Autophosphorylation within Insulin Receptor β-Subunits Can Occur as an Intramolecular Process†

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ABSTRACT: The insulin receptor is a complex membrane-spanning glycoprotein composed of two α-subunits and two β-subunits connected to form an $\alpha_2\beta_2$ holoreceptor. Insulin binding to the extracellular α-subunits activates intracellular β-subunit autophosphorylation and substrate kinase activity. The current study was designed to differentiate mechanisms of transmembrane signaling by the insulin receptor, specifically whether individual β-subunits undergo cis- or trans-phosphorylation. We compared relative kinase activities of trypsin-truncated receptors, αβ-half receptors, and $\alpha_2\beta_2$ holoreceptors under conditions that allowed us to differentiate intermolecular and intramolecular events. Compared to the insulin-stimulated holoreceptors, the trypsin-truncated receptor undergoes autophosphorylation at similar tyrosine residues and catalyzes substrate phosphorylation in the absence of insulin at a comparable rate. The truncated receptor sediments on a sucrose gradient at a position consistent with a structure comprising a single β-subunit attached to a fragment of the α-subunit and undergoes autophosphorylation in this form in the absence of insulin. Autophosphorylation of the truncated insulin receptor is independent of receptor concentration, and immobilization of the truncated receptor on a matrix composed of an anti-receptor antibody bound to protein A–Sepharose diminishes neither autophosphorylation nor receptor-catalyzed substrate phosphorylation. Therefore, true intramolecular (cis) phosphorylations, which occur within individual β-subunits derived from trypsin-truncated receptors, lead to kinase activation. However, insulin-stimulated autophosphorylation of insulin receptor αβ heterodimers is concentration-dependent, and both autophosphorylation and kinase activity are markedly reduced following immobilization. Therefore, interactions between isolated αβ heterodimers are necessary for high-affinity insulin binding, rapid autophosphorylation, and kinase activation. In combination, these data suggest that transmission of the insulin signal requires integrity of the $\alpha_2\beta_2$ structure, even though individual β-subunits have intrinsic kinase activity. While cis-phosphorylations can occur and may be the initiators of kinase activation, we cannot eliminate the importance of trans contributions to kinase activation.

In delineating mechanisms of insulin action, a major question has been how the insulin binding signal is transmitted through the receptor to other intracellular effector molecules. While an overall understanding of these events has remained enigmatic, selected molecular details are understood. Structurally, the insulin receptor is a membrane-spanning glycoprotein composed of two α-subunits and two β-subunits (Massague et al., 1981; Massague & Czech, 1982). The α-subunits are entirely extracellular and participate in insulin binding (Yip et al., 1978); the β-subunits contain extracellular, transmembrane, and intracellular kinase domains (Kasuga et al., 1982a; Ullrich et al., 1985; Ebina et al., 1985). The insulin binding site of the insulin receptor has been localized to the amino-terminal halves of the α-subunits by cross-linking studies (Yip et al., 1988; Waugh et al., 1989; Wedekind et al., 1989; DeMeyts et al., 1990). Although specific residues have not been identified, regions of the receptor that may participate in insulin binding include segments 20–120 (Wedekind et al., 1989) and 243–251 (Yip et al., 1988; Rafaelof et al., 1989). Little is known about structural changes that accompany insulin binding, although an immunochemically detectable conformational change may occur (Baron et al., 1990). Truncation studies suggest that the α-subunits exhibit an inhibitory influence over the β-subunit kinase domains of the receptor (Ellis et al., 1987; Shoelson et al., 1988; Herrara et al., 1988; Hsuan et al., 1989).

The intracellular kinase domain of the receptor can be separated into ATP binding, tyrosine autophosphorylation, and substrate binding domains. Insulin binding enhances the rate of receptor autophosphorylation (Kasuga et al., 1982a,b; Petruzzelli et al., 1984), which in turn accelerates the rate of substrate phosphorylation by the receptor (Rosen et al., 1983; Yu & Czech, 1984; White et al., 1985; Rees-Jones & Taylor, 1985; Kwok et al., 1986). The major sites of autophosphorylation are at tyrosine residues 1158, 1162, 1163, 1328, and 1334[1] (Tornqvist et al., 1987, 1988a,b; White et al., 1988; Tavare et al., 1988; Tavare & Denton, 1988). These phosphorylations occur sequentially, with phosphorylation of all three tyrosines in the 1160 region correlating best with full activation of the substrate kinase (White et al., 1988; Tavare

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[1] The numbering sequence for the insulin receptor described by Ebina et al. (1985) was used in this paper. To compare to the sequence described by Ullrich et al. (1985), subtract 12 from the residue number of β-subunit residues only.

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& Denton, 1988; Flores-Riveros et al., 1989). Autophosphorylation of the insulin receptor results in a conformational change which can be detected immunochemically (Herrera et al., 1986; Perlman et al., 1989).

In the current study, we attempt to further delineate how the insulin binding signal is propagated through the receptor, specifically asking whether each β-subunit phosphorylates itself in response to insulin binding (cis) or whether the β-subunits trans-phosphorylate one another. This question has been addressed previously with truncated versions of the receptor that were constructed by using in vitro mutagenesis and expression in transfected cell systems. However, with very similar constructs, two groups came to diametrically opposite conclusions (Herrera et al., 1988; Cobb et al., 1989; Villalba et al., 1989). As a correct answer is crucial to our overall understanding of insulin signaling, we have attempted to clarify this important issue by conducting experiments using fragments of *native* receptors derived by limited proteolysis (Shoelson et al., 1988) and limited reduction (Boni-Schnetzler et al., 1986, 1987, 1988; Sweet et al., 1987a,b; Morrison et al., 1988).

Treatment of intact cells with trypsin activates insulin-like bioeffects (Kuo et al., 1967; Kono, 1971; Kono & Barham, 1971; Kikuchi et al., 1981) which have been correlated with activation of the insulin receptor kinase (Tamura et al., 1983; Leef & Larner, 1987; Shoelson et al., 1988; Hsuan et al., 1989). The kinase-active fragment of insulin receptor, isolated following proteolytic treatment of intact cells, solubilization, and WGA purification, comprises an intact β-subunit attached by disulfide linkage to a small fragment of the α-subunit (Shoelson et al., 1988; Xu et al., 1990). Along with losing the bulk of the α-subunits, these fragments lose the ability to bind insulin and concomitantly become constitutively activated toward autophosphorylation and substrate phosphorylations. On the other hand, $\alpha\beta$-half receptors, which are functionally inactive as kinases, are prepared by mild reduction of the $\alpha_2\beta_2$ holoreceptor (Boni-Schnetzler et al., 1986, 1987, 1988; Sweet et al., 1987a,b; Morrison et al., 1988).

By comparing the relative abilities of the $\alpha_2\beta_2$ heterotetramer, the $\alpha\beta$ heterodimer, and the trypsin-truncated receptor forms to undergo intermolecular and intramolecular kinase reactions, we have found that insulin-stimulated kinase activation requires an intact $\alpha_2\beta_2$ heterotetrameric structure but the kinase phosphorylation per se can occur within the isolated β-subunits of truncated receptors. While our data cannot exclude trans contributions to phosphorylation in the intact receptor, kinase activation can occur as a true intramolecular process within isolated β-subunits.

Experimental Procedures

Materials. Tissue culture plasticware was from NUNC, and fetal bovine serum and RPMI medium were from Gibco. [γ-^{32}P]ATP was from New England Nuclear, *N*-tosylphenylalanine chloromethyl ketone treated trypsin was from Cooper Biochemicals, porcine insuline was from Elanco, and P81 phosphocellulose paper was from Whatman. Reagents for electrophoresis and the Bradford protein assay were purchased from Bio-Rad, and HEPES, aprotinin, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, *N*-acetylglucosamine, and dithiothreitol were obtained from Sigma. Pansorbin was from Calbiochem, wheat germ agglutinin–agarose (WGA) was from Vector, and protein A–Sepharose was from Pharmacia.

Preparation of Intact and Truncated Insulin Receptor from Fao Hepatoma Cells. Intact and modified insulin receptor preparations from Fao hepatoma cells were purified as reported previously (Shoelson et al., 1988). Briefly, tissue culture media were removed from confluent plates of Fao hepatoma cells; the cells were washed with phosphate-buffered saline (PBS) and either treated with 0.5 mg/mL (20 μM) trypsin in PBS at 4 °C for 30 min (truncated) or maintained in PBS without trypsin. All cells were then washed with 0.5% soybean trypsin inhibitor in PBS and solubilized with 1% Triton X-100 in 50 mM HEPES. Following centrifugation at 100000*g* for 1 h to remove insoluble material, the cell extracts were passed over columns of wheat germ agglutinin–agarose, and the columns were washed extensively with 0.1% Triton X-100 in 50 mM HEPES, pH 7.4 (buffer A). Bound glycoproteins were eluted with 0.25 M *N*-acetylglucosamine in buffer A, and protein concentrations of the eluates were determined by Bradford protein assay (Bio-Rad). Equivalent numbers of cells were either solubilized or treated with trypsin prior to solubilization. As the truncated receptors do not bind insulin, quantitations were relative rather than absolute. Aliquots of intact and trypsin-truncated receptor were stored at -70 °C for up to 1 month without significant loss of activity.

Preparation of $\alpha\beta$ Heterodimers from Human Placental Insulin Receptors. $\alpha\beta$ dimers were prepared as decribed before (Boni-Schnetzler et al., 1987, 1988). Briefly, microsomal membranes prepared from human placental tissue were treated with 1.25 mM DTT in 75 mM Tris buffer (adjusted to pH 8.5 with hydrochloric acid) for 30 min at room temperature. The membranes were washed with excess HEPES buffer to remove the DTT, solubilized with 1% Triton X-100, and centrifuged to remove insoluble material. Partially purified glycoproteins were obtained following affinity chromatography on WGA–agarose.

Velocity Sedimentation. Velocity sedimentation was performed in a linear 5–25% sucrose gradient containing 30 mM HEPES, 0.1% Triton X-100, and 0.02% sodium azide, adjusted to pH 7.6. Receptor preparations (250 μL) were layered over 4-mL gradients and centrifuged for 16 h at 180000*g* at 4 °C in a Beckman SW 56 rotor (Boni-Schnetzler et al., 1986). Fractions (100 μL) were collected from the bottom of the gradient. Aliquots (25 μL) of every other fraction were incubated with ($\alpha_2\beta_2$ and $\alpha\beta$) or without (truncated) 100 nM insulin and reacted with 25 μM [γ-^{32}P]ATP (25 μCi) and 5 mM $MnCl_2$ for 10 min at 22 °C. Reactions were terminated with sample buffer (Laemmli, 1970), and phosphorylated proteins were separated by SDS–polyacrylamide electrophoresis.

Receptor Dilution Studies. WGA-purified, trypsin-truncated insulin receptor (0.6 mg of protein/mL) was diluted with 50 mM HEPES buffer (pH 7.4) containing 0.1% Triton X-100 and 0.25 M *N*-acetylglucosamine. Each of the receptor dilutions (40 μL) was combined with 10 μL of 125 μM [γ-^{32}P]ATP (30 μCi) and $MnCl_2$ (5 mM final concentration) and allowed to react at 22 °C for 2, 5, and 10 min (the increase in autophosphorylation was nearly linear over this time). Reactions were terminated by adding 1 mL of 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, 2 mM sodium vanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 5 mM EDTA (stopping solution). The phosphorylated insulin receptor was immunoprecipitated by incubation with 2 μg of anti-phosphotyrosine antibody (Pang et al., 1985) for 8 h at 4 °C, immobilization on Pansorbin (10%, 50 μL), and washing of the precipitated receptor–antibody–Pansorbin complex 2 times with buffer A. Phosphoproteins were eluted from the Pansorbin with sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on

7.5% resolving gels, and identified by autoradiography. Initial rates, expressed as relative incorporation, were quantified by scanning the autoradiographs (Hoeffer, Model GS 300).

Dilution studies with $\alpha\beta$ dimers were performed in an analogous fashion with the following exceptions. Phosphorylation reactions were allowed to proceed for 2, 4, 6, 8, and 10 min and terminated by addition of sample buffer (Laemmli, 1970) containing 35 mM *N*-ethylmaleimide rather than DTT (Boni-Schnetzler et al., 1986). Phosphorylated proteins were separated by SDS–PAGE on 3–10% resolving gels and quantified following excision of the bands from the gel and counting Cerenkov radiation.

Immobilization of Receptors on Antibody–Protein A–Sepharose. Anti-peptide antisera, generated against the carboxyl-terminal 1326–1336 sequence of the insulin receptor, were prepared as described (Perlman et al., 1989). For experiments conducted here, the antibody (50 μg) was incubated with 200 μL (packed volume) of protein A–Sepharose (Pharmacia) for 8 h at 4 °C, and the antibody–protein A–Sepharose was washed with 10 mL of buffer A. A 50% slurry of the antibody–protein A–Sepharose (40 μL) was combined with trypsin-treated or untreated WGA-purified receptor (24 μg of protein/40 μL), incubated overnight at 4 °C, and washed extensively with buffer A. The receptor–antibody–protein A–Sepharose complex was aliquoted into microfuge tubes for autophosphorylation and substrate kinase assays.

Immobilized Receptor Autophosphorylation and Substrate Kinase Assays. Equivalent amounts of immobilized trypsin-treated or untreated receptor were incubated with or without 100 nM insulin for 1 h at 4 °C. For autophosphorylation reactions, immobilized receptor aliquots were reacted with 25 μM [γ-^{32}P]ATP (15 μCi) and 5 mM $MnCl_2$ for 60 min at 22 °C. Reactions were terminated by adding 1 mL of stopping solution, phosphorylated insulin receptor–antibody–protein A–Sepharose complexes were centrifuged, supernatant solutions were discarded, and the precipitated complexes were washed 2 additional times with stopping solution. Phosphoproteins were eluted from the protein A with sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol, separated by SDS–polyacrylamide gel electrophoresis on 7.5% resolving gels, and identified by autoradiography. For substrate phosphorylations, aliquots (20 μL) of immobilized receptors were incubated 1 h with and without 100 nM insulin and prephosphorylated for 60 min at 22 °C with 25 μM ATP and 5 mM $MnCl_2$. The immobilized receptor was then combined with 0.4 mM peptide 1142–1153 (Stadtmauer & Rosen, 1986; Shoelson et al., 1988), 25 μM [γ-^{32}P]ATP, and 5 mM $MnCl_2$ in buffer A. Substrate phosphorylation reactions were allowed to proceed for 2 min at 22 °C and terminated with 50 μL of 5% trichloroacetic acid. Incorporated phosphate was determined by a modification (Shoelson et al., 1988) of the phosphocellulose adsorption method (Glass et al., 1978).

Time Course of Autophosphorylation by Intact vs Truncated Receptors. Immobilized trypsin-treated with untreated receptor preparations were reacted as described above: ±100 nM insulin, 25 μM [γ-^{32}P]ATP (15 μCi), and 5 mM $MnCl_2$ at 22 °C. Reactions were terminated at the indicated times, and receptor-incorporated phosphate was determined by autoradiography following separation by SDS–polyacrylamide gel electrophoresis.

Results and Discussion

We have previously shown that treatment of whole cells with trypsin prior to purification of the insulin receptor results in proteolytic truncation of the receptor and activation of autophosphorylation and substrate kinase activities (Shoelson et al., 1988). The truncated receptor isolated following such treatment has a molecular mass of ≈116 kDa, estimated by SDS–PAGE, and is composed of an intact 92-kDa β-subunit that is disulfide-linked to an ≈24-kDa fragment of the α-subunit (Xu et al., 1990). While electrophoretic mobility in the presence of SDS assesses the covalent structure of the truncated receptor adequately, it provides little information about noncovalent interactions within or between trypsin-activated receptor forms. In fact, in related studies, it has been suggested that overall $\alpha_2\beta_2$ structural integrity could be maintained following tryptic activation (Pilch et al., 1981) and that a proteolytic clip within the α-subunit alone might activate receptor kinase activity (Hsuan et al., 1989). To determine the association state of isolated trypsin-activated receptors under phosphorylation assay conditions (0.1% Triton, pH 7.4–7.6), we conducted sucrose gradient sedimentation, immobilization, and concentration dependence studies.

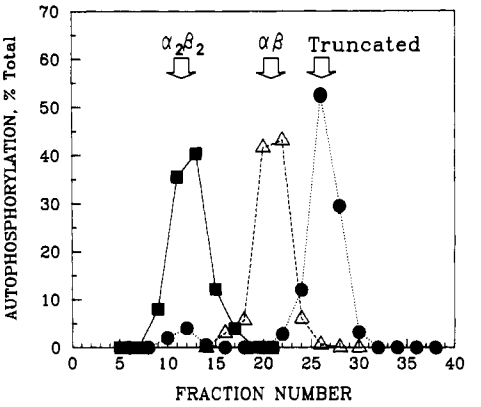

FIGURE 1: Sucrose gradient sedimentation of receptor forms. Trypsin-truncated (●), $\alpha\beta$-dimeric (△), and intact $\alpha_2\beta_2$ (■), insulin receptors were analyzed by velocity sedimentation on 5–25% sucrose density gradients containing 30 mM HEPES (pH 7.6), 0.1% Triton X-100, 100 mM sodium chloride, and 0.02% sodium azide as described under Experimental Procedures. Individual fractions were subjected to autophosphorylation in the presence (■, △) or absence (●) of 100 nM insulin. Phosphorylated proteins were separated by SDS gel electrophoresis under reducing conditions and identified by autoradiography of fixed and dried gels. The degree of β-subunit autophosphorylation in each fraction was determined by scanning the autoradiographs. The percentage of total autophosphorylation (ordinate) is the amount in each fraction divided by the sum in all fractions (multiplied by 100).

Sucrose Gradient Sedimentation. As demonstrated previously (Boni-Schnetzler et al., 1986), the ≈460-kDa $\alpha_2\beta_2$ tetramer and the ≈230-kDa $\alpha\beta$ dimer can be separated from one another by sucrose gradient centrifugation (Figure 1). Sedimentation coefficients of 10.3 and 6.2 S were determined for the $\alpha_2\beta_2$ and $\alpha\beta$ receptor forms, respectively, by comparisons with known protein standards and are in excellent agreement with published values (Pollet et al., 1981; Aiyer, 1983). Under similar conditions, the truncated receptor sedimented significantly more slowly than the $\alpha\beta$ dimer with a sedimentation coefficient of 4.6 S. If the trypsin-activated receptor remained associated as a noncovalent $\alpha_2\beta_2$ complex, it should sediment at or near the position of the intact holoreceptor (10.3 S). If, on the other hand, the truncated receptors formed stable noncovalent homodimers ($\alpha'_2\beta_2$), these would sediment near the position of the $\alpha\beta$ half-receptors (6.2 S). The observed position of the truncated receptor on sucrose gradient sedimentation (4.6 S) agrees well with the predicted sedimentation coefficient of 4.7 S. These results are consistent with an unassociated monomer structure rather than a noncovalently associated dimer or heterotetramer. Therefore, when the insulin receptor is activated by extensive treatment

of intact cells with trypsin and isolated by lectin chromatography, there is no evidence for either dimerization between truncated forms or maintenance of a noncovalent $\alpha_2\beta_2$ general structure, as judged either by gel electrophoresis under nonreducing conditions or by sedimentation characteristics from sucrose gradient centrifugation.

Immobilization Studies. To confirm that unassociated truncated receptors were active, receptors were immobilized by binding to antibodies on solid supports (antibody–protein A–Sepharose). The anti-insulin receptor antibodies chosen for this study were prepared against a peptide corresponding to residues 1326–1336 of the carboxyl terminus of the receptor; the antibodies do not interfere with the insulin binding or the substrate kinase activity of the receptor (Perlman et al., 1989; unpublished observations). As protein A binding sites were present in great molar excess (>50-fold) over anti-receptor antibodies, and these were present in molar excess (>50-fold) over receptors, immobilization on the matrix should prevent interactions between nonassociated receptors. Insulin stimulates autophosphorylation of intact immobilized receptors from Fao cells 10–20-fold (Figure 2A, lanes 1 and 2), confirming that the antibodies do not interfere with the assay. Under these conditions, the immobilized trypsin-truncated receptor was fully activated in the absence of insulin, and insulin had no additional stimulatory effect (Figure 2A, lanes 3 and 4). The patterns for autophosphorylation following immobilization were similar to those for intact and truncated receptors in solution (Shoelson et al., 1988) and demonstrate that autophosphorylation reactions within the $\alpha_2\beta_2$ and truncated receptor forms are intramolecular.

Receptor-catalyzed exogenous substrate phosphorylations were similarly analyzed. Phosphate incorporation into peptide substrate was enhanced in the presence of insulin when catalyzed by immobilized holoreceptors from either Fao cells (Figure 2C, lanes 1 and 2) or placental tissue (Figure 2D, lanes 5 and 6). Analogous to the results for receptor phosphorylation, the immobilized truncated receptor was a fully active catalyst for phosphorylating substrate in the absence of insulin (Figure 2C, compare lanes 2 and 3). Adding insulin to the immobilized, truncated receptor preparation had little additional effect on either receptor (Figure 2A, lanes 3 and 4) or substrate (Figure 2C, lanes 3 and 4) phosphorylation, as had been shown previously for truncated receptors in solution (Shoelson et al., 1988).

For comparison, $\alpha\beta$ dimers were immobilized and studied in the same manner. Unlike the trypsin-truncated receptor, autophosphorylation of the $\alpha\beta$ dimer was not activated in the absence of insulin (Figure 2B, compare lanes 6 and 7). Addition of insulin to the immobilized dimer did not activate autophosphorylation (Figure 2B, lane 8). By contrast, when a concentrated preparation of $\alpha\beta$ dimers was autophosphorylated in solution, there was significant insulin-stimulated phosphorylation concomitant with association of two $\alpha\beta$ halves (Sweet et al., 1987b; Boni-Schnetzler et al., 1988). Results from exogenous substrate phosphorylation experiments were parallel. Low levels of substrate phosphorylation were found in the absence and presence of insulin (Figure 2D, compare lane 6 to lanes 7 and 8). These data from experiments with immobilized $\alpha\beta$ dimers are consistent with results from additional studies suggesting that insulin binding and insulin stimulation of receptor kinase activity require association between $\alpha\beta$ halves (Boni-Schnetzler et al., 1986, 1987, 1988; Sweet et al., 1987a,b; Morrison et al., 1988; Chiacchia, 1988). Experiments with immobilized truncated receptors, on the other hand, clearly indicate that autophosphorylation

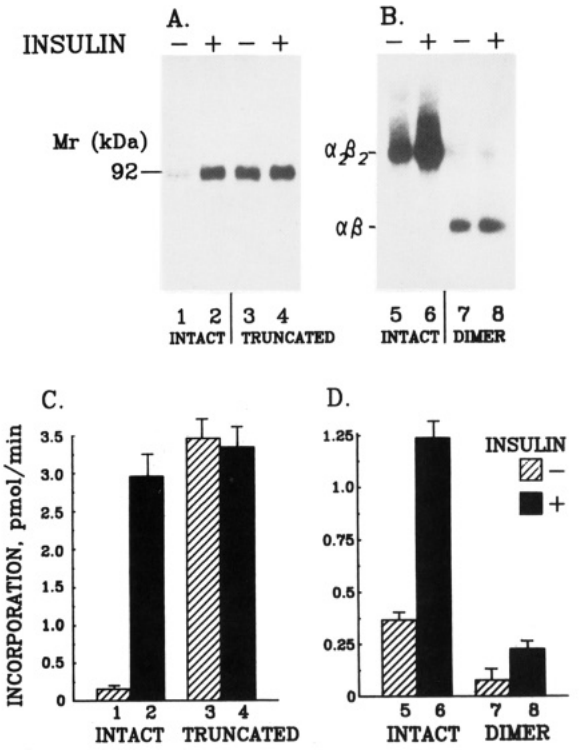

FIGURE 2: Immobilized receptor autophosphorylation and kinase activities. Trypsin-truncated, $\alpha\beta$-dimeric, and intact $\alpha_2\beta_2$ insulin receptor forms were immobilized on antibody–protein A–Sepharose matrices and either autophosphorylated or used as catalysts for substrate phosphorylations as described under Experimental Procedures. The antibody used in these studies is polyclonal and generated against the carboxyl terminus (1326–1336) of the insulin receptor. (A) Intact (lanes 1 and 2) and truncated (lanes 3 and 4) receptors from Fao hepatoma cells were immobilized and phosphorylated in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of insulin, and separated by SDS gel electrophoresis under reducing conditions on 7% resolving gels. (B) Intact receptor (lanes 5 and 6) and $\alpha\beta$ dimers (lanes 7 and 8) prepared from human placental tissue were similarly immobilized and phosphorylated in the presence (lanes 6 and 8) and absence (lanes 5 and 7) of insulin. In this case, phosphorylated proteins were prepared for SDS–PAGE in the presence of 35 mM *N*-ethylmaleimide and resolved on 3–10% gradient gels in the absence of DTT. (C and D) Phosphate incorporation into a synthetic substrate (peptide 1142–1153) was catalyzed by the immobilized receptors. Intact receptor from Fao hepatoma cells (lanes 1 and 2) or placenta (lanes 5 and 6), truncated receptor from trypsin-treated Fao cells (lanes 3 and 4), and $\alpha\beta$ dimers from DTT-treated placental membranes (lanes 7 and 8) were all used to catalyze substrate phosphorylations in the absence (lanes 1, 3, 5, and 7) and presence (lanes 2, 4, 6, and 8) of insulin.

itself can occur in the absence of intermolecular associations.

Concentration Dependence. To verify that the truncated receptor was active as a monomer and that $\alpha\beta$ halves were active only after association, concentration dependencies for receptor autophosphorylation were studied. Autophosphorylation of the holoreceptor has already been shown to be independent of concentration (Shia et al., 1983; Petruzzelli et al., 1984; White et al., 1984; Kwok et al., 1986), but two β-subunits are always associated within the covalent $\alpha_2\beta_2$ complex. Consequently, studies with the holoreceptor are unable to differentiate between cis and trans mechanisms of autophosphorylation. Concentration studies with constitutively activated truncated receptors should, however, differentiate between these mechanisms. If individual subunits self-phosphorylate, the relationship between incorporation and concentration should be linear (concentration independent). Conversely, if autophosphorylation requires associations between two truncated receptors, the relationship should be parabolic (concentration dependent). For the truncated re-

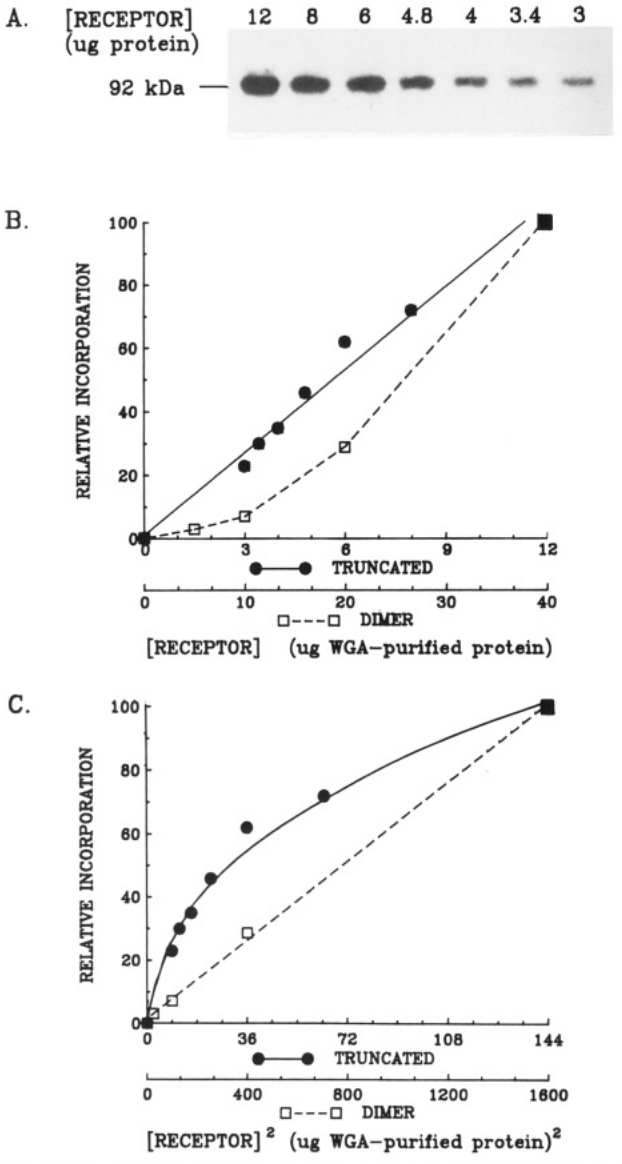

FIGURE 3: Concentration dependence for autophosphorylation of receptor forms. WGA-purified, truncated insulin receptor was diluted in 50-μL aliquots such that only the concentration of receptor varied. Each dilution of truncated receptor was autophosphorylated with 25 μM [γ-^{32}P]ATP and 5 mM $MnCl_2$ for 2, 5, and 10 min at 22 °C. Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies and separated by SDS–PAGE as described under Experimental Procedures. (A) A representative autoradiography from a dilution experiment shows the effect of dilution on relative phosphate incorporation into the β-subunit of the truncated receptor. (B) The relative amounts of phosphate incorporated into the truncated receptor aliquots were determined by gel-scanning, the data obtained were plotted as a function of receptor concentration (solid circles, solid line), and linear regression analyses were performed (r = 0.99). Various concentrations of $\alpha\beta$ dimers were autophosphorylated as described under Experimental Procedures (open squares, dashed line; r = 0.95). (C) Relative amounts of phosphate incorporated into truncated (closed circles, solid line) and $\alpha\beta$-dimeric (open squares, dashed line) receptors are plotted as functions of the square of the receptor concentrations (abscissa); linear correlation coefficients (r) were 0.91 for truncated receptors and 0.99 for $\alpha\beta$-dimeric receptors, respectively.

ceptor, the relation was clearly linear and consistent with intramolecular self-phosphorylation (Figure 3A,B). Results from similar experiments with $\alpha\beta$ dimers conducted in the presence of insulin do not demonstrate a linear relationship (Figure 3B). A plot of phosphate incorporation as a function of the square of the $\alpha\beta$ dimer concentration does show a linear relationship (Figure 3C), however, indicating a bimolecular

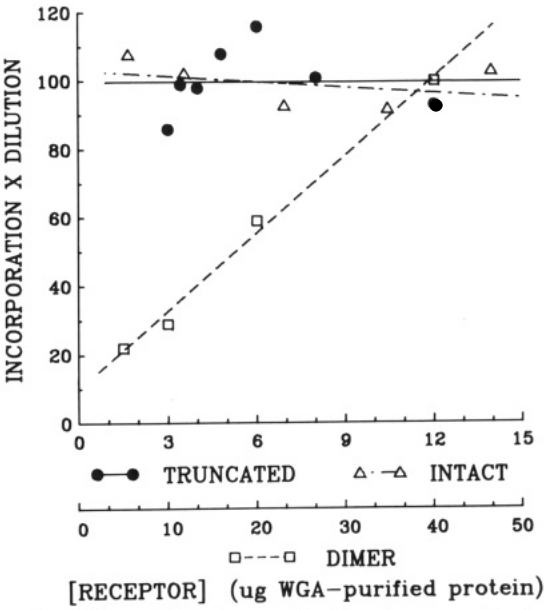

FIGURE 4: Effects of dilution on phosphate incorporation by the receptor forms. Receptor forms were diluted and phosphorylated as described under Experimental Procedures and in the legend to Figure 3. Relative incorporation was multiplied by the x-fold dilution to yield values plotted on the ordinate; receptor amount is on the abscissa. Data for intact (open triangles, dashed–dotted line) and truncated (solid circles, solid line) receptors from Fao cells and for placental dimers (open squares, dashed line) are shown. The lines represent linear regressional analyses through the points shown.

reaction and suggesting that in this case two $\alpha\beta$ halves must come together for insulin-stimulated autophosphorylation to occur. To test this further, $\alpha\beta$ dimers were treated with trypsin under conditions used previously for activation of solubilized preparations of holoreceptor (Leef & Larner, 1988; Shoelson et al., 1988; Hsaun et al., 1989). Upon mild proteolysis, the $\approx$230-kDa $\alpha\beta$ heterodimer was converted to a $\approx$110-kDa truncated receptor with partial activation of autophosphorylation, showing that truncated the $\alpha\beta$ dimer also activates autophosphorylation (data not shown).

A plot of the product of the degree of receptor phosphorylation and receptor dilution vs receptor concentration helps to differentiate mechanisms of phosphorylation by each of the receptor forms further (Figure 4). If autophosphorylation is intramolecular, the product of phosphate incorporation and the x-fold dilution should be constant (concentration independent); if phosphorylation requires interactions between receptor forms, the product should decrease at lower receptor concentrations. For the intact receptor, the product is constant, confirming an intramolecular mechanism for autophosphorylation (Shia et al., 1983; Petruzzelli et al., 1984; White et al., 1984). In a similar fashion, the product is constant for the truncated receptor, indicating that autophosphorylation also occurs as an intramolecular process within these individual β-subunits. The product is clearly not constant for the $\alpha\beta$ half-receptors, however, suggesting that in this case interactions must occur between dimer halves.

Mechanism of Phosphorylation. All results presented here are consistent with an intramolecular mechanism for autophosphorylation within the truncated insulin receptors, i.e., within individual β-subunits. Previous results from our laboratories and others have shown that in the intact holoreceptor an initial phosphorylation initiates a cascade of reactions that result in phosphorylation of five or six different tyrosine residues in each β-subunit (White et al., 1988; Tavare & Denton, 1988; Flores-Riveros et al., 1989). Our current data are consistent with the initial phosphorylation occurring as an

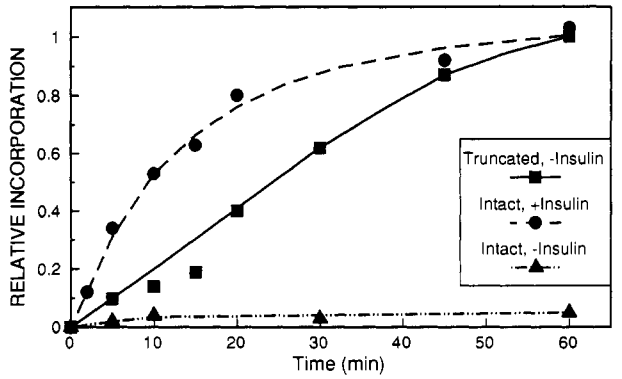

FIGURE 5: Time course for autophosphorylation of receptor forms. Truncated receptors isolated from trypsin-treated Fao hepatoma cells were autophosphorylated in the absence of insulin (squares. solid line) as described under Experimental Procedures. Intact receptors isolated from untreated Fao cells were autophosphorylated in the presence (circles, dashed line) and absence (triangles, dot–dash line) of insulin. Aliquots were removed from the reaction at the indicated time and quenched with "stopping solution", and the immunoprecipitated pellets were washed to remove excess [γ-^{32}P]ATP. Phosphorylated proteins were separated by polyacrylamide gel electrophoresis, and relative incorporation of phosphate into the β-subunits was determined by scanning the appropriate positions of fixed and dried polyacrylamide gels.

intramolecular event within a β-subunit. The insulin receptor can catalyze phosphorylation of and be phosphorylated by other kinases in an intermolecular fashion (Yu et al., 1985; Ballotti et al., 1989), so it may be that the initial phosphorylation enhances rates of subsequent phosphorylations of both β-subunits (in an intramolecular and intermolecular fashion). If this occurs to a significant degree, then the rate of autophosphorylation in the $\alpha_2\beta_2$ complex would be expected to be more rapid than the rate of truncated receptor phosphorylation. As shown in Figure 5, the initial rate of autophosphorylation by the holoreceptor is 2–3-fold greater than that of the truncated receptor. These studies are consistent with but do not prove a trans contribution to net phosphorylation following an initial cis event within the intact $\alpha_2\beta_2$ complex.

Previous attempts to delineate cis vs trans mechanisms of receptor activation have been ambiguous (Herrera et al., 1988; Cobb et al., 1989; Villalba et al., 1989). With expressed cytoplasmic insulin receptor domains, investigators from the Rosen laboratory observed intramolecular phosphorylation, analogous to our observations (Herrera et al., 1988; Villalba et al., 1989). By contrast, Cobb et al. (1989) concluded that their very similar construct exhibits intermolecular phosphorylation, although these studies were conducted in the presence of protamine. While we cannot resolve the different conclusions arrived at by other investigators, it is worthwhile pointing out that the receptor forms used in the current study are significantly different than the genetically engineered forms used to address this question previously. Trypsin-truncated receptors and $\alpha\beta$ dimers are both derived directly from native mammalian holoreceptors, so all protein folding patterns and posttranslational modifications are normal. In addition, the sites of autophosphorylation in the trypsin-truncated receptors are identical with those in intact holoreceptors (Shoelson et al., 1988), unlike mutagenized receptors (Herrera et al., 1988).

Importantly, treatment of intact cells with trypsin activates insulin-like bioeffects, suggesting that experiments presented here have functional relevance. Reiser and Reiser (1964) first showed increased sugar and amino acid transport in trypsin-treated muscle, and the insulin-mimicking effects of trypsin on isolated adipocytes have been well documented, including stimulation of glucose transport and oxidation, glycogen synthesis, pyruvate dehydrogenase activity, and lipogenesis and inhibition of lipolysis (Kuo et al., 1967; Kono, 1971; Kono & Barham, 1971; Kikuchi et al., 1981). Subsequent studies concluded that trypsin's effects on cellular bioeffects and receptor autophosphorylation followed similar mechanisms (Tamura et al., 1983; Leef & Larner, 1987; Shoelson et al., 1988).

Conclusion. On the basis of current and previous results, we can propose a working model for what we currently think are the structural requirements of insulin receptor activation. Insulin stimulation of receptor activation requires an association between two $\alpha\beta$ dimers. High-affinity binding, maximal autophosphorylation, and maximal kinase activity within $\alpha\beta$ halves all require this association. If, however, β-subunit kinase domains are relieved of the inhibitory constraints of α-subunit binding sites, intermolecular associations may not be necessary. Although the rate of truncated receptor activation is slower, full autophosphorylation and kinase activation do occur. On the basis of these results, we suggest that an initial phosphorylation within the 1158, 1162, 1163 cluster of tyrosine residues might occur as an intramolecular event. This may in turn initiate subsequent phosphorylations within the same β-subunit, and possibly in the adjacent β-subunit as well. These phosphorylations are known to activate the receptor kinase toward exogenous substrates (Rosen et al., 1983; Yu & Czech, 1984; Kwok et al., 1986; Stadtmauer & Rosen, 1986; Shoelson et al., 1988; White et al., 1988), but it is not yet clear whether receptor phosphorylation itself or receptor-catalyzed phosphorylations of other endogenous proteins carry the insulin signal into the cell.

While we continue to learn more about transmembrane signaling by the insulin receptor, our knowledge of conformational changes that occur and requisite interactions with other proteins remains scant. Despite this, differentiation of potential signaling mechanisms is crucial to our understanding of insulin action and possible pathogenic mechanisms in non-insulin-dependent diabetes mellitus.

Registry No. Insulin, 9004-10-8; insulin receptor tyrosine kinase, 88201-45-0.

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