Mutant Forms of Spectrin α-Subunits in Hereditary Elliptocytosis

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Abstract

Two variant spectrins have been described in hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP). Both are characterized by increased susceptibility of the αI (N-terminal) 80-kD domain to mild tryptic digestion, yielding peptides of 46–50 or 65–68 kD (T50a and T68 in our terminology). In this report we add a third unstable spectrin αI domain found in three kindreds with HE; αIT80 in this type of spectrin is cleaved by mild tryptic digestion to a 50-kD peptide (T50b) distinguished from T50a by its more basic isoelectric point. All three spectrins show impaired self-association to form oligomers.

Intermediate tryptic peptides of the three unstable αI domains from HE spectrins were characterized by monoclonal immunoblotting and I^{125} limit peptide mapping and affinity purified using polyclonal anti-αIT80. Partial amino acid sequences of αI domain peptides were obtained from two unrelated patients for each of the three variant spectrins. T50a results from cleavage at arginine 250 or lysine 252 of αIT80; a proline replaced the normal leucine or serine at residues 254 and 255, respectively. T50b and a 19-kD peptide result from cleavage at arginine 462 or arginine 464; a proline replaced the normal residue 465 (in T19b) in one of the two patients studied. T68 results from cleavage at arginine 131. In both 68-kD peptides examined, a leucine is inserted at residue 150. The relationship of the sequence changes to the new tryptic cleavages, to the current model of αI domain structure, and to defective spectrin self-association is discussed.

Introduction

Hereditary elliptocytosis (HE)[1] is an autosomal dominant disorder of erythrocytes in which clinical expression ranges from mild ovalocytosis on peripheral smear without evidence of hemolysis to striking elliptocytosis of all red cells, with laboratory evidence of hemolysis. Some affected individuals have erythrocytes which sphere, bud, and fragment, resulting in severe hemolytic anemia which has been termed hereditary pyropoikilocytosis (HPP).

HE has long been considered to be a disorder of the erythrocyte membrane based on the typical shape changes in the absence of an abnormal hemoglobin or altered enzyme content. The first evidence supporting this concept came from Zarkowsky et al. (1) who showed that HPP red cells fragment at 46°C instead of the normal fragmentation temperature of 49°C, and that thermal denaturation of spectrin extracted from the same cells is similarly shifted to lower temperatures (2). More recently, variant spectrins have been described in HE based on changes observed on two-dimensional (isoelectric focusing/SDS polyacrylamide gel electrophoresis [PAGE]) maps of intermediate sized tryptic peptides (3–8). These two-dimensional maps contain over 50 peptides with molecular weights ranging from 80,000 to 11,000 D, from which a chemical domain structure has been deduced from monoclonal immunoblots, I^{125} limit peptide mapping, and overlap chemical cleavages (9–11). The resulting model consists of five domains within the α-subunit and four domains within the β-subunit. The αI N-terminal domain is an 80,000-D peptide (αIT80), which is resistant to further tryptic cleavage under the mild conditions used. In contrast, spectrin from a subset of kindreds with HE has been shown to contain an unstable αI domain that undergoes tryptic cleavage under the same conditions to a ~ 46–50-kD peptide (3, 4, 8). Other kindreds with HE have been described in which the αIT80 is cleaved to a 65–68-kD fragment under the same conditions of mild tryptic digestion (5, 6, 12).

We have recently observed a third type of unstable αI domain in spectrin from affected members of four kindreds with HE (7). In these spectrins, αIT80 is cleaved by mild tryptic digestion to a fragment at ~ 50 kD that is distinguishable from the 46–50-kD peptide mentioned above by its more basic isoelectric point.

We present below the purification and partial amino acid sequence of peptides from each of the three unstable αI domains recognized to date in spectrin from HE erythrocytes, which we term αIT50a, αIT50b, and αIT68.

All three variant spectrins have impaired ability to undergo self association to form tetramers and higher oligomers, a process that is known to be dependent upon an intact αI domain, and one which is thought to be important in formation of the spectrin actin 4.1 network on the inner surface of the red cell membrane.

Methods

Blood collection, preparation of red cell ghosts and extraction of spectrin. Blood was collected by venipuncture into anticoagulant citrate dextrose (ACD); informed consent was obtained from all donors and the quantity of blood drawn was appropriate to the estimated blood volume and hematocrit of the subject as approved by the Yale Medical School Human Investigations Committee. Red cell ghosts were prepared by lysis of washed red cells for 30 min at 2°C in 15–20 vol of 5 mM sodium phosphate and 1 mM EDTA, pH 8.0, containing 0.1 mM DFP (diisopropyl fluorophosphate, Aldrich Chemical Co., Milwaukee, WI) followed by three washes with the same buffer. Spectrin was extracted by 48 h dialysis of ghosts at 4°C into 0.1 mM EDTA, pH 9.4, containing 130 μM PMSF (phenylmethylsulfonyl fluoride) and 0.1 mM DFP. The vesiculated ghosts

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1. *Abbreviations used in this paper:* ACD, anticoagulant citrate dextrose; DFP, diisopropyl fluorophosphate; IAEDANS, *N*-iodoacetyl-*N'*(5-sulfo-1-naphthyl) ethylene diamine; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl-sulfonyl fluoride; TPCK, *N*-tosyl phenylalanine chloromethyl ketone.

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were removed from the spectrin extract by two centrifugations at 48,000 *g* for 60 min at 4°C. The spectrin extract was then dialyzed into buffer containing 10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 30 μM PMSF at pH 7.4, and stored on ice.

Tryptic digestion of spectrin. Aliquots of spectrin extract were dialyzed against 20 mM Tris-HCl pH 8.0, and 1 mM β-mercaptoethanol for 20 h at 4°C. Trypsin (TPCK [*N*-tosyl phenylalanine chloromethyl ketone] treated, 206 U/mg; Cooper Biomedical, Malvern, PA) was added at an enzyme/substrate ratio of 1:20 wt/wt. The reaction was carried out for 90–180 min at 2°C and terminated by adding DFP to a concentration of 1 mM and cooling to −80°C. The digests were lyophilized and stored at −20°C before electrophoresis.

PAGE. Spectrin tryptic digests (200 μg) were solubilized in 75 μl of a solution containing 9 M urea, 2% Triton X-100, 5% β-mercaptoethanol and 2.4% ampholines (LKB Instruments, Inc., Rockville, MD) and focused on 3 × 125 mm polyacrylamide gels according to O'Farrell (13) for 5700 Vh. The pH gradient extended from 7.7 to 4.5 and was formed as previously described (3). After electrophoresis, the gels were equilibrated for 10 min in 10% glycerol, 3% SDS, 1 mM EDTA and 2% β-mercaptoethanol and then frozen at −60°C before electrophoresis in the second dimension on 10–15% acrylamide gradients using Laemmli buffers (14).

Analysis of spectrin oligomers. Formation of spectrin oligomers was studied by electrophoresis of extracted spectrin on 2–4% gradient nondenaturing polyacrylamide slab gels at 4°C. The gels were electrophoresed at 50 V for 48 h in buffer containing 40 mM Tris, 20 mM Na acetate, 2 mM EDTA pH 7.4 (15). Spectrin extracts prepared at 4°C as described above were concentrated up to 16 mg/ml by vacuum dialysis against 10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM β-mercaptoethanol, pH 7.4. Serial dilutions of spectrin in the same buffer were incubated for 3 h at 30°C prior to electrophoresis.

Quantitation of spectrin. Spectrin oligomers from nondenaturing gels and tryptic peptides from Coomassie Blue stained acrylamide gels were quantitated by elution of excised gel slices with 25% pyridine for 24–48 hrs until the gel slice was colorless. Optical density of the eluates at 590 nm was measured in a DMS 90 (Varian Associates, Inc., Palo Alto, CA) spectrophotometer. Mole fractions of peptides were calculated with appropriate correction for molecular weight and for the proportion of αIT80 degraded to αIT74.

I^{125} peptide maps. Two-dimensional maps of ^{125}I-labeled limit chymotryptic peptides were prepared from intermediate sized tryptic peptides cut from two-dimensional polyacrylamide gels. Na ^{125}I (carrier free, 350–600 mCi/ml) was obtained from Amersham Corp. (Arlington Heights, IL). Labeling of excised gel bands was performed by the method of Elder (16) with modifications (9, 10). Digestion with chymotrypsin and two-dimensional cellulose peptide maps were prepared as described (9, 10).

Preparation of rabbit anti-αIT80 and immunoaffinity purification of αI domain peptides. Anti-αIT80 polyclonal antibodies were raised in rabbits using monoclonal immunoaffinity purified αIT80 from normal human erythrocyte spectrin (17). Antibody titers of serum collected over the ensuing 4 mo were between 1:2,000 and 1:10,000 as measured by an ELISA method using Linbro microtiter plates (Flow Laboratories, McLean, VA) with spectrin as antigen. Anti-αIT80 immunoglobulin was purified from immune serum on a spectrin immunoaffinity column and the purified antibody was coupled to Staph A-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) by the method of Schneider et al. (18).

Lyophilized tryptic digests (see above) of normal and HE spectrin were solubilized in 100 mM Tris HCl, 300 mM NaCl, 1 mM β-mercaptoethanol, 0.05% Triton X-100, 1.5 M urea pH 7.4, and applied to 3–5 ml of rabbit anti-αIT80 immunoaffinity matrix. The column was washed before elution with the same buffer without urea or Triton X-100 and the bound peptides were eluted with 6 M urea in 100 mM Tris, 300 mM NaCl, pH 7.4. The bound fraction was collected and dialyzed against 10 mM Tris, 1 mM β-mercaptoethanol, 0.1 mM DFP pH 7.5 and lyophilized.

Immunoblots of two-dimensional acrylamide gels were made according to Towbin et al. (19) using monoclonal anti-αIT80 IID2 (11) and rabbit polyclonal anti-αIT80 described above.

Amino acid sequencing. αI domain peptides were purified as described above, labeled with IAEDANS (*N*-iodoacetyl-*N'* [5-sulfo-1-naphthyl] ethylene diamine) reagent (20), and electrophoresed in two dimensions. The fluorescent peptides were electroeluted into 5 mM NH_4HCO_3, 0.5 mM EDTA, 0.5 mM DFP, 0.1% β-mercaptoethanol; 0.05% SDS pH 8.2. After precipitation of SDS at 0°C, the peptides were dialyzed extensively against Milli-Q water before sequence analysis. The amount of peptide used for sequencing ranged from 400 to 800 pmol. Details of sequencing methods are as previously published (17, 21).

Results

Tryptic peptides of normal and HE spectrin. Fig. 1 *A* is a two-dimensional map of intermediate sized tryptic peptides of normal spectrin after digestion for 90 min at 0°–2°C at a 1:20 (wt/wt) enzyme to substrate ratio. In normal spectrin, (Fig. 1 *A*), the 80,000-D N-terminal domain of the α-subunit (αIT80) is remarkably stable to further cleavage under the mild digestion conditions used; the only significant cleavage is near the N-terminus at arginine 39 producing a 74-kD peptide (αIT74) at the same isoelectric point (21). Under the conditions stated, this cleavage occurs to varying degrees in normal and HE spectrins and may vary in sequential digests of a single spectrin sample. We have not observed any relationship between the presence or amount of αIT74 and elliptocytosis in the families we have studied, contrary to the results of Lawler et al. (4, 22).

Figs. 1 *B–D* show similar tryptic digests of spectrin from affected members of three kindreds with HE. In each digest, αIT80 is moderately reduced in amount, and a new peptide is present at ~ 50 kD (Fig. 1 *B* and *C*) or at ~ 68 kD (Fig. 1 *D*). The 50-kD peptides are distinguishable by their isoelectric points and are termed T50a (Fig. 1 *B*) and T50b (Fig. 1 *C*). (T50a is called Sp $\alpha^{1/46}$, and T68 is called Sp $\alpha^{1/65}$ in the terminology of Lawler et al. [4, 5].) In addition, Fig. 1 *B* and *D* show polymorphisms in the αII and αIII domains that occur commonly in Black subjects (8, 23). Other differences between the peptide maps are due to variability in the rates of tryptic cleavage of the remaining α- and β-spectrin domains inherent in the experimental system.

Peptides T50a, T50b, and T68 were shown to be derived from the αI domain by I^{125}-labeled limit chymotryptic peptide mapping shown in Fig. 2. The drawing at the right shows the major chymotryptic peptides of αIT80 and indicates which of these are found in T50a, T50b, and T68. While the maps of T50a, T50b, and T68 are all subsets of the parent αIT80, T50a and T50b do not overlap extensively. This suggested that the two 50-kD peptides are derived from opposite ends of the 80-kD domain, an impression that was confirmed by the similarity of maps of T50b to those of the 30- and 21-kD peptides (not shown), discussed further, which accompany the cleavage of αIT80 to T50a. The map of αIT68 includes three prominent peptides (left side) that upon direct comparison are present, but are less intense, on the other maps.

Tryptic digests of normal and HE spectrins were immunoblotted using anti-αI monoclonal antibody previously described (11) (Fig. 3). The monoclonal antibody recognizes αIT80, T50a, and T68, but not T50b, consistent with conclusions regarding the locations of the 50-kD peptides derived from the ^{125}I cellulose maps shown in Fig. 2.

Our laboratory has identified to date 12 affected members of 5 kindreds with HE in which spectrin αIT80 is cleaved to T50a (αIT80 → T50a), 7 members of three kindreds with spectrin αIT80 → T50b and 7 members of four kindreds with spec-

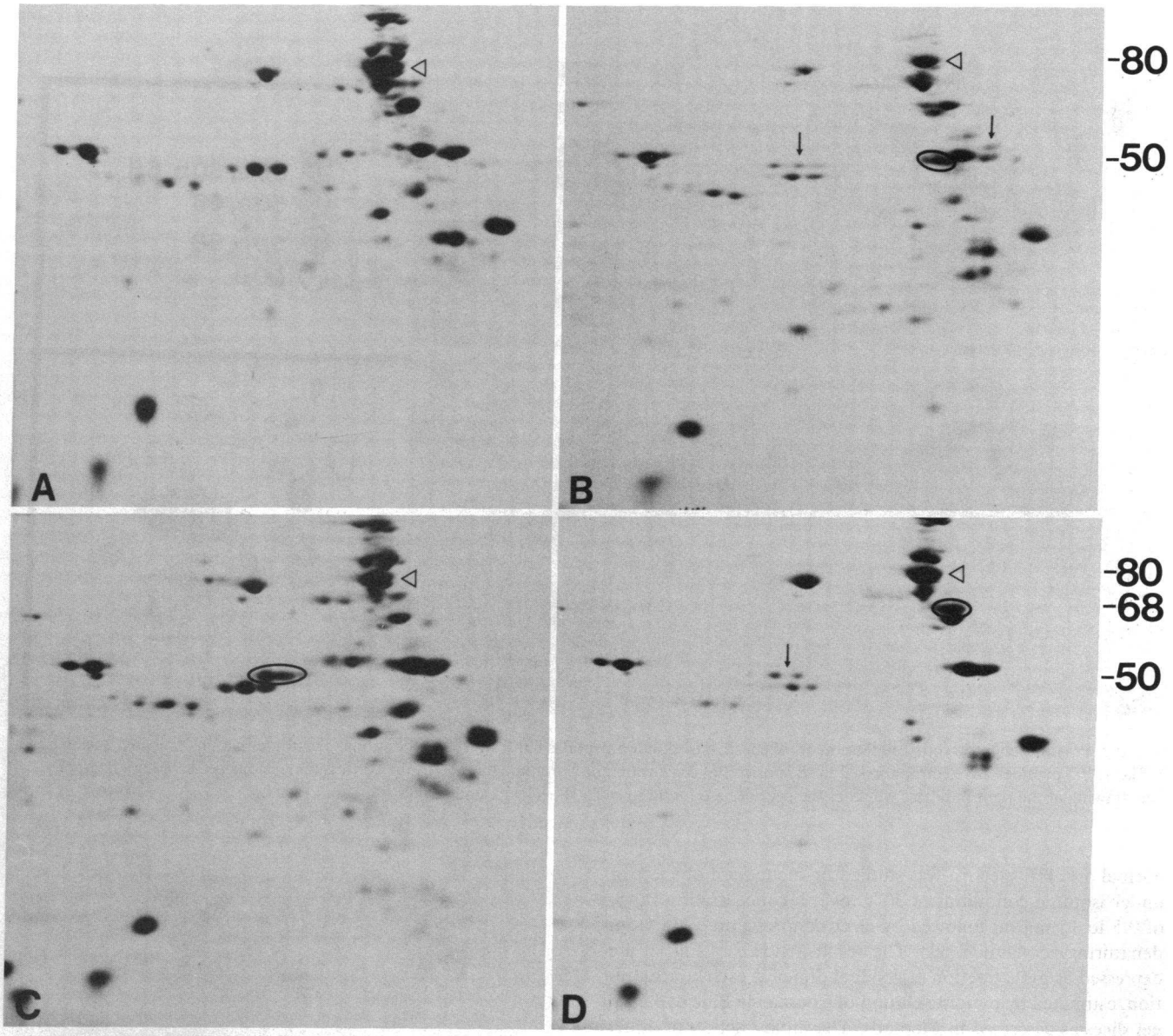

Figure 1. Intermediate tryptic digests of normal and HE spectrin. 200 μg of spectrin in 20 mm Tris, HCl pH 8.0 was digested with trypsin at 1:20 w/w ratio at 2°C for 90 min, electrophoresed in two dimensions (IEF/SDS PAGE), and stained with Coomassie Blue. The numbers on the right indicate apparent molecular weight (kD). (*A*) Normal spectrin. (*B–D*) Spectrin from 3 unrelated individuals with HE. The parent αI domain (αIT80) is marked in each digest by an open triangle. New peptides in *B* (T50a), *C* (T50b), and *D* (T68) are circled. The arrows in *B* and *D* point to polymorphic αII and αIII domains (23).

trin αIT80 → T68. All of the affected members of these families have unstable αI domains while 14 members of the 12 families with normal appearing erythrocytes have normal spectrin digests. Hematologic expression in affected individuals ranges from mild elliptocytosis without hemolysis to marked elliptocytosis with well-compensated hemolysis, and further to marked fragmentation and severe hemolytic anemic (HPP). All families are Black except for one Puerto Rican family with αIT80 → T50a. Four of the kindreds with spectrin αIT80 → T50a were previously reported in references (3) and (8). We have also studied five Caucasian families and one Black family with HE whose spectrin tryptic digests appear normal.

While spectrins of two siblings with HPP previously reported (3) showed complete cleavage of αIT80 to T50a, tryptic digests of the remaining 24 variant spectrins contained residual (35–70%) intact αIT80 measured by pyridine elution of Coomassie Blue dye from peptides excised from two-dimensional gels. Extending the duration of tryptic digestion to 24 h, at which time normal αIT80 begins to decline, did not change significantly the fraction of unstable αIT80 in each spectrin sample. Thus, all affected individuals but the siblings mentioned above (3) appear to be heterozygous for the abnormal αI domain.

Spectrin self-association. We and others have previously demonstrated that spectrin dimer-dimer interaction is impaired in spectrins with unstable αI domains of the types αIT80 → T50a (3, 4, 8, 24) and αIT80 → (5, 6). Oligomer formation by

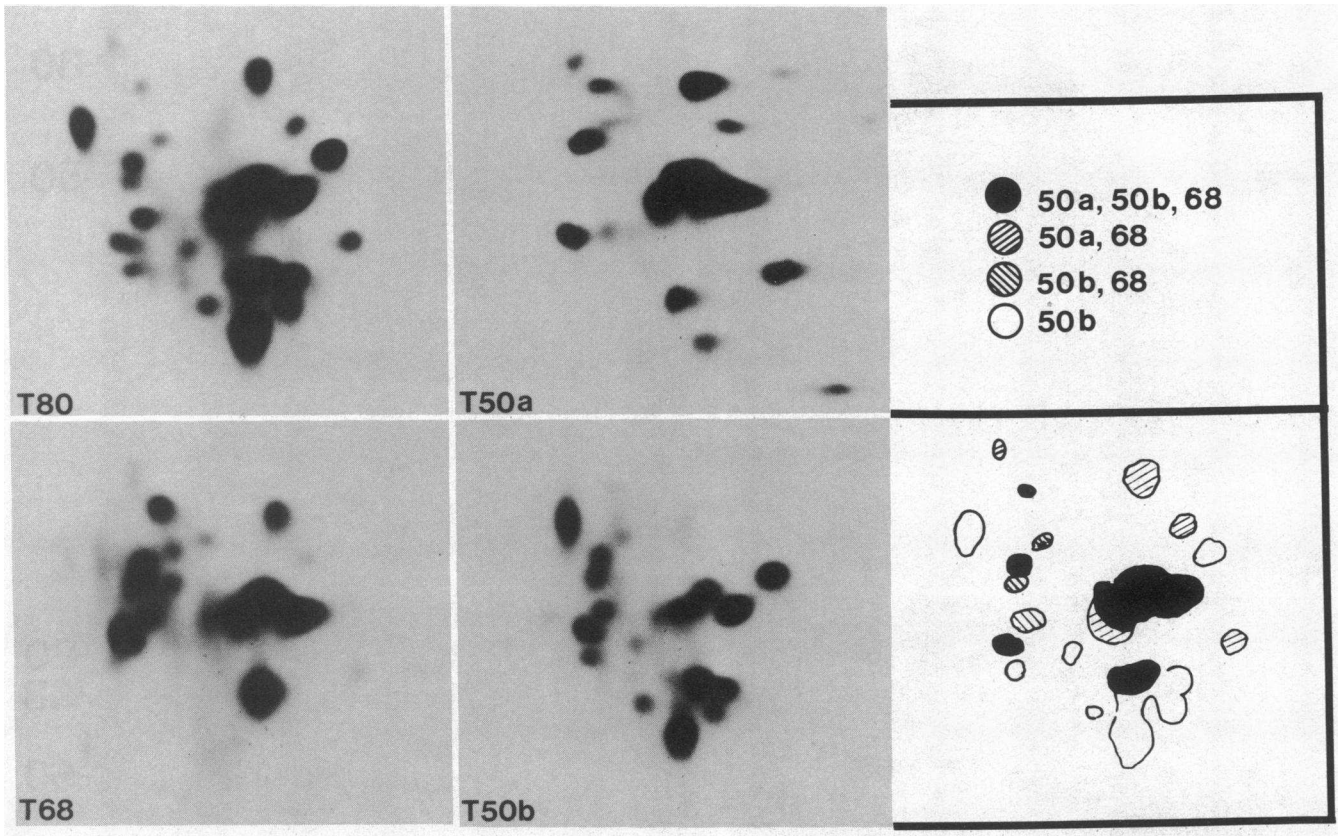

Figure 2. I^{125} limit chymotryptic cellulose maps of spectrin αI domain peptides cut from two-dimensional electropherograms prepared as described in Fig. 1. ^{125}I peptides are electrophoresed in the horizontal dimension and chromatographed in the vertical dimensions before autoradiography. The drawing at the right indicates which of the peptides are found in αIT50a, αIT50b, and αIT68.

normal and HE spectrin was studied by incubation of spectrin under isotonic conditions at 30°C over a concentration range of 0.5 to 15 mg/ml followed by electrophoresis on 2–4% non-denaturing acrylamide gels. The results, shown in Fig. 4, are expressed as mole fraction of residual dimer at each concentration, estimated by pyridine elution of Coomassie Blue dye from gel slices as described in Methods. The mole fraction of dimer from normal erythrocytes (controls and normal family members) is seen to fall from 0.5 to < 0.1 over this concentration range as tetramer and higher oligomers are formed. In contrast, dimer content is elevated at low concentrations in all HE and HPP spectrins with unstable αI domains and, while some oligomerization occurs as concentration increases, dimer content remains elevated at all concentrations. In three patients with HPP and αIT80 → T50a described earlier (3, 8) oligomer formation was essentially absent.

Isolation of αI peptides from HE spectrins. To purify and isolate all peptides from the unstable αI domains, a polyclonal antibody was raised in rabbits to normal αIT80 and purified on a spectrin immunoaffinity column (see Methods). The affinity purified rabbit anti-αIT80 was coupled to Staph A-Sepharose, which was then used to isolate αI domain peptides derived from normal and HE spectrin digests.

Fig. 5 shows two-dimensional (isoelectric focusing, IEF/SDS) electropherograms of purified αI peptides from normal spectrin and HE spectrins αIT80 → T50a, αIT80 → T50b, and αIT80 → T68. The normal αI domain (Fig. 5 *A*) is represented by the 80- (and 74-)kD peptides and several faint degradation

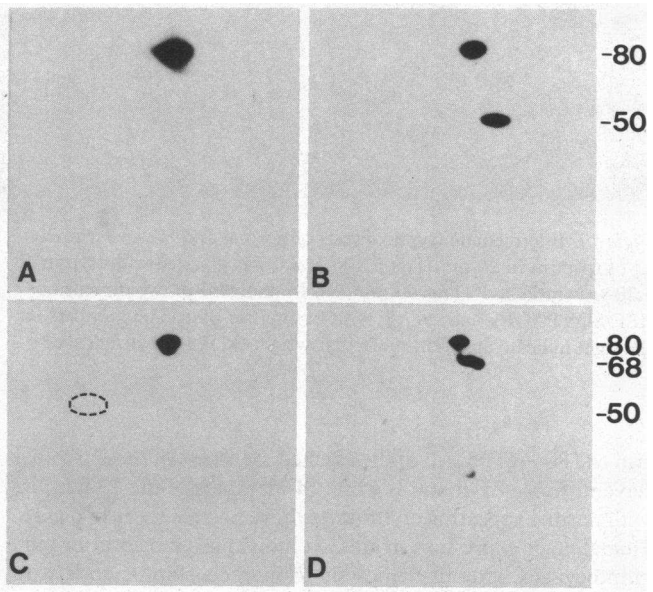

Figure 3. Monoclonal immunoblots of limited tryptic digests of normal and HE spectrin. Tryptic digests were electrophoresed in two dimensions as in Fig. 2 and immunoblotted using the anti-αI domain monoclonal antibody 2DII (11). (*A*) Normal spectrin. (*B–D*), spectrin from unrelated patients with HE and αIT80 → T50a (*B*) αIT80 → T50b (*C*) and αIT80 → T68 (*D*). The dotted circle in (*C*) shows expected location of T50b. The faint circles on the lower part of the blots are artifacts.

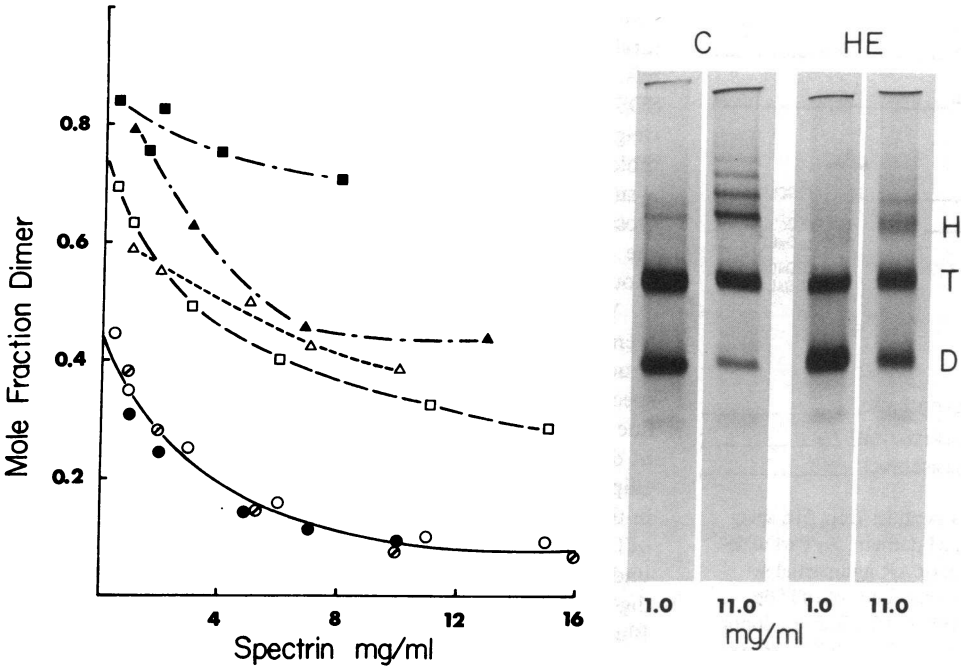

Figure 4. Concentration dependent oligomer formation by normal and HE spectrin dimers. Mole fraction of spectrin dimer is shown as a function of spectrin concentration estimated by dye elution from nondenaturing gels. Normal spectrin (○ ● ○). Child with HPP (■) and his mother (▲) with HE who have spectrin αIT80 → T50a; (△) woman with αIT80 → T68 and HPP; (□) man with αIT80 → T50b and HE. The 2–4% nondenaturing acrylamide gels shown on the right were those used to obtain plotted data for control and HE spectrin designated (●) and (□), respectively. D: dimer, T: tetramer, and H: hexamer.

products of lower molecular weight. Fig. 5 *B* shows purified αI peptides from spectrin αIT80 → T50a, which include 30- and 21-kD peptides in addition to the prominent αIT50a. I^{125} cellulose maps of the 30-kD peptide do not overlap the map of the 50-kD peptide and include all of the 21-kD peptide (data not shown). The αI domain peptides in Fig. 5 *C* are T50b and two peptides at ~ 19 and 17 kD, as well as intact αIT80. A peptide of ~ 55 kD not appreciated in the digests of whole spectrin of this type is also present and probably represents a precursor of T50b as deduced from the amino acid sequence results described below. Fig. 5 *D* shows αIT80 and T68.

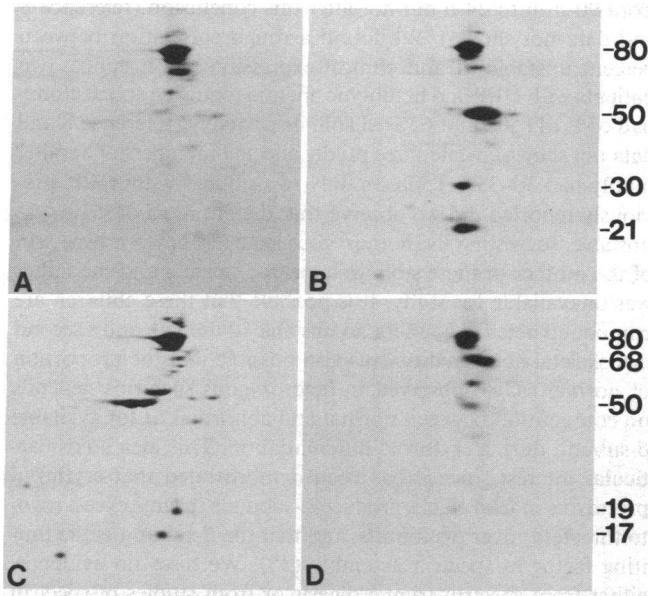

Figure 5. Two-dimensional (IEF/SDS PAGE) electrophoresis of affinity purified spectrin αI peptides from normal (A) and HE spectrins type αIT80 → T50a (B), αIT80 → T50b (C) and αIT80 → T68 (D). The gels are stained with Coomassie Blue.

Immunoaffinity purified αI peptides from HE spectrins were labeled with *N*-iodoacetyl-*N'*(5-sulfo-1-naphthyl) (IAEDANS), a fluorescent cysteine modifying reagent (20), and electrophoresed in two dimensions. The fluorescent αI peptides shown in Fig. 5 were cut from the gels, electroeluted, and, when necessary, were further purified by high performance liquid chromatography (HPLC) for amino acid sequencing. N-terminal sequence analysis was carried out on peptide samples ranging from 400 to 800 pmol and these sequences were compared with the previously determined sequence of the spectrin αI domain (21).

In Fig. 6 *A*, segments of the normal sequence of αIT80 are compared with N-terminal sequences of T50a, T30a, and T21a peptides from two unrelated individuals, AR and TS, both of whom have spectrin αIT80 → T50a. T30a and T21a from AR were found to originate at the N-terminus of αIT80; the N-terminal 20 amino acids of both peptides were identical to the normal sequence. T21a is presumably produced by cleavage of T30a near its C-terminus. The N-terminus of T50a from spectrin of AR is alanine (A) 253, tryptic cleavage having occurred at lysine (K) 252. In T50a from AR, a proline (P) replaces the normal serine (S) at residue 255. T50a from TS results from cleavage at arginine (R) 250 (the primary cleavage) and at lysine (K) 252 (secondary cleavage) producing an overlapping sequence that constitutes ~ 25% of the primary sequence. Both sequences show replacement of leucine (L) 254 by proline (P). Additional sequence of these peptides to residues 296 and 267, respectively, revealed no other differences from the normal sequence.

Fig. 6 *B* shows N-terminal sequence of peptides from HB and DF, two unrelated individuals with αIT80 → T50b. N-terminal sequence of T50b from patient HB establishes its location at the N-terminus of the αI domain; its position with respect to T50a, T30a, and T21a is consistent with the I^{125} mapping data discussed above. The first 25 residues of T50b from HB are identical to normal αIT80. The 19- and 17-kD peptides from HB spectrin which accompany digestion of αIT80 to T50b are produced by cleavage at arginine (R) 462 and thus have the same N-terminus. T17 is presumably derived from T19 by a

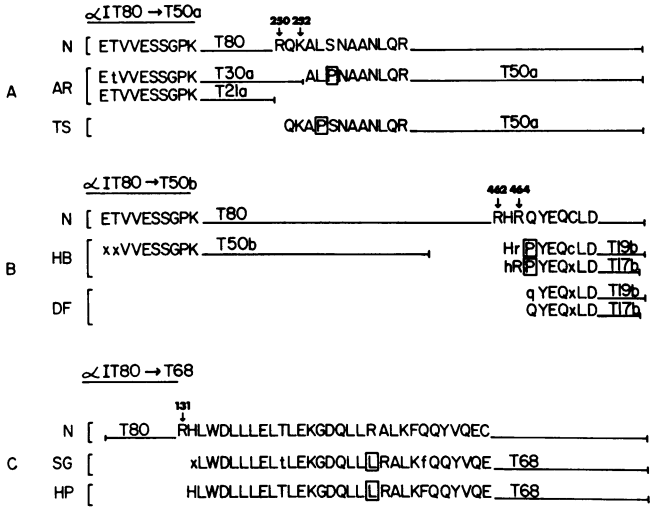

Figure 6. Partial amino acid sequence of αI peptides from HE spectrins: comparison with sequence of normal αI domain. A) Partial sequence of αIT50a, T30a and T21a from donor AR and partial sequence of αIT50a from donor TS. B) Partial sequence of αIT50b, T19b and T17b from donor HB, and of T19b and T17b from donor DF. C) Partial sequence of αIT68 from donors SG and HP. Arrows and numbers mark tryptic cleavage sites at lysine (K) or arginine (R) residues. Actual sequencing was continued to the residue number stated in the text. Unidentified residues are represented as x and tentatively identified residues are given in lower case letters. E:Glu, T:Thr, V:Val, S:Ser, G:Gly, P:Pro, K:Lys, R:Arg, Q:Gln, A:Ala, L:Leu, P:Pro, N:Asn, H:His, Y:Tyr, C:Cys, D:Asp, W:Try, F:Phe.

cleavage near its C-terminus. At residue 465 in both peptides, a proline replaces glutamine (Q) in the normal sequence. Sequence of an additional 18 and 11 residues, respectively, from the N-terminus of these two peptides otherwise shows no difference from normal spectrin.

The N-terminus of T19b and T17b from spectrin of patient DF is residue 465, tryptic cleavage having occurred at arginine 464. In contrast to spectrin of HB, the normal glutamine is present at residue 465 in both peptides and the following 28 and 21 residues, respectively, are normal.

Cleavage of αIT80 to T68 (Fig. 6 *C*) occurs at arginine 131 in spectrin from SG and HP, two unrelated patients with HE. The sequence of both 68-kD peptides is normal up to leucines (L) 149 and 150 at which point an additional leucine is inserted. Following the triple leucine at residues 149–151, sequence of both peptides is normal through residues 160 and 168, respectively.

Discussion

Hereditary elliptocytosis has repeatedly been described in the literature as a heterogeneous disorder, referring both to the variable red cell morphology and clinical expression of disease (25). The heterogeneity of HE is also apparent from genetic studies that show that HE is linked to genes for the Rh antigen located on chromosome one in some kindreds, while other kindreds do not show this linkage (26–28). It has also been suggested that there is a second locus for elliptocytosis linked to the Duffy (Fy) blood group antigens, also located on chromosome one (29). More recently, a variety of alterations in the structure and/or quantity of spectrin and of protein 4.1 in the cytoskeleton of HE red cells has been demonstrated. The alterations in spectrin structure that have been reported to date include the three structural variants of the αI domain described in detail here (and see refs. 3–8), an α-subunit with anomalous (rapid) migration on SDS (30), a shortened spectrin β-subunit (31), a high molecular weight β-subunit (32), deficiency of protein 4.1 (33), and a high molecular weight form of 4.1 (34). The genes for both the spectrin α-subunit and protein 4.1 have recently been assigned to chromosome 1 and further genetic linkage studies in association with the accumulating information on cytoskeletal abnormalities should prove of interest.

We have presented here data representative of 26 affected members of 12 kindreds with HE and HPP, all of whom have structural abnormalities within the N-terminal domain of the spectrin α-subunit (αIT80) recognized by instability of this peptide to mild proteolysis. The question arises as to whether the αI domain is indeed a specific site for mutations that result in elliptocytosis, or whether the frequency of mutations observed in the αI domain is an artifact of the technique used. Since the αIT80 domain is unusually resistant to further tryptic digestion under the mild conditions used, an increase in susceptibility to digestion is particularly easy to detect by standard Coomassie Blue staining of maps of intermediate tryptic peptides combined either with I^{125} cellulose mapping or immunoblotting. By contrast, altered digestion of a normally susceptible domain such as β4, which is cleaved under the same conditions to approximately 20 peptides, might be quite hard to detect.

Although HE is generally thought to be an autosomal dominant disease and most affected individuals to be heterozygotes, expression within an affected kindred in our experience may vary from mild HE to HPP in an unpredictable manner. We have asked the question whether the proportion of abnormal αIT80 in HE spectrins as determined by its susceptibility to mild tryptic digestion is an indication of abnormal gene dose and whether it correlates with the severity of clinical expression. Quantitation of αI peptides as described above revealed that 30–65% of αIT80 is unstable in most affected individuals, compatible with the heterozygous state; extension of the digestion period from 90 min to 24 h did not alter this conclusion (reference 8 and data not shown). We found a rough correlation between percent unstable αI and clinical expression; for instance, two patients with HPP and hemolytic anemia requiring splenectomy had 55% αIT50a and 65% αIT68, respectively (reference 8 and data not shown), while three mildly affected members of another family had 30–35% T50b. In only two siblings with 'HPP' previously reported did we observe that the entire αI domain was unstable, suggesting the homozygous state (3); however, spectrin of the mother of these siblings appeared normal and the father was unavailable for study. It is possible that these children are compound heterozygotes for an unstable αI domain and a second cytoskeletal abnormality. It is also possible that the proportion of normal αIT80 observed in heterozygous spectrins depends on competition between normal and abnormal αI for available β-subunit during erythroid differentiation. This idea holds particular interest since it has been demonstrated that erythroid precursors in fetal chicks produce α-subunits in large excess (two- to threefold) over β-subunits, and that the β-subunit is the limiting factor in spectrin assembly (35). We have no evidence, either from spectrin tryptic digests or from studies of spectrin self-association for an "asymptomatic carrier state" affecting expression of HE in the next generation, as suggested by other workers (36).

Impairment of dimer-dimer association to form higher

oligomers is characteristic of all of the HE spectrins with unstable αI domains described to date (3–6, 8, 22, 24). Since oligomer formation occurs as a function of concentration, we have measured and expressed spectrin self-association here as the proportion of dimer remaining as spectrin concentration increases. This seems appropriate, since the concentration of spectrin on the inner surface of the membrane has been calculated to be at least 200 mg/ml (15). Fig. 3 demonstrates that spectrins with unstable αI domains of all types have impaired oligomer formation over the entire range of concentrations tested. The most severe impairment of spectrin self-association that we have observed was in patients with HPP associated with αIT80 → T50a (3, 8).

The basis for the impairment in spectrin self-association is thought to be the requirement of intact αIT80 for this process, as shown by Morrow et al. (37, 38) who demonstrated that normal αIT80, but not its cleavage product αIT74, is able to bind to intact spectrin dimer and to inhibit tetramer and oligomer formation. Although the native oligomeric state of spectrin on the membrane is not known, the process of oligomer formation is thought to be important in the assembly and stability of the spectrin actin 4.1 lattice. The impairment of oligomer formation in HE spectrins with unstable αI domains seems likely to be at least one, if not the primary pathophysiologic result of the altered αI domain.

Three types of unstable αI domains are recognized in this report. All are characterized by their increased susceptibility to mild tryptic digestion, producing one of two 50-kD peptides (T50a and T50b) or a 68-kD peptide. It is important to note that the tryptic cleavage products of these three unstable αI domains are unique. That is, there is no common pathway of proteolysis that could result from nonspecific unfolding of the spectrin α-subunit. This suggests that the instability of each abnormal αI domain is the result of a specific event (mutation) resulting in a local conformational change that exposes a nearby arginine or lysine to tryptic cleavage.

N-terminal amino acid sequence data has been obtained on spectrin αI peptides from six patients with HE or HPP and unstable αI domains. The cleavage of αIT80 to T50a in spectrin of AR and TS occurs at lysine 252, as well as at arginine 250 in spectrin of TS; in each case, a proline is substituted close to the cleavage site, at serine 255 or leucine 254. Substitution of a proline at these sites is theoretically capable of disrupting helical structure sufficiently to allow exposure of lysine 252 and/or arginine 250 to tryptic cleavage. It is possible that in spectrins of αIT80 → T50a type, other nonconservative substitutions occur which could result in similar changes in conformation and instability to tryptic digestion.

Tryptic cleavage of αIT80 → αIT50b has not been previously reported. We have observed this cleavage in the spectrin of three kindreds and we report here sequence of the resulting peptides in members of two of these kindreds. The initial tryptic cleavage of αIT80 occurs at arginine 462 in the spectrin of HB and at arginine 464 in the spectrin of DF, producing the T19b peptides; a second clip near the C-terminus of both 19b peptides produces T17b. The large (55–60 kD) peptide produced by cleavage at arginine 462 or 464 is presumably clipped once or more near its C-terminus to produce the N-terminal 50b fragment. Substitution of proline for glutamine at residue 465 in spectrin of HB could, as discussed above for αIT80 → T50a, disrupt helical structure sufficiently to allow for the new cleavage at arginine 462. In spectrin from DF, cleavage occurs at arginine 464, and no amino acid substitution is detected. It is possible that in this

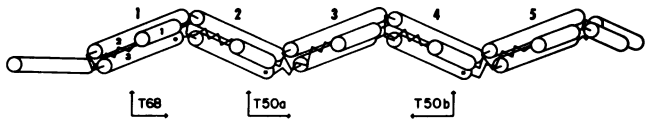

Figure 7. Model of spectrin αI domain (αIT80) according to Speicher (reference 40 and manuscript in progress). ▭: predicted helical segments, ⎯: nonhelical connectors and ⋀⋁: beta sheet. Arrows mark sites of tryptic cleavage and the locations of new tryptic peptides in HE spectrins; asterisks mark sites of amino acid substitution or insertion described in the text for spectrins αIT80 → T68, T50a, and T50b.

case, substitution of a proline or other nonconservative substitution has occurred at residue 463, preventing or limiting cleavage at arginine 462 (39) but allowing cleavage at arginine 464. There are of course other possible explanations for the instability of DF spectrin at residue 464 but the one offered has an interesting parallel in the sequences of the two T50a peptides described above.

The 68-kD peptides from the αI domain of subjects SG and HP result from cleavage at arginine 131. In both peptides, the double leucine at residues 148 and 149 becomes a triple leucine. While the inserted leucine is some distance from the cleavage site, the insertion disturbs the strictly conserved 106 amino acid repeat unit (40), and thus may perturb tertiary structure of the entire helix sufficiently to expose arginine 131 to tryptic cleavage. It is of interest that inside out membrane vesicles from patient HP were previously found to have decreased high affinity binding sites for ankyrin (41). It is not clear how this finding is related to the patient's mutant spectrin, but it is possible that the altered cytoskeleton resulted in rearrangement of the band 3 receptor site in these red cells.

Fig. 7 locates the new tryptic peptides and the amino acid sequence changes described in HE spectrins on a model of the αI domain developed by Speicher (ref. 40 and manuscript in preparation). In this model, the αI domain is comprised of five complete repeat units, each consisting of 106 amino acids, and parts of a sixth repeat unit at each end. Each repeat unit is comprised of three helical segments of different lengths connected by nonhelical segments. The figure shows that the major new tryptic cleavages (*arrows*) described in HE spectrins occur within helix 3 of repeat units 1, 2, and 4. The sites of amino acid replacement or insertion described in HE spectrin αI domains in Fig. 6 are indicated by asterisks. In this model, all of these occur near the C-terminus of helix 3 within their respective repeat units. It is interesting that the cleavage site of αIT80 to T74 at Arg 39 (21) is also within helix 3 of the partial repeat unit shown at the N-terminus of the αI domain in this model (40). This cleavage occurs commonly in normal spectrin in our experience, but has been associated with elliptocytosis in the experience of others (4, 22). We conclude that there are at least three variable regions within the αI domain, all near the C-terminus of helix 3 in this model. The manner in which the sequence changes in these variable regions impair spectrin self-association, and their relationship to the abnormal shape and survival of the elliptocyte remain to be determined.

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