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CYTOCHALASIN B BINDING TO EHRLICH ASCITES TUMOR CELLS AND ITS RELATIONSHIP TO GLUCOSE CARRIER

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Summary

Cultured Ehrlich ascites tumor cells equilibrate D-glucose via a carrier mechanism with a K_m and V of 14 mM and 3 μmol/s per ml cells, respectively. Cytochalasin B competitively inhibits this carrier-mediated glycose transport with an inhibition constant (K_i) of approx. $5 \cdot 10^{-7}$ M. Cytochalasin E does not inhibit this carrier function. With cytochalasin B concentrations up to $1 \cdot 10^{-5}$ M, the range where the inhibition develops to practical completion, three discrete cytochalasin B binding sites, namely L, M and H, are distinguished. The cytochalasin B binding at L site shows a dissociation constant (K_d) of approx. $1 \cdot 10^{-6}$ M, represents about 30% of the total cytochalasin B binding of the cell ($8 \cdot 10^6$ molecules/cell), is sensitively displaced by cytochalasin E but not by D-glucose, and is located in cytosol. The cytochalasin B binding to M site shows a K_d of $4—6 \cdot 10^{-7}$ M, represents approx. 60% of the total saturable binding ($14 \cdot 10^6$ molecules/cell), is specifically displaced by D-glucose with a displacement constant of 15 mM, but not by L-glucose, and is insensitive to cytochalasin E. The sites are membrane-bound and extractable with Triton X-100 but not by EDTA in alkaline pH. The cytochalasin B binding at H site shows a K_d of $2—6 \cdot 10^{-8}$ M, represents less than 10% of the total sites ($2 \cdot 10^6$ molecules/cell), is not affected by either glucose or cytochalasin E and is of non-cytosol origin. It is concluded that the cytochalasin B binding at M site is responsible for the glucose carrier inhibition by cytochalasin B and the Ehrlich ascites cell

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is unique among other animal cells in its high content of this site. Approx. 16-fold purification of this site has been achieved.

Introduction

A number of observations now available indicate that a highly active glucose transport system is common to the neoplastic as well as transformed animal cells [1—6]. Ehrlich ascites tumor cells transport glucose and other hexoses via a classical, carrier-mediated transport system [7]. Study on the nature of the increased glucose carrier activity in transformed cells may provide insight to understanding the molecular events during the transformation process as well as the mechanism of the carrier mediation itself.

Cytochalasin B, a fungal metabolite originally known for its effect on cell motility-related functions [8,9], has been shown to inhibit the transmembrane movement of sugars in a variety of cell types [10—15]. Studies on the human erythrocyte [14,15] have revealed three types of cytochalasin B binding proteins, and shown that one of them, namely site I, or the D-glucose sensitive cytochalasin B binding is intimately related, if not identical to the glucose carrier. This strongly indicated that cytochalasin B may be used as a molecular marker for the glucose transport protein. In the present report, we have demonstrated that cytochalasin B also inhibits glucose transport of the Ehrlich ascites tumor cell. Further, we have distinguished at least three different cytochalasin B binding sites in Ehrlich ascites cells, and assigned one of these sites as responsible for the inhibition of glucose transport by cytochalasin B. Approx. a 16-fold purification of the D-glucose sensitive cytochalasin B binding protein was achieved.

Materials and Methods

Materials. Ehrlich ascites tumor cells were grown in suspension culture in RPMI medium 1630 containing 10% fetal calf serum [9]. [^{3}H]Cytochalasin B was obtained from New England Nuclear and used without further purification. Cytochalasins B and E were obtained from Aldrich Chemical Company. D-[^{14}C]-Glucose and 3-*O*-[^{14}C]methyl D-glucose were from New England Nuclear. Triton X-100 was obtained from Sigma.

Flux measurement. Isotopic equilibrium exchange of sugars was measured using cells suspended at 5—6% cytocrit in 20 ml RPMI 1630 medium containing 5% fetal calf serum and 13.5 mM D-glucose or 13.5 mM D-glucose plus 1 mM 3-*O*-methyl glucose. The cells were preincubated for 30 min at room temperature to achieve complete chemical equilibrium. At $t = 0$, 1.0 ml medium containing 0.6 μCi D-[^{14}C]glucose or [^{14}C]methyl glucose were added to the cell suspension and 12 1-ml aliquots were taken at 5 s intervals. Each aliquot was quickly mixed with 8 ml prechilled isotonic NaCl/5 mM $HgCl_2$ and centrifuged at 480 × *g* for 5 min. Pellets were obtained free of medium by carefully wiping the interior of the centrifuge tube with cotton swabs. Both supernatants and pellets were assayed for radioactivity using a liquid scintillation counter (Mark II, Nuclear Chicago). The radioactivity associated with the pel-

lets were expressed as a percent of the total and plotted against the sampling time in a semilog scale from which the half equilibration time was estimated as described [15].

Equilibrium binding of cytochalasin B. For all of the binding assays, the cells were washed three times by centrifugation and resuspended in a buffer solution containing 150 mM NaCl adjusted to pH 7.4 with 10 mM Tris-HCl, and used immediately or frozen at −20°C until use. [^{3}H]Cytochalasin B was used as a tracer. The equilibrium binding of cytochalasin B was carried out essentially as described for the human erythrocyte system [15]. The assay mixture contained approx. 10^7 cells, or particulate fractions derived from them/150 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.02 μCi [^{3}H]cytochalasin B/10^{-8}—10^{-5} M cytochalasin B in 1.0 ml. Stock solutions of cytochalasin B were prepared in 10% ethanol, and used to give a final concentration of 0.1% ethanol in the assay mixture. Cytochalasin E and sugars were also included in the assay medium where indicated in the figure legends. The mixtures were equilibrated for 20 min and bound ligand was separated from free ligand by centrifugation (300 000 × *g* using SW 50.1 rotor in a Beckman ultracentrifuge for 30 min). Intact or ruptured cells were separated by centrifugation at 30 000 × *g* as indicated in the figure legends. 0.5 ml of the supernatant was removed and its radioactivity assayed in PCS (Amersham) scintillation fluid. The remainder was decanted, the tubes inverted and carefully wiped with cotton swabs. The pellet was then suspended in 1.0 ml water, and 0.5 ml counted using 5.0 ml PCS scintillation fluid.

EDTA extraction. Washed cells, frozen at a density of $1 \cdot 10^7$ cells/ml in 140 mM NaCl/10 mM Tris-HCl, pH 7.4 were thawed. The particulate material was resuspended with gentle homogenization with a glass-glass homogenizer (Bellco Co.), and the homogenate centrifuged 27 000 × *g* for 20 min. The pellet was resuspended in 0.1 mM EDTA pH 8.0, incubated at 37° C for 30 min and centrifuged 35 000 × *g* for 20 min. The pellet was assayed at a protein concentration of 1 mg/ml.

Fractionation procedures. Microsomes containing plasma membranes were prepared essentially by the method of Haldar et al. [16]. Cells were washed twice by centrifugation, resuspended in a buffer (10 mM Tris-HCl, pH 7.4), containing sucrose and 1 mM EDTA and kept frozen overnight at −20°C. The thawed cells were homogenized using a glass-Teflon homogenizer (Thomas), and the homogenate readjustment to pH 7.4 with the addition of NaOH. The homogenate was centrifuged for 5 min at 750 × *g*, and the pellet reextracted with the sucrose/EDTA buffer, the pooled extracts were centrifuged for 15 min at 10 000 × *g*, and the resulting supernatant was centrifuged for 60 min at 100 000 × *g*. The resulting pellets were resuspended in a buffer containing 1.0 mM Tris-HCl (pH 7.4)/15 mM NaCl. Electron microscopic examination of these 100 000 × *g* pellets showed microsomes with little nuclear and mitochondrial contamination. The D-glucose' sensitive cytochalasin B binding activity was stable for at least two weeks when stored at −20°C.

Other methods. Protein was measured by the method of Lowry et al. [17], with the absorbance reading taken at 750 nm after a 5 min 1000 × *g* centrifugation to minimize light scatter due to the high concentration of lipid in the later fractions. SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. [18].

Experimental results

Glucose flux inhibition by cytochalasin B. Ehrlich ascites tumor cells in culture medium equilibrated D-glucose with a half equilibration time ($t_{1/2}$) of approx. 7 s at 23°C when a sugar concentration of 13.5 mM was used (Fig. 1). The rate of equilibration was increased with an increasing concentration of D-glucose, following a simple Michaelis-Menten type of kinetics (Fig. 2). The K_m and V were 14 mM and 3 μmol/s per ml packed cells, respectively, at 23°C. Some significant deviation from this simple kinetic pattern was observed when the sugar concentration was lowered below 13.5 mM (Fig. 2). Since lower sugar concentrations were achieved by dilution of the culture medium with a sugar-free phosphate buffer, the possibility that this anomalous kinetic behavior may be artifact due to the dilution cannot be ruled out.

Cytochalasin B inhibited the glucose equilibration (Fig. 1). This inhibition was reversible by washing. The kinetics of the inhibition were apparently of the competitive type with an estimated inhibition constant, K_i of $5 \cdot 10^{-7}$ M (Fig. 2). Again, at glucose concentrations lower than 13.5 mM, the inhibition appeared to be non-competitive, and it is not clear if these non-competitive

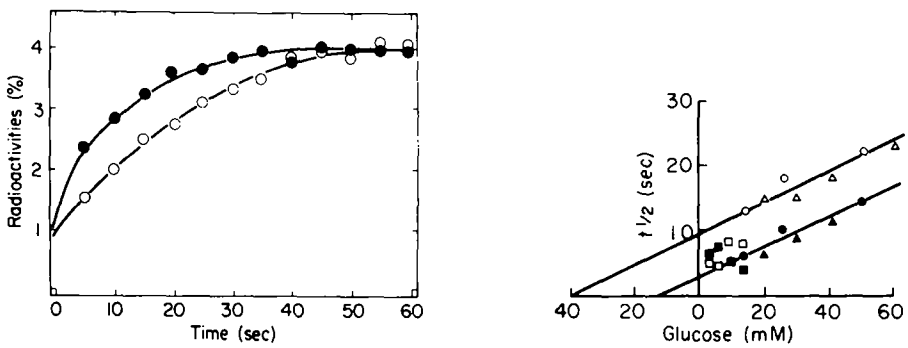

Fig. 1. Effect of cytochalasin B on time course of equilibrium exchange of D-glucose by ascites tumor cells. A 20 ml cell suspension (5—6% cytocrit) in Medium RPMI-1630 supplemented with 5% fetal calf serum containing 13.5 mM D-glucose in the absence (●———●) and in the presence of 10^{-6} M cytochalasin B (○———○) were preequilibrated at room temperature for 30 min. At $t = 0$, 1.0 ml of the medium containing 0.6 μCi of D-[^{14}C]glucose was added to the cell suspension and 12 1.0-ml aliquots were taken at 5 s intervals. The radioactivity associated with the packed cells was expressed as a percent of the total, and plotted against the sampling time. Solid lines are theoretical curves generated to best-fit the data based on a single, first-order process in a closed two compartment system. The lines represent $y = 4 - 3.1e^{-0.09x}$ (●———●) and $y = 4 - 3.1e^{-0.045x}$ (○———○).

Fig. 2. Equilibrium exchange of D-glucose by ascites tumor cells as a function of the D-glucose concentration. Each data point represents the flux measurement of a 30 s equilibrium exchange similar to the experiments of Fig. 1. The half equilibration time, $t_{1/2}$, was calculated from a semilog plot of the isotopic equilibration time as described in Experimental procedures. The $t_{1/2}$ values were plotted against glucose concentrations using the relationship $t_{1/2} = (0.693/V)\ [K_m(1 + (I)/K_i) + (S)]$, where V and K_m denote the usual meanings and (S), (I), and K_i denote the glucose concentrations, the inhibitor concentration and the inhibition constant, respectively. Closed symbols are without cytochalasin B and open symbols are with 10^{-6} M cytochalasin B, with different symbols representing different sets of flux measurements. The straight lines are drawn by eye to best-fit the data points, and represent $y = 3.3 + 0.24x$ and $y = 9.6 + 0.24x$ for the absence and presence of the inhibitor, respectively. From these values for K_m, V and K_i of 13.7 mM, 2.89 μmol/s per ml and $5.26 \cdot 10^{-7}$ M, was estimated, respectively.

kinetics are an artifact induced by the dilution of the culture medium by phosphate buffer. $1 \cdot 10^{-5}$ M cytochalasin E did not inhibit the glucose equilibration significantly (data not given).

Cytochalasin B binding to intact cells. Equilibrium binding of cytochalasin B to fresh Ehrlich ascites cells in isotonic NaCl was followed (Fig. 3). This binding revealed a non-linearity on the Scatchard analysis (Fig. 4a) indicating that there are at least two classes of cytochalasin B binding sites in the cell. Accordingly, components of a high affinity ($K_d = 2 \cdot 10^{-8}$ M) and of a low affinity

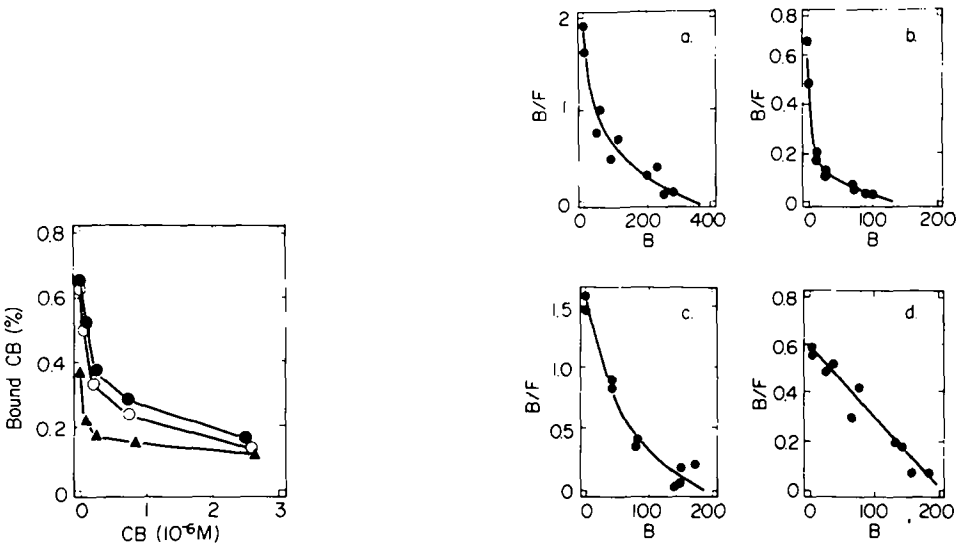

Fig. 3. Binding of cytochalasin B to fresh, intact Ehrlich ascites cells as a function of the free ligand concentration. Each ml of the incubation mixture contained $1 \cdot 10^7$ cells (1.94 mg protein), a tracer of [^{3}H]-cytochalasin B (0.02 μCi), and 10^{-7}—10^{-5} cytochalasin B. The cells were incubated at room temperature for 20 min, and the binding activity determined by centrifugation for 20 min at 23 000 $\times g$ (15 000 rev./min in a Sorvall SS34 rotor in RC2-B centrifuge) at 4° C. The supernatants were separated from the pellets and the radioactivities determined. The tubes were then inverted and drained, the residual supernatant carefully removed with cotton swabs. The pellets were then dissolved in 1.0 ml 1% SDS, and the radioactivities were determined. The cytochalasin B bound was calculated as percent of total. The difference between the control (●———●) tubes, and tubes containing 500 mM D-glucose (▲———▲) represents the D-glucose-sensitive portion. Similarly, the difference between the control and the 10^{-5} M cytochalasin E containing tubes (○———○) represents the cytochalasin E-sensitive portion.

Fig. 4. Scatchard analysis of cytochalasin B binding to intact ascites cells. (a) Overall cytochalasin B binding. The control data of Fig. 3 (●———●) were used to calculate the bound (B) and free (F) ligand for each ml of the mixture containing 10^7 cells. The solid curve was drawn by eye to best-fit the data from which two K_d values and overall B_0 were estimated by linear extrapolation of this curve at the upper and lower end S (see text). (b) D-glucose-insensitive binding. Cytochalasin B binding in the presence of D-glucose from Fig. 3 was plotted revealing a non-linear plot indicative of multiple components. Two sets of binding constants were estimated by the linear extrapolation of the curve at the two ends: they are for a low affinity site ($K_d = 10^{-6}$ M) constituting approx. 35% of the total saturable binding (120 pmol/10^7 cells) and high affinity site ($K_d = 2 \cdot 10^{-8}$ M), constituting less than 10% of the total sites (15 pmol/10^7 cells). (c) Cytochalasin E-insensitive binding. The cytochalasin B binding in the presence of 10^{-5} M cytochalasin E from Fig. 3 was plotted, revealing non-linearity, from which two sets of binding parameters were obtained by linear extrapolation at both ends of the curve, as shown in the text. (d) D-glucose-sensitive cytochalasin B binding. The difference between cytochalasin B bound in the presence of 500 mM D-glucose and the absence of D-glucose from Fig. 3 was plotted. The essentially linear nature of the plot indicates the presence of only one glucose-sensitive component, with a K_d of 5—6 $\cdot 10^{-7}$ M and a B_0 of 200 pmol/10^7 cells (approx. 60% of the total saturable cytochalasin B binding).

(K_d = 6—11 · 10^{-7} M) were identified. The total binding (B_0) was approx. 350 pmol/10^7 cells (Fig. 4a).

More than 50% of this binding was inhibited in the presence of 500 mM D-glucose (Fig. 3). This glucose-sensitive portion of the cytochalasin B binding showed an apparent linearity on the Scatchard plot (Fig. 4d) with a K_d of 5—6 · 10^{-7} and a B_0 of 190—200 pmol/10^7 cells. The cytochalasin B binding observed in the presence of 500 mM D-glucose, on the other hand, revealed a non-linearity on the Scatchard plots (Fig. 4b). This glucose-insensitive portion of the cytochalasin B binding also includes at least two classes of binding sites; one with an estimated K_d of 6—11 · 10^{-7} M and a B_0 of 80—120 pmol/10^7 cells, the other with a K_d of 1—3 · 10^{-8} M and a B_0 of 20—40 pmol/10^7 cells.

About 30% of the cytochalasin B binding to the intact cells was inhibited in the presence of 1 · 10^{-5} M cytochalasin E (Fig. 3). This cytochalasin E-sensitive cytochalasin B binding revealed what appears to be a single binding component upon Scatchard analysis (data not shown). The cytochalasin B binding observed in the presence of 1 · 10^{-5} M cytochalasin E (cytochalasin E-insensitive binding), on the other hand, showed non-linearity on Scatchard analysis (Fig. 4c). Two binding components are apparent in this analysis, one with a K_d of approx. 1—3 · 10^{-8} M, and the other with a K_d of approx. 2—7 · 10^{-7} M. The total B_0 of this cytochalasin E-insensitive binding was approx. 160—200 pmol/10^7 cells.

Together, these findings suggested as a working hypothesis that fresh ascites tumor cells possess three classes of cytochalasin B binding sites, which differ in affinity; a high affinity site (H site), a low affinity site (L site), and a site with an intermediate affinity (M site). They also differ in sensitivity to cytochalasin E and to D-glucose. The cytochalasin B binding to the M site is glucose-sensitive while that to the L site is cytochalasin E-sensitive. The fact that both the glucose-insensitive cytochalasin B binding and the cytochalasin E-insensitive cytochalasin B binding include the high affinity binding component indicates that the H site is insensitive to both cytochalasin E and D-glucose. The H site accounts for approx. 10% of the total saturable binding of the cell, whereas M and L sites account for 60 and 30% of the total cytochalasin B binding, respectively.

Cytochalasin B binding to cytosol-depleted Ehrlich cells. Ehrlich cells after depletion of cytosol by freeze-thawing lost 25—30% of the cytochalasin B binding. The particulate fraction was almost insensitive to cytochalasin E (Fig. 5), indicating that the loss occurs at the cytochalasin E-sensitive cytochalasin B binding site or L site. Consistent with this conclusion, the Scatchard analysis of the cytochalasin B binding to this cytosol-depleted preparation revealed the binding parameters characteristic of H and M sites, but not of L site (Fig. 5 insert). The majority of the cytochalasin B binding to this preparation, on the other hand, was inhibited by 500 mM D-glucose, and this effect is specific to D-glucose, L-glucose being ineffective (Fig. 6). A Scatchard plot of the glucose-sensitive portion of the cytochalasin B binding revealed a single binding component with a K_d of 5 · 10^{-7} M and a B_0 of more than 200 pmol/10^7 cells (Fig. 6 insert). The binding affinity is characteristic of the M site. The portion of the cytochalasin B binding which is not inhibited by D-glucose revealed on the Scatchard plot a set of binding parameters similar to that of the H site of the

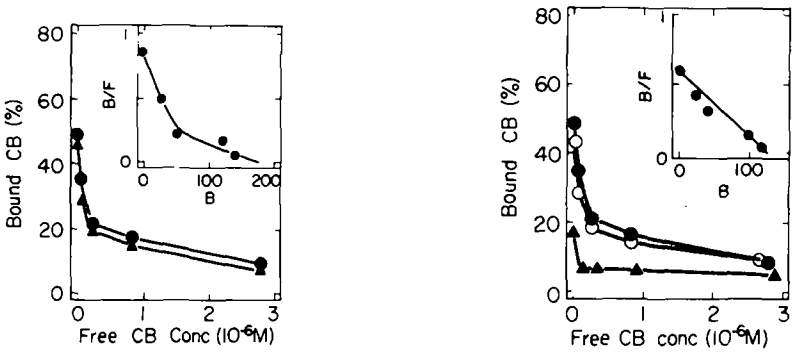

Fig. 5. Cytochalasin B (CB) binding to freeze-thawed ascites cells. The incubation mixture contained $1.0 \cdot 10^7$ freeze-thawed cells/ml, 0.02 μCi/ml [^{3}H]cytochalasin B, and 10^{-7}—10^{-5} cytochalasin B. The ruptured cells were incubated at room temperature for 20 min in the presence (▲——▲) and in the absence (○——○) of 10^{-5} M cytochalasin E. The incubation mixtures were centrifuged at 27 000 × *g* for 20 min (15 000 rev./min in SS34 rotor in RC2-B centrifuge), and the radioactivity of the pellet (bound cytochalasin B) is plotted against the free cytochalasin B concentrations. When the cytochalasin B binding in the presence of 10^{-5} M cytochalasin E is subjected to Scatchard analysis (insert), at least two binding components are evident: one with a K_d and B_0 of $6 \cdot 10^{-8}$ M and 40 pmol/10^7 cells, respectively, and the other with a K_d and B_0 of $5 \cdot 10^{-7}$ M and 160 pmol/10^7 cells, respectively.

Fig. 6. D-Glucose-sensitivity of cytochalasin B binding to cytosol-depleted Ehrlich ascites cells. The incubation mixture contained 10^7 cells/ml, 0.02 μCi/ml [^{3}H]cytochalasin B and 10^{-7} to $3 \cdot 10^{-5}$ M cytochalasin B. The assay was performed as in the legend to Fig. 5. The difference between the control (●——●) and the set with 500 mM D-glucose (▲——▲) was used to show (in insert) the Scatchard analysis of the glucose-sensitive cytochalasin B binding portion; the solid line gives a K_d of $5.0 \cdot 10^{-7}$ M and a B_0 of 260 pmol/10^7 cells. The cytochalasin B binding in the presence of 500 mM L-glucose (○——○) is also shown.

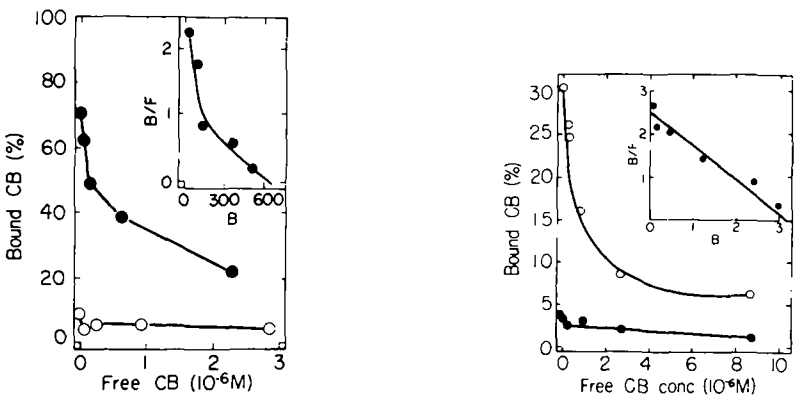

Fig. 7. Cytochalasin B binding to EDTA-extracted, cytosol-depleted cells. Cells (39 mg protein) were freeze-thawed and centrifuged at 27 000 × *g* for 20 min. The pellet was resuspended in 0.1 mM EDTA, pH 8.0. The cell fragments were incubated 30 min at 37°C and centrifuged for 20 min. Each incubation mixture contained 0.5 mg protein/ml, 0.02 μCi [^{3}H]cytochalasin B, and 10^{-7} to $3 \cdot 10^{-7}$ M cytochalasin B. The cytochalasin B binding activity (●——●) was assayed as in the legend to Fig. 5. Also shown is the cytochalasin B binding in the presence of 500 mM D-glucose plus 10^{-5} M cytochalasin E (○——○). Insert: shows the Scatchard plot of the cytochalasin B binding that is replaced by the sugar plus cytochalasin E. Two K_d values were estimated by linear extrapolations. B, bound; F, free.

Fig. 8. Cytochalasin B binding to a microsomal fraction (see Materials and Methods). The binding was assayed at a final protein concentration of 0.130 mg/ml with 0.2 μCi/ml [^{3}H]cytochalasin B and 10^{-7}—10^{-5} M cytochalasin B in 15 mM NaCl solution containing 1 mM Tris-HCl, pH 7.4. The D-glucose-sensitive portion was determined by the difference in binding in the presence (●——●) and absence (○——○) of 250 mM D-glucose). Insert: Scatchard analysis of the D-glucose-sensitive binding. The solid line is drawn by eye, which yields a B_0 of 330 pmol/0.13 mg protein and a K_d of approx. $1.3 \cdot 10^{-6}$ M.

TABLE I

PARTIAL PURIFICATION OF D-GLUCOSE SENSITIVE CYTOCHALASIN B BINDING ACTIVITY OF EHRLICH CELLS

This represents a typical result of M site protein isolation and purification using 10^9 intact cells as the starting material.

Preparation	Protein (mg)	Specific activity (pmol/mg protein)	Yield (%)	Purification factor
Intact cells	119	167	100 *	1.0
Cytosol-depleted cells	19	1200	105	7.2
Cytosol-depleted, EDTA-extracted cells	15	1400	88	8.4
Microsomes	7.1	2600	92	15.7

* This corresponds to approx. 200 pmol/10^7 intact cells of the total binding capacity (B_0) as estimated by the Scatchard analysis.

intact cells (data not shown). It is indicated that both H and M sites are particulate or membrane-bound, whereas L site is primarily of cytosol origin.

Partial purification of M site protein. Cytosol-depleted Ehrlich ascites cells retained most of the cytochalasin B binding activity after an EDTA extraction of extrinsic proteins (Fig. 7). The Scatchard analysis of the cytochalasin B binding to the EDTA-extracted, cytosol-depleted cells revealed at least two binding components with estimated K_d values of $3-5 \cdot 10^{-8}$ M and $3-7 \cdot 10^{-7}$ M (Fig. 7 insert). The total saturable binding to this preparation was approx. 700 pmol/0.5 mg protein (Fig. 7, insert).

A microsomal fraction was isolated by employing differential centrifugation procedures as described in the Materials and Methods section. This fraction was

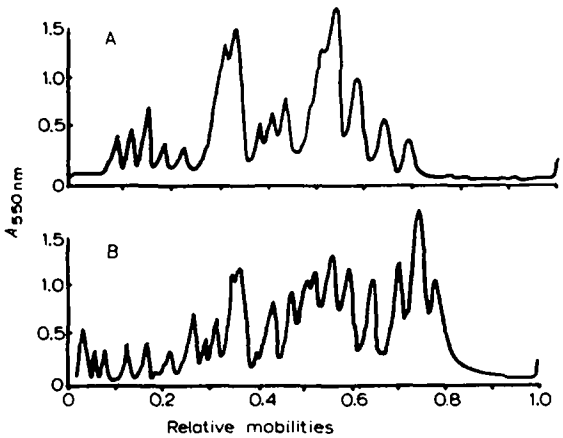

Fig. 9. SDS-polyacrylamide gel electrophoresis of Ehrlich ascites tumor cell preparations. A, microsomal fraction (100 000 × *g* fraction); B, washed whole cells. The electrophoretic mobility on abscissa was expressed as a relative quantity to that of tracking dye, pyronin Y. 5.6% acrylamide gels were used. Molecular weights of some bands were estimated by relating their motilities to those of selected polypeptide bands of human erythrocyte ghosts which was run in parallel. Relative masses of some of the polypeptides were estimated by the method described [25].

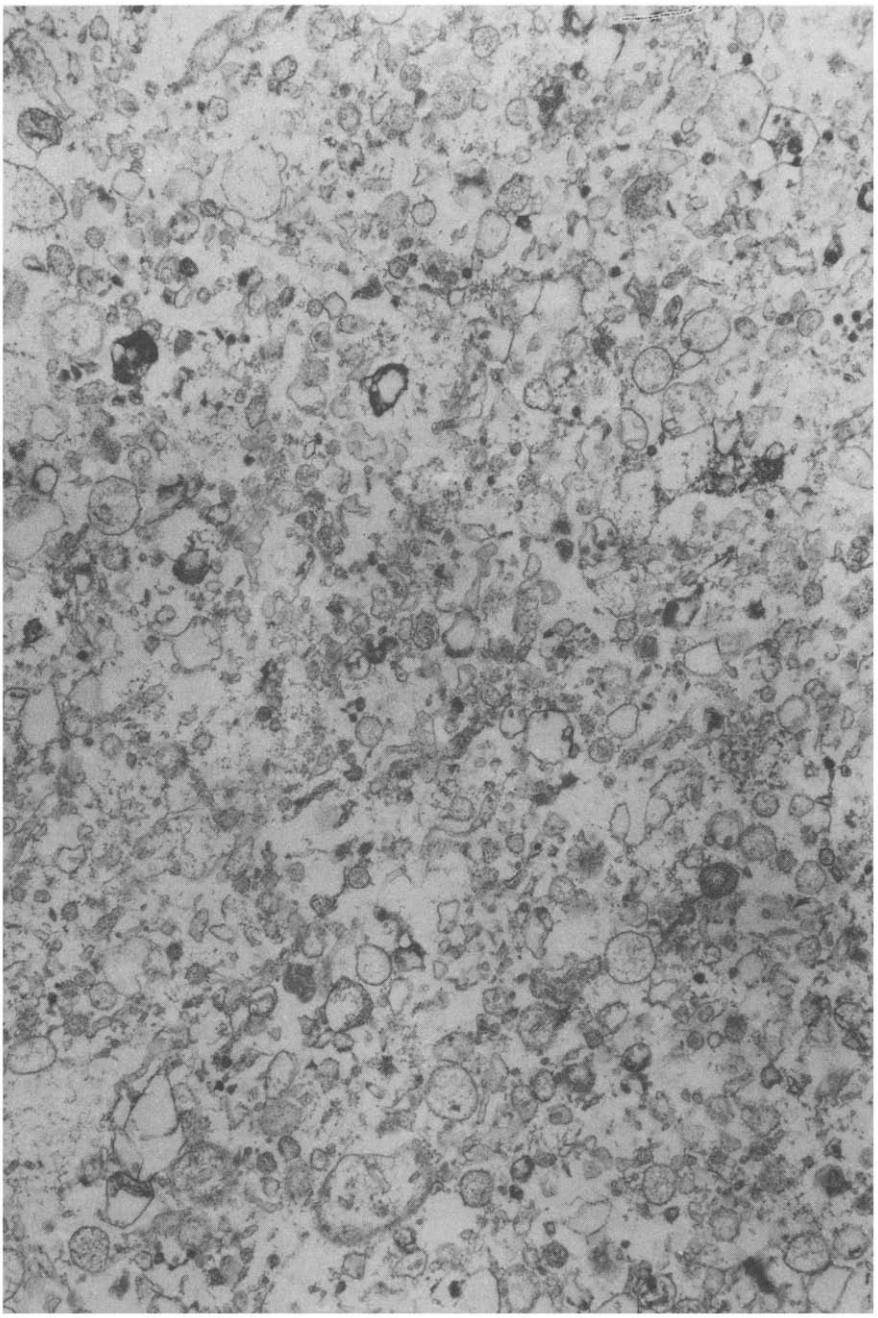

Fig. 10. Electron micrograph of thin section through the microsomal fraction (100 000 $\times g$ pellets). The specimens were fixed in 2% glutaraldehyde, post fixed in 1% OsO_4, with 0.5% uranyl acetate. The ultrathin sections were stained with uranyl acetate and lead acetate. The plasma membranes appear as many open and closed vesicular membranous profiles. Also seen are structures representing intracellular organelles such as endoplasmic reticulum and occasional mitochondrial fragments. Magnification X5850.

found to be enriched in cytochalasin B binding, virtually all of which was sensitive to D-glucose (Fig. 8). The Scatchard analysis of this glucose-sensitive cytochalasin B binding (Fig. 8, insert) revealed a major binding component. The binding affinity was significantly lower compared with the M site in intact cells. However, the B_0 indicated that more than 90% of the M site of intact cells was recovered in this fraction with a 16-fold increase in specific activity. This and other purification data are summarized in Table I.

SDS-polyacrylamide gel electrophoresis of the microsomal fraction is compared with that of whole cells in Fig. 9. The microsomal fraction was particularly enriched in two polypeptides with molecular weights of approx. 100 000 and 45 000, each of which represent more than 20% of the total microsomal protein. Beside these two major polypeptides, at least a dozen discrete polypeptide bands were readily distinguished. Electron microscopic examination of the microsomal fraction (Fig. 10) showed that it consisted mostly of plasma membranes and endoplasmic reticuli with little mitochondria and nuclear fragments.

Discussion

Heterogeneity in cytochalasin B binding to the Ehrlich ascites cells

Fresh Ehrlich ascites cells bind cytochalasin B in a saturable manner with the maximum binding of approx. 350 pmol/10^7 cells (Figs. 3 and 4). Three major classes of cytochalasin B binding sites, H, M and L, are distinguished on the basis of the affinity, sensitivity to glucose, sensitivity to cytochalasin E and subcellular location. The most abundant is the M site which accounts for some 60% of the total saturable sites. This site is probably on the glucose carrier. The following observations support this conclusion. The dissociation constant for the binding of cytochalasin B to this site ($K_d = 5 \cdot 10^{-7}$ M) is similar to the inhibition constant ($K_i = 5 \cdot 10^{-7}$ M) of cytochalasin B as an inhibitor of glucose transport (Fig. 2). The cytochalasin B bound at this site is stereospecifically displaced by D-glucose, a typical carrier substrate, but not L-glucose, a non-substrate. This glucose effect on the cytochalasin B binding is competitive (mutually exclusive). The linear patterns for cytochalasin B inhibition of glucose transport (Fig. 2) suggest a 1 : 1 stoichiometry between cytochalasin B binding and transport inhibition. Cytochalasin E, which does not inhibit glucose flux, does not displace cytochalasin B bound to the M site (Fig. 5). The lack of effects of cytosol depletion (Fig. 6), and of EDTA treatment (Fig. 7), indicate that the M site is an intrinsic membrane protein. These findings are similar to those observed with the glucose-sensitive cytochalasin B site of human erythrocytes [14].

The cytochalasin B binding to Ehrlich ascites cells includes a low affinity component or L site (K_d approx. 10^{-6} M and B_0 approx. 100 pmol/10^7 cells), which accounts for some 30% of the total binding. The binding to this site is insensitive to D-glucose (Fig. 4c), but is sensitive to cytochalasin E (Fig. 4a). The human erythrocyte [15], the HeLa Cell and SV40-transformed embryo fibroblast cells [9] have all been shown to contain a glucose-insensitive cytochalasin B binding which is sensitive to cytochalasin E. The fact that many of the motility-related functions of these cells are sensitive to cytochalasin E [9] would suggest involvement of L-site protein in these motility-related functions.

This binding is mostly abolished by depletion of cytosol (Fig. 6), suggesting its cytosol origin.

A small fraction (less than 10%) of the cytochalasin B binding to the Ehrlich cell (B_0 = 30 pmol/10^7 cell) is due to a high affinity site or H site (K_d = 2—5 · 10^{-7} M) which is insensitive to both glucose and cytochalasin E. Cytosol depletion (Fig. 5) and mild alkaline EDTA treatment (Fig. 7) do not affect the cytochalasin B binding to this site, indicating that H site is an intrinsic membrane protein. However, the H site does not appear in preparations that have been exposed to 1 mM EDTA for long periods in the cold (Fig. 8). The high affinity to cytochalasin B and insensitivity to glucose and to cytochalasin E of this site are similar to one of the high affinity cytochalasin binding sites, namely site III, of the human erythrocyte [15].

Partial purification of the M site protein

The results of the present study reveal that the Ehrlich cell is remarkably rich in glucose-sensitive cytochalasin B binding protein. The glucose-sensitive cytochalasin B binding protein (Site I protein) of human erythrocytes was suggested to be a peptide of 50 000 daltons [20]. If one assumes that the M site is a protein similar to that of the site I of the human erythrocyte (vide infra), the M site-protein in 10^7 cells could amount to approx. 10 μg. Since 10^7 Ehrlich cells contain 1.20 mg protein (an average of three determinations), and 3—5% of this is assumed in the plasma membrane [16], the M-site protein would amount to more than 20% of the plasma membrane protein. This is at least 4-fold greater than the estimate for the glucose-sensitive cytochalasin B binding site of transformed chicken embryo fibroblasts, which was approx. 4% of the membrane protein [6], or the similar estimate for the human erythrocyte which was approx. 5% of the membrane protein [15]. With other animal cells, the glucose-sensitive cytochalasin B binding appears to be far less abundant than in these cells [21,22]. It is plain that the Ehrlich cell membrane is a convenient source of the glucose-sensitive cytochalasin B binding protein for its isolation, purification and characterization.

The results of the present study indicate that cytosol depletion results in a major purification of the M and H sites while a major portion of the non-glucose sensitive sites (L sites) is lost. An EDTA treatment further removes approx. 25% of the protein associated with the cytosol-depleted preparation with some reduction in the M site cytochalasin B binding (Table I). Extended treatment with EDTA throughout the isolation procedure also results in the removal of most of the H site activity, leaving the M site mostly intact (Fig. 8). A 16-fold purification of the M site protein was achieved in microsomal fractions (Table I), with a yield of more than 90% of the binding activity. This would indicate that the M site protein in this microsomal preparation may amount to 13% of the total protein if it is 50 000 dalton protein, or 26% if 100 000 dalton protein. It is interesting to note that the SDS-polyacrylamide gel electrophoretic pattern of this microsomal fraction shows more than 14 discrete peptide bands, and includes two major bands with molecular weights of approx. 45 000 and 100 000, respectively (Fig. 9). These two bands also represent the cellular proteins which are greatly enriched during the purification (Fig. 9). Relative abundance of the 45 000 and 100 000 dalton peptides

were 26—32 and 24—29%, respectively (results of two independent estimations), both of which can meet the expected abundance of the M site protein. Further work is required for identification of M site peptide or peptides.

Comparison of the glucose carriers of the human erythrocyte and the Ehrlich cell

The glucose flux measurements reported here with the Ehrlich ascites tumor cell confirm the results obtained by previous workers [7,23,24]. The D-glucose flux is very fast (vide infra). The flux is best described as a reversible, first-order process with an equilibrium ratio of unity (Fig. 2). The kinetic parameters determined (Fig. 2) (K_d = 14 mM and V = 3 μmol/ms) are in fair agreement with those reported by other workers (K_d = 2—6 mM and V = 0.05—10 μmol/ms) [7,23]. These findings and other more exhaustive kinetic studies on the glucose-carrier activity of the Ehrlich ascites cell [7,23,24] point out that the glucose-carrier activity of the Ehrlich ascites cell and that of the human erythrocyte reside on similar proteins. The present study further demonstrates that the rapid, carrier-mediated exchange of D-glucose (Fig. 1) is competitively inhibited by cytochalasin B with a K_i of $5 \cdot 10^{-7}$ M (Fig. 2). This is similar to the inhibition by cytochalasin B of D-glucose transport in human erythrocytes [15,19] and 2-deoxy glucose transport in chick embryo fibroblasts [6]. The competitive nature of the inhibition as well as the value of the K_i strongly suggest that the D-glucose-sensitive cytochalasin B binding site or M site is responsible for this inhibition as discussed above.

The V of the glucose-carrier activity of the Ehrlich cell when expressed per unit volume (approx. 3 μmol/s per ml cells) (Fig. 2) is approximately equal to that of the erythrocyte measured under similar experimental conditions [15]. However, since the ratio of surface area-to-volume of the erythrocyte vs. the Ehrlich cell is 3 : 1 [7], the V of the Ehrlich cell would be about 3-times greater than that of the erythrocyte on a per unit membrane area basis. On the other hand, the D-glucose sensitive cytochalasin B binding proteins at the human erythrocyte are present at approx. $1.2 \cdot 10^5$ copies per cell [15], and that of the Ehrlich cell are present at $1.2—1.5 \cdot 10^7$ copies per cell (Table I). Since the surface area of the human erythrocyte and that of the Ehrlich cell are approx. $1.5 \cdot 10^{-6}$ M and $5.3 \cdot 10^{-6}$ cm^2/cell, respectively, the density of the binding sites per unit area in the Ehrlich cell is 30-times greater than that in the erythrocyte. Thus, a 10-fold discrepancy exists between the normalized V against area and the density of the glucose-sensitive cytochalasin B binding sites. This could be attributed either to a lower turnover number of the carrier in the Ehrlich cell or the existence of some glucose-sensitive cytochalasin B binding proteins in the Ehrlich cell that are non-functional as glucose-carrier. The turnover number of the chick embryo fibroblast glucose-carrier is shown to be 25-fold lower than the erythrocyte carrier turnover number [6]. One admittedly speculative explanation of the possible lower turnover number in the Ehrlich and chick embryo fibroblast cells is that the carrier, in the native state, is under control by some modifier protein of membrane or cytosol origin.

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