Band 3 Tuscaloosa: Pro\textsuperscript{327} → Arg\textsuperscript{327} Substitution in the Cytoplasmic Domain of Erythrocyte Band 3 Protein Associated With Spherocytic Hemolytic Anemia and Partial Deficiency of Protein 4.2

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Protein 4.2 is a major red blood cell (RBC) protein that interacts with the band 3 protein and with ankyrin. Inherited deficiencies of this protein are associated with spherocytic hemolytic anemia, but the molecular basis of this defect is unknown. We have studied the underlying defect in a patient with spherocytic hemolytic anemia whose RBCs had a partial (29% ± 5%) deficiency of protein 4.2. We have first studied the binding of normal ankyrin and protein 4.2 to patient inside-out vesicles (IOVs) stripped of peripheral proteins. While the binding of ankyrin was normal, the predicted maximal binding capacity of protein 4.2 was 20% to 33% lower than that of control IOVs, suggesting a possible abnormality in protein 4.2 to cdb3. An additional line of evidence pointing to a possible abnormality of protein 4.2 to cdb3 is an abnormal proteolytic digest of cdb3. To elucidate the underlying molecular defect, we have cloned and sequenced the cDNA coding for cdb3 from the patient. One band 3 allele was found to be normal, while clones corresponding to the other allele contained two mutations: substitution A → G in nucleotide 166, changing codon 56 from AAG to GAG (Lys → Glu), and substitution C → G in nucleotide 980, changing codon 327 from CCC to CGC (Pro → Arg). Since the Lys\textsuperscript{56} → Glu\textsuperscript{56} substitution is found in a common asymptomatic variant of the band 3 protein designated band 3 Memphis, we conclude that either the Pro\textsuperscript{327} → Arg\textsuperscript{327} substitution itself, or in combination with the band 3 Memphis polymorphism, underlies the abnormal binding of protein 4.2 to cdb3 and results in the spherocytic phenotype.

THE RED BLOOD CELL (RBC) membrane skeleton, a two-dimensional protein network underlying the membrane lipid bilayer, is composed of spectrin, actin, ankyrin, proteins 4.1 and 4.9, and several recently described minor proteins.\textsuperscript{1,3} The membrane skeleton is attached to the membrane by binding of ankyrin to the anion exchanger protein, band 3, and by interactions of protein 4.1 with several integral proteins, as well as with the negatively charged lipids of the inner membrane lipid layer.\textsuperscript{4,5} A number of hereditary deficiencies of the skeletal and skeleton-associated proteins, including deficiencies of spectrin, ankyrin, band 3 protein, and protein 4.2, have been found in congenital spherocytosis.\textsuperscript{6,8}

Protein 4.2 is a 72-Kd peripheral membrane protein\textsuperscript{9} that binds to the cytoplasmic domain of band 3 (cdb3), ankyrin,\textsuperscript{10,12} and, possibly, protein 4.1.\textsuperscript{13} Since only a few reports on deficiency of protein 4.2 are available,\textsuperscript{14-20} deficiency of the protein 4.2 constitutes one of the rarest, and most poorly characterized molecular defects. The molecular basis of this deficiency may be heterogenous, as evidenced by variable degree of the deficiency in the individual reports and, in one case, by the presence of another functional abnormality characterized by weakened ankyrin-band 3 interaction.\textsuperscript{17}

To elucidate the molecular basis of protein 4.2 deficiency, we have studied a patient with atypical spherocytic anemia with partial deficiency of protein 4.2. Taking a functional and structural approach, we have assigned the molecular defect to cdb3. Subsequent sequencing of cDNA corresponding to cdb3 showed the presence of two missense mutations in cis position in one of the two band 3 alleles, changing Lys\textsuperscript{56} to Glu\textsuperscript{56}, and Pro\textsuperscript{327} to Arg\textsuperscript{327}. Since we have previously detected the Lys\textsuperscript{56} → Glu\textsuperscript{56} substitution in carriers of a common band 3 protein polymorphism designated band 3 Memphis,\textsuperscript{21,22} we suggest that the Pro\textsuperscript{327} → Arg\textsuperscript{327}, either itself or in combination with the band 3 Memphis polymorphism, leads to the decreased binding of protein 4.2 to cdb3 and to the spherocytic RBC morphology.

MATERIALS AND METHODS

Subjects. The patient was referred to the University of Alabama Medical Center for evaluation of anemia. After obtaining informed consent, venous blood from the patient, the patient's mother and siblings, and control subjects was drawn and sent on ice overnight to Boston for evaluation of a possible defect of RBC membrane skeleton.

Preparation of erythrocyte ghosts and analysis of the membrane proteins. Erythrocyte ghosts were prepared from freshly drawn blood anticoagulated in acid citrate/dextrose by the method of Dodge et al\textsuperscript{23} with minor modifications. Whole blood was washed four times in 10 vol of phosphate-buffered saline (PBS) (5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA, 150 mmol/L NaCl) at 4°C. Disopropylfluorophosphate (DFP), 15 μL diluted in 400 μL of 1 mol/L Tris, pH 8.5, was added to 100 mL of packed cells and the mixture was incubated on ice for 1 hour. The cells were lysed in 10 vol of 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA, at 4°C, and washed repeatedly in the same buffer until white. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 3% to 17% exponential gradient Fairbanks\textsuperscript{24} and in 9% and 12% Laemmli gels.\textsuperscript{25}

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Limited tryptic digestion of spectrin and measurements of the ratio of spectrin dimers to spectrin tetramers in the low ionic strength spectrin extract, and test for stability of isolated membrane skeletons were performed as described.26

Preparation of inside-out vesicles stripped of ankyrin and protein 4.2. White ghosts were depleted of band 6 by incubation in 10 vol of PBS. Spectrin and actin were removed from the band 6-depleted ghosts by incubation of ghosts in 20 vol of 0.1 mmol/L EGTA, pH 8.5, at 37°C for 30 minutes. The resulting inside-out vesicles (IOVs) were centrifuged and washed once in 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA. The remainder of the peripheral membrane proteins, ankyrin, protein 4.1, and protein 4.2, were removed by incubation of the vesicles in 30 vol of 0.1 mmol/L EGTA titrated to pH 11 with 0.1N NaOH, followed by incubation at 25°C for 20 minutes. The membranes were centrifuged and washed once in 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA. These membranes are depleted of all peripheral membrane proteins (spectrin, ankyrin, actin, and proteins 4.1, 4.2, and 6).

Preparation and radioiodination of erythrocyte ankyrin and protein 4.2. Ankyrin was prepared according to the method of Bennett and Stenbuck.28 The protein migrated as a single band on SDS-PAGE and was greater than 90% pure as determined by quantitative densitometry of Coomassie blue-stained gels. Erythrocyte protein 4.2 was prepared as described previously,27 and was greater than 90% pure. Purified protein was radioiodinated with 125I-labeled Bolton-Hunter reagent as described.13

Binding of ankyrin and protein 4.2 to pH 11-stripped IOVs. 125I-labeled ankyrin or 125I-labeled protein 4.2 were incubated with 25 µg/ml pH 11-stripped IOVs in a final volume of 300 µL of 120 mmol/L KCl, 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA. 0.5 mmol/L DTT, 20 µL/mL phenylmethylsulfonyl fluoride (PMSF), 0.02% sodium azide, 1 mg/mL bovine serum albumin (Palo Alto, CA) at 17,000 rpm for 25 minutes. The supernatant of unbound protein was carefully aspirated with a Pasteur pipette and the tubes containing the membrane pellet with bound protein were counted in a gamma counter. For each concentration of 125I-protein incubated with membranes, a control sample lacking membranes was prepared. These samples were incubated, centrifuged, and aspirated as described above, and the amount of sedimented 125I-protein was subtracted from the corresponding sample-containing membranes. These values were generally between 30% and 60% of the total amount sedimented in the presence of membranes depending on the protein being used. Duplicate samples generally agreed to within 2%.

Limited proteolytic cleavage of the band 3 protein. Band 3 protein was cleaved in intact RBCs with pronase (Streptomyces griseus protease, Calbiochem, San Diego, CA). A 1-mL sample of whole blood was washed three times with PBS, and 0.2 mL of packed RBCs was transferred into 50 mL Sorvall (Wilmington, DE) tubes and 1.8 mL PBS added. A 3.5-µg/mL sample of stock solution of pronase was added to final concentration of 35 µg/mL and the suspension was incubated for 1 hour at 37°C. The digestion was terminated by 15 minutes incubation with 4 mmol/L PMSF and the cell suspension was washed three times with PBS. Erythrocyte ghosts were then prepared as described and analyzed by SDS-PAGE on 7.5% to 17% linear gradient Laemmli gels. The cdb3 was cleaved by trypsin. Erythrocyte ghosts were suspended in PBS and digested with N-tosyl-L-phenylalanine chloromethyl ketone-trypsin (1:250 to 1:2,000 wt/wt) for 6 hours at 0°C. The reaction was terminated by 1 mmol/L DFP and heating the samples to 100°C for 1 minute in the presence of 1% SDS and 20 mmol/L DTT. The samples were analyzed on 12% Laemmli gels.

Sequencing of band 3 cDNA. Total reticulocyte RNA, isolated by ammonium chloride lysis as described,29 was reverse-transcribed using a band 3-specific polymerase chain reaction (PCR) primer P134 (5’-TCCGACATCCCATCCTGGTT3’; bases 1316-1297) and PCR-amplified with primers P122 (5’-GGAAAGATGGGGACAGTA3’; bases 150 to 133) and P134 using the Perkin-Elmer (Norwalk, CT) GeneAmp PCR Reagents kit (35 cycles, 1 minute at 94°C, 1 minute at 60°C, 2 minutes at 72°C). An aliquot of the PCR product was cloned into plasmid pCR 1000 using the TA Cloning kit (Invitrogen, San Diego, CA) and inserts were sequenced in their entirety with the T7 Sequencing kit (Pharmacia, Uppsala, Sweden) and a set of nested sequencing primers.

Direct sequencing of amplified DNA. Genomic DNA was isolated as described22 and PCR-amplified using primers P193 (5’-CCCGATAATGCTCATAGTGG-3’; bases 882-901) and P184 (5’-AAGCTGAGTCTGGTTTGGCA-3’; bases 1073-1053), 30 cycles of 1 minute at 94°C and 1 minute at 57°C. Five microliters of the product of the first PCR were reamplified using primer P183 (5’TATGGCTGTCCTCCACCCAGT3’; bases 956-976), 30 cycles of 1 minute at 94°C and 1 minute at 57°C. The product of the asymmetric PCR was purified by three centrifugations through a Centricon 30 (Amicon, W. R. Grace, Danvers, MA) microconcentrator and sequenced using primer 184 and T7 DNA polymerase (Sequenase; US Biochemical Corp, Cleveland, OH). Similarly, cDNA was purified as described and PCR-amplified using primers P247 (5’-CCAGACATCCTCCCGAAOTCCC3’; bases 70-88) and P51 (5’-GAGAGGGTGCGCCGCCGCCCA-3’; bases 299-280), 30 cycles of 1 minute at 94°C and 1 minute at 55°C. Five microliters of the products of the first PCR was reamplified using primer P256 (5’TCTGGTTCTTTTCTGACAC3’; bases 215-191), 50 cycles of 1 minute at 94°C, 1 minute at 60°C, purified as described, and sequenced using primer P208 (5’-CGACACCGAGGCACCGC; bases 111-131).

Sequence analysis. DNA sequences of human, mouse, rat, and chicken band 3 proteins have been downloaded from the Genbank database, translated into amino acid sequences, and aligned using the multiple alignment program CLUSTAL (PCGene; Intelligenetics, Mountain View, CA). For comparison of two sequences, program ALIGN (DNAsel, Madison, WI) was used.

CASE REPORT

The patient is a 25-year-old African-American female who presented with a moderate anemia with hemoglobin (Hgb) of 10.3 to 12.3 and hematocrit (Hct) of 29% ± 1%. Reticulocyte counts were 24% and 25%, and the RBC volume distribution width (RDW) was 16.2. The peripheral blood film showed striking microspherocytosis with some microspherocytomatoses. The osmotic fragility was increased. The patient had a negative Coombs test and a normal radioimmunoassay for RBC IgG. Basic hematologic parameters of the mother, brother, and sister were all normal. The patient’s father and three additional siblings were unavailable for clinical evaluation. The patient was splenectomized 3 months after our initial studies were performed. After splenectomy, the anemia was corrected and the reticulocyte count decreased to 4% to 5%. The anisocytosis and the RBC morphology remained unchanged.

RESULTS

Protein composition of RBC membranes. Analysis of the RBC ghosts by SDS-PAGE electrophoresis on 3.5% to 17% exponential gradient gels according to Fairbanks14 and on
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12% gels according to Laemmli25 showed that the patient’s membranes were 29% ± 5% deficient in protein 4.2, as evidenced by a decrease in the ratio of protein 4.2 to the band 3 protein on the gel densitometric tracing (Fig 1). The protein 4.2 deficiency was detected both before and after splenectomy. To exclude the possibility that the protein 4.2 was either present in the cytoplasm or weakly bound to the membrane and, hence, lost during the preparation of ghosts, we solubilized whole erythrocytes and analyzed them by SDS-PAGE. Again, we detected less protein 4.2 in the patient’s solubilized erythrocytes (data not shown). All other protein ratios, i.e., spectrin to band 3, ankyrin to band 3, and protein 4.1 to band 3, were within the normal range. The SDS-PAGE of the patient’s mother, brother, and sister were all normal. Limited tryptic digestion of the spectrin extract yielded a normal tryptic cleavage pattern, suggesting a normal structure of the patient’s spectrin. The percentage of dimers in the patient’s low ionic strength spectrin extract (<10%) was within the normal range.

.Binding of protein 4.2 and ankyrin to stripped IOVs. To elucidate the molecular basis of the protein 4.2 deficiency, we first asked whether normal protein 4.2 bound normally to patient IOVs stripped of ankyrin and protein 4.2. Figure 2 shows that the predicted maximal binding capacity of patient IOVs for band 4.2 was 33% lower than that of control IOVs (208 ± 9 µg/mg compared with 312 ± 1 µg/mg for control membranes). The kd for binding to patient membranes was also decreased nearly twofold (2.4 ± 0.2 x 10⁻⁷ mol/L compared with 4.6 ± 0.3 x 10⁻⁷ mol/L for the control). Similar results were obtained in two other experiments; in one, the predicted binding capacity of patient membranes was 20% less than controls, and the kd was decreased by 1.2-fold; in the other experiment, a complete binding curve could not be plotted, but the patient membranes had from 14% to 60% lower band 4.2 binding than controls, over a range of band 4.2 concentrations.

Because of previous reports by others that ankyrin binding to IOVs from protein 4.2-deficient RBCs is reduced,13 we compared binding of normal ankyrin with patient and control IOVs. We found no significant differences between the patient and control IOV samples (not shown).

Structural abnormality of the cytoplasmic domain of band 3 as shown by proteolytic digestion. To address the possibility that the decrease in binding of protein 4.2 to the patient’s membranes was due to a defect in the binding site for protein 4.2 on cdb3, pronase digestion of band 3 in intact erythrocytes and limited tryptic digestion of band 3 in IOVs was used (Fig 3). Both the pronase digest and the tryptic digest of patient band 3 were abnormal. While pronase digestion of control cells produced one band of 60 Kd that contains the 43-Kd cdb3 and the 17-Kd transmembrane segment, pronase treatment of patient cells produced a doublet with equal amounts of two bands of 60 and 63 Kd. Limited cleavage of IOVs with trypsin produced a single band of 22 Kd in the control and two bands of 22 and 25 Kd in the patient. Since the 22-Kd fragment is derived from the N-terminal part of cdb3,30 the proteolytic data suggest presence of the defect within the N-terminal 180 amino acids, involving either an extension of the protein or a mutation that alters the charge and/or conformation of the protein and thus affects the protein electrophoretic mobility.
Cloning and sequencing of band 3 cDNA. Because the above studies suggested that the molecular defect causing reduced band 4.2 in patient membranes resides within cdb3, we isolated total reticulocyte RNA and reverse transcribed it into cDNA. PCR amplification of the cDNA with primers specific for band 3 and designated P122 (band 3 cDNA bases −150 to −133, the initiator methionine corresponding to bases 1 to 3) and P134 (bases 1297-1316) yielded a single PCR product of 1,466 nucleotides. We have cloned and sequenced this cDNA segment and obtained two types of clones. Approximately 50% of the clones had the previously reported band 3 sequence,31 the remainder of the clones contained two mutations in cis position (Fig 4): a substitution A → G in nucleotide 166, changing codon 56 from AAG to GAG (Lys → Glu), and a substitution C → G in nucleotide 980, changing codon 327 from CCC to CGC (Pro → Arg). We have further confirmed that these two mutations were present in the same allele by sequencing clones obtained from an independent reverse transcription and amplification of patient cDNA spanning both missense mutation sites.

Direct sequencing of genomic DNA. We have PCR-amplified and directly sequenced the region of cDNA flanking the Lys56 → Glu56 mutation and the region of genomic DNA flanking the Pro327*→ Arg327* mutation. We have confirmed that the patient is a heterozygote for both mutations (Fig 5), while we have not detected these mutations in the unaffected family members (not shown).

The positions of both mutations in the band 3 protein are schematically depicted in Fig 6. Figure 6 also shows approximate locations of the binding regions for ankyrin and RBC glycolytic enzymes,9 as well as of the previously described hinge region.24

Multiple alignment of cloned band 3 proteins. Previously cloned sequences of human, mouse, rat, and chicken band 3 proteins were aligned as described. Figure 7 shows the alignment in the vicinity of the Pro327*→ Arg327* mutation. The sequence is nearly identical among species, with prolines 322, 323, and the mutated proline 327 being perfectly conserved.

DISCUSSION

In contrast to a relatively high number of reported mutations and/or deficiencies of erythrocyte spectrin and

Fig 3. Proteolytic digestion of normal and patient membranes. (A) Pronase digestion of band 3 in intact RBCs. Lanes 1 and 2, Coomassie blue-stained 7.5% to 15% linear gradient Laemmli gel of the pronase digest shows a normal 60-kD band in the patient and a 60 + 63-kD doublet in the patient. Lanes 3 and 4, corresponding immunoblot with a polyclonal antibody raised against cdb3. (B) Tryptic digest of band 3 in IOVs. Lanes 5 and 6, Coomassie blue-stained 12% Laemmli gel shows the normal 22-kD fragment of cdb3 in the patient and a 22 + 25-kD doublet in the patient. Lanes 7 and 8, corresponding immunoblots with the same antibody.

Fig 4. Nucleotide sequence of patient PCR-amplified cDNA. Patient cDNA was amplified by PCR and the 1,466-nucleotide PCR product was cloned into plasmid pCR1000. Clones corresponding to both band 3 alleles were sequenced as described. One allele had the previously published sequence, the other contained two mutations. (A) Substitution A → G in nucleotide 166 changing codon 56 from AAG to GAG (Lys → Glu). (B) Substitution C → G in nucleotide 980 of the same allele changing codon 327 from CCC to CGC (Pro → Arg).
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**Fig 5.** Direct sequencing of patient cDNA and genomic DNA. To demonstrate the presence of the two mutations in the heterozygous state, the region of patient cDNA flanking the 166A → G mutation and the segment of patient genomic DNA surrounding the 980C → G substitution was amplified by asymmetric PCR and the obtained single-stranded DNA was sequenced. (A) Nucleotides A and G in position 166. (B) Nucleotides C and G in position 980.

protein 4.1, only a few reports of a deficiency of protein 4.2 have been published. The molecular basis of these apparently heterogeneous disorders remains unknown.

To elucidate the molecular basis of the protein 4.2 deficiency, we first asked whether the binding of normal 4.2 protein to patient IOVs, devoid of all peripheral proteins, is normal. We have found a decrease in the binding capacity of patient membranes for the normal 4.2 protein. Since band 3 is the principal binding site for band 4.2, this suggests that there is a defect within or in the vicinity of the protein 4.2 binding site in cdb3 protein. However, we cannot rule out that the binding of protein 4.2 to other, non-band 3, sites may also be affected.

Additional evidence pointing to a defect in cdb3 was the abnormal pronase and trypsin digest of patient band 3. However, the observed proteolytic pattern was identical with that observed in carriers of an asymptomatic band 3 variant designated band 3 Memphis who have normal band 4.2 content. As we and others have recently reported, the underlying polymorphism in band 3 Memphis is the Lys56 → Glu56 substitution. Therefore, after detecting this mutation in one allele of patient band 3 cDNA, we looked for an additional molecular defect and have found a second mutation in the same allele that substitutes Pro327 with Arg327 (CCC → CGC).

Pro327 is located in a highly conserved region of the cytoplasmic domain of band 3 (Fig 7). There is a 100% identity between mouse and human cDNA in amino acids 314 through 339, and an 88% identity and 100% similarity (DNAStar) in amino acids 306 through 347. Comparison with chicken band 3 cDNA in the same region yields 60% identity and 86% similarity. Prolines in positions 322, 323, 327, and 337 are conserved in chicken, rat, mouse, and human erythrocyte band 3 (Fig 7). The high degree of conservation of this part of cdb3 throughout evolution suggests an important structural and functional role of this segment of band 3. Mutation in this region is therefore likely to exert adverse effects on the normal properties of cdb3. Moreover, proline is known to play an important role in modifying the secondary structure of proteins in that it breaks both α-helix and β-sheet conformations and introduces regions of relative flexibility, while arginine is indifferent for formation of both α-helix and β-sheet. Thus, replacement of proline may considerably perturb the secondary structure of cdb3 and its normal

**Fig 6.** Position of the two mutations in the band 3 cDNA and in the band 3 protein. (A) Schematic depiction of cDNA corresponding to band 3 Tuscaloosa. (B) Schematic representation of the band 3 protein based on hydropathy plot of the sequence in Lux et al. The previously described binding regions and the position of the regulated hinge are shown.

**Fig 7.** Alignment of the previously cloned band 3 proteins in vicinity of the Pro327 → Arg327 mutation. Sequences of human, mouse, rat, and chicken erythrocyte band 3 proteins were retrieved from the Genbank database and aligned using program CLUSTAL (PCGene). Alignment of amino acids 314 through 339 is shown. The sequences are highly homologous, prolines 322, 323, 327, and the mutated proline 327 are perfectly conserved. The mutant arginine (R) in the patient sequence is boxed.

**Band 3**

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Fig 7. Alignment of the previously cloned band 3 proteins in vicinity of the Pro327 → Arg327 mutation. Sequences of human, mouse, rat, and chicken erythrocyte band 3 proteins were retrieved from the Genbank database and aligned using program CLUSTAL (PCGene). Alignment of amino acids 314 through 339 is shown. The sequences are highly homologous, prolines 322, 323, 327, and the mutated proline 327 are perfectly conserved. The mutant arginine (R) in the patient sequence is boxed.
interactions with other RBC proteins. Since we did not have an opportunity to study additional affected family members and have not sequenced band 3 cDNA in its entirety, we cannot exclude the possibility of another mutation being present in the transmembrane domain of the band 3 protein. However, the experimental data and the evolutionary conservation of Pro$^{327}$ suggest that the Pro$^{327} \rightarrow$ Arg$^{327}$ is the underlying molecular defect of spherocytosis in the patient studied.

In conclusion, we report one of the first mutation of the band 3 protein. The corresponding clinical phenotype is a typical hereditary spherocytosis. Work of several laboratories provided evidence that the 4.2 protein binds to cdb$^{31,13,49}$; however, the actual binding site remains to be defined. Our data suggest that the region of cdb3 containing Pro$^{327}$ and, possibly, Lys$^{56}$ is involved either directly or indirectly in the interaction of protein 4.2 with cdb3. Although there is a correlation between the appearance of the Pro$^{327} \rightarrow$ Arg$^{327}$ substitution, or of both Pro$^{327} \rightarrow$ Arg$^{327}$ and Lys$^{56} \rightarrow$ Glu$^{56}$ substitutions, and reduced binding of protein 4.2 to membranes, other, as yet undetected, consequences of these substitutions may also exist.

REFERENCES