An Endogenous Substrate for the Insulin Receptor-associated Tyrosine Kinase*

Robert W. Rees-Jones‡ and Simeon I. Taylor§

From the Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

(Received for publication, March 8, 1984)

Insulin binding to its receptor stimulates a tyrosine-specific protein kinase. This enzyme phosphorylates the insulin receptor, as well as a variety of exogenous substrates in vitro. In the present studies, we have identified an endogenous substrate for the insulin receptor-associated kinase. We studied insulin-stimulated protein phosphorylation in partially purified insulin receptor preparations from the livers of dexamethasone-treated rats. In this cell-free system, insulin stimulated the phosphorylation of its own receptor as well as of a phosphoprotein of apparent Mr = 120,000 (termed pp120). pp120 was not immunoprecipitated by three anti-receptor antisera, nor was the receptor immunoprecipitated by antisera raised against pp120, suggesting that pp120 is not antigenically related or tightly bound to the insulin receptor. Dose-response curves for receptor and pp120 phosphorylation stimulated by pork insulin were essentially identical, and showed the appropriate specificity (insulin > proinsulin) for a receptor-mediated event. Phosphoamino acid analysis revealed that insulin stimulated the incorporation of $^{32}$P predominantly into tyrosine residues of pp120. Casein, an artificial substrate for the insulin receptor kinase, competed with pp120 for insulin-stimulated phosphorylation. Phosphorylation of pp120 was rapid (half-maximal effect within 2 min at 24 °C) and, like receptor phosphorylation, was supported with Mn$^{2+}$ or Mg$^{2+}$ as divalent cation and ATP as the phosphate donor. While receptor autophosphorylation and artificial substrate phosphorylation were not altered by prior treatment of the rats with dexamethasone, insulin-stimulated pp120 phosphorylation was enhanced in preparations derived from dexamethasone-treated rats, suggesting an alteration of pp120, not the receptor, as a result of dexamethasone-treatment. Further studies of this newly identified endogenous substrate may help clarify the physiologic role of the insulin receptor-associated kinase.

The initial event in insulin action is binding to a specific receptor in the plasma membrane of the target cell (1). Recently, phosphorylation of the insulin receptor has been identified as an early biochemical event occurring after insulin binding (2-18). In a variety of cell-free systems, insulin stimulates a tyrosine-specific protein kinase which phosphorylates predominantly the $\beta$ (Mr = 95,000 daltons) subunit of the insulin receptor. Several lines of evidence suggest that this enzyme is part of the $\beta$ subunit: this subunit contains an ATP binding site (19-21); highly purified insulin receptor preparations retain the kinase activity (22, 23); and selective degradation of the $\beta$ subunit abolishes kinase activity (24, 25). Thus, the signal of insulin binding to the $\alpha$ subunit of its receptor (26-28) is transmitted to the activation of a protein kinase in the $\beta$ subunit.

The possibility has been raised that this tyrosine-specific kinase activity may mediate some or all of insulin’s biological actions. One mechanism for this would be the phosphorylation of cellular proteins by the tyrosine kinase. Compatible with this hypothesis is the demonstration that the insulin receptor-associated kinase is capable of phosphorylating a variety of exogenous substrates, including casein, histones, angiotensin II, actin, and synthetic tyrosine-containing polypeptides (10, 13, 15, 23, 29, 30). In addition, when studied in intact cells (4) and some cell-free systems (31), insulin binding stimulates receptor phosphorylation on serine as well as tyrosine residues. This raises the possibility of phosphorylation and activation of a serine kinase by the receptor-associated tyrosine kinase. The identification of cellular substrates for the insulin receptor’s tyrosine kinase might aid in the search for the physiologic role of this kinase activity.

During studies of insulin receptor kinase activity in dexamethasone-treated rats, we observed that insulin stimulated the phosphorylation of a protein with an apparent Mr of 120,000 (pp120). In this report, we present the initial characterization of this endogenous substrate for the tyrosine kinase associated with the insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male Sprague-Dawley rats weighing 200 g (Taconic Farms, Germantown, NY) were treated with dexamethasone, 1 mg subcutaneously daily, for four days prior to killing. Control animals were injected with normal saline. All animals had free access to food and water.

**Insulin Receptor Preparations**—Rats were decapitated, their livers were excised, and a microsomal fraction was obtained by differential centrifugation (32). The microsomes were solubilized in Triton X-100, and the insulin receptor was partially purified by affinity chromatography with wheat-germ agglutinin coupled to agarose (33). The

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address, Department of Medicine, Columbia-Presbyterian Medical Center, 622 West 168th Street, New York, NY 10032.

§ To whom correspondence should be addressed: National Institutes of Health, Building 10, Room 8N 250, Bethesda, MD 20205.

The abbreviations used are: pp120, insulin-stimulated phosphoprotein with an apparent Mr, 120,000; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaDODSO$_4$, sodium dodecyl sulfate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF1, insulin-like growth factor 1.
elutes (100-300 μg of protein/ml), containing partially purified insulin receptors, were stored at -80 °C until use.

The addition of protease inhibitors (1 mM N-ethylmaleimide, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, 0.1 inhibitor units/ml of a2 macroglobulin, 1000 KI units/ml of aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 0.8 mg/ml of bacitracin) to solutions during the insulin receptor preparation did not affect the subsequently observed pp120 phosphorylation.

Protein Phosphorylation—This was performed by minor modifications of previously described techniques (18). Receptor preparations (20 μl) were preincubated with hormones for 30 min at 24 °C in 75-μl solutions containing HEPES (50 mM, pH 7.6) and bovine serum albumin (15 μg/ml). Phosphorylation was initiated by adding 20 μl of a reaction mixture to give final concentrations of 50 μM [γ-32P]ATP (specific activity 70 Ci/mmol), 1 mM CTP, and 3 mM Mn2+ acetate. After 10 min at 24 °C, 75-μl aliquots were added to 25 μl of "stopping solution." (7) and 25 μl of 5 times electrophoresis sample buffer (8) and heated to 97 °C for 10 min.

Phosphoproteins were subsequently analyzed by NaDodSO4-polyacrylamide gel electrophoresis and autoradiography (18, 34). Phosphoprotein bands of interest were excised from the dried gels, and the proteins were separated by autoradiography. Phosphoamino acid standards were added to the proteins in partially purified insulin receptor preparations from the livers of dexamethasone-treated rats. In this cell-free system, insulin stimulated the phosphorylation of at least five endogenous proteins (Fig. 1, left). Four of these proteins were immunoprecipitated specifically by antiserum antibodies (Fig. 1, center). These included both the β (M, = 95,000) and α (M, = 135,000) subunits of the insulin receptor, as well as two minor receptor species (M, = 210,000 and 240,000) previously described (7, 11, 19, 31). The β subunit was the most heavily phosphorylated of these receptor proteins. Insulin also stimulated the phosphorylation of an additional protein (M, = 120,000) present in these receptor preparations (Fig. 1, left).

Unlike the receptor phosphoproteins, pp120 was not immunoprecipitated by antiserum antibodies (Fig. 1, center), suggesting that pp120 was not antigenically related or tightly bound to the insulin receptor. To further support this, we produced antisera to pp120 by injecting rabbits with the protein band cut from polyacrylamide gels (see "Experimental Procedures"). This antiserum specifically immunoprecipitated only pp120, and not the receptor phosphoproteins (Fig. 1, right). pp120 therefore appeared to be an endogenous protein unrelated to the insulin receptor, but phosphorylated in an insulin-stimulated manner.

To further demonstrate that pp120 was unrelated to the insulin receptor, we subjected the phosphoproteins to electrophoresis under both reducing and nonreducing conditions (Fig. 2). Electrophoresis under nonreducing conditions revealed a decrease in the amount of phosphorylated free β subunit and an increase in the amount of higher molecular weight phosphoproteins, as previously described for disulfide-linked insulin receptor oligomers (3, 22, 36). However, the amount of pp120 observed was similar in the presence and absence of reducing, suggesting that this protein is not disulfide-linked to itself or other proteins in its native state.

Finally, we performed phosphopeptide mapping (35) of the phosphoproteins present in our receptor preparations. Several phosphopeptides obtained from the β subunit were not seen in pp120, and vice versa (Fig. 3). Moreover, the phosphopeptides obtained from the two epidermal growth factor receptor proteins (at M, = 170,000 and 150,000 daltons) present in our preparations (15) also showed differences from those of pp120, suggesting that pp120 was not a degradation product of the EGF receptor. At the lower concentration of protease used at 3-week intervals with the same dose of antigen in incomplete Freund's adjuvant.

Phosphorylated insulin receptor preparations were immunoprecipitated, prior to electrophoresis, with antisera or the IgG preparation as previously described (7, 18), using anti-pp120 antisera at a 1:40 dilution, and the others at a 1:100 dilution.

Insulin-binding Determination—225I-Inulin binding to the lectin-purified insulin receptor preparations was determined as previously described (33).

Phosphopeptide Mapping—Peptide mapping of the major phosphopeptides present in the insulin receptor preparations was performed by the two-dimensional electrophoretic method of Border and Cret-tol-Järvinen (35). Insulin receptor preparations were phosphorylated by the protocol used for phosphoryoamino acid analysis, and the phosphoproteins were separated on a 7.5% NaDodSO4-polyacrylamide gel. After transfer of individual lanes to the top of the second dimension gel (4% stacking, 12 or 15% resolving), the phosphoproteins were digested with 2-30 μg of Staphylococcus aureus strain V8 protease (Miles Laboratories) during the stacking phase of the second gel (4 mA overnight). After completing the separating phase of the gel at 25 mA, the resulting phosphopeptides were visualized by autoradiography.

RESULTS

Insulin-stimulated Protein Phosphorylation—We studied the insulin-stimulated phosphorylation of endogenous proteins in partially purified insulin receptor preparations from the livers of dexamethasone-treated rats. In this cell-free system, insulin stimulated the phosphorylation of at least five endogenous proteins (Fig. 1, left). Four of these proteins were immunoprecipitated specifically by antiserum antibodies (Fig. 1, center). These included both the β (M, = 95,000) and α (M, = 135,000) subunits of the insulin receptor, as well as two minor receptor species (M, = 210,000 and 240,000) previously described (7, 11, 19, 31). The β subunit was the most heavily phosphorylated of these receptor proteins. Insulin also stimulated the phosphorylation of an additional protein (M, = 120,000) present in these receptor preparations (Fig. 1, left).

Unlike the receptor phosphoproteins, pp120 was not immunoprecipitated by antiserum antibodies (Fig. 1, center), suggesting that pp120 was not antigenically related or tightly bound to the insulin receptor. To further support this, we produced antisera to pp120 by injecting rabbits with the protein band cut from polyacrylamide gels (see "Experimental Procedures"). This antiserum specifically immunoprecipitated only pp120, and not the receptor phosphoproteins (Fig. 1, right). pp120 therefore appeared to be an endogenous protein unrelated to the insulin receptor, but phosphorylated in an insulin-stimulated manner.

To further demonstrate that pp120 was unrelated to the insulin receptor, we subjected the phosphoproteins to electrophoresis under both reducing and nonreducing conditions (Fig. 2). Electrophoresis under nonreducing conditions revealed a decrease in the amount of phosphorylated free β subunit and an increase in the amount of higher molecular weight phosphoproteins, as previously described for disulfide-linked insulin receptor oligomers (3, 22, 36). However, the amount of pp120 observed was similar in the presence and absence of reducing, suggesting that this protein is not disulfide-linked to itself or other proteins in its native state.

Finally, we performed phosphopeptide mapping (35) of the phosphoproteins present in our receptor preparations. Several phosphopeptides obtained from the β subunit were not seen in pp120, and vice versa (Fig. 3). Moreover, the phosphopeptides obtained from the two epidermal growth factor receptor proteins (at M, = 170,000 and 150,000 daltons) present in our preparations (15) also showed differences from those of pp120, suggesting that pp120 was not a degradation product of the EGF receptor. At the lower concentration of protease used...
**Substrate for the Insulin Receptor-associated Kinase**

4463

**Fig. 1.** Insulin-stimulated protein phosphorylation in insulin receptor preparations from dexamethasone-treated rats. Sprague-Dawley rats (200 g body weight) were treated with 1 mg of dexamethasone subcutaneously daily for 4 days prior to sacrifice. Insulin receptor preparations were prepared from hepatic microsomes by solubilization in Triton X-100 and lectin affinity chromatography, as described under "Experimental Procedures." Left, after preincubation (30 min, 24°C) in the absence (lane a) or presence (lane b) of insulin (10⁻⁷ M), the preparations were phosphorylated with [γ-³²P]ATP (10 min, 24°C) in the presence of Mn²⁺ (3 mM). Shown is an autoradiogram of phosphorylated receptor preparations electroeluted into the absence (lane a) or presence (lane b) of insulin (10⁻⁷ M). Center, autoradiogram of phosphorylated preparations (as in lane b) which were immunoprecipitated with control serum (lane c) or antireceptor antiserum B-10 (lane d) prior to electrophoresis. Similar results were obtained with antireceptor antisera B-2 and B-d. Although not well visualized here, the 210,000-dalton phosphoprotein was evident in the antireceptor antiserum immunoprecipitates with longer exposure of the autoradiograms. Even with overexposure of the autoradiograms, no pp120 was evident in the antireceptor antiserum immunoprecipitates. Right, autoradiogram of phosphorylated receptor preparations (as in lane b) which were immunoprecipitated with control serum (lane e) or anti-pp120 antisemum (lane f) prior to electrophoresis.

(Fig. 3, left), the α subunit of the receptor generated a single phosphopeptide not seen in pp120, and another which co-migrated with a phosphopeptide from both pp120 and the β subunit. At the higher concentration of protease, the α subunit was degraded completely (Fig. 3, right). Despite the use of multiple concentrations of protease and high specific activity labeling, no other phosphopeptides could be visualized from the α subunit.

**Mechanism of Insulin-stimulated pp120 Phosphorylation—** To determine whether insulin stimulated the phosphorylation of pp120 by a receptor-mediated process, we next studied the dose responses for procine insulin and proinsulin stimulation of receptor (β subunit) and pp120 phosphorylation. Pork insulin stimulated the phosphorylation of both proteins with essentially identical dose-response curves (Fig. 4). Maximal stimulation of phosphorylation (approximately 5-fold) was observed at 10⁻⁷ M insulin. Half-maximal phosphate incorporation into both proteins occurred at 10⁻⁸ M insulin. Pork proinsulin, which has less than 10% of insulin's affinity for the receptor (37), also stimulated the phosphorylation of both substrates. The dose-response curves for proinsulin were shifted approximately 30-fold to the right as compared to

**Fig. 2.** Effect of disulfide bond reduction on substrates for insulin-stimulated kinase activity (reducing versus nonreducing NaDodSO₄-polyacrylamide gel electrophoresis). Phosphorylated receptor preparations (as in Fig. 1, left) were denatured at 97°C in the presence (lanes a and b) and absence (lanes c and d) of β-mercaptoethanol (βME) and electrophoresed on 5% resolving gels.

**Fig. 3.** Phosphopeptide mapping of phosphoproteins. Phosphopeptide mapping of the major phosphoproteins in the lectin purified receptor preparations was performed as described under "Experimental Procedures." Left, the phosphoproteins were digested with 2 μg of S. aureus V8 protease, and the resulting phosphopeptides were separated on a 12% NaDodSO₄-polyacrylamide resolving gel. Right, the phosphoproteins were digested with 20 μg of protease and separated on a 15% gel. Shown are autoradiograms of the separated phosphopeptides. The positions of the original phosphoproteins are indicated by arrows at the top of the gels.
Preparations were preincubated with insulin and porcine proinsulin. Insulin receptor preparations were preincubated (30 min, 24 °C) with increasing concentrations of pork insulin (solid symbols) or porcine proinsulin (open symbols). Phosphorylation was performed for 10 min at 24 °C with [γ-32P]ATP as described under “Experimental Procedures.” The phosphoproteins were analyzed by electrophoresis and autoradiography. 32P incorporation into the β subunit of the insulin receptor (●, ○) and pp120 (▲, △) was determined by liquid scintillation of bands excised from the dry gel. The results shown represent means of three experiments, and are expressed as a per cent of the maximal phosphorylation observed for each protein (at 10−7 M pork insulin), which was 60, 64, and 36 units/μg of protein for the β subunit and 38, 41, and 20 units/μg of protein for pp120 (experiments 1, 2, and 3, respectively).

**FIG. 4.** Dose-response of protein phosphorylation stimulated with pork insulin and pork proinsulin. Insulin receptor preparations were preincubated (30 min, 24 °C) with increasing concentrations of pork insulin (solid symbols) or porcine proinsulin (open symbols). Phosphorylation was performed for 10 min at 24 °C with [γ-32P]ATP as described under “Experimental Procedures.” The phosphoproteins were analyzed by electrophoresis and autoradiography. 32P incorporation into the β subunit of the insulin receptor (●, ○) and pp120 (▲, △) was determined by liquid scintillation of bands excised from the dry gel. The results shown represent means of three experiments, and are expressed as a per cent of the maximal phosphorylation observed for each protein (at 10−7 M pork insulin), which was 60, 64, and 36 units/μg of protein for the β subunit and 38, 41, and 20 units/μg of protein for pp120 (experiments 1, 2, and 3, respectively).

**FIG. 5.** Phosphoamino acid analysis of insulin-stimulated phosphoproteins. The β subunit (left) and pp120 (right), phosphorylated in the absence or presence of insulin as indicated, were analyzed as described under “Experimental Procedures.” The positions of phosphoserine, phosphothreonine, and phosphotyrosine standards are indicated to the left of the autoradiogram and by dotted circles in the lanes. The result shown is representative of three experiments.

Those results suggested that insulin-stimulated phosphorylation of pp120 was mediated via the receptor-associated tyrosine kinase. In further support of this, we performed phosphoamino acid analysis on both the β subunit of the receptor and pp120 (Fig. 5). Under our conditions, tyrosine was the predominant amino acid phosphorylated in both substrates. Moreover, most of the stimulation by insulin was explained by an increase in phosphotyrosine content of both substrates. Minor increases were observed in phosphoserine and phosphothreonine contents of both substrates in response to insulin. Because of the low level of 32P incorporation into serine and threonine, the significance of this observation is unclear.

Finally, if pp120 were phosphorylated by the receptor-associated tyrosine kinase, other substrates for the kinase should be able to compete for pp120 phosphorylation. As expected, casein, which has been shown to serve as an artificial substrate for this kinase (15), competed effectively with pp120 for insulin-stimulated phosphorylation (Fig. 6). At the concentrations used in this experiment, casein did not appear to compete with β subunit phosphorylation.

**Characterization of pp120 Phosphorylation**—Phosphorylation of both the receptor and pp120 was rapid when assayed at 24 °C (Fig. 7). When stimulated with pork insulin (10−7 M), half-maximal incorporation of phosphate into pp120 was observed within 2 min, at a time when β subunit phosphorylation had reached its maximum. The lag in pp120 phosphorylation might be explained by the previous observation that auto-phosphorylation of the receptor enhances the activity of the receptor kinase towards other substrates (9). When assayed at 4 °C, pp120 phosphorylation proceeded more slowly and appeared less efficient relative to β subunit phosphorylation (Fig. 7).

When assayed at 24 °C, receptor and pp120 phosphorylation were supported with either Mn2+ or Mg2+ as the divalent cation in the reaction mixture (Fig. 8), as previously observed for the receptor itself (6, 12). However, the patterns of phosphorylation of both substrates were different with the two divalent cations. Increasing concentrations of Mg2+ enhanced predominantly insulin-stimulated kinase activity, while leav-
and with pork insulin
with dexamethasone-treated rats, but not in those with
nor-
tially, we observed the phosphorylation of pp120 in studies
caused by insulin relatively unchanged (Fig. 8). Such activa-
tion of the receptor kinase by Mn²⁺ has been observed previ-
ously (16).

Finally, both receptor and pp120 phosphorylation showed an absolute requirement for ATP as the phosphate donor, with other nucleotides (CTP and GTP) being ineffective (data not shown), as previously reported for the insulin receptor kinase (5, 7).

Effect of Dexamethasone on pp120 Phosphorylation—Initially, we observed the phosphorylation of pp120 in studies with dexamethasone-treated rats, but not in those with normal animals. However, in the present studies, by increasing the specific activity of the [γ-³²P]ATP and lengthening the exposure of autoradiograms, we were able to demonstrate insulin-stimulated phosphorylation of pp120 in both control and dexamethasone-treated rats. Receptor phosphorylation was quantitatively similar in normal and dexamethasone-
treated rats (Fig. 9, top panel, left) suggesting similar numbers of insulin receptors were present in the lectin purified preparations from both groups. In contrast, both basal and insulin-
stimulated phosphorylation of pp120 were increased in receptor preparations from dexamethasone-treated rats compared to the normals (Fig. 9, top panel, right).

This enhancement could be a result of either a change in the receptor itself (enhanced kinase activity towards other substrates) or in the substrate (increased substrate amount in our preparations and/or more phosphate acceptor sites). To distinguish between these possibilities, we compared the ability of these receptor preparations to phosphorylate two artificial substrates, (Glu 80-Tyr 20), and casein. Insulin-stimu-
lated phosphorylation of both substrates was similar in receptor preparations from normal and dexamethasone-treated rats (Fig. 9, bottom panel), suggesting that dexamethasone treatment did not increase the intrinsic activity of the insulin receptor-associated kinase. Thus, the enhanced phosphoryla-
tion of pp120 observed in receptor preparations from dexamethasone-treated rats reflects an alteration of pp120 as a substrate for the kinase, most likely an increased amount of pp120 recovered in our lectin purified preparations.

Fig. 7. Time course of insulin-stimulated protein phosphorylation. Insulin receptor preparations were preincubated (1 h, 4 °C) with pork insulin (10⁻⁷ M) and subsequently phosphorylated at 24 °C (top panel) or 4 °C (bottom panel). Aliquots were removed at the indicated times for determination of ³²P incorporation into the β (●) and α (○) subunits of the insulin receptor and into pp120 (▲).

Fig. 8. Divalent cation dependence of β subunit and pp120 phosphorylation. Receptor preparations were preincubated (30 min, 24 °C) in the presence (solid symbols) and absence (open symbols) of pork insulin (10⁻⁷ M). Phosphorylation was performed for 10 min at 24 °C with increasing concentrations of Mn²⁺ (top panels) or Mg²⁺ (bottom panels) and ³²P incorporation into the β subunit (left) and pp120 (right) was determined.

Fig. 9. Dexamethasone-treated rats show enhanced pp120 phosphorylation. Sprague-Dawley rats (200 g body weight) were given 1 mg of dexamethasone subcutaneously daily for 4 days prior to killing. Control rats received normal saline. Top panels, hepatic insulin receptor preparations were phosphorylated with [γ-³²P]ATP, and phosphate incorporation into the β subunit (left) and pp120 (right) were determined as described under “Experimental Procedures.” Bottom panels, hepatic insulin receptor preparations were assayed for insulin-stimulated phosphorylation of artificial substrates, (Glu 80-Tyr 20), (left) and casein (right). The results shown are mean ± S.E. for four pairs (control and dexamethasone) of animals. The results are expressed as phosphorylation units/³²P-insulin binding capacity of the individual receptor preparations, in order to minimize differences in phosphorylation resulting from differing numbers of insulin receptors in the individual preparations.
Possibilities for the Identity of pp120—The apparent molecular weight of pp120 suggested that pp120 might be either vinculin, which is known to be a substrate for other tyrosine kinases (38), or ATP citrate lyase, whose phosphorylation is stimulated by insulin (39). However, neither anti-vinculin IgG, nor anti-ATP citrate lyase antisera was capable of immunoprecipitating pp120 (data not shown).

DISCUSSION

Phosphorylation of pp120 by the Insulin Receptor—Previous investigations have shown that the tyrosine-specific protein kinase associated with the insulin receptor can phosphorylate the receptor and a variety of exogenous substrates (2–25). The present work identifies an endogenous protein, pp120, as a substrate for this kinase. This substrate is present in a partially purified insulin receptor preparation, derived from hepatic microsomes by solubilization in Triton X-100 followed by lectin affinity chromatography. Several lines of evidence demonstrate that the insulin receptor is responsible for the insulin-stimulated phosphorylation of pp120 as follows. 1) Pork insulin and proinsulin stimulated pp120 phosphorylation in parallel to receptor phosphorylation, and with potencies proportional to their binding affinities for the insulin receptor. 2) Insulin stimulated phosphorylation of tyrosine residues in pp120, as expected if mediated by the tyrosine-specific kinase associated with the receptor. 3) Insulin-stimulated phosphorylation of pp120 was competed for by casein, a known substrate for the receptor kinase. 4) Finally, the cation and nucleotide dependence of pp120 phosphorylation was similar to that for receptor phosphorylation. We conclude that the insulin-stimulated phosphorylation of pp120 is mediated by the tyrosine-specific protein kinase associated with the insulin receptor. Of course, we cannot rule out the possibility that another tyrosine kinase copurifies with the insulin receptor and is insulin-stimulated (perhaps via the receptor kinase) to phosphorylate pp120. This, however, seems less likely.

Identity of pp120—The identity of pp120 is not yet known. Its presence in a hepatic microsomal preparation suggests it is membrane-associated, either as an integral or peripheral membrane protein. It is soluble in Triton X-100 and is retained on a wheat-germ agglutinin column, raising the possibility that pp120 is a membrane glycoprotein. pp120 was not immunoprecipitated by several polyclonal antisera directed against the insulin receptor; furthermore, antiserum raised against pp120 did not immunoprecipitate the insulin receptor. pp120 thus appears unrelated to the receptor proteins present in these preparations. In addition, several lines of evidence suggest that pp120 is not a degradation product of the insulin receptor as follows: 1) the behavior of pp120 on electrophoresis under nonreducing conditions, 2) the extent of pp120 phosphorylation (compared to the higher molecular weight receptor phosphoproteins), and 3) the phosphopeptide maps obtained from the phosphoproteins.

Substrates for Other Tyrosine-specific Protein Kinases—As in the present work, others have previously identified membrane-associated proteins which serve as substrates for tyrosine-specific protein kinases. Substrates for the EGF receptor kinase (40, 41) and the PDGF-stimulated protein kinase (42) have been found in membrane preparations. Integral membrane proteins, such as erythrocyte band 3 (43) and human lymphocyte antigens (44), have been shown to be phosphorylated by tyrosine kinases. Furthermore, several cytoskeletal elements, including vinculin (38) and myosin (45), also serve as substrates. Of course, this localization of substrates may simply reflect the localization of the tyrosine kinases, which appear to be predominantly membrane-associated. Several are associated with plasma membrane receptors, including those for insulin, EGF, and PDGF as previously mentioned, and for IGFl (46, 47). The tyrosine kinase responsible for the transforming ability of Rous sarcoma virus (pp60\(^{c-src}\)) becomes membrane-attached, via its amino-terminal portion, after translation (48). Moreover, the majority of tyrosine kinase activity in normal tissues has been found in particulate fractions (49–52). So tyrosine phosphorylation (both kinases and substrates) appears to be centered largely around cell membranes. Thus, the existence of pp120 as a substrate for the insulin receptor kinase in a membrane glycoprotein preparation is entirely consistent with previous studies of other tyrosine kinases. However, pp120 does not appear to correspond to any of the previously described substrates for tyrosine kinases. pp120 does not appear to be vinculin or ATP citrate lyase, two proteins of similar molecular weight to pp120.

Regulation by Glucocorticoids—Dexamethasone treatment of the rats in this study increased the observed pp120 phosphorylation in our receptor preparations, most likely by increasing the amount of pp120 present in our lectin-purified receptor preparations. Studies of the effects of glucocorticoids on insulin binding to receptor and insulin action in humans and animals have documented a variety of effects, depending on such variables as the steroid used, length and dosage of administration, and tissue studied (53–59). These effects have included alterations in both receptor number and affinity, increases in receptor biosynthesis, and diminished insulin action at postreceptor sites. At present, we cannot define the relationship of pp120 phosphorylation to these events, or the exact mechanism of dexamethasone’s effect on pp120 in vivo.

Physiologic Role of the Receptor-associated Protein Kinase—Finally, the physiologic significance of the insulin receptor-associated tyrosine kinase is still unclear. Evidence has been presented that this enzyme may mediate the metabolic effects of insulin, as some agents which mimic insulin effects, such as vanadate (60) and trypsin (61), also stimulate the insulin receptor kinase. On the other hand, two monoclonal antireceptor antisera, which mimic insulin’s metabolic effects, did not stimulate the receptor kinase when assayed in a cell-free system (62) or in intact cells (63). Other proposed functions for the enzyme have included signaling in receptor biosynthesis (64) or in receptor down-regulation (24). With regard to the latter, an antireceptor antiserum which does not stimulate the receptor kinase (62, 63), was shown to be capable of inducing receptor down-regulation (65). Finally, in analogy to other tyrosine kinases, evidence has been presented suggesting that the insulin receptor kinase may be involved in regulation of cellular growth (66). Perhaps further study of pp120 phosphorylation will provide insight into the function of the insulin receptor-associated tyrosine kinase.

Acknowledgments—We thank Drs. Aidan McElduff, Phillip Gordon, and Jesse Roth for their helpful suggestions during the course of this work and for critical review of the manuscript, and Victoria Moncada for technical assistance.

REFERENCES
