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D-GLUCOSE-SENSITIVE AND -INSENSITIVE CYTOCHALASIN B BINDING PROTEINS FROM MICROVILLOUS PLASMA MEMBRANES OF HUMAN PLACENTA

IDENTIFICATION OF THE D-GLUCOSE TRANSPORTER

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Cytochalasin B was found to bind to at least two distinct sites in human placental microvillous plasma membrane vesicles, one of which is likely to be intimately associated with the glucose transporter. These sites were distinguished by the specificity of agents able to displace bound cytochalasin B. [^{3}H]Cytochalasin B was displaceable at one site by D-glucose but not by dihydrocytochalasin B; it was displaceable from the other by dihydrocytochalasin B but not by D-glucose. Some binding which could not be displaced by D-glucose + dihydrocytochalasin B was displaced by excess unlabeled cytochalasin B and may represent a third cytochalasin B binding site. Cytochalasin B can be photoincorporated into specific binding proteins by ultraviolet irradiation. D-Glucose specifically prevented such photoaffinity labeling of a microvillous protein component(s) of $M_r = 60\,000 \pm 2\,000$ as determined by urea-sodium dodecyl sulfate acrylamide gel electrophoresis. This D-glucose-sensitive cytochalasin B binding site of the placenta is likely to be either the glucose transporter or be intimately associated with it. The molecular weight of the placental glucose transporter agrees well with the most widely accepted molecular weight for the human erythrocyte glucose transporter. Dihydrocytochalasin B prevented the photoincorporation of [^{3}H]cytochalasin B into a polypeptide(s) of $M_r = 53\,000 \pm 2\,000$. This component is probably not associated with placental glucose transport. This report presents the first identification of a sodium-independent glucose transporter from a normal human tissue other than the erythrocyte. It also presents the first molecular weight identification of a human glucose-insensitive high-affinity cytochalasin B binding protein.

Sodium-independent, carrier-mediated glucose transport is an important cellular process in many cell types [1] including the human erythrocyte [2], placental syncytiotrophoblast [3,4], and lymphocyte [5]. Despite its widespread importance, unequivocal identification of this glucose transporter has proven difficult. Attempts to identify and obtain information on the physical characteristics of the human sodium-independent glucose transporter have involved the erythrocyte almost exclusively. Utilizing a variety of approaches, the erythrocyte glucose transporter has been identified as a glycoprotein with a monomeric M_r of 50 000–70 000 [6–15], 75 000–100 000 [16–20], or 180 000–200 000 [21,22]. Some investigators have suggested that the lower apparent molecular weight estimates are attributable to proteolysis [18,19]. However, antibodies generated against purified 55 kDa polypeptides do not react with larger polypeptides [13,23]. Most of the human erythrocyte data thus support a monomeric M_r of about 55 000. The sodium-independent glucose transporter of

HeLa cells also has an apparent M_r of about 55 000 [13].

A very useful tool in the study of sodium-independent glucose transport has been the fungal metabolite, cytochalasin B, a potent reversible inhibitor of glucose transport with an inhibition constant, K_i, of about 0.3–0.7 μM in the erythrocyte [11,16,24]. Three separate cytochalasin B binding sites, distinguishable by their specificity of cytochalasin B displacement, have been reported in the human erythrocyte [12,24–26]. Cytochalasin B binding at site I is displaceable by D-glucose but not by dihydrocytochalasin B, site II cytochalasin B binding is displaceable by dihydrocytochalasin B but not by D-glucose, and Site III binding is not displaceable by either D-glucose or dihydrocytochalasin B but is displaced by excess cytochalasin B. While cytochalasin B binding sites II and III appear to be primarily independent of the glucose transporter, a large body of evidence suggests that the D-glucose displaceable cytochalasin B binding site, site I, is, or is part of the glucose transporter [7,8,12,24,26–28]. Carter-Su et al. [29], Pessin et al. [30], and Shanahan [31] have recently reported cytochalasin B to be photoreactive under ultraviolet irradiation. By comparing the photoincorporation of [^{3}H]cytochalasin B into proteins in the presence and absence of D-glucose, Carter Su et al. [29], Shanahan [31], and Shanahan et al. [32] have identified the human erythrocyte glucose transporter as a polypeptide(s) of apparent M_r of 54 000, 50 000 and 47 000, and 55 000 and 46 000, respectively.

Plasma membrane vesicles derived from the microvillous surface of the human placental syncytiotrophoblast show stereospecific, sodium-independent hexose transport which is inhibited by cytochalasin B with a K_i of about 0.5 μM [3,4,33]. The human placental glucose transporter is thus similar to the transporter of the erythrocyte. Comparison of physical characteristics of the placental and erythrocyte glucose transporters is therefore of interest. Also, since glucose is the primary substrate for fetal oxidative metabolism and is transported by the placenta [34], an understanding of the molecular basis of placental glucose transport is of importance in itself.

We have utilized the cytochalasin B photoaffinity labeling technique of Carter-Su et al. [29], Pessin et al. [30], and Shanahan [31] to identify the D-glucose-sensitive, as well as D-glucose-insensitive, cytochalasin B binding sites of syncytiotrophoblast microvillous membrane vesicles. We report the apparent molecular weight of the human glucose transporter from a non-erythrocyte tissue, the placenta, and the first molecular weight of a D-glucose-insensitive, erythrocyte-like site II cytochalasin B binding protein.

Experimental procedures

Materials. [^{3}H]Cytochalasin B (15 Ci/mmol), D-[^{14}C]glucose (0.33 Ci/mmol), L-[^{3}H]glucose (11 Ci/mmol), and Protosol were obtained from New England Nuclear. ACS scintillation fluid was from Amersham. Cytochalasin B and dihydrocytochalasin B were obtained from Aldrich Chemical Co., Inc. Sigma Chemical Co. provided the molecular weight standards and glucose analogues. Ultrapure electrophoresis reagents were purchased from Bio-Rad Laboratories.

Methods. Plasma membrane vesicles from human placenta were prepared as described by Bissonnette et al. [4]. Glucose transport and binding of [^{3}H]cytochalasin B by vesicles were measured by Millipore filtration as described [4]. Uptake and binding studies were performed at 14°C and measured in quadruplicate. Phloretin was omitted in buffer solutions used for cytochalasin B binding studies.

Cytochalasin B photoaffinity labeling of vesicles was accomplished essentially by the method of Carter-Su et al. [29] and Pessin et al. [30]. Vesicles were suspended in 5 mM NaH_2PO_4, 1.0 mM EDTA, 250 mM sucrose, pH 7.4 (buffer A) with 250 mM D-glucose, L-glucose, or D-sorbitol at 1 mg/ml vesicle protein. After equilibration, the suspension was added to a test tube in which [^{3}H]cytochalasin B, with or without dihydrocytochalasin B, had been dried under N_2. Concentration of cytochalasin B in final suspension was 0.3 μM; dihydrocytochalasin B, when used, was 70 μM. Prior to ultraviolet irradiation, samples were equilibrated at room temperature for 30–45 min. Samples in glass Petri dishes on ice were irradiated from a vertically positioned Bausch and Lomb 150 watt zenon light source at a distance of 12 cm for 0, 5, 10, and 30 min. 0.5-ml aliquots were then

diluted with 6 ml ice-cold buffer A and centrifuged at 100000 × *g* for 90 min. The pellet was washed once by centrifugation in 6 ml cold buffer A. Protein concentrations were determined by the method of Lowry et al. [35].

Proteins were resolved on urea-SDS acrylamide gels basically by the method of Swank and Munkries [36] using some modifications from Downer et al. [37]. Vesicle proteins were solubilized in fresh 2% SDS, 4 M urea, 5% β-mercaptoethanol, 5 mM H_2PO_4, pH 8.3 and incubated at 37°C for 90 min. The stacking gel was 3.5% acrylamide, 4 M urea, 0.1% SDS, pH 5.8; the resolving gel was 7.5% acrylamide, 8 M urea, 0.1% SDS, pH 6.8. The resolving gel was 3 mm thick, 180 mm long and 200 μg protein was applied per gel lane. Radioactivity in various protein bands was determined by cutting the lanes into 3 mm slices with a Bio-Rad gel slicer. Slices were extracted with 1 ml Protosol and assayed in 14 ml ACS fluid with a Packard model 3380 liquid scintillation counter. Myosin, β-galactosidase, phosphorylase B, bovine serum albumin, rabbit muscle actin, and lysozyme were used as molecular weight standards.

Results

Effects of D-glucose and dihydrocytochalasin B on cytochalasin B binding are additive

The effects of D-glucose, dihydrocytochalasin B, and cytochalasin B on the binding of 0.2 μM [^{3}H]cytochalasin B to microvillous membrane vesicles were examined. In the presence of 70 μM unlabeled cytochalasin B, no vesicle-associated labeled cytochalasin B could be detected. 200 mM D-glucose * eliminated 53 ± 7% (S.D., $n = 4$), 70 μM dihydrocytochalasin B eliminated 27 ± 3% ($n = 4$), and 200 mM D-glucose + 70 μM dihydrocytochalasin B removed 80 ± 6% ($n = 4$) of the labeled cytochalasin B binding. Thus it appears that the effects of D-glucose and dihydrocytochalasin B are additive, suggesting that they displace cytochalasin B from different binding sites.

* The effect of D-glucose on 0.2 μM cytochalasin B binding is constant over a range of 200 to 500 mM, unpublished observations.

Stereospecific glucose uptake is unaffected by high concentrations of dihydrocytochalasin B

5 s stereospecific glucose uptake was examined in the absence and presence of 0.1 μM to 0.1 mM dihydrocytochalasin B. Initial uptake of carrier-mediated D-glucose transport (calculated as D-glucose uptake minus L-glucose uptake) for 5 s was 0.41 ± 0.23 ($n = 4$) nmol/mg protein. In the presence of 0.1 mM dihydrocytochalasin B, uptake was 0.43 ± 0.04 ($n = 4$). Stereospecific glucose uptake was therefore unaffected by relatively high concentrations of dihydrocytochalasin B.

Photoaffinity labeling with [^{3}H]cytochalasin B identifies 50–63 kDa proteins

Vesicle proteins photoaffinity labeled with [^{3}H]cytochalasin B were electrophoresed on urea-SDS acrylamide gels. Non-ultraviolet irradiated samples showed no discernible polypeptide labeling (data not shown). Exposure for 5, 10, and 30 min resulted in increasing specific [^{3}H]cytochalasin B-polypeptide labeling, however, the same molecular weight polypeptides were labeled at the various exposure times. A representative pattern of tritium distribution in a gel is shown in Fig. 1. A broad band of [^{3}H]cytochalasin B labeling was generally observed in the M_r range of 50000–63000 for photoaffinity labeling conducted in the presence of D-sorbitol, a non-transported sugar alcohol [38] (L-glucose, substituted for D-sorbitol, gave similar results). Most of this labeling was eliminated by ultraviolet irradiation in the presence of 250 mM D-glucose + 70 μM dihydrocytochalasin B (Fig. 1). Coomassie blue staining of polypeptides showed several prominent bands at $M_r = 69000$ and above, and at 43000 and below. Several minor bands did correspond to the region of maximal displaceable cytochalasin B binding.

D-Glucose and dihydrocytochalasin B displaceable cytochalasin B binding sites are associated with different polypeptides

To distinguish between D-glucose and dihydrocytochalasin B displaceable cytochalasin B binding sites, the following gel protocol was employed: in addition to molecular weight standards, microvillous vesicle proteins irradiated in the presence of (1) 250 mM D-sorbitol, (2) 250 mM D-glucose, (3) 250 mM D-sorbitol + 70 μM dihydrocyto-

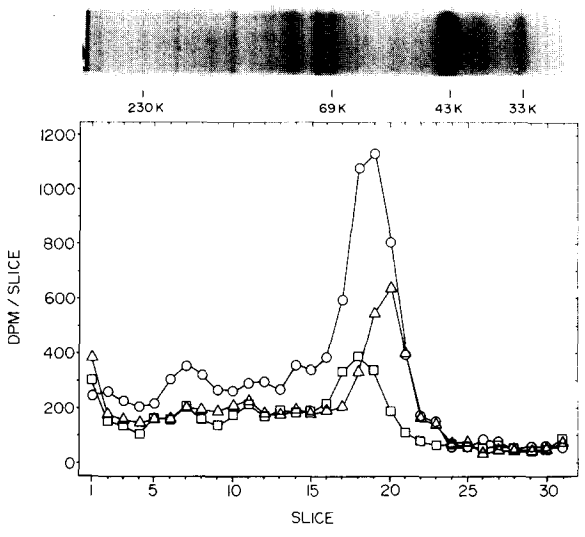

Fig. 1. Profile of Coomassie blue R staining of placental microvillous proteins in a urea-SDS acrylamide gel and corresponding distribution of polypeptides photoaffinity labeled with [^{3}H]cytochalasin B. Shown are the results of membrane vesicles incubated with 0.3 μM [^{3}H]cytochalasin B in the presence of 250 mM D-sorbitol (○), 250 mM D-glucose (△), or 250 mM D-glucose + 70 μM dihydrocytochalasin B (□). Samples were irradiated for 10 min, washed, and electrophoresed as described under Experimental Procedures. 200 μg membrane protein were applied per lane. Distribution of radioactivity within gel is shown only for the top half of the gel; previous data (not shown) indicated no significant radioactivity from mid-gel to the bromphenol blue tracking dye. Although actin has been reported to be a D-glucose insensitive, cytochalasin B binding protein [5], little radioactivity is present at the 43 kDa position of the gel.

chalasin B, and (4) 250 mM D-glucose + 70 μM dihydrocytochalasin B were electrophoresed simultaneously in adjacent gel lanes. Specific D-glucose displaceable cytochalasin B binding was localized by determining the difference in tritium labeling per gel slice between lanes of protein labeled in the presence of D-sorbitol and D-glucose then plotting that difference against slice number, i.e., position on gel. Such a difference plot shows molecular weight regions of the gel where photoincorporation of [^{3}H]cytochalasin B is prevented by one agent relative to another. In this case, the plot shows the specific prevention of photoaffinity labeling of microvillous membrane proteins of D-glucose relative to D-sorbitol corresponding to the specific D-glucose displaceable cytochalasin B binding site(s). A similar plot was generated for the difference in labeling between the D-sorbitol + dihydrocytochalasin B and D-glucose + dihydrocytochalasin B lanes. Both are shown in Fig. 2A. Maximal D-glucose displaceable cytochalasin B binding was found at a protein $M_r = 60\,000 \pm 2000$ ($n = 11$). (Data was obtained from two sets of experiments. In each set, a single gel was run for 5, 10, and 30 min ultraviolet irradiated vesicle sam-

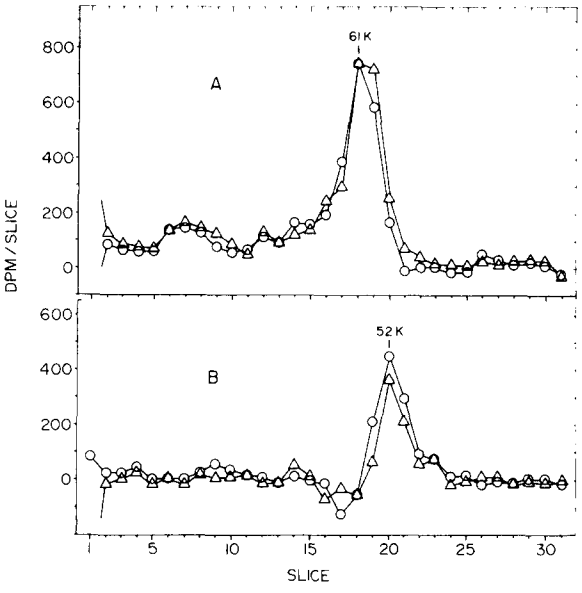

Fig. 2. (A) Specific D-glucose displaceable [^{3}H]cytocholasin B photoaffinity labeling of polypeptides resolved on a urea-SDS acrylamide gel. (Gel system is that shown in Fig. 1 and described under Experimental Procedures.) Ordinate is the difference in dpm per slice obtained by subtracting the dpm in the lane of vesicle protein irradiated with ultraviolet light in the presence of 250 mM D-glucose from the dpm in the lane of protein irradiated in the presence of 250 mM D-sorbitol (○) or obtained by subtracting dpm of the 250 mM D-glucose + 70 μM dihydrocytochalasin B lane from the dpm of the 250 mM D-sorbitol + 70 μM dihydrocytochalasin B lane (△). (B) Specific dihydrocytochalasin B displaceable [^{3}H]cytochalasin B photoaffinity labeling. Ordinate is the difference in dpm per slice between dpm values of the lanes of vesicle proteins irradiated in the presence of 250 mM D-glucose + 70 μM dihydrocytochalasin B and 250 mM D-glucose (○) or difference in dpm values of the 250 mM D-sorbitol + 70 μM dihydrocytochalasin B and 250 mM D-sorbitol lanes (○). All data shown in A and B were obtained from a single gel. The molecular weights correspond to location of the center of the slice position with the greatest radioactivity difference, i.e., greatest specific displaceable [^{3}H]cytochalasin B binding, and were calculated from molecular weight standards electrophoresed with the irradiated protein samples.

ples. Two placentas were pooled for each set.) Two difference plots were also generated for specific dihydrocytochalasin B displaceable cytochalasin B binding. One was generated as D-glucose minus D-glucose + dihydrocytochalasin B; a second as D-sorbitol minus D-sorbitol + dihydrocytochalasin B per urea-SDS gel. These plots, corresponding to the specific dihydrocytochalasin B displaceable cytochalasin B binding site(s) are shown in Fig. 2B. Maximal dihydrocytochalasin B displaceable cytochalasin B binding was found at a protein M_r = 53 000 ± 2 000 (n = 11).

Some D-glucose displaceable cytochalasin B binding was usually noted in the high molecular weight region of the gel, however, actual molecular weight determinations varied considerably over 140 000–180 000 and may represent artifactual aggregation of the 60 kDa component. Electrophoresis of proteins solubilized in the absence of β-mercaptoethanol showed little radioactivity within the gel; most of the labeled polypeptides appeared to remain on the stacking gel.

Discussion

Cytochalasin B is a potent reversible inhibitor of carrier-mediated glucose transport by the human erythrocyte. The transporter itself is therefore likely to contain a cytochalasin B binding site. Three major types of cytochalasin B binding sites have been characterized with respect to cytochalasin B affinity and specific displacement in the erythrocyte [12,24–26]. Human lymphocytes also appear to have three cytochalasin B binding sites with similar displacement characteristics [5]. In the present study, we have found that cytochalasin B binding (at 0.2 μM) to human placental membrane vesicles is partially displaceable by 200 mM D-glucose (53%) and partially by 70 μM dihydrocytochalasin B (27%). Displacement by D-glucose + dihydrocytochalasin B (80%) is greater than either and appears additive suggesting that D-glucose and dihydrocytochalasin B displace cytochalasin B from different binding sites. These findings correspond well to those from a study by Lin and Snyder [25] of the erythrocyte. Thus, erythrocyte-like type I and II cytochalasin B binding sites appear to exist in the placental microvillus. That D-glucose + dihydrocytochalasin B displaced about 80% of the total displaceable cytochalasin B binding suggests that an erythrocyte-like type III cytochalasin B binding site exists in the placental syncytiotrophoblast plasma membrane as well.

Cytochalasin B can be covalently linked to binding sites by ultraviolet irradiation. Agents which displace cytochalasin B from binding sites thus should tend to prevent such linking. Since D-glucose and dihydrocytochalasin B appear to displace cytochalasin B from distinctly different binding sites, we have examined the tendency of these agents to block the photoincorporation of labeled cytochalasin B to polypeptide chains. With this approach, it has been possible to localize the erythrocyte-like site I and II cytochalasin B binding sites, shown in Fig. 2A and B. The D-glucose displaceable cytochalasin B binding site is a polypeptide(s) of apparent mean M_r = 60 000 ± 2 000; the dihydrocytochalasin B displaceable cytochalasin B binding site is a polypeptide(s) of apparent mean M_r = 53 000 ± 2 000. The erythrocyte-like placental cytochalasin B binding sites I and II thus appear to be on distinctly different polypeptide chains. Furthermore, these chains do not coincide with major Coomassie blue staining bands observed by electrophoresis; they are apparently relatively minor components of the total microvillous protein complement. Vesicle irradiation in the presence of D-glucose + dihydrocytochalasin B still shows some [^{3}H]cytochalasin B incorporation into polypeptides of M_r of about 54 000–60 000 (Fig. 1). This may represent the erythrocyte-like site III cytochalasin B binding site or be due to incomplete inhibition of cytochalasin B binding by 250 mM D-glucose + 70 μM dihydrocytochalasin B.

Numerous findings suggest that the D-glucose displaceable cytochalasin B binding site, site I, of the erythrocyte is, or is a monomer of, the glucose transporter. These findings include the following: cytochalasin B prevents the irreversible inhibition of glucose transport by a variety of reagents [7,8,28], the dissociation constant, K_d, of cytochalasin B binding is approximately equal to the K_i for inhibition of transport, cytochalasin B is apparently displaced by D-glucose with 1 : 1 stoichiometry, and the extent of site I binding sites is similar to the estimated number of glucose transporters in the erythrocyte [24,27]. Additional findings summarized by Jung and Rampal [24]

further support the contention that the D-glucose displaceable cytochalasin B binding site of the erythrocyte is an integral part of the glucose transporter.

The placental microvillus also has a D-glucose displaceable cytochalasin B binding site identified as a 60 kDa polypeptide(s). It is likely that this component of the placental membrane is, or is intimately associated with, the human placental glucose transporter. This follows from the findings that as in the erythrocyte, cytochalasin B is a potent inhibitor of placental membrane glucose transport and that the placental microvillus contains a significant D-glucose displaceable cytochalasin B binding capacity. Furthermore, there is a marked analogy between the sodium-independent glucose transporters of the erythrocyte and placental microvillus. These include similar molecular weights, indistinguishable K_i values of transport inhibition by cytochalasin B, similar responses to the inhibition by cytochalasin B, similar responses to the inhibitors phlorizin and phloretin, and similar transport characteristics of glucose analogues [3,4,38].

The erythrocyte-like type II cytochalasin B binding site of the placental microvillus, the 53 kDa component, is unlikely involved in glucose transport. Bissonnette et al. [4] have reported that inhibition of the stereospecific glucose transport of placental membrane vesicles by cytochalasin B has a K_i of about 0.5 μM; virtually all glucose transport is inhibited at a concentration of 5 μM. Data from this study show that dihydrocytochalasin B has no influence on D-glucose transport up to a concentration of 0.1 mM. The dihydrocytochalasin B binding site thus appears independent of hexose transport activity. It is more likely associated with cell morphology [39].

Based on the findings of this study, it appears that the syncytiotrophoblast microvillus of the human placenta contains cytochalasin B binding sites apparently analogous to those of the erythrocyte. The placental cytochalasin B binding site sensitive to D-glucose and likely to be intimately associated with stereospecific glucose transport has been identified as a polypeptide(s) of $M_r = 60\,000$. This corresponds well to the probable molecular weight of the erythrocyte hexose transporter. The placental cytochalasin B binding site displaceable by dihydrocytochalasin B, and probably not involved with glucose transport, has been identified as a polypeptide(s) of $M_r = 53\,000$. Placental cytochalasin B binding sites analogous to sites I and II of the erythrocyte are thus distinctly different proteins distinguishable by urea-SDS acrylamide electrophoresis.

Note added in proof (Received January 14th, 1983)

Since the submission of the present communication for publication, a study using similar approaches has been published by Johnson and Smith [40]. Although the results appear qualitatively similar, there is a discrepancy on the estimation of M_r values of the cytochalasin B binding sites.

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