A Nonsense Mutation 1669Glu→Ter within the Regulatory Domain of Human Erythroid Ankyrin Leads to a Selective Deficiency of the Major Ankyrin Isoform (Band 2.1) and a Phenotype of Autosomal Dominant Hereditary Spherocytosis

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Abstract

We describe a nonsense mutation in the regulatory domain of erythroid ankyrin associated with autosomal dominant hereditary spherocytosis with a selective deficiency of the ankyrin isoform 2.1 (55% of normal), a deficiency of spectrin (58% of normal) proportional to the decrease in ankyrin 2.1, and a normal content of the other main ankyrin isoform, protein 2.2. PCR amplification of cDNA encoding the regulatory domain of ankyrin revealed a marked decrease in the ratio of ankyrin 2.1 mRNA to the ankyrin 2.2 mRNA. Sequencing of ankyrin gene in the region where the 2.1 and 2.2 mRNA differ detected a nonsense mutation 1669Glu→Ter (GAA→TAA) in one ankyrin allele. Only normal ankyrin 2.1 mRNA was detected in the reticulocyte RNA. Since the alternative splicing within the regulatory domain of ankyrin retains codon 1669 in ankyrin 2.1 mRNA and removes it from ankyrin 2.2 mRNA, we propose that the 1669Glu→Ter mutation decreases the stability of the abnormal ankyrin 2.1 mRNA allele leading to a decreased synthesis of ankyrin 2.1 and a secondary deficiency of spectrin. (*J. Clin. Invest.* 1995; 95:941–947.) Key words: congenital hemolytic anemia • spectrin • ankyrin • RNA splicing • erythrocyte membrane

Introduction

Erythroid ankyrin is a major red cell membrane protein that links the red cell membrane skeleton to the plasma membrane by interactions with spectrin, the major protein of the membrane skeleton, and band 3 protein, the major transmembrane protein of the red cell membrane (1–3). Ankyrin contains three distinct regions which differ in their function. These regions include the NH₂ terminal domain which contains 24 homologous repeats and which is involved in the binding of band 3 protein, the central spectrin-binding domain, and the COOH-terminal regulatory domain (4, 5) (Fig. 1 A). The regulatory domain of ankyrin is subject to extensive alternative splicing (4–8). As a result, separation of membrane proteins in SDS-PAGE reveals

a ladder of bands reacting with ankyrin antibodies. These bands include band 2.1, the full-size erythroid ankyrin, and additional bands designated as bands 2.2 to 2.6 (1, 9).

Proteins 2.1 and 2.2 represent the two major ankyrin isoforms produced by alternative splicing (Fig. 1 B). They differ in that the major portion of exon 38 (8) is deleted from the ankyrin 2.2 mRNA, due to a usage of an intraexon splice site (4, 5) (Fig. 1 C). This alternative splicing produces a major difference in the function of these two ankyrin isoforms: protein 2.2 is an activated form of ankyrin in that it has threefold higher affinity for spectrin and binds to twice the number of high affinity band 3 sites (10). The 2.2 isoform is the predominant isoform of developing erythroblasts while protein 2.1 is the major ankyrin isoform of mature erythrocytes (6).

Several studies have suggested that ankyrin mutations represent the underlying molecular defect in a subset of patients with hereditary spherocytosis (HS).¹ This common hereditary hemolytic disorder is heterogeneous in terms of inheritance, severity of hemolysis, and the underlying molecular defects which involve α spectrin, β spectrin, ankyrin, and the band 3 protein (for review see reference 11). The data implicating ankyrin as the underlying molecular defect involve a report of a deletion of ankyrin gene on chromosome 8 (12), restriction fragment length polymorphism study of a large family demonstrating a linkage of autosomal dominant HS to the ankyrin gene (13), the coinheritance of an abnormally migrating ankyrin designated as ankyrin^{PRAGUE} with the HS phenotype (14), and a severe defect of ankyrin mRNA expression and ankyrin biosynthesis (15). Recently, several ankyrin mutations have been reported in a preliminary form, which involves one nonsense mutation, and small insertions or deletions leading to a frameshift and premature chain termination (16).

In this report, we describe a unique mutation in the regulatory domain of ankyrin in a kindred with autosomal dominant HS and a marked and selective deficiency of the content of ankyrin isoform 2.1 and a normal content of the second main ankyrin isoform 2.2. This deficiency of the 2.1 ankyrin isoform is accompanied by a proportional deficiency of spectrin. We find that the underlying genetic defect involves a nonsense mutation 1669Glu→Ter (GAA→TAA) in one allele of the ankyrin gene. As a result, alternative splicing in the regulatory domain of ankyrin leads to a retention of codon 1669 in ankyrin 2.1 mRNA while it removes an exon containing this mutation from ankyrin 2.2 mRNA. The mutation could be detected in the genomic DNA, but it is nondetectable in the reticulocyte RNA, suggesting that the underlying cause of the selective ankyrin 2.1 deficiency involves instability of ankyrin 2.1 mRNA which con-

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1. Abbreviations used in this paper: arb.u., arbitrary units; E5M, eosin-5-maleimide; HS, hereditary spherocytosis.

Figure 1. Ankyrin isoforms 2.1 and 2.2 are produced by alternative splicing in the regulatory domain of ankyrin. (A) Full size erythroid ankyrin (protein 2.1) is a 206-kD protein consisting of three distinct domains: band 3 binding, spectrin binding, and regulatory. (B) The difference between ankyrins 2.1 and 2.2 is due to alternative splicing in the region encoding the regulatory domain. (C) In more detail, 486 bases of exon 38 are missing in the mRNA encoding protein 2.2 due to usage of an intraexon splice site within exon 38. Compiled from references 4, 5, 16, and 26. Positions of the PCR primers used in this study are indicated.

tains the 1669Glu→Ter mutation. We further find that the partial deficiency of the 2.1 ankyrin isoform is accompanied by a proportional decrease in spectrin content of the cells. We designate the mutant ankyrin allele, based on the city of origin, as ankyrin^{RAKOVNIK}.

Methods

Case report. A Czech kindred of three generations was studied. The proband, J. K., was examined at 2 mo of age for suspected hemolytic anemia. Tests revealed hemoglobin levels of 12.4 g/dl, hematocrit of 37.0, reticulocyte count of 3.5%, and mean cell volume of 74 fl. The presence of numerous spherocytes in the peripheral blood smear was noted. Total bilirubin was 1.2 mg/dl with direct bilirubin of 0.5 mg/dl. Red cell osmotic fragility was increased, while Coombs' test was normal. Diagnosis of congenital spherocytosis was made. Neonatal jaundice was not observed, and exchange transfusions were not needed.

Parents and grandparents of the proband were studied. Mother of the proband, A. K., and the maternal grandfather, V. P., were found to have decreased hemoglobin and hematocrit, increased reticulocyte count, bilirubin, osmotic fragility, autohemolysis, and abnormal Pink test (17) (Table I). In both of them, spleen was enlarged to +2 cm

below the costal margin. Numerous spherocytes and some anisocytosis were noted in the peripheral blood smear, however mean red cell volume was in the normal range. Bilirubin levels were increased in both patients to ~4 mg/dl. Patient A. K. recently developed cholelithiasis and her total bilirubin increased to 7.6–9.1 mg/dl. The liver function tests remained within normal limits. No growth retardation, leg ulcers, central nervous system disease, or myopathy was noted, and no clear hemolytic crises were diagnosed in any of the three patients. Based on the clinical and laboratory data, autosomal dominant HS was diagnosed in all three patients J. K., A. K., and V. P. After obtaining informed consent, venous blood of the three affected individuals was collected into acid citrate/dextrose solution and shipped on ice overnight to Boston for evaluation.

Preparation of erythrocyte ghosts and analysis of membrane proteins. Freshly drawn blood anticoagulated in acid citrate/dextrose was shipped on ice to Boston. Within 48 h of phlebotomy, erythrocyte ghosts were prepared by the method of Dodge et al. (18) with minor modifications described in reference 19. Proteins were analyzed by SDS-PAGE in 3.5–17% exponential gradient gels according to Agre's modification (20) of the original Fairbanks method (21) and in 12% Laemmli gels (22), and the relative abundance of the major red cell membrane proteins was analyzed by densitometry. The abnormal proteins detected by SDS-PAGE were further characterized by immunoblotting with rabbit polyclonal antibodies raised against α and β spectrins and ankyrin. The

Table I. Selected Hematological Parameters of the Affected Family Members

Patient	Year born	Hemoglobin	Hematocrit	Mean cell volume	Bilirubin		Reticulocyte count	Osmotic fragility [‡]	48-h autohemolysis		Pink test
					Total	Direct			Without glucose	With glucose	
		g/dl	%	fl	mg/dl		%		%		%
III/1 (J. K., proband)	1992	12.4	37.0	74	1.2	n.d. [§]	3.5	0.52–0.36	n.d.	n.d.	n.d.
II/1 (A. K., mother)	1970	10.7	32.0	92	3.8	0.7–0.8	3.6	0.54–0.42	13.2	3.0	66
I/1 (V. P., grandfather)	1934	12.8	37.7	90	3.4–4.1	0.9–1.0	4.5	0.52–0.46	8.5	3.7	59

[‡] Osmotic fragility is given as an interval between minimum osmotic resistance (normal range 0.48–0.44) and complete hemolysis (normal 0.34–0.30). ^{||} After development of cholelithiasis, total bilirubin levels increased to 7.6–9.1 mg/dl. [§] n.d., not determined.

electrophoresed proteins were transferred to nitrocellulose, immunoreacted with the primary antibody and peroxidase-coupled secondary antibody, and the blot was developed using hydrogen peroxide and α -chloronaphthol.

Quantitation of band 3 copies by cytofluorometry. Band 3 protein was quantitated in individual red cells by cytofluorometry of eosin-5-maleimide (E5M)-labeled erythrocytes, essentially as described (23, 24). Briefly, blood was washed three times with phosphate-buffered saline (PBS), and the buffy coat was carefully removed. 40 μ l of packed red cells was resuspended in 1.76 ml of PBS with 0.5% bovine serum albumin (BSA) and 200 μ l of a 1 mg/ml stock solution of E5M (Molecular Probes, Inc., Eugene, OR) was added. The suspension was incubated for 1 h in the dark at room temperature on a tube rocker, washed three times with PBS, and resuspended in PBS with 0.5% BSA. Erythrocytes were analyzed using a flow cytometer (PROFILE II; Coulter Corp., Hialeah, FL). Fluorescence at 525 nm and low angle and right angle scatter were measured for 20,000 cells in each sample, a histogram of fluorescence intensity was plotted, and the mean fluorescence intensity and the standard deviation were calculated.

Preparation of reticulocyte RNA and PCR amplification of ankyrin cDNA. Total reticulocyte RNA was isolated by ammonium chloride lysis (25) and reverse transcribed using random hexamers. A segment of cDNA coding for the regulatory domain of ankyrin was PCR amplified using a PCR reagents kit (GeneAmp; Perkin Elmer-Cetus, Norwalk, CT) and primers P401 (5'-ACATCACCATGCCCCCTGCGCTA-3'; nt 4163-4186) and P400 (5'-GCACCGCTGCGGTGGCCCTCA-3'; nt 5793-5773) (Fig. 1 B), 35 cycles, 1 min at 95°C, 1 min at 65°C, 2 min at 72°C. Such amplification should produce bands of 1,631 bp and 1,145 bp corresponding to the 2.1 and 2.2 mRNAs (5, 26) and numerous additional weaker bands corresponding to other products of alternative splicing (7).

Identification of the band migrating in the 2.2 position by restriction digestion. The patient's band migrating in the 2.2 position was identified by restriction digestion. Patient and control cDNA encoding the regulatory domain was PCR amplified, the PCR products were electrophoresed in an agarose gel, the 2.2 bands were cut out, purified using the GeneClean II kit (BIO 101, Inc., La Jolla, CA), and digested with BamHI and PstI. Digestion of the normal 2.2 product should produce bands of 781, 216 and 138 bp for BamHI and 604, 201, 192, and 138 for PstI.

Determination of nucleotide sequence of splice sites used in formation of the ankyrin 2.2 mRNA. While the acceptor splice site is located within ankyrin exon 38 (4, 5) and can be readily PCR amplified from genomic DNA using the known cDNA sequence, it was necessary to determine the sequence of the donor splice site, i.e., sequence the 3' boundary of intron 37. For that, genomic DNA was isolated from a healthy individual and digested with restriction enzymes TaqI and DpnII. Plasmid pGEM4Z (Promega Corp., Madison, WI) was digested with AccI and BamHI, and cohesive ends of DNA and plasmid were ligated. The unknown sequence of intron 37 adjacent to exon 38 was PCR amplified from this ligation mixture using PCR primer A2 (5'-TTCCAGAGAGCCCAACTCGG-3', nt 4941-4922) within exon 38 (Fig. 1 C) and pGEM4Z primer T7 (5'-TAATACGACTACTA-TAGGG-3'), 40 cycles, 1 min 94°C, 1 min 40°C, 2 min 72°C, and the product of the first amplification was reamplified with primer T7 and a nested primer A8 (5'-CACGTAGCGGAGAGGAAAGTGC-3', nt 4693-4672) in exon 38 (Fig. 1 C), 40 cycles, 1 min 94°C, 1 min 55°C, 2 min 72°C. The obtained PCR product of ~520 bp was cloned into plasmid pCR II using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced using Sequenase version 2.0 DNA sequencing kit (United States Biochemicals Corp., Cleveland, OH).

Sequencing of patient genomic DNA. After sequencing of the 3' end of intron 37, most of the patient ankyrin exon 38 and the adjacent acceptor splice site of intron 37 was PCR amplified (35 cycles, 1 min 94°C, 1 min 55°C) using primer A16 (5'-CTAGATGCATGCTCG-AGCGG-3'; nt -63 to -44) and primer A10 (5'-CTCCTCTCCGTC-ACCTGACTC-3'; nt 5171-5151) (Fig. 1 C), phosphorylated at the 5'-end (3 μ g primer, 1 mM ATP, 10 U T4 polynucleotide kinase in a total volume of 30 μ l of 10 mM Tris-acetate, 10 mM magnesium acetate,

Figure 2. Electrophoresis of membrane proteins reveals a combined spectrin and ankyrin 2.1 deficiency and a relative increase in ankyrin 2.2. (A) Red cell membranes from both affected individuals were prepared and analyzed by SDS-PAGE in 3.5–17% exponential gradient gels. Densitometry of Coomassie blue stained gel followed by calculation of spectrin to band 3, ankyrin 2.1 to band 3, and ankyrin 2.2 to band 3 ratios revealed a 15% relative decrease in spectrin,

an 18% relative decrease in ankyrin 2.1, and a 52% relative increase in ankyrin 2.2 (see also Table II). (B) Immunoblotting with anti-ankyrin antibodies detected increased staining of the 2.2 band, suggesting that the increase is due to an increased expression of ankyrin 2.2 or to the presence of a truncated ankyrin comigrating with ankyrin 2.2 and is not caused by the presence of an abnormal protein other than ankyrin.

50 mM potassium acetate, pH 7.5, 30 min at 37°C). Single-stranded DNA was prepared from the 613-bp PCR product using the PCR template kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and directly sequenced using the Sequenase version 2.0 sequencing kit and a set of nested sequencing primers.

MseI restriction digestion and sequencing of patient cDNA. Digestion with the MseI restriction endonuclease was used to verify the presence of the mutation in the additional two family members. A segment of both genomic DNA and cDNA was PCR amplified using primers A1 (5'-CACGAGTGGAAAGTTGGAGGG-3'; nt 4876-4895) and A10, 35 cycles, 1 min 95°C, 1 min 65°C, 1.5 min 72°C. 8 μ l of the PCR product was digested with 8 U of MseI (New England Biolabs, Beverly, MA) and electrophoresed in a 3% agarose gel. MseI digestion should leave the control PCR product of 296 bp intact, while the PCR-amplified mutant allele should be cleaved into two fragments of 213 and 83 bp. For direct sequencing, patient cDNA was amplified using primer A1 and phosphorylated primer A10 under conditions described for the MseI digestion, single-stranded template was prepared using the PCR template kit and sequenced using the Sequenase version 2.0 sequencing kit.

Results

Ankyrin^{RAKOVNIK} red cells are markedly deficient in ankyrin isoform 2.1 and spectrin while having a normal amount of the ankyrin 2.2 isoform. Red cell membranes from both affected individuals were prepared and analyzed by SDS-PAGE in 3.5–17% exponential gradient gels. Densitometry of Coomassie blue stained gel followed by calculation of spectrin to band 3, ankyrin 2.1 to band 3, and ankyrin 2.2 to band 3 ratios revealed a 15% decrease in spectrin to band 3 ratio, an 18% decrease in ankyrin 2.1 to band 3 ratio, and a 52% increase in ankyrin 2.2 to band 3 ratio (Fig. 2 A and Table II). In accordance with the above results of densitometric determination, immunoblotting with anti-ankyrin antibodies revealed a relative increase in staining of the 2.2 band (Fig. 2 B). In addition to the densitometric analysis, we used flow cytometry of E5M-labeled cells to quantitate band 3 in single cells. E5M binds specifically to band 3

Table II. Densitometric Quantitation of Red Cell Membrane Proteins Separated in 3.5–17% Exponential Gradient Fairbanks Gels and Cytofluorometric Quantitation of Band 3 Copy Numbers in Individual Red Blood Cells

	Densitometry of Coomassie blue stained gels, uncorrected values (relative to band 3)				Band 3 content measured by flow cytometry		Relative protein content after correction for decrease in band 3 protein			
	Spectrin to band 3	Ankyrin 2.1 to band 3	Ankyrin 2.2 to band 3	Ankyrins 2.1 and 2.2 to band 3	Fluorescence per red cell (arb. u.)	Relative band 3 content*	Spectrin	Ankyrin 2.1	Ankyrin 2.2	Ankyrins 2.1 and 2.2
Normal range	0.97±0.10	0.17±0.03	0.07±0.02	0.24±0.04	10.0±0.7	100%	0.97±0.10	0.17±0.03	0.07±0.02	0.24±0.04
III/1 (J. K.)	0.86	0.14	0.10	0.24	6.3	63%	0.54 (56%)	0.09 (53%)	0.06 (86%)	0.15 (63%)
II/1 (A. K.)	0.78	0.13	0.11	0.24	7.2	72%	0.56 (58%)	0.09 (53%)	0.08 (113%)	0.17 (71%)
I/1 (V. P.)	0.84	0.15	0.11	0.26	6.9	69%	0.58 (60%)	0.10 (59%)	0.08 (113%)	0.15 (63%)
Average	0.83 (85%)	0.14 (82%)	0.11 (152%)	0.25 (103%)	6.8	68%	0.56 (58%)	0.09 (55%)	0.07 (105%)	0.16 (65%)

Abnormal results are shown in bold. Average values are means±S.D. * Relative to the control samples.

protein at lysine 430 (27), and its single cell fluorescence intensity, measured by flow cytometry, is therefore directly proportional to the number of band 3 molecules in each cell (23). While the average fluorescence intensity of normal red cells was normalized to 10.0±0.7 arbitrary units (arb. u.) per red cell, the fluorescence intensity of red cells from the affected family members ranged from 6.3 to 7.2 arb. u. per red cell, demonstrating an average 32% decrease in the number of band 3 molecules per red cell (Table II). Consequently, recalculation of the spectrin and ankyrin content with respect to the decrease in band 3 protein yields the following absolute content of spectrin and ankyrin: spectrin 58% of normal values, ankyrin 2.1 isoform 55% of normal, and ankyrin 2.2 isoform 105% of normal (Table II), i.e., approximately the same decrease in spectrin and ankyrin 2.1, and a normal content of ankyrin 2.2.

Ankyrin^{RAKOVNIK} cDNA exhibits altered proportion of ankyrin 2.1 and 2.2 mRNAs. To elucidate the underlying molecular defect, we used reverse transcription PCR to study alternative splicing within the regulatory domain of ankyrin. In all control subjects, amplification of the cDNA corresponding to the regulatory domain produced two main products the size of which corresponded to the predicted size for the known ankyrin 2.1 and 2.2 mRNAs. Amplification of the patient cDNA produced bands of identical size. In control subjects, the intensity of the PCR-amplified 2.1 mRNA is considerably higher than that of 2.2 mRNA. In the ankyrin^{RAKOVNIK} red cells, the intensities of the 2.1 and 2.2 bands were reversed, with the band 2.2 more intense than the band 2.1 (Fig. 3). The increased intensity of the 2.2 band in the HS patients could be due to (a) decrease in the 2.1 mRNA and increased amplification of the competing 2.2 cDNA; (b) higher abundance of the 2.2 mRNA; or (c) comigration of a different PCR product with the 2.2 band. We therefore studied the 2.2 band from the patient by restriction digestion. Patient and control bands 2.2 were cut out from the agarose gel, purified, and digested with BamHI and PstI. Both patient and control 2.2 bands were completely cleaved into restriction pattern expected for the ankyrin 2.2 mRNA, demonstrating that the patient band 2.2 contains exclusively PCR-amplified ankyrin 2.2 mRNA (data not shown).

A nonsense mutation is detected in one ankyrin allele. Because of the selective and marked decrease in ankyrin 2.1 protein in the patients, as well as the decrease in the 2.1 ankyrin cDNA, we focused on the region of ankyrin gene in which the 2.1 and 2.2 mRNAs differ, i.e., a 486-nt segment of exon 38 which is present in the 2.1 mRNA and is spliced out in the 2.2

message. To sequence this whole region, we first sequenced the unknown 3'-end of the preceding intron 37. PCR amplification with primers T7 and A8 of control genomic DNA, digested with TaqI and DpnII and ligated into the pGEM4Z plasmid digested with AccI and BamHI, yielded a product of 517 bp that was cloned and sequenced. Its sequence comprised 69 bp of exon 38, the rest being derived from intron 37. We sequenced 70 bases at the 3' end of intron 37, prepared an intronic primer A16 (5'-CTAGATGCATGCTCGAGCGG-3'; nt -63 to -44) (Fig. 1 C), and PCR amplified a 613-bp segment of patient genomic DNA comprising most of exon 38 and the adjacent part of intron 37 using primer A16 and primer A10 phosphorylated at the 5'-end. Single-stranded template was prepared and directly sequenced. Sequencing revealed a transversion G→T in codon 1669 that replaces GAA (glutamic acid) by TAA (termination codon) (Fig. 4).

This mutation changes the normal sequence of codons 1668 and 1669 (4) from AATGAA to AATTAA and thus creates a MseI restriction site TTAA. Digestion with the MseI restriction endonuclease of a 296-bp PCR product, obtained by PCR amplification of genomic DNA using primers A1 and A10, was therefore used to evaluate the presence of the mutation in the studied kindred. Portion of the PCR product, corresponding presumably to one ankyrin allele, was cleaved into two bands of 213 and

Figure 3. PCR amplification of cDNA corresponding to the regulatory domain of ankyrin. Total reticulocyte RNA was isolated by ammonium chloride lysis and reverse transcribed using random hexamers. Segment of cDNA encoding the regulatory domain of ankyrin was PCR amplified using primers P400 and P401, and the products of amplification were electrophoresed in a

3.5% polyacrylamide gel and stained with ethidium bromide. In both patients shown, the intensity of the PCR product corresponding to band 2.2 is higher than that corresponding to protein 2.1. In contrast, in all controls studied so far, three of which are shown, the intensity of the 2.2 band has always been less than the intensity of the 2.1 band.

Figure 4. A nonsense mutation is detected in one allele of the ankyrin gene but not in the ankyrin 2.1 mRNA. (A) Direct sequencing of the PCR amplified exon 38 and the adjacent region of intron 37 reveals a transversion G→T in codon 1669 that changes codon 1669 from GAA (glutamic acid) to TAA (termination codon). The normal intraexon splice site, preceded by the obligatory AG, and the portions of exon 38 that are either spliced out or retained in the 2.2 mRNA are shown. (B) Direct sequencing of a segment of patient cDNA corresponding to protein 2.1 does not detect the 1669Glu→Ter substitution.

83 bp in all three affected family members (data for two patients are shown in Fig. 5, lanes 4 and 6), while the PCR-amplified control genomic DNA was left intact (Fig. 5, lanes 8 and 10). This result confirmed the presence of the G→T transversion in codon 1669 in all three affected family members.

The mutant ankyrin 2.1 mRNA is not detected in ankyrin^{RAKOVNIK} reticulocytes. The two most likely mechanisms leading to ankyrin 2.1 deficiency are (a) synthesis of a dysfunctional 182-kD peptide that is not stably incorporated into the membrane skeleton or (b) decrease in the mutant 2.1 mRNA content due to its instability. We did not detect any abnormally migrating protein in the cell membranes (Fig. 1) or in whole red cell lysates (not shown). Using primers A1 and A10, we PCR amplified a 296-bp segment of the 2.1 cDNA that should contain the 1669Glu→Ter mutation. We directly sequenced the PCR product and detected only the normal ankyrin cDNA sequence (Fig. 4). Similarly, using MseI restriction digestion, we detected only the normal allele in all three affected members of the studied kindred (results shown for two patients in Fig. 5, lanes 3 and 5). We conclude that the amount of the ankyrin 2.1 mRNA that would contain the nonsense mutation is decreased in the reticulocytes below the levels detectable either by direct sequencing or by restriction digestion of the PCR-amplified cDNA.

Discussion

We analyzed ankyrin genomic DNA in three patients from a kindred with autosomal dominant HS and detected in all three

Figure 5. The 1669Glu→Ter substitution is detected by MseI digestion in genomic DNA but not in cDNA. PCR amplification of patient genomic DNA using primers A1 and A10 produces a band of 296 bp (lane 2). Since the 1669GAA→TAA transversion creates a new MseI restriction site, a portion of the patient PCR-amplified genomic DNA (g) from family members I/1 and II/1 is cleaved into bands of 213 and 83 bp (lanes 4 and 6) while neither control genomic DNA nor cDNA is digested (lanes 7–10). MseI digestion of cDNA (c) amplified with the same primers A1 and A10 prepared from patients I/1 and II/1 does not detect the mutation (lanes 3 and 5).

of them a nonsense mutation 1669Glu→Ter in allele of the ankyrin gene. We designated the mutant ankyrin allele, based on the city of origin, as ankyrin^{RAKOVNIK}. The region of cDNA containing the mutation is spliced out from the mRNA encoding the ankyrin 2.2 isoform. Consequently, normal ankyrin 2.2 mRNA should be produced from both alleles, while both mutant and normal 2.1 mRNAs should be present. We detected only the normal ankyrin 2.1 mRNA in reticulocytes from the affected individuals. We found normal levels (105%) of protein 2.2 and a selective decrease (55% or normal) of protein 2.1.

In agreement with our inability to detect the mRNA containing the 1669Glu→Ter substitution, we likewise were unable to detect the presence of a truncated ankyrin protein (predicted size 182 kD, as opposed to 206 kD for the normal ankyrin 2.1 isoform). We therefore conclude that the most likely mechanism of selective ankyrin 2.1 deficiency involves either a major decrease or a complete absence of the mutant 2.1 mRNA. We suggest that this decrease in the mutant mRNA is caused by the instability of this message and leads to a decreased synthesis of the 2.1 protein, while synthesis of the protein 2.2 remains normal. It has been well documented that nonsense mutations in messenger RNAs, with the exception of the 3'-ends of the coding regions, cause a reduction in steady state mRNA levels by promoting rapid degradation of mutant mRNA molecules (for review see reference 28).

Another mechanism that could possibly account for decreased production of the protein 2.1 involves an increased usage of the altered intraexon splice site: the G→T transversion is 19 nt upstream from the normal intraexon splice site which deletes most of exon 38 from the 2.1 mRNA and thus leads to the production of ankyrin 2.2 message (Fig. 6) (4, 5). The substitution elongates the obligatory pyrimidine tract in the intraexon acceptor splice site from 10 to 11 pyrimidine residues. Consequently, the mutation could increase the relative use of

Figure 6. Hypothetical mechanism leading to the decreased content of ankyrin 2.1 and normal content of ankyrin 2.2. Alternative splicing in the regulatory domain of ankyrin produces normal ankyrin 2.1 and 2.2 mRNAs from the normal allele. Ankyrin 2.1 mRNA from the mutant heteroogenous nuclear RNA contains the termination codon. We believe that the mutant 2.1 mRNA is unstable and is degraded. In contrast, ankyrin 2.2 mRNA from the mutant allele should have normal stability since the stop codon is spliced out. The result is a normal amount of the 2.2 mRNA originating from both alleles and a decreased content of the 2.1 mRNA that originates only from the normal allele.

this intraexon splice site and thus could lead to increased production of the 2.2 message which is accompanied by a correspondingly diminished production of the 2.1 mRNA from the mutant allele. Since no mutant 2.1 mRNA was detected in the patients' reticulocytes, the mutation would have to lead to an absolute preference of the 2.2 splice site and, thus, an increased production of the protein 2.2. However, this possibility is unlikely as made evident by the presence of normal amounts of the 2.2 isoform in the red cell membrane.

Erythroid ankyrin is expressed in erythrocytes, muscle, brain, endothelial cells, and macrophages (1, 3, 29, 30), and its deficiency could therefore affect the normal structure and function of these tissues. As was shown by us (6, 7) and others (8, 31), the main ankyrin isoform in the brain and the erythroid progenitors is protein 2.2. Protein 2.1 is the predominant ankyrin isoform of mature red cells. We therefore expect that the mutation described here, which involves only the 2.1 isoform, affects selectively the membrane of mature erythrocytes and does not have a major effect on maturing erythroid cells or brain cells expressing erythroid ankyrin. The expression of the 2.1 and 2.2 ankyrin isoforms in the muscle, endothelial cells, and macrophages has not been studied. However, the absence in the carriers of the mutation of symptoms that could be related to the expression of ankyrin in the other above mentioned tissues supports our conclusion that the predominantly affected cells are the mature erythrocytes.

It has been shown that ankyrin deficiency is in most cases associated with spectrin deficiency (32, 33) and that a decreased synthesis of ankyrin leads to a decreased assembly of spectrin on the plasma membrane (15). The results presented here further demonstrate that a primary deficiency of ankyrin 2.1 leads to a secondary deficiency of spectrin which is of a similar magnitude as the ankyrin deficiency.

In conclusion, we report a nonsense mutation 1669Glu→Ter in the regulatory domain of ankyrin associated with a moderately severe autosomal dominant HS. We suggest that a decreased stability of the mutant ankyrin 2.1 message leads to a primary deficiency of ankyrin 2.1, which in turn causes a secondary deficiency of spectrin.

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