Insulin Binding Changes the Interface Region between α Subunits of the Insulin Receptor†

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ABSTRACT: The homobifunctional cross-linking reagent disuccinimidyl suberate (DSS) was used to probe the interface region between the two α subunits of the $\alpha_2\beta_2$ human insulin receptor. The two α subunits formed a covalent dimer when affinity-purified receptor or membrane-bound receptor was reacted with DSS. The α_2 species was detected on protein blots from SDS gels using an anti-α-subunit antibody or ^{125}I-concanavalin A. Alternatively, iodinated receptor was reacted with DSS and the α_2 species measured directly in an SDS gel. As shown by all three assay systems, more α_2 was formed when insulin was bound to receptor than when insulin was absent. These data indicate that the conformational change which occurs in the α subunit in response to insulin binding results in a change in the α–α interaction within the receptor complex. The results are consistent with a kinase activation mechanism involving communication between the two αβ receptor halves.

The human insulin receptor is a multisubunit, transmembrane protein of the type $\alpha_2\beta_2$ (Jacobs et al., 1979; Siegel et al., 1981; Massagué et al., 1980, 1981; Pessin et al., 1985). Each α subunit is disulfide bonded to a β subunit, and the two αβ halves are also linked by disulfide bonds, presumably from α to α (Shoelson et al., 1988), although this (these) disulfide(s) has (have) not been identified. The α subunits are entirely extracellular (Grunfeld et al., 1985; Ullrich et al., 1985; Ebina et al., 1985), which each β subunit has a single stretch of hydrophobic amino acids of length sufficient to span the cell membrane once (Ullrich et al., 1985; Ebina et al., 1985). The α subunit contains the insulin binding site (Yip et al., 1978; Pilch & Czech, 1980) while the β subunit is an insulin-stimulatable tyrosine kinase capable of autophosphorylation as well as exogenous substrate phosphorylation (Kasuga et al., 1982; Avruch et al., 1982; Shia & Pilch, 1983; Roth & Cassell, 1983; Van Obberghen et al., 1983; Petruzzelli et al., 1984). The autophosphorylation sites (Tornqvist et al., 1987; White et al., 1988) as well as the enzyme active site (Ullrich et al., 1985; Ebina et al., 1985) exist exclusively on the cytoplasmic domain of the β subunit. The kinase activity is essential to receptor function since its inhibition, either with an anti-β-subunit monoclonal antibody (Morgan & Roth, 1987) or by site-directed mutagenesis (Ellis et al., 1986; Ebina et al., 1987; Chou et al., 1987), also inhibits one or more of a number of intracellular responses to insulin including glucose transport, S6 kinase activity, glycogen synthesis, and thymidine incorporation into DNA. The central role of kinase activity in receptor function necessitates a more thorough understanding of the insulin-induced transmembrane activation mechanism. Evidence to date indicates that communication between the two αβ receptor halves of the holoreceptor is essential for the activation process (Boni-Schnetzler et al., 1986, 1988; Sweet et al., 1987; O'Hare & Pilch, 1988). In support of this concept, we show here that a change in the interface region between two α subunits occurs upon insulin binding.

Experimental Procedures

Materials

All electrophoresis reagents were electrophoresis grade from Bio-Rad. Disuccinimidyl suberate (DSS)[1] was obtained from Pierce Chemical Co. Sodium iodide (IMS30) was from Amersham. [γ-^{32}P]ATP was prepared from [^{32}P]orthophosphate (NEN Research Products) using a Gammaprep kit from Promega Biotech. ^{125}I-Labeled protein A was also from NEN. Porcine insulin was a generous gift from Dr. Ron Chance of Eli Lilly. The anti-peptide antibody, raised in rabbits against the synthetic peptide ProProProTyrTyrHisPheGlnAsp-TrpArgCys, was kindly provided by Drs. Alessandro Cama and Simeon Taylor of NIH. Prestained high molecular weight markers for SDS–PAGE were from Bethesda Research Laboratories.

Methods

Receptor Purification. Microsomal membranes were prepared from human placenta as previously described (Harrison & Itin, 1980). Receptor was solubilized from placental microsomes (10–15 mg of protein/mL) with 1% Triton X-100 in the presence of 1 mM phenylmethanesulfonyl fluoride for 1 h at 4 °C with stirring. The soluble fraction (30–40 mL) was obtained upon centrifugation at 100000*g* for 1 h and then applied to a gel filtration column (Williams & Turtle, 1979; Shia & Pilch, 1983) of Sephacryl S-400 (60 cm × 5 cm). The column was equilibrated and eluted with 30 mM Hepes, 0.1% Triton X-100, and 0.02% NaN_3, pH 7.6 (buffer A). The insulin binding fractions from this column were pooled, made 0.5 M in NaCl, and circulated over a 12-mL affi-gel insulin column at 4 °C overnight. The column was washed with 10 column volumes of buffer A containing 0.5 M NaCl; then the receptor was eluted with 50 mM sodium acetate, 1 M NaCl, and 0.1% Triton X-100, pH 5.0 (Fujita-Yamaguchi et al., 1983). Fractions (5 mL) were collected into tubes containing 1 mL of 0.3 M Hepes and 0.1% Triton X-100, pH 7.6, to neutralize the column eluate. This eluate was desalted and

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[1] Abbreviations: DSS, disuccinimidyl suberate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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concentrated on a 0.5-mL wheat germ agglutinin–agarose affinity column. After the column was washed with buffer A containing 0.5 M NaCl, the receptor was eluted with 3 mL of buffer A containing 0.3 M *N*-acetylglucosamine. This concentrated receptor was dialyzed overnight against buffer A before use.

Receptor halves containing a single $\alpha\beta$ functional unit were prepared by treatment of placental microsomes in 75 mM Tris, pH 8.5, with 1.25 mM dithiothreitol for 30 min at room temperature (Boni-Schnetzler et al., 1987). The reduced receptor was solubilized by treatment of the membranes for 1 h at 4 °C with 1% Triton X-100 in the presence of 1 mM phenylmethanesulfonyl fluoride. After centrifugation for 1 h at 100000*g*, the soluble fraction was applied to a 4-mL wheat germ agglutinin–agarose column equilibrated in buffer A. The receptor was eluted from the column with buffer A containing 0.3 M *N*-acetylglucosamine and 0.5 M NaCl. As a control, receptor from membranes not exposed to DTT was purified in parallel. The partially purified, untreated receptor or $\alpha\beta$ halves were used in the experiment described in Figure 2.

Cross-Linking. Cross-linking was performed as described by Pilch and Czech (1979) with some modifications. Briefly, affinity-purified insulin receptor (ca. 10–20 μg/mL) was incubated with the indicated concentrations of iodinated or noniodinated insulin for 1 h at room temperature. The buffer contained 30 mM Hepes, 0.08% Triton X-100, and 0.02% NaN_3, pH 7.6. Unless indicated, the cross-linking solutions also contained 0.4 M NaCl. The solution was cooled on ice. Stock DSS in dimethyl sulfoxide was added to give 0.2 mM DSS and 2% dimethyl sulfoxide. Following 5–135 min of reaction at 0 °C, an equal volume of Laemmli sample buffer (100 mM Tris, 2% SDS, and 20% glycerol, pH 6.8) was added and the mixture heated for 5 min at 60 °C. Unless indicated, the sample buffer contained 50–100 mM dithiothreitol.

For the experiments described in Figure 6, microsomal membranes from human placenta were used rather than purified receptor. Two aliquots of microsomes, each containing 11 mg of membrane protein in 3 mL of 30 mM Hepes, 150 mM NaCl, and 0.02% NaN_3, pH 7.6 (buffer B), were incubated 1 h at room temperature. One aliquot had been supplemented with 3.5×10^{-7} M insulin. The mixtures were then cooled on ice and made 0.4 mM in DSS from a 20 mM stock in dimethyl sulfoxide. After 30 min on ice, the cross-linking reaction was stopped by addition of 4 volumes of ice-cold buffer B containing 1 mM lysine, followed by immediate centrifugation for 30 min at 15000*g*. Upon removal of the supernatant, the membrane pellets were solubilized in 10 mL of 30 mM Hepes, 2% Triton X-100, and 0.02% NaN_3 for 45 min at room temperature. These mixtures were centrifuged for 1 h at 100000*g*, and the supernatants were diluted with 3 volumes of 30 mM Hepes and 0.02% NaN_3, pH 7.6, before being applied separately to 2-mL wheat germ agglutinin–agarose columns. The columns were washed with 10 volumes of buffer A, and each was eluted with 10 mL of buffer A containing 0.4 M *N*-acetylglucosamine. Each eluate was concentrated to 150 μL in a Centricon-30 miniconcentrator, diluted with an equal volume of electrophoresis solubilization buffer containing 50 mM dithiothreitol, and heated at 60 °C for 5 min before being applied to an SDS–polyacrylamide gel.

Protein Iodinations. The chloramine T concentration used here was lower than commonly used in protein iodinations in order to lessen oxidative damage to the receptor. Affinity-purified insulin receptor in buffer A (300 μL, ca. 3–6 μg) was mixed with 20 μL of 0.25 M sodium phosphate, pH 7.2, and 2 μL (0.2 mCi) of $Na^{125}I$. The reaction was initiated by addition of 1 μg of chloramine T in 2 μL of water and incubated for 30 s at room temperature. The reaction was stopped by addition of 4 μg of sodium metabisulfite in 4 μL of water followed immediately by 0.8 mL of buffer A. The receptor was bound to a 0.1-mL wheat germ agglutinin–agarose affinity column, washed with 20 volumes of buffer A, and eluted with buffer A containing 0.3 M *N*-acetylglucosamine.

Concanavalin A was iodinated with chloramine T and affinity purified on Sephadex G-50 exactly as described (Burridge, 1978).

Receptor Phosphorylation. Affinity-purified receptor was mixed with 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 100 mM [γ-^{32}P]ATP (1000 mCi/mmol). The receptor was allowed to autophosphorylate for 90 min at room temperature, followed by overnight dialysis at 4 °C to remove the cations and ATP.

Protein Blotting. Proteins in an SDS gel were electroblotted onto nitrocellulose as described by Towbin et al. (1979). Additional protein binding sites on the nitrocellulose were blocked with TBS (20 mM Tris, 0.4 M NaCl, and 0.02% NaN_3, pH 7.5) containing 3% gelatin for 1 h at room temperature. The nitrocellulose was then incubated overnight at 4 °C with a 1:100 dilution of the immune serum in TBS containing 2% bovine serum albumin. The blot was washed 3×10 min with 200 mL per wash of TBS containing 0.05% Triton X-100 and then incubated for 2 h at room temperature with 200 000 cpm/mL ^{125}I-protein A in TBS containing 2% bovine serum albumin. The blot was washed as before, dried, and autoradiographed.

For lectin blotting, the nitrocellulose was first blocked with 2% hemoglobin in buffer B (50 mM Tris, 200 mM NaCl, 0.02% NaN_3, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4). The blot was then incubated for 2 h at room temperature with buffer B containing 1% hemoglobin and ca. 5 nM ^{125}I-concanavalin A. The blot was washed 3×10 min with 200 mL each wash of buffer B, then dried, and autoradiographed.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with a Laemmli buffer system (Laemmli, 1970) using linear gradients of 3–10% acrylamide. For autoradiography, gels were dried and then exposed to Kodak X-Omat AR film using Cronex lightning plus enhancing screens.

Results

The homobifunctional cross-linking reagent disuccinimidyl suberate has been used extensively to cross-link peptide hormones to their respective receptors. Most notably, ^{125}I-insulin can be covalently cross-linked to the α subunit of the $\alpha_2\beta_2$ holoreceptor with DSS and related cross-linkers (Pilch & Czech, 1980a; Massaguē et al., 1981). The labeled α subunit migrates on a denaturing SDS gel as an ca. 125-kDa protein. In addition, a slower migrating, minor band is often seen at ca. 250 kDa. This band presumably represents two α subunits covalently cross-linked by virtue of their close proximity in the holoreceptor (Pilch & Czech, 1980a; Massaguē et al., 1981). When cross-linking experiments are performed on affinity-purified, soluble receptor, this α_2 species can also be readily seen. Interestingly, the presence of salt in the cross-linking mixture not only increased the amount of ^{125}I-insulin which could be cross-linked to the receptor but also increased the relative amount of the α_2 species formed (Figure 1). These studies were done with 0.2 mM DSS. Alternatively, higher cross-linker concentrations in the absence of salt can be used to increase α_2 formation, but DSS tends to precipitate from solution at millimolar concentrations. When cross-linked receptor was analyzed on SDS gels under nonreducing conditions, no species above the $\alpha_2\beta_2$ holoreceptor was seen on

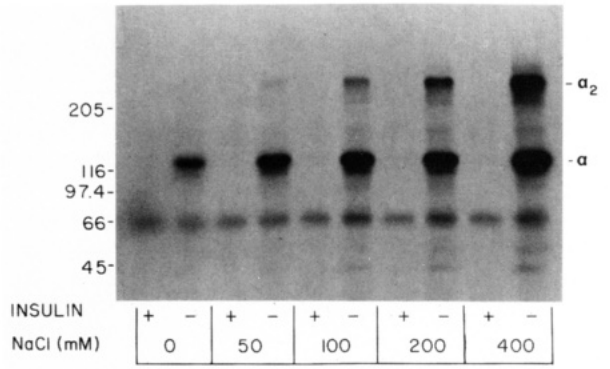

FIGURE 1: Cross-linking of α subunits by disuccinimidyl suberate is promoted by increasing salt concentration. Affinity-purified insulin receptor, with the indicated salt concentrations, was cross-linked to Tyr^{B26}-monoiodoinsulin using 0.2 mM DSS in the presence or absence of 3.5×10^{-6} M unlabeled insulin as described under Methods. The reaction was continued for 15 min on ice, quenched by addition of an equal volume of electrophoresis sample buffer containing 100 mM dithiothreitol, and heated for 2 min at 95 °C. Electrophoresis was performed on a 3–10% acrylamide gradient gel. Shown above is the autoradiograph of the dried gel.

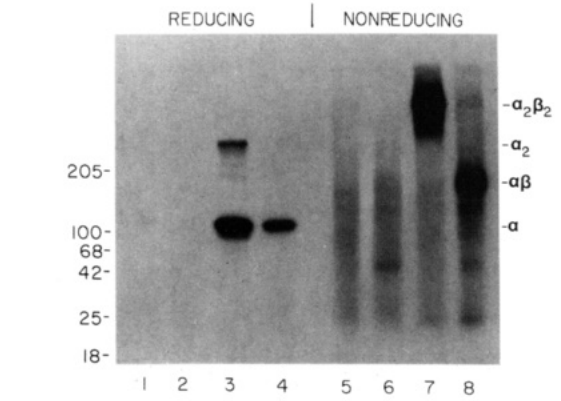

FIGURE 2: Affinity cross-linking of monoiodoinsulin to purified $\alpha_2\beta_2$ holoreceptor and $\alpha\beta$ receptor halves. Partially purified holoreceptor (lanes 1, 3, 5, and 7) or $\alpha\beta$ half-receptor (lanes 2, 4, 6, and 8) was cross-linked to Tyr^{B26}-monoiodoinsulin in the presence (lanes 1, 2, 5, and 6) or absence (lanes 3, 4, 7, and 8) of 3.5×10^{-6} M unlabeled insulin for 30 min on ice using 0.2 mM DSS as described under Methods. The cross-linking was stopped by addition of an equal volume of electrophoresis sample buffer which did (lanes 1–4) or did not (lanes 5–8) contain 50 mM dithiothreitol. After being heated for 5 min at 60 °C, the samples were electrophoresed on a 3–10% acrylamide gradient gel. Shown is the autoradiograph of the dried gel.

the gel (data not shown). This result indicates that α–α cross-linking occurs as an intramolecular rather than as an intermolecular process.

The ability of salt to promote α–α cross-linking was due to an increase in ionic strength rather than a specific ion effect, at least for salts composed of monovalent ions. When cross-linking was performed in the presence of 0.4 M NaCl, 0.4 M NaI, or 0.4 M CsCl, the α_2 signal represented 31%, 26%, and 29%, respectively, of the total signal versus 11% in the absence of salt ($\alpha + \alpha_2 = 100\%$) (data not shown). Hence, ionic strength alone increased α_2 formation to ca. the same proportion of total signal regardless of the specific ions involved.

The data in Figures 2 and 3 confirm that an α_2 species is being formed rather than other subunit combinations. Shown in Figure 2 are the positions of an $\alpha\beta$ dimer [prepared according to Boni-Schnetzler et al. (1987)] and the holoreceptor, as detected by cross-linking to ^{125}I-insulin. As expected, the α_2 species migrates between the holoreceptor and the $\alpha\beta$ dimer. Note that lane 8 contains a very small amount of the $\alpha_2\beta_2$

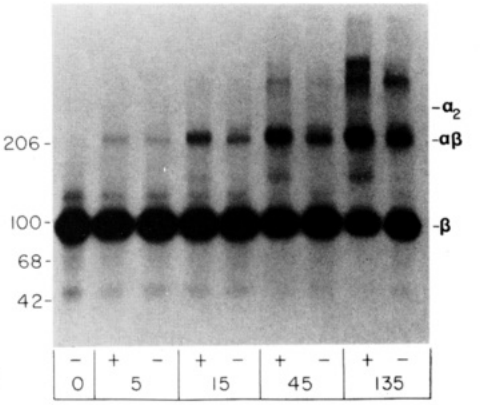

FIGURE 3: Cross-linking of phosphorylated insulin receptor with DSS. Insulin receptor was phosphorylated as described under Methods. The phosphorylated receptor in the presence or absence of 350 nM insulin was cross-linked for the indicated times on ice using 0.2 mM DSS. The reaction was stopped by addition of an equal volume of electrophoresis sample buffer containing 50 mM dithiothreitol and heating 5 min at 60 °C. Samples were electrophoresed on a 3–10% acrylamide gradient gel. Shown is the autoradiograph of the dried gel.

holoreceptor due either to incomplete reduction of class 1 disulfide bonds and/or chemical cross-linking of a small number of noncovalently associated $\alpha\beta$ receptor halves. Though not apparent in the photograph, a longer exposure reveals a very faint α_2 band in the autoradiograph of lane 4. Nevertheless, more effective cross-linking of α subunits occurs when the $\alpha_2\beta_2$ holoreceptor rather than $\alpha\beta$ half-receptors are treated with DSS. These data suggest that the $\alpha\beta$ half-receptors exist predominantly as monomers under our conditions, effectively lessening the chance for cross-linking to occur. The data in Figure 3 were generated by cross-linking phosphorylated receptor in the presence and absence of insulin. In the absence of added cross-linker, the only phosphorylated species in the gel is the 92-kDa β subunit (far left lane). Upon cross-linking with DSS, a labeled bond corresponding to an $\alpha\beta$ dimer appears in a time-dependent manner. As expected, the α_2 region of the gel contains no band, hence no β subunit. Since the experiments in the first five figures of this paper were conducted on affinity-purified receptor, the possibility that the α_2 species is actually an α subunit cross-linked to a nonreceptor protein can also be ruled out.

Our ability to effectively form the α_2 species allows the use of DSS to probe the interface region between two α subunits. More specifically, we wondered if there was a difference in our ability to cross-link the α subunits in the presence and absence of bound insulin. Purified receptor in the presence or absence of insulin was treated with 0.2 mM DSS for times ranging from 5 to 135 min. Following inhibition of the cross-linking reaction, the samples were electrophoresed and transferred to nitrocellulose. The α subunit on the blot was reacted with an anti-peptide antibody specific for residues 242–253 of this subunit. This was followed by labeling with ^{125}I-protein A and autoradiography. At all time points studied, more of the α_2 species was formed when insulin was present than when insulin was absent in the reaction mixture (Figure 4A). The α_2 species on protein blots was also detected with ^{125}I-concanavalin A (Figure 4B). The heavily glycosylated α subunit is readily seen in the absence of cross-linking (Figure 4B, far left lane) as well as a weaker signal from the less glycosylated β subunit. Again, upon DSS treatment, insulin binding is seen to promote α_2 formation. The result indicates that a conformational change occurs upon insulin binding, altering the interface region and allowing more effective cross-linking. All experiments presented here were performed

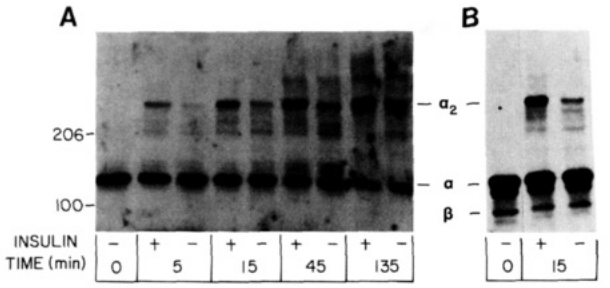

FIGURE 4: Formation of α_2 by DSS cross-linking is promoted by receptor-bound insulin. Purified insulin receptor in the presence or absence of 350 nM insulin was cross-linked for the indicated times on ice with 0.2 mM DSS. The reaction was stopped by addition of an equal volume of electrophoresis sample buffer containing 100 mM dithiothreitol and heating 10 min at 60 °C. Following SDS–PAGE, the proteins were electroblotted onto nitocellulose. (A) The α subunit was detected with an antibody specific for residues 242–253 of this subunit followed by reaction with ^{125}I-protein A as described under Methods. (B) The receptor subunits were detected on the blot with ^{125}I-concanavalin A as described under Methods. Only the 15-min time point is presented. Shown are the autoradiographs of the dried blots.

in 0.4 M salt, a condition which maximized α_2 formation for detergent-solubilized receptor. In fact, the excised radioactive bands from lane 8 of the immunoblot in Figure 4 were counted in a γ counter. The data indicated that after 135 min of cross-linking in the presence of insulin, 54% of the α subunit had been converted to the α_2 species. In the absence of salt, the same insulin effect upon α_2 formation could be observed, though the relative amount of α_2 formed was reduced (data not shown).

As a final method to verify our results, we performed the cross-linking experiment with iodinated receptor, a protocol that does not rely on protein blotting. In the absence of cross-linker, only the α and β subunits, along with a minor amount of β', are present (Figure 5A, far left lane). This confirms the purity of our receptor preparation and further emphasizes that any additional bands seen in a gel after DSS treatment must represent cross-linked receptor subunits. Upon cross-linking, the α_2 species appears with time (Figure 5A). Again, formation of the α_2 species was shown to be insulin responsive, as quantitated in Figure 5B.

Reaction of the receptor with DSS also resulted in cross-linking of an α subunit to a β subunit. This is most apparent when either the iodinated receptor (Figure 5A) or the phosphorylated receptor (Figure 3) is used. In the latter case, quantitation of the bands indicated that at best 30% of the β subunit became cross-linked to an α subunit (data from Figure 3, lane 9). This is less efficient than α–α cross-linking under the same conditions. Insulin is also seen to promote $\alpha\beta$ formation, but the relative difference in $\alpha\beta$ formation in the presence and absence of insulin is not as great as the relative difference in α_2 formation under identical conditions (compare Figure 5B to Figure 5C). Nevertheless, the insulin-induced conformational change in the α subunit is clearly more than a local perturbation. Rather, it is sufficiently long-range to affect both the α–β and α–α interfaces.

Our results indicate that α_2 formation in the presence and absence of insulin are kinetically distinct events. Hence, when insulin is bound to the receptor, the α subunits are more rapidly converted to α_2 than when insulin is absent. As discussed by Pilch and Czech (1984), nonproductive side reactions typically prevent cross-linking reactions from being 100% efficient. However, if these side reactions were eliminated, complete conversions of α to α_2 should occur whether insulin was present or not, the end result being that the relative differences in α_2

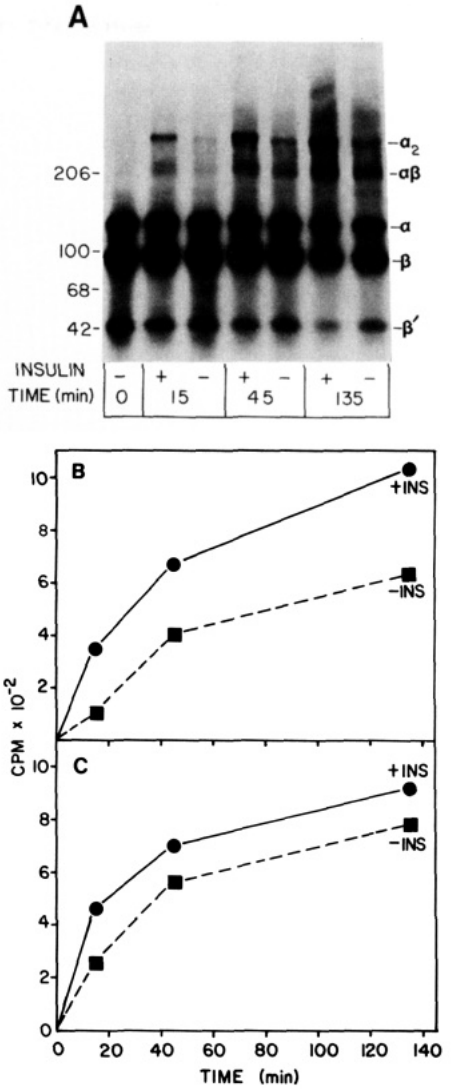

FIGURE 5: Insulin bound to iodinated receptor promotes α–α cross-linking. Affinity-purified receptor was iodinated as described under Methods. The iodinated receptor, in the presence or absence of 350 nM insulin, was cross-linked for the times indicated on ice with 0.2 mM DSS. The reaction was inhibited by addition of an equal volume of electrophoresis sample buffer containing 50 mM dithiothreitol and heating 5 min at 60 °C. The samples were electrophoresed on 3–10% acrylamide gradient gels. (A) Shown is the autoradiograph of the dried gel. Iodinated receptor in the absence of cross-linker treatment is shown in the far left lane. The dried gel was purposely overexposed to visualize the α_2 species clearly. (B) The α_2 species was quantitated by cutting this band from the dried gel and counting in a γ counter. Shown is a plot of α_2 formation with time when cross-linking was performed in the presence (solid line) or absence (dashed line) of 350 nM insulin. (C) Same as in (B) except that the $\alpha\beta$ cross-linked species is plotted.

formation in the presence and absence of insulin decrease as the reactions approach completion. This is perhaps most apparent in Figure 4A where at the early 5- and 15-min time points very little α_2 is detected in the absence of bound insulin. As the reactions near completion at 135 min, similar amounts of α_2 have formed in the presence and absence of insulin. The quantitative differences seen in the rates of α_2 formation plus and minus insulin are not great. However, a priori, there is no requirement that even major changes at the interface region (e.g., different sets of lysines could be cross-linked in the presence or absence of bound insulin) should result in large rate differences. Finally, it is assumed that the receptor population is homogeneous and what is true for the receptors that undergo cross-linking is also true for those that do not.

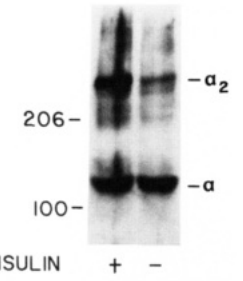

FIGURE 6: Formation of α_2 by DSS cross-linking of membrane-bound receptor is promoted by receptor-bound insulin. Placental microsomes, in the presence or absence of 350 nM insulin, were treated for 30 min on ice with 0.4 mM DSS as described under Methods. Receptor was then detergent solubilized and partially purified on WGA–agarose columns. Following concentration and SDS–PAGE, the proteins were electroblotted onto nitrocellulose. The α subunit was detected with an antibody specific for residues 242–253 of this subunit followed by reaction with ^{125}I-protein A. Shown above is the autoradiograph of the dried gel.

The iodinated receptor may be an exception since only ca. 21% of α was present as α_2 after 135 min of cross-linking. This suggests that iodinated tyrosine residues on the receptor and/or the reaction conditions used in iodination may have inactivated a significant portion of the receptors (Figure 5).

A final experiment was performed to ensure that the effect we see for detergent-solubilized, purified receptor also occurred with receptor in the more native membrane environment. The experiment necessitated starting with relatively large amounts of membrane protein (11 mg) in order to visualize the receptor on the final immunoblot. Two aliquots of placental microsomes, with or without added insulin, were reacted with disuccinimidyl suberate. After cross-linking, the membranes were solubilized with detergent, and the solubilized receptor preparations were partially purified on two identical WGA–agarose affinity columns. These two preparations were then concentrated and electrophoresed under reducing conditions. α-Subunit bands were detected by immunoblotting with the anti-peptide antibody described under Experimental Procedures. It is apparent from this single experiment that substantially more α–α cross-linking occurs when the membranes are preincubated with insulin. The streaking seen in the immunoblot may be at least partly due to the relatively high concentrations of protein and Triton X-100 present in our electrophoresis samples. The experiment was performed twice with similar results. The streaking prevented quantitative analysis of these data; nevertheless, the qualitative results entirely support our results with pure receptor.

The experiments performed in Figures 3–6 were performed at 350 nM insulin to ensure receptor saturation. However, we have confirmed that 35 nM but not 3.5 nM insulin shows a comparable effect on α–α cross-linking (data not shown). Numerous reports have indicated that insulin receptor activation occurs at similar insulin concentrations. For example, half-maximal insulin receptor autophosphorylation and exogenous substrate phosphorylation typically occur at insulin concentrations ranging from 3 to 70 nM depending upon experimental conditions (Shia & Pilch, 1983; Rosen et al., 1983; Shemer et al., 1987; Machicao et al., 1987).

Discussion

Several studies have demonstrated that a change in insulin receptor conformation occurs upon insulin binding (Pilch & Czech, 1980b; Donner & Yonkers, 1983; Maturo et al., 1983). The detailed nature of any conformational changes is unknown, but these are undoubtedly involved in the mechanism of transmembrane activation of β-subunit kinase. This is supported by several recent investigations. For example, trypsin treatment of cells can be used to generate an activated insulin receptor that contains an intact β subunit still disulfide bonded to a small (25 kDa) C-terminal fragment of the α subunit (Shoelson et al., 1988). Also, the cytoplasmic domain of the β subunit has been expressed in insect cells and purified to near-homogeneity (Herrera et al., 1988). These truncated receptor forms were constitutively activated in both autophosphorylation and exogenous substrate phosphorylation assays. These results suggest that the α subunit in the absence of insulin is a negative modulator of the intrinsic kinase activity of the β subunit. Therefore, in the intact receptor, insulin binding must induce a conformational change in the α subunit of the receptor which in turn is transmitted to the β subunit with resultant kinase activation. For insulin-induced activation, an $\alpha_2\beta_2$ holoreceptor is essential since insulin binding to preformed $\alpha\beta$ receptor halves does not activate kinase (Boni-Schnetzler et al., 1986). However, once autophosphorylation occurs, $\alpha\beta$ receptor halves can be generated which act independently to phosphorylate exogenous substrates (Boni-Schnetzler et al., 1986). Moreover, an $\alpha_2\beta\beta'$ form of the insulin receptor is incapable of insulin-induced autophosphorylation, where β' is a proteolyzed form of the β subunit lacking the cytoplasmic domain (O'Hare & Pilch, 1988). Hence, without discounting the possible importance of an insulin-induced change in α–β interaction, this interaction alone appears insufficient for kinase activation. It has therefore been proposed, on the basis of the above results and the fact that insulin can induce association of concentrated receptor halves (Sweet et al., 1987; Boni-Schnetzler et al., 1988), that interaction between $\alpha\beta$ receptor halves is crucial for insulin-induced receptor activation.

The results in this paper demonstrate that a change in the interface region between α subunits of the insulin receptor occurs in response to insulin binding and that this change can be monitored by using homobifunctional cross-linking reagents. Hence, the insulin-induced conformational change in the α subunit does in fact alter α–α interaction, a necessary first step if the above mechanism of kinase activation is correct. The cross-linking technique used here should also prove useful in investigating β-subunit interaction as well as subunit interaction in related growth factor receptors. In fact, a chemical cross-linking reagent was recently used to show that epidermal growth factor (EGF) induced EGF receptor dimerization in intact cells, in membrane preparations, or in preparations of purified receptors (Cochet et al., 1988; Boni-Schnetzler & Pilch, 1987). These authors suggested, as have others (Yarden & Schlessinger, 1987a,b), that interaction between two EGF receptors is necessary for kinase activation.

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Registry No. Insulin, 9004-10-8.

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