Glucose Transporter Asymmetries in the Bovine Blood-Brain Barrier*

The transport of glucose across the mammalian blood-brain barrier is mediated by the GLUT1 glucose transporter, which is concentrated in the endothelial cells of the cerebral microvessels. Several studies supported an asymmetric distribution of GLUT1 protein between the luminal and abluminal membranes (1-4) with a significant proportion of intracellular transporters. In this study we investigated the activity and concentration of GLUT1 in isolated luminal and abluminal membrane fractions of bovine brain endothelial cells. Glucose transport activity and glucose transporter concentration, as determined by cytochalasin B binding, were 2-fold greater in the luminal than in the abluminal membranes. In contrast, Western blot analysis using a rabbit polyclonal antibody raised against the C-terminal 20 amino acids of GLUT1 indicated a 1.5 luminal:abluminal distribution. Western blot analysis with antibodies raised against either the intracellular loop of GLUT1 or the purified erythrocyte protein exhibited luminal:abluminal ratios of 1:1. A similar ratio was observed when the luminal and abluminal fractions were exposed to the 2-N-[3H](1-azi-2,2,2,-trifluoroethyl)benzoxyl-1,3-bis-(d-mannos-4-yloxyl)-2-propylamine ([3H]ATB-BMPA) photoaffinity label. These observations suggest that either an additional glucose transporter isoform is present in the luminal membrane of the bovine blood-brain barrier or the C-terminal epitope of GLUT1 is “masked” in the luminal membrane but not in the abluminal membranes.

The mammalian brain depends on a continuous supply of glucose for normal function. The delivery of circulating glucose to the brain requires transport across the endothelial cells of the cerebral microvessels that constitute the blood-brain barrier. Several studies supported an asymmetric distribution of GLUT1 protein between the luminal and abluminal membranes (1-4) with a significant proportion of intracellular transporters. In this study we investigated the activity and concentration of GLUT1 in isolated luminal and abluminal membrane fractions of bovine brain endothelial cells. Glucose transport activity and glucose transporter concentration, as determined by cytochalasin B binding, were 2-fold greater in the luminal than in the abluminal membranes. In contrast, Western blot analysis using a rabbit polyclonal antibody raised against the C-terminal 20 amino acids of GLUT1 indicated a 1.5 luminal:abluminal distribution. Western blot analysis with antibodies raised against either the intracellular loop of GLUT1 or the purified erythrocyte protein exhibited luminal:abluminal ratios of 1:1. A similar ratio was observed when the luminal and abluminal fractions were exposed to the 2-N-[3H](1-azi-2,2,2,-trifluoroethyl)benzoxyl-1,3-bis-(d-mannos-4-yloxyl)-2-propylamine ([3H]ATB-BMPA) photoaffinity label. These observations suggest that either an additional glucose transporter isoform is present in the luminal membrane of the bovine blood-brain barrier or the C-terminal epitope of GLUT1 is “masked” in the luminal membrane but not in the abluminal membranes.

MATERIALS AND METHODS

Bovine microvessels were prepared according to Pardridge et al. (13) as modified by Sanchez del Pino et al. (14) and fractionated as previously described (8). Protease inhibitors (leupeptin, aprotinin, pepstatin, and 4-(2-aminoethyl)benzenesulfonyl fluoride) were included at 1 g/ml during the initial homogenization and then at 0.01 g/ml in all subsequent buffers. Characterization of the fractions was achieved by measuring the activities of luminal and abluminal markers, i.e. γ-glutamyltranspeptidase and System A amino acid transport activity with N-(methylylamilino)isobutyric acid as substrate, respectively (8). Rat microvessels and vascular-free rat brain membranes were prepared as previously described (15, 16).

Western Blot—Western blot analysis was performed as previously described (17) and quantified by chemiluminescence and image analysis (18). Human erythrocyte and rat brain membrane samples were included on all blots as internal GLUT1 standards for quantitation. The antibodies used were: 1) C, rabbit polyclonal anti-C-terminal peptide (20 amino acids, 472–492) (17); 2) H, rabbit polyclonal antibody raised against the purified human erythrocyte GLUT1 glucose transporter (H-sera (19, 20)); and 3) loop, a rabbit polyclonal antibody raised against residues 231–246 of the central cytoplasmic loop of GLUT1 (21), kindly provided by Dr. Steve Baldwin, Leeds University, Leeds, United Kingdom. Glucose transport was measured as described by Lee et al. (11).
Cytochalasin B (CB) Binding—CB binding was performed by a modification of the method of Wardzala et al. (22) as described in detail by Weber et al. (23). Briefly, aliquots of luminal and abluminal membranes (10–15 μg) were incubated in duplicate with varying concentrations of [3H]cytochalasin B (4–50 × 10^−6 M) in the presence of 2 mM cytochalasin E and with or without 500 μM D-glucose. Tracer [14C]urea was included to correct for trapping of radioactivity. The membranes were pelleted (Beckman Instruments LP 42 T Ti, 42 K, 220,000 × g, 30 min), and a sample was removed from the supernatant for the determination of radioactivity incorporated (including the lane of molecular markers), the slices were dried and exposed to UV light (Rayonet photochemical reactor with 300 nm lamps) for 2 min. The membranes were washed to remove free [3H]ATB-BMPA. Scatchard plots revealed the number of binding sites (B) and the Kd for cytochalasin B.

ATB-BMPA Photolabeling—ATB-BMPA is an impermeant, photactivatable ligand developed and kindly provided by Dr. Geoffrey Holman (25) that binds specifically to facilitative glucose transporters at the extracellular glucose-binding site. Membrane fractions were photolabeled essentially as described by Maher and Simpson (26). Briefly, 100 μCi of [3H]ATB-BMPA (final concentration 50 μM). The mixture was exposed to UV light (Rayonet photochemical reactor with 300 nm lamps) for 2 min. The membranes were washed to remove free [3H]ATB-BMPA, pelleted and solubilized in SDS/urea/dithiothreitol (3.5:6 m:100 mM), and separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels. The Coomassie Blue-stained gel was sliced (including the lane of molecular markers), the slices were dried and solubilized in 30% H2O2,2% NH4OH, and the radioactivity incorporated was determined in each fraction by measurement of radioactivity and a sample was removed from the supernatant for the determination of free CB. The pellet was solubilized in 0.1 M NaOH and neutralized, and the radioactivity associated with both pellets and supernatants was measured by liquid scintillation counting. The data were analyzed by subtraction of the binding curves obtained in the presence of D-glucose from those obtained in the absence of D-glucose (23, 24). The resultant Scatchard plots revealed the number of binding sites (Rn) and the Kn for cytochalasin B.

RESULTS

A typical membrane fractionation is depicted in Fig. 1A. The proportion of luminal versus abluminal membrane was determined in each fraction by measurement of γ-glutamyltranspeptidase activity (luminal) and System A amino acid transport activity (abluminal) (8). Glucose transport activity determined in the individual fractions is depicted in Fig. 1B and indicates a transport capacity in the luminal fraction greater than twice that of the abluminal fractions in agreement with the previous studies of Lee et al. (11). To analyze the GLUT1 content of these fractions, Western blot analysis was performed with three different antibody: an antiserum that recognizes the C-terminal 20 amino acids of GLUT1 (C), an antiserum against 15 amino acids of the intracellular loop (L), and an antiserum against the purified human erythrocyte GLUT1 protein (H). The relative concentrations of GLUT1 in each of the fractions as determined from image analysis of these blots are presented below the respective blots in Fig. 1C. It appeared, based on the results obtained with the C-terminal antibody, that there was a much lower concentration of GLUT1 in the abluminal fraction. However, this relationship was quite different when analyzed with the two other antisera. Whereas the ratio of F1 (luminal) to F3 (abluminal) obtained with the C-terminal antibody was approximately 1:5, this became 1:0.9 and 1:1.3 when analyzed with H and L antisera, respectively. Although the absolute luminal-abluminal content/luminal ratio varied, comparable ratios were obtained in all preparations (n = 5). Based on the distribution of luminal and abluminal markers detected in F1 (Fig. 1A), it appeared that the C-terminal antibody only detected the GLUT1 protein in the abluminal membrane. H sera provided an immediate response between the results obtained with C and loop. This may be explained by the studies of Lienhard et al. (27, 42) who demonstrated a significant C-terminal component to polyclonal antisera comparable with H. Using red cell membranes as an internal standard, the loop antisera provided the greatest estimate of total GLUT1 transporter in all fractions and suggested that the majority of the transporters in F1 and a significant proportion (50%) in F2-F5 express an epitope that is inaccessible to the C-terminal antibody. A simple explanation for this would be proteolysis during the fractionation; however, the identical observation in the unfractionated microvessel membrane samples refuted this possibility. Furthermore, proteolytic cleavage of GLUT1 in these samples should have resulted in a lower molecular weight protein detectable by both H and L sera, and this was not observed. These data suggest that the C terminus of luminal GLUT1 is somehow masked.

To determine whether the disparities in transport activity between fractions F1 (luminal) and F3 (abluminal) depicted in Fig. 1A were due to differences in concentration and/or intrin-
sic activity of GLUT1, the membranes were analyzed for glucose transporter concentration by CB binding (Fig. 2). Net \( \delta \)-glucose-inhibitable CB binding was determined by subtraction of the binding curves obtained in the absence and presence of \( \delta \)-glucose along the radial axes of equal free CB concentration radiating from the origin (23, 24). In this experiment, \( R_0 \) and \( K_d \) values were determined for luminal (F1) and abluminal (F3) fractions: F1, 630 pmol/mg, 509 \( \times \) 10^{-9} M; F3, 280 pmol/mg, 356 \( \times \) 10^{-9} M. The results depicted in Fig. 2 demonstrated a greater number of binding sites in the luminal fraction than in the abluminal fraction, which is in agreement with transport activity determinations (Fig. 1B) but distinct from the results obtained by Western blotting (Fig. 1C).

Fig. 2. Quantitation of glucose transporter concentration and affinity with CB binding. Fractions F1 (luminal) and F3 (abluminal) of the preparation described above were further analyzed for glucose transporter concentration by CB binding. Aliquots of luminal and abluminal membranes (10–15 \( \mu \)g) were incubated in duplicate with varying concentrations of \( \delta \)Hcytochalasin B (4–50 \( \times \) 10^{-8} M) in the presence of 2 mM cytochalasin E and with and without 500 nM \( \delta \)-glucose. Tracer \( \delta \)C] glucose was included to correct for trapping of radioactivity. The radioactivity associated with both pellets and supernatants was measured by liquid scintillation counting, and Scatchard plots were constructed. Net \( \delta \)-glucose-inhibitable CB binding was determined by subtraction of curves along the radial axes of equal free CB concentration radiating from the origin (23, 24). In this experiment, \( R_0 \) and \( K_d \) values were determined for luminal (F1) and abluminal (F3) fractions: F1, 630 pmol/mg, 509 \( \times \) 10^{-9} M; F3, 280 pmol/mg, 356 \( \times \) 10^{-9} M. The results depicted in Fig. 2 demonstrated a greater number of binding sites in the luminal fraction than in the abluminal fraction, which is in agreement with transport activity determinations (Fig. 1B) but distinct from the results obtained by Western blotting (Fig. 1C).

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Fig. 3. [\( \delta \)H]ATB-BMPA photolabeling was performed on fractions from a subsequent preparation, essentially as described for neuronal membranes (26). Luminal (F1) and abluminal membranes (F3), 100 \( \mu \)g each, were resuspended in isolation buffer (mannitol-HEPES) and incubated with 100 \( \mu \)Ci of [\( \delta \)H]ATB-BMPA (final concentration 50 \( \mu \)M). The mixture was exposed to UV light. The labeled transporters were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels, which were sliced, dried, and solubilized as described under "Materials and Methods." The area under the peak at 55 kDa was calculated by summing the slices included in the peak and subtracting the base line.

DISCUSSION

This study is the first demonstration that functional GLUT1 glucose transporter proteins may exist in different conformations in the luminal and abluminal membranes of the mammalian BBB. The GLUT1 transporters responsible for mediating transport from the blood across the luminal membrane into the endothelial cell appear to be modified such that the C terminus is no longer available to immunodetection by Western blot analysis.
The original purpose of this study was to systematically define the polarity of glucose transport across the cerebral microvascular endothelial cells that constitute the blood-brain barrier. This polarity has been characterized for some transport systems, i.e. amino acids, as involving different transporters, for instance sodium-dependent transport across the abluminal membrane but not the luminal membrane (10, 28). It has generally been agreed that GLUT1 is the sole mediator of glucose transport across the mammalian BBB and that the polarity of glucose transport has been derived from the observed asymmetry in the distribution of transporter numbers in the two membranes (12). However, actual measurements of transport activities in isolated luminal and abluminal membrane vesicles suggested equivalent levels of transport (11). Because the latter study did not include any specific measure of GLUT1 concentration in these vesicles, we undertook a comprehensive characterization of the glucose transport properties of these membrane vesicles by a variety of techniques. The results of the present study provide the first indication of a modification of the GLUT1 glucose transporter in the luminal membranes that is reflected in an inability to detect clearly functional transporters with an antibody directed against the C terminus of the protein.

Transport activities in luminal (F1) and abluminal (F2-F5) membrane vesicles in this study were ~2:1 (Fig. 1B). A similar ratio was obtained when these fractions were analyzed for glucose transporter content by CB binding to F1 and F3 (Fig. 2) suggesting a comparable number of transporters in the fractions. However, when the membrane fractions were blotted with the commonly used anti-C-terminal antibody, the ratio of luminal:abluminal GLUT1 was roughly 1:5, which is similar to the asymmetric distribution derived from electron microscopic immunogold studies (12). Further analysis of these fractions with H sera, which were raised against the entire purified human erythrocyte GLUT1 protein, yielded far more equivalent concentrations for GLUT1 in the respective membranes. This was corroborated in an additional Western blot employing an antisera directed against a peptide sequence within the intracellular loop of GLUT1. Thus, it is clear from the results of Figs. 1 and 2 that the glucose transporter protein in the luminal F1 fraction displays an epitope specificity quite different from the other fractions despite a greater capacity for transport and an apparent higher concentration of transporters as determined by cytochalasin B binding.

There could be several potential explanations for these findings. One possibility is that the epitope was lost due to proteolysis during preparation; however, several factors suggest that this is not the case. These include the use of multiple protease inhibitors throughout the preparation and the failure to identify any lower molecular weight proteolytic fragments with the antisera raised against the whole protein (H) or the loop antibody (L). Furthermore, we observed the same apparent loss of epitope in both unfractionated cow and rat microvessels by Western blot analysis (data not shown). Alternatively, the existence of other GLUT isoforms could contribute to increased transport activity and CB binding in the luminal membranes. Previous studies from our laboratory have shown both human and rat microvessels to be devoid of GLUT2-5 (17, 29, 30). There have been reports of GLUT3 in human, rodent, and canine BBB (31, 32). Subsequent studies refuted this observation for both rodent and human brain (29, 34). Others have detected trace levels of GLUT4 and GLUT5 in rat microvessels (33, 34). It should be noted that GLUT2 and GLUT5 have very low affinities for CB and glucose and thus are unlikely candidates to resolve the discrepancies. Our studies failed to detect GLUT4 in bovine vessels with an antibody that will recognize bovine GLUT4. However, as discussed above, we cannot use lack of immunodetection to rule out the presence of GLUT3 as our antibodies do not cross-react with the bovine isoform. The recent discovery of a new family of glucose transporters (35–37) offers additional candidates to explain these findings. Little is known about the transport kinetics of these proteins, although both GLUT1xs 1 and 2/GLUTs 8 and 9 appear to bind CB. The potential expression in the BBB has yet to be determined, although our preliminary studies have failed to detect GLUTX-1 or -2 mRNA in mouse BBB by in situ hybridization (data not shown).

Finally, the question arises as to whether there are any GLUT1 transporters in the luminal membrane that are detectable with the C-terminal-directed antibody or is this modification uniformly applied to luminal GLUT1? The results of our studies suggest the latter. In any given fractionation there is a certain degree of contamination of F1 with abluminal membrane (Fig. 1A) and the potential for further contamination with intracellular transporters, which may or may not fully interact with the C-terminal antibody. Thus, the results of this study confirm and extend our earlier report of a comparable level of glucose transport activity in luminal and abluminal fractions coupled with a modification of the luminal GLUT1 transporters that alters the C-terminal immunoreactivity.

It next becomes important to view these data in the context of previous electron microscopy studies on BBB GLUT1 localization especially with regard to the nature of the antisera used in the respective studies. The first report on canine microvessels used a C-terminal antibody and reported an equal distribution of GLUT1 transporters between the luminal and abluminal membranes with a relatively low level (13%) of intracellular transporters (3). Subsequent studies in rat, rabbit, and human found an asymmetrical distribution between luminal:abluminal membranes (1:4) and a substantial (40%) intracellular pool of GLUT1 (12, 38, 39). These studies also used a C-terminal-directed antibody, but in addition one study reported similar findings with an antibody raised against the purified human erythrocyte GLUT1 comparable with the H sera used in this study (12). When this human erythrocyte GLUT1 antisera was later used to describe the microvascular distribution of GLUT1-resected tissue from the brain of a patient with hemangioblastoma and seizures, there was a significant detection of the luminal transporters relative to abluminal (26.4:20.5%) (6). This observation was later confirmed in a subsequent, more extensive study (40). The authors attributed the redistribution to a pathologic adaptation of BBB GLUT1. However, in light of the results of this study it could reflect equally the physiologic distribution detected with an antisera to the whole protein.

A likely explanation for the differences in antibody binding would be a modification of the C-terminal epitope. The C terminus was first shown to be a target for protein kinase C in human erythrocytes by Witters et al. and has since been shown to be phosphorylated upon activation of protein kinase C in a variety of cells. However, in none of the above studies was the interaction of the phosphorylated protein with a C-terminal-specific antibody assessed. The GLUT1 C terminus is replete with serine and threonine residues, and in addition to the protein kinase C site there are two consensus casein kinase II phosphorylation sites. Thus the potential for a covalent modification that alters epitope accessibility is certainly plausible. Therefore the next step in elucidating such a regulatory mechanism is to confirm such a phosphorylation, determine the appropriate kinase responsible, and investigate the

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2 B. Thorens, personal communication.
role that the modification(s) play on the activity, localization, and recycling of the luminal GLUT1 protein.

Clearly, the results of this study demonstrate that the mechanism(s) of glucose transport into the mammalian brain are far from resolved. Rather, these initial findings support asymmetries in distribution as well as in protein conformation between luminal and abluminal vessel membranes, thus opening a spectrum of questions on the regulation of glucose transport across the BBB.

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