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Band 3 and Ankyrin Homologues Are Present in Eye Lens: Evidence for All Major Erythrocyte Membrane Components in Same Non-Erythroid Cell

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Summary. Although immunological homologues of erythrocyte membrane proteins have been individually discovered in a wide variety of tissues and cultured cells, the major structural components of the membrane have not yet been demonstrated simultaneously in the same cell type. Thus, considerable uncertainty continues to exist concerning whether the red cell homologues form elements of a structure which is similar to or unique from the framework which supports the erythrocyte membrane. Because the red cell cytoskeletal proteins, spectrin, actin and band 4.1, have been previously found in the superficial cortex of the lens, we decided to determine whether the corresponding membrane anchoring components of band 3 and ankyrin also occur in this cell type. Using antiserum specific for band 3 and ankyrin, we report the existence of immunologically cross-reactive proteins of similar molecular weight. Because these anchoring proteins appear and disappear coordinately with the aforementioned cytoskeletal proteins during the intermediate stages of lens cell maturation, it is conceivable that an erythrocyte-like membrane structural organization may occur transiently in the eye lens.

Introduction. Underlying the lipid bilayer of the erythrocyte membrane is a cytoskeletal network that is proposed to be involved in the maintenance and/or control of cell shape, deformability and stability (1-3). Spectrin, the most prominent component of this network, is a highly elongated, flexible heterodimer ($\alpha\beta$) which associates end to end mainly as heterotetramers $(\alpha\beta)_2$ in situ (4, 5). Several such spectrin filaments form a band 4.1 - stabilized interaction with each actin protofilament which consists of between 12 and 16 monomers of actin (6-8). This anastomosing filamentous network is attached to the lipid bilayer primarily via ankyrin, which binds both spectrin and the cytoplasmic domain of the major integral membrane protein, band 3 (9, 10). A second site of cytoskeletal attachment to the membrane may occur through an association of band 4.1 with either band 3 or a glycophorin species (11-13).

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Although the erythrocyte cytoskeletal organization has never been demonstrated in nonerythroid cells, the presence of homologous components in these other cell types has been well documented. Spectrin homologues, for example, have been found in brain, eye lens, thyroid gland, intestine, muscle, rod outer segments, etc. and in numerous cultured cells (14-20). These tetrameric molecules retain the ability to bind ankyrin, band 4.1 and actin, and exhibit a similar morphology to erythrocyte spectrin when viewed by electron microscopy after rotary shadowing. Nonerythroid ankyrin is similarly found in a variety of tissues where it exhibits many of the properties of its erythrocyte counterpart (21-24). However, unlike red cell ankyrin, this species may also interact in situ with microtubules and intermediate filaments (23, 24). Nonerythroid band 4.1 isotypes have been characterized mainly in lens and brain tissue (20, 25-28), however, they are also found in many other cell types (29). At least some of these band 4.1 homologues may arise from the same gene due to tissue-specific alternate splicing pathways (30). Band 3, though detected in a significant diversity of cells (31-38), appears to be most abundant in transport epithelia where its primary function is probably related to its role as an anion exchange enzyme rather than a cytoskeletal anchoring protein. Because the other major cytoskeletal attachment site in erythrocytes, i. e. glycophorin, is present only in cells of erythroid lineage (39, 40), it is conceivable that an entirely different membrane anchoring system has evolved for erythrocyte cytoskeletal homologues in nonerythroid cells. Thus, there is no evidence to date that nonerythroid band 3 contains a binding site for either ankyrin or band 4.1. In fact, no nonerythroid cell containing spectrin, actin and band 4.1 have been demonstrated to also express the erythrocyte membrane linkage proteins of band 3 and ankyrin.

In order to obtain an initial indication of whether the erythrocyte membrane architecture might also exist in nonerythroid cells, we decided to evaluate whether the essential cytoskeletal and linkage elements are found simultaneously in the same cell type. Human lens tissue was chosen for this

investigation for three reasons. First, it is not vascularized, and therefore, cross-contamination from red blood cells can be cleanly avoided. Second, the cells of the superficial cortex of the lens are already known to contain spectrin, actin, and band 4.1 (20, 25, 27, 41), i.e., the major components of the erythrocyte cytoskeleton. And third, like the erythrocyte, the lens cells lose their nuclei and other organelles during maturation, reducing their need for microtubules and other nonerythroid cytoskeletal elements. Using immunological techniques, we report here that proteins antigenically related to ankyrin and band 3 are indeed present in the same lens cells which contain spectrin, actin and band 4.1, and that these linkage proteins disappear concurrently with the three cytoskeletal proteins as the lens cell ages.

Materials and Methods. Human erythrocyte membranes were prepared as described by Dodge (42), except that the erythrocyte lysis buffer also contained 1 mM EDTA and 20 μg/ml PMSF. Human lens samples were prepared according to Aster (27). Human eyes were obtained from the Michigan Eye Bank. Lenses were excised from the eyes and carefully decapsulated to remove the nucleated anterior epithelial cells. The decapsulated lenses containing the fiber cells were then dissected into superficial and nuclear cell fractions. These fractions were disrupted in ice cold homogenization buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 20 μg/ml PMSF, 1 μg/ml Pepstatin A, 5 μg/ml Leupeptin, 0.5% β Mercaptoethanol pH 7.3) with a low clearance Dounce homogenizer. Crude membrane fractions were then collected by centrifugation at 37,000 xg for 10 minutes and were washed once with homogenization buffer. The crude membrane preparations in some cases were also extracted with 10 volumes of 8M urea dissolved in homogenization buffer. The stripped membranes were separated from the urea soluble supernatant by centrifugation at 90,000 xg for 30 minutes.

The various lens tissue fractions were solubilized and separated electrophoretically on acrylamide gels according to Laemmli (43)(Fig. 1). Gels were stained with Coomassie Blue. Proteins were transferred electrophoretically from gels to nitrocellulose paper (0.45 μm pore size) for 3 hours at 100V (-250 mA) using the buffer of Towbin (44). The resulting blots were quenched and treated with specific antibodies as described (27). The blots were then incubated with second antibody or protein A conjugated to horseradish peroxidase and stained with 4 chloro-1-naphthol according to the procedure of Hawkes (45).

Polyclonal antibodies to human erythrocyte cytoplasmic domain of band 3 (cdb3) and ankyrin were produced in New Zealand White rabbits. The cdb3 was prepared according to Bennett and Stenbuck (46) as modified by Appell (47). Rabbits were immunized with native or denatured cdb3 emulsified in complete Freund's adjuvant by intradermal injections at multiple sites. After boosting, the cdb3 antiserum was collected, affinity purified, and was shown on western blots of erythrocyte ghosts to stain exclusively band 3 (48). Human ankyrin was prepared as described by Bennett (49) and then subjected to preparative SDS-PAGE according to the method of Laemmli (47). The corresponding ankyrin band was excised from the gel, directly emulsified in

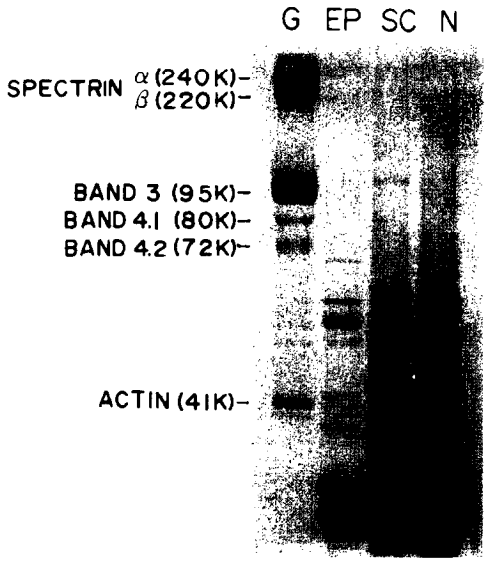

Figure 1. SDS-PAGE of human lens membrane fractions. Crude lens membrane fractions (EP, SC, and N) and human erythrocyte ghosts (G) were solubilized, electrophoretically separated on a 6-12% polyacrylamide gradient gel, and visualized by Coomassie blue staining. (G) human erythrocyte ghosts as molecular weight markers, (EP) lens epithelial fraction, (SC) lens superficial cortex fraction, and (N) lens nuclear fractions.

complete Freund's adjuvant and used for immunization as described above. The ankyrin antiserum was affinity purified, and by western blot analysis of erythrocyte ghosts, was shown to be monospecific to ankyrin (48).

Results. It has been reported previously that membranes of cells from the superficial cortex of the human lens contain immunological homologues of the three major erythrocyte membrane cytoskeletal proteins, spectrin, actin and band 4.1 (20, 25, 27, 41). In order to determine whether the membrane attachment system of erythrocytes is also present in these cells, the membranes of the superficial cortex were immunoblotted with antibodies to erythrocyte ankyrin and band 3. Figure 2A shows a control blot, conducted with antiserum to band 4.1, to confirm that the preparation isolated for the present studies indeed contains the cytoskeletal protein previously reported. The major cross-reactive species, as before, migrates slightly slower than its erythrocyte counterpart, with an apparent Mr-80 KDa. When the same material was blotted with antiserum to the cytoplasmic domain of band 3, a single component at Mr-100 KDa was observed (Fig. 2B, sample SC_2). This component has rougly the same molecular weight as erythrocyte band 3, but exhibits no detectable breakdown products and is considerably more sharply defined. That the staining pattern was not due to an artifact is evidenced by the observations that i) the same band appeared in the blot of a second

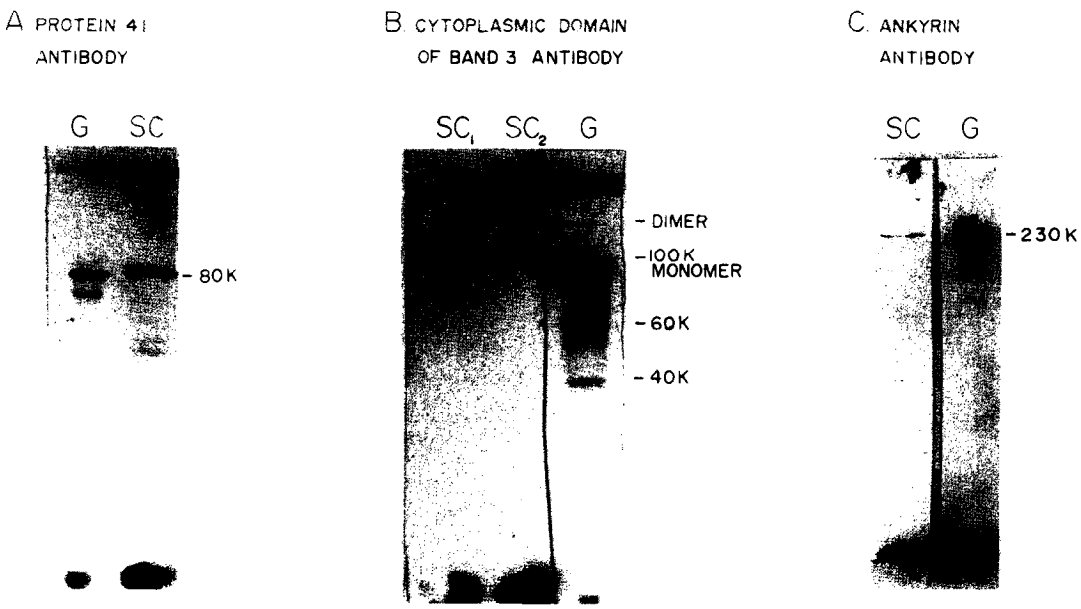

Figure 2. Western blot analysis of human lens superfical cortex membranes. Human lens superfical cortex (SC_1 and SC_2) and human erythrocyte ghost (G) membrane fractions were solubilized, electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and subjected to enzyme immunoassays (EIA) as described in Materials and Methods. Homologues of human erythrocyte membrane proteins were detected with antibodies to (A) protein 4.1, (B) the cytoplasmic domain of band 3 and (C) ankyrin. The lens superfical cortex samples SC_1 and SC_2 were obtained from adolescent (19 year old) and fetal lens, respectively. Cross-reactive bands seen in the ghost lanes below the parent bands are naturally occurring breakdown products.

preparation of superficial cortex membranes from a different individual (sample SC_1), ii) the two lens samples when blotted with preimmune serum displayed no cross-reactive material (not shown), and iii) the same or similar preparations revealed an identical 100 KDa band when blotted with a mouse monoclonal IgG to the cytoplasmic domain of band 3 or a different antibody raised against the denatured cytoplasmic domain of band 3 (not shown).

To determine whether the two lens preparations also contained an ankyrin homologue, i.e. the linkage protein which connects the cytoskeleton to band 3, the membrane homogenates were also blotted with antiserum to ankyrin. This antiserum, as documented previously (48), displays no cross-reactivity with spectrin or any other erythrocyte membrane protein except ankyrin. Importantly, the antiserum recognized a polypeptide in both lens samples with the same molecular weight (-230 KDa) as erythrocyte ankyrin (Fig. 2C, samples SC_1 and SC_2). Another blot with a different anti-ankyrin antibody yielded similar results (not shown). A minor cross-reactive band at -170 KDa was also seen in the lens cells of both the adolescent (SC_1) and fetus (SC_2)

TABLE I
DISTRIBUTION OF IMMUNOLOGICALLY RELATED PROTEIN UPON UREA EXTRACTION

PROTEIN	SC[a]	USF[a]	UIMF[a]
SPECTRIN	+ + + +	+ + +	(+)[c]
ANKYRIN	+ + +	+ +	(+)[c]
BAND 3	+ +	(+)[b]	+
4.1	+ + + +	+ + +	(+)[c]

a) SAMPLES WERE ANALYZED BY WESTERN BLOTTING/EIA METHODS
b) UREA EXTRACTED BAND 3 PROBABLY ARISES FROM IMPROPER COLLECTION OF THE UREA MEMBRANE FRACTION.
c) RESIDUAL PERPHERIAL PROTEINS PROBABLY EXISTS AS A RESULT OF MEMBRANES IN UREA INSENSITIVE ORIENTATIONS.

(Fig. 2c). Whether this minor band is a degradation product of the major 210 KDa ankyrin homologue or a second ankyrin isotype is not known. However, the usual erythrocyte ankyrin breakdown products of bands 2.2 and 2.3 seen in the ghost lane (Fig. 2C, sample G) were not detectable in either lens sample.

To determine the approximate disposition of the major erythrocyte membrane proteins in the membrane fraction of the superficial cortex cells, the isolated membrane material was treated with 8 M urea to strip off most peripheral proteins leaving a pelletable fraction enriched in integral membrane proteins. These urea-soluble (USF) and urea-insoluble fractions (UIF) were then each separated electrophoretically and analyzed for their polypeptide components by immunoblotting. As can be seen from Table I, spectrin, ankyrin and band 4.1 partitioned mainly into the urea-soluble fraction, while band 3 was concentrated predominantly in the pelletable material. Some cross-contamination was also evident, but we suspect this may have derived from resealing of part of the membrane material into urea-inaccessible compartments and/or from homogenization of the sample into fragments too small to easily pellet. Unfortunately, the limited supply of fresh lens did not permit a more detailed evaluation of this issue.

During maturation, epithelial cells at the anterior surface of the lens gradually differentiate into anuclear fiber cells which then elongate over the entire superficial cortex of the lens. As additional cells are later deposited, the more mature cells of the superficial cortex are gradually displaced into the deep cortex and ultimately to the nucleus of the lens. During this aging process, it has been reported that a gradual but complete disappearance of spectrin and band 4.1 occurs (27, 41), presumably due to

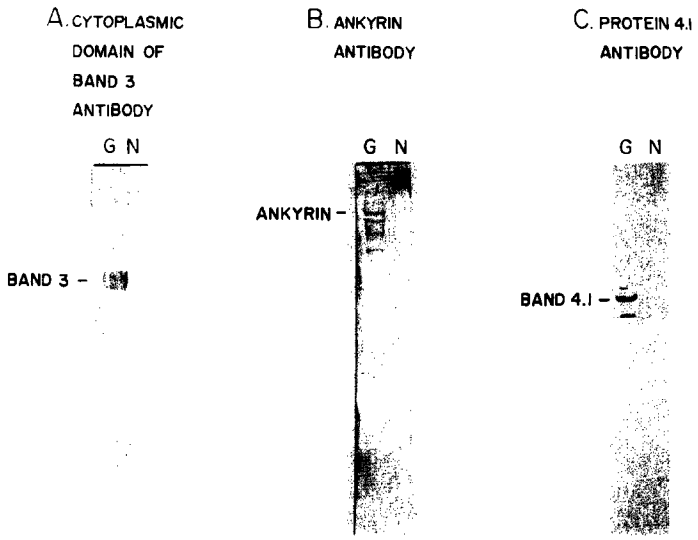

Figure 3. Western blot analysis of human lens nuclear membranes. Human lens nuclear membranes (N) and human erythrocyte ghosts (G) were separated and blotted electrophoretically as described in figure 2, except SDS-PAGE was carried out on a 6-12% polyacrylamide gradient gel. Enzyme immunoassays reveal the antibody staining pattern for (A) cytoplasmic domain of band 3, (B) ankyrin and (C) protein 4.1 on nitrocellulose immobilized erythrocyte ghosts (G) and lens nuclear membranes (N). Cross-reactive bands seen in the ghost lanes below the band of intact ankyrin and band 4.1 are naturally occurring breakdown products of their respective parent proteins.

degradation. Because a loss of the spectrin cytoskeleton would be expected to reduce or eliminate the need for its associated membrane attachment components, we decided to evaluate whether band 3 and ankyrin might also be absent in the older cells of the lens nucleus. Figure 3 shows the immunoblots of the membrane fraction of cells from the lens nucleus stained with antiserum to band 4.1, ankyrin and the cytoplasmic domain of band 3. As can be seen from the data, no evidence of any of the above erythrocyte membrane proteins could be detected in the older nuclear lens cells. However, since our antiserum to band 3 was raised against the isolated cytoplasmic domain of the protein, it is still possible that only this cytoskeletal anchoring site is degraded during aging, leaving the membrane-spanning anion transport domain to continue function.

Discussion. We have presented immunological evidence that isoforms of ankyrin and band 3 are present in cells of the superficial cortex of the human lens. Although no support was provided for an interaction among the erythrocyte homologues, the simultaneous presence of spectrin (bands 1 and 2), ankyrin (band 2.1), band 3, band 4.1, and actin (band 5) allows the hypothesis that the structural framework of the human erythrocyte might also

exist at least temporarily in the lens cell. To our knowledge, this is the first nonerythroid cell for which the simultaneous presence of these major essential elements of erythrocyte membrane structure has been demonstrated.

The possible occurrence of an erythrocyte membrane-like construction in a second anucleated cell suggests that the erythrocyte skeletal design may be useful for cells which do not accumulate cytoplasmic components. By the time the epithelial lens cell has matured to a fiber cell, it has lost most of its cytoplasmic organelles. Probably in an effort to further eliminate potential light-scattering particles from the visual light path, the fiber cell continues the voiding process by degrading its tubulin, vimentin and actin (27, 41, 50). Ultimately, with the loss of the erythrocyte structural components, the lens cell must rely largely on a high density of gap junctions (51) and an elaborate network of intercellular articulations (52) for its stability. The presence of the erythrocyte homologues during the transition to the highly differentiated transparent state may imply that the red cell components are present to stabilize the lens cell during reconstruction. The subsequent loss of both the anchoring proteins (i.e. ankyrin and band 3) and the cytoskeletal proteins (spectrin, actin and band 4.1) suggests further that when the cytoskeleton is degraded, the anchoring proteins become superfluous also.

Whether the erythrocyte homologues derive from expression of an erythrocyte or nonerythroid gene cannot be determined from the data. Although the staining patterns on the immunoblots appear grossly similar, a more careful scrutiny of the data reveals several significant differences. Thus, lens band 3 is less heterogeneous and of slightly higher molecular weight than erythrocyte band 3 (Fig. 2B). Likewise, lens ankyrin is accompanied by a lower molecular weight minor component at ~170 KDa, while red cell ankyrin is always trailed by cross-reactive bands 2.2 and 2.3 at ~190 KDa and 180 KDa. Antibodies or cDNA probes specific for the erythrocyte and nonerythrocyte isoforms will have to be employed to determine the relationship between the erythrocyte isoforms in the lens and red blood cell.

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