Tyrosine Phosphorylation of the Insulin Receptor \( \beta \) Subunit Activates the Receptor-associated Tyrosine Kinase Activity*

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The regulation of kinase activity associated with insulin receptor by phosphorylation and dephosphorylation has been examined using partially purified receptor immobilized on insulin-agarose. The immobilized receptor preparation exhibits predominately tyrosine but also serine and threonine kinase activities toward insulin receptor \( \beta \) subunit and exogenous histone. Phosphorylation of the insulin receptor preparation with increasing concentrations of unlabeled ATP, followed by washing to remove the unreacted ATP, results in a progressive activation of the receptor kinase activity when assayed in the presence of histone and \( [\gamma^32P]ATP \). A maximal 4-fold activation is achieved by prior incubation of receptor with concentrations of ATP approaching 1 mM. High pressure liquid chromatographic analysis of tryptic hydrolysates of the \( [\gamma^32P] \)-labeled insulin receptor \( \beta \) subunit reveals three domains of phosphorylation (designated peaks 1, 2, and 3). Phosphotyrosine and phosphoserine residues are present in these three domains while peak 2 contains phosphothreonine as well. Thus, at least seven sites are available for phosphorylation on the \( \beta \) subunit of the insulin receptor.

Incubation of the phosphorylated insulin receptor with alkaline phosphatase at 15°C results in the selective dephosphorylation of the phosphorytrosine residues on the \( \beta \) subunit of the receptor while the phosphoserine and phosphothreonine residues are retained. The dephosphorylation of the receptor is accompanied by a marked 65% inhibition of the receptor kinase activity. Almost 90% of the decrease in \([\gamma^32P]phosphate\) content of the receptor after alkaline phosphatase treatment is accounted for by a decrease in phosphotyrosine content in peak 2, while very small decreases are observed in peaks 1 and 3, respectively. These results demonstrate that the extent of phosphorylation of tyrosine residues in receptor domain 2 closely parallels the receptor kinase activity state, suggesting phosphorylation of this domain may play a key role in regulating the insulin receptor tyrosine kinase.

The hormone insulin modulates a large number of cellular functions by a mechanism that is initiated by its specific cell surface receptors. Studies during the past few years have significantly advanced our understanding about the molecular structure of the insulin receptor. Such studies have indicated that this receptor exists in a heterotetrameric disulfide-linked complex containing two \( M_r = 125,000 \) \( \alpha \) and two \( M_r = 90,000 \) \( \beta \) subunits (1, 2) as well as in other oligomeric forms (3). It is still unclear how the insulin receptor structure actually mediates the multiple characteristic biological responses to insulin and a number of mechanisms have been proposed (4). A recent discovery by Kasuga et al. (5, 6) showed that insulin stimulated the incorporation of \( [\gamma^32P]P \) into the \( \beta \) subunit of its own receptor in cultured human lymphocytes and rat hepatoma cells. Subsequent studies demonstrated that insulin also increased the phosphorylation of the insulin receptor in rat hepatocytes (7), human placenta (6–10), 3T3-L1 adipocytes (8), and rat adipocytes (11). In cell-free systems, the interaction of insulin with its receptor in the presence of \( [\gamma^32P]P \)ATP also resulted in increased phosphorylation of the insulin receptor (7–16). The increased phosphorylation of the insulin receptor in insulin-treated cells was predominately due to an increase in the level of phosphoserine with the simultaneous appearance of phosphotyrosine (6), whereas the increase by insulin added to detergent extracts of membranes or cells was primarily due to phosphotyrosine (8, 10, 12, 14).

Petruzelli et al. (8) demonstrated that insulin activated a tyrosine-specific protein kinase in Triton X-100 extracts of 3T3-L1 adipocytes and human placenta. The insulin-activated tyrosine-specific kinase was capable of phosphorylating added substrates such as histone and casein. Subsequent reports (17–20) have confirmed the ability of insulin to stimulate the phosphorylation of added substrates in detergent extracts of placenta, 3T3-L1 adipocytes, and liver cells. These studies raised the possibility that the insulin receptor itself is a protein kinase. The labeling of the \( \beta \) subunit of the insulin receptor with photoactive ATP analogues provided further support for the notion that the insulin receptor may possess intrinsic kinase activity (14–16). More recent studies using preparations of insulin receptor purified to near homogeneity indicated that the receptor retained the capability to autophosphorylate (21) and phosphorylate added substrates (22). These studies strongly suggest that the insulin receptor functions as a protein kinase.

The autophosphorylation of the insulin receptor has raised the question of whether the phosphorylation phenomenon is simply a reflection of the kinase activity of the receptor using its own \( \beta \) subunit as substrate versus the possibility that the phosphorylation event actually plays a role in regulating the function of the receptor. Recent studies by Rosen et al. (17) showed that the tyrosine kinase activity towards histone and angiotensin in placenta extracts was enhanced when the insulin receptor was first phosphorylated. Furthermore, once the receptor was phosphorylated, the kinase activity became independent of insulin. They suggested that phosphorylation of the insulin receptor activated its intrinsic kinase activity. This observation implies that the phosphorylation of the
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receptor is a major step in the regulation of a receptor function that is related to its kinase activity. The aim of the present studies was to examine the molecular basis of the regulation of the insulin receptor activity. Particular emphasis was placed on the nature and the location of the phosphorylation sites on the β subunit of the insulin receptor and their relationship to the activation of the receptor kinase. We demonstrate here the presence of several available phosphorylation sites on the insulin receptor that include serine, threonine, and tyrosine residues. At least seven sites of receptor phosphorylation can be identified using a partially purified receptor preparation and [γ-32P]ATP. Among these sites, the phosphorylation of a tyrosine residue(s) in a specific tryptic domain of the β subunit of the insulin receptor appears to correlate with activation of the receptor kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**

Leupetin, β-acylcholines, PMSF, bovine alkaline phosphatase Type VII-SA p-mercurphey phosphate, phosphonucleotide, phosphotyrosine, and phosphoinositol were purchased from Sigma. TPCK-treated trypsin was from Worthington, and [γ-32P]ATP was from New England Nuclear. Wheat germ agglutinin-agarse and CNBr-activated Sepharose 4B were from Pharmacia. Precast celulose plastic sheets were purchased from Brinkmann Instruments, Inc. Protein A-Sepharose was a gift from Dr. Donald Chance of Eli Lilly Co. Histone IIAs was a gift from Dr. T. Miller, University of Massachusetts Medical School. Molecular weight standards were from Bio-Rad Laboratories.

**Preparation of Insulin-Agarose and DTT-heat-inactivated Insulin-Agarose**—1 g of dry CNBr-activated Sepharose 4B was washed and swollen in 1 mM HCl. The resin was suspended in 7 ml of coupling buffer containing 0.1 M NaHCO3, 0.5 M NaCl, pH 8.4, 20 mg of porcine insulin, and a trace amount of [32P]labeled hormone and was mixed with 2% Triton X-100, 1 mM PMSF, and 10 µg/ml of leupeptin. The inclusion of bovine serum albumin (50 µg/ml) as coprecipitant at 4°C for 4 h. The precipitate was washed once with ethanol ether (1:1, v/v) at −20°C. The pellets were then hydrolyzed in 6 M HCl under vacuum at 110°C for 1 h. The hydrolysate was lyophilized, washed once with water, rehydrolyzed, and then dissolved in water containing 1 mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine. The samples were spotted on polyinositol cellulose plastic sheets and electrophoresed in pH 3.5 (pyridine/acetic acid/water, 1:10:189) at 1000 V for 2 h at 12°C. The position of the phosphoamino acids was located by ninhydrin staining and radioactivity was either detected by autoradiography using Kodak X-Omat film with enhancing screens or by Cherenkov counting of the excised spots.

**Phosphorylation Assays**—The washed insulin-agarose beads with or without bound insulin receptor were incubated in 50 mM Hepes, pH 7.6, containing 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml of leupeptin, and 0.5 M NaCl and then once in the same buffer without 0.5 M NaCl.

**Phosphorylation Assays**—The washed insulin-agarose beads with or without bound insulin receptor were incubated in 50 mM Hepes, pH 7.6, containing 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml of leupeptin, 10 mM MgCl2, and 3 mM MnCl2 in the presence and precipitated with 0.5 µg/ml of elastase at 22°C for 45 min. The phosphorylation was initiated by adding [γ-32P]ATP (final concentration, 5 µM; specific activity, 50 Ci/mmol). After the indicated period of incubation, the reaction was terminated by adding electrophoresis sample buffer containing 0.17 M Tris, 10% SDS, and 100 mM DTT, pH 6.8, and heating at 100°C for 5 min. The samples were electrophoresed on 8-10% SDS-polyacrylamide gradient gels. After electrophoresis, the gels were stained, destained, dried, and autoradiographed with Kodak X-Omat film and enhancing screens. Radioactivity in the 32P-labeled bands was determined by Cherenkov counting.

**Phosphoamino Acid Analysis**—The position of phosphoproteins on dried gels was located by aligning with the autoradiograms. The 32P-labeled bands were excised and extracted with 10 mM NH4HCO3 containing 5% mercaptoethanol and 0.1% SDS at 37°C according to the method of Hunter and Sefton (24). The extracted phosphoproteins were precipitated in 20% trichloroacetic acid with bovine serum albumin (50 µg/ml) as coprecipitant at 4°C for 4 h. The precipitate was washed once with ethanol ether (1:1, v/v) at −20°C. The pellets were then hydrolyzed in 6 M HCl under vacuum at 110°C for 1 h. The hydrolysate was lyophilized, washed once with water, rehydrolyzed, and then dissolved in water containing 1 mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine. The samples were spotted on polyinositol cellulose plastic sheets and electrophoresed at pH 3.5 (pyridine/acetic acid/water, 1:10:189) at 1000 V for 2 h at 12°C. The position of the phosphoamino acids was located by ninhydrin staining and radioactivity was either detected by autoradiography using Kodak X-Omat film with enhancing screens or by Cherenkov counting of the excised spots.

**Tryptic Peptide Mapping**—As these experiments required more radioactivity incorporated into the β subunit of the insulin receptor, the following modifications were made: 1) the ratio of placentals extracts to insulin-agarose was raised from the usual 10:1 to 125:1 (v/v) to increase the amount of insulin receptor bound to the insulin-agarose matrix and 2) the specific activity of [γ-32P]ATP was increased from 2-3 fold and the father reaction. After phosphorylation, the samples were washed and then placed and subjected as follows: 50 µl of insulin-agarose was mixed with 55 µl of 28 µM DTT in 10% SDS and incubated at 60°C for 15 min. The samples were cooled to 22°C followed by the addition of 27 µl of 800 mM iodoacetamide in 0.25 M Tris, pH 8.9, and incubated at 22°C for 10 min. Then 34 µl of 25% mercaptoethanol in 75% glycerol was added and incubation was continued for another 5 min at 60°C. The samples were electrophoresed on 8% SDS-polyacrylamide gel. After electrophoresis, the 32P-labeled β subunit of the insulin receptor in the unfixed gel was located by autoradiography and excised. The gel piece (1 x 1 cm) was then washed once in 2 ml of deionized water followed by 2 ml of 50 mM NH4HCO3 buffer at 22°C for 45 min. In later experiments, the volume of washing media was increased to 10 ml to ensure more effective removal of residual 32P-inorganic phosphate and nucleotides. The gel piece was then suspended in 3 ml of 50 mM NH4HCO3 buffer containing 50 µg/ml of TPA-treated trypsin and incubated at 37°C for 6 h. This was followed by 3 washes. TPA-treated trypsin and the incubation was continued for another 4 h at 37°C. The digestion buffer was then removed and the gel was rinsed in 3 ml of 50 mM NH4HCO3 buffer at 37°C for 5 h. The digestion and rinsing buffer were combined and lyophilized. The dried digest was then dissolved in 0.5 ml of 1% trifluoroacetic acid. The digest was applied to a Waters HPLC reverse-phase µBondapak C18 column pre-equilibrated in 0.1% trifluoroacetic acid. The 32P-labeled peptides were eluted in a 0-40% 1-propanol gradient at a flow rate of 0.5 ml/min. 1-min fractions were collected and radioactivity was determined by Cherenkov counting. The peak fractions were then pooled, lyophilized, and hydrolyzed in 6 M HCl at 110°C for 1 h. The
3P-labeled phosphoamino acids in the hydrolysates were analyzed as indicated above.

Dephosphorylation of Insulin Receptor by Alkaline Phosphatase—The phosphorylated receptor bound to 50 µl of insulin-agarose was washed four times, each in 4 ml of 50 mM Hepes containing 0.1% Triton X-100, 1 mM PMSF, and 10 µg/ml of leupeptin. The washed receptor having insulin-agarose was suspended in 80 µl of 200 mM Tris containing 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml of leupeptin, 5% glycerol, plus or minus 12 units/ml of bovine alkaline phosphatase, 80 mM MgCl₂, and 0.7 mg/ml of bovine serum albumin, pH 7.9, and incubated at 15 or 22 °C for 1 h. The receptor preparation was then washed three times, each in 50 mM Hepes containing 0.1% Triton, 1 mM PMSF, and 10 pg/ml of leupeptin. The washed receptor-bearing insulin-agarose was suspended in 80 mM Tris containing 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml of histone, 20 mM sodium phosphate, 5 mM nitrophenylphosphate, 1 mM DTT, and 100 µM sodium vanadate, pH 7.6, and incubated for either 15 or 30 min at 22 °C after the addition of [γ-33P]ATP to a final concentration of 5 µM (specific activity, 50 pCi/nmol). The reaction was terminated by adding SDS and DTT. The samples were electrophoresed on 8–16% SDS-polyacrylamide gradient gels and the histone bands were excised and counted.

RESULTS

Insulin-receptor Phosphorylation and Receptor-associated Kinase Activity—The ability of insulin-agarose to adsorb the insulin receptor and its associated kinase is shown in Fig. 1. Insulin-agarose preparations that were incubated with placental extract glycoproteins which had been affinity purified with wheat germ agglutinin-agarose and DTT-heat-inactivated insulin-agarose were used for assays of kinase activity. Incubation of such preparations of insulin-agarose with adsorbed placental membrane proteins in the presence of 5 µM [32P]ATP and histone at 22 °C for 60 min resulted in the phosphorylation of a species of Mr, 93,000 and histone (Fig. 1, lane A). The phosphorylation of the Mr, 93,000 band was still evident in the absence of histone, indicating that it is not a contaminant of the histone preparations used in these experiments (data not shown). The Mr, 93,000 32P-labeled band corresponds to the position expected for the β subunit of the insulin receptor in SDS-polyacrylamide gel.

The 32P-labeling of the Mr, 93,000 band was abolished and that of histone markedly inhibited when phosphorylation was performed with insulin-agarose preparations obtained after incubation with placental extracts in the presence of 100 µg/ml of free insulin (Fig. 1, lane B). This inhibition was specific for native insulin because desoctapeptide insulin was less effective and cytochrome c was without effect at the same molar concentration (Fig. 1, lanes C and D). Immunoprecipitation of the placental extracts with anti-insulin receptor immunoglobulin before adsorption to insulin-agarose resulted in the removal of the Mr, 93,000 phosphoprotein and inhibition of the phosphorylation of histone (Fig. 1, lanes E and F). The residual histone kinase activity exhibited by the insulin-agarose that was exposed to extract after immunoprecipitation with anti-insulin receptor antibodies was probably due to the presence of contaminating kinase activity present in the patient’s serum immunoglobulin fraction which absorbed nonspecifically to the insulin-agarose preparation. This possibility was confirmed when the phosphorylation of histone was performed with insulin-agarose preparations which were previously incubated with buffer containing no placental extracts but with control immunoglobulin or anti-receptor immunoglobulin. The extent of phosphorylation of histone incubated only with the patient’s immunoglobulin and insulin-agarose was 65% higher than that with control IgG (control 1037 cpn versus patient’s immunoglobulin = 1680 cpn).

Phosphoamino acid analysis indicated that the Mr, 93,000 species contained predominantly phosphotyrosine with a trace amount of phosphoserine (Fig. 2). The phosphoserine content appeared to be variable in different experiments and tended to be higher when higher concentrations of ATP were used for phosphorylation (see below). Both phosphotyrosine and phosphoserine could be detected in the 32P-labeled histone (Fig. 2). The inhibition of histone phosphorylation by excess free insulin was due to the decrease in tyrosine kinase activity with little change in the serine kinase activity, suggesting that the tyrosine kinase activity is insulin-receptor dependent.

The observations that the adsorption to insulin-agarose of the Mr, 93,000 phosphoprotein was inhibited by excess free insulin and by the removal of the insulin receptor by prior immunoprecipitation with anti-insulin receptor immunoglobulin indicates that the Mr, 93,000 phosphoprotein is the β subunit of the insulin receptor. The concurrent presence of the insulin receptor and histone tyrosine kinase activity suggests that either the insulin receptor itself or some tightly associated kinase(s) is responsible for the phosphorylation of the receptor and histone.

Activation of Receptor Kinase by ATP—In order to examine

FIG. 1. Specificity of adsorption of receptor-associated kinase by insulin-agarose. 0.5 ml of lectin-agarose and DTT-heat-inactivated insulin-agarose-enriched placental membrane extracts prepared as described under “Methods” was incubated in the absence (CON, lane A) or in the presence (lane B) of 17 µM porcine insulin (INS, lane B) 17 µM desoctapeptide insulin (DOP, lane C) or 17 µM cytochrome c (CYTO, lane D) at 22 °C for 1 h. The samples were cooled to 4 °C, mixed with 50 µl of insulin-agarose and incubated further at 4 °C for 15 h. After incubation, the insulin-agarose was separated from the extracts, washed, and suspended in 80 µl of 50 mM Hepes containing 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml of leupeptin, 0.3 mg/ml of histone, 20 mM sodium phosphate, 5 mM nitrophenylphosphate, 1 mM DTT, and 100 µM sodium vanadate, pH 7.6, and incubated for either 15 or 30 min at 22 °C after the addition of [γ-33P]ATP to a final concentration of 5 µM (specific activity, 50 pCi/nmol). The reaction was terminated by adding SDS and DTT. The samples were electrophoresed on an 8–16% SDS-polyacrylamide gel. The placental extracts in lanes E and F were first immunoprecipitated without or with patient’s immunoglobulin fraction B-9 containing anti-insulin receptor antibodies (AB) before incubation with insulin-agarose. Histone counts/min in lanes A–F were 2060, 680, 940, 2060, 1520, and 480 respectively. CON, control.
whether prior phosphorylation of the insulin receptor influences its kinase activity. Receptor-bearing insulin-agarose preparations were incubated with different concentrations of unlabeled ATP at 22°C for 1 h, washed to remove the unreacted ATP, and then assayed for phosphorylating activity with histone as substrate. The results shown in Fig. 3 indicate that the kinase activity of the receptor preparations was progressively activated when the concentrations of unlabeled ATP used for preincubation were increased from 5 to 1000 μM. The effect of ATP appears to be maximal at concentrations between 400-1000 μM. Half-maximal activation was achieved at 50 μM of ATP. The ATP-activated kinase activity of the insulin-agarose preparation is dependent on the presence of the insulin receptor as no such activation could be observed with a receptor-deficient insulin-agarose preparation obtained by including excess free insulin during the incubation of insulin-agarose with placental extracts. In the experiment depicted in Fig. 3, the amount of phosphate incorporated into the β subunit of the insulin receptor during the activation incubation was determined by using [γ-32P]ATP instead of unlabeled ATP. It is clear in Fig. 3 that the amount of phosphate incorporated into the β subunit of the insulin receptor increased as the concentrations of ATP used for activation were raised. A close parallel can be observed between the incorporation of phosphate into the insulin receptor and the activation of its associated kinase activity towards histone (Fig. 3).

Table I shows the specificity of activation of the insulin receptor kinase by ATP. Among the nucleosides and nucleotides tested, only ATP was effective in activating the receptor kinase.

In order to investigate the effects of ATP-activation on the kinetics of histone phosphorylation, experimental conditions were developed to ensure steady state phosphorylation of histone. As shown in Fig. 4, the phosphorylation of histone and the β subunit of the insulin receptor in the control and ATP-activated states remained linear up to 30 min at 22°C. When the concentration dependence of the receptor kinase was examined by serial dilution of the control and ATP-activated insulin receptor preparations, the rates of histone phosphorylation were linear over the range of 1/3 to 1/27 dilution (Fig. 5). Thus, the conditions that were chosen to examine the kinetic parameters of insulin receptor kinase preparations in subsequent experiments are those that fall within the linear portion of the time and receptor kinase concentration curves. No dephosphorylation of the 32P-labeled β subunit of the insulin receptor or of 32P-labeled histone
could be detected upon termination of the phosphorylation reaction by the removal of ATP and further incubation for up to 120 min at 22 °C. These results indicate that our experiments designed to examine the insulin receptor-associated kinase activity were performed under steady state conditions and that the histone and receptor phosphorylation values represent real increments of phosphate incorporation rather than increased phosphate turnover.

Table II shows the kinetic parameters of histone phosphorylation by insulin receptor bearing insulin-agarose preparations. Insulin receptor kinase activity was performed under steady state conditions and that the histone and receptor phosphorylation values represent real increments of phosphate incorporation rather than increased phosphate turnover.

To investigate the nature of the activation of histone and receptor phosphorylation in response to increasing concentrations of ATP, the phosphoamino acid contents of the 32P-labeled histone and the β subunit of the insulin receptor from the control and ATP-activated groups were analyzed. The results of the analyses indicated that the increase in histone phosphorylation by the receptor kinase activated by 400 μM of ATP was primarily due to an increase (500%) in the phosphotyrosine content. A small but consistent increase (30%) in the phosphoserine content was also observed. In contrast, the increase in phosphate incorporation into the β subunit of the insulin receptor was directed at both the tyrosine (880% increase) and serine (1500%) residues.

HPLC Tryptic Peptide Mapping of 32P-labeled β Subunit of the Insulin Receptor—In order to determine whether similar or different sites on the β subunit of the insulin receptor were phosphorylated by different concentrations of ATP used for activating the receptor kinase, the β subunit was intensively phosphorylated by 5, 20, and 400 μM of [γ-32P]ATP and was then digested with TPCK-treated trypsin. The tryptic phosphopeptides were resolved on a C-18 reverse phase HPLC column using a gradient of 1-propanol (Fig. 6). The HPLC...
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Fig. 6. HPLC tryptic peptide mapping of the \(^{32}\text{P}\)-labeled \(\beta\) subunit of the insulin receptor. Insulin receptor preparations containing higher amounts of insulin receptor were prepared as described under "Methods." The receptor preparations were phosphorylated at 22°C for 1 h with 5, 20, and 400 \(\mu\text{M}\) of \([\gamma^{32}\text{P}]\text{ATP}\) with specific activities of 1600, 400, and 200 \(\text{Ci/mol}\), respectively. The samples were then washed, reduced and alkylated, and electrophoresed as described under "Methods." Gel pieces containing the \(^{32}\text{P}\)-labeled \(\beta\) subunit of the insulin receptor were incubated with TPCK-treated trypsin for a total of 20 h at 37°C. The tryptic cleavage was loaded on a HPLC reverse-phase C-18 column and the \(^{32}\text{P}\)-labeled tryptic peptides were eluted in a linear 0-40% 3-propanol gradient. The radioactivity in each fraction was determined by Cherenkov counting.

Fig. 7. Effects of increasing concentrations of ATP on phosphate incorporation into the tryptic domains of the \(\beta\) subunit of the insulin receptor. The fractions making up peaks 1, 2, or 3 of the HPLC tryptic peptide maps shown in Fig. 6 were pooled and the combined radioactivity in each peak was plotted against the concentration of ATP used for phosphorylation. The amount of phosphate incorporated into each peak was calculated after correcting for changes in specific activity of ATP at each concentration.

The tryptic peptide maps of the \(^{32}\text{P}\)-labeled \(\beta\) subunit were quite similar when the insulin receptor was phosphorylated with either 5, 20, or 400 \(\mu\text{M}\) of ATP, although the ratio of peak 2 to peak 3 appears to increase somewhat with increasing \([\gamma^{32}\text{P}]\text{ATP}\) concentrations. Clearly, no new peptide peaks were demonstrated under conditions of high concentrations of \([\gamma^{32}\text{P}]\text{ATP}\). These results suggested that similar sites on the insulin receptor were phosphorylated irrespective of the concentration of ATP used. Four \(^{32}\text{P}\)-labeled peaks with different retention times can be identified. The first peak with a retention time of 5-6 min was found to co-migrate with inorganic phosphate. \([\gamma^{32}\text{P}]\text{ATP}\) was eluted at the position of the second peak with a retention time of 7-10 min. The second, third, and fourth peaks (designated peak 1, 2, and 3, respectively) contained phosphopeptides as shown by the presence of phosphoamino acid in each of them (see Fig. 8, below). The shoulder peak on the right of peak 2 derived from the \(\beta\) subunit phosphorylated with 5 \(\mu\text{M}\) of ATP was not consistently observed. The presence of this peak might represent some slight variations in tryptic digestion among experiments.

After allowing for the change in specific activity of the ATP used in these experiments, it was calculated that the net amount of phosphate incorporated into each peak was increased progressively as the concentration of ATP was raised (Fig. 7). The magnitudes of the increases in labeled phosphate incorporation into the three peaks differ from one another. The absolute amount of phosphate incorporated into each peak is in the order of peak 2 > peak 3 > peak 1. However, the order for the percentage increase in phosphorylation is peak 1 > peak 2 > peak 3. Analyses of the phosphoamino acids in the individual phosphopeptide peaks revealed that more than one phosphoamino acid residue was present in each (Fig. 8A). With the exception of peak 1, which contains phosphoserine residues that tend to be saturated at low concentrations of ATP, the phosphorylation of all other sites identified was increased with increasing concentrations of ATP (Fig. 8, B, C, and D).

The amounts of phosphoserine and phosphotyrosine found in peak 1 were much lower than that of the total phosphate observed in peak 1. Since \([\gamma^{32}\text{P}]\text{ATP}\) elutes with a retention time identical to that of peak 1, it is very likely that the majority of radioactivity in peak 1 actually represented carry-over contamination of \(^{32}\text{P}\)-labeled nucleotides which was not removed by the initial washing procedures. This possibility was confirmed when more extensive washing of the gel slices was used in subsequent experiments in which the magnitude of peak 1 was markedly reduced (see Fig. 10) while the ratio of peak 2 to peak 3 remained the same.

Relationship between the Phosphate Content of the Insulin Receptor and Its Associated Kinase Activity—The presence of phosphotyrosine, phosphoserine, and phosphothreonine in...
the insulin receptor preparation we employ prompted us to investigate which of these phosphoamino acid residues in the receptor structure is essential for the activation of the receptor kinase. Our strategy was to fully activate the receptor kinase by phosphorylating with high concentrations of ATP (400 μM) and then to selectively dephosphorylate the receptor with specific tyrosine and serine phosphatases. Recent studies demonstrated that alkaline phosphatase was capable of dephosphorylating histone and A-431 cell membrane proteins phosphorylated at tyrosine residues (25) and could effectively dephosphorylate the ³²P-labeled insulin receptor (17, 20). When the ³²P-labeled insulin receptor preparations were incubated in the presence and absence of 12 units/ml of alkaline phosphatase at pH 7.9 for 1 h at 15 and 22 °C, close to 55% of the ³²P-labeling on the β subunit was removed (Table III). Accompanying the decrease in the phosphate content of the receptor was a marked inhibition of the kinase activity of the insulin receptor-bearing insulin-agarose preparations (Table III). The dephosphorylation of the insulin receptor and inhibition of its kinase activity was not due to proteolysis of the receptor by some contaminating proteases in the alkaline phosphatase preparations. Thus, the alkaline phosphatase-treated insulin receptor could again be fully phosphorylated and its kinase activity was restored by subsequent incubation with ATP after the removal of the phosphatase by washing. Furthermore, when the alkaline phosphatase was inhibited by the inclusion of 500 μM vanadate and the omission of Mg²⁺,

K.-T. Yu and M. P. Czech, unpublished observation.

![Graph](image-url)

**Fig. 8. Analyses of the phosphoamino acid contents in peaks 1, 2, and 3 and the dose-dependent response of each phosphoamino acid residue to increasing concentrations of ATP.** The pooled peak fractions shown in Fig. 7 were lyophilized and their phosphoamino acid contents were analyzed as described under “Methods.” Only half of the sample at 5 μM ATP was used for the analysis. After electrophoresis and autoradiography, the phosphoamino acid spots were excised and their corresponding radioactivity determined by Cherenkov counting. A, autoradiogram of the ³²P-labeled phosphoamino acids in peaks 1, 2, and 3. B–D represent the respective dose-dependent phosphate incorporation into the threonine, tyrosine, and serine residues in each peak to increasing concentrations of ATP.

**Table III**

| Alkaline phosphatase | Temperature | % ³²P remaining in receptor | Decrease | Receptor-mediated phosphorylation of histone | Decrease |
|----------------------|-------------|-----------------------------|----------|---------------------------------------------|----------|
| –                    | 15          | 192                         | 100      | 1643                                        | 67       |
| +                    | 15          | 142                         | 51       | 538                                         | 67       |
| –                    | 22          | 282                         | 49       | 691                                         | 64       |
| +                    | 22          | 144                         | 49       | 253                                         | 64       |

Effect of alkaline phosphatase treatment on the phosphate content of the insulin receptor and its associated kinase activity

Insulin receptor-bearing insulin-agarose preparations were phosphorylated with either unlabeled or ³²P-labeled ATP (specific activity, 10 μCi/nmol) at 22 °C for 1 h. After extensive washing, the resin was incubated in the presence and absence of 12 units/ml of alkaline phosphatase for 1 h at 15 or 22 °C. Samples phosphorylated with [γ-³²P]ATP were used for the measurement of phosphate incorporation into the β subunit of the insulin receptor and sample treated with unlabeled ATP were used for the assays of receptor kinase activity as described under “Methods.” Each value represents the average of two experiments.
preparations were phosphorylated with 400 μCi [γ-32P]ATP (specific activity, 100 μCi/nmol) at 22°C for 1 h. After phosphorylation, the samples were washed and then incubated in the presence and absence of alkaline phosphatase as described under “Methods.” The control and dephosphorylated preparations were electrophoresed, followed by phosphoamino acid analyses of the 32P-labeled β subunit of the insulin receptor. Shown here is one representative experiment.

**TABLE IV**

Quantitation of the phosphoamino acid contents of the β subunit of the insulin receptor with or without alkaline phosphatase treatment

The phosphoserine and phosphotyrosine spots in the chromatogram shown in Fig. 9 were excised and their associated radioactivities were determined by Cherenkov counting. The counts in the phosphothreonine spots were too low to be determined accurately. However, visual examination of the autoradiogram shown in Fig. 9 indicated no difference between the control and phosphatase-treated groups.

| Treatment with alkaline phosphatase | Temperature | [32P]Ser Percentage change due to phosphatase | [32P]Tyr Percentage change due to phosphatase |
|------------------------------------|-------------|-----------------------------------------------|-----------------------------------------------|
|                                    | °C          | cpm                                          | cpm                                          |
|                                    | 15          | 15                                           | 15                                           |
|                                    | +           | 40                                           | 76                                           |
|                                    | −           | 35                                           | 196                                          |
|                                    | +           | 33                                           | 54                                           |

phosphatase selectively dephosphorylated the receptor on tyrosine residues without detectable effects on the serine phosphate residues. When the radioactivity of the phosphotyrosine spots were determined by Cherenkov counting, a decrease of approximately 65% in the alkaline phosphatase-treated group could be detected (Table IV). The decrease in phosphotyrosine content of the receptor correlates well with the decrease in the receptor kinase activity after alkaline phosphatase treatment (compare Tables III and IV). These results strongly suggest that tyrosine phosphorylation on the β subunit of the insulin receptor may be responsible for the activation of the insulin receptor kinase.

As phosphotyrosine residues were present in each of the three HPLC phosphopeptide peaks (Fig. 8), attempts were made to determine which phosphopeptide peak or peaks contained the phosphotyrosine residues that were associated with the activation of the receptor kinase. Fig. 10 shows the HPLC treatment.

**TABLE V**

Quantitation of the phosphoamino acid contents in the tryptic domains of the β subunit of the insulin receptor with or without alkaline phosphatase treatment

The pooled fractions of peaks 1, 2, and 3 shown in Fig. 10 were lyophilized and their phosphoamino acid contents were analyzed as described under “Methods.” Following the elution of the phosphoamino acid spots from the chromatogram, the radioactivity in each of them was determined by Cherenkov counting. Similar results were obtained when the analysis was performed on the peak fractions from another similar experiment with or without alkaline phosphatase treatment.

| Alkaline phosphatase | Phosphoamino acid | Peak 1 | Decrease due to phosphatase | Peak 2 | Decrease due to phosphatase | Peak 3 | Decrease due to phosphatase |
|----------------------|-------------------|--------|-----------------------------|--------|-----------------------------|--------|-----------------------------|
| −                    | Ser               | 35     | 425                         | 83     |
| +                    | Ser               | 24     | 11                          | 241    | 80                          | 3      |
| −                    | Thr               | 139    | 139                         | 6      |
| −                    | Tyr               | 173    | 2387                        | 545    |
| +                    | Tyr               | 121    | 52                          | 960    | 1427                        | 417    | 138                         |
The present study demonstrates that phosphokinase activity from detergent extracts of human placenta can be adsorbed to insulin-agarose. The chromatographic behavior of this kinase activity through wheat-germ agglutinin-agarose, DTT-heat-inactivated insulin-agarose, and native insulin-agarose columns is identical to that expected for the insulin receptor. The specificity of the insulin-agarose matrix to adsorb the kinase activity is demonstrated by its graded sensitivity to inhibition by native and desoctapeptide insulin and by the lack of inhibitory effects of cytochrome c. Furthermore, prior treatment of the placental extracts with anti-insulin receptor antibodies before adsorption to insulin-agarose completely abolished the phosphorylation of the M₁, 93,000 species and markedly inhibited the phosphorylation of histone. These results strongly suggest that the M₁, 93,000 species is the β subunit of the insulin receptor which can undergo autophosphorylation and can phosphorylate added substrate such as histone. Recent studies which demonstrated the retention of kinase activity by the insulin receptor purified close to homogeneity (21, 22), the affinity labeling of the β subunit of the insulin receptor by the ATP analog [α-32P]8-azidoadenosine 5'-triphosphate following UV irradiation (14, 16), and the enzymic similarity between casein kinase and insulin-stimulated kinase activity (26) are consistent with the hypothesis that the insulin receptor possesses intrinsic kinase activity. Our findings in the present study support those by other laboratories on this point.

Studies by Rosen et al. (17) have indicated that the lag period in the time course of insulin-stimulated histone phosphorylation in wheat germ aggluatinin-agarose-enriched placental extracts could be eliminated by prior incubation with unlabeled ATP. They suggested that the autophosphorylation of the insulin receptor is essential for the activation of its kinase activity towards histone. Our studies using purified insulin receptor have confirmed their findings. The observation that the activation is specific for ATP further substantiates the role of receptor phosphorylation in activating its intrinsic kinase activity. Significantly, the association between the marked inhibition of the receptor kinase activity and the dephosphorylation of the receptor provides additional support for the activation of the receptor kinase by phosphorylation.

Attempts to identify which phosphotyrosine residues are responsible for activating the receptor kinase were complicated by the general action of alkaline phosphatase in dephosphorylating the tyrosine residues in all these tryptic domains. On a qualitative basis, the deactivation of the receptor kinase appears to correlate best with the dephosphorylation of phosphotyrosine residues in peak 2. This is because the percentage decrease in phosphotyrosine content in peak 2 (60%) and that in the enzyme activity (65%) of the receptor kinase were similar (compare Table III with Table V), whereas the percentage decreases in phosphotyrosine content in peak 1 and peak 3 were smaller in magnitude. Furthermore, almost 90% of the decreased 32P content in the receptor associated with dephosphorylation and kinase deactivation is accounted for by receptor peptide in peak 2. Thus, it appears that the phosphotyrosine residue (or residues) in peak 2 is a likely candidate for receptor kinase activation. However, more extensive studies will be required to establish unequivocally whether the phosphorylation of the tyrosine residue or residues in peak 2 is linked to the activation process. The dephosphorylation of the trace phosphoserine residue in peak 1 raises the possibility that it might be responsible for the activation of the receptor kinase. However, because of its extremely low abundance relative to tyrosine phosphorylation and the observation that the phosphorylation of this serine residue became saturated at low concentrations of ATP, before full activation of the receptor kinase was achieved (Fig. 8D), it seems highly unlikely that the phosphorylation of this serine residue plays a critical role in kinase activation. It should be considered that pre-existing phosphoamino acids in the insulin receptor may also participate in the activation or regulation of the receptor kinase. However, the present approach does not allow the detection of these putative sites.

The role of phosphoserine and phosphothreonine residues in the regulation of the receptor has yet to be elucidated. The correlation between the phosphorylation of the tyrosine residues in the β subunit of the insulin receptor kinase does not preclude the possibility that serine and threonine phosphorylation may also be part of the activation mechanism. It is not unreasonable that the concerted phosphorylation of these three types of amino acids may be required for the full activation of the receptor kinase. However, it is also possible that the phosphoserine and phosphothreonine residues may represent some "silent" phosphorylation sites with no defined functions. Alternately, they may be involved in some subtle regulatory mechanism through which the phosphorylation and dephosphorylation of tyrosine residues in the β subunit can be modulated.

It should be noted that the present approach will not...
distinguish which portion of the insulin receptor phosphorylation is stimulated by insulin. This is because all our studies were performed on a receptor preparation already immobilized on insulin-agarose which presumably has fully activated the receptor. Because the major objective of our approach was to characterize the various phosphorylation sites on the receptor and their relationship to the regulation of the receptor kinase activity, the methodology used in the present study is quite suitable. The relationship between activation of insulin receptor kinase by insulin versus phosphorylation deserves further study, however.

The relative degree of serine phosphorylation in our receptor preparation contrasts with the findings by other laboratories that the phosphorylation is predominantly at tyrosine residues when the receptor is phosphorylated in vitro. One possible explanation for this difference may be the time period used to partially hydrolyze the $^{32}$P-labeled $\beta$ subunit. This is especially critical when the ratio of phosphoserine to phosphotyrosine is low. Thus, prolonged hydrolysis may lead to the lowering of phosphoserine to an undetectable level. Our experience is that 1-h hydrolysis with 6 M HCl at 110 °C seems to be optimal in releasing the phosphoserine and phosphotyrosine residues from the $\beta$ subunit of the receptor. The levels of phosphotyrosine and phosphoserine released tend to decrease when the hydrolysis period is extended beyond 1 h. Similar observations were reported previously (8). An alternate explanation for our detection of phosphoserine residues may be related to the level of pre-existing phosphoserine in the receptor when it is isolated. Thus, if the level is high, less serine residues may be available for phosphorylation when incubated with [y-$^{32}$P]ATP. The possibility that it may be due to the rapid turnover of phosphate on the serine residue in the $\beta$ subunit of the receptor caused by the presence of phosphatase is unlikely because no phosphatase activity could be detected in our receptor preparation.

It should be emphasized that the phosphoserine residues we detect are intrinsic to the receptor structure as indicated by the absence of phosphoserine when the receptor was prevented from adsorbing to the insulin-agarose matrix by excess free insulin (Fig. 2). When the degree of inhibition of serine, threonine, and tyrosine phosphorylation by excess free insulin were quantitated by direct scintillation counting of the phosphoamino acids, the phosphorylation of all three amino acid residues in the $M_r = 93,000$ band were decreased by approximately 75% compared to control levels (not illustrated). Further evidence for the presence of phosphoserine sites in the $\beta$ subunit was provided by the observation that when the insulin receptor was first phosphorylated for a brief period (<30 min) with high concentrations of unlabeled ATP and then phosphorylated with $^{32}$P-labeled ATP, the labeling of the receptor was enhanced by about 2-fold, both on the tyrosine and serine residues. No such stimulation could be observed when the experiment was performed with receptor-deficient insulin-agarose preparations. Of course, it has yet to be determined whether the phosphorylation of serine, tyrosine and threonine residues in the $\beta$ subunit of the insulin receptor is due entirely to intrinsic kinase activity of the receptor or to some contaminating kinases tightly bound to the receptor or insulin-agarose. This question as well as the full mechanism by which receptor phosphorylation leads to receptor-associated tyrosine kinase activation awaits the results of further investigation.

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