Spectrin β-Chain Variant Associated with Hereditary Elliptocytosis

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ABSTRACT An electrophoretically fast-moving variant of the spectrin β-chain was discovered in the erythrocyte membranes of a woman and her father who both exhibited elliptocytosis and mild hemolytic anemia. This abnormal β′-subunit (M_r = 214,000) coexisted with a decreased normal β-chain and represented about half of the total β-chains in the membrane. In contrast to the spectrin β-chain, the β′-chain was phosphorylated neither in the membrane by endogenous protein kinases nor in solution by pure membrane casein kinase whether or not the spectrin was dephosphorylated by erythrocyte cytosolic spectrin phosphatase.

The presence of the β′-chain was associated with a defective self-association of spectrin dimer to form tetramer as manifested by: (*a*) an excess of spectrin dimer in the 4°C spectrin crude extract, (*b*) a defective self-association of the spectrin dimer in the 37°C crude spectrin extracts.

Gel electrophoretic analysis of the tetramer and dimer species isolated from the proband's 4°C extract showed that the tetramer contained trace amounts of the β′-chain, whereas in contrast, a large proportion of β′-chain was present in the dimer. These results demonstrated the responsibility of the β′-chain for the defective reassociation of spectrin dimer into tetramer.

The study of this abnormal spectrin confirms the participation of spectrin β-chain in dimer-dimer association and strongly suggests that the phosphorylation sites of the normal β-chain are located at the end of the molecule involved in the dimer-dimer interactions.

INTRODUCTION

Hereditary elliptocytosis (HE)[1] is a genetic disorder characterized by the presence of elliptically shaped erythrocytes in peripheral blood. The erythrocyte shape is largely determined by a submembranous network of proteins, named the "membrane skeleton," which laminates the internal side of the membrane. The membrane skeleton is experimentally defined as the structure remaining after extraction of ghosts with Triton X-100 (1) and is mainly composed of spectrin, actin, and protein band 4.1. Spectrin, the major component, is a heterodimer composed of two subunits, α (M_r = 240,000) and β (M_r = 220,000). Several studies have shown that the spectrin dimer in the membrane is associated head-to-head to form tetramers that are linked into a two-dimensional network by actin oligomers and protein band 4.1 (2–11). In HE, elliptocytic shape is related to a membrane skeleton abnormality because elliptocytes are converted to elliptocytic ghosts or elliptocytic membrane skeletons (12). According to recent works, HE now appears to be a heterogeneous disorder associated with different molecular abnormalities of membrane proteins, mainly spectrin and protein band 4.1. The absence of protein band 4.1 in HE has been so far reported in three different families (13–16) and Tchernia et al. (14) recently developed arguments for a close relationship between the lack of protein band 4.1 and the erythrocyte membrane instability. A spectrin molecular abnormality was suspected in some cases of HE, because spectrin had an increased susceptibility to thermal denaturation (12)

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[1] *Abbreviations used in this paper*: HE, hereditary elliptocytosis; K_a, association constant; PMSF, phenylmethylsulfonylfluoride.

and its reassociation from dimer to tetramer was defective (17, 18). Coetzer and Zail (19) found that spectrin was more susceptible to tryptic digestion in some cases of HE, and Liu et al. (20) recently showed that partial tryptic digestion of spectrin in some variants of HE (type I) resulted in the increase of a 73,000-dalton component and in the concomitant decrease of an 80,000-dalton peptide that has been reported to represent the portion of the α-chain involved in spectrin tetramer formation (21).

In other cases of HE the primary defect could be due to a decreased number of ankyrin binding sites to protein band 3 leading to a defective anchorage of the skeleton to the integral proteins and lipid bilayer, which would account for the membrane instability and the hemolysis (22).

We report herein the first shortened spectrin β-chain variant (M_r = 214,000) associated with HE and characterized by the lack of a peptide bearing the phosphorylatable site of the chain and responsible for a defective self-association of the spectrin dimer.

METHODS

Case report

Elliptocytosis (80%) was discovered in 1978 in a White female born in 1952 who displayed chronic asthenia, frequent headaches, and splenomegaly. Her father, who is elliptocytic (see below) and her father's ancestors are from the "Département du Cantal" in the central part of France. Her mother and her mother's ancestors are from the "Département de la Lozère," a nearby department in the same region. The proband is unaware of jaundice in the neonatal period. At the time of elliptocytosis discovery, hematological parameters were as follows: hemoglobin, 11.6 g/100 ml; mean cell volume, 90 μm^3, mean cell hemoglobin concentration, 35.6 g/100 ml; reticulocytes, 163,000/mm^3; nonconjugated bilirubin 2.0 mg/100 ml. Radioisotopic analysis revealed an important shortening of the erythrocyte survival: $t_{1/2}$ Cr^{51} = 11 d (normal, 24–34 d) and splenic sequestration. Osmotic fragility was normal: initial hemolysis, 0.55% sodium chloride (controls, 0.43–0.52% sodium chloride); total hemolysis, 0.25% sodium chloride (controls, 0.25–0.31% sodium chloride); 50% hemolysis, 0.44% sodium chloride (controls, 0.37–0.43% sodium chloride). Hemoglobin analysis and erythrocyte enzymes G6PD and PK were normal. Splenectomy, which was carried out soon after discovery of the disease, showed an intrasplenic hematoma and greatly improved the clinical and hematological conditions of the patient (hemoglobin 13 g/100 ml, mean cell volume 78 μm^3, mean cell hemoglobin concentration 33.7 g/100 ml, reticulocytes 54,000 mm^3). The proband inherited elliptocytosis from her father born in 1920, in whom the condition is clinically silent (hemoglobin, 14.5 g/100 ml; mean cell volume, 89 μm^3; reticulocytes 150,000/mm^3). The proband's mother has normal erythrocyte shape and parameters (hemoglobin 14.5 g/100 ml mean cell volume 91 μm^3; mean cell hemoglobin concentration 33 g/100 ml). The sister, the two brothers, and one paternal aunt of the proband have normal hematological values without elliptocytosis.

Materials

Phenyl methyl sulfonyl fluoride (PMSF) was from Sigma. Acrylamide, ammonium persulfate, TEMED, sodium dodecyl sulfate (SDS), and Coomassie Brilliant Blue were from Bio-Rad Laboratories, Richmond, CA. $^{32}PO_4$ (1 mCi/ml) as Orthophosphate carrier-free, in dilute HCl and [γ^{32}P]ATP (1 mCi/ml, 3,000 Ci/mmol) were purchased from the Amersham International, Buckinghamshire, England, Sepharose CL4B was from Pharmacia Fine Chemicals, Uppsala 1, Sweden.

Methods

Blood samples were collected on heparin and used within 24 h. Morphology of the erythrocytes was studied on blood films stained with May Grunwald Giemsa.

Erythrocyte thermal stability study. Erythrocytes were washed in phosphate-buffered saline, pH 7.4, 290 mosmol, and resuspended in the same buffer; the cells (1% suspension) were incubated at various temperatures between 41 and 49°C in a temperature-controlled (±0.1°C) water bath. Cells were held at the selected temperature for up to 30 min and fixed immediately in an equal volume of 1% glutaraldehyde in phosphate-buffered saline. Morphology of fixed samples was determined by phase-contrast light microscopy.

Preparation of the erythrocyte membranes. Blood samples were centrifuged and the buffy coat was removed. Erythrocytes were washed thrice in 5 mM $NaPO_4$, 150 mM NaCl, pH 8.0. Ghosts were prepared according to Dodge et al. (23) except that 0.1 mM PMSF was added in the lysis buffer.

Polyacrylamide gel electrophoresis. Disk SDS-polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (24) with 0.1% SDS. Slab gel electrophoresis was performed according to Laemmli (25) using a 5–15% polyacrylamide gradient gel. Gels were stained by Coomassie Blue (24) and dried under vacuum with a Bio-rad apparatus. Autoradiographies were done by exposing the dried gel to a Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) with a Cronex lighting-plus intensifying screen (DuPont Instruments, Wilmington, DE) at −70°C in an x-ray exposure holder. Stained gels and autoradiograms were scanned at 550 and 750 nm, respectively, in a DU8 Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). M_r value of the abnormal spectrin β-chain was estimated by measuring its relative mobility and comparing it with a calibration plot of log M_r vs. relative mobility, established with the following proteins: spectrin (240,000 and 220,000), protein band 4.1 (80,000), actin (43,000) and glyceraldehyde-3-phosphate dehydrogenase (34,000).

Phosphorylation of the intact erythrocytes. Erythrocytes were washed three times in cold 5 mM $NaPO_4$ pH 7.4, 150 mM NaCl. Washed erythrocytes were mixed with a solution of equal volume containing 20 mM $NaPO_4$ pH 7.4, 130 mM NaCl, 5 mM glucose, and 100 μCi carrier-free $^{32}PO_4$ added for each ml of suspension. After a 4-h incubation time at 37°C the erythrocytes were washed in cold 5 mM $NaPO_4$, pH 7.4, 150 mM NaCl. Then the ghosts were prepared according to Dodge (23).

Phosphorylation of membrane proteins. Ghosts (23) were washed twice in 5 mM Tris–HCl, 1 mM EDTA pH 7.5. Autophosphorylation of membrane proteins was done with γ[^{32}P]ATP according to Guthrow et al. (26) with and without cyclic AMP. Incubations for phosphorylation of 100 μg membrane protein were carried out for 10 min at 30°C in 200

μl of a medium containing 50 mM acetate buffer pH 6.5, 10 mM magnesium acetate, 0.3 mM EGTA, 2.5 μM γ[^{32}P]ATP and when necessary 5 μM cyclic AMP. Membrane proteins were estimated spectrophotometrically at 280 nm in 1% (wt/vol) SDS assuming an $E^{1\%}_{1\,cm}$ (280 nm) of 10.0. For an overall phosphorylation assay, the reaction was stopped by addition of 4 ml of 7.5% trichloracetic acid and 0.2 ml of bovine serum albumin (6.3 mg/ml) was added as a carrier protein. After centrifugation the precipitate was dissolved in 0.2 ml of 1 N NaOH and the same procedure was repeated twice. Finally, 10 ml of Dimilume scintillation liquid (Packard Instrument Co., Inc., Downers Grove, IL) was added and radioactivity was counted in a ABAC SL 3000 Intertechnique scintillation counter (Roche Bioelectronic Kontron, Montigny le Bretonneux, France). Overall phosphorylation was expressed as picomoles of radiophosphorus transferred from ATP to 1 mg of membrane proteins per 10 min. For electrophoresis, the reaction was stopped by adding a stock solution giving final concentrations of: 2% SDS, 5% β-mercaptoethanol, and 10% sucrose. The samples were then boiled for 5 min.

Phosphorylation of purified spectrin dimer by purified membranous casein kinase of human erythrocyte was performed as previously described (27). When necessary, dephosphorylation of spectrin dimer prior to its phosphorylation was done by incubating the protein with human cytosolic erythrocyte phosphatase (28) during 1 h at 37°C in 20 mM Imidazol buffer pH 7.4, 100 mM KCl, 4 mM β-mercaptoethanol.

Gel filtration. Gel filtration of membrane extracts prepared by incubation of ghosts at low ionic strength at 4°C was carried out as follows: ghosts were washed twice in 0.3 mM $NaPO_4$, 0.3 mM PMSF, pH 7.6, dialyzed at 4°C for 18 h against the same buffer, and centrifuged at 38,000 rpm for 1 h at 4°C in a 50 Ti Beckman rotor. The supernatant was applied to a Sepharose CL4B column (50 cm × 1 cm) previously equilibrated with a 10-mM Tris HCl buffer containing 150 mM KCl, 5 mM EDTA, and 0.1 mM β-mercaptoethanol, pH 7.6. The column was eluted at 3 ml/h and 1.0-ml fractions were collected. Protein in the effluent was automatically monitored by absorbance at 280 nm.

Study of spectrin dimer-dimer association in solution and determination of equilibrium constant Ka. Ghosts were washed twice in 0.3 mM $NaPO_4$, pH 7.6. Packed membranes were then incubated in 3 vol of 0.3 mM $NaPO_4$, 0.3 mM PMSF, 0.1 mM EDTA, pH 7.6 for 30 min at 37°C. After cooling in ice, the vesicles were spun down at 150,000 *g* for 60 min at 4°C. The supernatant containing crude spectrin (37°C extract) was carefully removed and dialyzed overnight at 4°C against 100 vol of 5 mM $NaPO_4$, 0.3 mM PMSF, 150 mM NaCl, pH 7.6. After estimation of the protein concentration by absorbance at 280 nm, taking an $E^{1\%}_{1\,cm}$ (280 nm) of 10.7 (29) and adjustment of the required concentrations, each sample of dialyzed proteins was incubated for 180 min at 30°C, to induce spectrin dimer to tetramer transformation. After cooling, each incubated sample (mean volume 0.5 ml, concentration range 0.5–2 mg/ml) was applied to a 10–30% (wt/vol of dialysis buffer) linear sucrose gradient (with a 50% wt/vol sucrose cushion). Ultracentrifugation was performed in a SW 40 Beckman rotor at 40,000 rpm for 15 h at 4°C. Gradients were eluted at 4°C from the top to the bottom and the absorbance was automatically monitored at 280 nm. The fraction α of spectrin converted into tetramer after 180 min incubation at 30°C was determined from the sedimentation profile by measuring the areas under the peaks corresponding to spectrin dimer (D) and tetramer (T). α is given by the ratio T/D + T. Writing c for the total molar concentrations of spectrin (expressed as a dimer of molecular weight 460,000), K_a is given by $\alpha/2c\ (1-\alpha)^2$.

Binding of spectrin to spectrin-depleted membranes. Spectrin-depleted inside-out vesicles were prepared from normal ghosts by incubation at 37°C for 30 min in 30 vol of 0.5 mM Tris HCl, 1 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM PMSF, pH 8.0. After centrifugation at 38,000 rpm (50 Ti Beckman rotor) for 30 min, the pellet was passed five times through a 27-gauge needle. This method led to extraction of 90% of spectrin and formation of small vesicles that morphologically are inside-out (30). Assay of spectrin binding to membrane vesicles was performed essentially as described by Bennett and Branton (31) with some modifications. Spectrin dimer (20–50 μg) were incubated for 90 min at 4°C in a 0.6-ml vol. containing 200 μg of inverted vesicle membrane proteins, 10 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.2 mM dithiothreitol, pH 7.6. Free and vesicle-bound spectrin were separated by sucrose gradient velocity sedimentation, using the same method as described for study of spectrin dimer self-association, except that the sucrose gradient was made in 10 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.2 mM dithiothreitol, pH 7.6. The amount of free spectrin was estimated by measuring the area of the spectrin peak. Each patient's binding assay was compared to a control performed at the same time.

RESULTS

Blood cell morphology. As illustrated in Fig. 1, appreciable numbers of elliptocytes as well as fragmented erythrocytes are seen in the proband, in stained blood films. The same pattern is observed in the proband's father (not shown).

Erythrocyte thermal stability. Erythrocyte thermal stability of the Proband's elliptocytes has been measured at several temperatures (see Methods) and no abnormal fragmentation has been detected. However, at the temperature of 47°C, more echinocytic forms (which characterize the morphological step before fragmentation) were seen in the proband than in the control.

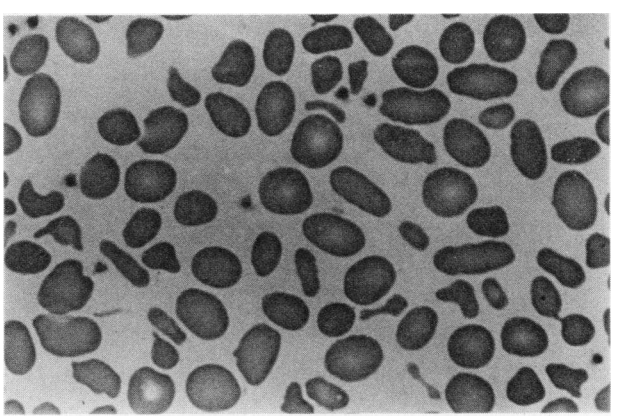

FIGURE 1 Morphology of erythrocytes from the proband (×600).

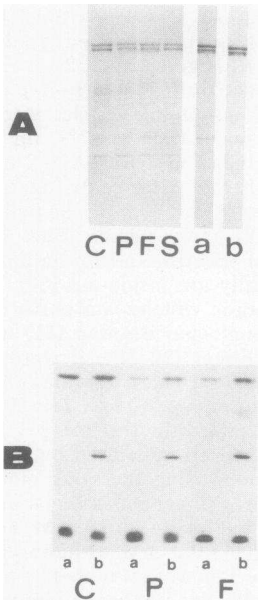

FIGURE 2 SDS polyacrylamide gel electrophoresis and autoradiography. A, Gel electrophoresis pattern using the 5–15% Laemmli gradient system of erythrocyte membranes from the control (C), the proband (P), the proband's father (F), the proband's sister (S); lanes a and b correspond respectively to the 4 and the 37°C extracts of the proband. B, autoradiograms of the autophosphorylated membranes from a control (C), the proband (P), the proband's father (F); a: without cyclic AMP, b: with cyclic AMP. Constant amount of membrane proteins and radioactivity were applied to the polyacrylamide gel.

Electrophoresis data. Slab-gel electrophoretic patterns of the red cell membranes of the proband and her father (Fig. 2) are characterized by the presence of an abnormal component migrating just below the spectrin doublet and by a decrease in the proportion of the spectrin β-chain. In disk SDS-PAGE performed according to Fairbanks with 0.1% SDS the abnormal component (M_r = 214,000) is clearly distinct from band 2.1 (ankyrin). Scanning of the disk gels (Fig. 3), limited to the spectrin region, shows in normal ghosts that spectrin α-chain represents 44%, β-chain 39.5%, and ankyrin 16.5% of the total spectrin area. In contrast, α-chain represents 41.5%, β-chain 23.5%, the abnormal component β′ 18.5%, and ankyrin 16.5% in whole ghosts of the proband and her father and the sum of normal and abnormal β-chains is equal to the percentage of β-chains found in controls. The β′-chain is also found in the 4°C and 37°C extracts obtained from the proband's ghosts (Figs. 2, 5). These data lead us to consider the abnormal component β′ as a shortened spectrin β-chain.

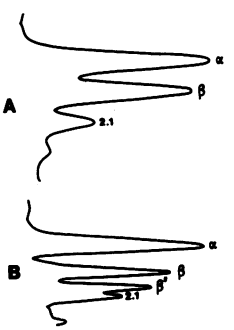

FIGURE 3 SDS polyacrylamide gel electrophoresis densitometric tracings (according to Fairbank's method) of: A, control ghosts, B, proband's ghosts.

A typical elution profile from a gel filtration column of normal 4°C extracts (prepared by dialysis of ghosts at 4°C) is shown in Fig. 5. The leading peak contains a complex of spectrin-actin-protein band 4.1, whereas the second peak consists mainly of spectrin tetramer. However, the analysis of the normal 4°C extract by sucrose gradient velocity sedimentation reveals that 10% of the total amount of protein layered on the gradient is a dimeric specie (result not shown). In contrast the elution profile from gel filtration of the proband's 4°C extracts shows three peaks (Fig. 4): the leading peak (33.6%) and the second peak (32%) correspond respectively to the oligomeric complex and to the spectrin tetramer; the third peak (34.4%) consists of spectrin dimer. Each peak was subjected to electrophoretic analysis and scanned (Fig. 5). The proband's oligomeric complex contains 50% spectrin α-chain, 40% normal β-chain, and 10% β′-chain; spectrin tetramer displays 51% α-chain, 46% β-chain, and 3% β′-chain. In spectrin dimer, α-chain represents 54%, β-chain 21%, and β′-chain 25%. However, separation

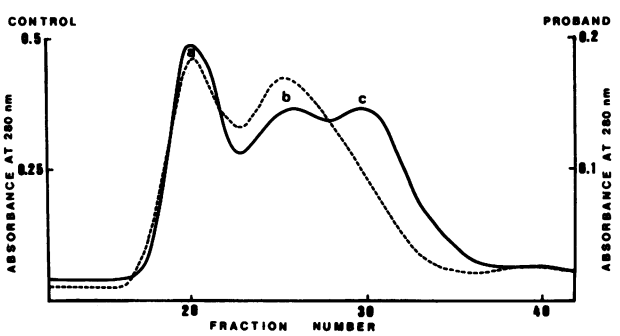

FIGURE 4 Gel filtration (Sepharose CL4B column) of normal and proband 4°C extracts. Elution profile of hydrosoluble proteins extracted at 4°C. Peaks a, b, c, contain respectively: oligomeric complexes, spectrin tetramer and spectrin dimer. The profile of normal ghosts is indicated by the broken line and is quantitated on the left-hand orinate (5.5 mg layered on the column). The profile of the proband's ghosts is indicated by the full line and is quantitated on the right-hand ordinate (2 mg layered on the column).

between tetramer and dimer species was not completely achieved and we further purified the proband's dimer spectrin peak by sucrose gradient velocity centrifugation. In such conditions pure dimer isolated from contaminated tetramer contains 51% α-chain, 13.4% β-chain and 35.6% β'-chain (Fig. 5), the β- to β'-ratio allows us to estimate that this pure dimer contains 27% of normal $\alpha\beta$-spectrin dimer, and therefore 73% of $\alpha\beta'$-spectrin dimer.

Spectrin dimer to tetramer transformation in solution. Crude spectrin extracted from ghosts incubated in low-ionic strength buffer at 37°C is mostly dissociated into dimer (32). The transformation of spectrin dimer into tetramer does not involve regulatory proteins (30) and is directly studied in 37°C extracts. After incubation at 30°C for 180 min (at which time equilibrium is reached in our hands) to induce dimer to tetramer transformation, the samples are cooled on ice and then ultracentrifuged in sucrose gradients, and eluted at 4°C. At this temperature interconversion between the two species (dimer and tetramer) is very slow (33) and separation of dimer from tetramer does not result in redistribution and gives the true proportions of the two species. Fig. 6 shows typical sucrose gradient distribution profiles of spectrin dimer and tetramer. These profiles are highly reproducible and using different concentrations of extracted proteins (0.5–2 mg/ml) we have found the association constant (K_a) for normal spectrin to be 5 ($\pm$0.27) $\times$ 10^5 M^{-1} (n = 38). In the two related cases of HE reported herein, the K_a was similarly decreased to a value of 2.3 ($\pm$0.3) $\times$ 10^5 M^{-1} (n = 4). Dimer and tetramer peaks isolated at equilibrium after dimer to tetramer conversion in the 37°C extracts of the proband's father was analyzed by gel electrophoresis and displayed, respectively, 19 and 7% β'-chain.

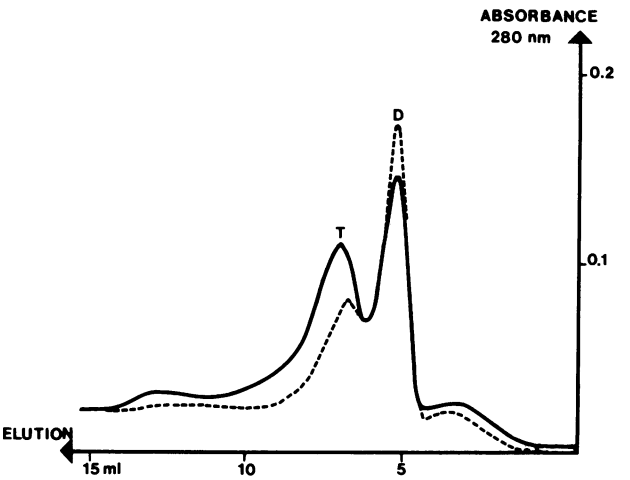

FIGURE 6 Elution profiles of sucrose gradient velocity centrifugation. 400 μl of a 37°C low ionic strength extract (1 mg/ml) were layered on the sucrose gradient; full line, normal 37°C extract; broken line, father's 37°C extract. A similar profile was obtained with the proband's 37°C extract (not shown). D, dimer peak; T, tetramer peak.

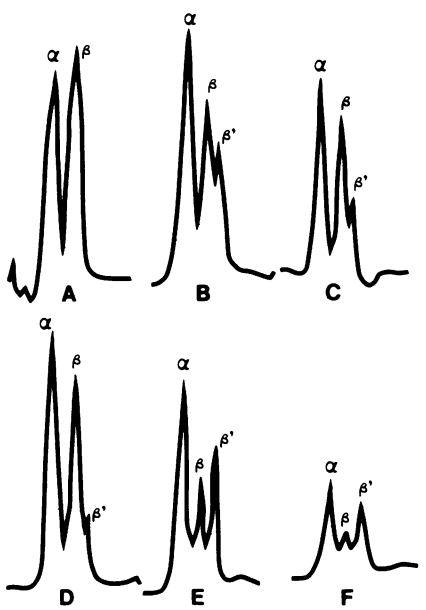

FIGURE 5 SDS polyacrylamide gel electrophoresis densitometric tracings of: A, control 4°C extract, B, proband's 4°C extract, C, D, and E, respectively: oligomer peak, spectrin tetramer peak, and spectrin dimer peak, each isolated by gel filtration of the proband's 4°C extract (see Fig. 4). F, Pure spectrin dimer further isolated from E by sucrose gradient velocity centrifugation.

Protein phosphorylation. Overall auto-phosphorylation of ghosts from the proband, her father and sister gives normal results (Table I). However autoradiograms of the proband's and her father's autophosphorylated membrane proteins (Fig. 2) show that β'-chain is not phosphorylated and that the spot given by the normal β-chain is decreased. The same results were obtained after phosphorylation of the intact erythrocytes of the proband with ^{32}Pi. In addition the percentage of β'-chain estimated on the Coomassie

TABLE I
Autophosphorylation of Ghosts

	μM ^{32}P incorporated by mg protein in 10 min	
	−cAMP	+cAMP
Proband	183	212
Proband's father	228	269
Proband's sister	175	211
Controls (20)	204 s = 27, 2 (E = 165–265)	219, 5 s = 30 (E = 170–265)

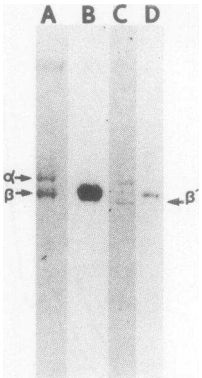

FIGURE 7 SDS polyacrylamide gel electrophoresis and the corresponding autoradiograms of proband's spectrin dimer. The proband's spectrin dimer was purified from the 4°C extract by gel filtration and sucrose gradient velocity sedimentation (see Fig. 4). Phosphorylation of the spectrin dimer with $\gamma[^{32}P]$ATP was performed using pure membrane casein kinase in solution (see ref. 27). In order to reach a better separation between α-, β-, and β'-bands longer running times than those described in Methods were used. A and C, gel electrophoretic patterns of control dimer and proband's dimer, respectively. B and D, corresponding autoradiograms.

stained gel remained constant (data not shown). Phosphorylation of the proband's sister's spectrin β-chain is normal. The proband's chromatographically isolated dimer, further purified by sucrose gradient velocity sedimentation, was phosphorylated by purified casein kinase obtained from human erythrocyte membranes (27). The autoradiogram shows that the β'-chain, as in the endogenous phosphorylation, is not phosphorylated (Fig. 7). After treatment with cytosolic human erythrocyte phosphatase in conditions where ~70% of $^{32}\gamma$-ATP prelabeled normal spectrin is dephosphorylated, the proband's spectrin β'-chain remained not phosphorylatable (data not shown).

Binding assay of spectrin dimer to spectrin-depleted normal inside-out vesicles. Binding assays were performed using spectrin dimer isolated by sucrose gradient velocity sedimentation after dimer to tetramer conversion of spectrin obtained (37°C extraction) from the proband and her father. These experiments were done using two concentrations of spectrin dimer (as described in Methods).

The binding of both patients spectrin is identical to the controls as shown in Table II. Furthermore polyacrylamide gel electrophoresis of the vesicles pelleted after binding shows a similar percentage of β'-chain in the bound-spectrin dimer and in the free spectrin dimer (Fig. 8).

DISCUSSION

SDS gel electrophoresis of the membrane proteins from the erythrocytes of a young woman with elliptocytosis and mild hemolytic anemia displayed the presence of an abnormal component with an M_r of 214,000. The same component was found in erythrocyte membranes of the proband's father, who also had elliptocytosis. We identified this subunit as an abnormal spectrin β chain for the following reasons: (*a*) this component and spectrin are both extracted in the hydrosoluble proteins; (*b*) it copurifies with spectrin dimer when hydrosoluble proteins are analyzed either by gel filtration or by sucrose gradient velocity centrifugation; (*c*) its presence on the electrophoretic gels is balanced by a decrease of the normal spectrin β-chain band since the sum of normal and abnormal β-chains (calculated from scanning) is equal to the control spectrin β-chain; (*d*) this component binds to spectrin-depleted normal inside-out vesicles as does normal β-chain. We concluded that this abnormal component was a shortened spectrin β-chain.

Extracts from normal erythrocytes membranes incubated at low ionic strength and 4°C, analyzed either by nondenaturating gel electrophoresis (34) or, in our work, by sucrose gradient velocity sedimentation, contain <10% spectrin dimer. In contrast, extracts of the proband's ghosts contain one-third of oligomer complex, one-third of tetrameric, and one-third of dimeric spectrin. In the 4°C extracts the spectrin dimer-tetramer equilibrium is kinetically trapped in its native state, as in the membrane. This allows us to conclude that the proband's membranes contain an abnormal amount of dimer with a concomitant decrease of tetramer. Furthermore, electrophoresis shows that the spectrin tetramer peak contains only trace amounts of β'-chain (3%), whereas the dimer peak is mainly composed of abnormal $\alpha\beta'$-dimer (73%). Self-association of this $\alpha\beta'$-dimer is obviously highly defective in the membrane and apparently explains the excess amount of dimer in the 4°C extracts; indeed the dimer peak that represents 34.4% of the total amount of the 4°C extract layered on the column, contains 27% of normal $\alpha\beta$-dimer. We can therefore estimate that 9% of normal $\alpha\beta$-dimer is present in the proband's 4°C extracts, which agrees with our results in normals.

TABLE II
Binding of Spectrin Dimer to Spectrin-depleted Inside-out Vesicles

	Spectrin dimer bound (mg/mg of membrane protein)			
Spectrin dimer-added	Control	Proband	Control	Proband's father
μg				
20			50	60
25	104	100		
40			85	85
50	177.5	180		

The defect of spectrin self-association suggested by the increase of spectrin dimer to tetramer ratio in the proband's 4°C extracts, was further investigated in solution. Pure spectrin dimer-tetramer interconversion in solution has been extensively studied (33) and the normal K_a value (in a physiological ionic strength buffer at 30°C incubation) was found to be 10^6 M^{-1}. Our normal K_a value (5×10^5 M^{-1}), determined with 37°C extracts, agrees with the value reported by Liu et al. (34) ($7 \pm 2 \times 10^5$ M^{-1}) and is the same in patients with reticulocytosis due to hemolytic anemias not caused by a primary membrane defect such as autoimmune hemolytic anemia, congenital hemolysis with pyruvate kinase deficiency or β-thalassemia. In our two cases, the K_a value was found to be similarly decreased (2.3×10^5 M^{-1}), and SDS gel electrophoresis of spectrin tetramer and spectrin dimer separated at equilibrium by sucrose gradient velocity centrifugation shows that tetramer contains about three times less β′-chain than dimer.

Several pieces of data (24, 35) suggest that spectrin tetramer is formed by duplicate but independent paired interactions between terminal peptide domains of the α- and β-subunits. An 80,000-dalton peptide isolated from a terminal portion of the α-chain was shown to bind preferentially to spectrin dimer and compete with spectrin tetramer (21). In the case reported herein, the defective self-association of spectrin dimer due to the presence of the β′-chain, demonstrates the participation of the normal β-chain in the tetramerization process, which is an argument for the bivalent nature of the dimer association proposed by Morrow and Marchesi (36).

Very likely, the abnormal dimer excess in the membrane due to defective self-association of the αβ′-dimer explains the membrane-skeleton instability and probably the hemolysis (2, 34). We do not know if the presence of the β′-chain is responsible for the elliptocytic shape. As pointed out before HE is associated with different molecular abnormalities such as defective spectrin self-association (17, 28) or protein 4.1 function (14). In addition, defective spectrin self-association has also been found in hereditary pyropoikilocytosis (34) a disease closely related to HE and characterized by an exceptional thermal sensitivity of the erythrocytes, which fragment at 45–46°C (instead of the usual 49°C) after a 15-min incubation. In our case, thermal sensitivity of the elliptocytes was normal. Liu et al. (20) recently showed that partial tryptic digestion of the self-association defective spectrin in variants of HE, as in hereditary pyropoikilocytosis, resulted in the increase of a 73,000-dalton component and in the concomitant decrease of a 80,000-dalton peptide suggesting a mutation that affects the dimer contact site of the α-chain involved in spectrin tetramer formation.

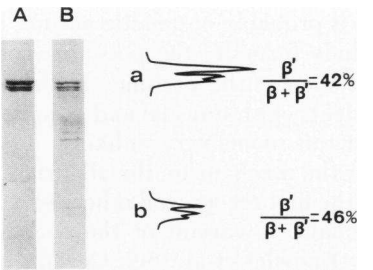

FIGURE 8 SDS polyacrylamide gel electrophoresis of the proband's free and bound spectrin dimer. Spectrin dimer was isolated by sucrose gradient velocity sedimentation from the proband's 37°C extract, after the extract had been incubated at 30°C for 180 min to induce spectrin dimer to tetramer conversion. A, free spectrin dimer; B, vesicle-bound spectrin dimer, (a, b) = corresponding densitometric tracings restricted to the spectrin area. The ratios of β′ to β + β′ calculated from these tracings were similar.

Overall phosphorylation of ghosts was normal but the autoradiograms revealed that the β′-chain was not phosphorylated. It seems very unlikely that all the phosphorylatable sites of the β′-chain are saturated for the following reasons: the β′-chain was neither phosphorylated in intact erythrocytes nor in solution after treatment of spectrin with human erythrocyte cytosolic spectrin phosphatase. These sites are probably lost along with the lacking peptide.

Both defective self-association and phosphorylation of the αβ′-dimer in this case of spectrin β′-variant imply that the phosphorylation sites are located at the end of the spectrin β-chain involved in the dimer-dimer interactions. This conclusion is in total agreement with the recent work of Speicher and Morrow (37), which demonstrates by peptide mapping studies the location of the β-chain phosphorylated domain at the end of the molecule involved in dimer-dimer interactions. Harris and Lux (38) have previously shown that the phosphorylation sites are located at the carboxy-terminus of the β-chain. Thus, it is possible to assign this terminus to the end of β-chain involved in the dimer-dimer association.

It is not surprising to find a normal operational ankyrin-binding site in the β′-chain since the high affinity membrane binding site is separated from the β-chain phosphorylated segment by a peptide of 28,000 dalton (37).

Elliptocytosis in Caucasians is classically inherited as a dominant trait; that was also the case of this patient. The father and daughter are heterozygotes for the same molecular abnormality since another daughter's erythrocytes are normal. The β′-chain represents ~44% of the total spectrin β-chains, which is rather close to the expected value of 50%. The mechanism leading to a shortened β′-variant chain is not yet un-

derstood. It is probably of genetic nature; however we cannot exclude formally the possibility of a postsynthetic event such as the presence in the erythrocytes of these patients of an unusual and specific proteolytic activity, but this seems very unlikely.

Whatever the mechanism, the abnormal β-chain we observed is the first recognized inherited variant of the spectrin β-chain. A variant of the α-chain has been reported by Knowles et al. (39). Certainly other variants will be described in the future; so we propose to give the present component the name of "variant β Le Puy," which refers to the town where the proband is living.

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REFERENCES

1. Lux, S. E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red cells. *Semin. Hématol.* **16:** 21–51.
2. Liu, S. C., and J. Palek. 1980. Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeleton. *Nature (Lond.).* **285:** 586–588.
3. Ji, T. H., D. J. Kiehm, and C. Russell Middaugh. 1980. Presence of spectrin tetramer on the erythrocyte membrane. *J. Biol. Chem.* **255:** 2990–2993.
4. Shotton, D. M., B. E. Burke, and D. Branton. 1979. The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopy studies. *J. Mol. Biol.* **131:** 303–329.
5. Ungewickell, E., P. M. Bennet, R. Calvert, V. Ohanan, and W. Gratzer. 1979. In vitro formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature (Lond.).* **280:** 811–814.
6. Tyler, J. M., W. R. Hargreaves, and D. Branton. 1979. Purification of two spectrin binding proteins: biochemical and electron microscopical evidence for site specific reassociation between spectrin and band 2.1 and 4.1. *Proc. Natl. Acad. Sci. USA.* **76:** 5192–5196.
7. Cohen, C. M., and S. F. Foley. 1980. Spectrin-dependent and independent association of F-actin with the erythrocyte membrane. *J. Cell Biol.* **86:** 694–698.
8. Brenner, S. L., and E. D. Korn. 1979. Spectrin actin interaction. Phosphorylated and dephosphorylated spectrin tetramer crosslink F-actin. *J. Biol. Chem.* **254:** 8620–8627.
9. Fowler, V., and D. L. Taylor. 1980. Spectrin plus band 4.1 crosslink actin. Regulation by micromolar calcium. *J. Cell Biol.* **85:** 361–375.
10. Sheetz, M. P. 1979. DNase I-dependent dissociation of erythrocyte cytoskeletons. *J. Cell Biol.* **81:** 266–270.
11. Nermut, M. V. 1981. Visualization of the "membrane skeleton" in human erythrocytes by freeze-etching. *Eur. J. Cell Biol.* **25:** 265–271.
12. Tomaselli, M. B., K. M. John, and S. E. Lux. 1981. Elliptical erythrocyte membrane skeletons and heat-sensitive spectrin in hereditary elliptocytosis. *Proc. Natl. Acad. Sci. USA.* **78:** 1911–1915.
13. Feo, C., S. Fischer, J-P. Piau, M. J. Grange, and G. Tchernia. 1980. Première observation de l'absence d'une protéine de la membrane érythrocytaire (bande 4.1) dans un cas d'anémie elliptocytaire familiale. *Nouv. Rev. Fr. Hématol.* **22:** 315–325.
14. Tchernia, G., N. Mohandas, and S. B. Shohet. 1981. Deficiency of skeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis. Implications for erythrocyte membrane stability. *J. Clin. Invest.* **68:** 454–460.
15. Mueller, T. J., and M. Morrison. 1981. Cytoskeletal alterations in hereditary elliptocytosis. *J. Supramol. Struct. Cell Biochem. Suppl.* **5:** 131.
16. Alloisio, N., E. Dorleac, R. Girot, and J. Delaunay. 1981. Analysis of the red cell membrane in a family with hereditary elliptocytosis. Total or partial absence of protein 4.1. *Hum. Genet.* **59:** 68–71.
17. Liu, S. C., J. Palek, J. Prchal, and R. P. Castleberry. 1981. Self-association of spectrin in abnormal red blood cells. *J. Supramol. Struct. Cell Biochem. Suppl.* **5:** 131.
18. Liu, S. C., J. Palek, and J. T. Prchal. 1982. Defective spectrin dimer-dimer association in hereditary elliptocytosis. *Proc. Natl. Acad. Sci. USA.* **79:** 2072–2076.
19. Coetzer, T., and S. S. Zail. 1981. Tryptic digestion of spectrin in variants of hereditary elliptocytosis. *J. Clin. Invest.* **67:** 1241–1248.
20. Liu, S. C., J. Lawler, J. Prchal, and J. Palek. 1981. Defective dimer-dimer association and altered tryptic digestion of spectrin (Sp) in variants of hereditary elliptocytosis (HE). *Blood.* **58**(Suppl. 1): 45a (Abstr.).
21. Morrow, J. S., D. W. Speicher, W. J. Knowles, C. Jennifer Hsu, and V. T. Marchesi. 1980. Identification of functional domains of human erythrocyte spectrin. *Proc. Natl. Acad. Sci. USA.* **77:** 6592–6596.
22. Agre, P., E. P. Orringer, D. H. K. Chui, and V. Bennett. 1981. A molecular defect in two families with hemolytic poikilocytic anemia. Reduction of high affinity membrane binding sites for ankyrin. *J. Clin. Invest.* **68:** 1566–1576.
23. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100:** 119–130.
24. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10:** 2606–2617.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:** 680–685.
26. Guthrow, C. E., Jr., J. E. Allen, and H. Rasmussen. 1972. Phosphorylation of an endogenous membrane protein by an endogenous membrane-associated cyclic adenosine 3′5′-monophosphate-dependent protein kinase in human erythrocyte ghosts. *J. Biol. Chem.* **247:** 8145–8153.
27. Boivin, P., and C. Galand. 1980. Compartmentalization of spectrin-phosphorylating enzyme in human erythrocytes. *Biochem. Biophys. Res. Commun.* **93:** 24–28.
28. Clari, C., and V. Moret. 1981. Partial purification and characterization of phosphoprotein phosphatase from human erythrocyte hemolysate. *Biochemistry Int.* **2:** 509–515.

29. Kam, Z., R. Josephs, H. Eisenberg, and W. B. Gratzer. 1977. Structural study of spectrin from human erythrocyte membranes. *Biochemistry.* **16:** 5568–5572.
30. Steck, T. L., and J. A. Kant. 1974. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Biomembranes. Part A. Methods Enzymol. **31:** 172–180.
31. Bennett, V., and D. Branton. 1977. Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. *J. Biol. Chem.* **252:** 2753–2763.
32. Raltson, G., J. Dunbar, and M. White. 1977. The temperature-dependent association of spectrin. *Biochim. Biophys. Acta.* **491:** 345–348.
33. Ungewickell, E., and W. Gratzer. 1978. Self-association of human spectrin. A thermodynamic and kinetic study. *Eur. J. Biochem.* **88:** 379–385.
34. Liu, S. C., J. Palek, J. Prchal, and R. P. Castleberry. 1981. Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeletons in hereditary pyropoikilocytosis. *J. Clin. Invest.* **68:** 597–605.
35. Burke, B. E. 1981. Structural studies of human erythrocyte spectrin—Ph.D. Thesis. Department of Biochemistry. Imperial College of Science and Technology. London, England.
36. Morrow, J. S., and V. T. Marchesi. 1981. Self-assembly of spectrin oligomers in vitro: A basis for a dynamic cytoskeleton. *J. Cell Biol.* **88:** 463–468.
37. Speicher, D. W., J. S. Morrow, W. J. Knowles, and V. T. Marchesi. 1982. A structural model of human erythrocyte spectrin: alignment of chemical and functional domains. *J. Biol. Chem.* In press.
38. Harris, W. H., Jr., and S. Lux. 1980. Structural characterization of the phosphorylation sites of human erythrocyte spectrin. *J. Biol. Chem.* **255:** 11512–11520.
39. Knowles, W. J., and V. T. Marchesi, 1981. Approaches to the study of molecular variations of spectrin. *J. Supramol. Struct. Cell Biochem. Suppl.* **5:** 130 (Abstr.).