Insulin Stimulates the Tyrosine Phosphorylation of a $M_r = 160,000$ Glycoprotein in Rat Adipocyte Plasma Membranes*

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The action of insulin on tyrosine phosphorylation of plasma membrane-associated proteins in rat adipocytes was investigated. Incubation of plasma membranes from insulin-treated adipocytes with [γ-32P]ATP results in a marked increase in tyrosine phosphorylation of $M_r = 160,000$ (P160) and $M_r = 92,000$ proteins when compared to controls. Based on the immunoreactivities of these two proteins with anti-insulin receptor antibodies, the $M_r = 92,000$ species is identified as the insulin receptor β subunit while P160 is unrelated to the receptor structure. P160 appears to be a glycoprotein as evidenced by its adsorption to wheat germ agglutinin-agarose.

The tyrosine phosphorylation of P160 exhibits a rapid response to insulin (maximal within 2 min at 37 °C) and is readily reversed following removal of the free hormone by anti-insulin serum. The time courses of insulin-stimulated phosphorylation as well as the dephosphorylation of P160 coincide with those of the activation and deactivation of the insulin receptor kinase in the same plasma membrane preparation. Concanavalin A and hydrogen peroxide mimic insulin stimulation of the insulin receptor kinase and enhance the tyrosine phosphorylation of P160. Isoproterenol, epidermal growth factor, and phorbol diester are without effect.

Analysis of the insulin dose-response relationship between P160 tyrosine phosphorylation and insulin receptor kinase activity reveals that maximal phosphorylation of P160 occurs when only a fraction (25%) of the receptor kinase is activated by the hormone. A similar relationship between these two parameters is observed for the insulinomimetic agent hydrogen peroxide. The close correlation between the level of P160 phosphorylation and insulin receptor kinase activity suggests that P160 may be tyrosine phosphorylated by the receptor kinase following receptor kinase activation by the hormone or insulin-like agents. This hypothesis is further supported by the finding that the insulin receptor kinase is the only insulin-sensitive tyrosine kinase detectable in adipocyte plasma membranes under the conditions of our experiments.

It has recently been documented that the purified insulin receptor is a tyrosine kinase and that its activity is greatly enhanced following the binding of insulin (1–6). Two laboratories have isolated full length cDNA clones encoding the receptor and have deduced the primary structure (7, 8). This structural information is consistent with earlier work which indicated that the native insulin receptor protein is a heterotetramer consisting of two α and two β subunits (9–11). The insulin receptor, like a number of other growth factor receptors (12–18), catalyzes autophosphorylation on its β subunit tyrosine residues as well as tyrosine phosphorylation of several artificial exogenous substrates.

The autophosphorylation reaction of the insulin receptor has been recognized to play a crucial role in regulating the tyrosine kinase activity of the receptor. It has been reported that there is a severafold difference in tyrosine kinase activity toward exogenous substrates between tyrosine-phosphorylated and unphosphorylated insulin receptor when assayed in vitro (19, 20). These data suggested that the insulin receptor kinase activity may also be activated in vivo following insulin-induced receptor autophosphorylation on tyrosine residues. More recently such a self-regulatory mechanism has been established in intact cells. Thus, insulin receptors isolated from insulin-treated cells by immobilization on insulin-agarose exhibit a marked increase (10–20-fold) in tyrosine kinase activity when compared to those isolated from control cells (21, 22).

The increase in kinase activity of the insulin receptor following insulin-stimulated receptor phosphorylation raises an important question regarding the identity of the putative substrates for the receptor kinase. Using antiphosphotyrosine antibodies, White and co-workers (23) have shown that insulin markedly stimulates the tyrosine phosphorylation of a $M_r = 120,000$ protein (P120) in the supernatant fraction of 32P-labeled hepatoma cell lysates. Earlier, Taylor and colleagues (24) have reported that the tyrosine phosphorylation of a $M_r = 185,000$ protein (P185) in the supernatant fraction of 32P-labeled hepatoma cells is elevated by the addition of insulin. More recently, Taylor and co-workers (25) have further shown using an antibody preparation generated against P120 that insulin also increases the tyrosine phosphorylation of this phosphoprotein in intact H-35 hepatoma cultures labeled with [32P]orthophosphate. The insulin-dependent increase in tyrosine phosphorylation of the two proteins described above suggests that they potentially represent physiological substrates for the insulin receptor kinase.

In light of the multiple bioeffects of insulin on intact cells, it seems possible that other insulin receptor kinase-phosphorylated proteins may also be present and have yet to be discovered. In this study, we have investigated whether other insulin receptor kinase substrates may be present in rat adipocyte plasma membranes. Our results indicate that the tyrosine phosphorylation of a $M_r = 160,000$ glycoprotein (P160) and the tyrosine kinase activity of the insulin receptor toward exogenous histone are markedly stimulated in plasma membranes prepared from insulin-treated fat cells when compared to controls. Significantly the tyrosine phosphorylation of P160 appears to be maximally stimulated when only a small
fraction (25%) of the insulin receptor kinase is activated by insulin. Furthermore, the extent of P160 phosphorylation appears to be modulated in parallel with the activity of the receptor kinase because both parameters are mimicked by concanavalin A and hydrogen peroxide. Together, these results suggest that P160 may be directly phosphorylated by the insulin receptor kinase and that this phosphoprotein may be involved in insulin signal transduction as evidenced by its high sensitivity to insulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMSF, histone VS, concanavalin A, phorbol 12-myristate 13-acetate, phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma. [γ-32P]ATP was obtained from Amersham Corp. Wheat germ agglutinin-agarose and protein A-agarose were obtained from Pharmacia P-L Biochemicals. Hydrogen peroxide was from J. T. Baker Chemical Co.; l-isoproterenol was from Aldrich. Precoated cellulose plastic sheets were purchased from Brinkmann Instruments, and peroxidase was a gift from Dr. Ronald Chance of Lilly. Molecular weight standards were from BioRad.

**Rat Adipocyte Isolation and Incubation**—Male rats weighing 100-120 g were used (S.D. strain, Tascon Farms, Germantown, NY). Adipocytes were prepared from the epididymal fat pads as described by Rodbell (26) in Krebs-Ringer-Hepes buffer, pH 7.4, containing 30 mg/ml BSA and 2 mM sodium pyruvate. Fat cells were equilibrated in the same buffer without collagenase at 37 °C for 10 min. After incubation, adipocytes were incubated in the presence and absence of different concentrations of insulin (10−7 to 10−3 M) at 37 °C for various periods of time and then homogenized according to the procedure described below.

**Preparation of Adipocyte Plasma Membranes**—Fat cells were homogenized in 10 mM Tris-HCl, pH 7.4, containing 225 mM sucrose, 5 mM EDTA, 10 mM NaF, 200 μM sodium vanadate, and 40 mM MgCl₂, 12 mM MnCl₂, 17 mM DTT, and 400 μM pyrophosphate, and 1 mM PMSF. Plasma membranes were prepared by differential centrifugation as described by McKeel and Jarett (27) with some modifications (28). Membranes were suspended in 25 mM Hepes, pH 7.4, containing 100 mM NaF, 200 μM sodium vanadate, 2 mM DTT, and 1 mM PMSF. Membrane protein concentrations were determined by BioRad protein reagents and normalized to 0.5-1 mg/ml.

**Phosphorylation of Adipocyte Plasma Membranes**—25 μl of the suspended plasma membranes from control and insulin-treated rat fat cells were mixed with 7 μl of phosphorylation mixture containing 40 mM MgCl₂, 12 mM MnCl₂, 17 mM DTT, and 400 μM [γ-32P]ATP (specific activity, 100 μCi/nmol). The labeling reaction was allowed to continue for 10 min at 22 °C. The samples were microcentrifuged for 1 min at 4 °C in order to separate the 32P-labeled plasma membranes from the excess 32P radioactivity remaining in the reaction mixture. The supernatant was removed, and the membrane pellet was dissolved by boiling in 0.1 ml of electrophoresis sample buffer containing 100 mM DTT and 10% SDS. The samples were then electrophoresed on a 7% SDS-polyacrylamide gel. The gel was fixed, dried, and autoradiographed at −70 °C. The intensities of the 32P-labeled protein bands visualized in the autoradiogram were measured by densitometric scanning at 750 nm using a Beckman DU-8 spectrophotometer.

**Assay of Insulin Receptor Kinase Activity in Adipocyte Plasma Membranes**—Plasma membranes from control and insulin-treated cells were solubilized in 1% Triton X-100. Insulin receptors in the membrane detergent extracts were absorbed onto wheat germ agglutinin-agarose in a ratio of 4:1 (v/v) at 4 °C for 30 min. The lectin-agarose was then sedimented at 600 × g and washed three times with 50 volumes of 25 mM Hepes, pH 7.4, containing 1% Triton X-100, 100 mM NaCl, and 0.5% PMSF. The membrane extract was then suspended in an equal volume of the same buffer. 20 μl of the lectin-agarose-immobilized insulin receptor preparation was mixed with 5 μl of histone VS. Phosphorylation was initiated by the addition of 5 μl of labeling mixture containing 40 mM MgCl₂, 12 mM MnCl₂, and 3.3 μM [γ-32P]ATP (specific activity, 3 μCi/nmol). The reaction was terminated after 10 min at 22 °C by the addition of 0.1 ml of electrophoresis sample buffer, and the samples were boiled for 5 min and then electrophoresed in a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed and autoradiographed. The amount of 32P radioactivity incorporated into histone was quantitated by laser densitometry. Control experiments (Fig. 8) indicate that the entire portion of insulin-stimulated tyrosine kinase activity bound histone in adipocyte plasma membranes can be specifically adsorbed by wheat germ agglutinin-agarose. Furthermore, the insulin-stimulated tyrosine kinase activity appears to be contributed solely by the insulin receptor. This is because the hormone-activated histone tyrosine phosphorylating activity in the plasma membrane fraction extract can be completely removed following immunoprecipitation of the membrane extracts with anti-insulin receptor sera (Fig. 9).

**In Vivo 32P Labeling of Adipocytes and Immunoprecipitation of 32P-Labeled Adipocyte Plasma Membrane Proteins with Antiphosphotyrosine Antibodies**—Two aliquots of rat adipocytes were 32P labeled in phosphate-free Krebs-Ringer-Hepes buffer containing 30 mg/ml BSA and 2 mg/ml carrier-free orthophosphate for 2 h at 37 °C. Insulin (final concentration, 10−7 M) was added to one sample, and the incubation was continued for another 10 min at 37 °C. The 32P-labeled adipocytes were homogenized in 6 volumes of 10 mM Tris-HCl, pH 7.4, containing 225 mM sucrose, 5 mM EDTA, 100 mM NaF, 200 μM sodium vanadate, and 1 mM PMSF. The homogenates were fractionated into plasma membranes, high and low density microsomes, and cytosol by differential centrifugation. The protein concentrations in the membrane suspensions were normalized. The membrane fractions were solubilized in 5 mM EDTA, 100 mM NaF, 200 μM sodium vanadate, 5 mM EDTA, and 1 mM PMSF. The samples were then diluted to 0.1% Triton X-100 with the same buffer without the detergent and mixed with 10 μl of monoclonal antiphosphotyrosine antibodies immobilized on Affi-Gel 10 (14 mg/ml) at 4 °C for 16 h. The resin was then washed extensively and then boiled in 0.1 ml of electrophoresis sample buffer containing SDS and DTT. The samples were electrophoresed on a 7% SDS-polyacrylamide gel. The gel was then fixed, dried, and autoradiographed at −70 °C.

**Phosphoamino Acid Analysis**—The 32P-labeled protein bands in the dry gel were located by autoradiography and excised. The phosphorylated proteins were eluted from the gel pieces by incubating in 50 mM NH₄HCO₃ containing 0.1% SDS. The extracted materials were precipitated in 20% trichloroacetic acid at 4 °C using 50 μg of bovine serum albumin as carrier. The precipitated proteins were washed with ether and then boiled in 0.5 ml of 6 M HCl at 110 °C for 1 h. The hydrolysates were lyophilized and then dissolved in 26 μl of H₂O containing 1 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine. The samples were spotted onto a cellulose precoated plastic sheet and electrophoresed at 1000 V for 2 h in pyridine/acetic acid/water (11:10:90, pH 3.5). The positions of the phosphoamino acids were visualized by ninhydrin staining, and the 32P radioactivity associated with each phosphoamino acid spot was monitored by autoradiography.

**RESULTS**

**Effects of Insulin on Protein Phosphorylation in Rat Adipocyte Plasma Membranes**—In an attempt to identify the cellular substrate(s) for the insulin receptor tyrosine kinase, plasma membranes from rat adipocytes were first treated with 10−4 M insulin and subsequently incubated with [γ-32P]ATP in vitro. As shown in Fig. 1A, the phosphorylation of a Mr = 92,000 protein is markedly stimulated (control cell panel). The Mr = 92,000 species is the insulin receptor β subunit because it can be immunoprecipitated by two different preparations of patient-derived anti-insulin receptor sera (Fig. 2). Phosphoamino acid analysis of the Mr = 92,000 species of insulin to plasma membranes from control adipocytes results in a marked increase in phosphotyrosine content of the insulin receptor β subunit (Fig. 3). Close examination of the autoradiogram shown in Fig. 1A reveals that the addition of insulin to plasma membranes from control adipocytes does not detectably alter the phosphorylation of other membrane proteins under the conditions of these experiments. The action of the hormone also appears to be confined to the phosphorylation of the insulin receptor β subunit when the 32P-labeled

1. The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol; EGF, epidermal growth factor.
samples from control and insulin-treated membranes were electrophoresed on polyacrylamide gels of different percentages (5 and 10%) to effect better resolution of the high and low molecular weight protein species (data not shown).

These results suggest that perhaps no insulin-dependent phosphorylation substrates other than the receptor β subunit itself are detectable or present in the plasma membrane. Alternately, the ability of the receptor kinase to phosphorylate its substrate may be dependent on the presence of other insulin-mediated changes which can only occur in intact cells. In order to address this possibility we have compared the insulin-mediated changes which can only occur in intact cells. When insulin is added directly to plasma membranes from control adipocytes and the insulin-stimulated phosphorylation occurs almost exclusively on tyrosine residues, the M, = 92,000 species is identified as the insulin receptor β subunit while the M, = 160,000 phosphoprotein is unrelated to the receptor structure (Fig. 2). When insulin is added directly to plasma membrane preparations from insulin-treated adipocytes, the phosphorylation of the receptor β subunit is further increased, whereas the P160 labeling of P160 remains essentially unchanged (Fig. 1A). These results are not unexpected in view of the finding that in plasma membranes from control cells insulin increases the phosphorylation of the receptor β subunit but not that of P160. Taken together, the results of these experiments illustrate the utility of employing membranes from insulin-treated cells in order to study the action of the hormone on membrane protein phosphorylation.

To investigate the phosphoamino acid contents of P160, the 32P-labeled M, = 92,000 bands (insulin receptor β subunit) in control and insulin (INS)-treated plasma membranes from control adipocytes and the M, = 160,000 phosphoprotein in membranes from control and insulin-treated cells as shown in Fig. 1 were excised. The phosphoamino acid contents in these two phosphorylated protein bands were analyzed according to the procedure described under "Experimental Procedures." SER(P), phosphoserine; THR(P), phosphothreonine; TYR(P), phosphotyrosine.

In order to estimate the amount of P160 in adipocyte
plasma membranes, the polyacrylamide gel was stained with silver reagents following electrophoresis of membrane preparations from control and insulin-treated cells. As shown in Fig. 1B, numerous protein bands can be visualized upon silver staining. The 32P-labeled P160 protein appears to correspond to one or two faintly stained species. Visual comparison of the relative staining intensities among the various protein species in the gel indicates that P160 represents a minor component of the total membrane proteins. Due to the qualitative nature of protein determination by silver staining, it is difficult to measure the actual amount of polypeptide material associated with P160. Furthermore, it is possible that more than one protein species may be present in a single stained band. For these reasons, the stoichiometry of P160 phosphorylation cannot at present be accurately determined.

P160 is a Glycoprotein—In view of the membrane-associated nature of P160, we have examined whether this phosphoprotein is glycosylated. Adipocyte plasma membranes from control and insulin-treated cells were labeled with [γ-32P]ATP and then solubilized in Triton X-100. The detergent membrane extracts were incubated with wheat germ agglutinin-agarose at 4 °C. The resin was washed extensively, and finally electrophoresed on SDS-polyacrylamide gel. The autoradiogram in Fig. 4 indicates that the insulin-sensitive P160 phosphoprotein is adsorbed by the lectin-agarose. As expected, the sugar-containing insulin receptor β subunit is also retained by the wheat germ agglutinin-agarose beads. These results indicate that P160, like other integral membrane proteins, contains carbohydrate moieties.

Time Course of Insulin Action on P160 Phosphorylation and Insulin Receptor Kinase Activity—As shown in Fig. 5 (panel A), the onset of the stimulatory effect of insulin on P160 phosphorylation in adipocyte plasma membranes is extremely transient and the reversibility of the insulin effects on P160 phosphorylation, adipocytes previously incubated with insulin antiserum were assayed according to the protocol described under “Experimental Procedures” following adsorption of the receptor to wheat germ agglutinin-agarose. The 32P-labeled histone was isolated by electrophoresis on a 7% SDS-polyacrylamide gel, and the magnitudes of histone phosphorylation were quantitated by taking the difference between the intensities of the P160 bands in insulin-treated and control groups. The insulin receptor subunit phosphorylation represents the net insulin effects calculated as the difference between the 32P radioactivities incorporated in insulin-treated and control groups.

FIG. 4. Adsorption of P160 to wheat germ agglutinin-agarose. Adipocyte plasma membranes (40 μl) from control and insulin (INS)-treated cells were labeled with [γ-32P]ATP according to the procedures described under “Experimental Procedures.” After 10 min at 22 °C, the phosphorylation reaction was terminated by the addition of EDTA to a final concentration of 20 mM. The 32P-labeled membranes were microcentrifuged and then dissolved in 20 μl of 25 mM Hepes, pH 7.4, containing 1% Triton X-100, 100 mM NaF, 200 μM sodium vanadate, 5 mM EDTA and 1 mM PMSF at 4 °C for 30 min. The solubilized membranes were diluted to 0.1% Triton X-100 with the same buffer without the detergent and microcentrifuged for 10 min at 4 °C. The supernatant was mixed with 20 μl of wheat germ agglutinin-agarose for 3 h at 4 °C. The agarose beads were washed extensively and then boiled in 0.1 ml of electrophoresis sample buffer containing SDS and DTT. Electrophoretic resolution of the samples was performed on a 7% gel.

FIG. 5. Time courses of the stimulatory effects of insulin on P160 phosphorylation and insulin receptor kinase activity and the reversal of the insulin effects by anti-insulin serum. In order to equalize the time of incubation at 37 °C, aliquots of rat adipocytes were incubated for a total period of 40 min. Insulin (final concentration, 10−7 M) was added at either 30, 15, or 2 min before the end of the incubation period so that cells were exposed to the hormone for 30, 15, or 2 min, respectively. Control cells were not treated with insulin. After incubation, adipocytes were homogenized and plasma membranes were prepared as described under “Experimental Procedures.” The membrane suspensions were phosphorylated with 100 μM [γ-32P]ATP and electrophoresed on a 7% SDS-polyacrylamide gel. The resultant gel was fixed and autoradiographed. For the reversal of the insulin effects on P160 phosphorylation, adipocytes previously incubated in the presence and absence of insulin for 30 min were separated from the incubation buffer by brief centrifugation and resuspended in fresh buffer containing excess guinea pig anti-insulin serum. The cell suspensions were incubated further for 0, 15, and 30 min at 37 °C. Following incubation, adipocytes were homogenized and plasma membranes were prepared. Phosphorylation of adipocyte plasma membranes was performed as described above. The magnitudes of P160 phosphorylation were quantitated by scanning at 750 nm following electrophoresis of the samples on SDS-polyacrylamide gels. The stimulatory effects of insulin at each time point were measured by taking the difference between the intensities of the P160 bands in insulin-treated and control groups. The insulin receptor kinase activities in plasma membranes from cells after different periods of incubation with insulin or insulin antisem were assayed using histone as phosphorylation substrate according to the protocol described under “Experimental Procedures” following adsorption of the receptor to wheat germ agglutinin-agarose. The 32P-labeled histone was isolated by electrophoresis on a 10% SDS-polyacrylamide gel, and the magnitudes of histone phosphorylation were quantitated by Cerenkov counting. The 32P radioactivity incorporated into the insulin receptor β subunit present in the same gel was also determined by Cerenkov counting. The data on histone and insulin receptor β subunit phosphorylation represent the net insulin effects calculated as the difference between the 32P radioactivities incorporated in insulin-treated and control groups.
rapid. Maximal stimulation of the \(^{32}\)P labeling of P160 is achieved when fat cells are incubated with 100 nM insulin for 2 min at 37 °C. The effect of insulin remains constant up to 30 min. Significantly, the insulin-stimulated P160 phosphorylation is readily reversed when guinea pig anti-insulin serum is added to the buffer to effect dissociation of the hormone from the cells. Thus, incubation of adipocytes in medium containing the hormone antisera results in a 50% decrease in \(^{32}\)P labeling of P160 after 10 min at 37 °C. The stimulatory effect of insulin is almost abolished after 30 min of incubation.

The ability of the receptor kinase to catalyze tyrosine phosphorylation of exogenous histone was monitored following partial purification of the receptor by wheat germ agglutinin-agarose chromatography from cells treated with insulin for different periods of time. Control experiments indicate that the insulin receptor tyrosine kinase activity can be specifically and completely adsorbed by the lectin agarose (see Figs. 8 and 9 below). Fig. 5 (panel B) shows that the insulin-dependent activation of the insulin receptor kinase and the hormone antisera-induced deactivation follow essentially the same time courses as the phosphorylation and dephosphorylation of P160 when the receptor kinase activities in plasma membranes were assayed using histone as phosphorylation substrate. The resemblance in the time-dependent modulation of P160 phosphorylation and insulin receptor kinase activity by insulin and anti-insulin antibodies suggests that these two biological parameters in fat cell plasma membranes are regulated in parallel by the hormone.

In a fashion similar to the time-dependent stimulation of insulin receptor histone kinase activities by insulin, the levels of receptor \(\beta\) subunit phosphorylation in the wheat germ agglutinin-agarose-purified receptor preparations are also rapidly increased by the hormone (Fig. 5, panel C). Furthermore, such an increase in receptor subunit phosphorylation can be readily reversed when the hormone is neutralized by anti-insulin immunoglobulin. These results are in agreement with previous findings that insulin receptor purified from insulin-treated cells exhibits enhanced kinase activity toward its own \(\beta\) subunit and exogenous substrates due to in vivo activation by the hormone (21, 22). Thus, the in vitro phosphorylation of the insulin receptor \(\beta\) subunit can serve as an indicator of the receptor kinase activity in intact cells.

**Specificity of Insulin-stimulated P160 Phosphorylation**—The specificity of the action of insulin on P160 phosphorylation is next examined. Table I shows that among the various agents tested, only insulin and concanavalin A are effective in enhancing the tyrosine phosphorylation of P160 and the insulin receptor \(\beta\) subunit. The maximal stimulatory effects of concanavalin A are identical to those of insulin. The ability of concanavalin A to stimulate the phosphorylation of the receptor \(\beta\) subunit indicates that the action of the lectin on the phosphorylation of P160 may be mediated through the receptor. Other biological factors (e.g., epidermal growth factor (EGF) and isoproterenol) or phorbol diesters did not alter the phosphorylation of either P160 or receptor \(\beta\) subunit. Taken together, these results indicate that the increase in P160 phosphorylation in rat adipocyte plasma membranes is highly specific for agents such as insulin and concanavalin A which interact with the insulin receptor. Furthermore, the mechanism(s) leading to the elevated phosphorylation of P160 appears to be unique for insulin and not shared by EGF, isoproterenol, and phorbol diester.

**Insulin Dose-response Relationship for P160 Phosphorylation and Insulin Receptor Kinase Activity**—A key question concerning the physiological relevance of P160 phosphorylation relates to the dose-response relationship of this phospho-protein to insulin. It is expected that the increase in phosphorylation of P160 should occur in response to physiological concentrations of insulin if it is involved in hormone action. In addition, it is important to define the relationship between the phosphorylation of P160 and the kinase activity of the insulin receptor in response to insulin. As discussed above, the level of the in *vitro* phosphorylation of the insulin receptor \(\beta\) subunit serves as an indicator of the receptor kinase activity in intact cells. A comparison between the phosphorylation of the insulin receptor \(\beta\) subunit and that of P160 should, therefore, adequately reflect the relative abilities of insulin to activate the receptor kinase and the phosphorylation of P160 in intact cells.

As shown in Fig. 6, the dose-response relationships between the insulin receptor \(\beta\) subunit and P160 phosphorylation are quite different. The increase in P160 phosphorylation is far more sensitive to insulin than that of the receptor subunit. Half-maximal and maximal stimulations of P160 phosphorylation occur at \(2 \times 10^{-10}\) M and \(10^{-8}\) M insulin, respectively. At least an order of magnitude higher concentrations of insulin are required for similar responses in the phosphorylation of the receptor \(\beta\) subunit. It is interesting to note that at \(10^{-9}\) M insulin the increase in P160 phosphorylation is nearly maximal while the insulin receptor kinase activity is only stimulated by 25%. The sensitivity of P160 phosphorylation to insulin is thus very similar to that of a number of insulin-stimulated cellular activities such as hexose and amino acid transport and glycogen synthase activity. On the other hand, the extent of elevation in insulin receptor kinase activity appears to be directly related to the degree of receptor occupancy by insulin as reported earlier (29).

**Effects of Hydrogen Peroxide on P160 and Insulin Receptor \(\beta\) Subunit Phosphorylation**—The results presented above demonstrate that the increase in the level of tyrosine phosphorylation of P160 is dependent on the interaction of insulin with its cell surface receptor. A question arises as to whether insulin mimicking agents can also stimulate the tyrosine phosphorylation of P160 independent of insulin binding. In view of the well documented insulin-like effects of hydrogen peroxide, the ability of this oxidant to stimulate the phospho-

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**Table I**

| Additions | P160 | \(\beta\) subunit |
|-----------|------|-----------------|
| None      | 0    | 0              |
| Insulin (10 nM) | 100 | 100            |
| Concanavalin A (50 \(\mu\)g/ml) | 100 | 100           |
| EGF (10 nM) | 0   | 0              |
| Isoproterenol (10 \(\mu\)M) | 0   | 0              |
| PMA (1 \(\mu\)M) | 0   | 0              |
Interestingly, the tyrosine phosphorylation of the insulin receptor β subunit is also increased in plasma membranes from hydrogen peroxide-treated adipocytes. Thus, incubation of fat cells with 1 and 10 mM hydrogen peroxide leads to respective increases in receptor β subunit phosphorylation to 9 and 28% of the maximal level attained with 10⁻⁷ M insulin (Fig. 7). A comparison of the relative sensitivities of P160 and insulin receptor β subunit phosphorylation in response to hydrogen peroxide indicates that, like insulin, the oxidant maximally stimulates the tyrosine phosphorylation of P160 while increasing the phosphorylation of only a fraction of the receptor subunits in adipocyte plasma membranes. Taken together, these results provide further support to the notion that activation of a small percentage of the insulin receptor kinase will lead to maximal phosphorylation of P160.

Effects of Insulin on Adipocyte Plasma Membrane-associated Tyrosine Kinase Activity—Our results indicate that the insulin receptor kinase is rapidly activated in adipocyte plasma membranes when fat cells are exposed to insulin. As a result, the activated insulin receptor kinase may in turn catalyze phosphorylation of P160. However, it is possible that other tyrosine kinases in adipocyte plasma membranes responsible for P160 phosphorylation may also be stimulated following insulin treatment. In order to address this issue, we have examined whether other tyrosine kinases besides the insulin receptor kinase may be activated by the hormone.

The experiments shown in Fig. 8 illustrate that all the insulin-stimulated tyrosine kinase activity toward exogenous histone can be adsorbed by wheat germ agglutinin-agarose, suggesting that the hormone-stimulated tyrosine kinase(s) is a glycoprotein like the insulin receptor. In contrast, the majority of the serine kinase activity in the detergent extracts of adipocyte plasma membranes is not retained by the lectin-agarose. Significantly, as depicted in Fig. 9, the insulin-stimulated histone tyrosine phosphorylating activity that can be measured by densitometric tracing of the autoradiogram. Results are expressed as percents of the maximal insulin effects on P160 and insulin receptor β subunit phosphorylation.

FIG. 7. Effects of hydrogen peroxide on P160 and insulin receptor β subunit phosphorylation. Rat adipocytes were incubated in the absence and presence of increasing concentrations of insulin (INS, 10⁻⁸ - 10⁻¹⁰ M) at 37 °C for 10 min. Plasma membranes were prepared and phosphorylated for 5 min at 22 °C with 100 μM [γ-³²P]ATP. Following electrophoresis of the ³²P-labeled samples, the gels were autoradiographed and the intensities of the P160 and insulin receptor β subunit bands were quantitated by scanning at 750 nm. The stimulatory effects of insulin at each hormone concentration were determined by taking the difference values obtained at 10⁻⁷ M insulin.

Fig. 6. Insulin dose-response relationships of P160 and insulin receptor β subunit phosphorylation. Suspensions of rat adipocytes were incubated in the absence and presence of increasing concentrations of insulin (INS, 10⁻⁸ - 10⁻¹⁰ M) at 37 °C for 10 min. Plasma membranes were prepared and phosphorylated for 5 min at 22 °C with 100 μM [γ-³²P]ATP. Following electrophoresis of the ³²P-labeled samples, the gels were autoradiographed and the intensities of the P160 and insulin receptor β subunit bands were quantitated by scanning at 750 nm. The stimulatory effects of insulin at each hormone concentration were determined by taking the difference between the intensities of the corresponding bands in insulin-treated and control cells. The results are expressed as percents of the maximal values obtained at 10⁻⁷ M insulin.

Tyrosine Kinase Activity—Our results indicate that the insulin receptor kinase is rapidly activated in adipocyte plasma membranes when fat cells are exposed to insulin. As a result, the activated insulin receptor kinase may in turn catalyze phosphorylation of P160. However, it is possible that other tyrosine kinases in adipocyte plasma membranes responsible for P160 phosphorylation may also be stimulated following insulin treatment. In order to address this issue, we have examined whether other tyrosine kinases besides the insulin receptor kinase may be activated by the hormone.

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Adsortion of insulin-stimulated tyrosine kinase activity in adipocytes.
tion can be detected in intact cells, adipocytes were labeled whether the insulin-stimulated P160 tyrosine phosphorylation sites.

The insulin receptor tyrosine kinase activities adsorbed by the anti-insulin receptor sera were also monitored following immobilization of the immunocomplex to Protein A-agarose. The insulin receptor bearing Protein A-agarose (PROT. A-AG) was suspended in 10 µl of the solubilization buffer, mixed with 5 µl of histone VS, and then 32P labeled as described under "Experimental Procedures." The samples were electrophoresed on a 10% SDS-polyacrylamide gel, and the 32P-labeled histone was visualized by autoradiography. The magnitudes of histone phosphorylation were determined by Cerenkov counting following excision of the 32P-labeled histone bands.

adsorbed by wheat germ agglutinin-agarose is almost totally depleted (95%) following immunoprecipitation of the plasma membrane detergent extracts with anti-insulin receptor serum (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 9). The small residual increase in histone kinase activity due to insulin after immunoprecipitation (Fig. 9, lanes 3 and 4) is most likely due to the incomplete removal of the insulin receptor by the receptor antisera as evidenced by the presence of a small amount of 32P-labeled insulin receptor β subunit remaining in the detergent extracts (Fig. 9, lane 4). Furthermore, the histone kinase activity that is stimulated by insulin is recovered in the immunoprecipitates (Fig. 9, lanes 7 and 8). In contrast, when anti-receptor immunoglobulins are omitted during immunoprecipitation of the plasma membrane detergent extracts no insulin-enhanced histone kinase activity can be detected in the Protein A-agarose immunocomplex (Fig. 9, lanes 5 and 6). The ability of the anti-insulin receptor antibodies to adsorb nearly all the insulin-stimulated tyrosine kinase activity in adipocyte plasma membranes suggests that the only membrane-associated tyrosine kinase that is activated by the hormone is the insulin receptor.

Effects of Insulin on the in Vivo Phosphorylation of Adipocyte Plasma Membrane Proteins—In order to investigate whether the insulin-stimulated P160 tyrosine phosphorylation can be detected in intact cells, adipocytes were labeled with [32P]orthophosphate for 2 h at 37°C followed by treatment with or without insulin. The adipocyte preparations are then homogenized in buffer containing phosphatase and protease inhibitors, and the 32P-labeled plasma membranes are isolated by differential centrifugation. The membrane proteins are resolved by SDS-polyacrylamide gel electrophoresis, and their phosphorylation patterns are visualized by autoradiography. As shown in Fig. 10A, no apparent increase or decrease in protein phosphorylation can be detected in the region expected for the P160 protein after allowing for background phosphorylation. The slight enhancement in background phosphorylation in the insulin-treated group is probably due to variation in carryover contamination by [32P]orthophosphate.

It is quite possible that the P160 protein may be a minor species in adipocyte plasma membranes so that its changes in phosphorylation state in response to insulin may be masked by the high level of background protein phosphorylation when whole 32P-labeled membranes are analyzed by gel electrophoresis. In order to circumvent this problem, we have attempted to immunoprecipitate the P160 phosphoprotein with monoclonal antiphosphotyrosine antibodies from plasma membrane of 32P-labeled adipocytes. As evidenced in Fig. 10B, a M5 = 92,000 32P-labeled species in membrane-derived insulin-treated adipocytes is isolated by the antiphosphotyrosine antibodies. Based on its immunoreactivity with patient-derived anti-insulin receptor immunoglobulins, the M5 = 92,000 phosphoprotein actually represents the β subunit of the insulin receptor (data not shown). Close examination of the autoradiogram reveals that insulin does not detectably alter the phosphorylation of other protein species around the P160 protein region in the gel. Taken together, these results suggest that the level of insulin-stimulated P160 phosphorylation may be too low to be detected in intact cells, perhaps due to rapid phosphate turnover. Alternately, the tyrosine-phosphorylated P160 may not be recognized by the antiphosphotyrosine antibodies because of steric or charge hindrance around the tyrosine phosphorylation sites.

DISCUSSION

The present study reports the stimulatory effect of insulin on the tyrosine phosphorylation of a plasma membrane-associated protein (P160) in rat adipocytes. A key feature in the design of experimental protocols used in this study is the inclusion of phosphatase inhibitors such as NaF and vanadate.
in the phosphorylation buffer such that the activities of essentially all the phosphatases present in the membrane are inhibited. Indeed, in control studies no dephosphorylation of \(^{32}\)P-labeled proteins can be detected when the phosphorylation reaction is terminated and chased with unlabeled ATP (data not shown). Thus, the \(^{32}\)P labeling of proteins under these conditions represents the actual magnitude of phosphorylation rather than the steady-state level of phosphate turnover. Most important, for some proteins the presence of phosphatase inhibitors amplifies the phosphorylation signal which would otherwise escape detection because of rapid dephosphorylation by phosphatases. The significance of this point is illustrated by the insulin-stimulated phosphorylation of P160 and insulin receptor \(\beta\) subunit as shown in Fig. 1. In the absence of phosphatase inhibitors, the stimulatory effects of insulin on these two protein species are barely detectable (data not shown).

The action of insulin on P160 appears to be very rapid (maximal after 2 min of incubation at 37°C) and is fully reversible following the removal of the hormone by anti-insulin serum (Fig. 4). Furthermore, the increase in P160 phosphorylation is highly specific for insulin and is not mimicked by other hormones such as epidermal growth factor and isoproterenol (Table I). Significantly, the tyrosine phosphorylation of P160 in adipocyte plasma membranes appears to be extremely sensitive to insulin. Half-maximal and maximal stimulation of P160 phosphorylation are achieved at \(2 \times 10^{-10}\) and \(10^{-8}\) M insulin, respectively. This hormonal response occurs well within the physiological range of the hormone concentrations and is indeed very similar to that of classic insulin responses such as hexose and amino acid transport and glycogen synthase activation. Together this body of evidence suggests that the insulin-stimulated tyrosine phosphorylation of P160 in plasma membranes of rat adipocytes represents a physiological event in cells treated with the hormone.

An interesting feature of the insulin-dependent tyrosine phosphorylation of P160 is that the stimulatory action of the hormone can only be detected in plasma membranes from cells which are exposed to insulin. Direct addition of the hormone to isolated membranes has no effect on the \(^{32}\)P labeling of this membrane-associated phosphoprotein, although the tyrosine phosphorylation of the insulin receptor \(\beta\) subunit is markedly enhanced under these conditions (Fig. 1). The reason for such a lack of direct action of insulin is unclear. Perhaps the insulin-stimulated tyrosine phosphorylation of P160 is dependent on other hormone-sensitive cellular processes which can only occur in intact adipocytes in response to the hormone. An example of this possibility is the \(M_r = 35,000\) subunit of the EGF receptor kinase in the A431 cell as described by Fava and Cohen (30). They reported that in isolated membrane systems the \(M_r = 35,000\) protein could only be phosphorylated by the EGF receptor kinase when it was derived from EGF-treated cells.

The precise mechanism mediating the stimulatory effects of insulin on P160 phosphorylation has yet to be delineated. Perhaps in a fashion analogous to the \(M_r = 35,000\) subunit for the epidermal growth factor receptor kinase (30), P160 may be directly phosphorylated by the insulin receptor kinase. This possibility is consistent with several observations reported here. First, there is a strong correlation between the activation of the insulin receptor kinase and P160 phosphorylation. Thus, the time courses of stimulation by insulin of insulin receptor kinase activity and P160 phosphorylation are indistinguishable from each other (Fig. 5). In addition, the time-dependent reversals of these two insulin-sensitive parameters by anti-insulin serum are also identical (Fig. 5). Furthermore, insulin-like agents such as concanavalin A and hydrogen peroxide which mimic insulin action on P160 phosphorylation also activate the insulin receptor kinase (Table I and Fig. 7). Second, the stoichiometry between the magnitudes of insulin receptor kinase activation and P160 phosphorylation is the same irrespective of the agents used to initiate these two responses. Thus, as shown in Figs. 6 and 7, the tyrosine phosphorylation of P160 is maximally stimulated when 25–28% of the insulin receptor kinase is activated by either insulin or hydrogen peroxide. Third, the evidences presented in Figs. 8 and 9 indicate that the only plasma membrane-bound tyrosine kinase that is activated by insulin is the insulin receptor. It is reasonable to suggest that if the increase in P160 phosphorylation is mediated through an elevation in tyrosine kinase activity in adipocyte plasma membranes, the insulin receptor kinase is perhaps the most likely candidate to serve such a role.

In view of the evidence that the insulin receptor is the sole detectable tyrosine kinase activated by insulin in adipocyte plasma membranes (Figs. 8 and 9), it can be argued that P160 is not phosphorylated by other membrane-associated tyrosine kinases stimulated indirectly as a result of insulin treatment. However, the possibility still remains that P160 is tyrosine phosphorylated by other membrane-bound tyrosine kinases distinct from the insulin receptor kinase as a result of substrate-directed changes in response to insulin. Such mechanisms may include intracellular movement of P160 to the target site of phosphorylation, the availability of essential cofactors for P160 phosphorylation following insulin treatment, or other insulin-mediated covalent modification of P160. It is also possible that the actual mechanism mediating the stimulatory action of insulin on P160 phosphorylation may involve a combination of one or more of the insulin-sensitive processes described above, including the activation of the insulin receptor kinase by the hormone. Perhaps a direct way to test whether P160 is indeed a substrate for the insulin receptor kinase is to investigate whether purified P160 can be directly phosphorylated by the receptor kinase in a reconstituted system. Studies are in progress in this laboratory to examine this important issue.

It can be argued that the increase in tyrosine phosphorylation of P160 in vivo in plasma membranes from insulin-treated adipocytes may result from hormone-induced dephosphorylation of this phosphoprotein in intact cells. Thus, more vacant tyrosine phosphate acceptor sites may become available for in vitro phosphorylation in plasma membranes from insulin-treated adipocytes when compared to control. However, as shown in Fig. 10, no apparent insulin-induced dephosphorylation of protein species can be detected in the region of the gel expected for P160 following electrophoretic resolution of whole plasma membranes and antiphosphotyrosine antibody immunopellets of membrane detergent extracts derived from \(^{32}\)P-labeled adipocytes. These results suggest that this alternate hypothesis is less likely. On the other hand, it is still possible that the analytic procedure employed in the present study may not be sensitive enough to detect such a hormone-induced change.

Whether or not P160 is indeed directly tyrosine phosphorylated by the insulin receptor kinase, it may be involved in signal transduction. This hypothesis is particularly attractive in light of the high sensitivity of P160 phosphorylation to insulin. If this hypothesis is correct, the phosphorylation of P160 may serve as an amplification step for the insulin signal because it can be maximally phosphorylated when only a
small percentage of insulin receptor kinase activity is activated by insulin.

The identity of P160 remains to be determined. It is unlikely that this phosphoprotein is related to the M1, = 185,000 protein (P185) which was shown to undergo increased tyrosine phosphorylation in rat Fao hepatoma cells in response to insulin (23). This conclusion is based on their differences in molecular weight and our results (Fig. 4) indicating that adipocyte P160 adsorbs to immobilized wheat germ agglutinin while hepatoma P185 does not (23). Furthermore, the dose-response relationships to insulin for these two phosphoproteins are quite different. The phosphorylation of P185 exhibits insulin dependence similar to that of the insulin receptor β subunit, whereas, as indicated in Fig. 6, P160 phosphorylation is much more sensitive to the hormone than the receptor subunit. In view of the lack of effects of EGF (Table I), P160 is unlikely to be the EGF receptor. P160 also appears to be unrelated to the c-fms and the neo proto-oncogene products because antisera to such oncogene-encoded polypeptides fail to immunoprecipitate this phosphoprotein (data not shown).

In the present study, we have been unable to detect the insulin-stimulated P160 tyrosine phosphorylation in intact adipocytes. These negative results do not necessarily invalidate the findings derived from in vitro studies. It is quite possible that insulin does stimulate the tyrosine phosphorylation of P160 in vivo. However, due to a rapid turnover in its phosphate content, the level of tyrosine phosphorylation may be too low to be detected above background by the gel electrophoresis method employed in the present study. Furthermore, the inability of antiphosphotyrosine antibodies to immunoprecipitate the P160 phosphoprotein from intact cells may be explained by the inaccessibility of the immunoglobulins to the recognition site, perhaps, due to steric or charge effects. Alternately, by using antibodies specific for the P160 protein molecule, it may be feasible to document the stimulatory effect of insulin on its tyrosine phosphorylation in intact adipocytes. Clearly such an approach will require the generation of antisera against the P160 protein. Attempts are in progress in this laboratory to pursue this important objective. The problem associated with demonstrating insulin-stimulated P160 phosphorylation in vivo highlights the significance of the in vitro approach in the present study. This is because by curtailing protein dephosphorylation with phosphatase inhibitors, the increase in phosphorylation signals of certain polypeptides in response to insulin can be greatly enhanced, thus facilitating the detection of these hormone-sensitive species.

In summary, our studies demonstrate the presence of a glycoprotein in adipocyte plasma membranes that is tyrosine phosphorylated in plasma membranes derived from intact adipocytes treated with low physiological concentrations of insulin. Further studies are now in progress to define a possible role of this phosphoprotein in the action of insulin.

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