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Characterization of the *N*-glycosylation phenotype of erythrocyte membrane proteins in congenital dyserythropoietic anemia type II (CDA II/HEMPAS)

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Abstract Congenital dyserythropoetic anemia type II (CDA II) is characterized by bi- and multinucleated erythroblasts and an impaired *N*-glycosylation of erythrocyte membrane proteins. Several enzyme defects have been proposed to cause CDA II based on the investigation of erythrocyte membrane glycans pinpointing to defects of early Golgi processing steps. Hitherto no molecular defect could be elucidated. In the present study, *N*-glycosylation of erythrocyte membrane proteins of CDA II patients and controls was investigated by SDS-Page, lectin binding studies, and MALDI-TOF/MS mapping in order to allow an embracing view on the glycosylation defect in CDA II. Decreased binding of tomato lectin was a consistent finding in all typical CDA II patients. New insights into tomato lectin binding properties were found indicating that branched polylactosamines are the main target. The binding of *Aleuria aurantia*, a lectin preferentially binding to α1-6 core-fucose, was reduced in western blots of CDA II erythrocyte membranes. MALDI-TOF analysis of band 3 derived *N*-glycans revealed a broad spectrum of truncated structures showing the presence of high mannose and hybrid glycans and mainly a strong decrease of large *N*-glycans suggesting impairment of *cis*, medial and *trans* Golgi processing. Conclusion: Truncation of *N*-glycans is a consistent finding in CDA II erythrocytes indicating the diagnostic value of tomato-lectin studies. However, structural data of erythrocyte *N*-glycans implicate that CDA II is not a distinct glycosylation disorder but caused by a defect disturbing Golgi processing in erythroblasts.

Keywords Hempas · CDA II · Congenital disorders of glycosylation · Dyserythropoesis · Erythrocyte membrane glycosylation

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Introduction

Congenital dyserythropoietic anemia type II (CDA II) [1], also known as *h*ereditary *e*rythroblastic *m*ultinuclearity with *p*ositive *a*cidified-*s*erum test (HEMPAS) [2] is the most frequent disorder in the group of congenital dyserythropoietic anemias. Ninety-eight patients have been reported to the international CDA II registry [3], and 205 patients from 160 families are known to the German CDA registry, including both published and unpublished cases [4]. Mild to moderate anemia and jaundice are the predominant symptoms. Hepatosplenomegaly and cholelithiasis are frequent consequences of increased red cell turnover with both ineffective erythropoesis and peripheral hemolysis. About 50% of the patients develop secondary hemochromatosis [4]. Autosomal recessive inheritance is indicated by

the observation that parents and offspring of CDA II patients show no hematological phenotype.

All types of CDA are characterized by ineffective erythropoesis and characteristic morphological abnormalities of red cell precursors. In CDA II, 10–40% of all erythroblasts are bi- or multinucleated [1, 4, 5]. Electron microscopy of red blood cells shows a second membrane close to the outer membrane consisting of residual endoplasmic reticulum [6]. Band 3 (anion exchange protein 1) and band 4.5 (glucose transporter 1), two abundant red blood cell membrane proteins [7], show sharper and faster migrating bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), caused by deficient glycosylation of these proteins [8]. Band 3 of wildtype red blood cells carries two polylactosamine chains ([-3Gal β1,4-GlcNac β1–]*n*) attached to a single N-linked biantennary asparagine-linked trimannosyl core structure. In CDA II, band 3 and other red blood cell membrane proteins carry truncated polylactosamine structures [8, 9] suggesting that CDA II might belong to the group of Congenital Disorders of Glycosylation (CDG) [10]. Hybrid- and high mannose oligosaccharides are more abundant on CDA II membrane proteins [8, 9]. Antibodies to linear polylactosamines (i-antigen) normally react strongly with *N*-glycans on fetal and neonatal erythrocytes but only weakly with the branched polylactosamines of erythrocytes of children and adults. Red cells of patients with CDA II retain strong anti-i binding throughout their life indicating decreased branching of the polylactosamine chains [2].

Up to now, the aberrant gene causing CDA II has not been identified and the biochemical mechanism causing CDA II remains unknown.

Materials and methods

SDS-Page, and lectin blots

Erythrocyte ghosts were prepared following standard procedures as described previously [11].

Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and either stained with Coomassie (15 μg protein/lane) or transferred to PVDF membrane (2 μg/lane). The blot was blocked with 1% bovine albumin in Tris-buffered saline with Tween20 0.1% (v/v) (TBST) at 4 °C overnight. Blots were incubated for 15 min in TBST with biotinylated tomato lectin (*Lycopersicon esculentum* agglutinin) (1 μg/ml), *Aleuria aurantia* (AAL) (1 μg/ml), or concanavalin A (Con A) (1 μg/ml) (Vector Lab, Burlingame, CA, USA) for 30 min, washed three times for 5 min, and incubated with streptavidin biotinylated peroxidase complex (1:1,500) (Amersham Pharmacia, Freiburg, Germany) in TBST for 15 min. After washing three times for 5 min the blots were stained using diaminobenzidine as substrate. Binding of tomato lectin to band 3 of healthy controls and CDA II patients was quantified determine the integrated density of the band using the program ImageJ (http://rsb.info.nih.gov/ij/), binding to control band 3 was set to 100%, background binding to 0%.

Deglycosylation of erythrocyte membrane proteins

Oligosaccharides were released from 20 μg protein of erythrocyte ghosts using PNGase F (New England Biolabs, Frankfurt, Germany) after denaturation with 0.5% SDS and 1% β-mercaptoethanol at 100 °C for 10 min. Incubation with PNGase F was performed for 12 h at 37 °C. Proteins were precipitated using ice-cold ethanol, air dried for 15 min and resuspended in running buffer for SDS-polyacrylamide gel electrophoresis.

Isolation of N-linked oligosaccharides from band 3 preparations

Aliquots corresponding to 50 μg protein of an erythrocyte membrane preparation were applied to 7.5% polyacrylamide gels. Protein bands were stained by colloidal Coomassie brilliant blue and destained in 30% MeOH/7% acetic acid. Gel areas corresponding to band 3 protein were cut and washed three times with 300 μl NH_4HCO_3 pH 8.0 for 30 min in a shaking water bath followed by 2×300 μl 60% acetonitrile/0.1% TFA. Gel slices were dried in a vacuum concentrator.

The dried gel pieces were soaked in 100 μl of a trypsin solution (2 μg/ml) in 50 mM NH_4HCO_3 at 37 °C. After 30 min another 50 μl of the trypsin solution was added and incubation was performed for 8 h at 37 °C.

Peptides were extracted with 250 μl of 20 mM NH_4HCO_3 pH 8.0 under sonication and then washed with 2×250 μl of 60% acetonitrile +0.1% TFA. Extracts were combined and dried in a vacuum concentrator. Subsequently peptides were dissolved in 50 μl of 0.5% formic acid and sonicated for 5 min.

N-linked oligosaccharides were released by incubation of the peptide mixture in 50 mM potassium phosphate buffer and the solution was adjusted to pH 7.0 by adding NaOH. 20 μl of recombinant *N*-glycosidase F (Roche, Germany) was added and incubation was performed over night.

The incubation mixture was applied onto a C_{18}-cartridge and was washed with 1 ml of 2% acetonitrile/0.1% TFA. The first flow through and the wash were combined and dried in a vacuum centrifuge. Oligosaccharides were further purified by adsorption onto 15 μl of porous graphite (Hypercarb, ThermoHypersil, Kleinostheim, Germany) filled into a small pipette tip. The hypercarb tip was

washed with 3×100 µl of H_2O and oligosaccharide material was eluted using 25% acetonitrile +0.1% TFA (2×25 µl).

Oligosaccharides were dried and were subjected to MALDI/TOF MS after reduction and permethylation as described below [12].

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF)

The reduced and permethylated oligosaccharides were subjected to positive ion matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a Bruker REFLEX time-of-flight (TOF) instrument equipped with delayed-extraction and reflectron systems and a N2 laser (337 nm) operating with 3 ns pulse width and 10^7–10^8 W/cm^2 irradiance at the surface of 0.2 mm^2 spots. One microliter of samples containing equal volumes of reduced and permethylated oligosaccharide solution (~10 pmol/µl) and α-cyano-4-hydroxycinnamic acid matrix was mixed and spotted on the target.

Results

Patients and samples

Diagnosis of CDA II was established in 15 patients from 13 unrelated families by bone marrow cytology, increased erythrocyte agglutination by anti-i sera, and a positive acid lysis test as described by Heimpel *et al.* [4]. All patients are included in the recent report of the German CDA registry [4]. Four families are of Italian, Greek, Turkish and Spanish origin. Seven blood samples of healthy controls and five blood samples of neonates (3–5 days of age) were used for either lectin binding studies or structural analysis of *N*-glycans.

Blood cells for *in vitro* studies were obtained by venipuncture after informed consent. EDTA was used as anticoagulant. Six CDA II patients had previously been splenectomized. Blood was shipped without cooling and processed not later than 36 h.

SDS-Page with and without PNGase F digestion

Erythrocyte membranes were investigated using SDS-gel electrophoresis (Fig. 1a). Healthy controls showed a broad band 3 which is due to the heterogeneity of the attached *N*-glycan. Band 3 of all 15 CDA II patients was narrower and had an increased mobility in the gel. PNGase F digestion of erythrocyte membrane ghosts, cleaving *N*-glycans from the protein, resulted in band 3 with identical apparent molecular weight in patients and healthy controls thus indicating that the migration difference was exclusively caused by the N-linked carbohydrate attached to band 3. The average apparent molecular weight of band 3 was decreased by 4,500 Da in CDA II patients compared to 9,500 Da in healthy controls. Ten obligatory heterozygous gene carriers showed no obvious abnormalities in band 3 mobility (data not shown).

Lectin binding

Tomato lectin is proposed to have a high affinity to polylactosamines ([-3Galβ1–4GlcNacβ1–]*n*) [13, 14] and binds strongly to normal human erythrocyte membrane proteins. Western blot analysis using tomato lectin showed a marked decrease of lectin binding to erythrocyte membrane proteins of all 15 CDA II patients investigated (Example in Fig. 1c). Integrated density analysis of tomato lectin binding to band 3 revealed a mean residual binding of CDA II patients of 7% (SD 2.9) compared to healthy controls.

In order to investigate the influence of polylactosamine β1-6-branching on the tomato lectin binding, we performed Western blots using erythrocyte samples of neonates, known to carry linear and only negligible amounts of branched polylactosamines [15]. Surprisingly, binding of tomato lectin to erythrocyte membrane proteins of healthy neonates was completely absent although the mobility of band 3 in SDS-PAGE was identical to healthy controls (Fig. 1c), revealing that branching of polylactosamines is crucial for tomato lectin binding.

Additionally, we performed Western-blots with Concanavalin A (Con A) a lectin preferentially binding to immature high mannose glycans that are present before early Golgi processing has been completed [16]. The presence of high mannose glycans on membrane proteins indicates incomplete early Golgi processing. In 11 of the 15 CDA II patients, Con A showed increased binding to band 3. The intensity of Con A binding was variable, indicating variable amounts of high mannose glycans in different patients. The intensity of Con A binding to membrane preparations of healthy controls was considerably lower even though a slight Con A binding could be detected (Fig. 1b).

The amount of core fucosylation bound in α1-6-linkage to the first GlcNAc of *N*-glycans can be investigated using *Aleuria aurantia* lectin and reflects a median Golgi processing step [17]. Western blot analysis of CDA II erythrocyte membranes showed a strong decrease of AAL binding compared to healthy controls (Fig. 1d). To rule out systematic errors due to lectin binding to oligosaccharides coding for blood group characteristics, the ABO blood group of every sample was determined. No association between blood groups and binding of the respective lectins was observed.

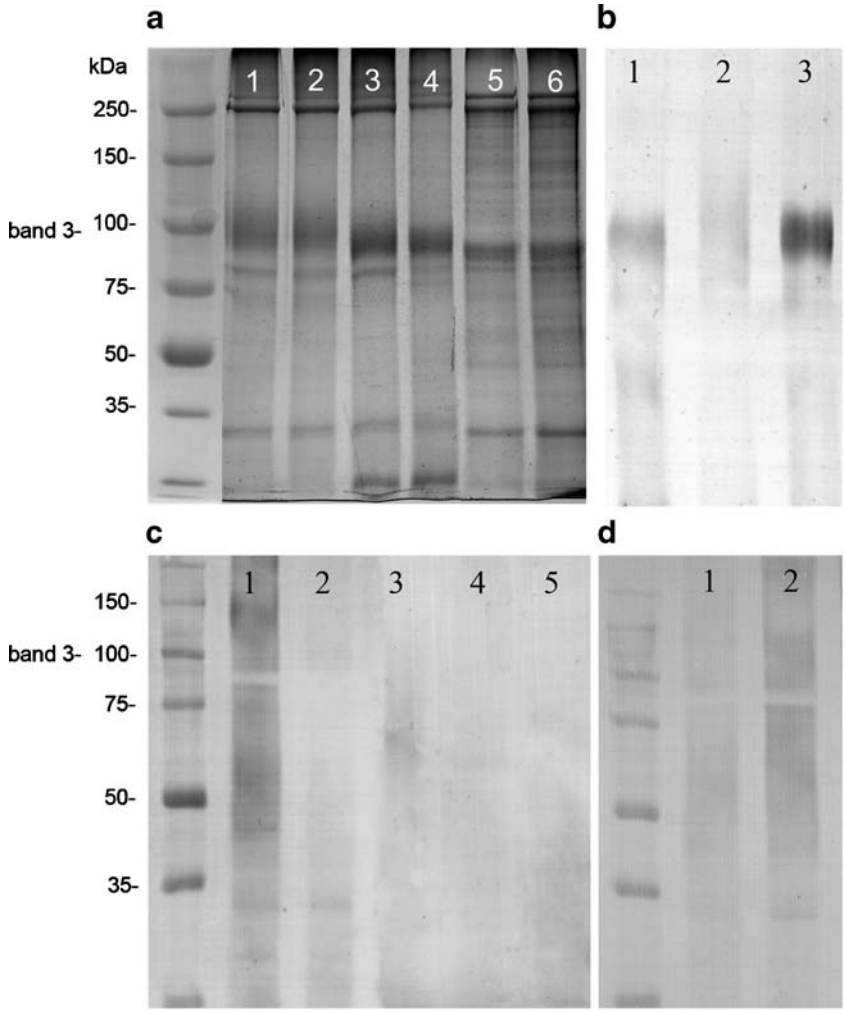

Fig. 1 SDS-PAGE, lectin and antibody binding to erythrocyte membrane proteins detected on western blot. **a** SDS-PAGE from two healthy controls [1, 2] and two CDA II patients [3, 4]. Mobility shift of band 3 after release of the *N*-glycans by PNGase F of a healthy control [5] and a CDA II patient [6]. **b** Western blot probed with Concanavalin A marking high mannose glycans. Two CDA II patients [1, 3] and a healthy control [2]. **c** Tomato lectin binding to erythrocyte membrane probing branched polylactosamines: Healthy control with a broad binding due to several glycoproteins and some glycolipids carrying polylactosamines [1]; two CDA II patients [2, 3]; healthy neonate [4]; control after cleavage of *N*-glycans using PNGase F treatment [5]. **d** Binding of *Aleuria aurantia* lectin to erythrocyte membrane proteins showing core fucosylation: CDA II patient [1] and healthy control [2]

MALDI-TOF analysis

Oligosaccharides were released from erythrocyte band 3 by in gel digestion and MALDI-TOF analysis of the desialylated and permethylated band 3 derived oligosaccharides of a 3 year old male CDA II patient (German CDA II registry, patient number UPN: 471/01) and a healthy control was performed. The oligosaccharide profiles revealed three major differences: (1) the existence of unprocessed high mannose glycans in CDA II band 3 glycans, (2) the presence of hybrid glycans in CDA II and (3) a markedly reduced size of band 3 derived *N*-glycans in CDA II (Fig. 2, Table 1). Glycans larger than 5 kDa were detectable only in the control. However, in MALDI-TOF spectra higher-molecular-weight structures are known to be detected at lower intensities due to a decreased signal to noise ratio and did not allow a quantitative evaluation of these glycans. The prevailing complex type *N*-glycans (8 and 9 in Fig. 2) were additionally analyzed by ESI-MS/MS (data not shown). Both carried a bisecting GlcNAc attached to the core mannose of the glycan. A fucose was attached to structure 9 which could be clearly identified as core fucose in the control as well as the CDA II patient.

Discussion

A mobility shift of erythrocyte membrane glycoproteins band 3 and band 4.5 [7, 18] is one of the key findings in CDAII. PNGase F digestion of CDA II erythrocyte membrane proteins, exclusively cleaving N-linked oligosaccharides, resulted in band 3 of apparent molecular weight identical to healthy controls, suggesting an abnormal *N*-glycosylation, whereas *O*-glycosylation seems to be negligible.

In the past, three different biochemical defects in the *N*-glycosylation pathway were proposed to cause for CDA II on the basis of enzyme activity data, western or southern blots, or expression studies [19–21]. All proposed defects were affecting glycosyltransferases. However, no mutation was found in any of the genes. In addition, a linkage analysis of seven Italian CDA II families mapped the CDA II locus to

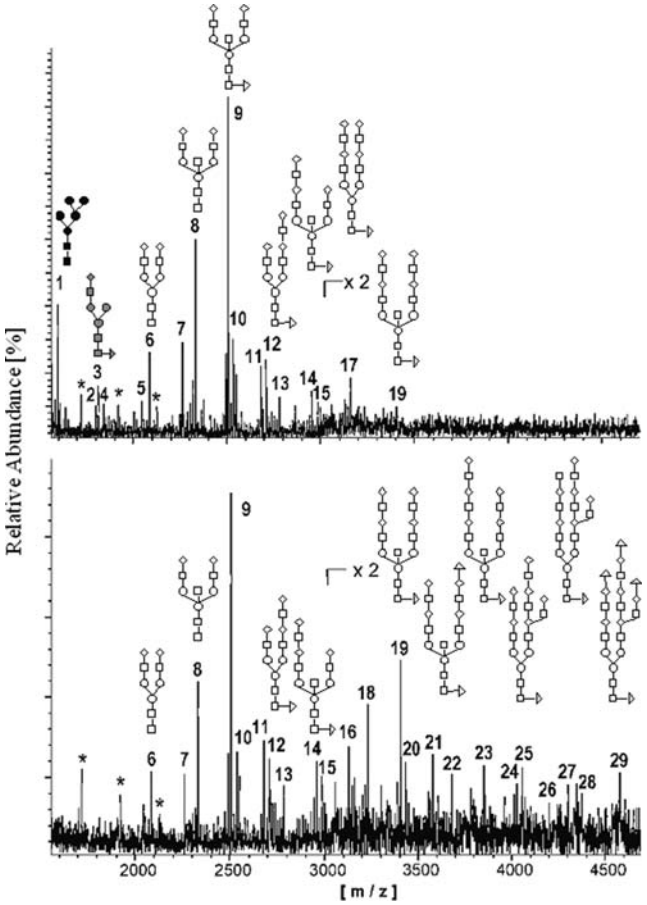

Fig. 2 MALDI-TOF analysis of *N*-glycans derived from erythrocyte band 3. *Upper panel* CDA II patient, *lower panel* healthy control. Proposed structures of some *N*-glycans are displayed above the peaks. Presence of high-mannose and hybrid glycans and truncation of complex type glycans on the patient's band 3. Mass and proposed structures of all peaks are given in Table 1. *Empty square* GlcNAc, *empty circle* mannose, *empty diamond* galactose, *triangle* fucose. * Signal from a contaminating hexose oligomer

chromosome 20q11.2 [22], thereby excluding the formerly proposed genes [23].

Up to now no gene known to be involved in *N*-glycosylation could be located to the mapping area. In addition several patients show no linkage to chromosome 20q11.2 [3, 23]. Recently, seven genes in the mapping area were sequenced and could be excluded as underlying defect for CDA II [24]. Although a *N*-glycosylation defect is common to all CDA II patients, the molecular basis of the disease and the affection of *N*-glycosylation is still unknown.

Erythrocyte band 3 has one *N*-glycosylation site carrying a large complex type oligosaccharide, which presents branched polylactosamines after the neonatal period in healthy subjects constituting the I-phenotype [25]. We found that tomato lectin, preferentially binding to polylactosamines [13, 14], showed negligible binding to erythrocyte membranes of CDA II patients, whereas healthy controls showed strong lectin binding. Tomato lectin almost exclusively binds to N-linked glycans since the enzymatic release of *N*-glycans using PNGase F completely abolished the binding to control samples.

This study revealed that tomato lectin did not bind to erythrocyte membrane proteins of newborn children containing large, but linear polylactosamines (i-phenotype) [15] providing evidence that branched polylactosamine structures are the binding target of tomato lectin. Decreased tomato lectin binding of CDA II membrane proteins, therefore reflect a decrease in the branching of polylactosamines.

Up to now, only limited structural data about CDA II band 3 derived *N*-glycans were available. All studies carried out so far used ionization methods leading to the

Table 1 Assignment of the peaks detected by MALDI/TOF–MS (Fig. 2) of the desialylated reduced and permethylated *N*-glycans isolated from erythrocyte band 3 (average masses are given) from a HEMPAS patient and a control person (lower panel of Fig. 2)

$[M+Na]^+$	Composition	Proposed Structure
1) 1596:	H_5 HN HNol	High mannose type (Man_5)
2) 1800	H_6 HN HNol	High mannose type (Man_6)
3) 1810:	H_4 HN_2 dH HNol	Hybrid type+F (Man_3)
4) 1841:	H_5 HN_2 HNol	Hybrid type (Man_4)
5) 2045:	H_6 HN_2 HNol	Hybrid type (Man_5)
6) 2086	H_5 HN_3 HNol	Diant.
7) 2261	H_5 HN_3 dH HNol	Diant.+F
8) 2332	H_5 HN_4 HNol	Diant.+bisect. GlcNAc[a]
9) 2506	H_5 HN_4 dH HNol	Diant.+F+bisect.GlcNAc[a]
10) 2536	H_6 HN_4 HNol	Diant.+Repeat
11) 2680	H_5 HN_4 dH_2 Hnol	Diant.+2F+bisect. GlcNAc
12) 2710	H_6 HN_4 dH HNol	Diant.+F+R
13) 2781	H_6 HN_5 HNol	Diant.+R+bisect. GlcNAc
14) 2956	H_6 HN_5 dH HNol	Diant.+R+F+bisect. GlcNAc
15) 2985	H_7 HN_5 HNol	Diant.+2R
16) 3131	H_6 HN_5 dH_2 HNol	Diant.+R+2F+bisect. GlcNAc
17) 3159	H_7 HN_5 dH HNol	Diant.+2R+F
18) 3231	H_7 HN_6 HNol	Diant.+2R+bisect. GlcNAc
19) 3405	H_7 HN_6 dH HNol	Diant.+2R+F+bisect. GlcNAc
20) 3435	H_8 HN_6 HNol	Diant.+3R
21) 3580	H_7 HN_6 dH_2 HNol	Diant.+2R+2F+bisect. GlcNAc
22) 3681	H_8 HN_7 HNol	Diant.+3R+bisect. GlcNAc
23) 3855	H_8 HN_7 dH HNol	Diant.+3R+F+bisect. GlcNAc
24) 4029	H_8 HN_7 dH_2 HNol	Diant.+3R+2F+bisect. GlcNAc
25) 4059	H_9 HN_7 dH HNol	Diant.+4R+F
26) 4203	H_8 HN_7 dH_3 HNol	Diant.+3R+3F+bisect. GlcNAc
27) 4305	H_9 HN_8 dH HNol	Diant.+4R+F+GlcNAc
28) 4377	H_8 HN_7 dH_4 HNol	Diant.+3R+4F+bisect. GlcNAc
29) 4581	H_9 HN_7 dH_4 HNol	Diant.+4R+4F

Proposed structure considering the data of Fukuda *et al.* [15, 25]
H hexose, *HN N*-acetylhexosamine, *dH* deoxyHexose, *HNol* reduced *N*-acetylhexosamine, *Diant*. diantennary complex type structure, *F* fucose, *GlcNAc N*-acetylglucosamine, *R N*-acetyllactosamine repeat.
[a] These structures were further analysed by ESI-MS/MS: Fucose, when present, was found to be predominantly linked to the proximal GlcNAc residue, and the additional GlcNAc residue to the inner mannose of the common core (bisecting GlcNAc) in both structures.

fragmentation of the carbohydrates. All proposed structures are therefore results of reconstruction processes [9, 20, 21].

To prevent the oligosaccharides from fragmentation we performed MALDI-TOF analysis of *N*-glycans released from band 3. The analysis revealed a broad spectrum of small, medium and large polylactosamine type structures in the control (Fig. 2), whereas the largest *N*-glycans released from CDA II band 3 were severely truncated, predominantly showing only one or two lactosamine repeats.

Recently, compositional analysis of glycans attached to band 3 of a CDA II patient showed an average *N*-glycan mass of about 3 kDa [26]. The structural data of our MALDI-TOF spectra showed a main peak at 2,506 Da, which would shift to 2,867 and 3,228 Da in its mono- or its desialylated native form, in the CDA II patient corresponding well with the former compositional analysis.

MALDI-TOF analysis did not detect very large polylactosamines in healthy controls as was expected from SDS-PAGE showing an average band 3 mobility shift of about 9,500 Da in healthy control after PNGase F digestion. This can be ascribed to the physicochemical properties of large polylactosamine which are less efficiently detected by MALDI-TOF. Moreover, the exact estimation of glycan masses by SDS-PAGE is rather imprecise.

In accordance with the Con A binding data, MALDI-TOF/MS showed the presence of high mannose (1,596 Da, 1,800 Da, Fig. 2) and hybrid glycans (1,810 Da, 1,841 Da, 2,045 Da, Fig. 2) in CDA II. Although the presence of high mannose glycans on erythrocyte band 3 was a consistent finding in CDA II patients detected by Con A staining, the quantity of these glycoforms was variable and low compared with the total amount of *N*-glycans of band 3. The truncation of erythrocyte *N*-glycans was the predominant finding in CDA II.

α1,6 linkage of fucose to the reducing GlcNAc of the *N*-glycan (core fucosylation) is a frequent but optional modification of numerous *N*-glycans [27]. α1,6 fucosyltransferase residing in the medial Golgi (Fut8) exclusively catalyzes this reaction. The core fucosylation of erythrocyte membrane proteins was decreased in CDA II patients as assayed by *Aleuria aurantia* lectin binding in western blots. In accordance with this, Zdebska *et al.* described a decreased fucose content of CDA II band 3 compared to healthy controls. However, Zdebska *et al.* did not investigate the specific glycosidic linkage of fucose as has been done in this study [26]. The same group could demonstrate, that obligatory heterozygous gene carriers also have a glycosylation phenotype that lies between healthy controls and CDA II patients [28].

The lectin binding studies indicated a decrease of core fucosylation whereas MALDI-TOF analysis revealed the presence of fucose in many band 3-derived oligosaccharides without providing information about the localization and linkage of fucose. The investigation of two major MALDI-TOF peaks by ESI-MS/MS showed that control and CDA II band 3 derived *N*-glycans were both core fucosylated and carried fucose coding for blood group determinants in comparable amounts.

The MALDI-TOF data revealed that the decreased binding of *Aleuria aurantia* lectin to CDA II erythrocytes is most probably a secondary effect due to the general shortening of the *N*-glycans. Core-fucosylation was most common, in large band 3 *N*-glycans of the healthy control which were absent in CDA II, whereas high mannose and hybrid type glycans, predominantly lacking proximal fucose, were present only in CDA II patients. Therefore, the detection of reduced *Aleuria aurantia* lectin binding might be due to a defect of median Golgi processing where the α1–6-fucosyltransferase is localized or is due to the decrease of complex type glycans in CDA II that are the preferred binding partner of AAL [17], if core fucosylated.

Increased binding of Con A to CDA II erythrocytes as well as the MALDI-TOF data demonstrate the presence of high mannose and hybrid glycans that are subject to early Golgi processing. In recent years the detection of high mannose and hybrid glycans lead to the suggestion of a defect in early Golgi processing steps and defects of distinct glycosyltransferases that could not be genetically proven [19–21].

However, MALDI-TOF data of band 3 derived *N*-glycans that are in accordance with the lectin binding studies of total erythrocyte membrane proteins provide evidence for a Golgi processing defect affecting *cis*- to *trans*-Golgi processing with a severe truncation of *N*-glycans as the major finding.

Regulation of *N*-glycosylation is complex and processing is supposed to be mainly regulated by the expression levels of different glycosyltransferases and their activities as well as competition for common substrates. Several regulatory effects on enzyme activities and creation of stop signals by competing glycosyltransferases are known [29–31].

A mutation in the band 3 ortholog of the zebrafish could be demonstrated to cause dyserythropoesis similar to CDA II [32]. The defect resulted in a decreased band 3 glycosylation not affecting the anion-exchange activity of the band 3 protein. The defect led to a differentiation arrest at the late erythroblast stage and the authors concluded that its N-terminal cytoplasmic domain plays a structural role in erythroid cytokinesis. Band 3 null mutants showed no dyserythropoesis. No mutations could be found in the human band 3 gene in CDA II patients. However, this cellular defect emphasizes the possible influence of altered band 3 glycosylation on erythroid cytokinesis.

In summary, our structural data of band 3-derived oligosaccharides using MALDI-TOF and lectin studies as well as the mobility shift of band 3 in SDS-PAGE upon

PNGase F digestion revealed that oligosaccharides on CDA II band 3 are markedly truncated. On the other hand MALDI-TOF of *N*-glycans showed no predominant structure pinpointing towards a specific glycosylation deficiency as present in other inherited disorders [10, 33]. Instead, a variety of immature oligosaccharides like high mannose, hybrid, and truncated complex type oligosaccharides were found representing several disturbed glycan processing steps from *cis*- to *trans*-Golgi.

As proposed in the past, the truncation of *N*-glycans in CDA II might be caused by mutations in glycosyltransferases. In fact, glycoproteins derived from mice embryos defect in α-mannosidase II activity show a tissue glycosylation pattern with increased hybrid glycans and decreased complex glycans [34] due to the partial compensation by α-mannosidase IIx. However, the oligosaccharide structural data with a defect starting with early Golgi processing but including processing steps located in the middle- and *trans*-Golgi associated with erythroblast differentiation and maturation containing a disturbance in the loss of organelles involved in *N*-glycosylation implicate a defect of the glycosylation apparatus in CDA II erythroblasts. Disorders affecting multiple glycosylation steps and that might be exemplary for a CDA II defect have recently been described [35–38]. Mutations in the multimeric conserved oligomeric Golgi-complex (COG) lead to altered retrograde transport kinetics of resident Golgi proteins ultimately affecting transport kinetics of nucleotide-sugar transporters and glycosyltransferases. As a result different glycosylation pathways are affected. We suggest that the glycosylation deficiency in CDAII bears analogy to COG-deficiency, where glycosylation is affected due to a defect in the glycosylation apparatus, but not due to a defect in a specific glycosyltransferase.

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