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RECONSTITUTION OF GLUCOSE TRANSPORT USING HUMAN ERYTHROCYTE BAND 3

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Band 3 and the diffuse component of zone 4.5, designated band 4.5.B, have been separately prepared from human erythrocyte membranes and incorporated into the membranes of 150 nm 1-palmitoyl-2-oleoyl phosphatidylcholine vesicles. The rates of glucose influx into these vesicles were measured under zero-*trans* conditions. Both sets of vesicles exhibited substrate-saturable transport which was inhibited by phloretin. However, the specific activity of the band 3 vesicles, 292 μmol·min^{-1}·(mg protein)$^{-1}$, was more than twice that of the band 4.5.B vesicles, and the turnover number of transporters in the band 3 vesicles was at least 4-fold greater than those in the 4.5.B vesicles. Very little background density was visible in the band 4.5 region of erythrocyte membranes protected from degradation. In unprotected membranes, band 4.5.B was abundantly present, could be purified, and had glucose transport activity. Previously we have shown (Biochemistry 19, 1205 (1980)) that maltosyl isothiocyanate, an affinity label for the glucose transporter, labelled a single 100000 M_r protein of the intact erythrocyte membrane. Based upon the results of both affinity labelling and reconstitution we suggest that the native glucose transporter is a component of band 3, and that band 4.5.B contains a partially active fragment of the native transporter.

Introduction

Conflicting evidence has been presented concerning the identity of the human erythrocyte monosaccharide transporter. Maltosyl isothiocyanate, a covalent affinity lable for the glucose transporter [1], was found to be incorporated into a single 100000 M_r protein component of Band 3 [2] (nomenclature based on electrophoretic mobility using the convention of Steck [3]), which was assumed to be the transporter. Lundahl and coworkers [4,5] have also concluded that the native transporter is a dimer of a band 3 component. On the other hand, ion exchange chromatography of detergent extracts of the erythrocyte membrane has led to the isolation of a heavily glycosylated protein fraction which on polyacrylamide gel electrophoresis migrates as a very broad, diffuse band without discrete substructure that has an average apparent M_r of 55000 [6]; it has been proposed that this substance is present in native erythrocyte membranes and accounts for the diffuse background density underlying several sharp, discrete bands in the 4.5 region of electrophoretograms. We shall refer to the diffusely stained component as 'band 4.5.B'. It has been reported that phospholipid vesicles containing band 4.5.B exhibit stereospecific glucose transport [7] and bind cytochalasin B [8], a potent inhibitor of glucose transport. Addition of purified band 4.5.B to planar bilayers also resulted in glucose transport, while addition of band 3 did not [9]. Quite recently it was reported that illumination with ultraviolet light caused covalent incorporation of cytochalasin B into the band 4.5.B region of erythrocyte ghosts [10,11] and into proteins of approximately the same molecular weight in fibroblasts and adipocytes [12].

We now report that glucose transport activity can be efficiently reconstituted into large, unilamellar phosphatidylcholine vesicles by incorporation into them of a fraction chromatographically enriched in band 3 but depleted of band 4.5.B. Vesicles containing band 4.5.B also transport glucose, but with a maximal specific activity less than those reconstituted with band 3-enriched material.

Materials and Methods

Phenylmethanesulfonyl fluoride, phloretin, Tris, sodium dodecyl sulfate, (SDS), glucose oxidase (type V), catalase, α-cellulose, Sigmacell type 50 microcrystalline cellulose, DEAE-cellulose and aminoethyl-cellulose were purchased from Sigma. Diisopropylfluorophosphate, dithiothreitol, acrylamide and mercaptoethanol were obtained from Aldrich. D-Glucose was from Pfanstiehl Laboratories, heparin from Organon, Inc., *n*-octyl-β-D-glucopyranoside (octylglucoside) from Calbiochem, *sn*-1-palmitoyl-2-oleoyl phosphatidylcholine from Avanti Polar Lipids, Sepharose CL-6B from Pharmacia, and dimethylsulfoxide from Pierce. Acrylamide was recrystallized from hot acetone and SDS was recrystallized from ethanol. Glucose oxidase was prepared for use by adding 8000 U catalase/ml, desalting on a Sephadex G-15 column equilibrated with 10 mM sodium phosphate buffer (pH 7) containing 0.1 mM EDTA, and concentrating 4-fold on a Millipore CX-10 ultrafilter.

Fresh venous blood was treated with anticoagulants (1 mg EDTA/16.7 U heparin/ml). Leukocytes were removed by passing the blood through a cellulose column as described by Beutler et al. [13]. Erythrocytes were collected by centrifugation at $500 \times g$ and were washed five times by resuspension in 150 mM NaCl buffered with 5 mM sodium phosphate, pH 8 (phosphate-buffered saline). Following passage through the cellulose column [13] the 'buffy coat' of leukocytes usually observed during the isolation of erythrocytes by centrifugation [14] was absent, and leukocytes were not found in the erythrocyte preparation by microscopic examination. After the final wash, the erythrocytes, suspended in an equal volume of phosphate-buffered saline, were stirred vigorously, and sufficient amounts of 0.1 M solutions of diisopropylfluorophosphate and phenylmethanesufonyl fluoride in anhydrous isopropanol were added slowly to give final concentrations of 0.1 mM each. After standing at room temperature for 20 min, the suspension was centrifuged at $500 \times g$, and the sedimented cells were washed once in phosphate-buffered saline. Membranes were prepared by the method of Fairbanks et al. [14]. The final membrane pellets were divided into small aliquots and stored at −70°C until used.

For fractionation of detergent solutions of erythrocyte membrane proteins to prepare band 3 the method of Yu and Steck [15] was used with modifications. To 0.5 ml white ghost suspension at 0°C was added 0.5 ml of 10 mM sodium phosphate buffer (pH 7)/0.1 mM EDTA/340 mM octylglucoside. After the mixture had stood for 20 min on ice it was centrifuged at $100\,000 \times g$ for 15 min at 4°C, and all subsequent steps were performed at this temperature. The clear supernatant solution, removed from a small, gelatinous pellet, was immediately applied to a 0.6×2.5 cm (total volume 0.7 ml) column of aminoethyl-cellulose which had been equilibrated with 10 mM sodium phosphate buffer (pH 8) containing 0.1 mM EDTA/34 mM octylglucoside. The column was washed with 1.5 ml of 44 mM sodium phosphate buffer (pH 8)/0.1 mM EDTA/34 mM octylglucoside, followed by elution of band 3 with 1.5 ml of 170 mM sodium phosphate buffer (pH 8)/0.1 mM EDTA/34 mM octylglucoside. The flow rate was maintained at 1.0 ml/min by applying nitrogen under pressure. Eluted fractions were concentrated to volumes of 0.75–1.0 ml using a Millipore CX-10 ultrafilter. Although the modifications introduced into the original method resulted in contamination of band 3 by band 4.2, band 6, and traces of other components, this was accepted in order to maintain transporter viability.

Isolation of band 4.5.B was attempted by following the procedure of Baldwin et al. [8] except that erythrocytes and membranes were prepared by the method described above and extraction with 2 mM sodium · EDTA (pH 12.1) was omitted. Eluates from the DEAE-cellulose column were concentrated up to 10-fold on Millipore CX-10 ultrafilters prior to reconstitution in attempts to demonstrate transport activity with this material. When it became obvious that neither significant

amounts of band 4.5.B nor transport activity could be detected in these preparations, the method of Baldwin et al. [8] was followed exactly to prepare the material from erythrocytes which had not been passed through a cellulose column, had not been exposed to serine protease inhibitors, but whose membranes had been extracted at pH 12.1.

Vesicles containing glucose oxidase in their internal volumes and ghost proteins in their membranes were prepared by detergent dialysis as described by Mimms et al. [16]. To 1.0 ml of unfractionated membrane solution (2 mg protein) in octylglucoside or of concentrated ion exchange column eluate (0.5 mg protein) was added 53 μmol *sn*-1-palmitoyl-2-oleoyl phosphatidylcholine in 1.0 ml of 10 mM sodium phosphate buffer (pH 7)/0.1 mM EDTA/340 μmol octylglucoside/0.5 ml of glucose oxidase solution. This was dialyzed at 4°C vs. 1 l of 10 mM sodium phosphate buffer (pH 7)/0.1 mM EDTA/0.25 mM mercaptoethanol for 36 h, with changes of dialysis solution at 12 h intervals. For reconstitution from band 4.5.B, the dialysis buffer was 50 mM Tris-HCl buffer (pH 7.4)/0.1 M NaCl/1.0 mM EDTA/0.25 mM mercaptoethanol as described by Baldwin et al. [8]. During dialysis all preparations became turbid due to the formation of vesicles. The dialyzed vesicle suspensions were passed through a 1.0×50 cm Sepharose CL-6B column equilibrated with dialysis buffer containing no mercaptoethanol. The turbid void volume fractions, which were completely separated from extra-vesicular glucose oxidase, were collected and used immediately for assay of glucose transport activity. Freeze-fracture electron micrographs of vesicles reconstituted with band 3-enriched fractions revealed rather uniform 150 nm unilamellar vesicles.

The rates of zero-*trans* influx of glucose into the glucose oxidase-containing vesicles were assayed using the method of Taverna and Langdon [17]. All measurements were carried out at 22°C in the same buffer as that used for Cl-6B chromatography. Phloretin was added as a 100 mM solution in dimethylsulfoxide; the final concentration of dimethylsulfoxide did not exceed 2%, and this concentration was found to have no effect on transport.

7.5% polyacrylamide gels were prepared as described by Laemmli [18]. Samples made 2% in SDS and 5 mM in dithiothreitol were heated at 100°C for 1 min. Gels were stained with silver using the technique of Oakley et al. [19] as modified by Eschenbruch and Burk [20].

Proteins were quantitatively assayed by amino acid analysis. Samples were hydrolyzed in constant boiling HCl for 18 h at 105°C, and the amino acids were separated on a modified Beckman Model 119 amino acid analyzer. Amino acids were detected using *ortho*-phthalaldehyde [21] and an Aminco Fluoro-monitor; peak areas were quantitated with a Hewlett-Packard 3390A integrator. Values of duplicate samples agreed within 10%. This method does not detect proline or cysteine, and tryptophan was destroyed during hydrolysis; however these usually account for less than 10 mol% of the amino acids in proteins.

Results

In preliminary experiments octylglucoside extracts of erythrocyte membranes were reconstituted into phosphatidylcholine vesicles without prior fractionation of their protein components. Plots of glucose influx rates into these vesicles vs. external glucose concentration clearly showed a saturable component, but at high glucose concentrations the rates became linear functions of substrate concentration. Results of a typical experiment are shown in Fig. 1. Because the intravesicular glucose concentrations, which were monitored continuously [17], were always less than 1 mM, zero-*trans* conditions were operative. The following relationship was proposed:

$$J_{in} = \frac{V_T[G]}{K_T + [G]} + k[G] \quad (1)$$

where J_{in} is the observed rate of influx at external glucose concentration [G], K_T is the half saturation constant, V_T is the maximal transport rate, and k is a constant. Values for V_T, K_T and k were calculated using a non-linear least-squares curve fitting program devised by Johnson et al. [22]. The excellent fit of the calculated curve to the experimental data is evident in Fig. 1. In a number of vesicle preparations reconstituted from erythrocyte membranes K_T was 15 (± 10) mM (average $\pm$ S.D.), which agrees well with the value of 10 mM

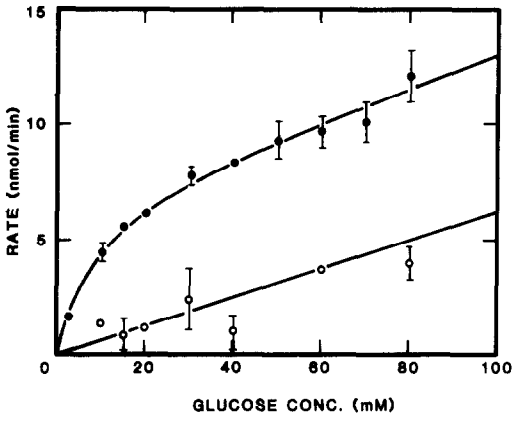

Fig. 1. Rate of glucose entry into vesicles reconstituted with unfractionated membrane extracts as a function of external glucose concentration. ●, control; ○, with 1.6 mM phloretin present. Error bars refer to standard deviation. Parameters for the control curve are: $K_T = 8.2$ (6.9–9.3) mM; $V_T = 10.4$ (10.1–10.9) nmol/min; $k = 0.086$ (0.076–0.095) μl/min. For the phloretin curve, slope = 0.064 (0.063–0.065) μl/min. Parenthetical values are 67% confidence intervals for the parameters [19].

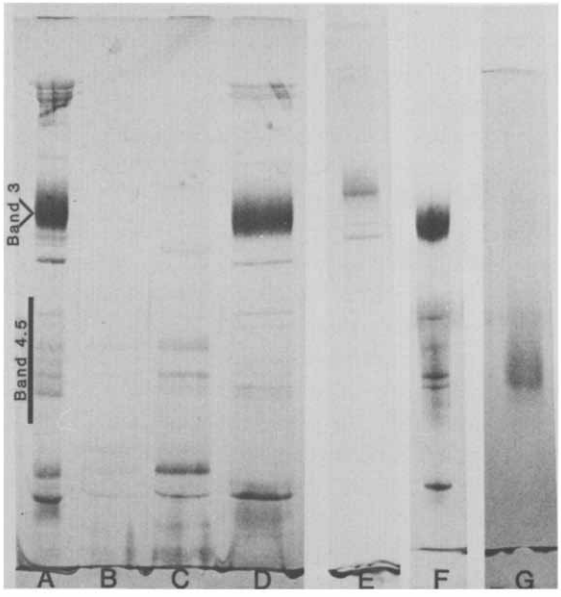

Fig. 2. SDS-polyacrylamide gels, silver stained. A, white ghosts. Aminoethyl-cellulose column fractions: B, loading effluent; C, 44 mM sodium phosphate (pH 8) wash; D, 170 mM phosphate (pH 8) eluate, containing band 3. E, reconstituted vesicles containing band 3-enriched fraction. F, white ghosts after treatment with 2 mM sodium·EDTA (pH 12.1). G, reconstituted vesicles containing band 4.5.B.

obtained for zero-*trans* influx into intact ghosts using the glucose oxidase method [17]. As observed with intact erythrocytes and resealed erythrocyte ghosts [17], glucose influx into the vesicles was inhibited by phloretin. As shown in Fig. 1, 1.6 mM phloretin abolished the saturable component; the residual rate was a linear function of glucose concentration the slope of which was very near the value calculated for k in the absence of inhibitor. It seems probable that saturable influx was mediated by the monosaccharide transporter, while the linear component was a 'leak'; vesicles prepared by detergent dialysis have been found to have low, but measurable, permeabilities to small molecules [16].

To determine whether transport activity in crude extracts resided in band 4.5.B or band 3 each was prepared essentially free of the other. Fig. 2. A–E illustrates the electrophoretic patterns of erythrocyte membranes, aminoethyl-cellulose chromatography fractions, and reconstituted vesicles from a typical preparation of band 3-enriched material. It should be noted that the diffuse component of the 4.5 region, band 4.5.B, is difficult or impossible to detect in any of these. We have consistently been unable to demonstrate significant quantities of band 4.5.B, even by ultrasensitive silver staining, in erythrocyte membranes or their fractions when erythrocytes are prepared from leukocyte-depleted blood and are treated adequately before lysis with the serine protease inhibitors diisopropylfluorophosphate and phenylmethanesulfonyl fluoride, membranes and extracts are kept cold, and extremes of pH are avoided. As expected, the chromatographic fraction containing band 3 (Fig. 2D) is contaminated with bands 4.2 and 6 as well as smaller amounts of other components, but band 4.5.B is not discernible. In reconstituted vesicles (Fig. 2E) bands 3 and 4.2 are the major protein constituents; other contaminating proteins are absent or are present in much reduced amount. In particular, band 4.5.B is not visible. On the other hand, following exposure to pH 12.1 in the procedure of Baldwin et al. [8], background density in the 4.5 region is present in much larger amounts relative to other membrane proteins (Fig. 2F). Chromatographic purification and reconstitution yielded vesicles in which the only major protein

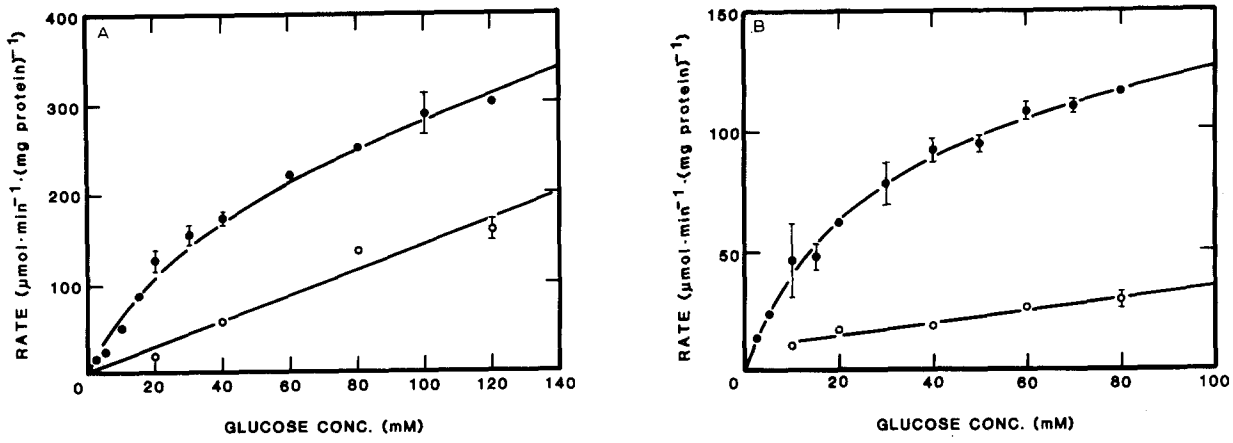

Fig. 3. Rate of glucose entry into vesicles as a function of external glucose concentration. For these graphs, observed rates were converted to specific activities. See Fig. 1. for explanation of statistics. ●, control; ○, in the presence of 1.6 mM phloretin. A, Vesicles containing band 3. For the control curve, $K_T = 33$ (24–42) mM; $V_T = 220$ (210–250) μmol·min^{-1}·mg protein^{-1}; $k = 1.14$ (1.07–1.21) ml·min^{-1}·mg protein^{-1}. For the phloretin curve, slope = 1.3 (1.1–1.6) ml·min^{-1}·mg protein^{-1}. B, Band 4.5.B vesicles. Control curve parameters are: $K_T = 22$ (17–28) mM; $V_T = 120$ (98–140) μmol·min^{-1}·mg protein^{-1}; $k = 0.28$ (0.10–0.43) ml·min^{-1}·mg protein^{-1}. For the phloretin curve, slope = 0.29 (0.26–0.33) ml·min^{-1}·mg protein^{-1}.

component was band 4.5.B (Fig. 2G).

Vesicles (Fig. 2E) reconstituted with the band 3-enriched fraction (Fig. 2D) displayed glucose influx kinetics which obeyed Eqn. 1. Data from a representative experiment are illustrated in Fig. 3A. In a series of 12 preparations the maximal specific acitivity, V_{TS}, of transport averaged 292 ($\pm$ 113) μmol·min^{-1}·mg protein^{-1}, and K_T was 28 ($\pm$ 10) mM. Saturable glucose transport was inhibited by phloretin (Fig. 3A).

Vesicles reconstituted with band 4.5.B also had phloretin inhibitable, substrate-saturable glucose transport activity as shown in Fig. 3B. The vesicles had average maximal specific activities of 120 ($\pm$20) μmol·min^{-1}·mg protein^{-1}. K_T was 22 ($\pm$ 5) mM, which is not significantly different from the half saturation constant for the band 3 preparation.

Discussion

We have efficiently reconstituted glucose transport into large unilamellar phospholipid vesicles using chromatographically purified band 3. In Table I comparative kinetic values are presented for glucose transport derived from the present experiments and from the literature. It is evident that we have achieved a 20–70-fold purification of transport activity. If it is assumed that each erythrocyte contains approx. 300 000 transporters [23] of molecular weight 100 000 [2] and 570 fg membrane protein [14], it can be calculated that measured under zero-*trans* conditions pure transporter should have a specific activity of 65–240 μmol·min^{-1}·mg protein^{-1} and a turnover number of 80 to 286 molecules of glucose/molecule of transporter per second. The turnover number of 465 observed for zero-*trans* influx into reconstituted vesicles is greater than the calculated value for either zero-*trans* exit or entry in intact cells. This may reflect uncertainties in the values, a difference in lipid environment, removal of inhibitory substances, or other unknown factors. Similar values for the turnover number have been obtained for vesicles reconstituted with band 3 purified by molecular sieve chromatography [4].

Vesicles reconstituted with purified band 4.5.B were also found to transport glucose with a 75-fold higher specific activity than reported by Wheeler and Hinkle [7] for zero-*trans* uptake and somewhat higher than the value they obtained for equilibrium exchange. However, the average maximal specific activity of our band 4.5.B preparation was approx. 40% that for vesicles containing band 3 but not band 4.5.B, and its turnover number was approx. one-quarter that of band 3.

TABLE I

SUMMARY OF HALF-SATURATION CONSTANTS, MAXIMAL VELOCITIES AND TURNOVER NUMBERS FOR THE GLUCOSE TRANSPORTER IN DIFFERENT MEMBRANES UNDER VARIOUS CONDITIONS

Values listed are from the literature and the present study. Transport in intact cells, isolated membranes and reconstituted vesicles was measured under conditions of zero-*trans* entry or exit, or equilibrium exchange. T_{TS} is the maximal transport rate/mg membrane protein. Turnover numbers for erythrocytes, ghosts and inside-out vesicles were calculated by assuming $3 \cdot 10^5$ transporters per cell [2,23].

	Ref.	Conditions	K_T (mM)	V_{TS} (μmol·min^{-1}·mg protein^{-1})	Turnover number (molecules·s^{-1}·transporter^{-1})
Erythrocytes	[24]	zt entry	1.6	4.2	80
Erythrocytes	[25]	zt exit	25	15	286
Erythrocytes	[26]	EE	34	144	2170
Erythrocyte ghosts	[17]	zt entry	10	12	240
Erythrocyte ghosts	[17]	zt exit	11	14	267
Band 4.5 vesicles	[7]	zt entry	1.2	1.6	1.5
Band 4.5 vesicles	[7]	EE	35	86	79
Band 3 vesicles		zt entry	28	292	465
Band 4.5 vesicles		zt entry	22	120	110

Reported values of the half saturation constant for glucose flux through erythrocyte membranes have varied considerably, depending upon the method of measurement and the states of the membranes; representative values are shown in Table I. It is clear that in intact cells both the maximal flux rates and the half-saturation constants are different for zero-*trans* entry and exit. A similar kinetic asymmetry has been reported for galactose transport [27]. A structural basis for kinetic asymmetry may be provided by the work of Barnett et al. [28], who have postulated that the transporter forms a channel whose inward and outward facing conformations have differing specificities and affinities for portions of the glucose molecule. However, an alternative explanation may be provided by the observation that the kinetic asymmetry observed in intact erythrocytes is not evident for zero-*trans* entry into erythrocyte ghosts or inside-out vesicles derived from them [17]; both had K_T values of about 10 mM and they had similar or identical rates of maximal transport. It is well known that band 3 proteins bind other proteins which are localized in the cytoplasm [15,29]. It seems possible that interactions with cytoplasmic proteins may alter the kinetic constants in intact cells, and that preparation of ghosts may disrupt these associations. In a reconstituted system the transporters are probably randomized with respect to any original orientation toward the cytoplasm or cell exterior. If the kinetic asymmetry were an intrinsic property of the native membrane based upon either structural asymmetry of the transporter and its asymmetric orientation in the membrane or upon interaction of the transporter with cytoplasmic proteins, a randomized orientation should yield kinetic properties intermediate between those for zero-*trans* entry and exit in the intact cell or ghost. Actually, when vesicles were reconstituted using unfractionated membrane extracts the average observed K_T was 15 mM, which is very near the mean value predicted from measurements on intact cells [24,25] or observed for ghosts [17]. On the other hand, vesicles reconstituted using either purified band 3 or band 4.5.B had half-saturation constants which were slightly greater than predicted. The reason for this is not known, although it might be related to removal of tightly-associated endogenous lipids or proteins by ion exchange chromatography or to conformational change in the transporter during purification and reconstitution.

Because both bands 3 and 4.5.B catalyze glucose transport when incorporated into phospholipid vesicles, the question arises whether both are of importance in carrying out glucose transport in

the native erythrocyte membrane. Following the initial report that about 300 000 molecules of cytochalasin B/cell were tightly bound to the erythrocyte membrane [30,33] and the suggestion that this number closely approximated the number of transporters, several groups have utilized cytochalasin B binding [31,32] or photoincorporation of this substance into membrane proteins [10–12] as means to purify or identify the glucose transporter of erythrocytes and other cells. These groups have all described a cytochalasin-binding protein which migrates on electrophoresis as band 4.5.B, and have concluded that this is the glucose transporter. Several pieces of evidence render this conclusion uncertain. Although it has been conclusively demonstrated that glucose and cytochalasin B compete for binding [31,32], it has been reported that cytochalasin B behaves as a noncompetitive inhibitor of glucose transport [33,34]. Furthermore, it has been shown rather convincingly by Krupka and coworkers that the substance must bind to a site remote from that for the transported substrate [35,36]. In addition, the number of cytochalasin molecules incorporated per erythrocyte by ultraviolet illumination [10,11] is orders of magnitude less than the number of transporters estimated by other methods [2,23], and the photolabelling has not been shown to affect transport kinetics. By contrast, the affinity label maltosyl isothiocyanate was found to inhibit glucose transport completely when 300 000 molecules per erythrocyte had been incorporated into a 100 000 M_r membrane protein [2]. We have observed that band 4.5.B is present in such trivial amounts in native membranes that it is difficult or impossible to detect by either electrophoresis or chromatography. Although the band 4.5 region has been reported to contain about 10% of the total membrane protein [23], most of this is resolved into a number of small discrete bands in Laemmli type gels (Fig. 2A). As pointed out by Jones and Nickson [23], it is doubtful that any of these alone contains sufficient protein to account for the number of transporters present in the erythrocyte membrane or for the number of cytochalasin molecules bound to the intact cell. Upon chromatography the small discrete bands elute in a fraction different from band 4.5.B (Fig. 2), and this fraction does not catalyze transport. Moreover, in octyl glucoside extracts from membranes which have been protected from degradation the chromatographic fraction which should contain band 4.5.B contains neither a significant quantity of stainable protein (Fig. 2B) nor transport activity. However, in membranes not protected from proteolysis or which have been exposed to pH 12.1 band 4.5.B is abundantly present (Fig. 2E), can be purified chromatographically and has glucose transport activity. Taken together, the results indicate that there is insufficient band 4.5.B in native erythrocyte membranes to account for transport or cytochalasin B binding; its presumed presence in native membranes and its role in glucose transport have probably resulted from poor resolution of region 4.5 proteins in Fairbanks [14] gels together with artefactual generation of this material during manipulation of membranes or membrane extracts. It seems clear that results obtained by binding or photolabelling with radioactive cytochalasin B may require reinterpretation.

A proteolytic origin of band 4.5.B from band 3 was suggested by Phutrakul and Jones [37], and conversion of band 3 to band 4.5.B was subsequently demonstrated directly [38]. Results consistent with this hypothesis were obtained by Acevedo et al. [4], who isolated by gel filtration a protein with band 4.5 mobility and glucose transport activity; this component was not present when the experiment was repeated under conditions which favored higher stability of the band 3 transporter. Using band 3 partially purified by affinity chromatography and reconstituted into phospholipid vesicles, Froman et al. [5] found a slow reduction of transport activity with time was associated with a decrease in the ratio of band 3 to band 4.5.B. Direct evidence that a band 3 component with glucose transport activity can be converted by proteolysis to a band 4.5 protein was reported by Mullins and Langdon [2]. Band 3 covalently labelled with [^{14}C]maltosyl isothiocyanate was converted to labelled material with band 4.5 mobility when cells were digested externally with chymotrypsin or were allowed to incubate at room temperature without added protease. Both could be prevented by the addition of a serine protease inhibitor. There are proteases endogenous to the erythrocyte membrane [39–41] which are presumed to be responsible for degradation of the membrane proteins. It may be signifi-

cant that preparation of band 4.5.B [8] utilizes an alkaline extraction step, and that some of the proteases are more active at elevated pH [40].

Although the transport activity observed in vesicles reconstituted with purified band 3 might be attributed to one of the minor protein components present in the preparation, this seems unlikely because there is no evidence implicating them in the transport process. However, there is strong evidence implicating a 100000 M_r component of band 3 [2]. The results of reconstitution experiments presented here and by others [4,5], together with the results of affinity labelling [2], strongly support the hypothesis that the native glucose transporter of the human erythrocyte membrane is a component of band 3, and that band 4.5.B contains a partially active fragment as previously proposed [2,4,37].

Band 3 appears to contain the glucose transporter, the anion transporter [42], and the ($Na^+ + K^+$)-ATPase [43], but all band 3 constituents seem to have certain common structural features [44]. It seems possible that band 3 constituents represent a family of transport proteins which have evolved to have differing substrate specificities but have retained structural homologies in a manner analogous to the serine proteases [45] and other protein families.

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