Interaction of the $\alpha\beta$ Dimers of the Insulin-like Growth Factor I Receptor Is Required for Receptor Autophosphorylation†

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ABSTRACT: We have recently found that association of the two $\alpha\beta$ dimers of the insulin-like growth factor I (IGF I) receptor is required for formation of a high-affinity binding site for IGF I [Tollefsen, S. E., & Thompson, K. (1988) *J. Biol. Chem. 263*, 16267-16273]. To determine the structural requirements for IGF I activated kinase activity, we have examined the effect of dissociation of the two $\alpha\beta$ dimers of the IGF I receptor on β subunit autophosphorylation. The $\alpha\beta$ dimers formed after treatment with 2 mM dithiothreitol (DTT) at pH 8.75 for 5 min were separated from IGF I receptor remaining as tetramers after DTT treatment by fast protein liquid chromatography on a Superose 6 gel filtration column. Purification of the $\alpha\beta$ dimers was confirmed by Western blot analysis using ^{125}I-labeled αIR-3, a monoclonal antibody to the IGF I receptor. Autophosphorylation of the IGF I receptor $(\alpha\beta)_2$ tetramer, treated without DTT or remaining after DTT treatment, is stimulated 1.6-2.9-fold by IGF I. In contrast, autophosphorylation of the $\alpha\beta$ dimers incubated in the presence or absence of IGF I (100 ng/mL) does not occur. Both IGF I receptor dimers and tetramers exhibit similar kinase activities using the synthetic substrate Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, indicating that the failure to detect autophosphorylation of the IGF I receptor dimers does not result from inactivation of the kinase by DTT treatment. We conclude that autophosphorylation of the IGF I receptor depends upon the interaction of the two $\alpha\beta$ dimers.

Insulin-like growth factor I (IGF I)[1] is a major mammalian postnatal growth factor regulated in part by pituitary growth hormone. The interaction of IGF I with a specific plasma membrane receptor results in both rapid insulin-like effects on intermediary metabolism and more long-term effects on growth (Froesch et al., 1985). The receptor for IGF I is a membrane of the tyrosine kinase family of growth factor receptors, other members of which include the EGF receptor, the insulin receptor, which is structurally homologous to the IGF I receptor, and the platelet-derived growth factor and colony-stimulating factor 1 receptors (Yarden & Ullrich, 1988). The extracellular ligand binding domain of these receptors is connected by a hydrophobic membrane-spanning segment to a cytoplasmic tyrosine kinase domain. IGF I binding causes the transmembrane activation of the intrinsic tyrosine kinase activity of the IGF I receptor, which results in β subunit autophosphorylation (Jacobs et al., 1983; Rubin et al., 1983; Zick et al., 1984; Sasaki et al., 1985; Jacobs & Cuatrecasas, 1986). The mechanism by which this occurs is not yet well understood.

We have purified the high-affinity IGF I receptor from human solubilized placental membranes by IGF I affinity chromatography (Tollefsen et al., 1987). The IGF I receptor is a heterotetramer, composed of two disulfide-linked $\alpha\beta$ dimers. Purification of the high-affinity IGF I receptor has made it possible for us to investigate the function of the apparently identical $\alpha\beta$ dimeric halves of the receptor. We have reported that the disulfide bonds linking the two $\alpha\beta$ dimers of the IGF I receptor can be reduced by incubation with 2 mM DTT at pH 8.75 for 5 min at room temperature (Tollefsen & Thompson, 1988). Scatchard plots of IGF I binding to receptor treated with or without DTT indicated that the $\alpha\beta$ dimer has a 6.1 ± 1.6 (mean ± SD) times lower affinity than the tetramer for IGF I. The total binding capacity of IGF I receptor treated with DTT was 1.6 ± 0.3 times higher than that of an equal amount of receptor treated without DTT. These results are consistent with a model in which the two $\alpha\beta$ dimers of the IGF I receptor associate to form a high-affinity binding site for IGF I.

In this report, we examine the structural requirements for IGF I receptor autophosphorylation. Phosphorylation of the $\alpha\beta$ dimer formed after DTT treatment, although minimal, was observed in our previous study. Phosphorylation of the $\alpha\beta$ dimer could have been effected by the $\alpha\beta$ dimer itself, by $\alpha\beta$ dimers which reassociate during the assay, or by intact $(\alpha\beta)_2$ tetramer remaining after DTT treatment since reduction of the disulfide bonds linking the $\alpha\beta$ dimers of the receptor is incomplete under the conditions of the protocol we employ. To distinguish these three possibilities, we have separated completely $\alpha\beta$ dimers from $(\alpha\beta)_2$ tetramers remaining after DTT treatment and examined the kinase activity of the purified $\alpha\beta$ dimers. Our studies indicate that interaction of the two $\alpha\beta$ dimers of the IGF I receptor is required for autophosphorylation.

Experimental Procedures

Materials. Sephadex G-50-80 and ATP from equine muscle were obtained from Sigma. Bio-Gel P-2 was obtained from Bio-Rad. DTT was obtained from Behring Diagnostics. Recombinant IGF I[Thr-59] was purchased from Amgen Biologicals. IGF I used to prepare the IGF I affinity adsorbent

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[1] Abbreviations: IGF I, insulin-like growth factor I; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; FPLC, fast protein liquid chromatography.

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was the generous gift of Dr. B. D. Burleigh (International Minerals and Chemicals Corp., Northbrook, IL). $Na^{125}I$ (13–17 mCi of $^{125}I/\mu g$ of I) and [γ-^{32}P]ATP (3000 Ci/mmol) were supplied by Amersham Corp. and DuPont/New England Nuclear Research Products, respectively. The monoclonal antibody to the IGF I receptor, αIR-3 (0.5 mg/mL), was generously provided by Dr. Steven Jacobs (The Wellcome Research Laboratories, Research Triangle Park, NC). Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (RR-SRC) was purchased from Peninsula Laboratories or was generously supplied by Dr. Linda J. Pike. Nitrocellulose sheets were obtained from Schleicher & Schuell.

Purification and Autophosphorylation of the IGF I Receptor from Human Placental Membranes. The high-affinity IGF I receptor was purified from normal full-term human placentas using wheat germ agglutinin–Sepharose chromatography, insulin affinity chromatography, and IGF I affinity chromatography, as previously described (Tollefsen et al., 1987). The purified IGF I receptor had a dissociation constant of 0.34–0.69 nM.

To monitor the elution of IGF I receptor forms during FPLC, purified IGF I receptor was ^{32}P-autophosphorylated by incubation with 5 μM ATP and 100 μCi of [γ-^{32}P]ATP in 20 mM imidazole hydrochloride buffer, pH 7.4, with 5 mM $MnCl_2$, 125 mM NaCl, 0.05% Triton X-100, and 5% glycerol (final volume, 100 μL) for 60 min at room temperature, as previously described (Tollefsen & Thompson, 1988).

Reduction of IGF I Receptor Disulfide Bonds with Dithiothreitol. IGF I receptor in 50 mM Tris-HCl buffer, pH 7.6, with 150 mM NaCl, 0.1% Triton X-100, 1 mg/mL BSA, and 0.02% NaN_3 was brought to pH 8.75 with 1.0 M Tris (final concentration, 140 mM), incubated for 25 min at room temperature, and then incubated for an additional 5 min with or without 2 mM DTT at room temperature. The samples were simultaneously neutralized and separated from the DTT by gel filtration on a Sephadex G-50 column (4 mL) equilibrated in 50 mM Tris-HCl buffer, pH 7.6, with 150 mM NaCl, 0.1% Triton X-100, 1 mg/mL BSA, and 0.02% NaN_3; 0.5-mL fractions were collected, and void volume column fractions containing IGF I receptor were pooled.

FPLC. The IGF I receptor, treated as described above, was concentrated to 100 μL in a Centricon 30 microconcentrator (Amicon) and analyzed with the Pharmacia FPLC system, using a Superose 6 HR 10/30 gel filtration column equilibrated in 10 mM Tris-HCl buffer, pH 7.6, with 150 mM NaCl and 0.1% Triton X-100. Chromatography was performed at a flow rate of 0.2 mL/min, and 0.2-mL fractions were collected. Column fractions were monitored for protein by recording the absorbance at 280 nm and for radioactivity by counting aliquots in a Packard Tri-Carb 4640 liquid scintillation counter, where indicated. Recovery of ^{32}P radioactivity from the Superose 6 column was typically >65%. Thyroglobulin (M_r 669 000), apoferritin (M_r 443 000), β-amylase (M_r 200 000), and BSA (M_r 66 000) were used for calibration.

Western Blotting. αIR-3 (10 μg) was radioiodinated by the chloramine T method (Greenwood et al., 1963), using 200 μCi of $Na^{125}I$ and a 15-s exposure to chloramine T. ^{125}I-Labeled αIR-3 was separated from free ^{125}I by gel filtration on a Bio-Gel P-2 column (1 × 28 cm) equilibrated in phosphate-buffered saline and was stored at −20 °C until use.

Proteins were electrophoretically transferred from 3–10% gradient acylamide resolving gels to nitrocellulose sheets according to the method of Towbin et al. (1979). Briefly, transfer was accomplished in a GENIE electrophoretic blotter (Idea Scientific Co.) using a transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol and applying a voltage of 20–24 V for 1.5 h. The blot was incubated overnight at room temperature in blocking buffer (50 mM Tris-HCl, pH 7.8, with 5% Carnation nonfat dry milk, 2 mM $CaCl_2$, 0.01% antifoam A emulsion, and 0.05% Triton X-100) with gentle shaking. ^{125}I-Labeled αIR-3 [(1.4–2.0) × 10^6 cpm] was then added for 2–3 h. The blot was washed in fresh blocking buffer and then twice in phosphate-buffered saline and dried. Autoradiographs were obtained by exposure of the dried blots to Kodak XAR-5 X-ray film in the presence of a Cronex Lightning Plus intensifying screen at −70 °C.

Autophosphorylation Assay. Autophosphorylation of the IGF I receptor forms was assayed as previously described (Tollefsen et al., 1987), with minor modifications. Briefly, IGF I receptor was incubated in the absence or presence of IGF I for 20 min and then with 5 μM ATP (containing 20 μCi of [γ-^{32}P]ATP/assay) and 5 mM $MnCl_2$ in 8.5 mM Tris-HCl buffer, pH 7.6, with 127.5 mM NaCl and 0.04–0.1% Triton X-100 (final volume, 100 μL) for 10 min at room temperature. The reaction was terminated by precipitation with 200 μL of 30% trichloroacetic acid. After centrifugation, the pellets were washed twice with acetone and resuspended in electrophoresis sample buffer.

RR-SRC Phosphorylation Assay. Kinase activity of the purified IGF I receptor was assayed by using the substrate Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (RR-SRC), as described by Pike (1987). IGF I receptor was incubated in the absence of IGF I with 200 μM ATP (containing 3 μCi of [γ-^{32}P]ATP/assay), 12 mM $MgCl_2$, and 2 mM RR-SRC in 7.5 mM Tris-HCl buffer, pH 7.6, with 112.5 mM NaCl and 0.05–0.625% Triton X-100 (final volume, 20 μL) for 30 min at room temperature. Assays were terminated by the addition of 30% acetic acid and spotted onto phosphocellulose paper squares which were washed and counted in a Packard Tri-Carb 4640 liquid scintillation counter. Data represent the mean counts of assays performed in duplicate. Background was measured by performing assays in the absence of receptor with or without RR-SRC. Initial experiments indicated that the inclusion of RR-SRC did not change the background counts on the paper, and it was therefore omitted from this measurement, unless otherwise indicated.

SDS–PAGE and Autoradiography. SDS–PAGE was performed according to the method of Laemmli (1970) with 5% or 3–10% gradient acrylamide resolving gels. Samples were treated with *N*-ethylmaleimide (final concentration, 125 mM) for 5 min at room temperature to block free sulfhydryl groups before SDS–PAGE, where indicated. Electrophoresis sample buffer was added to achieve a final concentration of 1% SDS, and the samples were heated for 2 min at 100 °C. Molecular weight standards (Bio-Rad) included myosin (200 000), β-galactosidase (116 250), and phosphorylase *b* (97 400). Prestained molecular weight standards (Bethesda Research Laboratories) included myosin (200 000), phosphorylase *b* (97 400), BSA (68 000), and ovalbumin (43 000). Gels were fixed, stained, and destained as previously described (Tollefsen & Kornfeld, 1983) and were then dried. Autoradiographs were obtained by exposure of the dried gels to Kodak XAR-5 X-ray film in the presence of a Cronex Lightning Plus intensifying screen at −70 °C and were scanned with an LKB Ultrascan XL laser densitometer.

Results

Purification of IGF I Receptor $\alpha\beta$ Dimers by FPLC. The disulfide bonds linking the two $\alpha\beta$ dimers of the IGF I receptor can be selectively reduced by incubation with 2 mM DTT at pH 8.75 for 5 min at room temperature (Tollefsen &

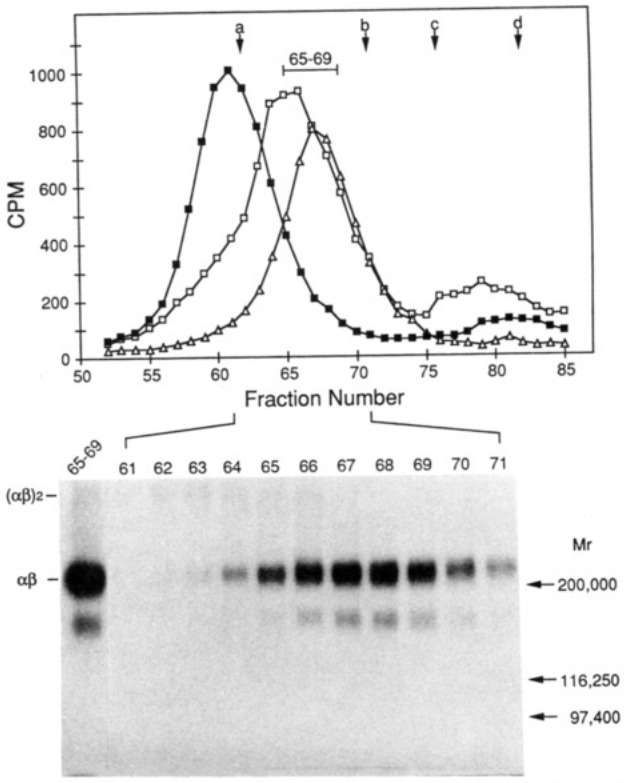

FIGURE 1: Separation of ^{32}P-autophosphorylated IGF I receptor $(\alpha\beta)_2$ tetramers and $\alpha\beta$ dimers by FPLC. ^{32}P-Autophosphorylated IGF I receptor, untreated or treated with DTT, was analyzed by FPLC, using a Superose 6 gel filtration column, as described under Experimental Procedures. Fractions 65–69 of ^{32}P-autophosphorylated IGF I receptor treated with DTT were pooled as indicated, concentrated, and reapplied to the column. (Top) Elution profiles of ^{32}P-autophosphorylated IGF I receptor untreated (■), treated with DTT (□), and pooled as noted and reapplied (△). The elution of the following standards is indicated by the arrows: a, thyroglobulin; b, apoferritin; c, β-amylase; d, BSA. (Bottom) SDS–PAGE analysis of the pooled fractions 65–69 (□) and of the indicated column fractions from rechromatography of this pool (△). Aliquots were treated with 125 mM *N*-ethylmaleimide for 5 min at room temperature and analyzed by SDS–PAGE (5% acrylamide resolving gel) under nonreducing conditions, as described under Experimental Procedures. The autoradiograph of the gel is shown. The migration of molecular weight standards is indicated.

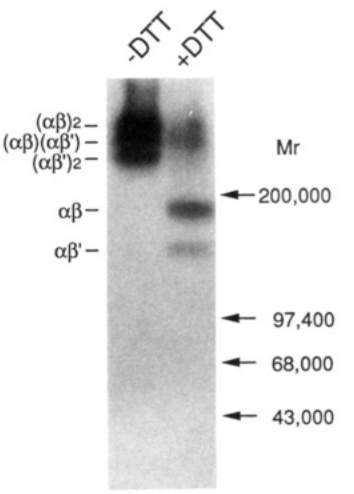

FIGURE 2: Western blot analysis of IGF I receptor treated with or without DTT. Equal amounts of unlabeled IGF I receptor were treated without (−DTT) or with (+DTT) DTT as described under Experimental Procedures. The samples were simultaneously neutralized and separated from the DTT by gel filtration, concentrated ~20 times, treated with 125 mM *N*-ethylmaleimide for 5 min at room temperature, and then analyzed by SDS–PAGE (3–10% gradient acrylamide resolving gel) under nonreducing conditions and Western blotting, using ^{125}I-labeled αIR-3, as described under Experimental Procedures. The autoradiograph of the nitrocellulose blot is shown. The migration of prestained molecular weight standards is indicated.

Thompson, 1988). After treatment under these conditions, ~75% of the IGF I receptor is in the $\alpha\beta$ dimeric form. We have separated the $\alpha\beta$ dimers from IGF I receptor remaining as $(\alpha\beta)_2$ tetramers after DTT treatment by FPLC using a Superose 6 gel filtration column, as described under Experimental Procedures. The elution profiles of ^{32}P-autophosphorylated IGF I receptor treated with or without DTT are shown in the top panel of Figure 1. The untreated ^{32}P-autophosphorylated IGF I receptor (closed squares) elutes in a single major peak. The ^{32}P-autophosphorylated IGF I receptor treated with DTT (open squares) elutes later in a broader peak; SDS–PAGE demonstrated the $(\alpha\beta)_2$ tetramer in early column fractions and the $\alpha\beta$ dimer in later column fractions (data not shown), as previously reported (Tollefsen & Thompson, 1988). To purify the $\alpha\beta$ dimer, fractions 65–69 in the trailing portion of this peak were pooled and concentrated. When reapplied to the column, these fractions elute somewhat later in a sharp peak (open triangles).

The identity of the IGF I receptor forms in column fractions was confirmed by SDS–PAGE under nonreducing conditions, as shown in the bottom panel of Figure 1. In pooled fractions 65–69 from FPLC of ^{32}P-autophosphorylated IGF I receptor treated with DTT, the receptor is present predominantly as $\alpha\beta$ dimers, although a small amount of $(\alpha\beta)_2$ tetramer is also present. SDS–PAGE of column fractions 61–71 from rechromatography of pooled fractions 65–69 confirmed that complete separation of the $\alpha\beta$ dimers (in later column fractions) from $(\alpha\beta)_2$ tetramers (in early column fractions) is achieved. Purified $\alpha\beta$ dimers were prepared by pooling these later column fractions for all subsequent experiments.

Autophosphorylation of IGF I Receptor $(\alpha\beta)_2$ Tetramers and Purified $\alpha\beta$ Dimers. In order to detect and quantify *unlabeled* IGF I receptor forms, we developed a Western blot method using αIR-3, a monoclonal antibody to the IGF I receptor (Kull et al., 1983). αIR-3 had been shown previously to immunoprecipitate the $\alpha\beta$ dimeric form of the IGF I receptor and to block IGF I binding to IGF I receptor treated with or without DTT (Tollefsen & Thompson, 1988). The immunological detection of the tetrameric and dimeric forms of the IGF I receptor immobilized on nitrocellulose sheets is shown in Figure 2. After incubation of the IGF I receptor at pH 8.75 without DTT (−DTT), the tetrameric forms of the receptor, $(\alpha\beta)_2$, $(\alpha\beta)(\alpha\beta')$, and $(\alpha\beta')_2$, are detected by Western blot analysis. The dimeric forms of the receptor, $\alpha\beta$ and $\alpha\beta'$, are detected after incubation of an equal amount of the IGF I receptor at pH 8.75 with DTT (+DTT). Densitometric scanning of the autoradiograph indicated that the amount of ^{125}I-labeled αIR-3 bound to the IGF I receptor treated with DTT is ~40% that bound to IGF I receptor treated without DTT. To establish whether the receptor forms are quantitatively transferred to the nitrocellulose sheet, ^{32}P-autophosphorylated receptor, treated with or without DTT, was analyzed, and the radioactivity recovered from the blot was compared directly to that recovered from the gel. Of the ^{32}P-radioactivity, 39.3–75.8% associated with the tetrameric forms and 93.8% associated with the dimeric forms were transferred to the nitrocellulose sheet. Hence, the smaller amount of ^{125}I-labeled αIR-3 bound to the dimeric forms appears to result from reduced recognition of these forms after SDS–PAGE by αIR-3 rather than from incomplete transfer to the nitrocellulose sheet.

In order to examine the effect of dissociation of the two $\alpha\beta$

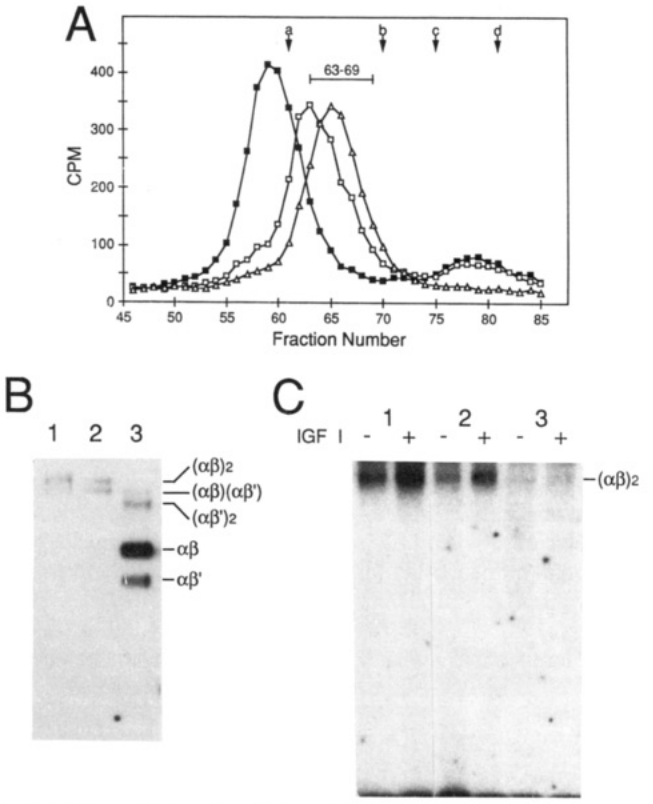

FIGURE 3: Autophosphorylation of IGF I receptor $(\alpha\beta)_2$ tetramers and purified $\alpha\beta$ dimers. Unlabeled IGF I receptor was treated with DTT, and $\alpha\beta$ dimers were purified by FPLC, using a Superose 6 column, as described under Results. ^{32}P-Autophosphorylated IGF I receptor was analyzed in a separate, parallel run. Fractions 63–69 of IGF I receptor treated with DTT were pooled, concentrated, and reapplied to the column. (A) Elution profiles of ^{32}P-autophosphorylated IGF I receptor treated without DTT (■), treated with DTT (□), and pooled as noted and reapplied (△). The elution of the following standards is indicated by the arrows: a, thyroglobulin; b, apoferritin; c, β-amylase; d, BSA. Fractions 58–59 (lanes 1) from FPLC of unlabeled IGF I receptor treated with DTT and fractions 58–59 (lanes 2) and 65–66 (lane 3) from rechromatography of pooled fractions 63–69 were concentrated ~5 times. (B) Western blot analysis of these fractions (75% of the total volume), using ^{125}I-labeled αIR-3, as described under Experimental Procedures. The autoradiograph of the nitrocellulose blot is shown. (C) Autophosphorylation of these fractions (25% of the total volume). Autophosphorylation assays were performed as described under Experimental Procedures, in the absence or presence of IGF I, as indicated. The autoradiograph of phosphorylated IGF I receptor forms, analyzed by SDS–PAGE (5% acrylamide resolving gel) under nonreducing conditions, is shown.

dimers of the IGF I receptor on β subunit autophosphorylation, unlabeled IGF I receptor was treated with DTT, and $\alpha\beta$ dimers were purified by FPLC. Fractions were pooled according to the elution of ^{32}P-autophosphorylated IGF I receptor established in separate, parallel runs, as described above. Figure 3A shows the elution profiles of ^{32}P-autophosphorylated IGF I receptor in this experiment. The Western blot of fractions of unlabeled receptor pooled for autophosphorylation assays is shown in Figure 3B. Western blot analysis confirmed that the $(\alpha\beta)_2$ and $(\alpha\beta)(\alpha\beta')$ tetrameric forms are present in fractions 58–59 (lane 1) from FPLC of IGF I receptor treated with DTT (corresponding to the open squares in panel A) and in fractions 58–59 (lane 2) from rechromatography of pooled fractions 63–69 (corresponding to the open triangles in panel A). The $\alpha\beta$ dimer is the major IGF I receptor form present in fractions 65–66 (lane 3) from rechromatography of pooled fractions 63–69 (corresponding to the open triangles in panel A), although smaller amounts of the $\alpha\beta'$ dimer and the $(\alpha\beta)(\alpha\beta')$ and $(\alpha\beta')_2$ tetrameric forms are also present.

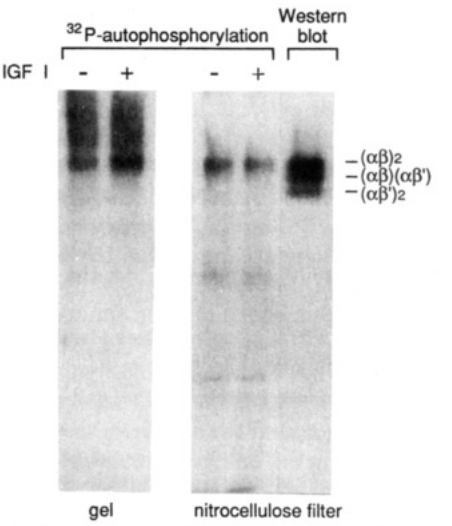

FIGURE 4: $(\alpha\beta)_2$ tetramer is the autophosphorylation-active IGF I receptor form. IGF I receptor was ^{32}P-autophosphorylated in the absence or presence of IGF I by incubation with 5 μM ATP (containing 20 μCi of [γ-^{32}P]ATP/assay) in 34 mM imidazole hydrochloride buffer, pH 7.4, with 5 mM $MnCl_2$, 187.5 mM NaCl, 0.075% Triton X-100, and 7.5% glycerol for 10 min at room temperature. The phosphorylated IGF I receptor together with unlabeled IGF I receptor in an adjacent lane was analyzed by SDS–PAGE (3–10% gradient acrylamide resolving gel) under nonreducing conditions and transferred to a nitrocellulose sheet. The portion of the sheet onto which the unlabeled IGF I receptor had been transferred was cut off and analyzed by Western blotting, using ^{125}I-labeled αIR-3. The sheet was then put back together, and an autoradiograph obtained by exposure of the entire sheet to X-ray film is shown. An autoradiograph of the gel from which the ^{32}P-autophosphorylated receptor had been transferred is also shown.

Figure 3C shows the autophosphorylation of IGF I receptor forms in these fractions. Autophosphorylation of a tetrameric form of the IGF I receptor, treated without DTT (not shown) or remaining after DTT treatment (lanes 1 and 2), is stimulated 1.6–2.9-fold by 100 ng/mL IGF I. Direct evidence that this is the $(\alpha\beta)_2$ tetramer is presented below (see Figure 4). In contrast, autophosphorylation of the $\alpha\beta$ dimers (lane 3) in the absence or presence of IGF I does not occur, despite the presence of much larger amounts of $\alpha\beta$ dimers than $(\alpha\beta)_2$ tetramers in these assays.

Three previously described forms of the IGF I receptor, the $(\alpha\beta)_2$, $(\alpha\beta)(\alpha\beta')$, and $(\alpha\beta')_2$ tetramers, are present in our preparations (Massague & Czech, 1982). It is likely that these forms result from limited proteolytic cleavage of the β subunit during the purification procedure, although it is possible that they occur in vivo. Only one form of the IGF I receptor is ^{32}P-autophosphorylated in our standard assay. To identify this tetramer, IGF I receptor, ^{32}P-autophosphorylated in our standard assay, was placed in an adjacent lane to unlabeled IGF I receptor, and these were analyzed by SDS–PAGE and transferred to a nitrocellulose sheet. The portion of the sheet onto which the unlabeled IGF I receptor had been transferred was cut off and analyzed by Western blotting, using ^{125}I-labeled αIR-3. The sheet was then put back together, and an autoradiograph was obtained by exposure of the entire sheet to X-ray film. This study is shown in Figure 4. Western blot analysis of the unlabeled receptor on the right demonstrates all three receptor forms. The gel on the left shows IGF I stimulated autophosphorylation of one of these forms. Autoradiography of this form after transfer to the nitrocellulose sheet demonstrates that it migrates in the position of the $(\alpha\beta)_2$ tetramer. The $(\alpha\beta)(\alpha\beta')$ form of the IGF I receptor, although present in similar amounts in our preparations, does not appear to be autophosphorylated.

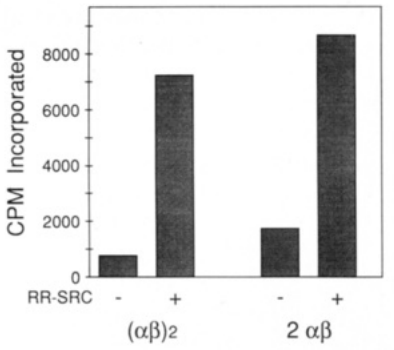

FIGURE 5: Phosphorylation of RR-SRC by IGF I receptor $(\alpha\beta)_2$ tetramer and purified $\alpha\beta$ dimers. Unlabeled IGF I receptor (300 μL) was mixed with ^{32}P-autophosphorylated IGF I receptor (23 200 cpm), reconstituted in 50 mM Tris-HCl buffer, pH 7.6, with 150 mM NaCl, 0.1% Triton X-100, 1 mg/mL BSA, and 0.02% NaN_3 by gel filtration, and concentrated to 600 μL; 478 and 96 μL were then treated with or without DTT, respectively, and analyzed by FPLC, using a Superose 6 column, as described under Experimental Procedures. Fractions containing $(\alpha\beta)_2$ tetramer (IGF I receptor treated without DTT) and fractions containing purified $\alpha\beta$ dimers [separated from $(\alpha\beta)_2$ tetramers remaining after DTT treatment] were pooled and concentrated for RR-SRC phosphorylation assays. Assays contained 136 and 140 cpm of ^{32}P-autophosphorylated IGF I receptor as tetrameric and dimeric forms, respectively, (corresponding to ~1.8 μL of unlabeled IGF I receptor tetramer or purified $\alpha\beta$ dimers) and were performed as described under Experimental Procedures. Background was 2300 cpm in this experiment and has been subtracted.

Phosphorylation of RR-SRC by IGF I Receptor $(\alpha\beta)_2$ Tetramer and Purified $\alpha\beta$ Dimers. We have measured IGF I receptor kinase activity using the substrate RR-SRC, a synthetic peptide based on the site of autophosphorylation in pp60$^{v\text{-}src}$ (Czernilofsky et al., 1981). To assay RR-SRC phosphorylation, IGF I receptor was incubated with 200 μM ATP (containing 3 μCi of [γ-^{32}P]ATP/assay), 12 mM $MgCl_2$, and 2 mM RR-SRC for 30 min at room temperature. IGF I was not routinely included in the incubation since IGF I stimulated autophosphorylation of the IGF I receptor appears to have a positive regulatory effect on receptor kinase activity toward exogenous substrates (Sasaki et al., 1985; Yu et al., 1986). Phosphorylation of this substrate by the untreated IGF I receptor is receptor concentration dependent (data not shown).

To determine if the failure to detect autophosphorylation of the IGF I receptor dimers resulted from inactivation of the kinase by DTT treatment, RR-SRC phosphorylation by equal amounts of $(\alpha\beta)_2$ tetramer or purified $\alpha\beta$ dimers was assayed. IGF I receptor was treated without or with DTT, and $(\alpha\beta)_2$ tetramers and purified $\alpha\beta$ dimers were prepared by FPLC. Unlabeled IGF I receptor was mixed with a trace amount of ^{32}P-autophosphorylated IGF I receptor in order to estimate the amount of IGF I receptor forms recovered. Figure 5 shows the results from a representative experiment. Both IGF I receptor $\alpha\beta$ dimers and $(\alpha\beta)_2$ tetramer exhibit similar kinase activities toward this synthetic substrate. In five experiments, purified $\alpha\beta$ dimers exhibited 0.818 ± 0.234 (mean ± SD) times as much kinase activity toward RR-SRC as an equal amount of $(\alpha\beta)_2$ tetramer. In addition, the activity of the $(\alpha\beta)_2$ tetramer and $\alpha\beta$ dimers recovered was similar to that of untreated IGF I receptor, indicating that minimal loss of activity occurred during their preparation. These results indicate that the receptor kinase is not inactivated by DTT treatment.

To confirm that the phosphorylation of RR-SRC by purified $\alpha\beta$ dimers did not result from their reassociation into $(\alpha\beta)_2$ tetramers during the assay, purified dimers were reapplied to the Superose 6 column after incubation under conditions of the RR-SRC phosphorylation assay. The top panel of Figure 6 shows the preparation of $(\alpha\beta)_2$ tetramer and $\alpha\beta$ dimers in this experiment.[2] SDS–PAGE of the column fractions pooled for RR-SRC phosphorylation assays, shown in the inset, confirms the purification of $\alpha\beta$ dimers. As shown in the bottom panel of Figure 6, the elution of purified $\alpha\beta$ dimers does not change after incubation under conditions of the RR-SRC phosphorylation assay, indicating that $\alpha\beta$ dimer reassociation does not occur.

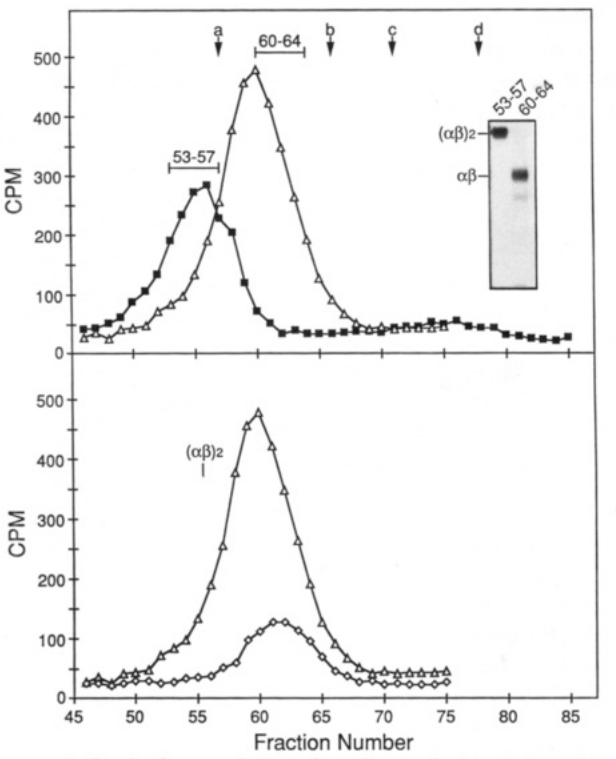

FIGURE 6: Lack of reassociation of $\alpha\beta$ dimers during the RR-SRC phosphorylation assay. Unlabeled IGF I receptor (450 μL of an IGF I receptor preparation different from that used in the study shown in Figure 5) was mixed with ^{32}P-autophosphorylated IGF I receptor (130 900 cpm), reconstituted in 50 mM Tris-HCl buffer, pH 7.6, with 150 mM NaCl, 0.1% Triton X-100, 1 mg/mL BSA, and 0.02% NaN_3 by gel filtration, and concentrated to 625 μL; 478 and 96 μL were then treated with or without DTT, respectively, and analyzed by FPLC, using a Superose 6 column, as described under Experimental Procedures. Fractions containing $(\alpha\beta)_2$ tetramer and purified $\alpha\beta$ dimers were pooled and concentrated to 60 and 80 μL, respectively, for RR-SRC phosphorylation assays. Twenty microliters of the purified $\alpha\beta$ dimers was incubated under conditions of the RR-SRC phosphorylation assay but without [γ-^{32}P]ATP and then reapplied to the Superose 6 column. RR-SRC phosphorylation assays contained 677 and 740 cpm of ^{32}P-autophosphorylated IGF I receptor as tetrameric and dimeric forms, respectively, and were performed as described under Experimental Procedures. 8890 cpm and 7320 cpm were incorporated into RR-SRC by $(\alpha\beta)_2$ tetramer and purified $\alpha\beta$ dimers, respectively, after subtracting background (2000 cpm in this experiment). (Top) Elution profiles of IGF I receptor treated without DTT (■) and of pooled fractions (▲) from FPLC of IGF I receptor treated with DTT. The column fractions pooled for RR-SRC phosphorylation assays are noted. The elution of the following standards is indicated by the arrows: a, thyroglobulin; b, apoferritin; c, β-amylase; d, BSA. Inset: An aliquot of the indicated pools, analyzed by SDS–PAGE and autoradiography. (Bottom) Elution profiles of pooled fractions (▲) from FPLC of IGF I receptor treated with DTT and of fractions 60–64 from rechromatography of these pooled fractions, after incubation under conditions of the RR-SRC phosphorylation assay (◇). The elution of IGF I receptor $(\alpha\beta)_2$ tetramers is noted.

[2] We have noted insignificant differences in elution of standards on the same Superose 6 column over time, cf. column profiles shown in Figures 1 and 3A, performed ~4 months apart. Small differences have been noted among different Superose 6 columns, cf. column profiles shown in Figures 1 and 3A with that shown in Figure 6.

Discussion

In this report, we have examined the structural requirements for IGF I receptor autophosphorylation. The IGF I receptor is composed of two $\alpha\beta$ dimers which are linked by disulfide bonds. These disulfide bonds can be selectively reduced by incubation with 2 mM DTT at pH 8.75 for 5 min at room temperature. After treatment under these conditions, ~75% of the IGF I receptor is in the $\alpha\beta$ dimeric form (Tollefsen & Thompson, 1988). We have used highly purified IGF I receptor solubilized from human placental membranes in our studies since the $\alpha\beta$ dimers formed after DTT treatment do not remain associated by noncovalent interactions. In the absence of detergent solubilization, the membrane environment maintains $\alpha\beta$ dimeric interactions despite reduction (Feltz et al., 1988). To investigate the tyrosine kinase activity of the $\alpha\beta$ dimeric halves of the IGF I receptor, we have separated completely $\alpha\beta$ dimers from $(\alpha\beta)_2$ tetramers remaining after DTT treatment by sequential FPLC using a Superose 6 gel filtration column. We show here that purified IGF I receptor $\alpha\beta$ dimers do not have intrinsic autophosphorylation activity. Under the same assay conditions, much smaller amounts of $(\alpha\beta)_2$ tetramers have β subunit autophosphorylation activity which is IGF I stimulated. We conclude that autophosphorylation of the IGF I receptor depends upon the interaction of the two $\alpha\beta$ dimers.

We had previously compared IGF I stimulated autophosphorylation of the IGF I receptor $(\alpha\beta)_2$ tetramer and $\alpha\beta$ dimer formed after treatment with DTT (Tollefsen & Thompson, 1988). Because treatment of the highly purified IGF I receptor with DTT under the conditions we employ results in the selective, but incomplete, reduction of the disulfide bonds linking the $\alpha\beta$ dimers of the receptor, both IGF I receptor tetramers and dimers were present in this study. Phosphorylation of the $\alpha\beta$ dimer formed after DTT treatment, although minimal, was observed in our previous study. Feltz et al. (1988) isolated IGF I receptor $\alpha\beta$ dimers from solubilized placental membranes by Bio-Gel A-1.5m gel filtration chromatography and showed that IGF I did not stimulate their phosphorylation; however, IGF I stimulated autophosphorylation of IGF I receptor $(\alpha\beta)_2$ tetramer in the dimer preparations. These results were consistent with IGF I induced covalent reassociation of $\alpha\beta$ dimers to $(\alpha\beta)_2$ tetramers, as the authors suggested, although interpretation is complicated by possible contamination of the $\alpha\beta$ dimer preparations with IGF I receptor tetramers. Our present studies have allowed us to distinguish among several possible explanations for the observed phosphorylation of the $\alpha\beta$ dimer in our previous study. Because the $\alpha\beta$ dimer does not have intrinsic autophosphorylation activity and because $\alpha\beta$ dimers do not reassociate during the autophosphorylation assay,[3] it is likely that the minimal phosphorylation of the $\alpha\beta$ dimer observed was effected by intact $(\alpha\beta)_2$ tetramer remaining after DTT treatment. Taken together, these results are consistent with a model in which autophosphorylation of the IGF I receptor occurs by cross-phosphorylation of $\alpha\beta$ dimers.

Studies with IGF I receptor preparations from rat BRL-3A2 liver cells (Zick et al., 1984; Sasaki et al., 1985) and from human placenta (Yu et al., 1986; Fujita-Yamaguchi et al., 1986; Sahal et al., 1988) have demonstrated that the IGF I receptor can catalyze the phosphorylation of exogenous tyrosine-containing substrates. We have used the substrate Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (RR-SRC), a synthetic peptide based on the site of autophosphorylation in $pp60^{v\text{-}src}$ (Czernilofsky et al., 1981), to measure the kinase activity of the purified IGF I receptor. IGF I receptor $(\alpha\beta)_2$ tetramer and purified $\alpha\beta$ dimers exhibit similar catalytic activities toward RR-SRC in our studies. The activity of the $\alpha\beta$ dimers does not result from their reassociation into $(\alpha\beta)_2$ tetramers during the assay. These results indicate that the failure to detect autophosphorylation of the IGF I receptor $\alpha\beta$ dimers does not result from inactivation of the kinase by DTT treatment. Two observations require further discussion. First, it is likely that the kinase activity of the purified $\alpha\beta$ dimers that we observe reflects a basal activity of the IGF I receptor β subunit itself. A basal kinase activity toward histone 2b has also been observed in insulin receptor preparations (Tornqvist & Avruch, 1988). Alternatively, it is possible that the IGF I receptor used in these studies is partially phosphorylated before or during its purification, although phosphate groups would be susceptible to endogenous phosphatase activity during the procedure since phosphatase inhibitors are not included. Second, autophosphorylation of the IGF I receptor appears to have a positive regulatory effect on receptor kinase activity toward exogenous substrates (Sasaki et al., 1985; Yu et al., 1986). Hence, in order to compare the basal kinase activities of the purified $\alpha\beta$ dimers and $(\alpha\beta)_2$ tetramer, it was necessary to select conditions under which autophosphorylation of the $(\alpha\beta)_2$ tetramer prior to and/or during the substrate phosphorylation assay does not occur. Since there is convincing evidence (Kohanski & Lane, 1986; Flores-Riveros et al., 1989) that only *insulin-dependent* receptor autophosphorylation is responsible for activation of insulin receptor kinase-catalyzed substrate phosphorylation, IGF I was not routinely included in the RR-SRC phosphorylation assay we employed. Interestingly, however, IGF I (20 min-preincubation without ATP) does not significantly enhance RR-SRC phosphorylation by the intact IGF I receptor under our assay conditions, and purified $\alpha\beta$ dimers exhibited 0.882 ± 0.373 (mean $\pm$ SD, $n = 5$) times as much kinase activity toward RR-SRC as an equal amount of $(\alpha\beta)_2$ tetramer in the presence of IGF I (data not shown). We have observed that minimal IGF I receptor autophosphorylation which is not stimulated by IGF I occurs during the RR-SRC phosphorylation assay (data not shown). It appears that this has little effect on substrate phosphorylation, suggesting that, in analogy to the insulin receptor, only IGF I dependent receptor autophosphorylation is responsible for activation of kinase-catalyzed substrate phosphorylation. A rigorous analysis of the relationship of IGF I receptor autophosphorylation to activation of kinase activity toward exogenous substrates in the presence and absence of IGF I is underway in our laboratory.

Recent studies have suggested that activation of the intrinsic kinase activity of the insulin receptor and the EGF receptor also depends upon the interaction of the two $\alpha\beta$ dimers of the insulin receptor (Böni-Schnetzler et al., 1986, 1987, 1988; Sweet et al., 1987a,b; Morrison et al., 1988) and the interaction of two EGF receptor monomers (Yarden & Schlessinger, 1987a,b; Böni-Schnetzler & Pilch, 1987; Cochet et al., 1988, Honegger et al., 1989), respectively. The function of the $\alpha\beta$ dimeric halves of the IGF I receptor can be compared with that of the $\alpha\beta$ dimeric halves of the structurally homologous insulin receptor. Böni-Schnetzler et al. (1986) isolated $\alpha\beta$ dimers of the insulin receptor in sucrose density gradients and showed that they do not demonstrate insulin-dependent autophosphorylation. Interestingly, insulin receptor $\alpha\beta$ dimers appeared to have basal autophosphorylation activity, whereas purified IGF I receptor $\alpha\beta$ dimers do not. Subsequent ex-

[3] R. M. Stoszek and S. E. Tollefsen, unpublished results.

periments demonstrated that autophosphorylation of more concentrated $\alpha\beta$ dimers could be stimulated by insulin (Böni-Schnetzler et al., 1988). Treatment of the $\alpha\beta$ dimers with glutathione in the presence of insulin resulted in reoxidation to $(\alpha\beta)_2$ tetramers, indicating that the $\alpha\beta$ dimers were in close proximity. These results suggested that insulin stimulated the autophosphorylation of $\alpha\beta$ dimers by inducing their *noncovalent* reassociation. In similar studies, Sweet et al. (1987a,b) isolated insulin receptor $\alpha\beta$ dimers by Bio-Gel A-1.5m gel filtration chromatography and showed that insulin stimulated the autophosphorylation of insulin receptor $\alpha\beta$ dimers and $(\alpha\beta)_2$ tetramers in the dimer preparations. These results suggested that insulin induced noncovalent and *covalent* reassociation of $\alpha\beta$ dimers to $(\alpha\beta)_2$ tetramers, although interpretation of these apparently conflicting results is complicated by possible contamination of the $\alpha\beta$ dimer preparations with insulin receptor tetramers. The function of the IGF I receptor $\alpha\beta$ dimer, uncontaminated by intact $(\alpha\beta)_2$ tetramer and analyzed under conditions in which no reassociation occurs, has been examined in this report. Our studies have demonstrated that interaction of the two $\alpha\beta$ dimers of the IGF I receptor is required for autophosphorylation. Interaction of receptor halves may be an important mechanism for modulating receptor function in the tyrosine kinase family of growth factor receptors.

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

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