Insulin Stimulation of the Insulin Receptor Kinase Can Occur in the Complete Absence of β Subunit Autophosphorylation*

Brian D. Morrison and Jeffrey E. Pessin‡

From the Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242

(Received for publication, June 5, 1986)

The glutamic acid:tyrosine (Glu:Tyr) synthetic polymer was observed to inhibit the insulin receptor β subunit autophosphorylation with an IC₅₀ of 0.20 mg/ml in the absence and 0.15 mg/ml in the presence of insulin. Even though complete blockade of β subunit autophosphorylation was observed at 4.0 mg/ml Glu:Tyr, insulin was still capable of stimulating the exogenous protein kinase activity of the insulin receptor toward Glu:Tyr. Ristone H2B (1.3 mg/ml) was also observed to inhibit the β subunit autophosphorylation by approximately 80% with an IC₅₀ of 0.31 and 0.35 mg/ml in the absence and presence of insulin, respectively. Similar to the results with Glu:Tyr, insulin was found to stimulate histone H2B phosphorylation under these conditions. Comparisons between the time courses of β subunit autophosphorylation with those of Glu:Tyr phosphorylation both in the presence and absence of insulin confirmed that insulin can stimulate the exogenous protein kinase activity of the insulin receptor in the complete absence of β subunit autophosphorylation.

Prephosphorylation of the insulin receptor (from 0 to 1.3 mol of phosphate/mol of insulin receptor) in the absence of insulin was found to have no significant effect on the exogenous protein kinase activity when assayed both in the presence and absence of insulin. Insulin was observed to stimulate the phosphorylation of Glu:Tyr approximately 3-fold independent of the extent of β subunit autophosphorylation. In contrast, prephosphorylation of the insulin receptors in the presence of insulin was observed to enhance the exogenous protein kinase activity dependent on the extent of autophosphorylation, such that by 1.4 mol of phosphate incorporated per mol of insulin receptor, insulin was found to maximally stimulate the initial rate of Glu:Tyr phosphorylation (approximately 9-fold). These results demonstrate that the insulin-dependent autophosphorylation of the insulin receptor results in an amplification of the insulin stimulation of the exogenous protein kinase activity, whereas the insulin-independent autophosphorylation does not.

Several growth factor receptors including those for insulin, insulin-like growth factor I, epidermal growth factor (EGF), and platelet-derived growth factor are all phosphoproteins containing phosphoserine, phosphothreonine, and phosphotyrosine in vitro (1-10). These receptors also share the common property of being hormone-stimulated protein kinases which specifically autophosphorylate on tyrosine residues in vitro (11-25). The functional consequences of the various specific receptor phosphorylation reactions with respect to growth factor receptor biological activity are fundamental issues which need to be elucidated to understand the molecular mechanism of transmembrane signal activation.

Autophosphorylation and pp60⁺⁻ kinase phosphorylation of the insulin receptor β subunit specifically on tyrosine residues in vitro have been correlated with the activation of the tyrosine protein kinase activity of the insulin receptor (26-28). Insulin treatment of rat adipocyte and H-35 hepatoma cells, followed by isolation of the insulin receptor kinase by insulin-agarose affinity chromatography, has demonstrated that tyrosine phosphorylation of the insulin receptor in vitro also results in the activation of the exogenous protein kinase activity of the insulin receptor in vivo results in the activation of the exogenous protein kinase activity of the insulin receptor in vitro (26, 30). Similarly, autophosphorylation of the insulin-like growth factor I receptor in the presence of insulin-like growth factor I has been observed to significantly activate its tyrosine specific protein kinase activity toward exogenous substrates (31, 32). Earlier studies of the tyrosine protein kinase activity of the Fujinami sarcoma and Rous sarcoma (pp60⁺⁻) viruses have also suggested that autophosphorylation may activate their respective kinase activities (33, 34). Recently, however, the tyrosine protein kinase activity of pp60⁺⁻ kinase was observed to be inhibited when the enzyme was phosphorylated on a carboxy-terminal tyrosine residue (35). In this regard, EGF-dependent autophosphorylation of the EGF receptor has been reported to have no effect (36, 37) as well as to activate (38) the exogenous protein kinase activity of this growth factor receptor.

In order to examine further the role of tyrosine-specific β subunit autophosphorylation on the activation of the exogenous tyrosine-specific protein kinase activity of the insulin receptor, we have made use of the observation that high concentrations of exogenously added substrates can effectively inhibit the β subunit autophosphorylation. In this communication, we demonstrate that insulin stimulation of the insulin receptor protein kinase activity can occur in the complete absence of β subunit autophosphorylation. Furthermore, prephosphorylation of the insulin receptor in the absence of insulin was observed to have no significant effect on the amount of insulin-stimulated exogenous protein kinase activity, whereas prephosphorylation in the presence of insulin...
dramatically increases the insulin-stimulated exogenous protein kinase activity of the insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Materials** — Triton X-100, Sephadex S-400, n-oclyl-β-D-glucopyranoside, protease inhibitors, alkaline phosphatase, and the Glu-Tyr (4:1) synthetic polymer were purchased from Sigma. [γ-32P]ATP (3000 Ci/mmol) and NCS tissue solubilizer were obtained from New England Nuclear and Amersham Corp., respectively. XAR-5 film and Cronex Lightning Plus intensifying screens were from Eastman Kodak and Du Pont. Reagents for SDS-polyacrylamide gel electrophoresis, No. 3MM filter paper, and histone H2B were purchased from Bio-Rad, Whatman, and Boehringer Mannheim, respectively. Mono-O[125I]iodoinsulin was kindly provided by the Diabetes and Endocrinology Research Center, The University of Iowa.

**Purification of Insulin Receptors** — Insulin receptors were purified to apparent homogeneity as previously described (39, 40). Briefly, membranes (10 mg/ml) prepared from freshly obtained human placenta (41) were solubilized for 1 h at 4°C in 0.25 M sucrose, 10 mM Tris, pH 8.0, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM benzamidine hydrochloride, 10 μM leupeptin, 50 trypsin inhibiting units aprotinin, 1 mM 1,10-phenanthroline, and 1 mM pepstatin A, with 2% Triton X-100. Following centrifugation at 104,000g for 1 h at 4°C, the soluble material was applied onto a 5 × 100-cm Sephacryl S-400 gel filtration column equilibrated with 50 mM Tris, pH 8.0, 0.1% Triton X-100, and 0.02% NaN3. The insulin binding activity eluted from the gel filtration column was directly applied to a 16 × 10-cm column of insulin coupled to Affi-Gel 10 (0.5 mg of insulin/ml of resin). The insulin receptor activity eluted from the gel filtration column was directly applied to a 16 × 10-cm column of insulin coupled to Affi-Gel 10 (0.5 mg of insulin/ml of resin). The insulin receptor activity was solubilized for 1 h at 4°C in 0.25 M sucrose, 10 mM Tris, pH 8.0, 10 mM NaCl, 0.6% n-oclyl-β-D-glucopyranoside. The insulin receptor was eluted from the resin with 50 mM sodium acetate, pH 8.0, 1.0 mg/ml G1u:Tyr was found to inhibit the insulin receptor protein kinase activity toward histone H2B under these conditions (Fig. 1B, lane 4). As observed for the Glu-Tyr synthetic polymer, partial blockade of the β subunit autophosphorylation by histone H2B did not significantly impair insulin from stimulating the insulin receptor protein kinase activity toward histone H2B under these conditions (Fig. 1B, lane 4).

In order to determine if the state of insulin receptor purity and/or the presence of Triton X-100 (as opposed to n-oclyl-β-D-glucopyranoside) would alter the ability of Glu-Tyr and histone H2B to inhibit the β subunit autophosphorylation, similar experiments were performed on purified insulin receptor preparations (Fig. 2). Although the degree of insulin-stimulated β subunit autophosphorylation (Fig. 2, A and B, lanes 1 and 3) was less than that observed for the partially purified insulin receptors (Fig. 1), both Glu-Tyr and histone H2B were capable of inhibiting the β subunit autophosphorylation (Fig. 2, A and B, lanes 2 and 4). Furthermore, insulin was still capable of activating the insulin receptor exogenous protein kinase activity toward Glu-Tyr and histone H2B (Fig. 2, A and B, lanes 2 and 4).

A summary of the effects of Glu-Tyr and histone H2B on the amount of 32P incorporated into the β subunit and into the exogenous substrates both in the presence and absence of insulin is presented in Table I. In the partially purified insulin receptor preparations, 1.0 mg/ml Glu-Tyr was found to inhibit the β subunit autophosphorylation 89 and 96% in the absence and presence of insulin, respectively. Under these conditions, insulin was observed to stimulate Glu-Tyr phosphorylation approximately 5-fold. Although 0.5 mg/ml histone H2B was only capable of inhibiting the β subunit autophosphorylation 73 and 68% in the absence and presence of insulin, respectively, the insulin stimulation of histone H2B phosphorylation was 2.5-fold. Similar results were also observed for the purified insulin receptor preparations even though the degree of insulin-stimulated Glu-Tyr (2.4-fold) and histone H2B (1.7-fold) phosphorylation was diminished. To quantitate further the effects of exogenous substrates on the β subunit autophosphorylation and exogenous protein kinase activities, the effect of increasing Glu-Tyr synthetic polymer concentrations on the purified human placental insulin receptor kinase was examined (Fig. 3). In the absence of insulin, the Glu-Tyr synthetic polymer completely inhibited
Autophosphorylation of the Insulin Receptor Kinase

FIG. 1. Inhibition of insulin receptor β subunit autophosphorylation by the Glu:Tyr synthetic polymer and histone H2B. Insulin receptors were partially purified by Sephacryl S-400 gel filtration and wheat germ agglutinin chromatography as described under “Experimental Procedures.” These insulin receptors were incubated with or without 200 nM insulin in the presence and absence of 1.0 mg/ml Glu:Tyr (A) or 0.5 mg/ml histone H2B (B) for 60 min at 23 °C. The reaction was initiated by the addition of [γ-32P]ATP (100 μM, 3 μCi/nmol) for 5 min and terminated by the addition of Laemmli sample buffer containing 100 mM dithiothreitol. The samples were run on a 7.0% SDS-polyacrylamide gel, stained, destained, and autoradiographed for 12 h.

FIG. 2. Inhibition of the purified insulin receptor β subunit autophosphorylation by the Glu:Tyr synthetic polymer and histone H2B. Purified insulin receptors (0.3 pg) were incubated with or without 200 nM insulin in the presence and absence of 1.0 mg/ml Glu:Tyr (A) or 0.5 mg/ml histone H2B (B) for 60 min at 23 °C. The samples were phosphorylated with [γ-32P]ATP, separated by SDS-polyacrylamide gel electrophoresis, and autoradiographed as described in Fig. 1.

TABLE I

Effects of Glu:Tyr and histone H2B on the protein kinase activity of the partially purified (wheat germ agglutinin (WGA)-Sepharose eluant) and purified (insulin-agarose eluant) human placental insulin receptor kinase

<table>
<thead>
<tr>
<th>Glu:Tyr</th>
<th>Histone H2B</th>
<th>Insulin</th>
<th>Insulin + Glu:Tyr</th>
<th>Insulin + histone H2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>20K</td>
<td>97K</td>
<td>66K</td>
<td>45K</td>
<td></td>
</tr>
</tbody>
</table>

The amounts of 32P incorporated into the β subunit and into 1.0 mg/ml Glu:Tyr or 0.5 mg/ml histone H2B in the absence and presence of insulin were determined from Figs. 1 and 2 as described under “Experimental Procedures.”

The insulin receptor β subunit autophosphorylation at 4.0 mg/ml, and half-maximal inhibition was observed at 0.20 mg/ml (Fig. 3B). Mirroring the inhibition of β subunit autophosphorylation, saturation of Glu:Tyr phosphorylation occurred at 1.0 mg/ml with half-maximal phosphorylation at 36 μM. Assuming an average Mr = 30,000 for the Glu:Tyr synthetic polymer, this corresponds to an apparent Kd of 1.2 μM and an IC50 of inhibition of β subunit autophosphorylation equal to 6.6 μM (Table II). In the presence of insulin, 4.0 mg/ml Glu:Tyr also completely inhibited the β subunit autophosphorylation with half-maximal occurrence at 27 pg/ml. Although there was essentially no β subunit autophosphorylation at saturating Glu:Tyr concentrations (4.0 mg/ml), insulin was observed to stimulate the protein kinase activity toward Glu:Tyr approximately 1.4-fold (compare Fig. 3A to 3B). This amount of insulin stimulation of the insulin receptor protein kinase activity is typical for purified insulin receptor preparations, particularly when assayed under long reaction time periods (43).

We next quantitated the effects of increasing concentra-
The apparent $K_m$ and $IC_{50}$ values in the presence and absence of insulin were determined as described in Figs. 3 and 4, assuming an average $M_r = 30,000$ for the Glu:Tyr synthetic polymer and $M_r = 12,000$ for histone H2B. These values represent the averages from three determinations for Glu:Tyr and two determinations for histone H2B with their respective standard errors of the mean.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$IC_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td>Glu:Tyr</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Histone H2B</td>
<td>6.0 ± 1.3</td>
<td>8.0 ± 1.9</td>
</tr>
</tbody>
</table>

Fig. 3. Concentration dependence of exogenous substrate phosphorylation and inhibition of insulin receptor β subunit autophosphorylation by Glu:Tyr. Purified insulin receptors (1.0 μg) were incubated in the presence (A) or absence (B) of 200 nM insulin with various Glu:Tyr concentrations for 60 min at 23 °C. The reaction was initiated by the addition of 100 μM [$\gamma$-32P]ATP (3 μCi/nmol) for 30 min and terminated as described in Fig. 1. The samples were run on a 7.0% SDS-polyacrylamide gel, stained, destained, and autoradiographed. The regions of the gel corresponding to the Glu:Tyr synthetic polymer (C) and insulin receptor β subunit (D) were excised, hydrated, solubilized in NCS, and counted for $^32$P incorporation. This is a representative experiment which was performed three times.

Fig. 4. Concentration dependence of exogenous substrate phosphorylation and inhibition of insulin receptor β subunit autophosphorylation by histone H2B. Purified insulin receptors (1.0 μg) were incubated in the presence (A) or absence (B) of 200 nM insulin with various concentrations of histone H2B for 60 min at 23 °C. The reaction was initiated by the addition of 100 μM [$\gamma$-32P]ATP for 30 min and terminated as described in Fig. 1. The quantitation of histone H2B phosphorylation (C) and β subunit autophosphorylation (D) was determined as described in Fig. 3. This is a representative experiment which was performed two times.

tions of histone H2B on the purified insulin receptor β subunit autophosphorylation and exogenous protein kinase activities (Fig. 4). At the highest concentration of histone H2B examined (1.3 mg/ml), the β subunit autophosphorylation both in the absence (Fig. 4B) and presence (Fig. 4A) of insulin was inhibited by approximately 80%. Although the extent of β subunit autophosphorylation was markedly diminished at these high concentrations of histone H2B, the exogenous protein kinase activity of the insulin receptor toward histone H2B was still insulin-responsive, approximately 1.6-fold (compare Fig. 4A to 4B). In the absence and presence of insulin, the half-maximal inhibition of the β subunit auto-

phosphorylation by histone H2B occurred at 310 and 350 μM/ml, respectively. Assuming an $M_r = 12,000$ for histone H2B, this corresponds to an $IC_{50}$ of inhibition of β subunit autophosphorylation in the absence and presence of insulin equal to 26 and 29 μM, respectively (Table II). Similar to that observed for the Glu:Tyr synthetic polymer, the apparent $K_m$ for histone H2B was approximately 4-5-fold less than the $IC_{50}$ of inhibition of β subunit autophosphorylation (Table II).

A time course of Glu:Tyr (1.0 mg/ml) phosphorylation and β subunit autophosphorylation in the absence and presence of insulin is shown in Fig. 5. In the presence of insulin, the exogenous insulin receptor protein kinase activity toward Glu:Tyr was found to be linear for approximately 15 min under these assay conditions. However, in the absence of insulin the receptor protein kinase activity was linear for the full length of the time course examined (Fig. 5A). In this experiment, the initial rate enhancement by insulin was observed to be approximately 5-fold. The addition of insulin (200 nM) to the untreated insulin receptors 5 min after the addition of [$\gamma$-32P]ATP resulted in the rapid stimulation (less than 1.0 min) of the exogenous protein kinase activity toward Glu:Tyr without any detectable lag period (Fig. 5, A and C). The initial rate enhancement by insulin, subsequent to insulin addition, was approximately 4-fold under these conditions.

In contrast, examination of the time course of β subunit autophosphorylation of the insulin receptor kinase under identical conditions demonstrated no measurable phospho-

ylation of β subunit in the absence of insulin (Fig. 5B). In the presence of insulin, a small amount of β subunit autophosphorylation was detected but was relatively insignificant for the first 15 min (less than 5.0% of the autophosphorylation which occurs in the absence of Glu:Tyr, Figs. 1–3). The addition of insulin to the untreated insulin receptors, 5 min
Fig. 5. Time course of exogenous substrate and insulin receptor β subunit autophosphorylation in the presence of the Glu:Tyr synthetic polymer. Purified insulin receptors (1.0 μg) were incubated in the absence (●) or presence (○) of 200 nM insulin plus 1.0 mg/ml Glu:Tyr for 60 min at 23 °C. The reaction was initiated by the addition of [γ-32P]ATP (100 μM, 3 μCi/nmol) and terminated at various times as described in Fig. 1. To one set of samples in the absence of insulin (●), 200 nM insulin was added 5.0 min subsequent to the addition of [γ-32P]ATP. The quantitation of Glu:Tyr phosphorylation (A) and insulin receptor β subunit autophosphorylation (B) was determined as described in Fig. 3. For clarity, an expanded time frame of Glu:Tyr phosphorylation and β subunit autophosphorylation is presented in C and D, respectively. This is a representative experiment which was performed two times.

subsequent to the addition of [γ-32P]ATP, did not result in any detectable β subunit autophosphorylation until 10 min after the addition of insulin (Fig. 5B). This is readily apparent when one expresses the data on an expanded time scale (Fig. 5D). In comparison, the ability of insulin to stimulate the phosphorylation of Glu:Tyr under these conditions was maximal within 1.0 min after the addition of insulin (Fig. 5C) and in the complete absence of β subunit autophosphorylation (Fig. 5D). These results clearly demonstrate that saturating insulin concentrations can stimulate the exogenous protein kinase activity of the insulin receptor in the complete absence of any additional β subunit autophosphorylation.

It has recently been observed that insulin receptors pretreated with ATP plus insulin have elevated exogenous protein kinase activity compared to insulin receptors pretreated with insulin in the absence of ATP (26, 27). We therefore examined the effects of β subunit autophosphorylation on the untreated and insulin-treated exogenous protein kinase activity of the insulin receptor (Fig. 6). A time course of β subunit autophosphorylation in the absence of exogenous substrates indicated an approximate 6-fold initial rate enhancement of β subunit autophosphorylation in the presence of insulin in this experiment (Fig. 6A). At the longest time point examined (60 min), the insulin receptor had incorporated approximately 3 mol of phosphate/mol of insulin receptor in the presence of insulin, whereas the insulin receptors in the absence of insulin had only incorporated approximately 1.3 mol of phosphate/mol of insulin receptor.

At various times subsequent to the addition of [γ-32P]ATP, corresponding to fixed extents of β subunit autophosphorylation, the insulin receptors were mixed with a saturating concentration of Glu:Tyr (1 mg/ml). As shown in Fig. 3, this concentration of Glu:Tyr inhibits any further β subunit autophosphorylation allowing the determination of the initial rate of Glu:Tyr phosphorylation as a function of mol of phosphate incorporated per mol of insulin receptor (Fig. 6B). Insulin receptors which were prephosphorylated in the absence of insulin (Fig. 6B) had a constitutive exogenous protein kinase activity that was completely unaffected by the extent of β subunit autophosphorylation. Similarly, the amount of insulin-stimulated Glu:Tyr phosphorylation from the insulin receptors prephosphorylated in the absence of insulin was also observed to be independent of the degree of β subunit autophosphorylation. In contrast, insulin receptors which were prephosphorylated in the presence of insulin became progressively more kinase-active as the extent of β subunit autophosphorylation increased (Fig. 6C). The effect of β subunit prephosphorylation in the presence of insulin resulted in an approximate 9.0-fold stimulation in the initial rate of Glu:Tyr phosphorylation compared to the 3-fold stimulation by insulin in the absence of prephosphorylation or for the insulin receptors prephosphorylated in the absence of insulin (Fig. 6, B and C).

These results become readily apparent when one expresses the data as the initial rate of insulin-stimulated Glu:Tyr phosphorylation as a function of the extent of insulin receptor autophosphorylation (Fig. 7). At the lowest extent of insulin-dependent insulin receptor β subunit autophosphorylation (0.14 mol of phosphate/mol of insulin receptor), the exogenous protein kinase activity was markedly activated compared to the nonphosphorylated insulin receptor preparation. The maximal insulin stimulation (9.0-fold) of the initial rate of Glu:Tyr phosphorylation occurred when the insulin receptors were prephosphorylated to approximately 1.4 mol of phosphate/mol of insulin receptor in the presence of insulin. However, insulin receptors which were prephosphorylated in the absence of insulin (up to a maximum of 1.3 mol of phosphate/mol of insulin receptor) did not exhibit any alteration in the insulin stimulation of Glu:Tyr phosphorylation. These results demonstrate that the insulin stimulation of the insulin receptor kinase is independent of the extent of the untreated insulin receptor autophosphorylation state and further that only β subunit autophosphorylation in the presence of insulin can maximally enhance the exogenous protein kinase activity of the insulin receptor.

It has been well established that β subunit autophosphorylation of the insulin receptor in cell-free systems occurs exclusively on tyrosine residues (11–19). However, in intact cells the insulin receptor is a phosphoprotein containing both phosphoserine and phosphothreonine in the absence of insulin (1–6). We therefore addressed the possible regulatory effects that the endogenous insulin receptor phosphorylation
Fig. 6. Effects of β subunit autophosphorylation on the exogenous protein kinase activity of the insulin receptor. A, insulin receptors were incubated with 100 μM ([γ-32P]ATP (3 μCi/nmol) for various times in the absence (○) and presence (●) of 200 nM insulin. The reaction was stopped by withdrawing aliquots (0.5 μg) and placing them into Laemmli sample buffer containing 100 mM dithiothreitol. B, aliquots of insulin receptors (0.5 μg) which were prephosphorylated for various times in the absence of insulin were removed and incubated with 1.0 mg/ml Glu:Tyr for 15 min in order to determine the initial rate of Glu:Tyr phosphorylation in the absence (△) and presence (●) of 200 nM insulin. The final [γ-32P]ATP concentration was 50 μM. C, aliquots of insulin receptors (0.5 μg) which were prephosphorylated for various times in the presence of insulin (200 nM) were removed and incubated for 15 min with 1.0 mg/ml Glu:Tyr in the presence (●) of 200 nM insulin. The final [γ-32P]ATP concentration was 50 μM. This is a representative experiment which was performed two times.

state may have on the activation of the insulin receptor kinase by the insulin-dependent tyrosine autophosphorylation (Table III). As previously observed (Fig. 6), insulin receptors which were not prephosphorylated demonstrated an approximate 2-fold increase in the insulin stimulation of Glu:Tyr phosphorylation in the presence of a saturating Glu:Tyr concentration (2.0 mg/ml). Prephosphorylation of the insulin receptor for 1 h in the presence of insulin resulted in a 10-fold stimulation of Glu:Tyr phosphorylation. These results are similar to those obtained when the insulin receptors were initially dephosphorylated with 5 units/ml of alkaline phosphatase for 60 min prior to autophosphorylation (Table III).

**FIG. 7.** The amount of insulin stimulation of the insulin receptor exogenous protein kinase activity as a function of the β subunit autophosphorylation state. The data from Fig. 6 are expressed as the amount of insulin stimulation of Glu:Tyr phosphorylation from insulin receptors prephosphorylated in the absence of insulin (○) (Fig. 6, △-△) and insulin receptors prephosphorylated in the presence of 200 nM insulin (●) (Fig. 6, △-△) compared to the mol ratio of phosphate incorporated into the insulin receptor αβ2 complex (M,= 400,000).

**TABLE III**

<table>
<thead>
<tr>
<th>Glu:Tyr phosphorylation</th>
<th>Second incubation</th>
<th>Untreated</th>
<th>Alkaline phosphatase-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
<td>Control</td>
</tr>
<tr>
<td>No prephosphorylation</td>
<td>288 ± 28</td>
<td>616 ± 23</td>
<td>301 ± 29</td>
</tr>
<tr>
<td>Prephosphorylation in</td>
<td>2913 ± 42</td>
<td>2492 ± 20</td>
<td>the presence of insulin</td>
</tr>
</tbody>
</table>

After alkaline phosphatase treatment, a 2-fold increase in the insulin stimulation of Glu:Tyr phosphorylation was also observed for the insulin receptor preparations which were not prephosphorylated. Furthermore, the alkaline phosphatase-treated insulin receptors also demonstrated an 8-fold increase in the insulin stimulation of Glu:Tyr phosphorylation when the insulin receptors were prephosphorylated in the presence of insulin. These data suggest that the endogenous phosphorylation state of the insulin receptor does not significantly influence the activation of the insulin receptor kinase by insulin-dependent autophosphorylation.

**DISCUSSION**

It has been suggested that the phosphorylation of cellular substrates by the insulin receptor kinase might be the initial event in the transmission of the insulin signal (13, 26). The effect of insulin on the protein kinase activity of the insulin receptor toward exogenous substrates has been mainly studied in cell-free systems. These studies have indicated that insulin stimulates the tyrosine-specific autophosphorylation of the insulin receptor on its β subunit, which results in the activation of its exogenous protein kinase activity (26, 27). More recently, in intact cells, isolation of the insulin receptors from insulin-treated or control cells also demonstrated that the
tyrosine-specific β subunit autophosphorylation in vivo results in an activated insulin receptor protein kinase in vitro (29, 30). These data have been interpreted to suggest that 1) insulin does not directly activate the insulin receptor kinase toward exogenous substrates but instead first activates the autokinase activity toward tyrosine residues of its own β subunit and 2) the autophosphorylated β subunit of the insulin receptor is required for the activation of the protein kinase activity toward exogenous substrates.

To investigate further the role of β subunit autophosphorylation on the protein kinase activity of the insulin receptor in vitro, we have made use of the observation that the high concentrations of the exogenous protein substrates, Glu:Tyr and histone H2B, can effectively inhibit the β subunit autophosphorylation (Figs. 1 and 2). We have observed that insulin is capable of stimulating the insulin receptor protein kinase activity toward these exogenous substrates in the complete absence (Fig. 3) or 80% blockade (Fig. 4) of the β subunit autophosphorylation. Interestingly, the apparent $K_a$ values for both the Glu:Tyr synthetic polymer and histone H2B demonstrate an approximately 4–5-fold decrease compared to their respective $I_C^{50}$ values of inhibition of β subunit autophosphorylation (Table II). This suggests that the exogenous substrates, Glu:Tyr and histone H2B, have Michaelis-Menten constants ($K_a$) which are probably not equivalent to the substrate binding constant ($K_a$). Studies are currently in progress to determine the individual rate and binding constants as well as the order of substrate binding and product release. It should be pointed out that one cannot examine the effect of exogenous substrates as a function of β subunit concentration, in order to determine the $K_a$, since the autophosphorylation of the β subunit is an intramolecular event (40, 44).

The ability of saturating concentrations of insulin to stimulate the insulin receptor protein kinase activity toward Glu:Tyr (1.0 mg/ml) is extremely rapid, with maximal rate enhancement occurring within 1.0 min after the addition of insulin (Fig. 5, A and C). However, under identical conditions, β subunit autophosphorylation was not observed until 10 min after the addition of insulin (Fig. 5, B and D). These data (Figs. 1–5) demonstrate conclusively that insulin can stimulate the exogenous protein kinase activity of the insulin receptor even in the complete absence of β subunit autophosphorylation. In contrast, a previous study has been interpreted to suggest that substrate phosphorylation (phosphorylation of reduced and carboxyamidomethylated lysozyme) is strictly dependent upon prior β subunit autophosphorylation (45). However, these studies are not directly comparable since a simultaneous incubation with saturating exogenous substrate in the presence and absence of insulin before the addition of [γ-32P]ATP was not performed.

Consistent with previous reports (26, 27), insulin receptors which were autophosphorylated in the presence of insulin were also observed to be markedly stimulated (approximately 9-fold) in their ability to phosphorylate the exogenous substrate Glu:Tyr (Fig. 6). However, the insulin receptors which were autophosphorylated in the absence of insulin displayed exogenous protein kinase activities that were essentially indistinguishable from the nonphosphorylated insulin receptors, even at relatively high extents of β subunit autophosphorylation (1.5 mol of phosphate/mol of insulin receptor). In contrast, insulin receptors autophosphorylated in the presence of insulin even at relatively low extents of autophosphorylation (0.14 mol of phosphate/mol of insulin receptor) were observed to be maximally activated toward exogenous substrate phosphorylation (Fig. 7). Similarly, only insulin-dependent β subunit autophosphorylation of the insulin receptor has been observed to activate the insulin receptor kinase activity toward reduced and carboxyamidomethylated lysozyme (45). These results indicate that autophosphorylation of the β subunit in an insulin-independent manner is kinetically distinguishable from the insulin-dependent β subunit autophosphorylation which results in maximal enhancement of the exogenous protein kinase activity of the insulin receptor. This further suggests that the insulin stimulation of the insulin receptor kinase under physiological conditions probably occurs as a result of an amplification cascade. Thus, we postulate that insulin binding stimulates the exogenous substrate protein kinase activity as well as β subunit autophosphorylation in vivo. However, it is the insulin-dependent autophosphorylation of the β subunit, presumably on a preferred site(s), that in turn allows maximal activation of the exogenous protein kinase activity of the insulin receptor by insulin.

In the intact cell, the insulin receptor has been found to be devoid of any phosphorylserine in the absence of insulin (1–6), although in cell-free systems autophosphorylation occurs exclusively on tyrosine residues (11–19). However, it is possible that the activation of the insulin receptor protein kinase activity by the insulin-dependent autophosphorylation observed in these experiments may have been modified due to the endogenous serine and threonine phosphorylation state of the purified insulin receptor preparations. In order to eliminate this possibility, insulin receptors were treated with alkaline phosphatase under conditions previously shown to dephosphorylate the insulin receptor (26, 27, 30). These alkaline phosphatase-treated insulin receptors displayed similar protein kinase activation by the insulin-dependent autophosphorylation (Table III). These results suggest that any possible heterogeneity in the endogenous phosphorylation state of these purified insulin receptor preparations did not influence the insulin-dependent activation by tyrosine-specific autophosphorylation.

The EGF-dependent autophosphorylation of the EGF receptor in vitro has been reported to activate (38) or to have no effect on its tyrosine-specific protein kinase activity toward exogenous substrates (36, 37). Although the effects of autophosphorylation on the EGF receptor kinase have not been clearly defined, the activation of the EGF receptor protein kinase has been interpreted to indicate that the EGF-dependant autophosphorylation may remove an inhibitory constraint of the receptor such that exogenous substrates can have greater access to the substrate binding region of the enzyme (38). This was suggested to be analogous to the autophosphorylation of the type II cyclic AMP-dependent protein kinase in which the regulatory and catalytic subunits associate, in part, via a substrate-like domain of the regulatory subunit (46, 47). The autophosphorylation of the regulatory subunit prevents this association and thus favors the formation of free catalytic subunits which can then express exogenous protein kinase activity (48).

In this regard, it has recently been reported that the EGF receptor kinase may exist as a kinase-inactive, noncovalently associated homodimer (49). The binding of EGF was reported to convert the inactive homodimer to a kinase-active monomer state. Although the intramolecular mechanisms of kinase activation by hormone-dependent autophosphorylation have not been elucidated to date, these data would suggest that subunit interactions are intimately involved. Assuming that the αβ heterodimeric form of the insulin receptor is a functional kinase unit, equivalent to the monomeric EGF receptor, then the insulin-dependent autophosphorylation of
the β subunit may alter these hypothesized β subunit interactions, whereas the insulin-independent autophosphorylation of the β subunit does not. Currently, we are testing this model with respect to the different consequences of β subunit autophosphorylation on the interaction of the insulin receptor subunits.

Acknowledgments—We wish to thank Dr. Laurel J. Sweet for helpful discussions during the course of these studies. We also thank Patsy McAtee and Mara O’Connell for their assistance in the preparation of this manuscript.

REFERENCES