Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2072–2076, March 1982
Medical Sciences

Defective spectrin dimer–dimer association in hereditary elliptocytosis

(spectrin transformation/membrane skeleton)

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Communicated by Ernest Beutler, December 30, 1981

ABSTRACT **We examined erythrocytes from 18 patients with hereditary elliptocytosis. Spectrin from eight patients (referred to as type 1) was defective in dimer–dimer association as demonstrated in two ways. First, there was an increased amount of spectrin dimer with a concomitant decrease in tetramer as measured in erythrocyte membrane preparations extracted at 0°C under low-salt conditions (the amount of spectrin dimer was 15–33% of total spectrin species compared with a normal range of 3–7%). Second, the equilibrium constants of spectrin dimer–dimer association were decreased in both solution and *in situ* membrane. Spectrin from the remaining 10 patients (referred to as type 2) showed normal dimer–dimer association. Membrane skeletons, produced from ghosts of both types of hereditary elliptocytosis by Triton X-100 extraction, were unstable when mechanically shaken. Because spectrin tetramers, but not dimers, can crosslink actin, we postulate that the defective spectrin dimer–dimer association in type 1 diminishes actin crosslinking and thus is responsible for membrane skeletal instability. A defective protein–protein association in type 2, however, remains to be identified.**

Spectrin, together with actin, band 4.1, and some minor components, forms a two-dimensional submembrane skeletal network that is thought to stabilize the membrane structure and maintain the discoid shape of normal erythrocytes (for reviews, see refs. 1 and 2). Recent studies suggest that membrane skeletal defects are involved in several erythrocyte disorders, including hereditary spherocytosis, pyropoikilocytosis, and elliptocytosis (2–5). In patients who have hereditary elliptocytosis (HE), it has been found that membrane skeletons derived from HE erythrocytes retain their abnormal elliptic shape and that purified spectrin from some, but not all, is abnormally heat sensitive (2). However, the pathogenesis of this disease is not understood. In a preliminary examination, we have observed a decreased stability of HE membrane skeletons on mechanical shaking. In a subpopulation of HE patients, this was associated with a concomitant decrease in spectrin tetramers and increase in spectrin dimers (6). We now demonstrate in these patients a defective association of spectrin dimers into tetramers in both the membrane and solution and point out the heterogeneity of such a defect among unrelated HE patients.

EXPERIMENTAL PROCEDURES

Clinical Material. We have studied 18 HE patients from 13 unrelated families. Blood films from all subjects showed 60% or more elliptical erythrocytes. Clinical review suggested minimal or absent hemolysis. Patients were 1.5–82 years old.

Venous blood from normal and HE patients was collected in sterile tubes containing citrate/phosphate/dextrose or citrate/dextrose. Specimens that were not obtained at St. Elizabeth's Hospital, Boston, were transported in insulated containers with ice to Boston where they were kept at ≈4°C and analyzed no more than 3 days later.

Spectrin Extraction. Ghosts were prepared from washed erythrocytes by the method of Dodge *et al.* (7). For low-ionic-strength extraction, erythrocyte ghosts were washed with 0.1 mM $NaPO_4$ (pH 8.0) and immediately centrifuged to reduce the ionic strength of the medium and obtain a smaller pellet. Ghosts were then incubated at 0°C (or at 37°C for the study of spectrin transformation in solution) with an equal volume of low-ionic-strength buffer containing 0.1 mM $NaPO_4$/0.1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride ($BzlSO_2F$)/0.1 mM N^{α}-tosyl-L-lysine chloromethyl ketone·HCl (Tos-$LysCH_2Cl$)/0.1 mM diisopropylfluorophosphate (iPr_2P-F)/0.1 mM 2-mercaptoethanol, pH 8.0. After incubation, the supernatant extracts and ghost residues were separated by centrifugation at 250,000 × *g* for 35 min.

Transformation of Spectrin Dimer to Spectrin Tetramer in Solution. Spectrin dimer in the 37°C extract was tested for ability to associate into spectrin tetramer in solution by incubation of the extract (0.2–0.8 mg/ml) at 30°C under isotonic conditions (5 mM $NaPO_4$, pH 7.4/150 mM NaCl/1 mM 2-mercaptoethanol/0.1 mM $BzlSO_2F$/0.1 mM iPr_2P-F/0.1 mM Tos-$LysCH_2Cl$/0.1 mM EDTA) (3). The mixture was subsequently analyzed by nondenaturing agarose/polyacrylamide gel electrophoresis (3).

Transformation of Spectrin Dimer to Spectrin Tetramer in the Membrane. To induce spectrin tetramer–dimer transformation in the membrane, ghosts were incubated hypotonically at 37°C for 20 min in 5 mM $NaPO_4$, pH 7.4/15 mM NaCl/0.1 mM $BzlSO_2FCl$/0.1 mM Tos-$LysCH_2Cl$/0.1 mM EDTA/0.1 mM iPr_2P-F/1 mM 2-mercaptoethanol (8). To reverse the transformation, ghosts were incubated in isotonic buffer/150 mM NaCl for 30 min at 37°C (8). Subsequently, crude spectrin was extracted (0°C, overnight) from these ghosts and analyzed for spectrin species by nondenaturing agarose/polyacrylamide gel electrophoresis (3).

Nondenaturing Agarose/Polyacrylamide Gel Electrophoresis. The low-ionic-strength extracts (≈15 μg of protein) were mixed with one-ninth volume of concentrated buffer to give a final solution of 40 mM Tris·HCl, pH 7.4/20 mM NaOAc/2 mM dithiothreitol/200 mM sucrose/2 mM EDTA and subjected to electrophoresis in 0.3% agarose/2.5% acrylamide gels as described (9) except that the electrophoresis temperature was 2–6°C and $NaDodSO_4$ was omitted from the gels. In this gel system, spectrin tetramers (M_r, 900,000) and dimers (M_r, 450,000) were separated into distinct bands with mobilities rel-

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Abbreviations: HE, hereditary elliptocytosis; $BzlSO_2F$, phenylmethylsulfonyl fluoride; Tos-$LysCH_2Cl$, N^{α}-tosyl-L-lysine chloromethyl ketone·HCl; iPr_2P-F, diisopropylfluorophosphate.

ative to bromophenol blue of ≈0.13 and 0.23, respectively.

The use of agarose/polyacrylamide composite gels in nondenaturing gel electrophoresis was particularly suited for quantitative analysis of spectrin species in the extracts. These composite gels could be prepared in large quantity and stored in the cold room for weeks. Large numbers of samples could be analyzed in parallel with good reproducibility. The relatively high porosity of these composite gels allowed the high molecular weight complexes to enter the gel. In addition, acrylamide was polymerized 1 to 2 mm below the agarose surface, yielding an extra layer of agarose at the top of the gel to trap the complexes during staining and destaining procedures. After electrophoresis, no protein was left in the sample layer above the gel. For quantitative estimation of protein complexes near the tops of the gels, gels were scanned at 545 nm in glass tubes (1.2 × 12 cm) filled with 10% acetic acid. The artifactual absorption at the edge of the gel, as measured in a gel blank, was <5% of that from high molecular weight complexes in a typical extract sample.

RESULTS

Spectrin Species in the 0°C Extracts from Fresh Erythrocyte Membranes. Crude spectrin extracted at 0°C from normal erythrocyte ghosts contained 56 ± 8% spectin tetramer, 39 ± 8% high molecular weight complexes that barely entered the gel, and a small amount (5 ± 2%) of spectrin dimer (Fig. 1 and Table 1), as measured by densitometric scans of Coomassie blue-stained agarose/polyacrylamide gels. This result is similar to that obtained previously by gel filtration on a 4% agarose column (8, 10, 11). In contrast, crude spectrin extracted from HE erythrocyte ghosts under identical conditions showed heterogeneity in the amounts of spectrin dimer. Eight out of 18 HE patients exhibited a marked increase in spectrin dimer (17–33%) with a concomitant decrease in spectrin tetramer, while the other patients had normal amounts of spectrin tetramer and spectrin dimer (Fig. 1 and Table 1). HE patients with increased amounts of spectrin dimer were designated type 1, while those with normal amounts of spectrin dimer were designated type 2. Statistical analysis of the differences in amounts of spectrin dimer between controls, type 1 HE, and type 2 HE (see Fig. 4*A*) shows that the increase in spectrin dimer in type 1 HE was highly significant ($P < 0.001$).

Analysis of the data in Table 1 shows that the decrease in spectrin tetramer that accompanied the increase in spectrin dimer in type 1 HE patients was not related to an increased propensity of spectrin tetramer to associate with the high molecular weight complex. Although the amounts of complex exhibited some variability (range: type 1 HE, 30–44%; type 2 HE, 25–41%), there were no differences in the amounts of high molecular weight complex measured in the control, type 1 HE, and type 2 HE groups. This conclusion is further supported by a lack of correlation between spectrin tetramer and high molecular weight complex among the patients studied.

Occasionally, we observed in the 0°C extracts from both normal and HE ghosts several slow-migrating bands corresponding to spectrin oligomers—e.g., hexamers, octamers, and decamers, similar to those reported recently by Morrow and Marchesi (12) in studies of spectrin association in solution. The amount of spectrin oligomers was found to increase after long (2 to 3 days) extraction of ghosts in low-ionic-strength-buffer at 0°C or in the absence of protease inhibitors during extraction (data not shown). The variability in the amount of spectrin oligomers in the extracts, nevertheless, did not affect the consistent observation of the dramatic increase in spectrin dimer in the extracts from type 1 HE patients. In addition, the increase of spectrin dimer in these patients was apparent with extraction

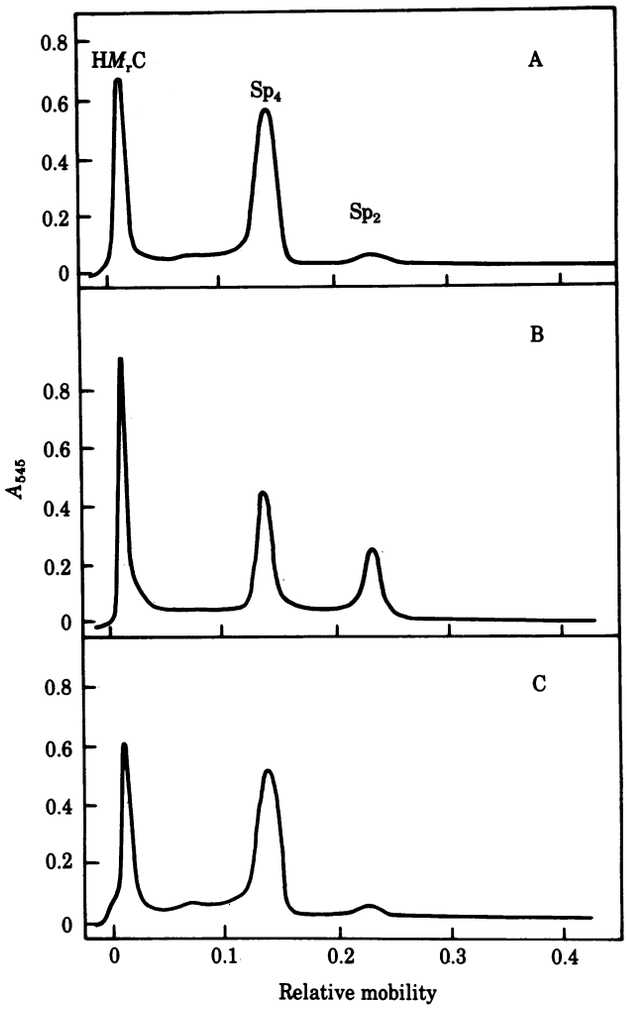

FIG. 1. Quantitative analysis of spectrin species in low-salt extracts (0°C, overnight) of erythrocyte membranes from normal individuals and HE patients. Low-salt extracts were prepared and analyzed by nondenaturing 0.3% agarose/2.5% polyacrylamide gel electrophoresis in the absence of $NaDodSO_4$ as described (3). Densitometric tracings of gel samples from a representative normal individual (*A*), a type 1 HE patient (M.S.) (*B*), and a type 2 HE patient (J.M.) (*C*) are shown. HM_rC, high molecular weight complex; Sp_4, spectrin tetramers; Sp_2, spectrin dimers. Patient M.S. (type 1 HE) shows a marked increase in spectrin dimer (20 ± 6%) as compared with the amount of spectrin dimer (5 ± 2%) in normal individuals and patient J.M. (type 2 HE).

times as short as 2 hr. Under these conditions, 40–60% of spectrin was eluted from the membrane containing no detectable spectrin oligomers (data not shown).

Association of Spectrin Dimers to Tetramers in Solution. To study the possibility that the increased spectrin dimer/spectrin tetramer ratio in the erythrocyte membrane extracts of type 1 HE patients results from a defect in spectrin self-association in these cells, we examined the ability of spectrin dimer from various patients to associate into spectrin tetramer in solution. This equilibrium process has been shown to be influenced by ionic strength, pH, and temperature (10). We extracted crude spectrin from ghosts in low-ionic-strength-buffer at 37°C, a temperature at which spectrin tetramer is mostly dissociated into spectrin dimer. Then, we restored the extract to isotonicity (150 mM NaCl) and incubated it at 30°C. The kinetics of the spectrin dimer–spectrin tetramer transformation in the crude spectrin extracts is shown in Fig. 2. Equilibrium of the dimer–tetramer transformation was achieved after 3 to 4 hr of incubation. The

Table 1. K_a of spectrin dimer–dimer association in HE erythrocyte membranes

Subject	Race	Clinical presentation	Dimer, %	Tetramer, %	Complex, %	Apparent K_a, M^{-1}	Determinations, no.
Normal (n = 15)			5 ± 2	56 ± 8	39	1–7 × 10^5	28
Type 1 HE*							
Patient W.W.	Black	HPP → HE†	26 ± 4	39 ± 5	35	6 × 10^3	3
Family D‡							
Proband (J.D.)	Black	HPP → HE†	25 ± 7	38 ± 4	37	6 × 10^3	3
Sister (D.D.)	Black	HE	20	40	40	3 × 10^4	1
Patient B.H.	White	HE	17 ± 3	48 ± 2	35	2 × 10^4	3
Family S							
Proband (M.S.)	Black	HE	20 ± 6	36 ± 8	44	9 × 10^3	5
Grandnephew (V.S.)	Black	HE	25 ± 7	32 ± 4	43	5 × 10^3	2
Grandniece (Z.S.)	Black	HE	33	37	30	3 × 10^3	1
Great grandniece (T.S.)	Black	HE	32	35	33	3 × 10^3	1
Type 2 HE*							
Patient T.A.	Black	HE	6 ± 3	53 ± 4	41	1 × 10^5	4
Patient M.P.	White	HE	5 ± 2	70 ± 4	25	3 × 10^5	2
Patient B.R.	Black	HE	3 ± 1	59 ± 1	38	6 × 10^5	2
Patient J.M.	White	HE	4 ± 1	61 ± 8	36	4 × 10^5	4
Family L							
Proband (J.L.)	Black	HE	7 ± 2	52 ± 4	41	1 × 10^5	3
Mother (A.L.)	Black	Asymptomatic	6 ± 2	56 ± 2	38	2 × 10^5	3
Sister (D.L.)	Black	HE	6 ± 2	55 ± 3	39	2 × 10^5	3
Brother (C.L.)	Black	Asymptomatic	6 ± 3	59 ± 4	35	3 × 10^5	3
Patient A.M.	Black	HE	4 ± 2	60 ± 4	36	1 × 10^5	2
Patient G.B.	White	HE	4	56	40	3 × 10^5	1
Patient R.P.	White	HE	4	56	40	3 × 10^5	1
Patient E.W.	Black	HE	5	69	25	3 × 10^5	1

Crude spectrin was extracted overnight from fresh untreated ghosts in low-ionic-strength buffer at 0°C and analyzed by nondenaturing gel electrophoresis. Results are expressed as mean ± SD (dimer and tetramer), mean (high molecular weight complexes), range (K_a for normal individuals), or mean of 1–5 determinations (each HE patient).

* Unrelated patients with mild HE with no or minimal hemolysis.

† HPP (hereditary pyropoikilocytosis)-like presentation (3) in the first year of life, subsequently converted to mild HE.

‡ Amounts of spectrin dimer and spectrin tetramer in the extracts from these patients were determined by gel filtration in an agarose column (8) instead of nondenaturing gel electrophoresis.

amount of high molecular weight complex in the 37°C extract did not change during isotonic incubation at 30°C for up to 4 hr, suggesting that these complexes were not involved in the spectrin dimer–spectrin tetramer transformation in solution. Based on the equilibrium concentrations of spectrin dimer and spectrin tetramer in the extracts after 4 hr of isotonic incubation at 30°C (Fig. 2), the K_a of spectrin dimer–dimer association in solution was estimated as follows: 7 ± 2 × 10^5 M^{-1} for normal, 3.5 ± 1.0 × 10^5 M^{-1} for patient M.S. (type 1 HE), and 7 ± 2 × 10^5 M^{-1} for patient J.M. (type 2 HE). These association constants were independent of the protein concentration used in our studies (0.2–0.8 mg/ml). Studies of four other type 1 HE patients (W.W., V.S., Z.S., and T.S.; see Table 1) showed a similar defect in spectrin dimer–dimer association in solution with K_a values of 2–5 × 10^5 M^{-1} while, for three type 2 HE patients (B.R., J.L., and D.L.), the K_a values were normal.

Spectrin Dimer–Tetramer Transformation in the Membrane. We further explored whether spectrin in type 1 HE patients transformed abnormally at high local concentration and in the membrane *in situ*. This was done by first incubating ghosts under hypotonic conditions to transform spectrin tetramer in part into spectrin dimer (8) and then determining the ability of spectrin dimer to reverse to spectrin tetramer by reincubation of the ghosts under isotonic conditions. The equilibrium concentrations of spectrin tetramer and spectrin dimer were measured from 0°C extracts derived from these incubated ghosts. When ghosts from normal, type 1 HE (patient M.S.), and type 2 HE (patient J.M.) erythrocytes were incubated in 5 mM Na phosphate, pH 7.4/15 mM NaCl at 37°C for 20 min, the amount of spectrin tetramer decreased while the amount of spectrin dimer increased proportionately (Fig. 1 and Fig. 3 *Left*). On subsequent restoration of isotonicity and incubation of ghosts at 37°C for 30 min, spectrin dimer in normal and type 2 HE ghosts reassociated to spectrin tetramer. In contrast, spectrin dimer in type 1 HE ghosts reversed to spectrin tetramer only partially (Fig. 3 *Right*) and reached approximately the same spectrin dimer/spectrin tetramer ratio as in the original untreated ghosts (Fig. 1). Studies of other patients (type 1 HE, W.W., V.S., Z.S., and T.S.; type 2 HE, B.R. and J.L.; see Table 1) gave similar results, indicating an abnormal spectrin dimer–spectrin tetramer transformation in the membrane of type 1 HE, but not type 2 HE, patients.

Prolonged (up to 1 hr) incubation of HE ghosts in isotonic buffer had no further effects on the spectrin dimer–spectrin tetramer transformation. The amount of high molecular weight complexes did not change significantly during hypotonic or isotonic incubation of normal or HE ghosts, suggesting that these complexes were not involved in the spectrin tetramer–spectrin dimer transformation. Assuming that the spectrin concentration in normal and HE ghosts is ≈230 mg/ml (2), the K_a of spectrin dimer–dimer interaction in the membrane at physiological conditions can be determined from the equilibrium concentrations of spectrin tetramer and spectrin dimer as measured in the crude extracts of isotonic-buffer-reincubated ghosts (Fig. 3 *Right*). Since such equilibrium concentrations are nearly identical to those found in cold extracts from fresh untreated ghosts (Fig. 1), we estimated the apparent K_a values for types 1 and 2 HE directly from the 0°C extract of untreated ghosts. The data

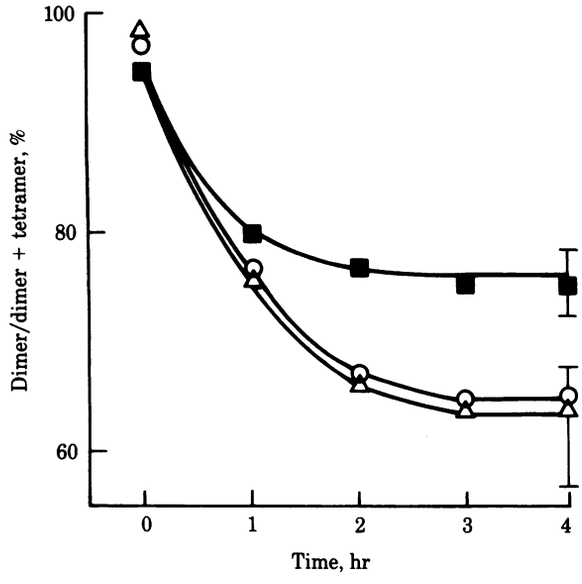

FIG. 2. Time dependence of spectrin dimer–tetramer conversion in solution at 30°C. Crude spectrin enriched in dimers was extracted at 37°C from normal and HE erythrocyte membranes in low-salt buffer. The extracts were restored to isotonic condition and adjusted to a protein concentration of 0.38 mg/ml. Spectrin dimer–spectrin tetramer conversion was carried out by incubation at 30°C for 0–4 hr. Aliquots were removed during the incubation and analyzed by nondenaturing agarose/polyacrylamide gel electrophoresis. Spectrin dimer was estimated from densitometric tracings of gel samples and plotted against incubation time. The degree of reversal of spectrin dimer prepared from patient M.S. (type 1 HE; ■) was less than those from a normal individual (△) or from patient J.M. (type 2 HE; ○). Estimated K_a values of spectrin in solution are $3.3 \times 10^6\ M^{-1}$ for patient M.S. and $6 \times 10^5\ M^{-1}$ for the normal individual and patient J.M., based on equilibrium concentrations.

are summarized in Table 1 and Fig. 4*B*. Striking differences are evident (type 1 HE patients $K_a = 3\text{–}20 \times 10^3\ M^{-1}$; normal individuals and type 2 HE patients, $K_a = 1\text{–}7 \times 10^5\ M^{-1}$).

DISCUSSION

We have previously shown that HE membrane skeletons exhibit marked mechanical fragility (6). This instability is not due to the elongated shape of HE skeletons because elongated membrane skeletons from irreversibly sickled cells are mechanically stable. We now show that, in a subpopulation of HE individuals (referred to as type 1), defective spectrin dimer–dimer association may be responsible for the skeletal instability (Fig. 2 and Table 1). In light of recent electron microscopic and biochemical evidence that spectrin tetramer of normal erythrocytes is formed by head-to-head association of two spectrin dimers (13) and that spectrin tetramer, but not spectrin dimer, can crosslink actin filaments (14–17), it is conceivable that, in type 1 HE membrane skeletons, the weakened crosslinking of spectrin to actin due to defective spectrin self-association may directly underlie the structural instability. In fact, we have recently shown that partial transformation of spectrin tetramer to spectrin dimer in normal erythrocyte membrane diminishes the stability of the membrane skeleton (8). In addition, a similar, although more profound, defect in spectrin dimer–dimer association has been found in another membrane disorder, hereditary pyropoikilocytosis (3).

However, in other HE individuals (type 2) examined by us, the spectrin dimer–dimer association appeared to be normal. The exact molecular defect responsible for the skeletal instability in the latter group of HE subjects is yet to be identified.

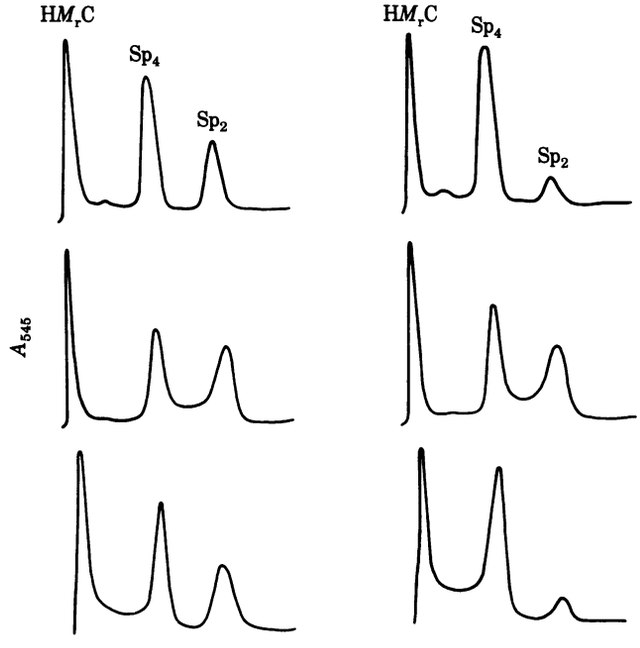

FIG. 3. Spectrin dimer (Sp_2)–tetramer (Sp_4) transformation in the membrane. Ghosts were incubated in 5 mM $NaPO_4$, pH 7.4/15 mM NaCl at 37°C for 20 min to initiate spectrin tetramer–spectrin dimer transformation (8) and then reincubated in isotonic buffer (150 mM NaCl) at 37°C to reverse the transformation. Equilibrium was reached after 30 min of incubation. Ghosts were subsequently washed and extracted overnight with low-salt buffer at 0–4°C. Spectrin species in the extracts from hypotonic-buffer-incubated ghosts (*Left*) and isotonic-buffer-incubated ghosts (*Right*) were analyzed by nondenaturing agarose/polyacrylamide gel electrophoresis and densitometry. Note the defective reversal of spectrin dimer in type 1 HE (patient M.S.; *Middle*) compared with type 2 HE (patient J.M.; *Bottom*) and normal (*Top*) erythrocyte membranes. HM$_r$C, high molecular weight complexes.

None of these patients except one individual exhibited a quantitative decrease in band 4.1 as recently identified in two HE patients (5, 18). Since HE is a clinically heterogeneous disorder

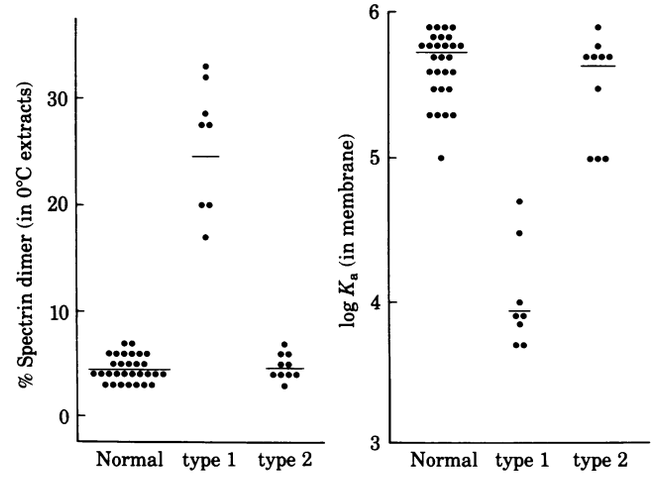

FIG. 4. Heterogeneity of spectrin dimer enrichment (*A*) and K_a values (*B*) in normal, type 1 HE, and type 2 HE erythrocytes. Spectrin dimer in 0°C low-salt extracts from fresh untreated ghosts were estimated as described in Fig. 1. K_a values for spectrin dimer–dimer association in the membrane were estimated as described in Table 1. Note the relatively large ranges among type 1 HE patients and that there are no overlaps of values between type 1 HE patients and type 2 HE patients or normal individuals. ——, Average values for group.

(2), it is likely that diverse alterations of membrane skeletal protein interactions (including those of spectrin dimer–dimer association) may underlie instability and possibly the shape alterations of erythrocytes in this disorder. In fact, recent limited tryptic mapping studies of HE spectrin by Coetzer and Zail (19) and by us (20) show considerable differences in the pattern of tryptic digests, further indicating a molecular heterogeneity of this disorder.

Patients in this study have been classified into types 1 and 2 on the basis of the relative proportion of spectrin dimer in 0°C extracts (Table 1), rather than on the basis of the ability of their spectrin dimer to reassociate into spectrin tetramer in solution. There are two main reasons for separating them this way. (*i*) Spectrin was found to undergo reversible transformation in solution as well as in the membrane. Thus, the distribution of spectrin species in 0°C extracts may directly correlate with the equilibrium of spectrin species *in vivo*. (*ii*) The relatively simple procedure of 0°C extraction followed by nondenaturing gel electrophoresis allows the detection of even a slight increase of spectrin dimer (e.g., 5%) in the extracts. In contrast, a slight abnormality may not be obvious in the reassociation study without extensive statistical analysis. In light of the complexity and heterogeneity of HE patients, it is still possible that some type 2 patients may have slightly altered spectrin dimer–dimer association that is beyond the detection limit of current methods. Because of different experimental conditions, it is also possible that alterations in spectrin species measured in 0°C extracts may not always correlate with the results of spectrin reassociation in solution or in isolated membranes. It has been shown that spectrin dimer–dimer association in solution is an intrinsic property of the molecules involving no regulatory components (10), but it is still not known whether this is the case in intact cells.

The defective association of spectrin dimers to tetramers in type 1 HE erythrocytes could result from various possible modifications of this protein. The most likely one, supported by preliminary results of limited tryptic digests of HE spectrin (19, 20), is an amino acid substitution near the dimer–dimer contact region. This region has recently been shown to involve an 80,000-dalton tryptic peptide located at the proximal end of the spectrin α chain (21). On the other hand, altered posttranslational modification of spectrin, associated with the state of phosphorylation, proteolytic damage, methylation, or acetylation appears highly unlikely, although it cannot be rigorously excluded at present. However, to date, no evidence indicates that spectrin phosphorylation is involved in dimer–tetramer transformation in normal erythrocyte either in solution (10) or in the membrane (8).

We thank Drs. M. L. Snyder and R. Sullivan for permission to study their patients. We thank James Monroe and Nelson Chu for technical assistance. This work was supported by National Institutes of Health Grants HL 15157, HL 27215, and F32-HL05557.

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