A Molecular Defect of Spectrin in a Subset of Patients with Hereditary Elliptocytosis

Alterations in the α-Subunit Domain Involved in Spectrin Self-Association

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Abstract. Hereditary elliptocytosis (HE) is a clinically and biochemically heterogenous group of diseases characterized by elliptically shaped erythrocytes and an autosomal dominant mode of inheritance. Whereas the self-association of spectrin heterodimers to tetramers is defective in a subpopulation of HE patients, designated HE[*SpD*-SpD], it is normal in others. We have examined the peptide pattern produced by limited tryptic digestion of spectrin extracts from patients with HE[*SpD*-SpD] to determine if the functional defects in spectrin self-association could be correlated with structural changes in the spectrin molecule. Although the peptide pattern produced by limited tryptic digestion of spectrin extracts from those HE patients with normal spectrin self-association was indistinguishable from the pattern from control normal volunteers, digestion of the spectrin extracts from the HE[*SpD*-SpD] patients showed a reproducible diminution in the 80,000-D domain of the α-subunit, which is involved in spectrin dimer self-association. The decrease in the 80,000-D fragment was associated with an increase in a 74,000-D fragment in eight of nine families, or, in one family, with an increase of fragments at 46,000 and 17,000 D. These atypical peptide patterns were similar to those previously reported in two variants of hereditary pyropoikilocytosis (HPP), which also had defective self-association of spectrin. These data indicate that two distinct structural variants of spectrin α-subunit are associated with the defective spectrin heterodimer self-association in a subpopulation of HE patients.

Introduction

Normal erythrocyte (RBC)[1] morphology and structural integrity are maintained in part by a submembrane skeleton comprised primarily of spectrin, actin, and polypeptide 4.1 (1–5). As recently reviewed, the prevailing evidence suggests that the major structural subunits of the skeleton are heterodimers of spectrin α- and β-subunits, which, by head-to-head self-association, form tetramers and higher oligomers (1–5). These, in turn, are thought to be assembled into a two-dimensional network via their interaction with band 4.1 and actin oligomers, which binds to the distal end of the heterodimers.

Recent studies indicate that the atypical morphology and predisposition to hemolysis observed in some forms of hereditary elliptocytosis (HE) may be associated with molecular defects or deficiencies in the RBC membrane skeletal components (6–12). On a molecular level, several spectrin variants, as well as a deficiency in band 4.1, have been reported to be associated with elliptocytosis (7–13). Liu et al. have found that the quantity of spectrin heterodimers in 0°C extracts is elevated in a subpopulation of HE patients (designated HE type 1 or HE[*SpD*-SpD]) (8). In the HE[*SpD*-SpD] patients, defective self-association of

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1. *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; HE, hereditary elliptocytosis; HE[*SpD*-SpD], hereditary elliptocytosis in which a defect in spectrin self-association has been identified; HPP, hereditary pyropoikilocytosis; RBC, erythrocyte(s); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

spectrin dimers was observed in solution and in the membrane (8). Defective spectrin self-association has also been reported in one family with homozygous HE (9). Using limited tryptic digestion as a structural probe of spectrin, Coetzer and Zail (10) observed an abnormal peptide pattern in one of four HE patients studied. They found that the β-subunit of spectrin extracted from HE erythrocytes was resistant to trypsin digestion, as compared with controls. A second β-subunit variant in HE was recently reported by Dhermy et al. (11). In addition, two apparently distinct variants of the spectrin α-subunit have been detected by limited tryptic digestion in hereditary pyropoikilocytosis (HPP) (14–16). Polymorphism of the α-II domain of spectrin has also been detected in the normal population by limited tryptic digestion (14, 17).

In this paper we report on the peptide patterns produced by limited tryptic digestion of spectrin from patients with HE. This group includes HE patients with either increased spectrin dimers in 0°C extracts (HE[*SpD*-SpD]) or normal levels of spectrin dimer in 0°C extracts, as well as three HE[*SpD*-SpD] patients who had HPP-like poikilocytic hemolytic anemia in their infancy.

Methods

Clinical material. We have studied 15 HE patients from six unrelated families. Their clinical data fulfilling the criteria of HE have been previously described (8, 18). Venous blood from these patients and their kindred was collected in sterile tubes that contained the anticoagulant citrate-phosphate-dextrose. Specimens that were not obtained at St. Elizabeth's Hospital, Boston, were transported in insulated containers with ice to Boston, where they were kept at 4°C and analyzed within 3 d. A control sample was sent along each time.

Spectrin extraction. Erythrocyte ghosts were prepared by the method of Dodge et al. (19). The ghosts were washed once in 0.1 mM $NaPO_4$ (pH 8.0) and resuspended in an equal volume of 0.1 mM $NaPO_4$ (pH 8.0), 0.1 mM EDTA, and 0.1 mM β-mercaptoethanol. Extracts that were subjected to column chromatography also contained 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl. After incubation at 0°C for 16 h or at 37°C for 20 min, the samples were centrifuged at 250,000 *g* for 35 min and the supernatants were decanted and adjusted to a final concentration of 40 mM Tris and 20 mM sodium acetate buffer (pH 7.4) containing 1 mM dithiothreitol (DTT). These extracts were subjected to limited tryptic digestion or further fractionated by gel-filtration chromatography on a column (2.1 × 49 cm) of Sepharose 4B or by density gradient centrifugation on linear 5–20% sucrose gradients for 15 h at 200,000 *g*. Both separations were performed at 4°C in 10 mM $NaPO_4$ (pH 7.4), 150 mM NaCl, 5 mM β-mercaptoethanol, and 5 mM EDTA.

Limited tryptic digestion. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard. The spectrin extracts and purified fractions were adjusted to the same protein concentration before treatment with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone-trypsin (1:10, 1:25, 1:50, 1:100, 1:200, or 1:400 wt/wt) for 20 h at 0°C. Digestion was terminated by adding 1 mM diisopropyl fluorophosphate (DFP) or by heating the samples at 100°C for 1 min in the presence of 1% sodium dodecyl sulfate (SDS) and 20 mM DTT.

Protein electrophoresis. Digests were electrophoresed on discontinuous SDS polyacrylamide tube or slab gels by the procedure of Laemmli (20). The stacking gel was comprised of 2.67% acrylamide and 0.10% bisacrylamide and the separating gel was comprised of 10% acrylamide and 0.38% bisacrylamide. The gels were stained for protein with Coomassie Brilliant Blue or by the silver staining procedure of Oakley et al. (21). Nondenaturing gel electrophoresis on 0.3% agarose-2.5% acrylamide gels was performed as described by Liu et al. (22).

Isoelectric focusing/SDS-polyacrylamide gel electrophoresis. Digestion of trypsin-treated samples was terminated by adding 1.0 mM DFP, and the samples were dialyzed against 9.5 M urea, 2% NP-40, and 5% β-mercaptoethanol for 16 h 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 h 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 (23). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension was performed on 10% acrylamide slab gels (0.3 × 12 × 80 cm) as described by Laemmli (20). This slab gel apparatus can accomodate six isoelectric focusing tube gels at one time. Perspective line plots of Coomassie blue-stained two-dimensional isoelectric focusing/SDS-PAGE were generated by a densitometer equipped with a stage that would step the gel 1 mm between scans. The densitometer was interfaced with a microcomputer (Apple II plus; Apple Computer Inc., Cupertino, CA) equipped with an analog-to-digital converter (Interactive Structures, Bala-Cynwyd, PA). 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.

Separation of spectrin subunits. The α- and β-subunits of spectrin were separated by SDS-PAGE of 2 mg of spectrin extracts on 3.5–10% polyacrylamide gradient gels. The bands were first visualized by incubating the gels in cold 250 mM KCl that contained 1.0 mM DTT and then were carefully excised. The minced gel slices were placed in dialysis tubing with 1 ml of 40 mM Tris and 20 mM sodium acetate buffer (pH 7.4) containing 0.1 mM DTT and were dialyzed against 1 liter of the same buffer for 5 d. The dialysis buffer was changed daily and contained 30 mM phenylmethylsulfonyl fluoride for the first 4 d. Densitometer scans of Coomassie Blue-stained gels indicated that the α-subunit preparations were 75–85% pure, whereas the β-subunit preparations were 80–90% pure.

Results

Tryptic digestion of spectrin from control normal adult volunteers. To control for minor variations in experimental conditions, control normal RBC were always extracted, digested, and electrophoresed concurrently with patient samples. Whereas the peptide pattern produced by limited tryptic digestion of 40 normal volunteers was similar and reproducible, some variability was found in polypeptides at 34,000 and 37,000 D (14). Some individuals were found to have either only the 34,000-D polypeptide, only the 37,000-D polypeptide, or a variable mixture of both polypeptides, with the total protein associated with these two bands remaining constant (14). Knowles et al. (17) have also observed this polymorphism in the normal black population and have shown that these peptides are derived from the α-II domain. Although the 37,000-D fragment was not observed in

any of our white control normal volunteers, >50% of the black normal volunteers had variable amounts of 37,000-D fragment in their spectrin digests. Our samples of normal volunteers were not chosen to provide statistically significant conclusions about the precise frequency of these two variants.

Tryptic digestion of HE[SpD-*SpD*]*spectrin.* Nondenaturing gel electrophoresis or gel filtration revealed that the level of spectrin dimers (13–33%) in 0°C extracts of the RBC membranes from some HE patients was increased as compared with controls (5±2%). On this basis they were designated HE[*SpD*-SpD] (8). All nine of these individuals produced limited tryptic digestion patterns that were different from controls. In all of these patients the 80,000-D domain involved in spectrin self-association was markedly decreased. In all of the patients except one (WW, see below), the decrease in the 80,000-D fragment was associated with a concomitant increase in the staining intensity of a 74,000-D fragment. These changes are typified by the members of the kindred shown in Figs. 1 and 3, in whom the atypical digestion pattern can be traced through four generations. A decrease in the staining intensity of a 22,000-D polypeptide occurred in all of the family members, and two of them (OC, JS) also showed a decrease in a band at 45,000 D. All of the individuals had 100% elliptocytes in their peripheral blood smear except OC, who had normal red cell morphology, and JS, who had ~50% elliptocytes. In addition, a sister of OC, designated MS, had a similar decrease in the 80,000-D polypeptide and increase in the 74,000-D polypeptide, but she also had a decrease in a 70,000-D fragment (Figs. 1 and 3). Densitometer scans of

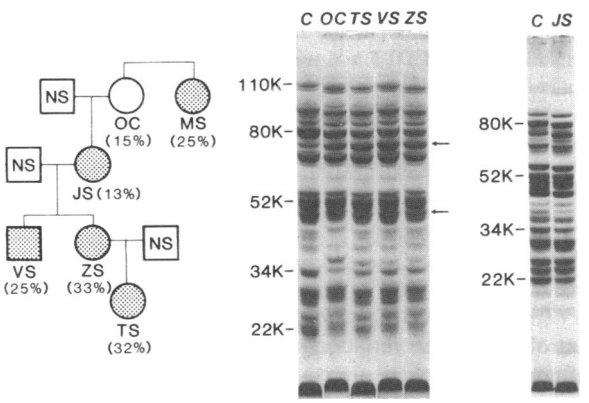

Figure 1. Limited tryptic digestion of HE[*SpD*-SpD] spectrin in the S kindred. The family pedigree is shown on the left. The individuals with HE are marked with the stippled pattern and the percentage of spectrin extracted as dimers is given in parentheses below the tested individuals. NS indicates that the individual was not available for study. The gels on the right show the limited tryptic digests of spectrin from a control normal volunteer (C) and from the members of the S kindred, with the position of variable bands indicated by arrows. The five gels in the middle and the two gels on the right represent different experiments and hence show slightly different digestion patterns.

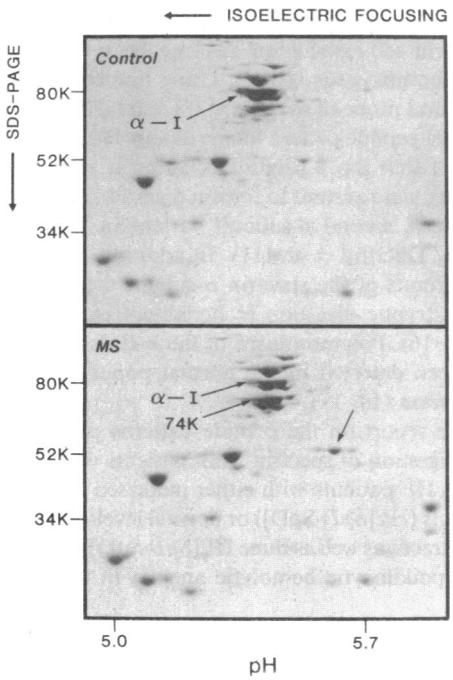

Figure 2. Two-dimensional isoelectric focusing/SDS-PAGE of tryptic digests of HE[*SpD*-SpD] spectrin. Tryptic digests of spectrin extracts from HE[*SpD*-SpD] patient MS and a control (C) normal volunteer were electrofocused on pH 4 to 6 gradients as described by O'Farrell (23). The focusing gels were equilibrated with Laemmli sample buffer and electrophoresed concurrently on a single 10% slab gel (20). The arrows indicate the position of the variable 52,000-D domain (see Results).

Coomassie Blue-stained gels indicated that the 80,000-D fragment was decreased from 19 to 38% in this kindred (Fig. 1).

To examine further the relationship between the presence of increased spectrin dimers in 0°C extracts and the increase in the 74,000-D fragment in tryptic digests, we separated spectrin dimers from tetramers in the HE[*SpD*-SpD] patient MS and subjected them to tryptic digestion. The ratio of the area of the 74,000-D peak to the area of the 80,000-D peak on densitometer scans was used to quantitate the relative increase in the 74,000-D fragment and decrease in the 80,000-D fragment. This ratio for the control individuals was 0.43±.09; for spectrin extracts from MS it was 1.15±.08. Digestion of separated dimers and tetramers from MS gave values of 0.88 for the tetramer pool and 1.60 for the dimer pool, indicating an enrichment of abnormal spectrin in the spectrin dimer pool of the patient. A similar enrichment was previously observed in our HPP patients (14).

Two-dimensional isoelectric focusing/SDS-PAGE of HE[SpD-*SpD*] *spectrin.* Two-dimensional analysis of normal spectrin resulted in a reproducible separation of the major tryptic fragments over a range between pH 5.0 and 6.0 (Fig. 2). Although some variations in the minor peptides were observed in the

normal volunteers, the principal fragments were reproducible and will be described here. The 80,000-D polypeptides consistently focused to multiple spots whose isoelectric points ranged from pH 5.3 to 5.4, with an additional spot at pH 5.65 (Fig. 2). Two-dimensional analysis of spectrin extracts from the members of the S kindred showed a diminution of the 80,000-D spots, with an increase in the staining intensity of spots at 74,000 D similar to that observed in one dimension (Fig. 2). Only the 80,000-D spots that focused to pH 5.3 to 5.4 were affected, and the 74,000-D polypeptides also focused to multiple spots in this range. In addition, an increase in the staining intensity of a 52,000-D polypeptide that focused to pH 5.5 was observed in MS (Fig. 2, arrow). The 52,000-D fragment was tentatively identified as the α-IV domain on the basis of its molecular weight and isoelectric point (24–26; Dr. David Speicher, personal communication). Variations in this fragment were frequently observed in control normal volunteers.

Tryptic digestion of the subunits of HE[SpD-*SpD*] *spectrin.* The α- and β-subunits of normal and HE[*SpD*-SpD] spectrins were isolated and subjected to limited tryptic digestion to determine which subunit gave rise to the abnormal peptides. The principal fragments produced by the control α-subunit were observed at 80,000, 74,000, 48,000, 29,000, and 25,000 D (Fig. 3). The principal fragments produced by the control β-subunit were observed at 52,000, 48,000, 31,000, and 26,000 D (Fig. 3). The α- or β-subunits from both the normal and HE[*SpD*-SpD] spectrin produced very similar peptide patterns. In both cases the 74,000-D polypeptide was a principal component of the α-subunit. In the case of the control subunit, the 74,000-D polypeptide was present in a considerably higher concentration in the isolated chain as compared with native spectrin, presumably owing to incomplete renaturation (Fig. 3, control lanes α and Sp; see Discussion). The staining intensity of a 38,000-D band of the β-subunit was increased in the HE[*SpD*-SpD] patient MS.

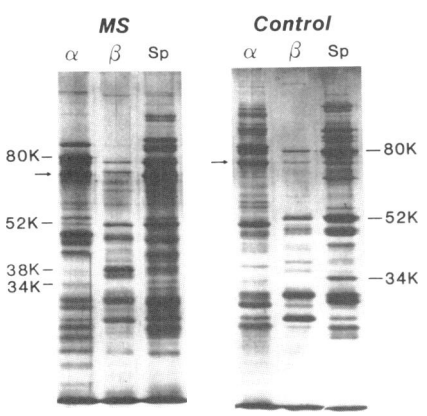

Figure 3. Limited tryptic digestion of the α- and β-subunits from control and HE[*SpD*-SpD] spectrins. The α- and β-subunits were isolated from spectrin (Sp) extracts of the HE[*SpD*-SpD] patient MS and a control normal volunteer by preparative SDS-PAGE.

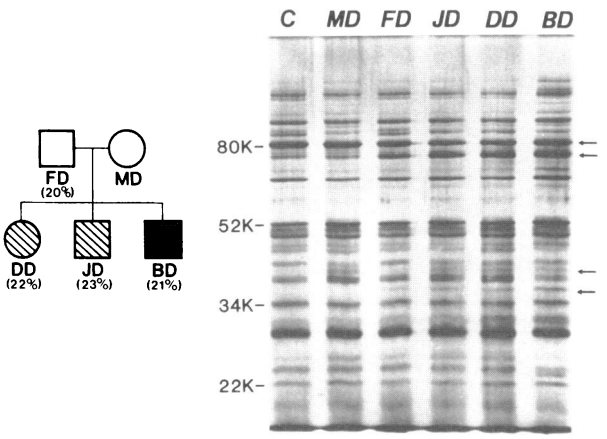

Figure 4. Limited tryptic digestion of HE[*SpD*-SpD] spectrin in the D kindred. The family pedigree is shown at the left, with the HE individuals who expressed HPP as infants marked by the hatched pattern and the sibling who made the transition from HPP to HE during this study marked by the solid pattern. The percentage of spectrin extracted as dimers is given in parentheses for those individuals who fell outside the normal range (5±2%). Spectrin from these individuals had an atypical pattern on nondenaturing gel electrophoresis: the dimer band was broadened. 10 μg of limited tryptic digests were electrophoresed on a 10% slab gel and the fragments were visualized with silver stain (right). The position of the affected bands is indicated on the right by arrows.

Analysis of kindreds containing HE and poikilocytosis in infancy. During infancy, a subpopulation of the HE[*SpD*-SpD] individuals (WW, BD, and JD) in this study expressed erythrocyte morphology and thermal sensitivity that were equivalent to those of the HPP phenotype, as previously reported (29). The patients BD and JD have a sister DD who now has HE but was not studied during infancy (Fig. 4). We have studied the patient BD from the age of 2 to 20 mo. While his RBC morphology has transformed from poikilocytic to elliptocytic during this time, no change was observed in the peptide pattern produced by limited tryptic digestion. All three siblings in the kindred had a similar increase in the staining intensity of the 74,000-D polypeptide and a concomitant decrease in the 80,000-D polypeptide (Fig. 4). All three siblings also had the 37,000- and 40,000-D polypeptides frequently observed in normal volunteers (14, 17). These polypeptides can be seen to segregate in the parents: the mother (MD) has only the 40,000-D polypeptide and the father (FD) has only the 37,000-D polypeptide (Fig. 4). The father also had an increase in the 74,000-D polypeptide and a diminution of the 80,000-D polypeptide, but to a lesser extent than his progeny (Fig. 4). The father's RBC morphology and hematological data were normal. Based on human leukocyte antigen typing, the probability that FD is the father of DD and JD is 99.0 and 98.1%, respectively (18, 27, 28). In addition to the above-mentioned variations, BD had a very slight increase in the amount of material associated with the high molecular

weight polypeptides, with the concomitant decrease in low molecular weight polypeptides ranging from 22,000 to 25,000 D (Fig. 4). These changes may indicate a slight decrease in the sensitivity of this patient's spectrin to trypsin.

Two-dimensional isoelectric focusing/SDS-PAGE of the fragments produced by limited tryptic digestion of spectrin from BD, JD, and DD revealed a diminution of the 80,000-D fragment and an increase in the 74,000-D fragment similar to that observed in the S kindred (Fig. 5). Quantitation of the volume under the 80,000-Dalton peak indicates that it was reduced by 40–60% in the patients BD, JD, and DD as compared with controls. The 74,000-D spot was increased by 150–200% for these patients. The father of the probands had an increase in the amount of protein associated with the 74,000-D spots and a decrease in the 80,000-D spots, although these changes were somewhat smaller than those of the probands. Limited tryptic digests of spectrin extracts from the mother's RBC were indistinguishable from controls.

Limited tryptic digestion of spectrin extracts from WW produced a peptide pattern that differed from those of the other HE[*SpD*-SpD] patients. There was again a decrease in the staining intensity of the 80,000-D fragment. However, the 74,000-D fragment was absent in this patient and there was an increase in bands at 46,000 and 17,000 D, which were not seen in the other HE[*SpD*-SpD] patients (Fig. 6). The patient's mother (MW), who is clinically normal, had a normal tryptic peptide pattern, with the 37,000-D fragment frequently observed in normal volunteers present (Fig. 6). The patient's father has HE but has been unavailable for this study. Based on paternity testing, there is a 99.9% probability that he is the father (18). Two-dimensional isoelectric focusing/SDS-PAGE of limited tryptic digests of spectrin from the HE patient WW revealed a decrease of the 80,000-D spots and an increase in spots at 46,000 and 17,000 D, which were similar to those observed in one dimension (Fig. 7). The 46,000-D fragment separated into multiple spots whose isoelectric points ranged from 5.25 to 5.35, and the 17,000-D fragment focused to a single spot at 5.4 (Fig. 7). We have recently reported similar structural alterations of spectrin in one of the subtypes of HPP (15).

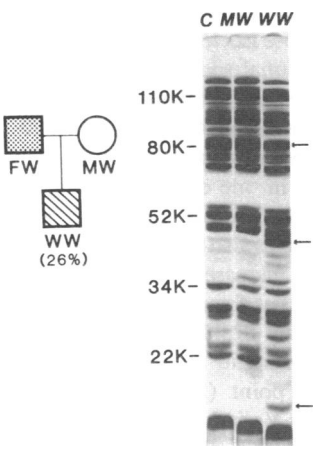

Figure 6. Limited tryptic digestion of HE[*SpD*-SpD] spectrin in the W kindred. The family pedigree is shown at the left, with the HE proband (WW) who expressed HPP as an infant marked by the hatched pattern and the HE parent marked by the dotted pattern. The percentage of spectrin extracted as dimers is given in parentheses for WW. The mother's (MW) level of spectrin dimers was normal and the father was unavailable for this study. The gels on the right show the limited tryptic digests of spectrin from these individuals; the position of variable bands is indicated by arrows.

Tryptic digestion of spectrin from neonatal RBC. Because of the difference in phenotypic expression of HE in the neonate in some patients, we have further studied spectrin self-association and tryptic peptides in neonatal red cells. These were isolated

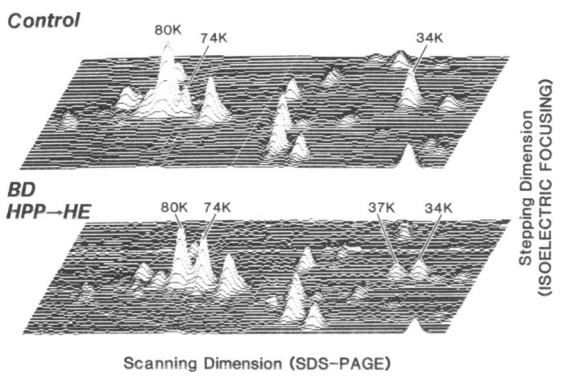

Figure 5. Perspective line plots of the two-dimensional separations of tryptic peptide from a control and the HE[*SpD*-SpD] patient BD. The gels were oriented so that the SDS-PAGE was the scanning dimension and isoelectric focusing was the stepping dimension.

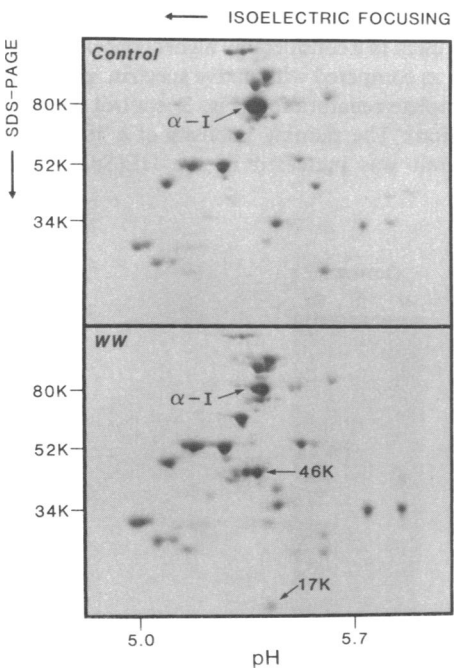

Figure 7. Two-dimensional isoelectric focusing/SDS-PAGE of tryptic digests from the HE patient WW and a control normal volunteer. The positions of the normal 80,000-D (α-I) and the abnormal 46,000- and 17,000-D fragments are indicated.

from human umbilical cords and compared with normal adult RBC in terms of the stability of their Triton shells (Rohm and Haas Co., Philadelphia, PA) (6), the quality of spectrin dimers in 0°C extracts, and the limited tryptic digestion of 0°C extracts. RBC were treated with 1% Triton X-100 and subjected to mechanical shaking at 4°C (6). Neonatal and adult Triton shells were found to be comparably resistant to fragmentation by mechanical shaking. Neonatal and adult RBC were also found to have an equivalent quantity of spectrin in the dimer state (5±2%). In addition, no differences were found in the peptide pattern produced by neonatal and adult spectrins over a wide range of trypsin concentrations (enzyme to substrate ratios 1:10 to 1:400; data not shown).

Tryptic digestion of spectrin from HE patients with normal spectrin self-association. In contrast to the HE[*SpD*-SpD] patients, some HE patients had levels of spectrin dimer in the 0°C extracts that fell in the normal range (5±2%). All six of these individuals produced limited tryptic digestion patterns that were indistinguishable from controls. In a kindred comprised of six individuals, those with HE (FR, TR, and CR) produced tryptic digestion patterns that were identical to the unaffected members (DR, PR, and KR) (Fig. 8). Similar peptide patterns were observed when limited tryptic digests of these HE spectrins and control normal volunteers were analyzed by two-dimensional isoelectric focusing/SDS-PAGE (Fig. 9). As shown in Fig. 9, an increase in the staining intensity of the 52,000-D fragment was also seen in the R kindred (Fig. 9). In this kindred, this change was selectively observed in the affected family members (FR, TR, and CR).

Discussion

The abnormalities of tryptic peptides reported here can be divided into three types: alterations that are directly associated

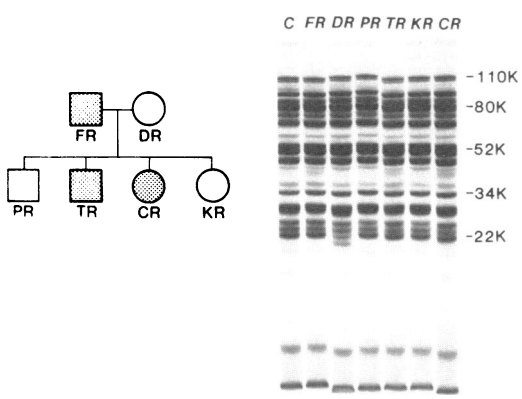

Figure 8. Limited tryptic digestion of spectrin from HE patients who do not have defective spectrin self-association. The family pedigree is shown on the left; the affected individuals are indicated by the dotted pattern. The gels on the right show the limited tryptic digests of spectrin from these individuals. Note that the difference in the 21,000-D fragment in DR was not reproducible.

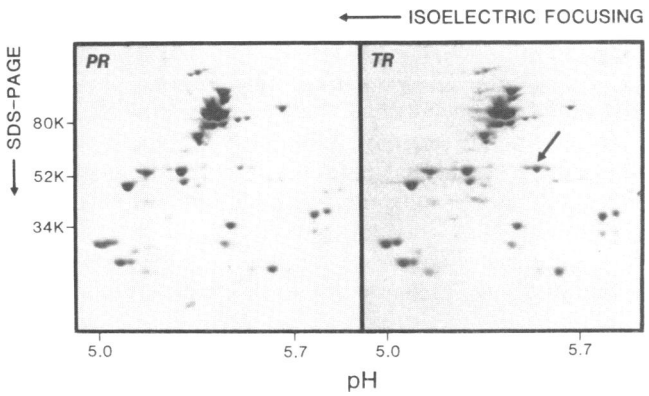

Figure 9. Two-dimensional isoelectric focusing/SDS-PAGE of tryptic digests of spectrin from the HE patient TR and his brother (PR), who had normal RBC morphology. The level of spectrin dimers in 0°C extracts fell in the normal range (5±2%) for both individuals.

with a functional defect of spectrin self-association; alterations associated with asymptomatic spectrin variants of the normal population; and alterations that are currently of unknown origin. Variations in the 80,000-D domain are examples of the first type. The 80,000-D terminal portion of the α-subunit has been reported to contain the domain involved in tetramer formation (25, 26). Further tryptic cleavage of the 80,000-D fragment to 74,000 D destroys the ability of this fragment to bind to native spectrin, suggesting that a terminal 6,000-D portion is essential to preserve function (26). These data are consistent with the observations that both the 80,000- and 74,000-D fragments in HE[*SpD*-SpD] are associated with the α-subunit in the present study and that the increase in the 74,000-D fragment is associated with a concomitant decrease in the 80,000-D fragment. We are now raising antibodies against the α-I domain to provide conclusive proof that the 74,000-D fragment is derived from the 80,000-D domain. A similar decrease in the 80,000-D fragment and increase in the 74,000-D fragment were found in most of the HPP patients we have studied (14, 15).

In one of the HE[*SpD*-SpD] individuals in the study (WW), the decrease in the 80,000-D fragment was accompanied by the appearance of bands at 46,000 and 17,000 D. The one- and two-dimensional peptide maps produced by spectrin from this individual (WW) were similar to those observed in a subpopulation of individuals with HPP (15, 16). Two-dimensional peptide maps of spectrin from these individuals indicate that the 46,000-D fragment is equivalent to the 50,000-D degradation product of the α-I (80,000 D) domain identified by Yurchenco et al. (24). These data suggest that the conformation of the 80,000-D domain of HE[*SpD*-SpD] and HPP spectrin is altered, rendering it less capable of forming tetramers and more susceptible to tryptic digestion.

The observation that the tryptic peptide maps of HE[*SpD*-SpD] and HPP spectrin are very similar suggests that these individuals carry the same or very similar spectrin defects,

whereas the phenotype (i.e., HE or HPP) is determined by other factors, such as the amounts of abnormal spectrin (Street, A., S. C. Liu, J. Lawler, and J. Palek, manuscript in preparation). This possibility is supported by two observations, which suggest a link between HPP and some forms of HE: (*a*) spectrin thermal stability and RBC heat stability are decreased in HPP and some forms of HE, particularly during infancy; and (*b*) both conditions are present in some families (7, 29). Studies on those patients with mild HE and poikilocytosis in infancy may help to elucidate these observations. A change in the peptide pattern produced by tryptic digestion of spectrin extracts from BD concurrent with the change in RBC morphology from poikilocytic to elliptocytic was not detected. The tryptic peptide patterns obtained for all three HE siblings in the family D (JD, DD, and BD) are very similar. In addition, the level of spectrin dimers, the mechanical stability of Triton shells, and the peptide pattern produced by tryptic digestion of neonatal RBC spectrin were indistinguishable from those of control normal adult RBC spectrin. Thus, it seems unlikely that spectrin in fetal cells is different from adult RBC spectrin or that the transition in RBC morphology observed during infancy in some forms of HE is due to changes in spectrin composition or conformation. This is consistent with recent findings of a lack of difference in the mechanical properties of HE RBC with neonatal poikilocytosis and mild HE (30), suggesting that these phenotypical differences may be related to an altered microenvironment of the neonate, rather than to an intrinsic RBC defect.

The variations in the 34,000-, 37,000-, and 40,000-D fragments are examples of the second type of alterations in the tryptic peptide patterns. These can be attributed to polymorphorism of normal spectrin. Knowles et al. have presented data that indicate that, on the basis of isoelectric point and molecular weight, there is a total of four distinct variants of the α-II domain (17).

The significance of the third type of alterations that we have observed in tryptic peptide maps of spectrin is unclear. This group is typified by the variations in the 52,000-D (α-IV) domain. An increase in this fragment is observed in MS and in the HE individuals of the R kindred. However, other HE patients do not appear to have an increase in this peptide, and the quantity of this peptide seems to vary somewhat in the normal population. We are currently studying the effect of several parameters on the production of this fragment.

The peptide composition of limited tryptic digests of the separated α- and β-subunits observed in this study agree well, except for minor variations in molecular weight, with data from other laboratories (17, 25, 26). The 31,000- and 26,000-D fragments of the β-subunit reported here apparently correspond to the 33,000-(β-III) and 28,000-D fragments described by Speicher and Marchesi (25). Some variations between the isolated subunits and the intact spectrin from which they were derived were seen (Fig. 4). The 34,000-D fragment observed in native spectrin and involved in an asymptomatic variant of the normal population cannot be identified in either subunit. The 74,000-D fragment is present at a higher concentration in the isolated α-subunit than in control normal spectrin. Presumably, the 80,000-D domain is more susceptible to proteolysis in these preparations of the α-subunit. These differences in peptide composition between the isolated subunits and native spectrin may indicate either that some of the domains are not completely renatured after SDS-PAGE or that the interchain associations between the two subunits influence the proteolytic sensitivity of each subunit. This latter possibility means that we cannot be absolutely certain that the primary molecular defect in HE is in the α-subunit. An alternative possibility is that a defect in the adjacent β-subunit increases the susceptibility of the α-subunit to digestion. This possibility is now being evaluated in our laboratory by reconstitution experiments.

The biochemical data presented here are consistent with clinical data that indicate that HE is a heterogenous disease characterized by an autosomal dominant mode of inheritance (7, 31). In part, this heterogeneity may result from the fact that several different membrane skeletal defects can give rise to elliptical morphology (7–13). Thus, it is not surprising that the limited tryptic peptide maps of spectrin produced by some HE patients are indistinguishable from those of normal volunteers, while others are atypical. Similarly, some HE spectrins are more sensitive to heat denaturation while others denature normally (13). In these HE individuals, who do not have a spectrin self-association defect, the molecular defect may involve another functional domain of spectrin or a structural or regulatory component of the cytoskeleton other than spectrin. Whereas the level of band 4.1 appeared normal in the patients described here, deficiencies of band 4.1 have been reported to produce elliptocytosis (12). Structural defects probably also occur in spectrin that cannot be detected by limited proteolysis.

The present data and results of our previous studies (14, 15) indicate that a similar, if not identical, functional and structural defect of the α-I domain of spectrin is present in a subpopulation of patients with HE and in all patients with HPP. Both the functional expression of the defect, that is, the defect in spectrin dimer-dimer self-association, and the structural expression of the defect are quantitatively different; they are more severe in HPP than in HE. This presumably reflects the fact that HPP RBC contain more of this abnormal spectrin than HE cells (14, 15). This question is now being investigated in our laboratory.

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