Distinct Variants of Erythrocyte Protein 4.1 Inherited in Linkage With Elliptocytosis and Rh Type in Three White Families

By Maura McGuire, Barbara L. Smith, and Peter Agre

Hereditary elliptocytosis is a heterogeneous disorder resulting from defects in the erythrocyte membrane skeleton. Although some cases of elliptocytosis result from defects in spectrin, the specific structural abnormality has yet to be identified in the majority of cases. Protein 4.1 plays an essential role in erythrocyte membrane physiology, and deficiencies have been implicated in only a few rare cases of elliptocytosis. By using 4.1 immunoblots and a 4.1 radioimmunoassay we identified distinct variants of protein 4.1 in 15 elliptocytic members of three US white families with the Rh-linked form of elliptocytosis. Elliptocytic members of family G were heterozygotes for a low–molecular weight (mol wt) 4.1 variant (65,000 to 68,000 daltons; normal, 80,000) inherited in linkage with the R, phenotype. Elliptocytic members of family C expressed a simple partial deficiency of protein 4.1 (63% of the normal level) that was inherited in linkage with the r phenotype. Elliptocytic members of family N were heterozygotes for a high–mol wt 4.1 variant (100,000 daltons) also inherited in linkage with the r phenotype. These studies indicate that mutant forms of protein 4.1 are not uncommon in elliptocytosis among whites and that different kindreds probably express different mutations. The observed linkage of elliptocytosis and Rh blood type most likely results from the close proximities of the 4.1 gene (site of the mutation) and the Rh gene, which is located nearby on the short arm of chromosome 1.

Materials and Methods

Patients. Family G is of Italian ancestry. Elliptocytosis was identified in the propositus at the age of 30 during evaluation of a transient aplastic episode associated with a viral illness. The episode required blood transfusion, and splenomegaly was noted. Before this episode the patient had been in excellent health aside from borderline anemia. The patient’s mother was elliptocytic with a history of anemia and had required blood transfusions. An elliptocytic brother had never been anemic.

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Family C is also of Italian ancestry. The propositus was a medical student who observed elliptocytes while examining her own peripheral blood smear. Her father informed her that he also had elliptocytosis, and subsequent evaluation of peripheral blood smears of several other asymptomatic family members revealed elliptocytes. Elliptocytic family members were found to have mild anemia with elevation of the reticulocyte count, but most were in good health.

Family N is of a large kindred of Scottish-Irish ancestry living near Hurdle Mills, NC. The propositus was a 65-year-old farmer who was hospitalized for a transient gastrointestinal illness when a spleen tip was palpated and elliptocytes were noted in the peripheral blood smear. Evaluation of seven of 15 family members also revealed elliptocytes. Anemia was observed in only one elliptocytic family member, a 72-year-old woman with renal failure and coronary artery disease.

**Laboratory procedures.** Samples of venous blood were obtained from elliptocytic and unaffected family members and controls, anticoagulated with acid-citrate-dextrose, and stored on ice for less than 24 hours before analysis. Erythrocyte membranes were prepared by hypotonic lysis in 7.5 mmol/L sodium phosphate (pH 7.4), 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L EDTA. Membrane proteins underwent electrophoresis on 9% polyacrylamide gels containing sodium dodecyl sulfate under reducing conditions by using the buffer system of Laemmli. Gels were stained with Coomassie brilliant blue R250 (Bio-Rad Laboratories, Rockville, NY) and scanned with a laser densitometer, and proteins were quantitated by cutting and weighing peaks. The amount of protein 4.1 in gel samples was corrected for sample application by normalization to bands 4.2 and band 3, an index of membrane surface area.

Proteins were blot transferred onto nitrocellulose sheets, incubated with affinity-purified antibodies, and visualized by autoradiography after labeling with 125I-protein A as previously described. Normal and mutant 4.1 proteins were purified according to a modification of the method of Tyler et al. by using a Mono Q fast-protein liquid chromatography anion-exchange column (Pharmacia, Inc, Piscataway, NJ) and elution with a 0 to 500 mmol/L KCl gradient in 7.5 mmol/L NaPO4 buffer (pH 7.4). The variant 4.1 proteins eluted with normal protein 4.1 at approximately 240 mmol/L KCl.

A protein 4.1 radioimmunoassay (RIA) measured competition between unlabelled 4.1 in detergent-lysed erythrocytes and 125I-labeled normal 4.1 for binding on protein A-bearing Staphyloococi coated with anti-4.1 IgG. Glycophorins were evaluated by using PAS-stained gels. Haptoglobins were quantitated by using a radial immunodiffusion kit (Behring Diagnostics, La Jolla, CA). Complete blood counts and bilirubin levels were determined by the hospital laboratory. Rh type was determined according to standard methods. The Rh phenotype indicated on the genealogies represents the most likely genotype of members as determined by gene frequency tables. Statistics and linear regression were performed on the Hewlett-Packard 41C StatPac.

**RESULTS**

**Clinical studies.** Peripheral blood smears from members of each of the three families G, C, and N were evaluated. Representative smears from affected members of each of the families contained multiple elliptocytes, but smears from unaffected members showed normal morphology (Fig 1). Erythrocytes from family G showed the most marked phenotypic abnormality, with many cells showing marked elongation compared with the more ovalocytic cells of families C and N. The percentage of elliptocytes in the three families was variable and ranged from 20% to more than 50% of the erythrocytes.

Clinical laboratory evaluations were performed on elliptocytic and unaffected members of each family (Table 1). Elliptocytic members of all families demonstrated a reduced mean haptoglobin level although only in families C and G was the reduction significant (P < .05). Only in family C was there a significant reduction in hematocrit and elevation in reticulocyte count in elliptocytic members compared with unaffected members. Bilirubin levels were normal in all patients. These results suggest there is a mild compensated hemolytic anemia in family C but minimal hemolysis in families G and N despite the morphological abnormality. Tryptic peptide maps of spectrin were normal in erythrocytes from elliptocytic members of the three families (S.L. Marchesi, personal communication).

**Protein 4.1 quantitation.** Erythrocyte membrane proteins were electrophoretically separated on polyacrylamide gels containing sodium dodecyl sulfate and stained with Coomassie blue. Preparations from elliptocytic members of the three families (S.L. Marchesi, personal communication). Complete blood counts and bilirubin levels were determined by the hospital laboratory. Rh type was determined according to standard methods. The Rh genotype indicated on the genealogies represents the most likely genotype of members as determined by gene frequency tables. Log odds (lod) scores, which establish the likelihood of genetic linkage, were calculated by using the LIPED program (IBM PC/AT version) using Rh gene frequencies obtained from published tables. Statistics and linear regression were performed on the Hewlett-Packard 41C StatPac.

**Table 1. Summary of Clinical Data**

<table>
<thead>
<tr>
<th>Family</th>
<th>Hematocrit* (%)</th>
<th>Reticulocyte* Number/100 RBCs</th>
<th>Haptoglobin* (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elliptocytic (n = 3)</td>
<td>40.7 ± 3.6</td>
<td>1.0 ± 0.2</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Unaffected (n = 1)</td>
<td>36.7</td>
<td>0.5</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Family C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elliptocytic (n = 3)</td>
<td>38.7 ± 1.8</td>
<td>3.1 ± 0.2</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>Unaffected (n = 2)</td>
<td>42.8 ± 0.6</td>
<td>1.0 ± 0.3</td>
<td>137 ± 63</td>
</tr>
<tr>
<td>Family N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elliptocytic (n = 7)</td>
<td>40.0 ± 5.0</td>
<td>0.7 ± 0.2</td>
<td>130 ± 83</td>
</tr>
<tr>
<td>Unaffected (n = 9)</td>
<td>43.2 ± 4.2</td>
<td>1.0 ± 0.5</td>
<td>161 ± 71</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Normal haptoglobin levels, 100 to 300 mg/dL.
protein 4.2 peaks to account for differences in sample application and membrane surface area (Table 2). Erythrocytes from elliptocytic members of family G had 62% of normal protein 4.1 levels when normalized to band 3, with 63% in family C and 60% in family N. PAS stains of erythrocyte ghosts revealed no reduction in the levels of glycophorins A, B, or C (not shown).

An RIA was developed to quantitate the amount of immunoreactive protein 4.1 in erythrocytes from elliptocytic and unaffected members from each family (Fig 3 and Table 2). Erythrocytes from the elliptocytic members of family G had 55% of the normal protein 4.1 levels by RIA compared with 63% by gel electrophoresis. The RIA showed no reduction in immunoreactive protein 4.1 levels in erythrocytes from the elliptocytic members of families G and N despite the apparent reductions on electrophoresis gels. The normal protein levels in families G and N as detected by RIA are due to the presence of immunoreactive mutant 4.1 proteins. These patients, like heterozygotes for a variety of mutations, express slightly more than half of the protein as the normal gene product.

Membrane immunoblots. Immunoblot analysis of erythrocyte membranes from elliptocytic and unaffected members of families G, C, and N are shown with controls in Fig 4. Membranes from elliptocytic members of family G have intense staining in the 65,000- and 68,000-dalton region of the blot in addition to staining at the 80,000-dalton region of normal 4.1 protein seen in the unaffected member and control. It is not clear why the low-mol wt doublet stained more intensely than the normal protein 4.1, since scanning densitometry suggested the variant constituted less than 50% of the total protein 4.1 content. Elliptocytic members of family G therefore appear to be heterozygotes for a low-mol wt variant of protein 4.1. Fragmentation patterns for immunoblots probed with antibodies to spectrin and ankyrin, which is highly susceptible to proteolysis, were identical in membranes prepared from elliptocytic and unaffected family members and controls, thus suggesting that generalized proteolysis was not the cause of the low-mol wt 4.1 variant (Fig 5). Whether this doublet represents a 4.1a/b equivalent in the variant protein is uncertain.

Erythrocytes from elliptocytic members of family C show the same autoradiographic pattern on immunoblots as an unaffected family member and a control, although a subtle decrease in intensity was apparent (Fig 4). Because both RIA and gel analyses indicate a reduced amount of protein, elliptocytic family members are likely to be heterozygotes for a simple partial deficiency of protein 4.1.

Erythrocyte membranes from elliptocytic members of family N show an intense band in a region of higher mol wt (100,000 daltons) on immunoblots in addition to the normal band seen in an unaffected family member and a control (Fig 4). Elliptocytic members therefore appear to be heterozygotes for a high-mol wt 4.1 variant. When intact erythrocytes from family N were subjected to limited chymotrypsin digestion to degrade band 3, a novel protein doublet of 100,000 daltons became apparent on Coomassie blue-stained gels (not shown).
Protein 4.1 purification. Protein 4.1 was purified from erythrocytes of elliptocytic members of the three families and analyzed by Coomassie blue staining and by immunoblots of electrophoretic gels. The purified protein confirmed results of whole membrane analysis, which revealed a doublet at 65,000 and 68,000 daltons in family G and a 100,000-dalton band in family N in addition to the normal 80,000-dalton band seen in all three families (Fig 6). Immunoblots of purified proteins confirmed that the Coomassie blue-stained bands corresponded to the high- and low-mol wt variants seen in immunoblots of erythrocyte membranes (not shown).

Rh linkage. Erythrocyte Rh phenotypes for members of all three families were determined (Fig 7). Erythrocytes from all elliptocytic members of family G bear the R_2 phenotype, whereas erythrocytes from all elliptocytic members of family C bear the r phenotype. Erythrocytes from all elliptocytic members of family N also bear the r phenotype, although the linkage is obscured by an unlinked r phenotype of the erythrocytes of some relatives. Patient N-III* is not elliptocytic and lacks the high-mol wt protein 4.1, but his erythrocytes express an Rh phenotype identical to that of his elliptocytic brothers. A recombination is therefore likely to have occurred in this individual. Elliptocytosis is expressed in autosomal dominant fashion, and elliptocytic members of the three families appear to be heterozygotes for mutant gene products. The combined maximum lod score for the three families is 3.01 at a distance of 5 centimorgans and indicates a high probability of linkage of the genes for protein 4.1 and Rh.
PROTEIN 4.1, ELLIPTOCYTOSIS, AND Rh TYPE

Fig 7. Genealogies of families G, C, and N. Elliptocytosis (half-shaded figures) is inherited in linkage with erythrocyte Rh type and 4.1 variants (LMW, low mol wt; HMW, high mol wt) or with a reduction in protein 4.1 levels (fractional values = patient/control) as determined by 4.1 RIA.

DISCUSSION

HE is a relatively common but heterogeneous disorder that has been attributed to variants in erythrocyte spectrin in many cases and to deficiencies of protein 4.1 in only a few cases. This report documents three American white families with HE. Two families have protein 4.1 variants, whereas the third has a simple partial deficiency of protein 4.1. Subtle differences in erythrocyte proteins may escape detection during polyacrylamide gel analysis of membranes but may be dramatically revealed by analysis using 4.1 immunobots or the 4.1 RIA. These techniques may be useful as standard methods in evaluating patients with elliptocytosis.

The identification of these families resulted from observation of subtle reductions in the intensity of protein 4.1 on Coomassie blue-stained polyacrylamide gels of erythrocyte membranes. However, erythrocyte membranes prepared from elliptocytic members of three other white families or from eight black families did not reveal protein 4.1 abnormalities when studied on polyacrylamide gels or by immunobots. These results suggest that 4.1 variants are not uncommon in HE among whites. Additional studies will be needed to determine whether these frequencies are representative of these and other racial groups.

The erythrocytes from families G and N have mutant 4.1 proteins associated with elliptocytosis, which suggests that the 4.1 variants were either dysfunctional or participated abnormally in membrane skeleton assembly. Protein 4.1 has a number of important functions in the membrane skeleton. It greatly enhances the spectrin-actin associations in the membrane skeleton. Protein 4.1 binds to the cytoplasmic domain of membrane glycophorins and to the cytoplasmic domain of band 3, the membrane anion channel, and may be important in linking the skeleton to the lipid bilayer although the physiological site of this suspected function is unclear.

The biochemical significance of protein 4.1 was confirmed by a recent study showing that mechanical stability was restored to 4.1-deficient membranes after incubation with purified protein 4.1. Multiple tissues including brain and lymphocytes contain proteins related to 4.1, which suggests that it may be functionally important in cells other than erythrocytes. More recent studies have suggested that multiple protein 4.1 isoforms resulting from alternative mRNA splicing may contribute to the many functions of this protein. Limited chymotryptic digestion of normal protein 4.1 has revealed four structural domains including a probable transmembrane protein binding site in the 30,000-mol wt amino-terminal domain and the spectrin-actin-binding site in a 10,000-mol wt domain near the midportion of the molecule. Because the mutant proteins observed in families G and N are likely to be dysfunctional, the sites of deletion and insertion may be in important functional domains of protein 4.1, and the variant proteins may serve as useful probes for understanding the structure of protein 4.1. Preliminary studies show the high-mol wt 4.1 variant of family N results from an elongated mRNA with the insertion peptide near the spectrin-binding site of the molecule. Binding studies using the mutant proteins of families G and N are underway as the next phase of this work. Protein variants do not necessarily result in functional changes. Only one 4.1 mutant has been described previously, and it did not result in morphological abnormality. The basis for the significant morphological variation between the elliptocytes of three families is uncertain but may indicate that more than one structural aberration can result in elliptocytic morphology.

Genealogical studies demonstrated linkage between genes coding 4.1 variants and Rh, although one recombination was noted in family N. It has been recognized that some cases of elliptocytosis are inherited in linkage with the Rh type while others are not. The linkage of the Rh phenotype and elliptocytosis in families N and G was determined after biochemical studies determined the high- and low-mol wt protein 4.1 variants in elliptocytic patients. Family C was known by clinically trained family members to have Rh-linked elliptocytosis, and the 4.1 RIA confirmed that partial deficiency was linked to the other two traits. These observations are consistent with the recent localization of the protein 4.1 gene to the short arm of chromosome 1 near the known site of the Rh gene and confirm the prediction of Conboy et al that Rh-linked elliptocytosis may result from 4.1 mutations. Elliptocytosis not linked to the Rh-phenotype may reflect variants of spectrin or other membrane proteins not yet known to be associated with elliptocytosis. Although the
α-spectrin gene has also been localized to chromosome 1, it is located on the long arm, distant from the Rh locus, and Rh linkage with the α-spectrin type of HE has not been reported.

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Distinct variants of erythrocyte protein 4.1 inherited in linkage with elliptocytosis and Rh type in three white families

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