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Reconstitution of the glucose transport activity of rat adipocytes

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Rat epididymal fat cell membrane proteins were extracted from adipocyte ghosts with octylglucoside and incorporated by detergent dialysis into unilamellar phosphatidylcholine vesicles approx. 200 nm in diameter. The rate of glucose transport into the vesicles under zero-trans conditions was substrate dependent, saturable and inhibited by phloretin and cytochalasin B. Their maximum specific transport activity was 35.6 μmol/min per mg protein, and their half saturation constant for glucose was 15 mM. Glucose transport into the reconstituted vesicles was inhibited by only those sugars which competitively inhibited glucose transport into intact adipocytes. A major protein component of the vesicles was a 100 kDa protein which we had previously found to react with the affinity label maltosyl isothiocyanate (Malchoff, D.M., Olansky, L., Pohl, S. and Langdon, R.G. (1981) Fed. Proc. 40, 1893). Removal of adipocyte ghost membrane extrinsic proteins with dimethylmaleic anhydride followed by extraction of the resulting membrane pellet with octylglucoside yielded a solution which contained two major proteins, of M_r 100000 and 85000, with very small quantities of lower M_r proteins. Vesicles into which these proteins were incorporated had average specific transport activities of 624 μmol/min per mg protein and half saturation constants of 22 mM. Our results strongly indicate that the native glucose transporter of the rat adipocyte, like that of the human erythrocyte (Shelton, R.L. and Langdon, R.G. (1983) Biochim. Biophys. Acta 733, 25–33), is a 100 kDa protein.

Introduction

Monosaccharides enter many mammalian cells passively by carrier-mediated diffusion. Identification of the glucose transport proteins of mammalian cells has been attempted using substrate analog affinity labels, photoactivated probes, cytochalasin B binding, and by reconstitution of detergent-extracted membrane proteins into phospholipid bilayer systems. Differential labelling experiments with fluorodinitrobenzene [1] and with *N*-ethylmaleimide [2], as well as studies on cytochalasin B binding [3,4] or photoincorporation [5,6] have all suggested that the glucose transporter of human erythrocyte membranes is a 45–55 kDa protein component of band 4.5. This component, which migrates as a very broad, diffuse band during polyacrylamide electrophoresis [7], has been prepared from erythrocyte membranes and found to have glucose transport activity when reconstituted into liposomes [7–9], unilamellar phospholipid vesicles [10–12] or planar bilayers [13]. Furthermore, the similar pattern of cytochalasin B photoincorporation into the membrane proteins of adipoctyes [14,15] and other mammalian cells [14,16–18] has been interpreted to mean that the glucose transporter of these cells is also a 45–55 kDa protein. On the other hand, affinity labelling of the erythrocyte membrane with the substrate analogs glucosyl [19] and maltosyl [20] isothiocyanate has indicated that the native transporter is

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a 100 kDa protein component of band 3 which can be degraded by endogenous proteolysis into a 55 kDa fragment which migrates during electrophoresis in the band 4.5 region. This interpretation has been strengthened by observations [21,22] which make it unlikely that there are sufficient quantities of the diffusely migrating protein or any of the small, discrete bands in the 4.5 region of carefully prepared erythrocyte membranes to account separately for either transport or cytochalasin B binding. Furthermore, unilamellar vesicles reconstituted with the purified 100 kDa transporter had specific transport activities much higher than those containing the purified band 4.5 material [23].

Although evidence obtained from cytochalasin B binding and photoincorporation experiments has been interpreted to mean that the adipocyte glucose transporter is a 50–55 kDa protein [14,15,24,25], covalent labelling of intact fat cells with the substrate analog probes [^{14}C]maltosyl isothiocyanate [26] and NAP-[^{3}H]glucosamine [27] have indicated that the native gluose transporter of adipocytes is an 80 kDa or 100 kDa protein.

Here we present methods for incorporating fat cell membrane proteins into large unilamellar phosphatidylcholine vesicles and show that the glucose transport properties of these vesicles closely resemble those of intact fat cells. Purification of the transporter and its reconstitution into vesicles has revealed that it is most probably a 100 kDa protein.

Materials and Methods

Male Sprague-Dawley rats weighing 150–225 g were obtained from Hilltop Laboratories. Collagenase was purchased from Worthington Diagnostic Systems, and bovine serum albumin fraction V was from Reheis Chemical Co. Sodium dodecyl sulfate, Tris, EDTA, phloretin, glucose oxidase (type V), glucose 6-phosphate, glucose 1-phosphate, 3-*O*-methylglucose, mannose, galactose, L-glucose, fructose, sucrose, maltose, 2-deoxyglucose and dimethylmaleic anhydride were purchased from Sigma Chemical Co. D-Glucose was from Pfanstiehl Laboratories. Dithiothreitol, acrylamide, 2-mercaptoethanol, silicone oil and cytochalasin B were purchased from Aldrich Chemical Co. Dimethylsulfoxide was from Pierce. Avanti Polar Lipids supplied *sn*-1-palmitoyl-2-oleoylphosphatidylcholine, the phospholipid used in all reconstitution experiments. *n*-Octyl-β-D-glucopyranoside (octylglucoside) was from Calbiochem. Sepharose CL-6B and Sephadex G-15 were Pharmacia products. Ammonium persulfate and *N,N,N′,N′*-tetramethylene ethylenediamine (TEMED) were from Bio-Rad Laboratories. D-[U-^{14}C]Glucose (1–5 mCi/mmol) was obtained from New England Nuclear, and EP scintillation cocktail was from Beckman Instruments. CX10 single use ultrafiltration units were supplied by Millipore. All other chemicals were reagent grade obtained from commercial sources. All aqueous solutions were prepared in water which had been deionized and then distilled in glass. Acrylamide was recrystallized from acetone, and sodium dodecyl sulfate was recrystallized from 95% ethanol before use. Prior to their use in reconstitution experiments, concentrated solutions of glucose oxidase and catalase were mixed and passed through a Sephadex G-15 column which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA.

Adipocytes and their membrane ghosts were prepared from rat epididymal fat pads [28,29]. The ghost membranes (1.0–1.5 mg protein) were extracted at 0°C with 340 μmol octylglucoside in 0.5 ml of 10 mM sodium phosphate (pH 7.0) containing 0.1 mM EDTA. After 20 min the mixture was centrifuged at 100 000 × *g* for 45 min at 4°C. The clear supernatant fraction was removed from a small gelatinous pellet and combined with 26 μmol *sn*-1-palmitoyl-2-oleoylphosphatidylcholine in 1.5 ml of 10 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 170 μmol octylglucoside, 1000 units of glucose oxidase and 5000 units of catalase. This solution was dialyzed at 4°C against 1 liter of 10 mM sodium phosphate (pH 7.0) containing 0.1 mM EDTA and 0.25 mM 2-mercaptoethanol. Dialysis was continued for 36 h, and the dialysis buffer was changed at 12-h intervals. During this time the preparation became turbid due to the formation of vesicles. After dialysis, the vesicle suspension was passed at 4°C through a 1 × 50 cm column of Sepharose CL-6B which had been 'conditioned' by passing through it a suspension of vesicles in dialysis buffer followed by extensive

washing with dialysis buffer containing no mercaptoethanol. The vesicles resulting from reconstitution emerged from the column near its void volume and were completely separated from extravesicular glucose oxidase and catalase.

Fat cell ghosts were extracted with dimethylmaleic anhydride according to Pillion et al. [30]. Briefly, membranes (4–10 mg protein per ml of 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA) were diluted to 56 ml with water, and 112 mg of dimethylmaleic anhydride was added with constant stirring; the pH was maintained between 8.0 and 8.5 by addition of 2 M NaOH. When the pH remained constant, the mixture was centrifuged at $32\,000 \times g$ for 35 min at 4°C. The supernatant was withdrawn, and the pellet was resuspended in sufficient 10 mM sodium phosphate buffer, 0.1 mM EDTA (pH 7.0) to give the desired protein concentration. The supernatant was concentrated at 4°C using a CX-10 ultrafilter until the desired protein concentration was attained. Both fractions were then used for reconstitution into phospholipid vesicles as described above.

Glucose transport into vesicles under zero-trans conditions was assayed at 25°C by measuring oxygen uptake in a miniature oxygen electrode chamber as previously described [23,31,32]. Sufficient volumes of 100 mM phloretin and 640 μM cytochalasin B in dimethylsulfoxide were added to give the final indicated concentrations. Addition of equivalent volumes of dimethylsulfoxide alone had no effect on transport. Hexose uptake into isolated adipocytes at 25°C was measured for 1 min using 1 mM [U-^{14}C]glucose and a rapid oil centrifugal flotation method [33] for separation of the adipocytes.

The protein content of fat cells and their ghost membranes was measured by the Lowry method [34] using bovine serum albumin as the standard. The protein content of reconstituted vesicles was measured by amino acid analysis as previously described [21,35]. Phospholipid content of vesicles was measured by the Bartlett method [36].

Electrophoresis was performed using 7.5% polyacrylamide gels prepared according to Laemlli [37]. Samples were made 2% in sodium dodecyl sulfate and 5 mM in dithiothreitol and heated at 100°C for 1 min. Separated proteins in the gels were visualized by the silver staining method of Morrisey [38]. Molecular weight markers, purchased from Sigma, were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, egg albumin and carbonic anhydrase, which had the molecular weights indicated in the figures.

Results

Large, unilamellar phosphatidylcholine vesicles with adipocyte membrane proteins in their membranes and glucose oxidase plus catalase in their interior volumes were formed by detergent dialysis. Electron micrographs, illustrated in Fig. 1, revealed that the vesicles had diameters of 191 ± 11 nm. In vesicles reconstituted in the absence of glucose oxidase and catalase we recovered $40 \pm 4\%$ of the added phospholipid, $18 \pm 6\%$ of the adipocyte ghost protein and $1.9 \pm 0.7\%$ of the total adipocyte protein. The rate of glucose influx into vesicles as a function of external glucose concentration in a typical experiment is illustrated in Fig. 2. The influx can be resolved into a major component which is saturated at high glucose concentrations and a minor linear component which represents a 'leak'. Since the amount of glucose oxidase incorporated into the interior vesicular space was sufficient to maintain intravesicular glucose near zero [31,32], zero-trans conditions were operative, and the kinetic equation describing the observed results is:

$$J_{in} = \frac{V_t[G]}{K_t + [G]} + k[G]$$

where J_{in} is the observed rate of glucose influx at the external glucose concentration [G], K_t is the half-saturation constant, V_t is the maximal transport rate, and k is a constant describing the leak rate. The values of the constants were computed using a nonlinear least-squares program [39]. Based on 14 separate reconstitution experiments $K_t = 15 \pm 4$ mM and $V_t = 35.6 \pm 0.9$ μmol glucose transported per min per mg membrane protein; the leak constant, k, was 0.1 ± 0.1 μl/min.

To ensure that the observed glucose influx into these vesicles was due to the insertion of adipocyte membrane proteins into their lipid bilayers, several control studies were undertaken. Fig. 3 shows one of two experiments in which vesicles were formed with adipocyte ghost extracts or with an equal

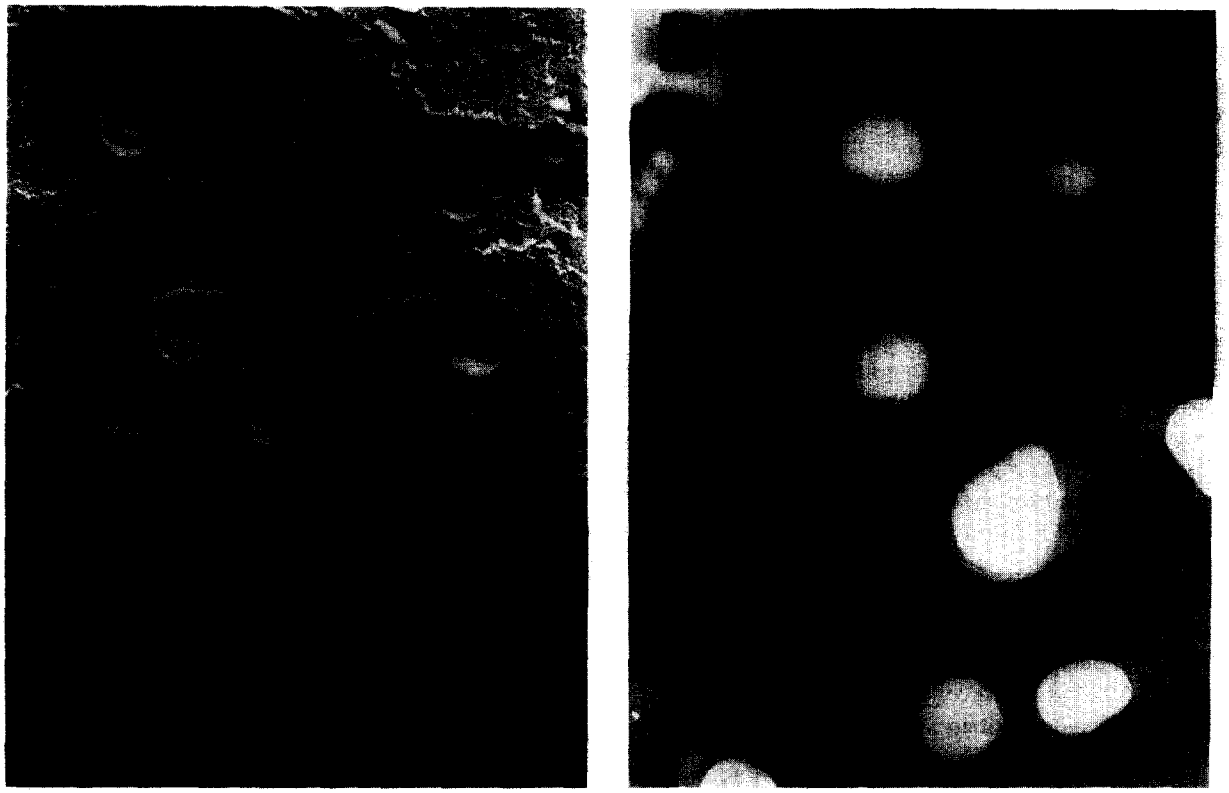

Fig. 1. Electron micrographs of phosphatidylcholine vesicles containing adipocyte ghost proteins. (A) Vesicles which had been freeze-fractured and etched. (B) Vesicles which had been negatively stained with 2% phosphotungstic acid.

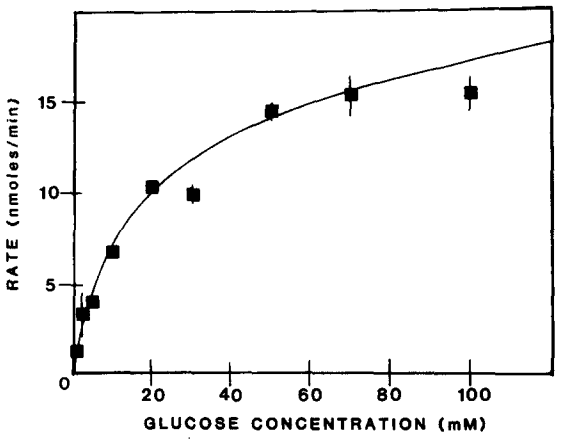

Fig. 2. The glucose transport activity of phosphatidylcholine vesicles containing adipocyte ghost proteins. This is a computer-generated plot of the data from a typical experiment. Error bars represent the standard deviations of each experimental point.

amount of bovine serum albumin. It is apparent that vesicles reconstituted with adipocyte proteins showed the usual transport rates, while those formed with serum albumin had no transport activity. Vesicles reconstituted with erythrocyte glycophorin or vesicles formed in the absence of test proteins also had no transport activity. However, all of these vesicles contained the same quantity of glucose oxidase, which could be liberated from their internal spaces by lysis with Triton X-100 [31,32]. To ensure that the activity observed in vesicles reconstituted with adipocyte proteins was not due to externally adherent glucose oxidase, vesicles were prepared with adipocyte proteins but without glucose oxidase or catalase; after the vesicles had formed, glucose oxidase and catalase were added, and the mixture was passed through a Sepharose CL-6B column in the usual manner, and the vesicle fraction was analyzed for glucose oxidase activity. None was found.

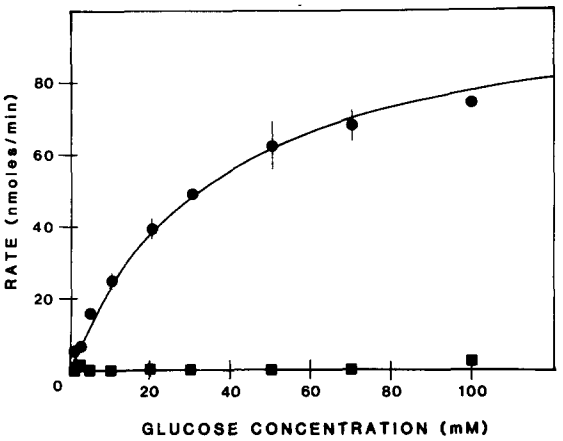

Fig. 3. The glucose transport rates of vesicles reconstituted with adipocyte ghost proteins (●) compared with vesicles formed in the presence of bovine serum albumin (■). Error bars as in Fig. 2.

Glucose transport into vesicles reconstituted with adipocyte membrane proteins was inhibited by compounds known to inhibit glucose transport into intact fat cells and by other transport substrates. As shown in Table I, transport into the vesicles was inhibited 40% by cytochalasin B and 34% by phloretin. The competitive sugars 3-*O*-methylglucose, 2-deoxyglucose, maltose and mannose inhibited glucose influx into both vesicles and intact adipocytes. Those sugars which did not significantly inhibit glucose entry into adipocytes also had little effect on glucose influx into vesicles. 2-*O*-Methylglucose, mannose and maltose are not substrates for the enzyme glucose oxidase; however, 2-deoxyglucose is oxidized at 20% the rate of glucose [40], and the observed extent of inhibition of glucose influx into vesicles by this sugar is probably less than actually occurred.

Vesicles reconstituted with adipocyte ghost proteins contained several of the proteins which are present in adipocyte ghosts. As shown in Fig. 4, vesicles reconstituted in the absence of glucose oxidase and catalase contained protein bands having M_r values ranging from approx. 50000 to 210000, although the protein pattern was somewhat simpler than that of adipocyte ghosts themselves. In vesicles reconstituted in the presence of glucose oxidase and catalase, the protein band having an M_r value of 85000 was obscured by glucose oxidase, M_r 82000.

No attempt had been made in the preceding

TABLE I

INHIBITORY EFFECTS OF VARIOUS COMPOUNDS ON GLUCOSE TRANSPORT ACTIVITY OF ISOLATED ADIPOCYTES AND RECONSTITUTED ADIPOCYTE GHOST VESICLES

Transport was determined at 25°C. Adipocyte uptake was assessed with 1 mM [U-^{14}C]glucose and 10 mM test sugar; vesicle transport was measured under zero-trans conditions using 5 mM glucose and 100 mM test sugar. The concentrations of cytochalasin B and phloretin were 52 μM and 500 μM, respectively. The percent inhibition was calculated as [(control rate − inhibited rate) × 100]/control rate. Values are the means ± S.D. of three experiments.

Additions	Inhibition (%)	
	adipocytes	vesicles
Cytochalasin B	100	40 ± 8
Phloretin	100	34 ± 5
Mannose	44 ± 0.1	36 ± 24
3-*O*-Methylglucose	57 ± 0.3	30 ± 5
Maltose	34 ± 0.1	32 ± 7
2-Deoxyglucose	44 ± 0.3	64 ± 13
Galactose	9 ± 0.2	12 ± 9
L-Glucose	18 ± 0.1	1 ± 29
Sucrose	8 ± 0.1	16 ± 4
Fructose	7 ± 0.1	8 ± 3
Glucose 1-*P*	5 ± 0.1	14 ± 1
Glucose 6-*P*	3 ± 0.2	0 ± 6

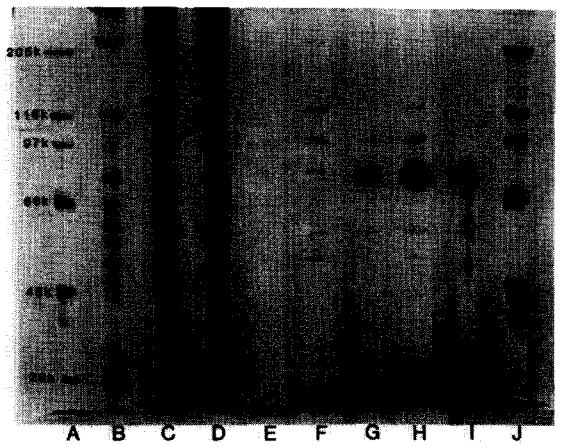

Fig. 4. Polyacrylamide gel analysis of adipocyte ghosts and phosphatidylcholine vesicles reconstituted with octylglucoside extract of adipocyte ghosts. Lanes A and J are molecular weight markers; B–D, 5, 10 and 20 μl of adipocyte ghosts, 4.9, 9.8, and 19.5 μg of protein; E and F, 30 and 50 μl aliquots of vesicles prepared in the absence of glucose oxidase and catalase, 0.46 and 0.76 μg protein; G and H, 30 and 50 μl of vesicles made in the presence of glucose oxidase and catalase; I, glucose oxidase and catalase. Proteins were stained with silver.

experiments to strip peripheral proteins from the adipocyte ghost membranes prior to octylglucoside extraction or to fractionate the proteins in the extracts. However, it obviously was desirable to attempt to identify which of the proteins visible in the polyacrylamide gels of the reconstituted vesicles was responsible for their transport activity. Dimethylmaleic anhydride extraction has been employed previously to strip membranes of peripheral proteins while leaving integral proteins with the membrane [41]. In four experiments we extracted fat cell ghosts with dimethylmaleic anhydride and centrifuged the mixture; in each, approx. 90% of the total membrane protein was recovered in the supernatant fraction, while 10% was recovered in the sedimented pellet. As shown in Fig. 5, the principal protein components of the pellet had M_r values of 100000 and 85000, while the supernatant fraction contained the remaining constituents. It should be noted that the minor protein components visible in the pellet were present much more abundantly in the supernatant fraction than in the pellet, but the M_r 85000 and 100000 proteins appeared to be present only in the pellet. The pellet and the supernatant fraction were each mixed with octylglucoside, and their solubilized components were separately reconstituted into phosphatidylcholine vesicles in the usual manner. The results of a representative experiment are illustrated in Fig. 6. Vesicles recon-

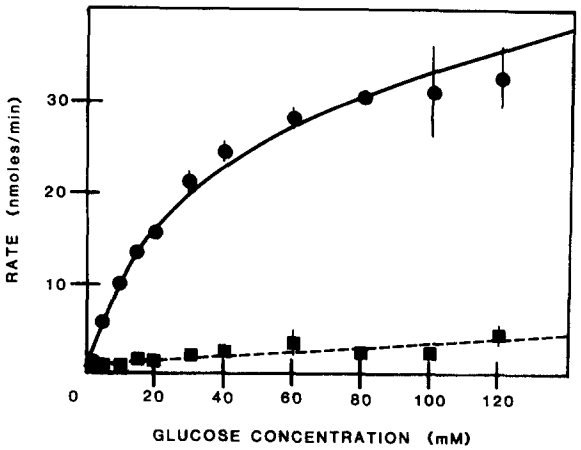

Fig. 6. The glucose transport rates of vesicles reconstituted with octylglucoside solutions of the pellet and supernatant fractions resulting from dimethylamleic anhydride extraction of adipocyte ghosts. This is representative of the results obtained by reconstituting phosphatidylcholine vesicles with the pellet (●) and the supernatant fractions (■). Error bars as in Fig. 2.

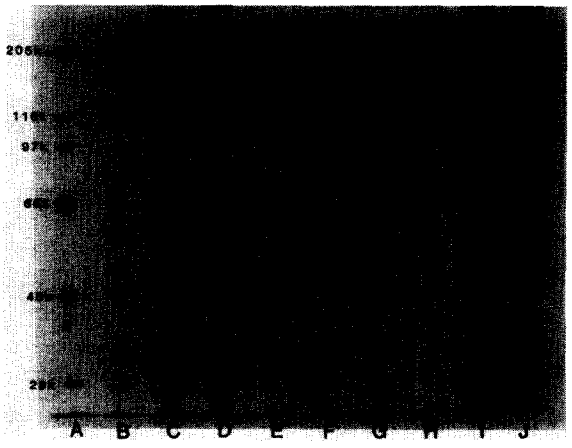

Fig. 5. Polyacrylamide gel electrophoresis of adipocyte ghosts, the proteins extracted by dimethylmaleic anhydride, and the pellet remaining after dimethylmaleic anhydride extraction. Lane A, molecular weight markers; B–D, adipocyte ghosts,2.1, 4.2, and 6.3 μg protein; E–G, pellet fraction, 0.93, 1.86, and 2.79 μg protein; H–J, supernatant fraction, 2.14, 5.35 and 10.7 μg protein.

Fig. 7. Polyacrylamide gel electrophoretic patterns of the supernatant and pellet fractions resulting from dimethylmaleic anhydride extraction of adipocyte ghosts and the vesicles reconstituted with these fractions. Lanes A and J, molecular weight markers; B, adipocyte ghosts, 47.8 μg protein; C, the supernatant fraction, 17.7 μg protein; D, pellet fraction, 4.5 μg protein; E and F, vesicles reconstituted with the supernatant fraction without (E) and with (F) glucose oxidase and catalase; G and H, vesicles reconstituted with the pellet fraction without (G) and with (H) glucose oxidase and catalase. Lane G contained approx. 60 ng of protein. Lane I, glucose oxidase and catalase.

stituted with proteins extracted from the pellet by octylglucoside transported glucose in a substrate-dependent, saturable manner, while those prepared with the supernatant fraction demonstrated no influx other than a leak. Analysis of the kinetics of uptake by vesicles reconstituted with octylglucoside extracts of the pellets from four experiments revealed that their K_t for glucose was 22 ± 5 mM and their maximum specific transport rate was 624 ± 460 μmol/min per mg membrane protein; this is approx. 18-times greater than the maximum transport rate obtained in vesicles reconstituted from octylglucoside extracts of adipocytes ghosts prior to dimethylmaleic anhydride extraction. The rate of glucose influx into these vesicles was decreased by 100 μM phloretin but not by 52 μM cytochalasin B. Electrophoresis of vesicles prepared from the supernatant and the pellet revealed, as shown in Fig. 7, that vesicles reconstituted with the supernatant fraction contained very little protein. On the other hand, approx. 5% of the total pellet protein was recovered in their reconstituted vesicles; electrophoresis showed that proteins of M_r 100000 and 85000 were the major ones in these vesicles, and as is evident from Fig. 7, the predominant component was the 100 kDa protein.

Discussion

Previous attempts to identify the glucose transporter of the adipocyte membrane have relied extensively on covalent affinity labelling, and the results have been discordant. The use of affinity labelling requires that the rather exacting established criteria for affinity labels [43] be satisfied if the results are to be meaningful, and these criteria have often been only partially satisfied. Carter-Su et al. [5] have employed photoincorporation of radioactive cytochalasin B into adipocyte membranes as a probe and have reported glucose-inhibitable labelling of a broad, diffuse protein band whose average M_r was around 50000. Similar results have been reported using other cells [11,16–18]. Cytochalasin B, while it is a potent inhibitor of glucose transport, is not a substrate analog. Moreover, its extent of incorporation into the M_r 50000 protein was so small that no inhibition of transport could be demonstrated. The kinetics of incorporation were not investigated sufficiently to tell whether the rate of incorporation showed saturation. Evidence that cytochalasin B binds to the adipocyte transporter is derived principally by analogy with experiments which showed that liposomes or planar bilayers containing a diffuse protein band average (M_r 45000–55000) derived from erythrocyte ghosts, which we have denoted band 4.5b [23], both bound cytochalasin B and transported glucose [3,4,7–9]. Very small amounts of cytochalasin B were also photoincorporated into this region of erythrocyte membranes [5,6]; like the fat cell, insufficient numbers of cytochalasin molecules were incorporated per cell so that inhibition of glucose transport, had it occurred, would have been detectable. On the other hand, affinity probes structurally related to glucose have labelled proteins of M_r 81000 and 100000 in the membranes of intact adipocytes [26,27]. Of the covalent affinity probes, only maltosyl isothiocyanate [20,42] has satisfied all of the rather stringent criteria for an affinity label; it reacted specifically and stoichiometrically with an M_r 100000 protein of intact erythrocyte membranes [20], and an M_r 100000 protein of intact adipocyte membranes was predominantly labelled [26]. Labelling of both cells was inhibited by glucose, and incorporation into the adipocyte membrane was stimulated by insulin.

Although affinity probes are useful for preliminary identification of transporters, final identification must depend upon purification of the putative transporter followed by its reconstitution in a functionally active form. Furthermore, sufficient activity must be recovered to account for transport in the intact cell in order to exclude the possibility that a minor component or a partially active degradation product of the native transporter has been isolated. Several laboratories have previously reconstituted glucose transport activity from rat adipocyte membranes into liposomes [44–46]. When Robinson et al. [45] incorporated extracts of adipocyte membranes into egg lecithin vesicles, the vesicles displayed glucose transport which was inhibited by cytochalasin B, phloretin and mercuric chloride; however, these investigators did not determine which membrane proteins had been incorporated. Phospholipid vesicles reconstituted with adipocyte plasma membrane

components by Carter-Su et al. exhibited glucose transport which was inhibited by cytochalasin B, but these investigators were unable to resolve which of the proteins incorporated was responsible for the transport activity [44]. Similar cytochalasin-inhibitable glucose transport was observed by Mora and Jones [46] in liposomes reconstituted from whole or partially purified plasma membranes; these liposomes contained three major proteins having relative mobilities between 0.5 and 0.75, but their molecular weights were not estimated. Another previous attempt to identify the transporter by extraction, purification and reconstitution also yielded inconclusive results [47].

We extracted adipocyte ghost membranes with octylglucoside, a nonionic detergent whose high critical micelle concentration facilitates its rapid removal by dialysis, a procedure which has been used successfully to form large unilamellar phospholipid vesicles with proteins embedded in their membranes [48] in a functionally active form [23,49]. As shown here, phosphatidylcholine vesicles containing adipocyte ghost proteins formed by detergent dialysis are large and unilamellar and possess glucose transport activity which we have compared with values for transport into intact adipocytes [50] and adipocyte plasma membrane vesicles [51,52] in Table II. Assuming that the specific transport activity of the total adipocyte ghost proteins was the same as that of the total plasma membrane proteins, it would appear that we achieved a 110-fold purification of the transporter by octylglucoside extraction and reconstitution alone. However, we recovered 18% of the ghost protein in the reconstituted vesicles; thus, the maximum increase in specific activity which could have been obtained by purification alone was 5.6-fold. We have considered, but not yet tested, several possible mechanisms which might account for this unexpected enhancement of activity. The lipid composition of the reconstituted vesicles is far different from that of the plasma membrane vesicles, and it is quite possible that this difference may affect the transporter's activity. It has been observed that the kinetic constants for glucose transport through the human erythrocyte membrane are considerably different in intact cells and in sealed ghosts; however, addition of the soluble cell contents to the ghosts prior to sealing them restored the original kinetic properties of intact cells [55]. Moreover, purification and reconstitution of the human erythrocyte glucose transporter [23] resulted in an apparent increase in its turnover number. These observations suggest the possibility that the turnover number of the glucose transporter may be modified by interactions with as yet unidentified soluble cytosolic constituents as well as components of the membrane. It also seems possible that some covalent modification of the transporter resulting in its activation may have occurred during its extraction and reconstitution. Finally, it is possible that the initial assumption of the equality of the specific activities of theplasma membrane vesicles and ghost membranes is incorrect, and that there is a larger pool of transporters residing in internal membranes of the cell, which are extracted from the ghosts and are functionally active when reconstituted, than previous work would suggest [45].

Vesicles reconstituted with unfractionated octylglucoside extracts of adipocyte ghosts contained a number of the proteins visible in polyacrylamide gels of ghosts themselves. The most abundant proteins in the vesicles had M_r values of 85 000 and 100 000, but variable amounts of other smaller proteins were present; these included proteins in the molecular weight range of the membrane constituent reported to be the site of cytochalasin B binding and photoincorporation [14,15,24,25]. We found that dimethylmaleic anhydride extracted 90% of the protein present in adipocyte ghosts, including the M_r 50 000 and 55 000 proteins. Attempts to reconstitute transport activity into vesicles with the extracted fraction were uniformly unsuccessful. By far the most abundant proteins retained in the membrane pellet fraction after dimethylmaleic anhydride extraction had M_r values of 100 000 and 85 000, with much smaller amounts of other proteins. In vesicles reconstituted with octylglucoside extracts of the pellet fraction the only detectable proteins were the M_r 100 000 and 85 000 components and much smaller quantities of other proteins which had molecular weights less than 40 0000. The maximum specific transport rate of these vesicles, 624 μmol/min per mg protein, was 18-fold greater than vesicles prepared from octylglucoside extracts of adipocyte ghosts prior to dimethylmaleic anhydride extrac-

tion. It is striking that, although the transport activity of these vesicles was inhibited by phloretin and had all the kinetic properties of the adipocyte glucose transporter, they were insensitive to cytochalasin B and did not contain the protein to which cytochalasin B has been reported to be bound [11,16,24,25]. This strongly suggests, but does not prove, that the cytochalasin B binding protein and the glucose transporter may be different proteins. Since cytochalasin B binding is competitively inhibited by glucose, and the cytochalasin binding site appears to be on the cytoplasmic side of the membrane [56] at a site remote from the transport site [57,58], it is tempting to speculate that this compound binds to a glucose-binding protein which normally modulates the activity of the glucose transporter and down-regulates it when intracellular glucose accumulates.

It is important in extraction and reconstitution experiments to calculate the total recovery of transport activity so that one may be assured that the activity recovered is of sufficient magnitude that it may be of importance in the intact cell. Jarett [59] has found 5.3 mg of protein per ml of packed fat cells. Whitesell and Gliemann [60] have estimated that the intracellular water volume of adipocytes is approx. 18 ml per liter of packed cell volume, and that the maximal entry rates for 3-*O*-methylglucose under equilibrium exchange conditions into basal and insulin stimulated cells are, respectively, 130 and 800 μmol/s per liter of cell water. When we began with adipocytes containing 80 mg of total protein, we recovered in reconstituted vesicles 0.04 mg of protein having a maximal specific transport rate of 10 μmol glucose/s per mg of protein or a maximal total activity of 0.4 μmol/s. It can be calculated that adipocytes containing 80 mg of protein would have a maximal total activity of 0.036 μmol/s in the basal state and 0.22 μmol/s after insulin stimulation. It is clear that on this basis a satisfactory recovery of total transport activity was obtained. However, because of uncertainties concerning the meaning and extent of the apparent increase in turnover number previously mentioned, these numbers must be interpreted with some caution.

In conclusion, the methods we have described for extracting the glucose transport activity of rat adipocytes and reconstituting it into phosphatidylcholine vesicles are rapid and efficient, and result in no obvious major modification of the transport protein. The predominant presence of a 100 kDa protein in reconstituted vesicles which transport glucose like fat cells suggests strongly that this protein is the transporter. As previously noted, this is consistent with the earlier observations that the substrate analog covalent affinity label maltosyl isothiocyanate labelled a 100 kDa protein of the adipocyte membrane in a stoichio-

TABLE II

KINETIC PARAMETERS OF GLUCOSE TRANSPORT BY ADIPOCYTES, MEMBRANE VESICLES AND VESICLES RECONSTITUTED FROM ADIPOCYTE GHOST EXTRACTS

ZT, zero-trans; EE, equilibrium exchange; DMMA, dimethylmaleic anhydride.

	Temp. (°C)	Conditions	K_t (mM)	V_t (μmol/min per mg protein)	Ref.
Adipocytes	37	ZT	8	0.027 [a]	59, 60
Plasma membrane vesicles	37	EE	9	0.192	52
Plasma membrane vesicles from insulinized adipocytes	37	EE	26	0.266	51
Vesicles reconstituted from adipocyte ghost extracts	25	ZT	15	35.6	present work
Vesicles reconstituted from DMMA pellet extracts	25	ZT	22.5	624	present work

[a] Calculated from values in Refs. 59 and 60.

metric manner; its binding was increased by insulin pretreatment of the cells and decreased by inclusion of D-glucose but not L-glucose during the labelling procedure [26]. The role in transport, if any, of the 85 kDa protein, which accompanied the 100 kDa protein during purification and which is probably identical with the 81 kDa protein described by Trosper and Levy [27], is not clear. Based on our present observations, we have concluded that the native glucose transporter of the rat adipocyte membrane, like that of the erythrocyte, is most probably a 100 kDa protein. However, participation of the 85 kDa protein cannot be excluded at this time.

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References

1 Lienhard, G.E., Gorga, F.R., Orasky, J.E. and Zoccoli, M.A. (1977) Biochemistry 16, 4921–4926
2 Batt, E.R., Abbott, R.E. and Schachter, D. (1976) J. Biol. Chem. 251, 7184–7190
3 Jung, C.Y. and Rampal, A.L. (1977) J. Biol. Chem. 252, 5456–5463
4 Pinkofsky, H.B., Rampal, A.L., Cowden, M.A. and Jung, C.Y. (1978) J. Biol. Chem. 253, 4930–4937
5 Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W. and Czech, M.P. (1982) J. Biol. Chem. 257, 5419–5425
6 Shanahan, M.F. (1982) J. Biol. Chem. 257, 7290–7293
7 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
8 Kahlenberg, A. and Zala, C. (1977) J. Supramol. Struct. 7, 287–300
9 Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914
10 Baldwin, J.M., Gorga, J.C. and Lienhard, G.E. (1981) J. Biol. Chem. 256, 3685–3689
11 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842
12 Carruthers, A. and Melchior, D.L. (1984) Biochemistry 23, 2712–2718
13 Nickson, J.K. and Jones, M.N. (1982) Biochim. Biophys. Acta 690, 31–40
14 Shanahan, M.F., Olson, S.A., Weber, M.J., Lienhard, G.E. and Gorga, J.C. (1982) Biochem. Biophys. Res. Commun. 107, 38–43
15 Oka, Y. and Czech, M.P. (1984) J. Biol. Chem. 259, 8125–8133
16 Pessin, J.E., Tillotson, L.G., Yamada, K., Gitomer, W., Carter-Su, C., Mora, R., Isselbacher, K.J. and Czech, M.P. (1982) Proc. Natl. Acad. Sci. USA 79, 2286–2290
17 Klip, A., Walker, D., Ransome, K.J., Schroer, D.W. and Lienhard, G.E. (1983) Arch. Biochem. Biophys. 226, 198–205
18 Johnson, L.W. and Smith, C.H. (1982) Biochem. Biophys. Res. Commun. 109, 408–413
19 Taverna, R.D. and Langdon, R.G. (1973) Biochem. Biophys. Res. Commun. 54, 593–599
20 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1205–1212
21 Shelton, R.L. and Langdon, R.G. (1984) Anal. Biochem. 140, 366–371
22 Jones, M.N. and Nickson, J.K. (1980) FEBS Lett. 115, 1–8
23 Shelton, R.L. and Langdon, R.G. (1983) Biochim. Biophys. Acta 733, 25–33
24 Wardzala, L.J., Cushman, S.W. and Salans, L.B. (1978) J. Biol. Chem. 253, 8002–8005
25 Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758–4762
26 Malchoff, D.M., Olansky, L., Pohl, S.L. and Langdon, R.G. (1981) Fed. Proc. 40, 1893
27 Trosper, T. and Levy, D. (1977) J. Biol. Chem. 252, 181–186
28 Rodbell, M. (1964) J. Biol. Chem. 239, 375–380
29 Rodbell, M. and Krishna, G. (1974) Methods Enzymol. 31, 103–114
30 Pillion, D.J., Shanahan, M.F. and Czech, M.P. (1978) J. Supramol. Struct. 8, 269–277
31 Taverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 298, 412–421
32 Shelton, R.L., Malchoff, D.M. and Langdon, R.G. (1984) in Methods in Diabetes Research, Vol. 1, Laboratory Methods Part B (Larner, J. and Pohl, S., ed.), pp. 63–74, John Wiley & Sons, New York
33 Gliemann, J., Osterlind, K., Vinten, J. and Gammeltoft, S. (1972) Biochim. Biophys. Acta 286, 1–9
34 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
35 Bohlen, P. and Schroeder, R. (1982) Anal. Biochem. 126, 144–152
36 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
37 Laemlli, U.K. (1970) Nature 227, 680–685
38 Morrissey, J.H. (1981) Anal. Biochem. 117, 307–310
39 Johnson, M.L., Correia, J.J., Yphantis, D.A. and Halvorson, H.R. (1981) Biophys. J. 36, 575–588
40 Pazur, J.H. (1966) Methods Enzymol. 9, 82–87
41 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
42 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1199–1205
43 Groman, E.V., Schultz, R.M. and Engel, L.L. (1977) Methods Enzymol. 46, 54–58
44 Carter-Su, C., Pillion, D.J. and Czech, M.P. (1980) Biochemistry 19, 2374–2385
45 Robinson, F.W., Blevins, T.L., Suzuki, K. and Kono, T. (1982) Anal. Biochem. 122, 10–19
46 Mora, J.E. and Jones, M.N. (1983) Biochem. J. 216, 113–120

47 Shanahan, M.F. and Czech, M.P. (1977) J. Biol. Chem. 252, 6554–6561
48 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840
49 Jackson, M.L. and Litman, B.J. (1982) Biochemistry 21, 5601–5608
50 Gliemann, J. and Rees, W.D. (1983) Curr. Top. Membranes Transp. 18, 339–379
51 Ludvigsen, C. and Jarett, L. (1980) Diabetes 29, 373–378
52 Ludvigsen, C. and Jarett, L. (1979) J. Biol. Chem. 254, 1444–1446
53 Karlish, S.J.D., Lieb, W.R., Ram, D. and Stein, W.D. (1972) Biochim. Biophys. Acta 255, 126–132
54 Lacko, L., Wittke, B. and Kromphardt, J. (1972) Eur. J. Biochem. 25, 447–454
55 Carruthers, A. and Melchior, D.L. (1983) Biochim. Biophys. Acta 728, 254–266
56 Basketter, D.A. and Widdas, W.F. (1978) J. Physiol. 278, 389–401
57 Krupka, R.M. and Deves, R. (1980) Biochim. Biophys. Acta 598, 137–133
58 Deves, R. and Krupka, R.M. (1980) J. Biol. Chem. 255, 11870–11874
59 Jarrett, L. (1974) Methods Enzymol. 31, 60–71
60 Whitesell, R.R. and Gliemann, J. (1979) J. Biol. Chem. 254, 5276–5283