A Molecular Study of Heterozygous Protein 4.1 Deficiency in Hereditary Elliptocytosis

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Protein 4.1 is an important component of the red cell membrane skeleton, which is a network of proteins underlying the plasma membrane and helping to maintain the shape and deformability of the red cell. Protein 4.1 forms a ternary complex with two major components of the skeleton, spectrin and actin, and greatly enhances their interaction. Protein 4.1 may also be involved in coupling the skeleton to glycoporphins in the plasma membrane, but the individual contribution of glycoporphins A and C to the protein 4.1 binding sites is controversial. The network is also anchored to the bilayer by ankyrin, which links the β subunit of spectrin with the anion transporter, an integral membrane protein.

In specific hereditary hemolytic anemias such as hereditary elliptocytosis (HE), hereditary pyropoikilocytosis, and hereditary spherocytosis, dysfunctions and/or deficiencies of membrane skeleton components have been implicated in the morphologic changes associated with these disorders. Structural alterations of spectrin that are associated with impairment of the spectrin oligomerization process have been reported in HE, and partial or total deficiencies of protein 4.1 have been shown to be an important cause of this disorder in certain population groups. The homozygous form of protein 4.1 deficiency in which there is a complete absence of protein 4.1 is associated with a severe hemolytic anemia. The heterozygous condition HE(4.1+) in which there is a partial deficiency of protein 4.1 is usually associated with prominent elliptocytosis without other clinical sequelae. Alloisio et al have described the heterozygous condition as a common cause of mild HE in cases originating from southeast France and North Africa. The expected 50% decrease in protein 4.1 was not found in all heterozygotes. When membrane proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the average deficit was approximately 30%, which is an underestimate due to the overlap of protein 3 with protein 4.1 on densitometric scanning of the gels. Similar findings with regard to the incidence of HE(4.1+) and the deficit of protein 4.1 have been found in white subjects from South Africa.

In a study of an HE kindred with members who were homozygous for protein 4.1 deficiency and had decreased membrane stability, Conboy et al using a cDNA probe for protein 4.1 identified a gene rearrangement upstream of the translation initiation codon. This rearrangement appeared to affect mRNA splicing by causing the production of a shortened protein 4.1 transcript that was probably not translated. In a second HE kindred with normal membrane stability, the underlying defect appeared to be an abnormal protein 4.1 with a molecular weight (mol wt) of 95 Kd (designated protein 4.1*). This kindred showed a restriction fragment length polymorphism (RFLP) segregating with the abnormal protein and an elongated protein 4.1 mRNA species. It was hypothesized that a mutation in the protein 4.1 gene had led to altered mRNA processing, with the consequent production of the abnormal protein 4.1.*

In the present study, we report a probable gene rearrangement in the DNA from a family with HE(4.1+), probed with a protein 4.1 cDNA. Altered DNA fragment patterns were observed with two restriction endonucleases in this family but were not observed in a number of other kindreds with partial protein 4.1 deficiency or in randomly chosen control subjects. The altered restriction fragment patterns appeared to be due to changes detected by the coding region of the protein 4.1 cDNA.

MATERIALS AND METHODS

Five kindreds and two individuals with HE who had been previously shown to have a partial deficiency of protein 4.1 were studied using Southern hybridization. The degree of protein 4.1 deficiency and relevant hematologic data for these patients are published elsewhere. In summary, a partial deficiency of protein 4.1 (mean, 30% reduction) was found to be inherited in an autosomal dominant fashion in five of 14 white HE kindreds studied. Protein 4.1 was quantitated relative to protein 3 after SDS-PAGE of isolated red cell membranes. This tends to underestimate the degree of deficiency in affected individuals because of the overlap of protein 3. Immuno-
blots of membrane proteins probed with a monoclonal antibody to protein 4.1 did not reveal any higher or lower mol wt fragments that could account for the reduction in protein 4.1 concentration. The pedigree of family Hu is shown in Fig 4 and is extended from that previously described.12 In this kindred, levels of protein 4.1 varied between 52% and 83% of control values (Fig 4, figures in parentheses), but there was no correlation between the degree of deficiency and clinical and hematologic status. All affected individuals had prominent elliptocytosis but were clinically asymptomatic with normal red cell indices and reticulocyte counts.

Genomic DNA was extracted from peripheral blood leukocytes obtained from these kindreds together with a number of control subjects using the method of Sykes.17 Blood was taken with informed consent and with the approval of the ethics committee of the South African Institute for Medical Research. DNA from family Hu was restricted with the endonucleases BglII, PvuII, EcoRI, HindIII, and TaqI (Boehringer Mannheim, Mannheim, FRG) and the restriction fragments separated by electrophoresis in 0.7% agarose gels.18 Fragments were transferred to nylon filters (Pall Ultrafine Filtration Corp, Glen Cove, NY) by using the method of Southern.18 Details of the preparation of a cDNA to protein 4.1 have been previously published.14 A 2.5-kilobase (kb) cDNA to protein 4.1 from the clone designated pHE 4.1-8 was restricted with the endonuclease HindIII to give three probe fragments (Fig 1). Probes A and B were identical to those described earlier.15 The third fragment, probe S, was a 1.4-kb fragment encompassing almost the entire coding region of the cDNA from a HindIII site just upstream of the initiation translation codon to 5' of the SstI site at the end of the coding region. Fragments B and S were radiolabeled by using the method of Feinberg and Vogelstein16 and hybridized to the nylon filters mentioned before.

Genomic DNA from other HE(4.1) kindreds and control subjects were restricted with the endonuclease BglII and transferred to nylon filters as before. These filters were hybridized with radiolabeled 2.5-kb pHE 4.1-8 cDNA.

**RESULTS**

*Southern hybridization of DNA from family Hu.* Figure 2 shows BglII restriction of genomic DNA from two separate experiments with five members of the Hu family and three control subjects that was hybridized with radiolabeled probe S. Heterozygous HE(4.1·) individuals (I1, I2, and I2) from this kindred showed a new restriction fragment at 5.5 kb and a reduction in the dosage of the band at 3.7 kb. The unaffected member of the Hu family (I1) had a restriction fragment pattern identical to that of the control and did not show the 7 kb fragment (not shown). Figure 3B shows the same HE(4.1·) individuals' DNA hybridized with probe B. No variation in the patterns could be observed between the HE(4.1·) patients and controls.

When using probe S, restriction fragment patterns obtained with the enzymes HindIII and EcoRI were the same in members of the Hu family and controls. Digestion with TaqI revealed an RFLP, but this did not segregate with the protein 4.1 deficiency in this family.

![Fig 2. Southern analysis of BglII digested genomic DNA from members of family Hu and controls that was hybridized with radiolabeled fragment S. The figure represents autoradiographs from two separate experiments. The identity of family members is shown above each lane (see Fig 4 for a full family pedigree). Controls are designated C.](image-url)
Figure 4 shows the pedigree of family Hu and reflects the inheritance of the 5.5 and 8.8 kb bands (large numbers) from the BglII digestion and the 7- and 3.7-kb bands (small numbers) from the PvuII digestion. In this family, HE(4.1<sup>+</sup>) is seen to segregate with new bands at 5.5 and 7 kb and with the decreased dosage of the restriction fragments at 8.8 and 3.7 kb as would be expected with a heterozygous condition. The same altered fragments were also observed when restricted genomic DNA from kindred Hu was hybridized to pHE 4.1-8 cDNA (not shown).

Southern hybridization of DNA from HE(4.1<sup>+</sup>) relatives. Figure 5 shows BglII-restricted genomic DNA from four other HE(4.1<sup>+</sup>) kindreds, a control, and two unrelated HE subjects (B.B. and A.T.) with a partial deficiency of protein 4.1; all DNA was hybridized with radiolabeled 2.5-kb pHE 4.1-8 cDNA. The restriction fragment pattern contained the bands seen in Fig 2 along with a 21.9 kb band that had previously been detected by fragment B. The 2.5 kb cDNA probe also detected an RFLP in these kindreds that was associated with the presence or absence of a 17.8-kb band not previously observed with probe B or probe S. However, this RFLP did not appear to segregate with protein 4.1 deficiency in these kindreds and was found to randomly segregate in BglII restrictions of genomic DNA from 20 control white subjects. The extra BglII restriction fragment at 5.5 kb observed in family Hu was not observed in these other HE(4.1<sup>+</sup>) kindreds or in the 20 random control subjects.

DISCUSSION

In this report, we describe a study of Southern hybridization with restricted genomic DNA from five HE(4.1<sup>+</sup>) kindreds and radiolabeled probes from a cDNA coding for protein 4.1. All of these kindreds were characterized by a deficiency of normal protein 4.1 rather than a qualitative change (eg, shortened or lengthened variants of protein 4.1). In one of these individuals, we were able to detect abnormal restriction fragments segregating with the HE(4.1<sup>+</sup>) condition by using two restriction enzymes (BglII and PvuII) but were unable to detect abnormal fragments with three other restriction endonucleases (EcoRI, HindIII, TaqI). The most tenable interpretation of these findings is that the BglII and PvuII polymorphisms reflect a gene rearrangement (eg, deletion or inversion) as the cause of the partial deficiency of protein 4.1 in this family. However, since we were unable to demonstrate abnormal fragments with three other enzyme systems, it is possible, although less likely, that the observed polymorphisms represent single base changes unrelated to the true defect in the protein 4.1 gene, which could occur on a different chromosomal haplotype.

By using fragments of the protein 4.1 cDNA we have been able to assign the gene rearrangement to the coding region of the cDNA. Conboy et al<sup>11</sup> have previously described a gene rearrangement when using a similar approach in a family with a rare homozygous form of protein 4.1 deficiency. However, in that case the gene rearrangement was assigned to the region upstream of the translation initiation codon. When using an identical probe (probe B) to that previously used,<sup>11</sup> we were unable to detect any abnormalities associated with this region in the HE(4.1<sup>+</sup>) kindreds in the present study.

The detection of a possible gene rearrangement in only one of five HE(4.1<sup>+</sup>) kindreds requires further comment. One might expect that the affected members of the other four kindreds, all of whom have a quantitative deficiency of protein 4.1, would also show evidence of a gene rearrangement in contrast to individuals with qualitative defects of protein 4.1 (eg, shortened or lengthened variants) in whom one might expect more subtle changes in DNA such as point mutations. By using the two enzymes that gave abnormal patterns in family Hu we have been unable to detect any RFLPs segregating with protein 4.1 deficiency in the remaining four kindreds. While this attests to the heterogeneous nature of quantitative deficiencies of protein 4.1, further studies using other enzyme systems will be necessary to adequately address this question.

REFERENCES

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