# Separation and Characterization of Three Insulin Receptor Species That Differ in Subunit Composition†

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ABSTRACT: Partially purified human placental insulin receptor preparations give rise to three distinct insulin-binding peaks when eluted from a Mono Q high-performance liquid chromatography anion-exchange column. We analyzed the basis for this phenomenon by affinity cross-linking of insulin to each peak, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. We find that the three insulin-binding peaks represent different molecular weight complexes with the following subunit composition: $(\alpha\beta)_2$, $(\alpha\beta)(\alpha\beta')$, and $(\alpha\beta')_2$, where $\beta'$ represents a proteolytically derived fragment of the $\beta$ subunit. This analysis of subunit composition was confirmed by silver staining of affinity-purified insulin receptor following resolution of the forms on a Mono Q column as described previously. We have characterized the three isolated insulin receptor forms with regard to ligand binding by LIGAND and Scatchard analysis. We also measured insulin-stimulatable autophosphorylation and exogenous kinase activity directed toward poly(Glu/Tyr) (4:1). The three forms of the insulin receptor exhibit similar $K_D$'s for insulin binding to the high- and low-affinity sites. The $(\alpha\beta)_2$ and $(\alpha\beta)(\alpha\beta')$ forms of the insulin receptor display superimposable curvilinear Scatchard plots. In contrast, only the intact holoreceptor $(\alpha\beta)_2$ form demonstrates insulin-stimulatable autophosphorylation and exogenous kinase activity. The $(\alpha\beta)(\alpha\beta')$ form has reduced basal kinase activity which was not increased by prior incubation with insulin. The $(\alpha\beta')_2$ form lacks a kinase domain and consequently demonstrated no kinase activity. These results demonstrate that only the intact $(\alpha\beta)_2$ form of the insulin receptor has the ability to undergo insulin-stimulated kinase activity. The conversion of only one $\beta$ subunit to the $\beta'$ subunit is enough to render the receptor incapable of ligand-induced autophosphorylation. However, the data also indicate that loss of one (or both) kinase domain(s) has no dramatic effect on the ability of the receptor's binding sites to interact with insulin.

The mammalian insulin receptor is an integral membrane protein comprised of two subunits ($\alpha$ and $\beta$) that are disulfide linked in a tetrameric complex of the form $(\alpha\beta)_2$. The $\alpha$ subunit ($M_r$ 130000) is thought to contain the insulin-binding site on the basis of chemical cross-linking (Pilch & Czech, 1979, 1980) and photoaffinity labeling (Yip et al., 1978) of $^{125}$I-insulin. The $\beta$ subunit ($M_r$ 90000) has been shown to become phosphorylated in response to insulin (Kasuga et al., 1982) and, by a number of criteria, has been shown to be an insulin-stimulated, tyrosyl-specific protein kinase (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983). The existence of a kinase domain in the $\beta$ subunit of the insulin receptor precursor was verified by cDNA cloning (Ebina et al., 1985; Ullrich et al., 1985). On the basis of the above-referenced data and data from affinity labeling studies of the holoreceptor (Jacobs et al., 1980; Massague et al., 1980; Kasuga et al., 1981) and biosynthetic labeling (Van Obberghan et al., 1981; Hedo et al., 1983; Salzman et al., 1984; Forsayeth et al., 1986), it is widely accepted that the biologically functional form of the insulin receptor residing in the plasma membrane is the disulfide-linked tetramer $(\alpha\beta)_2$ (Czech et al., 1981).

However, several investigators have demonstrated the existence of multiple insulin receptor species that differ in molecular weight when analyzed under conditions where internal disulfide bonds are intact (Massague & Czech, 1980, 1981; Yip & Moule, 1983). The apparent discrepancies in the reported molecular weight(s) of the insulin receptor have been attributed to proteolysis of the $\beta$ subunit (Massague et al., 1981; Fujita-Yamaguchi, 1984) and/or artifacts in the preparation of samples for electrophoresis (Boyle et al., 1985). Three high molecular weight forms of the insulin receptor have been observed in membranes derived from rat liver, adipocytes, kidney, and lung, and from human placenta (Massague et al., 1980). These three forms presumably correspond to intact receptor and proteolytically degraded forms where one or both $\beta$ subunits are affected to generate $(\alpha\beta)(\alpha\beta')$ and $(\alpha\beta')_2$, respectively. All three forms copurify on insulin–agarose columns, as evidence by silver staining (Fujita-Yamaguchi et al., 1986).[1]

It is not completely clear to what extent the formation of the $(\alpha\beta)(\alpha\beta')$ and $(\alpha\beta')_2$ forms occurs in vivo, and/or occurs during the purification process. However, the existence of multiple receptor forms is potentially complicating for the interpretation of functional studies of the insulin receptor in vitro and in vivo. It has been indirectly shown that the three high molecular weight forms of the insulin receptor have a grossly similar affinity for insulin (Lerea & Livingston 1983). With regard to the kinase function, it has been shown that proteolytic treatment of receptor with collagenase (Roth et al., 1983) or elastase (Shia et al., 1983) abolishes kinase activity without affecting the ability to cross-link $^{125}$I-insulin. The elastase digests give rise to receptor forms resembling those isolated from cells as described above (Pilch et al., 1981). Recently, domain-specific antibodies were used to probe affinity-purified insulin receptor (Kathuria et al., 1986). These data indicate that the cleavage site responsible for formation

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[1] T. O'Hare and S. M. Waugh, unpublished results.

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of the $\beta'$ subunit is located in the intracellular domain of the $\beta$ subunit, close to the putative transmembrane region.

To resolve some of the uncertainties of the previously reported studies employing mixtures of receptor forms, we isolated each of the three high molecular weight forms of the insulin receptor from human placenta, and we determined their functional properties with regard to ligand binding and ligand-activated kinase activity.

## Materials and Methods

*Materials.* Crystalline porcine insulin was a gift from Dr. R. E. Chance of Eli Lily Corp. and was stored at 4 °C as a stock solution in dilute HCl (pH 3.0) at 2 mg/mL. $Na^{125}I$ from Amersham was used to iodinate insulin using lactoperoxidase in 6 M urea (Gliemann et al., 1985). [A14-Tyr-$^{125}$I]-, [B16-Tyr-$^{125}$I]-, and [B26-Tyr-$^{125}$I]monoiodoinsulins were obtained following isocratic elution of isomers from a Vydac C4 column (25 × 0.46 cm) with triethylammonium formate buffer, pH 6.0, containing 27.2% acetonitrile (Welinder et al., 1984). Gamma-prep from Promega Biotech was used to convert [$^{32}$P]orthophosphate (New England Nuclear) to [$\gamma$-$^{32}$P]ATP. Disuccinimidyl suberate (DSS)[2] and bicinchoninic acid protein assay reagent were purchased from Pierce. Electrophoresis reagents including molecular weight standards were from Bio-Rad. HEPES and dithiothreitol were from United States Biochemical Corp. Triton X-100 and Sephacryl-S400 were from Fluka and Pharmacia, respectively. Pepstatin A and leupeptin were obtained from Vega. DEAE-Trisacryl was purchased from LKB. Insulin affinity columns were prepared using Affi-Gel 10 (Bio-Rad) according to the manufacturer as described in detail (Pilch, 1985). WGA–agarose was bought from EY-Labs.

*Insulin Receptor Preparations.* Full-term human placentas, obtained <1-h postdelivery, were placed in ice-cold phosphate-buffered saline (PBS). After the umbilicus, chorion, and amnion were removed, the placenta was cut into pieces and homogenized in four to five batches with a Tekmar Tissuemizer in homogenization buffer (30 mM HEPES, 0.25 M sucrose, 25 mM benzamidine, 10 $\mu$M leupeptin, 50 mTi units/mL aprotinin, 1 $\mu$M pepstatin A, and 1 mM 1,10-phenanthroline) at an approximate 1:4 (w/v) ratio of placenta to buffer. Each round of homogenization was immediately preceded by the addition of freshly prepared 1 M PMSF in dimethyl sulfoxide (to a final dilution of 1:1000). Placental microsomes were prepared by differential centrifugation (Harrison & Itin, 1980; Pilch, 1985), except that the membrane pellet obtained from the first 45000$g$ spin was rinsed 3 times in 30 mM HEPES (pH 7.6). Placental microsomes (10–20 mg/mL) were solubilized with 1% Triton X-100 at 4 °C, for 60 min in the presence of 1 mM PMSF. The supernatant, following ultracentrifugation at 100000$g$ for 50 min (4 °C), was applied to a Sephacryl S400 gel filtration column (5 × 60 cm), preequilibrated with 30 mM HEPES, 0.1% Triton X-100, and 0.02% $NaN_3$ (pH 7.6), and eluted at a flow rate of 70 mL/h. Those fractions which contain high insulin-binding activity and relatively low protein content were pooled and used as a starting material for anion-exchange chromatography, either on the Mono Q column or on a 1.0-mL DEAE-Trisacryl column. In addition, the pooled insulin-binding activity from the S400 column was applied to an insulin Affi-Gel 10 column (Pilch, 1985) and eluted as described (Fujita-Yamaguchi, 1984) to prepare homogeneous insulin receptor (Figure 3).

*Anion-Exchange Chromatography on Mono Q and DEAE-Trisacryl.* A Pharmacia 1.0-mL Mono Q HR 5/5 prepacked column (50 × 5 mm i.d.) was run on a Beckman HPLC system. Typically, 10 mL of the pooled fractions from the S400 column (3–5 mg of total protein) was applied at 1.0 mL/min and room temperature, in buffer A (20 mM Tris, pH 7.8, 0.1% Triton X-100, and 0.02% $NaN_3$). After application of the sample and washing the column with buffer A (20 mL total), a linear gradient was established from 0% to 45% buffer B (buffer A with 0.5 M $Na_2SO_4$), over a 30-min period. Fractions of 0.5–0.6 mL were collected, and insulin-binding peaks were pooled and dialyzed overnight against 30 mM HEPES (pH 7.6), 0.1% Triton X-100, and 0.02% $NaN_3$ (1:1000, two changes).

For the purpose of comparing the properties of the individual insulin receptor forms to a preparation containing all three forms, we ran 10 mL of the same preparation of S400 insulin-binding fractions over a conventional 1.0-mL DEAE anion-exchange column. The insulin receptor eluted as a single peak (Jacobs et al., 1977; Harrison & Itin, 1980) upon elution with a linear gradient of NaCl (0–0.45 M). The insulin-binding peak was pooled and dialyzed as described previously.

*Cross-Linking of $^{125}$I-Insulin.* Chemical cross-linking with DSS was performed similarly to that described by Pilch and Czech (1980). Insulin receptor preparations were incubated with [B26-$^{125}$I]- or [A14-$^{125}$I]monoiodoinsulin (1–2 nM) for 30 min at room temperature and then for 30 min on ice. DSS (10 mM freshly made in DMSO) was added to a final concentration of 0.2–0.25 mM and incubated with receptor for 15 min, or as indicated. The reaction was terminated by the addition of electrophoresis sample buffer.

*Insulin-Binding Assay and Scatchard Analysis.* Routine insulin-binding assays were performed with [B16-$^{125}$I]monoiodoinsulin (50–100 pM) in a final volume of 200 $\mu$L in 30 mM HEPES (pH 7.6) and 1 mg/mL BSA. After a 30-min incubation at room temperature, bound hormone was separated from free ligand by using the poly(ethylene glycol) precipitation assay (Hollenberg & Cuatrecasas, 1976). The precipitated $^{125}$I-insulin–receptor complex was pelleted for 10 min in a microcentrifuge. The supernatant was aspirated, and the pellets were counted in an LKB $\gamma$-counter. Nonspecific binding was determined in the presence of 5 $\mu$M unlabeled insulin.

Insulin binding for Scatchard plots and LIGAND analysis was performed with [A14-$^{125}$I]monoiodoinsulin in a competition assay, where a fixed amount of $^{125}$I-insulin is incubated with an insulin receptor preparation in the presence of increasing amounts of unlabeled insulin. The insulin receptor was incubated with labeled insulin, with or without unlabeled insulin, in triplicate, for 16–18 h at 4 °C, conditions in which steady state is reached and the dissociation rate is negligible (Haynes et al., 1986). Sequential addition of $\gamma$-globulin and poly(ethylene glycol), followed by centrifugation and aspiration, was carried out as described. The data were analyzed with the computer program LIGAND (Munson & Rodbard, 1980). Nonspecific binding was not subtracted and was used as a fitted parameter. Plots of bound/free versus bound were drawn according to Scatchard (1949).

*Electrophoresis and Autoradiography.* We carried out denaturing (SDS)–polyacrylamide gel electrophoresis using

[2] Abbreviations: BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DSS, disuccinimidyl suberate; bis(acrylamide), *N*,*N*′-methylenebis(acrylamide); WGA, wheat germ agglutinin; Ti, trypsin inhibitor.

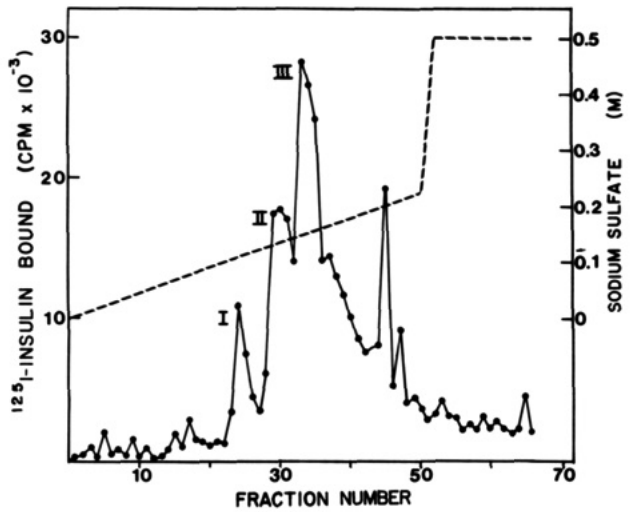


FIGURE 1: Insulin-binding profile of Mono Q column. Aliquots (20 μL) from all 0.5-mL fractions were subjected to a standard insulin-binding assay described under Materials and Methods. Insulin-binding activity (—) was eluted from the column in three distinct peaks (labeled I, II, and III) by a linear gradient of sodium sulfate (- - -).

a discontinuous buffer system (Laemmli, 1970). In order to effect separation of the high molecular weight insulin receptor complexes under nonreducing conditions, we ran samples on a 3–10% gradient gel (1.5 mm) using an acrylamide:bis-(acrylamide) ratio of 100:1; otherwise, the ratio was 37.5:1. All samples, unless running the gel under reducing conditions, were treated with 20 mM *N*-ethylmaleimide prior to addition of sample buffer. Autoradiography was performed at −70 °C using Kodak X-OMAT AR film and a Du Pont Cronex Lightning plus enhancing screen. The molecular weight markers used were myosin ($M_r$ 200K), β-galactosidase ($M_r$ 116K), phosphorylase *b* ($M_r$ 92.5K), BSA ($M_r$ 66K), and ovalbumin ($M_r$ 45K). Silver staining was performed as described by Oakley et al. (1980), a modification of the procedure by Switzer et al. (1979).

*Autophosphorylation and Exogenous Kinase Activity.* Insulin receptor autophosphorylation was determined by incubation of insulin receptor preparations with or without 5 μM insulin for 30 min at room temperature, in the presence of the divalent cations $Mg^{2+}$ (10 mM) and $Mn^{2+}$ (4 mM). The reaction was initiated by the addition of [γ-$^{32}$P]ATP (45 μM final concentration) and terminated by the addition of electrophoresis sample buffer. Electrophoresis under reducing conditions was then performed, and the appropriate bands of radioactivity were cut out and counted in an LKB liquid scintillation counter and read as Cerenkov radiation on the tritium channel. Exogenous kinase activity was examined with poly(Glu/Tyr)(4:1) as a phosphate acceptor. Insulin receptor was preincubated with or without insulin as above followed by a 20-min incubation with ATP (at room temperature) in order to fully activate the receptor toward reporter substrates (Rosen et al., 1983). Poly(Glu/Tyr)(4:1) was then added at a final concentration of 0.2 mg/mL. The reaction was stopped by spotting aliquots on 3 × 3 cm 3MM filter paper and immediate immersion in ice-cold 10% tricarboxylic acid in 10 mM sodium pyrophosphate. After several washes, the filter papers were counted for $^{32}$P as described.

## RESULTS

*Isolation of Insulin Receptor Forms by HPLC.* Pooled insulin-binding activity from the Sephacryl S400 column was adsorbed onto a 1.0-mL Pharmacia Mono Q anion-exchange column and eluted with a linear sodium sulfate gradient as

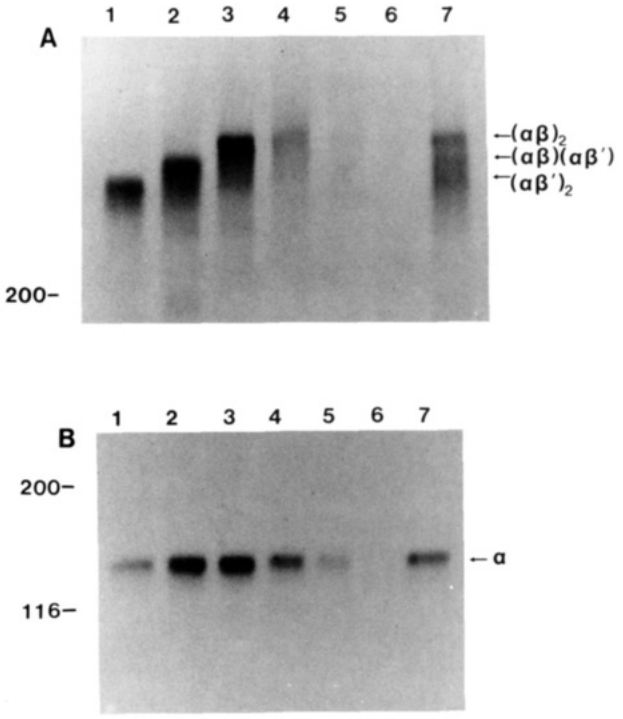


FIGURE 2: Autoradiogram of electrophoresed insulin receptor cross-linked to $^{125}$I-insulin. Aliquots from the different pooled peaks of Figure 1 were cross-linked either to [A14-$^{125}$I]monoiodoinsulin and electrophoresed on a nonreducing gel (A) or to [B26-$^{125}$I]monoiodoinsulin and electrophoresed on a reducing gel (B). In order to separate the high molecular weight insulin receptor forms (A), a 3–10% gel with a 100:1 ratio of acrylamide to bis(acrylamide) was used. DSS at 0.25 mM was used for cross-linking of ligand–receptor complexes at 0 °C for 15 min. The position of molecular weight markers is shown to the left.

depicted in Figure 1. Three major peaks of insulin binding can be observed eluting from the Mono Q column in contrast to the single peak of binding seen upon conventional anion exchange over DEAE (Jacobs et al., 1977). We have performed similar binding assays on 15 separate Mono Q runs employing 5 placental preparations. We reproducibly find three insulin-binding peaks and a shoulder to the right of peak III (fractions 37–41) as shown in Figure 1. However, the relative proportions of the three peaks vary according to the placental preparation and within preparations, depending on the age. With time, peaks 1 and 2 increase as peak 3 decreases. The single-fraction peaks (45, 47, and 60) shown in Figure 1, and observed in random fractions in some of our HPLC runs, are an artifactual result of errors from single-point binding determinations. No ligand–receptor complexes can be demonstrated by cross-linking of these fractions (see below). Note that the same placental preparation used in Figure 1 was also used for the experiment shown in Figure 2.

*Affinity Cross-Linking.* In order to determine the reason for multiple insulin-binding peaks eluting from the Mono Q column, we employed affinity cross-linking of $^{125}$I-insulin to the pooled and dialyzed peaks. In Figure 2A, an autoradiogram of a nonreducing SDS–polyacrylamide gel is shown in which the pooled peaks were cross-linked to $^{125}$I-insulin and electrophoresed. Lanes 1–3 correspond to peaks I–III of Figure 1, and lane 7 contains aliquots from peaks 1–3 added together prior to cross-linking. Three distinct bands can be seen (Figure 2A, lanes 1–3) in which each peak (I–III) represents a distinct insulin receptor complex. On the basis of the previous data already referenced, we believe the insulin receptor forms that have been cross-linked to $^{125}$I-insulin are as follows: $(\alpha\beta)_2$, peak III with $M_r$(app) 350 000; $(\alpha\beta)(\alpha\beta')$,

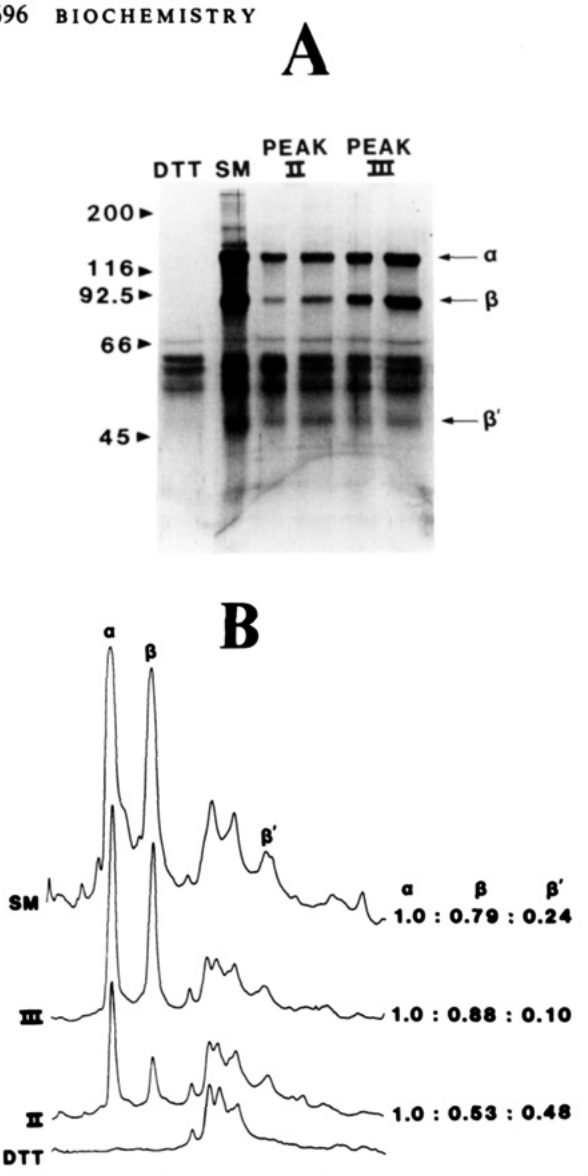


FIGURE 3: Silver staining (A) and densitometry (B) of affinity-purified insulin receptor separated by anion-exchange chromatography. One placenta was used to affinity purify insulin receptor by sequential chromatography over one Sephacryl S-400 column, two (10 × 1.6 cm) insulin–agarose columns, and one 0.5-mL WGA–agarose column. All of the eluate from the WGA–agarose column was used as a starting material (SM) for the anion-exchange column. Portions of the pooled peaks II (peak II) and III (peak III) were electrophoresed on a 3–10% gradient gel, as was the reducing sample buffer alone (DTT). Two lanes each of peaks II and III were run on SDS–PAGE corresponding to approximately 2 and 4 μg of protein, respectively, to ensure that densitometry could be performed in the linear absorbance range. Anion-exchange chromatography, electrophoresis, and silver staining were carried out as described under Materials and Methods. To determine the subunit ratios in (B), the α-subunit peak area was assigned a value of 1.00, and the areas of the other peaks were divided by the area of the α subunit to give the values indicated.

peak II with $M_r$(app) 320 000; and $(\alpha\beta')_2$, peak I with $M_r$(app) 290 000. The labeled bands for peaks II and III (lanes 2 and 3, Figure 2A) trail somewhat, probably due to the presence of small amounts of the lower molecular weight forms. The shoulder to the right of peak III contains an insulin receptor form similar to that of peak III (lane 4, Figure 2A,B). Fractions 45 and 47 (combined prior to cross-linking in lane 5) and 60 (lane 6), that show high insulin-binding capacity in a single-point assay, contain little receptor that can be cross-linked to $^{125}$I-insulin (Figure 2A,B) and are thus artifacts of the binding assay (see above). The small amount of α subunit labeled in lane 5 of Figure 2B (combined fractions 45 and 47) probably is the result of trailing peak III from the Mono Q column (Figure 1).

Table I: Scatchard Analysis of Mono Q Peaks[a]

| preparation | high-affinity site $K_1$ (nm) | high-affinity site $R_1$ (pmol/mg) | low-affinity site $K_2$ (nm) | low-affinity site $R_2$ (pmol/mg) | mean of squares[b] |
|---|---|---|---|---|---|
| peak I | 0.074 ± 0.02[c] | 1.3 ± 0.2 | 22 ± 10 | 23 ± 3 | 3.7 |
| peak II | 0.16 ± 0.02 | 9.3 ± 0.9 | 8.4 ± 3 | 26 ± 7 | 3.5 |
| peak III | 0.12 ± 0.04 | 21.2 ± 5 | 2.5 ± 1.5 | 72 ± 20 | 3.4 |
| DEAE | 0.071 ± 0.02 | 6.7 ± 1.4 | 2.4 ± 0.8 | 33 ± 5 | 8.1 |

[a] Insulin binding was performed as described under Materials and Methods, and the data were analyzed according to Munson and Rodbard (1980). $K_1$ and $K_2$ refer to the dissociation constants of insulin for the high- and low-affinity sites, respectively. $R_1$ and $R_2$ refer to $B_{max}$ (total binding capacity) of the high- and low-affinity sites, respectively. [b] These values for the mean of squares indicate a good fit of the data to a two-site model (Munson & Rodbard, 1980). [c] These values are the approximate standard error as determined by LIGAND and do not reflect the error involved for several competition experiments on a single preparation.

Figure 2B shows cross-linking of [B26-$^{125}$I]monoiodoinsulin to the receptor, followed by electrophoresis under reducing conditions, which results in only the α subunit being visible. There is no difference in α-subunit migration from any fraction, suggesting that this molecular weight differences seen in Figure 2A are a result of differences in β-subunit size. It seems likely that the three high molecular weight insulin receptor forms in Figure 2A correspond to those previously identified [for a review, see Czech (1985)].

*Anion-Exchange HPLC of Affinity-Purified Insulin Receptor.* In order to directly visualize the β subunit of the different insulin receptor forms, we chromatographed an affinity-purified insulin receptor preparation on the Mono Q column. The resultant chromatogram was qualitatively identical with Figure 1 and displayed three distinct insulin-binding peaks (data not shown) which were pooled and dialyzed as before. We subjected the applied receptor and pooled peaks II and III to SDS–PAGE under fully reducing conditions, and we silver stained the gel as shown in Figure 3A. The β subunit migrates to the same position for the starting material (SM) applied to the Mono Q column and for peaks III and II representing $\alpha_2\beta_2$ and $(\alpha\beta)(\alpha\beta')$, respectively. Thus, these data indicate that the β subunit of the insulin receptor form $(\alpha\beta)(\alpha\beta')$ does not contain a small clip in its carboxyl terminus that might render it kinase inactive (see below, Figure 5, and Discussion). The starting material is >90% pure insulin receptor if the staining due to DTT and sample buffer is subtracted (Figure 3A,B). We subjected the stained gels in Figure 3A to densitometric scanning (Figure 3B), and we found that the amount of β subunit was, as expected, reduced by half when comparing $(\alpha\beta)(\alpha\beta')$ to holoreceptor. This decrease was concomitant with a roughly equivalent increase in β' in the partially proteolyzed receptor form (ratios in Figure 3B).

*Scatchard Analysis.* The three forms of the nonreduced insulin receptor differ only in the overall structure of the β subunit. The possibility exists, however, that this cleavage can affect insulin binding, indirectly, by altering the way in which two αβ receptor halves interact. It has previously been determined that this interaction is necessary for high-affinity ligand binding (Böni-Schnetzler et al., 1987; Sweet et al., 1987). Therefore, we analyzed the affinity of each form of the receptor for insulin by LIGAND and Scatchard analysis of a competition protocol employing [A14-$^{125}$I]monoiodoinsulin and unlabeled insulin (see Materials and Methods). The data were then analyzed by the computer program LIGAND (Munson & Rodbard, 1980), a nonlinear model/curve-fitting program.

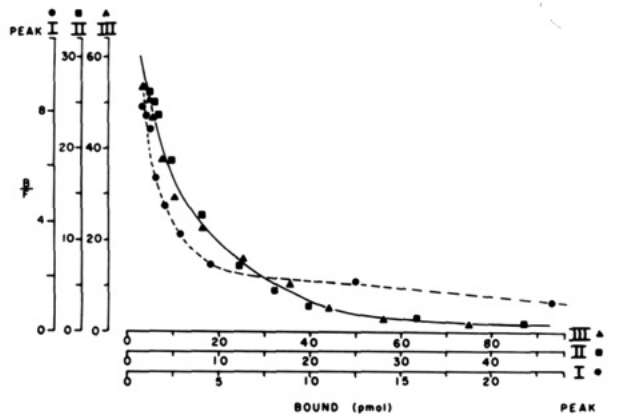


FIGURE 4: Scatchard plots for $^{125}$I-insulin binding to peaks I, II, and III from the Mono Q column. The pooled and dialyzed peaks I (●), II (■), and III (▲) from the Mono Q column were incubated with 52 pM $^{125}$I-insulin (specific activity 3.5 × $10^6$ dpm/pmol) with or without increasing concentrations of unlabeled insulin (0–700 pmol). Estimates of parameter values were determined by the program EBDA (McPherson, 1985) using dpm associated with the pellet in the presence of 700 pmol of unlabeled insulin as nonspecific binding. The range of values for $B/F$ (×$10^{-2}$) and "Bound" is different for each peak in order to directly compare the three curves.

The calculated final parameters of these pooled peaks are shown in Table I. The high-affinity binding site ($K_1$) is essentially indistinguishable for the different high molecular weight insulin receptor complexes (peaks I–III) and for the DEAE preparation. The values for the low-affinity site ($K_2$) vary to a greater degree; however, the approximate standard error is also greater. These data are similar to insulin-binding parameters of $^{125}$I-insulin binding to affinity-purified insulin receptor (Pessin et al., 1987) as analyzed by LIGAND, except that $R_1$ and $R_2$ are smaller (Table I) because these peaks are relatively impure, as evidenced by silver staining (data not shown). Scatchard plots of peaks I, II, and III are shown in Figure 4. The Scatchard plot for each peak is drawn with different scales (see figure legend) in order to directly compare the overall shape of each curve. Peaks II and III display Scatchard plots indistinguishable from each other, whereas the Scatchard plot for peak I is slightly different. It is clear that peak II, peak III, and the DEAE eluate display identical binding isotherms within the error of the analysis (Figure 4, Table I, and data not shown). However, the binding data for peak I deviate from the other insulin receptor preparations in that there is a greater percentage of low-affinity binding sites (see Discussion). A similar conclusion was drawn from data for the competition of $^{125}$I-insulin cross-linking to the three insulin receptor forms by unlabeled insulin, in that the three receptor forms exhibited similar dissociation constants (Lerea & Livingston, 1983). However, the latter results were qualitative in that cross-linking only detects a small portion of total ligand–receptor complexes (Pilch & Czech, 1980).[3]

*Kinase Activity.* There have been several studies that have correlated the integrity of the $\beta$ subunit with the retention of autophosphorylation and/or exogenous kinase activity (Roth et al., 1983; Shia et al., 1983; Kathuria et al., 1986). The authors of these studies concluded that the $(\alpha\beta')_2$ form was unable to phosphorylate exogenous substrates, but the techniques used precluded precise conclusions about the kinase competence of the $(\alpha\beta)(\alpha\beta')$ form. This latter form contains one intact kinase domain and could therefore account for at least some degree of basal kinase activity, if not ligand-stimulated activity. Separation of each insulin receptor complex

[3] S. W. Waugh, E. Dibella, and P. F. Pilch, submitted for publication.

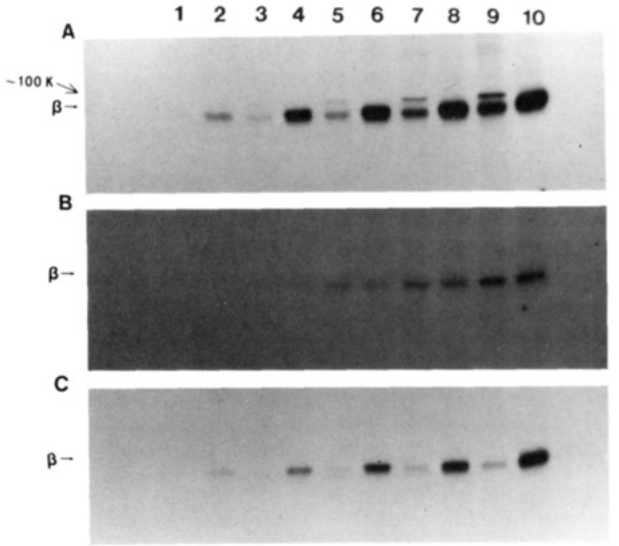


FIGURE 5: Time course of basal and insulin-stimulated autophosphorylation. The same amounts of insulin-binding capacity, as determined by Scatchard analysis (0.15 pmol), from peak III (A), peak II (B), and the pooled DEAE preparation (C) were incubated in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 3.5 × $10^{-7}$ M insulin for 30 min at room temperature. Autophosphorylation was initiated by adding [$\gamma$-$^{32}$P]ATP (3.75 dpm/fmol) to 45 $\mu$M, and the reaction was terminated by removing aliquots at the indicated times and adding them to sample buffer. For (A) and (C), the reaction was stopped after 15 s (lanes 1 and 2), 45 s (lanes 3 and 4), 90 s (lanes 5 and 6), 3 min (lanes 7 and 8), and 6 min (lanes 9 and 10). In (B) (peak II), the reaction was stopped after 0.5, 1, 4, 10, and 20 min (lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10, respectively). The samples were electrophoresed on a 7.5% gel and processed as described under Materials and Methods.

thus allows the direct determination of the intrinsic kinase activity of each form.

Figure 5 shows autophosphorylation activity ($^{32}$P incorporation into the $\beta$ subunit) of the $(\alpha\beta)_2$ form (Figure 5A), the $(\alpha\beta)(\alpha\beta')$ form (Figure 5B), and a DEAE preparation (Figure 5C). Basal autophosphorylation activity (lanes 1, 3, 5, 7, and 9) and insulin-stimulatable autophosphorylation activity (lanes 2, 4, 6, 8, and 10) were examined. A longer time course was used for Figure 5B as described in the figure legend. Since all three insulin-binding peaks from the Mono Q column and the peak from the DEAE column displayed essentially identical $K_D$ values for high- and low-affinity insulin-binding sites (Table I), we could compare the intrinsic kinase activity of each by adding equal amounts of insulin-binding capacity. We find that the $(\alpha\beta)_2$ form displays a substantial basal autophosphorylation rate and can be stimulated by insulin (≥3-fold) at all time points examined (compare lanes 1 and 2, 3 and 4, etc.; Figure 5A). In contrast, the $(\alpha\beta)(\alpha\beta')$ form of the insulin receptor exhibited a reduced basal kinase rate that was completely unresponsive to prior incubation with insulin, even when longer time points were examined [compare lanes 9 and 10 in Figure 5A (6-min time point) with lanes 9 and 10 in Figure 5B (20-min time point)]. The ability of the insulin receptor to respond to insulin with an increase in the rate of autophosphorylation is lost when one of the $\beta$ subunits is converted to the $\beta'$ form. In addition, this results in a decrease (5–10-fold) in the rate of basal autophosphorylation. As expected, the $(\alpha\beta')_2$ form, when assayed for autophosphorylation activity at the same time points as in Figure 5B, contained no detectable $^{32}$P at a position corresponding to the $\beta$ subunit (data not shown). We also compared the autophosphorylation activity of the $(\alpha\beta)_2$ form to a typical insulin receptor preparation from an anion-exchange column (DEAE) which contains all three high molecular weight insulin

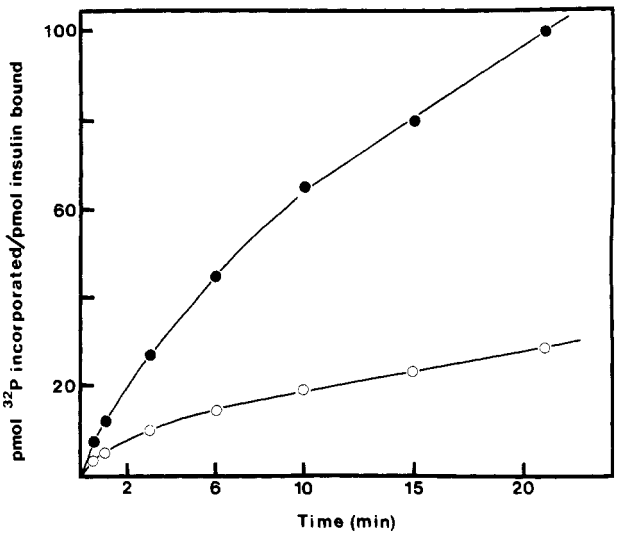


FIGURE 6: Peak III exogenous kinase activity. An aliquot of peak III (0.1 pmol of insulin-binding capacity) was used to assess exogenous kinase activity using poly(Glu/Tyr)(4:1) as a substrate. The insulin receptor preparation was allowed to autophosphorylate for 20 min prior to the addition of substrate. $^{32}$P incorporation into the substrate per picomole of insulin-binding activity is plotted on the ordinate, and time is plotted on the abscissa. The autophosphorylation and substrate phosphorylation were carried out in the absence (open circles) or presence (closed circles) of 3 nM insulin.

receptor complexes (Figure 5C, same time points as Figure 5A). Both basal and insulin-stimulatable kinase activities were lower in the DEAE preparation as compared to isolated holoreceptor (compare Figure 5A and Figure 5C), yet insulin was able to stimulate an identical fold increase in the rate of autophosphorylation as compared to isolated holoreceptor. These results are consistent with our observations that only the $(\alpha\beta)_2$ form is kinase active, and it is the only form responsible for the ligand-dependent kinase activity observed in the DEAE preparation. The same conclusion was also derived by Pang et al. (1984) based on the absence of $^{32}$P-labeled $(\alpha\beta)(\alpha\beta')$ or $(\alpha\beta')_2$ on nonreducing gels following autophosphorylation.

Note that there is a $^{32}$P-labeled protein migrating slower than the $\beta$ subunit (Figure 5, 100K) that is labeled in the absence of insulin, yet in the presence of insulin, it shows a reduced amount of $^{32}$P incorporation. A protein that migrates at 100K is also present primarily in peak III that is easily seen upon silver staining (data not shown). We have not identified this protein nor have we yet determined what type of phosphoamino acid this modification represents. However, in view of the importance of insulin-dependent dephosphorylation reactions in mediating insulin action, this is a potentially interesting observation.

We also compared basal and insulin-stimulated exogenous kinase activity using the synthetic copolymer Glu/Tyr (4:1). Under the conditions of Figure 6, we found that only the $(\alpha\beta)_2$ form had detectable basal exogenous kinase activity (open circles) and could be stimulated by insulin (closed circles). As expected, the $(\alpha\beta)(\alpha\beta')$ form displayed reduced basal kinase activity that was unresponsive to insulin, and the $(\alpha\beta')_2$ form was basal kinase inactive.

## Discussion

We have developed a novel procedure utilizing anion-exchange HPLC to separate the three high molecular weight insulin receptor complexes (Figure 1) that have been shown to exist in membranes isolated from several mammalian tissues (Massague et al., 1980). Our data together with the previous literature (Kathuria et al., 1986) are consistent with the notion that these forms represent, respectively, intact receptor and a deletion of most of the cytoplasmic portion of one and both $\beta$ subunits (Figures 2 and 3). All three forms show similar $K_D$ values for high- and low-affinity binding sites for insulin (Figure 4 and Table I), yet only the $(\alpha\beta)_2$ form can be stimulated by insulin to increase both autophosphorylation and exogenous kinase activity (Figures 5 and 6). We draw the following conclusions from these data. First, there appears to be little communication from the cytoplasmic portion of the $\beta$ subunit to the insulin-binding domain of the $\alpha$ subunit, since the affinity of the insulin-binding region of the $\alpha$ subunit is unaffected by the cleavage of a substantial portion of the cytoplasmic domain of the $\beta$ subunit (Table I). This notion is also supported by data which show that autophosphorylation of the $\beta$ subunit, which increases exogenous kinase activity, does not change the affinity of the insulin receptor for insulin (Rosen et al., 1983). The converse is not true because the interaction of insulin with the extracellular insulin-binding domain of the $\alpha$ subunit is responsible for increasing the rate of autophosphorylation of the intracellular kinase domain of the $\beta$ subunit [for a review, see Gammeltoft and Van Obberghen (1986)].

Peak I has a lower percentage of high-affinity binding sites compared to low-affinity binding sites (Table I) and visualized in Figure 4. This may be due to an artifact of the binding experiment such as an underestimation of the nonspecific component of total binding, which would lead to an overestimation of the number of low-affinity binding sites. However, it may be that cleavage of both $\beta$ subunits to the $\beta'$ form of the tetrameric receptor alters the ability of the $\alpha$ subunit to bind insulin: cleavage of only one $\beta$ subunit apparently has no effect (Table I and Figure 4). In order to compare the intrinsic kinase activity of the different receptor preparations, they were normalized for insulin-binding capacity. This is straightforward for peak II, peak III, and the eluate for the DEAE column, since they display almost identical binding data. The analysis of the binding data of peak I indicates that this receptor form behaves similar to the receptor forms of peaks II and III, except for the lower percentage of high-affinity binding sites. Regardless, the $(\alpha\beta')_2$ form of the insulin receptor is not expected to be kinase competent on the basis of the sensitivity of the $\beta$ subunit to proteolysis (Roth et al., 1983; Shia et al., 1983; Kathuria et al., 1986) and the fact that deletion mutants similar in structure to this form are kinase inactive (Ellis et al., 1986, 1987; Hari & Roth, 1987). Our main goal is to compare the functional properties of the insulin receptor forms contained within peaks II and III.

Our second conclusion relates to possible mechanisms by which insulin activates the receptor's kinase function. Only the holoreceptor is active in this regard and not the $(\alpha\beta)(\alpha\beta')$ form that retains one kinase domain (Figures 5 and 6). Therefore, it is likely that both $\beta$ subunits play a role in the ability of the holoreceptor to respond to insulin with an increase in the rate and extent of autophosphorylation. A similar conclusion was reached by Böni-Schnetzler et al. (1986) employing different experimental methodology; that is, the involvement of both $\alpha\beta$ halves seems to be necessary for insulin-stimulated kinase activity. However, these data do not address a possible mechanism by which the interaction of $\alpha\beta$ halves results in their ability to undergo phosphorylation reactions. The basal and insulin-stimulated autophosphorylation may involve the transfer of phosphate from ATP to one $\alpha\beta$ dimer catalyzed by the adjacent $\alpha\beta$ dimer. Alternatively, the interaction of $\alpha\beta$ halves (more specifically, $\beta$–$\beta$ interaction)

may involve conformational changes which alter the enzymatic properties of the kinase domain of one or both $\beta$ subunits. Data have been obtained showing that a monomeric insulin receptor derivative consisting of the intact $\beta$ subunit plus a 20K fragment of the $\alpha$ subunit can exhibit autophosphorylation and exogenous kinase activity identical with that of insulin-stimulated holoreceptor.[4]

Interestingly, it has been reported that the conversion of the intact $\beta$ subunit to a 85- or 70-kDa fragment, by cleavage at the C-terminus, does not result in loss of kinase competence (Goren et al., 1987). However, Kathuria et al. (1986) reported that when the $\beta$ subunit is converted to a 88-kDa form, by cleavage at the C-terminus, it is inactive as a kinase. Regardless of this apparent conflict, we determined that the remaining $\beta$ subunit of the $(\alpha\beta)(\alpha\beta')$, kinase-inactive insulin receptor form is indistinguishable from the $\beta$ subunit in the kinase-active holoreceptor (Figure 3). We conclude that the inability of the $(\alpha\beta)(\alpha\beta')$ insulin receptor form to respond to insulin by increasing the rate of autophosphorylation is not due to minor cleavage(s) of the intact $\beta$ subunit.

Since only one of the three insulin receptor forms is kinase active, yet all can bind insulin with similar affinity, data based on insulin-stimulated autophosphorylation or exogenous kinase activity normalized to insulin-binding capacity may be compromised. A similar conclusion was reached by Grunfeld et al. (1987) in that degradation of the $\beta$ subunit must be accounted for in studies examining kinase activity of the insulin receptor. Previous studies addressing intrinsic kinase activity of the insulin receptor normalized to insulin-binding activity (Freidenberg et al., 1985; Arsenis & Livingston, 1986; James et al., 1986) relied on cross-linking of $^{125}$I-insulin to examine the structural integrity of the different receptor preparations. The ability to isolate intact receptor by HPLC will allow a precise quantitation of the intrinsic kinase activity of receptors from different sources. For example, we have applied this technique to receptors from rat liver and have isolated the holoreceptor form (data not shown). Thus, it is likely that HPLC separation of insulin receptor forms could be used to compare intrinsic kinase activity of the holoreceptor isolated from different tissues, from different species, or from the same tissue treated with various pharmacological agents. Finally, the determination of precise kinetic constants for the various parameters of the insulin receptor kinase activity (relative to binding) obviously requires an intact holoreceptor, and our procedure can readily be applied to the placental receptor for this purpose.

**Registry No.** Insulin, 9004-10-8; insulin receptor kinase, 88201-45-0.

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# Effects of Chronic Exposure to Ethanol on the Physical and Functional Properties of the Plasma Membrane of S49 Lymphoma Cells†

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ABSTRACT: The effects of chronic exposure to ethanol on the physical and functional properties of the plasma membrane were examined with cultured S49 lymphoma cells. The β-adrenergic receptor-coupled adenylate cyclase system was used as a probe of the functional properties of the plasma membrane. Steady-state fluorescence anisotropy of diphenylhexatriene and the lipid composition of the plasma membrane were used as probes of the physical properties of the membrane. Cells were grown under conditions such that the concentration of ethanol in the growth medium remained stable and oxidation of ethanol to acetaldehyde was not detected. Chronic exposure of S49 cells to 50 mM ethanol or growth of cells at elevated temperature resulted in a decrease in adenylate cyclase activity. There were no changes in the density of receptors or in the affinity of β-adrenergic receptors for agonists or antagonists following chronic exposure to ethanol. The fluorescence anisotropy of diphenylhexatriene was lower in plasma membranes prepared from cells that had been treated with 50 mM ethanol than in membranes prepared from control cells. However, this change was not associated with changes in the fatty acid composition or the cholesterol to phospholipid ratio of the plasma membrane. There was a small but statistically significant decrease in the amount of phosphatidylserine and an increase in the amount of phosphatidylethanolamine. These changes cannot account for the decrease in anisotropy. In contrast to the effect of ethanol, a decrease in adenylate cyclase activity following growth of S49 cells at 40 °C was not associated with a change in anisotropy.

The effects of ethanol on biological systems can be studied at several different levels. Studies with whole animals can be influenced by effects of ethanol on the physiological state of the animal as well as by metabolites of ethanol produced in the organ being studied or elsewhere in the body. For example, it has been shown that the toxic effects of acetaldehyde, the primary metabolite of ethanol, are evident at much lower concentrations than are required to see the effects of ethanol (Koerker et al., 1976). Thus, the effects of ethanol in whole animals may be partially or entirely due to acetaldehyde rather than to ethanol. Furthermore, in the whole animal, it may be difficult to determine the concentration of ethanol to which a particular tissue is exposed, and fluctuations in the concentration of ethanol occur, which can further complicate interpretation of results. The use of isolated organs and organ

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