CONCISE REPORT

Spectrin-α1/4: A New Structural Variant of α-Spectrin in a Double-Heterozygous Form of Hereditary Pyrokoilocytosis

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Recent biochemical studies have led to the identification of abnormal spectrins in the erythrocytes of patients with hereditary pyrokoilocytosis (HPP) and hereditary elliptocytosis (HE). In this report we describe the biochemical characterization of the erythrocytes from a proband with severe HPP who is doubly heterozygous for two mutant spectrins (Sp): Spa‘74 and a new, previously undetected, mutant of α-spectrin designated Spa‘164. The proband’s erythrocytes are unstable when exposed to 45°C, and her membrane skeletons exhibit instability to shear stress. The content of spectrin in the proband’s erythrocyte membranes is decreased to 75% of control values. The amount of spectrin dimers in crude 4°C spectrin extracts is increased (58%) as compared with control values (6% ± 4%). Limited tryptic digestion reveals a marked decrease in the normal 80,000-dalton α1 domain, an increase in the 74,000-dalton fragment that is characteristic of Spa‘74, and an increase in a series of new fragments of 61,000, 55,000, 21,000, and 16,000 daltons. Both parents are asymptomatic, but they have increased amounts of spectrin dimers (17% to 25%). Limited tryptic digestion of the father’s spectrin demonstrates the presence of a previously identified abnormal spectrin (Spa‘74) that is characterized by a decrease in content of the 80,000-dalton peptide and an increase in concentration of the 74,000-dalton peptide. The mother’s spectrin digests show a decrease in the amount of 80,000-dalton peptide and the formation of new peptides of 61,000, 55,000, 21,000, and 16,000 daltons. The data indicate that this severe form of HPP is due to the inheritance of two distinct abnormal spectrins, Spa‘74 and a new spectrin mutant, Spa‘164.

Recent biochemical studies have led to the identification of abnormal spectrins in the erythrocytes of patients with hereditary pyrokoilocytosis (HPP) and hereditary elliptocytosis (HE). A defect in the ability of these spectrins to self-associate to form tetramers has been detected in all HPP patients and in a subset of the HE patients. In addition, the erythrocytes from HPP patients are deficient in spectrin. The 80,000-dalton tryptic fragment of the α-subunit, designated α1, has been identified as a functional domain for spectrin self-association. In α-spectrin from the HPP patients and a subset of HE patients, the α1 domain is structurally and functionally abnormal. When tryptic digest of patient spectrin are separated by two-dimensional isoelectric focusing (IEF)/sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), the 80,000-dalton peptide that is characteristic of the normal α1 domain is decreased or missing. To date, five distinct sets of abnormal peptides have been identified in different patients. These individuals may have an increase in (a) 74,000-dalton polypeptides with isoelectric points of 5.2 to 5.4 (Spa‘74), (b) 65,000-dalton polypeptides with isoelectric points of 5.1 to 5.3 (Spa‘164 or Spa‘74), (c) 46,000- to 50,000-dalton polypeptides with isoelectric points of 5.25 to 5.35 (Spa‘164 or Spa‘74), (d) 50,000-dalton polypeptides with isoelectric points of 5.6 to 5.7 (Spa‘74), or (e) 42,000- and 43,000-dalton polypeptides with isoelectric points of 5.75 to 5.85 (Spa‘74). In addition, several distinct low–molecular weight (mol wt) polypeptides are observed concomitant to the principal high–mol wt fragments listed before. The abnormalities observed on the tryptic peptide level are believed to reflect mutations in the α-spectrin gene. Amino acid sequencing of some of these abnormal spectrins has confirmed the existence of amino acid substitutions. In this report, we describe a kindred in which the asymptomatic mother carries a new spectrin variant, as judged by an abnormal limited tryptic peptide map, and the asymptomatic father carries the Spa‘164 variant. Like the Spa‘74 variant, the new variant, designated Spa‘164, is functionally defective in its ability to self-associate to form tetramers. The proband in this kindred is a double heterozygote for Spa‘164 and Spa‘74 and, as a result, has a severe form of HPP.

MATERIALS AND METHODS

Clinical material. We have studied an HPP patient (A.M.), her mother (R.M.), father (W.M.), and two sisters (T.M. and S.M.). Her clinical data fulfilling the criteria of HPP are summarized in the next paragraph. Venous blood from this kindred was collected into sterile tubes containing the anticoagulant citrate-phosphate-dextrose. Specimens were transported in insulated containers with ice and were kept at 4°C until analysis. A control sample was drawn, transported, and prepared along with the patient samples.

Patient A.M. is a 6-year-old black female presenting with severe hemolytic anemia since the neonatal period. A peripheral blood smear showed marked microcytosis and the presence of a high percentage of microspherocytes. Poststenectomy, the hematocrit and hemoglobin values were 31% and 10.5 g/dL, respectively, with a mean corpuscular volume of 56 and a reticulocyte count of 4.7%. The proband’s erythrocytes were markedly unstable at 45°C. The proband’s mother, father, and two sisters are hematologically normal.
Biochemical evaluation. The overall strategy for the biochemical evaluation of patient samples has been reviewed. Erythrocyte ghosts, Triton X-100 shells, and low-ionic strength spectrin extracts were prepared as described previously. The protein composition was determined by SDS-PAGE, and the content of spectrin dimers and tetramers was determined by nondenaturing gel electrophoresis. Triton shell stability was measured by using a concentric cylinder rod shearing apparatus as described by Liu and Palek. Limited tryptic digests of spectrin extracts were prepared and analyzed by SDS-PAGE as described previously. The peptides were electrophoretically transferred to nitrocellulose paper and probed with polyclonal rabbit anti-α′ antiserum as described.

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 HPP patient AM was found to be increased to 52% (data not shown). The Triton X-100–extracted ghosts from this patient were unstable, as compared with controls, when they were subjected to mechanical shearing. The spectrin–band 3 ratio (0.65) was significantly below the normal range (0.89 to 1.30). The contents of bands 2.1 and 4.1 were found to be within normal ranges, while the quantity of band 4.2 was slightly decreased.

Nondenaturing gel electrophoresis of the patient’s mother (R.M.) and father (W.M.) revealed that the quantity of spectrin dimers was increased to 17% and 25%, respectively. In addition, the quantity of spectrin dimers was increased to 17% in extracts prepared from one of the proband’s sisters (S.M.). In contrast, the proband’s other sister (T.M.) had normal dimer levels. All of these individuals were normal in terms of their red cell morphology, Triton shell stability, spectrin–band 3 ratio, and content of proteins 2.1, 4.1, and 4.2.

Analysis of limited tryptic digests of spectrin. To control for minor variations in experimental conditions, normal erythrocytes were always extracted, digested, subjected to electrophoresis and blotted concurrent to patient samples. The peptide pattern produced by normal control spectrin has been described previously. Limited tryptic digestion of the HPP patient AM showed a marked decrease in the 80,000-dalton band with a concomitant increase in a band at 74,000 daltons (Fig 1). When the peptide pattern was transferred to nitrocellulose paper and probed with a polyclonal rabbit anti-α′ antiserum, additional abnormalities were detected. Abnormal bands were present at 61,000 and 55,000 daltons, and a minor band that is present in normal digests at 46,000 daltons was decreased (Figs 1 and 2). In addition, a band that comigrated with the solvent front on a 10% gel was present. On a 14% gel, this band resolved into two bands with mol wts of 21,000 and 16,000 (Fig 2). The high-mol wt (55,000 and 61,000) bands had isoelectric points of 5.3 to 5.5, and the low-mol wt (16,000 and 21,000) bands had isoelectric points of 5.1 to 5.3. Limited tryptic digestion also showed the presence of a

Fig 1. Limited tryptic digestion of spectrin extracts from HPP patient AM. Spectrin extracts from the proband (P), her mother (M), her father (F), and a control (C) normal volunteer were prepared, digested, and subjected to electrophoresis concurrently. After electrophoresis the gels were stained with Coomassie blue (left) or electrophoretically transferred to nitrocellulose paper (right). The blots were probed with a rabbit antiserum against the α′ domain of spectrin. The mol wts of the peptides that are derived from the α′ domain are indicated at the right. The position of the 34,000- and 37,000-dalton variants of the α′ domain are indicated (arrows).

Fig 2. Immunoblotting of tryptic digests of spectrin from the HPP patient AM. (P) and a control (C) normal volunteer. The peptides were separated by two-dimensional IEF/SDS-PAGE using either 10% or 14% gels in the second dimension. After the second dimension the peptides were transferred to nitrocellulose paper and probed with anti-α′ antibody. The positions of the 74,000-, 61,000-, and 55,000-dalton peptides on the 10% gels and the 21,000- and 16,000-dalton peptides on the 14% gels are indicated.
variant of the αII domain of spectrin. This polymorphism of the αII domain is common in the normal black population. Tryptic digestion of the variant form of the αII domain produces a 37,000-dalton fragment instead of a 34,000-dalton fragment. In the proband A.M., there was an equal digestion of the variant form of the αII domain of spectrin. This polymorphism of the variant form showed a decrease in the 80,000-dalton peptide and increases in the 80,000-dalton band and an increase in the 37,000-dalton fragment instead of a 34,000-dalton peptide. The father's (W.M.) digests showed a decrease of spectrin isolated from both of the proband's parents was carried type 1 and 3 variants of the αII domain, which has been characterized by Knowles et al.

The peptide pattern produced by limited tryptic digestion of spectrin isolated from both of the proband's parents was also abnormal. The father's (W.M.) digests showed a decrease in the 80,000-dalton band and an increase in the 74,000-dalton band (Fig 1). The mother's (R.M.) digests showed a decrease in the 80,000-dalton peptide and increases in the abnormal peptides at 61,000, 55,000, 21,000, and 16,000 daltons (Fig 1). The mother also carried the αII variant, which is common in the black population. Approximately 11% of the spectrin was in the 37,000-dalton, type 3 form, and 89% was the most common, a 34,000-dalton type 1 form as judged by densitometer scans of Coomassie blue-stained gels. All of the father's spectrin was the most common form, type 1.

**DISCUSSION**

The data presented here indicate that the HPP patient A.M. is a double heterozygote for two mutant forms of α-spectrin. Both variants affect the αII domain and give rise to defective spectrin self-association. The defect in the father's spectrin appears to be identical to a previously reported normal polymorphism that we have designated Spa"61. In these individuals there is a decrease in the 80,000-dalton (α') peptide and an increase in a 74,000-dalton peptide. The clinical expression of this defect varies from asymptomatic carrier to severe HPP, depending upon the quantity of abnormal spectrin. Although specific abnormalities in the amino acid sequence of Spa"61 have not been identified to date, there are probably several primary structure defects that can give rise to this tryptic peptide pattern.

The defect in spectrin that the proband inherited from her mother has not been identified previously. The decrease in the 80,000-dalton peptide is associated with an increase in multiple minor bands. As a result, the detection of this defect requires a method that is specific and sensitive. We have used immunoblotting with a polyclonal anti-αII domain antibody. In accordance with the nomenclature of Palek, this abnormal spectrin has been designated as Spα"64.

Although the largest abnormal fragment produced by tryptic digestion of Spα"64 is similar in mass to the 65,000-dalton fragment produced by tryptic digestion of Spα"65, the complete peptide patterns are quite distinct. Spα"64 produces one major fragment of 65,000 daltons. By contrast, Spα"65 produces multiple fragments of varying mass.

Since the type 3 variant of the αII domain is present in both the proband and her mother, it appears that this variant occurs in the abnormal gene that the proband inherited from her mother. This conclusion is consistent with the observation that the proband's normal sister (T.M.) does not carry the αII variant. Thus, the quantity of the type 3 variant of the αII domain can be used as a measure of the quantity of abnormal spectrin (Spα"64) in the proband and her mother. The data indicate that the proband has approximately 50% Spα"64 and that the mother has approximately 11% Spα"64. It appears that more Spα"64 is incorporated into the membrane in the presence of a second abnormal spectrin (Spα"64) than in the presence of normal spectrin. These results suggest that normal spectrin is preferentially incorporated into the membrane and that the ability of spectrin to form tetramers is an important step in membrane assembly. These data also indicate that the composition of spectrin that is found on the membrane of mature erythrocytes may not reflect the composition of spectrin synthesized when a structural variant is present.

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**REFERENCES**


Spectrin-alpha I/61: a new structural variant of alpha-spectrin in a double-heterozygous form of hereditary pyropoikilocytosis

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