RECONSTITUTION OF D-GLUCOSE TRANSPORT IN VESICLES COMPOSED OF LIPIDS AND A PARTIALLY PURIFIED PROTEIN FROM THE HUMAN ERYTHROCYTE MEMBRANE*

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SUMMARY: The stereospecific efflux of D-glucose from liposomes formed upon sonication of erythrocyte lipid with various membrane fractions was measured in order to assess their D-glucose transport activity. Extrinsic and intrinsic membrane proteins were separated into soluble and membrane-bound fractions, respectively by extraction of ghosts with 2,3-dimethylmaleic anhydride. Reconstitution of D-glucose transport was catalyzed only by the intrinsic membrane proteins. Upon subsequent Triton X-100 extraction of these proteins, reconstitution of D-glucose transport was associated with both the extract and membrane residue separated by high-speed centrifugation. The membrane residue did not contain any periodic acid-Schiff-sensitive glycoprotein and consisted chiefly of a protein component of 95,000 molecular weight (Band 3), a portion of which was present in the Triton X-100 extract. These results support the proposal that Band 3 proteins are directly involved in D-glucose transport.

It is now generally accepted that the transport of D-glucose across the human erythrocyte membrane is mediated by a membrane protein (1). Recent attempts to identify this D-glucose transport protein (2, 3) suggest that it may be associated with a transmembrane protein of apparent molecular weight of 95,000 (commonly referred to as Band 3 from its position on SDS polyacrylamide gels (4)). The evidence is based on several considerations including the demonstration that an erythrocyte membrane preparation highly enriched with respect to Band 3 proteins retains the high-affinity binding sites for cytochalasin B, an inhibitor of D-glucose transport (2) and exhibits stereospecific D-glucose uptake activity (3). However, to further identify Band 3 protein as being associated with D-glucose transport, attempts were made to incorporate

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Abbreviations: SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff; DMMA, 2,3-dimethylmaleic anhydride.

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various preparations containing this protein into the lipid bilayer of liposomes. The effects of such reconstitution on the stereospecific transport of D-glucose across this otherwise impermeable artificial membrane barrier (5) were then determined.

MATERIALS AND METHODS: D-[2-^{3}H]-Glucose and L-[1-^{14}C]-glucose were obtained from New England Nuclear; sodium dodecyl sulfate from Pierce; polyacrylamide gel electrophoresis reagents and Bio-Gel P4 from Bio-Rad; inorganic salts and organic solvents from Fisher or Baker, and all other reagents from Sigma. Diaflo PM 10 ultrafiltration membranes were obtained from Amicon.

Hypotonic phosphate buffer contained 3.1 mM NaN_3 and 4.8 mM Na_2HPO_4 and was adjusted to pH 7.4. Liposome buffer contained 20 mM $MgCl_2$, 0.03 mM $CaCl_2$, 5 mM Na_2HPO_4, 3 mM NaN_3, 115.0 mM NaCl and was adjusted to pH 7.4. A stock solution of 5 mM D-[2-^{3}H]glucose (specific activity 160 μCi/mmole) and 5 mM L-[1-^{14}C]-glucose (specific activity 100 μCi/mmole) was prepared in this liposome buffer.

Hemoglobin-free erythrocyte ghosts were prepared from recently outdated transfusion blood by hypotonic hemolysis (6) and packed to contain 3-5 mg protein/ml. Selective extraction of extrinsic membrane proteins with 2,3-dimethylmaleic anhydride (DMMA) was performed as described by Steck and Yu (7), using 6-8 mg DMMA/ml packed ghosts. Following centrifugation at 30,000 x g for 30 min, the supernate was concentrated five-fold by ultrafiltration (DMMA extract) and the pellet was washed twice with 10 volumes of distilled water, once with 10 volumes of hypotonic phosphate buffer and was suspended in 0.6 volumes of this buffer (DMMA pellet). For the Triton X-100 extraction, 4 volumes of cold 0.125% Triton X-100 dissolved in hypotonic phosphate buffer were added to 1 volume of the DMMA pellet, and after mixing and incubating in ice for 10-20 min, the suspension was centrifuged at 10^5 x g for 1 hour. The supernate was immediately concentrated five-fold by ultrafiltration (Triton extract) and the pellet was washed once with a 25-fold volume of hypotonic phosphate buffer and suspended in 1 volume of this buffer (Triton pellet).

Polyacrylamide gel electrophoresis in the presence of 0.2% sodium dodecyl sulfate was performed as previously described (3).

Total red blood cell lipids for forming liposomes were prepared by the method of Rose and Oklander (8); all steps were performed under a nitrogen atmosphere. After flash evaporation of the solvent at 35°C, the lipids were redissolved in chloroform:isopropanol (7:11) and α-tocopherol was added as an anti-oxidant to a final concentration of 2% (w/w) of the total lipid.

Liposomes (5, 9) were prepared using the total red cell lipid extract. Aliquots of lipid solution containing 15 mg of total lipid were evaporated to dryness on the bottom of 22 x 140 mm Quickfit tubes. From 0.1 to 1.0 ml of ghosts or one of the above membrane fractions (commonly 0.2-0.4 ml) was added, followed by 1 ml of the stock solution of D-[2-^{3}H]-glucose and L-[1-^{14}C]-glucose and sufficient hypotonic buffer to bring the final volume to 2.0 ml. Two glass beads were then introduced and the tubes were flushed with nitrogen and sealed with parafilm. The lipids were suspended by vigorous shaking on a vortex mixer for 30 sec and the tubes were then transferred to a Bransonic 220 ultrasonic bath and sonicated at 35°C for 12-15 min. Tubes were automatically revolved in the bath to ensure uniform exposure to sonic irradiation.

Reconstitution of stereospecific D-glucose transport was measured by determining the difference in retention of D- and L-glucose in the intraliposomal volume after gel filtration of the liposome suspension. One ml of sonicated liposome suspension was placed at the top of a 1 x 13 cm column of Bio-Gel P4 equilibrated with liposome buffer and was eluted with this buffer at a flow rate of 1-2 ml/min; the passage time was normally 2-4 min. During passage, extraliposomal D- and L-glucose are separated from the liposomes, forming a concentration gradient, and resulting in the rapid loss of D-glucose from those

liposomes with functional carrier activity; this D-glucose is also separated from the liposomes during further gel filtration. Intraliposomal L-glucose is almost completely retained (10-12, and personal observations) and serves as a measure of intraliposomal volume and as a control for nonspecific loss. Twenty drops of turbid suspension were collected at the void volume; duplicate 0.1 and 0.4 ml samples of this suspension were assayed for ^{3}H and ^{14}C as previously described (3, 13), and for protein respectively. A 0.1 ml sample of the original liposome suspension was also assayed for ^{3}H and ^{14}C. Protein was determined by the method of Lowry et al. (14) modified so as to dissolve the liposomes and prevent interference by Triton X-100 (15). The lipids were found to interfere with the assay; however, a reagent blank consisting of a suspension of lipid sonicated as above in the absence of protein and passed through the Bio-Gel P4 column was used to correct for this interference.

From the values for the disintegrations per minute of ^{3}H and ^{14}C in the suspension before and after gel filtration, the nmoles of D-glucose lost (relative to L-glucose) from the liposomes per mg of membrane protein can be calculated:

$$\left[\frac{\text{dpm } ^{14}\text{C after}}{\text{dpm } ^{14}\text{C before}} - \frac{\text{dpm } ^{3}\text{H after}}{\text{dpm } ^{3}\text{H before}}\right] \times \frac{2500 \text{ nmoles D-glucose ml}^{-1}}{[\text{protein}] \text{ (mg ml}^{-1})}$$

The resultant value is defined as the D-glucose transport specific activity.

It should be noted that these data are obtained for an initial intraliposomal concentration of D- and L-glucose of 2.5 mM each, and that the use of other concentrations will give a transport activity proportional to those concentrations up to the point where carrier-bearing liposomes fail to lose all their D-glucose under the conditions of this method.

RESULTS AND DISCUSSION: D-Glucose was stereospecifically lost from liposomes formed by sonication of a suspension of red cell lipids with ghosts (Table I). In the absence of added ghost protein, sonication of the lipid suspension produced liposomes devoid of stereospecific transport activity. Ghosts sonicated in the absence of any additional lipids retained neither D- nor L-glucose within the limits of detection and consequently probably did not form sealed vesicles (data not shown).

Two serial passages of the reconstituted liposome suspensions through the Bio-Gel P4 columns at an interval of 1 hour did not result in any further loss of D- relative to L-glucose. In 25 trials, the ratio of L- to D-glucose after the second passage was not significantly different from that after the first (paired t-test : $P > 0.5$). It is likely, therefore, that during reconstitution two basic classes of liposomes are being produced: those whose membrane contains one or more functional carrier molecules, and those lacking a functional carrier The former class loses all D-glucose during the first column passage of 2-4 min,

TABLE I. D-Glucose transport activity of red cell lipid vesicles sonicated in the presence of various red cell membrane fractions.

Membrane Fraction*	D-Glucose Transport Specific Activity ** (nmoles/mg membrane protein)	R***
No additions (40)	-	1.017 ± 0.004
Whole ghosts (19)	65.7 ± 6.4	1.918 ± 0.143
DMMA extract (22)	6.0 ± 1.7	1.025 ± 0.007
DMMA pellet (22)	75.1 ± 5.9	2.212 ± 0.243
Triton extract (16)	26.5 ± 3.4	1.318 ± 0.056
Triton pellet (16)	174.3 ± 20.5	2.322 ± 0.255

*Membrane fractions were prepared and incorporated into liposomes which were assayed for D-glucose transport activity as described in METHODS. The protein composition of each fraction is shown in Figure 1.

**Transport specific activity refers to the nmoles of D-glucose stereospecifically lost from the liposomes per mg of membrane protein associated with the liposomes following gel filtration.

***R is the ratio of the intraliposomal concentration of L- to D-glucose following gel filtration.

Values are the mean ± S.E. of the results from the number of experiments shown in parentheses.

while completely retaining L-glucose; the latter type retains both D- and L-glucose. Since L-glucose is impermeant during the time of these experiments, the entrapment of L-glucose is proportional to the intraliposomal volume, which was normally between 0.4 and 1.0% of the volume of the sonicated suspension after dilution during column chromatography.

The polypeptide and glycoprotein composition of ghosts and the various membrane fractions assayed for their ability to reconstitute D-glucose transport is shown in Figure 1. As indicated previously (2, 3), any one of the nine major protein components of the erythrocyte membrane visualized on SDS-polyacrylamide gels is present in sufficient quantity to account for the estimated 2 to

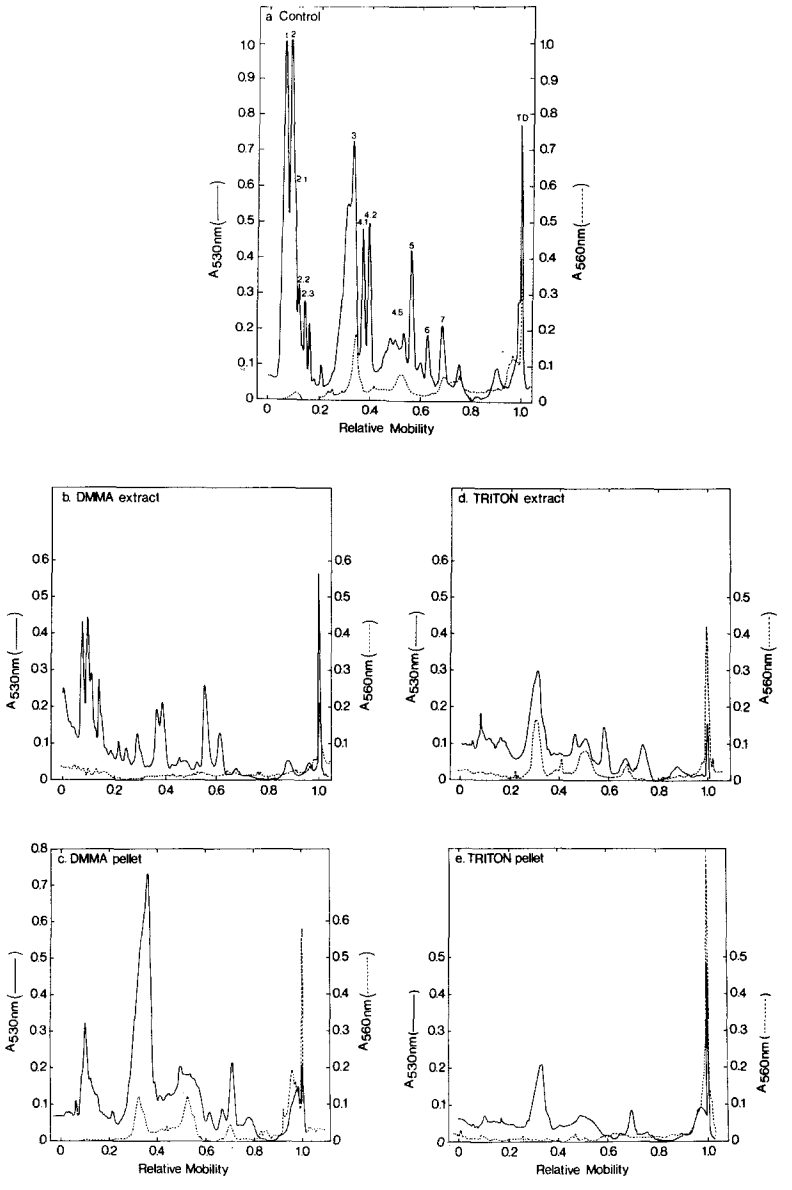

FIGURE 1. Protein composition of erythrocyte membrane fractions incorporated into liposomes.

Ghosts were extracted with DMMA and the resultant membrane residue (DMMA pellet) was extracted with Triton X-100 (see METHODS for details). Samples from the designated membrane fractions were analyzed electrophoretically on 5% polyacrylamide gels in 0.2% SDS. Solid lines, scans of Coomassie blue-stained gels at 530 nm; dashed lines, scans of periodic acid-Schiff-stained gels at 560 nm. The major Coomassie blue-stained bands were enumerated according to increasing electrophoretic mobility as described by Steck and Yu (7). TD, inked needle stab recording the position of the tracking dye.

3×10^5 D-glucose transport proteins per cell (13, 16, 17). Extraction of ghosts with DMMA resulted in the selective solubilization of the membrane proteins contained in Bands 1, 2, 2.1, 2.2, 4.1, 4.2, 5 and most of Band 6. The corresponding membrane pellet retained all of the proteins of Band 3, Zone 4.5, Band 7, and some of Band 6, as well as the full complement of the PAS-sensitive glycoprotein components of the original ghost preparation. When these membrane fractions were incorporated into liposomes, the DMMA pellet reconstituted D-glucose transport activity at a specific activity comparable to that of the original ghost preparation, while the DMMA extract was devoid of transport activity (Table I; the ratio of L- to D-glucose of liposomes plus DMMA extract was not significantly different from that of protein-free liposomes). Thus the involvement of the major extrinsic membrane proteins contained in the DMMA extract in D-glucose transport can be excluded. These results are in agreement with previous studies demonstrating the retention of the high-affinity binding sites for cytochalasin B, an inhibitor of D-glucose transport (2), and stereospecific D-glucose uptake (3) by DMMA-extracted membranes.

While this work was in progress, Kasahara and Hinkle (18) reported reconstitution of D-glucose transport by a Triton X-100 extract of ghosts incorporated into sonicated soybean phospholipid liposomes. The protein composition of this extract consisted of Bands 3, 4.1, 4.2 and the entire PAS-sensitive glycoprotein content of ghosts. In addition, some of the proteins of Bands 1, 2, 7 and Zone 4.5 were present. These results are confirmed and extended in the present study. Upon extraction of the DMMA pellet with 0.1% Triton X-100, the proteins of Bands 3 and 7 and Zone 4.5 were partially solubilized, while Bands 5, 6, and the PAS-sensitive glycoproteins were completely solubilized. The pellet after extraction contained only Band 3, Zone 4.5 and Band 7 proteins, and no PAS-sensitive material (Fig. 1). When incorporated into liposomes, both the Triton pellet and extract were capable of reconstituting stereospecific transport of D-glucose (Table I). However, the transport specific activity of liposomes reconstituted with the Triton extract was only one-third of the act-

ivity recorded for the DMMA pellet, suggesting either poor solubilization or destruction of carrier upon solubilization. By contrast, the liposomes reconstituted with the Triton pellet displayed a 2.3-fold increase in transport specific activity (Table I).

The Triton X-100 extraction experiments show that of the nine major protein components of the erythrocyte membrane, only Band 3 and Band 7 could contain D-glucose transport proteins. However, as indicated previously (2, 3), Band 7 is unlikely to contain these transport proteins. Band 7 proteins are known to be exposed entirely on the cytoplasmic surface of the erythrocyte membrane (4), whereas the transport of D-glucose is inhibited by nonpenetrating sulfhydryl reagents such as p-chloromercuribenzene sulfonic acid (19), indicating that a protein exposed on the extracellular surface, such as Band 3 (4), is part of the transport mechanism.

In addition to exhibiting substrate stereospecificity, the reconstituted D-glucose transport activity was inhibited by various inhibitors of the erythrocyte monosaccharide transport system (20). Pretreatment of ghosts with 2 mM 1-fluoro-2,4-dinitrobenzene or 5 mM N-ethylmaleimide for 2 hr at 37° inhibited the reconstituted transport activity by 60% and 37%, respectively (data not shown). A similar treatment of ghosts with 5 mM iodoacetate was without effect. Furthermore, the presence of 1 mM p-chloromercuribenzene sulfonic acid or 1 mM $HgCl_2$ during the formation and subsequent gel filtration of reconsituted liposomes completely abolished transport (data not shown). These observations are consistent with the idea that the reconstituted D-glucose transport activity is associated with glucose transport in erythrocytes.

Numerical values of transport activity of different fractions are subject to the uncertainty that the efficiency of incorporation of functional carriers into liposome membranes is unknown and may vary for each fraction. Hence, comparison of transport specific activity between different fractions must be made with caution.

A fundamental step in defining the molecular mechanism of mediated transport

of D-glucose across membranes is the isolation and characterization of the purified transport protein. Until recently, the attainment of this aim has been hampered by the necessity of using red cells, ghosts or a particulate fraction derived from ghosts to assay for carrier function (3, 13, 21-23). With the exception of the present work and that of Kasahara and Hinkle (18), attempts to reconstitute stereospecific D-glucose transport using red cell lipids (24, 25) or membrane protein fractions in a planar bilayer (26) have not been successful The present development of methods of reconstitution of D-glucose transport activity from a soluble fraction should greatly facilitate purification of the D-glucose transport protein.

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