Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 363–367, January 1983
Biochemistry

Expression of the β subunit of spectrin in nonerythroid cells

(immunofluorescence/immunoprecipitation/erythrocytes/plasma membrane/peptide mapping)

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Communicated by Norman H. Horowitz, October 7, 1982

ABSTRACT **Antibodies raised against electrophoretically purified chicken erythrocyte β subunit of spectrin, called "β-spectrin," have been used to demonstrate the presence of an immunoreactive form of this polypeptide in nonerythroid tissues. Immunoautoradiography shows that, in chicken erythrocytes, this antiserum reacts with β-spectrin (M_r 220,000) and another polypeptide (M_r 230,000) that, by two-dimensional tryptic peptide analysis, shows extensive homology with β-spectrin but not with the α subunit of spectrin, called "α-spectrin." Immunoautoradiography and immunoprecipitation of various chicken tissues with this antiserum shows that either one variant or both variants of β-spectrin are expressed. Indirect immunofluorescence reveals that the antiserum reacts with a plasma membrane-associated component of erythroid and some nonerythroid cells. Particularly strong fluorescence is observed in skeletal and cardiac muscle cells where β-spectrin appears to form a grid-like network along the inner surface of the sarcolemma. The noncoordinated distribution of α- and β-spectrin variants indicates that their expression may be tailored to the functional requirements of the plasma membrane in different cells.**

The fluid mosaic model for membranes, which was developed from early studies on the erythrocyte membrane (1, 2), has been extended to include the notion that the lateral mobility of proteins in the plane of the membrane is restricted by interactions between these proteins and the cytoskeleton (3, 4). The nature of these interactions remains, to a great extent, an unresolved problem despite the fact that several membrane-associated proteins have been implicated in this process. In mammalian erythrocytes, actin is attached to the plasma membrane through a high molecular weight protein, spectrin, which is found on the inner surface of the plasma membrane: it is composed of two nonidentical subunit polypeptides herein termed "α-spectrin" (M_r 240,000) and "β-spectrin" (M_r 220,000) (for reviews, see refs. 5–7). Spectrin forms a crosslinked network with actin filaments (8, 9) and is linked to the membrane through the association of the β subunit with ankyrin (10, 11). Ankyrin associates with the membrane by binding to a subset of the transmembrane anion transporters (5, 7). Thus, in the erythrocyte, spectrin appears to play a pivotal role in linking the plasma membrane to the cytoskeleton and in restricting the mobility of certain transmembrane polypeptides. In the past, the failure to detect immunoreactive forms of spectrin in nonerythroid tissues (12, 13) has limited generalizations regarding the role of spectrin in the highly specialized erythrocyte. Recently, however, several laboratories have identified a spectrin-like protein in nonerythroid cells (14–20); the protein shows several biochemical properties common with α-spectrin from erythrocytes (18–20). As the functional conformation of spectrin in the erythrocyte appears to be the $(\alpha,\beta)_2$ tetramer (refs. 21 and 22; for review, see ref. 7) it is important to determine whether β-spectrin is also expressed in nonerythroid tissues. In this respect it should be noted that immunoreactive forms of ankyrin have been detected in nonerythroid cells (23). In the studies reported here, we demonstrate that antibodies raised against avian erythrocyte β-spectrin react with a plasma membrane-associated protein in a variety of nonerythroid tissues.

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MATERIALS AND METHODS

Production of Antibody. Chicken erythrocyte membranes were prepared as described (24), and their proteins were separated by $NaDodSO_4$/10% or 12.5% polyacrylamide gel electrophoresis based on the system of Laemmli (25) as modified and described (26). The band corresponding to chicken β-spectrin (24) was excised from the gel, homogenized, and reapplied to a $NaDodSO_4$/10% polyacrylamide gel. After reelectrophoresis (see Fig. 1*A*, lane a), the β-spectrin band was excised from the Coomassie blue-stained gel and equilibrated in 0.15 M sodium phosphate (pH 7.4). The gel slices (ca. 20–30 μg of protein) were homogenized with a motor-driven Teflon pestle in a glass homogenizer (Potter–Elvehjem) and emulsified with Freund's complete adjuvant for the immunization of a New Zealand White rabbit (27); booster injections contained Freund's incomplete adjuvant and were administered 29 days and 39 days, respectively, after the initial immunization. Blood was collected 9 days after the second and third injections. The IgG fraction was partially purified by precipitation with ammonium sulfate at 50% saturation at 4°C. Serum from the first bleed was used in this study: preimmune serum was obtained from the same rabbit prior to antigen injection.

Immunoautoradiography. Various tissues were removed from adult White Leghorn chickens, minced, and washed in 20 mM Tris·HCl, pH 7.2/150 mM NaCl. The samples were boiled in $NaDodSO_4$ sample buffer (26). Immunoautoradiography (28) was performed as described (29). Intensifying screens were not used during film exposure to enhance the resolution of the immunoreactive polypeptides.

Immunoprecipitation. The immunoprecipitation of polypeptides from various chicken tissues with antisera against chicken β-spectrin was performed essentially by the method of Lingappa *et al.* (30) as modified by Levine and Willard (15) except that 10 μl of antisera was used per immunoprecipitation.

Immunofluorescence. Indirect immunofluorescence was performed as described (20) except that the primary serum was used at 1:10 serum dilution. For immunoadsorption of antisera, Coomassie blue-stained bands containing either α- or β-spectrin were excised from gels of chicken erythrocyte plasma membrane proteins, and the proteins were eluted electrophoretically from the gel into dialysis tubing. The eluted proteins were extensively dialyzed against 100 mM ammonium bicarbonate, lyophilized, extracted with acetone/HCl at −20°C, and relyophilized. The samples of α- and β-spectrin were incubated in diluted (1:100) β-spectrin antiserum; after centrifugation, the supernatant was used either for immunofluorescence or immunoprecipitation.

Two-Dimensional Tryptic Peptide Mapping. Radioiodination of fixed and stained proteins with $NaDodSO_4$ gel slices and tryptic release of peptides for mapping were performed as described by Elder *et al.* (31). Eluted peptides were separated on cellulose TLC plates (Eastman) by electrophoresis in the first dimension at 800 V for 40 min in acetic acid/formic acid/water, 11.4:10:379 (vol/vol) at pH 1.9. The plates were dried for 2 hr and chromatographed in butanol/pyridine/acetic acid/water 5.5:3.3:1:3 (vol/vol), in the second dimension.

RESULTS

Characterization of the Antibody. Previous studies from this laboratory (20, 24) have demonstrated that avian erythrocytes possess a protein that, by several biochemical parameters, appears to be analogous to mammalian erythrocyte spectrin. The polypeptide previously identified as β-spectrin (24) was electrophoretically purified and used as the immunogen (Fig. 1*A*, lane a). Immunoautoradiography of chicken erythrocyte membrane proteins revealed that this antiserum reacted with two polypeptides; one comigrated with the original β-spectrin immunogen (M_r 220,000), and the other, with a minor protein component (M_r 230,000) that migrated between α- and β-spectrin (Fig. 1*A*, lane c). The relative difference in intensity of the crossreactivity of the two bands appeared to correspond to their difference in quantity as shown by Coomassie blue staining. No crossreactivity was found with "goblin" (32) or α-spectrin (Fig. 1*A*, lane c). As previously shown, antibodies to chicken α-spectrin did not crossreact with either of these polypeptides (20). The series of weakly crossreacting bands beneath the M_r 220,000 polypeptide most likely represent degradation products of β-spectrin because they were not observed in immunoautoradiograms of freshly prepared whole erythrocyte extracts but became more evident during prolonged storage of samples (data not shown). Two-dimensional immunoautoradiography showed that, characteristically for β-spectrin (20), the polypeptides that reacted with the antiserum did not focus discretely in the system used in this study (data not shown). Furthermore, the antiserum did not react with synemin (data not shown), which also migrates between α- and β-spectrin on one-dimensional polyacrylamide gels of chicken erythrocyte membranes but which is clearly resolved from α- and β-spectrin on two-dimensional gels (24). To further characterize the two immunologically related polypeptides, two-dimensional tryptic peptide mapping was performed on proteins that had been excised from $NaDodSO_4$/polyacrylamide gels of chicken erythrocyte membranes and subsequently radioiodinated. The distribution of the iodinated tryptic peptides derived from the two polypeptides was strikingly similar (Figs. 1*B* and *C*). Although the relative intensity of individual spots varied slightly and there were some differences in the migration of some minor spots, the overall impression is that these two polypeptides are related and have similar primary sequences, thus substantiating the fact that they also are related immunologically. The tryptic peptide maps of these two polypeptides were different from peptide maps of α-spectrin (unpublished data). Therefore, the two polypeptides that crossreact with β-spectrin antibodies are identified tentatively as molecular weight variants of chicken β-spectrin.

The antiserum was further characterized by analyzing its crossreactivity against chicken fodrin (15). Fodrin is comprised of two high molecular weight polypeptides and was partially purified from 10-day chicken embryo brain by the method of Levine and Willard (15). As shown in Fig. 2, fodrin was immunoprecipitated with the antiserum raised against chicken α-spectrin (20) but not with the β-spectrin antiserum (lane d).

Immunoautoradiography of Nonerythroid Tissues. Fig. 3 shows a one-dimensional, Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel of various chicken tissues (Fig. 3*a*, lanes 1–8 and 11), partially purified human and rabbit erythrocyte spectrin (Fig. 3*a*, lanes 9 and 10, respectively; see also Fig. 3*b Inset*), and the resulting immunoautoradiograms (Fig. 3*b*). All tissues examined appeared to express either one or two polypeptides that have almost identical apparent molecular weights as the two variants of avian β-spectrin (see also Fig. 4, lanes e and f). The degree of crossreactivity varied considerably; in Fig. 3*b*, pectoral (lane 2) and cardiac (lane 3) muscle and cerebellum (lane 7) showed the strongest crossreactivity whereas whole gizzard (lane 4) and sciatic nerve (lane 6) were particularly weak. Closer examination of the molar ratios of the β-spectrin variants revealed differences from the ratio in chicken erythrocyte membranes (lane 1). Whereas the M_r 220,000 variant of β-spectrin was most prominent in chicken erythrocyte membranes, the M_r 230,000 variant was most prominent in pectoral and cardiac muscle and cerebellum. In duodenum and gizzard muscle only the M_r 220,000 variant was apparently present. This result also was confirmed by immunoprecipitation with anti-β-spectrin antibodies of $NaDodSO_4$-solubilized tissue, followed by immunoautoradiography to show the crossreacting antigens (Fig. 3*c*). The β-spectrin antiserum also crossreacted weakly with β-

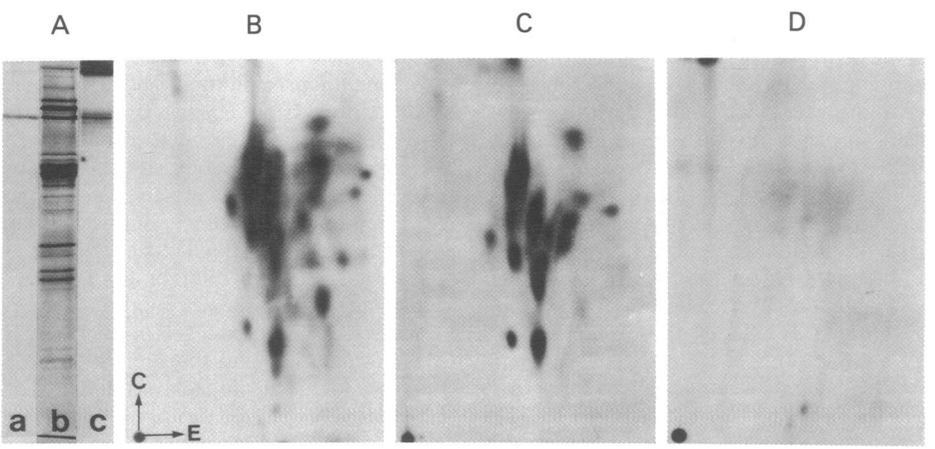

FIG. 1. Characterization of the polypeptides that crossreact with the β-spectrin antiserum. (*A*) Lanes: a, electrophoretically purified chicken β-spectrin used as the immunogen; b, Coomassie blue-stained gel of chicken erythrocyte membrane proteins; c, corresponding autoradiogram of lane b. (*B*) Two-dimensional tryptic peptide map of the M_r 230,000 variant of β-spectrin. (*C*) Two-dimensional tryptic peptide map of the M_r 220,000 variant of β-spectrin. (*D*) Blank gel, radioiodinated and treated with trypsin. The directions of electrophoresis (E) and chromatography (C) are shown in (*B*).

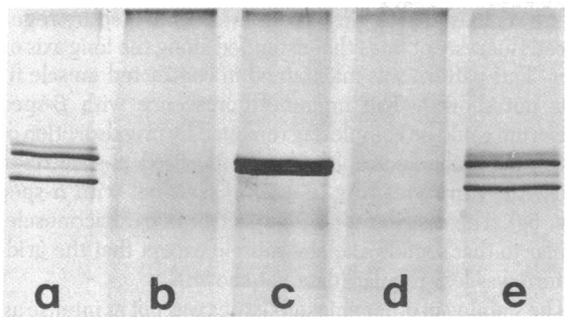

FIG. 2. Immunoprecipitation of partially purified fodrin from 10-day chicken embryo brains. Lanes: a and e, chicken erythrocyte plasma membrane proteins as markers; b, immunoprecipitate with preimmune serum; c, immunoprecipitate with α-spectrin antiserum (see ref. 20); d, immunoprecipitate with β-spectrin antiserum. Only part of the gel is shown; for identification of polypeptides, see the legend to Fig. 4.

spectrin (band 2) in partially purified human erythrocyte spectrin and in extracts of rabbit erythrocytes (Fig. 3*b Inset*).

The crossreaction of the β-spectrin antiserum with lens, which is avascular, indicates that this is not due to the presence of erythrocytes. Furthermore, the differences in the molar ratio of the β-spectrin variants in various tissues compared to that in erythrocytes indicates that the level of erythrocyte contamination was low.

Immunoprecipitation. Chicken cardiac (ventricular) muscle and erythrocyte membranes were solubilized in $NaDodSO_4$, and the particulate material was pelleted in an Eppendorf centrifuge; the supernatant was used for immunoprecipitation with α- and β-spectrin antiserum. Fig. 4 shows the proteins immunoprecipitated from chicken erythrocyte membranes with the preimmune serum (Fig. 4, lane a), α-spectrin antibodies (Fig. 4, lane b), and β-spectrin antibodies (Fig. 4, lane c). With either α- or β-spectrin antibodies, a complex was immunoprecipitated composed of α-spectrin and the two variants of β-spectrin, indicating that, even under the conditions for tissue solubilization used in this study, the spectrin complex appears to be either resistant to disruption or, more likely, the α and β subunits can reassociate to some extent to form a complex. The formation of a spectrin complex in the presence of $NaDodSO_4$ appeared to be specific, as no other polypeptide was specifically immunoprecipitated (see Fig. 4). Furthermore, preadsorption of the β-spectrin antiserum with purified β-spectrin inhibited the immunoprecipitation of the spectrin complex (data not shown). Lane f in Fig. 4 shows the immunoprecipitate of solubilized cardiac muscle with β-spectrin antiserum. A complex of polypeptides was immunoprecipitated with bands corresponding to α-spectrin and the two variants of β-spectrin. The two proteins marked with an arrowhead also were immunoprecipitated with the preimmune serum (data not shown) and, thus, represent nonspecific reactants with the β-spectrin antiserum. There appeared to be a slight difference in the molecular weights of the β-spectrin variants of erythrocyte membranes and cardiac muscle, although they had similar tryptic peptide maps (unpublished data).

Immunofluorescence. Indirect immunofluorescence was used to examine the distribution of β-spectrin in frozen sections of various nonerythroid tissues. As indicated by the immunoautoradiography of extracts of whole tissues (Fig. 3), cardiac and skeletal muscle revealed particularly strong fluorescence staining with the β-spectrin antiserum (Fig. 5). In longitudinal sections of pectoral (or anterior latissimus dorsi) muscle, the β-spectrin immunofluorescence (Fig. 5*b*) showed a distribution of the antigen preferentially at the area of the Z line, which was similar to that of α-spectrin (Fig. 5*d*). In cross sections of leg muscle (Fig. 5 *h* and *i*), strong fluorescence was observed at the sarcolemma; weaker fluorescence in the interior of the fiber showed a honeycomb pattern with β-spectrin antiserum (and α-spectrin antiserum; data not shown), which appeared to be located at the periphery of the Z disks. Cross sections of pectoral (or leg) muscle also showed strong fluorescence at the sarcolemma (Fig. 5*l*). In longitudinal frozen sections of relaxed an-

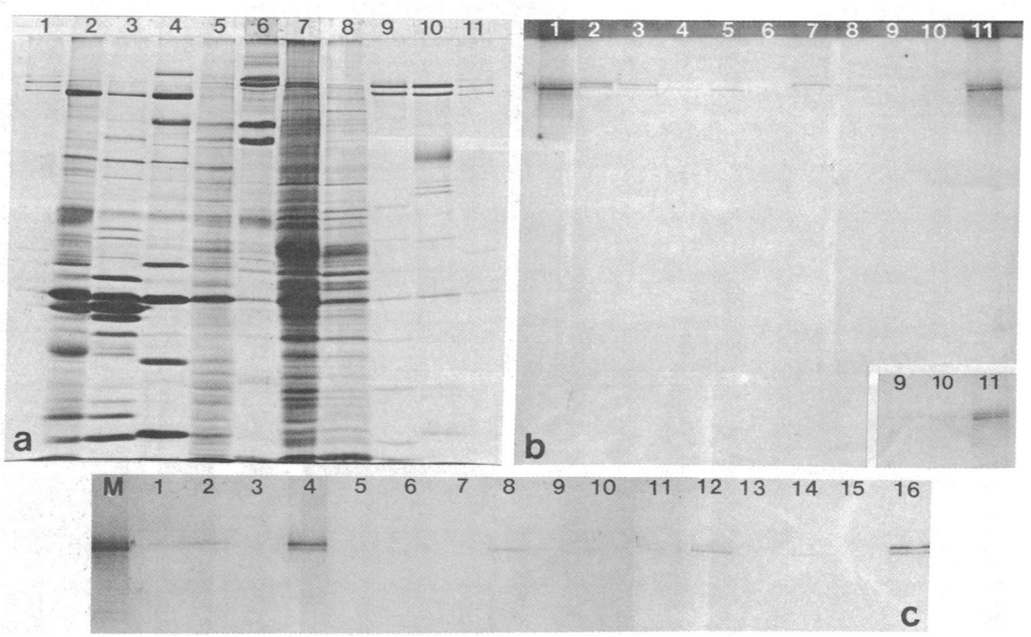

FIG. 3. Anti-β-spectrin immunoautoradiography of various chicken tissues (lanes 1–8 and 11 in *a* and *b*; all lanes in *c*) and human and rabbit erythrocytes: Coomassie blue-stained gel (*a*), corresponding immunoautoradiogram (b), and immunoautoradiogram after immunoprecipitation of various chicken tissues (*c*). Lanes in *a*, *b*, and *b Inset*: 1 and 11, chicken erythrocyte plasma membrane; 2, cardiac muscle; 3, pectoral muscle; 4, gizzard muscle; 5, duodenum; 6, sciatic nerve; 7, cerebellum; 8, spinal cord; 9, human erythrocyte ghosts; 10, rabbit erythrocyte ghosts. (*c*) From left to right, chicken tissue samples were immunoprecipitated in parallel with preimmune serum (odd numbers) and β-spectrin antiserum (even numbers). Lanes: M, chicken erythrocyte plasma membrane; 1 and 2, pectoral muscle; 3 and 4, cardiac muscle; 5 and 6, gizzard muscle; 7 and 8, duodenum; 9 and 10, lens; 11 and 12, spinal cord; 13 and 14, sciatic nerve; 15 and 16, cerebellum.

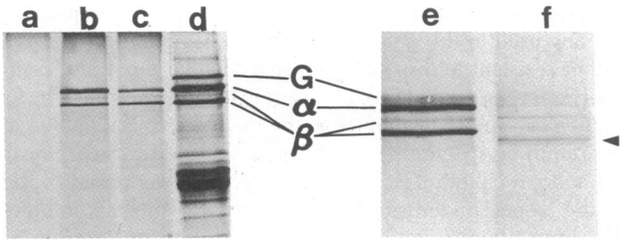

FIG. 4. Immunoprecipitation of solubilized chicken erythrocyte membrane proteins (lanes a–c) and cardiac muscle proteins (lane f) with preimmune serum (lane a), α-spectrin antiserum (lane b), and β-spectrin antiserum (lanes c and f). Lanes d and e are chicken erythrocyte plasma membrane proteins used as markers for goblin (G), α-spectrin (α), and β-spectrin (β). The arrowhead indicates a polypeptide in lane f that immunoprecipitates from cardiac muscle with the preimmune serum (data not shown).

terior latissimus dorsi (Fig. 5 *m* and *n*) and leg muscle (Fig. 5 *o* and *p*) the β-spectrin immunofluorescence was seen clearly as a punctate pattern in close association with the sarcolemma. The periodicity of the fluorescence, particularly in relaxed muscle, appeared to coincide with the Z and M lines. In longitudinal sections cut close to the sarcolemma of either anterior latissimus dorsi (Fig. 5 *j* and *k*), pectoralis (data not shown) or leg muscle (Fig. 5*q*), the immunofluorescence revealed an intricate grid-like pattern similar to that seen with α-spectrin antiserum (for comparison, see ref. 20). The rings of fluorescence transverse to the long axis of the fiber appeared to coincide with the Z- and M-line regions. These rings were interconnected by regularly spaced fluorescent lines that extended along the long axis of the fiber. This pattern was maintained in contracted muscle fibers (data not shown). The immunofluorescence with β-spectrin antiserum could be completely removed by preadsorption of the antiserum with electrophoretically purified β-spectrin from chicken erythrocytes (Fig. 5 *e* and *f*) but not with α-spectrin (Fig. 5*g*). The distribution of β-spectrin in cardiac muscle was similar to that seen in skeletal muscle except that the grid-like pattern was less regular (data not shown).

The staining of other chicken tissues was not as intense as that for skeletal muscle (data not shown), as indicated by the results of the immunoautoradiography (Fig. 2). In general, the staining of nerve tissue of the peripheral and central systems, epithelial tissues in the gastrointestinal tract, and smooth muscle showed diffuse staining, slightly above the level of the preimmune serum (data not shown). In these tissues, the erythrocytes stained very strongly at the plasma membrane, indicating that the weak fluorescence was not due to the inability of the antibodies to penetrate the tissue.

DISCUSSION

A previous immunological, biochemical, and electron microscopy investigation (20) established that avian erythrocyte plasma membranes possess a protein that is analogous to mammalian erythrocyte spectrin. In the present study, we have electrophoretically purified chicken β-spectrin and raised an-

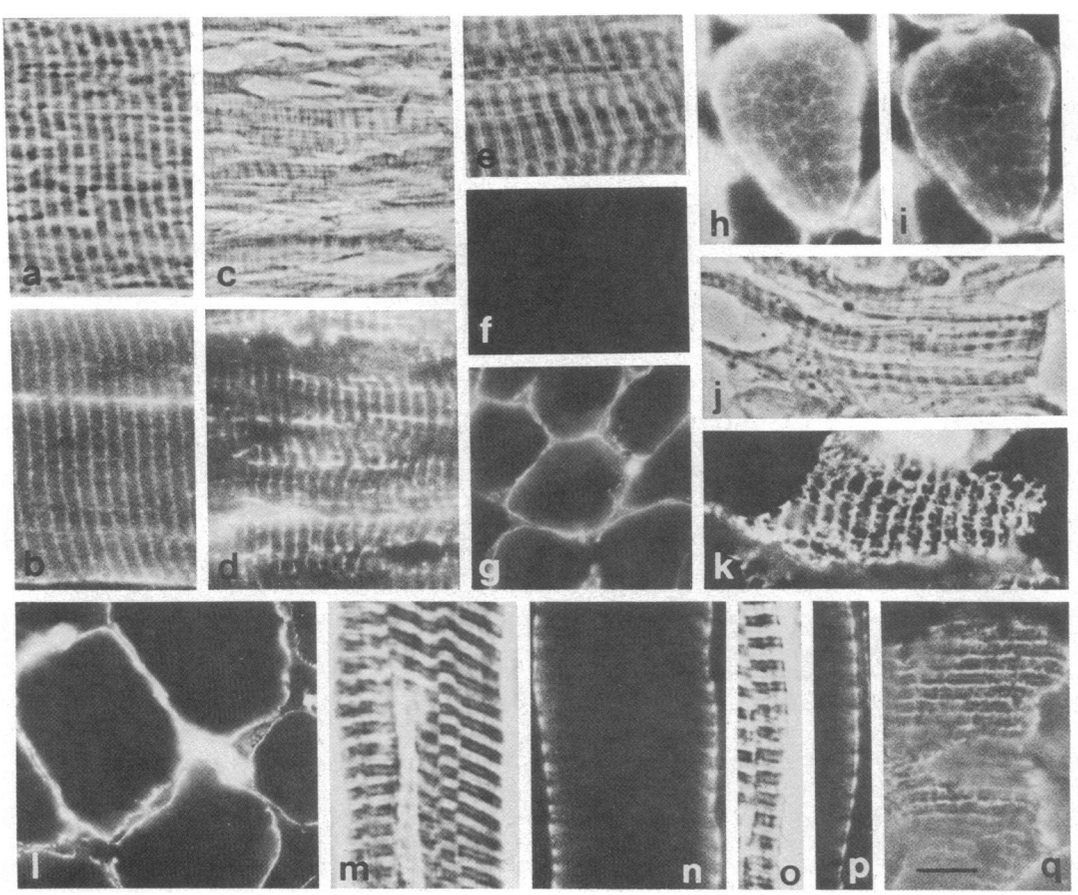

FIG. 5. Indirect immunofluorescence of frozen sections of chicken skeletal muscle with α-spectrin antiserum (*c* and *d*) and β-spectrin antiserum (*a*, *b*, and *e–q*). Fluorescence micrographs (*b*, *d*, *f–i*, *k*, *l*, *n*, *p*, and *q*) and phase-contrast micrographs (*a*, *c*, *e*, *j*, *m*, and *o*) of pectoral muscle, longitudinal sections (*a–f*) and cross-section (*g*); leg muscle, cross-section (*h* and *i*), sarcolemma surface section (*q*), and longitudinal section (*o* and *p*); anterior latissimus dorsi, sarcolemma surface section (*j* and *k*) and longitudinal section (*m* and *n*); and preadsorption of β-spectrin antiserum with purified β-spectrin (*e* and *f*) or purified α-spectrin (*g*). (Bar = 10 μm for *c* and *d*, 20 μm for *l*, 26 μm for *g*, and 8 μm for the rest.)

tibodies against this polypeptide. The resulting antiserum appears to react with two components of chicken erythrocyte membranes. That these two polypeptides are molecular weight variants of avian β-spectrin is based on the following observations. (*i*) Only the M_r 220,000 variant was used as an immunogen, and yet the antiserum also reacted with the M_r 230,000 variant. (*ii*) The polypeptides have strikingly similar two-dimensional tryptic peptide maps. (*iii*) The polypeptides have similar solubility properties (20). (*iv*) Both polypeptides coimmunoprecipitate in a complex with α-spectrin with either α- or β-spectrin antiserum. Turkey erythrocyte plasma membranes also possess both variants of β-spectrin (data not shown). In human and rabbit erythrocyte extracts, only one polypeptide, which corresponds to mammalian β-spectrin (band 2), cross-reacts with the avian β-spectrin antiserum. Therefore, the M_r 230,000 form may be limited to avian cells.

Direct immunoautoradiography of extracts of whole tissues or immunoautoradiography after immunoprecipitation with β-spectrin antibodies revealed immunoreactive forms of β-spectrin in various nonerythroid tissues. The polypeptides in cardiac and skeletal muscle crossreacted strongly with the β-spectrin antiserum both in polyacrylamide gels and in frozen sections. All other tissues examined, except cerebellum, showed weak crossreactivity; immunofluorescence microscopy of frozen sections of these tissues revealed a diffuse staining pattern that prevented the unequivocal localization of the antigen. Further studies are required to determine the distribution of β-spectrin in these tissues. It should be noted that all these tissues, except gizzard, crossreacted relatively strongly with α-spectrin antiserum in immunoautoradiograms of similar amounts of whole tissue extracts as used in this study (20). The molar ratios of the two β-spectrin variants also differed between tissues. Interestingly, those tissues that revealed weak fluorescence staining with β-spectrin antiserum appeared to express only the M_r 220,000 molecular weight variant of β-spectrin, whereas cerebellum and skeletal and cardiac muscle expressed both polypeptides, although in a different molar ratio to erythrocytes.

The finding of β-spectrin in nonerythroid tissues complements the recent results on the widespread occurrence of α-spectrin (14–20) and ankyrin (23). In this study we have shown that the distribution of α- and β-spectrin in avian skeletal muscle is very similar, if not identical. Furthermore, α-spectrin and both molecular weight variants of β-spectrin can be coimmunoprecipitated from cardiac muscle extracts with either α- or β-spectrin antiserum. Taken together these results clearly indicate that a spectrin complex similar to that found in the erythrocyte plasma membrane is present in certain nonerythroid tissues. This finding has important implications for the role of spectrin in membrane–cytoskeleton interactions in nonerythroid cells. An example of this is in skeletal muscle, where α- and β-spectrin are codistributed in a grid-like pattern on the inner surface of the sarcolemma. Particularly strong fluorescence was seen at the Z and M lines in the form of rings transverse to the long axis of the fiber. Several morphological studies have indicated that cytoskeletal elements at the periphery of the Z line connect to the sarcolemma (e.g., ref. 33). By analogy with the erythrocyte plasma membrane, therefore, spectrin may interact with structural elements transverse to the long axis of the fiber, thus linking the sarcolemma to the Z disk. Together with arrays of spectrin in the long axis of the fiber, which interconnect the rings around the Z and M lines, this system would confer structural integrity to the myofibril during contraction and relaxation.

Recently two sets of polypeptides have been identified in brain tissue (15) and intestinal brush borders (17), each of which is composed of two proteins termed "fodrin" (15) and "TW-260/240" (17), respectively. They share similar subunits of apparent molecular weight M_r 240,000 that correspond to α-spectrin from erythrocytes (19). It has been suggested (19) that the other variant subunits of fodrin and TW-260/240 may play a role analogous to β-spectrin. The results of this study show that fodrin is not immunoprecipitated with β-spectrin antiserum. It is apparent from our results (this study and ref. 20) that at least in brain, α-spectrin (the M_r 240,000 component of fodrin) is present in substantially greater amounts than is β-spectrin. Therefore, this raises the possibility that the cell can express a certain combination of spectrin polypeptides whose properties are tailored to the functional requirements of the plasma membrane.

We are grateful to Dr. B. L. Granger, J. J. Ngai, and R. Gomer for advice during various parts of this study and to Dr. B. L. Granger for his cogent comments on the manuscript. This work was supported by grants from the National Institutes of Health, the National Science Foundation, and the Muscular Dystrophy Association of America and by a Biomedical Research Support Grant to the Division of Biology, California Institute of Technology, W.J.N. also was supported by an international fellowship from the Cancer Research Campaign awarded by the International Union Against Cancer. E.L. is a recipient of a Research Career Development Award from the National Institutes of Health.

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