Defective Binding of Spectrin to Ankyrin in a Kindred with Recessively Inherited Hereditary Elliptocytosis

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Abstract. The interaction of spectrin with spectrin-depleted inside-out membrane vesicles was studied in a kindred with an atypical variant of hereditary elliptocytosis inherited in a recessive manner. The probands are characterized by prominent elliptocytosis, decreased erythrocyte thermal stability, an altered limited tryptic peptide pattern of spectrin digested at low ionic strength, and defective spectrin dimer-dimer association. The parents are normal. The spectrin/band 3 ratio determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of isolated membranes of the probands was decreased to ~70% of control values, and total erythrocyte spectrin content in one proband was also decreased on SDS-PAGE. When a monospecific antispectrin antibody was used, a faintly labeled fragment of molecular weight ~28,000 was detected on immunoblots of whole cell lysates of one proband and a control, but could not account for the decreased erythrocyte spectrin content of the proband on SDS-PAGE. Binding and competitive inhibition studies revealed an alteration in the spectrin-ankyrin interaction due to an abnormality of spectrin in the probands. No defect was found in the mother; the father's spectrin showed decreased binding affinity, although it was not so severe as in the probands. Separation of bound and unbound spectrin dimers from one proband and subsequent conversion to tetramers showed that the self-association defect was detectable only on the bound subpopulation of her spectrin. These findings demonstrate a hitherto undescribed functional abnormality of spectrin in this kindred which could result in decreased stability of the membrane skeleton and contribute to the elliptocytic shape of these erythrocytes.

Received for publication 19 September 1983 and in revised form 23 March 1984.

J. Clin. Invest.

0021-9738/84/09/0753/10 $1.00
Volume 74, September 1984, 753–762

Introduction

Hereditary elliptocytosis (HE)[1] is a heterogeneous disorder with several clinical phenotypes and molecular and genetic etiologies. Eight clinical syndromes have been delineated by Palek and Lux (1) in a recent comprehensive review: mild HE, HE with sporadic hemolysis, HE with infantile poikilocytosis, HE with abnormal erythropoiesis, homozygous HE, heterozygous and homozygous spherocytic HE, and stomatocytic HE. In addition, there is a close relationship between mild HE and hereditary pyropoikilocytosis (HPP). Since HE ghosts and membrane skeletons retain the shape of the parent cell, the basic membrane defects must reside in the membrane skeleton (2). The membrane-associated skeleton is a filamentous meshwork consisting of spectrin, actin, ankyrin, and band 4.1 (3). Spectrin exists as a dimer of α- and β-subunits of molecular weights 240,000 and 220,000, respectively. The dimers are thought to form tetramers by head-to-head associations through noncovalent interactions between an 80,000-D domain at one end of the α-subunit and the phosphorylated end of the β-subunit (4). Larger oligomers of spectrin may be formed by similar interactions (5). Spectrin forms the meshwork by crosslinking and binding to short filaments of actin (6), this interaction being enhanced by association with band 4.1 (7). The skeleton is bound to the membrane via ankyrin, which binds to one of the β-chains of a spectrin tetramer near the tetramer assembly site, as well as to a cytoplasmic portion of band 3, an integral membrane protein (8).

Dimer-dimer association has been found to be defective in some cases of mild HE (9) and in HPP (10, 11). In such affected cases, limited tryptic proteolysis of spectrin shows a defect of the 80,000-D domain at the end of the α-chain (11–

1. *Abbreviations used in this paper:* DTT, dithiothreitol; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; IOV, inside-out vesicle; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

13). A defect at the carboxyl terminus of the β-subunit of spectrin, which also results in altered spectrin tetramer assembly, has been recently described in a French kindred with mild HE (14). Deficiencies of band 4.1 (15) and band 4.1 and glycophorin C (16) have also been described in variants of HE. Two families have been described in which the number of effective high affinity ankyrin binding sites in ankyrin-depleted membranes is reduced by half and which is thought to reflect an altered arrangement of band 3 molecules in the membrane (17). Palek and Lux (1) postulate that repeated distortion of erythrocytes with mild membrane skeleton defects in the microcirculation leads to gradual elongation into the typical elliptocytic shape in contrast to normal cells, which realign rapidly in response to stress.

In the present study we report a new functional membrane skeleton defect in a kindred with an atypical variant of HE. This kindred, in which the defect is inherited recessively and in which the probands show some features of HPP, has been described in some detail previously (18–20). Spectrin isolated from the erythrocytes of the probands showed an abnormal pattern of peptides on limited tryptic hydrolysis in low ionic strength media, but this was qualitatively normal at high ionic strength (19). Altered spectrin dimer-dimer association was subsequently found in these subjects (20), although none of these defects could be demonstrated in either parent, both of whom are clinically and hematologically normal. We now report that both probands show an abnormality of spectrin that results in defective binding to spectrin-depleted membrane vesicles and also to a 72,000-D chymotryptic fragment of ankyrin in solution. No defect can be detected in the mother, but the father shows an altered affinity of binding of spectrin to membrane vesicles intermediate between the probands and normal controls.

Methods

Subject CG. The clinical and hematologic features of this patient have been described in detail (18–20). In summary, she is a 32-yr-old splenectomized white female with a well-compensated hemolytic anemia (hemoglobin, 13.5–14.5 g/100 ml; mean corpuscular volume, 80–82 fl; reticulocyte count, 4–7% over the past year). The smear shows predominant elliptocytosis with some budding and poikilocytosis. Osmotic fragility is increased and autohemolysis is increased but corrected by glucose. Incubation of heparinized venous blood for 15 min at 45°C shows membrane budding and cell fragmentation, which increases progressively up to 49°C. Both parents are clinically and hematologically normal, with a 98% likelihood of parentage as determined by HLA typing. The erythrocyte fragmentation and the apparent recessive inheritance suggest a relationship to the HPP syndrome, but the progressive fragmentation upon the heating of her erythrocytes to 49°C, the normal mean corpuscular volume, and the increased autohemolysis corrected by glucose are features against this diagnosis. We regard her as an atypical variant of HE, in keeping with the predominantly elliptocytic morphology of her erythrocytes.

Subject AD. She is the elder sister of CG who suffers from epilepsy and is at present committed to a state hospital for mentally defective individuals. She too has predominant elliptocytosis with hemoglobin values 13.5–14.5 g/100 ml. She does not appear to be actively hemolyzing as the reticulocyte count has consistently been found to be 1.0–1.5%. Erythrocyte glutamic oxaloacetate transaminase levels are also within the normal range. Osmotic fragility and autohemolysis are similar to those of CG, as is the pattern of erythrocyte fragmentation upon heating from 45–49°C.

Subject TM. This is a black male aged 3 yr with a 3–4 cm splenomegaly and hemolytic anemia who has been described in detail elsewhere (19–21). He too shows predominant elliptocytosis with increased thermal sensitivity of erythrocytes similar to, but less pronounced than, that of CG. The mother of TM is hematologically normal and the father is not available for study. TM probably represents a case of neonatal elliptocytosis with increased thermal sensitivity of erythrocytes. Previous studies of tryptic digests of crude spectrin extracts from his erythrocyte membranes in low ionic strength NH_4HCO_3 medium did not show any apparent abnormality (19). However, more recently repeated studies of such digests in normal ionic strength medium (10 mM Tris-HCl, 130 mM KCl, 20 mM NaCl, pH 8.0), show a consistent decrease of the 80,000-D α-subunit peptide with a reciprocal increase in a 74,000-D peptide similar to that described by Liu et al. (13) and Lawler et al. (12) in HE and HPP subjects, respectively. Spectrin extracts from TM also show a defect of dimer self-association and he has been used as an additional control for the spectrin vesicle binding studies outlined below.

Materials. ^{125}I-labeled Bolton-Hunter reagent (~2,000 Ci/mmol) and ^{125}I-labeled protein A were from The Radiochemical Centre LTD. (Amersham, England). Trypsin treated with L-one-tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK-trypsin) was from Worthington Biochemical Corp. (Freehold, NJ). Sepharose (Cl)4B and Pharmalyte, pH 4.0–6.5, were from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE cellulose (DE52) was from Whatman LTD. (Kent, England). Phenylmethylsulfonyl fluoride (PMSF), pepstatin A, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was from Merck (Darmstadt, West Germany). Heat-denatured formaldehyde-fixed protein A-bearing staphylococci were from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA). The protein assay kit and DEAE Affi-gel Blue were from Bio-Rad Laboratories (Richmond, CA).

Preparation of spectrin. Heparinized venous blood was collected on ice and processed within 1 h. Erythrocyte ghosts were prepared by hypotonic lysis of washed erythrocytes at 4°C in 30 vol of 10 mM Tris-HCl, 1 mM EDTA, 25 μg/ml PMSF (dissolved at 25 mg/ml isopropanol), pH 7.6, and centrifugation for 15 min at 30,000 *g*. In some experiments, pepstatin (2 μg/ml) and leupeptin (2 μg/ml) were also added to the lysis buffer. The ghosts were washed three times in the lysis buffer and then once in 0.25 mM EDTA, 25 μg/ml PMSF, pH 8.0. Spectrin was extracted by incubating the ghosts (2–3 mg/ml in 0.25 mM EDTA, 25 μg/ml PMSF, pH 8.0) for 30 min at 37°C. Pepstatin (2 μg/ml) and leupeptin (2 μg/ml) were included in the extracting medium in some experiments. The sample was centrifuged at 150,000 *g* for 30 min at 4°C and the supernatant collected. Spectrin dimers were purified by gel filtration of the supernatant at 4°C on a 1.6 × 100 cm Sepharose (Cl)4B column equilibrated in 10 mM sodium phosphate, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM NaN_3, 1 mM DTT, pH 8.0. The flow rate was 20 ml/h; fractions of 2.0 ml were collected and the absorbance was monitored continuously at 280 nm. Three protein peaks were resolved, the middle peak containing only spectrin as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below). Spectrin was concentrated

in a B15 cell (Amicon Corp., Danvers, MA) at 4°C to 1–3 mg/ml prior to labeling. Proteolytic degradation of either unlabeled spectrin or ^{125}I-labeled spectrin from patients or controls could not be detected. Pepstatin and leupeptin were not added routinely in all experiments, as it was found that the presence of EDTA and PMSF was sufficient to inhibit proteolysis of spectrin from both control and HE patients.

Preparation of 72,000-D fragment. The 72,000-D chymotryptic fragment of ankyrin was prepared as described by Bennett and Stenbuck (22). The purity of this fraction as determined by densitometric scans of SDS-polyacrylamide gels stained with Coomassie Blue varied between 60 and 75% in different experiments.

Labeling of skeletal proteins. Spectrin and the 72,000-D chymotryptic fragment of ankyrin were labeled with ^{125}I-Bolton-Hunter reagent by a modification of the method described by Litman et al. (23). 1 mg of spectrin or 72,000-D fragment was added to 65 μCi of dry Bolton-Hunter reagent and the reaction continued for 1 h at 0°C. After passage through a 1 × 10 cm Sephadex G-50 column equilibrated at 4°C with the binding assay buffer (10 mM sodium phosphate, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM NaN_3, 1 mM DTT, pH 7.5), the iodinated protein peaks were dialyzed against this buffer for 18 h at 4°C. Both proteins were at least 98% precipitable by trichloracetic acid. Specific activities obtained varied between 10,000 and 20,000 cpm/μg. Binding assays were carried out within 72 h of blood collection.

Preparation of spectrin-depleted inside-out vesicles (IOVs). Spectrin-depleted IOVs were prepared as described by Bennett and Stenbuck (24). Erythrocyte ghosts were incubated for 30 min at 37°C in 30 vol of 0.25 mM EDTA, 25 μg/ml PMSF, pH 8.0, and centrifuged at 50,000 *g* for 25 min. The vesicles were suspended in the binding assay buffer at a concentration of 1 mg/ml and stored overnight at 4°C. Sedimentation through a 10% (wt/vol) dextran barrier gradient to remove the small amounts of residual spectrin was omitted, as this has no effect on the properties of spectrin binding (24).

Preparation of antispectrin. Antibodies to purified spectrin heterodimers were raised in rabbits as described by Goodman et al. (25). The rabbit antiserum was dialyzed against 20 mM K_2HPO_4, 0.02% NaN_3, pH 8.0, at 4°C, and then passed through a column of DEAE Affi-gel Blue equilibrated in the same buffer. The protease-free gammaglobulin fraction was collected and concentrated. This fraction (1–2 mg/ml) was then incubated for 90 min on ice with spectrin-free IOVs to remove any antibodies that might cross-react with ankyrin in the IOVs. After centrifugation at 50,000 *g* for 25 min at 4°C, the antispectrin antibody was harvested and stored at −20°C.

Spectrin binding to IOVs. Binding of ^{125}I-spectrin heterodimers to IOVs was determined by use of the incubation conditions of Goodman and Weidner (26). Various concentrations of ^{125}I-spectrin (5–50 μg/ml) derived from a control and a test subject were incubated in parallel for 90 min at 0°C in a 250-μl volume containing 80 μg/ml IOV protein from a different control and 10 mM sodium phosphate, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM NaN_3, 1 mM DTT, pH 7.5. Membrane-bound ^{125}I-spectrin was determined after centrifugation at 50,000 *g* for 25 min at 4°C and aspiration of the supernatant. Nonspecific binding at each ^{125}I-spectrin concentration was determined by the use of heat-denatured (70°C, 15 min) spectrin, and this value (10–30% of total counts) was routinely subtracted. All binding data are shown as the mean of duplicates, which had ranges of $< \pm 5\%$. In all experiments (except in one crossover experiment) normal control IOVs were used so that any differences in binding isotherms of ^{125}I-spectrin between patients and controls must have been due to a change in the binding properties of spectrin itself.

With the use of the same conditions as those outlined above, competitive inhibition of binding of control ^{125}I-spectrin to control IOVs in the presence and absence of unlabeled spectrin from patients and controls was determined. Correction for nonspecific binding using heat-denatured spectrin was made as above.

Self-association of bound and unbound ^{125}I-spectrin dimers. ^{125}I-spectrin dimers derived from a control and subject CG were incubated with control IOVs under the same conditions as those described above except that the amounts of all constituents were increased 100-fold (total volume of incubation medium 25 ml). The unbound ^{125}I-spectrin dimers were collected, concentrated in a B-15 cell at 4°C to ~0.6 mg/ml and then incubated at 30°C for 2.5 h to transform free ^{125}I-spectrin dimers to tetramers. The equilibrium was stabilized by cooling to 0°C. The centrifuged IOVs with the bound ^{125}I-spectrin were suspended at a concentration of 1 mg membrane protein/ml in 5 mM sodium phosphate, 150 mM NaCl, 5 mM mercaptoethanol, 5 mM EDTA, and 25 μg/ml PMSF, pH 7.5, and incubated at 37°C for 30 min to convert bound ^{125}I-spectrin dimers to tetramers. The conditions are those described by Liu and Palek (27) for spectrin dimer-tetramer conversion in situ in whole ghosts. The IOVs were then cooled to 0°C, centrifuged and washed once at 4°C in 0.25 mM EDTA, 25 μg/ml PMSF, pH 8.0, and the ^{125}I-spectrin was extracted by incubation overnight in the same medium at 0°C. Both transformed ^{125}I-spectrin fractions (i.e., those derived from the initial bound and free ^{125}I-spectrin dimers) were then subjected to nondenaturing PAGE as described by Liu and Palek (27). The ^{125}I-labeled dimer and tetramer bands were visualized after staining with Coomassie Blue, and excised from the gels, and their relative proportions were determined after counting in a gamma counter.

Spectrin binding to 72,000-D ankyrin fragment. Binding of ^{125}I–72,000-D ankyrin fragment to spectrin was determined by the immunoprecipitation assay of Tyler et al. (28). Various concentrations of ^{125}I-labeled 72,000-D fragment (2.5–30 μg/ml) were incubated for 90 min at 0°C in a 200-μl volume containing 5 μg spectrin and 10 mM sodium phosphate, 1 mM EDTA, 130 mM KCl, 20 mM NaCl, 1 mM DTT, 0.5 mM NaN_3, 0.1% vol/vol Triton X-100, bovine serum albumin (1 mg/ml), pH 7.5. In each experiment, immunoprecipitation with antispectrin and staphylococcus A was monitored by the determination in quadruplicate of the percentage precipitation of ^{125}I-spectrin (from both control and test subjects) under the same conditions as those used for the binding assay. Nonspecific binding of ^{125}I-ankyrin fragment to staphylococci-bearing protein A was 20–30% and was corrected for by subtracting the values obtained when spectrin, but not antispectrin or staphylococcus protein A, was omitted from the assay.

Tryptic digestion of spectrin. Spectrin was extracted by incubating membranes at 4°C for 20 h in 0.1 mM EDTA, pH 8.0. The extracts were adjusted to 10 mM Tris-HCl, 20 mM NaCl, 130 mM KCl, 1 mM mercaptoethanol, pH 8.0, (31) and digested with 1:100 wt/wt TPCK-trypsin for 20 h at 0°C. Digestion was stopped by the addition of PMSF (final concentration 1 mM). The samples were lyophilized and stored at −20°C.

Protein electrophoresis. Analytical SDS-PAGE on tube gels (3% acrylamide) was performed by use of the conditions of Fairbanks et al. (32). Protein concentrations were measured by the method of Lowry et al. (33) or with the protein assay (Bio-Rad Laboratories), using bovine serum albumin as standard. One-dimensional gels stained with Coomassie Blue were scanned with a recording densitometer (SP8-100; Pye Unicam LTD., Cambridge, England). The areas under the spectrin and band 3 peaks were cut out and weighed and the

spectrin/band 3 ratio was determined. Samples digested with trypsin were reconstituted in 9.5 M urea, 2% wt/vol NP-40, 5% mercaptoethanol, and 6.25% ampholytes, pH 4.0–6.5. Prior dialysis to remove salts was omitted, as it does not affect the separation pattern (34). Samples (50 μg protein) were electrofocused for 16 h at 0.1 W/gel with a maximum of 400 V in 4% polyacrylamide gels containing 6.25% carrier ampholytes, pH 4.0–6.5, as described by O'Farrell (34). Second dimension SDS-PAGE was performed on 10% acrylamide slab gels according to Laemmli (35). Immunoblots of unstained 3.5 to 14% gradient SDS-PAGE slab gels of solubilized intact erythrocytes and erythrocyte cytosol (see below) were performed as described by Towbin et al. (36) and Davis and Bennett (37) using a monospecific antispectrin antibody isolated by affinity chromatography of immobilized spectrin. This antibody was kindly provided by Dr. V. Bennett (Johns Hopkins School of Medicine, Baltimore, MD). Absorbed antispectrin was detected by ^{125}I-protein A, and autoradiographs were prepared by use of XO-mat AR film (Eastman Kodak Co., Rochester, NY). Complete solubilization of washed intact erythrocytes was performed as follows: 1 vol of erythrocytes was added to 4 vol of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 25 μg/ml PMSF, pH 7.6) at 0°C. 1 vol of the lysate was then added to 1 vol of solubilizing solution to yield final concentrations of 10 mM Tris-HCl, pH 8.0, 5% sucrose, 1 mM EDTA, 3% SDS, 2% Triton X-100, 2% mercaptoethanol, and 4 M urea, and the solution was boiled for 3 min. This procedure was necessary to prevent high molecular weight aggregates from remaining at the top of the gel. Erythrocyte cytosol was obtained after lysis of 1 vol of packed erythrocytes with 15 vol of lysis buffer, centrifugation at 100,000 *g* for 20 min, and then recentrifugation of the supernate for 20 min at 150,000 *g*. Solubilization of the cytosol was achieved with the same procedure as for intact erythrocytes.

Results

Spectrin content of isolated membranes and intact erythrocytes. Spectrin/band 3 ratios determined by scanning densitometry of SDS-PAGE of isolated membranes of nine controls, and the two probands (CG and AD), their mother, and father are shown in Table I. Both probands show an ~30% decrease in this ratio, with the α- and β-chains of spectrin decreased by an equal amount. The mother and father have ratios within the normal range. SDS-PAGE of membranes of a control, CG, and her parents is shown in Fig. 1. Spectrin content of intact erythrocytes of CG is also decreased by a similar proportion, compared with the control, as determined by scanning densitometry of SDS-PAGE of erythrocyte lysates prepared from equal numbers of erythrocytes (mean of three separate preparations: 68% of control) (Fig. 2). An immunoblot (Fig. 3) of solubilized erythrocytes of CG confirms the decreased spectrin content noted with the Coomassie Blue stain. After prolonged exposure of the autoradiograph, a faintly labeled band (the arrow in Fig. 3) of molecular weight ~28,000 is detectable on immunoblots of control cell lysates. This band is even fainter in CG's lysates and in the control, and CG's cytosol and does not account for the decreased spectrin content of CG's erythrocytes noted on SDS-PAGE.

Table I. Spectrin/Band 3 Ratio (SDS-PAGE of Erythrocyte Membranes)

Subjects	Number of observations*	Mean±SD
Controls	9	1.02±0.06
CG	5	0.67±0.046
AD	1	0.74
Mother	2	0.99
Father	3	0.95

* Each observation refers to a separate membrane preparation.

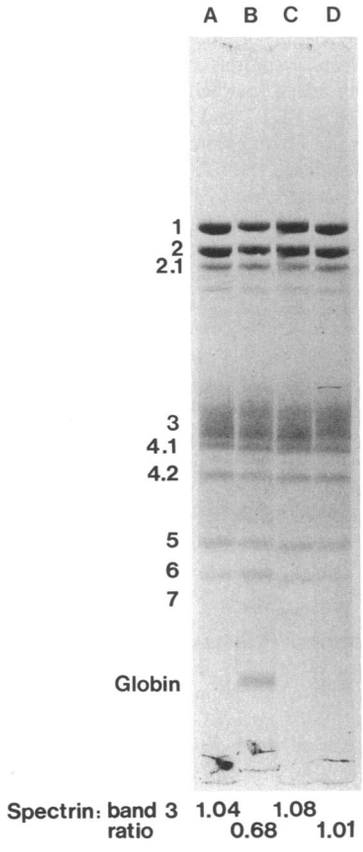

Figure 1. SDS-PAGE of erythrocyte membranes. (*A*) control; (*B*) CG; (*C*) Mother; (*D*) Father. 10 μg protein was loaded on each gel. Spectrin/band 3 ratios are given as subscripts on the gels.

Two-dimensional gel electrophoresis of limited tryptic digests of spectrin. Two-dimensional electrophoresis of tryptic digests of CG's and control spectrin (normal ionic strength medium at 0°C for 20 h) is shown in Fig. 4, *A* and *B*. The 80,000-D peptide is shown by the arrow. No significant difference in the peptide pattern between the control and CG is apparent.

Competitive inhibition of ^{125}I-spectrin binding. The competition of various concentrations of unlabeled spectrin from CG and a control with a constant amount of control ^{125}I-spectrin incubated with control IOVs is shown in Table II. Unlabeled spectrin from CG shows markedly less inhibition of ^{125}I-spectrin binding than does the control at all three concentrations tested. An even more pronounced defect was

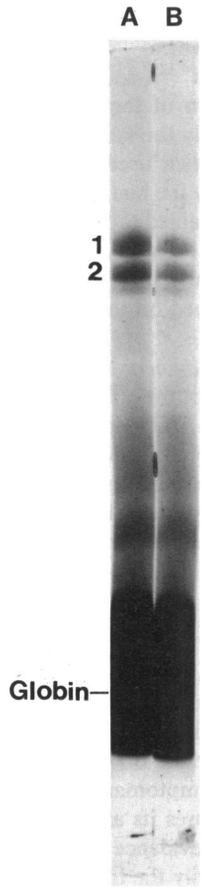

Figure 2. SDS-PAGE of intact erythrocytes. (*A*) control. (*B*) CG. Lysates derived from 2.2×10^7 erythrocytes were loaded on each gel.

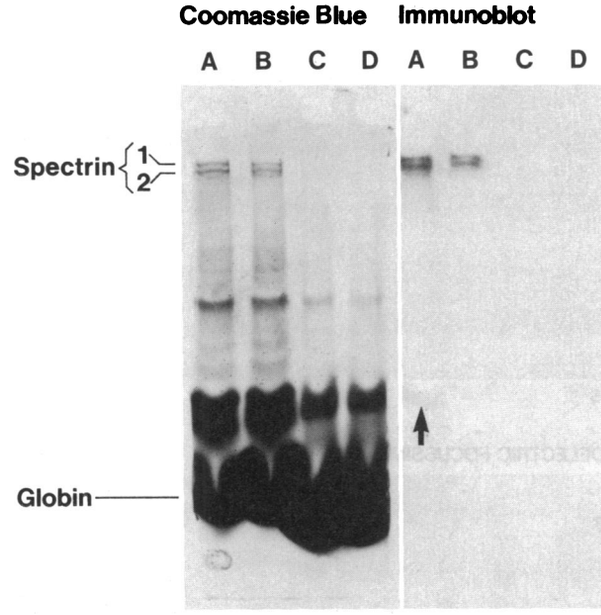

Figure 3. Immunoblots of intact erythrocytes and cytosol. *Left:* Coomassie Blue-stained SDS-PAGE slabs of (*A*) control erythrocytes; (*B*) CG's erythrocytes; (*C*) control cytosol; and (*D*) CG's cytosol. *Right:* Autoradiograph of immunoblot of SDS-PAGE slab. Sequence of labeling *A–D* as for left side. For explanation of arrow, see text.

found using unlabeled spectrin from AD at slightly different concentrations (not shown).

^{125}I-spectrin interaction with IOVs. Increasing amounts of ^{125}I-spectrin dimers from patients or controls were incubated with a constant amount of control IOVs. Three binding isotherms are shown for proband CG (Fig. 5, *A, B,* and *C*), one for proband AD (Fig. 5 *D*), and two for the father (Fig. 5, *E* and *F*) and mother (Fig. 5, *G* and *H*). In all test subjects and their respective controls, spectrin binds to normal IOVs in a saturable manner. The binding of CG's and AD's spectrin is, however, considerably decreased compared with the respective controls and indicates decreased affinity for normal IOVs. The binding of the father's spectrin is also decreased but is not as pronounced as in the probands. The mother has binding curves almost identical to those of the controls. The binding isotherm found in a crossover experiment, in which control spectrin is bound to IOVs prepared from CG's erythrocytes is almost identical to that obtained when simultaneously prepared IOVs from a control are used (data not shown), indicating that there is no abnormality of the ankyrin site in CG's membranes.

The binding isotherm for patient TM is shown in Fig. 5 *I* and is almost the same as the control. TM is a black child with neonatal elliptocytosis, high reticulocyte count (5–7%), and increased thermal sensitivity of erythrocytes. The findings in TM afford a useful additional control for vesicle binding studies of CG's spectrin in that he has a spectrin self-association defect with an abnormal 80,000-D α-subunit but has normal spectrin-IOV binding. The possibility that splenectomy might influence spectrin-IOV interaction was excluded by the finding of a normal binding isotherm of spectrin obtained from a healthy splenectomized subject (not shown). Although the binding curves of control IOV for control spectrin show some variation in different experiments, this may reflect differences in the amount of residual spectrin in the various control IOV preparations, and the variation is similar to that found by Agre et al. (17). However, within an experiment, preparations of spectrin from different controls show remarkably similar binding isotherms.

Self-association of bound and unbound ^{125}I-spectrin. The relationship between the functional abnormality of spectrin-ankyrin interaction and the previously reported spectrin dimer self-association defect in CG (20) was explored by separating the spectrin population that binds to control IOVs with relatively low affinity from the unbound spectrin population and then testing each population for heterodimer self-association. For the unbound spectrin the conversion was done in

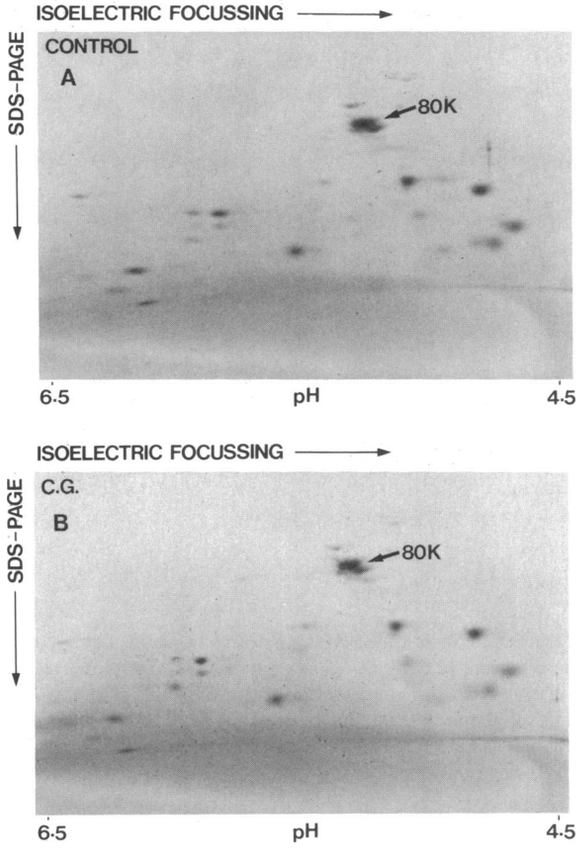

Figure 4. Two-dimensional electrophoresis of limited tryptic digests of spectrin from a control (*A*) and CG (*B*). Spectrin extracts, adjusted to 10 mM Tris-HCl, 20 mM NaCl, 130 mM KCl, 1 mM mercaptoethanol, pH 8.0, were digested with 1:100 wt/wt TPCK trypsin. 50 μg of protein was loaded on the first dimension gel. The 80,000-D α-subunit peptide is indicated by the arrow.

solution, and the bound fraction was transformed on the IOVs. The proportion of ^{125}I-spectrin dimers after conversion of bound and free ^{125}I-spectrin heterodimers is shown in Table III. Bound ^{125}I-spectrin dimers of CG show a striking defect of self-association when compared with control ^{125}I-spectrin dimers in which comparable amounts of ^{125}I-spectrin are bound to IOVs. In contrast, self-association of the free ^{125}I-spectrin heterodimers of CG is approximately the same as that of the control. In a second experiment, which used different incubation conditions, self-association of only the bound dimers was determined, and a similar result was obtained (not shown). These results indicate that the functional defect of spectrin heterodimer self-association is detectable only in a subpopulation of her spectrin that can be distinguished on the basis of binding to IOVs.

Table II. Competitive Inhibition of ^{125}I-Spectrin Binding

	% Inhibition of binding	
Unlabeled spectrin	Control	Patient CG
μg/ml		
25	44.6	8.5
50	64.5	18.0
75	79.5	26.7

1.25 μg ^{125}I-spectrin was incubated with vesicles (20 μg protein) in 0.25 ml for 90 min at 0°C in the presence and absence of unlabeled spectrin.

Spectrin interaction with the 72,000-D fragment of ankyrin in solution. The binding isotherms of control ^{125}I-labeled 72,000-D ankyrin fragment to control and CG's spectrin in solution are shown in Fig. 6. Although binding to control spectrin occurs in a saturable manner, the binding to CG's spectrin increases in a linear fashion and a high affinity interaction cannot be demonstrated. A second experiment shows an almost identical result.

Discussion

In this study we demonstrate a hitherto undescribed abnormality in the interaction of components of the erythrocyte membrane skeleton in a kindred with an atypical variant of HE. Both probands (CG and AD), as well as their asymptomatic father, have an abnormality of spectrin that decreases its affinity for spectrin-depleted IOVs. A large body of evidence (22, 24) indicates that this association reflects primarily the interaction of spectrin with ankyrin (band 2.1). We also demonstrated this defect by use of a purified chymotryptic fragment of ankyrin in one of the probands.

We have previously demonstrated (19) that limited tryptic digestion of the probands' spectrin for 24 h in low ionic strength bicarbonate medium leads to a grossly abnormal peptide pattern on one-dimensional electrophoresis. In normal ionic strength bicarbonate medium, the pattern at 24 h was similar to that of the control (19). CG has also been shown to have an abnormality of spectrin tetramer assembly (20) similar to that described for some patients with HE and HPP, in whom specific defects in the 80,000-D α-subunit peptide of spectrin have been found on two-dimensional gel electrophoresis (11–13). In order to better compare our kindred with similar cases in the literature, it was therefore important to document the limited tryptic digest pattern of CG's spectrin with the use of the higher resolution of the two-dimensional technique. With this technique we found no significant abnormality in the tryptic digest of CG's spectrin when we used a Tris buffer at normal ionic strength (Fig. 4, *A* and *B*). This indicates that the molecular defect responsible for altered spectrin tetramer assembly in this kindred is different from previously reported cases in the literature, although it is possible that a defect could still exist in the 80,000 α-subunit domain that would

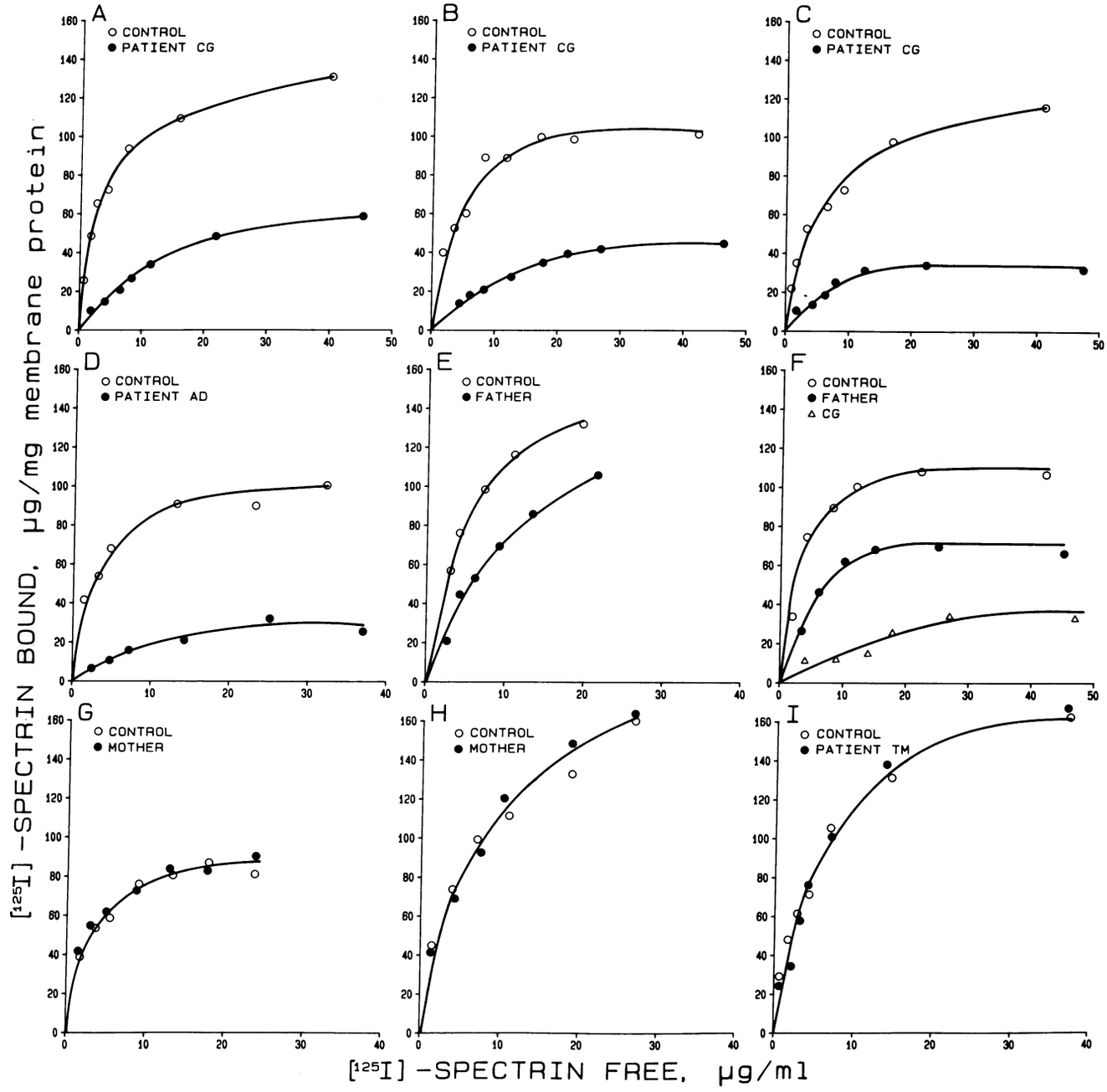

Figure 5. Binding of ^{125}I-spectrin to spectrin-depleted IOVs. Various concentrations of ^{125}I-spectrin from controls and patients were incubated in parallel with a constant amount of IOVs from a different control. Incubation conditions and determination of specific binding are described in Methods. (*A–C*) patient CG; (*D*) patient AD; (*E* and *F*) father of probands; (*G* and *H*) mother of probands; and (*I*) patient TM.

not affect its cleavage under conditions of limited tryptic proteolysis.

An important finding in the present study relates to the spectrin/band 3 ratio in both probands, which is diminished by ~30% (Table I and Fig. 1), and to the similarly decreased spectrin content of intact erythrocytes of CG when determined by SDS-PAGE (Fig. 2). Immunoblots of intact erythrocytes or cytosol of CG, although revealing a very faint band corre-

Table III. Self-Association of Bound and Unbound ^{125}I-Spectrin Dimers

	^{125}I-spectrin in medium	Bound ^{125}I-spectrin ($\mu g/mg$) IOV protein*	% Dimers after conversion of bound spectrin	% Dimers after conversion of unbound spectrin
	$\mu g/ml$			
Control	10	80	22.0	
	20	141	16.7	
	40	178	12.6	52.6
Subject CG	40	110	51.5	48.3

* The bound ^{125}I-spectrin has not been corrected for nonspecific binding, as the total bound ^{125}I-spectrin was subjected to conversion. After incubation at 0°C for 90 min the bound and unbound spectrin dimers were separated and transformed to tetramers (see Methods).

sponding to a molecular weight of ~28,000 (Fig. 4), could not account for the decreased spectrin content of her erythrocyte spectrin found on SDS-PAGE, since the same band was more prominent in a control lysate. The latter finding also excludes the possibility that the labeled band of molecular weight ~28,000 is a marker for the altered functional properties of CG's spectrin. Although we are unable to detect spectrin fragments on immunoblots of CG's lysates that would account for the decreased spectrin content of her cells on SDS-PAGE,

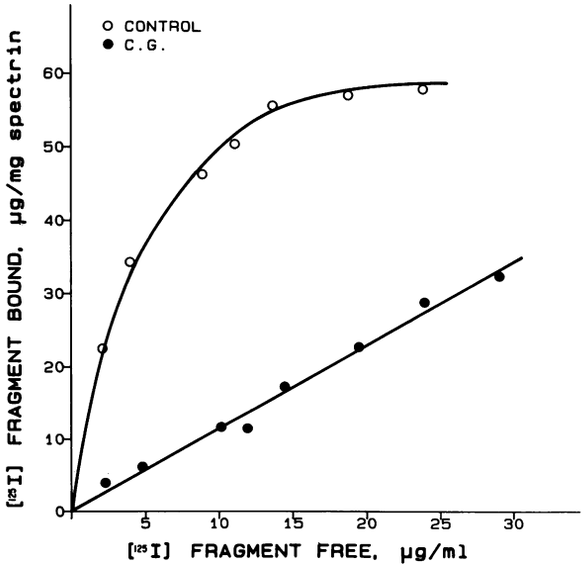

Figure 6. ^{125}I-labeled 72,000-D fragment interaction with spectrin. Various concentrations of ^{125}I-labeled 72,000-D fragment of ankyrin were incubated with 5 μg spectrin heterodimer from a control (○) and patient CG (●). Incubation conditions and determination of specific binding by immunoprecipitation are described in Methods.

it is possible that spectrin could be proteolyzed to peptides too small to permit detection, or that the antibody used for the immunoblot might have a relatively poor affinity for putative fragments that might be irreversibly denatured by the procedure used to solubilize her erythrocytes. We also cannot exclude the possibility of decreased synthesis of erythrocyte spectrin in the probands.

Our initial finding of a decreased membrane spectrin/band 3 ratio in the probands prompted us to study spectrin-ankyrin interaction in this kindred. Both probands show a marked decrease of spectrin affinity for normal IOVs (Fig. 5), and a crossover experiment excludes a defect of ankyrin in one of the probands. This defect of spectrin is also confirmed by our finding a decreased affinity of normal ^{125}I-ankyrin for CG's spectrin (Fig. 6) and by impaired inhibition of binding of normal ^{125}I-spectrin to IOVs (Table II). The decrease in the affinity of the probands's spectrin for control IOVs is also found in the father but is not as severe as in the probands. The mother's spectrin, however, shows normal affinity for IOVs. Since the clinical disorder is inherited in recessive fashion, some unknown factor in the mother probably influences the quantitative expression of the defect in the probands. In this regard it is also of interest to note that although the spectrin of CG's father has a decreased affinity for IOV that is intermediate between the control and the probands, his membranes do not show a decrease in the membrane spectrin/band 3 ratio. Decreased membrane or whole cell spectrin appears to manifest only in association with full expression of the functional spectrin defect.

We have previously reported that one of the probands (CG) has defective spectrin heterodimer self-association (20) and also abnormal spectrin phosphorylation in ghosts but not in intact erythrocytes (19). The other proband (AD) also has a defect of spectrin dimer self-association (Coetzer and Zail, unpublished observations), but spectrin phosphorylation studies have not been done on this subject. In the present study we show that both probands also have a defect in the binding of spectrin dimers to IOVs. It is thus of interest to determine how these defects relate to each other in molecular terms. Are all of the three functional alterations related to one molecular defect of spectrin or are there subpopulations of spectrin, each with a distinct molecular defect? We approached this problem by separating the spectrin population of proband CG that binds to control IOVs with relatively low affinity from the free or unbound spectrin. These two populations were then tested for spectrin heterodimer self-association. We have not tested these subpopulations for spectrin phosphorylation. The results shown in Table III indicate that the spectrin dimer self-association defect is detectable only in a subpopulation of CG's spectrin that binds to control IOVs and is not found in the unbound spectrin population. This is surprising, as one might expect these two functional defects to be related to one molecular defect of spectrin, particularly since both of these functions of spectrin have been linked to limit tryptic peptides

that spatially adjoin each other at the phosphorylated end of the β-chain (4).

The probands' spectrin-IOV binding defect does not correlate with splenic function. The present studies, however, do not exclude the possibility of a postsynthetic modification of spectrin, as we have not tested spectrin-ankyrin interaction in the light and dense erythrocyte fractions from the probands. The exact mechanism whereby the functional defects of spectrin in the probands lead to the elliptocytic shape of the erythrocytes is unknown. It is interesting, however, to note the preliminary studies of Platt and her co-workers (38), which show that sickle cells develop an abnormality of spectrin-ankyrin interaction, manifesting as a defect in high affinity spectrin binding sites of IOVs, and that the irreversibly sickled cells, which most closely resemble elliptocytes in shape, are the most severely affected.

Acknowledgments

We acknowledge the expert technical assistance of Mr. S. Lambert. We are indebted to Professor L. Macdougall for permission to study her patient, to Mrs. S. Woods for typing the manuscript, and to the Photographic Unit of The South African Institute for Medical Research for the photographic material.

This study was supported by a grant from the Medical Research Council of South Africa and the Atomic Energy Corporation.

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