Combined Spectrin and Ankyrin Deficiency Is Common in Autosomal Dominant Hereditary Spherocytosis

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The common autosomal dominant form of hereditary spherocytosis (HS) has been genetically linked to defects of the erythroid ankyrin gene in a few families; however, the frequency of ankyrin deficiency and its relationship to red blood cell (RBC) spectrin content are unknown. To test these questions, we measured RBC spectrin and ankyrin by radioimmunoassay in 39 patients from 20 families with dominant HS. Normal RBCs contained 242,000 ± 20,500 spectrin heterodimers and 124,500 ± 11,000 ankryns per cell. In dominant HS, RBC spectrin and ankyrin ranged from about 40% to 100% of normal and were continuously distributed. Measurements in the same patient on different occasions were reproducible (± 5% to 10%) and RBCs from affected members of a kindred contained similar amounts of spectrin and ankyrin (± 3% to 4%). Spectrin and ankyrin levels were almost always less than the assay controls, but were less than the normal range in only 75% and 80% of kindreds, respectively. Remarkably, the degree of RBC spectrin and ankyrin deficiency was very similar in 19 of 20 HS kindreds. One otherwise typical family differed, with marked ankyrin deficiency (45% of control) and a relatively mild spectrin defect (81%). We conclude that most patients with dominant HS have combined ankyrin and spectrin deficiency and that the two proteins are usually about equally deficient, suggesting that defects in ankyrin expression, ankyrin stability, or ankyrin band 3 (AEI) interactions may be common in dominant HS.

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an hematocrit of about 10%. The RBCs were counted with a Technicon H2 instrument (Tarrytown, NY) and the sample was stored at -80°C until used.

Preparation and iodination of spectrin and the 62-kD domain of ankyrin. Spectrin and the 62-kD spectrin binding domain of ankyrin were purified from normal erythrocytes as described. Their concentrations were determined at 280 nm using a measured extinction coefficient (E1%1cm = 1.07 for spectrin and calculated extinction coefficients, E1%1cm = 5.550 [no. of Trp/mol] + 1.340 [no. of Tyr/mol] + 150 [no. of Cys/mol]) × molecular weight (g/mol) (W.B. Grazer and J. Finder, personal communication), of 0.57 for ankyrin and 0.60 for 62-kD ankyrin. Pure proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were radiolabeled with [125]I to a specific activity of about 5 × 106 cpm/μg using Bolton-Hunter reagent (New England Nuclear, Boston, MA) and stored at 0°C at a concentration of about 0.1 mg/mL in radioimmunoassay (RIA) buffer (see below) for up to 3 weeks.

Preparation of antibodies. Polyclonal antispectrin antibodies (nos. 8000 and 8412) were raised in rabbits against a mixture of native and SDS-denatured α- and β-spectrin. They stained both spectrin subunits in immunoblots of human RBC ghosts (1:2,000 to 1:15,000 dilution) and did not react with purified ankyrin. Polyclonal rabbit antiankyrin antibodies (nos. 7000, 7608, and 7609) were developed using a mixture of native and SDS-denatured ankyrin and its 62-kD spectrin binding fragment. Antiserum nos. 7608 and 7609 are high titer antiserum and are effective at dilutions of 1:50,000 to 1:100,000. A dilution of 1:40,000 they detect ankyrin (band 2.1, 206 kD) and numerous ankyrin subspecies (ankyrin 2.2, 195 kD), 2.3 (175 kD), and 2.6 (145 kD), and minor ankyrin bands of 320 kD, 200 kD, 183 kD, 166 kD, 151 kD, and 117 kD on immunoblots of RBC membranes, but do not detect other membrane proteins, including purified spectrin.

RIAs. The spectrin and ankyrin contents of RBCs were determined using the competitive RIA described by Agre et al with some modifications. In general, protein A-bearing staphylococci were precoated with spectrin or ankyrin antibodies and mixed with iodinated spectrin or an iodinated fragment of ankyrin, the 62-kD spectrin binding domain. The number of counts bound under these conditions was taken as 100%. The assay was then standardized with increasing amounts of unlabeled spectrin or 62-kD ankyrin, which progressively displaced the radioactive protein. A similar displacement approach was used to measure the spectrin or ankyrin present in SDS-solubilized RBCs. The 62-kD fragment of ankyrin was used because whole ankyrin is more difficult to purify, tends to aggregate on storage, and is more sensitive to proteolysis.

To prepare the immunoadsorbent, protein A-bearing staphylococci (IgGisorb; The Enzyme Center, Malden, MA) were washed in 6 vol of 100 mmol/L NaCl, 20 mmol/L Na phosphate, 1 mmol/L EDTA, and 0.1% (vol/vol) Triton X-100, pH 7.5 (buffer A); resuspended in buffer A at a concentration of 10% (vol/vol); incubated with 0.5 vol of undiluted antisera at room temperature for 30 minutes; rewashed three times with buffer A; and stored for up to 3 weeks at 4°C as a 10% suspension in buffer A plus 0.05% NaN3.

Before the assay, the RBCs and all of the proteins, both labeled and unlabeled, were separately incubated at 60°C in 1% (wt/vol) SDS in phosphate-buffered saline for 10 minutes and then diluted ≥40-fold into RIA buffer (150 mmol/L NaCl, 10 mmol/L Na phosphate, 1 mmol/L EDTA, 1 mmol/L NaN3, 1% [vol/vol] Triton X-100, 1 mg/mL bovine serum albumin, pH 7.5).

The assay was performed in triplicate in 12-× 75-mm polystyrene tubes containing 0.8 mL of RIA buffer, 50,000 cpm of [125]I-labeled spectrin or [125]I-labeled 62-kD ankyrin, varying dilutions of unlabeled proteins or RBC lysate, and a 0.3% (vol/vol) suspension of antibody-labeled staphylococci (diluted 1:9 with washed, unlabeled staphylococci so that only about 40% of the added cpm precipitated before the addition of unlabeled proteins or RBC lysate). The samples were shaken vigorously at 4°C for 16 hours (spectrin) or 40 hours (ankyrin) and then washed twice with 4 mL of buffer B (2 mol/L urea, 1% [vol/vol] Triton X-100, 0.1 mol/L glycine) and counted.

Linear regression and other statistics were analyzed with the Statgraphics computer programs (Statistical Graphics Corp, Rockville, MD).

SDS gel electrophoresis. Erythrocyte membranes were analyzed by SDS-PAGE, using the Fairbanks buffer system and 3.5% SDS gel electrophoresis.5 SDS-PAGE was developed using a mixture of native and SDS-denatured ankyrin and its 62-kD spectrin binding fragment. Antisera nos. 7608 and 7609 are high titer antisera and are effective at dilutions of 1:50,000 to 1:100,000. At a dilution of 1:40,000 they detect ankyrin (band 2.1, 206 kD) and numerous ankyrin subspecies (ankyrin 2.2, 195 kD), 2.3 (175 kD), and 2.6 (145 kD), and minor ankyrin bands of 320 kD, 200 kD, 183 kD, 166 kD, 151 kD, and 117 kD on immunoblots of RBC membranes, but do not detect other membrane proteins, including purified spectrin.

RESULTS

Quantitation of ankyrin and spectrin in normal RBCs. Typical results of the ankyrin RIA are shown in the left panel of Fig 1. The displacement curves for 62-kD spectrin and RBCs are parallel, which indicates that the ankyrin antisera detects the same epitopes in the protein standard and solubilized cells. Under these conditions, the ankyrin content of an average RBC can be determined from the numbers of ankyrin molecules and RBCs that displace 50% of the labeled ankyrin fragments. Normal RBCs contain an average of 124,500 ± 11,000 (SD, n = 26) ankyrin molecules (Fig 1, right panel), which is similar to previous estimates. This measurement presumably includes all forms of ankyrin (ankyrin bands 2.1, 2.2, 2.3, 2.6, etc) that are detected by the ankyrin antisera.

In the spectrin RIA, the displacement curves for the standard and RBCs are also parallel (Fig 2, left panel). Normal RBCs contain 242,000 ± 20,500 (SD, n = 22) spectrin heterodimers per cell (Fig 2, right panel). This is also close to previous estimates.

Ankyrin and spectrin content of HS RBCs. RBC ankyrin and spectrin were also measured in 39 patients from 20 unrelated families with typical dominant HS. The results for each patient and the mean values for families containing more than one affected member are summarized in Fig 3A (ankyrin) and B (spectrin). Measurements in the same patient on different occasions (n = 18) averaged ±9.0% for ankyrin and ±5.4% for spectrin.

Most families had ankyrin-deficient RBCs, as the mean value of the kindreds was less than the simultaneous control in 19 of 20 families and was less than the lower limit of normal (±2 SD, ±82%) in 16 families (80%). The distribu-
tion of ankyrin values was broad (Fig 3A), ranging from 41% to 105% of simultaneous controls, and appeared to be continuous, which suggests there are many causes for ankyrin deficiency in these families or that other variables affect ankyrin expression. In contrast, affected members of a kindred only differed, on average, by 3.5% from the mean ankyrin value of the kindred, which was much less than the variation between kindreds.

Spectrin content of HS RBCs was also reduced, consistent with previous observations. Spectrin content for a kindred was less than the simultaneously run control in all 20 families and was below the lower limit of controls (−2 SD, ±84%) in 15 (75%). Individual spectrin levels ranged from 46% to 102% of simultaneous controls and, similar to ankyrin, varied much less within families than between families (Fig 3B).

Relationship of ankyrin and spectrin deficiencies. Remarkably, levels of ankyrin and spectrin were highly correlated in 19 of the 20 families (Fig 4). Within experimental error, the degree of spectrin and ankyrin deficiencies was essentially identical in these families, with one exception (Fig 4, ○) family no. 8, an otherwise typical family in which the RBCs were primarily ankyrin deficient.

To test whether the balanced deficit of spectrin and ankyrin in dominant HS was simply due to physical loss of membrane fragments, we determined the content of other membrane skeletal proteins by SDS gel electrophoresis in 4 unrelated dominant HS patients. The results, expressed as the ratio of each protein to band 3 (AE1), were spectrin/band 3 = 79% ± 7% (% of control ± SD), ankyrin/band 3 = 81% ± 4%, 4.1/band 3 = 102% ± 16%, 4.2/band 3 = 94% ± 6%, and actin/band 3 = 97% ± 24%. The data, although limited, indicate that the deficiency of spectrin and ankyrin is selective. It is unlikely that simple membrane fragmentation would produce this result.

**DISCUSSION**

This study shows that balanced, combined deficiency of erythrocyte spectrin and ankyrin is a common feature of dominant HS. It was present in at least 70% (14 of 20) of the kindreds we examined (Fig 4). One family showed predominant ankyrin deficiency (family 8). Spectrin and ankyrin...
levels were less than simultaneous controls in 4 of the other 5 kindreds, but the degree of deficiency did not exceed the normal range. Some of these families are probably also spectrin-ankyrin deficient, but this is impossible to prove without a more precise assay.

The fact that HS RBCs are spectrin deficient is well known from the pioneering work of Agre et al., but the frequency of ankyrin deficiency was not previously known. In retrospect, early observations of Hill et al. and Sawyer et al. hinted that problems in spectrin-ankyrin interactions might exist in HS. They observed slower and less complete dissociation of spectrin and actin from ghosts or membrane skeletons and a 40% reduction in the binding of a crude spectrin extract to spectrin-depleted membranes. The reduction was caused by the loss of spectrin binding sites rather than a decrease in binding affinity. However, the studies were performed with crude protein extracts and seemed to be contradictory. Moreover, direct measurements of spectrin re-binding to spectrin-depleted HS membranes were normal in two independent laboratories. This was probably caused by inherent limitations of the binding assay, which can vary by as much as 50%, even in practiced hands. The degree of ankyrin deficiency observed in this study varies from 0% to 60% (median, 22%) and is mild enough in most patients that it would be difficult to detect in a typical spectrin binding assay.

It is only recently that autosomal dominant HS has been associated with defects of erythroid ankyrin (Ank1). The initial reports dealt with patients who had unusually severe HS and atypical RBC morphology associated with unstable ankyrin or who had a syndrome of HS, psychomotor retardation, growth failure, and hypogonadism associated with heterozygous deletion of the ankyrin locus (at 8p11.1) and neighboring portions of chromosome 8. More recent studies have focused on typical dominant HS. In one large family, ankyrin was genetically linked to HS using a restriction fragment polymorphism. Members of this kindred have mild hemolysis and mild spectrin deficiency (~15%). RBC ankyrin was not measured, but, judging from SDS gels, it is normal or only
mildly decreased (P. Agre, personal communication). In another kindred, reported in preliminary form, affected individuals have a small ankyrin isoform (ankyrin Prague, 174 kD) that probably lacks a portion of the regulatory domain. Finally, rare families with typical dominant HS have balanced translocations that break at 8p11.1 and presumably involve the ankyrin locus.20-22

These reports, together with the present observations, are compatible with the hypothesis that ankyrin deficiency is the primary defect in the majority of the patients with dominant HS and that spectrin deficiency is secondary to the loss of ankyrin attachment sites.

The opposite view, that spectrin deficiency is primary (eg, caused by diminished spectrin synthesis or damage to the spectrin-ankyrin interaction) and fosters loss of the uncovered ankyrin, is not supported by known examples. Mice with complete or nearly complete spectrin deficiency and severe spherocytosis caused by homozygous defects at the α-spectrin (sph, sphβ4, sphβ′, and sphβ3 abnormalities) or β-spectrin (ja mutation) loci have roughly normal amounts of RBC ankyrin on SDS gels,39,40 as do humans with recessive HS and marked spectrin deficiency.41

Combined deficiency could result from decreased synthesis of ankyrin or band 3 or from impaired interaction of the two proteins. The wide variation in the degree of spectrin-ankyrin deficiency from kindred to kindred suggests there are many different mutations. However, the fact that spectrin and ankyrin are equally deficient in most kindreds suggests that these mutations may be qualitatively similar.

Two examples of decreased ankyrin synthesis are known. Ankyrin and spectrin are deficient in both conditions, but ankyrin deficiency predominates. For example, RBCs from a patient who is heterozygous for a deletion of the erythroid ankyrin locus (ANK1) contain 59% of the normal amount of ankyrin, 79% of the normal amount of spectrin, 74% of the normal amount of protein 4.2, and normal amounts of protein 4.1 and actin.19 Similarly, mice that are homozygous for the nb mutation and lack erythrocyte ankyrin39,40,42 have reduced but not absent levels of RBC spectrin (≈50% of normal). In both cases the actual numbers are probably about 20% lower than stated since the skeletal proteins were quantified relative to the amount of band 3; some band 3 is lost along with membrane lipids and other integral membrane proteins in spherocytes.4 Nevertheless, it is surprising that spectrin levels exceed ankyrin because ankyrin is thought to be the sole high-affinity spectrin-binding protein in mature RBCs.43 Perhaps some spectrin binds via protein 4.1 or actin. Alternatively, membrane proteins may exist early in erythrocyte development that have some affinity for erythrocyte spectrin, particularly when RBC ankyrin is deficient. Brain ankyrin (Ank2), which can bind erythrocyte spectrin,44 is present in mouse fetal liver cells,45 which are mostly erythrocytoid, and it (or other ankyrins) might also be expressed in adult marrow erythroblasts. In addition, nonankyrin proteins that bind spectrins to membranes are known46 and could exist in developing erythrocyte cells. Once bound, spectrin would establish lateral connections to other membrane proteins and tend to remain attached despite the absence of Ank1.

Examples of primary band 3 (AE1) deficiency may also exist. In about 10% to 15% of dominant HS patients, erythrocytes contain 25% to 40% less band 3 than normal.47-50 Band 3 synthesis is normal46 but the molecule is lost progressively as the cells circulate, particularly the “mobile” band 3 fraction.46 This fraction represents band 3 that is not bound to the skeleton, which suggests that the primary defect may affect self-association of band 3 rather than its interactions with ankyrin or other skeletal proteins. In support of this, we have quantified membrane proteins in three affected members of one band 3-deficient family47 (RBC band 3 ≈ 75% of normal), using both SDS gels and radioimmunoassays, and find normal levels of spectrin, ankyrin, actin, and proteins 4.1 and 4.2 (Savvides et al, unpublished observations). If the results are representative, band 3 deficiency could not be responsible for HS in the majority of the patients in our survey, although it could be present in the families with normal or near normal levels of RBC spectrin and ankyrin (Fig 4).

The phenotype of ankyrin-band 3 binding defects is unknown. An apparent defect in the ankyrin-band 3 interaction, associated with severe hemolysis and poikilocytosis, was described some years ago,41 but subsequent investigations showed these patients also had a defect in spectrin self-association. Theoretically, defects in ankyrin that alter band 3 binding could lead to balanced spectrin-ankyrin deficiency if RBC membranes self-assemble, as predicted by Lazarides.52 Lazarides suggests that newly synthesized membrane skeletal proteins such as spectrin and ankyrin spontaneously associate in the cytoplasm, as they do in vitro,43 and the prefabricated parts coalesce into a mature skeleton when band 3 is expressed, relatively late in erythroid differentiation. Failure of preformed spectrin-ankyrin complexes to bind to band 3 would lead to loss of both components. In contrast, simple absence of ankyrin might allow spectrin to bind to lower affinity sites, as noted earlier, and paradoxically lead to less severe spectrin deficiency.

Unfortunately, ankyrin-band 3 binding is difficult to measure in vitro, so it will not be easy to test for a functional defect in HS patients, particularly if binding is only decreased by 20% to 40%, as seems likely. It will not be easy to screen for ankyrin-band 3 binding defects by molecular methods either, because large portions of both molecules will have to be tested. The band 3 binding site in ankyrin is localized to the C-terminal half of the 89-kD domain, particularly the last two ankyrin repeats53; however, the 55-kD “regulatory” domain54 influences band 3 binding55,56 and could also be defective. The ankyrin binding site in band 3 involves widely scattered portions of the cytoplasmic do-
main that are probably brought together when the domain folds.

In principle, our patients could also have a defect of protein 4.2, because it binds to both ankyrin and band 3 and may help strengthen their interaction. However, this is not likely. Humans who lack protein 4.2 sometimes have a mild form of HS, but, with one exception, the disease is inherited as an autosomal recessive and is not detectable in the heterozygous state. Moreover, RBC spectrin and ankyrin are not deficient, even when 4.2 is absent.

Finally, it is important to add a comment regarding methodology. As noted by Agre et al., RIAs are more accurate than SDS gels for quantitating RBC spectrin, in part because band 3 is lost with other membrane surface components as spherocytes circulate, which causes spectrin (expressed as a spectrin/band 3 ratio) to be overestimated. This is even more true for ankyrin, which is present in smaller amounts and lies close to β-spectrin on SDS gels. If optimal conditions are used, reproducible and reasonably accurate results can be obtained when ankyrin deficiency is moderate (>30%). These conditions include the use of exponential gradient Fairbanks gels, multiple replicates, saturation staining of bands, and quantitation by dye elution and spectrophotometry. Gradient Fairbanks gels are needed to optimize the separation of spectrin and ankyrin. Laemmli gels are unsatisfactory because ankyrin lies between the spectrin bands in the Laemmli gel system. The dye elution technique is important, especially compared with densitometry, because it is easy to adjust the dye concentration so that one remains within the linear portion of a Beer’s Law plot. However, even when SDS gels are performed under optimal conditions, the spectrin and ankyrin RIAs are more accurate and less work, particularly when multiple samples are analyzed. Unfortunately, quantitation of spectrin and ankyrin in HS RBCs is arduous no matter what technique is used, because very small deficits must be measured. Better methods are needed if spectrin or ankyrin measurements are to become routine.

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REFERENCES

42. White RA, Birkenmeier CS, Lux SE, Barker JE: Ankyrin and the hemolytic anemia mutation, nb: map to mouse chromosome 8: Presence of the nb allele is associated with a truncated erythrocyte ankyrin. Proc Natl Acad Sci USA 87:3117, 1990


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