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Adipocyte Insulin-Binding Species: The Size and Subunit Composition of the Larger Binding Species

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Several investigators have reported that there are both large and small insulin-binding proteins in plasma membranes; the larger protein demonstrates nonlinear Scatchard binding, and the smaller protein has linear binding. We now present evidence that the larger insulin-binding species consists of four proteins of different sizes. Rat epididymal adipocyte plasma membranes were prebound with ^{125}I-insulin and then exposed to 1 mM disuccinimidyl suberate for 15 min at 2°C. The membranes were solubilized in 0.1% Triton X-100 and applied to a Sepharose 6B column. Peaks of radioactivity from the column were dialyzed, lyophilized, and analyzed by dodecyl-sulphate gel electrophoresis (5%, 100/1; mono/bisacrylamide). Autoradiograms of the gels were scanned with a densitometer. The Sepharose chromatogram revealed four radioactive peaks: peak 1 at column void volume; peak 2, K_{av} = 0.27; peak 3, K_{av} = 0.77; and peak 4, K_{av} = 1.09. Dodecyl sulphate electrophoresis of fractions in peak 2 demonstrated four bands on autoradiography; peak 1 did not enter the gel and peaks 3 and 4 ran with the dye front. Molecular weight estimates of the four insulin-binding species in peak 2 were 600, 500, 420, and 350 K. On dithiothreitol reduction each insulin-binding species yielded subunits of $M_r \cong$ 135 and 18 K. The three largest binding species demonstrated an additional 45-K dalton protein on dithiothreitol reduction, and the 500-K and 420-K binding species also yielded a 49-K dalton protein. These results suggest that the large insulin-binding protein in rat epididymal adipocytes contains several insulin-binding species, and that these insulin-binding species differ in the number of and the type of subunits they contain. In addition, it may be postulated that the nonlinear Scatchard binding associated with the larger binding protein is a consequence of the heterogeneity of the insulin-binding species in this Sepharose peak.

Key words: insulin receptor, rat adipocyte, insulin-binding species, disuccinimidyl suberate, dodecyl sulphate gel electrophoresis

Abbreviations: disuccinimidyl suberate, DSS; ethylene diamine tetraacetic acid, EDTA; sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE.

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The existence of large (Stoke's radius of approximately 70 Å for Triton X-100 solubilized receptor) and small (≅40 Å) insulin-binding species have now been reported in several laboratories [1–8]. Where steady-state insulin-binding measurements have been made, the smaller insulin-binding protein demonstrates linear Scatchard [9] binding, whereas the larger binding species demonstrates a curvilinear plot [2,3,5]. It was noted further that the two forms of the insulin-binding species were interconvertible [2,3,5,8]. This report describes that the 70-Å insulin-binding protein from rat adipocyte plasma membranes may contain as many as four insulin-binding species and that the 70-Å insulin-binding species are composed of subunits whose molecular weight estimates are similar to those reported for affinity-labeled [7,10,11], photoaffinity-labeled [12–14] and purified insulin receptors [15,16].

Many methods have been used to investigate the structure of the insulin receptor [eg, 1–19]. We have used an affinity-labeling procedure [10,11,20] where ^{125}I-insulin bound to adipocyte plasma membranes is exposed to disuccinimidyl suberate (DDS), a chemical cross-linking agent. The irreversibly bound ^{125}I-insulin-labeled proteins were separated by dodecyl sulphate/acrylamide gel electrophoresis, or gel permeation chromatography. The latter method yielded a labeled protein with an elution position consistent with previously reported affinity-labeled receptors [7,8,10]. When ^{125}I-insulin-labeled membranes or ^{125}I-insulin-labeled receptor from Sepharose 6B chromatography was applied to 5% (w/v) acrylamide (mono-/bis-acrylamide = 100/1) slab gels [21], and proteins were separated by electrophoresis [23] and detected by autoradiography, four bands were detected. Molecular-weight estimates for the four bands were based on their relative migration in the electrophoretic field and were read from a calibration curve of relative migration and molecular weight of several high-molecular-weight globular proteins. The molecular weight estimates, 600, 500, 420, and 350 K, are larger than those cited by some investigators [8,23,24] but are in agreement with others [25,26]. The subunits of the four insulin-binding species, however, are the same as those previously reported [23,24]. These results suggest that rat adipocytes may contain several insulin-binding species, which elute as a single peak in Sepharose chromatography (70 Å, Stoke's radius). Assuming that each insulin-binding species has a unique affinity for insulin, the nonlinear Scatchard analysis of insulin binding to the large insulin receptor would reflect heterogeneity of receptors and not cooperativity [27].

MATERIALS AND METHODS

Preparation of Fat Cells and Membranes

Adipocytes were isolated from epidiymal fat pads from Sprague-Dawley rats weighing 300–400 g [28,29]. Collagenase digestion proceeded for 40 min at 37°C. Isolated adipocytes were washed twice in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 2% bovine serum albumin, and twice in warm (37°C) homogenization buffer (10 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.25 M sucrose, 0.5 mM phenyl methyl sulfonyl fluoride, pH 7.4). Homogenization (Thomas glass homogenizer with serrated Teflon pestle) and centrifugation steps for plasma-membrane preparation were as previously described [30,31]. Plasma membranes were collected from the top of a 32% (w/v) sucrose phase following a 60 min 80,000g centrifugation step [10]. The membranes were washed and stored in 62.5 mM Tris-HCl, pH 6.8, buffer. Plasma membranes were stored at −70°C.

^{125}I-Insulin Labeling of Membranes

Plasma membranes were slowly thawed, suspended in Krebs-Ringer bicarbonate buffer, pH 7.4, centrifuged 40,000g 20 min, and resuspended in the same buffer containing ^{125}I-insulin. The final concentration of protein varied from 0.3 to 1.2 mg/ml, and ^{125}I-insulin varied from 0.5 to 4 nM. A second membrane preparation was similarly prepared except the final addition of buffer contained ^{125}I-insulin and 1.6 μM unlabeled insulin. The volumes of the binding mixtures were 0.5–1 ml. Binding proceeded for 30 min at 23°C and then the mixture was diluted to 5 ml with cold (4°C) Krebs-Ringer bicarbonate buffer and centrifuged 30,000g 20 min. The pellet was washed twice by resuspending in 5 ml cold Krebs-Ringer bicarbonate buffer followed by centrifugation. The washes removed bovine serum albumin and unbound ^{125}I-insulin. Radioactivity associated with the pellet in each wash step was determined (Picker Nuclear Twin Scales II). Following the final wash the membrane pellet was suspended in 0.15 ml Krebs-Ringer bicarbonate buffer and cooled to 4°C, and cold 0.15 ml Krebs-Ringer bicarbonate buffer containing 2 mM DSS was added. DSS had been dissolved in dimethyl sulfoxide (100 mM) just prior to dilution with buffer. Cross linking proceeded at 4°C (ice bath) with shaking for 15 min (except where noted). The reaction was stopped by dilution to 5 ml with cold 10 mM Tris-HCl, 1 mM EDTA buffer and stored at −70°C overnight.

Electrophoresis and Autoradiography

Affinity-labeled plasma membranes were subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of Laemmli [22]. SDS-PAGE was performed on 1.5-mm slab gels (Bio-Rad Model 220 Dual Vertical Slab Gel Electrophoresis System). "Porous" gels consisted of 10 cm of 5% (w/v) polyacrylamide (acrylamide/bis-acrylamide, 100/1) covered by a 1 cm 4% polyacrylamide (acrylamide/bis-acrylamide, 100/1) stacking gel. Gradient gels, 10 cm, consisted of 4–15% (w/v) polyacrylamide (acrylamide/bis-acrylamide, 100/2.7) covered by 1 cm of 4% (w/v) polyacrylamide stacking gel. The membranes were solubilized by boiling (2 min) in 62.5 mM Tris, pH 6.8, buffer containing 10% sucrose, 2% SDS, and 2 mM EDTA (solubilization buffer). Electrophoresis was run under constant current (Beckman DuoStat): 15 mA through stacking gel and higher currents (35–45 mA) through the analytical gels. Tracking dye, 0.1% (w/v) bromophenol blue in water, was added to solubilized membrane solutions.

In some instances single lanes from 5% "porous" SDS-PAGE analysis were excised, cut into 2-mm slices with a Bio-Rad 190 Gel Slicer, placed in microfuge tubes, and counted. To the slices containing individual insulin-binding species was added 100 μl "solubilization" buffer containing 100 mM DTT. The tubes were boiled for 4 min. The acrylamide strips were cut into smaller pieces and loaded into wells in a stacking gel of a 4–15% linear gradient gel.

Following electrophoresis, gels were fixed in 5% methanol/10% acetic acid, stained with Coomassie Brilliant Blue dissolved in 45% methanol/9% acetic acid, destained in 5% methanol/7.5% acetic acid, and then dried (Bio-Rad Slab Gel Dryer model 224). Autoradiography of gels used preflashed Kodak XR-1 x-ray film backed by either a Dupont Cronex Hi-Plus or Quanta III intensifying screen. Autoradiograms were scanned in an Ortec 4310 Densitometer or in a Beckman DU8 Spectrophotometer.

Molecular weights of radioactive bands are based on relative migration. A calibration curve relating relative migration and molecular weight of SDS-solubilized proteins was prepared with each SDS-PAGE analysis. Proteins used for calibration were immunoglobulin M (IgM), immunoglobulin A (IgA), and immunoglobulin G (IgG) from Behringwerke; thyroglobulin and ferritin from Pharmacia Chemicals; fibronectin from Bethesda Research; and β-galactosidase, α-chymotrypsinogen, and ribonuclease A from Sigma Chemicals.

Sepharose Chromatography

For Sepharose 6B chromatography 3 nM ^{125}I-insulin was cross-linked to 0.9 mg/ml plasma membrane protein as described above; 4.8% of the ^{125}I-insulin present in the binding reaction remained associated with the membrane protein following the DSS cross-linking and washing steps. ^{125}I-insulin-labeled plasma membrane (10^5 cpm in 250 μl, 10 mM Tris HCl, 1 mM EDTA, pH 6.8, buffer) was mixed with 380 μl "solubilization" buffer containing 12% (w/v) Triton X-100 instead of SDS, and the mixture was maintained at 23°C for 20 min. The solubilized membrane was applied to a Sepharose 6B column (2.5 × 32 cm) equilibrated with 25 mM Tris, 19 mM glycine, 0.1% (w/v) Triton X-100, pH 8.5, buffer. The column was eluted with the latter buffer at 25 ml/h. One-milliliter fractions were collected and radioactive content was determined in a Searle Automatic γ-Counter. The void volume (V_o) was the volume of dextran blue elution and the column volume (V_t) was the volume of elution of NaCl. The elution volume (V_e) of the radioactive peaks are expressed by their K_{av} [8], where

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Fractions were pooled, as illustrated in Figure 4, and dialyzed 16 h against 80 ml distilled water containing 0.1% (w/v) SDS. The fractions were lyophilized, and the residue was mixed with 50 μl "solubilization" buffer and boiled 2 min. The solubilized Sepharose chromatography fractions were then subjected to electrophoresis on a 5% "porous" slab gel as described above.

Reagents

Porcine zinc insulin (exp 491-4) was a gift from Dr J. Clement (Connaught Laboratories). Bovine serum albumin (lot No. T13607) was purchased from Armour Pharmaceutical; collagenase (lot No. 40C164P) from Worthington Biochemical; phenylmethyl sulfonyl fluoride from Pierce Chemicals; sodium ^{125}I-iodide from New England Nuclear. Other reagents were puchased from Fisher Chemicals or Bio-Rad. DSS was synthesized [31] and its structure was confirmed by mass spectrometry and NMR analysis. ^{125}I-insulin was prepared by the choramine-T method as previously described [33] to specific activities of 150–200 μCi/μg.

Protein concentrations were determined by the method of Lowry using bovine serum albumin as standards [34].

RESULTS

binding mixture also contained 1.6 μM insulin, radioactivity remaining bound (non-specifically) to the membrane after each step was 2.6%, 1.7%, 1.5%, and 1.3%, respectively. These results indicate that 5.9% of the initial ^{125}I-insulin was specifically bound, either reversibly or irreversibly, to membrane-insulin binding sites following the DSS cross-linking procedure. In 14 other labeling procedures the amount of ^{125}I-insulin remaining specifically bound varied from 2% to 10%; a correlation between percent bound and concentration of protein or ^{125}I-insulin was not apparent.

When ^{125}I-insulin-labeled membranes were solubilized in the absence of DTT and subjected to SDS-PAGE, several radioactive bands were observed on autoradiography (Fig. 1). The 5% "porous" gel suggested four specific insulin-binding species, I, II, III, and IV, since adipocyte membranes labeled in the presence of 1.6 μM insulin (nonspecific labeling) had no comparable ^{125}I-insulin-labeled proteins. The area marked 2 in the densitometry tracing (Fig. 1) may also be an insulin-binding species since it always ran as a trailing shoulder of band I. Area 1 represents proteins that did not enter the gel. Two additional radioactive bands were observed just above the dye front; however, only one of these proteins (the one closest to the dye front) appears to be labeled specifically.

Figure 2 illustrates that high-molecular-weight proteins (listed in Fig. 2 as well as fibronectin ($M_r \cong$ 660, 440, and 220 K (Fig. 5)) demonstrated a linear relationship between migration and log molecular weight in 5% "porous" acrylamide SDS-PAGE.

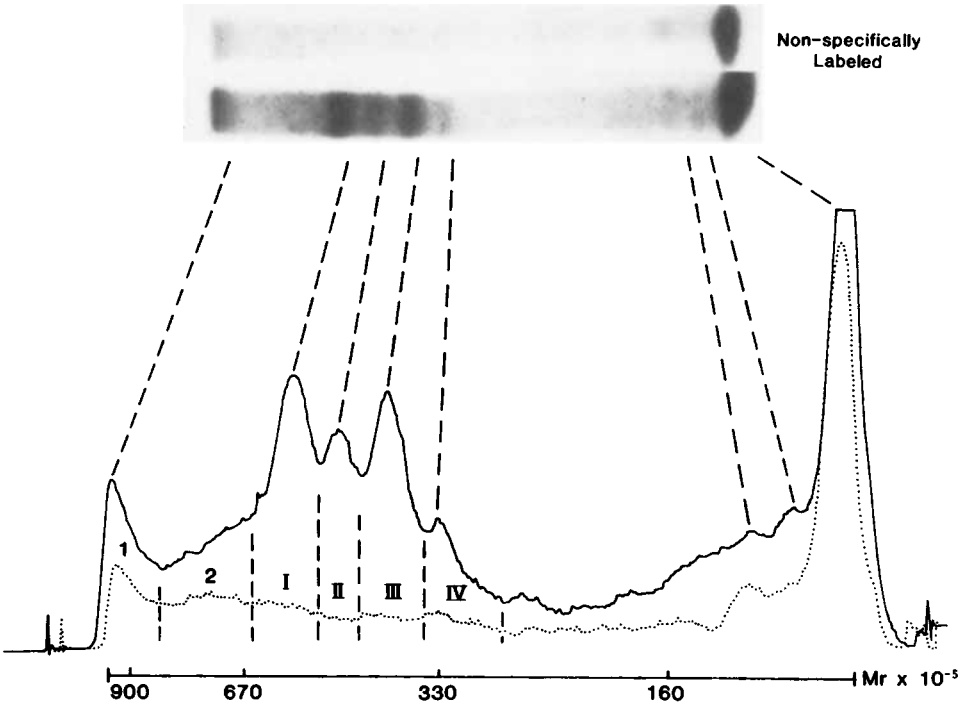


Fig. 1. Autoradiogram and densitometry scan of ^{125}I-insulin-labeled membrane proteins separated in 5% "porous" SDS-PAGE. Adipocyte plasma membranes with ^{125}I-insulin prebound were exposed to 1 mM disuccinimidyl suberate at 0°C for 20 min. Following solubilization in the absence of dithiothreitol, membranes were applied to a 5% "porous" polyacrylamide gel slab. Electrophoresis was 15 mA through the stacking gel and 35 mA through the analytical gel. Molecular weight standards (noted at bottom of figure) for this SDS-PAGE were IgM ($M_r \cong$ 900, 320, and 160 K [35] and thyroglobulin ($M_r \cong$ 670 and 330 K [36]). Gels were processed as described in Materials and Methods.

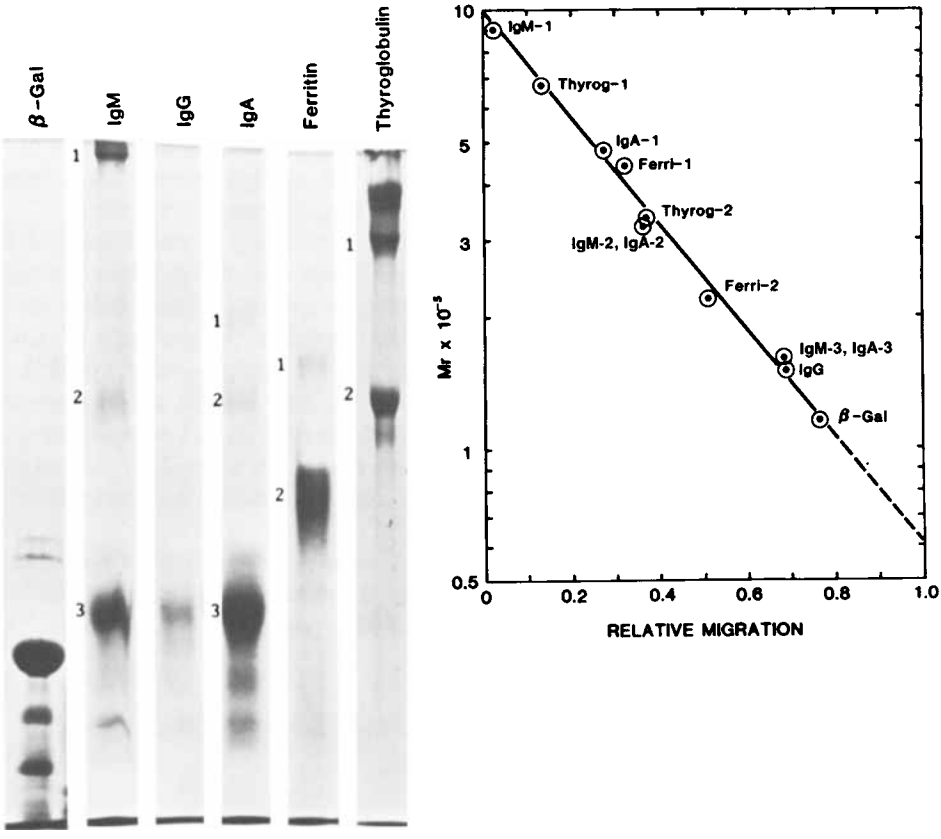


Fig. 2. Calibration of protein molecular weight and migration in 5% "porous" SDS-PAGE. Proteins were boiled 2 min in solubilization buffer and applied to 5% "porous" polyacrylamide slab gels. Electrophoresis was as described in Figure 1. M_r of proteins are as follows: immunoglobulin M, IgM-1 is a pentamer ≅ 900,000 [35], IgM-2 is a dimer ≅ 320,000 [35], IgM-3 is the monomer ≅ 160,000 [35], immunoglobulin A, IgA-1 is a trimer ≅ 480,000, IgA-2 and IgA-3 are dimer and monomer, and equivalent to IgM-2 and IgM-3, respectively [35]; immunoglobulin G, IgG is 155,000 [35]; β-Gal is 116,000 [21]; ferritin, Ferri-1 is 440,000 [37] and Ferri-2 is 220,000 [37]; and thyroglobulin, Thyrog-1 is 670,000 and Thyrog-2 is 330,000 [36]. The top band in the thyroglobulin lane may be a 900,000-dalton protein previously described [38].

On the basis of their relative migration and the above calibration curve, molecular weights were estimated for the four insulin-binding species: M_r (I) ≅ 600 K; M_r (II) ≅ 500 K; M_r (III) ≅ 420 K; and M_r (IV) ≅ 350 K.

Since these molecular weight estimates are larger than those reported by others [8,23,24], and since the method of labeling the insulin receptor involved the use of a nonspecific cross linker, DSS, intermolecular cross linking may be postulated. Two experiments have been performed to determine whether protein cross linking has occurred: 1) a time-course study in which ^{125}I-insulin labeling (cross-link reaction) was performed for 5, 10, 15 and 20 min at 1 mM DSS; and 2) a DSS concentration dependency study in which 0.1, 0.3, and 1 mM DSS were used in the cross-linking step. Figure 1 illustrates the results of 20 min of cross linking of DSS with membrane-bound ^{125}I-insulin. Less reaction time did not alter the relative intensities of bands I

→ IV. On the other hand, peak 1 and shoulder 2 (Fig. 1) did increase slightly with increasing reaction time. Figure 3 illustrates the autoradiogram of the second experiment where DSS concentration was varied. At 0.1 mM DSS (lane a, Fig. 3) insulin-binding species I, II, and III were seen, with insulin-binding species I being the most intense. Insulin-binding species IV was not detected until 1 mM DSS was used (lane c, Fig. 3). The results of the above two experiments suggested that the relative amounts of insulin-binding species I to IV reflected either their relative concentrations in plasma membrane or their ability to react with DSS, rather than a reactant (lower-molecular-weight insulin-binding species) to product (higher-molecular-weight insu-

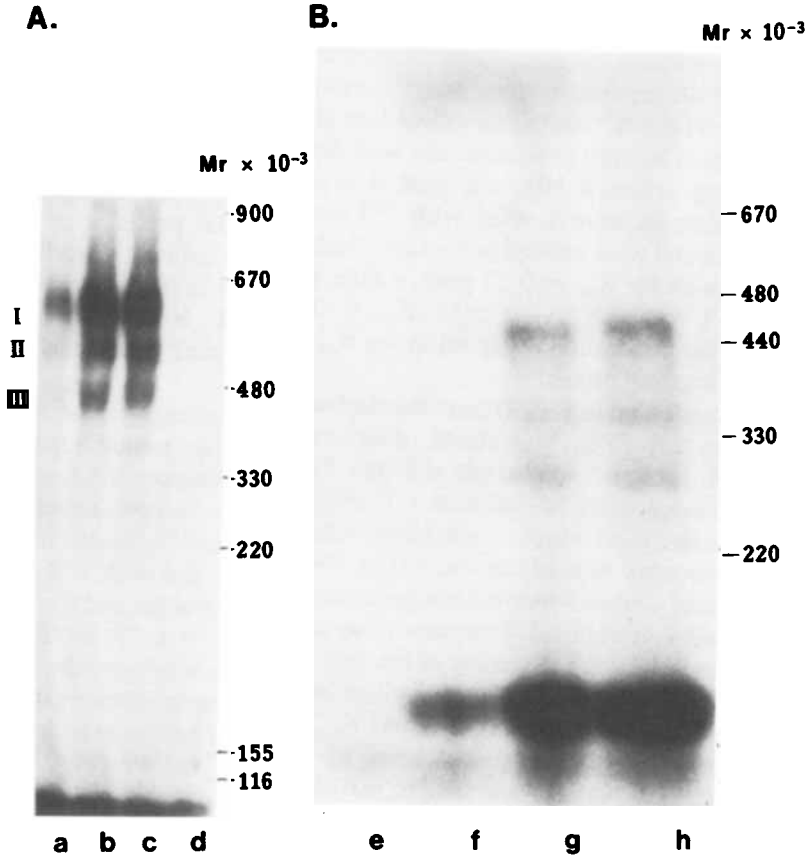


Fig. 3. The effect of disuccinimidyl suberate concentration on cross-linked products of ^{125}I-insulin and insulin-binding species. Adipocyte plasma membranes with ^{125}I-insulin prebound were exposed to 0.1 (lanes a and f), 0.3 (lanes b and g), and 1 mM (lanes c and h) disuccinimidyl suberate at 0°C for 15 min. Following solubilization in the absence (A) or presence (B) of 50 mM dithiothreitol, membranes were applied to a 5% "porous" polyacrylamide gel slab (A) and to a 4–15% polyacrylamide gel slab (B). Lanes d and e are the respective nonspecifically labeled plasma membranes; 1 mM disuccinimidyl suberate was used in the cross-link reaction. Except for the lanes containing nonspecifically labeled insulin-binding species (2×10^3 cpm), similar amounts of oxidized ($\sim 19 \times 10^3$ cpm) and reduced ($\sim 15.5 \times 10^3$ cpm) labeled proteins were analyzed. Electrophoresis was performed as described in the legend to Figure 1. Molecular weight standards were A) IgM, thyroglobulin, IgA, ferritin, and β-galactosidase; B) thyroglobulin, ferritin, and IgA. Gels were processed as described in Materials and Methods. The upper half of the 4–15% gradient gel is illustrated in B.

lin-binding species) relationship. Insulin-binding species I to IV did not appear to be a product of intermolecular cross linking between receptor and nonreceptor or receptor and receptor. On the other hand, peak 1 and area 2 (Figs. 1, 3) were seen following increased reaction time or higher DSS concentrations. These ^{125}I-insulin-labeled proteins may be intermolecularly cross-linked receptors or receptors to nonreceptor proteins.

Intramolecular cross-linking within insulin binding species did occur. Figure 3 (lanes f, g, h) illustrates that 50 mM dithiothreitol completely reduced the insulin-labeled proteins when the DSS concentration was 0.1 mM but did not do so when DSS was 0.3 or 1 mM. The products of dithiothreitol reduction of 0.3 and 1 mM DSS-treated ^{125}I-insulin membranes included three labeled proteins whose M_r were less than 500,000 and greater than 200,000 daltons. These latter proteins were smaller than the intact insulin-binding species (I and II) and were presumably prevented from dissociation into subunits because of intramolecular cross links formed by DSS.

Sepharose 6B chromatography of ^{125}I-insulin-labeled adipocyte membranes, solubilized with Triton X-100, demonstrated four labeled peaks; K_{av} = 0, 0.27, 0.77, and 1.09 (Fig. 4). The third peak coincides with the elution of ^{125}I-insulin mixed with buffer containing Triton X-100, and peak 4 is the elution of ^{125}I-insulin. When adipocyte membranes were labeled with ^{125}I-insulin in the presence of 1.6 μM unlabeled insulin and were applied to the same Sepharose 6B column, no radioactivity was associated with the K_{av} = 0.27 peak, a little was found in the void volume, but most of the ^{125}I was seen in the third (K_{av} = 0.77) peak. It may be concluded, therefore, that the ^{125}I-insulin observed in the K_{av} = 0.27 peak is associated with a specific insulin-binding protein.

Fractions (as noted in Fig. 4) from the Sepharose chromatography were pooled, dialyzed against 2% SDS, lyophilized, dissolved in "solubilization" buffer, and applied to a 5% "porous" acrylamide slab gel. Figure 5 illustrates the densitometry scans of the autoradiogram of fractions 1–8. Fractions 9 to 14 (not shown) demonstrated a radioactive band which on electrophoresis migrated with the dye front.

The densitometry scan of fraction 1 (Fig. 5) suggested that peak 1 (K_{av} = 0) is ^{125}I-insulin-labeled protein which did not penetrate the analytical gel and was probably the same as band 1 in Figure 1. Fractions from peak 2 (K_{av} = 0.27) on SDS-PAGE migrated as four bands; the migration of the four bands was similar to the migration of the insulin-binding species from affinity-labeled adipocyte membranes (bands I to IV, Fig. 1). Fraction 2, which had the lowest K_{av} of peak 2, had more of band I, and fraction 8, with the largest K_{av}, had more of bands III and IV (Fig. 5). Thus, Sepharose 6B chromatography was able to partially separate the four insulin-binding species.

When the individual binding species, I to IV, were boiled 4 min in "solubilization" buffer containing 100 mM DTT and the proteins were separated on a 4–15% gradient polyacrylamide gel, two heavily labeled proteins ($M_r \cong$ 300 and 135 K) and two slightly labeled proteins ($M_r \cong$ 45 and 18 K) were observed (Fig. 6). A densitometry scan of the same autoradiogram, but at an increased sensitivity (Fig. 6B), suggested that insulin-binding species I to III contained a subunit with M_r 45 K, whereas insulin-binding species II and III also contained a second labeled subunit with $M_r \cong$ 49 K; insulin-binding species IV did not appear to contain either the 45-K- or the 49-K-dalton subunits (Fig. 6B). Insulin-binding species III demonstrated a small amount of a 180-K-dalton intermediate (Fig. 6A). A 180-K-dalton ^{125}I-insulin-labeled intermediate is always seen when ^{125}I-insulin-labeled adipocyte membranes

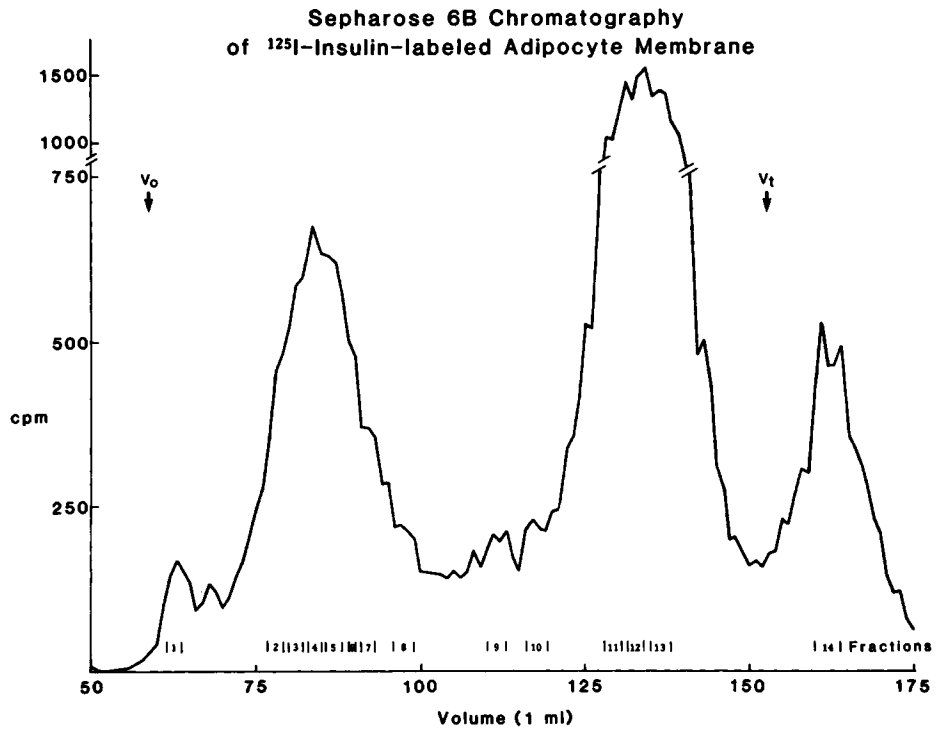
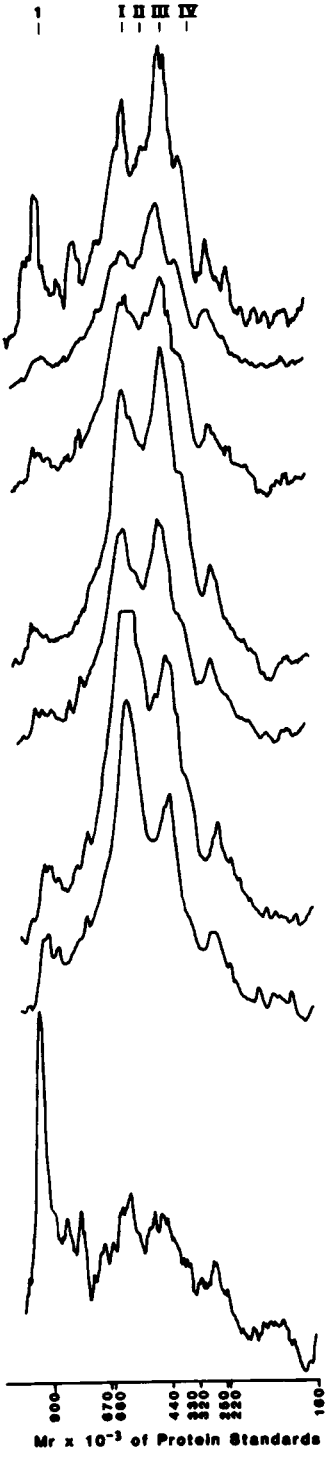


Fig. 4. Sepharose 6B chromatography of ^{125}I-insulin-labeled adipocyte membrane. ^{125}I-insulin-labeled adipocyte membrane (10^5 cpm) was solubilized with solubilization buffer containing 12% (w/v) Triton X-100, and applied to a Sepharose 6B column (2.5 × 32 cm). The column was eluted with Tris-glycine 0.1% Triton X-100, pH 8.5 buffer, and 1 ml aliquots were collected. The void volume (V_o) and total volume (V_t) were determined by the elution volume of dextran blue and NaCl, respectively. Fractions were pooled as noted at bottom of chromatogram.

are treated with 10 mM or less dithiothreitol (data not shown). The 180-K-dalton labeled protein present at low dithiothreitol concentrations, coupled with the observation that it was detected only in the reduction products of insulin-binding species III, suggested that the 180-K-dalton labeled protein was a disulfide reduction intermediate of insulin-binding species III.

The different insulin-binding species labeled in epididymal adipocyte plasma membranes may be a consequence of the homogenization and isolation procedure; that is, partial proteolysis may have occurred during the preparation of plasma membranes despite the presence of phenyl methyl sulfonyl fluoride (PMSF), a protease inhibitor, in all buffers. Accordingly, the following two control experiments were performed: 1) intact fat cells were labeled with ^{125}I-insulin; and 2) following homogenization, the homogenate was allowed to incubate for 10 min at 37°C, and the plasma membrane was isolated and then labeled with ^{125}I-insulin. Figure 7 illustrates the results of these experiments. Treatment of intact fat cells with prebound ^{125}I-insulin, with 1 mM DSS at 13°C for 15 min, yielded essentially two radioactively labeled proteins on SDS-PAGE (Fig. 7A). These labeled proteins corresponded to insulin-binding species I and III (Fig. 1). Insulin-binding species I and III were also observed in a parallel experiment when plasma membranes rather than intact fat cells were used as a source of insulin receptors (Fig. 7B). It was also noted that incubation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of whole-cell homogenates did not alter the size or apparent quantity of labeled insulin-binding species (Fig. 7B; cf lanes b and d). In addition, dithiothreitol-reduced insulin binding species demonstrated no differences in size or intensity of ^{125}I-insulin-labeled peptides when the plasma membranes were from incubated or nonincubated homogenized cells (Fig. 7C). These results are not consistent with the hypothesis that proteolysis occurred during plasma membrane preparation; they do suggest that the different insulin-binding species occur naturally.

DISCUSSION

When adipocyte membranes with bound ^{125}I-insulin were exposed to 1 mM disuccinimidyl suberate, at least four protein species were irreversibly and specifically labeled with insulin. These insulin-binding species were oligomeric since on treatment with dithiothreitol they were reduced in size. The oligomeric insulin-binding species had molecular weights, based on their migration in 5% "porous" SDS-PAGE, of 600, 500, 420, and 350 K (Fig. 1).

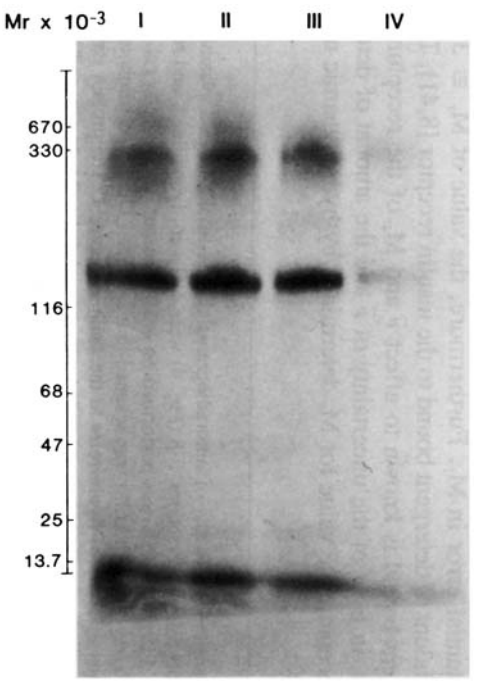
Generally, molecular weight (M_r) estimates for insulin-binding proteins are around 300 K [8,23,24]. Methods that have been used to determine M_r are chromatography on Sepharose 6B, coupled with sucrose-density centrifugation [8] and SDS-PAGE. There are generally two binding species observed on Sepharose 6B chromatography and their Stoke's radii are $\cong$70 and 40 Å [1–8] the 70 Å protein is estimated to be $M_r \cong 300$ K [8,41]. This value was estimated from the expression,

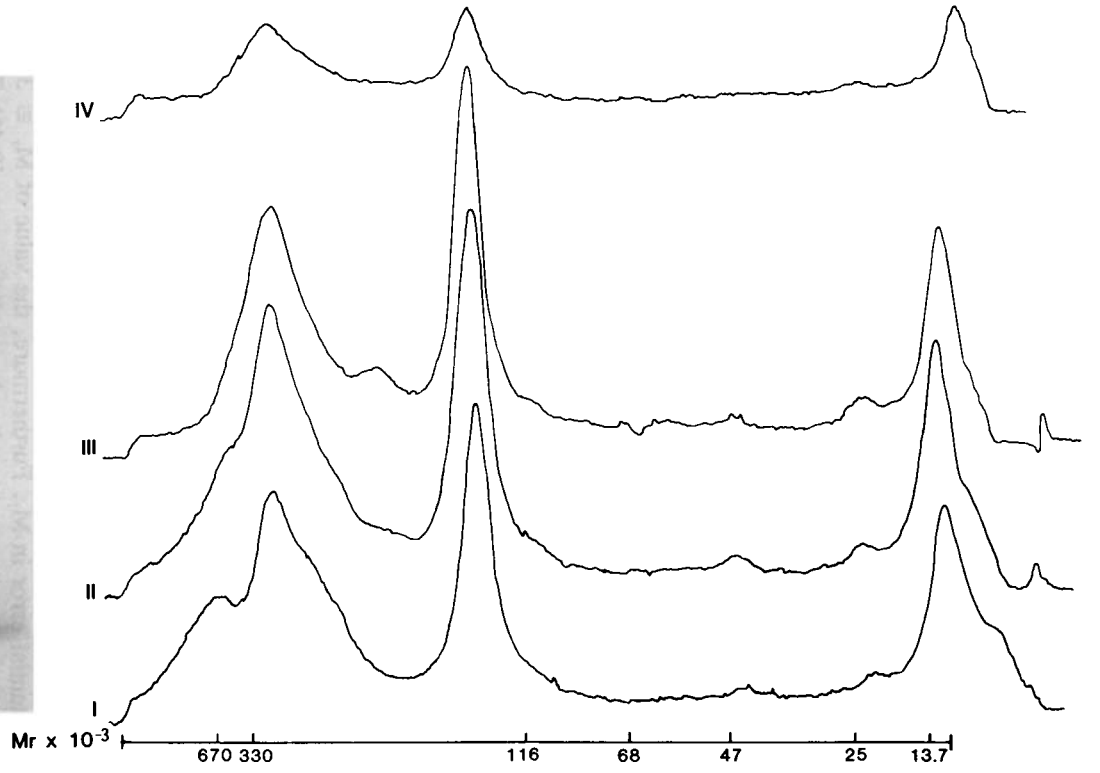
$$M_r = 6\pi\eta N\frac{as}{(1-\bar{\nu}\rho_0)}$$

where η is the viscosity of solution, N is Avagadro's number, a is the Stoke's radius, s is the sedimentation coefficient, $\bar{\nu}$ is the partial specific volume of the detergent-solubilized receptor and ρ_0 is the density of the medium. Experimentally, a (from Sepharose chromatography) and s (from sucrose-density centrifugation) are determined. The remaining terms are either constants or are estimated [eg, 8]. Substitution of appropriate values for $\bar{\nu}$ and ρ_0 demonstrates that $\bar{\nu}\rho_0 \cong 0.9$ and $(1-\bar{\nu}\rho_0) \cong 0.1$. Since $(1-\bar{\nu}\rho_0)$ appears in the denominator, then an incorrect estimate of $\bar{\nu}$ would lead to a substantial error in M_r. Furthermore, the value of $M_r \cong 300$ K is based on a certain amount of detergent bound to the insulin receptor [8,41]. The absolute amount of detergent bound is known to affect $\bar{\nu}$ and M_r of the receptor-detergent complex [8]. Thus, in light of the uncertainty of $\bar{\nu}$ and the amount of detergent bound to the insulin receptor, the value for M_r determined by hydrodynamic measurements is not definitive.

Fig. 5. Densitometry scans of autoradiograms of ^{125}I-insulin-labeled plasma membrane eluted off Sepharose 6B column. Fractions 1–8 (Fig. 4) were dialyzed, lyophilized, and redissolved in solubilization buffer. Electrophoresis was performed on a 5% "porous" acrylamide gel. The gel was dried and exposed to x-ray film. The film was scanned in a Beckman DU 8 Spectrophotometer. The Arabic and Roman numerals at top of figure refer to the same in Figure 1. The protein standards used in this slab gel were IgM ($M_r \cong$ 900, 320, and 160 K [35]), thyroglobulin ($M_r \cong$ 670 and 330 K [36]), ferritin ($M_r \cong$ 440 and 220 K [37]), and fibronectin ($M_r \cong$ 660, 440, and 220 K [39]). Scan of fraction 1 is bottommost scan, and fraction 8 is the topmost.

AUTORADIOGRAM AND DENSITOMETRY TRACING OF INDIVIDUAL INSULIN BINDING SPECIES
TREATED WITH 100mM DITHIOTHREITOL AND SEPARATED ON 4–15% ACRYLAMIDE GRADIENT GEL ELECTROPHORESIS





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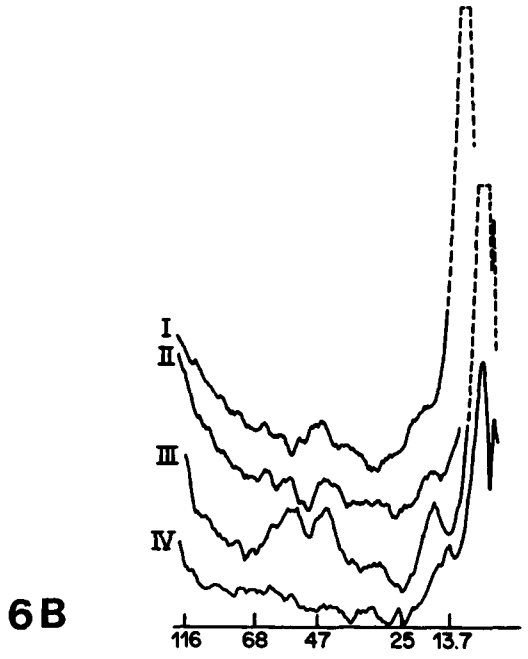

Fig. 6. Autoradiogram and densitometry tracing of individual insulin-binding species treated with 100 mM dithiothreitol and separated on 4–15% acrylamide gradient gel electrophoresis. Solubilized ^{125}I-insulin-labeled membrane proteins were separated on a 5% "porous" polyacrylamide gel (Fig. 1). Lanes from the latter gel were excised and cut into 2-mm slices, and radioactivity was determined. To the acrylamide slices containing the different insulin-binding species was added 100 μl solubilization buffer containing 100 mM dithiothreitol. Following a 4-min boiling, the gel slices were sliced into smaller pieces and inserted into wells of a gradient 4–15% acrylamide gel and electrophoresis proceeded 15 mA through the stacking gel and 35 mA through the gradient gel. Gels were then dried and exposed to x-ray film. The autoradiogram (Fig. 6A, left) was scanned in an Ortec Densitometer (Fig. 6A, center) and a Beckman DU8 Spectrophotometer (Fig. 6B). Proteins included as molecular weight standards (see relative migration at bottom of figure) were: thyroglobulin ($M_r \cong$ 670 and 330 K [36]), β-galactosidase ($M_r \cong$ 116 K [21]), bovine serum albumin ($M_r \cong$ 68 K [40]), ovalbumin ($M_r \cong$ 47 K [40]), α-chymotrypsinogen ($M_r \cong$ 25K [40]), and ribonuclease A ($M_r \cong$ 13.7 K [40]).

The SDS-PAGE method is an empirical method [42] and assumes a g-SDS to g-protein ratio [43]. Generally, proteins of known molecular weight are analyzed by SDS-PAGE and a calibration curve is generated. Previous reports have used myosin ($M_r \cong$ 200 K [10,11,21]) or filamin ($M_r \cong$ 250 K [44,45]) as the largest protein in preparing the calibration curve. Figure 2 illustrates that proteins up to an $M_r \cong 10^6$ may be detected on a 5% "porous" gel and that 10^5-dalton to 10^6-dalton proteins are separable in this electrophoretic gel. It should, however, be noted that, except for β-galactosidase, the proteins used in preparation of the calibration curve were not reduced. Since these proteins have naturally occurring disulfide cross links, it may be assumed that the ratio of SDS/protein would not be the same. Surprisingly, the calibration curve in Figure 2, as well as those generated in several other laboratories using nonreduced proteins [eg, 46,47] demonstrates a linear correlation between the log of molecular weight and relative migration in SDS-PAGE. Thus, if the assumption

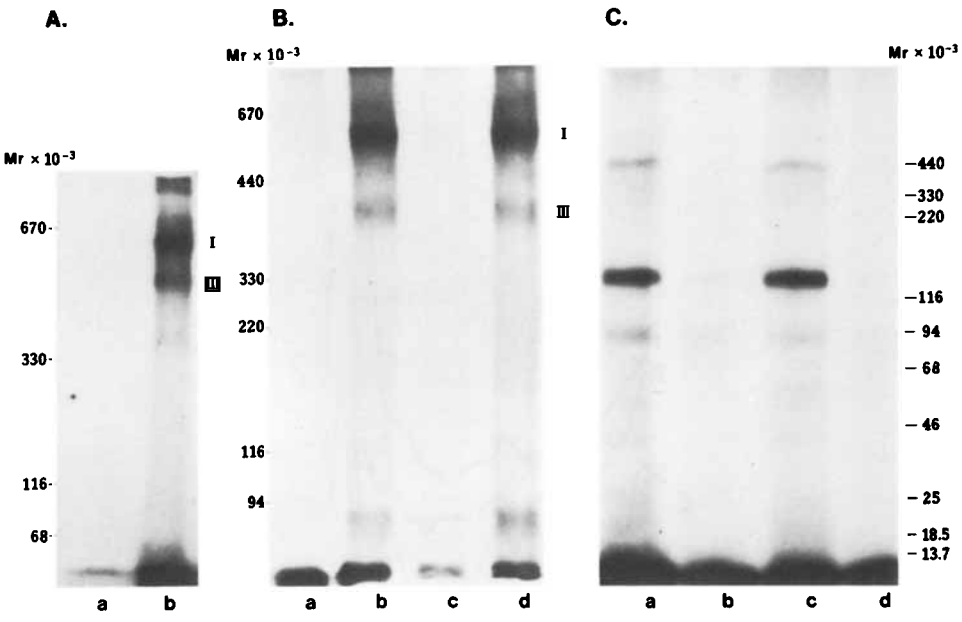

Fig. 7. Autoradiograms of ^{125}I-insulin-labeled insulin-binding sites from intact fat cells and from incubated adipocyte homogenates. A) Isolated fat cells from seven 135-g rats were suspended in Krebs-Ringer bicarbonate buffer containing 4% (w/v) BSA. ^{125}I-insulin (2 nM) was added and the mixture was incubated 30 min at 23°C. To 20% of fat cell suspension 2 μM insulin was also added (nonspecific labeling). The fat cells were washed and resuspended in Krebs-Ringer bicarbonate buffer at 13°C. Disuccinimidyl suberate in dimethyl sulfoxide was added to a final concentration of 1mM DSS and 0.5% dimethyl sulfoxide. After 15 min the cross-link reaction mixture was diluted with homogenization buffer. Plasma membranes were prepared, solubilized, and analyzed on a 6% (w/v) polyacrylamide (acrylamide/bis-acrylamide, 100/1) 1.5 mm slab gel (6% "porous" gel): a) nonspecifically labeled, adipocyte-derived membrane; b) specifically labeled, adipocyte-derived membranes. Molecular weight markers were thyroglobulin, β-galactosidase, and bovine serum albumin. B) Following adipocyte homogenization and centrifugation at 1,000g for 10 min at 4°C, the fat cake was removed from the adipocyte homogenate. Half of the homogenate was incubated for 10 min at 37°C while the other half was maintained in an ice bath. Plasma membranes were prepared then as described in Materials and methods. The membranes were labeled with ^{125}I-insulin, solubilized, and analyzed on a 6% "porous" gel: (a) nonspecifically and (b) specifically labeled membranes from 37°C incubated homogenates, (c) nonspecifically and (d) specifically labeled membranes from nonincubated homogenates. Molecular weight markers were thyroglobulin, ferritin, β-galactosidase, and phosphorylase b ($M_r \cong 94$ K [32]). C) ^{125}I-insulin-labeled adipocyte membranes as described in B were solubilized in "solubilization" buffer containing 30 mM dithiothreitol. Solubilized membranes were analyzed on a 4–15% gradient slab gel: (a) specifically and (b) nonspecifically labeled membranes from nonincubated homogenates; and (c) specifically and (d) nonspecifically labeled membranes from 37°C incubated homogenates. Molecular weight markers were ferritin, thyroglobulin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, α-chymotrypsinogen, and ribonuclease.

is made that insulin-binding species denature and bind SDS in a similar manner to the proteins in Figure 2, then the estimates of molecular weight given above for insulin-binding species I to IV are acceptable.

In addition to this report, two other reports [18,25] have estimated that the nonreduced insulin receptor has an $M_r > 350$ K. In both previous studies the M_r was based on receptor migration in Triton X-100-PAGE systems. Lang et al [18] concluded that the IM-9 lymphocyte receptor behaved in gel electrophoresis as a molecule with a radius of 68 Å; this radius is similar to that estimated from Sepharose 6B

chromatography. Kuehn et al [25] estimated the insulin receptor-Triton X-100 complexes to be about 600 K daltons.

A limitation to the use of DSS is that it may lead to intermolecular and intramolecular cross linking. Intramolecular cross linking presumably does occur since small amounts of oligomeric structures are detected on SDS-PAGE after extensive treatment of plasma-membrane proteins with dithiothreitol. Intermolecular cross linking may be responsible for the radioactivity seen at the top of the analytical gels. Intermolecular cross linking does not, however, contribute to insulin-binding species I to IV, since 1) these proteins elute on Sepharose 6B chromatography at a position where solubilized insulin receptors elute; 2) decreased reaction time with DSS or decreased concentrations of DSS did not decrease the amount of larger, or increase the number or amount of smaller, insulin binding species; and 3) purified human placental ^{125}I-insulin receptor analyzed by the 5% "porous" SDS-PAGE system demonstrated insulin-binding species I, II, and III on autoradiography [48]. Thus, insulin-binding species I to IV are not an artifact of the ^{125}I-insulin-labeling procedure.

The subunits contained in the different insulin-binding species are I) 135-K dalton, 45-K dalton, 18-K dalton; II and III) 135-dalton, 49-K dalton, 45-K dalton, and 18-K dalton; and IV) 135-K dalton and 18-K dalton. The 135-K-dalton subunit is presumably the same one reported by a number of other laboratories whose M_r estimates are 125 to 145 K [23,24]. Similarly, a 45-K-dalton [15] and a 49-K-dalton [11] protein have been reported as reduced subunits of larger oligomeric insulin-binding species. A 90-K-dalton subunit has also been detected in reduced insulin receptors [12,23,24,49,50]; however, as previously noted [10,11,21,31,32], the DSS-labeling procedure does not readily label the 90-K-dalton subunit. Thus, with the exception of the 90-K-dalton subunit, the subunits we have found are those seen by others.

Massague et al [11] suggest that the different insulin-binding species are a consequence of lysozomal degradation; that is, the 49-K-dalton subunit is a consequence of a proteolytic clip of the 90-K-dalton subunit. Since under our experimental protocol we are unable to detect a 90-K-dalton subunit, it is unlikely that the 49-K-dalton subunit we detect in insulin-binding species III and IV are lysosomal clips of the 90-K-dalton subunit. Alternatively, the 90-K-dalton subunit may be irreversibly cross-linked to other insulin-receptor subunits, and it migrates on SDS-PAGE with the 300-K-dalton nonreduced protein. In this instance the 49-K-dalton subunit would be reversibly attached (disulfide), and it could migrate on SDS-PAGE when reduced with dithiothreitol. Massague et al [11] also suggest that the 45-K-dalton subunit is a proteolytic fragment of the 135-K-dalton subunit. We have incubated adipocyte homogenates at 37°C for 10 min, prior to the preparation of plasma membranes, and have not observed any differences in radioactive content in the 135-K or 45-K bands (Fig. 7). Thus, although the 135-K and 45-K labeled peptides may yield similarly sized peptide fragments on chymotrypsin treatment [11], the 45-K-dalton subunit could be a distinct subunit of insulin-binding species I, II, and III.*

*β-Galactosidase and bovine serum albumin, two nonrelated proteins of different molecular weight, were subjected to papain, or Staphylococcus aureus protease, or trypsin. The digested proteins were subjected to electrophoresis in a 15% (w/v) polyacrylamide SDS-PAGE system. The proteins were stained with silver oxide. More than 75% similar banding patterns were obtained with papain digestion. Some similar peptide fragments were obtained with Staphylococcus aureus protease digestion, but trypsin digestion demonstrated no similarities.

In summary, the results described above suggest that there are minimally four insulin-binding species in isolated rat epididymal adipocyte plasma membranes. These insulin-binding species elute in the region of the insulin receptor on Sepharose 6B chromatography. If each of the insulin-binding species has a different affinity for insulin, then a Scatchard [9] analysis of steady-state insulin binding to the insulin receptor in the plasma membrane or to solubilized insulin receptors would demonstrate a nonlinear plot. It follows, therefore, that if insulin receptor-containing tissues possess several insulin-binding species with distinct affinities for insulin, then the curvilinear plots associated with insulin binding are due to heterogeneity of binding sites rather than negative cooperativity.

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