RAPID COMMUNICATION

Mutations of Conserved Arginines in the Membrane Domain of Erythroid Band 3 Lead to a Decrease in Membrane-Associated Band 3 and to the Phenotype of Hereditary Spherocytosis

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To elucidate the molecular basis of band 3 deficiency in a recently defined subset of patients with autosomal dominant hereditary spherocytosis (HS), we screened band 3 cDNA for single-strand conformation polymorphism (SSCP). In 5 of 17 (29%) unrelated HS subjects with band 3 deficiency, we detected substitutions R760W, R760Q, R808C, and R870W that were all coinherited with the HS phenotype. The involved arginines are highly conserved throughout evolution. To examine whether or not the product of the mutant allele is inserted into the membrane, we studied one HS subject who was doubly heterozygous for the R760Q mutation and the K56E (band $3^{MEMPHIS}$) polymorphism that results in altered electrophoretic mobility of the band 3 Memphis proteolytic fragments. We detected only the band $3^{MEMPHIS}$ in the erythrocyte membrane indicating that the protein product of the mutant, R760Q, band 3 allele is absent from the red blood cell membrane. These findings suggest that the R760Q substitution, and probably the other arginine substitutions, produce band 3 deficiency either by precluding incorporation of the mutant protein into the red blood cell membrane or by leading to loss of mutant protein from differentiating erythroid precursors.

BAND 3 is the most abundant protein of the red blood cell (RBC) membrane consisting of two domains with separate and largely independent functions. The N-terminal 45-kD cytoplasmic domain anchors the membrane skeleton to the membrane via interactions with ankyrin, protein 4.1, and protein 4.2.[1-5] In addition, the cytoplasmic domain binds hemoglobin and several glycolytic enzymes.[1,2,6] Most of the C-terminal 56-kD membrane domain consists of 12 to 14 membrane spanning α helices connected by endoplasmic and ectoplasmic loops. The main function of the membrane domain is to mediate anion exchange across the membrane.[2,7]

Several membrane protein mutations have recently been detected in hereditary spherocytosis (HS), a common inherited hemolytic anemia characterized by spheroidal shape of RBCs that have a reduced ratio of cell surface area to cell volume.[8] The principal biochemical abnormality of hereditary spherocytes is a partial deficiency of spectrin, the major protein of the RBC membrane skeleton, combined in most cases with a deficiency of ankyrin, a protein that anchors the spectrin-based membrane skeleton to the membrane by a β spectrin-ankyrin-band 3 linkage. In several kindred, the underlying molecular defects of spectrin and ankyrin have been defined.[8] Recently, two laboratories identified a novel subset of patients with autosomal dominant HS, characterized by an isolated deficiency of band 3 protein.[9,10] In one of the families with this phenotype the band 3 gene was found to contain a duplication of 10 bases (band 3^{PRAGUE}),[11] whereas in the remaining patients the molecular basis of this phenotype is unknown.

In addition to the above-noted band 3^{PRAGUE} mutation, several human band 3 mutations have been recently described producing several distinct phenotypes. Deletion of 9 amino acids from the interface of the cytoplasmic and membrane domains underlies the malaria-resistant Southeast Asian ovalocytosis.[12-14] Substitutions P327R (band $3^{TUSCALOOSA}$) and E40K (band $3^{MONTEFIORE}$) were found in association with atypical spherocytic hemolytic anemia and a deficiency of protein 4.2.[15,16] Mutation P868L (band 3^{HT}) has been detected in a subject with hereditary acanthocytosis and an increased anion transport.[17] Band 3 Memphis (K56E) is an asymptomatic polymorphism that alters electrophoretic mobility of band 3 and leads to an increase in the apparent molecular weight by 3 kD.[18-20]

In this study, we used single-strand conformation polymorphism (SSCP) detection to search for mutations of band 3 protein that underlie the phenotype of band 3–deficient HS. We report four different substitutions of three highly conserved arginine residues clustered in the C-terminal region of the membrane domain of band 3 protein, which are all coinherited with the HS phenotype. We detect both the mutant and normal mRNAs in total reticulocyte RNA whereas, in one case, we find the mutant band 3 protein absent from the membrane of mature erythrocytes, suggesting that the mutated arginines play a critical role either in the insertion of the mutant band 3 protein into plasma membrane of erythroid precursors or in its retention in the cytoplasmic membrane of maturing erythroid cells.

MATERIALS AND METHODS

Subjects. A total of 70 unrelated patients with autosomal dominant HS and 25 controls from Czech Republic were studied. The

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diagnosis of HS was based on the family history of hemolysis, presence of spherocytes in the peripheral blood smear, increased osmotic fragility, and exclusion of other causes of spherocytic hemolytic anemia. Freshly drawn blood anticoagulated in acid citrate/dextrose was shipped on ice to Boston.

Quantitation of erythrocyte membrane proteins. Within 48 hours of phlebotomy, erythrocyte ghosts were prepared by the method of Dodge et al[21] with minor modifications described in Jarolim et al.[15] Erythrocyte membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometry as described.[11] As an additional approach to quantitation of RBC proteins, we measured the relative number of band 3 copies per cell by labeling RBCs with eosin-5-maleimide[22,23] that binds to K430 in the membrane domain of band 3 protein.[24] In each quantitation, fluorescence of eosin-labeled band 3 protein in hereditary spherocytes was compared with fluorescence of a set of control samples.

SSCP analysis of band 3 cDNA. Total reticulocyte RNA was isolated by ammonium chloride lysis[25] and reverse transcribed using random primers. SSCP screening was performed as described[26,27] with minor modifications. Briefly, cDNA was polymerase chain reaction (PCR)-amplified in overlapping fragments of 300 to 400 bp. To each 10-μL PCR reaction, 2.5 μCi ^{32}P-dATP (3,000 μCi/mmol; ICN, Costa Mesa, CA) was added. The PCR products were diluted in formamide loading buffer (86% formamide, 10% glycerol, 20 mmol/L EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol), heat denatured by boiling for 5 minutes, and quickly cooled on ice. Samples were electrophoresed at 8 W for 16 hours at room temperature in a nondenaturing polyacrylamide MDE gel (25 mL MDE gel solution prepared according to manufacturer's specifications [AT Biochem, Malvern, PA], 69 mL deionized water, 6 mL 10× TBE, 0.4 mL 10% ammonium persulfate, 40 μL TEMED) and the gel was exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) overnight at −80°C with intensifying screens.

Sequencing of band 3 cDNA and genomic DNA. The segments of band 3 cDNA exhibiting the abnormal SSCP pattern were PCR-amplified using the same primers as for the initial SSCP screening. Single-stranded DNA template was prepared using the PCR Template Prep for ssDNA Sequencing kit (Pharmacia Biotech Inc, Piscataway, NJ) and sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, OH). Corresponding exons of the band 3 gene were PCR-amplified using intronic primers, cloned using the TA Cloning kit (Invitrogen, San Diego, CA) and individually sequenced using the Sequenase Version 2.0 DNA Sequencing Kit.

Sequence comparisons and structural predictions. Sequences of all 12 currently known sequences of human,[7,28] mouse,[29] rat,[30] chicken,[31,32] and trout[33] erythroid band 3 proteins (anion exchanger 1, AE1) and of the related anion exchangers AE2 from human,[34,35] mouse,[36] rat,[37] and rabbit[38] and AE3 from human,[39] mouse,[40] and rat[37] were retrieved from the Genome Sequence Data Base (National Center for Genome Resources, Santa Fe, NM) and aligned using the program CLUSTAL (PCGene; Intelligenetics, Mountain View, CA). The predictions of number and position of the band 3 transmembrane helices were made using programs RAOARGOS, HELIXMEM and SOAP (PCGene) based on the reported cDNA sequence.[7,28]

Sulfate fluxes. Cells were washed three times at 4°C in 140 mmol/L NaCl, 10 mmol/L Na phosphate, pH 7.4 (PBS), then three times in 84 mmol/L trisodium citrate, 1 mmol/L EGTA, pH 6.5. Washed cells were subjected to assays of unidirectional disodium ^{35}S-sulfate (ICN, Costa Mesa, CA) uptake at 20°C as described.[41] Each flux study in HS RBCs was performed in parallel using RBCs from unrelated healthy subjects. Each result is an average of three independent flux measurements.

Deglycosylation of band 3 protein. Erythrocyte membranes were prepared as described above and treated with recombinant peptide:N-glycosidase F (PNGase F; New England Biolabs, Beverly, MA). Typically, 10 μg of membrane proteins were digested with 300 U PNGase F for 30 minutes at 37°C in a total volume of 20 μL of 1× Reaction Buffer (50 mmol/L sodium phosphate, pH 7.5; New England Biolabs) with 1.25 mmol/L phenylmethylsulfonyl fluoride. The deglycosylated membranes were solubilized in 50 mmol/L Tris-HCl, pH 6.8, 4% glycerol, 1% SDS, 20 mmol/L dithiothreitol, 1% β-mercaptoethanol, 2 mmol/L EDTA, and the proteins were analyzed in a 12% Laemmli gel.[42]

RESULTS

A subset of HS patients is markedly deficient in band 3 protein. We studied 70 unrelated subjects with autosomal dominant HS and 25 controls. RBC membrane proteins were quantitated by two approaches. First, we directly measured the number of band 3 copies per cell by labeling RBCs with eosin-5-maleimide,[22,23] which specifically covalently binds to Lys 430 in the membrane domain of band 3 protein.[24] This approach showed a variable degree of band 3 deficiency (ranging from 47% to 92% of normal) in the HS subjects under study. The second approach involved electrophoretic separation of solubilized membrane proteins (SDS-PAGE), followed by gel densitometry and determination of the ratios of spectrin to band 3, and ankyrin to band 3. This approach allowed us to distinguish two major groups of HS subjects (Table 1).

The first group included 48 HS patients with a decreased ratio of spectrin to band 3, and, in 41 of these patients, a decrease in ankyrin to band 3 as well, indicating either a deficiency of spectrin alone or a combined deficiency of both spectrin and ankyrin, as previously reported.[43,44] The second group included 17 HS subjects in whom the SDS-PAGE densitometry showed an increase in the spectrin to band 3 and ankyrin to band 3 ratios, suggesting that the principal biochemical abnormality in this subset of HS involves a deficiency of band 3 protein. Indeed, calculation of the number of spectrin and ankyrin copies per cell (from the known spectrin to band 3 and ankyrin to band 3 ratios and the known number of band 3 copies per cell as determined by flow cytometry) showed that RBCs of these subjects contained a normal, or near normal, amount of spectrin and ankyrin (83% to 105% of normal values), whereas they were deficient in the band 3 protein. In this group of HS patients, the content of band 3 protein determined by flow cytometry ranged from 52% to 69% of the normal values and the band 3 content calculated from the spectrin/band 3 and ankyrin/band 3 ratios ranged from 55% to 72% of normal values with a good correlation between the results obtained by the two approaches in each patient. No biochemical abnormalities were detected in a third group of 5 HS patients (Table 1).

Clustered arginine substitutions are detected in band 3 cDNA from the band 3–deficient HS patients. To evaluate the possibility that the deficiency of the band 3 protein is caused by band 3 protein mutations, we isolated total reticulocyte RNA, reverse-transcribed it using random hexamers and screened the coding region of cDNA in overlapping fragment of 300 to 400 bp for SSCP. We detected a total of nine different SSCPs in the 95 studied individuals. We directly sequenced the segments of cDNA exhibiting SSCPs. In both control and HS subjects, we detected two silent polymorphisms (codon 417, CTG → TTG, Leu → Leu and

Table 1. Biochemical Phenotypes of the Studied Subjects and the Detected Amino Acid Substitutions

Subjects	Biochemical Phenotype	Subjects	Substitutions Detected	Subjects
Hereditary spherocytosis (n = 70)	Spectrin or combined spectrin and ankyrin deficiency (decrease in spectrin/band 3 and/or ankyrin/band 3 ratios by 15%-47%*)	48	E40K (Montefiore)	2
			K56E (Memphis)	2
			Codon 417 CTG → TTG (silent)	2
			Codon 441 CTG → CTA (silent)	8
	Band 3 deficiency (decrease in band 3 protein by 31%-48%†)	17	K56E (Memphis)	1
			Codon 441 CTG → CTA (silent)	2
			R760W (Hradec Kralove)	1
			R760Q (Prague II)	2
			R808C (Jablonec)	1
			R870W (Prague III)	1
	No abnormalities	5	Codon 441 CTG → CTA (silent)	1
Control (n = 25)	No abnormalities	25	Codon 441 CTG → CTA (silent)	4

Results of SSCP screening and sequencing of cDNA from 70 unrelated patients with hereditary spherocytosis and 25 healthy controls. The mutations associated with hereditary spherocytosis and band 3 deficiency are shown in bold.

• Decrease in the spectrin/band 3 and ankyrin/band 3 ratios shown in the table is based on densitometry of Coomassie blue–stained polyacrylamide gels.

† Based on flow cytometric quantitation of band 3 in eosin-labeled erythrocytes. Decrease in band 3 calculated from the gel densitometry data is proportional to that obtained by flow cytometry and ranges from 28% to 45%.

codon 441, CTG → CTA, Leu → Leu) and the previously described band 3 Montefiore mutation GAG → AAG (codon 40)[16] and band 3-Memphis polymorphism AAG → GAG (codon 56)[18] that are both asymptomatic in the heterozygous state. In contrast, in five unrelated HS patients with band 3 deficiency, we detected four missense mutations: R760W (CGG → TGG), R760Q (CGG → CAG), R808C (CGC → TGC), and R870W (CGG → TGG). The presence of these mutations was verified by PCR-amplification of the corresponding exons of human band 3 gene[45] from patient genomic DNA and separate sequencing of both alleles of the band 3 gene (Fig 1). The results of the SSCP screening and sequencing are summarized in Table 1.

Phenotype of band 3–deficient HS is coinherited with the arginine substitutions. We obtained blood samples from additional family members and PCR-amplified reverse-transcribed reticulocyte RNA using the same primers as for the SSCP screening. Taking advantage of the fact that all four arginine substitution abrogate the *Aci* I restriction sites, we digested the PCR amplified segments of cDNA with *Aci* I restriction endonuclease (Fig 2). We detected the arginine substitutions in all 13 affected members of the five studied families, a finding that was further verified by direct sequencing. We did not find the arginine substitutions in any of the nine healthy family members. We designated these mutations by the name of the cities of origin as band $3^{\text{HRADEC KRALOVE}}$, band $3^{\text{PRAGUE II}}$, band 3^{JABLONEC}, and band $3^{\text{PRAGUE III}}$. We change the designation of the previously reported band 3^{PRAGUE} mutation[11] to band $3^{\text{PRAGUE I}}$.

The mutated arginines are highly conserved. Alignment of the currently known sequences of human,[7,28] mouse,[29] rat,[30] chicken,[32,46] and trout[33] erythroid band 3 (anion exchanger, AE1) proteins and the related anion exchangers AE2 from human,[34,35] mouse,[36] rat,[37] and rabbit,[38] and AE3 from human,[39] mouse,[40] and rat[37] shows that R760 and R808 are conserved in all 12 proteins and R870 is conserved in all proteins except for mouse AE3 (Fig 3). The high degree of evolutionary conservation of the arginine residues and the position of arginines 760 and 808, and possibly also 870 at the cytoplasmic boundaries of predicted transmembrane helices suggests a crucial role of these arginine residues in the structure and function of the band 3 protein, and further supports our hypothesis that the R760W, R760Q, R808C, and R870W substitutions underlie band 3 deficiency in our HS patients.

mRNA corresponding to both band 3 alleles is detected in reticulocytes. Restriction digestion with *Aci* I allowed us to compare the relative amounts of the cDNA restriction fragments (Fig 2). We concluded that mRNAs encoded by both normal and mutant band 3 gene alleles are present in reticulocytes, suggesting that neither the transcription of the mutant gene nor the stability of the mutant mRNA are affected by the mutations.

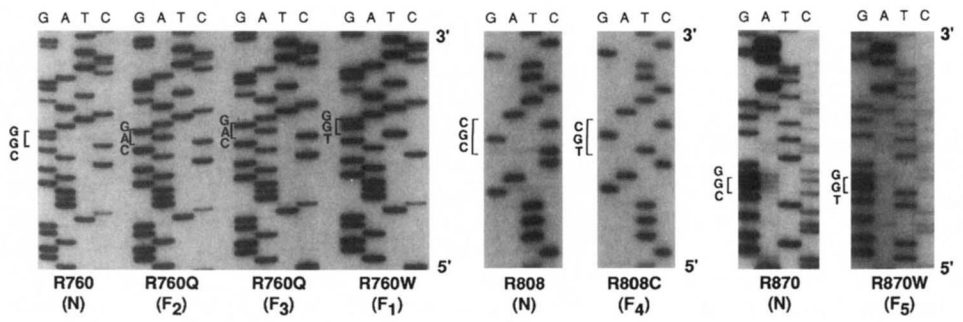

Fig 1. Detection or four arginine substitutions in the membrane domain of band 3. Sequencing of genomic DNA clones shows substitutions R760W, R760Q, R808C, and R870W in one allele of band 3 gene.

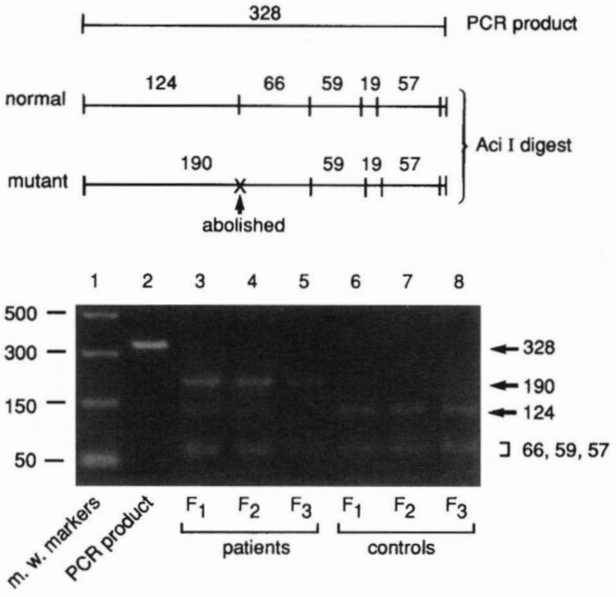

Fig 2. ***Aci*** **I restriction digestion of patient cDNA detects similar amount of cDNA for both the normal and the mutant alleles. Patient cDNA was PCR-amplified and completely digested with the** ***Aci*** **I restriction endonuclease. The normal product of 328 bp is completely digested (lanes 6 through 8). In contrast, the HS samples (lanes 3 through 5) contain three major bands. Two of these bands correspond in size to the fully digested product of the normal band 3 allele. The undigested 190-bp band and the mutant PCR bands represent the mutant allele, which is not digested because of the abrogation of** ***Aci*** **I restriction site. The relative amounts of both the digested products of the normal allele and the undigested product of the abnormal allele are approximately the same, indicating that mutant mRNA is present in the reticulocytes.**

The product of the R760Q band 3 allele is not detected in the erythrocyte plasma membrane. The quantitative analysis of the number of band 3 copies per cell by flow cytometry of eosin maleimide-labeled RBCs, described above, showed a marked decrease in the number of band 3 copies per cell, consistent with the possibility that the product of the mutant band 3 allele is not incorporated into the HS RBC membrane. We had the opportunity to directly verify this conclusion in a propositus from the family F3, who exhibited a 48% decrease in the band 3 content and who inherited two band 3 mutations in trans: the R760Q mutation, producing the phenotype of band 3–deficient HS inherited from the affected mother, and the band 3-Memphis polymorphism inherited from the asymptomatic father. The band 3-Memphis protein has a slower electrophoretic mobility, which is readily demonstrable after deglycosylation or proteolytic cleavage.[18] Therefore, we deglycosylated band 3 protein in erythrocyte ghosts using recombinant N-glycosidase F and electrophoresed the RBC membrane proteins in 10% Laemmli gels (Fig 4). The SDS-PAGE of the father showed two bands, which corresponded in their mobility to normal band 3 and band 3-Memphis, respectively. In the affected mother, who carried the R760Q mutation, only one band of normal mobility was detected. In contrast, examination of the propositus (their son) showed only the presence of the band 3-Memphis allele, inherited from the father. From this data, we conclude that the mutant band 3 protein containing the R760Q substitution is not present in the membrane of the proband. We further conclude that the band 3 polypeptide of normal mobility detected in the affected mother represents the product of the normal band 3 allele. Thus, the R760Q mutation either completely prevents the incorporation of the mutant band 3 protein into the plasma membrane of the erythroblasts, or destabilizes the mutant protein to prohibit its retention in mature RBCs.

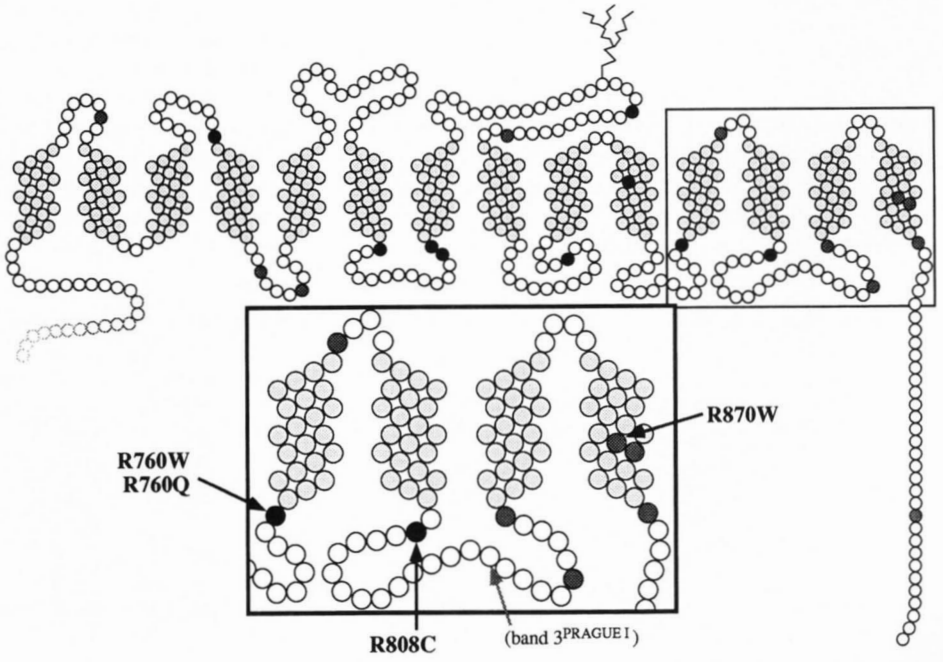

Fig 3. Mutated arginines are highly conserved throughout evolution. Scheme of the membrane domain of band 3 protein with 14 transmembrane segments (light shading) connected by endoplasmic and ectoplasmic loops. Arginine residues are marked by dark shading, those conserved throughout evolution of the anion exchanger proteins AE1, AE2, and AE3 are shown as filled circles. The drawing is based on the reported cDNA sequence[7,28] and on predictions of the secondary structure and transmembrane helices obtained using programs RAOARGOS, HELIXMEM, and SOAP (PCGene, Intelligenetics). The inset shows the C-terminal portion of band 3 protein with the position of the reported arginine substitutions and the previously reported band 3^{PRAGUE} mutation designated here as band 3$^{PRAGUE\ I}$.

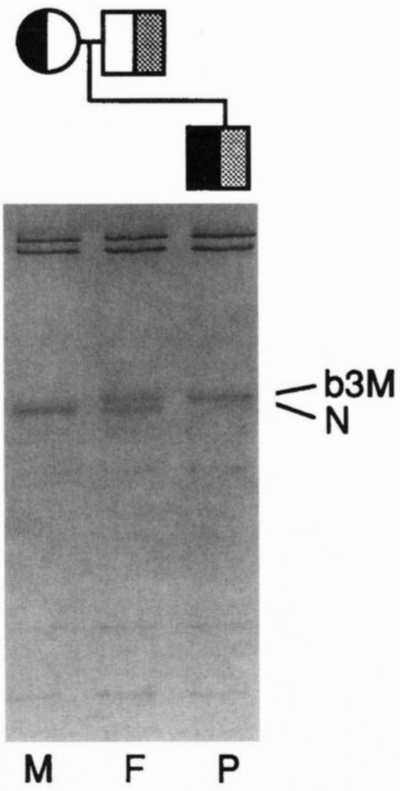

Fig 4. The product of the R760Q allele is not present in patient erythrocyte membranes. Band 3 protein was completely deglycosylated by recombinant peptide: N-glycosidase F in erythrocyte ghosts prepared from the propositus (P) and his parents (family F3). Both the normal band 3 allele (N, white) and the band 3 Memphis allele (b3M, shaded) with slower electrophoretic mobility are detected in the asymptomatic father (F). Normal mobility of band 3 protein is detected in the affected mother (M) who carries the R760Q substitution (black). This suggests that in her RBC membranes, either only normal band 3 protein is present, or that both normal and R760Q band 3 protein are present and have the same electrophoretic mobility. In the propositus, only the band 3 Memphis, inherited from the father, is detected, demonstrating that the R760Q allele, inherited from the mother, is not present in the RBC membranes of the propositus.

RBC anion transport is reduced in proportion to band 3 deficiency. Because band 3 is the principal anion exchange protein of the RBC membrane,[2] we compared anion fluxes in band 3–deficient HS and in normal RBCs. In eight independent measurements in five carriers of three different arginine substitution, we found a 48% ± 11% (52% ± 11% of control values) decrease in the influx of radiolabeled sulfate that serves as a measure of the transport function of band 3[47] (Table 2). There was a good correlation between the decrease in band 3 content determined by gel densitometry or flow cytometry and the decrease in sulfate flux in each individual patient studied (Table 2).

DISCUSSION

We studied a group of patients with autosomal dominant HS and isolated band 3 deficiency, and with normal content of RBC spectrin and ankyrin. We detected four distinct arginine substitutions in 5 of 17 unrelated patients (29%) but in none of the 53 other HS subjects, nor in 25 controls. We found that the mRNAs for both the normal and mutant band 3 are present in reticulocytes in approximately equal amounts (Fig 2), suggesting that the band 3 deficiency is caused by a defective insertion of the mutant protein into the membrane or by a loss of the protein from the membrane of erythroblasts during their terminal differentiation.

In one HS subject from family F_2, we were able to directly establish that the protein product of the R760Q band 3 allele is absent from the plasma membrane of circulating erythrocytes. This HS subject carried two band 3 mutations in trans: (A) K56E (AAG → GAG), producing a previously described asymptomatic Memphis polymorphism characterized by altered mobility of the band 3 protein, and (B) the spherocytogenic R760Q (CGG → CAG) mutation. Only the band 3-Memphis protein of slower electrophoretic mobility was detected in the membranes of the proband. Because the degree of band 3 deficiency in this HS proband is similar to that found in other patients carrying the arginine substitutions, we propose that the above outlined mechanism may be common to the other detected arginine substitutions.

The affected arginine residues are highly conserved during evolution, and are positioned at the boundaries of the putative transmembrane segments and may act as stop transfer signals during cotranslational insertion of band 3 protein into the endoplasmic reticulum membranes of erythroid precursor cells. Mutations of such amino acids are likely to have a major deleterious effect on structure and/or function of the protein. It is noteworthy that arginine residues have been implicated in the anion exchange function of band 3 by several lines of evidence. pH titration of Cl^-/Cl^- exchange in intact RBCs indicates the requirement of a functional group with pK_a of ~12 at 0°C.[48] Arginine-reactive reagents

Table 2. Band 3–Mediated $^{35}SO_4$ Fluxes and the Corresponding Band 3 Content Determined by Densitometry and Flow Cytometry

Family/Patient	Mutation	Band 3 Deficiency From Gel Densitometry (% of control)	Band 3 Deficiency From Flow Cytometry (% of control)	$^{35}SO_4$ Flux (% control)*	No. of Measurements*
F_1/I_1	R760W	57	53	46.0	1
F_2/II_1	R760Q	64	58	52.6	1
F_2/III_1	R760Q	72	69	63.4 ± 5.1†	2
F_3/I_1	R760Q	55	54	40.9 ± 16.5†	3
F_4/I_1	R808C	59	52	51.8	1
Average		61 ± 7†	57 ± 7†	52 ± 11†	

* Each measurement was performed in triplicate on blood samples drawn and shipped separately. The result is expressed as a ratio of the sulfate influx in the patient to the sulfate influx in a matched control.

† Average values are means ± SD.

such as phenylglyoxal[49] and cyclohexanedione[50] irreversibly inactivate anion exchange, and have been hypothesized to bind to sites in postulated inner or outer vestibules of band 3, or within the anion translocation pathway itself. Most recently, mutagenesis of individual lysine residues of the membrane domain, selected for their documented binding of distinct covalent inhibitors of transport, has been shown not to inactivate anion transport.[51] Thus, the mutated arginine residues are candidates for direct participation in the anion translocation process. Although we were able to show the absence of the mutant protein from the plasma membrane in one patient from family F_2, carrying the R760Q mutation, other mutations may not completely prevent the incorporation of the mutant protein into the plasma membrane. Presence of some dysfunctional band 3 protein could account for the small difference between the amount of band 3 obtained by densitometric (61% ± 7% of control) and flow cytometric (57% ± 7% of control) quantitation and the somewhat lower than expected values of the sulfate flux (52% ± 11% of control).

The mechanism leading to the spherocytic phenotype in the studied patients remains hypothetical. Band 3 protein is thought to stabilize the membrane by interacting with the adjacent boundary lipid molecules.[52] As was proposed by Lux et al,[10] a decrease in the content of band 3 could lead to formation of protein-free areas of the lipid bilayer lacking the stabilizing protein-lipid and protein-protein interactions. Such areas of the membrane might be unstable and susceptible to release from the membrane. We propose that a release of such band 3–free membrane regions represents the mechanism leading to surface area deficiency and spherocytosis in the studied subjects. Such a hypothesis is supported by our recent finding of decreased density of intramembrane particles in HS subjects with band 3 protein deficiency.[11]

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