

Phosphorylation and Dephosphorylation of the Insulin Receptor: Evidence against an Intrinsic Phosphatase Activity[†]

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ABSTRACT: We have studied the reversibility of insulin receptor phosphorylation to establish the relation between this autophosphorylation reaction and the initiation of insulin action and between dephosphorylation and the termination of insulin effects in cells. In cultured Fao hepatoma cells labeled with $^{32}\text{PO}_4^{3-}$, insulin increased 5-fold the phosphorylation of the β -subunit of the insulin receptor at serine, threonine, and tyrosine residues. Addition of anti-insulin antiserum to cells incubated with insulin caused dissociation of insulin from the receptor and concurrent dephosphorylation of the β -subunit. $^{32}\text{PO}_4^{3-}$ associated with the insulin-stimulated receptor could be decreased by the addition of sodium phosphate to the medium but with a slower time course. Insulin stimulated phosphorylation of insulin receptor purified partially on immobilized wheat germ agglutinin. This reaction utilized $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and occurred exclusively on tyrosine residues. Ad-

dition of unlabeled ATP caused a decrease in the amount of PO_4^{3-} associated with the receptor. Insulin-stimulated phosphorylation was also observed if the receptors were further purified by immunoprecipitation with anti-insulin receptor antibody prior to the phosphorylation reaction; however, addition of unlabeled ATP to this system did not chase the labeled $^{32}\text{PO}_4^{3-}$ from the β -subunit. These data are consistent with the notion that phosphorylation and dephosphorylation of the insulin receptor parallel the onset and termination of insulin action. Phosphatase activity involved in the dephosphorylation of the insulin receptor appears to be a glycoprotein because it was retained after partial purification of the receptor on wheat germ agglutinin-agarose; however, this phosphatase activity is distinct from the insulin receptor because it was not retained after immunoprecipitation of the receptor with anti-insulin receptor antibodies.

The precise molecular events that transmit intracellularly the metabolic signal initiated by insulin binding at the extracellular surface of the plasma membrane are not known. One possibility involves phosphorylation or dephosphorylation of certain cellular proteins following insulin binding (Jungas, 1970; Avruch et al., 1976; Benjamin & Clayton, 1978; Seals et al., 1979; Smith et al., 1979; Alexander et al., 1982; Brownsey & Denton, 1982; Le Cam, 1982; Sheorain et al., 1982). These covalent modifications could be important for transmission of the insulin signal beyond the membrane receptor. This concept is further supported by the finding that insulin stimulates the phosphorylation of the M_r 95000 subunit of its own receptor in both intact cells and cell-free systems (Kasuga et al., 1982a-c; Avruch et al., 1982; Haring et al., 1982b; Machicao et al., 1982; Petruzzelli et al., 1982; Van Obberghen & Kowalski, 1982; Kasuga et al., 1983a,b; Zick et al., 1983a,b). This autophosphorylation reaction appears to result from the activation by insulin of a tyrosine-specific protein kinase that is contained in the β -subunit of the receptor. This kinase activity is retained in a highly purified preparation of placental insulin receptors (Kasuga et al., 1983a), and the β -subunit can be labeled with ATP affinity reagents (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983). The insulin receptor kinase also catalyzes at tyrosine residues the phosphorylation of other proteins (Petruzzelli et al., 1982; Kasuga et al., 1983b). In the intact cell, insulin stimulates phosphorylation of the β -subunit at tyrosine,

threonine, and serine residues (Kasuga et al., 1982c); however, serine and threonine phosphorylations of the β -subunit, although insulin stimulated in intact cells, are lost from the receptor after purification.

In this report, we continue to characterize in the hepatoma cell line Fao the phosphorylation and dephosphorylation of the β -subunit of the insulin receptor. Comparisons are made between results obtained in both intact cells and a solubilized preparation. The role of receptor phosphorylation for cellular insulin action is uncertain at the present time. Possibly, receptor autophosphorylation is an early step in the intracellular transmission of the insulin signal in the intact cell that is initiated by extracellular hormone binding.

Experimental Procedures

Materials. The following materials were obtained from the sources indicated: $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, $^{32}\text{PO}_4^{3-}$, and Triton X-100 were from New England Nuclear; HEPES, aprotinin, phenylmethanesulfonyl fluoride, *N*-acetylglucosamine, bovine serum albumin, bovine γ -globulin, phospho amino acids, and xylene cyanol were from Sigma; poly(ethylene glycol) (PEG 6000) was from Fisher Scientific; pyridine was from Aldrich; porcine insulin (lot no. 1JM95AN) was from Elanco; reagents for SDS-PAGE¹ and the Bradford protein assay were purchased from Bio-Rad; Pansorbin and bacitracin were from Calbiochem; wheat germ agglutinin-agarose was from Vector or Miles. $[\text{Tyr}^{A14}][^{125}\text{I}]\text{moniodoinsulin}$ was prepared in our laboratory (Grigorescu et al., 1983); cellulose thin-layer plates (20 × 20 cm) were obtained from Analtech (G1140). All other reagents were of the best grade commercially available.

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¹ Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosylphenylalanine chloromethyl ketone.

Cell Culture. The Fao cell was selected from the rat hepatoma H4-II-EC₃ (Pitot et al., 1964; Descharette & Weiss, 1974). This cell line was derived from the Reuber H-35 hepatoma (Reuber, 1961). The Fao cell line was kindly provided by Dr. M. C. Weiss (Guf-sur-Yvette, France). It exhibits a number of liver-specific properties: the secretion of rat serum albumin and the synthesis of the liver isomers of alcohol dehydrogenase and aldolase (Descharette & Weiss, 1974). The Fao cell has many insulin receptors and is very sensitive to insulin as measured by stimulation of glycogen synthase, tyrosine aminotransferase, and aminoisobutyric acid influx (Crettaz & Kahn, 1983). Stock monolayer cultures of Fao cells were grown to confluence in 150-cm² plastic dishes (Nunc) or plastic bottles (Corning) containing 50 mL of Coon's modified F12 medium (Gibco) supplemented with 5% fetal bovine serum (Hyclone), 100 units/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). During cell culture, the monolayers were detached from the plastic dishes (Nunc or Corning) with a solution of 0.05% trypsin and 0.02% EDTA and replated 3–5 days prior to an experiment. The cells were used at confluence. Confluent 35-mm dishes contained (3–5) × 10⁶ cells, 100-mm dishes contained (15–20) × 10⁶ cells, and 150-mm dishes contained (60–80) × 10⁶ cells.

Insulin Binding. Insulin binding assays were done at 37 °C in Krebs–Ringer bicarbonate buffer (pH 7.8) containing 1% bovine serum albumin and 0.8 mg/mL bacitracin in 35-mm six-well culture dishes. Confluent Fao cells were incubated with 1 mL of buffer containing 10–30 pM carrier-free [¹²⁵I]insulin and 10 nM unlabeled insulin for various time intervals as described in the legend of Figure 3. At the end of these incubations, the monolayers were washed 3 times with 2 mL of cold phosphate-buffered saline and solubilized with 1 mL of 0.01% SDS, and the cell-associated radioactivity was quantified in a Tracer 1290 autogamma counter.

Insulin Receptor Phosphorylation with Intact Cells. Confluent monolayer cultures in 100-mm dishes were washed twice with a solution containing 50 mM HEPES, pH 7.4, and 0.1% bovine serum albumin. The cells were then incubated for 2 h at 37 °C in a humidified atmosphere composed of 5% CO₂/95% air with 5 mL of phosphate-free Krebs–Ringer bicarbonate containing ³²PO₄³⁻ (0.125 mCi/mL) and 0.1% dialyzed bovine serum albumin. Insulin was added at the indicated concentrations, and the incubation was continued for 1–120 min at 37 °C. The reactions were stopped by aspirating the incubation medium and washing the monolayers twice with cold Krebs–Ringer bicarbonate buffer containing 10 mM sodium pyrophosphate, 100 mM NaF, and 4 mM EDTA.

The cell monolayers were solubilized immediately at 4 °C with 5 mL of 50 mM HEPES buffer (pH 7.4) containing 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, aprotinin (1000 trypsin inhibitor units/mL), and 2 mM phenylmethanesulfonyl fluoride. After a 10-min interval, the cells were removed from the culture dish and the insoluble material was sedimented by ultracentrifugation for 60 min at 200000g. Each supernatant was applied onto a 1 mL packed volume wheat germ agglutinin affinity column. The agarose was washed with 50 mL of 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, and 4 mM EDTA. The glycoproteins bound to the immobilized wheat germ agglutinin were eluted with 5 mL of this solution supplemented with 300 mM *N*-acetylglucosamine. Five 1-mL fractions were collected, and the two fractions containing the most radioactivity were combined and used for immunopre-

cipitation. In a typical experiment, 3–4 µL of B-2 serum or 6–8 µL of control serum was added to 600–800 µL of eluate and incubated at 4 °C for at least 3 h in microfuge tubes. A 10% (w/v) suspension of Pansorbin (0.2 mL) was added to this mixture, and the incubation was continued for at least 1 h at 4 °C. After this incubation, the Pansorbin was sedimented by centrifugation (10000 rpm, 500g) of the microfuge tubes in a Microfuge B (Beckman) that was equilibrated at 4 °C in a cold room. The pellets were washed twice at 4 °C by resuspension and centrifugation with 1 mL of 50 mM HEPES (pH 7.4) containing 1% Triton X-100 and 0.1% SDS and once with 50 mM HEPES containing 0.1% Triton. After this wash procedure, the pellets were suspended in 80 µL of sample buffer containing 2% SDS, 0.1 M dithiothreitol, 0.01% bromophenol blue, 10% glycerol, and 10 mM sodium phosphate (pH 7.0). About 75 µL of each sample was applied to the SDS–polyacrylamide slab gel, and the phosphoproteins were separated by electrophoresis and detected by autoradiography. Electrophoresis was performed according to Laemmli (1970) with 4% stacking gels and 5% or 7.5% resolving gels. The phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-omat film.

In Vitro Phosphorylation. Sixty confluent 150-mm culture dishes of hepatoma cells were washed at 22 °C with calcium- and magnesium-free phosphate-buffered saline (20 mL). The cells were solubilized by adding to each dish at 22 °C a solution containing 50 mM HEPES, pH 7.4, 1% Triton X-100 with aprotinin (1000 trypsin inhibitor units/mL), and 2 mM PMSF. The cells were scraped from the dishes, and the insoluble material was sedimented by centrifugation at 50000 rpm in a Beckman 70.1Ti rotor for 40 min. The supernatant was applied onto a wheat germ agglutinin–agarose column. After sample application, the column was washed with 500 mL of 50 mM HEPES, pH 7.4, and 0.1% Triton X-100. Finally, the bound glycoproteins were eluted (1–3 mg of protein) with 10 mL of this solution supplemented with 300 mM *N*-acetylglucosamine.

Aliquots of the eluate from the immobilized wheat germ agglutinin column (4–6 µg of protein) were incubated in a solution containing 100 mM HEPES buffer, pH 7.4, 10 mM MgCl₂, and 2–10 mM MnCl₂ with or without insulin. Phosphorylation was studied by incubation of this mixture with [γ -³²P]ATP (5–50 µM) at 4, 22, or 37 °C for various time periods. The phosphorylation was stopped with PAGE sample buffer at 100 °C or by addition of NaF (100 mM), sodium pyrophosphate (10 mM), EDTA (5 mM), and ATP (5 mM) at 0 °C. The insulin receptor subunits were identified by SDS–PAGE and autoradiography as described above.

Identification of Phospho Amino Acids. The phospho amino acids were analyzed by a modification of the method of Hunter & Sefton (1980). Fixed, stained, destained, and dried polyacrylamide gel fragments containing phosphoprotein located by autoradiography were washed for 12 h at 37 °C with 20 mL of 10% methanol. The adsorbent paper was removed from the gel fragment. The gel was dried at 70 °C for 60 min and rehydrated in 2 mL of 50 mM NH₄HCO₃ containing 100 µg of TPCK-treated trypsin. This mixture was incubated for 24 h at 37 °C, and then the supernatant was clarified by centrifugation and lyophilized. The residue was dissolved in 300 µL of 6 N HCl. Acid hydrolysis was performed for 2 h at 110 °C. The sample was diluted with 2 mL of water and lyophilized. The residue was dissolved in 0.5 mL of water and re-lyophilized. The samples were dissolved in water containing 1 mg/mL each of phosphotyrosine, phosphoserine, and phosphothreonine and applied onto cellulose thin-layer plates.

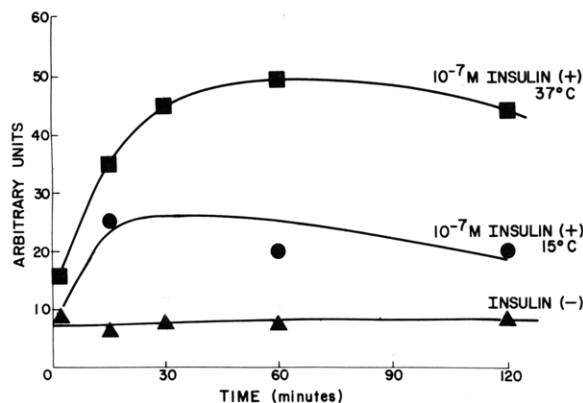


FIGURE 1: Time course of the insulin effect on ^{32}P labeling of the M_r 95 000 subunit of the insulin receptor in Fao cells. The cells were labeled with $^{32}\text{PO}_4^{3-}$ for 2 h and then incubated with (+) 10^{-7} M insulin for the indicated time intervals at 37 °C (■) or 15 °C (●) or without (-) insulin (▲) at 37 °C. The insulin receptors were partially purified on wheat germ agglutinin-agarose and immunoprecipitated with anti-insulin receptor serum or normal human serum. The immunoprecipitated protein was solubilized, reduced with dithiothreitol, and separated by SDS-PAGE. An autoradiogram was prepared, and relative labeling of the β -subunit was determined by densitometry.

These plates were sprayed uniformly with a solution composed of pyridine-acetic acid-water (1:10:89). Electrophoresis was performed on a Pharmacia flat-bed electrophoresis apparatus at 500–1000 V for 70 min at 15 °C. Xylene cyanol was used as a tracking dye. The phospho amino acid standards were identified by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

Results

Time Course and Specificity of Receptor Phosphorylation in Intact Cells. The kinetics of insulin receptor phosphorylation are shown in Figure 1. In the absence of insulin, $^{32}\text{PO}_4^{3-}$ incorporation into the receptor was low and remained constant during a 120-min incubation period. Insulin (10^{-7} M) stimulated receptor phosphorylation. At 37 °C, this effect was detectable 1 min after addition of insulin and a steady state of phosphorylation was reached between 15 and 30 min. Since insulin binding is nearly instantaneous at this temperature and insulin concentration, the stimulated phosphorylation occurring between 1 and 30 min should reflect the increased rate of phosphorylation rather than the kinetics of insulin binding. Our method of phospho amino acid analysis detected insulin stimulation of phosphorylation predominantly at serine residues of the β -subunit and, to a lesser extent, at tyrosine and threonine residues (Figure 2, left).

At 15 °C, insulin stimulation of receptor phosphorylation was less than observed at 37 °C (Figure 1). This characteristic is different from insulin binding, which shows the opposite temperature dependence, that is, higher binding at 15 °C than at 37 °C (Haring et al., 1981a). Most metabolic effects of insulin parallel the result with phosphorylation and decrease at lower temperatures (Haring et al., 1981a). Internalization of the receptor is almost not detectable at 15 °C (Gorden et al., 1980).

Insulin stimulation of receptor phosphorylation was detectable at 10^{-9} M, half-maximal stimulation occurred at 5×10^{-9} M, and maximum stimulation was reached at 10^{-7} M (Figure 3). Higher insulin concentrations caused a decrease in the level of phosphorylation. As expected from binding characteristics, proinsulin (Freychet et al., 1971), multiplication stimulating activity (Kasuga et al., 1981), and epidermal growth factor (Hollenberg & Cuatrecasas, 1975) stimulated poorly phosphorylation of the insulin receptor.

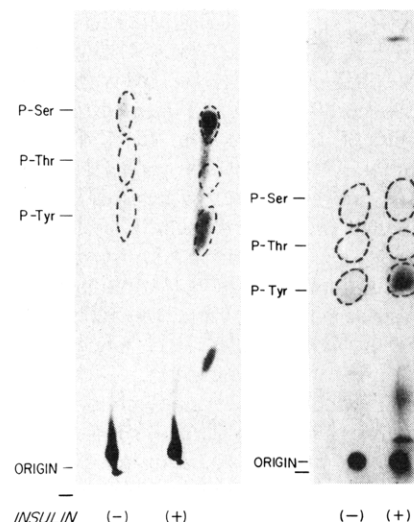


FIGURE 2: Identification of phospho amino acids in the β -subunit. (Right) The insulin receptor, purified on immobilized wheat germ agglutinin, was phosphorylated in the presence (+) or absence (-) of insulin. The β -subunit was separated by SDS-PAGE under reducing conditions and located by autoradiography. The phospho amino acids were identified as described under Experimental Procedures. (Left) The Fao cell was labeled with $^{32}\text{PO}_4^{3-}$ for 2 h, and the phosphorylation of the insulin receptor was stimulated with insulin (+) or without insulin (-) for 15 min. The insulin receptor was partially purified on immobilized wheat germ agglutinin followed by immunoprecipitation. The β -subunit was separated by SDS-PAGE under reducing conditions, and the phospho amino acids were identified.

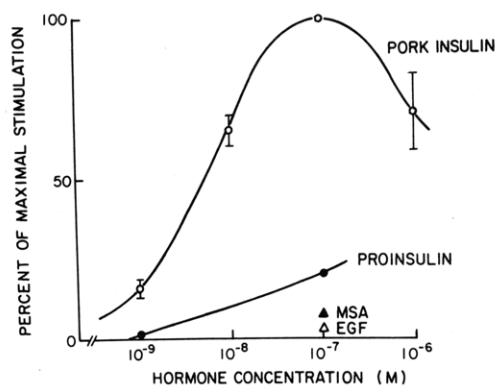


FIGURE 3: Hormonal dose-response and specificity of insulin receptor phosphorylation. Fao cells were labeled with $^{32}\text{PO}_4^{3-}$ for 2 h. Insulin (○), proinsulin (●), epidermal growth factor (Δ), or multiplication stimulating activity (▲) was added for an additional 30 min at 37 °C. The insulin receptor was purified by affinity chromatography and immunoprecipitation, and the β -subunit was identified by SDS-PAGE. The labeling of the β -subunit was quantified by scanning densitometry of the corresponding autoradiograms. The dose-response is expressed as a percentage of the maximum phosphorylation that was found at 10^{-7} M insulin.

Dephosphorylation of the Insulin Receptor in Intact Cells.

The reversibility of insulin-stimulated phosphorylation of the insulin receptor was studied by using anti-insulin antibody, which caused dissociation of insulin from the receptor. Labeled and unlabeled insulin (final concentration, 10^{-8} M) were allowed to bind to the Fao cells for 15 min at 37 °C. Addition of an aliquot of anti-insulin serum (1:400 dilution) caused dissociation of [^{125}I]insulin with a half-life of 8 min (Figure 4, left). In parallel experiments cells labeled with $^{32}\text{PO}_4^{3-}$ for 2 h at 37 °C were stimulated with insulin (10^{-8} M) for 15 min, and then guinea pig anti-insulin serum (1:400 dilution) was added to some aliquots of cells. Figure 4, right, shows an autoradiogram of one of these experiments. In the basal state, anti-receptor serum specifically immunoprecipitated one

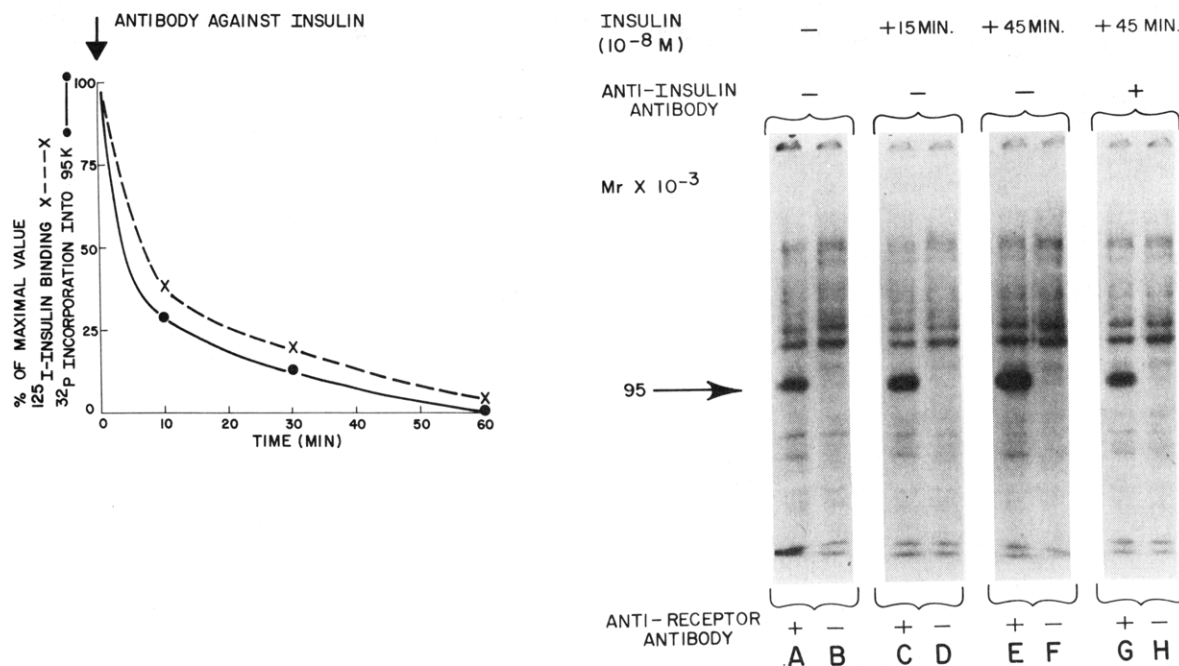


FIGURE 4: Anti-insulin serum induced dissociation of insulin and dephosphorylation of the insulin receptor. (Left) A comparison of the effect of anti-insulin serum on the dissociation rate of [¹²⁵I]insulin from the receptor and dephosphorylation of the β -subunit. [¹²⁵I]Insulin and insulin (10^{-8} M) were incubated with Fao cells for 15 min, followed by addition of anti-insulin serum (1:400 dilution) for the time intervals indicated on the abscissa. The dissociation of insulin from the receptor is expressed as a percentage of the value measured before addition of antibody (X). In parallel experiments (●), cells were labeled with $^{32}\text{PO}_4^{3-}$ for 2 h and then insulin (10^{-8} M) was added to each reaction mixture for 15 min. Finally, anti-insulin serum (1:400 dilution) was added. At the time intervals indicated on the abscissa, the reactions were stopped and the insulin receptor was prepared for SDS-PAGE. The phosphorylation of the M_r 95 000 subunit was determined by scanning densitometry of the corresponding autoradiograms. The 100% value represents the level of phosphorylation after 15 min of insulin treatment only; the value measured in the absence of insulin has been deducted. The values found after the addition of antibody were also corrected for the phosphorylation found in the absence of insulin and are shown in the figure as a percentage of the insulin effect before addition of antibody. (Right) Autoradiograms showing the effect of anti-insulin serum on the insulin-stimulated phosphorylation of the β -subunit of the insulin receptor in Fao cells labeled with $^{32}\text{PO}_4^{3-}$. Four panels are shown: the first lane in each autoradiogram shows the phosphoproteins immunoprecipitated with anti-insulin receptor serum from the immobilized wheat germ agglutinin purified cell extract; the second lane of each experiment shows the immunoprecipitation obtained with normal human serum. The Fao cells were incubated (left to right) in the absence of insulin (-) or in the presence of 10^{-8} M insulin (+) for 15 and 45 min. In the panel at the far right, Fao cells were stimulated with insulin (10^{-8} M) for 15 min, followed by addition of anti-insulin serum (1:400 dilution) for 30 min.

phosphoprotein of M_r 95 000 (Figure 4, right, lane A), which was not detected with normal human serum (lane B). In previous reports we established that this protein is the β -subunit of the insulin receptor (Kasuga et al., 1982a,c). Insulin (10^{-8} M) stimulated the phosphorylation of this band 3-fold after 15 min (Figure 4, right, lane C) and 4-fold after 45 min (lane E) as measured by scanning densitometry. Incubation of the cells with insulin antibody (Figure 4, right, lane G) reduced receptor phosphorylation almost to the level found in the absence of insulin. Furthermore, receptor dephosphorylation and dissociation of insulin from the receptor caused by anti-insulin antibody followed nearly identical time courses with a half-life of about 8 min (Figure 4, left).

The flux of $^{32}\text{PO}_4^{3-}$ bound to the receptor was demonstrated by an isotope exchange experiment. The insulin receptor was phosphorylated to steady state in the presence of 10^{-8} M insulin. When the medium was changed to replace the labeled phosphate with 10 mM unlabeled phosphate, $^{32}\text{PO}_4^{3-}$ was lost from the β -subunit with a half-life of 20 min (Figure 5). The slower rate of dephosphorylation in the chase experiments compared to antibody-induced dephosphorylation was due presumably to the time required for dilution of the specific activity of the cellular [γ - ^{32}P]ATP. If insulin (10^{-7} M) were added to the medium containing the unlabeled phosphate in order to keep the receptor occupancy constant, the apparent rate of dephosphorylation was slower. This difference could reflect an insulin effect on the phosphatase or could simply be the effect of, and a persisting activation of, the kinase and a partially labeled ATP pool. These experiments indicate for

the intact cell that phosphatases act on the insulin receptor.

Characterization of Phosphorylation in Vitro. Stimulation of receptor phosphorylation in the cell-free system can be demonstrated by using solubilized and partially purified receptors from several tissues (Avruch et al., 1982; Petruzzelli et al., 1982; Van Obberghen & Kowalski, 1982; Kasuga et al., 1983b; Zick et al., 1983b). A highly purified preparation of receptor from human placenta also retains the kinase activity (Kasuga et al., 1983a). Figure 6 shows insulin receptor phosphorylation in the solubilized and wheat germ agglutinin purified fraction from Fao cells. In the absence of insulin, a faint band of M_r 95 000 specifically precipitated with anti-insulin receptor antiserum was detected on autoradiograms of SDS-polyacrylamide gels under reducing conditions (Figure 6, left). Insulin (10^{-7} M) stimulated the phosphorylation of the β -subunit up to 50-fold in vitro (Figure 6, left). Separation of the same samples by SDS-PAGE under nonreducing conditions indicated that insulin stimulated phosphorylation of a prominent band of M_r 520 000 and to a smaller extent bands of M_r 350 000 and 95 000 (Figure 6, center). This pattern resembles in vivo phosphorylation of the receptor separated by nonreducing SDS-PAGE (Kasuga et al., 1982b). The proteins of M_r 520 000 and 350 000 are the major forms of immunoprecipitable insulin receptor observed in Fao cells under nonreducing conditions.

Insulin stimulation of the phosphorylation of the M_r 95 000 subunit in vitro was detectable at a concentration of 10^{-10} M, and the half-maximal effect was reached at 3×10^{-9} M (Figure 6, right). The maximal insulin effect occurred near

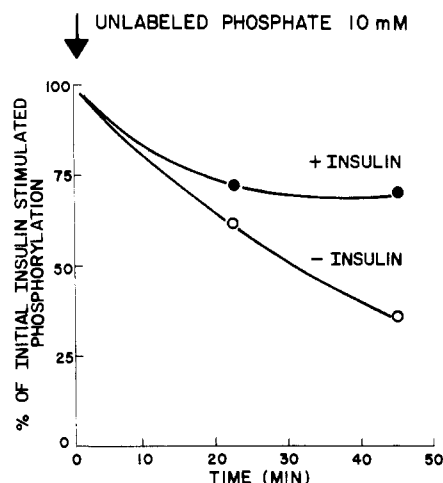


FIGURE 5: Pulse-chase of the $^{32}\text{PO}_4^{3-}$ incorporated into the β -subunit of the insulin receptor from Fao cells. Fao cells were labeled at 37°C for 2 h with $^{32}\text{PO}_4^{3-}$ and then incubated in the same medium for an additional 30 min in the presence of insulin (10^{-7} M). A cell sample was also incubated in the absence of insulin for 30 min to provide a measure of the basal phosphorylation. After this time interval, the basal phosphorylation reaction and one insulin stimulated reaction were stopped. The radioactive incubation media were removed from the other insulin-stimulated cells and replaced by a medium containing 10 mM unlabeled phosphate and either 10^{-7} M insulin (\bullet) or no insulin (\circ). These reactions were stopped after the time intervals indicated on the abscissa, the insulin receptor was purified and immunoprecipitated, and the proteins were separated by SDS-PAGE under reducing conditions. The labeling of the β -subunit was quantified by scanning densitometry of autoradiograms. The basal phosphorylation was deducted from the amount of phosphorylation found after 30 min of insulin stimulation only, and this value was taken as 100%. The values found after addition of unlabeled phosphate in the presence (+) and absence (-) of insulin were also corrected for the basal phosphorylation and are expressed as a percentage of the insulin effect before addition of unlabeled phosphate.

10^{-7} M, and a slight decrease was seen at higher insulin concentrations. Insulin stimulated *in vitro* only the phosphorylation at tyrosine residues (Figure 2, right).

Dephosphorylation of the Insulin Receptor *in Vitro*. The loss of $^{32}\text{PO}_4^{3-}$ from the partially purified insulin receptor was studied by using pulse-chase experiments. Solubilized receptor was preincubated for 60 min at room temperature with 10^{-8} M insulin at 4, 25, and 37°C . Phosphorylation was initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After a 10-min time interval, a 1000-fold excess of unlabeled ATP was added. After a slight delay, dephosphorylation of the insulin receptor was observed (Figure 7). The rate of loss of $^{32}\text{PO}_4^{3-}$ from the receptor ($t_{1/2} = 20$ min at 37°C) was considerably slower than the rate of phosphorylation (White et al., 1984). The slow chase of $^{32}\text{PO}_4^{3-}$ was even more evident when these experiments were performed at 4°C . The reversibility of $^{32}\text{PO}_4^{3-}$ incorporation into the β -subunit with a pulse-chase protocol suggests that phosphatases which recognize phosphotyrosine copurify with the insulin receptor kinase through the wheat germ affinity column.

Phosphorylation and Dephosphorylation of the Insulin Receptor *in Vitro* after Immunoprecipitation. To determine if the insulin receptor kinase is distinct from the phosphatases present in the wheat germ agglutinin purified preparation, the insulin receptor was immunoprecipitated from the mixture with anti-insulin receptor antiserum B-2 and the pulse-chase experiments were repeated. A single band of phosphoprotein (M_r 95000) was detected by SDS-PAGE and autoradiography when the immunoprecipitated insulin receptor, that is, a complex of Pansorbin, antibody, and receptor, was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence of insulin (Figure 8, lane D).

This band was more prominent than that observed in the partially purified receptor before immunoprecipitation, suggesting that some stimulation of phosphorylation of the β -subunit had occurred. Insulin further stimulated the phosphorylation of the β -subunit in the immunoprecipitated receptor 2–3-fold (Figure 8, lane F). No phosphoproteins were detected in the immunoprecipitate when the receptor was omitted (Figure 8, lanes A and B) or when anti-insulin receptor serum was substituted with normal human serum (Figure 8, lanes C and E).

In contrast to the partially purified receptor preparation, addition of 10 mM unlabeled ATP to the immunoprecipitated insulin receptor after the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ did not decrease the $^{32}\text{PO}_4^{3-}$ labeling of the β -subunit (Figure 7). This result suggests that the insulin receptor kinase has been separated by immunoprecipitation from the phosphatase activities which copurified on the immobilized wheat germ agglutinin. These results further support the notion that the insulin receptor possesses an intrinsic kinase activity rather than a phosphatase activity.

Discussion

Insulin receptor phosphorylation has been found in all tissues containing the insulin receptor thus far studied (Avruch et al., 1982; Haring et al., 1982b; Kasuga et al., 1982a–c; Machicao et al., 1982; Petruzzelli et al., 1982; Van Obberghen & Kowalaski, 1982; Kasuga et al., 1983a,b; Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983; Zick et al., 1983a,b). Evidence that the insulin receptor is itself a protein kinase includes the following facts: (1) the kinase activity of the receptor is retained after purification of the receptor protein to near homogeneity (Kasuga et al., 1983a); (2) the kinase activity is retained in the receptor after immunoprecipitation by a serum containing antibodies against the insulin receptor (present study); (3) the partially purified receptor preparations from several tissues (Kasuga et al., 1983a; Petruzzelli et al., 1982) and the highly purified receptor from human placenta membranes catalyze the phosphorylation of tyrosine residues of several artificial substrates; (4) the β -subunit of the insulin receptor is labeled with ATP affinity reagents, suggesting that the catalytic domain is located in this subunit (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983).

The notion that the insulin receptor is a tyrosine-specific plasma membrane protein kinase parallels findings for the receptors of two other growth-regulating peptides, namely, epidermal growth factor (Carpenter et al., 1979; Cohen et al., 1980; Ushiro & Cohen, 1980; Pike et al., 1982; Ernoux et al., 1983) and platelet-derived growth factor (Ek & Heldin, 1982; Ek et al., 1982; Pike et al., 1983). These receptors, like the transforming gene products of the Rous sarcoma virus (Erikson et al., 1979; Hunter & Sefton, 1980; Levinson et al., 1980), the feline sarcoma virus (Reynolds et al., 1980), and the Abelson murine leukemia virus (Hunter et al., 1981), are tyrosine kinases. In each system, phosphorylation of proteins at tyrosine residues is suspected to serve a signal transmitting function, although proof of this hypothesis and identification of the natural substrates involved are still lacking.

In this study, we have characterized insulin receptor phosphorylation and dephosphorylation in the intact cell. Kinetic properties, specificity, and the dose-response of receptor phosphorylation are consistent with the concept that this modification of the receptor might be a regulatory step in the transmission of the insulin signal. Receptor phosphorylation follows immediately insulin binding; it is clearly detectable in the intact cell within 1 min after onset of insulin binding. Even rapid insulin effects in intact cells, such as

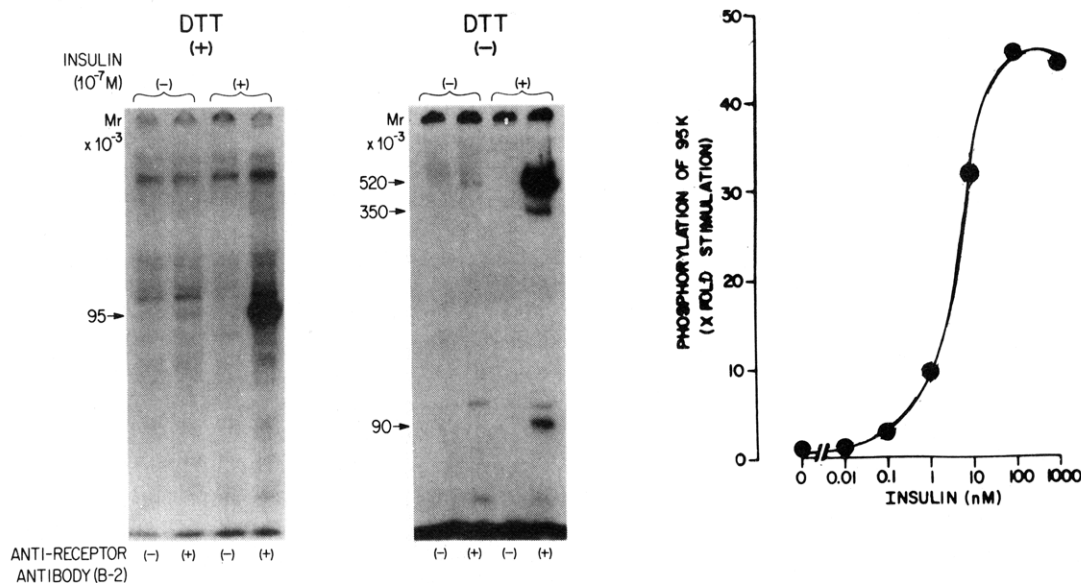


FIGURE 6: Phosphorylation of the insulin receptor from Fao cells purified on immobilized wheat germ agglutinin. Phosphorylation of the insulin receptor was carried out at 4 °C with 5 μ M ATP for 15 min. The reaction was stopped, the phosphorylated receptor was immunoprecipitated with anti-receptor antibody (+) or normal human serum (-), and the proteins were separated by SDS-PAGE under reducing conditions (left autoradiogram, 100 mM DTT, 7.5% polyacrylamide) or nonreducing conditions (center autoradiogram, no DTT, 5% polyacrylamide). The last two lanes in each autoradiogram show the effect of 10⁻⁷ M insulin. (Far right) Insulin dose-response on phosphorylation of the β -subunit. Partially purified insulin receptor (4 μ g of protein) was incubated for 1 h at 22 °C with the concentration of insulin indicated on the abscissa. Phosphorylation was measured at 25 °C with 15 μ M [γ -³²P]ATP for 10 min. The reaction was stopped by adding PAGE sample buffer and heating the mixture. The phosphoproteins were separated by SDS-PAGE. The M_r 95 000 phosphoprotein was located by autoradiography, the band was cut from the gel, and the radioactivity was quantified by liquid scintillation counting. The background was estimated by measuring the radiation in another piece of the gel judged by the autoradiography to be free of discrete phosphoproteins. The relative stimulation after subtraction of the background is shown in the figure.

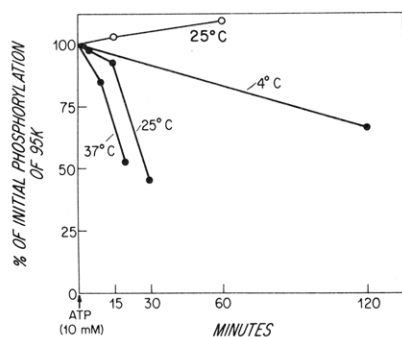


FIGURE 7: Pulse-chase labeling of the solubilized insulin receptor from the Fao cell. Insulin receptor purified from the Fao cell by chromatography on immobilized wheat germ agglutinin was incubated for 1 h at 22 °C with insulin (10⁻⁸ M). Aliquots containing 5–10 μ g of protein were phosphorylated with [γ -³²P]ATP (10 μ M, 0.01 mCi) at 4, 25, and 37 °C for 10 min. Then 10 mM ATP was added to the reaction mixtures. The reactions were stopped at the time intervals indicated on the abscissa by adding PAGE sample buffer at 100 °C for 3 min. Phosphoproteins were separated by SDS-PAGE under reducing conditions and located by autoradiography. The phosphorylation of the β -subunit was quantified by scanning densitometry. The level of phosphorylation of the M_r 95 000 band before addition of unlabeled ATP was taken as 100% and the values found after addition of unlabeled ATP are expressed as a percentage of this value. In one experiment (O), the insulin receptor before phosphorylation was incubated for 2 h at 4 °C with anti-insulin receptor serum (1:300). The immunoglobulin was precipitated by addition of Pansorbin, and the protein A-IgG-receptor complex was washed 3 times with 50 mM HEPES buffer containing 0.1% Triton. Insulin (10⁻⁷ M) was added to this suspension at 22 °C for 1 h. Then the phosphorylation reaction was started by adding [γ -³²P]ATP (5 μ M, 0.01 mCi). After a 10-min incubation 10 mM ATP was added to the mixture. This reaction was stopped at the time intervals indicated on the abscissa, and the samples were processed as described above.

activation of glucose transport and depletion of cAMP levels, show an initial lag phase of 15 s–1 min (Haring et al., 1978).

The dose-response of receptor phosphorylation parallels the dose-response of insulin binding, and both properties show the

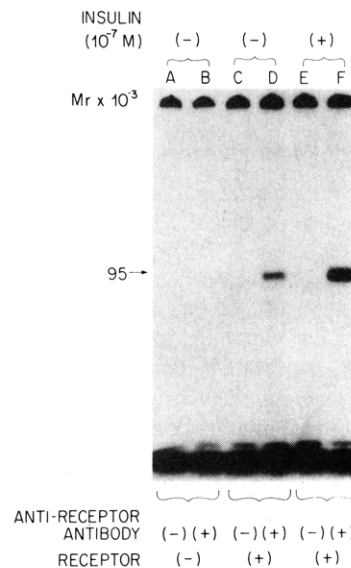


FIGURE 8: An autoradiogram showing the phosphorylation of the β -subunit of the insulin receptor in vitro after immunoprecipitation. The insulin receptor purified on immobilized wheat germ agglutinin (4 μ g) was incubated for 2 h with anti-insulin receptor serum or normal human serum. The immune complex was subsequently precipitated on Pansorbin. The protein A-IgG-receptor complex was washed 3 times with HEPES buffer containing 0.1% Triton and suspended in this buffer. Insulin was added at room temperature for 1 h, followed by the addition of [γ -³²P]ATP (5 μ M, 0.01 mCi) at 4 °C for 10 min. The reaction was stopped by adding PAGE sample buffer at 100 °C for 3 min. The phosphoproteins were separated by SDS-PAGE under reducing conditions, and an autoradiogram was prepared. Lanes A and B show phosphorylated immunoprecipitates in the absence of added insulin receptor. Phosphorylation of the β -subunit in the absence of insulin is shown following immunoprecipitation of the receptor with normal human serum (C) or anti-receptor serum (D); lanes E and F show a parallel experiment in the presence of insulin (10⁻⁷ M). same hormonal specificity. Proinsulin has a small effect similar to its binding potency (Freychet et al., 1971), whereas mul-

tiplication stimulating activity (Kasuga et al., 1981) and epidermal growth factor (Hollenberg & Cuatrecasas, 1975), which bind to the insulin receptor with very low affinity or not at all, do not stimulate phosphorylation. Receptor phosphorylation in the intact cell, that is, both serine and tyrosine phosphorylation, is not stimulated by cAMP. This result parallels our earlier findings in the cell-free system (Haring et al., 1982b), which showed that tyrosine phosphorylation of the receptor is cAMP independent.

Dissociation of insulin from the receptors parallels dephosphorylation of the β -subunit. This dephosphorylation reaction occurs rapidly enough to initiate the loss of insulin effects in the intact cell. When receptor occupancy is high, the termination of the insulin effect lags behind dissociation of insulin from the receptor (Haring et al., 1982a). This is in part due to spare receptors and, in part, probably reflects the half-time of the cellular processes activated by insulin.

Half-maximal stimulation of glycogen synthase, activation of amino acid uptake, and induction of tyrosine aminotransferase occur at markedly lower insulin concentrations than half-maximal stimulation of receptor phosphorylation (Crettaz & Kahn, 1983). Other metabolic events in intact cells reach half-maximal stimulation at $(2-5) \times 10^{-10}$ M insulin depending on the effect and the cell type studied (Kahn, 1976). The dose-response of receptor phosphorylation is less sensitive and follows closely the dose-response of receptor occupancy. Receptor phosphorylation is detectable at 10^{-9} M insulin, a high physiologic concentration at which about 5% of receptors are occupied; half-maximal insulin binding and receptor phosphorylation are obtained at about $(3-5) \times 10^{-9}$ M. This relation might be expected for a cellular insulin effect that is the first reaction in a series of events which amplify a signal initiated by the binding of insulin to the receptor. The maximal metabolic effect of insulin in some target tissues, particularly in fat cells (Kono & Barham, 1971), is reached when 2-5% of the total receptors on the cell are occupied. This finding strongly suggests the existence of a signal amplification system at the postreceptor level. Furthermore, in a cell-free system in which the basal phosphorylation is low, activation of the receptor kinase by insulin can be detected at concentrations around 10^{-10} M, a low physiological concentration. It is likely that insulin activation of phosphorylation occurs at this low concentration in the intact cell as well; however, our assay is not sensitive at low insulin levels due to concurrent dephosphorylation and high background of basal phosphorylation of the region of the M_r 95 000 band that is seen in the absence of insulin.

Receptor dephosphorylation in the intact cell occurs at a similar rate as phosphorylation, which suggests that phosphorylation of receptor is a dynamic process. This turnover of phosphate is most likely the result of a steady state between a kinase and phosphatase reaction, although it is possible that dephosphorylation is due to transfer of phosphate from the receptor to a substrate or ADP. Recent studies have shown that the energy-rich phosphate bond present in the phosphotyrosine residue in phosphorylated immunoglobulin to Rous sarcoma virus can be transferred to ADP (Furami & Lipman, 1983). Two findings in this paper argue against this mechanism. We know from *in vitro* studies that insulin increases V_{max} of the receptor kinase (White et al., 1984). Therefore, if receptor dephosphorylation reflects phosphate transfer, we would expect that in pulse-chase experiments isotope exchange of the receptor would be faster in the presence of insulin than in the absence of insulin. In fact, the effect of insulin is just the opposite: an apparent decreased rate of $^{32}\text{PO}_4^{3-}$ loss is

observed in the presence of insulin. This conclusion is also supported by the *in vitro* chase experiments with the partially purified receptor. If the pulse-chase experiment at 4 °C were performed with an insulin concentration of 10^{-7} M, no receptor dephosphorylation was detectable over the time period of 2 h (data not shown). The second argument against phosphate transfer is that receptor dephosphorylation is not detectable in a further purified system, though the assay conditions are identical with those of the less purified system. For these reactions, we believe that receptor dephosphorylation is catalyzed by phosphatases.

The receptor in the intact hepatoma cell is not only a substrate for a tyrosine-specific autophosphorylation reaction but also a substrate for a serine kinase, the latter being separate from the receptor itself (Kasuga et al., 1982c). Therefore, in the intact cell, receptor dephosphorylation must involve both serine- and tyrosine-specific phosphatases. After solubilization and wheat germ purification, the serine kinase is lost and phosphorylation occurs only at tyrosine. Phosphorylation is reversible in the partially purified preparation, suggesting that a tyrosine-specific phosphatase is retained in the glycoprotein fraction after the wheat germ agglutinin affinity purification.

Several phosphatases specific for tyrosine have been described (Carpenter et al., 1979; Brautigan et al., 1981; Gallis et al., 1981; Swarup et al., 1981; Leis & Kaplan, 1982). Carpenter et al. (1979) first suggested the existence of a tyrosine-specific phosphatase for the EGF receptor in A431 cells. Brautigan et al. (1981) and Gallis et al. (1981) have demonstrated tyrosine-specific phosphatases that are specifically inhibited by Zn^{2+} . Swarup et al. (1981) have reported that alkaline phosphatases also dephosphorylated phosphotyrosine specifically. Some of this alkaline phosphatase activity was present in membrane-bound glycoproteins with selective specificity for phosphoproteins phosphorylated at tyrosine residues. Acid phosphatase specific for phosphotyrosine has also been found in the plasma membranes prepared from astrocytoma cells (Leis & Kaplan, 1982). The copurification of the phosphatase activity on immobilized wheat germ agglutinin chromatography suggests phosphatases that interact with insulin receptor are glycoproteins as well. The finding that the phosphorylation reaction in the *in vitro* system is still reversible is in contrast to the findings of Zick et al. (1983b), obtained with receptors partially purified from liver membranes. This discrepancy might be due to a difference between the systems or due to a loss of phosphatase activity in the process of the membrane preparation.

The copurification of kinase and phosphatase activity through the wheat germ step raised the possibility that the insulin receptor protein might contain both catalytic properties. The presence of both kinase and phosphatase activity in a single protein has been described in the case of glycogen synthase kinase 3 (Hemmings et al., 1982). The same dual enzyme function was recently suggested for the insulin receptor on the basis of studies with a receptor preparation from human placenta (Machicao et al., 1982). Our findings argue clearly against this possibility. After purification of the receptor protein by immunoprecipitation, there is still an effect of insulin on receptor phosphorylation, whereas dephosphorylation is no longer observed. Furthermore, pulse-chase experiments with a highly purified placenta receptor preparation demonstrated insulin-stimulated receptor phosphorylation; however, no dephosphorylation of the receptor upon addition of unlabeled ATP was detected. These results support the concept that the insulin receptor is a tyrosine-specific protein kinase intrinsic to the receptor itself (Kasuga et al., 1983a), whereas the

tyrosine phosphatase is located in a separate glycoprotein.

There is now growing reason to suspect a crucial role of protein phosphorylation in both the growth-promoting and metabolic effects of insulin on cells. Insulin action on glucose transport in fat cells requires cellular ATP (Haring et al., 1981a,b). Insulin stimulates phosphorylation of a number of cellular proteins at serine residues including ATP-citrate lyase (Alexander et al., 1982), acetyl-CoA carboxylase (Brownsey & Denton, 1982), ribosomal protein S6 (Smith et al., 1979), and other unidentified proteins (Avruch et al., 1976; Benjamin & Clayton, 1978; Seals et al., 1979; LeCam, 1982). Insulin also stimulates dephosphorylation of pyruvate dehydrogenase (Jungas, 1970) and glycogen synthase (Sheorain et al., 1982). Insulin stimulates in intact Fao cells phosphorylation of the insulin receptor also at serine (Kasuga et al., 1982c). As the receptor possesses no serine kinase activity (Kasuga et al., 1982b) after purification, it seems possible that the receptor kinase activates a serine kinase which phosphorylates the receptor and other substrates in the cell.

A role of receptor kinase phosphorylation in the growth regulation effect of insulin has been suggested by our studies with melanoma cells. Insulin inhibits cell growth in a mouse melanoma cell line, the Cloudman S91 cell line. Mutants of this cell line were derived in which the insulin effect is lost. We have found that the loss of insulin action on cell growth was accompanied by a defect in receptor phosphorylation (H. U. Haring, M. F. White, C. R. Kahn, M. Kasuga, V. Lauris, R. Fleischmann, M. Murray, and J. Pawelek, unpublished results). Our present study also adds data that support a role of receptor phosphorylation in insulin action.

Registry No. Insulin, 9004-10-8; phosphatase, 9013-05-2.

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Neoglycoproteins: Preparation of Noncovalent Glycoproteins through High-Affinity Protein- (Glycosyl) Ligand Complexes[†]

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ABSTRACT: This work was undertaken as part of a search for well-characterized glycoprotein models in which both the oligosaccharide structure, the number of oligosaccharide chains, and the precise location of these chains in the protein are known. On the basis of the fact that high-affinity ligand binding sites have been defined precisely for several proteins in terms of both number and relative location, the hypothesis to be tested was that if oligosaccharide chains were covalently attached to such high-affinity ligands, they would be specifically bound in the ligand sites of the appropriate protein, thus permitting the preparation of neoglycoproteins of precise predetermined oligosaccharide valency and topography. To test this hypothesis, pyridoxal 5'-phosphate was reductively (NaBH_4) aminated with the α -amino group of the asparagine oligosaccharide $\text{Man}_6\text{-GlcNAc}_2\text{-Asn}$ from ovalbumin. When the resulting phosphopyridoxylated oligosaccharide (PG) was added to the apo form of aspartate aminotransferase (AAT;

EC 2.6.1.1, the cytosolic enzyme from pig heart, consisting of two subunits and containing two coenzyme binding sites), a 2:1 (PG-AAT) complex was formed which could be characterized on the basis of tritium content, the absorbance and fluorescence of the pyridoxamine phosphate moiety of PG, and the concanavalin A binding properties acquired by AAT through the incorporation of the oligosaccharide. As expected from the established properties of the holoenzyme, the AAT-PG complex is stable in the absence of phosphate or vitamin B_6 derivatives and can be dialyzed for 24 h without any significant loss of PG. According to the three-dimensional model of AAT, the oligosaccharide chain of PG should be partially masked in the coenzyme binding pocket. This was confirmed by exposing the complex to α -mannosidase. Under conditions which gave complete removal of all five α -mannosyl residues in free PG, only one α -mannosyl residue was removed from the AAT-PG complex.

Synthetic glycoconjugates are important tools in biochemistry and cell biology (Stowell & Lee, 1980). For example, sugars covalently attached to proteins have been used as immunogens for eliciting antibodies specifically directed against carbohydrates (Goebel & Avery, 1929; Lonngren & Goldstein, 1978). Solid supports bearing glycosides have been prepared as column materials for affinity chromatography (Pazur, 1981) or as culture surfaces for studying cell adhesion to carbohydrate-derivatized matrices (Pless et al., 1983). A wide variety of neoglycoconjugates have been extensively employed as model compounds in studying sugar-lectin interactions ranging from the characterization of individual binding activities

(Goldstein et al., 1977) to the investigations of cell surface receptor mediated uptake of macromolecules (Lee & Lee, 1982). However, synthetic glycoconjugates so far reported mainly consist of covalently bound saccharide moieties randomly dispersed over the surface of the matrix. These may not serve as accurate models for naturally occurring glycoproteins in which the carbohydrates are attached at specific locations (Sharon & Lis, 1982). Indeed, it has been postulated that the unique patterns of glycosylation in proteins may provide a basis for specificity in sugar-lectin interactions (Ashwell & Morell, 1974; Mencke & Wold, 1982). Therefore, it is desirable in a model system to be able to attach oligosaccharides of diverse structures to a matrix at defined locations and stoichiometry. One way to realize this is to conjugate an oligosaccharide to a protein via a ligand which possesses high affinity for specific loci on that protein. As an example of this approach for neoglycoprotein preparation, we have explored the binding of a glycosylated coenzyme (pyridox-

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