Relationship between Insulin Receptor Subunit Association and Protein Kinase Activation: Insulin-Dependent Covalent and Mn/MgATP-Dependent Noncovalent Association of $\alpha\beta$ Heterodimeric Insulin Receptors into an $\alpha_2\beta_2$ Heterotetrameric State†

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ABSTRACT: The purified human placenta $\alpha_2\beta_2$ heterotetrameric insulin receptor was reduced and dissociated into a functional $\alpha\beta$ heterodimeric complex by a combination of alkaline pH and dithiothreitol treatment. In the presence of Mn/MgATP, insulin binding to the isolated $\alpha\beta$ heterodimeric insulin receptor was found to induce the formation of a covalent disulfide-linked $\alpha_2\beta_2$ heterotetrameric complex. In the absence of insulin, a noncovalent association of the $\alpha\beta$ heterodimeric insulin receptor complex into an $\alpha_2\beta_2$ heterotetrameric state required the continuous presence of both a divalent metal ion (Mn or Mg) and an adenine nucleotide (ATP, ADP, or AMPPCP). Thus, Mn/MgATP binding and not insulin receptor autophosphorylation was responsible for the noncovalent association into the $\alpha_2\beta_2$ heterotetrameric state. However, the divalent metal ions or NaATP separately was ineffective in inducing the noncovalent association between the $\alpha\beta$ heterodimers. The specific sulfhydryl agent iodoacetamide (IAN) was observed to inhibit the insulin-dependent covalent association of the $\alpha\beta$ heterodimers without affecting the Mn/MgATP-induced noncovalent association into the $\alpha_2\beta_2$ heterotetrameric state. Insulin treatment of the isolated $\alpha\beta$ heterodimeric complex in the presence of IAN demonstrated that the Mn/MgATP-induced noncovalent association into the $\alpha_2\beta_2$ heterotetrameric state was sufficient for insulin stimulation of both β-subunit autophosphorylation and exogenous substrate protein kinase activity. These data indicate that although interaction between the individual insulin receptor $\alpha\beta$ heterodimers is necessary for insulin stimulation of protein kinase activity it does not require covalent disulfide bond formation.

The insulin receptor is a disulfide-linked multisubunit integral membrane glycoprotein composed of two extracellular M_r 130000 (α) subunits and two transmembrane M_r 95000 (β) subunits (Czech, 1985; Kahn, 1985; Ebina et al., 1985; Ullrich et al., 1985). The α subunit is generally accepted to contain the high-affinity insulin binding site (Yip et al., 1978, 1980; Jacobs et al., 1979; Pilch & Czech, 1980a,b; Wang et al., 1982), whereas the intracellular portion of the β subunit encompasses an adenosine 5′-triphosphate (ATP)[1] binding domain (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983) as well as tyrosine-specific autophosphorylation acceptor sites (Avruch et al., 1982; Kasuga, 1982a–c, 1983a,b; Petruzzelli et al., 1982, 1984; Shia & Pilch, 1983; Tamura et al., 1983; Zick et al., 1983). The molecular mechanism by which extracellular insulin binding to the α subunit(s) elicits a transmembrane signal capable of activating the intracellular β-subunit protein kinase domain is a central problem related to the signal transduction properties of several growth factor receptors including those for EGF, PDGF, CSF, and IGF-1. In each of these receptors, the propogation of the ligand binding signal is limited by the presence of only a single transmembrane sequence within the polypeptide chain (Ullrich et al., 1984, 1985, 1986; Ebina et al., 1985; Coussens et al., 1986; Yarden et al., 1986) and is further constrained by the apparent lack of any major requirement for domain separation by a phospholipid bilayer (Sweet et al., 1985; Panogoton et al., 1985).

In the analogous EGF receptor system, it has been observed that EGF activation of the receptor protein kinase activity was dependent upon a ligand-induced noncovalent dimerization of receptor monomers (Yarden & Schlessinger, 1987a,b; Boni-Schnetzler & Pilch, 1987). Assuming that the EGF receptor monomer is functionally equivalent to the $\alpha\beta$ heterodimeric insulin receptor complex, then insulin activation of the insulin receptor protein kinase activity may require subunit–subunit interactions. In this regard, methods have recently been established to isolate a functional $\alpha\beta$ heterodimeric insulin receptor complex from the native $\alpha_2\beta_2$ heterotetrameric disulfide-linked state (Boni-Schnetzler et al., 1986). The $\alpha\beta$ heterodimeric complex was observed to covalently associate, in an insulin-dependent manner (Boni-Schnetzler et al., 1986; Sweet et al., 1987b; Morrison et al., 1988), into the $\alpha_2\beta_2$ heterotetrameric state which directly correlated with the extent of protein kinase activation (Boni-Schnetzler et al., 1988; Morrison et al., 1988). However, the denaturing method used to distinguish the covalent association, SDS–polyacrylamide gel electrophoresis, did not allow for determining the role of a possible noncovalent association between the $\alpha\beta$ heterodimers

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[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CSF, colony stimulating factor; IGF-1, insulin-like growth factor 1; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; AMPPCP, adenosine 5′-(β,γ-methylenetriphosphate); GMPPNP, guanosine 5′-(β,γ-iminotriphosphate); Mn/Mg, 2 mM $MgCl_2$ plus 10 mM $MnCl_2$; IAN, iodoacetamide; DTT, dithiothreitol; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate.

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(Sweet et al., 1987b; Morrison et al., 1988).

In this paper, we have observed that a combination of Mn/MgATP induces a noncovalent association of the $\alpha\beta$ heterodimeric insulin receptor complex into an $\alpha_2\beta_2$ heterotetrameric state. Further, the insulin-dependent covalent association, but not the divalent metal ion/ATP-induced noncovalent association, was observed to be blocked by sulfhydryl reagents, thus allowing examination of the protein kinase activity of the noncovalently associated $\alpha_2\beta_2$ heterotetrameric insulin receptor.

Experimental Procedures

Materials. Triton X-100, *n*-octyl β-D-glucopyranoside, IAN, DTT, Sephadex G-50, Sephacryl S-400, and protease inhibitors were obtained from Sigma Chemical Co. [γ-^{32}P]ATP (3000 Ci/mmol) and Cronex lightening plus intensifying screens were purchased from New England Nuclear. Centricons and XAR-5 film were obtained from Amicon and Kodak, respectively. Affi-Gel 10, Bio-Gel A-1.5m resin, and SDS–polyacrylamide gel electrophoresis reagents were from Bio-Rad. Monoiodinated ^{125}I-[A^{14}]insulin was provided by the Diabetes and Endocrinology Research Center, The University of Iowa.

Purification of the Insulin Receptors. Insulin receptors were purified from freshly obtained full-term human placenta as previously described (Sweet et al., 1985; Boyle et al., 1985). Briefly, placenta membranes were prepared as described by Harrison and Itin (1980). The membranes were solubilized with 2.0% Triton X-100 for 1 h at 4 °C in 10 mM Tris-HCl (pH 8.0), 250 mM sucrose, 2 mM EDTA, and protease inhibitors. The insulin receptors were then purified by sequential gel filtration chromatography on Sephacryl S-400 and affinity chromatography on insulin–agarose. The eluant from the insulin–agarose column was concentrated on Centricon 300 miniconcentrators, washed with 50 mM Hepes (pH 8.0), 10% glycerol, and 0.6% *n*-octyl β-D-glucopyranoside, and stored at 4 °C until used.

Isolation of $\alpha\beta$ Heterodimeric Insulin Receptors. The formation of the $\alpha\beta$ heterodimeric insulin receptor complex from the purified human placenta $\alpha_2\beta_2$ heterotetrameric complex was previously reported to occur with an approximate 55% efficiency (Sweet et al., 1987a). Minor modifications of this procedure were employed in this study to yield a higher percentage of isolated $\alpha\beta$ heterodimeric insulin receptor complexes. Briefly, relatively large amounts (75–100 μg) of the purified insulin receptors (pH 7.8) were adjusted to pH 8.40–8.50 by the addition of 1.0 M Tris (pH 10.5) for 25 min at 23 °C, followed by a 5-min treatment in the presence or absence of freshly prepared 2.0 mM DTT. The reaction was terminated by application of the samples to a Sephadex G-50 gel filtration column (1.6 × 10 cm) equilibrated in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Triton-100, and 0.1% bovine serum albumin at 23 °C. The peak column fractions (2.4 mL) which contained the control $\alpha_2\beta_2$ heterotetrameric complex or the dissociated $\alpha\beta$ heterodimeric insulin receptors were identified by insulin binding. This combination of alkaline pH and DTT treatments resulted in greater than 95% conversion of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex into the $\alpha\beta$ heterodimeric state with the retention of insulin binding (Sweet et al., 1987a) and protein kinase activities (Sweet et al., 1987b). Under these conditions, the isolated $\alpha\beta$ heterodimeric insulin receptor complex was <5% contaminated with the DTT-treated but nondissociated $\alpha_2\beta_2$ heterotetrameric insulin receptor complex. In addition, the DTT-treated nondissociated $\alpha_2\beta_2$ heterotetrameric complex has been previously documented to be kinase inactive with greatly reduced insulin binding affinity (Sweet et al., 1987b).

^{125}I-Insulin Binding. The insulin receptor complexes were incubated in the presence of 0.25 nM monoiodinated ^{125}I-[A^{14}]insulin for 20 h at 4 °C with a final volume of 200 μL in Krebs–Ringer–Hepes buffer [50 mM Hepes (pH 7.6), 130 mM NaCl, 5.1 mM KCl, and 1.3 mM $MgSO_4$] plus 0.1% bovine serum albumin. The binding assay was terminated by the sequential addition of 0.5 mL of 0.1 bovine γ-globulin and 0.5 mL of 25% poly(ethylene glycol) at 4 °C (Cuatrecasas, 1972). The samples were collected by centrifugation at 12000*g* at 4 °C for 10 min. The pellets were washed and counted for the amount of ^{125}I-insulin bound. Nonspecific binding was determined in the presence of 1.0 μM unlabeled insulin and represented less than 5% of the total binding activity. Although the insulin binding properties of the $\alpha\beta$ heterodimeric and $\alpha_2\beta_2$ heterotetrameric insulin receptor complexes are different, equal insulin receptor occupancy occurs at tracer (0.25 nM) insulin concentrations (Sweet et al., 1987a).

Insulin Receptor Autophosphorylation. Insulin receptors were incubated for 1 h in the absence or presence of 200 nM insulin in 50 mM Hepes (pH 8.0), 10 mM $MgCl_2$, and 10 mM $MnCl_2$. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1.0 mM ATP, 3.0 μCi/nmol) to give a final concentration of 100 μM ATP. The reaction was allowed to continue for 5 min at 23 °C and was terminated by the addition of Laemmli sample buffer [50 mM Tris-HCl (pH 6.9), 10% glycerol, 0.05% bromophenol blue, and 1.0% SDS] followed by nonreducing SDS–polyacrylamide gel electrophoresis.

Exogenous Substrate Phosphorylation. The insulin receptor complexes were treated as described in the figure legends, and the kinase reactions were initiated by the addition of 2 mg/mL poly(Glu/Tyr) (4:1) and [γ-^{32}P]ATP as described above. The reactions were terminated after 15 min by spotting the samples on Whatman 3MM filter paper and immersion into ice-cold 10% (v/v) trichloroacetic acid plus 10 mM Na_2HPO_4. The filters were extensively washed in the above buffer, dried, and counted for ^{32}P. Filter papers treated as above in the absence of Glu/Tyr polymer or insulin receptors were always subtracted as background and accounted for less than 2% of the total radioactivity incorporated.

Bio-Gel A-1.5m Gel Filtration Chromatography. Insulin receptor complexes after Sephadex G-50 gel filtration chromatography were resolved on Bio-Gel A-1.5m gel filtration columns (1.6 × 42 or 50 cm, depending upon the experiment) equilibrated at 4 °C in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.02% NaN_3 (Bio-Gel buffer) plus any additions as described in the individual figure legends. After application of the appropriate samples to the columns, approximately 20 mL was voided before collection of 0.40-mL fractions at a flow rate of 20 mL/h. Insulin binding was performed as described above. This protocol has been previously established to separate the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes obtained by the alkaline pH and DTT treatments (Sweet et al., 1987a).

SDS–Polyacrylamide Gel Electrophoresis. One-dimensional SDS–polyacrylamide gel electrophoresis was performed by using a 3–10% gradient separating gel and a 3% stacking gel [acrylamide/bis(acrylamide) 30:0.8) as described by Laemmli (1970). The insulin receptor samples were prepared as described above, and the gels were fixed in 10% trichloroacetic acid/10 mM Na_2HPO_4, followed by staining with Coomassie Brilliant Blue R250. The gels were then dried and subjected to autoradiography on Kodak XAR-5 film using

Cronex lightening plus intensifying screens. The bands were then excised, rehydrated, and hydrolyzed using 0.5 mL of tissue solubilizer at 55 °C overnight. The samples were then prepared for scintillation counting by the addition of neutralizer scintillation cocktail in order to determine the amount of total β subunit incorporated ^{32}P.

Results

It has recently been reported that treatment of the insulin receptor with a combination of alkaline pH and DTT results in the formation of a functional $\alpha\beta$ heterodimeric insulin receptor complex from the native $\alpha_2\beta_2$ heterotetrameric disulfide-linked state (Boni-Schnetzler et al., 1986; Sweet et al., 1987a). Previous studies have observed that insulin treatment of the isolated $\alpha\beta$ heterodimeric insulin receptor complex induced the covalent association into a disulfide-linked $\alpha_2\beta_2$ heterotetrameric complex (Boni-Schnetzler et al., 1986; Sweet et al., 1987b). However, autophosphorylation of the $\alpha\beta$ heterodimeric complex in the absence of insulin demonstrated ^{32}P labeling of the $\alpha\beta$ heterodimeric complex by SDS–polyacrylamide gel electrophoresis which migrated with identical mobility as the $\alpha_2\beta_2$ heterotetrameric complex by Bio-Gel A-1.5m gel filtration chromatography (Morrison et al., 1988). These results suggested that the isolated $\alpha\beta$ heterodimers in the presence of divalent metal ions and ATP could noncovalently associate into an $\alpha_2\beta_2$ heterotetrameric state in the absence of insulin.

We therefore examined the ability of Mn/MgATP to induce the association of the $\alpha\beta$ heterodimeric insulin receptors into an $\alpha_2\beta_2$ heterotetrameric state (Figure 1). The isolated $\alpha\beta$ heterodimers were initially incubated for 1 h with Mn/MgATP, in the presence or absence of insulin, followed by rapid Sephadex G-50 and Bio-Gel A-1.5m gel filtration chromatography in columns equilibrated in the Bio-Gel column buffer without insulin in the presence or absence of Mn/MgATP. As previously reported (Sweet et al., 1987a), the $\alpha\beta$ heterodimeric complex incubated in the absence of insulin migrated with an approximate K_{av} = 0.33 in the Bio-Gel A-1.5m columns equilibrated in the absence of Mn/MgATP, corresponding to the mobility of the $\alpha\beta$ heterodimers (Figure 1A). The $\alpha\beta$ heterodimeric complex incubated in the presence of insulin for 1 h was observed to migrate with a K_{av} = 0.17, corresponding to the mobility of the $\alpha_2\beta_2$ heterotetrameric complex. However, the $\alpha\beta$ heterodimers incubated in the presence of Mn/MgATP and subjected to gel filtration chromatography in the Bio-Gel A-1.5m columns equilibrated with Mn/MgATP consistently migrated with identical mobility as the $\alpha_2\beta_2$ heterotetrameric complex whether or not they were pretreated with insulin (Figure 1B). These data demonstrate that the noncovalent association of the $\alpha\beta$ heterodimeric insulin receptors into an $\alpha_2\beta_2$ heterotetrameric state was dependent upon the continuous presence of divalent metal ions/ATP.

The fact that the Mn/MgATP-induced noncovalent association of the $\alpha\beta$ heterodimers into an $\alpha_2\beta_2$ heterotetrameric state required the continuous presence of Mn/MgATP (Figure 1) indicated that this noncovalent association resulted from the binding of Mn/MgATP to the $\alpha\beta$ heterodimeric complex and was not due to insulin receptor autophosphorylation. Consistent with this interpretation, the formation of the noncovalent $\alpha_2\beta_2$ heterotetrameric complex from the $\alpha\beta$ heterodimers occurred in the continuous presence of both Mn/MgADP and Mn/MgAMPPCP without any detectable $\alpha\beta$ heterodimers remaining (data not shown). As a control, the $\alpha\beta$ heterodimers maintained in the absence of divalent metal ions and adenine nucleotides migrated in the expected position corresponding to the mobility of the $\alpha\beta$ heterodimeric

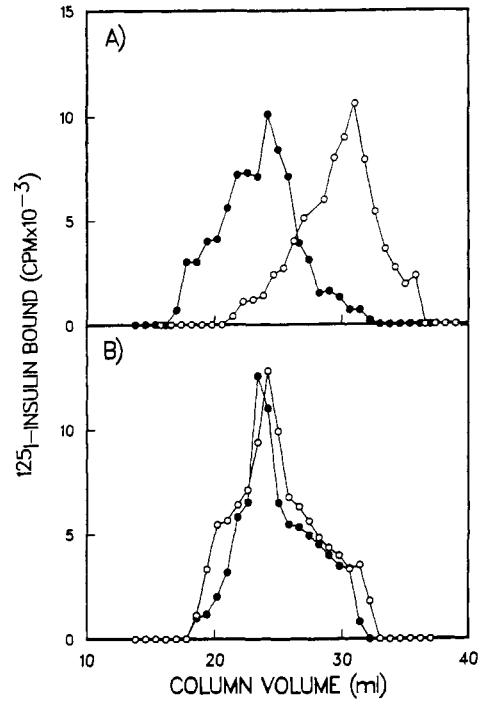

FIGURE 1: Mn/MgATP-induced association between $\alpha\beta$ heterodimeric insulin receptors into an $\alpha_2\beta_2$ heterotetrameric state. (A) The $\alpha\beta$ heterodimeric complex (12 μg) was isolated as described under Experimental Procedures and incubated for 1 h at 23 °C with 2 mM $MgCl_2$, 10 mM $MnCl_2$, and 100 μM NaATP in the presence (●) or absence (○) of 200 nM insulin. The samples were then subjected to rapid Sephadex G-50 (1.6 × 10 cm) and Bio-Gel A-1.5m (1.6 × 42 cm) gel filtration chromatography in columns equilibrated in the standard Bio-Gel buffer in the absence of Mn/MgATP. Aliquots of every other fraction were assayed for ^{125}I-insulin binding as described under Experimental Procedures. (B) The isolated $\alpha\beta$ heterodimeric insulin receptor complex (12 μg) was incubated for 1 h at 23 °C with 2 mM $MnCl_2$, 10 mM $MgCl_2$, and 100 μM NaATP in the presence (●) or absence (○) of 200 nM insulin. The samples were then subjected to rapid Sephadex G-50 and Bio-Gel A-1.5m gel filtration chromatography in columns equilibrated in the standard Bio-Gel buffer containing 2 mM $MnCl_2$, 10 mM $MgCl_2$, and 100 μM NaATP. ^{125}I-Insulin binding was determined for every other column fraction as described under Experimental Procedures.

complex. Other nucleotides which are not substrates for the insulin receptor kinase, Mn/MgAMP and Mn/MgGMPPNP, were unable to induce the noncovalent association of the $\alpha\beta$ heterodimeric insulin receptors into the $\alpha_2\beta_2$ heterotetrameric complex (data not shown).

We next examined the effect of the individual divalent metal ions as well as ATP to induce the noncovalent association between the $\alpha\beta$ heterodimeric complexes to form the $\alpha_2\beta_2$ heterotetrameric state (Figure 2). The isolated $\alpha\beta$ heterodimers continuously maintained in the presence of Mn/Mg alone or NaATP alone did not associate into the $\alpha_2\beta_2$ heterotetrameric complex (Figure 2A). However, the combination of Mn/MgATP was fully effective in inducing the noncovalent $\alpha_2\beta_2$ heterotetrameric formation as analyzed by Bio-Gel A-1.5m gel filtration chromatography (Figure 2A). In contrast, no apparent selectivity was observed between MnATP or MgATP to induce the association of the $\alpha\beta$ heterodimers into the noncovalent $\alpha_2\beta_2$ heterotetrameric state (Figure 2B). These data demonstrate that a divalent metal ion in the presence of an adenine nucleotide can induce the noncovalent association of $\alpha\beta$ heterodimers into the $\alpha_2\beta_2$ heterotetrameric state independent of β-subunit autophosphorylation.

One method to distinguish the relative contributions of the covalent and noncovalent association of the $\alpha\beta$ heterodimers into the $\alpha_2\beta_2$ heterotetrameric state on the insulin stimulation

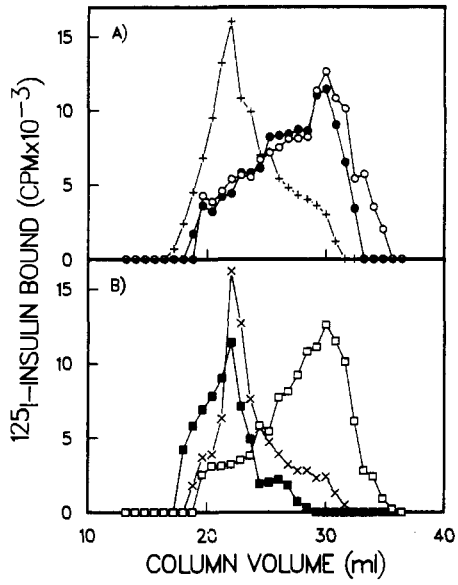

FIGURE 2: Effects of the individual divalent metal ions and NaATP on the noncovalent association of the $\alpha\beta$ heterodimeric insulin receptors into the $\alpha_2\beta_2$ heterotetrameric state. (A) The $\alpha\beta$ heterodimeric insulin receptor complex (12 μg) was incubated in the presence of 2 mM $MnCl_2$ plus 10 mM $MgCl_2$ (○), 100 μM NaATP (●), or 2 mM $MnCl_2$ and 10 mM $MgCl_2$ plus 100 μM NaATP (+) for 1 h at 23 °C. The samples were then subjected to rapid Sephadex G-50 and Bio-Gel A-1.5m gel filtration chromatography in columns equilibrated with the standard Bio-Gel buffer containing the same ions as the initial treatments. ^{125}I-Insulin binding to every other column fraction was determined as described under Experimental Procedures. (B) The $\alpha\beta$ heterodimeric insulin receptor complex (12 μg) was incubated in the absence (□) and in the presence of either 10 mM $MgCl_2$ plus 100 μM NaATP (×) or 2 mM $MnCl_2$ plus 100 μM NaATP (■) for 1 h at 23 °C. The samples were then subjected to rapid Sephadex G-50 and Bio-Gel A-1.5m gel filtration chromatography in columns equilibrated with the standard Bio-Gel buffer containing the same ions as the initial treatments. ^{125}I-Insulin binding to every other column fraction was determined as described under Experimental Procedures.

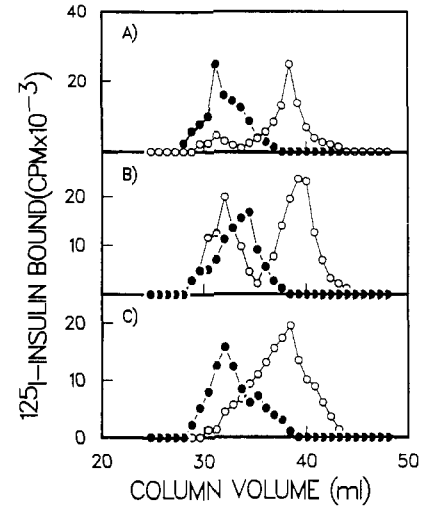

FIGURE 3: IAN inhibition of the insulin-dependent covalent association between $\alpha\beta$ heterodimeric insulin receptors into the $\alpha_2\beta_2$ heterotetrameric complex. (A) The isolated $\alpha\beta$ heterodimeric insulin receptors (25 μg) were incubated for 1 h at 23 °C in the absence (○) or presence (●) of 200 nM insulin. The samples were then subjected to Bio-Gel A-1.5m (1.6 × 50 cm) gel filtration chromatography in columns equilibrated with the standard Bio-Gel buffer in the absence of Mn/MgATP. Aliquots of every other column fraction were assayed for ^{125}I-insulin binding as described under Experimental Procedures. (B) The isolated $\alpha\beta$ heterodimeric insulin receptors (25 μg) were incubated for 1 h at 23 °C with 1 mM IAN either prior (○) or subsequent (●) to the addition of 200 nM insulin for 1 h. ^{125}I-Insulin binding was determined for every other column fraction as described in (A). (C) The isolated $\alpha\beta$ heterodimeric insulin receptors (25 μg) were incubated for 1 h at 23 °C with 10 mM IAN either prior (○) or subsequent (●) to the addition of 200 nM insulin for 1 h. ^{125}I-Insulin binding was determined as described in (A). The apparent difference in the column profiles presented in Figures 3 and 4 compared to Figures 1 and 2 reflect the use of longer Bio-Gel A-1.5m columns (1.6 × 50 cm versus 1.6 × 42 cm) to provide increased resolution of the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor species.

of protein kinase activity would be to specifically block the insulin-dependent disulfide bond formation between the $\alpha\beta$ heterodimers. Unlike *N*-ethylmaleimide, the specific sulfhydryl alkylating agent iodoacetamide (IAN) has been previously documented to have no significant effect on insulin binding or insulin stimulation of protein kinase activity in the $\alpha_2\beta_2$ heterotetrameric complex (Wilden & Pessin, 1987). Therefore, we incubated the isolated $\alpha\beta$ heterodimeric insulin receptor complex with 1 and 10 mM IAN in the absence or presence of insulin (Figure 3). The control $\alpha\beta$ heterodimeric complex incubated in the absence or presence of insulin, prior to Bio-Gel A-1.5m gel filtration chromatography, migrated with the typical mobility of the $\alpha\beta$ heterodimeric (K_{av} = 0.33) and $\alpha_2\beta_2$ heterotetrameric (K_{av} = 0.17) complexes, respectively (Figure 3A). It should be noted that the apparent difference in the column profiles presented in Figures 1 and 2 compared to Figures 3 and 4 were due to the use of longer Bio-Gel A-1.5m columns (1.6 × 50 cm as opposed to 1.6 × 42 cm) to provide increased resolution of the two insulin receptor species. Nevertheless, the $\alpha\beta$ heterodimeric complex incubation with 1 mM IAN (Figure 3B) or 10 mM IAN (Figure 3C) subsequent to insulin treatment consistently migrated in the Bio-Gel A-1.5m columns with the relative mobility of the $\alpha_2\beta_2$ heterotetrameric complex (K_{av} = 0.17). In contrast, incubation of the $\alpha\beta$ heterodimeric complex with 1 mM IAN prior to insulin treatment resulted in the appearance of two species by Bio-Gel A-1.5m gel filtration chromatography corresponding to the mobilities of the $\alpha\beta$ heterodimeric and $\alpha_2\beta_2$ heterotetrameric complexes (Figure 3B). Incubation of the $\alpha\beta$ heterodimeric complex with 10 mM IAN before insulin treatment completely blocked the insulin-dependent conversion to the $\alpha_2\beta_2$ heterotetrameric complex, resulting in a single broad peak (K_{av} = 0.33) with the mobility of the $\alpha\beta$ heterodimeric complex (Figure 3C). These data demonstrate that 10 mM IAN can fully inhibit the insulin-induced covalent association of $\alpha\beta$ heterodimeric insulin receptors into the $\alpha_2\beta_2$ heterotetrameric complex.

To determine the effect of IAN on the Mn/MgATP-induced noncovalent association of the $\alpha\beta$ heterodimers into the $\alpha_2\beta_2$ heterotetrameric state, a series of Bio-Gel A-1.5m gel filtration chromatography studies were performed in the absence of insulin (Figure 4). Similar to Figure 1, the control $\alpha\beta$ heterodimeric complex applied to Bio-Gel A-1.5m gel filtration columns equilibrated in the absence of Mn/MgATP was observed to migrate with the expected mobility of the $\alpha\beta$ heterodimeric complex (K_{av} = 0.33) whereas it migrated with the mobility of the $\alpha_2\beta_2$ heterotetrameric complex (K_{av} = 0.17) when the Bio-Gel A-1.5m column was equilibrated in the presence of Mn/MgATP (Figure 4A). In contrast to the ability of IAN to inhibit the insulin-induced covalent association of the $\alpha\beta$ heterodimeric complex into the $\alpha_2\beta_2$ heterotetrameric state (Figure 3), incubation of the $\alpha\beta$ heterodimeric complex with 1 or 10 mM IAN, prior to Bio-Gel A-1.5m gel filtration chromatography in the presence of Mn/MgATP, persistently migrated with the mobility of the $\alpha_2\beta_2$ heterotetrameric complex (Figure 4B). These data demonstrate that although IAN treatment can block the insulin-dependent covalent association of the $\alpha\beta$ heterodimeric complex into the $\alpha_2\beta_2$ heterotetrameric state, it does not affect

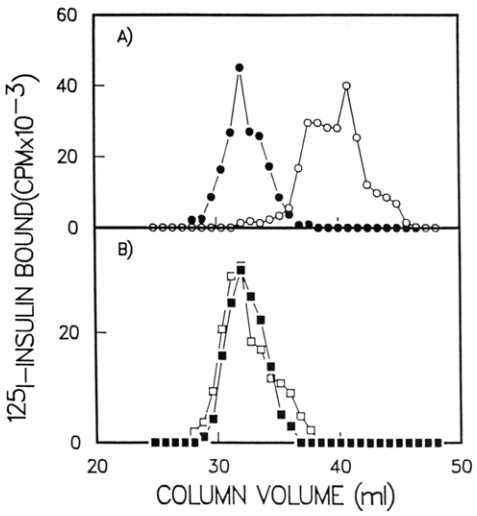

FIGURE 4: Effect of IAN on the Mn/MgATP noncovalent association of the αβ heterodimeric insulin receptors into the $\alpha_2\beta_2$ heterotetrameric complex. (A) The isolated αβ heterodimeric insulin receptors (40 μg) were incubated for 1 h at 23 °C in the absence (○) or presence (●) of Mn/MgATP and subjected to Bio-Gel A-1.5m gel filtration chromatography on columns equilibrated in the standard Bio-Gel buffer with or without Mn/MgATP. Aliquots of every other column fraction were assayed for ^{125}I-insulin binding as described under Experimental Procedures. (B) The isolated αβ heterodimeric insulin receptors (25 μg) were incubated for 1 h at 23 °C with 1 mM IAN (■) or 10 mM IAN (□) prior to an additional 1-h incubation in the presence of Mn/MgATP. The samples were then subjected to Bio-Gel A-1.5m gel filtration chromatography in columns equilibrated with the standard Bio-Gel buffer containing Mn/MgATP. ^{125}I-Insulin binding to every other column fraction was determined as described in (A).

the noncovalent association induced by Mn/MgATP.

The ability of IAN to block the insulin-dependent covalent but not the Mn/MgATP-dependent noncovalent association of the αβ heterodimers was used to address the role of noncovalent subunit association in the insulin activation of the insulin receptor kinase (Figure 5). Similar to earlier studies (Wilden & Pessin, 1987), incubation of the $\alpha_2\beta_2$ heterotetrameric complex with 1 mM IAN or 10 mM IAN in the presence or absence of insulin had no significant effect on β-subunit autophosphorylation (data not shown). Autophosphorylation of the αβ heterodimeric complex in the absence of insulin demonstrated the ^{32}P labeling of the αβ heterodimeric complex as well as the appearance of a trace amount of labeled $\alpha_2\beta_2$ heterotetrameric complex (Figure 5A, lane 1). The presence of the ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex in the autophosphorylated αβ heterodimeric preparation is probably due to a small amount of Mn/MgATP-induced covalent association (5–8%) and is not a result of any contaminating $\alpha_2\beta_2$ heterotetrameric complexes (Sweet et al., 1987a,b). As previously reported (Sweet et al., 1987b), autophosphorylation of the αβ heterodimeric complex in the presence of insulin not only increased the ^{32}P labeling of the αβ heterodimeric complex approximately 2-fold but also increased the extent of labeling in the $\alpha_2\beta_2$ heterotetrameric complex greater than 10-fold (Figure 5A, lane 2, and Table I). Incubation of the αβ heterodimeric complex with 1 mM IAN subsequent to a second control (Figure 5A, lane 5) or insulin treatment (Figure 5A, lane 6) had no significant effect on β-subunit autophosphorylation (Table I). However, incubation of the αβ heterodimeric complex with 1 mM IAN prior to a second control (Figure 5A, lane 3) or insulin treatment (Figure 5A, lane 4) significantly decreased the extent of autophosphorylated $\alpha_2\beta_2$ heterotetrameric complex with the concomitant increase in the amount of ^{32}P-labeled αβ heterodimeric complex. This occurred such that the overall insulin stimulation of β-subunit autophosphorylation was essentially unchanged (5–7-fold) under all three experimental conditions (Table I).

Similarly, preincubation of the αβ heterodimeric complex with 10 mM IAN subsequent to control or insulin treatments (Figure 5B, lanes 5 and 6) was not significantly different than the basal or insulin-stimulated autophosphorylation in the

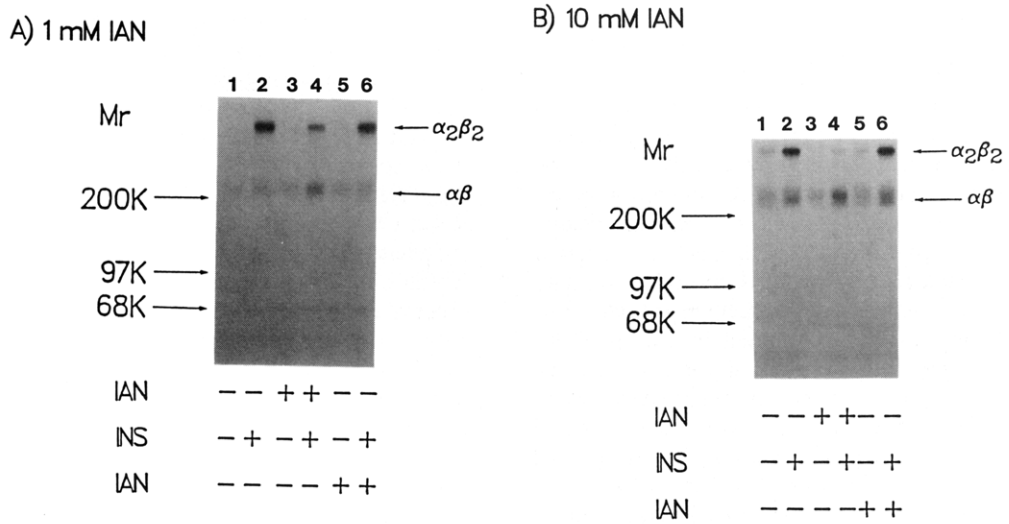

FIGURE 5: Insulin-dependent autophosphorylation of the αβ heterodimeric insulin receptor complex in the presence of IAN. (A) The isolated αβ heterodimeric insulin receptors (1.2 μg) were initially incubated for 1 h at 23 °C in either the absence (lanes 1, 2, 5, and 6) or the presence of 1 mM IAN (lanes 3 and 4). The samples were next incubated for 1 h in either the absence (lanes 1, 3, and 5) or the presence of 200 nM insulin (lanes 2, 4, and 6). The samples were then incubated for an additional 1 h in either the absence (lanes 1–4) or the presence of 1 mM IAN (lanes 5 and 6). The samples were then autophosphorylated by the addition of 100 μM [γ-^{32}P]ATP for 5 min and subjected to nonreducing SDS–polyacrylamide gel electrophoresis and autoradiography as described under Experimental Procedures. (B) The isolated αβ heterodimeric insulin receptors (1.2 μg) were initially incubated for 1 h at 23 °C in either the absence (lanes 1, 2, 5, and 6) or the presence of 10 mM IAN (lanes 3 and 4). The samples were next incubated for 1 h either in the absence (lanes 1, 3, and 5) or in the presence of 200 nM insulin (lanes 2, 4, and 6). The sample were then incubated for an additional 1 h in either the absence (lanes 1–4) or the presence of 10 mM IAN (lanes 5 and 6). The samples were then autophosphorylated for 5 min, subjected to nonreducing SDS–polyacrylamide gel electrophoresis, and prepared for autoradiography as described under Experimental Procedures. In both groups, the order of IAN and insulin (INS) additions is directly indicated on the figure.

Table I: Effect of Iodoacetamide (IAN) on the Insulin Stimulation of β-Subunit Autophosphorylation of the Isolated αβ Heterodimeric Complex[a]

	cpm incorporated		insulin stimulation (x-fold)
	control	insulin	
	Experiment A (1.0 mM IAN)		
untreated	188 ± 8.6	1280 ± 68.2	6.8 ± 0.1
IAN plus insulin	208 ± 19.6	1025 ± 105.3	4.9 ± 0.8
insulin plus IAN	227 ± 32.1	1222 ± 90.2	5.4 ± 1.1
	Experiment B (10 mM IAN)		
untreated	221 ± 12.7	898 ± 75.2	4.1 ± 0.6
IAN plus insulin	174 ± 25.1	670 ± 140.1	3.8 ± 1.1
insulin plus IAN	147 ● 60.3	775 ± 115.0	5.3 ± 1.3

[a] The isolated αβ heterodimeric insulin receptor complexes (1.0 μg) were incubated for 60 min with various ordered additions of insulin and IAN as described in Figure 5. The extent of β-subunit autophosphorylation was determined by the addition of [γ-^{32}P]ATP for 5 min and subjecting the samples to SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. The ^{32}P-labeled band composed of both the αβ heterodimeric and $\alpha_2\beta_2$ heterotetrameric insulin receptor complexes were excised from the gel, pooled, and quantitated by scintillation counting as described under Experimental Procedures. The cpm values presented represent the ^{32}P incorporation into both the αβ heterodimeric and $\alpha_2\beta_2$ heterotetrameric insulin receptor complexes. This experiment was independently performed 4 times with similar results. The values represent the mean of four trials with their standard deviations from the mean.

absence of IAN incubation (Figure 5B, lanes 1 and 2). However, preincubation of the αβ heterodimeric complex with 10 mM IAN prior to the addition of insulin completely inhibited the appearance of the autophosphorylated $\alpha_2\beta_2$ heterotetrameric complex with a significant increase in the ^{32}P-labeled αβ heterodimeric band (Figure 5B, lane 4). Although there was an apparent 20% decrease in the overall extent of autophosphorylation in the samples initially incubated with 10 mM IAN, the amount of insulin-stimulated β-subunit autophosphorylation was essentially unchanged, approximately 4-fold (Table I).

The effect of IAN on exogenous substrate protein kinase activity of the αβ heterodimeric insulin receptor complex was next examined (Figure 6). Insulin stimulation of the initial rate of Glu/Tyr (2 mg/mL) phosphorylation was approximately 8-fold under these conditions. Incubation of the αβ heterodimeric complex with 1 mM IAN or 10 mM IAN subsequent to insulin treatment displayed a 9-fold stimulation of Glu/Tyr phosphorylation (Figure 6). Incubation of the αβ heterodimeric complex with 1 mM IAN prior to insulin treatment again had no effect on the insulin stimulation of substrate kinase activity. In contrast, 10 mM IAN preincubation of the αβ heterodimeric insulin receptors caused a 50% decrease in the extent of basal substrate kinase activity and a 65% decrease in the amount of insulin-stimulated substrate kinase activity. However, similar to the effect of autophosphorylation (Figure 5B), the amount of insulin-stimulated protein kinase activity (5-fold) was only marginally affected under these conditions (Figure 6). Incubation of the $\alpha_2\beta_2$ heterotetrameric complex with these concentrations of IAN had no effect on exogenous substrate protein kinase activity (data not shown). Further, identical results were obtained when the initial rate of Glu/Tyr (0.1 mg/mL) phosphorylation was determined (data not shown).

Discussion

Several recent studies have suggested that the native EGF receptor exists primarily in a kinase-inactive monomeric state (M_r 170 000) and that EGF binding activates the tyrosine-specific protein kinase activity by inducing the formation of

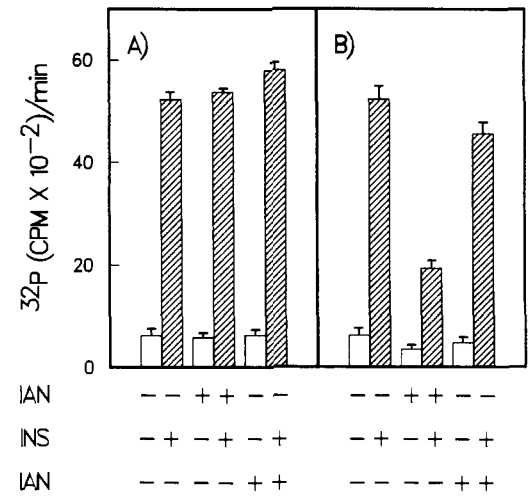

FIGURE 6: Insulin-dependent exogenous protein kinase activity of the αβ heterodimeric insulin receptor complex in the presence of IAN. The isolated αβ heterodimeric insulin receptors (1.2 μg) were incubated for 1 h at 23 °C with the sequential additions of insulin (INS, open box) and IAN (hatched box) in the order described in Figure 5 and directly indicated on the figure. The initial rate of Glu/Tyr (2 mg/mL) phosphorylation was determined by the addition of [γ-^{32}P]ATP for 15 min as described under Experimental Procedures. The error bars represent the range of values of assays performed in duplicate. Similar results were obtained when the initial rate of 0.1 mg/mL Glu/Tyr phosphorylation was determined.

a noncovalent homodimeric complex (Yarden & Schlessinger, 1987a,b; Boni-Schnetzler & Pilch, 1987). To investigate the role of subunit interactions in the insulin-dependent regulation of the insulin receptor protein kinase activity, we and others have developed methods to isolate a functional αβ heterodimeric complex from the native $\alpha_2\beta_2$ heterotetrameric state (Boni-Schnetzler et al., 1986, 1987; Sweet et al., 1987a,b; Koch et al., 1986; Deger et al., 1986). The availability of a functional αβ heterodimeric insulin receptor complex has allowed detailed characterization of several intrinsic properties of the insulin receptor. Initial studies have demonstrated that the high-affinity curvilinear insulin binding typically observed for the $\alpha_2\beta_2$ heterotetrameric complex requires the interaction between αβ heterodimeric species (Deger et al., 1986; Sweet et al., 1987a; Boni-Schnetzler et al., 1987). More recently, it was reported that insulin can induce the covalent association of the isolated αβ heterodimeric complex into a disulfide-linked $\alpha_2\beta_2$ heterotetrameric state (Boni-Schnetzler et al., 1986; Sweet et al., 1987b; Morrison et al., 1988). The formation of the $\alpha_2\beta_2$ heterotetrameric complex apparently correlated with the insulin stimulation of both β-subunit autophosphorylation and exogenous substrate phosphorylation (Boni-Schnetzler et al., 1986, 1988; Sweet et al., 1987b; Morrison et al., 1988). These data clearly demonstrated that insulin activation of the insulin receptor protein kinase requires interactions between the individual αβ heterodimers within the holoreceptor complex. However, the methods used in these previous studies (denaturing SDS–polyacrylamide gel electrophoresis) were unable to distinguish any possible contributions of noncovalent association between the αβ heterodimers in this signal transduction process.

In the present studies, we have documented that the isolated αβ heterodimeric insulin receptor complex undergoes an Mn/MgATP-dependent noncovalent association into an $\alpha_2\beta_2$ heterotetrameric state (Figure 1). This noncovalent association was found to be dependent on the continuous presence of both divalent metal ions and ATP. Further, a nonhydrolyzable ATP analogue, AMPPCP, in the continuous presence of Mn/Mg was fully capable of supporting the noncovalent association

of the $\alpha\beta$ heterodimers into the $\alpha_2\beta_2$ heterotetrameric state (data not shown). Similarly, Mn/MgADP also induced the noncovalent association of the $\alpha\beta$ heterodimers. Interestingly, both MnATP and MgATP were independently found to be fully functional with regard to the induction of this noncovalent association at saturating divalent metal ion concentrations (Figure 2). It should be noted that both MnATP and MgATP can support the tyrosine-specific protein kinase activity of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex, albeit with different K_m values (White et al., 1984; Kwok et al., 1986). Even though the consequences of Mn/MgATP binding on the regulation of the insulin receptor kinase have not yet been clearly defined, Mn/MgATP binding has also been implicated in the regulation of the monomer–dimer equilibrium of the EGF receptor kinase system (Basu et al., 1986). We postulate that in the native $\alpha_2\beta_2$ heterotetrameric state MgATP may also serve as an allosteric regulator of insulin receptor function by maintaining the insulin receptor subunits in an appropriate conformation for insulin activation of the protein kinase activity.

Previously, we have demonstrated that the specific sulfhydryl alkylating agent IAN, although it directly reacts with the insulin receptor, does not inhibit insulin binding or insulin stimulation of protein kinase activity (Wilden & Pessin, 1987). Further, this agent was found to completely inhibit the insulin-induced covalent disulfide bond formation between the $\alpha\beta$ heterdimeric insulin receptors when added prior to ^{125}I-insulin affinity cross-linking (Morrison et al., 1988). In order to distinguish between the insulin-dependent covalent association and the Mn/MgATP-induced noncovalent association on the insulin stimulation of the insulin receptor protein kinase activity, we therefore used IAN to specifically block the insulin-dependent covalent association (Figure 3). Under these conditions, 1 mM IAN partially inhibits the insulin-dependent covalent association (Figure 3B) whereas 10 mM IAN completely blocked the covalent disulfide bond formation (Figure 3C). In contrast, the Mn/MgATP-induced noncovalent association between the $\alpha\beta$ heterodimers was totally unaffected by the presence of IAN (Figure 4).

This experimental paradigm was then used to examine the relationship between insulin stimulation of protein kinase activity with the covalent and noncovalent association of the insulin receptor $\alpha\beta$ heterodimers (Figures 5 and 6, Table I). Complete blockade of the insulin-dependent covalent association with 10 mM IAN, although it slightly decreased the overall extent of β-subunit autophosphorylation (20%), had no significant effect on the magnitude of insulin-stimulated autophosphorylation (Table I). Similarly, IAN treatment of the $\alpha\beta$ heterodimeric insulin receptor complex was unable to prevent the insulin stimulation of exogenous protein kinase activity (Figure 6). These data indicate that only noncovalent interactions between the individual $\alpha\beta$ heterodimeric complexes were necessary for insulin stimulation of protein kinase activity. Although the insulin-dependent covalent association of the $\alpha\beta$ heterodimeric complex was an intrinsic function of insulin binding, the role of the insulin-induced disulfide bond formation in the activation of the $\alpha\beta$ heterodimeric insulin receptor protein kinase activity remains unknown at the present time.

Recently, it has been reported that although insulin can induce $\alpha\beta$ heterodimeric subunit interactions in partially purified insulin receptors this apparently occurred in a noncovalent manner. Insulin-dependent covalent association of the $\alpha\beta$ heterodimers into an $\alpha_2\beta_2$ heterotetrameric state was only detectable in the presence of oxidized glutathione (Boni-Schnetzler et al., 1988). In addition, incubation of the $\alpha\beta$ heterodimers with Mn/MgATP in the absence of insulin were found to be incapable of associating into the covalent $\alpha_2\beta_2$ heterotetrameric state by oxidized glutathione. In contrast, we have observed an insulin-induced covalent association of the $\alpha\beta$ heterodimeric insulin receptors into an $\alpha_2\beta_2$ heterotetrameric state in the absence of added oxidizing agents (Sweet et al., 1987b; Morrison et al., 1988; Figure 5) as well as noncovalent interactions in the presence of Mn/MgATP (Morrison et al., 1988; Figures 1–4). One likely reason for these differences may reflect the use of purified insulin receptors at significantly higher insulin receptor concentrations (Sweet et al., 1987b; Morrison et al., 1988; this study) compared to the use of partially purified insulin receptor preparations (Boni-Schnetzler et al., 1986, 1988). Alternatively, slight differences in the alkaline pH and DTT incubations conditions were employed as well as in the isolation method for the insulin receptor $\alpha\beta$ heterodimers. Nevertheless, all these studies directly support the necessity of $\alpha\beta$ heterodimeric subunit interaction in the insulin-dependent activation of the insulin receptor protein kinase activity.

In summary, our data demonstrate that although insulin in the presence of Mn/MgATP can induce the formation of a covalent disulfide linkage between the $\alpha\beta$ heterodimers, the covalent $\alpha_2\beta_2$ heterotetrameric complex is not necessary for the insulin stimulation of the insulin receptor protein kinase activity. However, these data support the conclusion that, at minimum, noncovalent interactions between the individual $\alpha\beta$ heterodimeric insulin receptor complexes are required for the insulin stimulation of protein kinase activity.

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Registry No. ATP, 56-65-5; AMPPCP, 3469-78-1; ADP, 58-64-0; Mn/MgATP, 117471-94-0; Mn, 7439-96-5; Mg, 7439-95-4; insulin, 9004-10-8; insulin receptor protein kinase, 88201-45-0.

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