

Identification and characterization of the rat adipocyte glucose transporter by photoaffinity crosslinking

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The photoaffinity crosslinking agent hydroxysuccinimidyl-4-azidobenzoate has been used to attach [³H]cytochalasin B to a rat adipocyte low-density microsomal membrane protein of 45–50 kDa. The characteristics of the [³H]cytochalasin B-labeled protein are consistent with those of the adipocyte glucose transporter. The low-density microsomes from cells incubated without insulin incorporate twice the amount of radioactivity per mg membrane protein than low-density microsomes derived from insulin-stimulated cells. This value agrees with the distribution of glucose transporters measured in this intracellular membrane fraction prepared from basal and insulin-treated cells by [³H]cytochalasin B binding. Preincubation of membranes with 500 mM D-glucose reduces the photoaffinity crosslinking by 48% relative to that observed with 500 mM L-glucose. Isoelectric focusing of low-density microsomes containing the photoaffinity crosslinked transporter yields three bands of radioactivity focusing at pH values of 5.5, 4.5, and 4.2 respectively. Following isolation from the isoelectric focusing gel and SDS–polyacrylamide gel electrophoresis, all three peaks can be shown to contain a band of 45–50 kDa which crossreacts with an antiserum raised against the purified human erythrocyte glucose transporter. These results suggest that the identification, isolation and purification of the adipocyte glucose transporter is now possible using the techniques described above.

<i>Adipocyte</i>	<i>Glucose transporter</i>	<i>Insulin</i>	<i>Photoaffinity labeling</i>
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1. INTRODUCTION

Insulin stimulates glucose transport in rat adipocytes and diaphragm by inducing a rapid and reversible translocation of glucose transporters from an intracellular membrane pool to the plasma membrane [1–6]. Further insights into the nature of the translocation process require the isolation and purification of glucose transporters from these tissues.

Cytochalasin B, a potent inhibitor of glucose transport, binds to specific sites on the glucose transporter and has thus provided the means for measuring the amount of glucose transporters in

various membrane preparations [1–7]. Recently it has been shown that cytochalasin B forms covalent linkages with the glucose transporter when bound and exposed to a high-intensity ultraviolet light source [8]. Although this is a simple method for labeling the glucose transporter, we have found that this technique suffers from both high levels of non-specific crosslinking and extensive radiation damage to the transporter and other membrane proteins. As a result, attempts to purify further the glucose transporter covalently labeled with cytochalasin B have failed.

As an alternative, we have chosen to crosslink cytochalasin B to the glucose transporter with the heterobifunctional crosslinking agent *N*-hydroxy-succinimidyl-4-azidobenzoate. This crosslinker has been employed for covalently linking peptide hor-

Abbreviation: SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

mones to their receptors [9,10]. We demonstrate here the efficacy and specificity of this technique for labeling the insulin responsive glucose transporter of the rat adipocyte.

2. MATERIALS AND METHODS

2.1. *Materials*

[³H]Cytochalasin B (spec. act. 10–15 Ci/mmol) was obtained from New England Nuclear. Carrier ampholytes and all other reagents for isoelectric focusing and electrophoresis were from Bio-Rad.

2.2. *Preparation of subcellular membrane fractions*

The low-density microsomal membrane fraction from isolated rat adipocytes was prepared by differential ultracentrifugation as in [11].

2.3. *Photoaffinity crosslinking*

Low-density microsomal membranes prepared from rat adipocytes were resuspended in 30 mM Tris-HCl (pH 7.4), to a final concentration of 1–5 mg membrane protein/ml. The membranes were incubated for 10 min at 4°C with [³H]cytochalasin B at a final concentration of 1–10 μM. The cytochalasin B was previously dried under nitrogen to evaporate the ethanol. Hydroxysuccinimidyl-4-azidobenzoate was then added to the membrane suspension at a final concentration of 390 μM. The membrane suspension was placed in wells on a porcelain plate which was cooled on ice. The membranes were photolysed for 2 min with a Rayonet RMR-400 photochemical reactor (254 nm) with the lamp placed 2 cm over the wells. Following photolysis, the membranes were separated from non-covalently bound cytochalasin B by centrifugation at 300 000 × *g*_{max} for 75 min in 30 mM Tris-HCl (pH 7.4). Experiments involving D- or L-glucose were carried out as above except that the sugar (final concentration 500 mM) was incubated with the membranes for 10 min prior to the addition of the cytochalasin B. Photolysis times were identical in all cases.

2.4. *SDS-PAGE*

Low-density microsomal membranes covalently crosslinked to [³H]cytochalasin B were solubilized by boiling for 5 min in Laemmli sample buffer [12]. The solubilized membranes were then cen-

trifuged at 160 000 × *g*_{max} for 15 min, and the supernatant was analyzed by SDS-PAGE on 5 mM (internal diameter) cylindrical gels. Resolving gels of 8% polyacrylamide were used. Electrophoresis was carried out at 3 mA per gel until tracking dye, bromphenol blue, had migrated a measured distance. After electrophoresis, the gels were serially sliced with a Hoefer vibrating gel slicer. The slices were incubated in 20 ml scintillation vials, 2 slices per vial, with 1 ml NCS (Amersham) for 2 h at 60°C. The solubilized gel slices were cooled, 10 ml scintillation fluid 3a20 (Research Products International) and 50 μl acetic acid added, and the vials counted in a scintillation counter. The radioactive profile obtained was used as a guide to locate the position of the glucose transporter in duplicate gels. Those slices containing the glucose transporter together with the two preceding and two following slices were extracted electrophoretically as in [13]. Prestained *M*_r marker proteins (Bethesda Research Labs) were used to estimate the *M*_r of the ³H-labeled membrane proteins.

2.5. *Isoelectric focusing*

The extracted and concentrated SDS-solubilized glucose transporter was subjected to isoelectric focusing on cylindrical polyacrylamide gels. Gel composition and isoelectric focusing conditions were as in [14]. Duplicate gels were run; one was processed for scintillation counting as above, while slices from the other gel were extracted for 1 h in 1 ml water (4 slices per tube) and the pH determined [15]. In addition to the analytical isoelectric focusing described above, we also subjected the photoaffinity labeled low-density microsomal membranes to preparative isoelectric focusing on Sephadex IEF (Pharmacia). A horizontal slab apparatus (PBE 3000, Pharmacia) was used. Catholyte and anolyte were those in [14]. Wicks were immersed in catholyte or anolyte, blotted, and placed in a gel trough. The Sephadex slurry of 200 ml containing 8 M urea, 2% octylglucoside, and 2% Pharmalyte (Pharmacia, *pI* range 5–8) was poured, and allowed to air-dry until 25% of the weight of the gel was lost. The gel was prefocused for 2 h at a constant voltage (1500 V) until the current fell to 20 mA. The photoaffinity labeled low-density microsomal membranes (20 mg) were solubilized in SDS, made to a volume

of 4 ml with water, and applied to the gel bed as described (Instruction Manual, PBE 3000, Pharmacia, pp.9–12). Isoelectric focusing was then carried out for 3 h at 1500 V. Two centimeter fractions were scooped out using a fractionation grid, and 9 ml of water were added to each. After centrifugation and a 2-fold repetition of the gel extraction, the combined supernatants (24–27 ml) were analyzed for pH and radioactivity. The recombinated supernatants were saturated with ammonium sulfate by the addition of 14 g solid ammonium sulfate and left overnight at 4°C. The protein precipitates were collected by centrifugation at $105\,000 \times g_{\max}$ for 30 min. The ammonium sulfate-precipitated proteins were washed with water, solubilized in 1% SDS by boiling for 5 min and desalted on a column of Sephadex G-25 equilibrated in 0.1% SDS. The desalted protein fractions (3.5 ml) were concentrated in 0.6 ml by vacuum centrifugation and were stored frozen at -20°C until required.

2.6. Immunological identification of the glucose transport

Aliquots of the protein fractions isolated from preparative isoelectric focusing as described above were taken up in Laemmli sample buffer and analyzed by SDS-PAGE on 3 mm slab gels containing 9% resolving gel. The gels were run at 100 mA for 2.5 h. The proteins were transferred electrophoretically to nitrocellulose paper as in [16]. Crossreacting protein was visualized using an antiserum against the purified human erythrocyte glucose transporter, ^{125}I -protein A and the procedure in [17]. Autoradiography was carried out at -20°C using Kodak XAR5 film and a Dupont Cronex Lightning-Plus Intensifying screen.

3. RESULTS

The low-density microsomal membrane fraction contains the largest pool of glucose transporters [1] and reconstitutable glucose transport activity [3] of all the subcellular fractions derived from rat adipocytes in the basal state. Consequently, this membrane fraction was used routinely in the photolabeling experiments described here.

Incubation of low-density microsomes with [^3H]cytochalasin B followed by addition of crosslinker resulted, after irradiation, detergent ex-

traction, and SDS-PAGE, in the appearance of a broad band of radioactivity with an apparent M_r ranging from 45 000 to 50 000 (fig.1). Incorporation of radioactivity required photolysis and the addition of the crosslinking agent (not shown) indicating that the radioactive material represents cytochalasin B crosslinked to membrane proteins. Cytochalasin E which does not bind to the glucose transporter [1], did not inhibit the covalent linkage of cytochalasin B to the 45–50 kDa protein, under identical conditions (not shown). We attempted to increase the specific labeling of the protein by extending the photolysis time beyond 2 min but found that this procedure resulted only in increased non-specific labeling of the protein.

To test whether the 45–50 kDa protein represented the glucose transporter, we preincubated the microsomal membranes in the presence of 500 mM D- or L-glucose prior to irradiation (fig.2). Photolabeling of the 45–50 kDa band was reduced 48% by D-glucose, but not at all by the presence of L-glucose. This preferential inhibition of cytochalasin B labeling of the protein is consistent with the stereoselectivity of the glucose transporter for D-glucose [1].

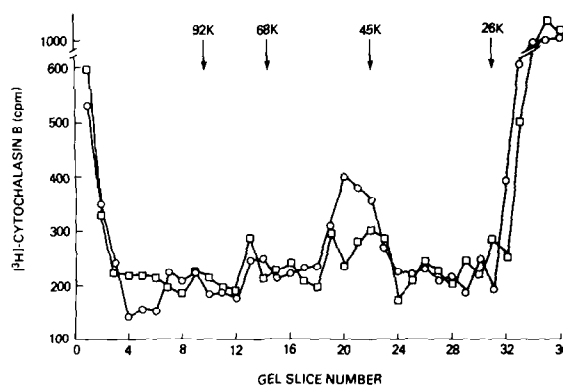


Fig.1. Electrophoretic profile of [^3H]cytochalasin B-labeled low-density microsomal membrane proteins from basal and insulin-treated rat adipocytes. Low-density microsomes from basal (○) and insulin-treated (□) rat adipocytes were incubated with [^3H]cytochalasin B for 10 min at 4°C. Hydroxysuccinimidyl-4-azidobenzoate was then added and the membranes were irradiated with UV light at 254 nm for 2 min as described in section 2. Membrane protein (200–300 μg) was applied to 8% gels and analyzed by SDS-PAGE. Arrows indicate the positions of M_r marker proteins and the tracking dye. K, kDa.

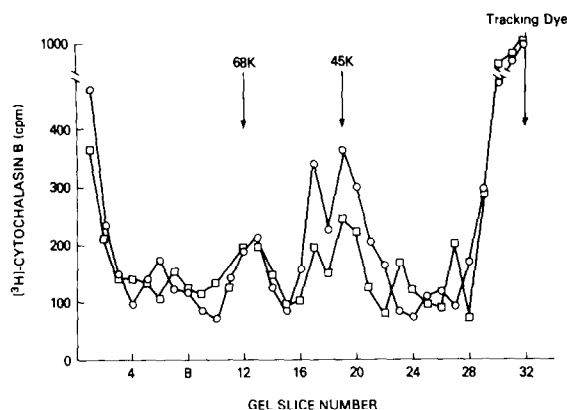


Fig. 2. Electrophoretic profile of [^3H]cytochalasin B-labeled low-density microsomal membrane proteins photolabeled in the presence of D- or L-glucose. Low-density microsomes from basal cells were incubated with 500 mM D-glucose (\square) or L-glucose (\circ) prior to incubation with [^3H]cytochalasin B for 10 min at 4°C . Hydroxysuccinimide-4-azidobenzoate was then added and the membranes were irradiated with UV light at 254 nm for 2 min as described in section 2. Membrane protein (200–300 μg) was applied to 8% gels and analyzed by SDS-PAGE. Arrows indicate the positions of M_r marker proteins and the tracking dye. K, kDa.

Insulin has been shown to induce the translocation of glucose transporters from the low-density microsomal membrane fraction used in this study, to the plasma membranes [1–4]. As shown in fig. 1, insulin pretreatment of adipocytes, followed by homogenization and labeling of the intracellular membrane pool of glucose transporters resulted in a 50% reduction in the incorporation of [^3H]cytochalasin B into the 45–50 kDa fraction. Under the conditions utilized in this experiment, insulin also reduced the number of glucose transporters in the low-density microsomal membrane fraction by 50% as measured by the more conventional procedure of determining the binding capacity of the membranes for [^3H]cytochalasin B [1–4].

As a means of characterizing the nature of the material in the 45–50 kDa band, we excised and isolated this band from an SDS gel and subjected the eluted proteins to isoelectric focusing in polyacrylamide tube gels; results from a typical experiment are shown in fig. 3. Isoelectric focusing of the radiolabeled protein yielded one major band of radioactivity, focusing at pH 5.4, and two minor

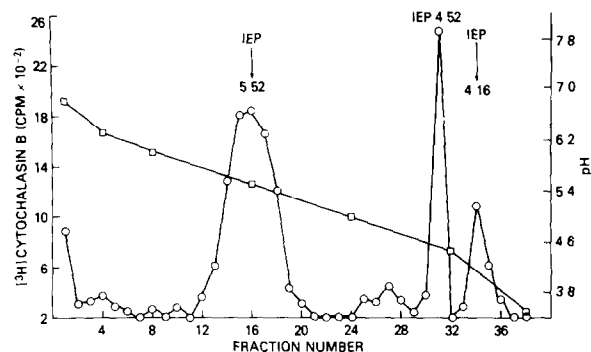


Fig. 3. Isoelectric focusing of the SDS-PAGE-purified, photoaffinity-labeled rat adipocyte glucose transporters. The [^3H]cytochalasin B-labeled low-density microsomal membrane proteins migrating at 40–55 kDa were excised from an SDS-polyacrylamide gel and, after elution from the gel, were subjected to isoelectric focusing as described in section 2. (\circ) [^3H]Cytochalasin B (cpm); (\square) pH. IEP, isoelectric point.

bands focusing at pH 4.5 and 4.2, respectively. To obtain more material for further characterization, we repeated the isoelectric focusing on a preparative scale using a flat bed Sephadex gel and the conditions described in section 2. The isolated fractions corresponding to the labeled proteins focusing at pH 5.4, 4.5 and 4.2 were then subjected to SDS-PAGE, and the resolved proteins transferred via electrophoretic blotting to nitrocellulose. Fig. 4 illustrates the crossreactivity of these resolved proteins with an antiserum prepared against the purified human erythrocyte glucose transporter, as detected by labeling with ^{125}I -protein A and autoradiography. Lane 1, which represents basal low-density microsomes (starting material) and thus contains the glucose transporter by the previously described criteria, reacts quite strongly with the antiserum. The prominently labeled 45–50 kDa band has previously been shown to contain the glucose transporter [17]. Lane 2 represents material isolated from one region of the isoelectric focusing gel (fig. 3) lacking material labeled with cytochalasin B. This fraction contained high- M_r bands which crossreact with the antiserum. However, since this fraction was not labeled with cytochalasin B, it very likely represents material which interacts non-specifically with the antiserum. Lane 3 corresponds to material focusing at pH 5.4 (fig. 3). This fraction, as the original low-density microsomes, shows strong

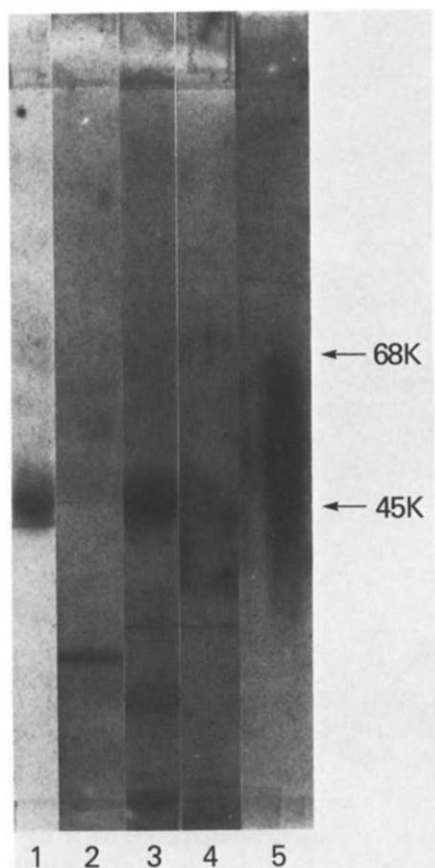


Fig.4. Immunological detection of the rat adipocyte glucose transporter. Low-density microsomal membrane proteins were fractionated by preparative isoelectric focusing and eluted as described in section 2, analyzed by SDS-PAGE on 9% resolving gels, and transferred to nitrocellulose. The nitrocellulose blots were then incubated with antiserum raised against the purified human erythrocyte glucose transporter, and the crossreacting proteins identified using ^{125}I -protein A and autoradiography. Conditions were as described in section 2. Lane 1, low-density microsomes (control); lanes 2, 3 and 4, low-density microsomes fractionated by isoelectric focusing (see fig.3); lane 2, pooled fractions (8–12); lane 3, pooled fractions (13–19); lane 4, pooled fractions (30–36); lane 5, human erythrocyte membranes (2 μg). K, kDa.

reactivity with the antiserum in the region 45–50 kDa. Lane 4, representing the combined material focused at pH 4.5 and 4.2 (fig.3), also reacted with the antiserum. Hence, these experiments provide conclusive evidence that the

45–50 kDa band labeled with cytochalasin B focuses at the 3 discrete pH values, each representing a subpopulation of identifiable glucose transporters. Lane 5 represents human erythrocyte membranes shown for comparison.

4. DISCUSSION

We have shown here the feasibility of covalently coupling cytochalasin B to rat adipocyte glucose transporters using a photosensitive bifunctional crosslinking agent. On SDS-PAGE, a broad band of proteins with apparent M_r values of 45 000–50 000 was labeled. Labeling of this band was sensitive to unlabeled cytochalasin B, providing one indication that the proteins in this band represent glucose transporters. Additional evidence supporting this suggestion is the finding that labeling was blocked by D-glucose to the extent of 48% whereas L-glucose was ineffective at the same concentration (500 mM) (fig.2). This agrees with the K_m values of the two sugars for transport (10 mM for D-glucose and 300 mM for L-glucose) [18]. Moreover, insulin treatment of intact adipocytes led to a 50% reduction in the amount of [^3H]cytochalasin B incorporated into the 45–50 kDa band (fig.1); this reduction correlates well with a similar reduction in the number of transporters as measured in previous studies by either cytochalasin B binding [2] or the reconstituted glucose transport activity itself [3,4]. Finally, we have also shown that cytochalasin E, which does not block the binding of cytochalasin B to glucose transporters, similarly does not inhibit covalent labeling of the 45–50 kDa band. Cytochalasin E, like cytochalasin B, binds to actin [8]. Hence, the inability of the former to block incorporation of cytochalasin B into the 45–50 kDa band makes it unlikely that the proteins in this band represent actin.

The broadness of the band of cytochalasin B-binding proteins suggests heterogeneity of glucose transporter species. This heterogeneity is confirmed by isoelectric focusing which resolves at least 3 distinct peaks of cytochalasin B-labeled proteins having different pH profiles (fig.3). In addition, each of these peaks crossreacted with an antiserum to the human erythrocyte glucose transporter (fig.4). Thus, the adipocyte glucose transporter appears to exist in different forms perhaps depending on the degree of glycosylation. Authors in [19]

have shown that the human erythrocyte glucose transporter is heavily glycosylated, and that variations in the level of glycosylation appear to explain the broadness of the band with which this purified protein migrates on SDS-PAGE. However, the possibility should also be considered that the pH heterogeneity derives from protein phosphorylation.

In any event, the present experiments with the antiserum reactive material show conclusively that cytochalasin B can be covalently coupled to the glucose transporter in a selective manner and that this procedure can be usefully applied in monitoring the rat adipocyte glucose transporter. We are now in the process of using this technique for purifying the rat adipocyte glucose transporter with the specific aim of preparing antibodies against this protein with higher specific activities than those seen with any of the currently available antisera to the human erythrocyte glucose transporter.

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REFERENCES

- [1] Wardzala, L.J., Cushman, S.W. and Salans, L.B. (1978) *J. Biol. Chem.* 253, 8002-8005.
- [2] Cushman, S.W. and Wardzala, L.J. (1980) *J. Biol. Chem.* 255, 4758-4762.
- [3] Suzuki, K. and Kono, T. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2542-2545.
- [4] Kono, T., Robinson, F.W., Blevins, T.L. and Ezaki, O. (1982) *J. Biol. Chem.* 257, 10942-10947.
- [5] Wardzala, L.J. and Jeanrenaud, B. (1981) *J. Biol. Chem.* 256, 7090-7093.
- [6] Wardzala, L.J. and Jeanrenaud, B. (1983) *Biochim. Biophys. Acta* 730, 49-56.
- [7] Bloch, R. (1973) *Biochemistry* 12, 4799-4801.
- [8] Shanahan, M.F., Olson, S.A., Weber, M.J., Lienhard, G.E. and Gorga, J.C. (1982) *Biochem. Biophys. Res. Commun.* 107, 38-43.
- [9] Johnson, G.L., MacAndrew, V.I. and Pilch, P.F. (1981) *Proc. Natl. Acad. Sci. USA* 78, 875-878.
- [10] Puma, P., Buxser, S.E., Watson, L., Kelleher, D.J. and Johnson, G.L. (1983) *J. Biol. Chem.* 258, 3370-3375.
- [11] Karnieli, E., Zarnowski, M.J., Hissin, P.J., Simpson, I.A., Salans, L.B. and Cushman, S.W. (1981) *J. Biol. Chem.* 256, 4772-4777.
- [12] Laemmli, U.K. (1970) *Nature* 227, 680-685.
- [13] An der Lan, B., Horuk, R., Sullivan, J.V. and Chrambach, A. (1983) *Electrophoresis*, in press.
- [14] Horuk, R. and Wright, D.E. (1983) *FEBS Lett.* 155, 213-217.
- [15] Beeley, J.A., Stevenson, S.M. and Beeley, J.G. (1972) *Biochim. Biophys. Acta* 285, 293-300.
- [16] Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.
- [17] Wheeler, T.J., Simpson, I.A., Sogin, D.C., Hinkle, P.C. and Cushman, S.W. (1982) *Biochem. Biophys. Res. Commun.* 105, 89-95.
- [18] LeFevre, P.G. (1961) *Pharmacol. Rev.* 13, 39-70.
- [19] Sogin, D.C. and Hinkle, P.C. (1978) *J. Supramol. Struct.* 8, 447-453.