External Labeling of Human Erythrocyte Glycoproteins

STUDIES WITH GALACTOSE OXIDASE AND FLUOROGRAPHY*

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Glycoproteins of the human erythrocyte membrane were labeled with tritiated sodium borohydride after oxidation of terminal galactosyl and N-acetylgalactosaminyl residues with galactose oxidase. After separation of the polypeptides on polyacrylamide slab gels, a scintillator was introduced into the gel, and the radioactive proteins were visualized by autoradiography (fluorography). The following results were obtained. (a) The erythrocyte membrane contains at least 20 glycoproteins, many of which are minor components. (b) The carbohydrate of all the labeled glycoproteins is exposed only to the outside, since no additional glycoproteins can be labeled in isolated unsealed ghosts. (c) The membrane contains two major groups of glycoproteins. The first group of proteins contains sialic acids linked to the penultimate galactosyl/N-acetylgalactosaminyl residues, which are efficiently labeled only after pretreatment with neuraminidase. The second group has terminal galactosyl/N-acetylgalactosaminyl residues which can be easily labeled without neuraminidase treatment. The glycoproteins from fetal erythrocytes all belong to the first group, whereas only five glycoproteins of erythrocytes from adults belong. (d) Trypsin cleaves the proteins containing sialic acids, and fragments containing carbohydrate remain tightly bound and exposed in the membrane. (e) Pronase cleaves Band 3 in addition to the sialic acid containing glycoproteins, but most of the glycoproteins still remain unmodified in the membrane. (f) No difference is seen between membrane glycoproteins from cells of different ABH blood groups.

There is increasing evidence that cell surface glycoproteins are involved in a variety of surface-mediated processes such as intercellular adhesion (1), cell recognition (2), lectin agglutinability (3-8), and ion transport (9-11). However, the molecules involved and their detailed structures are still largely unknown. This is partially due to the difficulties encountered in purifying plasma membranes from nucleated cells. By contrast, the membranes of the human erythrocyte are easy to obtain in large quantities, and many generalizations about membrane structure and function are derived from results obtained using this membrane.

One important aspect of membrane structure is the location and properties of the glycoproteins in the membranes. At least 4 major glycoproteins seem to span the membrane (12-18), the sialoglycoproteins, PAS1, PAS2, and PAS3, and Band 3 (see Table II). The major sialoglycoprotein has been the most extensively studied, and it is known that the sugar-containing portion is located externally (19), the intramembranous part is hydrophobic in nature (14, 17, 20), and the COOH-terminal cytoplasmic end interacts with peripheral membrane proteins on the inside of the membrane (21, 22).

To locate external proteins, a number of techniques have recently been developed (23-28). In most of these techniques, the reagents are specific for the protein part of glycoproteins. Because most, if not all, surface proteins are glycoproteins, a technique to label this part of these molecules specifically was developed (29, 30). Terminal galactosyl or N-acetylgalactosaminyl residues are oxidized to the corresponding C-6 aldehydes by the enzyme galactose oxidase (31). The resulting aldehydes are then reduced under physiological conditions with tritiated borohydride. The large size of the enzyme molecule inhibits its penetration into the cell. Because sialic acids often are linked to subterminal galactosyl residues, more efficient labeling can often be achieved after pretreatment of cells with neuraminidase (29).

MATERIALS AND METHODS

Cells—Human erythrocytes from adults, obtained from citrated blood by centrifugation, were washed in phosphate-buffered saline, pH 7.4. The cells were used within 3 days of blood donation. Care was taken to remove theuffy coats. ABH blood groups were determined by standard techniques. Umbilical cord blood erythrocytes and fetal erythrocytes were obtained from the Department of Obstetrics and Gyneceology, University of Helsinki.

Enzymes—Galactose oxidase with a specific activity of 130 units/mg of protein was purchased from Kabi AB, Stockholm, Sweden. It contained no neuraminidase or protease activities when measured as described previously (29). Neuraminidase (Vibrio cholerae, 500 units/ml) was obtained from Behringwerke AG, Marburg-Lahn, Germany.
Pronase (Streptomyces griseus protease type V, 0.9 unit/mg solid) was obtained from Sigma. Crystalline trypsin was bought from Merck AG, Darmstadt, Germany.

Chemicals—Tritiated sodium borohydride (8.2 Ci/mM) was obtained from the Radiochemical Centre Ltd., Amersham, England. Two hundred fifty millilitres were dissolved in 0.5 ml of 0.01 N NaOH, divided into five tubes, and immediately frozen at -70°. Each tube was diluted with 2 ml of 0.1 N NaOH and then divided into 20 equal portions, which were immediately frozen. Usually, one of these tubes was used for the labeling experiments. When handled in this manner, the isotope remained active for at least 12 months.

Acrylamide and N,N'-methylenebisacrylamide were purchased from Eastman Kodak; 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyl-oxazolyl)]benzene (POPOP) from New England Nuclear.

Labeling Procedure—The labeling was done essentially as previously described (29, 32) but was slightly modified. Higher concentrations of galactose oxidase were used, and incubation was at room temperature. The specific activity of NaB³H₄ was about 60 times higher than that used for erythrocytes previously. Treatment of cells with pronase, trypsin, and neuraminidase and the isolation of membranes have been described previously (20). Aliquots of the membrane preparations were counted for radioactivities in a dioxane-based scintillation fluid (33) in a Wallac liquid scintillation counter 8120.

Protein Assay—The method of Lowry et al. (40) was used with bovine serum albumin as standard.

RESULTS

Incorporation of Label into Erythrocyte Membranes

Cells from Adults—The incorporation of label from NaB³H₄ into erythrocyte membranes depends on the time of incubation with galactose oxidase and reaches a plateau after 1 to 2 hours (Fig. 1A). High concentrations of galactose oxidase suppress the labeling of the membranes (Fig. 1B). It is not possible to saturate the oxidized membrane with tritium from NaB³H₄, because after acrylamide gel analysis the stained protein pattern resembles that due to proteolysis (Ref. 30, and Footnote 2).

Initial treatment of the cells with neuraminidase results in a 3-fold increase in incorporation of label over those cells which are not treated. Pronase and trypsin remove 46% and 37%, respectively, of the radioactivity from prelabeled cells (Table I). The label in cells which are not pretreated with neuraminidase is hardly susceptible to the action of pronase or trypsin. If cells are digested with the proteolytic enzymes before labeling, less label is incorporated than when cells are digested after labeling. Control cells without galactose oxidase show very little label.

Umbilical Cord Blood and Fetal Cells—The membranes of these cells are not efficiently labeled with galactose oxidase/NaB³H₄ alone. However, if the cells have been treated with neuraminidase prior to labeling, the label is strongly enhanced (Table I).

Carbohydrate Left in Membrane after Proteolytic Digestion

Chemical determinations show that even after prolonged proteolysis a substantial amount of carbohydrate, which cannot be extracted with chloroform/methanol, is left in the membrane (Fig. 2, A and B). After pronase or trypsin digestion more than 50% of the protein-bound galactose remains in the membrane.

Membrane Proteins Stained by Coomassie Blue

Fig. 3 shows the stained membrane proteins after separation on a polyacrylamide slab gel. The main components are numbered according to Fairbanks et al. (41). All membranes have been reduced with NaB³H₄ after enzymatic treatments. The trypsin-treated membranes (c and d) do not show any obvious change, whereas in the pronase-treated membranes (e and f), Band 3 is cleaved and a new polypeptide P appears. This fragment has an apparent molecular weight of 64,000.
TABLE I
Total label in erythrocyte membranes

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Cells from adults</th>
<th>Cord blood cells</th>
<th>Fetal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase + galactose oxidase</td>
<td>100</td>
<td>92.6</td>
<td>88.2</td>
</tr>
<tr>
<td>Neuraminidase + galactose oxidase + pronase</td>
<td>54.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase + galactose oxidase + trypsin</td>
<td>62.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose oxidase</td>
<td>32.8</td>
<td>16.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Galactose oxidase + pronase</td>
<td>30.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose oxidase + trypsin</td>
<td>32.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pronase + neuraminidase + galactose oxidase</td>
<td>25.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin + neuraminidase + galactose oxidase</td>
<td>26.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No enzyme used</td>
<td>0.8</td>
<td>1.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Tritium label could not be visualized from polyacrylamide gels by conventional autoradiography and so a scintillator was introduced into the gel before drying. Membranes from cells labeled after neuraminidase and galactose oxidase treatment show the presence of a large number of labeled polypeptides. The apparent molecular weights of some of these are presented in Table II. The major components are GP7, GP8, GP13, GP14, and GP16. If neuraminidase treatment is omitted, proteins GP8 and GP14 are weakly labeled, and GP13 and GP16 are not detected at all. This indicates that in GP8, GP13, GP14, and GP16 sialic acids are linked to most of the penultimate galactosyl/N-acetylgalactosaminyl residues, whereas none of the other labeled glycoproteins contain neuraminidase-susceptible sialic acids. If cells are treated with trypsin before labeling, Bands GP8, GP13, GP14, and GP16 completely disappear (Fig. 4, A-c). Instead, Bands T1, T2, and T3 appear. These bands are not observed without pretreatment with neuraminidase. Pronase degrades Bands GP7, GP8, GP13, GP14, and GP16 (Fig. 4, A-d), and a strong diffuse band, PG1, appears, and in the region of the previous band, GP7, Bands GP7a-d appear. Bands GP7a-d are not fragments of larger proteins because no larger glycoproteins than GP7 are degraded. Band PG1 is very weak without pretreatment with neuraminidase (not shown). This indicates that Band PG1 is mostly derived from sialic acid-containing...
proteins are glycoproteins. Fig. 4, B-c, is the control where the enzymes have been omitted. No labeled bands are observed in Fig. 4, B-c. Thus the labeled peptides of membranes labeled after neuraminidase and galactose oxidase was omitted. Fig. 4, B-a, shows the polygalactose oxidase treatment; Fig. 4, B-b, shows the labeled are glycoproteins, it was necessary to include a control where relatively weak label. To test whether these proteins actually glycoproteins. Many of the labeled glycoproteins contain a (~) of radioactivity is bound to the numerous minor glycoproteins. To quantitate the amount of label in the minor glycoproteins, cells were labeled with galactose oxidase only, and the membranes were isolated and subjected to electrophoresis on cylindrical acrylamide gels. The gels were then sliced and counted as described previously (29). Twenty-four percent of the total radioactivity in the proteins was recovered from the slices between the origin and Band 3. Band 3 contained 28%, PAS1 contained 10%, and the remaining 40% of the radioactivity was rather evenly distributed between the positions of PAS1 and the lipid peak. Thus a substantial amount of radioactivity is bound to the numerous minor glycoproteins (see Fig. 4a in Ref. 29).

**Table II**

Labeled glycoproteins, their apparent molecular weights, and synonyms used

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>MW × 10⁶</th>
<th>Synonym</th>
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</thead>
<tbody>
<tr>
<td>GP1</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>GP2</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>GP3</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>GP4</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>GP5</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>GP6</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>GP7</td>
<td>100</td>
<td>Band 3 (41), Band a (13).</td>
</tr>
<tr>
<td>GP8</td>
<td>65</td>
<td>Major sialoglycoprotein, MN glycoprotein (15, 20), PAS1 (41, 18), glycophorin (14), Band b (12).</td>
</tr>
<tr>
<td>GP9</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>GP10</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>GP11</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>GP12</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>GP13</td>
<td>50</td>
<td>PAS2 (41, 18)</td>
</tr>
<tr>
<td>GP14</td>
<td>47</td>
<td>PAS2 (41, 19)</td>
</tr>
<tr>
<td>GP15</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>GP16</td>
<td>24</td>
<td>PAS3 (41, 18)</td>
</tr>
<tr>
<td>GP17a</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>GP17b</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>GP17c</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GP17d</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>FG1</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>62</td>
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</tr>
<tr>
<td>T2</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

glycoproteins. Many of the labeled glycoproteins contain a relatively weak label. To test whether these proteins actually are glycoproteins, it was necessary to include a control where the galactose oxidase was omitted. Fig. 4, B-a, shows the polypeptides of membranes labeled after neuraminidase and galactose oxidase treatment; Fig. 4, B-b, shows the labeled polypeptides after treatment with galactose oxidase only, and Fig. 4, B-c, is the control where the enzymes have been omitted. No labeled bands are observed in Fig. 4, B-c. Thus the labeled proteins are glycoproteins.

To quantify the amount of label in the minor glycoproteins, cells were labeled with galactose oxidase only, and the membranes were isolated and subjected to electrophoresis on cylindrical acrylamide gels. The gels were then sliced and counted as described previously (29). Twenty-four percent of the total radioactivity in the proteins was recovered from the slices between the origin and Band 3. Band 3 contained 28%, PAS1 contained 10%, and the remaining 40% of the radioactivity was rather evenly distributed between the positions of PAS1 and the lipid peak. Thus a substantial amount of radioactivity is bound to the numerous minor glycoproteins (see Fig. 4a in Ref. 29).

**Labeling of Umbilical Cord and Fetal Erythrocytes**

Identical amounts of membranes from erythrocytes from adults, umbilical cord blood, and fetuses, labeled after neuraminidase and galactose oxidase treatments or after galactose oxidase treatment only, were subjected to electrophoresis (Fig. 5). After neuraminidase treatment, the glycoprotein patterns are similar, although some minor differences exist: GP4 and GP10 of cord blood cells are relatively strong (Fig. 5c). If cells are labeled without neuraminidase treatment, the total glycoproteins are very weakly labeled (Fig. 5). This shows that in the glycoproteins of fetal erythrocytes only a few galactosyl/N-acetylgalactosaminyl residues are available for labeling in the native state. Instead, sialic acids are linked to penultimate galactosyl/N-acetylgalactosaminyl residues.

**Labeling of Intact Cells and Isolated Membranes**

To study the distribution of glycoproteins between the two halves of the lipid bilayer and to see whether a reorganization of the membrane can be observed after isolation, the following experiments were done. (a) Intact cells were treated with galactose oxidase followed by reduction with NaBSH,. (b) Intact cells were treated with galactose oxidase, and the membranes were isolated and then reduced. (c) The membranes were first isolated, then treated with galactose oxidase, and reduced. Table III shows the specific activities in the membranes and Fig 6 shows the fluorography patterns of the labeled glycoproteins. The incorporation of label into intact membranes is less than that of reduced ghosts. In addition, the individual glycoproteins are less efficiently labeled than those of isolated ghosts. Other qualitative or quantitative changes are not observed. It is obvious that regardless of whether the membrane has been treated with galactose oxidase from the outside only or from both sides of the membrane, all individual glycoproteins are equally efficiently labeled. The lower label in the membranes of cells reduced while intact may be due to intracellular consumption of borohydride.

**Labeling of Cells of Different ABH Blood Groups**

Intact cells of the blood groups A, A, A, A, A, A, A, B, B, and O were labeled. The total radioactivities were similar and no obvious difference in the fluorography patterns could be observed (data not shown).

**Discussion**

Development of the galactose oxidase method to a very high sensitivity by combining it with fluorography of slab gels permitted analysis of red cell membrane glycoproteins with a resolution previously unavailable. The labeling is very specific: galactose oxidase reacts only with galactosyl and N-acetylgalactosaminyl residues (29, 31). Under these conditions no labeled protein bands were observed when the galactose oxidase treatment was omitted. Therefore, the labeled proteins must represent glycoproteins. However, it is possible to label isolated proteins in vitro by NaBSH, alone. Morell et al. (42) treated ceruloplasmin with NaBSH, both after incubation with neuraminidase and galactose oxidase, or without enzyme treatment. After enzyme treatment, the specific radioactivity was 25 times higher. van Lenten and Ashwell (43) labeled orosomucoid with NaBSH, after oxidation of sialic acids with periodate. Compared to the nonoxidized control, the specific radioactivity was 21 times higher. I have labeled, without using galactose oxidase, the glycoproteins, transferrin, and ovalbumin, and the nonglycoproteins, bovine serum albumin and hemoglobin, with NaBSH,. In all cases a low labeling was obtained. Therefore, it was always necessary in the membrane-labeling experiments to include a control in which no enzymes were used. In some types of cells some proteins are preferentially labeled by NaBSH, alone (32).

I have separately labeled human serum proteins. Without neuraminidase treatment, no labeled protein band is observed on electrophoresis. After neuraminidase plus galactose oxidase treatment, a few proteins are labeled but they do not corre-
FIG. 4. A, Fluorography pattern of \textsuperscript{3}H-labeled erythrocyte membranes. Packed erythrocytes (0.5 ml), were labeled as follows: a, membranes from cells labeled after treatment with neuraminidase and galactose oxidase; b, membranes from cells labeled with galactose oxidase only; c, membranes from cells first treated with trypsin and then labeled after neuraminidase and galactose oxidase treatments; d, membranes from cells first treated with pronase and then labeled after neuraminidase and galactose oxidase treatments. For labeling details see Fig. 3. b, dependency of label on enzyme treatment. Packed erythrocytes (0.5 ml) were labeled as follows: a, membranes from cells labeled after treatment with neuraminidase and galactose oxidase; b, membranes from cells labeled with galactose oxidase only; c, membranes from cells labeled without enzyme treatment. For labeling details see Fig. 3.

TABLE III

<table>
<thead>
<tr>
<th>Total label in membranes treated with galactose oxidase from outside only or from both sides of membrane</th>
<th>cpm/\mu g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells + galactose oxidase + NaB\textsuperscript{3}H\textsubscript{4} → membranes\textsuperscript{*}</td>
<td>5275</td>
</tr>
<tr>
<td>Intact cells + galactose oxidase → membranes + NaB\textsuperscript{3}H\textsubscript{4}</td>
<td>10642</td>
</tr>
<tr>
<td>Intact cells → membranes + galactose oxidase + NaB\textsuperscript{3}H\textsubscript{4}</td>
<td>10208</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Packed intact cells (0.5 ml) were treated with 5 units of galactose oxidase for 1 hour in 1 ml of PBS, pH 7.0, at room temperature, and washed by centrifugations three times. The membranes were isolated and reduced with 0.5 mCi of NaB\textsuperscript{3}H\textsubscript{4}.

It is evident that the glycoprotein pattern of human erythrocyte membrane is unexpectedly complex. At least 20 glycoproteins are reproducibly found on the outer surface and it is reasonable to assume that there are additional glycoproteins which either do not serve as substrate for galactose oxidase, or co-migrate with other glycoproteins, or are represented by too few copies per cell to be visualized.

In contrast to the glycoproteins from erythrocytes of adults, the fetal glycoproteins are labeled with a very low efficiency if treated with galactose oxidase and NaB\textsuperscript{3}H\textsubscript{4}, only (29). If these cells are pretreated with neuraminidase they are, however, strongly labeled. This indicates that most of the fetal glycoproteins contain penultimate galactosyl or N-acetylgalactosaminyl residues, which are linked to sialic acids in the native state.

The effects of proteolytic enzymes on the surface proteins of
the red cell have been extensively studied (12, 19, 26, 44). It is known that trypsin and pronase cleave the major sialglyco-
protein (PAS1) and that pronase cleaves Band 3 as well in addition to this. Chemical determinations show that approximately one-half of the nonlipid carbohydrate can be released from the membranes by pronase or trypsin. The rest of the protein-bound carbohydrate is firmly associated with the membrane and is not removed by 2 M KC1. It is possible that protein-bound carbohydrate is firmly associated with the polypeptide backbones of these proteins are located deeper in the membrane than their constituent carbohydrate chains. The carbohydrate may then protect the proteins from proteolytic attack. No glycoproteins are more easily labeled after protease treatment, which shows that their carbohydrate always is exposed to galactose oxidase.

One major conclusion from recent erythrocyte membrane research is the asymmetrical distribution of both proteins and lipids (for recent reviews see Refs. 45 to 47). The sugar chains of both glycoprotein and glycolipid have been suggested to be found exclusively on the outer surface. Treatment of cells with neuraminidase removes all the sialic acid (48), and lectin-ferritin complexes bind only to the outer surface of isolated ghosts (49). The galactose oxidase method has been used to approach this problem before (29, 30), but the limited resolution of the glycoproteins on cylindrical gels and the low radioactivities did not permit clear-cut conclusions. The results reported here strongly confirm previous results. All of the labeled glycoprotein oligosaccharides reside on the outer surface of the cells and no extensive reorganization of the membrane seems to take place during isolation. The question then arises whether all external proteins are glycoproteins. To my knowledge all well characterized mammalian and viral surface proteins are glycoproteins. Previous work and present data indicate that no glycoproteins are located on the cytoplasmic side of the lipid bilayer. How the absolute asymmetrical distribution of the carbohydrate-containing proteins is accomplished during the biosynthesis of the membrane is not completely understood. Recently, Component 3 was implicated in anion transport (10, 11). It is also possible that many of the minor glycoproteins also span the lipid bilayer and have important functions. This problem is currently being investigated.

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C G Gahmberg


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