Morimoto, K., & Harrington, W. F. (1974) *J. Mol. Biol. 88*, 693-709.
Murray, J. M., Weber, A., & Bremel, R. D. (1975) in *Calcium Transport in Contraction and Secretion* (Carafoli, E., et al., Ed.) pp 489-496, North-Holland, Amsterdam.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628-4633.
Sellers, J. R., Chantler, P. D., & Szent-Györgyi, A. G. (1980) *J. Mol. Biol.* (in press).
Sillen, L. G., & Martell, A. E. (1971) in *Stability Constants of Metal-Ion Complexes*, The Chemical Society, London.
Simmons, R. M., & Szent-Györgyi, A. G. (1978) *Nature (London) 273*, 62-64.
Simmons, R. M., & Szent-Györgyi, A. G. (1980) *Nature (London) 286*, 626-628.
Stafford, W. F., Szentkiralyi, E. M., & Szent-Györgyi, A. G. (1979) *Biochemistry 18*, 5273-5280.
Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1-5.
Szent-Györgyi, A. G. (1975) *Biophys. J. 15*, 707-723.
Szent-Györgyi, A. G., Cohen, C., & Kendrick-Jones, J. (1971) *J. Mol. Biol. 56*, 239-258.
Szent-Györgyi, A. G., Szentkiralyi, E. M., & Kendrick-Jones, J. (1973) *J. Mol. Biol. 74*, 179-203.
Taussky, H. M., & Schorr, E. (1952) *J. Biol. Chem. 202*, 675-685.
Tawada, Y., & Tawada, K. (1975) *J. Theor. Biol. 56*, 269-283.
Weber, A., Herz, R., & Reiss, I. (1964) *Proc. R. Soc. London, Ser. B 160*, 489-501.

Chromatographic Resolution of Insulin Receptor from Insulin-Sensitive D-Glucose Transporter of Adipocyte Plasma Membranes†

Christin Carter-Su,* Paul F. Pilch, and Michael P. Czech

ABSTRACT: The chromatographic properties of the affinity-labeled adipocyte insulin receptor and the insulin-sensitive hexose transporter were evaluated in order to test the hypothesis that the transporter might be composed of receptor polypeptides. [^{125}I]Insulin was linked specifically and covalently to the high-affinity insulin receptor in dimethylmaleic anhydride extracted rat adipocyte plasma membranes by using the cross-linking reagent disuccinimidyl suberate [Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375-3381]. The membranes were solubilized with sodium cholate and resolved into three peaks of protein following hydroxylapatite chromatography. The first or void volume peak contained 10-20% of the protein, no receptor-bound [^{125}I]insulin, and no cytochalasin B sensitive D-glucose transport activity when reconstituted into phospholipid vesicles. The second major peak contained about half of the protein and as much as 87% of the affinity-labeled receptor but no hexose transport activity. The third peak contained 30-40% of the protein, 13-20% of the receptor-bound [^{125}I]insulin, and all of the recoverable D-glucose transport activity. Similar results were obtained by using diethylaminoethylcellulose (DEAE-cellulose) anion-exchange chromatography. More than 90% of the recovered receptor-linked [^{125}I]insulin eluted from this column at concentrations of NaCl below 1 M. These fractions contained no recoverable hexose transport activity. All of the hexose transporter recovered from the DEAE-cellulose column was eluted at higher NaCl concentrations. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the column fractions that contained all of the recovered D-glucose transporter from hydroxylapatite or DEAE-cellulose chromatography showed after autoradiography only traces of the affinity-labeled insulin receptor. These data indicate that the insulin receptor and D-glucose transporter represent distinct polypeptides in the fat cell membrane and imply that a transduction event must mediate hexose transport activation by insulin.

Although significant advances have been made in understanding how hormones interact with receptor proteins and what changes in cell function are brought about as a result of hormone–receptor interactions, little is known about the spatial relationship of membrane-bound hormone receptors and their effector systems. Two of the hormone receptor–effector systems for which such information is available are the acetylcholine and β-adrenergic receptor–effector systems. Huganir et al. (1979) have recently reported that the acetylcholine-dependent Na^+ channel appears to be part of the same protein complex as the acetylcholine receptor. The complex which can be isolated by using a choline carboxymethyl affinity column (Huganir et al., 1979) or α-neurotoxin affinity column (Heidmann & Changeux, 1978) is composed of four tightly associated but nondisulfide-linked peptides. These four peptides can be dissociated only following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Huganir et al., 1979; Heidmann & Changeux, 1978). When this purified acetylcholine receptor complex was reconstituted into phospholipid vesicles, carbamoylcholine-dependent, neurotoxin-sensitive Na^+ transport was observed (Huganir et al., 1979). In contrast to this observation is the finding that the β-adrenergic receptor is structurally distinct from its effector protein, adenylate cyclase. Sahyoun et al. (1977) separated erythrocyte ghost fragments enriched in β-adrenergic receptor from fragments enriched in adenylate cyclase by using discontinuous sucrose gradients. Vauquelin et al. (1977) found that the β-adrenergic receptor was retained by an alprenolol–agarose affinity column while adenylate cyclase activity

† From the Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912. *Received May 23, 1980.* This work was supported by Grants AM 17893 and HD 11343 from the National Institutes of Health. C.C.-S. is a postdoctoral fellow of the Juvenile Diabetes Foundation. P.P. is a postdoctoral fellow of the U.S. Public Health Service. M.C. is a recipient of Research Career Development Award AM 00249 from the U.S. Public Health Service.

0006-2960/81/0420-0216$01.00/0

passed unretarded. Limbird et al. (1980) report a differential elution on gel filtration columns of the β-adrenergic receptor, the adenylate cyclase modulating G protein, and adenylate cyclase activity.

Jarett & Smith (1979) have suggested that the insulin receptor and the insulin-sensitive D-glucose transporter in adipocyte plasma membranes may resemble the acetylcholine–Na^+ channel system in that both the receptor and transport protein are part of the same protein complex. Using ferritin–insulin to visualize insulin receptors in adipocyte plasma membranes by electron microscopy, they observed dissociation of insulin receptor clusters with low concentrations of cytochalasin B, a potent inhibitor of D-glucose transport. Cytochalasin D, which does not block hexose transport, had no effect on receptor clusters in these studies. A high correlation between the percent increase in single receptors and the percent decrease in glucose transport caused by cytochalasin B was found. Jarett and Smith suggested that groups of insulin receptors were therefore needed to form glucose transport pores. Cytochalasin B was hypothesized to inhibit transport by dispersing the receptors which resulted in disruption of the transport pore.

A technique to label covalently the high-affinity insulin receptor of rat adipocyte membranes by specifically cross-linking [^{125}I]insulin to its receptor with disuccinimidyl suberate has recently been developed in this laboratory (Pilch & Czech, 1979). We have also reported membrane reconstitution methodology for measuring D-glucose transport activity of detergent-solubilized adipocyte membrane proteins (Shanahan & Czech, 1977b). We have used these two techniques to study the chromatographic properties of the insulin receptor and the glucose transport protein in detergent solution. The data presented provide strong evidence that the adipocyte insulin receptor and the glucose transporter are distinct polypeptides which do not form a complex in mild detergent solution.

Materials and Methods

Preparation of Fat Cells and Membranes. Fat cells were isolated from the omental and parametrial adipose tissue of 250–500-g female Sprague–Dawley rats by collagenase digestion according to Rodbell (1966). Plasma membranes were prepared and extracted with dimethylmaleic anhydride as described previously (Carter-Su et al., 1980).

[^{125}I]Insulin Binding and Cross-Linking. Membranes suspended in 10 mM Tris[1]-HCl and 1 mM EDTA, pH 7.4, were pelleted (15 min at 30000*g*) and resuspended in Krebs–Ringer phosphate buffer containing 1% bovine serum albumin. This membrane suspension was incubated with 3 nM [^{125}I]insulin for 30 min at 24 °C, cooled to 0 °C, and washed by centrifugation as described above. The membrane pellet was resuspended in Krebs–Ringer phosphate at 0 °C and was treated with disuccinimidyl suberate [0.1 M in dimethyl sulfoxide, prepared according to Pilch & Czech, (1979)] to a final concentration of 1 mM. After 15 min, the cross-linking reaction was quenched by the addition of several volumes of 10 mM Tris and 1 mM EDTA, pH 7.4, and the membranes were collected by centrifugation (15 min at 30000*g*). Cross-linked membranes (2–6 mg in 1–4 mL) were solubilized with 2–4% (w/v) sodium cholate. Following a 30–60-min incubation on ice, the solubilized membranes were centrifuged at 40000*g* for 45 min. The supernatant routinely contained 60–80% of both the membrane protein and [^{125}I]-insulin. When adipocyte plasma membranes are cross-linked to [^{125}I]insulin under these conditions, an estimated 25% of the occupied [^{125}I]insulin binding sites are cross-linked. This amounts to 12.5% of the saturable binding sites at the insulin concentration used (Pilch & Czech, 1979).

Chromatography. In Figures 1 and 2, solubilized protein was added to a column (28 × 1 cm) containing hydroxylapatite (HTP, Bio-Rad Laboratories) equilibrated in 10 mM K_2HPO_4–KH_2PO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. The solubilized protein was eluted with a linear K_2HPO_4–KH_2PO_4 gradient. Prior to ion-exchange chromatogaphy shown in Figure 3, solubilized cross-linked membranes were passed through a column (26 × 1 cm) containing Sephadex G-75 (Sigma Chemical Co.) equilibrated with 10 mM NaCl, 10 mM K_2HPO_4–KH_2PO_4 and 0.5% sodium cholate, pH 7.4, in order to separate free [^{125}I]insulin from the proteins which had [^{125}I]insulin cross-linked to the high-affinity receptor. This procedure routinely removed 40–50% of the total radioactivity.

Ion-exchange chromatography was carried out by using DEAE-cellulose (15 × 1 cm, Cellex D, Bio-Rad) equilibrated in 10 mM NaCl and 10 mM K_2HPO_4–KH_2PO_4, pH 7.4. The solubilized protein was eluted with a linear NaCl gradient (0.01–1.0 M) in 0.5% sodium cholate and 10 mM K_2HPO_4–KH_2PO_4, pH 7.4. Further protein was removed with addition of 4 M NaCl, 10 mM K_2HPO_4–KH_2PO_4, and 0.5% sodium cholate, pH 7.4. All chromatography was carried out at 4 °C.

Sample Concentration and Reconstitution of D-Glucose Transport Activity. Chromatography column fractions were assayed for protein (see below) and pooled as indicated in the figures. The pooled samples were concentrated and/or dialyzed in the presence of exogenous phospholipids [2.5 mg of egg phosphatidylcholine (Sigma) and 2.5 mg of egg phosphatidylethanolamine (Supelco)] by using vacuum dialysis (25 000 molecular weight cutoff collodion bags, Arthur H. Thomas Co.) at 4 °C (Figures 1 and 2) or ultrafiltration followed by diafiltration with the Amicon Corp. MMC Multiphor (PM 10 membranes) at room temperature (22 °C) (Figure 3).

Concentrated proteins (250 μL) were added to exogenous phospholipids (2.5 mg of egg phosphatidylethanolamine and 2.5 mg of egg phosphatidylcholine) previously dispersed by sonication in 250 μL of 2% sodium cholate, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4 (Shanahan & Czech, 1977b). Liposomes were prepared from this mixture by using the method of Brunner et al. (1976) as modified by Shanahan & Czech (1977b).

Glucose Transport in Extracted Membranes and Liposomes. D-Glucose uptake was assayed by using rapid filtration (Amicon filters, 0.2-μm pore size, 25-mm diameter) to separate vesicular glucose from medium glucose as described in detail previously (Shanahan & Czech, 1977b). Uptake of label was expressed as the amount of radioactivity accumulated at a given time minus the radioactivity bound on filters containing membranes to which D-[^{3}H]glucose and 1 mL of cold buffer were added simultaneously. Net D-glucose transport has been corrected for diffusional entry of glucose as measured by D-glucose uptake in the presence of 20 μM cytochalasin B, a potent transport inhibitor (Shanahan & Czech, 1977b; Czech, 1976). Figures 1 and 3 and Table I give the result of a representative experiment in which the transport values were determined in triplicate (Table I) or quadruplicate (Figures 1 and 3).

Electrophoresis. The membrane protein composition of the various column fractions was analyzed by using the NaDod-

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Table I: D-Glucose Transport of Dimethylmaleic Anhydride Extracted Membranes Cross-Linked to Insulin[a]

	control membranes		cross-linked membranes	
uptake condition	D-[^{3}H]-glucose uptake (pmol/mg of protein)	% equilibration	D-[^{3}H]-glucose uptake (pmol/mg of protein)	% equilibration
2 min	83 ± 1	62	57 ± 2	57
2 min plus cytochalasin B	52 ± 1	39	37 ± 1	38
60 min	132 ± 5	100	99 ± 9	100

[a] Dimethylmaleic anhydride extracted membranes were resuspended in Krebs–Ringer phosphate buffer and cross-linked to insulin by using 1 mM disuccinimidyl suberate as described under Materials and Methods. Control membranes, which were treated in an identical fashion but without disuccinimidyl suberate, and cross-linked membranes were incubated for 2 min or 60 min in the presence of 250 μM D-[^{3}H]glucose. The 60-min D-glucose value was used to determine percent equilibration on the basis of the observation that D-glucose is equilibrated or nearly equilibrated by 60 min (Carter-Su et al., 1980). Corrections for diffusional entry and nonspecific binding were made by using the D-[^{3}H]glucose uptake values in the presence of 25 μM cytochalasin B. The data shown are the mean ± SEM of triplicate determinations.

SO_4–polyacrylamide (7.5%) gel electrophoresis procedure described by Laemmli (1970). The molecular weight standards routinely run were β-galactosidase (M_r = 116 000, gift of Dr. Boris Rotman, Brown University), phosphorylase *b* (M_r = 94 000, Sigma), bovine serum albumin (M_r = 68 000, Armour), and ovalbumin (M_r = 45 000, Sigma). Gels were stained, destained, and dried prior to autoradiography on Kodak XR-5 film used with a Du Pont "Cronex" (TM) "Lightning plus" enhancing screen.

Membrane protein was estimated by a modification (Geiger & Bessman, 1972) of the method of Lowry et al. (1951) using as a standard a 5:3 mixture of human albumin and human globulin (Sigma). $NaDodSO_4$ was added in sufficient quantities to clarify phospholipid-containing samples. Phosphate concentrations of the fractions eluted from the hydroxylapatite column were determined by using the method of Ames (1966).

Results

The methodology for resolving the insulin receptor and the glucose transporter required the measurement of glucose transport in protein fractions from plasma membranes cross-linked to insulin by using disuccinimidyl suberate. We reported previously that treating intact adipocytes with the cross-linking reagent did not alter basal hexose transport activity (Pilch & Czech, 1979). As shown in Table I, we tested whether cross-linking insulin to the high-affinity receptor altered D-glucose transport activity in dimethylmaleic anhydride extracted adipocyte plasma membranes. These membranes are reported to be an enriched source of transport protein compared to nonextracted plasma membranes (Shanahan & Czech, 1977a). D-Glucose uptake was measured in both cross-linked and non-cross-linked membranes at 2 min in the presence and absence of 20 μM cytochalasin B, a potent inhibitor of glucose transport (Czech, 1976). The cytochalasin B sensitive transport was taken to represent carrier-mediated uptake. Significant D-glucose transport activity was preserved in the extracted membranes cross-linked to insulin. If D-glucose transport activity in the two membrane preparations is calculated in terms of percent equilibration by using the assumption that 60 min is sufficient time for D-glucose to equilibrate across the extracted membranes (Shanahan & Czech, 1977a), it is seen that D-glucose equilibrates across the cross-linked memranes at the same rate as in the control membranes. The cross-linking reaction does not appear to significantly alter glucose transport activity in dimethylmaleic anhydride extracted membranes under the conditions of these studies.

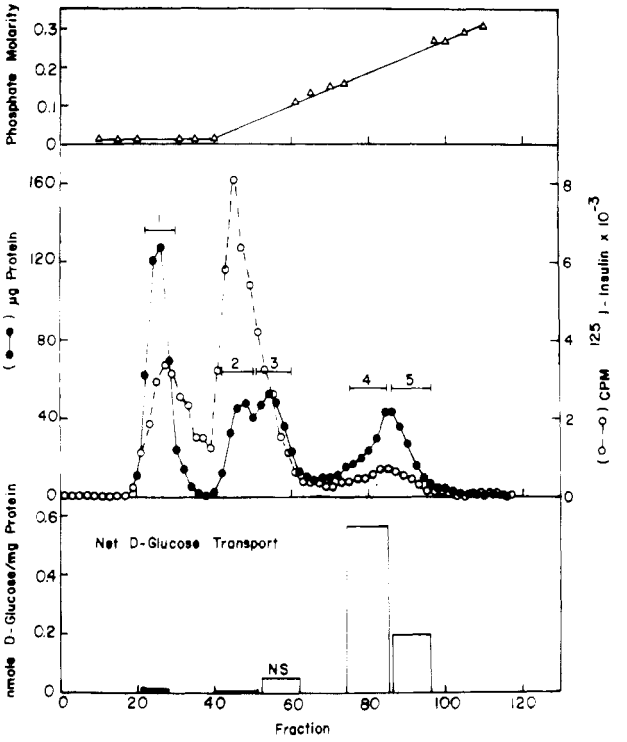

FIGURE 1: Use of hydroxylapatite chromatography to separate the hexose transporter from the high-affinity insulin receptor. [^{125}I]Insulin was cross-linked to dimethylmaleic anhydride extracted membranes, which were subsequently solubilized for 80 min on ice in 4 mL of 2% sodium cholate, 10 mM K_2HPO_4–KH_2PO_4, 0.02% NaN_3, and 5 mM dithiothreitol, pH 7.4. A portion (230 μg) of the solubilized protein was diluted to 5 mL with the above buffer containing 0.5% sodium cholate (no dithiothreitol) and kept overnight at 4 °C as the control. The remainder (1.8 mg in 3 mL) was applied to a hydroxylapatite column and eluted with a linear phosphate gradient. Fractions (1 mL) were collected, pooled, concentrated, and dialyzed along with the control against 10 mM Tris-HCl, pH 7.5, in the presence of exogenous phospholipids by using vacuum dialysis. The upper panel shows the phosphate concentration at which each of the fractions eluted. The middle panel shows the protein profile and the [^{125}I]insulin profile obtained following hydroxylapatite chromatography, and the fractions pooled for electrophoresis (see Figure 2). The lower panel shows the transport activity of pooled fractions from a parallel experiment using nonradioactive insulin which were reconstituted into artificial liposomes as described under Materials and Methods. Uptake was measured in the presence and absence of 20 μM cytochalasin B at 2 min. Standard errors of the mean for glucose transport in the presence or absence of cytochalasin B ranged between 0.03 and 0.12 nmol of D-glucose/mg of protein. The net transport shown for fractions 2 and 3 was found to be statistically insignificant at the 95% confidence level (Student's *t* test). Control uptake was 0.29 nmol of D-glucose/mg of protein.

In order to fractionate the proteins in the dimethylmaleic anhydride extracted membranes, we first cross-linked the extracted membranes to [^{125}I]insulin and then solubilized them in 2% sodium cholate in the presence of 5 mM DTT. The solubilized protein was added to a hydroxylapatite column equilibrated in 10 mM KH_2PO_4–K_2HPO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. Bound protein was eluted with a linear phosphate gradient. The middle panel of Figure 1 shows the protein and [^{125}I]insulin elution profiles of a representative fractionation. The upper panel shows the phosphate concentration at which each fraction eluted. As

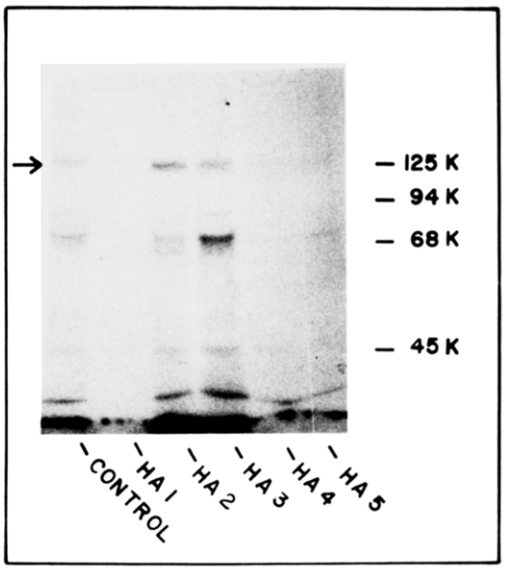

FIGURE 2: Autoradiography of solubilized membrane proteins cross-linked to [^{125}I]insulin and fractionated by using hydroxylapatite chromatography. Dimethylmaleic anhydride extracted membrane proteins were cross-linked to [^{125}I]insulin, solubilized, fractionated by using hydroxylapatite chromatography, pooled, and concentrated as described for the previous figure and under Materials and Methods. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by using a 7.5% Laemmli gel system. Samples were boiled in 1% $NaDodSO_4$ and 50 mM dithiothreitol. The molecular weight standard β-galactosidase (M_r = 116 000) was run but is not indicated in the figure. All samples contained 100 μg of protein except HA1 which contains 40 μg of protein. The lanes contained varying amounts of [^{125}I]insulin as follows: control, 3700 cpm; HA1, 3600 cpm; HA2, 9400 cpm; HA3, 4700 cpm; HA4, 1800 cpm; HA5, 1500 cpm. The arrow indicates the only high-affinity insulin binding protein (M_r = 125 000) present in these fractions. The other autoradiographic bands represent low-affinity or nonspecific binding sites (Pilch & Czech, 1980).

observed in experiments using solubilized proteins from non-cross-linked membranes (Carter-Su et al., 1980), the protein eluted from a hydroxylapatite column in three major peaks: a void peak (fraction HA1) and two additional peaks (fractions HA2 plus HA3 and fractions HA4 plus HA5). Total recovery of [^{125}I]insulin and protein added to the column was achieved.

The void peak (fraction HA1) routinely contained 30–40% of the total [^{125}I]insulin and 20–40% of the protein. Proteins in this fraction exhibited no detectable cytochalasin B sensitive uptake when concentrated and reconstituted into phospholipid vesicles (Figure 1). The void fraction also appeared to contain little or no insulin receptor on the basis of the following observations. When treated with 50 mM DTT in the presence of 1% $NaDodSO_4$, the high-affinity insulin receptor is known by previous studies (Pilch & Czech, 1979, 1980) to migrate as a major labeled band of M_r = 125 000, the band noted by the arrow in Figure 2. The other labeled bands in lanes 1 and 3–6 in Figure 2 represent low-affinity binding sites as evidenced by the disappearance of only the M_r = 125 000 band when the cross-linking reaction takes place in the presence of excess (1 μM) unlabeled insulin [Pilch & Czech (1979, 1980) and data not shown]. When the proteins in the void volume were concentrated by using vacuum dialysis and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography (Figure 2), all radioactivity ran at the dye front. This suggests that the [^{125}I]insulin in fraction HA1 as seen in Figure 2 was bound to proteins of very low molecular weight (<25 000) or was not covalently attached to membrane protein. When the proteins in the void peak were concentrated by using lyophilization rather than vacuum dialysis to ensure that no label was lost by adsorption to or passage through the dialysis bags, no [^{125}I]insulin ran behind the dye front whether DTT was present or absent during sample preparation (data not shown). When [^{125}I]insulin and solubilized dimethylmaleic anhydride extracted membranes were chromatographed simultaneously on a hydroxylapatite column under the same conditions as described in Figure 1, all of the radioactivity eluted in the same fraction as the first peak of radioactivity observed in Figure 1 (data not shown).

Greater than 87% of the remaining [^{125}I]insulin was found in fractions HA2 plus HA3 which eluted between 15 and 150 mM KH_2PO_4–K_2HPO_4 and routinely contained 30–40% of the total protein. A significant amount of [^{125}I]insulin was found to migrate at M_r = 125 000 when fractions HA2 and HA3 were concentrated and subjected to electrophoresis and autoradiography (Figure 2). These fractions contain more high-affinity insulin receptor per microgram of protein than the starting material (lane 1) as judged by the presence of darker bands at M_r = 125 000. Transport activity associated with these fractions was assessed by using protein fractions from a parallel experiment in which dimethylmaleic anhydride extracted membranes were cross-linked to unlabeled insulin (Figure 1, lower panel). The net D-glucose transport values exhibited are the cytochalasin B sensitive uptakes at 2 min. The proteins from fractions HA2 and HA3 contained no statistically significant hexose transport activity when they were concentrated and reconstituted into phospholipid vesicles. This is consistent with the finding that solubilized proteins from non-cross-linked membranes that elute at these phosphate concentrations do not exhibit D-glucose transport activity upon reconstitution into phospholipid vesicles (Carter-Su et al., 1980).

The final peak of protein in Figure 1 (fractions HA4 plus HA5) eluted between 150 and 300 mM K_2HPO_4–KH_2PO_4 and routinely contained 20–25% of the total protein and 10–15% of the protein bound [^{125}I]insulin. As seen in Figure 2, the two fractions comprising this peak (HA4 and HA5) contained little, if any, M_r = 125 000 receptor subunit per 100 μg of protein. Because the M_r = 125 000 receptor protein does not represent a larger percentage of the [^{125}I]insulin label in fractions HA4 or HA5 compared to fractions HA2 or HA3, we used the [^{125}I]insulin distribution in the different fractions as a rough estimate of the distribution of the high-affinity insulin receptor. When fractions HA4 or HA5 from membranes cross-linked to unlabeled insulin were concentrated and reconstituted into phospholipid vesicles, significant D-glucose transport activity was observed (lower panel, Figure 1). This recovery of transport activity in the protein fractions eluting between 150 and 300 mM K_2HPO_4–KH_2PO_4 is again consistent with the results obtained by using solubilized proteins that have not been cross-linked to insulin (Carter-Su et al., 1980). The data contained in Figures 1 and 2 show, therefore, that all recoverable D-glucose transport activity is found in protein fractions that contain <15% of the [^{125}I]insulin cross-linked to protein (HA4 and HA5). Conversely, no statistically significant transport activity was found in those fractions (HA2 and HA3) that contained the major proportion of the high-affinity insulin receptor.

The conclusion that the cholate-solubilized high-affinity insulin receptor can be separated from the cholate-solubilized glucose transporter was confirmed by using DEAE-cellulose chromatography. In the experiments depicted in Figure 3, dimethylmaleic anhydride extracted membranes were cross-linked to [^{125}I]insulin by using disuccinimidyl suberate and solubilized in 4% sodium cholate, 10 mM NaCl, and 10 mM

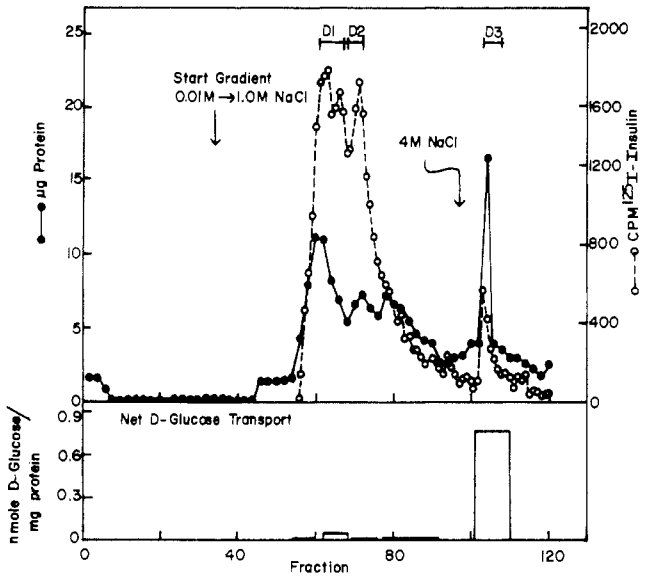

FIGURE 3: Resolution of D-glucose transport activity from the high-affinity insulin receptor following DEAE-cellulose chromatography. [^{125}I]Insulin was cross-linked to 2.3 mg of dimethylmaleic anhydride extracted membranes which were then solubilized for 30 min on ice in 1 mL of 4% sodium cholate, 10 mM NaCl, 10 mM K_2HPO_4–KH_2PO_4, and 5 mM DTT, pH 7.4. The soluble protein was added to a Sephadex G-75 column equilibrated in the above buffer containing 0.5% sodium cholate and no DTT. The void volume containing 60% of the radioactivity was pooled and added to a DEAE-cellulose column equilibrated in the above buffer without cholate (no DTT). Protein was eluted by using a linear NaCl gradient (0.01–1.0 M) in 0.5% sodium cholate. Additional protein was eluted with 4 M NaCl in 0.5% sodium cholate. The lower panel shows the transport activity of pooled fractions from a parallel experiment using membranes not cross-linked to [^{125}I]insulin. Dimethylmaleic anhydride extracted membranes (5.3 mg) were solubilized in 1.4 mL of 4% sodium cholate, 10 mM NaCl, 10 mM K_2HPO_4–KH_2PO_4, and 5 mM DTT, pH 7.4. A portion (100 μL) of the solubilized protein was diluted to 5 mL final volume of the above buffer in 0.5% sodium cholate (no DTT). The remainder (1.2 mL) was added to a DEAE-cellulose column equilibrated and eluted as above. Fractions (900 μL) were collected, pooled, concentrated, and dialyzed with the control against 10 mM Tris-HCl, pH 7.5, in the presence of exogenous phospholipids by using Amicon ultrafiltration. D-Glucose uptake was measured at 2 min in the presence and absence of 20 μM cytochalasin B. Standard errors of the mean for glucose transport in the presence and absence of cytochalasin B ranged between 0.05 and 0.15 nmol of D-glucose/mg of protein. Control uptake was 1.1 nmol of D-glucose/mg of protein.

K_2HPO_4–KH_2PO_4, pH 7.4. The solubilized proteins were chromatographed on a Sephadex G-75 column equilibrated in the above buffer containing 0.5% sodium cholate to remove free [^{125}I]insulin. The void volume fractions containing the solubilized protein (60% of the total [^{125}I]insulin) were pooled and added directly to a DEAE-cellulose column equilibrated in the solubilizing buffer without cholate. In experiments using non-cross-linked proteins, the presence or absence of cholate in the column prior to protein addition did not appear to significantly alter the final protein or transport activity elution profiles (data not shown). Protein was eluted from the DEAE-cellulose column with a linear NaCl gradient (0.01–1.0 M) in the presence of 10 mM K_2HPO_4–KH_2PO_4 and 0.5% sodium cholate, pH 7.4. Additional protein was eluted with a 4 M NaCl wash. As seen in Figure 3, 77% of the recovered protein eluted at NaCl concentrations <1 M. These fractions combined contained 93% of the recovered [^{125}I]insulin. In contrast, the 4 M NaCl wash eluted 23% of the recovered protein and 7% of the recovered [^{125}I]insulin. Autoradiographs of the concentrated column fractions following $NaDodSO_4$–polyacrylamide gel electrophoresis confirmed the presence of large amounts of receptor-bound [^{125}I]insulin in fractions D1 and D2 with little or no receptor in fraction D3 (data not shown).

Transport activity of the various fractions was assayed by using equivalent pooled fractions derived from solubilized non-cross-linked extracted membranes. The bottom panel of Figure 3 illustrates the finding that none of the fractions that eluted at <1 M NaCl contained any significant D-glucose transport activity when they were reconstituted into phospholipid vesicles. In contrast, significant transport activity was associated with the protein eluting with the 4 M NaCl wash. Thus, separation of the high-affinity insulin receptor from the D-glucose transporter was even more pronounced with the DEAE-cellulose anion-exchange column than with hydroxylapatite chromatography.

Discussion

The experiments described in this paper underscore the divergent chromatographic properties of the insulin receptor and the glucose transporter. As shown in Figures 1 and 3, cholate-solubilized hexose transporter binds more tightly to both hydroxylapatite and DEAE-cellulose than the high-affinity insulin receptor cross-linked to [^{125}I]insulin. These two proteins also appear to differ in their affinity for the lectin concanavalin A. Cuatrecasas & Tell (1973) reported that the Triton X-100 solubilized liver plasma membrane insulin receptor binds to concanavalin A and can be subsequently eluted with α-methyl mannoside. Jacobs et al. (1977) made use of this property as a basis for a receptor purification procedure. We have confirmed this result for the adipocyte insulin receptor by using the [^{125}I]insulin-labeled receptor and immobilized concanavalin A. When proteins were solubilized in Triton X-100, over 85% of the [^{125}I]insulin was bound by the immobilized lectin (data not shown). In contrast to what was observed with the insulin receptor, transport activity in cholate-solubilized membranes is recovered in the fraction that does not bind to concanavalin A (Carter-Su et al., 1980). Little or no transport activity is found associated with the protein that elutes from the column with 400 mM α-methyl mannoside.

The fact that glucose transport activity can be physically separated from the [^{125}I]insulin-labeled receptor in mild detergent solution suggests that the hexose transporter is not composed of receptor aggregates as suggested by Jarett & Smith (1979). The results of this paper would be compatible with Jarett and Smith's conclusion if the DTT used during the cholate solubilization of the membrane dissociated disulfide-linked receptor aggregates. Intact disulfide-linked receptor aggregates proposed by these workers to serve as transport pores may chromatograph differently than individual receptors. However, experiments not shown indicate that solubilization in the presence or absence of 5 mM DTT does not significantly alter the chromatographic properties of either the transport protein or the [^{125}I]insulin–receptor complex. These data also argue against the possibility that DTT cleaves off an [^{125}I]insulin-labeled portion of the receptor that migrates in the "receptor" peak from an unlabeled portion which migrates as the transporter. The experiments involving concanavalin A described above were also performed in the absence of a reducing agent, providing further evidence that the chromatographic properties of the receptor and the transporter differ even in the absence of DTT in the solubilizing buffer.

Our results show that most of the insulin receptor material in the extracted adipocyte membranes is associated with fractions that are free of hexose transport activity. However, small amounts of receptor did elute in the transport fraction (Figures 1 and 3). It could be argued that only this very small

fraction of the insulin receptor is responsible for all observable hexose transport activity. However, present evidence suggests that the D-glucose transporter represents as much of the membrane protein as the insulin receptor, or more. Scatchard plots of insulin binding indicate the receptor represents 0.01% (w/w) of the total adipocyte membrane protein (Pilch & Czech, 1980). With the assumption of a molecular weight of 300 000–350 000 for the native insulin receptor in the absence of DTT (Cuatrecasas, 1972; Jacobs et al., 1979; Pilch & Czech, 1980), there are roughly 0.3 pmol of receptors/mg of protein. The best estimate for the number of glucose transporters, based on the number of cytochalasin B binding sites as measured by Wardzala et al. (1978), is 4 pmol/mg of protein. This amount represents over 10-fold more transporters than the known number of receptors. These calculations employ the assumption that each D-glucose-specific cytochalasin B binding site reflects the presence of one glucose transport protein. Documentation of this point is not available for the adipocyte transporter. However, present evidence suggests that the physiologically similar erythrocyte hexose transporter binds at most one cytochalasin B molecule per band 4.5 protein, the putative glucose transporter (Sogin & Hinkle, 1978). Although exact quantitation of the recovery of transport activity is difficult because of the nature of the reconstitution assay (Carter-Su et al., 1980), estimates based on the 2-min glucose uptake values in vesicles made from control membranes vs. vesicles made from fractions HA4 and HA5 (Figure 1) indicate a total hexose transport recovery of at least 25%. This is a significantly greater recovery than would be expected if only the fraction of [^{125}I]insulin-labeled receptor found in the transporting fraction were responsible for all of the recoverable transport activity (Figure 1).

The results from these studies would be compatible with the results of Jarett & Smith (1979) if cytochalasin B's abilities to cause receptor dispersion and transport inhibition are not causally related. Rat adipocyte membranes have been shown to have at least three classes of high-affinity binding sites, only one of which is glucose displaceable (Wardzala et al., 1978). The nature of the non-glucose-displaceable binding sites in adipocyte membranes has not been studied in any detail.

The experiments described in this paper provide strong evidence that the high-affinity insulin receptor and insulin-sensitive glucose transporter exhibit diverse chromatographic properties and exist as separate polypeptides in the rat adipocyte plasma membrane. Further studies are necessary to determine whether these two components interact directly or whether some intermediary cytoplasmic or membrane component is necessary to mediate the receptor–transporter coupling event.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Lynne Rozek and thank Diane Goldman and Judy Carter for preparing membranes.

References

Ames, B. (1966) *Methods Enzymol. 8*, 115–117.
Brunner, J., Skrabal, P., & Hauser, H. (1976) *Biochim. Biophys. Acta 455*, 322–331.
Carter-Su, C., Pillion, D. J., & Czech, M. P. (1980) *Biochemistry 19*, 2374–2385.
Cuatrecasas, P. (1972) *J. Biol. Chem. 247*, 1980–1991.
Cuatrecasas, P., & Tell, G. P. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 485–489.
Czech, M. P. (1976) *J. Biol. Chem. 251*, 2905–2910.
Geiger, P. J., & Bessman, S. P. (1972) *Anal. Biochem. 49*, 467–473.
Heidmann, T., & Changeux, J.-P. (1978) *Annu. Rev. Biochem. 47*, 317–357.
Huganir, R. L., Schell, M. A., & Racker, E. (1979) *FEBS Lett. 108*, 155–160.
Jacobs, S., Schechter, Y., Bissell, K., & Cuatrecasas, P. (1977) *Biochem. Biophys. Res. Commun. 77*, 981–988.
Jacobs, S., Hazum, E., Schecter, Y., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4918–4921.
Jarett, L., & Smith, R. M. (1979) *J. Clin. Invest. 63*, 571–579.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Limbird, L. E., Gill, D. M., & Lefkowitz, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 775–779.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3381.
Pilch, P. F., & Czech, M. P. (1980) *J. Biol. Chem. 255*, 1722–1731.
Rodbell, M. (1966) *J. Biol. Chem. 241*, 130–139.
Sahyoun, N., Hollenberg, M. D., Bennett, V., & Cuatrecasas, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2860–2864.
Shanahan, M. F., & Czech, M. P. (1977a) *J. Biol. Chem. 252*, 6554–6561.
Shanahan, M. F., & Czech, M. P. (1977b) *J. Biol. Chem. 252*, 8341–8343.
Sogin, D. C., & Hinkle, P. C. (1978) *J. Supramol. Struct. 8*, 447–453.
Vacquelin, G., Geynet, P., Hanoune, J., & Strosberg, A. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3710–3714.
Wardzala, L. J., Cushman, S. W., & Salans, L. B. (1978) *J. Biol. Chem. 253*, 8002–8005.