CONCISE REPORT

Sp α1/65: A New Variant of the α Subunit of Spectrin in Hereditary Elliptocytosis

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Recent studies revealed molecular defects or deficiencies in the red cell membrane skeletal components in a number of patients with hereditary elliptocytosis (HE) (reviewed1,2). On a molecular level, several spectrin variants, as well as a deficiency in band 4.1, have been reported.2 The quantity of spectrin heterodimers (SpD) in 0 °C extracts is elevated in a subpopulation of HE patients (designated HE type 1 or HE SpD-HEP).3 In the HE SpD-HEP patients, defective self-association of spectrin dimers was observed both in solution and in the membrane.3 Defective spectrin self-association has also been reported in one family with homozygous HE.4 Using limited tryptic digestion as a structural probe of spectrin, two apparently distinct variants of the spectrin α subunit have been detected in HE.5 In both variants, a decrease in the 80,000-dalton αI domain was detected by two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).6 In one, designated Sp α1/674, an increase in a 74,000-dalton fragment is observed and in the other, designated Sp α1/646, increases in fragments at 46,000 and 17,000 daltons are observed.7 Similar peptide patterns are produced by limited tryptic digestion of spectrin from patients with hereditary pyropoikilocytosis (HPP).8,9 In contrast to the HE patients, the HPP patients also exhibit a decreased spectrin to band 3 ratio.9

In this article, we describe a third type of abnormal peptide map of spectrin from three patients with HE. In two of these individuals, sporadic hemolysis was associated with increased circulatory stress. These individuals have a decrease in the 80,000-dalton αI domain and an increase in fragments of 65,000 daltons. The 65,000-dalton fragment was shown to be derived from the αI domain by immunologic methods.

MATERIALS AND METHODS

Clinical material. We have studied three unrelated HE patients. Their clinical data fulfilling the criteria of HE are summarized below. Venous blood from these patients and their kindred was collected into sterile tubes containing the anticoagulant citrate-phosphate-dextrose. Specimens that were not obtained at St. Elizabeth’s Hospital were transported in insulated containers with ice to Boston, where they were kept at 4 °C and analyzed within three days. A control sample was sent along each time.

Patient D.E. was a 40-year-old black female with mild common HE. She was referred to the University of Minnesota Hospital for evaluation of chronic renal transplantation. The creatinine level had risen to 4.7 mg/dL and a graft biopsy documented chronic rejection with interstitial fibrosis and edema and vascular intimal proliferation and fibrosis. The hemoglobin level, which had been 11 g/dL two months previously, had dropped to 6.4, the platelet count was 50,000, and a mild consumption coagulopathy was documented with a prothrombin time of 15.2, partial thrombin time (PTT) of 50.1, and thrombin time of 23 seconds. However, fibrin split products were not increased, and fibrinogen and factor V levels remained normal. A peripheral blood smear at that time revealed marked fragmentation of red cells with only modest numbers of intact elliptocytes. The clinical course was subsequently complicated by recurrent infections, to which the patient ultimately succumbed.

Patient A.S. is a 63-year-old black woman from Haiti with mild chronic anemia (hematocrit, 33%; hemoglobin, 13 g/dL) with marked microcytosis (mean corpuscular volume [MCV] of 64), elliptocytosis, and poikilocytosis. The reticulocyte count was in the range of 2% to 4%. Total iron binding capacity (TIBC), hemoglobin, A2, and F were all within normal limits. She has two children, both with mild HE.

Patient T.E. is a 12-month-old black child who originally presented to the Children’s Hospital National Medical Center with severe anemia (hematocrit, 19.2%; hemoglobin, 6.4 g/dL) with MCV of 79 and 4.5% reticulocytes. A blood smear revealed marked microcytosis, elliptocytosis, and poikilocytosis. The anemia initially required transfusion, but throughout the year there was a gradual improvement of anemia. At twelve months, the hematocrit and hemoglobin values were 29.9% and 10.5 g/dL, respectively, with MCV of 80.6 and a reticulocyte count of 4.7%. The proband’s...
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motherside all have mild HE.

Biochemical evaluation. The strategy for biochemical evaluation of patient samples has recently been reviewed.2 Erythrocyte spectrin extracts from patients and controls were prepared as described previously.3,4 The protein composition was determined by SDS-PAGE, and the content of spectrin dimers and tetramers was determined by nondenaturing gel electrophoresis.5,6 Triton shell stability was measured using a concentric cylinder rod shearing apparatus as described by Liu and Palek.7 Limited tryptic digests of spectrin extracts were prepared and analyzed by SDS-PAGE and two-dimensional isoelectric focusing/SDS-PAGE as described previously.8

Isolation of the aI domain. The 80,000-dalton fragment of the α subunit of normal spectrin was isolated by the procedure of Hanspal and Ralston9 with minor modifications. Low ionic strength extracts of spectrin were digested with L-(tosylamido 2-phenyl) ethyl chloride (TPCK)-trypsin (1:100 wt/wt) for 20 hours at 0 °C. These digests were applied to a column (1.9 × 70 cm) of Sepharose 4B. The peak containing the 80,000-dalton fragment was concentrated and rechromatographed on the same column. The 80,000-dalton fragment was again concentrated and applied to a high-performance liquid chromatography (HPLC) column (75 × 7.5 mm) of Bio-Gel TSK DEAE-5-PW (Bio Rad, Richmond, Calif) equilibrated with 10 mmol/L of Na3PO4 (pH 7.2) and the column was developed with a gradient of 0 to 0.6 mol/L of NaCl. The 80,000-dalton fragment produced by this procedure was homogenous as judged by SDS-PAGE.

Production of antisera. Antisera was produced in New Zealand white rabbits by subcutaneous injections at multiple sites of purified 80,000-dalton tryptic fragment emulsified with an equal volume of Freund's complete adjuvant.

Protein blotting. After either SDS-PAGE or two-dimensional isoelectric focusing/SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose paper (BioRad) at 50 V for 24 to 72 hours in pH 8.3 buffer containing 25 mmol/L of Tris, 192 mmol/L of glycine, and 20% methanol. The nitrocellulose paper containing a replica of the gel was washed briefly in 0.15 mol/L of NaCl containing 0.015% H2O2, 20% (vol/vol) methanol, and 3.36 mmol/L of 4-chloro-1-naphthol for one to three minutes for the detection of horseradish peroxidase-conjugated anti-rabbit IgG.

RESULTS

Functional and biochemical characterization. When 0 °C extracts of normal red cells were analyzed by nondenaturing gel electrophoresis, 5% ± 3% of the spectrin was in the dimer state. This value represents the amount of spectrin dimer as a percentage of the total dimer plus tetramer pool, and does not include oligomeric forms of spectrin. The quantity of spectrin dimers in the HE patient D.E. was found to be increased to 17% (data not shown). The Triton X-100–extracted ghosts from this patient were markedly unstable, as compared to controls, when they were subjected to mechanical shearing. The spectrin-band 3 ratio and the content of bands 4.1 and 4.2 were found to be within normal range.

Nondenaturing gel electrophoresis of the patients T.E. and A.S. revealed that the quantity of spectrin dimers was increased to 19% and 20%, respectively. An increase in spectrin dimers was also found in the grandmother (G.H., 4%), mother (S.H., 11%), and uncle (G.W., 13%) of the patient T.E. All of these individuals had mild HE. The proband’s father was morphologically and biochemically normal. In contrast to the HE patient D.E., both A.S. and T.E. were found to have normal Triton shell stability. The spectrin-band 3 ratio and the content of bands 4.1 and 4.2 were also within the normal range.

Analysis of limited tryptic digest by SDS-PAGE. To control for minor variations in experimental conditions, control normal erythrocytes were always extracted, digested, and electrophoresed concurrent to patient samples. The peptide pattern produced by normal control spectrin has been described previously.9,10 Limited tryptic digestion of the patients D.E., T.E., and A.S. revealed a decrease in the 80,000-dalton band and a concomitant increase in a band at 65,000 daltons (Fig 1). In addition, those relatives of T.E. with HE (G.H., S.H., and G.W.) displayed a similar decrease in the 80,000-dalton fragment and an increase in a fragment at 65,000 daltons. Limited tryptic digests of spectrin from the father of HE patient T.E. were indistinguishable from those produced by spectrin from control normal volunteers.

The HE patient A.S. and some of the relatives of T.E. (G.W. and S.H.) also showed some variability in bands in the 48,000- to 52,000-dalton range (Fig 1). The appearance of these variable bands did not correlate with the expression of HE. The presence of these bands is the subject of a separate report.11

Analysis of limited tryptic digests by two-dimensional isoelectric focusing/SDS-PAGE. The peptide pattern produced by two-dimensional isoelectric focusing/SDS-PAGE of control normal volunteers has been described previously.9,12 The domains of spectrin have been identified, using the nomenclature of Speicher et al.,14 on the basis of molecular weight and isoelectric point. Two-dimensional analysis of the HE patients D.E., T.E., and A.S. revealed a diminution of the αI domain (80,000 daltons; pl 5.2 to 5.4) and the concomitant increase of a spot at 65,000 daltons (Fig 2). In the patients T.E. and A.S., the 65,000-dalton spot is immediately adjacent to and partially overlapping the βII domain (65,000 daltons; pl 5.1 to 5.3) (Fig 2). To confirm that the abnormal 65,000-dalton spots were derived from the αI domain, the peptide pattern was transferred to nitrocellulose paper and was probed with a rabbit antiserum that was raised against the αI domain. The control blots produced staining almost exclusively in the 80,000-dalton range (Fig 2B). The low-molecular weight portion of this spot probably represents a small quantity of the 74,000-dalton fragment, which was not resolved from the 80,000-dalton fragment at
these protein loadings. The 74,000-dalton fragment is also derived from the αI domain. When the peptide pattern produced by limited tryptic digestion of the HE patients A.S. or T.E. are probed with this antibody, there is a diminution of the staining in the 80,000-dalton region, and the appearance of new spots, which represent the abnormal 65,000-dalton spots (Fig 2D).

**DISCUSSION**

The data presented here indicate that Sp α165 represents a third type of structural defect in the spectrin molecule, which may give rise to the clinical presentation of HE. Sp α165 is similar to Sp α174 and Sp α146 in that the αI domain is affected and the ability of these spectrins to form tetramers is
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defective. In this study, we have shown that the 65,000-
dalton peptides observed in the HE patient spectrin digests are
derived from the αI domain by demonstrating immunologic
crossreactivity with an antisera directed against the αI
domain. This antiserum also crossreacts with the 74,000- and
46,000-dalton fragments observed in other HE and HPP
patients (unpublished data). The production of abnormal
peptides is thought to be due to a conformational change
which renders the αI domain more labile to proteolysis. This
conformational change would also lead to changes in the
orientation of the dimer–dimer contact site and a decreased
ability to form tetramers.

In this study, the patient T.E. is similar to the HE patients
B.D. and W.W. in our previous study, in that she exhibited
marked hemolytic anemia and poikilocytosis as an infant. In the
patients T.E. and B.D., we did not detect any biochemical
changes in their skeletal proteins concurrent to the change
from an HPP-like to an HE phenotype. These data suggest
that poikilocytosis during infancy is related to other superim-
posed abnormalities of neonatal erythrocytes, such as con-
comitant destabilization of the spectrin 4.1 contact due to
elevation of free 2,3-diphosphoglycerate or differences in
the microcirculation of the neonate. In this study, the
patient D.E. developed severe poikilocytic hemolytic anemia
resembling HPP during renal transplant rejection. In this
instance, we speculate that the increased circulatory stress that
occurred concomitant to organ rejection is responsible
for the change in phenotype.

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NOTE ADDED IN PROOF

Lecomte et al have recently described similar tryptic
peptide patterns for the spectrin from seven HE patients.

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