

Identification and characterization of the glucose-transport protein of the bovine blood/brain barrier

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The glucose-transport protein from bovine cerebral-cortex microvessels has been identified and characterized by virtue of its ability to bind the ligand [4-³H]cytochalasin B. Microvessel membranes were found to contain a single set of glucose-inhibitable high-affinity cytochalasin B-binding sites [113 ± 16 (S.E.M.) pmol/mg of membrane protein], with an association constant of 6.8 ± 1.8 (S.E.M.) μM^{-1} . D-Glucose inhibited the binding to these sites with a K_i of 31 mM. The transport protein was identified by photoaffinity labelling with [4-³H]cytochalasin B and was found to migrate as a broad band of apparent M_r 55000 on SDS/polyacrylamide gels. Labelling was inhibited by D-glucose, but not by L-glucose. Treatment with endoglycosidase F yielded a sharper band of apparent M_r 46000, indicating that the transport protein is glycosylated. However, in contrast with the human erythrocyte glucose transporter, digestion with endo- β -galactosidase had little effect on the electrophoretic mobility of the microvessel protein. Tryptic digestion of the photolabelled protein yielded a radioactive fragment of apparent M_r 18000, similar to that of the fragment produced by digestion of the labelled human erythrocyte glucose transporter. In addition, a protein of M_r identical with that of the photolabelled transporter was labelled on Western blots of microvessel membranes by antisera raised against the intact erythrocyte transporter and against synthetic peptides corresponding to its N- and C-terminal regions. It is concluded that the glucose-transport protein of bovine cerebral-cortex microvessel endothelial cells shows structural homology with the human erythrocyte glucose transporter.

INTRODUCTION

The mechanism by which glucose passes across the mammalian blood/brain barrier is of considerable interest in view of the brain's dependence on glucose as its main energy source [1]. It is now generally accepted that the barrier function is provided by brain capillary endothelial cells, which are joined by tight junctions, and that to pass from the blood to the brain's extracellular space, glucose must traverse both the luminal and the contraluminal membranes of the endothelial cell [2,3]. Because glucose is a polar water-soluble molecule, its passage across the hydrophobic lipid bilayer of the plasma membrane is mediated by specific transport systems in most animal cells. The best characterized of these transport systems is that of the human erythrocyte membrane, where transport is stereospecific for D-glucose, is neither Na⁺ or energy-dependent, is unaffected by insulin, and is reversibly inhibited by the fungal metabolite cytochalasin B [4]. Studies on the transport of glucose and other monosaccharides across the mammalian blood/brain barrier *in vivo* have shown that this process exhibits many similarities to transport in the erythrocyte [2,3]. These similarities suggest that erythrocytes and endothelial cells may share a common mechanism of transport.

The glucose transporter of human erythrocytes has been isolated and shown to comprise a membrane-

spanning glycoprotein that migrates as a broad band of average apparent M_r 55000 on SDS/polyacrylamide-gel electrophoresis [5,6]. Recently the complete amino acid sequence of the glucose transporter from a human hepatoma cell line (HepG2) was deduced from the nucleotide sequence of a cDNA clone [7]. Protein-chemical studies have indicated that the erythrocyte transporter probably has an identical sequence [7]. The predicted transporter sequence comprises 492 amino acids, corresponding to an M_r of 54117, with a single site of glycosylation at asparagine-45, where an oligosaccharide chain of the poly(N-acetyl-lactosamine) type is attached [7,8]. Deglycosylation of the erythrocyte protein by treatment with endoglycosidase F from *Flavobacterium meningosepticum* causes the protein to migrate as a sharp band of apparent M_r 46000 on electrophoresis [9]. However, hydrophobic polypeptides such as the transporter are known to migrate anomalously on SDS/polyacrylamide gels [6].

Recently, photolabelling with [4-³H]cytochalasin B has proved useful in identifying the glucose-transport proteins of a variety of cell types [10,11]. Although the binding of this inhibitor to glucose-transport proteins is normally reversible, on illumination with u.v. light a covalent linkage is formed between the two molecules [12,13]. Not only has this phenomenon been employed to identify transport proteins, but it has also enabled the site of cytochalasin B binding to the transporter

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polypeptide to be investigated. Tryptic cleavage of the native membrane-bound human erythrocyte glucose transporter after photolabelling has been found to yield an unlabelled glycosylated fragment of apparent M_r 23000 and a non-glycosylated fragment of apparent M_r 18000 that bears the label [14,15]. The two fragments are now known to be derived from the *N*- and *C*-terminal halves of the protein respectively [16].

Photoaffinity labelling with cytochalasin B has enabled the identification of putative glucose-transport proteins in the blood/brain barrier of the sheep [17] and of the pig and the rat [18]. In each case, the labelled protein migrated as a broad band of apparent approx. M_r 55000, very similar to the human erythrocyte glucose transporter. A protein of similar M_r was also labelled in pig cerebral microvessels by an antibody raised against the human glucose transporter [18]. However, the preparation of transporter used to raise the antibodies is known to contain a variety of other proteins, including a nucleoside-transport protein [19], so the antiserum may not be entirely specific. Another recent advance has been the preparation from whole rat brain of a cDNA clone encoding a glucose-transport protein of sequence almost identical with that of the human erythrocyte transporter [20]. However, it is not known whether the encoded protein corresponds to the neuronal-, glial- or endothelial-cell transporter. The aim of the experiments described here was therefore to compare the structures of the glucose transporters from the bovine blood/brain barrier and the human erythrocyte by enzymic-digestion studies and the use of antibodies raised against specific regions of the human transport-protein sequence.

EXPERIMENTAL

Materials

Endoglycosidase F from *Flavobacterium meningosepticum* was kindly given by Dr. A. D. Whetton (University of Manchester Institute of Science and Technology, Manchester, U.K.). It was prepared by the method of Elder & Alexander [21], and was the concentrate from the Ultrogel AcA 54 chromatography step, in 50 mM-EDTA/50% (v/v) glycerol. Endo- β -galactosidase from *Bacteroides fragilis* was generously provided by Dr. P. R. Scudder (Clinical Research Centre, Harrow, Middx., U.K.). Diphenylcarbamoyl chloride-treated trypsin was obtained from Sigma (Poole, Dorset, U.K.). [4-³H]Cytochalasin B (7.1 Ci/mmol) was obtained from New England Nuclear Corp. (Southampton, Hants, U.K.). Bio-Rad (Watford, Herts, U.K.) supplied an Immun-Blot assay kit and other materials for Western blotting. All other chemicals were from Sigma or BDH (Poole, Dorset, U.K.) and were the highest grade available. Outdated human blood was provided by the blood bank of the Royal Free Hospital. Bovine brains were kindly provided by Zif Meats.

Preparation of erythrocyte and microvessel membranes

Erythrocyte membranes were prepared from both fresh bovine blood and from outdated human blood by the procedure of Steck & Kant [22]. Bovine cerebral-cortex microvessels were prepared by the method previously described for sheep brain microvessels [17]. The cerebral vasculature of fresh bovine brains obtained from a slaughterhouse was flushed with ice-cold 15 mM-

Hepes (pH 7.4)/147 mM-NaCl/4 mM-KCl/3 mM-CaCl₂/1.2 mM-MgCl₂/5 mM-sodium pyruvate/0.2 mM-sodium DL- β -hydroxybutyrate (Hepes/saline), until apparently cleared of blood. The pia mater and superficial vessels were then removed, and the grey matter was separated. The latter was homogenized at 600 rev./min with a Tissuemizer (Tekmar) for 90 s in four times its volume of Hepes/saline, and the mixtures centrifuged at 2500 *g* (r_{av} 13 cm) for 30 min at 4 °C. The supernatant was discarded and the pellet resuspended in Hepes/saline containing 1 mM-EDTA and 20% (w/v) Ficoll, before centrifuging at 15000 *g* (r_{av} 8 cm) for 50 min at 4 °C. This procedure separated the myelin and neuronal elements, which floated to the top and were discarded, from a pellet containing the microvessels and arterioles, venules and amorphous debris. The pellet was resuspended in Hepes/saline and then passed through a 300 μ m-pore-size nylon mesh. Large vessels were retained and discarded, and the microvessels were collected from the filtrate on a 30 μ m-pore-size nylon mesh. The purity of the microvessel preparation was routinely monitored at this stage by phase-contrast microscopy. Only occasional erythrocytes were present in the lumina of the microvessels, and few pericytes or other contaminants were seen. We estimate that the preparation comprised more than 95% microvessel endothelial cells.

Endothelial-cell plasma membranes were prepared by osmotic lysis of the microvessels overnight at 4 °C in 7.5 mM-sodium phosphate (pH 7.4)/10 mM-NaCl, containing 1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride and 1.5 μ M-pepstatin A to prevent proteolytic degradation. The lysed microvessels were collected by centrifugation at 2500 *g* (r_{av} 13 cm) for 10 min at 4 °C, then washed twice with the lysis buffer by centrifugation. They were next washed three times with 50 mM-sodium phosphate, pH 7.4, containing 100 mM-NaCl and 1 mM-EDTA, and finally resuspended in this buffer by sonication for 2 min using an MSE Soniprep sonicator at full power. The purity of the membrane preparation was assessed by measuring the activity of γ -glutamyltransferase, a specific marker of the endothelial-cell plasma membrane [23], by the method of Orłowski & Meister [24]. The specific activity of this enzyme was found to be 18-fold higher in the microvessel membrane preparations than in the crude brain homogenate.

Measurement of cytochalasin B binding

The binding of [4-³H]cytochalasin B to erythrocyte and microvessel membranes was measured by equilibrium dialysis using 40 nM-cytochalasin B, essentially as described by Zoccoli *et al.* [25]. Under these conditions the ratio of bound to free cytochalasin B is approximately equal to the concentration of binding sites divided by the dissociation constant for cytochalasin B, and is referred to as the 'cytochalasin B-binding activity'. Correction for non-specific binding of cytochalasin B was made by making the measurements in the presence and absence of 400 mM-D-glucose. For more accurate determination of the concentration of binding sites and the association constant for binding, measurements were made over a range of cytochalasin B concentrations and the data analysed by the LIGAND procedure of Munson & Rodbard [26]. In all cases the best fit of the data was provided by a model comprising a single set of high-

affinity binding sites plus non-specific binding. The latter was estimated from the data by the LIGAND procedure and was not independently measured by performing the binding in the presence of 400 mM-D-glucose. All measurements were made in the presence of 10 μ M-cytochalasin E to minimize the binding of cytochalasin B to sites other than the glucose transporter [12,13].

Photoaffinity labelling

The glucose-transport proteins of bovine and human erythrocytes, and of bovine cerebral-cortex microvessel endothelial cells, were identified by photoaffinity labelling with cytochalasin B. Microvessel membranes (1.73 mg of protein/ml) and erythrocyte membranes (0.87 mg of protein/ml) were suspended in 50 mM-sodium phosphate (pH 7.4)/100 mM-NaCl/1 mM-EDTA and then incubated for 30 min at 4 °C with 0.51 μ M-[4-³H]cytochalasin B, 10 μ M-cytochalasin E and 500 mM-D-glucose or -L-glucose or -D-sorbitol. Samples were then transferred to quartz cuvettes of 1 cm path length, on ice, and exposed to high-intensity u.v. light from a 100 W mercury lamp at a distance of 10 cm for 10 min. Non-covalently bound radioactivity was removed by diluting the samples in 50 mM-Tris/HCl, pH 6.8, containing 20 μ M unlabelled cytochalasin B, and then collecting the membranes by centrifugation at 126000 *g* (r_{av} . 5.6 cm) for 1 h at 4 °C.

Enzymic digestion of microvessel membranes

Tryptic digestion of photoaffinity-labelled microvessel membranes (0.96 mg of protein/ml) was performed in 50 mM-sodium phosphate (pH 7.4)/100 mM-NaCl/1 mM-EDTA at 25 °C. Trypsin was added to give a concentration of 11 μ g/ml initially, and again after 2 and 4 h to ensure the completeness of the digestion. After 6 h, the digestion was halted by the addition of a 2-fold excess (by weight) of bovine lung aprotinin over trypsin.

Before digestion with endoglycosidase F, photoaffinity-labelled microvessel membrane proteins (1.1 mg/ml) were first solubilized by incubation for 30 min at 25 °C in 100 mM-sodium phosphate (pH 6.1)/50 mM-EDTA/75 mM-2-mercaptoethanol/0.05% (w/v) SDS/1.5% (v/v) Triton X-100. After centrifugation for 15 min at 11500 *g* (r_{av} . 5 cm) to remove the basement membranes, samples were incubated for 18 h at 25 °C with and without endoglycosidase F (15 μ l enzyme/200 μ l sample). Digestion was terminated by denaturing the proteins with SDS in preparation for polyacrylamide-gel electrophoresis.

Photoaffinity-labelled microvessel membranes were also solubilized before digestion with endo- β -galactosidase from *Bacteroides fragilis*. The membranes (3.1 mg of protein/ml) were first dissolved in 50 mM-sodium phosphate (pH 6.0)/1 mM-EDTA/1 mM-dithiothreitol containing 1.35% (v/v) Triton X-100. After centrifugation to remove the basement membranes, as described above, the soluble proteins were digested with 0.6 unit of endo- β -galactosidase/ml [27] for 18 h at 25 °C. Digestion was halted as described for endoglycosidase F digestion.

SDS/polyacrylamide-gel electrophoresis

Samples of photolabelled membranes were prepared for electrophoresis by dissolution in 50 mM-Tris/HCl (pH 6.8)/1 mM-EDTA/1% (w/v) SDS at 22 °C. Heating was avoided to minimize aggregation of the transport proteins [11]. Solubilized microvessel membranes were next centrifuged at 11500 *g* (r_{av} . 5 cm) for 3 min to

remove undissolved fragments of basement membrane. Samples of the supernatant were then taken for protein estimation by the procedure of Lowry *et al.* [28] (with bovine serum albumin as standard) and for gel electrophoresis.

Samples (50 μ g) of photolabelled membrane proteins were electrophoresed in 10 or 12% SDS/polyacrylamide gels by the procedure of Laemmli [29]. Proteins of known M_r used as markers were as previously described [14]. After fixing and staining the separated proteins with Coomassie Blue, the distribution of radioactivity on gels was determined by solubilizing 2 mm slices of gel by the method of Goodman & Matzura [30], followed by liquid-scintillation counting with quench correction by the external-standards-ratio procedure.

Western blotting

Electrophoretic transfer of proteins from SDS/polyacrylamide gels to nitrocellulose and staining for protein were as previously described [31]. The binding of antibodies to the transporter on the nitrocellulose was detected using goat anti-rabbit IgG conjugated to alkaline phosphatase, with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as chromagens, as described by the manufacturers of the Immuno-Blot assay kit. Antisera were used at a dilution of 1:300. Three different antisera were used that had been raised in rabbits against the intact glucose transporter from human erythrocytes and against synthetic peptides corresponding to residues 1–15 (*N*-terminal peptide) and residues 477–492 (*C*-terminal peptide) of this protein. The synthesis of the peptides and the production of antibodies has been described in detail elsewhere [16].

RESULTS

The bovine cerebral-cortex microvessel preparations used in the present study comprised almost entirely endothelial cells, with only a few trapped erythrocytes. In order to assess the contribution of the latter to the total number of glucose-transport proteins in the preparation, the cytochalasin B-binding activity of bovine erythrocyte membranes was measured. The bound/free ratio for cytochalasin B binding to these membranes (2 mg of protein/ml) was 0.32 (corrected for non-specific binding). This value can be compared with 1.35, 1.50, 1.44 and 1.40 for binding to four typical preparations of microvessel membranes (2 mg of protein/ml) measured under the same conditions. These findings suggest that trapped erythrocytes make a negligible contribution to the total number of cytochalasin B-binding sites in the microvessel preparations and are compatible with reports that adult bovine erythrocytes are relatively impermeable to glucose [32]. In contrast, the bound/free ratio for cytochalasin B binding to human erythrocyte membranes (2 mg of protein/ml), which are known to be very rich in glucose transporters [33], was measured to be 9.97, suggesting that these cells contain approx. 30 times as many glucose transporters as bovine erythrocytes.

Cytochalasin B binding to the glucose-transport proteins in the bovine brain microvessels was characterized by measuring the binding over a range of cytochalasin B concentrations in the presence and absence of D-glucose. The results are shown in the form of Scatchard plots in Fig. 1. Analysis of the data by the LIGAND procedure indicated that, in the absence of glucose, the

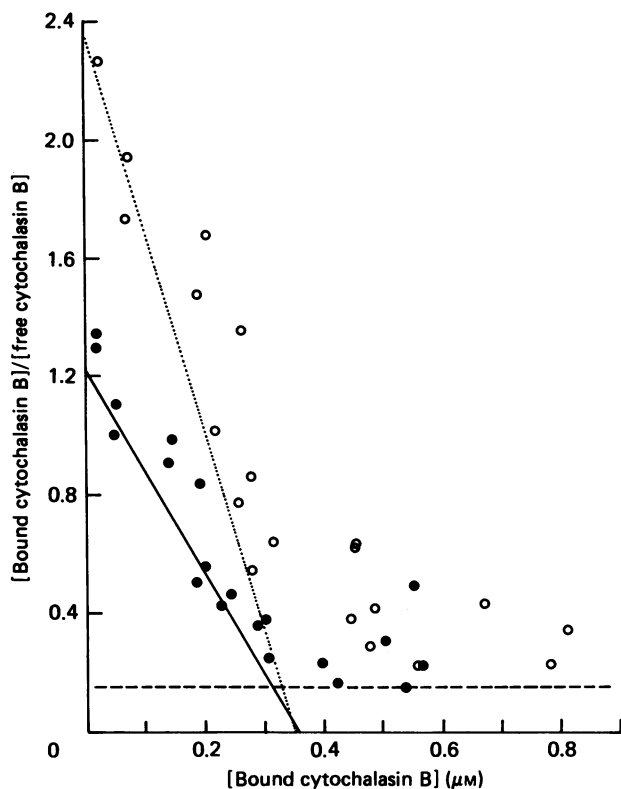


Fig. 1. Scatchard-plot analysis of cytochalasin B binding to bovine cerebral-cortex microvessel membranes

Binding to microvessel membranes (3.1 mg of protein/ml) was measured in the absence (○) and presence (●) of 30 mM-D-glucose. Straight lines are computerized best fits determined by the LIGAND procedure [26]. Binding in the absence of glucose; —, binding in the presence of glucose; ----, non-specific binding.

best fit was provided by a model consisting of a single set of high-affinity binding sites with an association constant (\pm S.E.M.) of $6.8 \pm 1.8 \mu\text{M}^{-1}$. The concentration of sites (\pm S.E.M.) was 113 ± 16 pmol/mg of membrane protein. The presence of D-glucose (30 mM) did not alter the concentration of sites (115 ± 24 pmol/mg of membrane protein) but decreased the apparent association constant to $3.4 \pm 1.0 \mu\text{M}^{-1}$, indicating that D-glucose inhibited binding with a K_i of approx. 31 mM. A similar K_i value was obtained when the effect of D-glucose on cytochalasin B binding was examined over a range of glucose concentrations using a single low concentration (40 nM) of cytochalasin B: the continuous line through the closed circles in Fig. 2 is a theoretical inhibition curve calculated by the method of Zoccoli *et al.* [25] using a K_i value of 30 mM. In contrast, L-glucose had little effect on the binding at any concentration tested (Fig. 2).

The glucose-transport protein of the bovine blood/brain barrier was identified by photoaffinity labelling the cytochalasin B-binding sites in the microvessel membranes. Membranes were photolabelled with [^3H]cytochalasin B in the presence of either 500 mM-L-glucose or 500 mM-D-sorbitol, neither of which is a substrate for transport across the barrier. The labelled proteins were then analysed by SDS/polyacrylamide-gel electrophoresis. In each case a broad peak of labelling

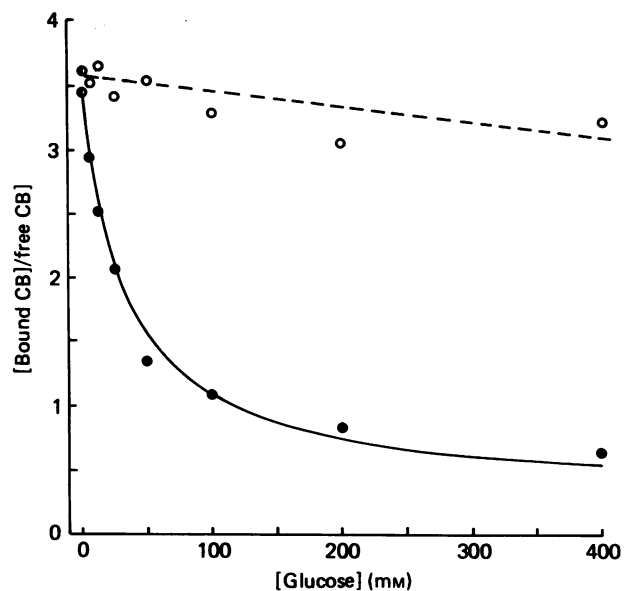


Fig. 2. Effects of D- and L-glucose on the binding of cytochalasin B (CB) to bovine cerebral-cortex microvessel membranes

Binding to microvessel membranes (3.6 mg of protein/ml) was measured in the presence of various concentrations of D-glucose (●) or of L-glucose (○). The results are the means for triplicate and duplicate samples respectively. The continuous line through the closed circles was calculated by using a K_i for glucose of 30 mM, according to the procedure of Zoccoli *et al.* [25].

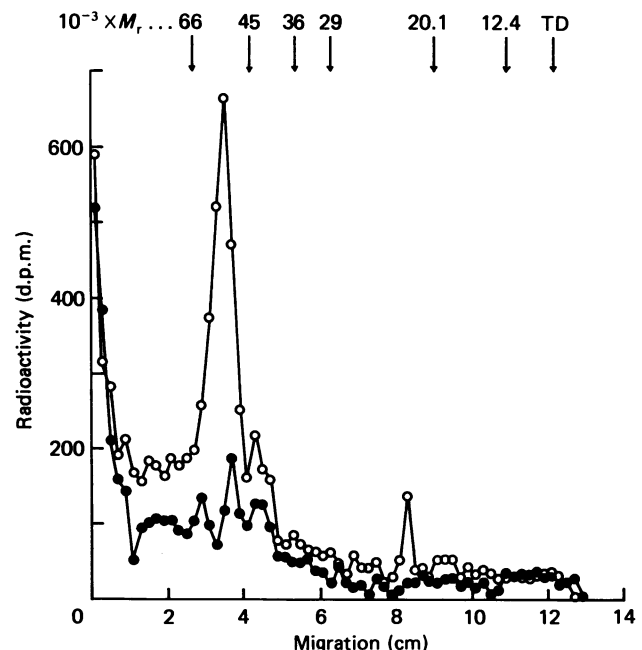


Fig. 3. Photoaffinity-labelling of bovine cerebral cortex microvessel membranes in the presence of D- or L-glucose

Microvessel membranes were photoaffinity-labelled with [^3H]cytochalasin B as described in the text, in the presence of either 500 mM-L-glucose (○) or 500 mM-D-glucose (●). The labelled proteins (50 μg) were then electrophoresed on an SDS/12% (w/v)-polyacrylamide gel. Arrows indicate the positions of M_r markers. Abbreviation: TD, tracking dye.

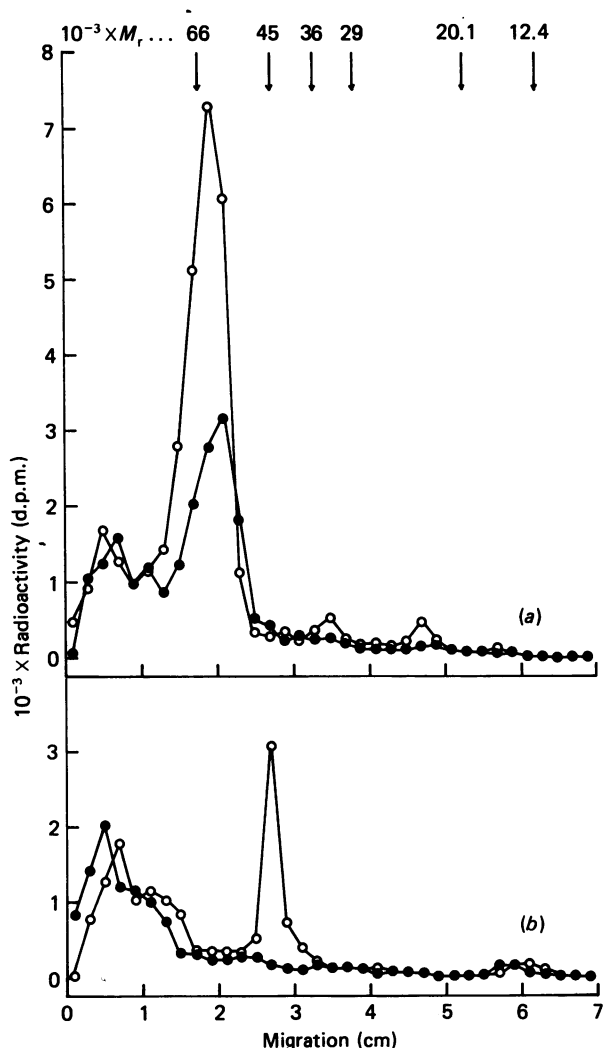


Fig. 4. Photoaffinity-labelling of human and bovine erythrocyte membranes

Human (a) and bovine (b) erythrocyte membranes were photoaffinity-labelled with [^3H]cytochalasin B as described in the text, in the presence of either 500 mM-L-glucose (○) or 500 mM-D-glucose (●). The labelled proteins were then electrophoresed on a 10% SDS/polyacrylamide gel. Arrows indicate the positions of M_r markers.

was found, corresponding to the M_r range 45000–66000, with a maximum at 55000 [Figs. 3 (above) and 7 (below)]. This pattern of labelling closely resembled that seen for photolabelled human erythrocyte membranes (Fig. 4a). The incorporation of cytochalasin B into the peak in the presence of L-glucose and D-sorbitol was 3.7 and 3.3 pmol/mg of microvessel membrane protein respectively, representing an efficiency of labelling of about 3%. In the presence of 500 mM-D-glucose, the photolabelling of the peak was inhibited by 68% (Fig. 3).

In addition to the main peak of labelling seen for microvessel membranes, a smaller D-glucose-inhibitable peak was also present, with an apparent M_r of 45000 (Fig. 3). In previous studies we had observed a peak of similar M_r in photolabelled sheep erythrocytes (M. T. Cairns, R. M. Gardiner & S. A. Baldwin, unpublished

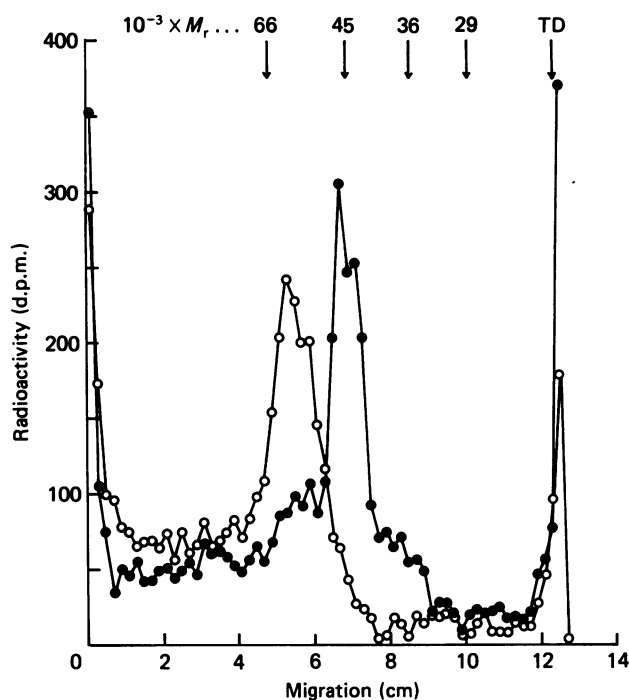


Fig. 5. Effect of endoglycosidase F on the electrophoretic mobility of the bovine cerebral-cortex microvessel glucose transporter

Microvessels photoaffinity-labelled with [^3H]cytochalasin B in the presence of D-sorbitol were incubated with (●) or without (○) endoglycosidase F as described in the Experimental section. Samples (50 μg) of the labelled proteins were electrophoresed on a 10% SDS/polyacrylamide gel. Arrows indicate the positions of M_r markers. Abbreviation: TD, tracking dye.

work). We therefore investigated the possibility that, despite their low numbers, erythrocytes trapped in the microvessel preparations were contributing to the pattern of labelling. Human and bovine erythrocyte membranes were photolabelled in the presence of 500 mM-D- or L-glucose, in precisely the same way as microvessel membranes. Human erythrocyte membranes yielded the expected pattern, with a broad peak of labelling of average apparent M_r 55000 on SDS/polyacrylamide gels (Fig. 4a). The incorporation of cytochalasin B into the peak in the presence of L-glucose was 30.5 pmol/mg of membrane protein, and the labelling was inhibited by 50% in the presence of 500 mM-D-glucose (Fig. 4a). No labelling was seen in this region of SDS/polyacrylamide gels of photolabelled bovine erythrocyte membranes (Fig. 4b), indicating that the main peak of labelling seen with the microvessel membranes did not arise from contaminating erythrocytes. However, the erythrocytes did exhibit a substantial peak of labelling with an apparent M_r of 45000, corresponding to the incorporation of 5.6 pmol of cytochalasin B/mg of membrane protein. In the presence of 500 mM-D-glucose, the incorporation of cytochalasin B into this peak was almost completely inhibited (Fig. 4b).

The broadness of the main radioactive peak seen on SDS/polyacrylamide gels of microvessel membrane proteins photolabelled with [^3H]cytochalasin B suggested that the blood/brain-barrier glucose transporter might be glycosylated. Fig. 5 shows the distribution of

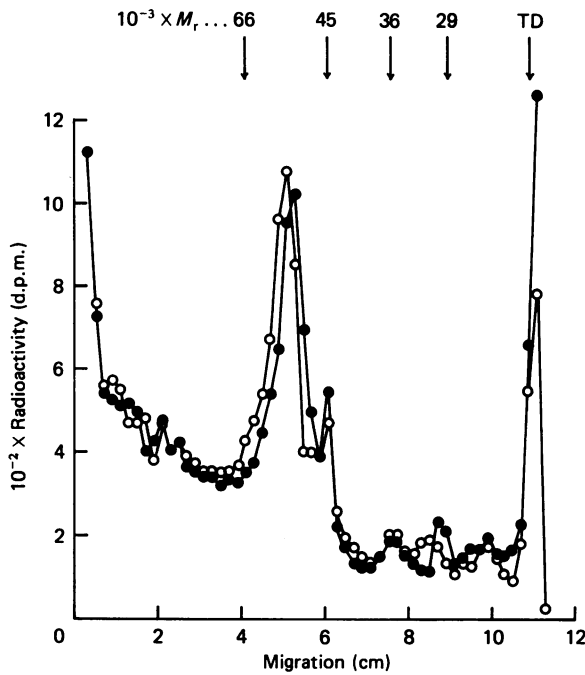


Fig. 6. Effect of endo- β -galactosidase on the electrophoretic mobility of the bovine cerebral-cortex microvessel glucose transporter

Microvessels photoaffinity-labelled with [^3H]-cytochalasin B in the presence of D-sorbitol were incubated with (●) or without (○) endo- β -galactosidase as described in the Experimental section. Samples of the labelled proteins were electrophoresed on a 10% SDS/polyacrylamide gel. Arrows indicate the positions of M_r markers. Abbreviation: TD, tracking dye.

radioactivity in SDS/polyacrylamide gels of photo-labelled microvessel membranes that had been incubated in the presence or absence of endoglycosidase F. This enzyme removes *N*-linked oligosaccharides from glycoproteins by cleaving the glycosidic bond of *N*-acetylglucosamine(1-4)*N*-acetylglucosamine linked to protein asparagine residues [21]. Enzyme treatment of microvessel membranes not only sharpened the peak of labelling, but also caused it to migrate with an increased mobility corresponding to an apparent M_r of 46000. In contrast, incubation of labelled microvessel membranes with endo- β -galactosidase had little effect on the electrophoretic mobility of the labelled peak (Fig. 6). Endo- β -galactosidase hydrolyses the internal β -galactosidic linkages of oligosaccharides belonging to the poly-(*N*-acetylglucosamine) series [27]. Enzyme treatment of photo-labelled human erythrocyte membranes, carried out under identical conditions, did cause the labelled glucose-transport protein to migrate as a sharper band of apparent M_r 46000 (results not shown).

Fig. 7 shows the effect of tryptic digestion, carried out under non-denaturing conditions, on the radioactive profile of photolabelled microvessel membrane proteins electrophoresed on SDS/polyacrylamide gels. Tryptic digestion led to the loss of about 80% of the radioactivity from the M_r -55000 peak, and the appearance of an equivalent amount of radioactivity in bands of lower M_r , principally in a peak of apparent M_r 18000. The pattern of radioactive fragments closely resembled that

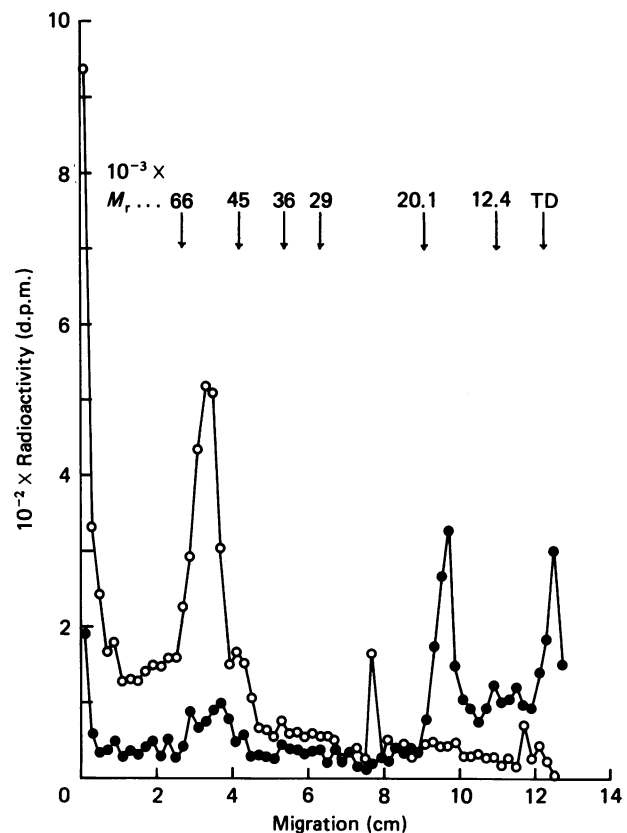


Fig. 7. Effect of trypsin on the electrophoretic mobility of the bovine cerebral-cortex microvessel glucose transporter

Microvessels photoaffinity-labelled with [^3H]-cytochalasin B in the presence of D-sorbitol were incubated with (●) or without (○) trypsin as described in the Experimental section. Samples (50 μg) of the labelled proteins were electrophoresed on a 12% SDS/polyacrylamide gel. Arrows indicate the positions of M_r markers. Abbreviation: TD, tracking dye.

seen in tryptic digests of the photolabelled human erythrocyte glucose-transport protein [14,15].

Further investigation of the similarities between the bovine blood/brain-barrier glucose transporter and the human erythrocyte glucose transporter was carried out by using antibodies to the latter. The antisera were raised against the intact transporter and against synthetic peptides corresponding to the *N*-terminal 15 residues and to the *C*-terminal 16 residues of the protein. All three antisera have been shown to recognize the human erythrocyte glucose transporter on Western blots [16]. Each antiserum labelled a single broad band of apparent M_r 55000 on Western blots of bovine microvessel membrane proteins (Fig. 8). In its broadness and apparent M_r this band closely resembled that labelled by the antisera on Western blots of human erythrocyte membrane proteins (Fig. 8). The mobility of the labelled band also closely corresponded to that identified by photoaffinity labelling with cytochalasin B.

DISCUSSION

The concentration of glucose-transport proteins in the plasma membranes of the endothelial cells that form the bovine blood/brain barrier was found to be similar to

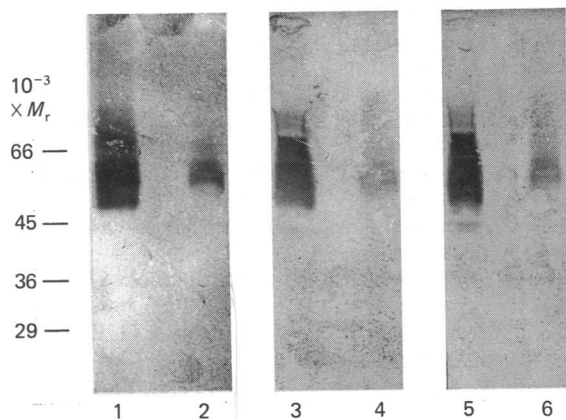


Fig. 8. Binding of antibodies to bovine endothelial cell and human erythrocyte glucose-transport proteins on Western blots

Samples of human erythrocyte membranes (100 μ g, lanes 1, 3 and 5) and of bovine cerebral-cortex microvessel membranes (100 μ g, lanes 2, 4 and 6) were electrophoresed on a 10% SDS/polyacrylamide gel, electrophoretically blotted on to nitrocellulose paper and then immunologically stained with anti-transporter serum (lanes 1 and 2), anti-(*N*-terminal peptide) serum (lanes 3 and 4) or anti-(*C*-terminal peptide) serum (lanes 5 and 6) as described in the text. The positions of M_r markers are indicated.

that previously reported for sheep [17] and about twice that reported for pig and rat [18]. It is about 15-fold greater than that found in the plasma membranes of many other cells, such as the rat adipocyte [34], the rat skeletal-muscle cell [35] and the chick-embryo fibroblast [36]. It is also considerably greater than the concentration found in other brain cells [37], reflecting the fact that the microvessels must supply the entire brain with glucose, despite representing less than 1% of its mass [38]. The value of $7 \mu\text{M}^{-1}$ for the association constant of cytochalasin B binding was similar to that of $10 \mu\text{M}^{-1}$ previously observed in sheep [17], but was about 5-fold greater than the values reported for pig and rat [18]. It closely resembled the value of $8.3 \mu\text{M}^{-1}$ found for the binding of cytochalasin B to the glucose transporter in human erythrocytes [25]. The K_i for inhibition of cytochalasin B binding by D-glucose, 31 mM, was also similar to the values of 21–38 mM reported for the human erythrocyte [25].

Further similarities to the human erythrocyte glucose transporter were revealed by photoaffinity labelling. The photolabelled bovine transporter migrated as a broad band of apparent M_r 55000 on SDS/polyacrylamide gels, closely resembling the human protein [12,13]. The D-glucose-inhibitable labelling of higher- M_r material, seen in Fig. 3, probably resulted from aggregation of labelled protein: the glucose-transport proteins of several other cell types show a tendency to aggregate in SDS [6,11]. The presence of an additional, minor, peak of labelling at M_r 45000 may reflect contamination of the microvessel preparation with erythrocytes. Although few erythrocytes were apparently present, the extent of their photolabelling seen in Fig. 4(b) was more than 5-fold greater than had been expected from the results of non-covalent cytochalasin B-binding assays. The efficiency of labelling of the bovine erythrocyte glucose transporter may thus be substantially greater than that of the blood/

brain barrier or human erythrocyte glucose transporters. However, the presence of two peaks of labelling similar to those seen with the microvessel membranes has previously been observed in another cell type, the chick-embryo fibroblast [10,36]. The lower- M_r peak thus may correspond to a less heavily glycosylated form of the endothelial cell glucose-transport protein itself. In some experiments, a very small peak of D-glucose-inhibitable labelling of apparent M_r approx. 22000 was also seen (Fig. 3). The origin of this peak is not clear, but it may have comprised a fragment of the transporter produced by endogenous proteolytic cleavage. Similar minor labelled fragments of low M_r have been seen after photolabelling the human erythrocyte glucose transporter [14].

Endoglycosidase F-treatment of the labelled microvessel protein of M_r 55000 increased its electrophoretic mobility, indicating that, like the human erythrocyte glucose transporter [19], it contained *N*-linked oligosaccharide. Furthermore, the apparent M_r of the deglycosylated bovine glucose transporter was identical with that of the deglycosylated human protein [19,39]. However, the oligosaccharide chain of the human erythrocyte glucose transporter is also partially removed by treatment with endo- β -galactosidase [8,14,39]. This enzyme had no effect on the electrophoretic mobility of the bovine endothelial-cell glucose transporter, indicating a difference from the human glycoprotein in oligosaccharide structure.

A further similarity between the protein structures of the bovine endothelial and human erythrocyte glucose transporters was revealed by tryptic digestion of the former, which yielded a photolabelled fragment of apparent M_r 18000. Fragments of similar M_r are characteristically produced by digestion of the human protein with either chymotrypsin or trypsin, reflecting the presence of accessible hydrophilic regions near the centre and at the *C*-terminus of the amino acid sequence [7,14,15]. The apparent similarity in structure of the two transporters was confirmed by the observation that antibodies raised not only against the intact human protein, but also against its *N*- and *C*-terminal regions recognized the bovine transporter on Western blots. The amino acid sequences of the two proteins must therefore be highly homologous in these two regions. The other similarities observed in the structure and properties of the two proteins suggest that this homology may extend throughout the sequences.

It is now clear that at least two classes of passive-glucose-transport protein are found in different mammalian tissues [20]. The human erythrocyte glucose transporter is representative of the type of transporter found in most cell types, including adipocyte and muscle cells [34,35]. The transporter encoded by a cDNA recently prepared from whole rat brain is also a member of this class [20]. In contrast, the liver glucose transporter, although functionally similar, appears to be unrelated or only distantly related, immunologically and genetically, to the glucose transporters of other cells [20]. The studies we have reported here indicate that the blood/brain-barrier glucose transporter is a member of the class of transport proteins typified by that from the human erythrocyte membrane.

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