Abnormal Glycosylation of Red Cell Membrane Band 3 in the Congenital Disorder of Glycosylation Ig

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ABSTRACT

A description is provided of the clinical presentation in an infant of the recently described congenital disorder of glycosylation type Ig, and the changes affecting glycosylation of red cell membrane band 3, the anion exchanger. It has been shown that the condition stems from a homozygous mutation within the human ortholog of yeast *ALG12* gene, which encodes a dolichol-P-mannose:Man$_7$GlcNAc$_2$-PP-dolichol $\alpha,1-6$ mannosyltransferase of the endoplasmic reticulum. The clinical phenotype included prominent central and peripheral manifestations in the CNS. Although the infant studied had no anemia, band 3 abnormally separated into two fractions upon electrophoresis. The chemical composition of the glycans of both fractions was analyzed in detail. The fraction with low electrophoretic mobility was moderately hypoglycosylated (by 27%) and its mannose content was normal. The fraction with high electrophoretic mobility was deeply carbohydrate deficient (by 64%) and had 1 mol mannose in excess but only three residues of *N*-acetylglucosamine. Glycophorin A was hypoglycosylated with respect to *O*-linked glycans. Glycosphingolipids of red cells were normal. We suggest that the incomplete biosynthesis of the *N*-linked glycan of band 3 was largely caused by the persistence of the 3-linked mannose residue on the 6-mannose arm of the trimannosyl moiety of the glycoprotein. It is remarkable that the changes recorded in band 3 have no clinical consequences. Band 3 alteration might serve as an additional indicator (some serum *N*-glycoproteins of hepatic origin are also indicative) of the congenital disorder of glycosylation type Ig. (***Pediatr Res* 54: 224–229, 2003)**

Abbreviations
ALG12, gene encoding the dolichyl-P-mannose:Man7GlcNAc2-PP-dolichol mannosyltransferase of the endoplasmic reticulum
CDG, congenital disorder of glycosylation
LLO, lipid-linked oligosaccharide
Gal, galactose
GalNAc, *N*-acetylgalactosamine
GlcNAc, *N*-acetylglucosamine
Man, mannose
P, phosphate
PP, pyrophosphate

CDG designates a group of rare inherited diseases of metabolism characterized by a variety of glycosylation defects of glycoproteins that are involved in different metabolic functions. CDG I comprises all defects in the assembly of the dolichol-linked glycan and its transfer to the protein, whereas CDG II refers to defects in the processing of the protein-bound glycans. Their clinical picture is severe and associated with multisystemic lesions, particularly central and peripheral nervous system deficiencies. Serum *N*-glycoproteins of hepatic origin are abnormally glycosylated.

Thus far, six types of CDG I have been identified and characterized on a molecular basis: i) CDG Ia, which accounts for 80% of CDG I cases as presently known, is associated with deficient phosphomannomutase, encoded by the *PMM2* gene (1); ii) CDG Ib is associated with deficient phosphomannose isomerase, encoded by the *MPI* gene (2); iii) CDG Ic with deficient glucosyltransferase, encoded by the *hALG6* gene (3);

iv) CDG Id, with deficient mannosyltransferase, encoded by the *hALG3* gene (4); v) CDG Ie, with deficient dolichol-phosphate-mannose synthase-1, encoded by the *DPM1* gene (5); and vi) CDG If, with deficient Lec35 protein, encoded by the *Lec35* gene (6). CDG I cases for which the genetic origins remain unknown are defined as CDG subtype Ix. Their clinical presentations are heterogeneous.

We recently described the novel CDG Ig, involving still another endoplasmic reticulum enzyme—a mannosyltransferase. The latter is encoded by the human ortholog of yeast gene *ALG12* (7). We report here the clinical presentation and the altered glycosylation of red cell membrane band 3 in the first patient described with this condition. It is odd that there were no gross hematological signs among so many manifestations and despite pronounced alterations of the band 3 glycan moiety. Previously, aberrant glycosylation of band 3 was described in one patient with a CDG II (8), and abnormalities of both band 3 and glycophorin A were reported in three patients with CDG Ia (9).

METHODS

Patient. The investigation was undertaken after informed consent of the parents was obtained.

The patient is a Tunisian infant. She is the third child of consanguineous parents. Neonatal weak suckling and hypotonia were the first clinical manifestations. Facial dysmorphic features (prominent forehead, large ears, thin upper lip) were also present. During the clinical course, severe psychomotor retardation appeared as the main symptom. At the age of 3, the girl was unable to sit and walk and needed enteral feeding with a percutaneous endoscopic gastrostomy. Failure to thrive and strabismus were noted. The concentration of serum electrolytes and creatinine, and liver function were normal. Blood cell count revealed monocytosis ranging from 1 to 2.5×10^9/L, and normal leukocyte and platelet counts. Red cell indices appeared essentially normal (Hb: 11.6 g/dL; mean corpuscular volume: 75 fL; mean corpuscular Hb concentration: 32 g/dL; mean corpuscular Hb: 24 pg/L). Bilirubinemia was normal. Some coagulation factors were decreased, including factor XI (26%), antithrombin (33%), protein C (52%), and protein S (37%). Serum IgG was reduced (2 g/L; normal, >4 g/L). The serum blood serum contained no unusual anti–red cell antibodies according to the Coombs' test using blood group O cells.

At 6 mo of age, a diagnostic procedure was performed. The Western blot profile, as well as isoelectric focusing of transferrin, showed a hypoglycosylation pattern characteristic of CDG. Cranial magnetic resonance imaging was normal. Constitutional karyotype showed no abnormalities. Amino acids and organic acids in the urine were normal. Nevertheless, phosphomannomutase and phosphomannose isomerase activities, known to be reduced in CDG Ia and Ib, respectively, were normal in the patient's leukocytes and cultured fibroblasts. However, skin biopsy fibroblasts were deficient in their capacity to add the eighth Man residue onto the LLO precursor.

We found that the cDNA corresponding to the human ortholog of the yeast *ALG12* gene carried a base substitution: T571G in the homozygous state. One would expect the protein product, the dolichol-P-Man:Man$_7$GlcNAc$_2$-PP-dolichol α 1–6 mannosyltransferase, to show the amino acid substitution F142V. The pathologic phenotype of the patient's fibroblasts was largely normalized upon transfection with the wild-type gene. Accordingly, this change was considered the cause of a novel disease, which was termed CDG Ig (7).

Methods. Red cell membrane proteins were analyzed using PAGE in the presence of SDS (10, 11).

Carbohydrate molar composition of glycoproteins was determined as previously described (12). The method involves simultaneous quantification of carbohydrate and protein contents of glycoproteins separated by SDS-PAGE and thereafter electroblotted onto polyvinyl difluoride membranes. Bands of the glycoproteins under study were excised and sequentially hydrolyzed *in situ* with 0.2 M trifluoric acid, 2 M trifluoric acid, and 6 M HCl to release sialic acid, neutral sugars with hexosamines, and amino acids. The carbohydrates were analyzed by high pH anion exchange chromatography with pulsed amperometric detection using the Dionex system (Dionex Corporation, Sunnyvale, California, U.S.A.) and PA-1 column (13), and protein was determined as amino acids by the fluorescamine method (14).

Glycophorin A was analyzed by the same method. Before being subjected to electrophoresis, it was purified by extraction with aqueous phenol (15). Purification was followed by dialysis.

Oligoglycosylceramides were extracted from the lyophilized erythrocyte membranes successively with 5 mL portions of chloroform-methanol 1:2 and 2:1 by volume, respectively, and thereafter fractionated to pure compounds as described previously (13). Polyglycosylceramides were extracted from the insoluble residue of the organic solvent–extracted membranes after a preliminary acetylation step (16). Both oligo- and polyglycosylceramides were quantified as carbohydrates and sphingosine after hydrolysis of individual glycosphingolipids with 2 M trifluoroacetic acid for 4 h at 100°C.

RESULTS

Electrophoresis of serum glycoproteins and enzyme activities. In comparison with controls, the patient's serum exhibited the presence of two additional bands of lower molecular weight on transferrin Western blot profile (not shown). The isoelectrofocusing pattern showed a marked elevation of the tri-, di-, mono-, and asialotransferrin fraction. A generalized glycosylation defect was confirmed by Western blot patterns of α-1-antitrypsin, α-1-acid glycoprotein, and haptoglobin (not shown). Taken together, these results were characteristic of CDG I patients. Normal activities of phosphomannomutase and phosphomannose isomerase in fibroblasts as well as in leukocytes from the patient and her parents ruled out CDG Ia and CDG Ib (7).

Electrophoresis of red cell membrane proteins. Band 3 was markedly altered. It consisted of two peaks instead of the normal single broad peak, suggesting a glycosylation abnormality. The intensity of the peak with low electrophoretic mobility (LM) was slightly lighter as compared with the peak displaying the high mobility (HM). It was also slightly larger

than normal (Fig. 1). The upper edge of the LM fraction was smoother and shifted upward. The lower edge of the HM fraction had a normal aspect but was shifted downward. Densitometric quantification of Coomassie blue–stained gel indicated a normal surface area. A profile from a patient with congenital dyserythropoietic anemia (CDA II) is presented as a distinct abnormality of band 3 glycolylation (Fig. 1).

Chemical analysis of band 3 glycan moiety. The two fractions of band 3 were separately analyzed for carbohydrate molar composition (Table 1). The LM fraction exhibited a significantly decreased number of carbohydrate residues per mole but had a normal level of three residues of Man. The HM fraction was deeply deficient in carbohydrates and contained about 1 mol Man in excess. Its content of GlcNAc was close to three residues. These results, as well as the known order of addition of GlcNAc residues to the two branches of the trimannosyl moiety of *N*-glycans with the 3-Man arm being substituted first (for review, see Ref. 17), suggest that the core portion of the HM fraction had the structure depicted in Figure 2.

In normal controls, there was also a difference between the leading and trailing edges of band 3 but it amounted to only several sugar residues. In addition, Man contents of the two fractions were identical (not shown). We cannot explain the higher than expected amount of Gal relative to GlcNAc in HM fraction. A small amount of GalNAc occurred in all preparations of band 3, and is probably an impurity. In blood group A

individuals, there is an additional amount of GalNAc that is a part of blood group A epitope (for review, see Ref. 18).

Chemical analysis of glycophorin A glycan moiety. We failed to observe any gross change in the mobility of glycophorin A in SDS-PAGE (not shown). This glycoprotein has normally about 12 *O*-linked glycans, as shown by its content of GalNAc (15). This number is preserved in glycophorin A of the patient (Table 2). The *O*-linked glycans of the patient's glycophorin A were, however, hypoglycosylated, because its contents of Gal and *N*-acetylneuraminic acid were significantly decreased. Gross abnormalities in the amounts of GlcNAc and Man, the components of the single *N*-linked glycan of glycophorin A, were not observed, although the amounts of the two sugars were on the low and high side of normal values, respectively.

Chemical analysis of glycosphingolipid glycan moiety. Contents of most glycosphingolipids in patient's erythrocytes, including polyglycosylceramides, were normal (Table 3). The polyglycosylceramides also had the normal carbohydrate molar composition, although a polylactosamino type glycan very similar to that of band 3 was present (for review, see Ref. 18).

DISCUSSION

Abnormal electrophoretic profile of band 3. The present case is probably the first example of a clear separation of band 3 into two subfractions of different electrophoretic mobility in
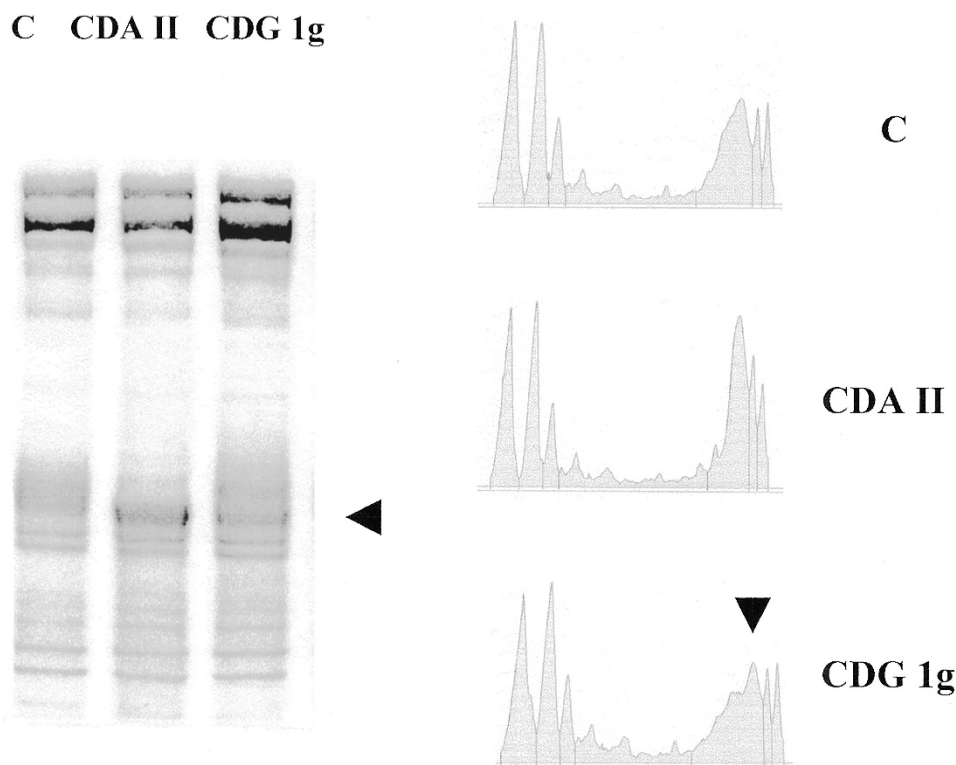

Figure 1. SDS-PAGE of red cell membrane proteins. *Left panel*: *Arrowhead* indicates band 3. *Right panel*: C (*top*) is the normal control. CDA II (*middle*) is the control with congenital dyserythropoietic anemia, chosen to illustrate another marked, yet distinct, abnormality of band 3 (band 3 was narrower and had a higher electrophoretic mobility). CDG Ig (*bottom*) is from the patient under study. Band 3 was wider (*arrowhead*) and was duplicated into an upper fraction (low electric mobility) and a lower fraction (higher electrophoretic mobility [HM]). The lower edge of the HM fraction was lower (as in CDA II) than that in the normal control. Scanning showed the amount of band 3 was normal in CDG Ig (as in CDA II).

Table 1. *Carbohydrate molar composition of band 3*

Material analyzed	Fuc	GalNAc	GlcNAc	Gal	Man	NeuAc	Total CHO (%)
Band 3, CDG Ig							
LM fraction (mol/mol)	2.5	0.4	**11.3**	8.6	**2.9**	0.4	**26.1 (5.5)**
HM fraction (mol/mol)	0.5	0.7	**3.4**	3.4	**4.3**	0.5	**12.8 (2.7)**
Band 3, normal adults ($n = 9$) (mol/mol)	2.4	1.2	14.7	14.1	2.8	0.8	36.0 (7.6)
± SD (mol/mol)	0.5	0.5	0.8	1.4	0.3	0.5	1.1 (0.2)

Each monosaccharide species was expressed as moles per mole of protein. The most salient features appear in bold type. CHO, carbohydrate.
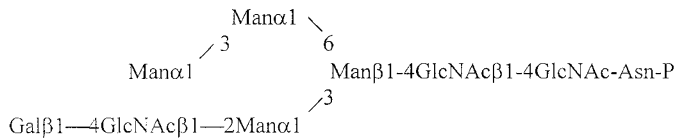
Figure 2. Proposed structure of *N*-linked glycan of HM fraction.

a condition with aberrant glycosylation (for review, see Ref. 19). The alteration was very different from that observed in CDA II, producing another dramatically abnormal pattern of band 3 (20, 21). The explanation for the double peak in the presently studied case of CDG should be sought in the sequence and nature of events occurring on the biosynthetic path of glycans from endoplasmic reticulum to the Golgi. A reduced activity of the dolichol-P-Man:$Man_7GlcNAc_2$-PP-dolichol $\alpha 1{-}6$ mannosyltransferase of the endoplasmic reticulum results in the production of a curtailed form of LLO with the peripheral $\alpha 1{-}6$ mannosyl branch missing (7) (Fig. 3*A*). Nevertheless, this compound is still transferred from dolichol onto protein as *N*-linked $Glc_3 Man_7GlcNAc_2$ oligosaccharide. Such a transfer was reported previously in yeast (22). Surprisingly, nonglucosylated $Man_7GlcNAc_2$ oligosaccharide is also transferred onto protein (7). In addition, the mutated *ALG12* gene in the patient is leaky and does not prevent the formation of a normal LLO with nine Man and three glucose residues (7). These three oligosaccharides are transferred onto protein in approximately equimolar proportions.

On its path to the Golgi, the normal *N*-linked glycan is trimmed of all $\alpha 1{-}2$ Man residues down to a total of five Man residues (Fig. 3*B*) (23) and in the patient's band 3 to four Man residues, presumably. Both *N*-linked glycans should be good substrates for *N*-acetylglucosaminyltransferase I (GnT 1), which adds GlcNAc residue to the 3-Man arm of the trimannosyl core (24), thus initiating the first, less complex antenna (25) and at the same time providing a signal for mannosidase II to cleave two mannosyl residues from the 6-Man arm of the core. In the case of the normal *N*-linked glycan of the complex type, the exposed 6-Man arm of the core becomes a substrate for *N*-acetylglucosaminyltransferase II (GnT II) (for review, see Ref. 17), thereby initiating biosynthesis of the second, a more complex antenna of band 3 (25). It is very probable, however, that mannosidase II is unable to cleave the single peripheral Man-3 linked to the 6-Man arm of the core of the patient's band 3 because mannosidases II from various animal and plant sources hydrolyze the peripheral 6-linked Man (6-Man) before the peripheral 3-linked Man (3-Man) (26, 27). A recent proposal for the reaction mechanism suggests that only after the cleavage of the peripheral 6-Man is the peripheral 3-Man brought into the active site cavity of the enzyme (28).

Thus, in the absence of the peripheral 6-Man in the patient, the hydrolysis of the peripheral 3-Man may well be hampered. It is, however, possible that the peripheral 3-Man is hydrolyzed by mannosidase II even in the absence of the peripheral 6-Man, but with a lower efficiency. In such a situation, only a part of the peripheral 3-Man would be cleaved, thus providing the substrate for GnT II, in addition to that supplied by the leaky, mutated *ALG12* gene. In conclusion, we propose that LM fraction of band 3 resulted from a portion of the protein that was initially glycosylated in the endoplasmic reticulum with a normal LLO, whereas the HM fraction resulted from another portion initially glycosylated with $Glc_3 Man_7GlcNAc_2$ oligosaccharide or nonglucosylated $Man_7GlcNAc_2$ oligosaccharide or both. We consider it more likely, however, that *N*-linked glycan of HM fraction of band 3 originated from LLO with a more physiologic $Glc_3 Man_7GlcNAc_2$ structure. It appears that SDS-PAGE of band 3 glycan moiety constitutes a biochemical stigmata for the diagnosis of this CDG on par with transferrin isoelectric focusing or Western blotting of a variety of proteins.

Glycophorin A. Glycophorin A of the present CDG patient shows hypoglycosylation of *O*-linked chains. In contrast, this glycoprotein is partly unglycosylated (nonglycosylated) in CDG Ia (9) and in several patients with CDA I and CDA II (15, 29, 30). In all these cases, glycosylation of band 3 was reduced. In subjects with the so-called En (−) phenotype, glycophorin A is largely missing but band 3 is overglycosylated (for review, see Ref. 31). Conversely, when the amount of glycophorin A is increased, the size of band 3 glycan is reduced. Aberrant glycosylation of glycophorin A in many diverse conditions affecting band 3 is difficult to rationalize, thus raising the issue of their partnership in biosynthesis.

Glycosphingolipids. Normal contents of most glycosphingolipids, including polyglycosylceramides, in red cells of the patient sharply separate CDG Ig from CDA I-III, because, in the latter group of diseases, lactotriaosylceramide, neotetraosylceramide, and polyglycosylceramides are invariably elevated (29, 32, 33). Polyglycosylceramides are, in addition, hypoglycosylated (29, 34). It has been proposed that the increased content of polyglycosylceramides in erythrocytes of CDA II patients is of a compensatory type because band 3 and polyglycosylceramides have glycans of the same polylactosamino type that are synthesized, presumably, by the same glycosyltransferases (for review, see Ref. 35). Thus, when the amount of protein-bound substrate is diminished, the enzymes overglycosylate ceramides. This explanation should not apply to the present patient, in whom the glycosylation of band 3 was reduced by about 50%, yet the content and glycosylation of polyglycosylceramides were normal.

Table 2. *Carbohydrate (CHO) molar composition of glycophorin A*

Material analyzed	Fuc	GalNAc	GlcNAc	Gal	Man	NeuAc	Total CHO (%)
Glycophorin A, CDG Ig (mol/mol)	1.6	**11.7**	5.4	**17.7**	3.5	**14.5**	**54.4 (39.1)**
Glycophorin A, normal adults, ($n = 9$) (mol/mol)	2.9	12.2	6.8	23.2	3.0	21.2	69.3 (50.5)
± SD (mol/mol)	0.6	1.2	0.8	1.09	0.2	1.2	1.8 (1.4)

Each monosaccharide species was expressed as moles per mole of protein. The most salient features appear in bold type.

Table 3a. *Contents of glycosylceramides in erythrocyte membranes: oligoglycosylceramides*

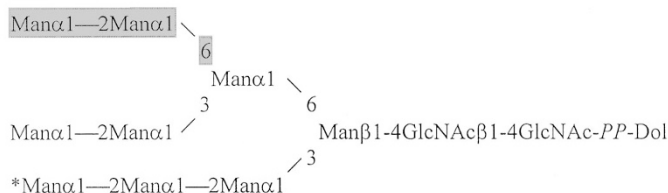| Material analyzed	GlcCer	LacCer	Lc3Cer	Gb3Cer	Gb4Cer	nLc4Cer
Oligoglycosylceramides, CDG 1g	0.5	3.5	0.1	2.5	9.1	0.01
Oligoglycosylceramides, normal adults ($n = 15$)	0.8	3.4	0.1	3.1	11.6	0.006
± SD	0.07	0.2	0.05	0.3	1.2	0.004

Measurements expressed as nanomoles per mg membrane proteins.

Table 3b. *Contents of glycosylceramides in erythrocyte membranes: polyglycosylceramides (PGC)*

Material analyzed	a	b
PGC, CDG 1g	35.6	29.4
PGC, normal adults ($n = 9$)	35.6	33.8
± SD	3.1	2.6

Measurements expressed as nanomoles per 100 mg membrane proteins (a), or as the sum of all sugar residues per mole of PGC (b).
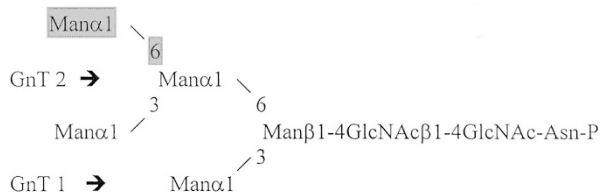

Figure 3. Structures of LLO (*A*) and of *N*-linked glycan in the middle Golgi (*B*). In both *A* and *B*, the *shaded areas* depict structures that should be missing in HM fraction. The *asterisk* marks the mannosyl residue to which the triglucosyl tag is attached. *Arrows* mark acceptor Man residues for GnT 1 and GnT 2.

CDG Ig and CDA II display opposite statuses in other respects as well. In keeping with the electrophoretic aspect of band 3 in CDA II (see above), the latter protein shows dramatic glycosylation changes (for review, see Ref. 35). However, several genes encoding glycan metabolism enzymes had been excluded (36). The responsible gene for CDA II has been mapped (37), but remains unidentified. Would CDA II nonetheless stem from a glycan metabolism gene, CDA II would stand in sheer contrast with CDG Ig. The former is restricted to the red cells whereas the latter is associated with multifaceted clinical manifestations. Why should the glycan alteration of band 3 be deleterious in CDA II, or at least be associated with dyserythropoiesis and fragile mature red cells, and remain innocuous in CDG Ig erythroid precursors and mature erythrocytes?

Apart from this study, glycosylation abnormalities of band 3 were reported only in CDG Ia (9) and CDG IIa (8). No hematological signs were recorded. In the first instance, the abnormalities were due to a deficiency of the enzyme that supplies Man-1-phosphate for the biosynthesis of GDP-Man. Inadequate supply of GDP-Man resulted in an impaired biosynthesis of LLO and, consequently, of *N*-linked glycoproteins. Some of them, like band 3, were hypoglycosylated (9), whereas others, like transferrin, were partially or fully unglycosylated (38, 39). Recently, we have found an alteration of electrophoretic mobility of band 3 of red cells in a patient with CDG II. The patient was again devoid of hematological symptoms (unpublished data). In patients with CDG IIa, the observed hypoglycosylation of band 3 is due to a deficiency of *N*-acetylglucosaminyl transferase II, the enzyme that transfers GlcNAc to the 6-arm of the trimannosyl core of the glycoprotein, thus initiating the biosynthesis of its second antenna (8).

CONCLUSION

In the present CDG Ig, band 3 glycosylation abnormalities are easy to show, based on SDS-PAGE. If such an observation was to be generalized in CDG, then band 3 would become a mirror of a general glycosylation defect on par with the glycosylation defect of serum *N*-glycoproteins of hepatic origin. Misglycosylation of band 3 also asks the question why it fails to induce any changes in the red cell physiology. We proposed an explanation for the misglycosylation of band 3 in CDG Ig.

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