Molecular and Functional Changes in Spectrin from Patients with Hereditary Pyropoikilocytosis

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ABSTRACT The structural and functional properties of spectrin from normal and hereditary pyropoikilocytosis (HPP) donors from the two unrelated families were studied. The structural domains of the spectrin molecule were generated by mild tryptic digestion and analyzed by two-dimensional electrophoresis (isoelectric focusing; sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The αI-T80 peptide (M_r 80,000) is not detectable in two related HPP donors; instead, two new peptides (M_r 50,000 and 21,000) are generated and have been identified as fragments of the normal αI-T80. A third sibling has reduced levels of both the normal αI-T80 and the two new peptides.

A similar analysis of spectrin from another HPP family indicates that their spectrins contain reduced amounts of the αI-T80 and the 50,000 and 21,000 fragments of the αI domain. The HPP donor also has other structural variations in the αI, αII, and αIII domains.

The αI-T80 domain of normal spectrin has been shown to be an important site for spectrin oligomerization (J. Morrow and V. T. Marchesi. 1981. *J. Cell Biol.* 88: 463–468), and in vitro assays indicate that HPP spectrin has an impaired ability to oligomerize. Ghost membranes from HPP donors are also more fragile than membranes from normal erythrocytes when measured by ektacytometry. In both the oligomerization and fragility assays, the degree of impairment is correlated with the amount of normal αI-T80 present in the spectrin molecule.

We believe that a structural alteration in the αI-T80 domain perturbs normal in vivo oligomerization of spectrin, producing a marked decrease in erythrocyte stability.

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INTRODUCTION

Recent studies of membrane structure and function have drawn attention to the fact that all cells have a unique array of membrane-associated proteins that serve several important functions. These proteins form what has come to be known as a membrane skeleton, which confers stability and deformability to the overlying lipid bilayer. In some cases, the membrane skeleton may also serve as a connecting link between the surface membrane and other cytoplasmic organelles.

A number of erythrocyte abnormalities have been described that are believed to be due to primary structural defects of the membranes (1, 2). In most of these conditions, the cells develop abnormal shapes and the membranes appear more fragile in response to mechanical and/or osmotic stresses. Many investigators have suggested that such properties are likely to be related to abnormalities of the membrane skeleton.

The proteins that make up the membrane skeleton of the human erythrocyte have been studied in some detail (3–6). The principal components are spectrin and an erythrocyte form of actin (7). These proteins are believed to form specific complexes with each other and are attached to the membrane in company with other intermediary components (8, 9). Spectrin is a huge, rodlike molecule composed of two nonidentical subunits, which are themselves composed of multiple chemical domains (10). Spectrin also has a remarkable capacity to self-associate and can form large oligomeric units under appropriate conditions (11, 12).

Hereditary pyropoikilocytosis (HPP)[1] is a relatively rare hemolytic disorder characterized by markedly fragile cell membranes and an unusual sensitivity to thermal disruption. The cells form microcytes and poikilocytes in vivo, and the spectrin extracted from HPP erythrocyte membranes shows enhanced sensitivity to thermal denaturation in vitro (13, 14) and an altered dimer-tetramer ratio (15).

The results presented here indicate that spectrin isolated from erythrocytes of patients with HPP is chemically different from that isolated from normal controls. A modified segment of HPP spectrin has been mapped to a specific chemical domain of the α-subunit. This segment of the α-subunit is known to be involved in regulating the capacity of spectrin to self-associate into higher oligomeric forms. The capacity of HPP spectrin to form higher oligomers is also markedly reduced. These findings suggest that HPP represents a hemolytic disorder caused by an abnormal membrane skeleton.

We postulate that the functional defect in HPP may be related to the reduced capacity of spectrin to form oligomeric complexes.

METHODS

Patients. Blood from normal donors and affected patients (from two unrelated families) was drawn into vacutainer tubes containing either heparin or acid citrate dextrose as anticoagulant. Donors from the A family consisted of the mother and her three children. The mother (Mrs. A.) shows no hematologic abnormality. Two of her children, M.A. (male) and V.A. (female), have classic pyropoikilocytosis according to standard hematologic criteria and have been described previously (13). Both children had severe anemia at birth (7–9 g of hemoglobin) and substantial reticulocytoses (20–25%), and underwent splenectomy at an early age. After splenectomy, both had improved hemoglobin values (11–12 g) and reduced reticulocyte counts (3–7%). Although they do not require transfusions, they clearly still have residual hemolytic anemia. Erythrocytes from these patients fragmented extensively at 45°C, as described previously (13). Patient A.A., the brother of M.A. and V.A., is not anemic, but his peripheral blood cells show moderate numbers of elliptocytes and ovalocytes (~20%).

The B family (Black) consisted of a mother and her child, K.B. (female), now 19 yr old. Severe anemia (hemoglobin 3.8 g/dl, mean corpuscular volume 68 μ^3, reticulocyte count 14%) was first noted in K.B. at 6 wk of age. Persistent anemia and reticulocytosis (5–27%) required transfusion on four occasions before splenectomy at 22 mo of age. After splenectomy, hemoglobin levels rose to 10–12 g/dl, the mean corpuscular volume fell to 50–57 μ^3, and reticulocytosis continued to be noted, but at a lower level (2–10%). K.B.'s erythrocytes exhibited morphologic changes and fragmented when heated to 46°C (normal 49°C). In addition, spectrin became inextractible from K.B.'s erythrocytes heated to 46°C (normal 49°C) (16). These findings plus a clinical pattern identical to that of M.A. and V.A. established the diagnosis of pyropoikilocytosis in K.B. The mother (Mrs. B.) is hematologically normal, except for iron deficiency. Blood from neither father could be obtained.

All blood samples (including control samples) were maintained at 4°C and either processed immediately—e.g., for the membrane fragility measurements—or sent by air freight to New Haven for the biochemical determinations. Blood from all patients was studied on at least two separate occasions.

Extraction of spectrin. Erythrocyte membranes were prepared as previously described (17). Specific problems encountered with the preparation of HPP erythrocytes are described in Results.

Spectrin was extracted by dialyzing the membranes for 40 h against 0.1 mM EDTA, pH 9.0, containing 120 μM phenylmethylsulfonyl fluoride at 4°C. After dialysis, the samples were centrifuged twice at 48,000 *g* for 60 min to remove membrane vesicles. The supernatants containing both spectrin and actin were dialyzed against isotonic KCl buffer (10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.2 mM diisopropylfluorophosphate [DFP], pH 7.4). All of the above procedures were performed at 0–4°C.

Tryptic digestion of spectrin. Spectrin (0.8–1.4 mg/ml) was dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol (Tris buffer) for 20 h at 4°C. Trypsin (treated with 206 μ/mg L-1-tosylamide-phenyl-ethylchloromethyl ketone, Worthington Biochemical Corp., Freehold, NJ) was added to an enzyme-substrate ratio of 1:20 (wt/wt). The reaction was terminated after 90 min at 0°C by adding DFP to a final concentration of 1 mM and cooling to −80°C. Digests were lyophilized and stored at −20°C until further use.

Denaturation-renaturation of spectrin. Spectrin (0.8–1.4 mg/ml) was denatured in Tris buffer with 8 M urea (Ultra-pure, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) and 50 mM dithiotreitol (DTT) for 30 min at 37°C under N_2. After denaturation, the urea and the DTT were removed by dialysis against Tris buffer. Denatured-renatured spectrin was digested with trypsin and the reaction then inhibited as described above.

Thermal denaturation of spectrin. The structural regions of spectrin used for thermal denaturation studies were prepared by digesting spectrin with trypsin at an enzyme-substrate ratio of 1:200 for 20 h at 0°C. The reaction was inhibited by adding DFP to 1 mM once every 24 h for 4 d. The digest was dialyzed into 100 mM NaCl, 20 mM Tris HCl, pH 8.0, 1 mM 2-mercaptoethanol, and heated to various temperatures for 15 min. After cooling for 2 h at 0°C, a second trypsin digestion (enzyme-substrate ratio 1:20) for 60 min at 0°C was performed. The reaction was inhibited by DFP and lyophilizing as described above.

Polyacrylamide gel electrophoresis (PAGE). Samples for one-dimensional electrophoresis were solubilized in 2% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol and electrophoresed according to Laemmli (18). Samples for two-dimensional electrophoresis (isoelectric focusing-SDS) were solubilized in 9 M urea, 2% Triton-X100, 5% 2-mercaptoethanol, and 2.4% ampholines and then isoelectric focused on 3 × 120-mm polyacrylamide gels according to O'Farrell (19). The pH gradient extended from 7.2 to 4.5 and was formed by mixing equal volumes of LKB ampholytes (LKB Instruments, Inc., Rockville, MD) at pH 4–6, pH 5–7, and pH 3.5–10 (2.4% final ampholyte concentration). After elec-

[1] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; DI, deformability index; DTT, dithiotreitol; HPP, hereditary pyropoikilocytosis; PAGE, polyacrylamide gel electrophoresis.

trophoresis for 5,700 V/h, the gels were removed and equilibrated for 10 min in 10% glycerol, 3% SDS, 1 mM EDTA, and 2% 2-mercaptoethanol and then electrophoresed in the second dimension on a 10–15% acrylamide gradient using Laemmli buffers (18). Molecular weights were determined by reference to known protein standards.

^{125}I-Peptide mapping. Peptide maps were prepared from ^{125}I-labeled Coomassie Blue-stained peptides removed from two-dimensional polyacrylamide gels as previously described (10).

Analysis of spectrin oligomers. The distribution of spectrin oligomers was determined by nondenaturing 2–4% gradient polyacrylamide gel electrophoresis at 4°C as previously described (11, 12, 20).

Measurement of membrane stability. The stability of intact erythrocyte membranes was measured by ektacytometry (21). In brief, 100 μl of ~40% ghost suspension was thoroughly mixed with 3 ml of dextran medium (35 g/100 ml, wt/vol of dextran 40,000 mol wt in phosphate-buffered saline (PBS) with a viscosity of 97.5±2.5 cp). The cell suspension was subjected to a constant shear stress of 575 dyn/cm^2 in the ektacytometer and the decay of the deformability index (DI) signal was recorded continuously as a function of time. It has been previously shown that the loss of cell deformability represented by the decay in the DI signal is due to fragmentation of intact deformable resealed ghosts into small, nondeformable spherical fragments (22). Hence, the rate of decay in the DI as measured in this assay, is directly proportional to the fragility of the membranes.

RESULTS

HPP erythrocytes are more fragile than normal. Erythrocytes from normal and HPP patients were washed with PBS to remove serum proteins, platelets, and leukocytes. During these washes, a significant percentage of erythrocytes from patients M.A. and V.A. fragmented into small hemoglobin-containing vesicles that appeared similar to HPP microcytes formed in vivo. These microcytes did not sediment at 2,300 *g* used to pellet intact erythrocytes. To recover these vesicles, the PBS supernates were pooled and centrifuged at 30,000 *g* for 15 min. The vesicles were resistant to further fragmentation and could be washed with PBS and pelleted with the increased centrifugal forces. After the PBS washes, the vesicles were hypotonically lysed and processed as normal erythrocyte ghosts.

HPP ghosts contained the normal complement of membrane proteins in addition to more hemoglobin and band 8 than are usually associated with normal ghosts (Fig. 1A) HPP microcyte vesicles had an additional protein with a molecular weight of 67,000 (presumed to be albumin) and varying amounts of hemoglobin (Fig. 1B). The albumin from the HPP microcyte membranes was not released even after prolonged washings in 5 mM phosphate at pH 8.0. HPP microcyte membranes also have increased amounts of proteins with mobilities similar to band 4.5 and band 8.

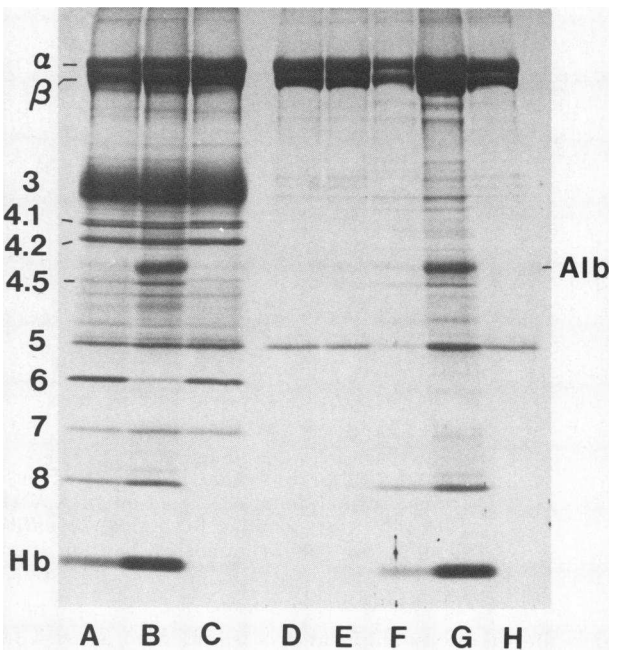

FIGURE 1 SDS-PAGE analysis of membrane proteins and spectrin extracts from normal and HPP erythrocytes. Membrane ghosts (A) and HPP microcyte membranes (B) were from patient V.A. The erythrocyte ghost profile from a typical normal donor is in C. Spectrin and actin preparations extracted from normal ghost membranes during low ionic strength incubation are shown in D, E, and H. The low ionic strength extracts from HPP ghosts and HPP microcytes (patient V.A.) are shown in F and G. The proteins are numbered according to standard nomenclature. The separating gel is a 7–15% gradient, 80 μg of ghost protein (A–C) or 15 μg of low ionic strength extract (D–H) are loaded per lane.

Low-ionic strength extracts of the above membrane preparations are shown in Fig. 1. Extracts from normal ghosts contained primarily spectrin and actin (Fig. 1D, E, and H), whereas HPP ghost extracts have increased levels of band 8 and hemoglobin as well (Fig. 1F). HPP microcyte extracts contain albumin, band 4.5, band 8, hemoglobin, and other minor components (Fig. 1G). Two-dimensional gel electrophoresis (2-4% nondenaturing at 4°C, SDS) (11) of HPP microcyte extracts indicates that the contaminating-proteins are not associated with spectrin (data not shown).

Limited tryptic digestion of HPP spectrin does not yield an 80,000-mol wt α-subunit peptide. Limited tryptic digestion of spectrin under controlled conditions generates a highly reproducible pattern of peptides ranging in molecular weight from 80,000 to 12,000 (10, 23). These peptides can be separated by two-dimensional gel electrophoresis (Fig. 2). One of the most prominent peptides generated by tryptic digestion of normal spectrin has a molecular weight of 80,000 (Fig. 2A). This peptide occupies a terminal

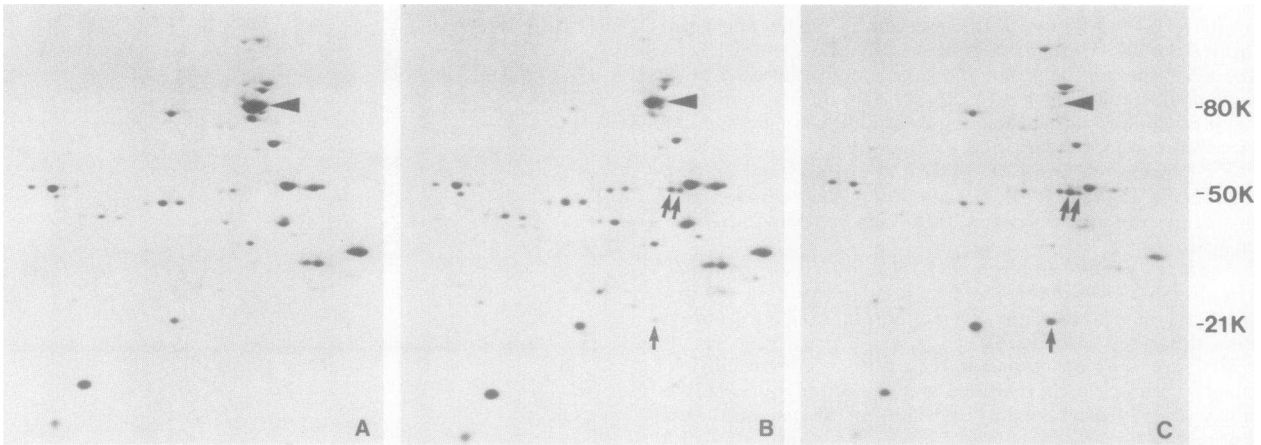

FIGURE 2 Two-dimensional (isoelectric focusing-SDS) separation of a tryptic digest of spectrin from a normal donor (A), from donor A.A. (B) and from HPP patient V.A. (C). Digests from patient M.A. are identical to C. Arrows identify HPP αI-T50 and HPP αI-T21 peptides. Triangle identifies αI-T80 or the normal position of αI-T80.

position on the α-chain and is referred to as αI-T80 (23). In contrast to results obtained from studies of over 85 normal donors, tryptic digestion of spectrin from patients V.A. and M.A. did not produce an αI-T80 peptide (Fig. 2C). The lack of detectable αI-peptide was not a reflection of a different rate of digestion, since altering the time of digestion from 5 to 120 min did not generate this fragment.

Our inability to generate an αI-peptide from patients V.A. and M.A. was coupled with the appearance of three new peptides. Two of these had identical molecular weights of 50,000, but slightly different isoelectric points. The third peptide had a molecular weight of 21,000 (Fig. 2C). High-resolution peptide maps of the two 50,000-mol wt peptides were identical (data not shown) and also indicated that they were fragments of the αI-T80 region of the spectrin molecule (Fig. 3). Peptide maps of the 21,000-mol wt peptide (αI-T21) showed that it was also a fragment of the αI-T80 region (Fig. 3). Co-electrophoresis and chromatography of the peptides generated from the 50,000-mol wt peptide (HPP-αI-T50) and the 21,000-mol wt peptide (HPP-αI-T21) produced a peptide map that was virtually identical to that of normal αI-T80 (data not shown).

Spectrin extracted from HPP microcytes derived from patients V.A. and M.A. appeared identical in its tryptic sensitivity when compared with spectrin from the same ghosts (data not shown).

In contrast to the complete absence of αI-T80 from patients V.A. and M.A., their sibling (A.A.) had a reduction in the Coomassie Blue staining intensity of αI-T80, along with the presence of smaller amounts of the 50,000- and 21,000-mol wt fragments (Fig. 2B). The mother (Mrs. A.) had spectrin indistinguishable from normal donors.

Limited tryptic digests of spectrin from patient K.B. has decreased amounts of the αI-T80 peptide along with increased αI-T74, and the αI-T50 and αI-T21 fragments (Fig. 4C). In addition, this donor has a structural variation in the αII domain (Fig. 4C) unrelated to HPP (W. J. Knowles et al., manuscript in prepa-

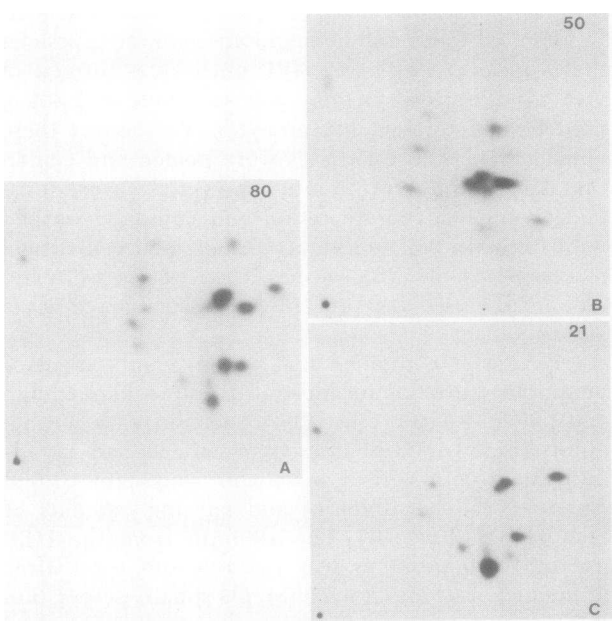

FIGURE 3 Chymotryptic peptide maps of αI-T80 (A), HPP αI-T50 (B), and HPP αI-T21 (C).

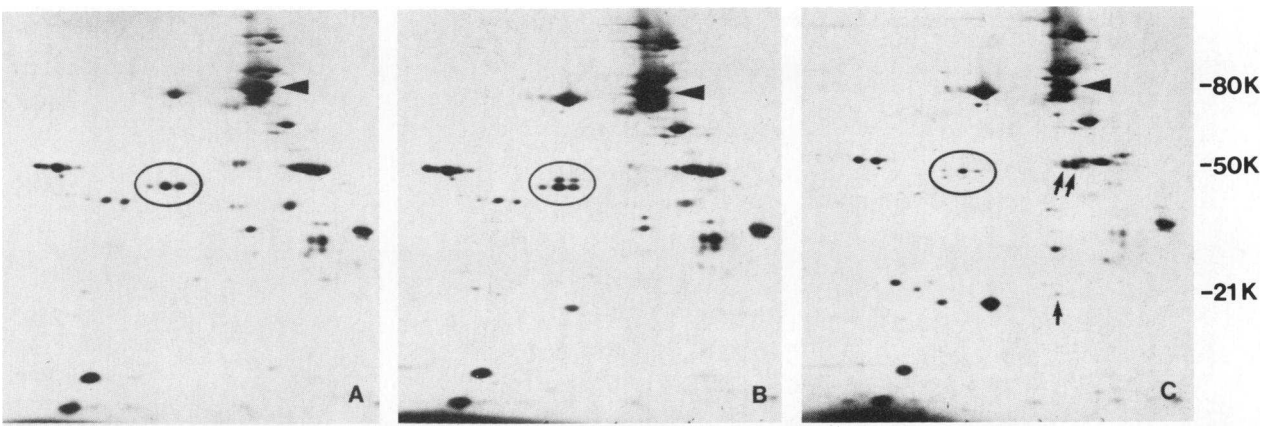

FIGURE 4 Two-dimensional separation of a tryptic digest of spectrin from a normal donor (A), from donor Mrs. B. (B), and from HPP donor K.B. (C). Peptides are labeled as in Fig. 2. Encircled area identifies the αII-peptides.

ration). Several other alterations in the digest pattern were also detectable. In particular, peptides from the αIII-, αIV-, and αV-domains were much lower in Coomassie Blue intensity than controls. The αIII-peptide was also slightly higher in molecular weight (48,000) than the normal αIII-T46. In contrast, the β-chain peptides are identical in molecular weight, pI, and in yield, when compared with normal spectrin digests. The mother, Mrs. B., heterozygous for the αII-variant, has increased amounts of αI-T74 and lacks any detectable HPP αI-T50 and αI-T21 (Fig. 4B). The control spectrin shown in Fig. 4A has normal αI-T80 and is homozygous (common type) for the αII region.

Denaturation-renaturation does not alter the HPP digestion pattern. Experiments using limited tryptic digestion of spectrin to produce the intermediate-size peptides shown in Fig. 2 rely on the fact that many potential proteolytic cleavage sites are masked in the native molecule. These can be exposed by denaturants allowing complete digestion of both subunits (24). Removal of the denaturants permits refolding of the individual chains and restores the original tryptic insensitivity (24). From this, one might infer that subtle changes in peptide structure may be detected if they modify the protein conformation at critical sites. It also follows that an altered proteolytic digestion pattern of HPP spectrin could be due to conformational changes in the αI-segment of the spectrin molecule. To explore this possibility, spectrin was denatured in 8 M urea containing 50 mM DTT and then allowed to refold by dialyzing away the urea. Incubation with urea results in disruption of the noncovalent associations between subunits and reduces the α-helical content to extremely low levels. After removal of the urea, spectrin regains its α-helicity as measured by circular dichroism and refolds into its protease resistant conformation (24). Tryptic digestion of spectrin treated in this way from HPP donors M.A. and V.A. produced a cleavage pattern of the αI-region identical to that found in the native HPP spectrin (Fig. 5C). Denatured-renatured normal spectrin generated the normal αI-T80 (Fig. 5A). Thus, we conclude that the digestion pattern of HPP spectrin in family A is due to a conformational alteration of the spectrin molecule. Since HPP spectrin renatures into the conformationally altered form, it is believed that abnormal conformation is the result of a covalent modification of the HPP spectrin molecule (i.e., primary sequence change or posttranslation modification). Denatured-renatured spectrin from family B donor K.B. also produced the αI-T50 and αI-T21 peptides, as well as the same increased levels of αI-T74 seen previously. The molecular-weight increase in the αIII-domain and the variation in αII-domain is identical to that seen in the native digest from this donor. The denaturation-renaturation cycle also produced αIV- and αV-peptides that are identical in molecular weight, pI, and Coomassie Blue staining intensity when compared with the same peptides produced from normal spectrin.

The αI-T80 peptide domain denatures in the 48°–52°C temperature range. Previous studies using circular dichroism and differential scanning calorimetry indicated that some parts of the normal spectrin molecule thermally denature with a transition midpoint of 49°C (transition A, reference 25) and do not renature upon cooling. A similar analysis of HPP spectrin has shown that the A transition has shifted to a midpoint of 44°C (14), the same temperature at which the erythrocytes fragment. To identify the parts of the normal spectrin molecule that might be responsible for this A transition, a digest of normal spectrin peptides was heated to various temperatures, cooled, and again

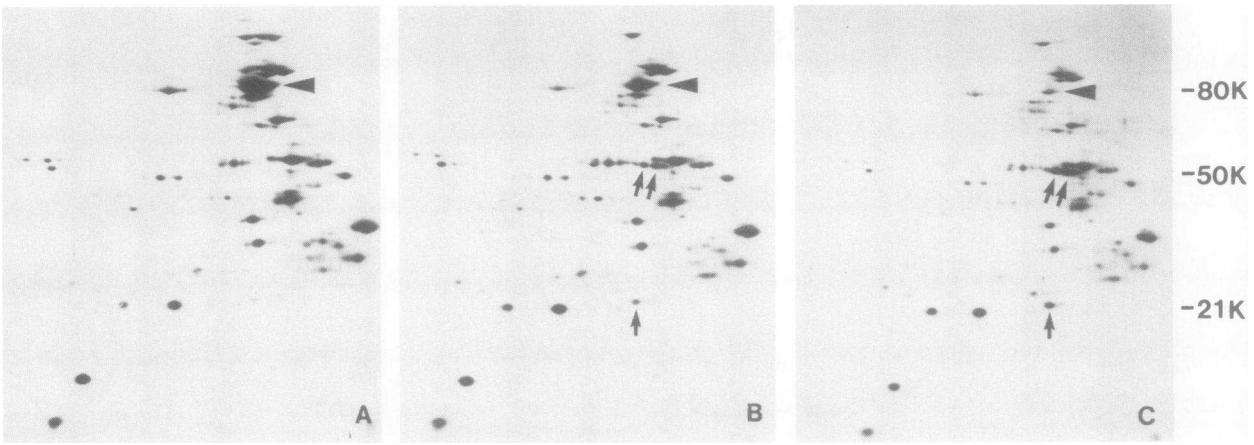

FIGURE 5 Two-dimensional separation of a tryptic digest of spectrin after urea denaturation and renaturation by dialysis of a normal donor (A), A.A. (B), and V.A. (C). Peptides are identified as in Fig. 2.

digested with trypsin at 0°C. The segments of the spectrin molecule that refold after heating retain their resistance to protease, in contrast to the irreversibly denatured regions, which should be digested completely by the second trypsin treatment. The results, shown in Fig. 6, indicate that the αI-T80 peptide and related αI-T74 peptide do not survive a second tryptic digestion after being heated above 52°C. At 49°C, ~40% of the αI-region of spectrin (αI-T80 and αI-74) was irreversibly denatured. These results suggest that the A transition described earlier (25) is probably due to the irreversible denaturation of the αI-segment of the normal spectrin molecule. Since this αI-domain is also modified in HPP spectrin, it is likely that changes in this segment are responsible for this shift in the A transition of HPP spectrin (14).

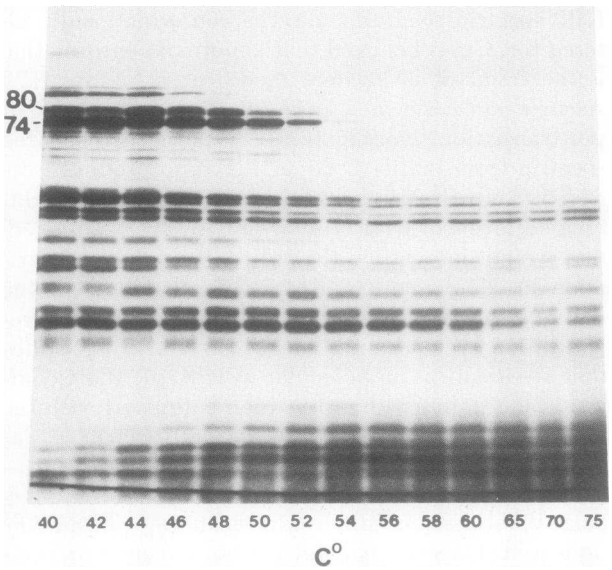

FIGURE 6 Thermal denaturation of different structural regions of the spectrin molecule. Spectrin peptides were heated to the indicated temperature for 15 min, cooled to 0°C, and then digested with trypsin at 0°C a second time (enzyme-substrate ratio, 1:20) for 60 min. The structural regions that are irreversibly thermally denatured are digested to low-molecular weight peptides. The peptides that did not denature or denatured reversibly do not change in molecular weight or Coomassie Blue intensity. Spectrin peptides are identified according to molecular weight.

HPP and normal erythrocytes both contain spectrin oligomers in vivo. A striking characteristic of normal spectrin is its ability to form oligomers by the concentration-dependent self-association of α,β dimers (12, 20). Previous studies have established that spectrin oligomerization in vitro is a concentration-driven process characterized by a high energy of activation barrier, so that interconversion between oligomers below 4°C is slow (12, 20, 26). Low ionic strength extracts of ghosts prepared at 4°C contain spectrin oligomers, which are presumed to have preexisted on the intact membrane (12).

Since the αI-T80 peptide region is crucial for the oligomerization process (12), the existence of spectrin oligomers in HPP erythrocytes was investigated (Fig. 7). Erythrocyte ghosts from HPP patients V.A. and M.A. (Fig. 7A, lanes 1 and 2) and from their sibling (patient A.A., lane 3) were extracted at 4°C as described, and the resulting extracts were analyzed at 4°C by 2–4% nondenaturing PAGE. All extracts display a similar distribution of oligomers and the patterns are indistinguishable from normal control subjects.

HPP spectrin has impaired ability to form oligomers in vitro. An approximate association constant for oligomer formation in normal spectrin is 10^5 mol^{-1}.

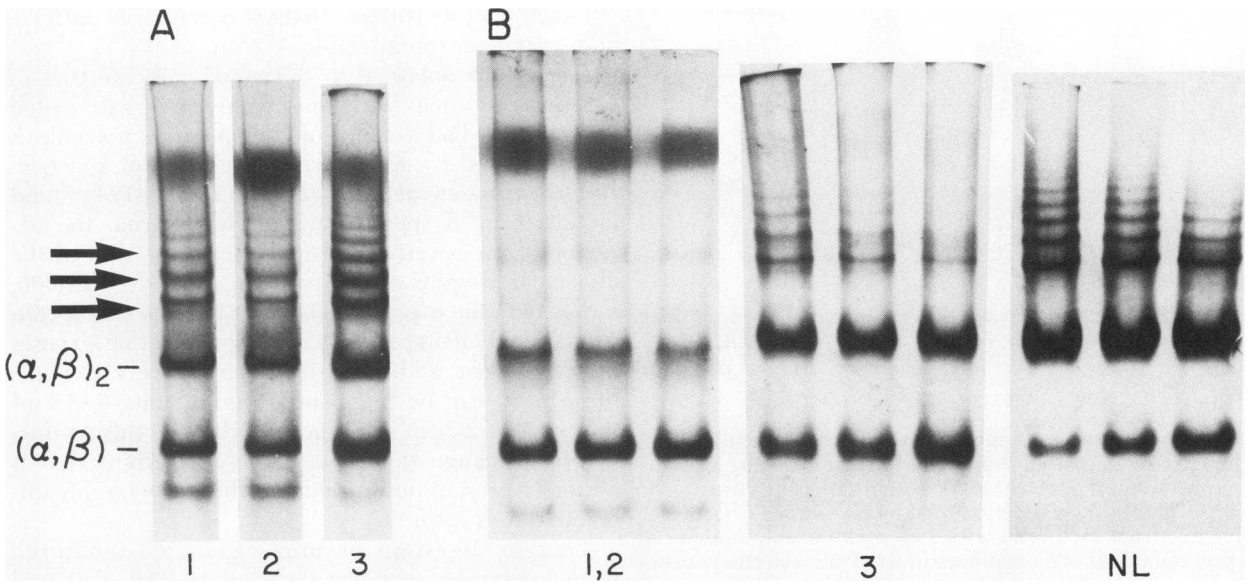

FIGURE 7 Nondenaturing gel electrophoresis of spectrin dimers and oligomers of normal and HPP spectrin. (A) Low ionic strength extracts (prepared at 4°C) of ghosts from donors M.A.-1, V.A.-2 and A.A.-3. Note the presence of spectrin oligomers in each extract. These extracts are comparable to similar extracts from normal donors (data not shown). (B) Low ionic strength extracts concentrated in vitro and equilibrated at 30°C for 3 h. Concentrations are 12.5 mg/ml (left lane), 8 mg/ml (middle lane), and 4 mg/ml (right lane) for donors M.A.-1, V.A.-2, A.A.-3, and a normal donor (NL). (Spectrin from donors M.A.-1 and V.A.-2 behaved similarly; only the result of V.A. is shown.) Note the reduced oligomer formation at each concentration of spectrin from donor V.A. (1, 2). Spectrin from donor A.A. (3) shows intermediate behavior compared with the normal control (NL). $(\alpha_1\beta)$, dimer; $(\alpha_1\beta)_2$, tetramer; higher-molecular weight oligomers are indicated by arrows. The diffuse band at the top of A1-3 and B1,2 is hemoglobin.

Thus, by concentrating normal spectrin in vitro to 10 or 20 mg/ml (10^{-5} M), extensive oligomer formation can be demonstrated (12). In vivo, high local concentrations of spectrin are probably maintained at the membrane surface by a high-affinity interaction with its membrane receptor, identified as band 2.1 or ankyrin (27). It is likely that HPP erythrocytes also have a highly concentrated submembraneous array of spectrin. To explore the possibility that the very high in vivo concentrations of spectrin obscured differences between HPP and normal spectrin oligomerization, the spectrin from HPP patient V.A. and sibling A.A., as well as a normal control, were equilibrated at 30°C. An analysis of the resulting oligomer distributions is shown in Fig. 7B. When equilibrated at concentrations up to 12 mg/ml, spectrin from HPP patient V.A. (Fig. 7B, 1) and M.A. (data not shown) formed only modest amounts of tetramer and trace amounts of hexamer, whereas normal spectrin at 12 mg/ml (Fig. 7B, NL) existed almost entirely as tetramer and higher oligomers. The spectrin of patient A.A. showed intermediate behavior (Fig. 7B, 3), correlating with the reduced amount of αI-T80 peptide generated from the spectrin of this patient. Quantitation of these oligomeric forms is shown in Fig. 8. Preliminary estimates suggest that the oligomer association constant of HPP spectrin is reduced by over an order of magnitude ($\sim 2 \times 10^4$ mol^{-1}, vs. 5×10^5 mol^{-1} for normal spectrin in these experiments).

These results are not due to thermal denaturation of HPP spectrin, since a similar result is obtained by equilibrating the samples at 25°C (data not shown). Other parameters (protease resistance, circular dichroic spectra, and fluorescence depolarization data) are also inconsistent with denaturation (data not shown).

Decreases in membrane deformability correlate with structural changes in spectrin. To measure directly the physical stability of the erythrocyte membrane under shear stress, the membrane ghosts from each donor were studied by ektacytometry (21). The results (Fig. 9) show that Mrs. A., who has no apparent structural changes in spectrin, has erythrocyte membranes with normal fragility. Her two children M.A. and V.A. have a marked decrease in membrane stability, along with a complete absence of normal αI-T80. Her third child, A.A., has a membrane stability that is intermediate between normal donors and M.A.

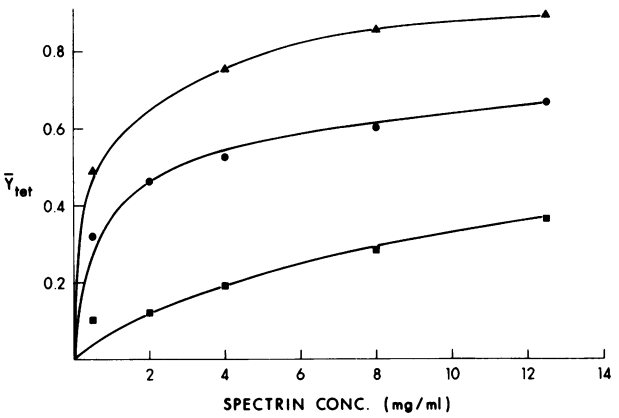

FIGURE 8 Approximate mole fraction as spectrin tetramer. The relative amount of dimer and tetramer existing at each concentration of spectrin after equilibration in vitro for 3 h at 30°C (Fig. 7) was determined by eluting the Coomassie Blue dye from each band with 25% pyridine and then measuring the OD at 605 nm. Spectrin was from a normal donor (▲), from A.A. (●), and from M.A. and V.A. (■).

$$Y_{tet} = \frac{OD_{tet}}{OD_{dimer} + OD_{tet}}.$$

and V.A. This donor (A.A.) has ~50% normal of αI-T80 and lesser amounts of the αI-T50 and αI-21 fragments.

Donor K.B. has membranes that are as fragile as those seen from M.A. and V.A. This donor (K.B.) has decreased αI-T80 increased levels of αI-T74, along with the αI-T50 and αI-T21 peptides, and variations in the αII and αIII domain. The mother (Mrs. B.) has intermediate membrane stability with increased amounts of the αI-T74 peptide and has a variation in the αII domain.

DISCUSSION

Spectrin is a complex molecule having nine structural domains, many with known functions or associations. To identify these domains from normal spectrin, we used mild tryptic digestion at 0°C. The generation of the intermediate-sized peptides is dependent upon the native conformation of the molecule. The spectrin dimer (α,β) contains ~525 lysine and arginine residues (10), but only a small percentage of those in native spectrin are reactive toward trypsin. Small changes in conformation, produced by primary sequence alterations, chemical oxidation, or abnormal subunit associations, could expose or bury additional lysine or arginine and result in an altered digestion pattern. Modified peptides (altered in pI and/or molecular weight) can be identified by comparison with two-dimensional electrophoretic analysis of controls. Using this approach, we studied spectrin from over 100 donors and obtained peptide patterns that are remarkably uniform and extremely reproducible.

The results obtained in this study indicate that in certain individuals with the hemolytic disease called HPP the αI-T80 domain of the spectrin molecule is not generated by mild tryptic digestion of spectrin. Instead, two new peptides (αI-T50 and αI-T21) found in this form of spectrin are produced from the αI-region of the spectrin molecule. Since the sum of the molecular weights of the two fragments is ~71,000, rather than the expected 80,000, it is likely that a conformational change exists in this spectrin that exposes more than one additional trypsin reactive site. Since the αI-T50 can be found in trace amounts (~1% of that in Fig. 2C) in some normal digests, this conformational change exposes a lysine or arginine that is normally present but buried and therefore largely unreactive to trypsin.

Trypsin digestion of urea-denatured-renatured spectrin samples produces the same αI-T80, T50, and T21 fragments as found in the native molecule from the same donor. In normal spectrin, the αI-region renatures into a functionally active domain and trypsin digestion after renaturation produces only the αI-T80 and small amounts of the αI-T74 peptide. It is there-

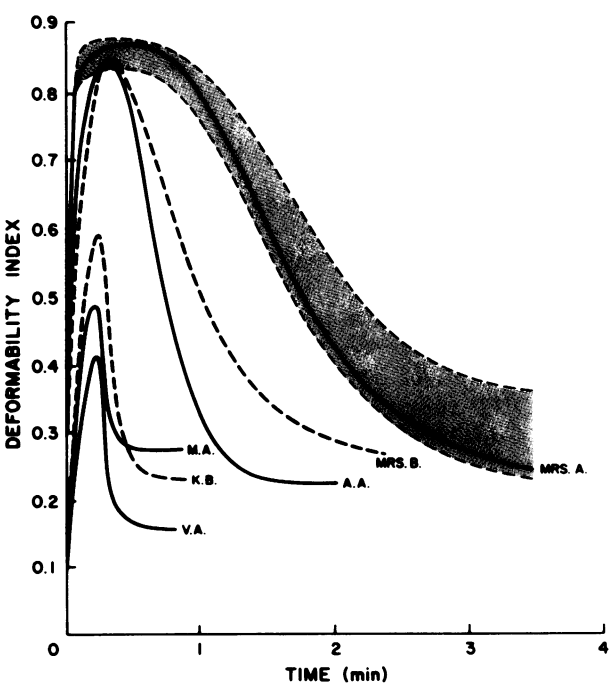

FIGURE 9 Fragmentation assay for resealed ghosts prepared from whole blood samples. The shaded area represents the variation seen in 80 normal subjects. In this assay, the decay of DI with time is directly related to membrane fragility.

fore likely that the modified digestion pattern seen in spectrin from HPP donors is due either to a change in the primary sequence or to a posttranslational modification (i.e., amidation, oxidation) of the αI-domain. This alteration results in an abnormal conformation of spectrin in the HPP erythrocyte and the abnormal renaturation pattern in vitro.

In support of the αI-domain being the site of the defect in HPP spectrin is the fact that αI domain from normal donors thermally denatures between 48° and 52°C. This denaturation is likely to be at least partially responsible for the A transition seen using circular dichroism (14, 25). Since the A transition shifts from 49°C in normal spectrin to 44°C in HPP spectrin, it seems likely that the αI-domain contains the defect that results in both HPP spectrin and HPP erythrocyte thermal instability.

These structural variations in spectrin correlate with a clinically significant disease. The αI-T50 and αI-T21 peptides have been found only in four donors, and have not been seen in over 100 others. The mother (Mrs. A.), without clinical evidence of an erythrocyte abnormality and with normal erythrocyte stability, does not have these peptides. Further support comes from the finding that erythrocytes from patient V.A. and M.A. are severely defective in their membrane stability and also have a complete absence of αI-T80. Erythrocytes from patient A.A. have a membrane stability intermediate between that of normal donors and patients M.A. and V.A. This patient has ~50% of the normal amount of αI-T80 and 50% of the αI-T50 and αI-T21 peptides. These combined results suggest that the conformational change in spectrin that alters the tryptic sensitivity of the αI-region is connected with clinically manifested HPP in family A. However, we are left with a paradox—a clinically and biochemically normal mother with three affected children. At the present time, we can only speculate on this finding. Since multiple genes encode for proteins such as actin (28), the possibility exists that the spectrin gene(s) expressed by Mrs. A. is not the same as those expressed by her children. A second possibility is that the HPP condition in this family is the result of two independently variable genes. One gene may code for a structurally altered spectrin molecule without producing any clinical consequences (e.g., Mrs. A.). The offspring could be normal, heterozygous, or homozygous for this spectrin, depending on the genotype of the parents. A second gene may code for an enzyme that posttranslationally modifies only the altered form of spectrin, producing the HPP phenotype. In support of the latter interpretation, the spectrin of Mrs. A. is chemically different (in the αI-domain) from normal spectrin when compared by two independent chemical cleavages (research in progress).

The results of studies on family B are also complex. Donor K.B. has reduced amounts of αI-T80 and the αI-T50 and αI-T21 peptides. The αI-T50 has a pI slightly more basic than donors M.A., V.A., and A.A. In addition, donor K.B. has increased αI-T74 and variations in the αII- and αIII-domains. How these variations modify the stability of the membrane skeleton is not known. It is possible that one or more of these variations can act synergistically with the αI-T50, αI-T21 alteration in perturbing the normal erythrocyte membrane skeleton and result in the significant decrease in membrane deformability seen in donor K.B.

The mother (Mrs. B.) does not have the αI-T50 or αI-T21 peptides, but has increased levels of αI-T74 and a variation in the αII-domain. One or both of these variations may produce the intermediate membrane stability observed with her erythrocyte ghosts. Since her child (K.B.) has these variations plus the αI-T50, αI-T21 change, the erythrocytes may display the classical properties of HPP erythrocytes, but the underlying structural defect in her spectrin is not identical to that of donors V.A. and M.A.

The differences in spectrin structure of donors from families A and B may explain why the genetics of HPP are not understood. In most cases the parents of HPP children are clinically and morphologically normal (e.g., Mrs. A.). Her child (A.A.) with a partial variation in the αI-domain (heterozygote?) has symptoms that are mild at best and would most likely go unnoticed, whereas M.A. and V.A. have HPP and a total variation in the αI-domain.

In at least one previously studied family, a parent has been shown to have mild hemolytic elliptocytosis while the child has HPP (2). This again supports the possibility that the molecular basis of HPP and possibly other hemolytic anemias will prove to be due to combinations of structural changes in the spectrin molecule. In the case of family B, a defective gene from the mother (Mrs. B.) plus an altered paternal gene could produce the severe erythrocyte instability in their child.

It is also of interest that most known HPP patients are black and that genetic variations within the αII-domain (Mrs. B. and K.B.) have only been found in the black population (W. J. Knowles et al., manuscript in preparation).

HPP spectrin also provides insight into the functional role of spectrin in the normal erythrocyte membrane skeleton. Evolving concepts of the erythrocyte membrane skeleton attribute a significant role to spectrin oligomers. The clear demonstration of such oligomers in fresh, low temperature extracts of both HPP and normal erythrocyte ghosts, under conditions in which they are not likely to form, provides compelling evidence for the existence of such oligomeric forms

in vivo. Although the reduced association constant for oligomer formation characteristic of HPP spectrin nearly eliminates oligomers at the concentrations attainable in vitro, it apparently still allows the assembly of the membrane skeleton when driven by the higher local concentrations induced at the membrane surface by concomitant binding of spectrin to ankyrin (a process termed affinity-modulated assembly [12]). Nevertheless, these oligomeric associations are probably less stable in HPP than in normal cells, perhaps contributing to overall membrane instability. Similar observations on decreased spectrin tetramer formation of HPP spectrin have been made by Palek and coworkers (15, 29, 30) but the molecular basis for this is apparently different than that described in this paper (31).

Another previously characterized function of the αI-T80 region is the noncovalent association of the α- and β-chains (11). The possibility that such associations between α- and β-subunits may also be perturbed in HPP spectrin cannot be excluded. Similarly, the manner in which these specific functional changes in spectrin translate into the observed abnormalities in shape remains a mystery, although they strongly imply a role for the αI-region of the spectrin molecule.

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