Defective Glycosylation of Erythrocyte Membrane Glycoconjugates in a Variant of Congenital Dyserythropoietic Anemia Type II: Association of Low Level of Membrane-Bound Form of Galactosyltransferase

By Michiko N. Fukuda, Khaled A. Masri, Anne Dell, Eugene J.-M. Thonar, George Klier, and Ray M. Lowenthal

Congenital dyserythropoietic anemia type II (CDA II) or HEMPAS is a genetic disease caused by plasma membrane abnormality. The enzymic defect of HEMPAS has been suggested to be the lowered activity of N-acetylgalactosaminyltransferase II, resulting in lack of polylactosamine formation on proteins and leading to accumulation of polylactosaminyl lipids. In contrast to typical HEMPAS cases, cell-surface labeling of the erythrocytes of a HEMPAS variant G.K. showed an absence of polylactosamines either on proteins or on lipids. Fast-atom bombardment mass spectrometry analysis of G.K.'s erythrocyte glycopeptides detected a series of high mannose-type oligosaccharides, which were not detected in erythrocyte N-glycans of normal cells or of other HEMPAS cases: The former contains polylactosaminoglycans and the latter contains hybrid-type oligosaccharides. Keratansulfate (sulfated polylactosamines) in this patient's serum was abnormally low. The galactosyltransferase activity in microsomal membranes prepared from G.K.'s mononucleated cells was 24% of the normal level, whereas this enzyme activity in G.K.'s serum was comparatively higher than normal. Western blotting of G.K.'s membranes using antigalactosyltransferase antibodies showed that G.K. has reduced amounts of this enzyme present. The results collectively suggest that variant G.K. is defective in polylactosamine synthesis owing to the decreased quantity of the membrane-bound form of galactosyltransferase.

Preparation of glycopeptides and carbohydrate analysis by fast-atom bombardment mass spectrometry. Membranes were prepared from 36 mL erythrocytes from patient G.K. and were first extracted with ten times the volume of chloroform/methanol (2:1, vol/vol) to remove lipids. The residues were extensively digested with pronase (Calbiochem, La Jolla, CA), and glycopeptides were isolated using gel filtration and affinity chromatography on Concanavalin A (Con A)-Sepharose as described previously. About 80% of glycopeptides were bound to Con A and eluted with 200 mmol/L methyl-a-glucoside. This major glycopeptide fraction was methylated and subjected to analysis by fast-atom bombardment mass spectrometry as described previously.

Cell-surface labeling. Erythrocytes were incubated with galactose oxidase followed by reduction with NaB[3H]4, so that terminal galactose and N-acetylgalactosamines of glycoproteins and glycolipids were labeled with tritium. Cell-surface sialic acids were also labeled by the periodate-NaB[3H]4 method. Membranes were prepared from the surface-labeled erythrocytes, and glycoproteins and polyglycosylceramides were analyzed by SDS-PAGE followed by fluorography as described previously.

Measurement of keratansulfate. Keratansulfate in serum was quantitated by enzyme-linked immunosorbent inhibition assay

MATERIALS AND METHODS

Cells. Peripheral blood samples obtained from HEMPAS patients were provided by Dr P. Scartezzini, Galliera Hospital, Genoa, Italy, and Dr P. Izzo, University of Bari, Italy; and Dr G. F. Gaetani, University of Genoa, Italy. A blood sample of HEMPAS variant G.K., with coolant, was sent to San Diego. The blood samples were kept at 4°C for three to four days before being subjected to analyses. Normal blood samples, either fresh or stored at 4°C for three to four days, were obtained from healthy volunteers in the laboratory.
Galactosyltransferase whose sequence was deduced from the nucleotide sequence of human galactosyltransferase cDNA2 was synthesized by an automatic peptide synthesizer (430A peptide synthesizer, Applied Biosystems, Frost City, CA). The N-terminus of the peptide, Cys-Ser-Arg-Asp-Arg, was conjugated to keyhole limpet hemocyanin through meta-maleimidobenzoyl-N-hydroxysuccimide ester.10 A rabbit was immunized with this peptide conjugate, and an immune serum was obtained. Western blotting was performed as described previously.12 Total blood cell membrane proteins (150 μg) were separated by 10% PAGE. Proteins in the gel were transferred to a nitrocellulose filter electrothermically. The filter was soaked with 20 mmol/L Na-phosphate buffer, pH 7.4, containing 0.12 mol/L NaCl and 1% bovine serum albumin (BSA) at 4°C overnight and then reacted with diluted (1:100) antiserum at room temperature for two hours. After being washed with the buffer described above, the filter was soaked with [125I]protein A (10 cpm/mL) for 30 minutes and was then exposed to x-ray film (XAR-5, Kodak) backed by an intensifying screen at −100°C overnight to obtain an autoradiogram.

Glycosyltransferase assay. Mononucleated cells were obtained from the peripheral blood of variant G.K., typical HEMPAS cases, and normal individuals by Histopaque (Sigma, St Louis) centrifugation. Microsomes were prepared as described previously.13 Assays of N-acetylglucosaminyltransferase I were performed as described previously11 with a synthetic oligosaccharide, Manol−6Manol−3Manol−1→O-(CH2)3COOMe, provided by Dr O. Hindsgaul, University of Alberta, Canada. A reaction mixture (20 μL) was composed of 20 mmol oligosaccharide, 0.1 mol/L sodium-cacodylate buffer, pH 7.6, containing 10 mmol/L MnCl2 and 1% Triton X-100, 12.5 mmol UDP-[3H]N-acetylglucosamine (2 × 106 cpm), 0.75 mg 2-deoxy-2-acetamidoglucosamine and 2.65 μg enzyme protein. After incubating at 37°C for four hours, the reaction mixture was applied to a Sep-Pack C18 reverse-phase column (Waters Associates, Milford, MA). After the column was washed with water, [3H]-labeled product was eluted with methanol and measured for radioactivity. N-Acetylglucosaminyltransferase VIII (polylactosamine extension enzyme) in microsome membranes was assayed with asialo α-1-acid glycoprotein as described previously.14 This enzyme activity in serum was assayed according to the method of Piller and Carton15 with lactose used as an acceptor. Galactosyltransferase was assayed as follows: The reaction mixture (33 μL) was composed of 2.1 mmol di-N-acetyllactosamine, 25 mmol UDP-[3H]galactose (5.8 × 106 cpm), 2.65 μg microsome protein, and 0.1 mol/L sodium cacodylate buffer, pH 7.6, containing 10 mmol/L MnCl2 and 1% Triton X-100. After incubation at 37°C for 60 minutes, a 0.4-mL suspension (resin/water, 1:5 vol/vol) of AG 1 x 8 (100 to 200 mesh, Cl− form, BioRad) was added, the suspension was mixed with rotation for ten minutes and centrifuged, and the supernatant (0.2 mL) was measured for radioactivity. Controls were performed in the same manner except that the di-N-acetyllactosamine was omitted in the reaction mixture. Galactosyltransferase in serum was measured in the same manner as described above.

Immunoelectronmicroscopy. Blood cells were fixed at room temperature in freshly prepared 10% v/v phosphate buffer containing saponin (Sigma) for one hour. The cells were washed overnight with or without (control) anti-band 3 antibodies diluted (1:200) with phosphate buffer containing saponin at 4°C.16 After being washed three times with phosphate buffer containing saponin, the cells were incubated with a second antibody complexed to colloidal gold for one hour at room temperature. The cells were washed three times with phosphate buffer containing saponin and twice with phosphate buffer. Finally, the cells were treated with glutaraldehyde and processed for transmission electron-microscopy. A Hitachi K-600 electron microscope was used.

BRIEF CASE REPORT

The HEMPAS CDA II variant G.K., a man now aged 53 years, is a son of a first-cousin Anglo-Saxon marriage. The detailed clinical documentation of this patient was presented by Lowenthal et al.17 The patient is of short stature with prominent bossing of the frontal skull. He and his twin brother had anemia since early childhood. He has developed complications that include bile pigment gall stones, hemosiderosis proven on liver biopsy, and severe tophaceous gout. He also has diabetes and, at the age of 41 years, spontaneously developed a nontraumatic retinal detachment. Since publication of the clinical details,18 he has developed biventricular cardiac failure and atrial fibrillation owing to cardiomyopathy, the cause of which is not clear but which may be caused by hemosiderosis. He has also developed severe, recurrent Bowen's disease (intraepithelial carcinoma), which has required treatment by plastic surgery. Electronmicroscopic examination of his blood cells revealed membrane duplication in erythrocyte cells. Unusual to CDA II; however, granulocytes and platelets also showed peripheral cisternae.

Cell-surface labeling of erythrocyte glycoconjugates. On SDS-PAGE, erythrocyte membrane components from HEMPAS patients show specific anomalies. Band 3 glycoprotein is less heterogenous and migrates slightly faster than normal band 3.44 The alteration in molecular weight (mol wt) of HEMPAS band 3 results from incomplete glycosylation of this molecule.7-11 Coomassie blue staining of SDS-PAGE, containing band 3 of variant G.K. and normal band 3, showed that the band 3 glycoprotein of variant G.K. migrates slightly faster than normal band 32 (Fig 1, lanes 1 and 2) as expected. Cell-surface labeling using galactose oxidase/NaB[3H]4 efficiently labels polylactosamine conjugates. Thus, in normal erythrocytes, band 3 and band 4.5 are labeled4 (lanes 3 and 5). In HEMPAS erythrocytes, however, these glycoproteins are not labeled, and accumulated polyglycosylceramides usually produce a diffuse band in the 20- to 30-Kd region on SDS-PAGE3 (Fig 1, lane 6); G.K. erythrocytes revealed no radioactive component on SDS-PAGE (lane 4). Thus, polyglycosylamides appear to be absent from the surface of G.K.'s erythrocytes. On the other hand, sialoglycoproteins (glyco- phorins) detected by periodate/NaB[3H]4 surface labeling show a normal pattern in G.K. cells (lanes 7 and 8), indicating that glycosylation of O-linked carbohydrates occurs normally.

Structural analysis of N-glycans. To determine the carbohydrate structures of glycoproteins, we prepared
A VARIANT OF HEMPAS

Fig 1. SDS-PAGE of surface-labeled erythrocyte membrane components that were separated on a 10% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue (lanes 1 and 2) and/or subjected to fluorography, (lanes 3 through 8). Lanes 1 through 6: Erythrocytes were treated with galactose oxidase followed by NaB[3H] so that terminal galactose and N-acetylgalactosamine of glycoproteins and glycolipids Incorporated radioactivity. Lanes 1 and 3 are normal; lanes 2 and 4 are variant G.K. Lanes 3 and 4 are fluorograms of the stained gel shown in lanes 1 and 2, respectively. Lanes 5 and 6 are fluorograms of normal and HEMPAS samples, respectively. Lanes 7 and 8: Erythrocytes were treated with sodium periodate followed by NaB[3H] so that terminal sialic acid incorporated radioactivity. Lanes 7 and 8 are fluorograms of normal and variant G.K. samples, respectively.

G.K.'s erythrocyte membranes and isolated glycopeptides from the membranes. We noticed during the preparation that G.K.'s major N-glycans were eluted at the position of the high mannose-type oligosaccharides in the Sephadex G-50 gel-filtration column and in the Con A-Sepharose affinity chromatography.

Figure 2 shows the fast-atom bombardment mass spectrum of G.K.'s glycopeptides. The most prominent peak, m/z 1280 for Hex5 HexNAc is indicative of the high mannose-type structure as follows:

\[
\text{Man}1 \xrightarrow{\text{Man}3} \text{Man}1 \xrightarrow{\text{Man}3} \text{Man}1 \xrightarrow{\text{GlcNAc}1} 4\text{GlcNAc} \rightarrow \text{Asn}
\]

The presence of high mannose-type oligosaccharide is also shown by the ions m/z 1,484 for Hex4 HexNAc and m/z 1,688 for Hex5 HexNAc. The spectrum also shows small amounts of hybrid-type oligosaccharides and complex type oligosaccharides (Fig 2). These results indicate that the G.K. erythrocyte membrane is characterized by an accumulation of high mannose-type oligosaccharides.

Keratansulfate concentration in serum. Keratansulfate is a sulfated polylactosamine present in cartilage and cornea. The level of keratansulfate in serum was measured by monoclonal antikeratansulfate antibody and existed in a much lower concentration (51 ng/mL) in G.K.'s serum than in normal controls (range 103 to 496 ng/mL, mean ± SD: 251 ± 78 ng/mL, n = 45).

Glycosyltransferase activity. Accumulation of the five mannosyl oligosaccharide was previously shown in a lectin-resistant mutant cell line defective in N-acetylgalactosaminyltransferase. Therefore, we tested this enzyme activity in G.K.; the enzyme activity was higher than normal levels (Table 1). The decrease of polylactosamines not only in glycoproteins but also in glycolipids and keratansulfates led us to suspect a deficiency of either galactosyltransferase or N-acetylgalactosaminyltransferase VIII in G.K. The galactosyltransferase activity in G.K. microsomal membranes prepared from mononucleated cells was only 24% of normal levels (Table 1), whereas N-acetylgalactosaminyltransferase VIII was as high as the normal level. To evaluate whether the decreased activity of the galactosyltransferase resulted from the quantitative decrease of this enzyme in membranes, we performed Western blotting of total blood cell membranes using antigalactosyltransferase serum. The apparent mol wt of the membrane-bound form of galactosyltransferase is 65 Kd and, as the Western blot shows, the 65 Kd band reactive to the antibodies is missing in G.K.'s membranes (Fig 3). The galactosyltransferase and the N-acetylgalactosaminyltransferase VIII activities in G.K.'s serum were 28.0 and 3.0 nmol/h/mL, respectively, whereas these values in normal sera were 16.7 ± 3.2 (n = 18) and 1.99 ± 0.42 (n = 41), respectively. These results indicate that the membrane-bound form of galactosyltransferase is decreased in variant G.K.

Distribution of band 3 glycoproteins. We previously...
showed anomalous clustering of band 3 glycoprotein in HEMPAS erythrocyte membranes. Variant G.K.’s erythrocytes were examined by immunogold electron microscopy with anti-band 3 antibodies. Band 3 in G.K.’s erythrocytes were apparent as clustered gold particles (Fig 4) rather than single and double gold particles. Such a staining pattern is similar to those previously obtained for HEMPAS erythrocytes, suggesting an uneven distribution of band 3 glycoprotein in G.K.’s membranes. G.K.’s erythrocytes often show fingerlike projections, and band 3 appears to be enriched in such an area. Band 3 was also noted along the membranes of blebs.

DISCUSSION

In normal erythrocyte membranes, band 3 and band 4.5 are glycosylated by polylactosaminoglycans, side chains of which are made of repeats of galactose and N-acetylglucosamines. Because the core structure of polylactosaminoglycans the same as those of complex-type oligosaccharides, polylactosaminoglycans are assumed to be synthesized from high mannose-type oligosaccharides, precursor form for complex-type and hybrid-type N-glycans (Fig 5). Previously, we showed that HEMPAS band 3 contains truncated tri- and tetra-anten 

<table>
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<th>Type</th>
<th>Normal</th>
<th>HEMPAS</th>
<th>Variant G.K.</th>
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<tbody>
<tr>
<td>N-acetylglucosaminyltransferase</td>
<td>4.92</td>
<td>7.88</td>
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<td>N-acetylglucosaminyltransferase VIII</td>
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<tr>
<td>Galactosyltransferase</td>
<td>108.87</td>
<td>121.07</td>
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The nomenclature of N-acetylglucosaminyltransferase I-VII has been given by Schachter et al.27 Galactose: β1 → 3N-acetylglucosaminyltransferase, which is involved in the synthesis of polylactosamines, is called N-acetylglucosaminyltransferase VIII in this report.

The present study of variant G.K. showed accumulation of high mannosetype oligosaccharides (Fig 2) not detected in the HEMPAS erythrocytes. Moreover, G.K. erythrocytes appear to lack polylactosamines both on glycoproteins and glycolipids, in contrast to HEMPAS (Fig 1). These characteristics of variant G.K. lead us to suspect a deficiency in galactosyltransferase or N-acetylglucosaminyltransferase VIII. Enzyme assays (Table 1) and Western blotting (Fig 3) showed significantly decreased levels of membrane-bound galactosyltransferase. Because the galactosyltransferase is involved in all steps of the biosynthesis of complex-type, hybrid-type, and polylactosamine saccharides, the displaced galactosyltransferase probably results in accumulation of high mannose-type precursors (Fig 5). Based on previous studies and the present study, schematic figures for band 3 glycoproteins and glycolipids in normal, HEMPAS, and variant G.K. erythrocytes are presented (Fig 6).

The N-acetylglucosaminyltransferase II defect most prominently affects polylactosamine formation of bianten 

Fig 2. Fast-atom bombardment spectrum of permethylated glycopeptides of variant G.K. erythrocyte membranes. Signals attributable to high manno 

Other signals are assigned as follows: m/z 1,382 (Hex5 · HexNAca2), m/z 1,688 (β-cleavage ion, [OH] NeuNAc, · Hex4 · HexNAca2), and m/z 1,770 (Hex4 · HexNAca2), m/z 1,811 (Hex4 · HexNAca2), m/z 1,913 (β-cleavage ion, [OH] NeuNAc, · Hex4 · HexNAca2).
**Fig 3.** Western blotting of total blood cell membranes by antigalactosyltransferase antibodies. Lane 1, normal; lane 2, variant G.K.; lane 3, galactosyltransferase from bovine milk (Sigma).

**Fig 4.** Immunogold staining of band 3. HEMPAS variant G.K. erythrocytes were treated with sheep anti-band 3 antibodies followed by gold-coated rabbit anti-sheep IgG antibodies. (A) Fingerlike projection stained heavily with gold particles. (B) Small open blebs stained with gold particles. (C) Peripheral cisternae (arrows) and cytoplasmic vacuoles. Clustered gold particles are evident together with plasma membranes.
Fig 5. Biosynthesis of N-glycans of normal, HEMPAS, and variant G.K. In normal erythrocytes (left), major N-glycans are polylactosaminoglycans and complex type oligosaccharides. In HEMPAS (center), the normal glycosylation pathway is blocked at N-acetylglucosaminyltransferase II step, which shifts the glycosylation pathway to hybrid type oligosaccharides. Galactosyltransferase is involved in the formation of all three types of N-glycans (right). Particularly, multiple galactosylations are needed in polylactosamine formation. Lowered activity of galactosyltransferase in variant G.K. hinders the glycosylation pathways to all directions, resulting in accumulation of high mannose-type oligosaccharides.

G.K.'s erythrocytes is not restricted to hematopoietic cells. Serum glycoproteins, which are synthesized in liver cells, also contain significant amounts of high mannose-type oligosaccharides in variant G.K. (M.N. Fukuda and A. Dell, unpublished observations). This abnormal glycosylation of serum glycoproteins may cause the secondary tissue siderosis and cirrhosis of this patient because serum proteins with high mannose-type oligosaccharides should be recognized and trapped by the mannose binding protein in reticuloendothelial cells. Abnormally low levels of keratansulfates in G.K. also suggest that the connective tissue cells are affected by the galactosyltransferase defect. His short stature and diabetes may also be related to incomplete glycosylation of growth factor receptor and insulin receptor: Growth factor receptor and insulin receptor become less abundant at the cell surface if glycosylation is inhibited by tunicamycin. The development of nontraumatic detachment of the retina that occurred in this patient suggests involvement of cell-surface galactose-terminated glycoconjugates in intercellular adhesion.

Galactosyltransferase is primarily present in the membranes of the trans-Golgi. The soluble form, which is found in milk, colostrum, and serum, is produced by a proteolytic cleavage of the membrane-bound enzyme. We recently identified the full-length coding sequence of human galactosyltransferase. Shaper et al also deduced full-length coding sequences of murine galactosyltransferase. These translated sequences show only one prominent hydrophobic segment near the NH₂-terminus, and this segment is most likely a combination signal-membrane anchor domain. A similar (type II) membrane topology with a membrane anchoring-uncleavable signal peptide near the NH₂-terminus was shown for α₂,6-sialyltransferase. Because the variant G.K. demonstrates high levels of soluble galactosyltransferase but reduced amounts of membrane-bound enzyme, the genetic mutation may have occurred in the membrane-binding domain, changing the uncleavable signal peptide into a sequence susceptible to signal peptidase. The mutation of G.K.'s galactosyltransferase may also result in increased susceptibility at the proteolytic cleavage site, which is identi-
fied as Arg 77 by our sequence data. However, an enhancement of protease activity in G.K.'s cells may cause production of more of the soluble form of galactosyltransferase, and other structural and functional anomalies of the Golgi apparatus that affect glycosylation machinery may exist. Nucleotide sequencing of G.K.'s DNA encoding the galactosyltransferase will provide a clearer answer as to whether G.K. has a mutation in the galactosyltransferase gene.

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