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THE MONOSACCHARIDE TRANSPORTER FROM HUMAN ERYTHROCYTES IS HETEROGENEOUSLY GLYCOSYLATED

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SUMMARY. Labeling of intact erythrocytes with galactose oxidase/NaB[^{3}H]$_4$ resulted in the incorporation of radioactivity into the monosaccharide transporter. When the purified labeled protein was subjected to SDS gel electrophoresis, the peak of radioactivity migrated more slowly than the peak of Coomassie Blue-staining material. Endo-β-galactosidase treatment of the purified labeled transporter led to partial loss of the label, sharpening of the stain profile, and a change in the apparent molecular mass of the polypeptide from 55,000 to 46,000 daltons. Approximately 50% of the transporter bound to a column of *Ricinus communis* agglutinin I-agarose. These findings demonstrate that the transporter is heterogeneously glycosylated and, in conjunction with other data, show that it is a transmembrane protein and probably a source of erythroglycan.

INTRODUCTION

The purification of the monosaccharide transporter (or a component thereof) from human erythrocytes has been described by others (1,2) and ourselves (3). The purified transporter runs as a very broad band upon SDS gel electrophoresis. It is unlikely that this broadness is due to impurities, since differential radiolabeling gives the same pattern (4,5). Because the purified transporter contains approximately 15% carbohydrate by weight (6), heterogeneity in the carbohydrate structure could account for this electrophoretic behavior. Herein, we report that such is the case. In addition, our results establish a transmembrane structure for the transporter and show that it is probably a source of erythroglycan, the recently discovered mixture of large glycopeptides that is obtained by protease digestion of erythrocyte membranes and that carries ABH determinants (7,8).

MATERIALS AND METHODS

Sodium [^{3}H]borohydride was obtained from Amersham. *Dactylium dendroides* galactose oxidase (EC 1.1.3.9), purchased from Worthington, was heated at 50°

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for 30 min to destroy protease activity (9). Purified, protease-free endo-β-galactosidase from *Escherichia freundii* (10) was a gift from Dr. M. Fukuda. *R. communis* agglutinin I (4 mg/ml) linked to agarose was a product of Vector Laboratories.

Erythrocytes were labeled with galactose oxidase and NaB[^{3}H]$_4$ by a modification of published methods (9,11). Cells (35 ml), from outdated units, were washed with 80 mM NaCl/50 mM NaPi (pH 8.0), suspended to 50% hematocrit, and treated with 4 units/ml of galactose oxidase for 2 hr at 22°. In a control mixture, the enzyme was omitted. The cells were then washed with the buffer, adjusted to pH 8.0, and reacted at 90% hematocrit with NaB[^{3}H]$_4$ (100 µl of 43 mM (0.9 Ci/mmole) in 10 mM NaOH). After 30 minutes the cells were lysed by adding them to 10 volumes of water, and ghosts were prepared by the method of Steck and Kant (12). Transporter was then purified from the ghosts and reconstituted with soybean phospholipids, as described previously (3), with the exception that the lipids were added at 2 mg/ml with 0.5% Triton X-100 rather than as a sonicated dispersion at 5 mg/ml.

More recently we have substituted the nonionic detergent Nikkol (octaethylene glycol dodecyl ether, supplied by Nikko Chemicals Ltd., Tokyo) for Triton X-100 in our purification procedure. The experiments with the *R. communis* lectin were carried out with transporter isolated in this detergent. Transporter at 60 µg/ml, purified in 0.5% Nikkol/50 mM TrisCl (pH 7.4) without dithiothreitol, was taken directly from the DEAE-cellulose column (see reference 3) and made 100 mM in NaCl; 3.5 ml of this material was applied to a column of *R. communis* agglutinin I-agarose (0.5 x 6 cm) equilibrated at 4° with 0.5% Nikkol/2.5 mM dioleoyl phosphatidylcholine/100 mM NaCl/50 mM TrisCl (pH 7.4). The column was eluted with 5 ml of this buffer and then with buffer containing 100 mM D-galactose. The fractions (1 ml) were made 2.5 mM in dithiothreitol, 2 mM in $MgCl_2$, and 1 mM in EDTA; and those fractions that lacked dioleoyl phosphatidylcholine were made 2.5 mM in it (added with 0.5% Nikkol). The transporter was reconstituted by the removal of the Nikkol with 400 mg/ml of Bio•Beads SM2 (3). The reconstituted fractions were dialyzed against 100 mM NaCl/1 mM EDTA/20 mM NaPi (pH 6.0).

Electrophoresis in 10% acrylamide gels was performed according to Laemmli (13). Samples were prepared with 3% SDS/1 mM EDTA/5 mM dithiothreitol/50 mM TrisCl (pH 6.8), held at 100° for 3 min, alkylated with 15 mM N-ethylmaleimide, and made 8% in sucrose. The methods for staining of the gels and protein assay were as described elsewhere (3,14).

RESULTS

Upon SDS gel electrophoresis the purified glucose transporter runs as a broad band in the 55,000 molecular weight region (1,3) (Fig. 1). In addition, there are small amounts of band 7 polypeptide and of higher molecular weight species, which arise from aggregation of the monomer (6, unpublished results). When erythrocytes were labeled externally with galactose oxidase/NaB[^{3}H]$_4$, the purified transporter was found to have incorporated radioactivity. Analysis by SDS gel electrophoresis revealed that the major peak of radioactivity (R_f, 0.32) migrated slightly behind the major peak of protein, as stained by Coomassie Blue (R_f, 0.39) (Fig. 1).

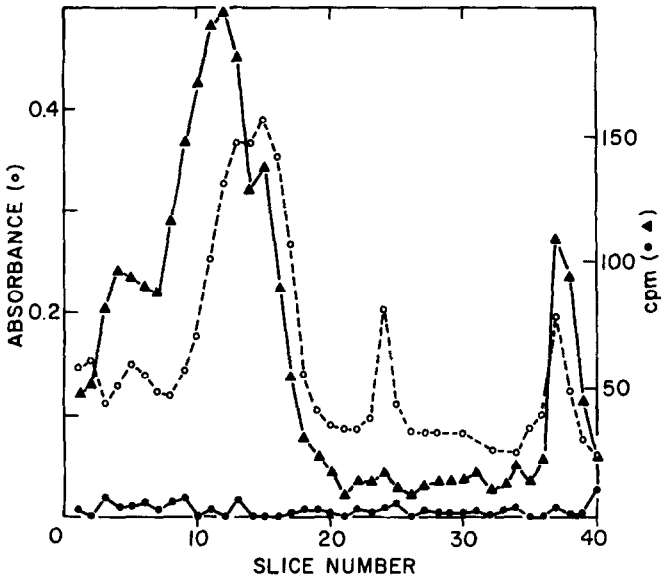

Figure 1. SDS gel electrophoresis of transporter purified from erythrocytes labeled externally with galactose oxidase/NaB[^{3}H]$_4$. The gels, stained with Coomassie Blue, were sliced into 2 mm sections, and the absorbance of each slice at 550 nm was measured in a semimicro cuvette. The slices were then digested with hydrogen peroxide and the radioactivity determined (▲, cpm from cells treated with oxidase; ●, cpm from the control without oxidase). The absorbance profile (○) was the same for both gels. 7 and TD indicate the positions of band 7 and the tracking dye.

Treatment of the purified labeled transporter with endo-β-galactosidase led to a sharpening of the major peak of Coomassie Blue stain and a shift to a higher electrophoretic mobility, corresponding to an apparent molecular weight of 46,000 (Fig. 2A). At the same time approximately 70% of the tritium label was lost (Fig. 2B). Digestion of the transporter with twice the amount of enzyme used for these experiments sharpened the peak of Coomassie Blue stain only slightly more. Thus, the results shown here are those for almost limit action of the endo-β-galactosidase on the polysaccharide of the transporter.

The purified, reconstituted transporter binds the compound cytochalasin B with high affinity (3,6). The effect of endo-β-galactosidase digestion on this function was examined by reconstituting the transporter (3) in the presence of the enzyme. SDS gel electrophoresis showed that digestion of the

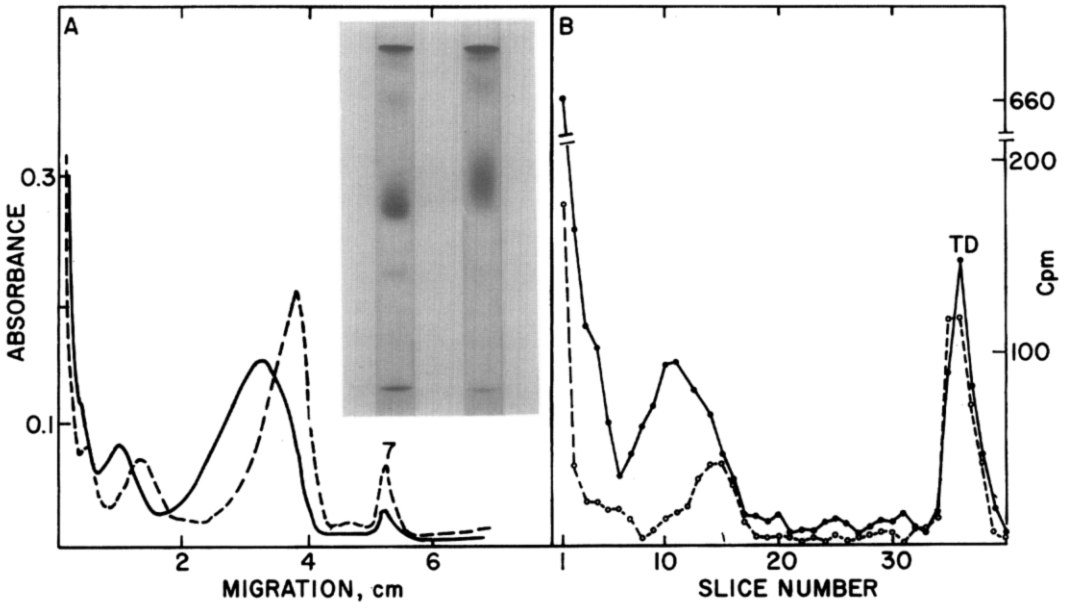

Figure 2. SDS gel electrophoresis of labeled transporter treated with endo-β-galactosidase. Labeled transporter, at 70 μg/ml with 3 mg/ml phospholipid, in 1 mM dithiothreitol/0.1 mM EDTA/100 mM sodium acetate (pH 6.0) was solubilized by the addition of 0.6% Nikkol, digested with 168 milliunits/ml of enzyme (10) at 37° for 30 min, and then prepared for electrophoresis. The control received no enzyme. Panel A shows the Coomassie Blue staining of treated transporter (---, left of insert) and untreated transporter (——, right of insert). Panel B shows the radioactivity of treated (●) and untreated (○) transporter. The greater amount of aggregation seen here (*vs* Fig. 1) arises from the conditions of digestion. Endo-β-galactosidase runs with almost the same mobility as band 7.

polysaccharide had occurred; the cytochalasin B binding activity (14), however, was the same as that of the corresponding control.

Chromatography of solubilized, purified transporter on a column of *R. communis* agglutinin I-agarose, which is specific for galactose residues (15), resulted in the retention of about one half of both the recovered cytochalasin B binding activity and the protein (Fig. 3). The total binding activity and protein that were recovered amounted to 50 and 100% of the applied amounts, respectively. The loss of binding activity can be explained by a time-dependent inactivation that occurs in the solubilized state (unpublished results). The heterogeneous behavior upon chromatography is probably not due to an overloading of the column, since when twice as much transporter protein was applied, about 1.6 times as much was retained. Upon SDS gel electrophoresis

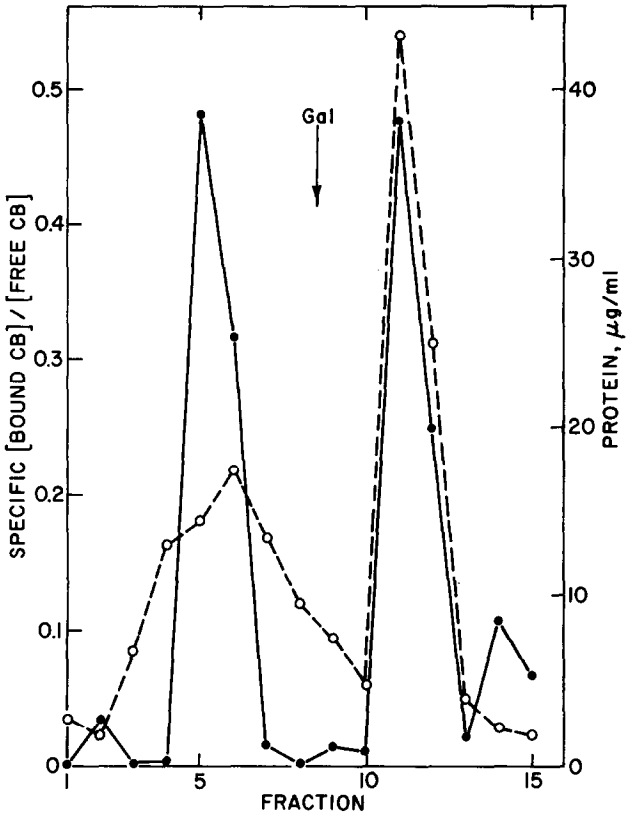

Figure 3. Chromatography of purified transporter on *R. communis* agglutinin I-agarose. See Materials and Methods. After dialysis, the fractions were assayed for cytochalasin B (CB) binding activity (14) expressed as specific [Bound CB]/[Free CB] (●), and for protein (O).

both the retained and the unretained fractions of the transporter showed a rather broad band of Coomassie Blue staining material, similar to that of the unfractionated transporter; however, the average mobility of the unretained fraction was slightly greater than that of the retained fraction.

DISCUSSION

The evidence for the conclusion that the monosaccharide transporter is heterogeneously glycosylated is of three types. First, the profile of radiolabel introduced with galactose oxidase/NaB[^{3}H]$_4$ is not coincident with that of Coomassie Blue stain (Fig. 1). Second, hydrolysis of a portion of the protein-linked carbohydrate with endo-β-galactosidase sharpens the peak

of protein stain (Fig. 2). Third, only half of the transporter is absorbed by the *R. communis* lectin (Fig. 3).

The finding that tritium is incorporated into the transporter purified from cells labeled externally shows that the protein is exposed at the outer surface. Trypsin has been found to cleave and inactivate the transporter only at the cytoplasmic surface of the cell (16, manuscript submitted for publication). Thus, the transmembrane disposition of the polypeptide is established.

The carbohydrate associated with the transporter has the same properties as those recently described for erythroglycan (see Introduction). Both are susceptible to hydrolysis by the endo-β-galactosidase (8, data herein) and both contain large fractions of galactose and N-acetylglucosamine (6-8). Thus it seems likely that a portion of erythroglycan arises from proteolysis of the transporter.

Very recently it has been reported that galactose oxidase/NaB$[^3H]_4$ treatment of erythrocytes labels, among other polypeptides, one or more polypeptides that migrate in the region of the transporter upon SDS gel electrophoresis, and that the endo-β-galactosidase releases this label (17,18). Our results with purified transporter establish that it is one of the substrates.

Interestingly, the anion transporter of the human erythrocyte, band 3, shows very similar properties to those described here (17-20). It may be that all transport systems of the human erythrocyte are heterogeneously glycosylated with the same type of carbohydrate structures.

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