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PURIFICATION OF THE CYTOCHALASIN B BINDING COMPONENT OF THE HUMAN ERYTHROCYTE MONOSACCHARIDE TRANSPORT SYSTEM

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Summary

The cytochalasin B binding component of the human erythrocyte monosaccharide transport system has been purified. The preparation appears to contain one major protein with an apparent polypeptide chain molecular weight of 55 000 and about 0.4 binding sites per chain. Cytochalasin B binds to the reconstituted preparation with a dissociation constant of $1.3 \cdot 10^{-7}$ M, a value which is similar to that reported for the transport system in the intact erythrocyte.

The unequivocal identification and isolation of the polypeptide(s) that constitute(s) the human erythrocyte monosaccharide transport system has proved difficult. Kasahara and Hinkle [1] have shown that a preparation from the erythrocyte membrane containing largely a 55 000-dalton polypeptide catalyzes the entry of D-glucose into phospholipid vesicles. Unfortunately, the specific activity of transport in this reconstituted system is only about 0.3% of the value expected for the purified system on the basis of the rate of transport in intact erythrocytes [1,2]. Kahlenberg and Zala [3] have described a 13-fold purification of transport activity, relative to erythrocyte membranes, in a similar preparation. However, the nature of their assay is such that the measured activity cannot be directly related to the rate of transport in the intact erythrocyte. Thus, these results do not exclude the possibility that the transport system is only a minor component of the preparations. In order to resolve this question, we have developed an assay for a stoichiometric function of the transport system, the binding of

the competitive inhibitor cytochalasin B [2]. Herein, we describe the use of this assay to purify the cytochalasin B binding component of the transport system and to show that it is the 55 000 dalton polypeptide.

Purification of the cytochalasin B binding component. Erythrocyte membranes in 5 mM sodium phosphate, pH 8, were isolated by the procedure of Steck and Kant [4]. Peripheral proteins were removed from the membranes by treatment with EDTA at pH 12 [5,6]. This procedure led to the nearly complete removal of protein bands 1, 2, 4.1, 4.2, 5 and 6 (Fig. 2A). This preparation is referred to as protein-depleted membranes.

The purification of the cytochalasin B binding component was achieved by a modification of previously reported procedures [1,2]. All operations were at 4°C. Triton X-100 was added to protein-depleted membranes (2 mg protein/ml) in 50 mM Tris-HCl/ 1 mM dithiothreitol (pH 7.4) at 4°C, to give a final concentration of 0.5% (w/v). After shaking for 20 min, the mixture was centrifuged at 130 000 × *g* for 1 h. The extract was subjected to chromatography. A sample (20 ml) was applied to a column of DEAE-cellulose (1.5 × 5.7 cm) equilibrated with 0.5% Triton X-100 in 50 mM Tris-HCl/1 mM dithiothreitol, pH 7.4, and the column was eluted with this buffer. The fractions that contained the cytochalasin B binding sites, which eluted between 15 and 40 ml from initiation, were pooled and are referred to as the purified preparation. Typically, it contained about 40 μg/ml of protein and 400 μg/ml of erythrocyte phospholipid. Protein was assayed by the Peterson [7] modification of the method of Lowry et al. Appropriate blanks were included to correct for the color produced by phospholipid. Phospholipid was determined as described previously [2].

Reconstitution and assay of the solubilized cytochalasin B binding component. Before being assayed for cytochalasin B binding, samples of the extract and purified preparation were treated with Bio·Beads SM2 to remove Triton [8]. This is necessary, since Triton is a potent competitive inhibitor of binding [2]. The samples were made 2.5 mM in dithiothreitol, 1 mM in EDTA, 3 mM in $MgCl_2$, and 100 mM in NaCl; then 40 mg of moist beads per mg Triton in the original mixture were added and the mixtures were shaken for 6 h at 4°C. After removal of the samples from the beads, the pH was adjusted to 6 with NaH_2PO_4, in preparation for the binding assay. Acetone-washed soybean phospholipids [9] were added to some samples before removal of detergent. These were introduced as a sonicated dispersion to a final concentration of 5 mg/ml.

[^{3}H]Cytochalasin B binding was assayed by equilibrium dialysis, according to the method described previously [2]. The dialysis buffer was 50 mM Tris-HCl/3 mM $MgCl_2$ /100 mM NaCl/1 mM EDTA/1 mM dithiothreitol/10 mM NaH_2PO_4, pH 6.

Scatchard plot analysis (Fig. 1A) indicated that the protein-depleted membranes contain a single set of high affinity binding sites for cytochalasin B. The value of the dissociation constant, $1.4 \cdot 10^{-7}$ M, is similar to the ones reported for D-glucose-sensitive sites in erythrocyte membranes [10—12] The binding of cytochalasin B, at $4 \cdot 10^{-8}$ M, to the protein-depleted membranes was inhibited stereospecifically by glucose; 100 mM D-glucose produced an inhibition of greater than 90%, whereas L-glucose at the same con-

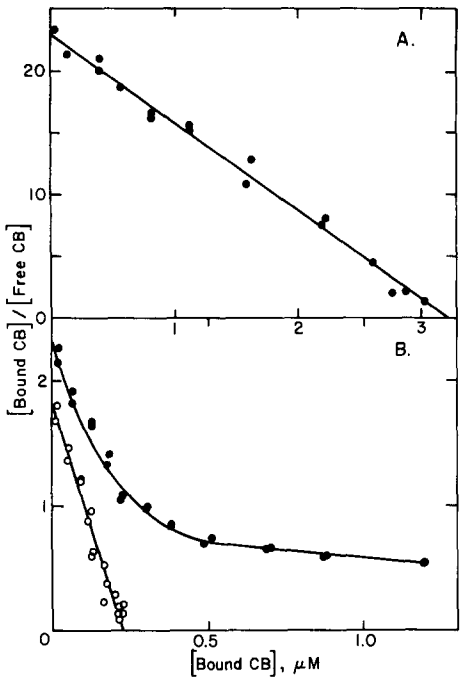

Fig.1. Scatchard plots for the binding of cytochalasin B (CB) to protein-depleted membranes (A) and to the purified preparation of the cytochalasin B binding component (B). The higher concentration of phospholipid present in B due to the addition of soybean lipid results in significant low affinity absorption of cytochalasin B; the value of [Bound cytochalasin B] /[Free cytochalasin B] for phospholipid alone at the concentration present (3.6 mg/ml) was found to be 0.4 over a range of cytochalasin B concentrations. The open symbols represent values that have been corrected for this non-specific binding by the procedure described in ref. 2. Low-affinity binding is not apparent in A because of the larger concentration of binding component and lower one of lipid.

centration inhibited by less than 4%. This finding shows that the high-affinity sites are the ones associated with the monosaccharide transport system.

Treatment of the protein-depleted membranes with 0.5% Triton X-100 solubilized about 20% of both the protein and the cytochalasin B binding component (Table I). Scatchard plot analysis (data not presented) showed that the dissociation constant for the extract, $1.2 \cdot 10^{-7}$ M, was about the same as that of the protein-depleted membranes.

Chromatography of the Triton extract on DEAE-cellulose at first gave low and variable recovery of cytochalasin B binding sites. It was then discovered that about 50% of both the protein and erythrocyte phospholipid in the purified preparation were absorbed by the Bio·Beads. The addition of soybean phospholipids to the purified preparation before removal of the Triton with Bio·Beads reduced the absorption of protein and phospholipid to about 15%, and led to a greater and consistent recovery of sites. In this regard, treatment of the Triton X-100 extract itself with Bio·Beads did not result in significant removal of protein or lipid, nor did the addition of soybean phospholipid increase the amount of cytochalasin B binding sites. The difference in behavior is probably due to the much higher protein

TABLE I

PURIFICATION OF THE CYTOCHALASIN B BINDING COMPONENT

The number of sites and their dissociation constant (K_D) for cytochalasin B were determined from Scatchard plots. Soybean phospholipids (5 mg/ml) were added to the extract and to the purified preparation before removal of the detergent. Their Scatchard plots were corrected for the resultant low-affinity binding of cytochalasin B by the procedure of Zoccoli et al. [2].

Preparation	Protein (mg)	Sites (nmol)	Sites (nmol) per mg protein	K_D (M)
Ghosts	109	68*	0.62*	$1 \cdot 10^{-7}$*
Protein-depleted membranes	40	86	2.2	$1.4 \cdot 10^{-7}$
Extract	8	21	2.6	$1.2 \cdot 10^{-7}$
Purified preparation	1.5	10	6.7	$1.3 \cdot 10^{-7}$

*Obtained from literature values [10,17].

concentration in the extract (400 μg/ml) as compared to the purified preparation (40 μg/ml).

With the realization that added phospholipid was needed for maximal yield, it was found that about 50% of the sites applied to the DEAE-cellulose column were recovered in the eluate (Table I). The eluate contained about 20% of the protein and 90% of the erythrocyte phospholipids applied to the column. The remainder of the protein and lipid but no more binding activity could be eluted with 1 M NaCl.

Characterization of the purified preparation. Scatchard plot analysis of the purified preparation (Fig. 1B) showed a dissociation constant for cytochalasin B binding of $1.3 \cdot 10^{-7}$ M, a value very similar to that in the intact membranes. There were 6.7 nmol binding sites per mg protein; this value represents about an 11-fold purification relative to the ghosts. Binding to the purified preparation was inhibited by 80% in the presence of 200 mM D-glucose and was unaffected by 200 mM L-glucose.

A single major band was found upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the pooled fractions (Fig. 2B). In its migration as a broad band of apparent molecular weight 55 000, it closely resembles the previously described preparations of purified transport system [1,3]. On the basis of its staining intensity, it comprises about 82% of the protein on the gels. The second broad peak with an apparent molecular weight of 120 000 that contains about 11% of the staining material has been sugested by Kahlenberg and Zala [3] to be an aggregate of the major species. In addition, there is a small amount of band 7 protein. Staining for glycoprotein with periodic acid-Schiff reagent showed a faint band coincident with the major band found with Coomassie Blue staining.

It remains to be determined whether the major gel band comprises a single polypeptide species. Its broadness may be due to heterogeneity in glycosylation. This has been found to be the case for the anion transport protein of the human erythrocyte membrane [13]. The fact that the same broad band has been observed in differential labeling studies of the mono-

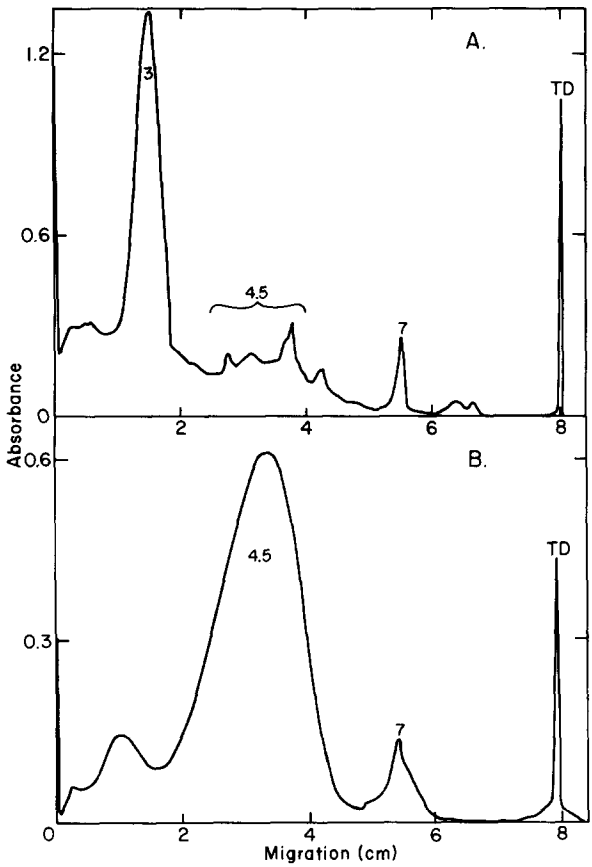

Fig.2. The polypeptide patterns obtained upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis of protein-depleted membranes (A, 17 μg) and the purified preparation of cytochalasin B binding component (B, 7 μg). Electrophoresis was performed as described previously [2]. Gels were stained with Coomassie Blue and scanned at 550 nm. Bands are designated according to Steck [18]. TD indicates the position of the tracking dye.

saccharide transport system suggests that the band does comprise a single polypeptide species [14,15].

The high specific activity for binding of cytochalasin B to the purified preparation suggests that the major gel band is the cytochalasin B binding protein. If its molecular weight is taken to be 55 000 and it comprises 93% of the total protein, then there are 0.39 binding sites per polypeptide chain in the preparation. The difference from unity may be due to the presence of some denatured protein, to errors in the estimation of the protein concentration and molecular weight, or to a true stoichiometry for binding of one cytochalasin B molecule per two polypeptide chains. A recent abstract [16] that appeared while this research was in progress reports a similar stoichiometry for cytochalasin B binding.

Thus, assay of a stoichiometric function of the erythrocyte monosaccharide transport system provides strong evidence for the participation

of the 55 000-dalton polypeptide in transport. The vagaries of reconstitution and transport assays may be responsible for the low specific transport activity obtained by Kasahara and Hinkle [1]. However, it is possible that the transport system consists of several components, only one of which has been purified. The preparation described herein should permit the investigation of this question.

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