The Cisternae Decorating the Red Blood Cell Membrane in Congenital Dyserythropoietic Anemia (Type II) Originate From the Endoplasmic Reticulum


We studied 20 individuals from 17 unrelated families with congenital dyserythropoietic anemia (type II; CDAII). The clinical phenotype was mildly to moderate. The inheritance pattern was invariably recessive. Coomassie blue stained gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) show that band 3 was thinner and migrated slightly faster than usual. In addition, staining showed two unknown minor bands (in the patients), but not in normal controls, the obligate carrier parents, or in patients with other anemic syndromes. These minor proteins were studied using partial digestion, amino acid sequencing, Western blotting, immunofluorescence, and immunogold electron microscopy. They were identified as the glucose-regulated protein GRP78 and calreticulin that are resident proteins of the endoplasmic reticulum (ER). Using specific antibody, we showed that protein disulfide isomerase (PDI), a third major protein of the ER, was also present on the SDS-PAGE of red blood cell (RBC) ghosts. Immunofluorescence colocalized PDI with the dense discontinuous ring decorating the RBC membrane. Immunogold electron microscopy showed that PDI was localized in the lumen of the cisternae, confirming that these originate from the smooth ER. From a practical point of view, screening the above minor proteins in RBC membranes appears to be straightforward and reliable diagnostic test for CDAII.

© 1996 by The American Society of Hematology.

CASE REPORTS

We investigated 20 CDAII patients from 17 unrelated families originating from France (1 family), Italy (13 families), and Spain (3 families; Table 1). In Italy, all but 1 of the probands resided in the southern part of the country. In most cases, anemia was diagnosed early in childhood based on classical criteria. The parents were clinically normal and presented with no detectable change in the RBC indices. The father, ROI.1, and the mother, ROI.2, of children ROI.1 and ROI.2 were also investigated biochemically as obligate CDAII carriers. We had no proof that consanguinity existed, although this was suspected in some families. In addition to samples from normal individuals, we investigated samples from patients with CDAI (unpublished data), hereditary spheroctysis (HS), and hereditary elliptocytosis (HE), respectively.

From CNRS URA 1171, Institut Pasteur de Lyon, Lyon, France; the Service Central d’Analyse, CNRS, Vernaison, France; the Service de Pédiatrie, Centre Hospitalier, Oyonnax, France; the Département de Pédiatrie, Hôpital ‘12 de Octubre’, Instituto Nacional de la Salud, Carretera de Andalucia, Madrid, Spain; INSERM U91, Hôpital Henri-Mondor, Créteil, France; and INSERM U362, Institut Gustave Roussy, Villejuif, France.

Submitted July 10, 1995; accepted January 8, 1996.

Supported by the ‘Institut Pasteur de Lyon,’ the ‘Centre National de la Recherche Scientifique’ (URA 1171), the ‘Université Claude Bernard Lyon-I, the ‘Institut National de la Santé et de la Recherche Médicale’ (CRE 930405 and INSERM U91), the ‘Conseil de la Région Rhône-Alpes,’ the ‘Association Française contre les Myopathies,’ the ‘Association de Recherche contre le Cancer,’ and Grant, DEP90-0227 from the ‘Comité de Investiga’ion Científica y Desarrollo Tecnológico.’

Address reprint requests to N. Alloisio, PhD, CNRS URA 1171, Institut Pasteur de Lyon, avenue Tony Garnier, 69365 Lyon Cedex 07, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8710-0002$3.00/0

Among the HS patients (unpublished results), several displayed a reduction in band 3, as has been presented by others.\textsuperscript{27,33}  

\section*{MATERIALS AND METHODS}

\subsection*{Protein Chemistry}

Erythrocyte ghost protein analysis was performed using SDS-PAGE according to Laemmli\textsuperscript{34} (acrylamide concentration, 7%). Fifteen micrograms of protein, assayed using the Lowry procedure, was loaded in each lane. We verified that the surface area of all of the Coomassie blue-stained peaks was very similar for each sample. Peak areas of individual major proteins (spectrin, protein 4.1, protein 4.2, and actin, but not P-spectrin and ankyrin, which comigrated on the Laemmli gels) were expressed with respect to all membrane protein peaks. However, to improve the estimate, we subtracted the optical density of the corresponding area from the membranes, and microsequenced with an Applied Biosystems Model 470A gas-phase sequenator (Model 470A; Applied Biosystems Inc, Foster City, CA) coupled on line with a PTH-amino acid analyzer (Applied Biosystems Inc), as previously described.\textsuperscript{36} However, because we were unable to sequence the 74-kD protein (presumably due to blocked N-terminal residue), we performed in situ protease cleavage on the PVDF membrane, followed by peptide separation using reverse-phase high-performance liquid chromatography (HPLC).\textsuperscript{37} Specifically, tryptic digestion (trypsin-TPCK Worthington Biochemical Corp, Freehold, NJ) was performed for 6 hours at 37°C. The supernatant and the five subsequent washing solutions (about 600 $\mu$L) were loaded on a C8 reverse-phase column (0.46 \texttimes 22 cm; Applied Biosystems Inc.), and the peptides were eluted with a linearly increasing gradient of acetonitrile in 0.05% trifluoroacetic acid. Peptides were selected for amino acid sequencing on the basis of peak height and resolution.  

Amino acid sequence comparisons were performed using the FASTDB Computer program of Intelligenetics (Mountain View, CA). Two protein data bases were screened: the Protein Identification Resources (release 30) from the National Biochemical Research CA). Two protein data bases were screened: the Protein Identification Resources (release 30) from the National Biochemical Research Foundation and the Genbank (release 70).  

\subsection*{Western Blots}

Western blots were performed after SDS-PAGE.\textsuperscript{38} Equivalent amounts of proteins (15 $\mu$L) were loaded on the gel for each sample. In addition, we assessed that equivalent amounts of proteins were transferred onto the nitrocellulose membrane using Ponceau S red staining by comparing the intensities of the invariant protein bands: spectrin, proteins 4.1 and 4.2, and actin. We used (1) a monoclonal
Fig 1. SDS-PAGE and Western blots of red blood cell membrane proteins. (Lane a) Coomassie blue staining. (*) Band 3 was thinner and migrated slightly faster than normal band 3. The 74-kD and the 58-kD components were GRP78 and calreticulin, respectively. The open arrow indicates the faint band shown in lane c (GRP947). (Lane b) Western blot using anticalreticulin polyclonal antibodies. (Lane c) Western blot using an anti-GRP78 monoclonal antibody. The open arrow indicates a nonspecific reactivity with a-spectrin. (Lane d) Western blot using anti-PDI polyclonal antibodies. (Lane e) Western blot using an anti-PDI monoclonal antibody showing both PDI and calreticulin. C, control; PB, patient (see Table 1).

antibody (dilution 1/500) against a synthetic peptide of rat GRP78 exhibiting cross-reactivity with human GRP78 and GRP94 (Stress Gen Biotechnologies Corp, Victoria, British Columbia, Canada), (2) a polyclonal rabbit antibody (dilution 1/100) against calreticulin (courtesy of Dr W. Kiesel, Albuquerque, NM), (3) a polyclonal rabbit antibody (dilution 1/1,000) against bovine PDI exhibiting a cross-reactivity with human PDI (Stress Gen Biotechnologies Corp), and (4) a monoclonal antibody (dilution 1/500) against a synthetic peptide of bovine PDI exhibiting a cross-reactivity with human PDI and human calreticulin (Stress Gen Biotechnologies Corp).

**Phase Contrast Microscopy**

Red blood cells from a control and from a CDAII patient (PMII. 6) were lysed using distilled water and examined using phase-contrast microscopy as previously described.

**Immunofluorescence Microscopy**

Red blood cells from a control and a CDAII patient (PMII.6) were cytospun on a slide and fixed for 5 minutes using methanol. The monoclonal antibody against PDI, diluted at 1/250, was incubated with the samples at room temperature for 30 minutes. After three washes in phosphate-buffered saline (PBS), a fluorescein-conjugated antimouse IgG sheep Fab\(^{\text{c}}\) (Silenus, Hawthorn, Australia) was applied at a 1/100 dilution for 30 minutes. After washing and mounting, cells were examined under a fluorescence microscope.

**Immunogold Electron Microscopy**

Normal and pathologic (from patient PMII.6) red blood cells were prepared for immunogold electron microscopy by fixing them in 1% glutaraldehyde in 0.1 mol/L phosphate buffer (PB), pH 7.4, for 1 hour at 22°C, washing them three times with the same buffer, embedding them in 15% polyvinylpyrrolidone (PVP; Sigma, St Louis, MO; 0.3 mmol/L PVP, 1.1 mol/L Na\(_2\)CO\(_3\), 2 mol/L sucrose in PB), and freezing them in liquid nitrogen. The immunochemical reactions were performed on ultra-thin sections collected on copper grids according to the method of Slot et al. Briefly, the sections were labeled by a first incubation with the monoclonal antibody against PDI used at 1/500 dilution in PBS containing 1% of bovine serum albumin.

### Table 2. Amino Acid Sequencing Data

<table>
<thead>
<tr>
<th>Molecular Weight (kD)</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>74*</td>
<td>NDPSVQDD</td>
</tr>
<tr>
<td></td>
<td>(S)IFSTASDNOPTV</td>
</tr>
<tr>
<td></td>
<td>?ELE(S)YAIYS</td>
</tr>
<tr>
<td></td>
<td>D(A)GT(I)(A)GLNVMR</td>
</tr>
<tr>
<td></td>
<td>VMEH(FJILK)</td>
</tr>
<tr>
<td></td>
<td>N-ter XPXVFKEQFLDG</td>
</tr>
<tr>
<td>58*</td>
<td></td>
</tr>
</tbody>
</table>

The data correspond to the partial amino acid sequences of the 74- and 58-kD components seen in CDAII samples. Question marks indicate the positions at which residues could not be identified with certainty. Residues in parentheses are the most probable assignment.

* Molecular weight as determined on our gels.
Fig 2. Serial Western blot analysis in CDAII patients and various controls. Polyclonal antibodies against human calreticulin was used. Two determinations were performed with an increased staining revelation time regarding the bottom gel, which explains the higher intensity of the bands. Red blood cell membrane samples from all CDAII patients displayed a positive reaction, but this was stronger in splenectomized patients (*). No reaction was observed with membrane samples from normal controls (C1, C2, C3, and C4) or with samples from parents RO1.1 and RO1.2 (CDAII obligate carriers) of children RO1.1 and RO1.2.

albumin (Sigma) for 20 minutes at 22°C, washed, and incubated with goat antimouse antibodies (IgG) coupled to 10-nm gold particles (Bio Cell, Cardiff, UK) used at 1/100 dilution in PBS containing 1% bovine serum albumin for 20 minutes at room temperature. The sections were then counterstained with 2% uranyl acetate, pH 7.0, and methyl cellulose uranyl and samples were observed using a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands). Some additional samples were prepared using a monoclonal antibody against band 3 (Sigma), which was diluted 1/50.

RESULTS

Protein Chemistry

The samples from all the patients with CDAII had a thinner band 3 than usual that also migrated slightly faster on SDS-PAGE, as has been previously reported (Fig 1). Nonetheless, in agreement with a previous publication, the amount of band 3, measured through Coomassie blue staining, was within the normal range (band 3/total spectrin ratio 1.08 ± 0.09 [n = 20] v 1.10 ± 0.07 in normal controls [n = 27]). However, a recent report of two CDAII cases showed that band 3 was present in quantities ranging from 60% to 75% of normal. In the CDA I sample, band 3 was qualitatively and quantitatively normal (data not shown). In the sample HS with band 3 deficiency, there was 20% to 40% less band 3, but the band retained its normal width and position on SDS-PAGE (data not shown).

In all CDAII patients, two minor proteins of 74 and 58 kD were observed on Coomassie blue-stained gels (Fig 1). The amount of these proteins represented 0.7% to 1.7% (74-kD protein) and 1% to 2% (58-kD protein) of the total normal membrane proteins. The 74-kD band was not present in normal controls; in CDAII obligate carriers (individuals RO1.1 and RO1.2); in patients presenting with CDAI, HS (with or without band 3 deficiency), and various cases of HE (data not shown). In all these individuals, a very faint 58-kD band could be seen; however, it did not react with anticalreticulin antibodies (see below and Fig 1).

Amino acid sequencing of the N-terminal region of the 58-kD component and sequencing of several peptides obtained after partial digestion of the 74-kD component yielded the data presented in Table 2. Homology searches showed that the 74-kD protein was the glucose-regulated protein referred to as GRP78. This protein belongs to the family of heat-shock proteins HSP70. The apparent molecular weight (74 kD) that we determined using SDS-PAGE was in good agreement with those reported previously and with the actual molecular weight of GRP78 (73 kD). The 58-kD protein was calreticulin, a high-affinity Ca2+ binding protein. The molecular weight that we determined (58 kD) was substantially different from the actual molecular weight of the protein (48 kD). This aberrant electrophoretic mobility has been noted before and is attributed to the high acidic amino-acid content of calreticulin. Both GRP78 and calreticulin are major resident proteins of the ER lumen.

Western Blots

Calreticulin. Polyclonal antibodies against human calreticulin reacted strongly with CDAII samples but not with...
normal samples (Fig 1). This reaction was observed with samples from all CDAII patients (Fig 2), although the intensity of the response was variable. Two subgroups of patients emerged depending on whether splenectomy had been performed. Splenectomized patients yielded a stronger response than did nonsplenectomized patients. As shown below, calreticulin reflects the presence of cisternae of the ER. It may be assumed that the red blood cells that are the richest in ER were those most readily destroyed by the spleen. In the CDAII obligate carriers RO 1.1 and RO 1.2, the antibodies failed to show calreticulin (Fig 2). This fact emphasizes the strictly recessive inheritance pattern of CDAII.

**GRP78.** Monoclonal antibody against rat GRP78 showed a strong band in CDAII samples but not in normal samples (Fig 1). A faint additional band was also shown in CDAII samples only. This band may correspond to GRP94, a third major protein of the ER lumen, based on its electrophoretic mobility and its cross-reactivity with the monoclonal antibody, as reported by the manufacturer (Stress Gen Biotechnologies Corp). A band migrating just above band 3 in CDAII patients could be seen on the Coomassie blue-stained gels and this possibly corresponds to GRP94 (Fig 1). In controls, this region was blurred by the larger band 3, but we can expect that there is no band. Unexpectedly, the anti-GRP78 monoclonal antibody displayed a strong cross-reaction with α-spectrin, although the manufacturer had not reported this; therefore, the possibility that the 94-kD band is a spectrin degradation product that, for an undetermined reason, is present in CDAII membranes and not in controls cannot be disregarded, although it sounds very unlikely.

**PDI.** Besides calreticulin and GRP78 (and GRP94), the ER contained another major protein, the PDI. Specific polyclonal antibodies against PDI showed a strong reaction with CDAII samples only (Fig 1). The measured molecular weight (59 kD) of the band is close to the actual molecular weight (57 kD) of PDI. This protein migrated just above calreticulin in our hands. As stated by the manufacturer, the monoclonal antibody against PDI cross-reacts with calreticulin (Fig 1). There was less PDI than calreticulin in Western blots, which may explain why only one band (calreticulin) was clearly seen on the Coomassie blue-stained gels.

**Phase Contrast and Immunofluorescence Microscopy**

In all normal red blood cell ghosts, the cell membrane had a uniform contrast. However, in CDAII red blood cell ghosts, many cells exhibited a discontinuous peripheral dense ring that was often split into several fragments (Fig 3A), as has been reported previously.

The majority of CDAII red blood cells were labeled using an anti-PDI monoclonal antibody. A long cisternae was located beneath the CDAII red blood cell membrane. The ER lumen was heavily labeled with the gold particles coinciding with the presence of a gray substance; when the lumen appeared clear (no grey substance), no labeling was observed.

**Immunogold Electron Microscopy**

Using the anti-PDI monoclonal antibody, a strong and specific labeling was observed only in the lumen of the ER when dense material was preserved (Fig 3C). No gold particles were seen where the lumen was clear, on the cell membrane, or between the latter and the ER. When the cisternae were dilated, dense material concentrated at one or both extremities and the labeling was restricted to these zones. In many red blood cells, one or two globular structures were heavily labeled (data not shown). They could correspond to the dense granules observed by phase contrast and to the dots seen by immunofluorescence. There was no specific labeling in the control.

Using an anti-band 3 antibody in CDAII red blood cells, a weak but specific labeling was present both in the cisternae of ER and in the cell membrane. This usually appeared to be close to the ER membrane or to the plasma membrane (data not shown).

**DISCUSSION**

We have detected three additional minor proteins that are all resident proteins of the ER lumen associated with CDAII red blood cell membranes: GRP78, calreticulin, and PDI. Using a variety of microscopic techniques, we showed that PDI is localized inside the cisternae of the ER, which are variable in shape and number and run parallel to and beneath the plasma membrane. This provides formal evidence that the cisternae associated with CDAII originate from the ER, as has been previously suggested.

![Image](https://via.placeholder.com/150)
It was interesting that some band 3 molecules also appeared at the level of the cisternae membrane and the plasma membrane. The amount of normal band 3 determined using SDS-PAGE suggests that some band 3 molecules are retained in the ER instead of being incorporated in the plasma membrane. If this is so, purified plasma membrane would be expected to be deficient for band 3; further studies will be necessary to investigate this hypothesis.

It remains unclear how all the abnormal features recorded in CDAII can have a coherent explanation. The synthesis of polyglactosaminoglycans increases significantly at the stage of erythroblast. Fukuda has suggested that defects in various glycosyltransferases or glycosidases could affect other glycoproteins besides band 3. Some glycoproteins may be involved in cytokinesis and/or the clearance of intracellular organelles, but no such glycoproteins has been identified.

CDAII is not a rare condition, but it can be wrongly diagnosed, because no easy reliable test exists for its diagnosis. We suggest that the altered characteristics of band 3 and the nassed, because no easy reliable test exists for its diagnosis.

We have shown that proteins of the ER (GRP78, calreticulin, and PDI) migrate with red blood cell membrane proteins in samples from CDAII patients on SDS-PAGE. PDI is located in the lumen of the cisternae that decorate the outer surface of the CDAII erythrocyte membrane. This provides strong evidence that the cisternae, reported a long time ago in CDAII patients, originate from the ER.

ACKNOWLEDGMENT

We thank the patients and their families for their cooperation, Dr W. Kiesel for providing us with specific antibody to human calreticulin, and M.C. Haugh for checking the English wording and syntax, and N. Connan for preparing the manuscript.

REFERENCES

The cisternae decorating the red blood cell membrane in congenital dyserythropoietic anemia (type II) originate from the endoplasmic reticulum

N Alloisio, P Texier, L Denoroy, C Berger, E Miraglia del Giudice, S Perrotta, A Iolascon, F Gilsanz, G Berger and J Guichard