Molecular Heterogeneity of Hereditary Pyropoikilocytosis: Identification of a Second Variant of the Spectrin \( \alpha \)-Subunit

By Jack Lawler, Jiri Palek, Shih-Chun Liu, Josef Prchal, and William M. Butler

In hereditary pyropoikilocytosis (HPP), the red cell membrane skeletons exhibit a mechanical instability that can be correlated to defective self-association of spectrin heterodimers. To determine the underlying molecular defect, we have subjected HPP spectrin to limited tryptic digestion, followed by one- and two-dimensional separations of the peptides. Two of the HPP kindreds exhibited a marked decrease in 80,000-dalton peptide (previously identified as the spectrin dimer–dimer contact domain of the \( \alpha \)-subunit) and a concomitant increase of the 74,000-dalton polypeptide (presumably derived from the 80,000-dalton domain) and a decrease in a 22,000-dalton polypeptide. We now report tryptic digests of two other HPP kindred that are characterized by a decrease or complete absence of the 80,000-dalton tryptic fragment, with a concomitant increase in fragments at 46,000 and 17,000 daltons. The 46,000-dalton fragment separated into multiple spots on isoelectric focusing, ranging in isoelectric point from 5.25 to 5.35, and the 17,000-dalton fragment focused to a single spot at 5.4. Minor fragments at 56,000 and 22,000 daltons were also decreased, while a 38,000-dalton fragment increased. Limited tryptic digestion of the separated \( \alpha \)- and \( \beta \)-subunits revealed that the 74,000-dalton fragment in the first group of patients and the 46,000-dalton fragment in the second group of patients were derived from the \( \alpha \)-subunit. Both subtypes exhibited a similar defect of spectrin self-association, with 30%–38% of spectrin dimers in 0°C extracts. The results indicate that at least two distinct forms of structurally defective spectrin may give rise to the clinical presentation of HPP.

NORMAL RED CELL MORPHOLOGY and structural integrity is maintained in part by a submembrane skeleton comprised primarily of spectrin, actin, and polypeptide 4.1. The prevailing evidence suggests that the major structural elements of the skeleton are approximately 1,000 \( \text{Å} \) long fibers of spectrin \( \alpha \)- and \( \beta \)-subunits, twisted around each other to form heterodimers. \(^1\) \(^4\) These in turn associate, in a head-to-head fashion, to form tetramers and, possibly, higher oligomers. \(^1\) \(^4\) The tetramers are thought to be assembled into a two-dimensional network via their interaction with oligomeric actin. This interaction is greatly facilitated by protein 4.1, which binds to the distal end of the heterodimers. The skeleton is attached to the transmembrane protein, band 3, via ankyrin (protein 2.1). \(^4\)

Several molecular defects of the red cell membrane skeletal proteins have been identified in a group of hemolytic anemias, including hereditary spherocytosis, elliptocytosis, and pyropoikilocytosis. \(^5\) \(^12\) These include (1) complete and partial deficiency in skeletal proteins 4.1 and spectrin, respectively, \(^5\) \(^6\) (2) several spectrin variants characterized by defective self-association of spectrin heterodimers or defective binding of spectrin to protein 4.1, \(^7\) \(^11\) and (3) an incompletely defined defect in band 3 resulting in defective binding of ankyrin to the membrane. \(^15\) In hereditary pyropoikilocytosis (HPP), we have previously identified a defect in the self-association of spectrin heterodimers. \(^9\) We have subsequently characterized this defect by limited tryptic peptide mapping of spectrin in three patients in two HPP kindreds. \(^13\) We have found a decrease in the 80,000-dalton fragment of the spectrin \( \alpha \)-subunit, which represents the heterodimer contact site (\( \alpha \)-I domain) and the concomitant increase in a fragment of 74,000 daltons, both in the patients and to a lesser degree in the carrier. \(^13\) We now report the results of limited tryptic digestion of two other HPP families in which the spectrin heterodimer self-association defect was associated with a decrease in the 80,000-dalton domain and a concomitant appearance of fragments at 46,000 and 17,000 daltons. Similar findings were recently reported in preliminary form by Knowles and Marchesi. \(^14\) These results raise the possibility that the spectrin self-association defect in HPP may result from at least two different structural defects in spectrin.

MATERIALS AND METHODS

Clinical Materials

Two HPP patients from two unrelated families were examined. Clinical data fulfilling the criteria of HPP for these individuals and those in our previous study have been described. \(^13\) \(^15\) \(^16\) A summary of the clinical data is shown in Table I. Venous blood from these patients and their kindred was collected into sterile tubes containing citrate-phosphate-dextrose or anticoagulant-citrate-dextrose. Specimens were transported in insulated containers with ice to Boston.
The ghosts were washed once in 0.1 mM NaPO (pH 8.0) and 0.1 mM DTT, and subjected to limited tryptic digestion. The bands were visualized by incubating the gels in cold 250 mM DII and were dialyzed against 1 liter of the same buffer for 5 days. The dialysis buffer was changed daily and contained 30 mM phenylmethylsulfonyl fluoride for the first 4 days. Densitometer scans of Coomassie-blue-stained gels indicated that the α-subunit preparations were 75%–85% pure, while the β-subunit preparations were 80%–90% pure.

Spectrin Extraction

Erythrocyte ghosts were prepared by the method of Dodge et al. The ghosts were washed once in 0.1 mM NaPO (pH 8.0) and resuspended in an equal volume of 0.1 mM NaPO (pH 8.0), 0.1 mM EDTA, and 0.1 mM β-mercaptoethanol. After incubation at 0°C for 16 hr, the samples were centrifuged at 250,000 g for 35 min, the supernatant was decanted, adjusted to a final concentration of 40 mM Tris and 20 mM sodium acetate buffer (pH 7.4) containing 10 mM DTT, and subjected to limited tryptic digestion.

Limited Tryptic Digestion

The protein concentration was determined with the Bio Rad protein assay kit with bovine serum albumin as the standard. The spectrin extracts and purified fractions were adjusted to the same protein concentration prior to treatment with TPCK-trypsin (Worthington, Freehold, NJ, 1:10 1:25, 1:50, 1:100, 1:200, or 1:400 w/w) in 0.1 mM DTT, and were dialyzed against 9.5 mM urea, 2% NP-40, and 5% β-mercaptoethanol for 6 hr at 22°C. Carrier ampholytes (1.6% pH 5–7 and 0.4% pH 3–10, final concentrations) were added, and the samples were electrofocused for 16 hr at 400 V in 4% polyacrylamide tube gels (0.4 × 10 cm) containing 1.6% pH 4–6 and 0.4% pH 3–10 carrier ampholytes, as described by O’Farrell. SDS-polyacrylamide gel electrophoresis in the second dimension was performed on 10% acrylamide slab gels (0.3 × 12 × 80 cm) as described by Laemmli. The slab gel apparatus can accommodate six isoelectric focusing tube gels at one time. Perspective line plots of Coomassie-blue-stained two-dimensional isoelectric focusing/SDS-PAGE were generated using a densitometer equipped with a stage that would step the gel 1 mm between scans. The total protein in a spot was determined from the summation of the integrated volume elements in successive scans. The gels were oriented so that SDS-PAGE was the scanning dimension and isoelectric focusing was the stepping dimension.

RESULTS

Tryptic Digestion of Spectrin From Control Normal Adult Volunteers

In order to control for minor variations in experimental conditions, control normal red cells were always extracted, digested, and electrophoresed concurrent to patient samples. As previously reported, the peptide patterns produced by limited tryptic digestion of 40 normal volunteers were similar and reproducible. Some variability was found in polypeptides at

### Table 1. Summary of the HPP Clinical Data

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Hemoglobin (g/dl)</th>
<th>Percentage Reticulocytes (%)</th>
<th>Mean Corpuscular Volume (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPP [SpDα₁₈⁴-SpD]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.P.</td>
<td>9</td>
<td>M</td>
<td>Pre-sp 5.7</td>
<td>22</td>
<td>71</td>
</tr>
<tr>
<td>T.N.</td>
<td>13</td>
<td>M</td>
<td>Pre-sp 5.9</td>
<td>2.7</td>
<td>65</td>
</tr>
<tr>
<td>N.E.</td>
<td>9</td>
<td>F</td>
<td>Pre-sp 6.4</td>
<td>31.3</td>
<td>—</td>
</tr>
<tr>
<td>D.B.</td>
<td>4</td>
<td>M</td>
<td>Post-sp 10.9</td>
<td>4.9</td>
<td>44</td>
</tr>
<tr>
<td>A.B.</td>
<td>2</td>
<td>M</td>
<td>Post-sp 10.9</td>
<td>4.9</td>
<td>44</td>
</tr>
</tbody>
</table>

HPP [SpDα₁₈⁴-SpD] | | | | | |
| N.E. | 9 | F | 8.4 | 13 | 56 |
| D.B. | 4 | M | 8.2 | 16 | 50 |
| A.B. | 2 | M | 7.5 | 20 | 81 |

*All of the subjects were black, exhibited a characteristic red cell morphology (micropoikilo, spheroid), red cell thermal instability (fragmented at 46°C as compared to 50°C for normals), and an autosomal recessive mode of inheritance.

†In accordance with the nomenclature of Palek and Lux, we have designated those individuals from our previous study (N.E., D.B., and A.B.) with decreased 80,000-dalton tryptic fragment and increased 74,000-dalton tryptic fragment as HPP[SpDα₁₈⁴-SpD]. The individuals reported here for the first time (A.P. and T.N.) with increased 46,000- and 17,000-dalton tryptic fragments (see Results) were designated HPP[SpDα₁₈⁴-SpD]. Note that in this notation, SpD-SpD in brackets indicates that these HPP patients have defective heterodimer self-association.

‡Values for A.P. and T.N. are given before (pre-sp) and after (post-sp) splenectomy.

where they were kept at 4°C and analyzed within 3 days. A control sample was sent along each time.
34,000, 37,000, 40,000, and 47,000 daltons. In the case of the 34,000- and 37,000-dalton polypeptide, all of the white individuals were found to have only the 34,000-dalton polypeptide, while the normal black individuals had either only the 34,000-dalton fragment (approximately 50% of control individuals were in this group), only the 37,000-dalton fragment, or a variable mixture of both polypeptides, with the total protein associated with these two bands remaining constant. Knowles and Marchesi have also observed this polymorphism in the normal black population and have shown that it occurs in the α-II domain. The frequencies of these variants as well as the relationship between the 40,000- and 47,000-dalton fragments and the polymorphism in the α-II domain is currently under investigation. All of the patients and controls shown in the present study contained equal quantities of 34,000, 37,000, 40,000 and 47,000 dalton fragments. Thus, the changes observed in HPP described in this article cannot be attributed to the polymorphism observed in the normal population.

Tryptic Digestion of Spectrin From the HPP Kindreds

One-dimensional peptide maps of limited tryptic digests of HPP patients T.N. and A.P. revealed a decrease in the 80,000-dalton fragment, with a concomitant appearance of fragments at 46,000- and 17,000-daltons (Figs. 1 and 2). In both HPP patients, changes in minor peptides were also observed. Minor bands at 56,000, 26,000, and 24,000 daltons were absent in patient A.P. (Figs. 1 and 2). Patient T.N. showed a decrease in bands at 56,000 and 22,000 daltons and an increase in a band at 40,000 daltons (Fig. 1). The changes in major and minor peptides occurred each time these individuals were studied over a 12-month period. Limited tryptic digestion of spectrin extracts from the asymptomatic mother (M.P.) and sister (L.P.) of the HPP patient A.P. had one-dimensional tryptic peptide maps that were indistinguishable from control normal volunteers (data not shown). The patient’s father was unavailable for study, but the limited tryptic digestion of spectrin from the paternal uncle (J.R.) revealed a decrease in the 80,000-dalton fragment and an increase in the 46,000- and 17,000-dalton fragments (Figs. 1 and 2). Quantitation of the 80,000- and 46,000-dalton fragments was imprecise because of the small separations between these bands and neighboring bands. This problem is eliminated in the two-dimensional separations, which will be described below. The 17,000-dalton fragment was sufficiently separated from other bands to allow

Fig. 1. Limited tryptic digestion of HPP spectrin. (Left) Spectrin extracts from the HPP patient A.P., his paternal uncle (J.R.), and a control normal volunteer were treated with TPCK-trypsin (1:100, w/w) at 0°C for 20 hr. Eighty micrograms of protein was loaded onto each gel. The molecular weights are indicated on the left and the position of variable bands are indicated by arrows on the right. (Right) Spectrin extracts from HPP patient T.N. and a control normal volunteer were treated with trypsin as described above.
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precise quantitation. This fragment was absent in the control digests, while it was increased in the asymptomatic carrier J.R. (60%–70% less 17,000-dalton fragment than in the HPP patient A.P.). In the N kindred, limited trypsic digestion of spectrin from the asymptomatic mother (M.N.) and brother (C.N.) of the HPP patient T.N. revealed a decrease in the 80,000-dalton fragment and an increase in the 46,000- and 17,000-dalton fragments (data not shown).

Two-Dimensional Isoelectric Focusing/SDS-PAGE

Two-dimensional isoelectric focusing/SDS-PAGE resulted in a reproducible separation of the major trypsic fragments over a range between pH 5.0 and 6.0 (Fig. 3). Two-dimensional analysis of HPP patients A.P. and T.N. spectrin revealed a diminution of the 80,000-dalton spots and a concomitant increase in spots at 46,000 and 17,000 daltons, similar to those observed in one dimension (Fig. 3). In some experiments, the 80,000-dalton spots were almost completely absent. The 46,000-dalton fragment separated into multiple spots, ranging in isoelectric point from 5.25 to 5.35 and the 17,000-dalton fragment focused to a single spot at 5.4 (Fig. 3). Additional faint spots were also observed at 43,000 daltons and 5.25–5.35 (Fig. 3).

The asymptomatic carriers of HPP in this study (J.R., M.N., and C.N.) had changes in the two-dimensional peptide patterns, which were intermediate in magnitude between the HPP patients and controls (Fig. 4). Two-dimensional densitometer scans (perspective line plots, see Materials and Methods) indicate that the carriers of HPP (J.R., M.N., and C.N.) contained 70%–85% less 46,000-dalton fragment than the HPP patients. The two-dimensional peptide patterns of the asymptomatic mother (M.P.) and sister (L.P.) of A.P. were indistinguishable from control normal volunteers.

Tryptic Digestion of the α- and β-Subunits of HPP Spectrin

The α- and β-subunits of normal and abnormal spectrins were isolated and subjected to limited trypsic digestion to determine which subunit gave rise to the abnormal peptides. These studies include an HPP patient reported here for the first time (T.N.) and two HPP patients from our previous study (N.E. and D.B.). Although some variations in the minor peptides were observed in the digests of separated subunits from normal volunteers, the principal fragments were reproducible and will be described here. The principal fragments produced by the control α-subunit were observed at 80,000, 74,000, 48,000, 29,000, and 25,000 daltons (Fig. 5). The principal fragments produced by the control β-subunit were observed at 52,000, 48,000, 31,000, and 26,000 daltons (Fig. 5). The 74,000-dalton fragment was observed in the α-subunit digests of the HPP patients N.E. and D.B. and the control. In the case of the control α-subunit, the 74,000-dalton fragment was present in considerably higher concentrations in the isolated chain as compared to native spectrin (Fig. 5, control lanes α and Sp). The staining intensity of the 52,000- and 48,000-dalton fragments of the β-subunit of HPP patient N.E. was decreased as compared to controls (data not shown). The 46,000-dalton fragment was observed in the α-subunit digests of the HPP patient T.N. (Fig. 5).

Functional Characterization and Clinical Expression of HPP Spectrin

The level of spectrin dimers in 0°C extracts of the HPP patients are given in Table 2. When 0°C extracts of normal red cells were analyzed by nondenaturing gel electrophoresis, 5% ± 2% of the spectrin is in the dimer state. In contrast, 0°C extracts of HPP red cells contained 30%–38% of the spectrin in the dimer state (Table 2). In the nomenclature of Palek and Lux, all of the HPP patients in our studies will be designated as HPP [SpD-SpD] to indicate that these individuals have defective heterodimer self-association (Table 2). To distinguish the two subtypes of HPP, we will identify those patients (N.E., A.B., and D.B.) in whom the 80,000-dalton α-1 domain is diminished and the 74,000-dalton fragment is increased as HPP.
Isoelectric Focusing

Control

HPP Patient AP

[SpDa\textsuperscript{174}-SpD], while those patients (A.P. and T.N.) with increased 46,000- and 17,000-dalton fragments will be designated HPP [SpDa\textsuperscript{46}-SpD]. The asymptomatic mother (M.P.) and sister (L.P.) of the HPP patient A.P. were normal in terms of the quantity of spectrin dimers in 0\degree C extracts. While the patient’s father was unavailable for study, the paternal uncle (J.R.) was found to have 15% of the spectrin extracted at 0\degree C as dimers. In the N kindred, the asymptomatic mother (M.N.) and brother (C.N.) of the HPP patient T.N. had 23% and 21% of spectrin as dimers, respectively. The father in this kindred was unavailable for study.

Although a detailed comparison is not possible due to the limited number of HPP patients, the HPP [SpDa\textsuperscript{46}-SpD] patients presented here had lower hematocrits (presplenectomy) and more severe anemia than the HPP [SpDa\textsuperscript{174}-SpD] patients in our previous study\textsuperscript{13} (Table I). The hemolytic anemia was ameliorated by splenectomy in the HPP [SpDa\textsuperscript{46}-SpD] patients (Table I).

DISCUSSION

Limited tryptic digestion of spectrin has been used to identify some of the functional domains of normal spectrin.\textsuperscript{22,23} An 80,000-dalton terminal portion of the \(\alpha\)-subunit, designated \(\alpha\)-1, has been reported to contain the domain involved in spectrin dimer–dimer association.\textsuperscript{24} We have found that this peptide is decreased in all of the HPP patients studied. In the HPP...
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Fig. 4. Two-dimensional isoelectric focusing/SDS-PAGE of tryptic digests of HPP carrier J.R. (bottom) and a control normal volunteer (top). Tryptic digests of spectrin extracts were electrofocused on pH 4–6 gradients as described by O’Farrell.2 The positions of the 46,000- and 17,000-dalton fragments are indicated.

[SpDα1/4-SpD] patients, this decrease in the 80,000-dalton fragment was associated with a concomitant increase in a 74,000-dalton fragment. As shown by tryptic digestion of purified α- and β-subunits, both the 80,000- and 74,000-dalton fragments were derived from the α-subunit (Fig. 5). These data are consistent with findings that further tryptic cleavage of the 80,000-dalton fragment to 74,000 daltons destroys the ability of this fragment to bind to native spectrin, suggesting that a terminal 6,000-dalton portion is essential for preservation of function.24 Thus, the decrease in the 80,000-dalton fragment and increase in the 74,000-dalton fragment observed in the HPP [SpDα1/4-SpD] patients suggests that this site is more sensitive to trypsin in these spectrins. In HPP [SpDα1/4-SpD] patients, the decrease in the 80,000-dalton fragment was accompanied by the appearance of bands at 46,000 and 17,000 daltons. The 46,000-dalton fragment is also associated with digests of the α-subunit. Using monoclonal antibodies to the α-I (80,000 dalton) domain, Yurchenco et al.25 have identified a fragment that migrates at 50,000 daltons in their system, which is derived from the 80,000-dalton domain. The position of this fragment on two-dimensional peptide maps is very similar to that of the 46,000-dalton fragments in our study.25 In addition, Knowles and Marchesi14 have reported an increase in 50,000- and 21,000-dalton fragments in tryptic digests
of HPP patient spectrin. The 21,000-dalton fragment is probably equivalent to our 17,000-dalton fragment. We hypothesize that, in this variant, a different conformational change has occurred and different cleavage sites have become more labile to trypsin.

The peptide composition of limited tryptic digests of the separated \( \alpha \) - and \( \beta \) -subunits observed in this study agree well with data from other laboratories, with the exception of minor variations in molecular weight.\(^{26}\) The 31,000- and 26,000-dalton fragments of the \( \beta \) -subunit reported here apparently correspond to the 33,000-dalton (\( \beta \)-III) and 28,000-dalton fragments described by Speicher and Marchesi.\(^{26}\) Some variations are observed between the isolated subunits and the intact spectrin from which they were derived (Fig. 5). The 34,000-dalton fragment (\( \alpha \)-II) observed in native spectrin and involved in an asymptomatic variant of the normal population cannot be identified in the isolated \( \alpha \) -subunit. The 17,000-dalton fragment observed in HPP [SpD\(^{146}\)-SpD] spectrin digests is present at very low levels in the digests of the \( \alpha \) -subunit. The 74,000-dalton fragment is present at a higher concentration in the isolated \( \alpha \) -subunit than in control normal spectrin; presumably, the 80,000-dalton domain is more labile to proteolysis in these preparations of the \( \alpha \) -subunit. These differences in peptide composition between the isolated subunits and native spectrin could be due to the fact that (1) some of the domains are not completely renatured after SDS-PAGE, or (2) the interchain associations between the two subunits influence the proteolytic sensitivity of each subunit. The latter possibility is presently being evaluated through reconstitution experiments.

Based on the fact that HPP patients have two clinically normal parents, HPP is considered to be a recessive disease; both parents are presumed heterozygous carriers of HPP and their affected progeny would be homozygous.\(^{7}\) The data presented here indicate that one asymptomatic parent in each family contains a structural defect in spectrin that is similar to their HPP offspring and that the other parent does not carry the same spectrin defect. Thus, the HPP patients in our studies are not simply homozygotes and may have inherited a second, as yet unidentified, defect from their noncarrier parent.\(^{13}\) This second defect may involve another structural or regulatory component of the membrane skeleton or may vary the structure of spectrin in a way that is undetectable by peptide mapping of limited tryptic digests.\(^{5,12}\) Wiley and Gill\(^{16}\) have previously shown that the HPP patient T.N. has an elevated level of membrane-associated calcium.

![Fig. 5. Limited tryptic digestion of the \( \alpha \) - and \( \beta \) -subunits from HPP patients D.B. and T.N. and control normal spectrin. The \( \alpha \) - and \( \beta \) -subunits were isolated from spectrin (Sp) by preparative SDS/PAGE (see Materials and Methods). The digests were electrophoresed on 10% gels and stained with silver. The molecular weights, as judged from the electrophoretic mobility of standards, for the three different experiments are indicated on the left.](image-url)
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but, as discussed elsewhere, it is unlikely that this represents another primary defect contributing to the disease expression.

While the clinical severity of the disease differed, the five HPP individuals described in this study are similar in terms of (1) red cell morphology, (2) red cell cytoskeletal instability, (3) defective spectrin dimer–dimer association, and (4) apparent autosomal recessive mode of inheritance. These patients could be divided into two distinct subpopulations on the basis of differences in their tryptic peptide maps of spectrin. In both forms of HPP, the 80,000-dalton domain of the α-subunit is affected and the dimer–dimer association is defective. In HPP [SpDa1/46–SpD], this domain is partially degraded to 74,000 daltons. In HPP resistant domain involved in spectrin self-association. J Clin Invest 70:1019, 1982

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