Functional Properties of an Isolated $\alpha\beta$ Heterodimeric Human Placenta Insulin-like Growth Factor 1 Receptor Complex†

Stephen M. Feltz, Michael L. Swanson, John A. Wemmie, and Jeffrey E. Pessin*
Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242
Received September 9, 1987; Revised Manuscript Received December 22, 1987

ABSTRACT: Treatment of human placenta membranes at pH 8.5 in the presence of 2.0 mM dithiothreitol (DTT) for 5 min, followed by the simultaneous removal of the DTT and pH adjustment to pH 7.6, resulted in the formation of a functional $\alpha\beta$ heterodimeric insulin-like growth factor 1 (IGF-1) receptor complex from the native $\alpha_2\beta_2$ heterotetrameric disulfide-linked state. The membrane-bound $\alpha\beta$ heterodimeric complex displayed similar curvilinear ^{125}I-IGF-1 equilibrium binding compared to the $\alpha_2\beta_2$ heterotetrameric complex. Triton X-100 solubilization of the alkaline pH and DTT-pretreated placenta membranes, followed by Bio-Gel A-1.5m gel filtration chromatography, was found to effectively separate the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor species. ^{125}I-IGF-1 binding to both the isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes demonstrated a marked straightening of the Scatchard plots, compared to the placenta membrane-bound IGF-1 receptors, with a 2-fold increase in the high-affinity binding component. Similar to the membrane-bound IFG-1 receptor species, the ^{125}I-IGF-1 binding properties between the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes were not significantly different. IGF-1 stimulation of IGF-1 receptor autophosphorylation indicated that the ligand-dependent activation of $\alpha\beta$ heterodimeric protein kinase activity occurred concomitant with the reassociation into a covalent $\alpha_2\beta_2$ heterotetrameric state. These data demonstrate that (i) a combination of alkaline pH and DTT treatment of human placenta membranes results in the formation of an $\alpha\beta$ heterodimeric IGF-1 receptor complex, (ii) unlike the insulin receptor, high-affinity homogeneous IGF-1 binding occurs in both the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes, and (iii) IGF-1-dependent autophosphorylation of the $\alpha\beta$ heterodimeric IGF-1 receptor complex correlates with an IGF-1-dependent covalent reassociation into an $\alpha_2\beta_2$ heterotetrameric disulfide-linked state.

Insulin and insulin-like growth factor 1 (IGF-1)[1] are structurally related polypeptide hormones with a high degree of amino acid homology as well as overlapping biological activities (Rinderknecht & Humbel, 1976, 1978; Van Wyk & Underwood, 1978; Zapf et al., 1978; Froesch et al., 1985). Each of these hormones binds with high affinity to its own specific cell-surface receptor but can also bind to the heterologous receptor albeit with a substantially reduced binding affinity (Van Wyk et al., 1980; Bhaumick et al., 1981a; Kasuga et al., 1981; Massague & Czech, 1982). Human placenta membranes are rich in both the insulin and IGF-1 (type 1) receptors which, similar to insulin and IGF-1, also share structural and functional properties (Marshall et al., 1974; Jacobs et al., 1980; Massague et al., 1980; Bhaumick et al., 1981b; Chernausek et al., 1981; Kasuga et al., 1981, 1982; Jacobs & Cuatrecasas, 1981; Massague & Czech, 1982). These receptor species are both composed of two nonidentical subunits with approximate molecular weights of 135 000 (α) and 95 000 (β) disulfide-linked into an $\alpha_2\beta_2$ heterotetrameric complex (Czech, 1985; Kahn, 1985; Jacobs, 1985; Rechler & Nissley, 1985). The α subunit(s) contain(s) the high-affinity ligand binding domain(s) and exist(s) exclusively on the extracellular face of the plasma membrane (Ebina et al., 1985; Grunfeld et al., 1985; Ullrich et al., 1985, 1986; Pilch et al., 1986). The α subunits are covalently anchored to the cell surface by disulfide bonds with the transmembrane β subunits which encode for the intracellular tyrosine-specific protein kinase domain, ATP binding site(s), and several phosphotyrosine acceptor sites (Avruch et al., 1982; Kasuga et al., 1982b,c; Jacobs et al., 1983; Roth & Cassell, 1983; Rubin et al., 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983; Zick et al., 1984; Sasaki et al., 1985; Fujita-Yamaguchi et al., 1986; Yu et al., 1986). Although extensive structural information has been accumulated for both the insulin and IGF-1 receptors, the molecular mechanism by which extracellular ligand binding to the α subunit results in the transmembrane activation of the β-subunit intracellular protein kinase domain has not been elucidated to date. Recently, a method has been developed to obtain functional insulin receptor $\alpha\beta$ heterodimers from the $\alpha_2\beta_2$ heterotetrameric disulfide-linked complex using a combination of alkaline pH and DTT (Boni-Schnetzler et al., 1986, 1987; Sweet et al., 1987a,b). Several studies examining the properties of the $\alpha\beta$ heterodimeric insulin receptor complex have demonstrated homogeneous insulin binding (linear Scatchard plot) whereas the typically observed high-affinity curvilinear insulin binding apparently required the presence of the $\alpha_2\beta_2$ heterotetrameric complex (Deger et al., 1986; Koch et al., 1986; Boni-Schnetzler et al., 1987, Sweet et al., 1987a). Further, the $\alpha\beta$ heterodimeric insulin receptor complex was found to be relatively kinase inactive and insulin independent (Shia et al., 1983; Boni-Schnetzler et al., 1986) but displayed

†Supported by Research Grants DK33823 and DK25295 from the National Institutes of Health and a Basil O'Connor research award from the March of Dimes Birth Defects Foundation. J.E.P. is a recipient of a Research and Development Award from the American Diabetes Association.

[1] Abbreviations: IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2; DTT, dithiothreitol; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate; IAN, iodoacetamide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

0006-2960/88/0427-3234$01.50/0

insulin-stimulated protein kinase activity when accompanied by covalent reassociation into a disulfide-linked $\alpha_2\beta_2$ heterotetrameric state (Boni-Schnetzler et al., 1986; Sweet et al., 1987b).

In this paper, we have further extended these studies to determine the IGF-1 binding properties and protein kinase activity of the $\alpha\beta$ heterodimeric IGF-1 receptor complex isolated from human placenta membranes. The relationship between IGF-1 binding, IGF-1 stimulation of autophosphorylation, and IGF-1-dependent covalent reassociation of the $\alpha\beta$ heterodimeric complex into an $\alpha_2\beta_2$ heterotetrameric state has been examined.

Experimental Procedures

Materials

Bovine serum albumin, bacitracin, protease inhibitors, and DTT were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear. Triton X-100, Bio-Gel A-1.5m resin, and electrophoresis reagents were obtained from Bio-Rad. DSS, XAR-5 film, and Cronex lightning-plus intensifying screens were obtained through Pierce, Kodak, and Du Pont Chemical Co., respectively. Molecular weight SDS–polyacrylamide gel standards were obtained from Bethesda Research Laboratories. Porcine insulin was graciously provided by Dr. R. Chance, Eli Lilly Co., and recombinant IGF-1 was purchased from Toyobo. ^{125}I-IGF-1 (100–150 Ci/g) was prepared by the Diabetes and Endocrinology Research Center, The University of Iowa.

Methods

Membrane Preparation. Human placenta membranes were prepared from freshly obtained human placentas by the procedure of Harrison and Itin (1980). Placental membranes (10 mg/mL) were stored at −70 °C in 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 25 mM benzamidine hydrochloride, 10 μM leupeptin, 50 trypsin inhibitor units of aprotinin, 1 mM 1,10-phenanthroline, and 1 μM pepstatin A. Protein concentration was determined by a modification of the method described by Lowry et al. (1951).

Treatment of Membranes with DTT. Placenta membranes (10 mg/mL) were incubated at 23 °C for 25 min at pH 8.5 by the addition of the appropriate amount of 1.0 M Tris. Membranes were then incubated for 5 min at 23 °C in the presence or absence of freshly prepared 2.0 mM DTT in water. The placenta membranes were immediately diluted with a 10-fold excess of 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM EDTA, and 0.02% NaN_3 (TEN buffer) at 4 °C and centrifuged for 20 min at 48000*g*. The supernatant following centrifugation was aspirated, and the membrane pellets were washed with the corresponding TEN buffer, pH 8.5. The membrane pellets were then resuspended with TEN buffer. This procedure effectively brings the DTT concentration below detectable levels (data not shown). Samples of the resuspended pellets were solubilized with 1.0% Triton X-100 and 10 μM leupeptin for 30 min at 4 °C followed by microcentrifugation at 13000*g* for 30 min at 4 °C.

IGF-1 Binding. IGF-1 binding to placenta membranes, Triton X-100-solubilized membranes, or Bio-Gel A-1.5m gel filtration column fractions was performed by the addition of 0.5 nM ^{125}I-IGF-1 for 16 h at 4 °C in a final volume of 0.2 mL with KRH buffer (50 mM Hepes, pH 7.6, 130 mM KCl, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$) plus 0.1 mg/mL bacitracin and 0.1% bovine serum albumin. Free ^{125}I-IGF-1 was separated from the bound hormone by the addition of 0.5 mL of 0.1% bovine γ-globulin and 0.5 mL of 25% poly(ethylene glycol) at 4 °C, followed by microcentrifugation at 13000*g* for 10 min. The supernatant was aspirated off, and the pellets were washed with 1.0 mL of 10% poly(ethylene glycol). Nonspecific binding was determined in the presence of 100 nM unlabeled IGF-1 in the binding assays. Competition of ^{125}I-IGF-1 binding was generated by incubating the various IGF-1 receptor samples with 0.5 nM ^{125}I-IGF-1 plus increasing concentrations of unlabeled IGF-1 (0.1–30 nM) or unlabeled insulin (0.1–1000 nM). Scatchard analysis (Scatchard, 1949) was performed by using a modification of the computer program LIGAND provided by the Diabetes and Endocrinology Research Center, The University of Iowa. The concentration of Triton X-100 in all the soluble IGF-1 binding assays was maintained at 0.025%.

Bio-Gel A-1.5m Gel Filtration Chromatography. Solubilized IGF-1 receptors, after various treatments as described in the figure legends, were resolved on separate Bio-Gel A-1.5m (1.6 × 46 cm) gel filtration columns equilibrated with 50 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 150 mM NaCl, and 0.02% NaN_3 at 4 °C. The columns were run at a flow rate of 15 mL/h, and 18 mL was voided before collecting 0.4-mL fractions. Fractions from the Bio-Gel A-1.5m gel filtration columns were then assayed for ^{125}I-IGF-1 binding and affinity cross-linking.

IGF-1 Affinity Cross-Linking. IGF-1 receptor samples (membranes, solubilized or gel filtered) were incubated for 16 h at 4 °C in the presence of 0.5 nM ^{125}I-IGF-1 in KRH buffer plus 0.1 mg/mL bacitracin and 0.1% bovine serum albumin. Affinity cross-linking was initiated by the addition of 5.0 mM DSS in 10% dimethyl sulfoxide/45% ethanol to yield a final DSS concentration of 0.1 mM. The samples were then incubated for 5 min at 4 °C followed by the serial addition of 1.0 M Tris, pH 10.5, 0.5 mL of 0.02% bovine γ-globulin plus 0.1% bovine serum albumin, and 0.5 mL of 25% poly(ethylene glycol). The samples were centrifuged, washed as described earlier for IGF-1 binding assays, resuspended in water, and subjected to SDS–polyacrylamide gel electrophoresis under nonreducing conditions.

Autophosphorylation of the IGF-1 Receptors. The Bio-Gel A-1.5m column isolated IGF-1 receptor complexes were incubated for 1 h at 23 °C in the absence or presence of 100 nM IGF-1 or 100 nM insulin in 50 mM Hepes, pH 7.8, 10 mM $MnCl_2$, and 10 mM $MgCl_2$. The autophosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP (100 μM, 3 μCi/nmol) and terminated after 5 min by the addition of Laemmli sample buffer (50 mM Tris-HCl, pH 6.9, 10% glycerol, 0.05% bromophenol blue, and 1.0% SDS).

SDS–Polyacrylamide Gel Electrophoresis. IGF-1 affinity-cross-linked and [γ-^{32}P]ATP-autophosphorylated samples were run under nonreducing conditions using 3–10% linear gradient SDS–polyacrylamide gels containing a 3% stacking gel [acrylamide:bis(acrylamide) ratio of 37.5:1.0] according to the procedure of Laemmli (1970). ^{125}I-IGF-1 affinity-cross-linked and ^{32}P-autophosphorylated receptors were mixed with Laemmli sample buffer and applied to the 3–10% gels without heating. The gels were stained with Coomassie Brilliant Blue R, dried, and autoradiographed with Kodak XAR-5 film using Cronex lightning-plus intensifying screens.

Results

It has been previously established that treatment of human placenta membranes at pH 8.5 (Boni-Schnetzler et al., 1987), but not at pH 7.6 (Sweet et al., 1986, 1987), in the presence of 1–2 mM DTT results in the formation of a functional $\alpha\beta$ heterodimeric insulin receptor complex upon detergent solu-

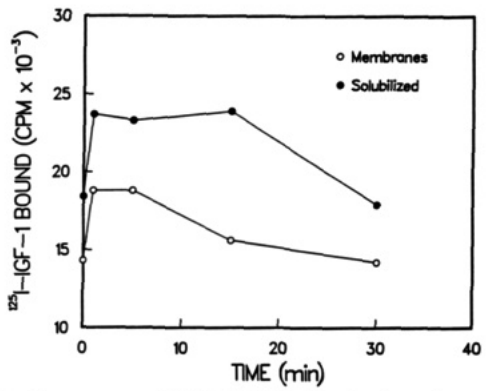

FIGURE 1: Time course of ^{125}I-IGF-1 binding to placenta membranes and solubilized membranes. Placenta membranes (10 mg/mL) were incubated at pH 8.5 in the absence or presence of 2.0 mM DTT at 23 °C for the times indicated. The reaction was stopped by dilution, centrifugation, and resuspension of the membranes as described under Experimental Procedures. Aliquots of the membranes (40 μg) were either used directly (O) or solubilized (●) in 1.0% Triton X-100 prior to ^{125}I-IGF-1 binding.

bilization. In contrast, in the absence of detergent solubilization, the membrane environment was found to maintain $\alpha\beta$–$\alpha\beta$ heterodimeric subunit interactions despite reduction of the class I disulfide bonds responsible for the covalent assembly of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (Boni-Schnetzler et al., 1987). In order to characterize the DTT-dependent formation of an $\alpha\beta$ heterodimeric IGF-1 receptor complex, we initially examined the time dependence of ^{125}I-IGF-1 binding to placenta membranes and detergent-solubilized placenta membranes pretreated with 2.0 mM DTT at pH 8.5 (Figure 1). Placenta membranes were incubated for various times with 2.0 mM DTT at pH 8.5, and the DTT was removed by dilution, centrifugation, and resuspension. Aliquots of these pretreated membranes were then subjected to detergent solubilization with 1.0% Triton X-100. The combination of alkaline pH and DTT treatments was observed to initially increase tracer ^{125}I-IGF-1 binding to both the placenta membranes and the detergent-solubilized membranes by approximately 30% within 5 min. However, after longer incubation times (30 min), the ^{125}I-IGF-1 binding was found to progressively decrease and returned to the initial binding activity observed in the absence of DTT.

To determine the molecular identities of the IGF-1 binding species present after the alkaline pH and DTT treatments, ^{125}I-IGF-1 affinity cross-linking was performed on both the placenta membranes and the detergent-solubilized membranes (Figure 2). ^{125}I-IGF-1 labeling of placenta membranes (Figure 2A, lanes 1–3) and detergent-solubilized membranes (Figure 2B, lanes 1–3) incubated at pH 8.5 in the absence of DTT demonstrated the presence of the M_r 400 000 $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor complex. The affinity labeling of the two lower molecular weight bands of approximately M_r 350 000 and 300 000 reflects the presence of the proteolytic receptor products $\alpha_2\beta\beta_1$ and $\alpha_2(\beta_1)_2$, respectively. ^{125}I-IGF-1 affinity cross-linking performed in the presence of 50 nM unlabeled insulin (lanes 2,5, and 8) was unable to compete for the specific labeling of these receptor bands whereas 50 nM unlabeled IGF-1 (lanes 3, 6, and 9) completely inhibited the ^{125}I-IGF-1 cross-linking. Pretreatment of the placenta membranes and detergent-solubilized membranes for 5 min (lanes 4–6) or 30 min (lanes 7–9) at alkaline pH in the presence of DTT demonstrated the appearance of the M_r 210 000 $\alpha\beta$ heterodimeric IGF-1 receptor complex with a decrease in the amount of the M_r 400 000 $\alpha_2\beta_2$ heterotetrameric complex. In several experiments, near-maximal formation

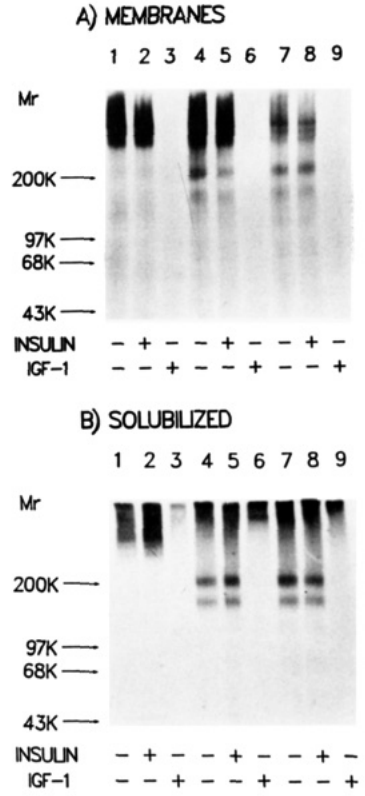

FIGURE 2: Effect of DTT treatment on ^{125}I-IGF-1 affinity cross-linking of placenta membranes and solubilized membranes. (A) Placenta membranes (10 mg/mL) were incubated at pH 8.5 in the absence (lanes 1–3) or presence of 2.0 mM DTT at 23 °C for 5 min (lanes 4–6) or 30 min (lanes 7–9). The reaction was stopped by dilution, centrifugation, and resuspension of the membranes. Aliquots of the membranes (40 μg) were affinity cross-linked after incubation with ^{125}I-IGF-1 for 16 h at 4 °C in the absence (lanes 1, 4, and 7) or presence of 50 nM unlabeled insulin (lanes 2, 5, and 8) or 50 nM unlabeled IGF-1 (lanes 3, 6, and 9) as described under Experimental Procedures. (B) The placenta membranes incubated as above (A) were solubilized with 1.0% Triton X-100 prior to ^{125}I-IGF-1 affinity cross-linking in the absence (lanes 1, 4, and 7) or presence of 50 nM unlabeled insulin (lanes 2, 5, and 8) or 50 nM unlabeled IGF-1 (lanes 3, 6, and 9).

of the $\alpha\beta$ heterodimeric IGF-1 receptor complex with complete preservation of IGF-1 binding activity occurred when the placenta membranes were Triton X-100 detergent solubilized subsequent to the 5-min DTT treatment at pH 8.5. Reducing SDS–polyacrylamide gel electrophoresis demonstrated that all the ^{125}I-IGF-1 affinity-cross-linked bands migrated with the expected mobility of the M_r 135 000 α subunit (data not shown).

The ability of unlabeled IGF-1 and insulin to compete for ^{125}I-IGF-1 binding to placenta membranes incubated at pH 8.5 in the presence or absence of DTT for 5 min is presented in Figure 3. Competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 for both membrane preparations demonstrated saturable binding with half-maximal inhibition occurring at approximately 1.5 nM IGF-1 and with maximal inhibition requiring 30 nM IGF-1. In contrast, insulin was only able to partially compete for ^{125}I-IGF-1 binding, with half-maximal inhibition occurring at 500 and 800 nM insulin for the untreated (Figure 3A) and DTT-treated (Figure 3B) placenta membranes, respectively. Thus, the ability of unlabeled IGF-1 and insulin to compete for tracer ^{125}I-IGF-1 binding was similar whether or not the placenta membranes were pretreated with DTT at pH 8.5 and are analogous to previous studies performed in the absence of DTT (Marshall et al., 1974). Similarly, no differences were observed between placenta membranes incubated

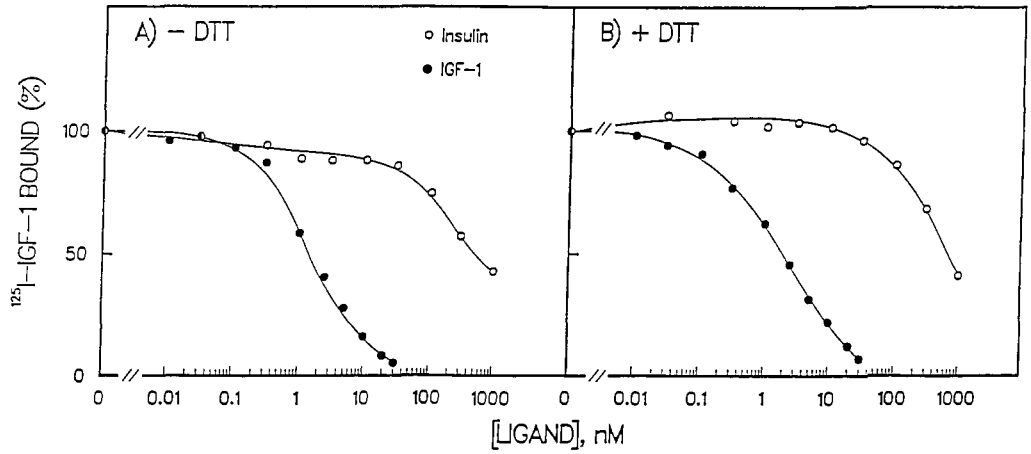

FIGURE 3: Competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 and insulin to control and DTT-treated placenta membranes. Placenta membranes (10 mg/mL) were incubated at pH 8.5 in the absence (A) or presence (B) of 2.0 mM DTT for 5 min as described in Figure 1. ^{125}I-IGF-1 (0.5 nM) was then added to the placenta membranes (40 μg) for 16 h at 4 °C in the presence of increasing concentrations of unlabeled IGF-1 (●) or unlabeled insulin (○). The specific binding of ^{125}I-IGF-1 was determined as described under Experimental Procedures. The maximal percent specific binding in these experiments was 26.3% and 29.0% in the absence and presence of DTT, respectively.

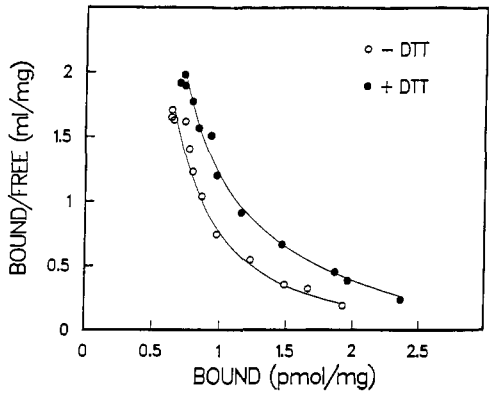

FIGURE 4: Scatchard analysis of ^{125}I-IGF-1 binding to control and DTT-treated placenta membranes. Placenta membranes (10 mg/mL) were incubated at pH 8.5 in the absence (○) or presence (●) of 2.0 mM DTT as described in Figure 3. The competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 was then analyzed by the method of Scatchard as described under Experimental Procedures.

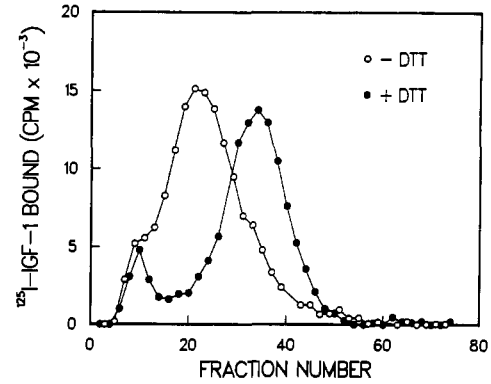

FIGURE 5: Bio-Gel A-1.5m gel filtration column profiles of IGF-1 binding to placenta membranes incubated in the absence or presence of DTT. Placenta membranes (10 mg/mL) were incubated in the absence (○) or presence (●) of 2.0 mM DTT as described in Figure 3. The membranes were then solubilized with 1.0% Triton X-100 and applied to Bio-Gel A-1.5m gel filtration columns (1.6 × 46 cm). Approximately 18 mL was voided before 0.4-mL fractions were collected. Aliquots (50 μL) of column fractions were then assayed for ^{125}I-IGF-1 binding activity as described under Experimental Procedures.

in the presence or absence of DTT at pH 7.6 (data not shown).

Scatchard analysis of the competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 generated curvilinear binding isotherms for both the untreated and DTT-treated placenta membranes (Figure 4). Although the IGF-1 Scatchard plots deviated significantly from linearity, they were never observed to be as concave as the corresponding insulin binding curves in human placenta membranes (Harrison & Itin, 1980). Scatchard analysis of IGF-1 binding to the untreated and DTT-treated membranes revealed identical (0.28 nM) high-affinity IGF-1 dissociation constants. However, the total number of IGF-1 binding sites was found to be consistently increased approximately 20% by the DTT treatment. This apparently occurred due to an increase in the number of low-affinity binding sites subsequent to DTT treatment. The ability of DTT at pH 8.5 to enhance ^{125}I-IGF-1 binding to human placenta membranes is similar to that previously observed at pH 7.6 (Jonas & Harrison, 1986).

Recent studies have demonstrated that although alkaline pH and DTT treatment of placenta membranes results in the reduction of the disulfide bonds necessary for the covalent attachment between the αβ heterodimeric insulin receptors, the αβ heterodimers remain associated and can still functionally interact (Boni-Schnetzler et al., 1987). In order to examine the properties of the αβ heterodimeric IGF-1 receptor complex, we next employed Bio-Gel A-1.5m gel filtration chromatography to ensure separation of the $\alpha_2\beta_2$ heterotetrameric and αβ heterodimeric IGF-1 receptor species in the Triton X-100 solubilized placenta membranes (Figure 5). Triton X-100 solubilized placenta membranes incubated at pH 8.5 in the absence of DTT migrated in the Bio-Gel A-1.5m gel filtration columns as a single broad symmetrical peak with a K_{av} of approximately 0.18. Placental membranes incubated at pH 8.5 in the presence of DTT prior to Triton X-100 solubilization migrated with a decreased mobility in these columns with a K_{av} of approximately 0.33. A peak of ^{125}I-IGF-1 binding activity was also consistently observed at the void volume in the Bio-Gel A-1.5m columns and probably reflects the presence of a small amount of aggregated IGF-1 receptors.

To confirm that the materials migrating in the Bio-Gel A-1.5m gel filtration columns with the K_{av} = 0.18 and 0.33 were the $\alpha_2\beta_2$ heterotetrameric and αβ heterodimeric IGF-1 receptor complexes, respectively, ^{125}I-IGF-1 affinity cross-linking was performed on these column fractions (Figure 6). ^{125}I-IGF-1 affinity cross-linking of the Bio-Gel A-1.5m gel filtration column fractions from the detergent-solubilized placenta membranes incubated at alkaline pH in the absence of DTT only identified the M_r 400 000 $\alpha_2\beta_2$ heterotetrameric

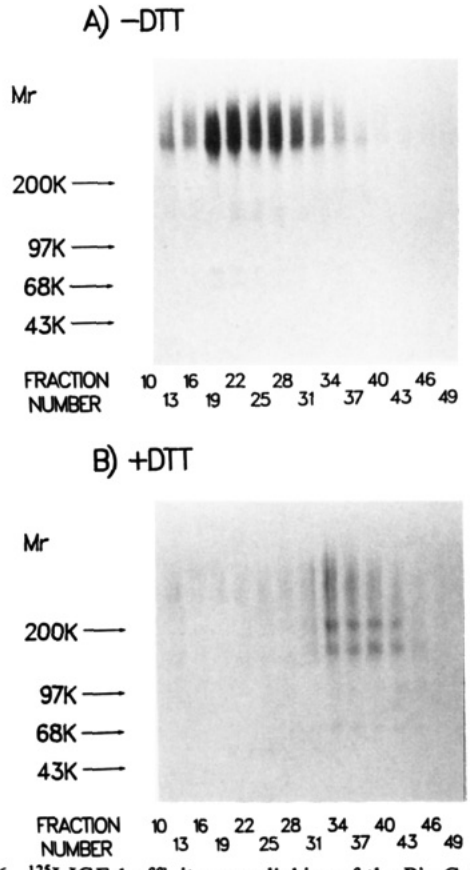

FIGURE 6: ^{125}I-IGF-1 affinity cross-linking of the Bio-Gel A-1.5m gel filtration column fractions. Aliquots (50 μL) from the fractions obtained by Bio-Gel A-1.5m gel filtration chromatography (Figure 5) were incubated for 16 h at 4 °C in the presence of 0.5 nM ^{125}I-IGF-1. The samples were then affinity cross-linked with 0.1 mM DSS and subjected to nonreducing SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. (A) ^{125}I-IGF-1 affinity cross-linking profiles from placenta membranes previously incubated in the absence of DTT, followed by detergent solubilization and Bio-Gel A-1.5m gel filtration chromatography. (B) ^{125}I-IGF-1 cross-linking profiles from placenta membranes previously incubated in the presence of 2.0 mM DTT, followed by detergent solubilization and Bio-Gel A-1.5m gel filtration chromatography.

IGF-1 receptor complex by nonreducing SDS–polyacrylamide gel electrophoresis (Figure 6A). The maximal amount of ^{125}I-IGF-1 affinity labeling occurred in fractions corresponding to the peak position (K_{av} = 0.18) of the $\alpha_2\beta_2$ heterotetrameric complex in the Bio-Gel A-1.5m gel filtration columns (Figure 5). In contrast, ^{125}I-IGF-1 affinity cross-linking of the Bio-Gel A-1.5m gel filtration column fractions from the detergent-solubilized placenta membranes previously incubated at alkaline pH in the presence of 2.0 mM DTT identified both the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes (Figure 6B). Under these conditions, the peak of ^{125}I-IGF-1 labeling occurred in the column fractions corresponding to the mobility of the $\alpha\beta$ heterodimeric complex (K_{av} = 0.33) observed by ^{125}I-IGF-1 binding (Figure 5B). Surprisingly, almost no $\alpha_2\beta_2$ heterotetrameric complex was detected in the fractions corresponding to the mobility of the $\alpha_2\beta_2$ heterotetramers (K_{av} = 0.18) even though ^{125}I-IGF-1 affinity cross-linking identified both the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes in the fractions with the mobility of the $\alpha\beta$ heterodimers (K_{av} = 0.33). Similar to Figure 2, the material migrating with an increased mobility compared to the $\alpha\beta$ heterodimeric complex was due to the presence of proteolyzed $\alpha\beta_1$ complexes.

We next determined if the appearance of the ^{125}I-IGF-1 affinity cross-linked $\alpha_2\beta_2$ heterotetrameric complex in the peak $\alpha\beta$ heterodimeric fractions from the Bio-Gel A-1.5m gel filtration columns (Figure 6B) was due to the presence of a small amount of contaminating $\alpha_2\beta_2$ heterotetrameric complex or occurred as a result of an IGF-1-induced covalent reassociation of the $\alpha\beta$ heterodimeric complex. To address these possibilities, the isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes were ^{125}I-IGF-1 affinity cross-linked in the presence or absence of the specific sulfhydryl alkylating agent IAN (Figure 7). As previously observed in the absence of IAN (Figure 6B), ^{125}I-IGF-1 affinity cross-linking of the $\alpha\beta$ heterodimer preparation resulted in the affinity labeling of the $\alpha_2\beta_2$ heterotetrameric complex (Figure 7B, lane 1). In contrast, ^{125}I-IGF-1 affinity cross-linking of the $\alpha\beta$ heterodimeric preparation preincubated with 1.0 (lane 2) or 10 (lane 3) mM IAN significantly blocked the appearance of the ^{125}I-IGF-1-labeled $\alpha_2\beta_2$ heterotetrameric complex. ^{125}I-IGF-1 affinity labeling of the control $\alpha_2\beta_2$ heterotetramer preparation, either untreated or pretreated with IAN, uniquely identified the M_r 400000 $\alpha_2\beta_2$ heterotetrameric complex (Figure 7A, lanes 1–3). Similar results were also obtained when the specific sulfhydryl agent *N*-ethylmaleimide was used (data not shown). These data demonstate that the appearance of the ^{125}I-IGF-1 affinity-labeled $\alpha_2\beta_2$ heterotetrameric complex in the $\alpha\beta$ heterodimer preparation was due to an IGF-1-induced covalent reassociation of the $\alpha\beta$ heterodimer and not a result of cross-contamination with the $\alpha_2\beta_2$ heterotetrameric complex.

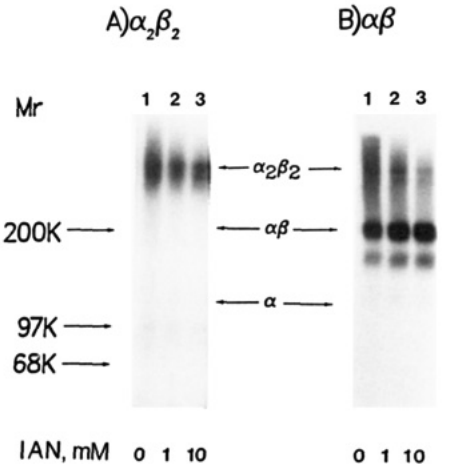

FIGURE 7: Effect of sulfhydryl alkylation on the ^{125}I-IGF-1 affinity cross-linking of the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes. Aliquots (50 μL) of the pooled $\alpha_2\beta_2$ heterotetrameric (A) and $\alpha\beta$ heterodimeric (B) peak fractions from Bio-Gel A-1.5m gel filtration chromatography, as described in Figure 5, were incubated in the absence (lane 1) or presence of 1.0 mM (lane 2) and 10 mM (lane 3) IAN for 30 min. The samples were then incubated with 0.5 nM ^{125}I-IGF-1 for 16 h at 4 °C followed by DSS affinity cross-linking and nonreducing SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. Quantitation of labeling intensity was determined by scanning laser densitometry.

Competition of ^{125}I-IGF-1 binding to the Bio-Gel A-1.5m gel filtration column peak fractions of the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes is shown in Figure 8. Unlabeled IGF-1 (30 nM) completely inhibited tracer ^{125}I-IGF-1 binding with half-maximal inhibition occurring at 0.8 nM IGF-1 for the isolated $\alpha_2\beta_2$ heterotetrameric complex (Figure 8A). In contrast, half-maximal inhibition by unlabeled insulin occurred at approximately 70 nM, similar to that reported for the purified IGF-1 receptor (Casella et al., 1986). ^{125}I-IGF-1 binding to the isolated $\alpha\beta$ heterodimeric IGF-1 receptor complex also was completely inhibited by 30 nM unlabeled IGF-1 with half-maximal inhibition occurring at approximately 1 nM (Figure 8B). Similar to the control and DTT-treated placenta membranes (Figure 3B), unlabeled insulin was able to half-maximally displace ^{125}I-IGF-1 binding

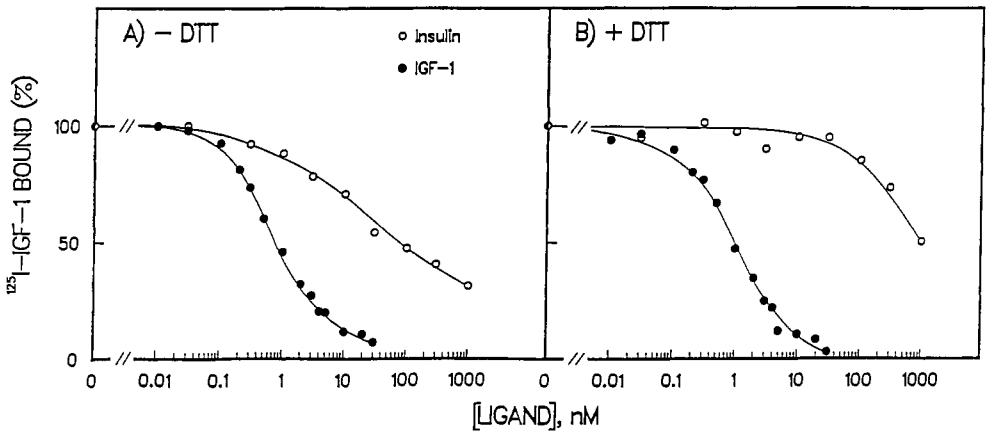

FIGURE 8: Competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 and insulin to the Bio-Gel A-1.5m gel filtration column isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes. The $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric receptor complexes were isolated as described in Figure 5. (A) ^{125}I-IGF-1 (0.5 nM) in the presence of unlabeled insulin (O) or unlabeled IGF-1 (●) was incubated for 16 h at 4 °C with the isolated $\alpha_2\beta_2$ heterotetrameric complex. (B) ^{125}I-IGF-1 (0.5 nM) in the presence of unlabeled insulin (O) or unlabeled IGF-1 (●) was incubated for 16 h at 4 °C with the isolated $\alpha\beta$ heterodimeric complex. The amount of specifically bound ^{125}I-IGF-1 was determined as described under Experimental Procedures. The maximal percent specific binding in these experiments was 10.9% and 9.9% in the absence and presence of DTT, respectively.

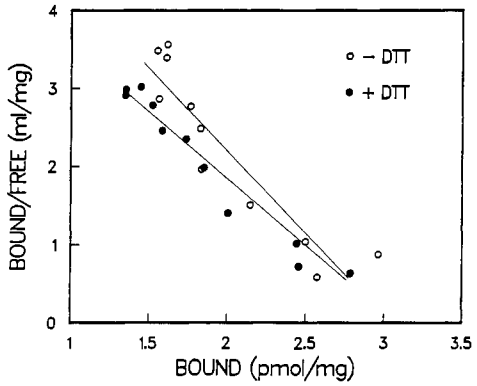

FIGURE 9: Scatchard analysis of ^{125}I-IGF-1 binding to the Bio-Gel A-1.5m gel filtration column isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes. The $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes subjected to competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 (Figure 8) were analyzed by the method of Scatchard as described under Experimental Procedures.

to the $\alpha\beta$ heterodimers with an approximate 3 log unit shift to the right compared to unlabeled IGF-1.

Scatchard analysis of ^{125}I-IGF-1 binding to the Bio-Gel A-1.5m gel filtration column isolated $\alpha\beta$ heterodimeric IGF-1 receptor demonstrated a linear (homogeneous) binding isotherm (Figure 9). Similarly, ^{125}I-IGF-1 binding to the isolated $\alpha_2\beta_2$ heterotetrameric complex also displayed a marked straightening of the binding isotherm compared to the placenta membrane bound IGF-1 receptor complexes (Figure 9). The high-affinity dissociation constant was increased approximately 2-fold in both the $\alpha_2\beta_2$ heterotetrameric (0.45 nM) and $\alpha\beta$ heterodimeric (0.62 nM) complexes isolated by Bio-Gel A-1.5m gel filtration chromatography compared to the placenta membrane bound receptor complexes (0.28 nM). No significant differences were observed in the overall IGF-1 binding affinity or number of IGF-1 binding sites between the Bio-Gel A-1.5m gel filtration column isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes.

A summary of the IGF-1 binding parameters to the placenta membrane bound and Bio-Gel A-1.5m gel filtration column purified $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes is presented in Table I. Although it was possible to fit the membrane binding data to both a one- and a two-site model, the analysis would only accept a one-site model for the Bio-Gel A-1.5m gel filtration column purified $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes.

Table I: IGF-1 Binding Parameters[a]

	Two-Site Binding Model dissociation constant (nM)		binding sites (pmol/mg)	
	$1/K_{11}$	$1/K_{12}$	R_1	R_2
placenta membranes				
−DTT	0.28 ± 0.11	8.7 ± 4.1	1.0 ± 0.2	2.7 ± 0.3
+DTT	0.28 ± 0.10	7.1 ± 2.1	1.1 ± 0.2	3.3 ± 0.2
	One-Site Binding Model dissociation constant (nM) $1/K_{11}$		binding sites (pmol/mg) R_1	
placenta membranes				
−DTT	0.57 ± 0.08		1.6 ± 0.1	
+DTT	0.69 ± 0.09		2.0 ± 0.1	
Bio-Gel A-1.5m				
−DTT	0.45 ± 0.16		3.2 ± 0.5	
+DTT	0.62 ± 0.10		3.3 ± 0.3	

[a] Analyzed by both a two-site and a one-site binding model (Munson & Rodbard, 1980) for the control and DTT-treated placenta membranes (Figure 4) and by a one-site model only for the Bio-Gel A-1.5m gel filtration column isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes (Figure 9).

Autophosphorylation of the isolated $\alpha_2\beta_2$ heterotetrameric complex in the presence of 100 nM IGF-1 demonstrated an approximate 3-fold increase in the initial rate (5 min) of IGF-1 receptor self-phosphorylation (Figure 10A, lane 2). Since these fractions also contain the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex, 100 nM insulin was observed to stimulate the initial rate of the insulin receptor self-phosphorylation approximately 4-fold (Figure 10A, lane 3). Autophosphorylation of the Bio-Gel A-1.5m gel filtration column isolated $\alpha\beta$ heterodimers, in the absence of ligand, demonstrated not only the phosphorylation of the $\alpha\beta$ heterodimeric complex but also the presence of a trace amount of the ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex in three experiments (Figure 10B, lane 1). Autophosphorylation of the $\alpha\beta$ heterodimeric preparation in the presence of 100 nM IGF-1 had essentially no effect on the extent of $\alpha\beta$ heterodimer phosphorylation but markedly increased (4-fold) the amount of ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex present (Figure 10B, lane 2). On the other hand, 100 nM insulin increased the amount of $\alpha\beta$ heterodimer phosphorylation approximately 5-fold as well as increasing the extent of ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex 5-fold

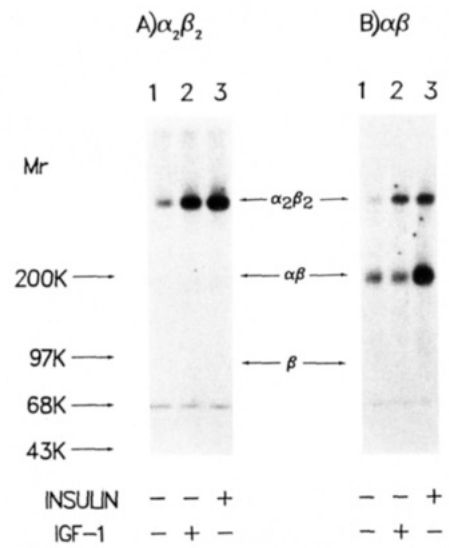

FIGURE 10: IGF-1- and insulin-dependent autophosphorylation of the Bio-Gel A-1.5m gel filtration column peak fractions of the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric receptor complexes. The isolated $\alpha_2\beta_2$ heterotetrameric (A) and $\alpha\beta$ heterodimeric (B) receptor complexes, as described in Figure 5, were incubated in the absence (lane 1) or presence of 100 nM IGF-1 (lane 2) or 100 nM insulin (lane 3) for 1 h at 23 °C. The autophosphorylation reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (3 μCi/nmol) for 5 min and terminated with Laemmli sample buffer. The samples were then separated by nonreducing 3–10% SDS–polyacrylamide gel electrophoresis. Quantitation of the labeling intensity was determined by scanning laser densitometry.

(Figure 10B, lane 3). On the basis of the competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 and insulin (Figures 3 and 8), the concentrations of IGF-1 and insulin used in these phosphorylation reactions were specific for their homologous receptors.

Discussion

It has previously been observed that reduction of both the class I and class II disulfide bonds necessary for the covalent assembly of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex does not result in receptor subunit dissociation under normally used nondenaturing buffer conditions (Sweet et al., 1986). However, it has been recently observed that a simultaneous treatment of the human placenta $\alpha_2\beta_2$ heterotetrameric insulin receptor complex with 1–2 mM DTT at pH 8.5 results in the formation of a functional $\alpha\beta$ heterodimeric complex (Boni-Schnetzler et al., 1987). In this study, we have examined the ability of this procedure to generate IGF-1 receptor $\alpha\beta$ heterodimers from the human placenta $\alpha_2\beta_2$ heterotetrameric disulfide-linked IGF-1 receptor complex. Preincubation of placenta membranes at alkaline pH in the presence of DTT was found to initially increase (30%) the extent of tracer ^{125}I-IGF-1 binding to both membranes and detergent-solubilized membranes (Figure 1) similar to that observed by Jonas and Harrison (1986). This is presumably due to a direct effect on the IGF-1 receptor since the membranes are incubated with DTT and subsequently washed to remove the reducing agent before the addition of ^{125}I-IGF-1. Longer incubation times with DTT (30 min) were found to decrease the amount of tracer ^{125}I-IGF-1 binding to approximately the same level as observed in the absence of DTT. These data indicate that the IGF-1 receptor at alkaline pH is stable to short-term exposure to DTT but upon longer incubation the receptor apparently begins to undergo denaturation presumably as a result of disulfide bond reduction. Thus, although the DTT treatment may reduce additional disulfide bonds not directly involved in IGF-1 receptor subunit assembly, this can be effectively minimized by short-term incubation with reductant.

^{125}I-IGF-1 affinity cross-linking of the DTT-treated placenta membranes demonstrated that the reduction of the class I disulfide bonds responsible for the maintenance of the covalent $\alpha_2\beta_2$ heterotetrameric structure had occurred under these conditions (Figure 2). However, the formation of the $\alpha\beta$ heterodimeric complex was only partially efficient, and a significant amount of the $\alpha_2\beta_2$ heterotetrameric complex was present. In contrast, a greater proportion of the $\alpha\beta$ heterodimeric complex was generated when the pretreated membranes were initially detergent solubilized before ^{125}I-IGF-1 affinity cross-linking was performed. These data are consistent with the ability of the membrane-bound $\alpha\beta$ heterodimeric IGF-1 receptors to more easily reassociate into a covalent $\alpha_2\beta_2$ heterotetrameric complex in an IGF-1-dependent manner, as has been observed for the $\alpha\beta$ heterodimeric insulin receptor complex (Boni-Schnetzler et al., 1986; Sweet et al., 1987b). Further, ^{125}I-IGF-1 binding to the DTT-treated and control placenta membranes was similar, suggesting no functional alterations of the interactions between the $\alpha\beta$ heterodimers to generate high-affinity curvilinear binding (Figure 3). Similarly, high-affinity curvilinear insulin binding has also recently been observed to require subunit interaction between the $\alpha\beta$ heterodimeric insulin receptor complexes which can be maintained by the plasma membrane environment despite reduction of the class I disulfides (Boni-Schnetzler et al., 1987).

Physical separation of the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric complexes was readily achieved by Triton X-100 solubilization and Bio-Gel A-1.5m gel filtration chromatography. IGF-1 binding to the Bio-Gel A-1.5m gel filtration isolated $\alpha\beta$ heterodimeric complex yielded a straight-line Scatchard plot (Figure 9). Scatchard analysis of IGF-1 binding to the $\alpha_2\beta_2$ heterotetrameric complex also resulted in a straightening of the binding isotherm compared to the membrane-bound $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor (Figure 9). IGF-1 binding to membrane-bound, partially purified, and purified IGF-1 receptors has been previously reported to generate both linear (Bala et al., 1983; Baxter & Williams, 1983; Casella et al., 1986) and also curvilinear (Jonas & Harrison, 1985) Scatchard plots similar to those observed in Figures 4 and 9.

These results are similar to several studies demonstrating heterogeneous insulin binding in the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex whereas homogeneous linear insulin binding occurred in the $\alpha\beta$ heterodimeric insulin receptor state (Deger et al., 1986; Koch et al., 1986; Boni-Schnetzler et al., 1986, 1987; Sweet et al., 1987a). Insulin binding to the $\alpha\beta$ heterodimeric complex was also observed to have an intermediate binding affinity, compared to the high and low binding affinities of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex, with a 2-fold increase in the number of insulin binding sites (Sweet et al., 1987a). In contrast to the insulin receptor, both the binding affinities (0.45 versus 0.62 nM) and also the number of IGF-1 binding sites (3.2 versus 3.3 pmol/mg) between the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric IGF-1 receptor complexes were found to be similar (Figure 9, Table I). These data suggest either that the $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor complex contains two equivalent noninteracting IGF-1 binding sites or that the $\alpha_2\beta_2$ heterotetrameric complex is composed of nonidentical $\alpha\beta$ heterodimers. This latter possibility would be consisent with the observation that the purified IGF-1 receptor displays high-affinity IGF-2 binding which is immunologically distinct from the IGF-1 binding site (Casella et al., 1986).

It is interesting to note that the $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor displays increased affinity for insulin (approximately 7-fold) following detergent solubilization and Bio-Gel A-1.5m gel filtration chromatography of the control placenta membranes (compare Figure 3A with Figure 8A). In contrast, no significant difference in the binding affinity for insulin was observed between the DTT-treated placenta membrane bound and the detergent-solubilized $\alpha\beta$ heterodimeric IGF-1 receptor complexes (compare Figure 3B with Figure 8B). Although we do not have an explanation for this phenomenon at the present time, similar results have previously been observed by others for the $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor complex (Marshall et al., 1974; Casella et al., 1986).

Examination of the IGF-1-stimulated autophosphorylating activity of the $\alpha\beta$ heterodimeric complex demonstrated the formation of a ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex (Figure 10). Even though this preparation contains both the IGF-1 and insulin $\alpha\beta$ heterodimeric complexes, the patterns of IGF-1- and insulin-dependent stimulation of β-subunit autophosphorylation were different. Insulin-dependent autophosphorylation resulted in increased ^{32}P labeling of the $\alpha\beta$ heterodimeric as well as the $\alpha_2\beta_2$ heterotetrameric complexes. In contrast, IGF-1-dependent stimulation of autophosphorylation had no effect on the ^{32}P incorporation into the $\alpha\beta$ heterodimeric complex but resulted in the increased formation of the ^{32}P-labeled $\alpha_2\beta_2$ heterotetrameric complex. These data demonstrate that insulin and IGF-1 stimulation of the $\alpha\beta$ heterodimeric receptor autophosphorylation activity occurs through their respective homologous receptors and not via heterologous interactions.

Consistent with IGF-1 inducing the covalent reassociation of the $\alpha\beta$ heterodimeric complex into the $\alpha_2\beta_2$ heterotetrameric state, ^{125}I-IGF-1 cross-linking of the Bio-Gel A-1.5m gel filtration column isolated $\alpha\beta$ heterodimers (K_{av} = 0.33) demonstrated the presence of the $\alpha_2\beta_2$ heterotetrameric complex in these fractions (Figure 6). The $\alpha_2\beta_2$ heterotetrameric complex was only observed in the $\alpha\beta$ heterodimeric fractions and was not detected in the fractions corresponding to the mobility of the $\alpha_2\beta_2$ heterotetrameric complex (K_{av} = 0.18). Further, pretreatment of the isolated $\alpha\beta$ heterodimeric IGF-1 receptor complex with specific sulfhydryl alkylating agents prior to ^{125}I-IGF-1 affinity cross-linking decreased the appearance of the $\alpha_2\beta_2$ heterotetrameric complex by approximately 50% (Figure 7). These results indicate that IGF-1 induces the reassociation of the $\alpha\beta$ heterodimers into a covalent $\alpha_2\beta_2$ heterotetrameric state and that IGF-1 stimulation of autophosphorylation is not due to cross-contamination with DTT-treated but nondissociated $\alpha_2\beta_2$ heterotetrameric IGF-1 receptor complexes. The presence of the ^{32}P-autophosphorylated $\alpha_2\beta_2$ heterotetrameric complex (25%) in the isolated $\alpha\beta$ heterodimeric preparation (Figure 10B, lane 1) probably results from a small amount of (Mn/Mg/-ATP-dependent covalent reassociation that occurs in the absence of IGF-1.

In summary, these studies have defined the methodology for isolating a functional $\alpha\beta$ heterodimeric IGF-1 receptor complex from the disulfide-linked human placenta $\alpha_2\beta_2$ heterotetrameric state. The ability to isolate functional $\alpha\beta$ heterodimeric IGF-1 and insulin receptor complexes will allow us to further examine the role of subunit interaction in the high-affinity curvilinear binding and in the hormone-dependent transmembrane activation of protein kinase activity in these analogous growth factor receptors.

ACKNOWLEDGMENTS

We thank Patsy McAtee for her skillful assistance in the preparation of the manuscript.

Registry No. IGF-1, 67763-96-6; IGF-1 protein kinase, 103843-29-4.

REFERENCES

Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., & Osathanondh, R. (1982) *J. Biol. Chem. 257*, 15162.

Bala, R. M., Baumick, B., Armstrong, G. H., & Hollenberg, M. D. (1983) in *Insulin-like Growth Factors/Somatomedins* (Spencer, E. M., Ed.) p 491, Walter de Gruyter, Berlin.

Baxter, R. C., & Williams, P. F. (1983) *Biochem. Biophys. Res. Commun. 116*, 62.

Bhaumick, B., Goren, H. J., & Bala, R. M. (1981a) *Horm. Metab. Res. 13*, 515.

Bhaumick, B., Bala, R. M., & Hollenberg, M. D. (1981b) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4279.

Boni-Schnetzler, M., Rubin, J. B., & Pilch, P. F. (1986) *J. Biol. Chem. 261*, 15281.

Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E., & Pilch, P. F. (1987) *J. Biol. Chem. 262*, 8395.

Casella, S. J., Han, V. K., D'Ercole, A. J., Svoboda, M. E., & Van Wyk, J. J. (1986) *J. Biol. Chem. 261*, 9268.

Chernausek, S. D., Jacobs, S., & Van Wyk, J. J. (1981) *Biochemistry 20*, 7345.

Czech, M. P. (1985) *Annu. Rev. Physiol. 47*, 357.

Deger, A., Kramer, H., Rapp, R., Koch, R., & Weber, U. (1986) *Biochem. Biophys. Res. Commun. 135*, 458.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985) *Cell (Cambridge, Mass.) 40*, 747.

Froesch, E. R., Schmid, C., Schwander, J., & Zapf, J. (1985) *Annu. Rev. Physiol. 47*, 443.

Fujita-Yamaguchi, Y., LeBon, T. R., Tsubokawa, M., Henzel, W., Kathuria, S., Koyal, D., & Ramachandran, J. (1986) *J. Biol. Chem. 261*, 16727.

Grunfeld, C., Shigenaga, J. E., & Ramachandran, J. (1985) *Biochem. Biophys. Res. Commun. 133*, 389.

Harrison, L. C., & Itin, A. (1980) *J. Biol. Chem. 255*, 12066.

Jacobs, S. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) p 31, Plenum, New York.

Jacobs, S., & Cuatrecasas, P. (1981) *Endocr. Rev. 2*, 251.

Jacobs, S., Hazum, E., & Cuatrecasas, P. (1980) *J. Biol. Chem. 255*, 6937.

Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J., & Cuatrecasas, P. (1983) *J. Biol. Chem. 258*, 9581.

Jonas, H. A., & Harrison, L. C. (1985) *J. Biol. Chem. 260*, 2288.

Jonas, H. A., & Harrison, L. C. (1986) *Biochem. J. 236*, 417.

Kahn, C. R. (1985) *Annu. Rev. Med. 36*, 429.

Kasuga, M., Van Obberghen, E., Nissley, S. P., & Rechler, M. M. (1981) *J. Biol. Chem. 256*, 5305.

Kasuga, M., Van Obberghen, E., Nissley, S. P., & Rechler, M. M. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1864.

Kasuga, M., Zick, Y., Blith, D. L., Karlsson, F. A., Haring, H. U., & Kahn, C. R. (1982b) *J. Biol. Chem. 257*, 9891.

Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., & Kahn, C. R. (1982c) *Nature (London) 298*, 667.

Koch, R., Deger, A., Jack, H. M., Klotz, K. N., Schenzle, D., Kramer, H., Kelm, S., Muller, G., Rapp, R., & Weber, U. (1986) *Eur. J. Biochem. 154*, 281.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Marshall, R. N., Underwood, L. E., Voina, S. J., Foushee, D. B., & Van Wyk, J. J. (1974) *J. Clin. Endocrinol. Metab. 39*, 283.
Massague, J., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 5038.
Massague, J., Pilch, P. F., & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7137.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220.
Pilch, P. F., O'Hare, T., Rubin, J., & Boni-Schnetzler, M. (1986) *Biochem. Biophys. Res. Commun. 136*, 45.
Rechler, M. M., & Nissley, S. P. (1985) *Ann. Rev. Physiol. 47*, 425.
Rinderknecht, E., & Humbel, R. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2365.
Rinderknecht, E., & Humbel, R. E. (1978) *J. Biol. Chem. 253*, 2769.
Roth, R. A., & Cassell, D. J. (1983) *Science (Washington, D.C.) 219*, 299.
Rubin, J. B., Shia, M. A., & Pilch, P. F. (1983) *Nature (London) 305*, 438.
Sasaki, N., Rees-Jones, R. W., Zick, Y., Nissley, S. P., & Rechler, M. M. (1985) *J. Biol. Chem. 260*, 9793.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 666.
Shia, M. A., & Pilch, P. F. (1983) *Biochemistry 22*, 717.
Shia, M. A., Rubin, J. B., & Pilch, P. F. (1983) *J. Biol. Chem. 258*, 14450.
Sweet, L. J., Wilden, P. A., & Pessin, J. E. (1986) *Biochemistry 25*, 7068.
Sweet, L. J., Morrison, B. D., & Pessin, J. E. (1987a) *J. Biol. Chem. 262*, 6939.
Sweet, L. J., Morrison, B. D., Wilden, P. A., & Pessin, J. E. (1987b) *J. Biol. Chem. 262*, 16730.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756.
Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., LeBon, T. R., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503.
Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 945.
Van Wyk, J. J., & Underwood, L. E. (1978) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Vol. 5, p 101, Academic, New York.
Van Wyk, J. J., Svoboda, M. E., & Underwood, L. E. (1980) *J. Clin. Endocrinol. Metab. 50*, 206.
Yu, K.-T., Peters, M. A., & Czech, M. P. (1986) *J. Biol. Chem. 261*, 11341.
Zapf, J., Rinderknecht, E., Humbel, R. E., & Froesch, E. R. (1978) *Metab. Clin. Exp. 27*, 1803.
Zick, Y., Sasaki, N., Rees-Jones, R. W., Grunberger, G., Nissley, S. P., & Rechler, M. M. (1984) *Biochem. Biophys. Res. Commun. 119*, 6.

Ribonuclease T_1 Is Stabilized by Cation and Anion Binding†

C. Nick Pace* and Gerald R. Grimsley
Biochemistry Department, Texas A&M University, College Station, Texas 77843
Received October 5, 1987; Revised Manuscript Received December 24, 1987

ABSTRACT: The stability of the folded conformation of ribonuclease T_1 is increased by 0.8, 1.8, and 3.3 kcal/mol in the presence of 0.1 M NaCl, $MgCl_2$, and Na_2HPO_4, respectively. This remarkable increase in the conformational stability results primarily from the preferential binding to the native protein of one Mg^{2+} or two Na^+ ions at cation-binding sites and by the binding of one HPO_4^{2-} ion at an anion-binding site. Only modest binding constants, 6.2 (Na^+), 155 (Mg^{2+}), and 282 M^{-1} (HPO_4^{2-}), are required to account for the enhanced stability. One important goal of the modification of proteins through genetic engineering is to increase their stability. Our results suggest that the creation of specific cation- and anion-binding sites on the surface of a protein through amino acid substitutions might be a generally useful way of achieving this goal. The design of these sites will be aided by the recent availability of detailed structural information on cation- and anion-binding sites.

Ribonuclease T_1 (RNase T_1) is a small (104 amino acids), well-characterized enzyme excreted from *Aspergillus oryzae* that cleaves single-stranded RNA molecules at guanine residues (Heinemann & Saenger, 1982; Takahashi et al., 1970). The three-dimensional structure has recently been refined to 1.9-Å resolution (Arni et al., 1987). RNase T_1 has many properties that make it an excellent model for studying various aspects of protein folding (Pace & Creighton, 1986). Oobatake et al. (1979) first showed that RNase T_1 is stabilized by NaCl. The melting temperature of RNase T_1 is increased 20 °C in the presence of 2 M NaCl as compared to an increase of about 1.5 °C for ribonuclease A (RNase A) (von Hippel & Wong, 1965). The aim of the experiments reported here was to reach a better understanding of how salts stabilize RNase T_1.

We show that the marked stabilization of RNase T_1 at moderate salt concentrations results mainly from the relatively weak binding of cations and anions by the native, folded conformation of the protein. We suggest that constructing ion-binding sites on the surface of globular proteins through genetic engineering may be an easier way to increase their conformational stability than adding disulfide bonds or other stabilizing interactions.

† Supported by grants from the NIH (GM 37039) and the Robert A. Welch Foundation (A 1060).

0006-2960/88/0427-3242$01.50/0