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CHARACTERIZATION AND SOLUBILIZATION OF THE CYTOCHALASIN B BINDING COMPONENT FROM HUMAN PLACENTAL MICROSOMES

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Human placental microsomes exhibit uptake of D-[^{3}H]glucose which is sensitive to inhibition by cytochalasin B (apparent $K_i = 0.78$ μM). Characterization of [^{3}H]cytochalasin B binding to these membranes reveals a glucose-sensitive site, inhibited by D-glucose with an $ED_{50} = 40$ mM. The glucose-sensitive cytochalasin B binding site is found to have a $K_d = 0.15$ μM by analysis according to Scatchard. Solubilization with octylglucoside extracts 60–70% of the glucose-sensitive binding component. Equilibrium dialysis binding of [^{3}H]cytochalasin B to the soluble protein displays a pattern of inhibition by D-glucose similar to that observed for intact membranes, and the measurement of an $ED_{50} = 37.5$ mM D-glucose confirms the presence of the cytochalasin B binding component, putatively assigned as the glucose transporter. Further evidence is attained by photoaffinity labelling; ultraviolet-sensitive [^{3}H]cytochalasin B incorporation into soluble protein (M_r range 42 000–68 000) is prevented by the presence of D-glucose. An identical photolabelling pattern is observed for incorporation of [^{3}H]cytochalasin B into intact membrane protein, confirming the usefulness of this approach as a means of identifying the presence of the glucose transport protein under several conditions.

Introduction

The use of the fungal metabolite cytochalasin B has been instrumental in efforts leading to the current understanding of monosaccharide transport by facilitated diffusion systems. The inhibition of glucose transport by cytochalasin B has been described in several cell types, and has been correlated to a glucose-sensitive [^{3}H]cytochalasin B binding component, as observed in human erythrocyte membranes [1], rat fat cell membranes [2], chick embryo fibroblast membranes [3], rat-liver plasma membranes [4] and rat skeletal muscle membranes [5]. The erythrocyte membrane transport protein has been purified by assaying reconstitution of D-glucose transport activity [6]. Baldwin et al. [7] established the use of cytochalasin B binding activity as criteria for purification by isolating a protein identical to that described by Kasahara and Hinkle [6] that has an M_r value of 55 000. Furthermore, the glucose-sensitive cytochalasin B binding protein in erythrocyte membranes has been characterized as an M_r 55 000 species by direct photoaffinity labelling of [^{3}H]cytochalasin B to erythrocyte membranes [8,9]. This technique has also been used to identify the cytochalasin B binding protein in rat adipocyte and chick embryo fibroblast membranes [10,11].

Recently, monosaccharide transport has been studied in microvillous membranes of human placenta [12,13]. Cytochalasin B was shown to be a

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

potent inhibitor of the transport process. Identification of a glucose-sensitive cytochalasin B binding protein was accomplished by photocoupling [^{3}H]cytochalasin B to an M_r 52000 moiety present in this membrane [14,15]. This evidence prompted the current investigation into the solubilization and characterization of the glucose-sensitive cytochalasin B binding component, putatively assigned as the glucose transport protein, present in human placental microsomes.

D-Glucose uptake by placental microsomes displayed a response to cytochalasin B similar to that previously described in other systems. The cytochalasin B binding activity of the membranes from placental microsomes was further characterized in order to develop a means to assay the solubilization of this component. Equilibrium dialysis binding of [^{3}H]cytochalasin B defined a soluble binding complex with a response to D-glucose identical to that observed in intact membrane. A second method of characterizing the soluble cytochalasin B binding component exploited the use of photoaffinity labelling techniques. Upon exposure to ultraviolet light, [^{3}H]cytochalasin B was incorporated into the soluble preparation, labelling a species in the M_r range 42000–68000. The photolabelling of [^{3}H]cytochalasin B was prevented by the presence of D-glucose. These results confirm the solubilization and resolution of the cytochalasin B binding protein from human placental microsomes.

Methods

Preparation of placental microsomes and membranes. Isolation of placental microsomes was performed essentially as described by Cuatrecasas [16] for rat-liver microsomes. Amnion and chorion were dissected from human placenta after extensive washes with ice-cold phosphate-buffered saline. The remaining placenta was homogenized in 50 mM Hepes (pH 7.5)/0.25 M sucrose/1 mM benzamidine/1 mM phenylmethylsulfonyl fluoride, employing a Tekmar tissuemizer. Following centrifugation of the homogenate for 30 min at $10000 \times g$, the supernatant was adjusted to be 100 mM NaCl and 0.2 mM $MgCl_2$ and centrifuged 45 min at $40000 \times g$. Pellets, consisting of microsomes, were subjected to three washes in 50 mM Hepes (pH 7.6)/0.25 M sucrose, with centrifugation for 45 min at $40000 \times g$. Microsomes were resuspended at a final concentration of about 8 mg of protein/ml. Visualization by electron microscopy showed this preparation to consist mainly of a population of sealed, intact vesicles (data not shown). It was not necessary to maintain the integrity of the microsomes for membrane preparations, and the final washes were accomplished with 50 mM Tris (pH 7.4). All preparations were stored frozen at −20°C until use.

Solubilization of membrane preparation. The preparation of membranes was centrifuged for 20 min at $25000 \times g$ and resuspended in 1% octylglucoside/50 mM Tris (pH 7.4) with a final protein concentration of 2 mg/ml. Solubilization occurred with gentle rocking of this sample for 30 min at 4°C, after which the preparation was centrifuged for 60 min at $100000 \times g$. The supernatant containing soluble protein was removed for immediate use. About 40% of the membrane protein was routinely extracted by this procedure.

Glucose uptake measurements. Placental microsomes were washed with Krebs-Ringer phosphate buffer (128 mM NaCl/5.2 mM KCl/1.4 mM $CaCl_2$/1.4 mM $MgSO_4$/10 mM Na_2HPO_4, pH 7.4) and dispersed with a Tekmar tissuemizer for 5 s. Aliquots of the microsomal suspension were incubated in the absence or presence of cytochalasin B for 20 min at ambient temperature. Uptake was initiated by mixing 90 μl microsomes with 10 μl buffer containing 2 μCi L- or D-[^{3}H]glucose. Transport was stopped by the addition of 3 ml ice-cold Krebs-Ringer phosphate buffer containing 2 mM $HgCl_2$ and rapid vacuum filtration of this medium through a membrane filter (Millipore, 0.45 μM), followed by two more 3-ml washes of the stopping solution. Radioactivity on the filters was measured by scintillation counting.

Cytochalasin B binding assays. Measurement of cytochalasin B binding to membranes was performed by a centrifugation assay described in detail by Axelrod and Pilch [4]. Binding was performed using 50 mM Tris (pH 7.4) buffer with 5 μM cytochalasin E, 0.2 μCi [^{3}H]cytochalasin B, appropriate amounts of unlabeled cytochalasin B, D-glucose, or sorbitol, and 250–300 μg protein in 1 ml. This mixture was incubated 30 min at ambient temperature, after which membranes were pelleted

by a 10-min microfuge spin (approx. $15000 \times g$). Immediately after centrifugation, the supernatant was aspirated and ends of the microfuge tubes were cut off and equilibrated with 4 ml scintillation cocktail before counting to determine bound [^{3}H]cytochalasin B. Preliminary experiments showed that the 30 min incubation time was ample, as binding attains equilibrium within a 10-min period.

Equilibrium dialysis was performed to determine cytochalasin B binding to soluble protein. Spectrapor 2 dialysis membrane which had been boiled in 20 mM Na_2CO_3/1 mM EDTA, and then boiled with three changes of water, was used to separate the two 1-ml chambers of each dialysis cell. Solubilized protein was diluted with 50 mM Tris (pH 7.4) and concentrated using an Amicon device with type YM10 membranes such that the final concentration of octylglucoside was 0.85 mM in the dialysis cells. One chamber of each dialysis cell was filled with 1 ml containing 250–300 μg soluble protein. The opposing chamber was loaded with 1 ml 50 mM Tris (pH 7.4) containing 1 μCi [^{3}H]cytochalasin B, with appropriate amounts of unlabelled cytochalasin B, D-glucose or sorbitol. Dialysis cells were then gently rocked overnite at 4°C. Aliquots were removed from each side, and triplicate 200-μl samples were measured for radioactivity by scintillation counting. An initial experiment determined that bound cytochalasin B rose to a maximum within 10 h of dialysis time and remained constant over the next 48-h period. Therefore, the soluble binding component appeared stable over the time-course of the dialysis experiments, with routine dialysis times of 17–18 h.

Nonspecific [^{3}H]cytochalasin B binding was measured as binding in the presence of $4.2 \cdot 10^{-5}$ M unlabelled cytochalasin B in both the equilibrium dialysis and centrifugation binding assays. Data were analyzed by the method of Scatchard [17] where appropriate.

Photoaffinity labelling. Membranes were washed into 5 mM phosphate buffer (pH 8.0) with a final concentration of 1 mg protein/ml. Soluble membrane protein was concentrated to 2–3 mg/ml using an Amicon device (type YM10 membrane filter) and dialyzed overnite against 5 mM phosphate buffer (pH 8.0). Aliquots were incubated for 20 min at room temperature with 0.1 mM cytochalasin E and 0.5 M D-glucose or sorbitol. [^{3}H]Cytochalasin B (17 Ci/mmol) was dried at the bottom of a jacketed glass test-tube such that the final concentration was 1 μM. Incubated samples were added to these test tubes and vigorously vortexed. Samples were then placed in an ice-cold water bath and photolyzed for 30 min with a focused 200 watt mercury lamp (Oriel Corp.) at a distance of 20 cm. Membrane samples were pelleted with a 10-min microfuge spin and washed once with buffer. Soluble protein samples were taken directly from the test-tube. Samples of 200–400 μg of protein were prepared for gel electrophoresis. Gel lanes were sliced into 3-mm sections, which were then boiled in 0.5 ml 30% H_2O_2 until dissolved. Radioactivity was measured by scintillation counting of the dissolved samples. Incubation of [^{3}H]cytochalasin B itself with warm H_2O_2 under conditions that dissolved gel slices did not lead to any loss of radioactivity.

Other procedures. Protein concentrations were determined according to the method of Lowry et al. [18] using bovine serum albumin as the standard. Raidoactivity was measured by liquid-scintillation counting, with Altex Readi-Solv MP as the counting solution. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [19]. Electrophoresis samples were prepared with 1% SDS/50 mM dithiothreitol/10% glycerol/50 mM Tris (pH 6.8) and incubated for 5 min at 95°C. The following molecular weight standards were used: galactosidase (M_r 116000), phosphorylase *b* (M_r 97000), bovine serum albumin (M_r 68000), actin (M_r 42000), ovalbumin (M_r 43000) and carbonic anhydrase (M_r 29000). Gels were stained with Coomassie blue, and gel densitometry was performed using an Isco UA-5 spectrophotometer, with a 0.25 mm slitwidth, at 580 nm.

Materials

D-[^{3}H]Glucose and L-[^{3}H]glucose were purchased from New England Nuclear. [^{3}H]cytochalasin B was obtained from Amersham. Aldrich was the source of unlabelled cytochalasin B. Octylglucoside was purchased from Calbiochem-Boehring. Hepes was purchased from Research

Organics. Electrophoresis reagents were obtained from Bio-Rad. All other chemicals were reagent grade and purchased from Sigma.

Results

Initial experiments were performed to verify the presence of functional D-glucose transport in the preparation of human placental microsomes. Fig. 1 illustrates the time-dependent vesicular uptake of D-[^{3}H]glucose which is very rapid, equilibrating extra- and intravesicular glucose in 15–30 s. Uptake of D-glucose in the presence of 0.15 mM cytochalasin B was equivalent to the uptake of L-[^{3}H]glucose and thus represents vesicle leakage and nonspecific association. In subsequent experiments, a 2.5 s time-point of uptake was assayed. Although this time is probably not representative of initial velocity [20], it represents a compromise between convenience and kinetics and allows a rough determination of K_i (see below).

The dose-response relationship of cytochalasin B inhibition of D-glucose transport was investigated. Microsomes were incubated with concentrations of cytochalasin B from $5 \cdot 10^{-9}$ to $5 \cdot 10^{-5}$ M

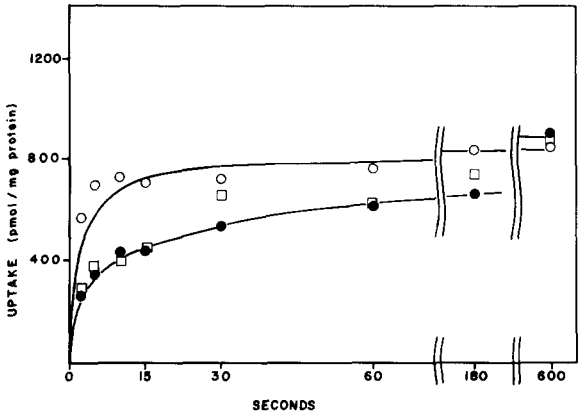

Fig. 1. Time-course of L- or D-glucose uptake into placental microsomes. 90 μl microsomes (190 μg protein) in Krebs-Ringer phosphate buffer (pH 7.4) at 22°C were used to assay uptake in the presence or absence of 0.15 mM cytochalasin B. Mixing with 10 μl buffer containing L- or D-[^{3}H]glucose initiated transport, which was stopped, after appropriate incubation times, by the rapid filtration technique, as described in Methods. The time-courses for uptake of D-[^{3}H]glucose in the absence (○) and presence (□) of cytochalasin B and for uptake of L-[^{3}H]glucose (●) in the absence of cytochalasin B are presented.

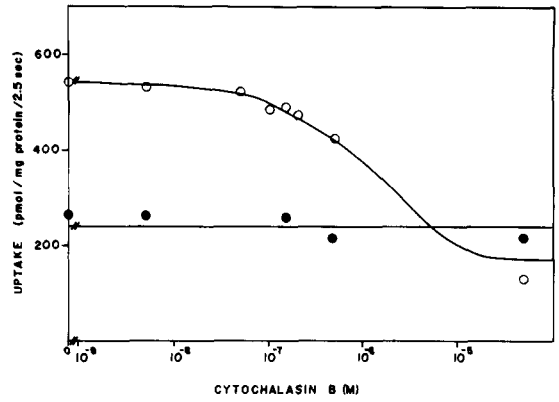

Fig. 2. Inhibition of D-glucose uptake by cytochalasin B. Uptake assays were performed in the presence of appropriate concentrations of cytochalasin B in order to determine the dose-response relationship of transport inhibition by cytochalasin B. Uptake of D-[^{3}H]glucose (○) and L-[^{3}H]glucose (●) by microsomes (175 μg protein) was measured at a 2.5 s time-point as described under Methods.

and uptake of D-[^{3}H]glucose was measured at 2.5 s. The plot of velocity as a function of cytochalasin B concentration is shown in Fig. 2. Cytochalasin B inhibited D-[^{3}H]glucose uptake with an apparent $K_i = 0.78$ μM and, under identical conditions, had no effect on L-[^{3}H]glucose uptake, which was measured as a control for nonspecific uptake and binding to membrane surface. Since uptake of D-[^{3}H]glucose is extremely rapid, the measured velocities are likely to be underestimates of true initial velocities. Therefore, the apparent K_i derived may as well be a low value relative to the true K_i.

To analyze further cytochalasin B interactions with the glucose transporter, binding assays were performed using the placenta microsomal membrane preparation, as intact vesicles were no longer required to monitor binding. Other systems [1,2,4] that have been investigated display a cytochalasin E-sensitive cytochalasin B binding component ascribed as actin. Furthermore, in another placental preparation cytochalasin E was demonstrated to prevent photoaffinity labelling of cytochalasin B to an M_r 46000 protein [14]. A preliminary experiment (results not shown) determined that less than 20% of total cytochalasin B binding was displaced at 1 μM cytochalasin E with no further reduction at concentrations up to 10 μM. There-

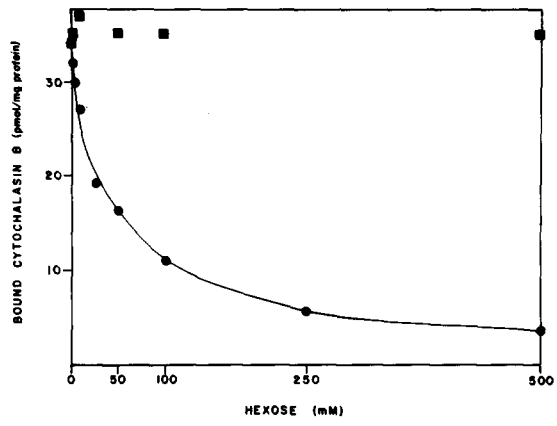

Fig. 3. Glucose displacement of [^{3}H]cytochalasin B binding to membranes. The centrifugation binding assay described under Methods was employed to determine the dose-response relationship of D-glucose inhibition of [^{3}H]cytochalasin binding to the placenta microsomal membrane preparation. Membranes (285 μg protein) were incubated for 30 min at 22°C in the presence of $5 \cdot 10^{-6}$ M cytochalasin E, $5 \cdot 10^{-8}$ M [^{3}H]cytochalasin B, and appropriate amounts of D-glucose or Sorbitol. Bound [^{3}H]cytochalasin B was separated from free ligand by pelleting the membranes and removing the supernatant. Radioactivity in the pellets was measured as discussed in Methods. Bound [^{3}H]cytochalasin B per mg protein in the presence of D-glucose (●) and sorbitol (■) is plotted as a function of hexose concentration.

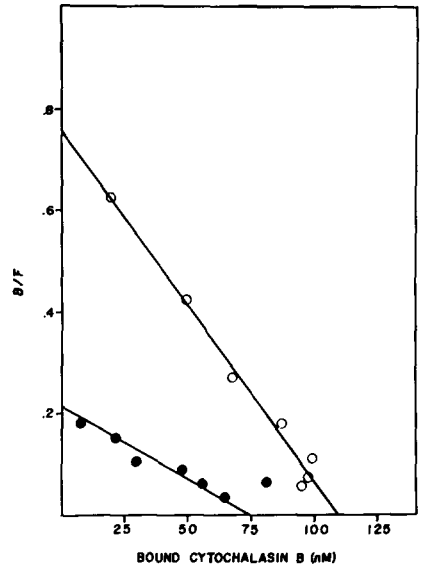

Fig. 4. Scatchard plot of cytochalasin B binding to placental membranes in the presence and absence of D-glucose. Placental membranes (333 μg) were incubated with $5 \cdot 10^{-6}$ M cytochalasin E, $5 \cdot 10^{-8}$ M [^{3}H]cytochalasin B, 50 mM D-glucose (●) or 50 mM sorbitol (○), and different amounts of unlabelled cytochalasin B. The centrifugation method outlined in Methods was used to measure bound and free [^{3}H]cytochalasin B. The data are plotted according to Scatchard [14].

fore, cytochalasin B binding assays were routinely performed in the presence of 5 μM cytochalasin E in order to investigate D-glucose-sensitive sites.

Fig. 3 illustrates D-glucose displacement of [^{3}H]cytochalasin B binding. Membranes were incubated with $5 \cdot 10^{-8}$ M [^{3}H]cytochalasin B in the presence of 5 μM cytochalasin E and concentrations of D-glucose or sorbitol up to 500 mM. The centrifugation method was employed to assay binding, with nonspecific binding measured by the addition of $4.2 \cdot 10^{-5}$ M unlabelled cytochalasin B. The presence of D-glucose dramatically reduced bound [^{3}H]cytochalasin B, with no effect observed in the control with sorbitol present. The dose dependency curve yields ED_{50} of 40 mM for D-glucose inhibition of cytochalasin B binding.

Further characterization of cytochalasin B binding exploited the use of Scatchard analysis. Fig. 4 shows the results of a centrifugation assay in which membranes were incubated with 5 μM cytochalasin E, 50 mM D-glucose or sorbitol, $5 \cdot 10^{-8}$ M [^{3}H]cytochalasin B and appropriate amounts of unlabelled cytochalasin B. The presence of D-glucose produced the expected shift in K_d, measured as apparent affinity, K_{app}. The control, with sorbitol present, represents the K_d of the D-glucose-sensitive site. The inhibition constant, K_i, for D-glucose inhibition of cytochalasin B binding can be calculated from the equation:

$$K_{app} = K_d\left(1 + \frac{[I]}{K_i}\right)$$

with [I] = 50 mM D-glucose as inhibitor concentration. Slopes of the lines from the Scatchard plot gave values of $K_d = 1.45 \cdot 10^{-7}$ M for cytochalasin B binding, and $K_{app} = 3.71 \cdot 10^{-7}$ M. A $K_i = 32.1$ mM D-glucose was derived from the given equation, which is in close agreement with the ED_{50} of 40 mM for D-glucose displacement of cytochalasin B binding determined from the results shown in Fig. 3.

A series of experiments was performed in a similar fashion to measure [^{3}H]cytochalasin B

binding to membranes in the presence of 5 μM cytochalasin E. The dissociation constant, K_d, for cytochalasin B binding was determined by Scatchard analysis and found to be $1.53 \cdot 10^{-7}$ ($\pm 0.07 \cdot 10^{-7}$) M ($n = 3$). This value is readily comparable to values of $0.7 \cdot 10^{-7}$ to $4 \cdot 10^{-7}$ M for the cytochalasin B dissociation constant found in other systems: erythrocytes [1,21], adipocytes [2] and chick embryo fibroblasts [3,11]. The total number of binding sites, B_{max}, measured from the Scatchard plots was determined to be 297 (± 64) pmol/mg protein. Based on a molecular weight of 55 000 and a single binding site per transporter, the estimate can be made that 1.6% of total protein in the placenta microsomal membrane is comprised of the glucose transport protein.

Upon characterization of the glucose-sensitive cytochalasin B binding present in this rich source, efforts were focused on solubilization and resolution of the protein based on the criteria of [^{3}H]cytochalasin B binding activity established by Baldwin et al. [7] in their purification of the erythrocyte glucose transporter. Protein was solubilized from placenta microsomal membranes as described in Methods. Approx. 40% of total membrane protein was extracted, and centrifugation binding assays performed on the protein-depleted membranes (data not shown) indicated 60–70% of glucose-sensitive cytochalasin B binding activity was solubilized. In order to assay [^{3}H]cytochalasin B binding activity in the soluble preparation, the equilibrium dialysis technique, discussed in Methods, was employed. A preliminary experiment resulted in the finding that cytochalasin E has no effect on cytochalasin B binding; in doses up to 20 μM, cytochalasin E did not displace [^{3}H]cytochalasin B bound to the soluble protein. This indicates that upon solubilization, the cytochalasin E-sensitive cytochalasin B binding site remains unextracted. Binding assays involving the soluble material were therefore accomplished in the absence of cytochalasin E.

Fig. 5 presents a comparison of the D-glucose effect for soluble cytochalasin B binding sites with that observed for intact membranes. Aliquots of soluble protein were subjected to equilibrium dialysis against $1 \cdot 10^{-7}$ M [^{3}H]cytochalasin B and appropriate concentrations of D-glucose, with a final concentration (across both sides of dialysis

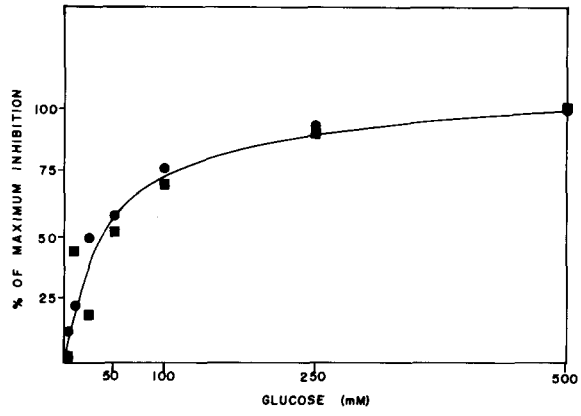

Fig. 5. Inhibition of [^{3}H]cytochalasin B binding by D-glucose. Binding of [^{3}H]cytochalasin B to soluble placental membrane protein (268 μg) was measured by the equilibrium dialysis method detailed in Methods. Medium in the assay contained 0.85 mM octylglucoside, $5 \cdot 10^{-8}$ M [^{3}H]cytochalasin B, and appropriate amounts of D-glucose or sorbitol. Binding data for placental membranes (●) measured by the centrifugation assay (285 μg protein) are presented along with results for soluble material (■) as percentage of maximum inhibition as a function of D-glucose concentration.

cell) of 0.85 mM octylglucoside. Upon addition of the control, sorbitol, samples showed no decrease in [^{3}H]cytochalasin B bound. However, in the presence of D-glucose, significant inhibition of binding occurred in a dose-dependent fashion identical to that observed for membranes. Results are presented as maximum inhibition of [^{3}H]cytochalasin B binding as a function of D-glucose concentration. An ED_{50} of 37.5 mM D-glucose was found for inhibition of cytochalasin B binding in the soluble membrane preparation. This value is virtually identical to the ED_{50} of 40 mM D-glucose previously determined for intact membranes (Fig. 3).

To characterize further the cytochalasin B binding component in soluble protein, equilibrium dialysis experiments were performed in order to construct Scatchard plots. Difficulties arise, however, in attaining an accurate value of K_d for cytochalasin B binding. The soluble preparation must be maintained in the detergent, octylglucoside, in order to stabilize the protein in solution and retain cytochalasin B binding activity. Unfortunately, octylglucoside acts to inhibit cytochalasin B binding, as shown by our results. However,

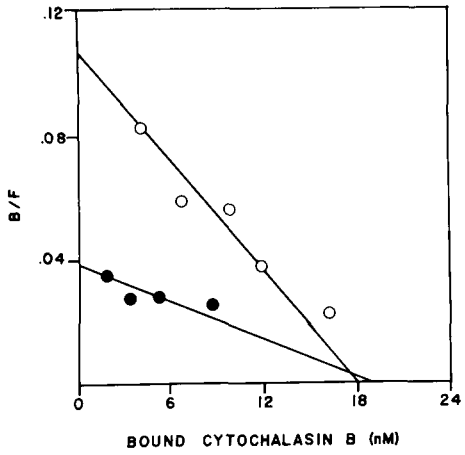

Fig. 6. Scatchard plot of cytochalasin B binding to soluble placental membrane protein. Equilibrium dialysis binding of [^{3}H]cytochalasin B was measured with soluble protein (270 μg) in the presence of two different concentrations of octylglucoside (see text). Media contained $6 \cdot 10^{-8}$ M [^{3}H]cytochalasin B, appropriate amounts unlabelled cytochalasin B and 0.85 mM octylglucoside (○) or 2.55 mM octylglucoside (●). Data were analyzed by the method of Scatchard (1949).

Triton X-100 has a much greater effect on cytochalasin B binding, and octyl glucoside represents a convenient alternative detergent. Fig. 6 presents a Scatchard plot showing the results of an experiment in which [^{3}H]cytochalasin B binding was measured in the presence of two concentrations of octylglucoside. Soluble membrane protein, with 0.85 or 2.55 mM octylglucoside present, was dialyzed against $1.2 \cdot 10^{-7}$ M [^{3}H]cytochalasin B and appropriate quantities of unlabelled cytochalasin B. The decrease in slope observed in the presence of 2.55 mM octylglucoside is indicative of the detergent's inhibition. Analysis of the data in a fashion similar to that described for Fig. 4 yields an inhibition constant, K_i, of 0.4 mM octylglucoside. The dissociation constant, K_d, for cytochalasin B binding, corrected for the presence of octylglucoside, was determined to be $5.34 \cdot 10^{-8}$ M. The total number of binding sites, B_{max}, was measured as 133 pmol/mg protein. These are discrepant results, in view of the fact the centrifugation-binding data collected for protein-depleted membrane predict a 1.5-fold increase in binding activity in the soluble preparation. Either there has been a loss in functional-binding sites and the intrinsic K_d has been altered in the solubilization process, or the presence of octylglucoside has obscured the correct values of K_d and B_{max}, resulting in the depressed values cited above. We feel that the latter is the case, since solubilization of erythrocyte membranes with octylglucoside maintained the K_d for cytochalasin B binding [22] and enabled reconstitution of functional D-glucose transport [23].

In an effort to gain further insight into the cytochalasin B binding component present in the soluble preparation, use was made of [^{3}H]cytochalasin B as a photoaffinity radiolabel. Johnson and Smith [14] and Ingermann et al. [15] have described the photoincorporation of this label as identification for the glucose transport protein in microvillous membrane of human placenta. This work was extended by photoaffinity labelling soluble protein as well as intact microsomal membranes. Figs. 7 and 8 present the results of the photoincorporation of [^{3}H]cytochalasin B. Membranes (1 mg of protein/ml) in 5 mM phosphate buffer (pH 8.0) were exposed to ultraviolet light in the presence of 1 μM [^{3}H]cytochalasin B, 0.5 M hexose and 0.1 mM cytochalasin E. After photolysis for 30 min, samples were subjected to SDS-

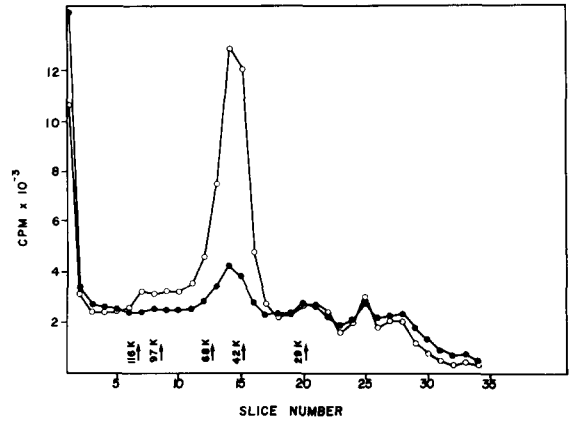

Fig. 7. Photoaffinity labelling of placental membrane. [^{3}H]Cytochalasin B, 1 μM, was crosslinked to membranes (1 mg protein/ml) incubated with 0.5 M D-glucose (●) or 0.5 M sorbitol (○), in the presence of 0.1 mM cytochalasin E. After a 30-min exposure under ultraviolet light, aliquots (470 μg protein) were washed free of excess radioactivity and prepared for electrophoresis on a 7.5–15% gradient polyacrylamide gel. Lanes were sliced into 3-mm pieces and prepared for scintillation counting as described under Methods. Radioactivity (cpm) is plotted against corresponding slice number.

polyacrylamide gel electrophoresis. Lanes were sliced into 3-mm segments and prepared as described in Methods. Data are presented as cpm vs. slice number. The presence of 0.5 M D-glucose blocks the photoincorporation of [^{3}H]cytochalasin B into a protein in the M_r range 42 000–68 000 which is observed in the control with 0.5 M sorbitol present (Fig. 7). Similarly, photoaffinity labelling of the soluble membrane protein was accomplished. The soluble material was prepared for photolysis by dialysis overnite against 5 mM phosphate buffer (pH 8.0) in order to remove detergent, and was subjected to identical conditions as for the membrane photoaffinity labelling experiment. The data for photolabelled soluble protein presented in Fig. 8 show results identical to those obtained for membranes. Again, D-glucose blocks labelling [^{3}H]cytochalasin B which is observed in the presence of sorbitol in the M_r range 42 000–68 000. These results clearly indicate a glucose-sensitive cytochalasin B binding constituent in the soluble preparation.

Fig. 9 summarizes the solubilization and resolution of the glucose transporter. SDS-polyacrylamide gel electrophoresis was performed on samples of placenta microsomal membrane, soluble membrane protein, and photoaffinity-labelled membrane. Panels A and B in Fig. 9 depict gel densitometry scans of the Coomassie-stained regions of membrane and soluble protein, respectively. Panel C depicts the [^{3}H]cytochalasin B photoaffinity labelling pattern obtained from membranes. The solubilization of placenta microsomal membrane obviously depletes the region of interest, M_r 42 000–86 000, of several proteins. However, it is not possible to assign a particular Coomassie-stained peak as the glucose transporter at this time, particularly in light of evidence showing that the erythrocyte glucose transport protein as a poorly Coomassie-stained species that migrates over a broad region of the gel [24]. When the

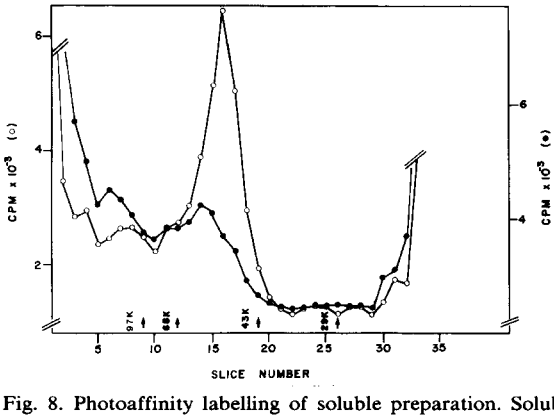

Fig. 8. Photoaffinity labelling of soluble preparation. Soluble placental membrane protein (2 mg/ml) in 5 mM phosphate buffer (pH 8.0) was incubated with 0.1 mM cytochalasin E, 0.5 M D-glucose (●) or 0.5 M sorbitol (○). [^{3}H]Cytochalasin B, 1 μM, was photocoupled to soluble protein with a 30-min exposure to ultraviolet light. Samples of 300 μg protein were directly applied to a 7.5–15% gradient polyacrylamide gel. Lanes were sliced into 3-mm sections and treated as described under Methods in preparation for counting. Plot represents cpm measured versus slice number.

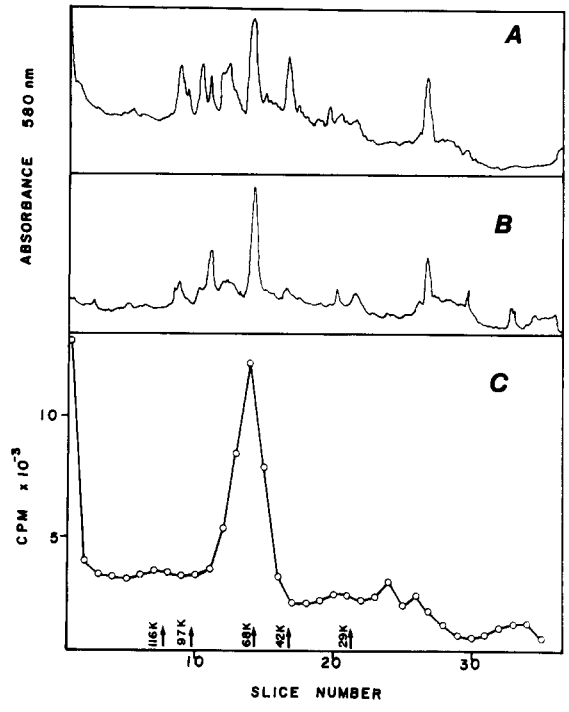

Fig. 9. Gel electrophoresis of placental membrane and soluble protein. SDS-polyacrylamide gel electrophoresis (7.5–15% gradient gel) was performed using samples of placenta microsomal membrane, soluble membrane protein, and [^{3}H]cytochalasin B photoaffinity-labelled intact membrane. The gel was stained with Coomassie blue and densitometry was performed at 580 nm. Radiolabel was measured in 3-mm slices as detailed in Methods. Panel A represents the densitometric scan of 100 μg of intact membrane protein. Panel B is the trace of 40 μg of soluble protein. Panel C is the profile of distribution of [^{3}H]cytochalasin B crosslinked to intact membranes.

purified red cell transporter is treated with endoglycosidase F, SDS-polyacrylamide gel electrophoresis reveals a sharp, tightly defined band of M_r 46000 [25]. Isoelectric-focusing experiments performed on [^{3}H]cytochalasin B-photolabelled glucose transporter from rat adipocyte low-density microsomes uncovered three distinct species at pH values 5.5, 4.5 and 4.2 [26]. Furthermore, SDS-polyacrylamide gel electrophoresis of the glucose transport protein from chick embryo fibroblasts displays a pattern of [^{3}H]cytochalasin B photolabelling over a broad range [10,11]. Johnson and Smith [14] and Ingermann et al. [15] have determined $M_r = 52000$ and 60000, respectively, for the photolabelled glucose transporter from placental microvillous membrane. Our results are consistent with these values. However, it is not clear from the present data if one or more species is present in placental microsomes, and/or if the broad peak is due to heterogenous glycosylation as seems to be the case for the erythrocyte [25].

Discussion

Cytochalasin B is well-characterized as a specific inhibitor of glucose transport. The transport protein has been implicated to be identical to the glucose-sensitive cytochalasin B binding component described for several systems: human erythrocyte [1,21], rat adipocyte [2], chick embryo fibroblast [3], rat hepatocyte [4] and rat muscle [5]. We determined in this laboratory that there were unique characteristics of cytochalasin B binding in rat-liver plasma membranes [4]. With this exception, the glucose-sensitive cytochalasin B binding components from different cell types appear similar in dissociation constant, K_d. The present investigation extends this generality to cytochalasin B interactions with the glucose transporter in placental microsomes, which are found to have a $K_d = 1.53 \cdot 10^{-7}$ M for ligand binding. The K_i for inhibition for this binding was determined to be 32.1 mM D-glucose, a value which is also in agreement with those found for other membrane types. Furthermore, it appears that the placental microsome preparation is an abundant source of transport protein, with an estimated 1.6% of total protein implicated as D-glucose-sensitive cytochalasin B binding sites.

These results indicate the likelihood that purification of the glucose transport protein from human placenta may be readily feasible. The human red blood cell glucose transporter has been purified on the basis of reconstitution of transport activity [6] as well as on the criteria of cytochalasin B binding activity [7]. In the present study, this latter convenient assay of cytochalasin B binding was utilized to monitor the solubilization of this component from placental microsomes. This was accomplished by equilibrium-dialysis measurements of [^{3}H]cytochalasin B binding activity. It was seen that a cytochalasin E-sensitive cytochalasin B binding site remained insoluble. Characterization of [^{3}H]cytochalasin B binding in response to D-glucose confirmed the presence of a glucose-sensitive site, identical to one present in intact membranes. The presence of octylglucoside was shown to inhibit cytochalasin B binding; values of K_d ($5.34 \cdot 10^{-8}$ M cytochalasin B) and B_{max} (133 pmol/mg protein) are probably inaccurate measurements in view of this evidence and represent artifactually low values for these parameters. It is clear, however, that a functional, glucose-sensitive, cytochalasin B binding component is solubilized. The soluble preparation represents a 1.5-fold enrichment of this binding site over intact membranes, based on centrifugation-binding measurements assayed on protein-depleted material.

Further confirmation of the solubilization of the glucose-sensitive cytochalasin B binding protein is shown by the photoaffinity labelling technique. [^{3}H]Cytochalasin B has been photocoupled to erythrocyte membranes [8,9] rat adipocyte and chick embryo fibroblast membranes [10,11] as well as to microvillous membrane of human placenta [14,15]. In this investigation, [^{3}H]cytochalasin B was photolabelled to placenta microsomal membranes, producing a major, broad peak shown to be D-glucose-displaceable. The labelled protein was in the M_r range 42000–68000, consistent with other results for various membrane types. In particular, the crude microsome preparation compares well with the microvillous placental membrane preparation previously studied [14,15]. This technique was extended to identify the presence of this species in the soluble preparation. A single D-glucose-displacable peak in the M_r range 42000–68000 was observed by exposing the solu-

ble protein in the presence of [^{3}H]cytochalasin B to ultraviolet light. The photoaffinity labelling technique provides a convenient means of assaying the presence of the cytochalasin B binding component and gives evidence to support the data characterizing the soluble protein. This investigation establishes the initial stages toward purification of the glucose transport protein found in human placental microsomes. Convenient methodology was developed in order to achieve this end. The realization of this goal will aid in understanding the molecular basis of the transport process. Biochemical differences between the placental and erythrocyte glucose transport proteins have already been observed in the course of our investigations. Attempts were made to isolate further the placental glucose transporter by employing DEAE ion-exchange chromatography. This step results in nearly complete purification of the erythrocyte glucose carrier [24] and we were able to repeat this result (data not shown). However, DEAE ion-exchange chromatography is unable to resolve the placental glucose carrier from the bulk of placental microsomal proteins (data not shown). We are presently exploiting other techniques to purify and characterize the placental glucose transporter. Characterization of differences in transport systems at the molecular level may lead to the elucidation of factors involved in regulation by hormones such as insulin [27], and in the response to altered cellular states such as transformation [3,11].

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