An Alanine-to-Threonine Substitution in Protein 4.2 cDNA Is Associated With a Japanese Form of Hereditary Hemolytic Anemia (Protein 4.2^{NIPPO\text{N}})

By Eric E. Bouhassira, Robert S. Schwartz, Yoshihito Yawata, Kazuyuki Ata, Akio Kanzaki, Judy J.-H. Qiu, Ronald L. Nagel, and Anne C. Rybicki

Erythrocyte (RBC) protein 4.2 (P4.2)-deficiency observed in Japanese individuals results in a hemolytic anemia associated with abnormally shaped (spherocytic, ovalocytic, and elliptocytic), osmotically fragile RBCs, the clinical presentation of which resembles hereditary spherocytosis (HS). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, P4.2-deficient individuals contain less than 1% of the normal membrane content of P4.2 and immunologic analysis shows that the P4.2 present exists as an equimolar doublet of 74-Kd and 72-Kd bands, in contrast to normal RBC membranes where a discrete 74-Kd band is not observed. RBC membranes from both of the biologic parents of a P4.2-deficient individual contained both the 74-Kd and the 72-Kd bands, demonstrating their heterozygosity for the P4.2 defect. The molecular basis of Japanese P4.2-deficiency was investigated by reverse transcription of total reticulocyte RNA, followed by polymerase chain reaction (PCR) amplification, subcloning, and sequencing. The complete cDNA sequence of a P4.2-deficient patient showed a single point mutation that changes codon 142 from GCT (alanine) to ACT (threonine) (Protein 4.2^{NIPPO\text{N}}). The mutation also eliminated an Hgal restriction site, therefore allowing rapid screening for the presence of the mutation. Screening of PCR-amplified genomic DNA showed that the mutation was present in the homozygous state in four (eight chromosomes) unrelated Japanese P4.2-deficient individuals and absent in 35 (70 chromosomes) P4.2-normal controls (including 15 Japanese [30 chromosomes]). The presence of the mutation was confirmed by allele-specific hybridization. The mutation occurred in an alternatively spliced exon that is present in two of four P4.2 mRNA splicing isoforms. These results demonstrate that Japanese P4.2-deficiency is closely associated with the P4.2 gene and does not arise secondarily to a defect in another membrane protein, and further suggest that the P4.2-deficiency is related to the pathogenesis of the hemolytic anemia in this variant form of recessively inherited spherocytosis.

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The pathophysiology of hereditary spherocytosis (HS) involves abnormally shaped, osmotically fragile red blood cells (RBCs) that are selectively removed by the spleen, resulting in anemia.\textsuperscript{1} The RBCs are spherocytic and osmotically fragile due to a loss of membrane surface area, which results from a defect in the interaction between the membrane and its underlying protein skeleton.\textsuperscript{1} The defective protein(s) responsible for destabilizing the membrane may, in theory, be any one of the skeletal or peripheral membrane proteins that underlie the cytoplasmic side of the membrane, or one of the integral membrane proteins to which the skeletal proteins are bound.\textsuperscript{2} The RBC skeleton, whose major components include α- and β-spectrin, actin, protein 4.1, and protein 4.9,\textsuperscript{1} is attached to the integral membrane proteins band 3 and glycophorin by the linking proteins, ankyrin and protein 4.1.\textsuperscript{1}

P4.2 is an RBC peripheral membrane protein that binds strongly to the transmembrane protein band 3 (anion exchange protein) and weakly to both ankyrin and protein 4.1.\textsuperscript{2} The function of this protein is still largely unknown, although a role for stabilizing ankyrin in the membrane has been proposed.\textsuperscript{4} P4.2-deficiency has been reported by several investigators and most of these deficiencies have been found in the Japanese population. At present, it is unclear whether all P4.2-deficient phenotypes share identical genotypes. This assignment has been complicated by different descriptions of the P4.2-deficiency by different investigators. For example, Hayashi et al\textsuperscript{5} described two groups of Japanese HS individuals, one group with a nearly complete deficiency of P4.2 and another group in which P4.2 was partially deficient. These individuals were classified as having recessively inherited HS based on the presence of microspherocytes on peripheral smears and increased RBC osmotic fragility. Nozawa et al\textsuperscript{6} also described the RBCs of P4.2-deficient patients as spherocytes by scanning electron microscopy (SEM). In another early report, Iida et al\textsuperscript{7} observed a transitory disappearance of P4.2 in patients with hiliar obstruction that reappeared after removal of the obstruction; these RBCs appeared as target cells by SEM. Rybicki et al\textsuperscript{8} described a Japanese anemic individual with less than 1% of the normal amount of RBC P4.2, as detected by immunoblotting with P4.2 antibodies. The P4.2 present in this subject's RBC membranes was unusual in that it was composed of a protein doublet of 74 Kd and 72 Kd. More recently, Ata et al\textsuperscript{9} described a P4.2-deficiency in patients with two different hereditary hemolytic anemias, HS and hereditary elliptocytosis (HE), which were characterized by increased membrane leakiness to sodium and stomatocytic changes. Yawata et al\textsuperscript{10} characterized the morphology of these P4.2-deficient RBCs as ovalostomatocytes based on scanning electron micrographs. Ideguchi et al\textsuperscript{10} observed increased RBC membrane protein phosphorylation in Japanese patients with P4.2-deficiency that they classified as HS by the presence of spherocytes on peripheral blood smears and increased RBC osmotic fragility.
Recent reports have also described P4.2-deficiency in non-Japanese individuals: Jarolim et al, in a preliminary report, described a non-Japanese HS individual with a partial deficiency of P4.2 that was suggested to arise secondarily from a defect in the cytoplasmic domain of band 3, the major P4.2 membrane binding site. Ghanem et al suggested a recessively inherited hemolytic syndrome associated with a complete deficiency of P4.2 in two Tunisian siblings. RBCs of these individuals, born from a consanguineous marriage, were morphologically normal and mechanically stable, despite a complete P4.2-deficiency. Based on these observations, Ghanem et al suggested that the Tunisian P4.2-deficiency exemplified a unique clinical syndrome distinct from HS and HE.

The cDNA for P4.2 has recently been cloned and sequenced. In addition, four mRNA splice isoforms (I through IV) characterized by the presence or absence of a 90-bp or 234-bp insert and potentially coding for proteins of predicted MW of 80 Kd, 78 Kd, 71 Kd, and 69 Kd, respectively, have been described and antibodies directed to specific isoforms have been prepared. This information has allowed us to investigate the molecular basis for the P4.2-deficiency. We now report that unrelated Japanese individuals whose RBCs are greater than 99% deficient in P4.2 are homozygous for a nucleotide substitution at position +424 in the P4.2 cDNA that results in an alanine to threonine replacement at amino acid 142 of the protein.

**EXPERIMENTAL PROCEDURES**

**Clinical studies.** After obtaining informed consent, venous blood was obtained from Japanese P4.2-deficient subjects, unaffected family members, and normal controls. These P4.2-deficient subjects were characterized by the presence of mild to moderate anemia, splenomegaly, the presence of spherocytes, ovalocytes, and some elliptocytes on peripheral blood smears, and, in one patient, aggravation of hemolysis during viral infections. Routine hematologic parameters were determined on an automated cell counter and incubated osmotic fragility studies performed by the method of Dacie and Lewis. No genetic relationship was found between any of the affected probands with P4.2-deficiency. P4.2-deficient subject S1 was American born of Japanese ancestry and P4.2-deficient subjects S2 through 4 were native Japanese.

**Quantitation of protein 4.2 by gel electrophoresis and immunoblotting.** RBC ghost membranes were prepared by the hypotonic lysis method of Dodge et al. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli buffer system and Western immunoblotting was performed according to the procedure of Towbin. The preparation of affinity purified polyclonal antibodies to human RBC P4.2 was described previously.

**Isoform-specific antibody production.** Decapetides specific for the 90-bp exon in the large P4.2 splicing isoform (GEP-SORSTGL), or from a region closer to the 3' end that is the same in all of the isoforms (GVERVEKEMER) (see also Fig 2), were synthesized (Laboratory for Macromolecular Analysis, Albert Einstein College of Medicine, Bronx, NY) to contain a C-terminal cysteine residue for subsequent conjugation to keyhole limpet hemocyanin (KLH; Pierce Scientific, Rockford, IL) as suggested by the manufacturer. The KLH-conjugated peptides were injected subcutaneously into female New Zealand rabbits to produce polyclonal antibodies as follows: An initial inoculation with 2 mg KLH-peptide (in an equal volume of complete Freund’s adjuvant [Difco Laboratories, Detroit, MI]) followed 4 weeks later by a booster of 1 mg KLH-peptide (in an equal volume of Freund’s incomplete adjuvant [Difco Laboratories]). A second 1-mg KLH-peptide booster was given 4 weeks after the first booster. Four weeks after the second booster, blood was collected from the marginal ear vein, serum was separated from clotted blood and complement inactivated at 56°C for 30 minutes. Antibody specificity was documented by Western immunoblotting using synthetic P4.2 peptides (data not shown). IgG was prepared from serum by ammonium sulfate precipitation. IgG fractions were concentrated by lyophilization and reconstituted in 5 mmol/L phosphate-buffered saline, pH 8.0 (PBS). Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) and adjusted to be uniform by making the appropriate dilutions in PBS.

**RNA extraction, cDNA synthesis, and PCR amplification.** Total RNA was prepared from peripheral blood reticulocytes by the buffered ammonium chloride method of Temple et al. Reticulocyte cDNA was prepared from the RNA by reverse transcription and amplified by the polymerase chain reaction (PCR), essentially as described by Saiki et al. Briefly, 1 μg of total RNA was treated for 1 hour at 42°C in the presence of 10 U 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 55°C and 90 seconds at 72°C) in a DNA thermal cycler (Perkin-Elmer Cetus Corp, Emeryville, CA), using primers A/C, B/E, B/G, D/G, or F/I. The sequences and positions of the primers are depicted in Table 1.

**DNA sequencing.** PCR fragments representing the entire coding sequence of P4.2 and the variable portion of the splicing isoforms were subcloned into either the pGEM-3zf plasmid (Promega Biotech, Madison, WI) or the PCR 1000 plasmid (TA cloning Kit; Invitrogen, San Diego, CA) and sequenced by the dideoxy nucleotide (nt) method. To detect and rule out artifacts generated during the PCR reaction, nt substitutions were qualified as genuine mutations either by restriction digestion of the unfractated PCR reaction or by detection a second time by sequencing at least eight independently isolated subclones. By these criteria, nine nt substitutions found in isolated subclones were ruled out as PCR artifacts.

**Southern blot, restriction analysis, and allele-specific hybridization.** Total genomic DNA was extracted from peripheral blood leukocytes. Southern blot analysis was performed as described by Sambrook et al using as a probe the complete P4.2 cDNA. Detection of the point mutation at nt +424 was accomplished by PCR amplification of 1 μg of genomic DNA with primers J and H.
RESULTS

Clinical and biochemical features of the Japanese P4.2-deficient subjects. Clinical and biochemical screening of approximately 250 anemic Japanese individuals has allowed us to identify five unrelated individuals whose RBCs are P4.2-deficient. Clinical parameters of four of these subjects are presented in Table 2. The detailed description of the RBC morphology of these P4.2-deficient individuals have been reported previously by Rybicki et al. and Yawata et al. The protein composition of RBC ghost membranes from all of these P4.2-deficient subjects, as well as the parents of P4.2-deficient subject S1, were examined by SDS-PAGE and Western immunoblot analysis using affinity-purified polyclonal antibodies to human RBC P4.2. As shown in Fig 1A, Coomassie blue-stained gels showed that P4.2 is absent in all of the P4.2-deficient ghost membranes and is present in normal amounts in both of the biologic parents of P4.2-deficient subject S1. Western immunoblotting showed a small amount of an immunoreactive P4.2 doublet consisting of similar amounts of both a 74-Kd band and a 72-Kd band that appeared in all of the P4.2-deficient subjects tested (Fig 1B). In contrast, in normal individuals, the major RBC membrane P4.2 band is 72 Kd and a discrete 74 Kd band is not observed. Also in Fig 1B, Western immunoblotting of RBC ghost membranes of the clinically normal biologic parents of P4.2-deficient subject S1 demonstrated that their RBC membranes contained both the 74-Kd and 72-Kd bands, strongly suggesting that the parents were heterozygous for the P4.2 defect. Further evidence for the parents’ heterozygosity came from immunologic studies using antibodies directed against specific P4.2 mRNA splicing isoforms (see below). (It should be noted that the presence of the P4.2 74-Kd protein band has not determined.

Analysis of P4.2 mRNA isoforms. As stated above, we have described four P4.2 mRNA splicing isoforms that differ by the presence or absence of a 90-bp exon between nt +10 and nt +100, and a 234-bp exon between nt +286 and nt +520 (Fig 2). The four isoforms (types I through IV) code for protein of predicted MW of 80 Kd, 78 Kd, 71 Kd, and 69 Kd, respectively. Polyclonal antibodies were prepared against peptides contained within the 90 bp exon (anti-P1) or the invariant 3' end (anti-P2) (Fig 2). Anti-P1 recognizes isoforms I and III while anti-P2 recognizes all P4.2 isoforms. When RBC membranes from P4.2-deficient subject S1 were Western blotted and tested with anti-P1 (Fig 3A, lane 1), only the 74-Kd band reacted, while testing with anti-P2 (Fig 3A, lane 2) resulted in both the 74-Kd and 72-Kd bands reacting. This suggests that the 74-Kd band is either isoform I or isoform III. Similar analysis using anti-P2 (Fig 3B, lanes 1 through 4) and anti-P1 (Fig 3B, lanes 5 through 8) with RBC membranes from the biologic parents of subject S1 showed that both parents had the 74-Kd band (Fig 3B, lanes 2, 3, 6, and 7) whereas normal controls (including nine Japanese, not shown) did not contain the 74-Kd band (Fig 3B, lanes 1 and 5). Taken together, these results provide direct evidence for the heterozygosity of the P4.2 defect in the parents and confirms that the 74-Kd polypeptide is authentic P4.2 and not an immunologic analogue.

Therefore, the phenotype of this Japanese form of hereditary hemolytic anemia can be characterized by the following distinct features: (1) the amount of P4.2 in RBC membranes from homozygotes is less than 1% of normal, and the P4.2 present exists as a protein doublet of 74 Kd and 72 Kd; and (2) RBC membranes of the clinically normal heterozygotes contain normal amounts of P4.2 but additionally contain small amounts of the 74-Kd peptide.

Molecular analysis of the P4.2 gene in Japanese P4.2-deficient subjects. To determine if a large rearrangement of the P4.2 gene was responsible for the P4.2-deficiency, restriction analysis of genomic DNA from P4.2-deficient individuals and normal controls was performed. Restriction digests of genomic DNA with Taq I, Hind III, EcoRI, BamHI, Xba I, and Rsa I, followed by Southern blot analysis using the complete P4.2 cDNA as a probe, showed identical

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Table 2. Clinical Parameters of P4.2-Deficient Japanese Subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
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<tbody>
<tr>
<td>Age, sex</td>
<td></td>
<td>21, F</td>
<td>37, F</td>
<td>17, F</td>
<td>64, F</td>
</tr>
<tr>
<td>RBC (10(^6)/µL)</td>
<td>4.50 ± 0.65</td>
<td>3.66</td>
<td>1.80</td>
<td>3.59</td>
<td>3.18</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>14.3 ± 1.8</td>
<td>10.9</td>
<td>6.1</td>
<td>11.2</td>
<td>9.6</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>80.0 ± 5.5</td>
<td>86.4</td>
<td>92.0</td>
<td>81.9</td>
<td>90.9</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>34.5 ± 1.5</td>
<td>34.5</td>
<td>33.3</td>
<td>33.9</td>
<td>34.2</td>
</tr>
<tr>
<td>Reticulocyte (%)</td>
<td>1.0 ± 0.5</td>
<td>8.0</td>
<td>4.4</td>
<td>7.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>0.50 ± 0.05</td>
<td>0.65</td>
<td>0.63</td>
<td>0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>FEP</td>
<td>&lt; 90</td>
<td>59</td>
<td>ND</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>200 ± 100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

S1-S4 refer to P4.2-deficient subjects. All values are pre-splenectomy. Osmotic fragility was defined as the percent sodium chloride required for 50% RBC hemolysis at 37°C.

Abbreviations: MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; FEP, free erythrocyte protoporphyrin; ND, not determined.
Fig 1. (A) SDS-PAGE analysis of RBC ghost membranes. Protein, 30 µg, was applied to each lane and the gel was stained with Coomassie brilliant blue. Lanes 1 and 2, P4.2-normal controls; lanes 3 and 4, biologic parents of P4.2-deficient subject S1; lanes 5 through 7, P4.2-deficient subjects S1, S2, and S3, respectively. RBC membranes from both parents of S1 contain normal amounts of P4.2 whereas those from S1, S2, and S3 contain no visible P4.2. The identities of the membrane proteins are indicated. (B) Western immunoblot of gel in (A). The blot was developed with polyclonal affinity purified anti-P4.2 IgG. Lanes 1 and 2 (control) contained 8-µg ghosts, lanes 3 and 4 (biologic parents of S1) contained 13-µg ghosts, and lanes 5 through 7 (S1, S2, and S3, respectively) contained 138-µg ghosts. The MW, of the P4.2 bands (Kd) are indicated. P4.2 in control RBC membranes has a major band at 72 Kd; P4.2 in membranes from both of the biologic parents of S1 has both a 72-Kd band and a small amount of an additional 74-Kd band; P4.2 from S1, S2, and S3 contain small equimolar amounts of both the 72-Kd and 74-Kd bands.
RBC membranes with α-chymotrypsin and papain in normal and P4.2-deficient individuals resulted in similar but nonidentical proteolytic peptide patterns; both enzymes generated a large number of identical peptide fragments; however, two of the major P4.2 proteolytic fragments present in normal RBC membranes were missing in the P4.2-deficient RBC membranes, suggesting that some or all of the P4.2 present in these membranes is structurally different from normal P4.2, and thus that a mutation was present in the P4.2 coding sequence.

**P4.2 cDNA sequence of a P4.2-deficient subject.** Sequencing the entire P4.2 cDNA of P4.2-deficient subject S1 and comparison with the two published sequences (see note in the legend to Fig 4) showed a single point mutation at nt +424 that resulted in a G → A transition introducing a threonine (Thr) in place of an alanine (Ala) (P4.2mut). We have now described four P4.2 mRNA splicing isoforms (see Fig 2, refs 13 and 15, and data presented above), and sequencing of the relevant portion of these isoforms showed that the junctions of the alternatively spliced exons were identical in both the normal and P4.2-deficient P4.2 mRNA.

**Fig 2.** Structural map of P4.2 mRNA isoforms. The four isoforms (types I, II, III, and IV) and their predicted MW are indicated. The locations of the synthetic peptides used to generate the isoform-specific antibodies are indicated (▼, anti-P1; ●, anti-P2). Location and direction of the PCR primers are indicated by arrows (see also Table 1). Numbering is with the initiation codon as nt +1.

Restriction fragments in the P4.2-deficient subjects and normal controls (data not shown), making it very unlikely that there was a large P4.2 gene rearrangement.

Rybicki et al4 previously reported that partial digestion of

**Fig 3.** RBC ghost membranes (118 µg) from P4.2-deficient subject S1 Western blotted and reacted with antibodies directed against the 90-bp exon (lane 1) (specific for P4.2 mRNA isoforms I and III) or with antibodies reactive against all isoforms (lane 2). MW, (Kd) of the P4.2 bands is indicated. (B) Similar analysis of normal control (lanes 1 and 5), P4.2-deficient subject S1 (lanes 4 and 8), biologic mother of S1 (lanes 2 and 6), and biologic father of S1 (lanes 3 and 7) using P4.2 peptide antibodies reactive against all P4.2 isoforms (-P2, lanes 1 through 4), or P4.2 peptide antibodies directed against the 90-bp exon (-P1, lanes 5 through 8). All lanes contained 35-µg membranes except for lanes 4 and 8, which contained 100-µg membranes. Membranes from S1 and both biologic parents contained a discrete 74-Kd band whereas normal RBC membranes (including nine Japanese not shown) did not contain this band. Note that the results shown here with -P2 (lanes 1 through 4) are similar to those shown in Fig 1B using antibodies to the whole P4.2 molecule.
The mutation at nt +424 also eliminated an HgaI restriction site. To determine if this point mutation was closely associated with P4.2-deficiency, two oligonucleotide primers J and H, specific for the amplification of a 200-bp fragment of P4.2 that contained the HgaI site, were synthesized and used to amplify genomic DNA from the four unrelated P4.2-deficient individuals as well as from 35 controls (15 Japanese, 10 African Americans, and 10 whites). Presence or absence of the mutation was then determined by digestion with HgaI (Fig 5A) and confirmed by hybridization with allele-specific oligonucleotides (Fig 5B). The results (Table 3 and Fig 5) demonstrated that the four P4.2-deficient subjects were all homozygous for A at nt +424 (ACT, coding for Thr) while the 35 normal controls were all homozygous for G at this position (GCT, coding for Ala). These results strongly suggest that the G → A mutation is closely associated with the P4.2-deficiency. It is noteworthy that the portion of the P4.2 cDNA sequence that contains the mutation occurs within a highly conserved region in both P4.2 and guinea pig liver transglutaminase (71% identity in a 14 amino acid stretch) (Fig 4), a protein known to be homologous to P4.2.13,14

DISCUSSION

Molecular analysis of four unrelated Japanese subjects with hereditary hemolytic anemia and P4.2-deficiency has allowed us to identify a single point mutation in the P4.2 cDNA that changes codon 142 from GCT (Ala) to ACT (Thr) (Protein 4.2NPON). All of the four unrelated P4.2-deficient Japanese subjects were homozygous for this mutation, whereas 0 of 35 normal controls tested (including 15 Japanese) had this mutation. The mutation appears to be inherited in an autosomal recessive manner because all of the P4.2-deficient subjects tested were homozygous for the mutation and neither of the biologic parents tested were P4.2-deficient. Moreover, RBC membranes from the biologic parents of P4.2-deficient subject S1 contained both the 74-Kd and 72-Kd P4.2 bands whereas RBC membranes from normal individuals (Japanese and non-Japanese) did not contain the 74-Kd band. The clinical phenotype of this Japanese disorder is distinct from the “classical” autosomal dominant HS and different from the severe, recessively inherited HS.25 In contrast to the HS phenotype associated with spectrin deficiency where the predominant RBC morphology observed on blood smears is spherocytic, the phenotype of the Japanese P4.2-deficiency (Protein 4.2NPON) is associated with spherocytic, ovalocytic, and elliptocytic RBCs. Furthermore, the hereditary pattern in the Japanese phenotype is recessive but quite dissimilar clinically from the spectrin-deficient recessive disorder.25 We suggest that the phenotype of the Japanese form of hereditary hemolytic anemia associated with Protein 4.2NPON is distinct from the HS phenotypes associated with spectrin deficiency.

The absence of the Ala→Thr mutation in 15 normal Japanese (30 chromosomes) and 20 normal African Americans and whites (40 chromosomes) strongly argues against this mutation being a common polymorphism, particularly in light of its presence in the homozygous state in four unrelated Japanese P4.2-deficient individuals. These findings imply either that the mutation is a rare polymorphism in linkage disequilibrium with the determinant of the
P4.2-deficiency (which further implies that all the Japanese P4.2-deficiency cases described here are unicentric in origin), or more likely, that the mutation is directly involved in the P4.2-deficiency.

Any mechanism invoked to explain the P4.2-deficiency must account for both a decrease in the amount of P4.2 and an alteration in the structure of P4.2 (because earlier studies showed that limited proteolytic digestion patterns of P4.2 present in P4.2-deficient individuals were abnormal4). It is also possible that an alteration in the structure of P4.2 constitutes a signal for its elimination. How could the Ala → Thr mutation described here result in a P4.2-deficiency? The P4.2-deficiency could arise by a number of mechanisms, for example: (1) the mutation impairs binding of P4.2 to its membrane attachment site (cytoplasmic domain of band 3), and after failure to assemble normally is proteolyzed, as is the case for α-spectrin in β-spectrin-deficiency5; (2) the mutation affects P4.2 mRNA transcription or the efficiency of translation; (3) the mutation affects isoform processing; (4) the mutation results in an unstable P4.2 mRNA or protein. At present we do not know the mechanism resulting in the P4.2-deficiency. Interestingly, the mutation is located in an alternatively spliced exon (the 234-bp exon found in isoforms I and III), raising the possibility that the P4.2-deficiency could be related to specific decreases of these isoforms. However, preliminary studies using PCR-amplified reticulocyte RNA from controls and P4.2-deficient subject S1 do not demonstrate differences in the relative amounts of P4.2 mRNA isoforms (data not shown), implying that the steady-state level of the mutant P4.2 mRNA isoforms is not decreased.

That the heterozygotes have apparently normal amounts of P4.2 in their RBC membranes could be due to the existence of a feedback mechanism that causes upregulation of P4.2 expression in the heterozygotes. Alternatively, it could be that the P4.2-deficiency is not related to a protein defect that affects the ability of the mutant P4.2 to associate normally with the membrane. This might imply that the number of P4.2 membrane attachment sites are

### Table 3. P4.2 mRNA Ala → Thr Mutation Is Present Exclusively in P4.2-Deficient Japanese Individuals

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>Clinical Status</th>
<th>P4.2 Deficiency</th>
<th>No. Individuals Tested</th>
<th>Hgal Digest + → Ala142</th>
<th>− → Thr142</th>
</tr>
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<tr>
<td>Japanese</td>
<td>Hemolytic anemia</td>
<td>Yes</td>
<td>4</td>
<td>−/−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>No</td>
<td>15</td>
<td>+/+</td>
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<tr>
<td>African American</td>
<td>Normal</td>
<td>No</td>
<td>10</td>
<td>+/+</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>Normal</td>
<td>No</td>
<td>10</td>
<td>+/+</td>
<td></td>
</tr>
</tbody>
</table>

P4.2 content in RBC membranes was determined by SDS-PAGE. Genomic DNA was prepared from peripheral blood leukocytes and PCR-amplified with primers C/D followed by overnight digestion (100 ng) with Hgal. Sequencing was by the dideoxy nucleotide method following subcloning in pGEM 3zf plasmid.
fewer compared with the amount of P4.2 molecules synthesized, a situation similar to that described previously for RBC β-spectrin.\textsuperscript{26} The relative increase in the amount of 74-Kd peptide observed in the homozygotes and heterozygotes could be due to differences in the relative rates of disappearance of the P4.2 isoforms before their integration into the membrane. This leaves open the possibility that the mutation affects the cellular content of P4.2 mRNA or protein by affecting their intracellular stability or the efficiency of translation of P4.2 mRNA. That the mutation occurs in a highly conserved region in both human P4.2 and guinea pig liver transglutaminase further suggests that the mutated area may have some important structural or functional role, although the importance of this is difficult to assess due to our limited knowledge of P4.2 function. Studies in progress using recombinant P4.2 may be useful in better defining these mechanisms.

It is not surprising that a conservative mutation (Ala $\rightarrow$ Thr) could affect the content or function of P4.2. Physically, Thr is larger in size, is more polar, and has a different affinity for the $\alpha$-helix than does Ala. An example of this sort has been described in human hemoglobin (Hb) where an identical Ala $\rightarrow$ Thr mutation generates a high ligand affinity variant (Hb Saint Jacques).\textsuperscript{27}

Although these results firmly ascertain that the P4.2 cDNA mutation is associated with the P4.2-deficiency (based on its presence in the homozygous state in four unrelated P4.2-deficient individuals and its absence in normal individuals), in vitro expression and analysis of the membrane binding properties and/or stability of the mutant P4.2 will be necessary to definitively establish that this substitution is responsible for the P4.2-deficiency in these Japanese individuals. The variation in the phenotypic expression of the various P4.2-deficient subjects reported in the literature could arise from heterogeneity in the molecular mechanisms involved in the deficiency, or could indicate that additional factors modulate the expression of the defective gene and abnormal protein. The mutations generating the P4.2-deficiency could be different in different ethnic groups and/or even within the same ethnic group, as has been observed in spectrin and Hb abnormalities.\textsuperscript{28,29} In addition, the same mutation could be associated with variations in RBC morphology and extent of anemia due to epistatic effects from subtle polymorphic variations in the quantity or function of other interacting cytoskeletal proteins. It is noteworthy that the mutation occurs in a CpG dinucleotide, which are known to be mutational “hot spots.”\textsuperscript{30-32} Whatever the mechanism that is ultimately responsible for the P4.2-deficiency, we have now demonstrated that the protein deficiency is strongly associated with the P4.2 gene (in these Japanese subjects), thus adding the P4.2 gene product to the list of proteins implicated in the clinical etiology of HS and its related disorders.

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