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A Cascade of Tyrosine Autophosphorylation in the β-Subunit Activates the Phosphotransferase of the Insulin Receptor*

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We identified the major autophosphorylation sites in the insulin receptor and correlated their phosphorylation with the phosphotransferase activity of the receptor on synthetic peptides. The receptor, purified from Fao hepatoma cells on immobilized wheat germ agglutinin, undergoes autophosphorylation at several tyrosine residues in its β-subunit; however, anti-phosphotyrosine antibody (α-PY) inhibited most of the phosphorylation by trapping the initial sites in an inactive complex. Exhaustive trypsin digestion of the inhibited β-subunit yielded two peptides derived from the Tyr-1150 domain (Ullrich, A, Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* 313, 756–761) called pY4 and pY5. Both peptides contained 2 phosphotyrosyl residues (2Tyr(P)), one corresponding to Tyr-1146 and the other to Tyr-1150 or Tyr-1151. In the absence of the α-PY additional sites were phosphorylated. The C-terminal domain of the β-subunit contained phosphotyrosine at Tyr-1316 and Tyr-1322. Removal of the C-terminal domain by mild trypsinolysis did not affect the phosphotransferase activity of the β-subunit suggesting that these sites did not play a regulatory role. Full activation of the insulin receptor during *in vitro* assay correlated with the appearance of two phosphopeptides in the tryptic digest of the β-subunit, pY1 and pY1a, that were inhibited by the α-PY. Structural analysis suggested that pY1 and pY1a were derived from the Tyr-1150 domain and contained 3 phosphotyrosyl residues (3Tyr(P)) corresponding to Tyr-1146, Tyr-1150, and Tyr-1151. The phosphotransferase of the receptor that was phosphorylated in the presence of α-PY at 2 tyrosyl residues in the Tyr-1150 domain was not fully activated during kinase assays carried out with saturating substrate concentrations which inhibited further autophosphorylation. During insulin stimulation of the intact cell, the 3Tyr(P) form of the Tyr-1150 domain was barely detected, whereas the 2Tyr(P) form predominated. We conclude that 1) autophosphorylation of the insulin receptor begins by phosphorylation of Tyr-1146 and either Tyr-1150 or Tyr-1151; 2) progression of the cascade to phosphorylation of the third tyrosyl residue fully activates the phosphotransferase during *in vitro* assay; 3) *in vivo*, the 2Tyr(P) form predominates, suggesting that progression of the autophosphorylation cascade to the 3Tyr(P) form is regulated during insulin stimulation. Thus, multisite phosphorylation in the Tyr-1150 domain may be an important control site for transmission of the insulin signal in the intact cell.

Tyrosine autophosphorylation of the β-subunit of the insulin receptor is one of the earliest cellular responses to insulin binding. In the intact Fao rat hepatoma cell (1), 3T3-L1 cells (2), and Chinese hamster ovary cells expressing a high concentration of the human insulin receptor (3), the β-subunit is maximally phosphorylated in less than 20 s after exposure to insulin. This rapid stimulation is also observed *in vitro*[1] after purification of the insulin receptor from Fao cells (4), and other cells and tissues (5). *In vitro*, autophosphorylation is an intramolecular reaction (4, 6) occurring at several sites within the β-subunit (4, 7). Certain tyrosine residues are phosphorylated faster than others suggesting that a cascade of autophosphorylation occurs during insulin stimulation (4, 8). In the intact cell, the autophosphorylation reaction appears to be more selective since fewer major phosphotyrosine-containing peptides are observed (1, 9); however, the significance of this selectivity and its consequences on the activity of the insulin receptor *in vivo* are unknown.

Autophosphorylation of the insulin receptor is important because it activates the phosphotransferase in the β-subunit which then catalyzes tyrosine phosphorylation of other proteins (10–13). Although the mechanism of insulin action is not fully understood, autophosphorylation of the insulin receptor (14–16) and possibly substrate phosphorylation (3, 17–20) may be required to transmit the insulin signal to metabolic pathways in the intact cell. Mutation of the insulin receptor at the presumed ATP binding site (15, 21) or substitution of the tyrosine residues 1150 and 1151[2] with phenylalanine (14) results in a complete loss or a decrease of insulin-stimulated kinase activity and failure to activate fully the cellular insulin response.

In this report, some of the tyrosine residues which undergo autophosphorylation in the β-subunit of the solubilized insulin receptor from Fao rat hepatoma cells have been deduced. By using a combination of antiphosphotyrosine antibodies, mild proteolysis, immunoprecipitation, radiosequenation, and kinetic studies, we have identified the first residues that

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[1] In this report, the term *in vivo* refers to intact cells, whereas the term *in vitro* refers to a cell-free system.

[2] The numbering sequence used in this paper was described by Ullrich *et al.* (35) and is based on the position of the amino acids in the precursor of the human insulin receptor.

undergo autophosphorylation during insulin stimulation as Tyr-1146 and either Tyr-1150 or Tyr-1151. The phosphorylation of these two residues in the so-called Tyr-1150 domain[3] appears to be an obligate first step in the insulin-stimulated autophosphorylation cascade. However, autophosphorylation at these two tyrosine residues did not activate the insulin receptor kinase during *in vitro* assays at saturating substrate concentrations. Subsequent phosphorylation of the third tyrosine residue which generates the 3Tyr(P) form[4] of the Tyr-1150 domain was required for full activity. In the intact Fao cell, the 2Tyr(P) form of the Tyr-1150 domain predominated during insulin stimulation, and the 3Tyr(P) form was scarcely detected. Consistent with this finding, the receptor purified from insulin-stimulated cells was not fully activated when assayed at saturating substrate concentrations *in vitro*. These results suggest that other regulatory mechanisms are operating on the insulin receptor in the intact cell to restrain the activation induced by insulin binding.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: [^{32}P]orthophosphate, [γ-^{32}P]ATP, and Triton X-100 were from Du Pont-New England Nuclear; HEPES,[5] aprotinin, phenylmethylsulfonyl fluoride, *N*-acetylglucosamine, and bovine serum albumin were from Sigma. Inorganic reagents were purchased from Fisher unless indicated otherwise. Reagents for SDS-PAGE and the reverse phase HPLC column (RP-318) were purchased from Bio-Rad, and the HPLC apparatus was from Waters Associates. Porcine insulin (Lot 1JM95AN) was from Elanco. Pansorbin was from Behring Diagnostics and purified as previously described (22). The synthetic peptide, Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (Thr-12-Lys),[6] which contains the amino acid sequence between residues 1142 and 1153 of the β-subunit was purchased from Dr. David Coy, Tulane University, New Orleans, LA; Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (Asp-10-Lys) was from Peninsula Laboratories, Inc. Tissue culture medium and fetal bovine serum were obtained from GIBCO, and all tissue culture supplies were from NUNC or Costar.

The anti-insulin receptor antibody was obtained from patients with severe insulin resistance (22), and the anti-phosphotyrosine antibody (α-PY) was prepared by immunization of rabbits as previously described (12). Antibodies against specific domains of the β-subunit were prepared by immunization of rabbits with synthetic peptides coupled to keyhole limpet hemocyanin as previously described (26). The αPep-1, αPep-3, and αPep-4 were raised against residues 1314–1324, 1143–1152, and 952–962, respectively, of the human insulin receptor described by Ullrich *et al.* (35). The αPep-7 was raised against residues 718–727 of the human insulin receptor sequence described by Ebina *et al.* (23).

Cell Culture and Purification of the Insulin Receptor—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 (24, 25). The Fao cell cultures were maintained at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2, and grown in plastic tissue culture dishes (15-cm diameter) containing 30 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. The insulin receptor was partially purified from these cells by wheat germ agglutinin affinity chromatography (22). Confluent Fao cells in ten 15-cm dishes were solubilized at 22 °C with 3 ml of 50 mM HEPES (pH 7.4) containing 1% Triton X-100, 0.1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Following centrifugation to remove the insoluble material, the cell extract was passed over a WGA-agarose column, and the insulin receptor was eluted with 1–2 ml of 0.3 M *N*-acetylglucosamine in 50 mM HEPES (pH 7.4), 0.1% Triton X-100 as previously described (22).

In Vitro Autophosphorylation Assay—WGA-purified insulin receptor (0.24 μg/μl) was incubated at 22 °C for 10 min in a solution (100 μl) containing 50 mM HEPES and 5 mM $MnCl_2$ in the absence or presence of 100 nM insulin (4). Phosphorylation was initiated by adding 25 μM [γ-^{32}P]ATP (2.5 mCi/ml) and was continued for the time intervals indicated in the figure legends. In certain experiments, 3 μg of the α-PY was added to the reaction mixture before the addition of the [γ-^{32}P]ATP. The phosphorylation reaction was terminated by adding a 0.5-ml portion of 50 mM HEPES containing 0.1% Triton X-100, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, and 2 mM sodium vanadate (Aldrich) at 4 °C.

In some experiments, the insulin receptor was mildly digested with trypsin before or after the autophosphorylation reaction as described by Goren *et al.* (26). The TPCK-treated trypsin (final concentration, 5 μg/ml) or buffer was added to the reaction mixture before or after phosphorylation and incubated for 1 min at 22 °C. Digestion was stopped by adding aprotinin to a final concentration of 10 μg/ml. Trypsin was found to completely convert the 95-kDa β-subunit to an 85-kDa fragment that lacked the C-terminal domain (26).

The phosphorylated insulin receptor was immunoprecipitated from each reaction with the α-PY. The purified α-PY (2 μg) was mixed with phosphorylated insulin receptor preparations and allowed to incubate for 2 h at 4 °C. The antibody was immobilized on Pansorbin (10%, 50 μl) and washed 3 times with 1 ml of HEPES (50 mM, pH 7.4) containing Triton X-100 (1.0%), SDS (0.1%), NaCl (150 mM), NaF (100 mM), and Na_3VO_4 (2 mM). The phosphoproteins were eluted from the Pansorbin with Laemmli sample buffer (27) containing 100 mM dithiothreitol, separated by SDS-PAGE using 7.5% resolving gels (22), and identified by autoradiography. The phosphoamino acids were identified in the proteins by a modification (28) of the method of Hunter and Sefton (29).

^{32}P-Phosphorylation of the Insulin Receptor in the Intact Fao Cells—Confluent Fao cells in 15-cm dishes were incubated at 37 °C 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) (1). Insulin was added, and the incubation was continued at 37 °C for 1 min and then stopped quickly by removing the incubation medium and freezing the cell monolayers with liquid nitrogen. The monolayers were thawed and homogenized immediately at 4 °C with 2 ml of a solution containing 50 mM HEPES (pH 7.4), 1.0% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride (1). The cells were scraped from the dishes, and the detergent-insoluble material was sedimented by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor for 60 min. Each supernatant was applied onto a 0.2-ml WGA column which was washed with 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100 and the inorganic reagents described above. The bound glycoproteins were eluted with 1 ml of the column wash buffer supplemented with *N*-acetylglucosamine (300 mM). Half of the eluate was incubated with 10 μg/ml trypsin for 1 min at 22 °C to remove the C-terminal domain of the β-subunit (26). The insulin receptor and the proteolytic fragments were immunoprecipitated from the eluate with anti-insulin receptor antibody (22). The phosphoproteins were solubilized in Laemmli buffer, reduced with 100 mM DTT, separated by SDS-PAGE, and identified by autoradiography of the stained and dried gels with Kodak X-Omat film. The radioactivity in gel fragments was measured by Cerenkov counting.

HPLC Separation of the Tryptic Phosphopeptides and Identification of the Phosphorylated Tyrosine Residues—The β-subunit was digested with trypsin as previously described (4). The fixed and dried gel fragments containing the β-subunit were rehydrated and incubated in 1 ml of 50 mM NH_4HCO_3 (pH 8.2) containing 100 μg of TPCK-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 (about 95%) were separated with a Waters high performance liquid chromatography system equipped with a wide-pore C_{18} reverse-phase column (Bio-Rad, RP-

[3] We call the Tyr-1150 domain the amino acid sequence in the β-subunit of the insulin receptor which extends from arginine residue 1143 to the lysine residue 1153 and includes tyrosine residues 1146, 1150, and 1151.

[4] In this report, 2Tyr(P) (read as bis-phosphotyrosyl) indicates two phosphotyrosyl residues in the Tyr-1150 domain, whereas 3Tyr(P) (read as tris-phosphotyrosyl) indicates three phosphotyrosyl residues in the Tyr-1150 domain.

[5] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGF, epidermal growth factor; IGF, insulin-like growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; wheat germ agglutinin agarose was from Vector; DTT, dithiothreitol; WGA, wheat germ agglutinin; α-PY, anti-phosphotyrosine antibody; αIR, anti-insulin receptor antibody.

[6] In this report, synthetic peptides are abbreviated by indicating their first amino acyl residue, the number of amino acyl residues, and the last residue.

318). The phosphopeptides were applied to the column which was washed for 5 min at a flow rate of 1.1 ml/min with 0.05% trifluoroacetic acid, 95% water, and 5% acetonitrile. The peptides were eluted by a gradient of acetonitrile increasing linearly from 5 to 25% during 85 min. Fractions (1.1 ml) were collected at 1-min intervals in 1.5-ml polypropylene microcentrifuge tubes, and the radioactivity in each tube was measured with 40% efficiency as Cerenkov radiation using a Beckman scintillation counter. The percentage of acetonitrile in each sample had no effect on the efficiency of the Cerenkov radiation. Greater than 95% of the radioactivity in the trypsin digest was routinely recovered from the reverse-phase HPLC column.

In some cases, the tryptic phosphopeptides obtained from the reverse-phase HPLC column were further digested with *Staphylococcus aureus* V8 protease (5 μg) in 50 μl of NH_4HCO_3 (pH 8.2) (30); these fragments were separated by high voltage electrophoresis (65 V/cm) on cellulose thin layer plates (Analtech) saturated with 30% formic acid (pH < 1). The intensity of the spots on the autoradiogram was measured by scanning densitometry using a Hoeffer GS-300 scanning densitometer. In other cases, the peptides were separated by anion exchange HPLC on a Waters Protein Pac DEAE 5P column; the column was equilibrated with 20 mM Tris (pH 8.5) and eluted with 20 mM Tris (pH 7.0) containing 300 mM NaCl increasing linearly to 100% during 100 min.

The identity of the tryptic phosphopeptides was determined by immunoprecipitation with the specific antibodies αPep-1, αPep-3 and αPep-4, and α-PY. The fractions containing the peptides were dried *in vacuo*, and the residue was dissolved in 0.5 ml of 50 mM HEPES containing 0.1% Triton X-100. Antibody (1 μg) was added to each sample and incubated for 12 h at 4 °C, and then it was precipitated by addition of 50 μl of a 10% suspension of Pansorbin. The precipitate was washed three times with a solution containing HEPES (50 mM, pH 7.4), Triton X-100 (1%), SDS (0.1%), NaCl (150 mM), NaF (100 mM), and Na_3VO_4 (2 mM). The radioactivity in the precipitate was measured by Cerenkov counting.

The positions of the ^{32}P-phosphorylated tyrosine residues in the tryptic peptides were located by automated Edman sequence analysis in a Beckman model 890C Sequencer. The Edman degradation was carried out in the presence of 1 mg of myoglobin as an inert carrier (31). The amount of [^{32}P]phosphate in the butyl chloride-extracted anilinothiazolinone derivatives obtained at each cycle was measured in scintillation fluid. Aliquots from successive cycles were also converted to the phenylthiohydantoin derivative for quantification of myoglobin recovery as an internal standard. The phosphorylated tyrosine residues were deduced by comparing the Edman cycle number with the amino acid sequence of presumed tryptic peptides in the human insulin receptor. To show that the method works, we sequenced the synthetic peptide Thr-12-Lys after its phosphorylation by the insulin receptor. The results indicated that the tyrosine residue at position 9 was phosphorylated which was consistent with previous results (9). The total recovery of the [^{32}P]phosphate from the sequenator in this case and subsequent experiments was between 5 and 10%. An additional problem with this method was the persistent occurrence of asymmetric peaks because of the poor elution of the anilinothiazolinone derivative of phosphotyrosine.

Tyrosine Kinase Assay—The kinase activity of the insulin receptor was measured by an initial velocity experiment using Thr-12-Lys as the peptide substrate (26, 32). The activity of the insulin receptor was measured under various conditions: (i) the WGA-purified receptor was allowed to autophosphorylate to near steady state in the presence of [γ-^{32}P]ATP for 2 min before addition of the substrate; (ii) the reaction mixture containing the intact insulin receptor was incubated for 1 min with 5 μg/ml trypsin to form the 85-kDa fragment of the β-subunit before autophosphorylation and substrate phosphorylation (26); (iii) receptor autophosphorylation was carried out with nonradioactive ATP in the absence or presence of the α-PY, and then the phosphorylated receptor was purified by immunoprecipitation on immobilized protein A (Pierce Chemical Co.) and eluted with 20 mM *p*-nitrophenyl phosphate; (iv) intact cells were stimulated with 100 nM insulin for 1 min and then the receptor was extracted as described for the *in vivo* labeling experiments, purified by WGA agarose, and assayed for kinase activity after immunoprecipitation and elution from α-PY. Generally, the receptor preparation (4 μg of protein) was diluted to 50 μl with 50 mM HEPES, pH 7.4, and a final concentration of 0.1% Triton X-100, 5 mM $MnCl_2$, and 25 μM [γ-^{32}P] ATP. Various concentrations of the substrate Thr-12-Lys were added as indicated for each experiment, and the reaction was stopped after 10 min by adding 20 μl of 1% bovine serum albumin followed immediately by 50 μl of 10% trichloroacetic acid. The precipitated protein was sedimented by centrifugation, and the supernatant which contained the phosphorylated peptide was applied to a 2 × 2-cm piece of phosphocellulose paper (Whatman). The paper was washed with four changes of 1 liter each of 75 mM phosphoric acid, and the retained radioactivity was measured by Cerenkov counting. When reported, the K_m and the V_{max} for the substrate phosphorylation were determined by nonlinear least squares analysis using the FORTRAN programs called HYPER or SUBIN as described by Cleland (33).

RESULTS

The Effect of Substrate Concentrations on Autophosphorylation and Insulin-stimulated Kinase Activity of the Purified Insulin Receptor—As we have previously reported (4), in the absence of insulin stimulation, autophosphorylation of the WGA-purified insulin receptor from Fao cells was barely detectable during a 1-min incubation (Fig. 1, *lanes a–c*). Insulin (100 nM) produced more than a 10-fold stimulation of autophosphorylation of the β-subunit which reached 90% of the maximum within 1 min (Fig. 1, *lanes d–f*). Insulin-stimulated autophosphorylation increases the activity of the tyrosine kinase in the β-subunit of the receptor toward exogenous substrates (10–13). Thus, in the absence of insulin, the V_{max} for receptor-catalyzed phosphorylation of Thr-12-Lys was about 0.25 pmol/min (Fig. 2*A*). This rate was increased 2.5-fold when the insulin-stimulated receptor was maximally phosphorylated by incubation with [^{32}P]ATP before the addition of the substrate (Fig. 2*A*); importantly, the substrate had only a small inhibitory effect on the level of the prior β-subunit autophosphorylation (Fig. 2*B*). However, phosphorylation of Thr-12-Lys by the insulin-stimulated receptor that was not incubated with ATP before addition of the substrate exhibited a biphasic kinetic curve (Fig. 2*A*). The phosphorylation of Thr-12-Lys at nonsaturating concentrations was stimulated normally by insulin, and autophosphorylation of the β-subunit reached normal levels. However, above the K_m of Thr-12-Lys *de novo* autophosphorylation of the receptor was strongly inhibited during the kinase assay (Fig. 2*B*) and the phosphotransferase not fully activated by insulin (Fig. 2*A*). At 5 mM Thr-12-Lys receptor autophosphorylation was inhibited 80%, and kinase activity was equal to that of the basal receptor (Fig. 2, *A* and *B*). We conclude that during *in vitro* kinase assays, saturating concentrations of substrates inhibited autophosphorylation of the β-subunit which blocked the activation of the phosphotransferase.

The Effect of Anti-phosphotyrosine Antibodies on Insulin-stimulated Autophosphorylation and Kinase Activity of the β-

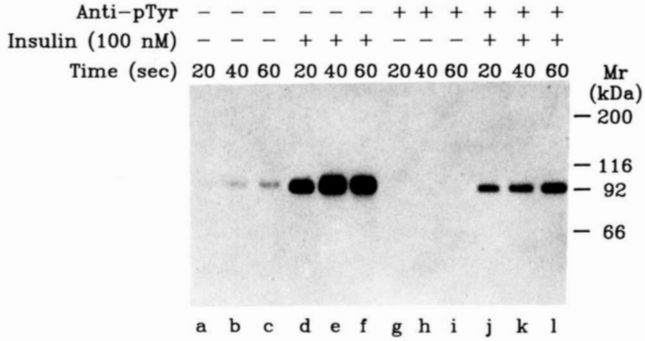

FIG. 1. **Inhibition of autophosphorylation with the anti-phosphotyrosine antibody.** Partially purified insulin receptor was incubated with [γ-^{32}P]ATP in the absence (−) or presence (+) of 100 nM insulin for 20 min at 22 °C. The α-PY (labeled *Anti-pTyr* in the figure) was added to some of the reactions (+) at a concentration of 40 μg/ml. Phosphorylation was initiated by adding 25 μM [γ-^{32}P]ATP for the indicated time intervals. The reaction was terminated and the phosphorylated receptor was immunoprecipitated with the α-PY, reduced with DTT, and separated by SDS-PAGE.

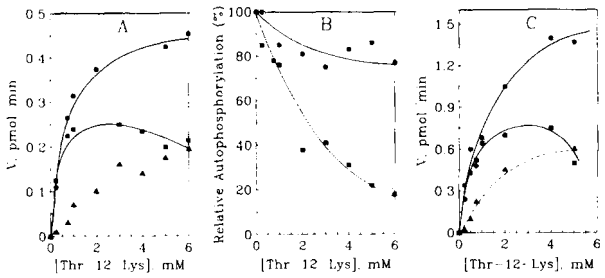

FIG. 2. **Inhibition of β-subunit activation by Thr-12-Lys.** *Panel A*, WGA-purified insulin receptor (4 μg) was incubated without (▲) or with 100 nM insulin (■, ●) for 20 min. Half of the insulin-stimulated reaction mixtures were allowed to autophosphorylate in the presence of 25 μM [γ-^{32}P]ATP for 2 min before the addition of substrate (●). Then the indicated concentration of Thr-12-Lys was added to all of the reaction mixtures and incubated for 10 min. The reaction was stopped by adding trichloroacetic acid and the Thr-12-Lys was collected on phosphocellulose paper, and the precipitated β-subunit was separated by SDS-PAGE. The phosphate content of Thr-12-Lys and the β-subunit was determined by measuring Cerenkov radiation, and the initial velocity of phosphorylation of Thr-12-Lys is shown. *Panel B*, the level of β-subunit phosphorylation which occurred during the experiment described in *panel A* is shown for the insulin-stimulated receptors. The receptor was incubated with [γ-^{32}P]ATP before and after addition of the Thr-12-Lys (●), or it was exposed to [γ-^{32}P]ATP only in the presence of Thr-12-Lys (■). *Panel C*, the WGA-purified insulin receptor was incubated without (▲) or with (■, ●) 100 nM insulin for 20 min. The insulin-stimulated receptor was incubated with 50 μM unlabeled ATP for 10 min in the absence (■) or presence of α-PY (●), and then immunoprecipitated with α-PY and eluted with 20 mM *p*-nitrophenyl phosphate, 5 mM $MnCl_2$, and 100 nM insulin. The kinase activity of these phosphorylated receptors and an equal portion of unstimulated receptor (▲) were measured during a 10-min incubation with the indicated concentrations of Thr-12-Lys. The *lines* are drawn by hand through the points.

Subunit—Prior to insulin stimulation, the insulin receptor extracted from Fao cells contains phosphoserine and phosphothreonine but does not contain phosphotyrosine and does not bind α-PY (1, 3, 18, 34). However, addition of α-PY (0.04 μg/μl) to an *in vitro* autophosphorylation reaction before the [γ-^{32}P]ATP caused a 65–70% inhibition of the phosphorylation of the β-subunit in both the basal and insulin-stimulated states (Fig. 1, *lanes g–l*). Prolonged incubation with [γ-^{32}P]ATP did not overcome the inhibition by the α-PY, and concentrations of α-PY 100-fold higher did not further inhibit autophosphorylation (data not shown). These results suggested that the α-PY bound to the newly phosphorylated tyrosine residues in the β-subunit and inhibited complete autophosphorylation.

The phosphotransferase of the insulin-stimulated receptor that was partially autophosphorylated in the presence of α-PY revealed a biphasic kinetic curve. In this experiment, the receptor was incubated with insulin, ATP and α-PY for 10 min, and then the receptor was immunoprecipitated on protein A Sepharose, washed and eluted from the α-PY with *p*-nitrophenyl phosphate. The activity of this partially autophosphorylated receptor was assayed with Thr-12-Lys. At nonsaturating concentrations of Thr-12-Lys (<1 mM) the phosphotransferase was stimulated normally by insulin (Fig. 2*C*). However, it was not fully activated during incubation of the partially autophosphorylated receptor with [γ-^{32}P]ATP and saturating concentrations of Thr-12-Lys (Fig. 2*C*). In fact, the initial rate of substrate phosphorylation at 5 mM was not greater than that seen with the noninsulin-stimulated receptor. Thus, partial autophosphorylation of the β-subunit that occurred during incubation with the α-PY did not activate the phosphotransferase under conditions in which further autophosphorylation was inhibited by saturating substrate concentrations.

The Effect of the Antiphosphotyrosine Antibody on the Autophosphorylation Sites in the β-Subunit—The phosphorylation sites in the β-subunit obtained in the absence and presence of α-PY were identified by exhaustive trypsin digestion of the phosphorylated β-subunit followed by separation of the peptides on reverse-phase HPLC (Fig. 3*A*). As previously reported (4, 26), five or six major phosphopeptides were observed by this separation system that are designated pY1, pY1a, pY2, pY3, pY4, and pY5 (3, 26). In the presence of α-PY, the tryptic phosphopeptides corresponding to pY1, pY1a, pY2, and pY3 were completely inhibited, and only two peaks were detected by HPLC that are designated pY4* and pY5* (Fig. 3*B*). These peptides corresponded exactly to pY4 and pY5 obtained during phosphorylation in the absence of α-PY as a mixture of pY4 and pY4* migrated together on reverse-phase HPLC (Fig. 3*C*) and by high voltage electrophoresis (Fig. 3*D*). A similar correspondence was found for pY5 and pY5*. The pY5 (pY5*) was more positively charged as it migrated during electrophoresis toward the cathode more rapidly than pY4 (pY4*). These data suggest that α-PY bound to the initial tyrosine phosphorylation sites in the β-subunit which are found in pY4 and pY5 and inhibited autophosphorylation at the other tyrosine residues. Together with our kinetic results, phosphorylation of the tyrosine residues in pY4 and pY5 only was insufficient to activate the kinase when further autophosphorylation was inhibited by high concentrations of exogenous substrates (Fig. 2*C*).

Consistent with previous observations (4, 8), the phosphorylation of the various sites reached a steady state at distinct time intervals (Fig. 4*A*). The presence of α-PY completely inhibited the phosphorylation of pY1, pY1a, pY2, and pY3 but had no effect or a stimulatory effect on the phosphorylation of pY4* and pY5* (Fig. 4*B*). The total phosphate incorporated into pY4 and pY5 (pY4 + pY5) reached about 85% of the steady state in less than 20 s following addition of the [γ-^{32}P]ATP (Fig. 4*C*). Identical results were obtained for the phosphorylation of pY4* and pY5* (pY4* + pY5*) in the presence of the α-PY. Relative to these peptides, the total incorporation of phosphate into pY2 and pY3 (pY2 + pY3), and pY1 and pY1a (pY1 + pY1a) occurred more slowly requiring about 30 s for half-maximal phosphorylation (Fig. 4*C*). Together with the α-PY inhibition experiments described above, these results suggest that pY4 and pY5 contain the first sites of tyrosine autophosphorylation in the β-subunit.

Identification of the First Phosphorylation Sites in the Insulin Receptor—The domains of the β-subunit that contain the autophosphorylation sites were further defined by mild tryptic digestion of the insulin receptor. Mild trypsinolysis of the solubilized receptor before autophosphorylation converted the β-subunit into a catalytically active 85-kDa fragment (Fig. 5*A*, *lanes e* and *f*) that contained about 40% of the phosphotyrosine originally found in the intact 95-kDa subunit (Fig. 5*B*, *bars e* and *f*). We have shown previously that the phosphotyrosine lost by mild trypsin digestion (Fig. 5*A*, *lanes a* and *b*) is in phosphopeptides pY2 and pY3 which were tentatively localized to the C-terminal domain of the β-subunit since the 85-kDa fragment lost reactivity with an antibody (αPep-1) directed at the C-terminal domain (26). In the presence of α-PY, there was a 30–35% inhibition of autophosphorylation in the 85-kDa fragment whether the fragment was prepared before (Fig. 5*B*, *bars f* and *h*) or after phosphorylation (Fig. 5*B*, *bars b* and *d*). Peptide mapping indicated that both pY4 and pY5 were obtained from the 85-kDa fragment and that α-PY inhibited phosphorylation of

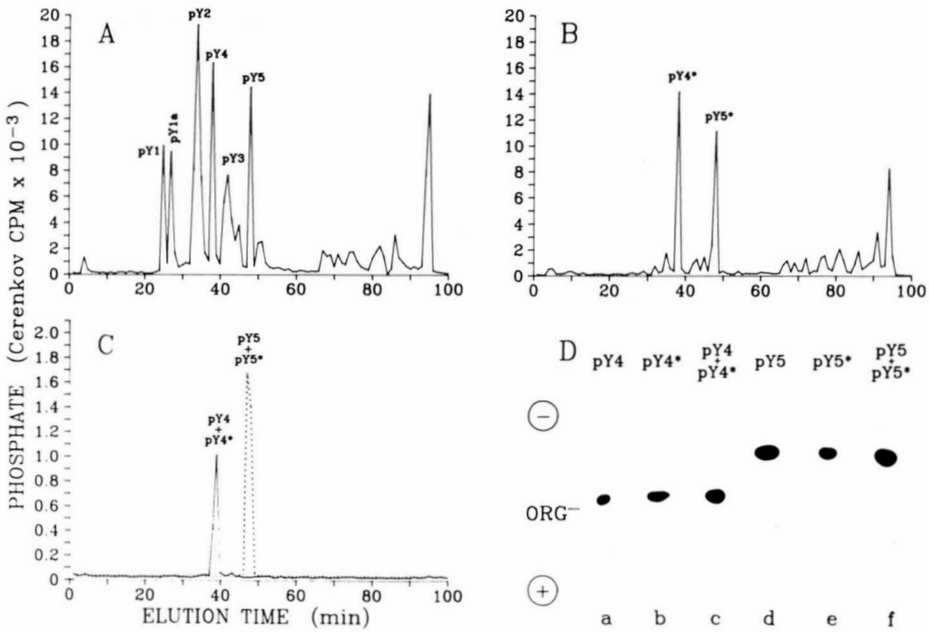

FIG. 3. **Analysis of the *in vitro* autophosphorylation sites in the β-subunit of the insulin receptor by tryptic peptide mapping.** The WGA-purified insulin-stimulated receptor was phosphorylated for 1 min in the absence (*A*) or presence of α-PY (*B*). The phosphorylated receptor was immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. The tryptic phosphopeptides were separated by reverse-phase HPLC. Equal portions of pY4 and pY4* were mixed, and equal portions of pY5 and pY5* were mixed. The mixtures were separated by reverse-phase HPLC (*C*) and by high voltage thin-layer electrophoresis (*D*). For the electrophoresis, the origin (*ORG*−), anode (+), and the cathode (−) are shown.

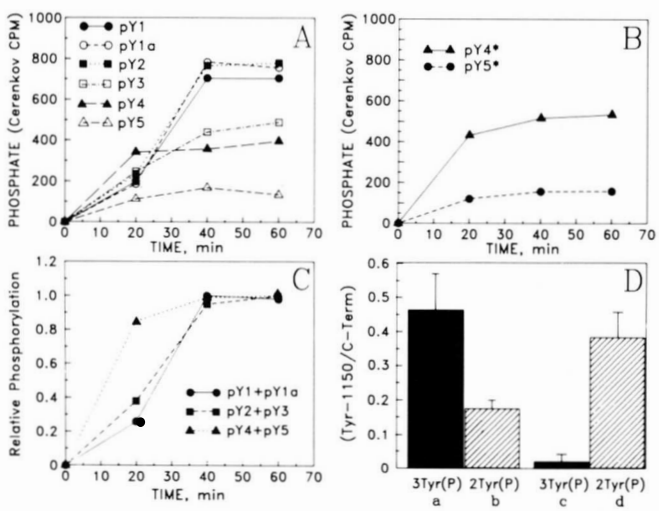

FIG. 4. **The time course of insulin receptor autophosphorylation at the various sites in the β-subunit.** The WGA-purified insulin-stimulated receptor was phosphorylated for 20, 40, or 60 s in the absence (*panel A*) or presence of 40 μg/ml α-PY (*panel B*). The phosphorylated receptor was immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. The tryptic phosphopeptides were separated by reverse-phase HPLC, and the radioactivity in each peak was measured and plotted as a function of time. *Panel C*, the phosphate in pY4 and pY5 from *panel A* was totaled for each time interval (▲), and the phosphate in pY1 and pY1a (●), and pY2 and pY3 (■) was also totaled. The phosphorylation of each set of peaks relative to the maximum for that set was calculated and plotted in this figure. *Panel D*, the insulin receptor was stimulated with insulin and incubated with [γ-^{32}P]ATP for 10 min in the absence (*a* and *b*) or presence (*c* and *d*) of the α-PY. Phosphorylation sites were identified by separation of the tryptic phosphopeptides as described above. This chart shows the amount of Tyr-1150 domain in the 3Tyr (P)-form (*a* and *c*) and the 2Tyr (P)-form (*b* and *d*) relative to the amount of the C-terminal (*C-Term*) domain obtained in the absence of the γ-PY. The values for the ratio Tyr-1150/C-terminal (*C-Term*) are calculated as follows: C-terminal = (pY2 + pY3)/2); Tyr-1150 for 3Tyr(P) = (pY1 + pY1a)/3; Tyr-1150 for 2Tyr(P) = (pY4 + pY5)/2. The results are an average ± S.D. for four determinations.

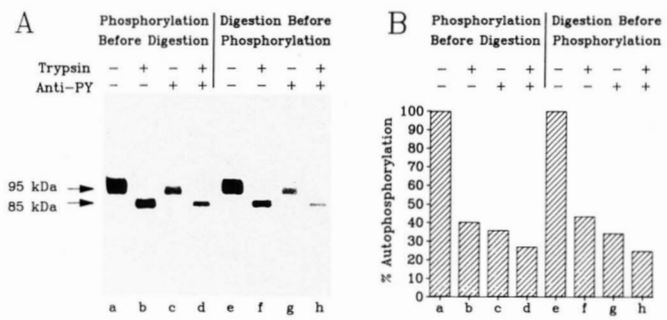

FIG. 5. **The effect of anti-phosphotyrosine antibodies on the phosphorylation of the intact insulin receptor and the 85-kDa tryptic fragment.** WGA-purified insulin receptor was stimulated with insulin (100 nM) for 20 min and labeled with 25 μM [γ-^{32}P]ATP for 10 min in the absence (*a, b, e,* and *f*) or presence (*c, d, g,* and *h*) of 40 μg/ml anti-phosphotyrosine antibody (*anti-PY*). The insulin receptor was digested briefly with trypsin after phosphorylation (*b* and *d*) or before phosphorylation (*f* and *h*). The phosphorylated receptor was immunoprecipitated, reduced with DTT, and separated by SDS-PAGE. *Panel A* shows the autoradiogram and *panel B* shows the relative amount of radioactivity in the β-subunit or the 85-kDa fragment relative to the intact and inhibited β-subunit.

pY1 and pY1a. The loss of phosphate in pY1 and pY1a during α-PY inhibition was partially compensated by an increase in the phosphorylation of pY4 and pY5 (data not shown).

The exact location of the tyrosine residues undergoing phosphorylation in the β-subunit of the insulin receptor was deduced by automated Edman degradation of each labeled peptide in a Beckman liquid-phase sequenator (31). The radioactivity released at each cycle of degradation was measured, and the peak elution positions were compared to the expected position for tyrosine residues in the tryptic peptides of the amino acid sequence of the normal human insulin receptor (35, 36). Comparisons between the rat and human insulin receptor are justified for two reasons. First, the tryptic peptide map of the human insulin receptor is identical to the peptide map of the receptor from the Fao cells (data not shown). Second, the amino acid sequences of the intracellular domain of the human and the rat insulin receptor show nearly 94% positional identity, and the relative positions of the

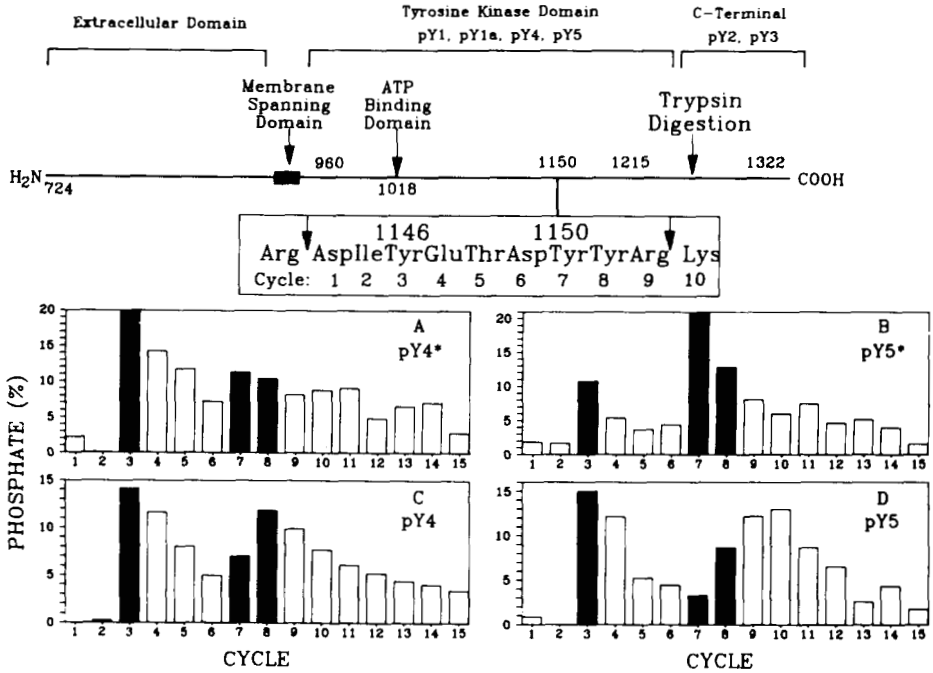

FIG. 6. **Radiosequenation of tryptic phosphopeptides from the β-subunit.** The primary structure of the β-subunit is represented as a *line*. The *solid box* indicates the position of the presumed transmembrane spanning region and the lysine residue at position 1018 is involved in ATP binding (see Refs. 15 and 21). The amino acid sequence in the *box* illustrates the likely sites of tyrosine phosphorylation identified by Edman degradation. The WGA-purified insulin-stimulated receptor was phosphorylated for 10 min in the presence (*panels A* and *B*) or absence (*panels C* and *D*) of α-PY. The phosphorylated receptor was immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. The tryptic phosphopeptides were separated by reverse-phase HPLC and subjected to automated Edman degradation. Based on the amino acid sequence in the *box*, the expected cycles for phosphotyrosine recovery during Edman degradation are *shaded*.

tryptic cleavage sites and the putative phosphorylation sites are identical.[7]

For both pY4 and pY5, radioactivity was first detected at cycle 3 (Fig. 6, *C* and *D*). Since pY4 and pY5 were not located in the C-terminal domain, Tyr-1146 is the only residue in the intracellular portion of the β-subunit that could be found in a tryptic phosphopeptide at cycle 3 (Fig. 6 and Ref. 35). Further Edman degradation of pY4 and pY5 yielded a second domain of radioactivity at cycle 7 and/or 8 (Fig. 6, *C* and *D*). This domain corresponds to phosphorylation of Tyr-1150 and/or Tyr-1151. Similar results were obtained during sequenation of pY4* and pY5* which occurred during inhibition of autophosphorylation with α-PY (Fig. 6, *A* and *B*). Thus, the first autophosphorylation sites in the β-subunit of the insulin receptor occurred in the Tyr-1150 domain at tyrosine residue 1146, and tyrosine residues 1150 and 1151.

The number of phosphotyrosine residues in the Tyr-1150 domain was determined by cleavage of pY4 and pY5 at glutamate residues with *S. aureus* V8 protease (30). Digestion at pH 8 is expected to separate Tyr-1146 from the pair of tyrosyl residues at 1150 and 1151 by cleavage after the glutamyl residue at position 1147 (Fig. 6 and Ref. 35). Both pY4 and pY5 were converted into two fragments. One of the fragments of pY4 and pY5 ($pY4^0$ and $pY5^0$, respectively) was neutral at pH < 1 since it remained during high voltage electrophoresis near the origin. The other fragments ($pY4^+$ and $pY5^+$, respectively) were positively charged as they migrated toward the negatively charged electrode. Each pair of fragments ($pY4^0$/$pY4^+$ and $pY5^0$/$pY5^+$) contained approximately equal amounts of [^{32}P]phosphate (Figs. 7*A* and 9*D*). Since phosphorylation of two tyrosine residues is expected to yield a phosphate ratio of 1:1, these results suggest that only 2 of the 3 tyrosine residues were phosphorylated, always Tyr-1146 and either Tyr-1150 or Tyr-1151. We conclude that pY4 and pY5 were derived from the 2Tyr(P) form of the Tyr-1150 domain.

Although pY4 and pY5 are apparently derived from the same receptor domain that contains two phosphotyrosine residues, they migrated distinctly on HPLC and thin-layer electrophoresis. This may be due to the formation of two tryptic peptides from the β-subunit by digestion after Arg-1143 and after either Arg-1152 or Lys-1153 (Fig. 6). This is indicated since pY5 was more positively charged (*i.e.* migrated faster toward the negatively charged cathode at pH < 1) than pY4 (Fig. 7*B*). These characteristics are consistent with the following peptide structures at pH < 1: pY4, $(Asp^{(+)}$-Ile-Tyr(P)$^{(-)}$-Glu-Thr-Asp-(Tyr(P)Tyr)$^{(-)}$-$Arg^+)^0$; pY5, $(Asp^{(+)}$-Ile-Tyr(P)$^{(-)}$-Glu-Thr-Asp(Tyr(P)Tyr)$^{(-)}$-Arg^+-$Lys^{(+)})^+$. Furthermore, the V8-protease fragments of pY4 and pY5, designated $pY4^0$ and $pY5^0$, were neutral at pH < 1 suggesting that they were the same peptide, $(Asp^{(+)}$-Ile-Tyr(P)$^{(-)}$-$Glu)^0$, which contains Tyr-1146 and is neutral at pH < 1. In contrast, $pY5^+$ migrated toward the cathode faster than $pY4^+$ suggesting that $pY5^+$ was the more positively charged fragment, $(Thr^{(+)}$-Asp-(Tyr(P)Tyr)$^{(-)}$-$Arg^{(+)}$-$Lys^{(+)})^{2+}$, whereas $pY4^+$ was $(Thr^{(+)}$-Asp-(Tyr(P)Tyr)$^{(-)}$-$Arg^{(+)})^+$ which lacks the C-terminal lysine residue. The detection of pY4 and pY5 in the tryptic digests of the β-subunit was variable between experiments, and in extreme cases only pY4 or only pY5 was detected, whereas in others, both peptides were equally observed. Since each tryptic peptide contained 2 phosphotyrosyl residues, this variability is attributed to variable digestion of the β-subunit by trypsin.

The migration of the phosphorylated form of the synthetic peptide Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (Asp-10-Lys) which is composed of the predicted sequence of pY5, was compared by electrophoresis to the migration of pY5 and pY4. The insulin receptor phosphorylated Asp-10-Lys on a single residue which corresponded to Tyr-1150 (data not shown) as previously described (9). Consistent with the presence of 1 phosphotyrosine residue, Asp-10-Lys migrated during electrophoresis more rapidly toward the cathode than either pY4 or pY5 (Fig. 7*B*, *lanes c–e*); however, a small component of Asp-10-Lys co-migrated with pY5 suggesting that some of it contained 2 phosphotyrosine residues. These data suggest that phosphorylation of 2 tyrosine residues is preferred in the intact receptor, whereas a single phosphorylation predominated in the synthetic peptide.

Identification of the Phosphorylated Domain That Correlates with the Activation of the Insulin Receptor Kinase—Since the 2Tyr(P) form of the Tyr-1150 domain did not activate the

[7] R. Lewis, M. Tepper, and M. P. Czech, submitted for publication.

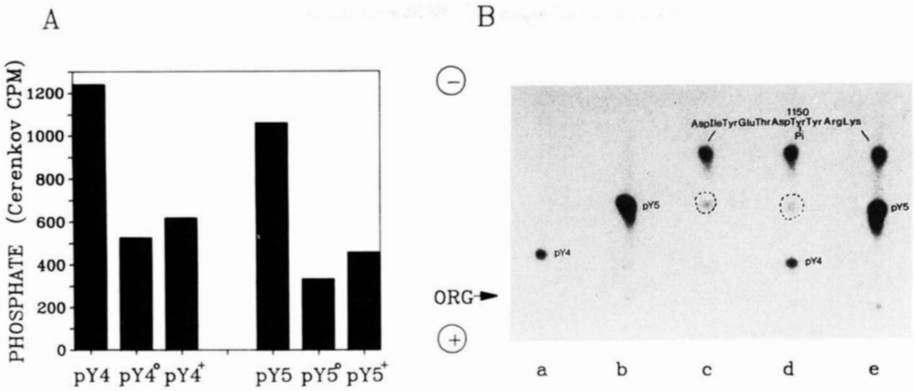

FIG. 7. **Characterization of pY4 and pY5.** The tryptic phosphopeptides were separated by reverse-phase HPLC, and pY4 and pY5 were digested with V8 protease and separated by high voltage thin-layer electrophoresis (*panel A*). Two unique fragments were obtained from pY4 and pY5; the fragments $pY4^0$ and $pY5^0$ were nearly neutral as they barely migrated from the origin (*ORG*) toward the negatively charged electrode. The fragments designated $pY4^+$ and $pY5^+$ were positively charged as both migrated toward the negatively charged electrode (cathode). The $pY5^+$ migrated further than $pY4^+$ indicating that $pY5^+$ was more positively charged than $pY4^+$. The radioactivity in pY4 and pY5 and in the V8 protease fragments obtained from an equal amount of pY4 and pY5 was measured by Cerenkov counting. *Panel B* shows a high voltage electrophoresis separation of pY4 (*lane a*) and pY5 (*lane b*) relative to the migration of the phosphorylated synthetic peptide, Asp-Ile-Tyr-Glu-Thr-Asp-Tyr(P)-Tyr-Arg-Lys, alone (*lane c*), or mixed with pY4 (*lane d*) or pY5 (*lane e*). The synthetic peptide was phosphorylated as described for the kinase assay.

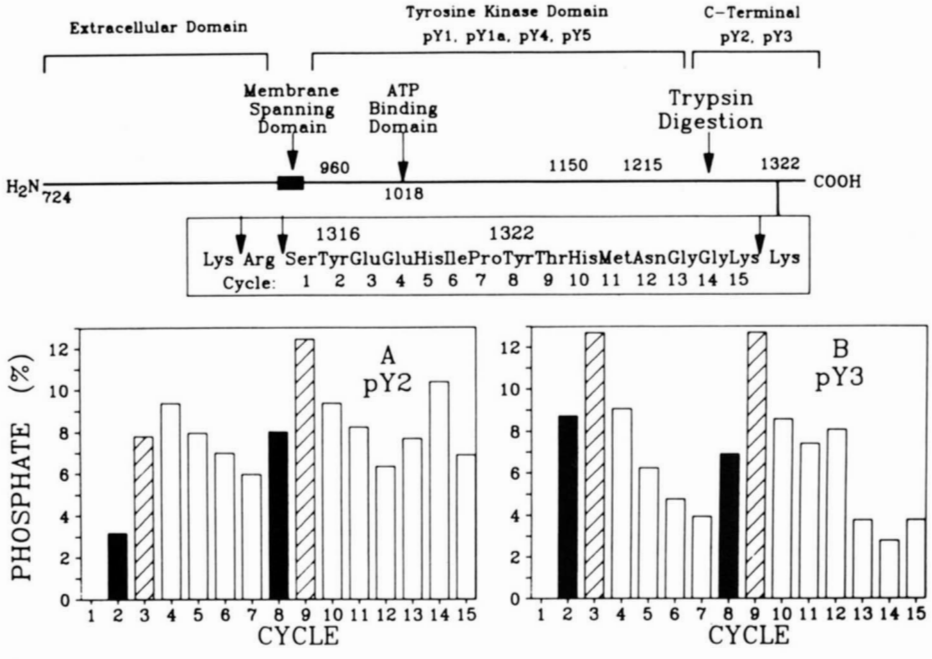

FIG. 8. **Radiosequenation of tryptic phosphopeptides pY2 and pY3.** The tryptic phosphopeptides were analyzed as described in the legend to Fig. 6. The amino acid sequence in the *box* illustrates the likely sites of tyrosine phosphorylation identified by Edman degradation. The *solid bars* indicate the predicted cycle for the appearance of Tyr(P)-1316 if tryptic cleavage occurred after Arg-1314, whereas the *striped bars* indicate the predicted cycle for Tyr(P) if cleavage occurred after Lys-1313.

phosphotransferase of the β-subunit (Fig. 2*C*), we have investigated the regulatory role of the subsequent phosphorylation events that occurred at pY2 and pY3 (C-terminal), and at pY1 and pY1a. Edman degradation of pY2 and pY3 indicated that two domains of radioactivity existed in each peptide (Fig. 8, *A* and *B*). The first domain began at cycles 2 and 3, and the second domain began at cycles 8 and 9. From the predicted amino acid sequence of the insulin receptor (Fig. 8 and Ref. 35) and the fact that pY2 and pY3 are located in the C-terminal domain (26), these peptides probably arise from tryptic cleavage of the Arg-Lys residues at positions 1313 and 1314 and the Lys-Lys residues at positions 1329 and 1330. High voltage electrophoresis indicated that both pY2 and pY3 contained a mixture of two unique peptides (data not shown) which is consistent with the formation of four distinct peptides by variable tryptic cleavage in this domain. Thus, depending on the site of cleavage at the N terminus, radioactivity is expected to be found at cycles 2 and 8 (Fig. 8, *solid bars*) or cycles 3 and 9 (Fig. 8, *striped bars*). These data and our previous report (26) are consistent with phosphorylation at Tyr-1316 or Tyr-1322, or both. Further analysis of these peptides to determine definitively the sites of tryptic cleavage in each peptide was not carried out.

Removal of the C-terminal phosphorylation sites by mild trypsinolysis of the 95-kDa β-subunit to form the 85-kDa fragment did not alter the insulin-stimulated tyrosine kinase activity (26). In the presence of α-PY, autophosphorylation of the 85-kDa fragment was restricted to the 2Tyr(P) form of the Tyr-1150 domain during incubation with insulin and [γ-^{32}P]ATP. After elution of the partially phosphorylated 85-kDa fragment from the α-PY, the kinase was not fully activated during assays with saturating concentrations of Thr-12-Lys; however, it was fully active at low concentrations of Thr-12-Lys which did not inhibit further autophosphoryla-

tion. Following complete autophosphorylation of the 85-kDa fragment in the absence of the α-PY, the phosphotransferase was fully activated during assay at saturating concentrations of Thr-12-Lys. The kinetic curves for the truncated receptor were superimposable with those shown for the intact receptor in Fig. 2*A*. We conclude that autophosphorylation of the β-subunit at Tyr-1316 and/or Tyr-1322 in the C-terminal domain was not required to activate the phosphotransferase of the insulin receptor *in vitro*.

Since autophosphorylation of pY4 and pY5, and pY2 and pY3 did not activate the receptor, our data suggest that autophosphorylation of tyrosine residues in the tryptic peptides called pY1 and pY1a activated the receptor. They were not located in the C-terminal domain (26), although their phosphorylation was inhibited by the α-PY (Fig. 3). The pY1 and pY1a, as well as pY4, were precipitated specifically with αPep-3, our antibody raised against the Tyr-1150 domain (Fig. 9*A*). In contrast, pY2 and pY3 were not precipitated by αPep-3 but were precipitated by αPep-1 which reacts with the C-terminal domain of the β-subunit. Furthermore, antibodies that react with the receptor domain containing tyrosine residues 953 and 960 (αPep-4) or an α-subunit domain (αPep-7) did not react with any of these phosphopeptides, whereas the α-PY reacted with all of them. Thus, the pY1 and pY1a were derived from the Tyr-1150 domain which contains tyrosine residues 1146, 1150, and 1151.

We carried out several experiments to determine the stoichiometry of phosphorylation in pY1 and pY1a. Edman degradation of pY1a indicated that phosphate existed at cycle 3 which corresponds to Tyr-1146 (Fig. 9*B*); however, because of inefficient elution intrinsic to the methods, further Edman degradation did not clearly identify a second domain of radioactivity in pY1a, and similar analysis of pY1 was unsuccessful. The pY1a was negatively charged at pH < 1 as it migrated toward the positively charged electrode during high voltage electrophoresis; pY1 remained at the origin suggesting that it was nearly neutral (Fig. 9*D*). During anion exchange chromatography, pY1a was more strongly retained than pY1 which confirmed this result (Fig. 9*C*). These characteristics are consistent with the following related structures for pY1 and pY1a at pH < 1: pY1, $(Asp^{(+)}\text{-}Ile\text{-}Tyr(P)^{(-)}\text{-}Glu\text{-}Thr\text{-}Asp\text{-}Tyr(P)^{(-)}\text{-}Tyr(P)^{(-)}\text{-}Arg^{(+)}\text{-}Lys^{(+)})^0$; pY1a, $(Asp^{(+)}\text{-}Ile\text{-}Tyr(P)^{(-)}\text{-}Glu\text{-}Thr\text{-}Asp\text{-}Tyr(P)^{(-)}\text{-}Tyr(P)^{(-)}\text{-}Arg^{(+)})^-$. To test the validity of these assignments, we digested pY1 and pY1a with V8 protease and separated the products by high voltage electrophoresis. The digestion of pY1 yielded a neutral peptide at pH < 1 called $pY1^0$ ($(Asp^{(+)}\text{-}Ile\text{-}Tyr(P)^{(-)}\text{-}Glu)^0$) and a positively charged peptide $pY1^+$ ($(Thr^{(+)}\text{-}Asp\text{-}Tyr(P)^{(-)}\text{-}Tyr(P)^{(-)}\text{-}Arg^{(+)}\text{-}Lys^{(+)})^+$) with a [^{32}P]phosphate ratio of 1 to 2, respectively (Fig. 9*D*). Furthermore, the [^{32}P]phosphate ratios of pY1 to $pY1^0$ and pY1 to $pY1^+$ were 3 to 1 and 3 to 2, respectively (Fig. 9*D*, *lanes a* and *b*). These results indicated that pY1 was derived from the 3Tyr(P) form of the Tyr-1150 domain. Digestion of pY1a with V8-protease yielded one major neutral peptide[8] which was expected for the two uncharged products of pY1a: $(Asp^{(+)}\text{-}Ile\text{-}Tyr(P)^{(-)}\text{-}Glu)^0$ and $(Thr^{(+)}\text{-}Asp\text{-}Tyr(P)^{(-)}\text{-}Tyr(P)^{(-)}\text{-}Arg^{(+)})^0$ (Fig. 9*D*, *lanes c* and *d*). For comparison, pY4 yielded on V8 digestion a neutral peptide, $pY4^0$ (which is equivalent to $pY1^0$ and $pY1a^0$), and a positively charged peptide, $pY4^+$, described above; both of these fragments had equal levels of phosphorylation (Fig. 9*D*, *lanes e* and *f*).

The effect of the α-PY on the relative amount of the

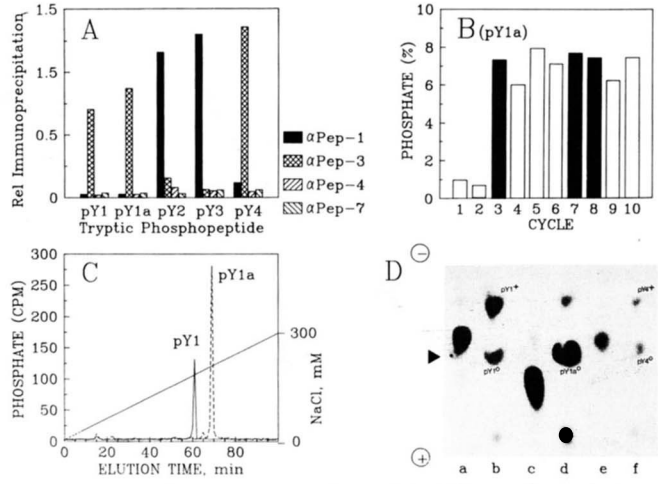

FIG. 9. **Specific immunoprecipitation, Edman degradation, ion exchange chromatography, and V8 digestion of pY1 and pY1a.** *Panel A*, the insulin receptor was phosphorylated for 1 min and then immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. Tryptic phosphopeptides were separated by reverse phase HPLC and immunoprecipitated with the indicated domain-specific antibody as described under "Experimental Procedures." The amount of radioactivity precipitated is expressed relative to that recovered by an identical immunoprecipitation with the α-PY. *Panel B*, the pY1a was prepared as described above and subjected to automated Edman degradation. Based on the amino acid sequence shown in Fig. 6, the expected cycles for phosphotyrosine recovery during Edman degradation are shown by *solid bars*. *Panel C*, separation of pY1 and pY1a by HPLC ion exchange chromatography was carried out as described under "Experimental Procedures." *Panel D*, high voltage electrophoretic separation of pY1 (*lanes a* and *b*) and pY1a (*lanes c* and *d*) and pY4 (*lanes e* and *f*), and the related V8 protease fragments. The phosphopeptides were separated before (*lanes a, c,* and *e*) or after V8 protease digestion (*lanes b, d,* and *f*). Equal amounts of radioactivity were applied in *lanes a* and *b* (1057 cpm each) and in *lanes c* and *d* (1734 cpm each); in a separate experiment shown here for comparison, unequal amounts of radioactivity were applied in *lanes e* (300 cpm) and *f* (200 cpm). The origin is indicated with an *arrow*, and the anode (+) and the cathode (−) are shown. The intensities of pY1, $pY1^0$, and $pY1^+$ were measured by two separate scans: the pY1 and $pY1^0$ were scanned together and yielded relative values of 22,394 and 7,024, respectively; the pY1 and $pY1^+$ were scanned together and yielded values of 26,084 and 16,841, respectively. The following ratios were calculated: $pY1/pY1^0 = 3.2{:}1$; $pY1/pY1^+ = 3.1{:}2$; $pY1^+/pY1^0 = 2.1{:}1$. The intensities of pY4, $pY4^0$, and $pY4^+$ were measured by two separate scans: the pY4 and $pY4^0$ were scanned together and yielded relative values of 6,989 and 2,474; the pY4 and $pY4^+$ were scanned together and yielded values of 6,634 and 2,177, respectively. After correcting for the amount of radioactivity applied in each lane, the following ratios were calculated: $pY4/pY4^0 = 1.9{:}1$; $pY4/pY4^+ = 2.0{:}1$; $pY4^+/pY4^0 = 0.95{:}1$.

2Tyr(P) and 3Tyr(P) forms of the Tyr-1150 domain generated *in vitro* was measured in four separate experiments. In the absence of the α-PY, the 3Tyr(P) form predominated over the 2Tyr(P) form by more than 2 to 1 (Fig. 4*D*, *bars a* and *b*). In contrast, the 2Tyr(P) form predominated during α-PY inhibition by more than 10-fold (Fig. 4*D*, *bars c* and *d*). Furthermore, the relative amount of the 2Tyr(P) form was reduced by 50% in the absence of the α-PY. These results support the notion that the α-PY blocked the autophosphorylation cascade in the Tyr-1150 domain after phosphorylation of 2 out of the 3 tyrosyl residues.

In conclusion, since the kinetic curves for phosphorylation of Thr-12-Lys were not biphasic under conditions in which pY1 and pY1a were detected in the digestion of the β-subunit, we suggest that the 3Tyr(P) form of the Tyr-1150 domain activated fully the phosphotransferase of insulin receptor.

[8] Other smaller peptides (<10% of the total) were also observed in this lane which may be dephosphorylated forms generated during the digestions.

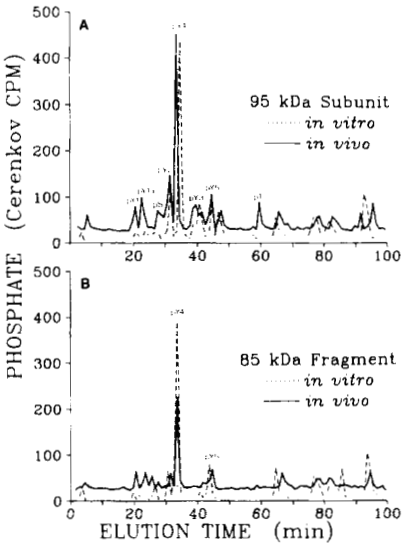

FIG. 10. **Comparison of the phosphorylation sites of the β-subunit that occur *in vivo* and *in vitro* during inhibition with α-PY.** The phosphorylated insulin receptor was obtained from insulin-stimulated (1 min, 100 nM) [^{32}P]orthophosphate-labeled Fao cells by purification on immobilized WGA-agarose (——). Before immunoprecipitation and SDS-PAGE, half of the eluate was digested with trypsin (5 μg/ml) for 1 min to form the 85-kDa fragment. The tryptic phosphopeptides were obtained by exhaustive trypsin digestion of the intact β-subunit (*panel A*) and the 85-kDa fragment (*panel B*), and separated by reverse-phase HPLC. The receptor was also labeled after WGA purification from unstimulated and unlabeled Fao cells during incubation with [^{32}P]ATP in the presence of insulin and the α-PY (– – –). Before immunoprecipitation and SDS-PAGE, half of the reaction was digested with trypsin (5 μg/ml) for 1 min to form the 85-kDa fragment. Then the β-subunit (*panel A*) and the 85-kDa fragment (*panel B*) were digested exhaustively with trypsin and separated by HPLC (*panel A*).

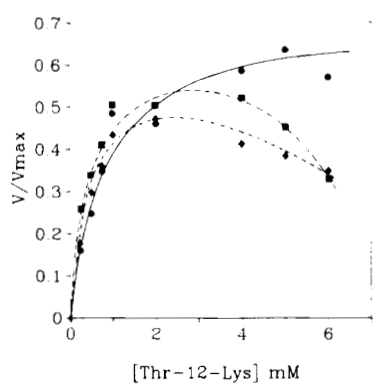

FIG. 11. **The kinase activity of the insulin-stimulated insulin receptor autophosphorylated *in vitro* or *in vivo*.** The WGA-purified insulin receptor was incubated with 100 nM insulin for 20 min and then phosphorylated with unlabeled ATP in the absence (●) or presence of α-PY (♦). The insulin receptor was also purified from insulin-stimulated (100 nM) Fao cells by WGA affinity chromatography (■). In each case, the phosphorylated receptors were immunopurified on immobilized α-PY and eluted with 20 mM phosphotyramine, 5 mM $MnCl_2$, and 100 nM insulin. The kinase activity of these phosphorylated receptors was measured during a 10-min incubation with the indicated concentrations of Thr-12-Lys and 25 μM [γ-^{32}P] ATP. The ratio of v/V_{max} is plotted against the substrate concentration. The values of V_{max}, K_m, and K_i for each receptor preparation were determined by fitting the data with program SUBIN to the equation for substrate inhibition (33); *in vivo* insulin stimulation (■): V_{max} = 0.5 ± 0.1 pmol/min, K_m = 0.9 ± 0.4 mM, K_i = 4 ± 1 mM; *in vitro* phosphorylation without α-PY (●): V_{max} = 0.2 ± 0.1 pmol/min, K_m = 1.2 ± 0.2 mM; *in vitro* phosphorylation with α-PY (♦): V_{max} = 0.33 ± 0.01 pmol/min, K_m = 1.1 ± 0.1 mM, K_i = 3.7 ± 0.4 mM.

Comparison of the Tryptic Phosphopeptides and the Activity of the Receptor Obtained in Vivo and in Vitro—The β-subunit of the insulin receptor in the intact Fao cell is phosphorylated on serine and threonine residues before insulin stimulation (1, 37). After insulin stimulation of the cells for 1 min, phosphorylation of the β-subunit increases 5-fold mainly on tyrosine residues (34). The tryptic peptide map of the insulin receptor phosphorylated *in vivo* is distinct from that phosphorylated *in vitro* (1). This is due partly to the presence of phosphoserine and phosphothreonine in the β-subunit labeled *in vivo*, but arises primarily from differences in the intensity of the tyrosine phosphorylation sites which occur *in vivo* (1). Since the yield of radioactivity obtained during *in vivo* labeling is low, we could not sequence the tryptic phosphopeptides directly but tentatively identified these sites by comparing the migration of the phosphopeptides on HPLC obtained after *in vivo* and *in vitro* autophosphorylation.

Insulin stimulated the phosphorylation of tyrosine residues in a major phosphotyrosine-containing tryptic peptide which migrated at the same position as pY4 (pY4*) obtained during *in vitro* autophosphorylation in the presence of α-PY (Fig. 10*A*). The 85-kDa fragment obtained by mild tryptic digestion of the β-subunit phosphorylated *in vivo* still contained pY4 (Fig. 10*B*). In some *in vivo* experiments, pY5 was also observed as a major peptide, but not here, and as noted above this is probably dependent on the exact site of tryptic cleavage. Thus, during insulin stimulation of the receptor *in vivo*, the β-subunit contained primarily the 2Tyr(P) form of the Tyr-1150 domain.

The pY1, pY1a, pY2, and pY3 were minor peptides in the β-subunit labeled *in vivo* as determined by comparison of their migration to the corresponding peptides found *in vitro*. This was true even though care was taken to avoid dephosphorylation during purification as previously described (1, 38). Furthermore, the pY2 and pY3 were removed from the β-subunit by mild trypsin digestion as expected for these C-terminal phosphorylation sites (Fig. 10*B*) (26). Both pY1 and pY1a were resistant to removal by mild trypsin digestion; however, their intensity of labeling was much less than that seen in the purified receptor. Thus, the 3Tyr(P) form of the Tyr-1150 domain did not accumulate during insulin stimulation *in vivo*.

The near absence of the 3Tyr(P) form of the Tyr-1150 domain suggested that most of the insulin receptors may not be activated in the intact Fao cell during insulin stimulation. To test this prediction, the activity of the insulin receptor purified from insulin-stimulated cells was compared to the activity of the insulin-stimulated receptor phosphorylated *in vitro* with ATP in the absence or presence of α-PY. Before the addition of Thr-12-Lys, these three preparations of receptors were immunopurified with α-PY and eluted with 20 mM phosphotyramine. The receptor phosphorylated *in vitro* in the absence of α-PY was fully activated as the kinetic curve for phosphorylation of Thr-12-Lys was not biphasic (Fig. 11). However, the kinetic curve was biphasic when the receptor used in the assay was partially phosphorylated *in vitro* in the presence of α-PY before the addition of substrate. Similarly, when [γ-^{32}P]ATP and Thr-12-Lys were added together to the receptor purified from insulin-stimulated cells, the kinetic curve for Thr-12-Lys phosphorylation was biphasic (Fig. 11). In each case, the half-maximal inhibition of activation occurred at 4 mM peptide. These results suggest that the receptor phosphorylated *in vivo* was not fully activated during *in vitro* assays because the β-subunit contained mainly the 2Tyr(P) form of the Tyr-1150 domain. Interestingly, it could be activated at nonsaturating substrate concentrations which allowed complete autophosphorylation of the Tyr-1150 domain

to occur during the assay (Fig. 11). We do not know how the Tyr-1150 domain is inhibited from progressing to the 3Tyr(P) form *in vivo*.

DISCUSSION

There is increasing evidence that transmission of the insulin signal inside cells is mediated by tyrosine phosphorylation of the insulin receptor kinase itself (14–16) or its intracellular substrates (3, 17, 18, 20). Insulin-stimulated autophosphorylation of the receptor activates the phosphotransferase in the β-subunit so that substrates are phosphorylated more rapidly (2, 8, 10, 11, 13, 39–41). Many tyrosine residues undergo phosphorylation during insulin stimulation (4), but the exact events that activate the β-subunit have not been resolved. In this report, we identified some of the tyrosine residues in the β-subunit of the insulin receptor that undergo tyrosine phosphorylation during insulin binding and correlated their phosphorylation to the catalytic activity of the receptor kinase.

In vitro, α-PY inhibits insulin-stimulated autophosphorylation of the β-subunit by 70–80% and traps one of the first phosphotyrosine intermediates. Since the α-PY does not bind to the insulin receptor purified from unstimulated cells (3, 12, 18), the α-PY probably binds to newly phosphorylated tyrosine residues which occur during the incubation with insulin and ATP. Tryptic peptide mapping indicates that the autophosphorylation sites in peptides pY4 and pY5 are not inhibited by the α-PY and actually increase, whereas all of the other phosphorylation sites in peptides pY1, pY1a, pY2, and pY3 are completely inhibited. Structural analysis indicates that pY4 and pY5 are derived from a single region of the receptor containing Tyr-1146, Tyr-1150, and Tyr-1151, called the Tyr-1150 domain. In the presence of the α-PY, this domain is phosphorylated at 2 of the 3 tyrosine residues, always Tyr-1146 and either Tyr-1150 or Tyr-1151. We call this the 2Tyr(P) form of the Tyr-1150 domain. Since we never observed a monophosphotyrosyl form of this domain, two tyrosine residues apparently undergo autophosphorylation before the α-PY binds to the domain and stops the cascade. Possibly, formation of the 2Tyr(P) form induces a conformational change in the β-subunit which allows immediate binding of the α-PY.

A major domain of β-subunit autophosphorylation *in vitro* is in the tryptic peptides designated pY1 and pY1a (1, 26). Their phosphorylation was inhibited by the α-PY which suggest that they are generated after pY4 and pY5. Immunological and structural analysis suggests that pY1 and pY1a are derived from the Tyr-1150 domain and contain 3 phosphotyrosyl residues at positions 1146, 1150, and 1151. This is called the 3Tyr(P) form of the Tyr-1150 domain. When the formation of the pY1 and pY1a is inhibited by the α-PY, the level of pY4 and pY5 increases suggesting that the 2Tyr(P) form of the Tyr-1150 domain is the immediate precursor of the 3Tyr(P) form.

Phosphorylation of Tyr-1150 or -1151 was previously predicted because of a close homology of the surrounding amino acid sequence to the major autophosphorylation site, Tyr-416, in $pp60^{v\text{-}src}$ (5, 42). Antibodies directed against the amino acid sequence surrounding Tyr-1150 of the insulin receptor immunoprecipitated phosphorylated peptides from a CNBr digest of the β-subunit (8). Replacement by oligonucleotide-directed mutagenesis of Tyr-1150 and Tyr-1151 with phenylalanine decreased the phosphorylation of the β-subunit *in vivo*, suggesting that they play a role in receptor function (14). However, several reports suggest that phosphorylation of the tyrosine residues in the corresponding domain of other tyrosine kinases does not always occur. For example, Tyr-416 is not the major site of autophosphorylation in $pp60^{c\text{-}src}$ even though the sequence around Tyr-416 is identical to that in $pp60^{v\text{-}src}$ (43, 44). Although the epidermal growth factor receptor contains Tyr-845 in a homologous domain (5, 45), its phosphorylation has not been reported (46, 47). In contrast, the preferred phosphorylation site in both $pp60^{c\text{-}src}$ (43) and the EGF receptor (47) is near the C-terminal domain, Tyr-527 and Tyr-1173, respectively.

The purified insulin receptor also undergoes tyrosine autophosphorylation in the C-terminal domain on Tyr-1316 and Tyr-1322. These sites are phosphorylated after the 2Tyr(P) form of the Tyr-1150 domain and coincide with the appearance of the 3Tyr(P) form. Phosphorylation of one or both of these C-terminal tyrosine residues was previously predicted from the use of specific antibodies which recognize this domain (8) and from proteolytic mapping of the β-subunit (26). Removal of the C-terminal phosphorylation sites by mild trypsin digestion had no detectable effect on the phosphorylation of the remaining residues in the truncated (85 kDa) β-subunit (26). The autophosphorylation cascade in the Tyr-1150 domain of the 85-kDa β-subunit fragment is stopped at the 2Tyr(P) form during incubation with the α-PY suggesting that the C terminus does not play a major role in the function of the Tyr-1150 domain.

The major insulin-stimulated tyrosine autophosphorylation sites *in vivo* are the 2Tyr(P) forms of the Tyr-1150 domain. The 3Tyr(P) form is barely detected *in vivo*, confirming our previous observations (1, 3). Stadtmauer and Rosen (9) suggested that autophosphorylation in this domain occurs during insulin stimulation of IM-9 lymphocytes, but the exact tyrosine residues were not deduced. In the Fao cells, autophosphorylation also occurs at the C-terminal sites, but the level of phosphorylation is considerably less than that observed *in vitro*. The cause of the differences between *in vivo* and *in vitro* phosphorylation, especially the near absence of the 3Tyr(P) form, is unknown. The *in vitro* phosphorylation conditions which employ high concentrations of Mn^{2+} to compensate for a low ATP concentration are very different from the whole cell and may alter the pattern of phosphorylation (4). Endogenous phosphotyrosine phosphatases may be acting on the receptor in the cell but are separated from the receptor during purification. Possibly, substrates exist in the cell at concentrations above their K_m which inhibit autophosphorylation as described for Thr-12-Lys during *in vitro* experiments. Other mechanisms involving specific inhibitors, steric hindrance, or serine/threonine phosphorylation may also play a role (48).

The kinase activity of the purified insulin receptor increases during insulin stimulation due to autophosphorylation of the β-subunit. This has been shown with various substrates including histone, poly(Glu:Tyr), and various tyrosine-containing synthetic peptides. Removal of insulin from the phosphorylated receptor does not inactivate it, as long as the phosphorylation state is retained (10). Dephosphorylation of the β-subunit with alkaline phosphatase reduces the kinase activity of the receptor (11), and inhibition of insulin-stimulated autophosphorylation by high concentrations of substrate blocks activation of the tyrosine kinase (13, 39). Yu and Czech (11) suggested that phosphorylation of tyrosine residues in a single domain is responsible for activation, and Herrera and Rosen (8) suggested that phosphorylation of tyrosine residues in the Tyr-1150 domain correlated best with the rate of receptor catalyzed histone phosphorylation. In contrast, Kwok *et al.* (13) suggested that most or all of the autophosphorylation of the β-subunit is functionally related to autoactivation. Thus, autophosphorylation appears to be required to

activate the tyrosine kinase in the β-subunit, but the exact events involved remained obscure.

Our approach to determine the phosphorylation sites involved in activation of the phosphotransferase has been to use peptide substrates and α-PY as inhibitors of autophosphorylation. We find that high concentrations of Thr-12-Lys, like histone and poly(Glu:Tyr) reported previously (13, 39), inhibit insulin-stimulated tyrosine autophosphorylation and prevent the activation of the insulin-stimulated kinase. However, insulin-stimulated kinase activity is not blocked by concentrations of substrate below K_m, since nonsaturating substrate does not inhibit autophosphorylation. Consistent with these results, when autophosphorylation is carried out to completion before addition of saturating ($>K_m$) substrate levels, no inhibition of kinase activity is seen at any substrate concentrations. Thus, the rate of substrate phosphorylation measured at concentrations which inhibit receptor autophosphorylation provides a means to estimate the activity of the insulin receptor at a fixed state of autophosphorylation reached before addition of the substrate.

Activation of the insulin receptor kinase *in vitro* best correlates with the occurrence of the 3Tyr(P) form of the Tyr-1150 domain. Our results are consistent with previous reports suggesting that autophosphorylation in this domain correlates with the activation of the receptor (8) but emphasizes the need for phosphorylation of Tyr-1146, Tyr-1150, and Tyr-1151 to activate fully the kinase. Removal of Tyr-1150 and Tyr-1151 by oligonucleotide-directed mutagenesis strongly decreases the kinase activity which is consistent with our results (14).

Most tyrosine kinases undergo autophosphorylation, and some of them are clearly activated by it (49), but evidence for activation of all kinases is not available. Clearly, the insulin receptor is in a unique class in that it contains 3 closely spaced tyrosyl residues, all of which play a role in activation. The receptor for insulin-like growth factor I is activated by autophosphorylation (50). It contains tyrosine residues in an identical domain as the insulin receptor (51), and it is likely that a cascade of tyrosine autophosphorylation which generates the 3Tyr(P) form of its Tyr-1135 domain activates its phosphotransferase. A similar argument can be made for the gene product of v-ros (52). However, activation of the epidermal growth factor receptor kinase by autophosphorylation is unclear. Bertics and Gill (53) reported that autophosphorylation increases the kinase activity of the receptor, but other reports have found no effect (47, 54). However, applying some of the strategies described in this report, Shoelson *et al.*[9] suggest that both the EGF receptor and the pp60$^{c\text{-}src}$ are activated by autophosphorylation.

The possibility that phosphorylation of other tyrosine residues is required to activate the β-subunit cannot be ruled out by our experiments. A role for the phosphorylation of Tyr-960 has been suggested since stimulation by insulin of the kinase activity was blocked by an antibody that reacts with this domain (55). However, none of the major tryptic phosphopeptides were immunoprecipitated with αPep-4, our antibody which reacts with this domain. Furthermore, substitution of Tyr-960 with phenylalanine did not affect the autophosphorylation of the β-subunit or change the tryptic peptide map suggesting that this tyrosine may not be a major autophosphorylation site.[10]

Although the C-terminal residues, Tyr-1316 and Tyr-1322, are major sites of autophosphorylation *in vitro*, they do not appear to play a role in the activation of the phosphotransferase of the insulin receptor *in vitro*. This is consistent with previous kinetic correlations (8, 26) and with similar observations for the epidermal growth factor receptor kinase (56). In contrast, the C-terminal tyrosine autophosphorylation site in pp60$^{c\text{-}src}$ (Tyr-517) appears to inhibit its tyrosine kinase activity (43). An inhibitory role for the C-terminal of the insulin receptor β-subunit may also exist. Our preliminary results indicate that a serine and threonine phosphorylation site may be located in the 10-kDa portion of the β-subunit that is removed by mild trypsin digestion. Since serine and threonine phosphorylation decreases the kinase activity of the insulin receptor in Fao cells, a regulatory role may exist for this domain (12, 48). However, the C-terminal domain of the β-subunit may play other roles required for signal transmission such as binding specific cellular proteins, a function similar to that of the C-terminal domain of pp60$^{c\text{-}src}$ which binds to the middle T antigen of the polyoma virus (57).

The insulin receptor purified from insulin-stimulated cells contains primarily 2 phosphotyrosine residues in its Tyr-1150 domain. Following purification from insulin-stimulated Fao cells, the receptor was not fully activated during *in vitro* kinase assays because saturating substrate concentrations inhibit the additional autophosphorylation required to stimulate the phosphotransferase. However, the receptor is fully activated during *in vitro* assays at low substrate concentrations as the Tyr-1150 domain of the β-subunit is able to proceed rapidly to the 3Tyr(P) form. These results are consistent with our conclusion that the 3Tyr(P) form is the active form of the kinase and that this form is present at low levels *in vivo*. It is not known whether full activation of the phosphotransferase of the β-subunit by generation of the 3Tyr(P) form of the Tyr-1150 domain is required for transmission of the insulin signal *in vivo*. Perhaps only a small percentage of this form is necessary for insulin action. Of course, the possibility that the insulin signal is not transmitted by substrate phosphorylation at all cannot be excluded. A conformational change like the one that allows the α-PY to bind to the 2Tyr(P) form of the Tyr-1150 domain may be sufficient.

The mechanism by which full autophosphorylation of the insulin receptor is blocked *in vivo* is unknown. Presumably, the regulatory components are removed from the Fao cell extract by purification on WGA-agarose. Previous reports indicate that the receptor purified from insulin-stimulated adipocytes and H35 hepatoma cells are fully activated during *in vitro* assay with histone H2b (38, 58). This inconsistency may arise from the assay conditions employed that did not prevent subsequent *in vitro* autophosphorylation; alternatively, the regulation of the autophosphorylation cascade initiated by insulin binding may vary in different cell types and under distinct physiologic conditions.

In conclusion, antiphosphotyrosine antibodies trap the first autophosphorylation sites in the β-subunit. We have used this observation to identify the sequence of autophosphorylation events that occur in the β-subunit during insulin stimulation. Autophosphorylation of the insulin receptor begins by phosphorylation of Tyr-1146 and either Tyr-1150 or Tyr-1151. *In vitro*, this 2Tyr(P) form of the Tyr-1150 domain proceeds rapidly to the 3Tyr(P) form, whereas in the intact cell, the 2Tyr(P) form is the predominant phosphorylated species. Progression to the 3Tyr(P) form appears to be necessary for full activation of the phosphotransferase during *in vitro* assays. The low level of the 3Tyr(P) form of the Tyr-1150 domain suggests that regulation of the autophosphorylation cascade may play an important role in the transmission of

[9] S. E. Shoelson, M. F. White, and C. R. Kahn, submitted for publication.

[10] M. F. White, J. N. Livingstone, J. Backer, T. Dull, A. Ullrich, and C. R. Kahn, manuscript in preparation.

the insulin signal in the intact cell.

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