Structure and Composition of the Cytoskeleton of Nucleated Erythrocytes

I. The Presence of Microtubule-associated Protein 2 in the Marginal Band

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Abstract The marginal band (MB) of nucleated erythrocytes is composed of a bundle of microtubules that encircles the cell immediately beneath the plasma membrane. When cells are lysed in buffer containing Triton X-100 the MB microtubules remain intact, and the nucleus remains suspended at the cell center by a filamentous network called the trans-MB material that connects the nucleus to the peripheral MB. When these lysed cells are prepared for indirect immunofluorescence by use of an antibody to chick brain microtubule-associated protein 2 (MAP 2), intense staining of the MB results; no staining is evident in the areas occupied by the nucleus or the trans-MB material. Controls demonstrate that the staining is specific, because no staining occurs with fluorescent goat antirabbit serum alone or when nonimmune serum is used as the first antibody. Furthermore, the fluorescence of the MB is not affected by pretreatment of the immune serum with purified tubulin, but staining is prevented by pretreatment of the immune serum with purified MAP 2. To determine which protein component of the MB was responsible for the positive immunofluorescence results, 125I-protein A staining was used after the protein components of the isolated cytoskeleton had been resolved by SDS-polyacrylamide gels. Controls showed that the antiserum could react on SDS gels with MAP 2 from purified chick brain microtubules. The results with the cytoskeletal proteins demonstrated that the antiserum reacted only with a high molecular weight protein having a molecular weight similar, but not identical, to that of chick brain MAP 2. Thus, it is concluded that a protein with antigenic characteristics similar to those of chick brain MAP 2 is a component of the MB. The results are discussed in terms of the possible function of MAP 2 in the MB.

MATERIALS AND METHODS

Organisms and Isolation of Erythrocytes

For the experiments reported here, erythrocytes were isolated from the following sources: baby chicks, the Eastern red spotted newt, Notophthalmus viridescens, etc.
cens, and the toad, *Bufo marinus*. Chicks were obtained from Hall Brothers Hatchery, Wallingford, Conn., and were used between 1 and 5 d after hatching. Newts were caught from a pond on the Sherrard Farm in nearby Norwich, Vt., and were returned to the pond after use. Toads were purchased from Carolina Biological Supply Co., Burlington, N. C., and were maintained in a moss-bottomed aquarium for up to 1 mo; they were fed a diet of crickets and fruits and vegetables. Blood was obtained by decapitation (chicks), tail snipping (newts), or heart puncture after pithing (toads). The blood was then washed in one of two ways, depending on the source, because of variations in response to the standard lysis medium. Blood from the chick and toad was suspended in 10–20 ml of wash medium: 20 mM PIPES, pH 6.9, 1 mM MgSO₄, 5 mM EGTA, 150 mM NaCl, and either 1 mM Na-tetrathionate (as a sulfhydryl protease inhibitor; see reference 17) or 1 mM p-tosyl-L-arginine methyl ester (TAME; see also reference 8). The cells were washed twice by resuspension in fresh wash medium after centrifugation at 500 g for 30 min in 30 ml Correx centrifuge tubes (Corning Glass Works, Scientific Products Div., Corning, N. Y.). The cells were resuspended in lysis medium (wash medium made 0.4% in Triton X-100) at maximum dilution per tube and were again centrifuged to sediment the semisuspended cells. They were then resuspended in fresh wash medium and washed two times to remove the detergent, released hemoglobin, and other soluble cytoplasmic constituents.

Newt erythrocytes tend to clump more easily after lysis than either chick or toad erythrocytes. For this reason, and because of the small volume of blood obtained, the newts were bled directly into a solution containing one part wash medium to one part lysis medium. The resulting lysed cells were then washed and centrifuged to remove released soluble proteins. To counteract aggregation and clumping of the cells of all species, dilutions of the lysed cells in wash medium were kept at a maximum and the solutions were maintained at room temperature for the duration of the experiment.

### Immunofluorescence

Lysed cells suspended in wash medium were placed on coverslips and allowed to adhere for 30 s; the coverslips were then washed free of excess cells, using a stream of wash medium. Without drying, the coverslips were placed in 3.7% formaldehyde in wash medium at 37°C for 30 min. They were then rinsed in wash medium followed by cold acetone and placed in fresh cold acetone, on ice, for 10 min. The coverslips were again rinsed in wash medium and placed in a humid chamber, made by lining a petri dish with moist filter paper. The coverslips were flooded with an appropriate dilution of anti-MAP 2 serum (the preparation of which has been described previously; see reference 30) and allowed to stand at room temperature for 45 min. The coverslips were then returned to the humid chamber and were flooded with an appropriate dilution of fluorescein isothiocyanate-labeled goat antirabbit serum. The coverslips were flooded with an appropriate dilution of fluorescein isothiocyanate-labeled goat antirabbit serum (Miles Laboratories, Inc., Elkhart, Ind.) and were allowed to stand for 45 min. The coverslips were then returned to the humid chamber and were flooded with an appropriate dilution of fluorescein isothiocyanate-labeled goat antirabbit serum (Miles Laboratories, Inc., Elkhart, Ind.). They were allowed to stand for 45 min, washed extensively as before, and mounted in 50% glycerol in wash medium.

The cells were observed with phase-contrast and epifluorescence optics. Using a Nikon Optiphot microscope equipped with a X40, 0.85 NA Zeiss phase-contrast oil-immersion objective. Images were recorded on Tri-X film developed in Diafine.

### Marginal Band Isolation

Lysed erythrocytes of the chick or toad in wash medium were fragmented by two 5-s bursts of a Lab-Line sonicator (Lab-Line Instruments, Inc., Melrose Park, Ill.) set at maximum output for the microtubes. The sonicated fragments were then layered over a discontinuous sucrose gradient containing steps of 1.0, 1.5, and 2.0 M sucrose in wash medium, in a 15 ml Corex centrifuge tube. The tube was then spun at 500 g for 15 min in a Sorvall HB-4 rotor (DuPont Co., Sorvall Biomedical Div., Newtown, Conn.) at 21°C. The fragments of the cytoskeleton formed by sonication were recovered at the top of the 1.0 M sucrose and larger fragments appeared at the 1.0/1.5 M sucrose interface. Nuclei were recovered from the 1.5/ 2.0 M interface and also from the pellet, which contained cells.

### Electron Microscopy

For negative-stain electron microscopy, lysed cells in wash medium were placed on carbon-over-Formvar-coated grids that were rinsed with 0.2% cytochrome c in 1% amyl alcohol, stained with 1% uranyl acetate, and stained to near-dryness with filter paper. For thin sectioning, lysed cells in wash medium were fixed for 1 h in 2% glutaraldehyde in wash medium, postfixed, embedded, and sectioned as previously described (10, 30). All observations were made with a JEOL 100CX electron microscope operated at 80 kV.
Immunofluorescence Localization of MAP 2

When lysed erythrocytes were first treated with an antibody to MAP 2 and then with fluorescein-labeled goat antirabbit serum as described in Materials and Methods, intense fluorescence of the MB was observed as demonstrated in Fig. 4. In this figure, several newt cells either containing or lacking nuclei are shown in phase-contrast optics on the left, while the corresponding fluorescent image is shown on the right. In all cases studied, the fluorescence was localized in the area of the lysed cells occupied by the MB. Little staining was evident in the area occupied by the nucleus; nor was there any fluorescence detected in the area of the cytoskeleton between the nucleus and the MB. Similar results have been obtained with erythrocytes of chicks and toads.

The following controls (Fig. 5) showed that the localization obtained in Fig. 4 above was specific, because of the presence of a MAP 2-like protein in the MB of these erythrocytes. First, when the cells were treated with the fluorescent goat serum alone, no areas of the cells were stained when viewed in the fluorescence microscope (Fig. 5a and b). Second, no fluorescence was noted when the cells were treated with nonimmune serum followed by fluorescent goat antirabbit serum (Fig. 5c and d). Finally, the fluorescence of the MB was completely blocked by pretreatment of the immune serum with purified MAP 2 (Fig. 5e and f) but it was not affected by pretreatment of the immune serum with purified tubulin (Fig. 5g and h).

These controls, together with the experimental data provided in Fig. 4, strongly suggested that a protein with antigenic characteristics analogous to those of vertebrate brain MAP 2 was a component of the MB of those nucleated erythrocytes studied thus far.

Proteins of the MB

To characterize further the MB and associated filamentous components of the cytoskeleton, sonication and sucrose gradient centrifugation were used to separate the nuclei from the remaining components of the cytoskeleton (MB and trans-MB material) as described in Materials and Methods. When these isolated fragments of cytoskeleton were analyzed on SDS-polyacrylamide gels, proteins with molecular weights similar to those of spectrin, goblin (3), tubulin, and actin were found to be the major components (Fig. 6). In addition, a high molecular weight polypeptide with a relative migration similar to that of brain MAPs was also observed (arrow, Fig. 6).

To determine whether this protein similar in molecular weight to brain MAPs was responsible for the positive immunofluorescence results (Fig. 4), iodinated protein A was used to determine which protein in the MB was capable of reacting specifically with the MAP 2 antibody. For this technique, proteins of the cytoskeleton were first separated by SDS-poly-
FIGURE 2  Thin section through a lysed toad erythrocyte showing the MB in cross section (arrows 1 and 2) and its connections to the central nucleus. The MB in the area of arrow 1 has folded back on the nucleus during processing of the cells for electron microscopy. Bar, 1 μm. × 37,000.
FIGURE 3 Negative-stain whole-mount electron micrographs showing the microtubules of the MB system of the newt erythrocyte, as well as the filamentous nature of the trans-MB material that appears to anchor the nucleus at the cell center. (a) Low power view of a lysed newt erythrocyte. N denotes the area previously occupied by the nucleus, which has been dislodged by mild agitation. (b) Higher power of the area outlined by the rectangle in Fig. 3 a. The filamentous nature of the trans-MB material is readily apparent. Bars, = 0.5 μm. a, × 13,000; b, × 23,000.
Corresponding phase (left) and fluorescence (right) micrographs of lysed newt erythrocytes stained with anti-MAP 2 and prepared for indirect immunofluorescence with fluorescein-labeled goat antirabbit antibodies. (a and b) Lysed cells with nuclei; (c and d) isolated MB from which the nucleus has been removed by vortexing. Bar, 20 μm. x 875.

acrylamide gels, which were then reacted with antibody. The gels were next treated with iodinated protein A, which is capable of binding tightly and specifically to the Fc portion of immunoglobulins (18, 20). After the second reaction, the gels were dried and autoradiographed. Thus, in a manner analogous to indirect immunofluorescence, one can detect the presence of specific antigens in SDS gels by noting the distribution of the radioactivity associated with protein A.

Fig. 7 demonstrates the results obtained when a control sample containing MAPs and tubulin was used in the protein A staining technique. Lanes a and c show Coomassie Blue-stained samples of purified chick brain microtubules that contain tubulin and MAPs. Lanes b and d show the corresponding autoradiographs of these samples obtained after staining either with nonimmune serum (lane b) or with immune immunoglobulins containing anti-MAP 2 (lane d). The autoradiograph reveals only the iodinated protein A. In this example, a specific reaction was observed with MAP 2. No reaction occurred with the nonimmune serum, and the immune serum reacted only with MAP 2, not with tubulin or any other major protein component of the preparation. The only other reaction product detected was with two to three bands having molecular weights slightly below that of MAP 2, and it has been determined that these are hydrolytic cleavage products of the MAP 2 polypeptide (footnote 1, see also references 30 and 35).

Having established that the MAP 2 antibody was capable of reacting with its respective antigen in SDS gels, we employed the same technique to determine which protein component of the MB was antigenically similar to chick brain MAP 2. Lysed toad erythrocyte cytoskeletons were reduced and run on a 20-cm-long acrylamide slab gel after the method of Laemmli (21). The top third of the gel lane containing the sample was then used for the iodinated protein A staining technique. A large slab gel was run to ensure that enough protein would be loaded to show a distinct MAP 2-like band when stained with Coomassie Blue; only the top third of the gel was used because it was easier to handle during the subsequent steps of the protein A technique. The results shown in Fig. 8 demonstrate clearly that the MAP 2 antibody reacted specifically with a protein component of the MB having a molecular weight similar (but not identical) to that of MAP 2, because this band (arrow, Fig. 8) is the only protein that has radioactivity, and therefore protein A and ultimately anti-MAP 2 antibody, associated with it.

DISCUSSION

The data presented here demonstrate that the cytoskeleton of nucleated erythrocytes contains a protein that has antigenic characteristics similar to those of chick brain MAP 2. This protein is associated with the microtubules of the MB system of these erythrocytes, a conclusion supported by the following evidence: First, indirect immunofluorescence, using an anti-

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body to MAP 2, demonstrated intense and specific staining of the MB; no other component of the lysed erythrocytes (nuclear membrane or trans-MB material) stained with the antibody (Fig. 4). Second, a series of controls (Fig. 5), using nonimmune serum, fluorescent goat antirabbit serum alone, or preabsorbed antibodies showed that the fluorescence noted in Fig. 4 was attributable specifically to the presence of a MAP 2-like protein in the MB. Third, ¹²⁵I-protein A staining of SDS gels containing the suspected antigen (Fig. 8) demonstrated that the antibody was reacting with a protein of the MB having a molecular weight similar, but not identical, to that of chick brain MAP 2. Furthermore, control protein A staining showed that the antibody was not reacting with tubulin, the major component of the MB microtubules, an observation confirmed independently by ¹²⁵I-protein A staining (Fig. 7), immunoelectrophoresis (30), and immunodiffusion (data not shown).
The results summarized above confirm the proposal that the cytoplasmic microtubules have associated with their surfaces one or more characteristic proteins called MAPs, a term coined previously by Sloboda et al. (32) to refer to a class of high molecular weight proteins that are stoichiometrically associated with the tubulin subunit lattice of in vitro assembled vertebrate brain microtubules (10, 19, 24, 30, 33). The term MAP has since come to refer to a general class of proteins that associate with and appear to modulate some of the characteristics of in vitro assembled brain microtubules. Thus, MAPs can currently be divided into three subclasses: (a) the high molecular weight (~300,000) MAPs (6, 32); (b) the tau polypeptides described by Weingarten et al. (36); and (c) the tubulin assembly protein (TAP) described by Lockwood (22). Moreover, the term MAP has also been used to refer to similar molecules in cultured cells.

Indirect immunofluorescence results from several other laboratories have also shown that MAPs are associated with cytoplasmic microtubules in situ. Using an antiserum to the high molecular weight MAPs, Sherline and Schiavone (27, 28) noted fluorescent staining of cytoplasmic microtubules in rat neuroblastoma cells and 3T3 cells as well as staining of the microtubules of the mitotic apparatus; an identical observation has been made by Sheterline (29) working with both in vitro assembled pig brain microtubules and ovarian granulosa cells. Similarly, Connolly et al. (9) reported staining of the microtubule networks in rat glial cells, using both antibodies to MAPs 1 and 2 and to tau. Lockwood (22) has also reported that anti-TAP antibodies stain the microtubule networks of several lines of tissue culture cells. In all of the above cases, specific association of the respective MAPs was inferred by the following type of experiment. When the cells were treated with colcemid to disrupt the cytoplasmic microtubules, the cytoplasm no longer stained specifically with the MAP antiserum being used. Because the microtubules were depolymerized by the drug treatment, the MAPs, which were associated with the wall of the microtubules, also lost their characteristic distribution throughout the cytoplasm.

An analogous experiment with the MB microtubules is not possible because the microtubules of this organelle are extremely stable relative to the other examples of cytoplasmic microtubules discussed above. The erythrocytes can be lysed in low ionic strength buffers and the MB microtubules remain intact; no precautions, such as microtubule-stabilizing solutions, are necessary as with the cytoplasmic microtubules of other cell types. Thus, the technique of 125I-protein A staining (1) was used instead to demonstrate the existence of a MAP 2-like antigen among the protein components of the cytoskeleton of nucleated erythrocytes (Fig. 8). It is clear from the results of the immunofluorescence data as well as the protein A data that the nucleated erythrocyte contains a MAP 2-like protein as a component of the MB, presumably bound specifically to the

![Figure 6](image_url)  
**Figure 6** Coomassie Blue-stained samples to show the protein components of the cytoskeleton of nucleated erythrocytes. Lane a: Chick brain microtubules containing tubulin (T) and MAPs (M) 1 and 2. Lane b: human erythrocyte ghosts, showing bands! and 2 (spectrin, S) (see reference 12). Lane c: lysed chick erythrocytes, showing, among others, bands having molecular weights similar to those of actin, spectrin, and goblin (G) (see reference 3). The arrow points to a polypeptide having a molecular weight similar to that of chick brain MAP 2.

![Figure 7](image_url)  
**Figure 7** Control data for the 125I-protein A staining of SDS gels with anti-MAP 2 antibodies. Lanes a and c: Coomassie Blue-stained samples of chick brain microtubules containing tubulin and MAPs. Lanes b and d: the corresponding autoradiographs obtained after staining with nonimmune serum (lane b) or with anti-MAP 2 antibodies (lane d), followed by iodinated protein A. The anti-MAP 2 antibodies react specifically with MAP 2, the lower molecular weight polypeptide of the high molecular weight doublet labeled MAPs in this figure.
polypeptides could have a specific binding site at one end for microtubules and at the other end for organelles as structurally diverse as membranes, microtubules, and microfilaments.

When one considers the existence of tubulin in the membrane (5, 34), however, the first two possibilities listed above are not all that difficult to imagine. For example, MAPs could mediate tubule-tubule cross-bridging and also link microtubules to the membrane by anchoring them to membrane tubulin. In the case of the MB and adjacent plasma membrane, connections to the membrane via membrane tubulin are a distinct possibility because at least one report has suggested that tubulin is a component of the plasma membrane of pigeon erythrocytes (37). Moreover, viscometric analysis (15) has suggested an interaction between microtubules and actin-containing microfilaments mediated by the MAPs. Thus, the MAPs may be involved in the interaction of the microtubules of the MB with the trans-MB material (which preliminary results suggest contains intermediate filaments and actin filaments). Therefore, the MB, overlying plasma membrane, and associated trans-MB material provide an easily obtained and manipulated system to study these interactions further. In this regard, experiments are currently under way, using reversible protein cross-linkers in an attempt to differentiate among the three possibilities listed above.

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REFERENCES

[References list is omitted for brevity.]

Figure 8  Iodinated protein A staining of the cytoskeletal proteins of newt erythrocytes. For this figure the proteins were separated on a slab gel and the top third of the gel was used for the subsequent steps of the protein A staining technique. Lane a: Coomassie Blue-staining pattern showing erythrocyte spectrin (bands 1 and 2), goblin (the band labeled G, whose phosphorylation has been shown to be correlated with catecholamine-stimulated transport of sodium and potassium ions across the plasma membrane; see reference 3), and a high molecular weight protein (arrow) whose mobility is similar, but not identical, to that of chick brain MAP 2. Lane b: The corresponding autoradiograph, which demonstrates a specific reaction between the protein identified by the arrow and antibodies to chick brain MAP 2.

Another possible function of MAP 2 in the MB that should be considered is that the protein may cross-link the microtubules (a) to each other, (b) to the plasma membrane, or (c) to a component of the trans-MB material. Any of these examples are possibilities. The literature abounds with electron-microscope examples of microtubule-microtubule, microtubule-membrane, and microtubule-filament interactions (cf. references 13, 15, and 26 for some examples); and it is entirely possible that MAPs could be performing a similar function in the MB, although it is difficult to understand how a single
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.

22. Lockwood, A. H. 1978. Tubulin assembly protein: immunochemical and immunofluorescent studies on its function and distribution in microtubules and cultured cells. *Cell.* 13:613-627.

23. Matsudaira, P. T. and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386-396.

24. Murphy, D. B. and G. G. Borisy. 1975. Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 72:2696-2700.

25. Murphy, D. B., K. A. Johnson, and G. G. Borisy. 1977. Role of tubulin-associated protein in microtubule nucleation and elongation. *J. Cell Biol.* 77:33-52.

26. Ross, J. J. B. Omlund, and J. L. Rosenbaum. 1973. The ultrastructure of mouse neuroblastoma cells in tissue culture. *Tissue & Cell.* 7:107-136.

27. Sherline, P. and K. Schiavone. 1977. Immunofluorescence localization of high molecular weight proteins along intracellular microtubules. *Science (Wash. D. C.)* 198:1038-1040.

28. Sherline, P. and K. Schiavone. 1978. High molecular weight MAPs are part of the mitotic spindle. *J. Cell Biol.* 77:R9-R12.

29. Sherline, P. 1978. Localization of the major high molecular weight protein on microtubules in vitro and in cultured cells. *Exp. Cell Res.* 115:460-464.

30. Sloboda, R. D., D. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic-AMP dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. U. S. A.* 72:177-181.

31. Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic-AMP dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. U. S. A.* 72:177-181.

32. Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic-AMP dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. U. S. A.* 72:177-181.

33. Sloboda, R. D., and J. L. Rosenbaum. 1979. Decoration and stabilization of intact, smooth-walled microtubules with microtubule-associated proteins (MAPs). *Biochemistry.* 18:48-55.

34. Stephens, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry.* 16:2047-2058.

35. Vallee, R. B., and G. G. Borisy. 1977. Removal of the projections from cytoplasmic microtubules in vitro by digestion with trypsin. *J. Biol. Chem.* 252:377-382.

36. Weingarten, M. D., A. H. Lockwood, S. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U. S. A.* 72:1858-1862.

37. Zezza, H. P., and T. Pfeuffer. 1976. Microtubular proteins in pigeon erythrocyte membranes. *Eur. J. Biochem.* 71:177-184.