Human Erythrocyte Protein 4.2 Deficiency Associated With Hemolytic Anemia and a Homozygous 40Glutamic Acid→Lysine Substitution in the Cytoplasmic Domain of Band 3 (Band 3\textsuperscript{Montefiore})

By Anne C. Rybicki, Judy J.H. Qiu, Sylvia Musto, Norman L. Rosen, Ronald L. Nagel, and Robert S. Schwartz

Red blood cell (RBC) protein 4.2 deficiency is often associated with a moderate nonimmune hemolytic anemia, splenomegaly, and osmotically fragile RBCs resembling, but not identical to, hereditary spherocytosis (HS). In the Japanese type of protein 4.2 deficiency (protein 4.2\textsuperscript{Nippon}), the anemia is associated with a point mutation in the protein 4.2 cDNA. In this report, we describe a patient with moderate and apparently episodic nonimmune hemolytic anemia with splenomegaly, spherocytosis, osmotically fragile RBCs, reduced whole cell deformability, and abnormally dense cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the proposita’s RBC membrane proteins showed an 88% deficiency of protein 4.2 and a 30% deficiency of glyceraldehyde-3-phosphate dehydrogenase (band 6). Structural and molecular analyses of the proposita’s protein 4.2 were normal. In contrast, limited tryptic digestion of the proposita’s band 3 showed a homozygous abnormality in the cytoplasmic domain. Analysis of the pedigree disclosed six members who were heterozygous and apparently episodic nonimmune hemolytic anemia associated with a point mutation in protein 4.2. The molecular lesion responsible for this deficiency is associated with the homozygous state for band 3\textsuperscript{Montefiore} (40glutamic acid→lysine) and a decreased RBC membrane content of protein 4.2. We speculate that band 3 structural abnormalities can result in defective interactions with protein 4.2 and band 6, and in particular, that the region of band 3 containing 40glutamic acid is involved directly or indirectly in interactions with these proteins.

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MATERIALS AND METHODS

Clinical studies. After obtaining informed consent, venous blood was drawn from the proposita and hematologically normal individuals. Blood from the proposita was studied at several times, approximately 2 months before splenectomy, and then at approximately 5 months, 11 months, 17 months, and 22 months after splenectomy. For some studies, blood was also drawn from race-matched controls and the pedigree. Routine hematologic parameters were determined...

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RBC osmotic fragility, whole RBC deformability (osmotic gradient
ekactometry) and density gradient separation. RBC osmotic fra-
gility was performed on heparinized blood that had been incubated
at 37°C for 24 hours.1 Whole RBC deformability was measured as a
continuous function of suspending medium osmolality using an
ekactometer (Technicon Instruments, Tarrytown, NY). Whole
RBC deformability is expressed as a deformability index equivalent
to the ellipticity of the deformed cells. Normal ranges were obtained
from hematologically normal volunteers. For density separations,
RBCs in whole blood were separated by density on Stratex-Percol
continuous density gradients, according to Fabry and Nagel.11

Preparation of RBC ghosts and membrane vesicles. RBC ghosts
were prepared from freshly drawn blood anticoagulated in acid citrate/
dextrose by the hypotonic lysis procedure of Dodge et al,12 except
that dispropyli fluorophosphate (DFP) was added to the lysis step to
inhibit proteolysis. Spectrin and actin-depleted inside-out vesicles
(IOVs) were prepared by the method of Bennett and Branton,13 with
some modifications; ghost membranes were incubated in 0.1 mmol/
L Tris/HCl, pH 8.0, at 37°C for 30 minutes to induce membrane
vesiculation. For some experiments, NaOH-stripped membranes were
prepared from IOVs by incubation in 0.1 mmol/L EDTA, pH 11.0,
at 37°C for 30 minutes, as described by Korsgren and Cohen.14

Limited tryptic digestion of band 3. RBC mem-
bones (25 to 50

The proposita was first told of her anemia during her third
pregnancy. She became symptomatic (tiredness, weakness,
and extreme fatigue, and blurry vision) about
months after de-

RNA extraction, cDNA synthesis and polymerase chain reaction
(PCR) amplification. Total RNA was prepared from peripheral
blood reticulocytes by the buffered-ammonium chloride method
of Temple et al.20 Reticulocyte cDNA was prepared from the RNA by
reverse transcription and amplified by PCR, essentially as described
by Saiki et al.21 Briefly, 1 µg of total RNA was treated for 1 hour at
42°C in the presence of 10 U of avian myeloblastosis virus (AMV)
reverse transcriptase (Promega Biotech, Madison, WI) and 100 ng
of oligo dT [12-18] (Pharmacia-LKB, Piscataway, NJ). One-tenth of
the reaction mixture (2.5 µL) was then amplified for 35 cycles (60
seconds at 94°C, 60 seconds at 60°C, and 90 seconds at 72°C) in a
DNA thermal cycler (Perkin-Elmer Cetus Corp, Emeryville, CA),
using appropriate oligonucleotide primers derived from band 3 cDNA
sequence.22

DNA sequencing. PCR fragments representing portions of the
coding sequence of the cytoplasmic domain of band 3 were subcloned
into the PCR 1000 plasmid (TA Cloning Kit, Invitrogen, San Diego,
CA) and sequenced by the deoxy nucleotide (nt) method.23

Restriction analysis and ASO hybridization. For restriction
analysis, 200 ng of PCR-amplified cDNA from the proposita and an
unrelated race-matched control were digested for 2 hours at 37°C
with 8 U of Styl I (Stratagene, LaJolla, CA), according to the man-
facturer’s directions. For ASO hybridization, total genomic DNA
was extracted from peripheral blood leukocytes24 and PCR-amplified
using the following primers: 5’-CTGGAACAGAGGAAATATGAG-
T and 5’-GGCCCTCAATCATCTACGCT3’ (numbering from Lux
et al25). This DNA was hybridized with allele-specific oligonucleotides
recognizing either the mutant (22CAGACACCGAGGCAACACG) or
normal (22CAGACACCGAGGCAACAG) sequence.26

RESULTS

Clinical studies. The proposita is a 33-year-old Gravida
IV Para III female with a history of mild nonimmune (neg-
ative Coombs, polynuclear, and polycylinphroblastic tests) ep-
isodes of clinically apparent hemolytic anemia, in coincidence
with pregnancies, and exhibiting sphenogamoly (Table 1). Both
of the proposita’s parents, who were first cousins, were born
in the Dominican Republic and are largely of Spanish origin,
although with some Black admixture. The proposita’s iron
status (peripheral blood and bone marrow) and platelet count
were normal and Heinz bodies were not observed. Hemo-
globin (Hb) electrophoresis (by cellulose acetate) was normal,
as were glucose-6-phosphate dehydrogenase, vitamin B12, and
folic acid levels. In addition, antinuclear antibody, rheumatoid
factor, cold agglutinins, mononucleosis spot test, and Epstein-
Bar viral titers were all within normal levels.

The proposita was first told of her anemia during her third
pregnancy. She became symptomatic (tiredness, weakness,
extreme fatigue, and blurry vision) about 4 months after de-

SPHEROCYTOSIS ASSOCIATED WITH BAND 3 MUTANT 2157

Table 1. Proposita Hematologic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
<th>Pre-Spx Pre-Tx</th>
<th>Pre-Spx Post-Tx</th>
<th>Post-Spx 5 mo</th>
<th>Post-Spx 10 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10⁶/μL)</td>
<td>4.2-5.4</td>
<td>2.16-2.27</td>
<td>2.52-3.55</td>
<td>4.65</td>
<td>4.67</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>12-16</td>
<td>6.8-7.1</td>
<td>8.0-12.0</td>
<td>13.8</td>
<td>14.3</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>37-47</td>
<td>19.3-20.2</td>
<td>23.5-36.0</td>
<td>40.1</td>
<td>43.5</td>
</tr>
<tr>
<td>MCV (FL)</td>
<td>78-96</td>
<td>88.5-89.2</td>
<td>91.0-101</td>
<td>86.3</td>
<td>93.0</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>27-31</td>
<td>31.3-32.0</td>
<td>32.5-34.8</td>
<td>33.2</td>
<td>30.6</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32-36</td>
<td>35.2-36.1</td>
<td>34.3-36.5</td>
<td>34.4</td>
<td>32.8</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.8-2.2</td>
<td>9.5-13.4</td>
<td>10.6-25.8</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>C₂⁺⁻RBC survival (t½ d)</td>
<td>28-30</td>
<td>ND</td>
<td>12.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12-14</td>
<td>16.7-17.0</td>
<td>17.2-20.2</td>
<td>13.9</td>
<td>12.8</td>
</tr>
</tbody>
</table>

All values except reticulocytes and C₂⁺⁻RBC survival were from the Coulter model STKR automated cell counter. Reticulocyte counts were performed manually. Presplenectomy (Pre-Spx) values are presented as the range from analysis on 3 consecutive days before transfusion (Tx), or 13 days post-Tx. An exception to this was for Pre-Spx/Pre-Tx reticulocytes (2 values) and Pre-Spx/Post-Tx reticulocytes (11 values). The proposita was transfused with 2 U of packed cells approximately 2 months before Spx.

Abbreviations: HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; RDW, RBC distribution width; ND, not determined.

high reticulocyte count, and splenomegaly; Table 1) for which she received 2 U of blood. We first studied her approximately 2 months after this hospitalization. RBC survival (t½d), measured by ⁵¹Cr-labeling approximately 2 weeks posttransfusion, was 12.6 days, compared with 28 to 30 days for normal patients, which demonstrated increased splenic sequestration of RBCs. Peripheral blood smears before splenectomy contained 10% typical spherocytes (Fig 1A) and the RBCs were osmotically fragile (Table 1 and Fig 2B). It is likely that some transfused cells were still present at this initial examination. As mentioned above, prior medical histories showed that the proposita was found to be anemic during her two previous pregnancies. She was asymptomatic between pregnancies and did not consult a physician; thus, no documentation of her hematologic values exist for those periods. Various members of the proposita’s family (husband, son, daughter, mother, father, sister, and two brothers) were also studied and all were hematologically normal. The proposita was splenectomized approximately 1 month after these initial studies. In the postsplenectomy period, the proposita’s anemia was corrected (Table 1) and a marked reduction of peripheral blood spherocytes was noted (Fig 1B). Pathologic studies of the spleen showed an enlarged organ (308 g) with prominent lymphoid follicles with some germinal centers and areas of lipid granulomata. Of note was a considerable expansion of the red pulp and the presence of brown deposits (most likely lipid granulomata). Densitometric scans of eight pedigree members were all within the normal range (data not shown). Five months after splenectomy, both the ΔIₘₖ and ΔIₘₙ were within the normal range. This pattern was corroborated by incubated osmotic fragility analysis (Fig 2B) that demonstrated a clear improvement of RBC osmotic fragility postsplenectomy, although a small population of osmotically fragile cells persisted. Density separation of the proposita’s RBCs by Stractan-Percoll centrifugation¹¹ showed a large population of dense cells presplenectomy (Fig 3), a finding consistent with the increased RBC mean corpuscular Hb concentration (MCHC). Consistent with the postsplenectomy improvement of RBC deformability and osmotic fragility, numbers of dense RBCs were also reduced 5 months postsplenectomy (Fig 3). Subsequent analysis of the proposita’s RBCs 17 months and 22 months postsplenectomy gave similar results (data not shown).

RBC membrane protein composition. Densitometric analyses of RBC ghost membranes obtained both presplenectomy and postsplenectomy by SDS-PAGE showed that the proposita’s RBCs were 88% deficient in protein 4.2 (Fig 4). Immunoblots of the proposita’s ghost membranes using rabbit polyclonal affinity-purified human RBC protein 4.2 antibodies⁴ confirmed the protein 4.2 deficiency (Fig 4). SDS-PAGE analysis of proposita intact RBCs similarly showed a decreased content of protein 4.2 and immunologic analysis did not show degraded protein 4.2, suggesting that the decreased content of protein 4.2 was not the result of protein 4.2 loosely bound to the membrane or degraded during ghost preparation (data not shown). Because the presplenectomy sample likely contained some transfused cells, only the postsplenectomy results were quantitated. Levels of all other membrane proteins were normal except for band 6 (glycer-
aldehyde-3-phosphate dehydrogenase), which was 30% decreased. This is different from other cases of HS in which an increased membrane content of band 6 is found. The proposita and eight family members were studied simultaneously and only the proposita had a protein 4.2 deficiency (data not shown). Immunoblots of the proposita’s ghost membranes were also tested with antibodies to human RBC ankyrin and no quantitative or qualitative abnormalities were found (data not shown). Proposita PCR-amplified genomic DNA was further digested with Hpa I, which differentiates between normal protein 4.2 and protein 4.2Nippon, which showed a normal digestion pattern demonstrating that the proposita did not have the protein 4.2Nippon mutation (data not shown).

RBC membrane limited tryptic digestion. A partial deficiency of protein 4.2 in the proposita’s RBC membranes could be caused by a structural abnormality in the protein 4.2 molecule and/or as the result of an abnormality in band 3, its major membrane attachment site. To explore these possibilities, we performed limited tryptic digestions of the proposita’s post-splenectomy ghost membranes and examined the proteolytic digestion pattern of the water-soluble fragments of protein 4.2 and band 3 produced. Limited tryptic digestion and collection of the water-soluble fragments followed by immunoblotting and probing with polyclonal affinity-purified antibodies to protein 4.2 showed a normal tryptic digest, suggesting that the proposita’s protein 4.2 was

Fig 1. Peripheral blood smear from proposita (A) pre-splenectomy and (B) 17 months postsplenectomy. Spherocytes, elliptocytes, ovalocytes, “mushroom” shaped cells (upper left corner of A), fractured cells, and some cells exhibiting membrane exocytosis are observed pre-splenectomy. Postsplenectomy RBC morphology shows a significant decrease in spherocytes, but some anisocytosis and poikilocytosis is apparent.
SPHEROCYTOSIS ASSOCIATED WITH BAND 3 MUTANT

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SPHEROCYTOSIS ASSOCIATED WITH BAND 3 MUTANT
where control Dlh (deformability minimum) was 150 mosmol/kg
suspending medium osmolality. Composite scan was drawn to scale
at a constant applied shear stress as a continuous function of the
onstrated increased susceptibility to osmotic lysis, whereas 5
osmotic fragility analysis. Proposita presplenectomy RBCs dem-
splenectomy, the Dlh is shifted to the right and the DI, is de-
fragility, except for a small tail of osmotically susceptible cells. Nor-
postsplenectomy
and DI, (deformability maximum) was 0.9. In the proposita pre-
range in postsplenectomy RBCs. (B) Twenty-four hour incubated
months postsplenectomy RBCs had markedly improved osmotic
mal range is indicated by shaded area.

Fig 2. (A) Osmotic gradient ektacytometric scans of controls
(shaded area) and proposita presplenectomy (-----) and 5 months
postsplenectomy. The deformability index (DI) was measured
at a constant applied shear stress as a continuous function of the
suspending medium osmolality. Composite scan was drawn to scale
where control DI,min (deformability minimum) was 150 mosmol/kg
and DI,max (deformability maximum) was 0.9. In the proposita pre-
splenectomy, the DI,min is shifted to the right and the DI,max is de-
creased, indicating an increase in RBC osmotic fragility and a loss
of membrane surface area. These parameters are within the control
range in postsplenectomy RBCs. (B) Twenty-four hour incubated
osmotic fragility analysis. Proposita presplenectomy RBCs dem-
strated increased susceptibility to osmotic lysis, whereas 5
months postsplenectomy RBCs had markedly improved osmotic
fragility, except for a small tail of osmotically susceptible cells. Nor-
mal range is indicated by shaded area.

structurally normal (data not shown). Similar limited trypic
digestion followed by probing with polyclonal antibodies to
the cytoplasmic domain of band 3 showed an abnormal trypic
pattern in the proposita’s digest. In contrast to the limited
trypic digestion of control RBC membranes that generated
water-soluble fragments of 43 Kd, 41 Kd, 22 Kd, and 19
Kd,26 the proposita’s limited trypic digest contained little of
the 43-Kd and 41-Kd fragments and instead generated new
abnormal fragments of 37 Kd and 35 Kd (at the expense of
the 43-Kd and 41-Kd fragments) as well as an abnormal 16-
Kd fragment (Fig 5). Similar results were obtained using
monoclonal antibodies to the cytoplasmic domain of band
3 (data not shown). To determine whether the abnormal
trypic fragmentation was a genetic polymorphism unique
to the proposita’s family, we tested 7 pedigree members, in-
cluding the proposita’s son, daughter, sister, two brothers,
father, and mother, along with the proposita’s husband and
13 unrelated race-matched (Caribbean) controls. We found
that 6 pedigree members generated both the normal 43-Kd
and 41-Kd fragments, as well as the abnormal 37-Kd, 35-
Kd, and 16-Kd fragments (Fig 5). The proposita’s sister, hus-
band, and all controls generated only the normal 43-Kd and
41-Kd fragments (Fig 5). Similar results were obtained with
the monoclonal band 3 antibody (data not shown). These
data suggest that the unique abnormality in the proposita’s
band 3 molecule is the exposure of a trypic site that results
in the near complete conversion of the 43-Kd and 41-Kd
fragments to the 37-Kd, 35-Kd, and 16-Kd fragments. The
abnormal 37-Kd, 35-Kd, and 16-Kd trypic fragments were
observed whether ghost membranes or IOVs were digested
(data not shown).

N-terminal sequencing of the abnormal 37/35-Kd trypic
peptides showed that the first 10 residues were identical to
residues 41-50 of band 3.27 Similar sequencing of the first 10
N-terminal residues of the abnormal 16-Kd trypic peptide
showed that it was identical to the N-terminal of the 37/35-
Kd peptides, suggesting that it was derived from these pep-
tides. Taken together, these results suggested that the struc-
tural abnormality in the proposita’s band 3 molecule that
generates a new trypic cleavage site is located at or near
residue 40 (digestion data summarized in Fig 6).

Band 3 cDNA sequence. Sequencing the portion of band
3 cDNA between nts 227 and 498 (numbering according to
Lux et al22) showed a single point mutation at nt 232 in the
proposita that resulted in a G → A substitution introducing
a lysine (Lys) (AAG) in place of a glutamic acid (Glu) (GAG)
at amino acid 40 (Fig 7A). We have designated this mutation
band 3Monteiro.

The mutation at nt 232 generated an Sty I restriction site
that was exploited to confirm the presence of the mutation
at nt 232 in the proposita (Fig 7B). The presence or absence
of the mutation was also determined by hybridization of PCR-
amplified genomic DNA with allele-specific oligonucleotides.
This analysis showed that the proposita was homozygous for
A at nt 232, whereas 13 of 13 (26 chromosomes) unrelated
Caribbean controls were all homozygous for G at this position
(Fig 7C). Furthermore, 6 pedigree members who were phe-
notypically heterozygous for the band 3 structural abnor-
mality (Fig 5) were also genotypically heterozygous, contain-
ing both A and G at nt 232, whereas the proposita’s sister
and husband, who were phenotypically normal, were also
genotypically normal (Fig 7C). A summary of the pedigree
is shown in Fig 8.

We also sequenced the portion of band 3 cDNA between
nts 998 and 1184 containing the region in which the recently
described band 3Tuscalao mutation was found.27 The proposita
did not have this mutation (data not shown).

Protein 4.2 binding studies. To ascertain whether the
structural alteration in the proposita’s band 3 was responsible
for the protein 4.2 deficiency, we determined the in vitro binding of protein 4.2 purified from control RBCs to control and proposita protein 4.2-stripped IOVs. In two separate experiments, using protein 4.2 purified and radiolabeled by different methods,\textsuperscript{14,17} we found 30\% and 8\% decreased protein 4.2 binding to proposita IOVs (data not shown). SDS-PAGE analysis of the reassociated IOVs indicated that no protein degradation had occurred. Considering the inherent level of

Fig 3. Stractan-Percoll continuous density gradient separation of control (lane 2), proposita presplenectomy (lane 3), and proposita 5 months postsplenectomy (lane 4) RBCs. The numbers at the left refer to density (g/mL) of marker beads (lane 1). The proposita presplenectomy blood sample contained many abnormally dense RBCs that were substantially reduced in the postsplenectomy sample.

Fig 4. (A) Protein composition of control (C) and proposita (P) RBC ghost membranes (25 µg) separated on a 9\% Laemmli SDS-PAGE gel and Western blotted or stained with Coomassie brilliant blue (CBB). Position of protein bands are indicated. By densitometry, proposita membranes are 88\% deficient in protein 4.2 and 30\% deficient in band 6 (G3PD). The Western blot was developed with affinity-purified antiprotein 4.2 IgG. Arrow indicates the position of protein 4.2 on the Western blot.
POLYCLONAL ANTI-BAND 3

Fig 5. Immunoblot of limited tryptic digest of control (C) and proposita (P) ghost membrane proteins (25 μg) separated by 12% Laemmli SDS-PAGE, electrophoretically transferred to nitrocellulose and developed with polyclonal antibodies to the cytoplasmic domain of human RBC band 3. S indicates molecular weight standards (kilodaltons). Analysis of the pedigree is shown on the right. Pedigree members are: proposita's father (lane 1), mother (lane 2), brother (lane 3), brother (lane 4), sister (S; lane 5), daughter (lane 6), husband (H; lane 7), son (lane 8), and proposita (P; lane 9).

DISCUSSION

Although protein 4.2 constitutes approximately 5% of the total protein of human RBC membranes, little is known about its functional role. Recently, several laboratories described individuals whose RBCs were completely or partially deficient in protein 4.2. Most of these individuals were moderately to severely anemic, although some were hematologically normal. The molecular basis for the protein 4.2 deficiency in a subset of Japanese protein 4.2-deficient individuals has recently been found to be a protein 4.2 cDNA point mutation (protein 4.2Nip0). In this report we describe a patient with a nonimmune hemolytic anemia whose RBCs were 88% deficient in protein 4.2. These RBCs were spherocytic and osmotically fragile, suggesting a phenotype similar to HS. Consistent with HS, these RBCs were also less deformable (as determined by ektacytometry) and contained a large population of abnormally dense cells (as determined by Stractan-Percoll density centrifugation).

Noteworthy, however, in coincidence with splenectomy, the proposita's anemia was considerably improved with greatly reduced numbers of peripheral blood spherocytes and...
Fig 7. (A) Sequence analysis of control (left) and proposita (right) band 3 cDNA. Total reticulocyte RNA was reverse-transcribed, PCR-amplified, subcloned, and sequenced by the dideoxy method. The proposita has a point mutation changing amino acid 40 (numbering from Lux et al) from GAG (Glu) to AAG (Lys). (B) Sty I restriction digest of PCR-amplified band 3 cDNA. One hundred nanograms of control (C) or proposita (P) cDNA was PCR-amplified and 20% of the total PCR product was digested for 2 hours at 37°C with 8 U of Sty I. − or + indicates cDNA before and after Sty I digestion, respectively. PCR-amplified cDNA from the control was undigested by Sty I (187 bp), whereas PCR-amplified cDNA from the proposita yielded Sty I digestion fragments of 117 bp and 70 bp. (C) ASO hybridization: 100 ng of PCR-amplified genomic DNA was applied in duplicate to nitrocellulose and hybridized with a 32P-labeled oligonucleotide specific for either the normal (N) or the mutant (M) band 3 sequence. PCR-amplified genomic DNA from the proposita (P, lane 24) hybridized only to the M-specific oligonucleotide, whereas PCR-amplified genomic DNA from 13 unrelated race-matched (Caribbean) controls (lanes 3 through 15) hybridized only to the N-specific oligonucleotide. Lanes 16 through 23 contain PCR-amplified genomic DNA from the pedigree: lane 16, sister; lane 17, son; lane 18, husband; lane 19, brother; lane 20, mother; lane 21, brother; lane 22, father; lane 23, daughter. Lane 1 contains proposita (P) and lane 2 contains control (C) band 3 cDNA.

marked improvement of RBC osmotic fragility, deformability, and density. We are not convinced that splenectomy “cured” her hematologic picture because the removal of the spleen could have coincided with an amelioration of an episodic course in hemolysis (postpregnancy). The postsplenectomy blood features could actually be the proposita’s steady state. In any case, the clinical phenotype of this individual is distinct from classical HS in which these abnormalities are rarely improved (indeed, are often exaggerated) by splenectomy.31

In contrast to previously described cases of protein 4.2 deficiency associated with a protein 4.2 cDNA point mutation,4 there was no evidence that the proposita’s protein 4.2 was abnormal. However, analysis of the proposita’s band 3 cytoplasmic domain (the major membrane attachment site for protein 4.2) showed a structural abnormality that, along with direct protein sequencing, demonstrated a new trypsin cleavage site located at or near residue 40. This was confirmed by sequencing the proposita’s reticulocyte band 3 cDNA, which identified a single point mutation...
SPHEROCYTOSIS ASSOCIATED WITH BAND 3 MUTANT

changing amino acid 40 from $^{40}$Glu $\rightarrow$ Lys ($^{232}$GAG $\rightarrow$ AAG) (band $^{3}$Montefiore).

The $^{40}$Glu $\rightarrow$ Lys mutation occurs within an extremely acidic region of the N-terminus (of the first 40 amino acids, 20 are acidic and none are basic) that contains binding sites for other proteins, including native and denatured hemoglobins,$^{32,33}$ glyceraldehyde-3-phosphate dehydrogenase (band 6),$^{34}$ aldolase,$^{35}$ and phosphofructokinase,$^{36}$ all of which interact with band 3 through electrostatic interactions. Therefore, any sequence alteration that results in the substitution of a basic (lysine) for an acidic (glutamic acid) amino acid might be expected to disturb the binding of proteins that interact with band 3 in that region. That band $^{3}$Montefiore membranes were 30% deficient in band 6 further supports the contention that the $^{40}$Glu $\rightarrow$ Lys mutation affects band 3 N-terminal electrostatic interactions.

Although protein 4.2 is known to bind to the cytoplasmic domain of band 3, the precise location of the binding site has not been determined. Whereas our studies suggest that $^{40}$Glu is important for protein 4.2 binding, it is not known whether this residue is directly involved in binding or plays an allosteric role. Our sequence analysis showed that band $^{3}$Montefiore does not contain the band $^{3}$Memphis polymorphism ($^{56}$Lys $\rightarrow$ Glu) 57 nor does it contain the recently described band $^{3}$Tuscaloosa mutation (327Pro $\rightarrow$ Arg) also associated with RBC protein 4.2 deficiency. 27 Neither does band $^{3}$Montefiore contain the mutation described by Jarolin et al 18 in Southeast Asian ovalocytosis (deletion of 9 amino acids at the cytoplasmic domain-membrane domain interface). It is noteworthy that the band $^{3}$Montefiore mutation occurs in a CpG dinucleotide, which is known to be a mutational "hot spot." 39

If band 3 $^{40}$Glu is necessary for protein 4.2 binding, then should not the proposita, who is homozygous for the defect, have a complete protein 4.2 deficiency instead of the observed 88% protein deficiency? Not necessarily, because there could be multiple protein 4.2 binding sites on the membrane and/or $^{40}$Glu may be involved indirectly in protein 4.2 binding, perhaps by stabilizing a conformation that maximizes the availability of the protein 4.2 binding site. Regarding the former possibility, both protein 4.1 40 and ankyrin 41 interact with multiple sites on band 3. While the proposita's ghost membranes were 88% deficient in protein 4.2, in vitro binding studies showed only small (30% and 8%) decreases in the binding of normal protein 4.2 to the proposita's protein 4.2-stripped IOVs. Considering the level of error involved in these type of in vitro binding measurements, small differences are inconclusive and, moreover, are insufficient to explain the proposita's 88% protein 4.2 deficiency.

Pedigree studies showed that the band $^{3}$Montefiore mutation was present in the homozygous state in the proposita, in the heterozygous state in six members who were clinically normal but phenotypically heterozygous for the band 3 structural abnormality, and not present in one pedigree member whose band 3 was structurally normal. The mutation was not present in 26 chromosomes from unrelated race-matched controls nor in the proposita's sister or husband. These findings imply either that the mutation is a rare polymorphism in linkage disequilibrium with the determinant of the protein 4.2 deficiency or, more likely, that the mutation is directly involved in the protein 4.2 deficiency. Pedigree studies further suggest that the hemolytic anemia phenotype associated with the band $^{3}$Montefiore mutation was inherited in an autosomal recessive manner. Definitive proof that the band $^{3}$Montefiore mutation is directly responsible for the protein 4.2 deficiency will require finding both the mutation and protein deficiency in unrelated pedigrees, which, as is the case for every other cytoskeletal protein defect that is first identified in one pedigree, cannot be rigorously satisfied by studies in a single pedigree.

Heterozygotes for the band $^{3}$Montefiore mutation have normal RBC membrane protein 4.2 content. One possibility for this could be the redundancy of band 3-protein 4.2 binding sites. The ratio of band 3 to protein 4.2 in RBC membranes is approximately 4:1, 1 allowing for a potential fourfold excess of band 3-protein 4.2 binding sites (assuming that protein 4.2 associates principally with band 3 and that band 3 and protein 4.2 associate as monomers). Thus, a 50% reduction in band 3-protein 4.2 binding sites may have only negligible effects on the membrane content of protein 4.2. Alternatively, factors other than, or in addition to, the band 3 abnormality may be responsible for the protein 4.2 deficiency, although these other factors would necessarily be closely linked with the band $^{3}$Montefiore mutation. Moreover, because we did not sequence the proposita's entire band 3 cDNA, nor did we sequence the entire protein 4.2 cDNA, we cannot exclude the possibility of other mutations being present, although in the case of the latter, it would necessarily be different from protein 4.2$^{Nippon}$.

Recently, a point mutation in the protein 4.2 cDNA (protein 4.2$^{Nippon}$) of four Japanese individuals having nearly complete (<1% of normal levels) deficiencies of protein 4.2 has been described. 6 Thus, it is likely that a protein 4.2 deficiency can result from at least two independent mechanisms.
one involving a mutation in the protein 4.2 cDNA and the other involving a structural abnormality in band 3.

After splenectomy, the proposita’s RBC morphology, osmotic fragility, whole cell deformability, and cell density improved, despite the continued protein 4.2 deficiency. This phenotype is significantly different from other cases of protein 4.2 deficiency due to a mutation in the protein 4.2 gene in which the phenotype (spherocytosis and abnormal RBC osmotic fragility) does not improve after splenectomy. As mentioned before, we cannot exclude the possibility that the proposita’s postsplenectomy phenotype actually represents this patient’s steady state. Nevertheless, in classical HS, splenectomy, while improving the anemia, does not normally improve RBC morphology and osmotic fragility, which either remains the same or, more likely, worsens.

In conclusion, we have identified a band 3 mutation (Glu → Lys, band 3\textsuperscript{Mnemonic}) that is associated with an 88% deficiency of RBC membrane protein 4.2, a 30% deficiency of band 6, and episodic hemolysis. The homozygous phenotype of this abnormality is distinct from classical HS as demonstrated by significant postsplenectomy improvement in RBC morphology, instead of the expected increases in abnormal RBC morphology. Our results suggest that the region of band 3 containing Glu may be involved in the interaction of band 3 with proteins 4.2 and band 6.

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SPHEROCYTOSIS ASSOCIATED WITH BAND 3 MUTANT

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