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CHARACTERIZATION AND IDENTIFICATION OF THE GLUCOSE TRANSPORTER OF HUMAN ERYTHROCYTES *

SHIN'ICHI SASE [a], KUNIAKI TAKATA [b], HIROSHI HIRANO [b] and MICHIHIRO KASAHARA [a]

[a] *Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo and* [b] *Department of Anatomy, Kyorin University, School of Medicine, Mitaka, Tokyo (Japan)*

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The glucose transporter was purified from human erythrocytes (Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390). The following results support the conclusion that a major protein in the purified transporter fraction, zone 4.5 is the glucose transporter (or a part of the transporter) and is different from band 3: (1) peptide maps of zone 4.5 were similar throughout the broad band in sodium dodecyl sulfate-gel electrophoresis and were different from those of band 3, (2) specific binding of cytochalasin B was found to the transporter fraction, but not to a band 3 fraction, (3) the N-terminal amino acid analysis of the transporter fraction showed a single N-terminal of lysine, whereas the band 3 fraction showed no clear N-terminal, and (4) the rabbit antibody raised against the transporter fraction formed a precipitation line with the transporter fraction, but not with the band 3 fraction. A filtration apparatus was devised for quick and accurate measurement of cytochalasin B binding, with which results comparable to those from equilibrium dialysis were obtained.

Introduction

Reconstitution of the glucose transporter of human erythrocytes in liposomes [1,2] has stimulated the studies on the identification of the transporter. Of the several proteins identified as the transporter [1,3], zone 4.5 was considered most probable in reconstitution studies. The results are in contrast to several other studies in which band 3 is claimed to be the transporter (see Refs. 1 and 2). In the latter studies the origin of zone 4.5 was ascribed to a degradation product of band 3.

In this study, we present four lines of evidence which support the conclusion that zone 4.5 is the transporter (or a part of the transporter) and is not related to band 3. In addition, a new apparatus for binding assays is shown.

Experimental procedures

Materials

[^{3}H]Cytochalasin B was purchased from New England Nuclear, ^{125}I from Amersham, dansyl chloride from Pierce, anti-rabbit-IgG serum (goat) from MBL, Nagoya, Japan, anti-rabbit-IgG IgG (goat) from Miles and complete Freund's adjuvant from Difco. Other reagents were as described [4,5] and of analytical grade.

* Data supplementary to this article are deposited with, and can be obtained from, Elsevier Biomedical Press, B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD/234/71424/693 (1982) 253–260. The supplementary information includes: A photograph and a schematic drawing of the filtration apparatus described in Fig. 1.

Abbreviations: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate.

Methods

Analytical methods. Protein was measured by the method of Lowry et al. [6] with bovine serum albumin as a standard. When significant amounts of interferring materials were in protein samples, they were pretreated with 5% trichloroacetic acid in the presence of 0.05% Triton X-100 which was used as a coprecipitant, washed once with 1 ml of 5% trichloroacetic acid, mixed with 10 μl of 20% SDS and subjected to the protein assay. Lipid was estimated by phosphorus determination by the method of Gerlach and Deutlich [7]. SDS-gel electrophoresis was carried out by the method of Fairbanks et al. [8,9]. N-terminal analysis of zone 4.5 and band 3 was carried out as described previously [10]. Protein samples (15–30 μg) were pretreated with acetone-ether in the following way. After protein was precipitated with 5% trichloroacetic acid in the presence of 0.05% Triton X-100, it was washed twice with 1 ml of acetone/ether (1 : 1, v/v) and solubilized with 50 μl of 1% SDS at 100°C for 5 min, and subjected to dansylation.

Preparation of glucose transporter and band 3. Triton extract (a crude solublized membrane fraction) and the glucose transporter of human erythrocytes were prepared as described [5] with minor modifications [11]. The purified transporter fraction contained zone 4.5 as a major protein component, a minor amount of band 7 and lipids [5]. A band 3 fraction was obtained by eluting with 1 M NaCl, the DEAE-cellulose column used for purification of the transporter, followed by Bio-Beads treatment and dialysis overnight against 10 mM Tris-HCl (pH 7.5). Purity of band 3 was estimated to be more than 70% by Coomassie blue staining of SDS-gels. Reconstitution of glucose transporter and measurement of glucose transport activity were performed as described [5] with minor modification [11].

Preparation of IgG. Rabbit antibody was raised against the purified transporter fraction. The protein fraction (250 μg) was mixed with an equal amount of complete Freund's adjuvant and injected to each of four rabbits, followed by two injections at intervals of 2 and 4 weeks. During the following 3 months rabbit serum was obtained three times from these rabbits and the titer of the antigen-antibody binding reaction, as observed by the Ouchterlony double diffusion test, did not significantly change during that period. An IgG fraction was prepared by ammonium sulfate precipitation and DEAE-cellulose column chromatography as described [12]. Control IgG was prepared from a preimmune rabbit.

Electron microscopy. Reconstituted liposomes were mixed with the anti-transporter antibody or control IgG which has been centrifuged at 100 000 $\times g$ for 2 h at 4°C, incubated at 30°C for 30 min and centrifuged at 100 000 $\times g$ for 2 h at 4°C. The precipitate was suspended in a solution containing 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5) (Mg buffer) and washed once with the Mg buffer. The antibody-treated liposomes were suspended in the Mg buffer and incubated overnight at 5°C with ferritin-labeled anti-rabbit-IgG IgG (goat) [13]. The mixture was centrifuged at 15 000 $\times g$ for 30 min at 4°C and the precipitate was suspended in the Mg buffer, centrifuged at 100 000 $\times g$ for 2 h at

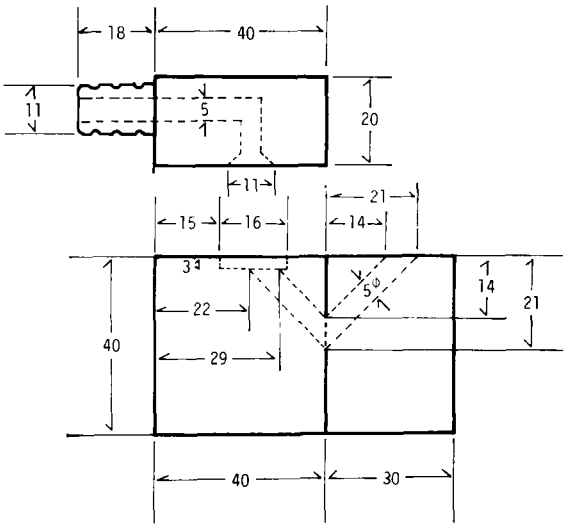

Fig. 1. A new instrument for binding assay. A filtration apparatus was constructed for cytochalasin B binding, which is also generally applicable to binding assays as a replacement for equilibrium dialysis. The apparatus is made of plexiglass and consists of three parts. Two slanted holes are separated by a ultrafiltration membrane (or a membrane filter). A transverse plane through one of ten holes is drawn. Lengths are indicated in mm. Pressure is applied to filter a small amount of assay mixture (ordinarily 20 μl out of 140 μl) for measurement of free concentration of the binding substrate. The amount of the substrate bound to macromolecules is calculated from the concentration of substrate in the assay mixture and the concentration in the filtrate.

4°C, fixed with 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) and subjected to electron microscopy [14].

Cytochalasin B binding. Binding of cytochalasin B was measured by equilibrium dialysis or with the aid of a newly devised instrument (Fig. 1), the instrument made use of ultrafiltration for separation of unbound [^{3}H]cytochalasin B. Assay mixture containing protein sample, 30 to 700 nM [^{3}H]cytochalasin B and 10 mM Tris-HCl (pH 7.5) in 140 μl was incubated for 20 min at 23°C. After 10 μl of the assay mixture was removed for the determination of total radioactivity, ultrafiltration of the assay mixture was carried out through a ultrafiltration membrane (YM-10U, Amicon) by the pressure of nitrogen gas. Selection of ultrafiltration membranes was found essential (Table I). 10 μl of the filtrate was removed. The radioactivity in these aliquots was counted in a scintillation counter. Comparable results were obtained by equilibrium dialysis and by the new filtration apparatus. A preparation of Triton extract showed a maximum binding of 0.31 nmol/mg protein and K_d 150 nM with equilibrium dialysis and a maximum binding of 0.31 nmol/mg protein and K_d 110 nM with the new instrument.

Peptide mapping of zone 4.5, band 7 and band 3. Peptide mapping of small amounts of proteins in acrylamide gels was performed as described [15] with minor modifications. After staining with Coomassie blue a necessary portion of cylinder gels were cut out by a razor blade. The iodination

Fig. 2. Peptide maps of zone 4.5 and band 3. The glucose transporter of human erythrocytes was purified [5] and SDS-gel electrophoresis of the transporter fraction was carried out. After staining with Coomassie blue R the broad zone 4.5 was cut out into three equal pieces, a high-molecular weight range (A), middle range (B) and a low-molecular weight range (C), and subjected to radioiodination and peptide mapping by trypsin treatment [15]. In the same way peptide mapping of band 3 (D) was carried out from a high-molecular part of band 3 prepared as described in Methods. (Arrows in (B) indicate common spots seen in A–C.)

TABLE I

FILTRATION OF BINDING SUBSTRATE THROUGH VARIOUS SEPARATION MEMBRANES FITTED TO A NEW BINDING APPARATUS

An assay mixture consisted of 20 nM [^{3}H]cytochalasin B or 30 μM D-[^{14}C]glucose and 10 mM Tris-HCl (pH 7.5). An aliquot of 10 μl was withdrawn for counting of radioactivity. The rest was applied to the hole in the side where pressure was to be applied. Pressure was applied to collect about 20 μl filtrate in 15 min at 23°C. An aliquot of 10 μl was taken from the filtrate and radioactivity was counted.

Membrane	Percent of filtration	
	Cytochalasin B	D-Glucose
YM10U (Amicon)	97	103
PM10 (Amicon)	4	94
XM50 (Amicon)	31	95
XM100A (Amicon)	70	97
Nitrocellulose filter (0.22 μm, Millipore)	58	98

reaction was stopped by the addition of sodium bisulfite, followed by the addition of 200 μl of 0.1 M sodium iodide to dilute radioactive iodide. Autoradiography was performed with Fuji Rx film at −70°C for one to several days.

Results

Peptide mapping of zone 4.5, band 7 and band 3

Peptide mapping of membrane proteins was performed by a highly sensitive method which made use of radioiodination of proteins in polyacrylamide gels. The broad zone 4.5 region of SDS-gels was cut into three equal pieces and peptide maps were made from each of the gel pieces. These three maps showed similar patterns, in which only a few spots were different (Fig. 2). No clear correspondence was found in peptide maps of band 3 and zone 4.5 (Fig. 2). When band 3 was cut into three pieces, peptide maps of these three pieces were the same in respect to the position and the density of each spot which has already been noted in other studies [16]. Maps of band 3 contained fewer spots than maps of zone 4.5. Similarity was, however, found between maps of zone 4.5 and band 7 in that several characteristic spots found on maps of zone 4.5 were found on

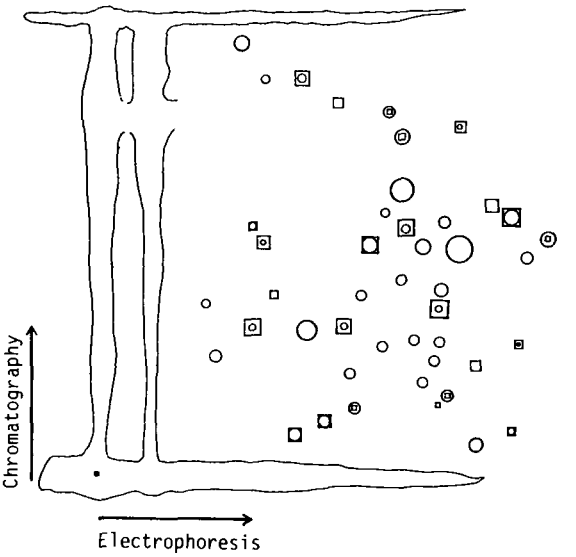

Fig. 3. Peptide maps of zone 4.5 and band 7. Peptide mapping was performed as described in Methods. SDS-gel electrophoresis of the transporter fraction was carried out and zone 4.5 at low-molecular weight range and band 7 was cut out from the gel and peptide maps were prepared as described in Methods. Circles indicate spots seen in the map of zone 4.5 and squares indicate spots in the map of band 7. The sizes of those marks are rough indications of relative darkness of spots seen in peptide maps.

TABLE II

CYTOCHALASIN B BINDING AND D-GLUCOSE TRANSPORT

Triton extract, the transporter fraction and the band 3 fraction were prepared as described in Methods. Cytochalasin B binding of each fraction was measured with a new filtration apparatus described in Methods. Reconstitution of membrane protein fractions was carried out by the freeze-thaw/sonication method. Transport of D-glucose or L-glucose was measured at 0.2 mM sugar for 15 s. D-Glucose-specific transport was calculated by subtracting L-glucose transport. Other procedures are described in Methods.

	Cytochalasin B binding		Transport (nmol/mg protein/min)
	Max. binding (nmol/mg protein)	K_d (nM)	
Triton extract	0.18	95	4.5
Transporter Fr.	0.85	150	28
Band 3 Fr. [a]	–	–	0.4

[a] Non-saturable binding, 0.12 pmol/mg protein per nM.

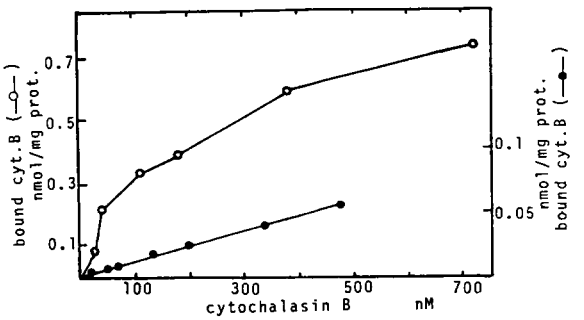

Fig. 4. Cytochalasin B binding to the transporter fraction or the band 3 fraction. Cytochalasin B binding was measured by a new instrument in which assay mixtures were ultrafiltered through a membrane (YM10U, Amicon) and the radioactivity in 10 μl of filtrate or assay mixture was counted. Other procedures were described in Methods. The left ordinate applied to cytochalasin B binding of the transporter fraction (○) and the right one to that of the band 3 fraction (●).

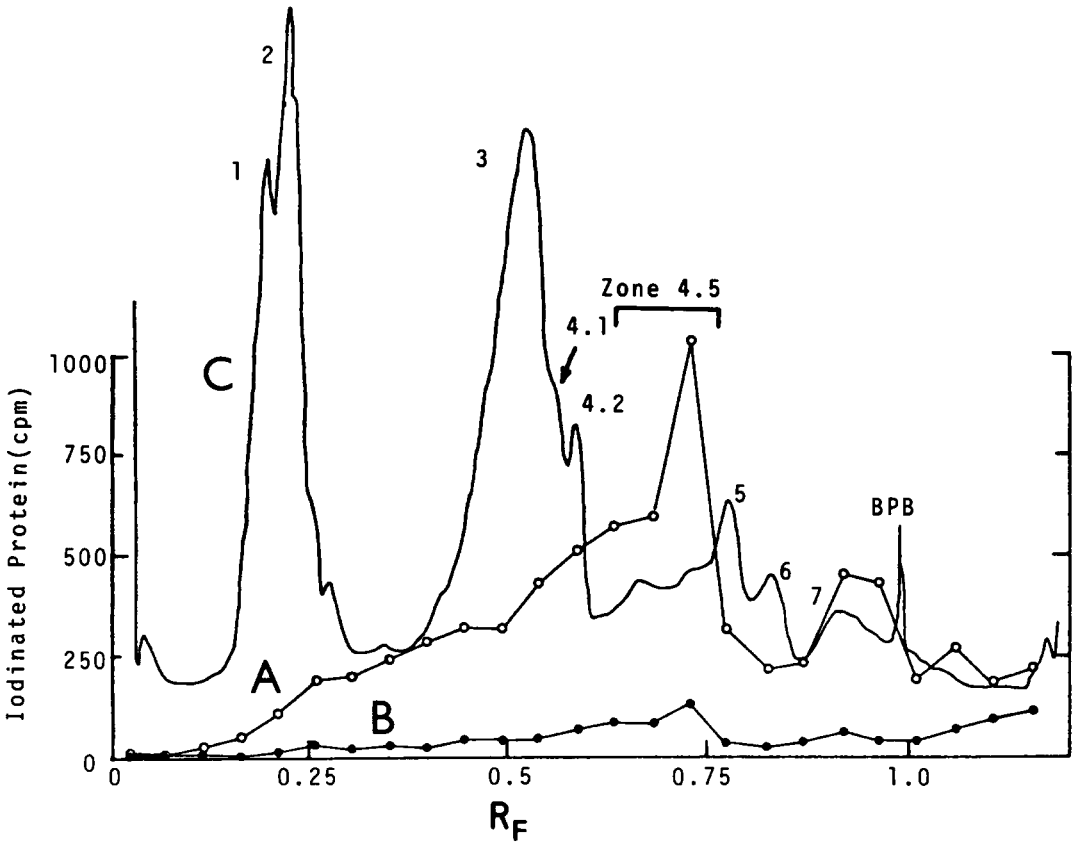

Fig. 5. Immunoprecipitation by the antibody against the purified transporter fraction. Radioiodination of the transporter fraction with ^{125}I by the chloramine T method [15] was employed for detection of antigenic counterpart of immune complex, since the molecular weights of the H and L chains of IgG were close enough to mask zone 4.5 and band 7, respectively, in SDS-gels. The reaction mixture containing the transporter fraction (140 μg), 10 μl carrier-free ^{125}I, 10 μl of 10 mg/ml chloramine T and 50 mM sodium phosphate (pH 7.5) in 800 μl was incubated at 25°C for 1 h, followed by successive additions of 1 ml of 1 mg/ml $Na_2S_2O_5$ and 200 μl of 0.1 M NaI. The mixture was centrifuged at 100000 × *g* for 30 min and the precipitate was washed twice with 10 mM Tris-HCl (pH 7.5) and stored at −70°C. (A) The iodinated transporter fraction (90 μg) was solubilized with 0.5% Triton X-100 and centrifuged at 15000 × *g* for 30 min. The supernatant (160 μl), the anti-transporter antibody (1.3 mg) and 0.5% Triton X-100 were mixed and incubated overnight at 5°C. After incubation with anti-rabbit-IgG antibody (0.75 mg) for 2 h, the mixture was centrifuged at 10000 × *g* for 20 min and the precipitate was washed once with 10 mM Tris-HCl (pH 7.5) and subjected to SDS-gel electrophoresis. After electrophoresis, the gel was frozen at −70°C for more than 3 h and cut into 2 mm pieces with razor blades. The radioactivity of each piece was counted in a gamma counter (JDC 751, Aloka). (B) Control IgG was used instead of the antibody against the transporter fraction. Other procedures are the same as in A. (C) Ghost (40 μg) was subjected to SDS-gel electrophoresis in parallel with proteins shown in A and B and stained with Coomassie blue. The gel was scanned by a densitometer (200/201, Joyce-Loebl). Minor difference in the length of gels was adjusted by the origin of gel and the Bromophenol blue (BPB) position.

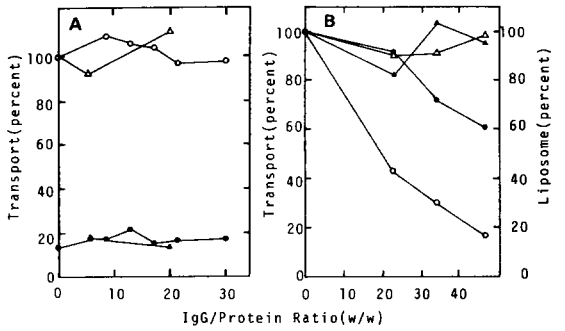

Fig. 6. Precipitation of the glucose transport activity in reconstituted liposomes by the anti-transporter antibody. In A, reconstituted liposomes (5 μg protein) were mixed with 10 mM Tris-HCl (pH 7.5) and appropriate amounts of anti-transporter IgG or control IgG to obtain indicated ratios of IgG/protein. The mixture was incubated at 25°C for 30 min. Transport of D-glucose (○, the anti-transporter IgG and △, control IgG) or L-glucose (●, the anti-transporter IgG and ▲, control IgG) was measured with 2 μg reconstituted liposomes for 15 s by the membrane filter method. Uptake of D-glucose without IgG was taken as 100% (0.058% of total radioactivity). In B, reconstituted liposomes were centrifuged at 10000 × *g* for 30 min. The supernatant was mixed with the anti-transporter IgG or control IgG in 10 mM Tris-HCl (pH 7.5) and incubated for 4 h at 25°C. Goat anti-rabbit-IgG serum (an equal amount to primary IgG) was added to the mixture and incubation was continued for 3.5 h at 25°C, followed by centrifugation at 10000 × *g* for 20 min. The supernatant was used for glucose transport assays and the measurement of liposomes remained in the transport assay. D-Glucose-specific transport after treatment with anti-transporter antibody (○) or with control IgG (△) is expressed as the difference of D-glucose uptake and L-glucose uptake for 15 s. The amount of liposomes remained after the treatment with anti-transporter IgG (●) or with control IgG (▲) is expressed as percent of control which was treated in the same way but without IgG.

that of band 7 (Fig. 3). Peptide pattern of band 7 derived from ghost preparations was similar to that of band 7 from the purified transporter fraction and showed similarity to zone 4.5 maps (data not shown).

N-terminal amino acid analysis of the transporter fraction and band 3

After the transporter fraction was treated with acetone-ether, N-terminal analysis of the fraction was performed. Dansylation of the N-terminal and hydrolysis with 6 M HCl showed a clear dansylated N-terminal of lysine with only a faint spot corresponding to tyrosine. Parallel experiments with band 3 showed no clear N-terminal, which is consistent with the observation that the N-terminal of band 3 is blocked [17].

Binding of cytochalasin B to the purified transporter fraction

Cytochalasin B binding of the purified transporter fraction showed close association of the activity to the transport activity (Table II) and inhibited by D-glucose with K_i 30 mM, 3-*O*-methyl-D-glucose with 31 mM and mercuric chloride with 3 μM, which is consistent with the previous reports [18,19]. The specific binding activity

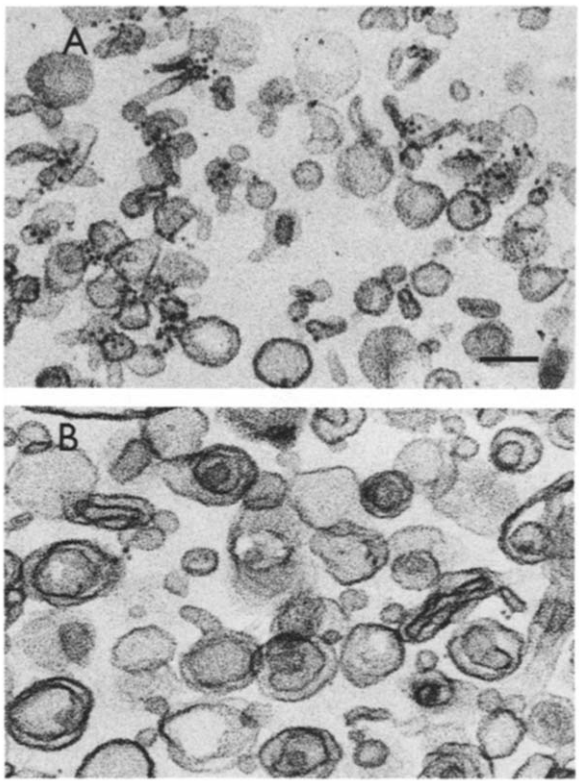

Fig. 7. Distribution of antigenic site(s) on reconstituted liposomes. (A) The anti-transporter antibody (1 mg) was mixed with reconstituted liposomes (210 μg) and incubated at 30°C for 2 h. After washing with a solution containing 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5), the liposomes were incubated with ferritin-labelled goat anti-rabbit-IgG (0.14 mg), fixed with 1% osmium tetroxide in 100 mM cacodylate butter (pH 7.4) and subjected to thin section electron microscopy. Other procedures are described in Methods. (B) Control IgG was used instead of the anti-transporter antibody. The bar in A indicates 100 nm and applies to both figures.

was found in the purified fraction and Triton extract, but not in the band 3 fraction (Table II and Fig. 4).

Characterization of the antibody against the purified transporter fraction. Rabbit antibody was raised against the purified transporter fraction. The antibody formed an immune complex with the ^{125}I-labeled transporter fraction. SDS-gel electrophoresis of the immune complex showed that the antibody was indeed raised against zone 4.5 (Fig. 5). Band 7 was also found in the immune complex. The antibody precipitated out the reconstituted liposomes when goat anti-rabbit-IgG serum was added as a secondary antibody, but no inhibitory effect of the antibody was found when the rabbit antibody alone was used (Fig. 6). When Triton extract, a crude solubilized membrane fraction, was treated with the anti-transporter antibody in the presence of 0.5% Triton X-100 and the resultant immune complex formed was removed by centrifugation at $100000 \times g$ for 60 min, the transport in reconstituted liposomes made with the treated Triton extract showed less than 20% of the control in which Triton extract was treated in the same way except for the addition of the antibody (data not shown). By electron microscopy, evidence was obtained that some of the antigenic site(s) were exposed on the outer surface of liposomes (Fig. 7). On Ouchterlony plates, the antibody showed a precipitation line with the purified transporter fraction but not with the band 3 fraction (Fig. 8).

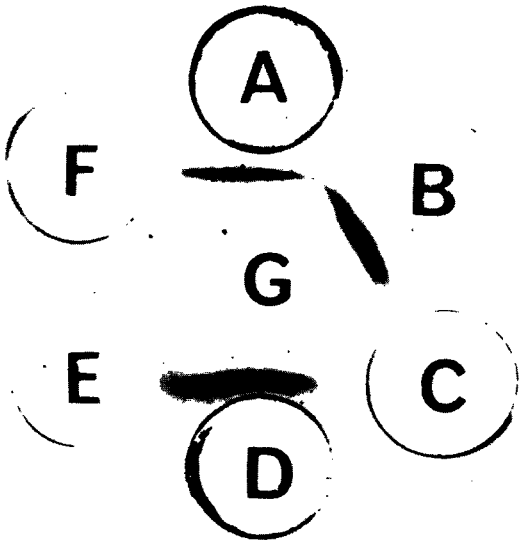

Fig. 8. The Ouchterlony double diffusion test of the antibody against the transporter fraction. Ghost (42 μg in A and 21 μg in D), the transporter fraction (1.7 μg in B and 0.7 μg in E), and the band 3 fraction (6.3 μg in C and 3.2 μg in F) were dissolved in 1.0% Triton X-100 and applied to indicated peripheral wells. The central well contained the antibody against the transporter fraction (21 μg in G). Agarose plate was made from 1% agarose, 0.1% Triton X-100 and 25 mM Tris-HCl (pH 7.5).

Discussion

Four lines of studies have been performed to identify the glucose transporter of human erythrocytes [1–3]; covalent labeling of proteins by glucose analog or inhibitors for glucose transport, purification and reconstitution and radiation target analysis. In those studies several membrane proteins in the molecular weight regions of 50000, 100000 and 200000 were suggested as the glucose transporter. Of those proteins, zone 4.5 was indicated in most reconstitution studies [1,2]. Other studies, however, indicated band 3 as the transporter and suggested that zone 4.5 is a proteolytic product of band 3 [20–22]. This study supports the conclusion that zone 4.5 is the transporter (or a part of the transporter) and is not related to band 3. First, peptide maps of zone 4.5 and band 3 were compared. Since zone 4.5 and band 3 are broad in SDS-gel electrophoresis, peptide maps were made from the high molecular, the middle and the low molecular regions of each band. Maps of band 3 were the same throughout the broad band, whereas those of zone 4.5 were similar in each region, but showed several different spots (Fig. 2). The numbers of major spots were more in band 3 maps than in zone 4.5 maps. No similarity was found in maps of band 3 and those of zone 4.5, indicating those two bands are different identities. It is not clear at present why maps of band 3 were the same and those of 4.5 were slightly different while both band 3 [23] and zone 4.5 [24] are claimed to be heterogeneously glycosylated. Secondly, cytochalasin B which is an inhibitor of glucose transport in intact erythrocytes [25–27], ghost [27] and reconstituted liposomes [4,28] was found to bind to the transporter fraction but not to the band 3

fraction. The binding activity showed good correlation with the glucose transport activity (Table II). A weak binding activity found in the band 3 fraction was linearly dependent on cytochalasin B concentration, indicating a non-specific binding. Thirdly, the N-terminal amino acid analysis of the transporter fraction showed a N-terminal of lysine with only a faint spot of tyrosine. These two amino acids were two of three amino acids found in similar dansylation analysis by others [29], but are different from methionine indicated by Edman degradation [29]. Finally, the antibody raised against the purified transporter fraction did not show cross-reactivity on the band 3 fraction (Fig. 8). The antibody did not inhibit the glucose transport activity, but it reacted with zone 4.5 and moderately with band 7 and precipitated the reconstituted liposomes (Figs. 5–7). Presence of antigenic site(s) on the outer surface of the liposomes was also observed by ferritin antibody electron microscopy. These results indicate antigenic site(s) of the transporter is remote from the active site for transport. Previously, two groups raised antibody against the glucose transporter and showed that band 3 did not cross-react with the antibody. The antibody used in this study seems similar to one obtained by Baldwin and Lienhard [30] in which the antibody precipitated the cytochalasin B binding activity, but is somewhat different from one described by Sogin and Hinkle [31] which inhibited 50% of the glucose transport activity in reconstituted liposomes. Taken together, those results, as well as the observation that the zone 4.5 fraction, but not the band 3 fraction is active in reconstitution [5], strongly support the conclusion that zone 4.5 is the glucose transporter (or a part of the transporter).

Some similarity was found in zone 4.5 and band 7. Antibody raised against the transporter fraction precipitated zone 4.5 as well as band 7. Peptide maps showed several common spots in zone 4.5 and band 7. These results raise the possibility that band 7 is a component of the glucose transporter and have similar amino acid sequence to zone 4.5. This explanation is consistent with the finding that during maturation of guinea pig reticulocytes the amount of zone 4.5 and band 7 went down concomitantly with the decrease of the glucose transport activity [33]. A multicomponent transport system was noted in the acetylcholine receptor of *Torpedo californica* where the receptor consisted of four subunits having closely related amino acid sequences [34]. However, a possibility that band 7 is a proteolytic product of zone 4.5 is not completely excluded at this stage of the study.

A filtration instrument was devised for the measurement of cytochalasin B binding. Binding of small molecules to macromolecules have been measured with equilibrium dialysis or other means including gel filtration [35], flow dialysis [36], centrifugation (see, for example, Ref. 37) and centrifuged-column [38,39]. Ultrafiltration was used in two occasions [40,41]. The new instrument is a modification of 'Poulus Cell' [40] and allows collection of filtrates. The present procedures are rapid and require less assay solution. A major problem of nonspecific binding of ligands to separation membranes [41] was overcome by the use of a ultrafiltration membrane, YM10U. We point out the following advantages over other methods. This method requires less time than equilibrium dialysis, gel filtration and flow dialysis. Binding assays with this instrument are quasi-equilibrium in that the protein concentration increases slightly (less than 15%) with filtration whereas a considerable change in the assay solution occurs during the assay with centrifugation or centrifuged-columns. This method is, however, subjected to the same limitation as equilibrium dialysis since the affinity of the binding reaction should be high so that a certain amount of the ligand binds to macromolecules.

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