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ISOLATION AND INCORPORATION INTO LIPID VESICLES OF A CONCANAVALIN A RECEPTOR FROM HUMAN ERYTHROCYTES

DWIGHT G. BARRATT, FRANCIS J. SHAROM, ANNE E. THEDE and CHRIS W.M. GRANT *

Department of Biochemistry, University of Western Ontario, London, Ontario, N6A 5C1 (Canada)

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Summary

Affinity chromatography has been used to isolate a concanavalin A receptor portion of Band 3 from humen erythrocytes in the presence of the readily-dialysable detergent, dodecyltrimethylammonium bromide. Addition of phospholipids to the isolated fraction and removal of detergent by dialysis leads to formation of vesicles containing the receptor. Intramembranous particles similar in size and shape to those seen in intact erythrocytes are a characteristic of the reconstituted preparations. Vesicles containing receptor bind concanavalin A with high affinity.

Introduction

The behaviour and metabolism of mammalian cells can be profoundly affected by contact with specific agents such as lectins, hormones and other cells (e.g. refs. 1—3). An important initial event in the triggering of a variety of cellular processes appears to be ligand binding to surface receptors [1,3—5]. Glycolipids and glycoproteins have been implicated as receptors in a number of these phenomena; yet much remains to be learned about the molecular basis of their involvement. We feel that useful information concerning such triggering mechanisms (e.g. ligand-receptor interactions, receptor redistribution phenomena, receptor interaction with other membrane components and ligand perturbation of such interactions) may be obtained by studying isolated receptors in model membrane systems. With this in mind, we report here a method for the isola-

* To whom correspondence should be addressed.
Abbreviations: SDS, sodium dodecyl sulfate; DTAB, dodecyltrimethylammonium bromide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tion and incorporation into lipid vesicles of the concanavalin A receptor from human erythrocytes.

The concanavalin A receptor of human erythrocytes is known to be associated with a rather broad Coomassie blue-staining band [6] (Band 3) on polyacrylamide gels run in sodium dodecyl sulfate (SDS). The exact nature of species covered by this designation is unclear. The major component seems to be a transmembrane glycoprotein [7—9] of mol. wt. about 95 000 [10] but several minor components are also claimed [11]. Certainly the designation "Band 3" does not describe a completely homogeneous molecular entity [6,10,12]. An isolation procedure for the receptor was first reported by Findlay [6] who used affinity chromatography following solubilization of erythrocyte membranes with Triton X-100. We have preferred to use the readily-dialysable detergent, dodecyltrimethylammonium bromide (DTAB) in this work because of the difficulty of removing Triton for the purpose of reconstitution (however, see refs. 13,14).

Materials and Methods

Materials

Human erythrocytes were obtained as outdated blood from Victoria Hospital, London, Ontario. Erythrocyte lipid was extracted from freeze-dried ghosts with chloroform/methanol (2 : 1). The lipid was dried and redissolved several times to precipitate protein contaminants prior to being stored as a stock solution at − 20°C. L-α-Dipalmitoyl phosphatidylcholine was obtained from Calbiochem. Phosphatidylcholines were pure as judged by thin-layer chromatography on silica gel G (Stahl).

DTAB was obtained from Eastman and was recrystallized several times from acetone/methanol before use. Concanavalin A-Sepharose (4B) was from Pharmacia and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Sigma.

SDS polyacrylamide gels (5%) were run in phosphate buffers and stained with Coomassie blue.

Isolation of concanavalin A receptor

Ghosts were prepared from outdated bank blood using the procedure of Dodge et al. [15]. The affinity column buffer was basically that described by Findlay [6] (0.1 M NaCl, 0.7 mM in each of $CaCl_2$, $MnCl_2$ and $MgCl_2$ and buffered at pH 7.4 with 10 mM Tris) except that the Triton X-100 was replaced with 50 mM DTAB.

For the solubilization step a 30—50 ml volume of column buffer was made 300 mM in DTAB and mixed on ice with an equal volume of packed ghosts. After stirring for 30 min at 0°C the solution was centrifuged at 15 000 × *g* for 20 min and the (very small) pellet discarded. The supernatant was run onto a concanavalin A-Sepharose column at 4°C (25 ml bed volume) and washed through with column buffer. Bound protein was subsequently eluted with column buffer contaning 0.1 M α-methyl-D-mannoside (Sigma). Tubes were read at 280 nm and used immediately. Protein determination was by ninhydrin assay [16].

Incorporation of receptor into lipid vesicles

Fresh column eluent containing the glycoprotein was concentrated 3× by vacuum dialysis at 4°C. This was added to an appropriate amount of lipid dissolved in 10 mM phosphate (pH 7.0), 300 mM DTAB and quickly dialysed against 5 mM HEPES buffer (pH 6.6). Dialysis was carried out at 4°C against four changes of a 200-fold excess of buffer. Samples for freeze-fracture were quenched on gold discs in freon cooled in liquid nitrogen prior to replication on a Balzers BA 501 instrument. Replicas were cleaned in commercial bleach followed by ethanol prior to examination on a Phillips EM 200.

Hemagglutination inhibition

The effectiveness of vesicles containing purified glycoprotein at binding concanavalin A was tested by their ability to inhibit concanavalin A-mediated hemagglutination of rabbit erythrocytes. Several sets of serial two-fold dilutions of concanavalin A were made into 100 μl of cold phosphate buffered saline * in a disposable micro-titre plate. In a typical test 3 rows of wells were used: to the first row there were no additions, to the second row was added 10 μl per well of an appropriate lipid vesicles suspension (5 mg lipid per ml), and to the third 10 μl per well of vesicles of the same lipid (at the same concentration) containing purified concanavalin A receptor. The micro-titre plate was incubated at 4°C for 30 min. 100 μl of a 0.5% suspension of fresh rabbit erythrocytes in phosphate buffered saline was then added to each well and the plate was incubated overnight at 4°C. End points were read as the highest dilution of concanavalin A at which the erythrocytes were not completely agglutinated.

Results and Discussion

A column profile for the affinity chromatography step is shown in Fig. 1. Unlike Triton, DTAB does not absorb at 280 nm and monitoring column effluent is relatively simple. The fraction of Band 3 collected in this fashion was variable but typically ranged about 15% (given that Band 3 material makes up some 25% [17] of the erythrocyte membrane protein). This is similar to the yield reported by Findlay and apparently does not reflect inefficiency in the affinity chromatography step but rather heterogeneity in the carbohydrate portion of Band 3 [6]. Yu and Steck have used an ion exchange column [10] to purify Band 3 (rather than selecting a concanavalin A receptor fraction) but they also report a rather low overall yield of 15—20%.

Fig. 2 shows an SDS polyacrylamide gel of the pooled central portion of the peak eluted with α-methyl-D-mannoside. There was no appreciable periodic acid-Schiff-staining material. Preparations were often slightly contaminated with a small amount of Band 6 (the protomer of glyceraldehyde-3-phosphate dehydrogenase). Other workers have reported a tendency of Band 6 (and Band 4.2) to co-purify [18] with Band 3.

Figs. 3a,c,d are freeze-fracture electron micrographs of lipid vesicles (egg phosphatidylcholine/dipalmitoyl phosphatidylcholine (50 : 50)) containing con-

* Phosphate buffered saline used as diluent contained 3.5 mg/ml bovine serum albumin.

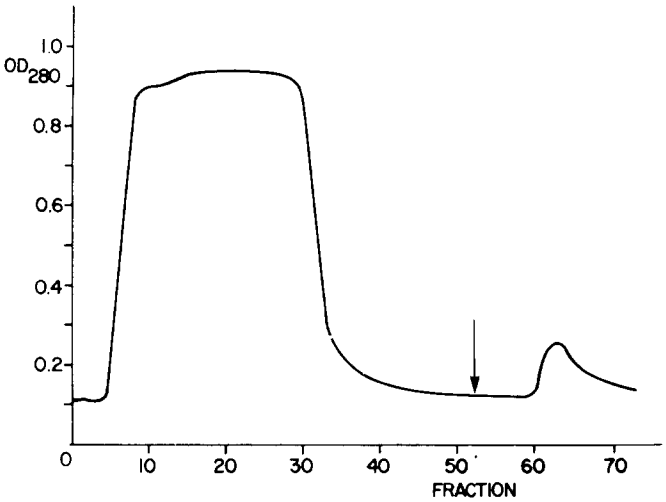

Fig. 1. Elution profile for concanavalin A affinity chromatography of human erythrocyte ghosts solubilized in DTAB. Arrow indicates the point at which α-methyl-D-mannoside was added.

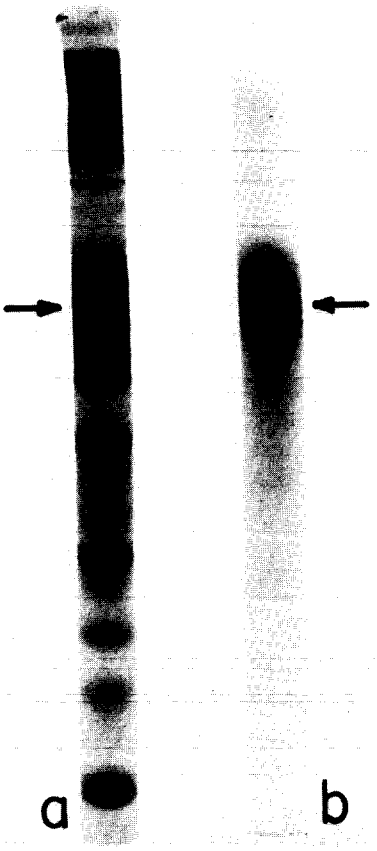

Fig. 2. SDS polyacrylamide gels of (a) washed human erythrocyte ghosts and (b) pooled fraction eluted from the concanavalin A affinity column with α-methyl-D-mannoside. Arrows indicate Band 3. Origin at top.

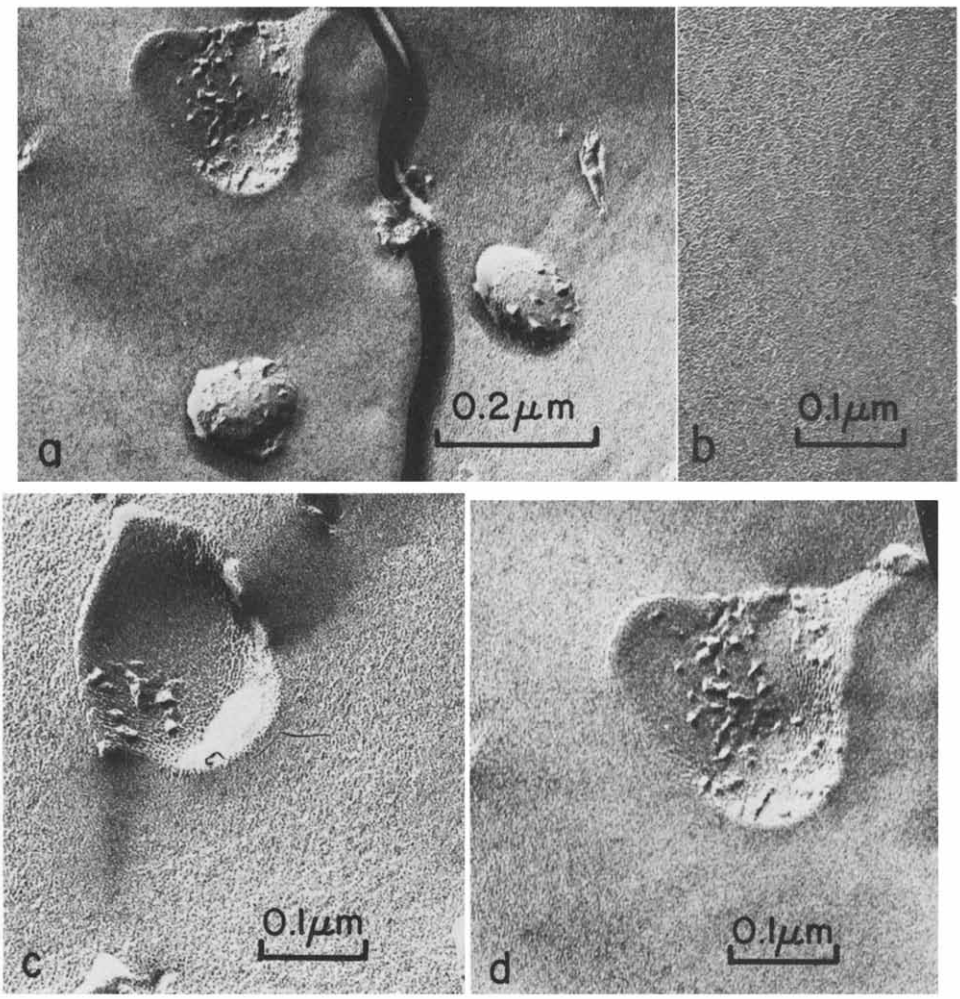

Fig. 3. (a) Freeze-fracture electron micrographs of egg phosphatidylcholine/dipalmitoyl phosphatidylcholine vesicles (50 : 50) contaning purified human erythrocyte concanavalin A receptor incorporated by dialysis from detergent solution as described in the text (magnification 104 500X). (b) shows an enlarged (142 000X) section of the fracture face of a typical vesicle generated in the same way but without protein. (c) and (d) are views at 142 000X of vesicles containing the concanavalin A receptor. Direction of shadow from bottom to top.

canavalin A receptor in a lipid/protein ratio of about 8 : 1 by weight. The particles seen are similar in size (80–125 Å) and appearance to those seen in intact erythrocyte membranes [19]. Such preparations also contain appreciable amounts of non-vesicular, jumbled, multilamellar lipid fragments containing the same intramembranous particles. Similar results have been obtained by incorporating concanavalin A receptor preparations into vesicles of widely different lipid composition such as extracted erythrocyte lipid and dioleoyl phosphatidylcholine. As shown in Fig. 3b, fracture faces of lipid bilayers produced by dialysis from detergent solutions containing no protein show no evidence of intramembranous particles.

The ability of integral membrane proteins to give rise to particles in fracture faces of lipid bilayers reconstituted from detergent solutions has been reported for other systems in recent years (e.g. rhodopsin [20], the adenosine triphosphatase of sarcoplasmic reticulum [21] and glycophorin [22]). The lack of such particles in bilayers of lipid alone has generally been interpreted to indicate that they are protein-related: for instance that intramembranous particles may represent points at which protein (or protein clusters [23]) penetrates the lipid hydrophobic region. However, this has not been absolutely proven, and the exact nature of such particles remains unresolved in the literature.

Two protein constituents of the human erythrocyte membrane have received attention in recent years as candidates for the classical erythrocyte intramembranous particles [12]. Band 3, with a major component mol. wt. of approx. 95 000 [10] and glycophorin with a mol. wt. of 23 000—31 000 [24,25] are both possiblities in that they traverse the membrane [7—9,26,27]. One of us (C.W.M.G.) has already reported [22,28] that purified glycophorin incorporated into lipid vesicles by exactly the same process as that described here for the concanavalin A receptor produces very small particles (on the order of 40 Å, uncorrected for shadow thickness [22]). Hence it seems quite likely that both glycophorin and certain components (at least) of Band 3 are responsible for some freeze-fracture intramembranous particle characteristics of the intact erythrocyte. It has been suggested that the two glycoproteins are physically associated in the membrane [12,17,29].

Preparations of lipid vesicles containing purified concanavalin A receptor were capable of effectively inhibiting concanavalin A-mediated rabbit erythrocyte agglutination. Rabbit erythrocytes (100 μl of a 0.5% suspension as described in Materials and Methods) were agglutinated by concanavalin A at a concentration of 1.56—3.12 μg/ml, a value which agrees well with those reported by other workers [30]. Addition to the microtitre plate wells of vesicles of lipid alone had no effect on the agglutination end point. However, in the presence of the same amount of lipid containing concanavalin A receptor (lipid/protein ratio 1 : 1), agglutination of the rabbit erythrocytes required 12.5—25.0 μg/ml concanavalin A. One can use such numbers in an attempt to calculate the fraction of exposed concanavalin A receptors which retain their ability to bind concanavalin A. Presumably the isolation procedure itself does minimal damage to this function since the receptors are isolated by virtue of their capacity to bind concanavalin A. However, one is reduced to making a rough eastimate by such problems as: the possible existence of receptor multimers which bind concanavalin A at a receptor/concanavalin A ratio greater than 1 : 1, and the fact that (especially at high lipid/protein ratios) there are a lot of multilamellar structures which may internalize up to 90% of the lipid surface area (our freeze fracture observation, see also ref. 31). Ignoring the first problem, but allowing roughly for receptor internalization as described above, it appears that a large fraction of the receptors exposed at lipid structure outer surfaces bind concanavalin A.

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