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Radiation Inactivation Experiments Predict That a Large Aggregate Form of the Insulin Receptor Is a Highly Active Tyrosine-Specific Protein Kinase†

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ABSTRACT: The technique of radiation inactivation has been used on a highly purified insulin receptor in order to determine the functional molecular size responsible for tyrosine-specific protein kinase activity. When both insulin binding and kinase activities were analyzed with the same receptor preparations, the functional size for kinase activity was found to be larger than that for insulin binding activity. The radiation inactivation curve for kinase activity was multiphasic. This indicates that at least two components contribute to total kinase activity. The average minimal functional size for the kinase was 370 000 ± 60 000 daltons ($n = 7$) which corresponds to the $\alpha_2\beta_2$ form of the insulin receptor. The average functional size for larger forms was estimated to be $\sim 4 \times 10^6$ daltons. (To minimize the complexity of the model used in this analysis, we have analyzed the radiation inactivation curves of the insulin receptor kinase activity with a two-component model. However, we believe that the larger component, $>1 \times 10^6$ daltons, is probably not a single molecular weight species but rather represents a continuum of sizes or aggregates of the $\alpha_2\beta_2$ form of the receptor.) These larger forms contributed 93% of the total activity. Mild reduction of the insulin receptor preparation with dithiothreitol (DTT) activated the total kinase activity by 3.5-fold. Under this condition, the minimal functional kinase size was 380 000 ± 30 000 daltons ($n = 6$) while the average functional size for the larger forms was $\sim 3 \times 10^6$ daltons. However, the contribution of the larger forms to the total kinase activity was reduced to 39%. When the 3.5-fold increase in the total kinase activity was taken into account, the activity of the minimal form increased ~30-fold (7% to 213%) whereas that of the larger forms did not change significantly (93% to 137%). These studies suggested that the minimal functional kinase is an $\alpha_2\beta_2$ receptor which can be activated ~30-fold by mild DTT treatment. The minimal kinase size did not change to that of an $\alpha\beta$ form under the condition that reduces disulfide bonds between the two α subunits, indicating that a free $\alpha\beta$ form is not an active kinase. Furthermore, radiation inactivation studies indicated that the kinase forms larger than the $\alpha_2\beta_2$ receptor appear to be extremely active. The presence of high molecular weight kinase was detected as a major ^{32}P peak at the void volume when autophosphorylated receptors were fractionated by Sepharose CL-6B chromatography. This peak was dissociated from major peaks corresponding to either insulin binding or receptor protein. These studies suggested that the larger forms of the insulin receptor, $(\alpha_2\beta_2)_{8-10}$, which are minor components in the receptor population, contribute the majority of the kinase activity. This conclusion derived from the present studies may be relevant to the notion that receptor aggregation with a concomitant increase in kinase activity plays an important role in the signal transduction of insulin.

The insulin receptor is a tyrosine-specific protein kinase. The kinase which resides in the cytoplasmic domain of the receptor β subunit is activated immediately after the binding of insulin to the extracellular domain (α subunit). This activation process appears to be essential for insulin signal transduction (Ullrich et al., 1985; Ebina et al., 1985; Rosen, 1987).

Structural requirements for the insulin receptor kinase activation have been studied in a variety of ways: (i) Binding of insulin to the outer surface domain activates the cytoplasmic kinase, which in turn autophosphorylates. This autophosphorylation process further activates the receptor kinase (Tornquist & Avruch, 1988). (ii) Mild dithiothreitol (DTT)[1]

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[1] Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mrads, megarads; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; WGA, wheat germ agglutinin; EGF, epidermal growth factor; DTT, dithiothreitol.

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treatment of the receptor activates the kinase, suggesting that the monomeric $\alpha\beta$ kinase has a higher specific activity than the intact $\alpha_2\beta_2$ kinase (Fujita-Yamaguchi & Kathuria, 1985; Sweet et al., 1986). (iii) Mild trypsin treatment activates the insulin receptor kinase (Tamura et al., 1983; Shoelson et al., 1988). (iv) When the insulin receptor kinase domain is expressed in Chinese hamster ovary cells, the kinase is constitutively activated (Ellis et al., 1987). Thus, it is likely that kinase activity is suppressed in an intact $\alpha_2\beta_2$ form of the receptor and that the role of insulin, DTT, and trypsin is to change the conformation of the intact receptor to kinase-active forms.

The technique of radiation inactivation can be used to measure the molecular size responsible for a certain biological activity. We have previously analyzed the size for insulin binding activity using purified insulin receptor preparations, which revealed that there is a monomer ($\alpha\beta$)–dimer ($\alpha_2\beta_2$) equilibrium and that monomers have higher binding activity than dimers (Fujita-Yamaguchi & Harmon, 1988). In this study, we describe the functional size for kinase activity. The kinase activity measured in the presence of insulin or DTT suggests that the minimally functional molecular size is an ($\alpha_2\beta_2$) or $(\alpha\beta)_2$ form[2] of the receptor. The results also suggest that the insulin receptor aggregates with $(\alpha_2\beta_2)_n$ (n = 8–10), which are minor components in the purified receptor preparations, are responsible for the majority of the kinase activity.

Materials and Methods

Materials. Crystalline porcine insulin was supplied by Eli Lilly; Sepharose CL-6B and Sephadex G50 were purchased from Pharmacia; thyroglobulin, catalase, γ-globulin, and calf intestine alkaline phosphatase were obtained from Sigma. A synthetic peptide resembling the tyrosyl phosphorylation site of $pp60^{src}$ (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) was purchased from Peninsula Laboratories. Molecular size markers for SDS–PAGE were obtained from Bio-Rad. [γ-^{32}P]ATP and ^{125}I-labeled insulin were obtained from New England Nuclear. All other chemicals used were reagent grade.

Purification of the Insulin Receptor. The insulin receptor was purified 2400-fold with a yield of 40% from the Triton X-100 extracts of human placental membranes by sequential affinity chromatography on wheat germ agglutinin–Sepharose and insulin–Sepharose columns as previously described (Fujita-Yamaguchi et al., 1983).

Irradiation Procedure. The samples in sealed ampules were irradiated with 13-MeV electrons from a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD) as previously described (Harmon et al., 1985). The temperature of the samples during irradiation was maintained at −135 ± 5 °C.

Insulin Binding Assay. Insulin binding activity was measured by incubating the receptor preparation at 4 °C for 16 h with ^{125}I-labeled porcine insulin (20 000 cpm, 0.2 ng) in a final volume of 0.4 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 0.1% bovine serum albumin in the absence and presence of excess unlabeled porcine insulin (20 μg/mL). The receptor–^{125}I-insulin complex was separated from free ^{125}I-insulin by adding 0.1 mL of 0.4% bovine γ-globulin and 0.5 mL of 20% poly(ethylene glycol) 6000, followed by centrifuging at 1500g for 20 min at 4 °C. The supernatants were aspirated, and the pellets were counted with a γ counter.

Tyrosine-Specific Protein Kinase Assay. The purified receptor (~0.1 μg), which had been irradiated in the presence or absence of 1 μM insulin and/or 0.5 mM DTT, was incubated at 25 °C for 40 min in 30 μL of 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM $MnCl_2$, 15 mM $MgCl_2$, 1 mM src-related peptide, [γ-^{32}P]ATP (40 μM, 5–10 μCi/nmol), and 0.1% Triton X-100. The reaction was terminated by adding 50 μL of 5% trichloroacetic acid and 20 μL of bovine serum albumin (10 mg/mL). After incubation of this solution at 0 °C for 30 min, the proteins were pelleted by centrifugation for 5 min. A 35-μL aliquot of the supernatant was spotted on a piece of phosphocellulose paper (Whatman, P81). The paper was extensively washed in 75 mM phosphoric acid and placed in a vial containing Aquasol (New England Nuclear). Duplicate papers were prepared from each reaction mixture, and ^{32}P incorporated into the peptide was counted in a liquid scintillation counter.

Autophosphorylation and SDS–PAGE Analysis. The phosphorylation[3] of the receptor (~0.5 μg) was carried out in the presence of 1 μM insulin and/or 0.5 mM DTT at 25 °C for 40 min in 40 μL of 50 mM Tris-HCl (pH 7.4) containing [γ-^{32}P]ATP (40 μM, 10 μCi), 2 mM $MnCl_2$, 15 mM $MgCl_2$, and 0.1% Triton X-100. The reaction was terminated by adding 20 μL of 50 mM Tris-HCl (pH 6.8) containing 6% SDS, 3 mM ATP, 60 mM dithiothreitol (DTT), and 50% (w/v) sucrose, followed by boiling for 5 min. Insulin receptor subunits were separated by SDS–PAGE (7.5%) under reducing conditions (Laemmli, 1970) and were stained with silver. The gel was autoradiographed.

Gel Filtration Chromatography on Sepharose CL-6B. The purified insulin receptor (~5–10 μg) was autophosphorylated and first applied to a Sephadex G50 column (5 mL). The phosphorylated insulin receptor eluted at the void volume of the Sephadex G50 column was applied to a Sepharose CL-6B column (1.5 × 50 cm) and eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100. Fractions of 1 mL each were collected and counted in a liquid scintillation counter; 100-μL aliquots were assayed for specific insulin binding. For molecular size determinations, thyroglobulin, catalase, γ-globulin, and calf intestine alkaline phosphatase were used and assayed as described (Le Bon et al., 1986). The receptors (~5 ng) which had been iodinated with Na^{125}I were applied to the column and analyzed as described (Fujita-Yamaguchi & Harmon, 1988).

Theory and Calculations. The results of radiation inactivation studies were analyzed by classical target theory as previously described (Harmon et al., 1980, 1985). Briefly, the logarithm of the fraction of remaining tyrosine-specific protein kinase activity or ^{32}P incorporation into the β subunit was plotted versus exposure dose. The following models were considered:

(*A*) *Single-Site Model.* In the simplest situation when only one component is responsible for the total activity, the inactivation curve is assumed to be single exponential. In this case, the molecular mass (in daltons) of the kinase-active component can be calculated as previously described by using the formula:

$$M = (6.4 \times 10^5) S_t K$$

where S_t is a temperature correction of 2.8 and K is the slope of the radiation inactivation curve with the dose measured in

[2] Parentheses indicate disulfide-linked subunit complexes; for example, $(\alpha\beta)_2$ indicates that a disulfide-linked $\alpha\beta$ half is noncovalently associated with another disulfide-linked $\alpha\beta$ half.

[3] Since purified receptors were used, phosphorylation of the β subunit occurred at only tyrosine residues under our conditions (Kasuga et al., 1983).

Table I: Summary of Radiation Inactivation Experiments on Kinase and Autophosphorylation Activities of the Purified Insulin Receptor

	functional sizes (daltons)		
	minimal form	larger forms (av distribution)	act.$_{\text{larger form}}$/total act.
(1) kinase activity			
+Ins, −DTT (n = 7)[a]	370 000 ± 60 000	(3.5 × 10^6) ± (0.5 × 10^6)	0.93 ± 0.04
+Ins, +DTT (n = 6)	380 000 ± 30 000	(2.6 × 10^6) ± (1 × 10^6)	0.39 ± 0.09
(2) autophosphorylation			
+Ins, −DTT (n = 2)	80 000 ± 50 000[b]	(1.9 × 10^6) ± (0.4 × 10^6)	0.80 ± 0.06
+Ins, +DTT (n = 3)	260 000 ± 60 000	(1.8 × 10^6) ± (0.9 × 10^6)	0.59 ± 0.02

[a] Number of determinations. [b] The small size cannot accurately be determined since the initial ^{32}P incorporation to the β subunit was very low (e.g., 460 cpm).

megarads (Mrad). The slope of the radiation inactivation curve is obtained from the best-fit line generated by the use of a computer program (RS/3, BBW Research Systems, Cambridge, MA) for weighted nonlinear regression.

(*B*) *Multisite Model.* When several components of different functional sizes contribute to the total activity, the radiation inactivation curve is multiexponential. The data can be fit to the equation:

$$A/A_0 = F_1e^{-K_1r} + F_2e^{-K_2r} + \ldots + F_ne^{-K_nr}$$

where A is the activity measured at given radiation dose, A_0 is the activity of the unirradiated control, F_1, F_2, ..., F_n are the fractions of activity contributed by each functional unit ($F_1 + F_2 + \ldots + F_n = 1.0$), K_1, K_2, ..., K_n are the slopes of the inactivation curve for each n unit, and r is the radiation dose. For an apparent single-exponential inactivation curve, n equals 1.

Results

Comparison of Radiation Inactivation Curves of Insulin Binding and Kinase Activities. Purified insulin receptor was irradiated in the absence of insulin at −135 °C. The receptor was thawed, and then tyrosine-specific kinase activity was assayed in the absence or presence of insulin by measuring ^{32}P incorporation into the src-related peptide. The results of a typical experiment are shown in Figure 1. A striking difference was observed between the radiation inactivation curve of insulin binding activity and that of kinase inactivation of the same receptor preparation. The insulin binding inactivation was consistent with our previous results; an average functional size for insulin binding was estimated to be 227 000 ± 39 000 daltons by the monomer–dimer model (Fujita-Yamaguchi & Harmon, 1988). In contrast, the functional size for kinase activity was estimated to be (4.9 × 10^6) ± (1.1 × 10^6) daltons (n = 6) and (5.7 × 10^6) ± (2.3 × 10^6) daltons (n = 4) in the presence and absence of insulin, respectively.[4]

Functional Size for Tyrosine-Specific Kinase Activity. As mentioned above, since the tyrosine-specific kinase activity is relatively low in purified receptor preparations in the absence of insulin, the loss of activity seen in the irradiated samples was difficult to measure. However, in the presence of insulin, there was a ~5-fold increase in the tyrosine-specific kinase activity, but there was no apparent change in the functional size of the tyrosine-specific kinase activity when the receptor preparations were irradiated in the absence or presence of insulin during radiation. Therefore, insulin receptor preparations were irradiated in the presence of insulin (1 μM) to more accurately follow the loss of tyrosine-specific kinase activity. For the same reason, more receptor (4× control) was irradiated and assayed in samples exposed to doses of greater than 3 Mrad. The results of a typical experiment are shown

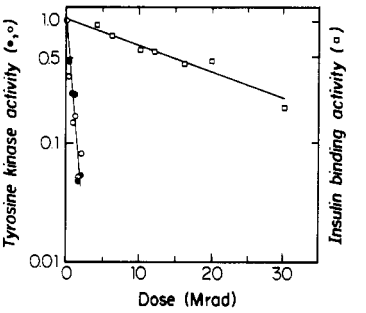

FIGURE 1: Inactivation of tyrosine-specific protein kinase and ^{125}I-insulin binding activities of the insulin receptor. Purified insulin receptor (0.1 mg/mL) was irradiated in the absence of insulin at −135 °C with high-energy electrons. The receptor was thawed, and then tyrosine-specific kinase activity was assayed in the absence (○) and presence (●) of 1 μM insulin by measuring ^{32}P incorporation into the src-related peptide as described under Materials and Methods. The logarithm of the fraction of remaining kinase activity is plotted versus exposure dose. Insulin binding activity was measured as described under Materials and Methods. The fraction of remaining insulin binding $(B/F)/(B/F)_0$ is plotted versus exposure dose (□).

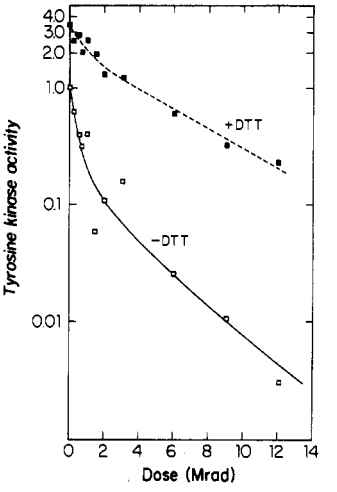

FIGURE 2: Radiation inactivation of tyrosine-specific protein kinase activity of the insulin receptor. Purified insulin receptor (0.1 mg/mL) was irradiated either in the presence of 1 μM insulin and 0.5 mM DTT (■) or in the presence of 1 μM insulin but the absence of DTT (□). The receptor was thawed and assayed for kinase activity as described under Materials and Methods. During assays, the concentrations of insulin and DTT were kept the same as those during irradiation. To enhance accurate measurement, more receptor (4× control) was irradiated and assayed in samples exposed to doses of greater than 3 Mrad. All results have been corrected to the amount of the receptor kinase in the nonirradiated control. The logarithm of the fraction of remaining kinase activity is plotted versus exposure dose: (solid line) −DTT; (dashed line) +DTT.

[4] These data were derived from the initial phase of this study. Later experiments showed multiphasic curves indicating the coexistence of a small form, which could not be accurately detected in these initial experiments. Activities less than 10% of the control were not accurate enough to estimate the small form. Also, since basal kinase activity was quite low, it was not possible to accurately estimate the kinase size. Therefore, these estimated values offer only a rough estimate of the functional size for the kinase activity.

Table II: Schematic Presentation of Structure–Function Relationships of the Insulin Receptor Kinase

−DTT		+DTT	
structure	% kinase act.	structure	% kinase act.
$(\alpha_2\beta_2)_{8-10}$[a]	93	$[(\alpha\beta)(\alpha\beta)]_{8-10}$	39 (39 × 3.5 = 137%)
$(\alpha_2\beta_2)$	7	$(\alpha\beta)(\alpha\beta)$	61 (61 × 3.5 = 213%)
$(\alpha\beta)$	0	$(\alpha\beta)$	0
ratio of total kinase act.	1 (100%)		3.5

[a] Parentheses indicate disulfide-linked subunit complexes; for example, $(\alpha\beta)(\alpha\beta)$ indicates that a disulfide-linked $\alpha\beta$ half is noncovalently associated with another disulfide-linked $\alpha\beta$ half.

in Figure 2, solid line. The radiation inactivation curve was clearly multiphasic. This indicates that at least two components of differing size contributed to the total tyrosine-specific kinase activity. The minimal component had an apparent mass of 370 000 ± 60 000 daltons (n = 7) which appears to correspond to an intact $\alpha_2\beta_2$ receptor.[5] The component larger than the minimal component, inactivated at lower radiation doses, contributed 93% ± 4% of the total tyrosine-specific kinase activity and had an apparent mass of (3.5 × 10^6) ± (0.5 × 10^6) daltons (n = 7) (Table I).[6]

Effect of Dithiothreitol on the Functional Sizes for Tyrosine-Specific Kinase Activity. Since treatment of the insulin receptor with 0.5 mM DTT increases kinase activity 3–5-fold (Fujita-Yamaguchi & Kathuria, 1985), radiation inactivation experiments were also performed using the same amount of the same receptor preparation as above in the presence of 0.5 mM DTT and 1 μM insulin. DTT treatment increased the total kinase activity of the insulin receptor ~3.5-fold; the activities of the nonirradiated controls with and without DTT treatment were 1.71 and 0.51 pmol min^{-1} $assay^{-1}$, respectively. In Figure 2, the logarithm of the fraction of remaining kinase activity of the DTT-treated receptor preparation is expressed as a percent of nonirradiated control sample without DTT treatment (dashed line). This inactivation curve showed a multiphasic nature similar to that of the receptor preparation without DTT treatment (solid line). The functional sizes estimated for the minimal and larger forms were 380 000 ± 30 000 and (2.6 × 10^6) ± (1 × 10^6) daltons, respectively (n = 6) (Table I). However, when compared to the data without DTT treatment, a marked difference was seen in the proportion of activity contributed by each of the two components. In the presence of DTT, the contribution of the minimal component to the total kinase activity increased to 61% while it was 7% in the absence of DTT. When the 3.5-fold increase in the total kinase activity was taken into account, the activity of the minimal form increased ~30-fold (7% to 213%) whereas that of the larger forms did not change significantly (93% to 137%) (Table II).

Size of the Autophosphorylation Activity Measured in the Absence and Presence of DTT. Receptor preparations, with and without DTT, were exposed to increasing doses of radiation. Upon thawing, the samples were autophosphorylated and subjected to SDS–PAGE (7.5% gel) under reducing conditions. The gels were stained with silver and prepared for autoradiography. Figure 3 shows one of the experiments: (A) the silver-stained gel containing receptor samples irradiated in the presence of insulin and DTT; (B) the autoradiogram of the gel shown in (A); and (C) the autoradiogram of the gel containing receptor samples irradiated in the presence of insulin but the absence of DTT.

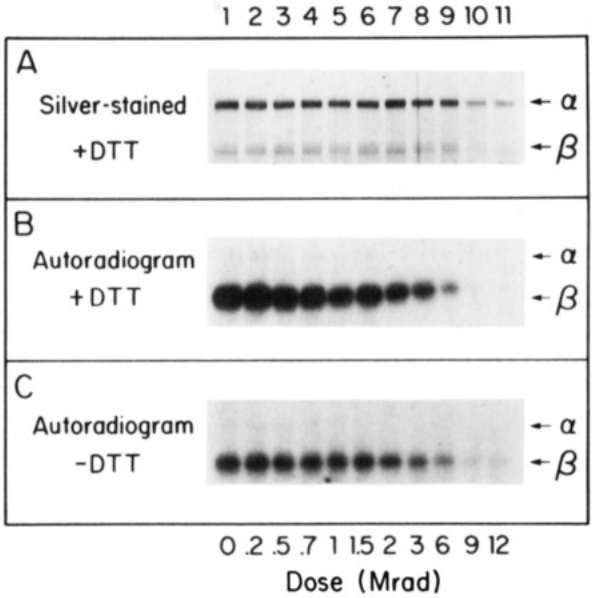

FIGURE 3: Effect of radiation on the insulin receptor subunits and insulin-stimulated autophosphorylation activity. Purified insulin receptor was irradiated, at −135 °C, either in the presence of 1 μM insulin and 0.5 mM DTT (A and B) or in the presence of 1 μM insulin but the absence of DTT (C). Upon thawing, the samples were autophosphorylated as described under Materials and Methods and subjected to SDS–PAGE (7.5% gel) under reducing conditions. The gels were stained with silver (A) and autoradiographed (B and C).

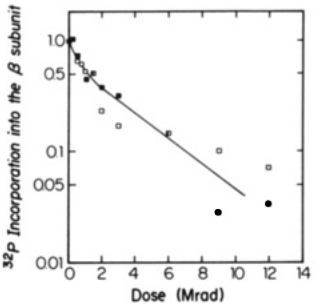

FIGURE 4: Radiation inactivation of autophosphorylation activity of the insulin receptor. Incorporation of ^{32}P into the β subunit shown in Figure 3B (+DTT; ■) and Figure 3C (−DTT; □) was quantitated by liquid scintillation counting. The initial radioactivities (at 0 Mrad) of DTT-treated and -untreated receptors were 2530 and 460 cpm, respectively. The logarithm of the fraction of remaining autophosphorylation activity is plotted versus exposure dose.

The same protein amount of purified insulin receptor was analyzed in each lane. As clearly seen in Figure 3B,C, phosphorylation of the β subunit was stimulated by DTT treatment. Incorporation of ^{32}P into the β subunit[7] was quantitated by liquid scintillation counting. The initial radioactivities (at 0 Mrad) representing β subunit phosphorylation of DTT-treated and -untreated receptor preparations

[5] The basic unit of the insulin receptor, $\alpha_2\beta_2$, has an apparent molecular mass of 350–400 kDa (Massague et al., 1980; Fujita-Yamaguchi, 1984).

[6] To minimize the complexity of the model used in this analysis, we have analyzed the radiation inactivation curves of the insulin receptor kinase activity with a two-component model. However, we believe that the larger component (>1 × 10^6 daltons) is most probably not a single molecular weight species but rather represents a continuum of sizes or aggregates of the $\alpha_2\beta_2$ form of the receptor.

[7] In Figure 3A, the silver-stained β subunit looks diffused or like a doublet. We have previously found that the upper band of the β subunit is the band predominantly phosphorylated (Kathuria et al., 1986). Therefore, radiation inactivation curves obtained from autophosphorylation experiments represent the decrease in the phosphorylation of the intact β subunit. The crooked radioactive bands seen in Figure 3B were caused by a slight distortion of the wet gel during autoradiography.

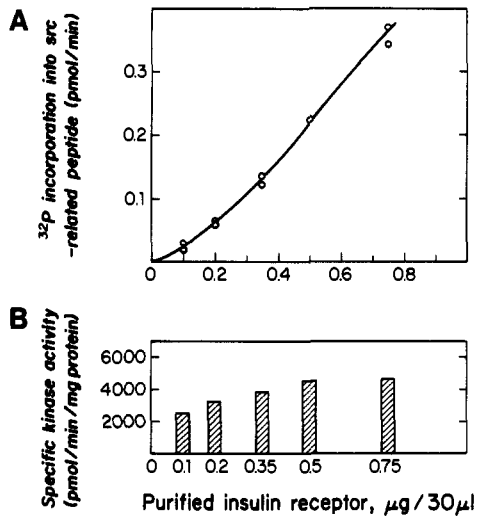

FIGURE 5: Effect of insulin receptor concentration on the specific kinase activity. Purified insulin receptor was assayed for kinase activity by using the src-related peptide as an exogenous substrate. Panel A shows kinase activity (picomoles per minute) measured at different concentrations of the receptor. Panel B represents the specific kinase activity (picomoles per minute per milligram of receptor protein) at the respective receptor concentration shown in panel A.

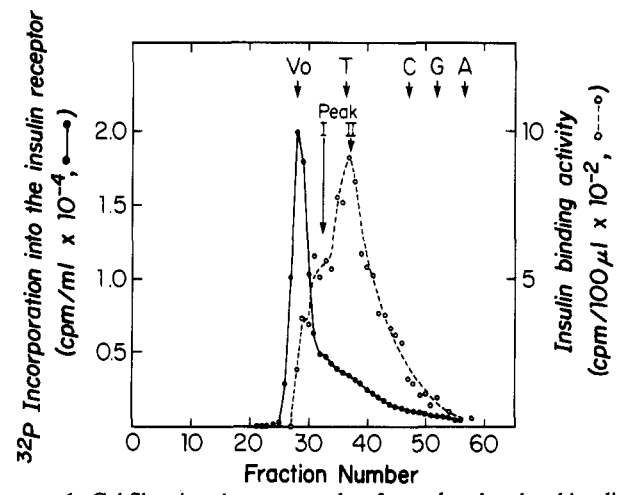

FIGURE 6: Gel filtration chromatography of autophosphorylated insulin receptor on Sepharose CL-6B. Purified insulin receptor (~10 μg) was autophosphorylated as described under Materials and Methods and applied to a Sephadex G-50 column (0.6 × 18 cm). Phosphorylated receptors eluted at the void volume were combined, concentrated 2.5-fold, applied to a Sepharose CL-6B column (1.5 × 50 cm), and eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100. The radioactivity in each tube (1 mL/tube) was measured by Cherenkov radiation (●), and 100-μL aliquots were assayed for specific insulin binding (○). Molecular size markers indicated are thyroglobulin (T, 669000 daltons), catalase (C, 224000 daltons), γ-globulin (G, 169000 daltons), and calf intestine alkaline phosphatase (A, 100000 daltons).

were 2530 and 460 cpm for Figure 3B and Figure 3C, respectively, indicating that the β subunit phosphorylation was stimulated 5.5-fold by DTT in this experiment. Figure 4 presents the radiation inactivation curve of the autophosphorylation activity measured in the absence and presence of 0.5 mM DTT as shown in Figure 3.

Because of the limitations in the assay for the measurement of the autophosphorylation activity, only qualitative results can be ascertained from these radiation inactivation curves. Thus, it appears that these curves are also multiphasic and suggestive of at least two functional components. It would also appear that the sizes of these components are similar to those obtained by measuring total kinase activity (Table I).

Destruction of the α and β Subunits during Irradiation. Since both kinase and autophosphorylation activities appear to be associated with extremely large forms of the receptor, the destruction of insulin receptor subunit structure was examined. The fraction of remaining subunits, either α or β, was estimated from silver-stained protein bands as seen in Figure 3A. The sizes, ~160000 daltons, roughly coincide with α + β, which indicates that the β subunit remains intact under conditions where radiation induces a loss of kinase activity (Figures 2 and 3A). This evidence supports the notion that the larger forms are responsible for the majority of the kinase activity and that the destruction of the β subunit per say is not required for inactivation of the majority of total kinase activity.

Separation of the Larger Forms from $\alpha_2\beta_2$ and αβ Forms. In order to verify the presence of larger forms in the insulin receptor preparations, the purified receptor was fractionated by Sepharose CL-6B chromatography. Low but significant kinase activity without insulin binding activity was detected in the void volume fractions whereas other fractions with demonstrable insulin binding activity had no kinase activity (data not shown). This is mostly attributed to dilution of the receptor since the kinase assay using the src-related peptide requires approximately 100 times more concentrated receptor in the assay mixture as compared to binding assays. In addition, it is possible that dilution during and after the chromatography may have caused the dissociation of receptor aggregates, which may result in a reduction of kinase activity.

In order to examine if receptor concentration causes changes in the specific kinase activity, the purified receptor preparation was assayed for kinase activity at different concentrations. The results show that ^{32}P incorporation into the src-related peptide (picomoles per minute) is not linearly related to the receptor concentration (Figure 5A). This indicates that specific kinase activity (picomoles per minute per milligram of receptor protein) increases as the receptor concentration increases (Figure 5B).

Alternatively, the receptor preparation was first autophosphorylated and then fractionated. In this case, even though dilution occurs during and after gel filtration, ^{32}P incorporation into the receptor can be detected. Figure 6 shows one of two similar experiments. The insulin binding peak showed at least two peaks which had been previously described as peak I ($\alpha_2\beta_2$ rich) and peak II (αβ rich) (Fujita-Yamaguchi & Harmon, 1988). The major ^{32}P peak was seen at the void volume of the Sepharose column and was almost completely dissociated from insulin binding activity peaks. Although there was a shoulderlike insulin binding activity peak around the void volume, this activity was very low when compared with the major insulin binding peaks (peaks I and II). The major ^{32}P peak was followed by a tailing of radioactivity presumably reflecting phosphorylated peak I receptors ($\alpha_2\beta_2$ form) and peak II receptors (αβ form). To identify the receptor species, ^{125}I-labeled insulin receptors were fractionated by Sepharose CL-6B chromatography. Receptor elution was monitored by counting each fraction with a γ counter (Figure 7A). The fractions marked with italic numbers were analyzed by SDS–PAGE under nonreducing conditions (Figure 7B). The first major peak represents the labeled receptor while the second peak is ^{125}I-labeled Triton X-100 micelles. The radioactive bands seen in Figure 7B clearly show that $\alpha_2\beta_2$ receptors are in the majority and the receptors recovered at the void volume represent a minor portion of the total receptor species although there is a possibility that iodination may dissociate receptor aggregates. The relative proportions of aggregates, $\alpha_2\beta_2$ receptors, and αβ forms were estimated to

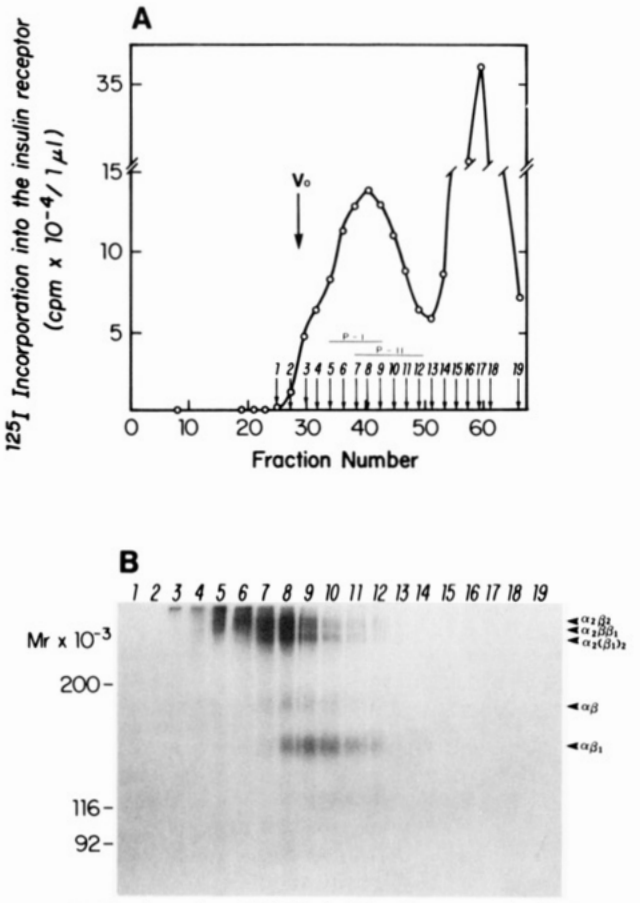

FIGURE 7: Fractionation of ^{125}I-labeled insulin receptor by Sepharose CL-6B chromatography. ^{125}I-labeled insulin receptor (~5 ng) was fractionated by Sepharose CL-6B chromatography. One microliter of each fraction was counted in a γ counter (A). The fractions marked with italic numbers were analyzed by SDS–PAGE (5% gel) under nonreducing conditions (B).

Table III: Summary of Radiation Inactivation Results on both Insulin Binding and Kinase Activities

insulin receptor in different forms	insulin binding act.[a]	kinase act.,[b] [A] (%)	protein amount,[c] [B] (%)	sp kinase act., [A]/[B]
$(\alpha_2\beta_2)_{8-10}$	±	93	6.3	14.8
$(\alpha_2\beta_2)$	+	7	69.8	0.10
$(\alpha\beta)$	+++	0	23.9	0

[a] Relative activity is summarized from the results of this and previous studies (Fujita-Yamaguchi & Harmon, 1988). [b] Relative activity is derived from Table I. [c] Relative protein amount is estimated from SDS–PAGE analysis of size fractionated ^{125}I-labeled receptors shown in Figure 7B.

be 6.3%, 69.8%, and 23.9%, respectively, by densitometric scanning of the autoradiogram.

The results indicate that larger forms of the receptor (M_r >2 × 10^6) exhibit much higher specific kinase activity (^{32}P/insulin receptor) than an $\alpha_2\beta_2$ form or an $\alpha\beta$ form of the receptor (Table III).

Discussion

By the use of radiation inactivation methods, we have consistently observed that the functional size for the purified insulin receptor kinase is extremely large. Present studies have suggested that there are at least two functional sizes for kinase and autophosphorylation activities. The summary is shown in Table I. The larger forms have a range of sizes with (~3–6) × 10^6 daltons, and the minimal form is ~400000 daltons. The former accounts for 93% of the total kinase activity of the purified insulin receptor. DTT treatment enhances the total kinase activity by 3.5-fold (Figures 2 and 3; Fujita-Yamaguchi & Kathuria, 1985) and changes the percentage of activity attributed to the large form to 39%. The minimal form which corresponds to an $\alpha_2\beta_2$ receptor appears to be the minimal functional unit for kinase activity. DTT treatment activates kinase without changing the functional size of this form, indicating that a free $\alpha\beta$ form which could be generated by radiation is not an active kinase.

We have previously reported that monomeric $\alpha\beta$ receptor kinase generated by DTT treatment is more active than the intact $\alpha_2\beta_2$ receptor kinase (Fujita-Yamaguchi & Kathuria, 1985). As we stated, this does not necessarily mean that the $\alpha\beta$ halves are free from other $\alpha\beta$ halves. In the present studies, by employing the radiation inactivation method, we found DTT-reduced $\alpha_2\beta_2$ receptors, in which two $\alpha\beta$ halves are still noncovalently associated in an $(\alpha\text{–}\beta)(\alpha\text{–}\beta)$ form, exhibit higher kinase activity than the intact $\beta\text{–}\alpha\text{–}\alpha\text{–}\beta$ receptor. Others also noted that the gel filtration profiles of the DTT-reduced ^{32}P-phosphorylated insulin receptor are indistinguishable from that of nonreduced receptor (Pike et al., 1986; Sweet et al., 1986). The results of present radiation inactivation studies are consistent with their observation that the DTT-reduced $\alpha_2\beta_2$ receptor complex remains tightly associated in buffers containing 0.1% Triton X-100 and dissociates only upon the addition of a denaturating reagent such as 0.1% SDS. Moreover, recent studies suggested that insulin-induced reassociation of $\alpha\beta$ forms into $\alpha_2\beta_2$ forms correlates with kinase activation (Morrison et al., 1988; Böni-Schnetzler et al., 1988).

The mechanism by which DTT activates insulin receptor kinase is not known. It seems that DTT potentiates conformational changes in the receptor molecule by releasing structural constraints produced by the disulfide linkages between the two $\alpha\beta$ halves and/or by reducing intramolecular disulfide linkages.

The large aggregate form could be artificially produced due to concentrating by 100-fold of the insulin–Sepharose eluates since this process also concentrates Triton X-100. Therefore, the functional size for kinase activity of the receptor in the buffer containing 0.1% Triton was analyzed by using two receptor preparations: (i) receptor partially purified by WGA–Sepharose chromatography and (ii) the purified receptor which had been concentrated by WGA–Sepharose chromatography instead of using our routine pressure dialysis method. In both cases, the functional kinase size was estimated to be approximately 2 × 10^6 daltons (data not shown). These results are consistent with the idea that the large kinase size obtained by using our purified receptor preparations is not an artifact but represents the kinase size of the solubilized insulin receptor in the other situations. In agreement with our observation, Yanaihara et al. (personal communications) consistently detected a small immunoreactive peak at the void volume when Triton X-100 extracts of placental membranes were applied to a Sepharose 6B column and assayed by radioimmunoassay using rabbit antibodies against peptides corresponding to the domains of the insulin receptor.

The purified insulin receptor preparation thus consists of at least three types of the receptor. Characteristics of each form are summarized in Table III. (i) The large forms, $(\alpha_2\beta_2)_{8-10}$, are the most active kinase but show little binding activity. These are apparently minor components. (ii) The $(\alpha_2\beta_2)$ form is the major component and shows both insulin binding and kinase activities. However, as previously described (Koch et al., 1986; Fujita-Yamaguchi & Harmon, 1988), the

binding activity of this form is much lower than that of the $\alpha\beta$ form under our assay conditions. The kinase activity of the $(\alpha_2\beta_2)$ form is significant but appears to have a lower specific activity than that of the larger aggregate forms. (iii) The $\alpha\beta$ form is the minimal functional unit for binding activity (Fujita-Yamaguchi & Harmon, 1988), but the free $\alpha\beta$ form does not appear to have significant kinase activity unless this form is associated with another $\alpha\beta$ half in an $(\alpha\beta)(\alpha\beta)$ form which is a much more active kinase than the $(\alpha_2\beta_2)$ form.

Receptor aggregation has been proposed as an important step for transmembrane signaling in various systems. Early studies include cytochemical observations such as microaggregation and patching of the receptor molecules induced by ligands or receptor antibodies (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978; Schecter et al., 1979). Anti-receptor antibodies bind specifically to the receptor and mimic biological effects of the hormone (Kahn et al., 1978; Schreiber et al., 1981). Monovalent (Fab') antibody lacks an ability to induce the biological responses while cross-linking of anti-receptor Fab's with anti-IgG restores its ability (Kahn et al., 1981; Schreiber et al., 1983). In extending this observation, O'Brien et al. (1987) reported monoclonal antibodies to the insulin receptor which stimulate the kinase activity by cross-linking receptor molecules. They observed, by applying sucrose gradient centrifugation, the formation of antibody–receptor complexes due to both intramolecular cross-linking and aggregates cross-linked intermolecularly although they suggested that kinase activation primarily depends on the intramolecular cross-linking.

The larger forms with kinase activity identified by our present studies have not been previously detected by using other biochemical methods. It is the radiation inactivation technique that first indicated the presence of receptor aggregates with extremely high kinase activity (Fujita-Yamaguchi et al., 1985). As described under Results, size fractionation of the receptor forms induced dilution of the receptor concentration which most likely resulted in dissociation of the receptor aggregates so that high kinase activity expected at the void volume of the Sepharose column was hardly measurable. In fact, we have observed good kinase activity at the void volume in one such experiment, but when fractions were assayed again, this activity peak was reduced to nearly zero. In order to overcome the dilution problem, the receptors which had first been phosphorylated were fractionated. We identified the major radioactive peak at the void volume as the aggregate form for two reasons: (i) its molecular size and (ii) its clear dissociation from major insulin binding peaks which correspond to the $\alpha_2\beta_2$ and $\alpha\beta$ receptors.

Two groups have reported gel filtration chromatographs of ^{32}P-phosphorylated insulin receptors (Pike et al., 1986; Sweet et al., 1986). In one case, the ^{32}P peak eluted at a position between thyroglobulin and ferritin markers (Pike et al., 1986) whereas we observed that the $\alpha_2\beta_2$ receptors (peak I) eluted earlier than the thyroglobulin marker on Sepharose CL-6B chromatography. In the second case, the elution profile of ^{32}P-labeled receptor (Figures 3 and 4; Sweet et al., 1986) is nearly identical with that of ours shown in Figure 5. However, in contrast to our interpretation, they concluded that the major ^{32}P peak corresponds to $\alpha_2\beta_2$ receptors. It was not unreasonable for them to assume that the major peak corresponded to $\alpha_2\beta_2$ receptors since the $\alpha_2\beta_2$ receptor exhibits a modest kinase activity and the idea of receptor aggregates having a higher specific kinase activity is not intuitively obvious.

Autophosphorylated receptor bands that migrate slower than the $\alpha_2\beta_2$ receptor have been seen in SDS–PAGE under nonreducing conditions (Kasuga et al., 1983). However, these bands have been neglected. When we analyzed autophosphorylated receptors eluted at the void volume of the Sepharose column by SDS–PAGE under nonreducing conditions, receptor species with higher molecular weight than the $\alpha_2\beta_2$ form were detected. The evidence suggests that cross-linking of $\alpha_2\beta_2$ receptors by disulfide linkages may partially be involved in the formation of the aggregates. However, it is not possible to describe the exact nature of the receptor aggregates at the present time. Chemical cross-linking of the receptors would be appropriate to generate receptor aggregates and examine kinase activity. Preliminary experiments using disuccinimidyl suberate resulted in an inactivation of the kinase, which suggested that appropriate conditions for cross-linking have to be carefully established.

An unexpectedly large size for the kinase function of the purified insulin receptor remains to be elucidated especially in relation to the physiological role of kinase activation in insulin signal transduction. These studies are currently under investigation.

Added in Proof

After submission of the manuscript, studies relevant to ours, namely, oligomeric forms of insulin receptor with 580- and 1200-kDa (Kubar & Van Obberghen, 1989), were reported.

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Lipoprotein Lipase Gene Expression in THP-1 Cells†

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ABSTRACT: Lipoprotein lipase (LPL) mRNA levels are under the control of signals that activate phospholipase C, resulting in activation of protein kinase C (PKC) and mobilization of intracellular Ca^{2+} in the human monocytic leukemia cell line THP-1. Induction of LPL in THP-1 cells appears to be mediated by PKC since it was affected by both phorbol 12-myristate 13-acetate (PMA) and a diacylglycerol analogue. This induction was blocked by the specific PKC inhibitor H-7. Although Ca^{2+} mobilization by the ionophore A23187 also induced LPL mRNA, the mechanism is most likely independent of activation of the Ca^{2+}/calmodulin protein kinase. Depletion of cells of PKC made them refractory to induction by A23187, suggesting that Ca^{2+} mobilization acts by activating PKC. Addition of cycloheximide (CHX) to undifferentiated THP-1 cells resulted in a transient increase in steady-state mRNA levels (3-fold). Sustained superinduction of LPL mRNA occurred when PMA and CHX were added simultaneously. These results suggest that the level of LPL mRNA is regulated either by a labile regulatory protein, which represses transcription of the LPL gene, or by a protein affecting mRNA stability.

Lipoprotein lipase (LPL)[1] is a glycoprotein that, after secretion, becomes bound to glycosaminoglycans on the luminal surface of capillary endothelial cells (Olivecrona & Bengtsson-Olivecrona, 1987). While attached to the endothelium, LPL binds its cofactor apoprotein CII, which is present on the surface of triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins), and hydrolyses the core triglycerides of these lipoproteins to glycerol and free fatty acids (Olivecrona & Bengtsson-Olivecrona, 1987). By this action LPL plays a major role in the maturation and processing of several different classes of lipoproteins. The free fatty acids generated during the hydrolysis of core triglycerides are utilized for energy by muscle tissues or can be stored in adipose tissue, making LPL a pivotal enzyme for energy utilization and storage (Eckel, 1987). The enzyme is synthesized by a number of different parenchymal cells including adipocytes, skeletal and cardiac muscle cells, and Kupffer's cells (Nilsson-Ehle et al., 1980). Macrophages and several macrophage-like cell lines, which secrete a variety of biologically active products (Nathan, 1987), also secrete LPL (Khoo et al., 1981; Chait et al., 1982; Ma-

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[1] Abbreviations: CHX, cycloheximide; di-C8, 1,2-dioctanoyl-*rac*-glycerol; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; IP_3, inositol 1,4,5-trisphosphate; LPL, lipoprotein lipase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate.

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