacetic acid precipitation technique makes possible analysis of insulin receptor tyrosine kinase activity in organs and tissues of the intact animal under physiological conditions, as described below.

Hepatic Insulin Response—To determine the rate of insulin receptor autophosphorylation and its relationship to tyrosine phosphorylation of cellular proteins ***in vivo,*** insulin (10^{-5} M) was infused into the portal vein of anesthetized rats (Fig. 2A). In the absence of insulin only one major phosphotyrosyl protein (120 kDa) is present. At the earliest time point sampled after initiating insulin infusion (+ ***lane,*** at $t = 0.5$ min) the insulin receptor @-subunitappeared at 95 kDa and was already maximally autophosphorylated. Similarly, at $t = 0.5$ min the endogenous substrate of the insulin receptor (pp185) was also maximally tyrosine phosphorylated. Despite continuous insulin infusion the level of insulin receptor β-subunit tyrosine phosphorylation decreased slowly, with a $t_{1/2} = 6$ min (determined by densitometry of autoradiographs from three replicate experiments). Under these same conditions, pp185 was even more rapidly dephosphorylated and reduced to nearly base-line intensity after only **2–3** min. No additional insulin-stimulated phosphotyrosyl proteins were detected in

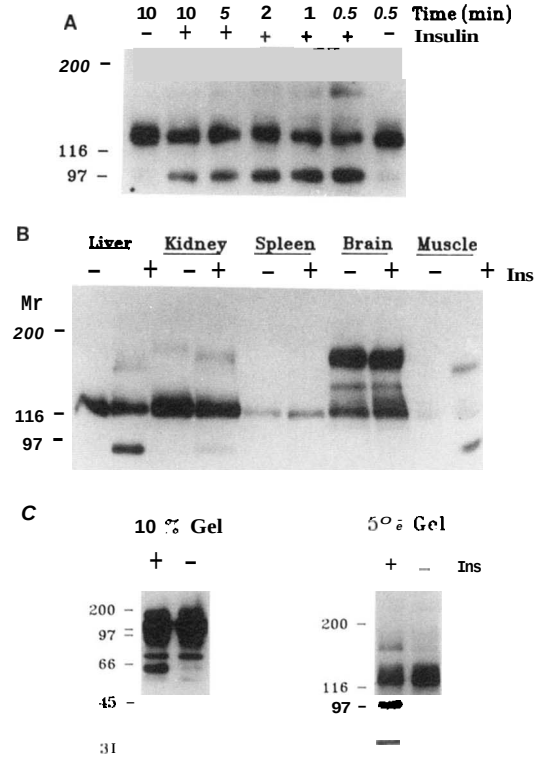

FIG. 2. *A,* insulin-stimulated tyrosine phosphorylation in intact rat liver: time course. Male Sprague-Dawley rats, fed *ad libitum,* were anesthetized and saline without (−) or with (+) 10^{-6} M insulin was infused into the portal vein for the indicated times. One animal was used for each time point, and the whole liver was taken for phosphotyrosyl protein analysis as described under "Experimental Procedures." The results shown are representative of three replicate experiments. *B*, tissue distribution of phosphotyrosyl proteins. Male rats were anesthetized, and saline without (−) or with (+) 10^{-6} M insulin was infused into the portal vein (for liver) or the inferior vena cava (other tissues) for **1** min at 1 ml/min. Tissues were excised and phosphotyrosyl proteins analyzed as described under "Experimental Procedures." C, insulin-stimulated phosphotyrosyl proteins in adipose tissue. Saline without (−) or with (+) 10^{-6} M insulin was infused into the inferior vena cava for 1 min at 1 ml/min. Epididymal fat pads were excised and phosphotyrosyl proteins separated by either *5%* T *(right)* or 10% T *(left)* SDS-PAGE for immunoblotting as described under "Experimental Procedures."

liver over this time period even when gel electrophoresis conditions were adjusted to resolve low M_r proteins (T acrylamide = 10–15%), or when autoradiographs were overexposed, or when the insulin-stimulated liver was initially quick frozen and powdered in liquid N_2 prior to further processing, or when two alternate preparations of anti-phosphotyrosine antibodies were used. Essentially identical patterns of tyrosine phosphorylation of the 185- and 95-kDa bands were observed (Fig. 2B) in hindlimb skeletal muscle and epididymal fat pads after acute insulin infusion into the inferior vena cava. However, in rat fat pads, an additional, insulin-sensitive phosphotyrosyl protein of 60 kDa is also detected readily (Fig. 2C). No consistent insulin-related change in the tyrosine phosphorylation of the 120-kDa band has been observed.

The ***in vivo*** insulin sensitivity of hepatic insulin receptor kinase activation and substrate phosphorylation was examined by varying the concentration of insulin infused into the portal vein from 10^{-12} to 10^{-6} M (Fig. 3). With increasing insulin, the intensity of insulin receptor @-subunitand pp185 tyrosine phosphorylation increased in parallel although the pp185 band was **30%** as intense as the @-subunitband. Half-maximal phosphorylation in three replicate experiments occurred at 1–5 **x** 10^{-8} M insulin. Based on dilution of the

infusate by portal blood flow (portal flow in 200-g anesthetized rat, 8.9 ml/min (54); insulin infused at 0.2 ml/min) the effective insulin concentration within the hepatic sinusoids and at the cell surface is at least 45-fold lower than the infused concentration. Thus, the insulin sensitivity is in good agreement with the reported binding constant of the hepatic insulin receptor (53). The observed results are not dependent on the anti-phosphotyrosine antibody immunoprecipitation/immunoblotting assay, as control experiments demonstrated the linearity of the method over the range of antibody concentrations used (Fig. 4).

Purification of pp185—To identify pp185 directly, we purified pp185 from liver using the SDS denaturation/trichloroacetic acid precipitation method coupled with preparative scale anti-phosphotyrosine antibody immunoaffinity chromatography. This purification was facilitated by using animals deprived of food for 48–72 h, for in preliminary experiments (not shown) we observed that prolonged fasting increased the insulin-stimulated tyrosine phosphorylation of pp185 about 2.5-fold. After acute intraportal infusion of either saline or 10^{-6} M insulin for 0.5 min, total liver extracts of denatured proteins were prepared from SDS-homogenates, using 50 livers for each condition as the starting material. After dissolution in base and neutralization each liver extract was passed through a 12-ml column of immobilized anti-phosphotyrosine antibody. The column was washed, and the adsorbed phosphotyrosyl proteins were eluted from the affinity matrix with 100 mM pNPP. Fig. 5 shows the profile of the eluted phosphotyrosyl proteins, both by anti-phosphotyrosine Western blotting and by direct silver staining.

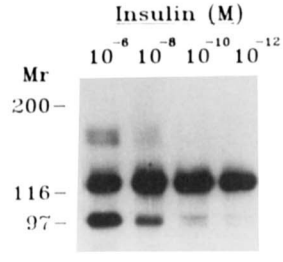

FIG. 3. **Insulin-stimulated tyrosine phosphorylation in intact rat liver: insulin concentration dependence.** Male Sprague-Dawley rats, fed *ad libitum*, were anesthetized, and saline with the indicated insulin concentrations was infused into the portal vein at 1 ml/min for 0.5 min. The entire liver was excised and processed as described under "Experimental Procedures." The results shown are representative of three experiments.

The pNPP-eluted proteins in the Western blot contain the same phosphotyrosyl proteins described previously: the 120-kDa insulin-insensitive protein (both − and + *lanes*) and also the insulin-stimulated pp185 and the 95-kDa insulin receptor β-subunit (+ *lane*). When these same pNPP-eluted phosphotyrosyl proteins were visualized with a sensitive silver stain (*right panel, supn't*) intense, broad bands appear which correspond to the 120-kDa protein and to the insulin-receptor β-subunit. However, at the position corresponding to pp185 there appear two comigrating bands: one broad band that stains weakly, the other quite narrow. This latter band is also equally evident in the absence of insulin (*supn't, − lane*). We assumed that the broad, poorly staining band detected only after insulin stimulation was authentic pp185. We assumed further that the narrow band, comigrating at 185 kDa, was a copurifying contaminant (not containing phosphotyrosine) that partially and nonspecifically eluted from the anti-phosphotyrosine antibody column together with the authentic phosphotyrosyl proteins. These assumptions were supported by the presence of this same sharp contaminant band at 185 kDa among those other proteins that were nonspecifically absorbed to the affinity column matrix and were not removed by the column washing procedures but which were dissociable from the affinity matrix by directly heating the matrix in Laemmli sample buffer (*pellet*, both − and + *lanes*).

To estimate recoveries of the phosphotyrosyl proteins, we performed total amino acid analysis on a portion of each of the phosphotyrosyl bands. After separation by one-dimensional SDS-PAGE, the phosphotyrosyl bands were electrotransferred to PVDF membranes, located by Coomassie Blue stain, and each band excised. The total amino acid content and composition of each band were obtained after *in situ* hydrolysis on an automated amino acid analyzer. From 50 insulin-stimulated livers we recovered 340 pmol of the 95-kDa insulin receptor β-subunit, 196 pmol of the 120-kDa band, and 108 pmol of the combined 185-kDa bands. Based on silver-stained gels and colloidal gold-stained nitrocellulose electroblots (not shown) we estimated that about half of the protein in the combined 185-kDa band was pp185.

Proteolytic Digestion and Sequencing of pp185—Because soluble proteins often have blocked amino termini which prevent Edman degradation (74, 79) and since pp185 was contaminated by an unknown protein of the same M_r, our strategy for amino acid sequencing depended on analysis of both basal and insulin-stimulated bands. The 185-kDa proteins eluted from the anti-phosphotyrosine affinity column

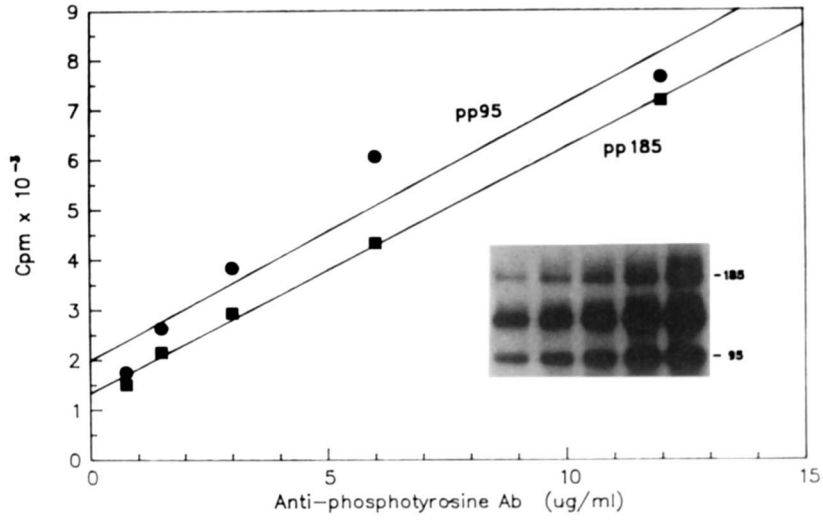

FIG. 4. **Anti-phosphotyrosine antibody concentration dependence of immunoblotting assay.** A total protein extract was prepared from a liver infused with 10^{-6} M insulin for 0.5 min as described under "Experimental Procedures." Protein was dissolved in Tris buffer (17 mg of protein/ml) and anti-PY antibody added at the indicated concentrations for 18 h. Immunocomplexes were analyzed by immunoblotting with anti-PY antibody/^{125}I-protein A detection. Bands on the nitrocellulose blot were localized by autoradiography, excised, and ^{125}I-cpm determined. Lines fit by linear least-squares regression. Equivalent results were obtained by optical scanning densitometry of autoradiograms.

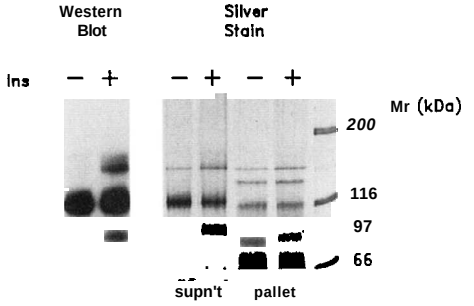

FIG. 5. Analysis of of anti-phosphotyrosine immunoaffinity-purified phosphotyrosyl proteins: Western blotting ***versus*** silver staining. Proteins eluted from the aPY Ab column with pNPP, resolved by 6.5% T SDS-PAGE, were either detected by aPY Ab immunoblotting and "'I-protein A ***(left panel)*** or directly silver-stained ***(rightpanel, supn't).*** Also shown are the proteins not eluting with pNPP, which were solubilized by heating an aliquot of the affinity matrix in Laemmli sample buffer at 100°C ***(right panel, pellet).*** − ***Lanes,*** control; + ***lanes,*** insulin-treated liver proteins.

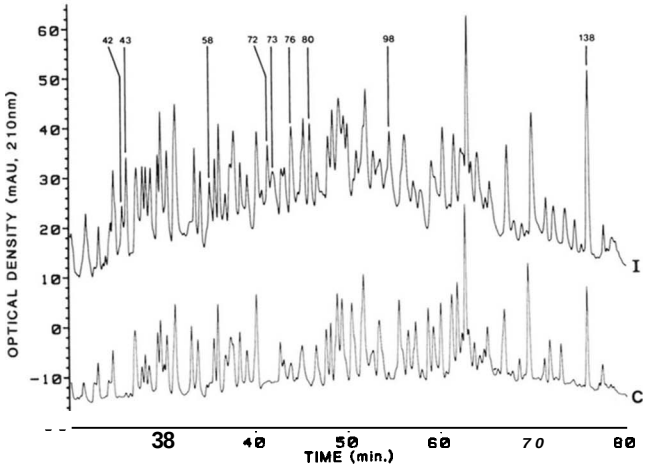

FIG. 6. Tryptic peptide maps of 185-kDa proteins. The 185-kDa proteins, purified by aPY antibody immunoaffinity chromatography from either control ***(lower trace,*** C) or insulin-stimulated livers ***(upper trace,*** *I)* were cleaved with trypsin and each digest resolved by reverse phase HPLC, as described under "Experimental Procedures." These two independent maps are aligned for comparison, with specific peaks identified by the ***numbers*** at the ***top.***

were separated by one-dimensional SDS-PAGE, transferred to nitrocellulose, and then digested with trypsin ***in situ.*** The resulting tryptic polypeptide fragments were then resolved on a reverse phase C18 HPLC column and the basal and insulin-stimulated tryptic peptides compared.

Close comparison of the two peptide maps revealed eight distinct peaks that were present only in the insulin-treated sample (peaks 42, 43, 58, 72, 73, 76, 80, and 98), and these were provisionally assigned to pp185 (Fig. 6). These peak fractions were subjected to direct amino acid sequence analysis in an automated sequenator. In addition, three peaks common to both the control and the insulin-treated maps were sequenced. The amino acid sequence data for all peptides are summarized in Table I.

Peak 42 yielded a single 8-residue sequence. Searching of gene and protein seauence registries reveals that this sequence is not identical to any previously reported sequence. Peak 43 yielded an interesting sequence of 18 residues, beginning with glutamic acid followed by 10 consecutive glutamine residues. Peak 43 also contained a secondary (less abundant) sequence of 11 residues. Neither the primary nor the secondary sequence of peak 43 is identical with reported sequences. Novel

TABLE I

Summary of sequence data for pp185 polypeptides

Tryptic peptides were subjected to amino-terminal protein sequence analysis as described under "Experimental Procedures." Designations for tryptic fragments correspond to the column peaks described in Fig. 6. Where multiple sequences were identified in a given peak fraction the sequences are designated primary (1") and then 2" and 3° in order of decreasing molar yield.

Fragment	Sequence[a]	Identity
From +insulin sample		
42	1° LEYYENEK	None
43	1° EQQQQQQQQqqSiLXPpE	None
	2" LSSETFSAPXp	None
58	1" VVAVDXGIK	CPS[b] @ 220
72'	1° EETGStXYMNMDLGPGea	None
	2" XLPDAemgXspaXT	None
76[d]	1° SVSAPQQIINPI	None
	2° NLIGIY	CPS @ 91
	3" GQXLTMAN	
80	1" YIPGATMGTSPALTGDEAr	None
	2" SFAFvS	CPS @ 1,263
98	1° XISHAISEHVEDSGVHs	CPS @ 1,187
	2" XLGASPpNAXTAPXXXr	None
	3° XPPXTFQXVXXR	None
138"	1" SAVTGPGEFWMQVDDSVVAQNmXe	None
	2" QADAVYFLPITPQFVTEVIX	CPS @ 478
From −insulin sample		
53	1" TFEESFQk	CPS
115	1° LFATEATSDW	CPS @ 1,388
	2° TADdSXIXIl	CPS @ 1,455

[a] Residues in lower case letters represent assignments of less than full confidence. Positions in which no assignment was possible are indicated by X.

[b] CPS, carbamyl phosphate synthetase.

'Residues clearly observed at positions 6 and 7 could not be assigned unambiguously to the major ***versus*** minor sequence, as they were of equal yield.

[d] The relative molar yield **of** phenylthiohydantoin derivatives for 1°:2°:3° sequences is approximately 2:2:1. Therefore, residues clearly observed at positions 1–6 of the 1° and 2" sequences could not be assigned unambiguously to the major ***versus*** minor sequence, as they were of equal yield.

[e] Equimolar abundance **of** 1" and 2" sequences. Deduced carbamyl phosphate synthetase tryptic peptide @ 478 are listed as 2".

sequences were also derived from peaks 72, 76, 80, 98, and 138. Although peak 58 appeared in the tryptic map of the insulin-treated sample, the sequence of this peak matched that of an internal tryptic fragment of carbamyl phosphate synthetase (75). Carbamyl phosphate synthetase is an inner mitochondrial matrix protein that is abundant in fasted rat liver (76). This result suggested that carbamyl phosphate synthetase was the protein that nonspecifically copurified with pp185. Additional sequence data supporting this identification of carbamyl phosphate synthetase as the contaminant was provided by the sequencing of peak 53, and peak 115 from the control sample. Carbamyl phosphate synthetase was also present in the secondary and tertiary sequences of peaks 80 and 76, respectively, and also as the primary sequence in peak 98. Control experiments with anti-carbamyl phosphate synthetase antiserum confirmed that carbamyl phosphate synthetase is not tyrosine phosphorylated in liver after insulin

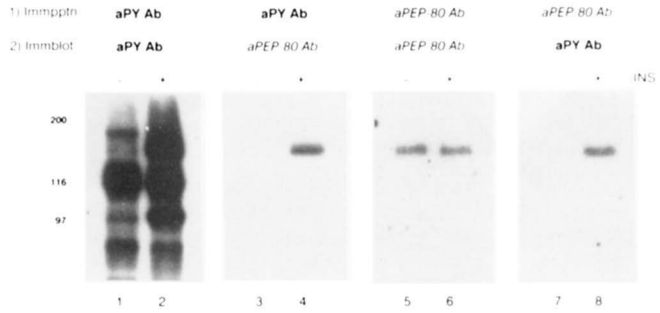

FIG. 7. **Specificity of anti-peptide 80 antibodies.** Liver extracts were prepared from control (− *lanes*) or insulin-treated (+ *lanes*) livers, divided, and incubated with either anti-phosphotyrosine antibody (*lanes 1, 2, 3,* and *4*) or with anti-peptide 80 antibody (*lanes 5, 6, 7,* and *8*). The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-phosphotyrosine antibody (*lanes 1, 2, 7,* and *8*) or with anti-peptide 80 antibody (*lanes 3, 4, 5,* and *6*) and then ^{125}I-protein A for autoradiography.

stimulation.[2]

As evidenced by these results, any individual tryptic peak is not necessarily homogeneous and may contain more than a single polypeptide species. To assess possible peak heterogeneity the ultraviolet absorbance of each peak was monitored with a diode array detector, allowing simultaneous detection at 210, 277, and 292 nm. Absorbance at 210 and 277 nm monitors for peptide bonds and aromatic residues, respectively, whereas detection at 292 nm distinguishes peptides containing tyrosine from those containing tryptophan. Tyrosine-containing peptides have a low 292/277 nm absorbance ratio, whereas tryptophanyl peptides have a high 292/277 nm ratio. One tryptic peak, 138, was present in the peptide maps of both control and insulin-treated material (Fig. 6), absorbing at 210 nm and 277 nm. However, this peak lacked absorbance at 292 nm in the control map but did absorb significantly at 292 nm in the insulin-treated map. Automated Edman degradation of peak 138 (from the insulin-treated map) resulted in identification of two different residues at each of the first 19 sequenator cycles. These residues were of equal yield, thus precluding direct assignment of residues to a primary or secondary sequence. However, analysis of the predicted tryptic cleavage products of carbamyl phosphate synthetase revealed one carbamyl phosphate synthetase peptide sequence (at carbamyl phosphate synthetase residue 478) that uniquely matched one of each pair of residues present in the first 19 sequenator cycles of peak 138. Therefore, we were able to deduce subtractively the second, novel peptide sequence (which contained the predicted tryptophan) from the data obtained from peak 138 and assign this sequence to pp185.

Antipeptide Antibody Studies—If the novel peptide sequences we assigned to pp185 are contained within the primary structure of this insulin receptor substrate, then antibodies to these peptides should recognize a protein with properties expected of pp185. To test this prediction, we raised polyclonal antibodies to a synthetic peptide containing the first 15 residues of the primary sequence of peak 80 (Table I). The anticipated specificity of these antipeptide antibodies is demonstrated in Fig. 7. Anti-peptide 80 antibodies clearly reacted on immunoblots with a single 185-kDa band which had first been immunoprecipitated with anti-phosphotyrosine antibodies from extracts of insulin-stimulated liver (*lane 4*). Antipeptide 80 antibodies do not recognize such a band in the anti-phosphotyrosine immunoprecipitate obtained from control (no insulin treatment) liver extracts (*lane 3*). This result is consistent with the presence of phosphotyrosine in pp185 only after the insulin receptor tyrosine kinase has been activated. Anti-peptide 80 antibodies also immunoprecipitate from both control and insulin-stimulated liver extracts a single 185-kDa protein that is recognized equally well on immunoblots by anti-peptide 80 antibodies (compare *lanes 5* and *6*) but which is reactive with anti-phosphotyrosine antibodies only when precipitated from insulin-stimulated livers (compare *lanes 7* and *8*). Additional control experiments demonstrated that anti-peptide 80 antibody immunoprecipitation of the 185-kDa protein was completely blocked when 1 μM synthetic peptide 80 was added to the original liver extract (not shown). These results support strongly the derivation of the novel peptide sequences of Table I from authentic pp185.

DISCUSSION

These studies had two complementary goals: first, to evaluate the role of insulin receptor kinase activation and receptor substrate tyrosine phosphorylation in the insulin signal transduction process in intact animal tissues under physiological conditions, and second, to purify and identify pp185, the major endogenous substrate of the insulin receptor and a putative intracellular effector of insulin action. To accomplish these objectives, we developed a new method of phosphotyrosyl protein isolation employing SDS denaturation of whole tissues, detergent removal, and subsequent anti-phosphotyrosine antibody absorption. In most prior animal studies of the physiological chemistry of insulin receptor function, the receptors were first partially purified, and then *in vitro* kinase assays were performed using exogenous phosphoacceptor substrates such as histones (reviewed in 22). Although certainly informative, this approach is susceptible to biochemical artifacts resulting from cell homogenization and receptor purification procedures, *e.g.* proteolysis (61) and/or dephosphorylation of the receptor by contaminating phosphoprotein phosphatases (60) as well as removal of the receptor from the plasma membrane where interactions with other cellular components can influence receptor activity (62, 63, 77). Moreover, important differences in receptor kinase activity may be manifest *in vitro* with only certain, specific phosphoacceptor substrate proteins but not with others (64). The *in vivo*, SDS denaturation method employed to make the observations reported in this paper is not subject to such complications and permits a direct assessment of insulin-stimulated tyrosine phosphorylation of endogenous proteins of potential physiological significance and in small samples from diverse tissue types. Moreover, as we have demonstrated, this method is applicable on a preparative scale for the purification of phosphotyrosyl proteins and thus will be of general use to those studying other novel protein substrates of tyrosine kinase-linked hormone receptors.

The detection sensitivity of the SDS denaturation method coupled with aPY Ab immunoprecipitation and immunoblotting is comparable to metabolic labeling procedures. In control experiments (not shown) we could detect insulin-stimulated pp185 tyrosine phosphorylation in cultured FaO hepatoma cells equally well using either the SDS denaturation/immunoblotting method or when using [^{32}P]orthophosphate-labeled cultures with direct autoradiography of SDS-PAGE resolved aPY immunoprecipitates (41). However, the difficulties associated with metabolically labeling live animals with high levels of [^{32}P]orthophosphate makes the SDS denaturation method much more appropriate for *in vivo* animal studies.

Using this technique we have observed tyrosine phosphorylation in only two distinct proteins in rat liver after acute

[2] P. L. Rothenberg, A. Karasik, and C. R. Kahn, unpublished data.

insulin infusion, and these are similar to those observed in cultured hepatoma cells. The 95-kDa band is most certainly the autophosphorylated β-subunit of the insulin receptor, based on its characteristic electrophoretic mobility, tissue distribution, rapid and graded response to varied insulin concentrations and its adsorption to a wheat germ agglutinin lectin column (not shown). We identify the broad band between 170 and 185 kDa as the putative endogenous cellular substrate of the insulin receptor kinase, pp185. This protein has been described in several insulin-stimulated cultured cell types (47–51), but its function and role in insulin action are as yet unknown. Previous studies suggested that pp185 is not a high molecular weight form of the insulin or other growth factor receptor, as it can be extracted from cells without detergents, is not labeled during cell surface iodination, does not bind to wheat germ agglutinin lectin or anti-insulin receptor antibodies, and has a unique tryptic phosphopeptide map (41). Our results indicate that pp185 is phosphorylated in all the major insulin target organs (liver, skeletal muscle, and adipose tissue), with an insulin dose dependence similar to the insulin receptor itself. These direct *in vivo* observations are consistent with a significant role for pp185 in linking the membrane-bound insulin receptor to intracellular metabolic pathways.

The rapidity of pp185 phosphorylation is also consistent with a role for pp185 in the very earliest stages of postreceptor insulin signal transduction. However, the relatively quick onset of pp185 dephosphorylation in intact liver (despite continued insulin receptor autophosphorylation) raises the possibility that the tyrosine-phosphorylated form of pp185 serves as labile, transient intermediary element that initiates (but may not be required to perpetuate) the persistent metabolic effects and/or growth-promoting effects of insulin. A similar transient course of pp185 tyrosine phosphorylation has been noted in FaO hepatoma (65), in N18 neuroblastoma (66), and in intact rat epididymal fat pads *in vivo*.[3] In contrast, a maintained, persistent tyrosine phosphorylation of pp185 in rat liver has been reported by Tobe *et al.* (78). The experiments of these workers, although *in vivo*, differed from ours. Tobe *et al.* (78) employed a single bolus injection of insulin whereas we employed a steady, maintained insulin infusion. Also, the specific methodology for phosphotyrosyl protein analysis differed. Using our SDS denaturation procedure the transient tyrosine phosphorylation of pp185 which we observed is not an artifactual consequence of differential extraction of pp185, for in unpublished experiments using anti-peptide 80 antibodies we found equivalent yields of nonphosphorylated pp185 at all times during the insulin infusion. Moreover, using our technique with both cultured H35 hepatoma cells and Chinese hamster ovary cells transfected with human insulin receptors (a kind gift from Dr. R. Roth) we did observe equivalent levels of pp185 tyrosine phosphorylation at all times between 0.5 and 30 min after insulin addition. Thus, the basis for the discrepancy between our observations and those reported by Tobe *et al.* is unclear. In our experiments, continuous insulin infusion with persistent receptor activation might be associated with a desensitization process involving rapid pp185 dephosphorylation, and this process might not be induced in liver during the transient receptor activation of a bolus insulin injection. A protein of 165 kDa, perhaps related to pp185, is rapidly tyrosine phosphorylated and also briskly dephosphorylated within 10 min in isolated adipocytes (67). However, a comparable 160-kDa putative protein substrate remains maximally tyrosine phosphorylated for up to 1 h in the continued presence of insulin in cultured 3T3-L1 adipocytes (68). Understanding the biochemical basis and functional consequences of this close temporal regulation of insulin receptor substrate tyrosine phosphorylation will require further investigation.

A protein of 120 kDa in rat liver has been reported to undergo insulin-stimulated tyrosine phosphorylation (80) and may be an endogenous substrate of the insulin receptor kinase (81). This putative substrate (pp120) has recently been immunologically identified as HA4, an integral membrane glycoprotein associated with the canalicular membrane of the hepatocyte and apparently involved in transmembrane bile acid transport (82). In our studies we consistently observed a 120-kDa protein that constitutively contained phosphotyrosine and which was unaffected by insulin. This 120-kDa band that we observed is not likely the same pp120 (HA4) bile canalicular protein described by others because in addition to its insulin insensitivity *in vivo*, we found this band is not liver specific but appears in muscle, kidney, spleen, brain, adipose tissue, and several cultured cell lines (results not shown). Moreover, the hepatic 120-kDa band we observed is not a sialylated glycoprotein like the pp120 (HA4) protein, as it does not adhere to a wheat germ agglutinin lectin affinity column (experiments not shown). In addition, partial sequencing of the 120-kDa constitutive phosphotyrosyl protein from liver reveals it is a novel protein, distinct from HA4.[4] The pp120 (HA4) bile canalicular protein may become tyrosine phosphorylated in insulin-stimulated liver, but this process could be obscured in our studies by a much greater abundance of the constitutive phosphotyrosyl protein of similar molecular mass.

We also observed an additional, insulin-stimulated phosphotyrosyl protein at 60 kDa. This band appeared in addition to pp185 but only in epididymal fat pads, and it was not evident in liver or muscle. This pp60 may therefore be an adipocyte-specific substrate and subserve some insulin action unique to the fat cell. A 60-kDa phosphotyrosyl protein has been reported previously in isolated adipocytes (55, 56), but the identity and function of this protein and its possible relationship to pp185 remain to be determined. A 46-kDa membrane protein has been reported to undergo tyrosine phosphorylation in insulin-treated intact adipocytes (83), and a 40-kDa microtubule-associated kinase is slowly tyrosine phosphorylated in 3T3-L1 cells treated with insulin (84), but insulin-sensitive phosphotyrosyl proteins in this lower molecular mass range were not evident in our experiments. And still other proteins have been described which undergo insulin-stimulated tyrosine phosphorylation in intact cells, *e.g.* a 15-kDa protein in 3T3-L1 adipocytes pretreated with the dithiol reagent phenylarsine oxide (85); several proteins (180, 150, 114, 100, 85, 68, and 56 kDa) in FaO hepatoma pretreated with sodium orthovanadate and/or hydrogen peroxide (65), and additional low molecular mass proteins in transfected NIH 3T3 cells overexpressing the human insulin receptor (87). It remains possible that such proteins are indeed tyrosine phosphorylated during insulin infusion into the live rat during our experiments but that they escape detection because of extremely low abundance or rapid dephosphorylation and/or failure to be bound by anti-phosphotyrosine antibodies. Alternatively, phosphorylations of these proteins might only occur under the special experimental conditions used in the aforementioned studies.

Based on the results of our *in vivo* analyses and employing the SDS denaturation/trichloroacetic acid precipitation/anti-phosphotyrosine immunoaffinity method for isolating phosphotyrosyl proteins, we identified pp185 as the predominant

[3] P. L. Rothenberg and B. Gill, unpublished data.

[4] P. L. Rothenberg and C. R. Kahn, unpublished data.

insulin-stimulated phosphotyrosyl protein in rat liver, skeletal muscle, and adipose tissue. We partially purified pp185 from liver and obtained the amino acid sequences of internal tryptic peptide fragments of this protein. These amino acid sequences do not match any reported gene or protein sequences. pp185 is apparently a novel, previously unknown protein. The use of antipeptide antibodies raised against the peptide sequence of peak 80 (Table I) also confirms the correct identification of pp185. These antibodies specifically recognize a 185-kDa protein that is present in liver, skeletal muscle, and adipose tissue and which is phosphorylated on tyrosine after insulin-stimulation. In preliminary immunocytochemical studies we have observed a diffuse cytoplasmic distribution of pp185, consistent with earlier reports describing the soluble nature of pp185 (41). Anti-pp185 antibodies will be useful in further investigations of the functional role of pp185 in the molecular mechanism of insulin signal transduction. Furthermore, using the pp185 peptide sequences (from peaks 80 and 138) we have synthesized oligonucleotide probes and have begun to screen successfully a rat liver cDNA library to obtain pp185 cDNA clones, and we have identified several of the novel peptide sequences from Table I within these clones.

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