A Marginal Band-associated Protein Has Properties of Both Microtubule- and Microfilament-associated Proteins

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Abstract. The marginal band of nucleated erythrocytes is a microtubule organelle under rigorous quantitative and spatial control, with properties quite different from those of the microtubule organelles of cultured cells. Previous results suggest that proteins other than tubulin may participate in organizing the marginal band, and may interact with elements of the erythrocyte cytoskeleton in addition to microtubules. To identify such species, we raised mAbs against the proteins that assemble from chicken brain homogenates with tubulin. One such antibody binds to a single protein in chicken erythrocytes, and produces an immunofluorescence pattern colocalizing with marginal band microtubules. Several properties of this protein are identical to those of ezrin, a protein isolated from brush border and localized to motile elements of cultured cells. A significant proportion of the antigen is not soluble in erythrocytes, as determined by extraction with nonionic detergent. This cytoskeleton-associated fraction is unaffected by treatments that solubilize the marginal band microtubules. The protein has properties of both microtubule- and microfilament-associated proteins. In the accompanying manuscript (Goslin, K., E. Birgbauer, G. Banker, and F. Solomon. 1989. *J. Cell Biol.* 109:1621–1631), we show that the same antibody recognizes a component of growth cones with a similar dual nature. In early embryonic red blood cells, the antigen is dispersed throughout the cell and does not colocalize with assembled tubulin. Its confinement to the marginal band during development follows rather than precedes that of microtubules. These results, along with previous work, suggest models for the formation of the marginal band.

ALTHOUGH the ultrastructure of microtubules is highly conserved, they are organized into a wide variety of structures in differentiated cells. The molecular determinants that specify these organizations are not known. For several reasons, nucleated erythrocytes, and the microtubule organelle they contain, present an appropriate system for studying this issue. The cells themselves are lentiform, and somewhat flattened to produce an equator. The microtubules in them are confined to a marginal band at that equator, very near to the plasma membrane, and in one plane only (Behnke, 1970*a*, *b*). No microtubules are found between the nucleus and the position of the marginal band. The microtubules curve with the profile of the cell. In the nucleated erythrocytes of any particular species, the number of microtubules in the marginal band is the same, although from species to species they differ significantly (Goniakowska-Witalinska and Witalinski, 1976). In these ways, the marginal band is a distinct microtubule organelle which is under tight spatial and quantitative control.

We are using chicken erythrocytes, which are available both as adult cells and as embryonic precursors, to study how the properties of the marginal band are specified. Several previous results suggested that proteins other than tubulin may be involved in determining the marginal band structure. Murphy and colleagues have shown that the tubulin from chicken erythrocytes has a distinctive primary structure (Murphy and Wallis, 1983) and different assembly properties in vitro (Rothwell et al., 1985). They have also demonstrated that the isotype of beta-tubulin specific to the erythropoietic lineage in chickens is incorporated into several sorts of microtubule structures (Murphy et al., 1986). Therefore, the sequence of this isotype may be necessary for formation of the marginal band in vivo, but is unlikely to be sufficient. Additional evidence of a role for interacting proteins is derived from analyses of the marginal band microtubules themselves. In an in vitro reconstruction experiment, extraction of erythrocytes preincubated in the cold produces a detergent-extracted cytoskeleton preparation devoid of microtubules. When these extracted cells are incubated with calf brain tubulin lacking associated proteins, microtubules reform in the position and morphology of the original marginal band, in numbers equal to or less, but not greater, than that found in the original marginal band (Swan and Solomon, 1984). This result suggests that proteins remaining after release of the assembled tubulin might form sites that determine the position, shape, and number of microtubules. Conversely, it also suggests that no protein solubilized by the

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extraction is necessary for the in vitro reformation. Since a significant proportion of the polymerized actin colocalizes with the marginal band (Kim et al., 1987), there may be an interaction between actin filaments and microtubules.

We are interested in identifying polypeptides in erythrocytes with properties expected for a marginal band associated protein. Antibodies raised against the proteins that coassemble with chicken brain tubulin were used to identify a protein that might also interact with marginal band microtubules. One such antibody, here called 13H9, was found. The 13H9 antigen may be identical to ezrin. It colocalizes with assembled tubulin in these cells, but remains associated with the cytoskeleton even after the microtubules are completely depolymerized. The experiments in this manuscript suggest that this protein has properties of both a microtubule- and a microfilament-associated protein, which may reflect the localization of both assembled tubulin and actin at the marginal band. In the accompanying manuscript (Goslin et al., 1989), evidence is presented that the same antigen colocalizes with F-actin in growth cones of primary neurons, in a manner which depends upon microtubules.

Materials and Methods

Isolation of Chicken Microtubule Protein

Chicken heads were obtained from Mayflower Poultry (Cambridge, MA). The brains were removed and homogenized and microtubule protein was prepared by two cycles of assembly and disassembly in the absence of glycerol (Borisy et al., 1975). The microtubule protein was chromatographed over a phosphocellulose column under conditions where tubulin does not bind (Weingarten et al., 1975). The associated proteins were then eluted with 0.8 M sodium chloride in column buffer.

Monoclonal Antibody Generation

Four mice were injected with phosphocellulose purified chicken brain microtubule-associated proteins emulsified with Freund's complete adjuvant, and were boosted three times with antigen in Freund's incomplete adjuvant. Their response was monitored by blotting with tail bleeds. 3 d after the last boost, the mice were killed and the spleens were removed, and hybridomas formed by fusion to P3X63-Ag8.653 cells, according to the method of Kohler and Milstein (1975) as modified by Manser and Gefter (1984). The fusion products were plated out by limited dilutions and selected in hypoxanthine/aminopterin/thymidine (HAT). Supernatants were screened, and selected clones were grown in RPMI-1640 medium (Whittaker M A. Bioproducts, Walkersville, MD) with 10% FCS (defined; Hyclone Laboratories, Logan, UT). Hybridoma 13H9 was subcloned twice, and supernatants obtained for immunofluorescence and immunoblots.

Preparation of Cells for Immunofluorescence

Mature erythrocytes were obtained and plated as described (Miller and Solomon, 1984). Cells were extracted in the presence of taxol (kindly provided by M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) according to Kim et al., 1987. After extraction, cells were fixed in 0.5% glutaraldehyde in the extraction buffer for 1 min, followed by subsequent fixation for 30 min in 3.7% formaldehyde in extraction buffer. After washing, the coverslips were treated with sodium borohydride in PBS to quench the glutaraldehyde reactivity.

The preparation of embryonic red blood cells was as described (Kim et al., 1987). Cells plated on glass coverslips were extracted for 5 min with 0.5% NP-40 in 0.1 M Pipes, 2 mM EGTA, 1 mM magnesium sulfate, 2 M glycerol, pH 6.9 (PM2G)[1] in the presence of 3.7% paraformaldehyde (prepared according to Osborn and Weber, 1982). The cells were subsequently fixed for 30 min with 3.7% paraformaldehyde in PM2G.

Chicken embryo fibroblast cells were grown and extracted as described (Solomon et al., 1979). Cells were fixed for 30 min with 1% glutaraldehyde in extraction buffer. The coverslips were washed and then quenched twice with sodium borohydride in PBS.

Immunofluorescence

Immunofluorescence staining of coverslips was as previously described (Kim et al., 1987). Coverslips were mounted onto slides using gelvatol mounting media containing 15 mg/ml 1,4-diazabicyclo[2.2.2]octane, an antifade agent obtained from Aldrich Chemical Co., Milwaukee, WI. Antitubulin antibody (429) is a rabbit polyclonal previously described (Kim et al., 1987). FITC conjugated goat anti-mouse and Texas red-conjugated goat anti-rabbit were obtained from Organon Teknika-Cappel, Malvern, PA. Rhodamine-labeled phalloidin was obtained from Molecular Probes Inc. (Junction City, OR). Photography was performed on an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) with a 100× lens (1.3 NA) using film (Hypertech) from Microfluor, Ltd., Stonybrook, NY.

Chicken Erythrocyte Preparation for Protein Analysis

Blood was obtained from chickens and washed (Miller and Solomon, 1984). 0.5 ml of packed erythrocytes were incubated at 39°C or 0°C for 90 min in PBS with 0.025 M glucose to obtain cells with or without microtubules. All extractions were performed at 39°C. Cells were pelleted and were extracted for 5 min with 0.1% NP-40 in PM2G (15 ml) with protease inhibitors (0.03 TIU/ml aprotinin, 1 microgram/ml leupeptin, and 2 mM PMSF). The extracted cells were pelleted and the supernatant was collected to give the "unassembled" fraction. The pellet was resuspended in 15 ml of the same extraction buffer and incubated for 5 min. The cytoskeletons were again pelleted, and the supernatant discarded. The pellet was subsequently extracted for 5 min in 0.75 ml PBS with protease inhibitors. PBS induces microtubule disassembly in these preparations even in the absence of calcium ions, so they are left out because they activate an associated protease. The cytoskeletons were pelleted in a microfuge and the supernatant was collected as the "assembled" fraction. This pellet was finally extracted with 0.75 ml 8 M urea in PBS with protease inhibitors. This extract was spun in the microfuge to remove insoluble material, and the supernatant was collected as the "cytoskeletal" fraction.

Purified chicken intestinal ezrin was a gift from P. Matsudaira (Whitehead Institute and M.I.T., Cambridge, MA).

Protein Blotting

For Western blots, samples separated on a 7.5% polyacrylamide gel with a 5% polyacrylamide stacker, according to the method of Laemmli (1970), were transferred to nitrocellulose electrophoretically and probed by slight modifications of standard procedures (Tobin et al., 1979). The nitrocellulose filters were blocked with 2% hemoglobin in PBS with 0.05% sodium azide. Antibody incubations were done in the same buffer. Following the first and second antibody incubations, the blot was washed in blocking buffer which contained 0.1% SDS and 0.05% NP-40. ^{125}I-Labeled goat anti-mouse Ig and protein A were obtained from New England Nuclear (Boston, MA).

Two-dimensional electrophoresis was done according to the method of O'Farrell (1975) as modified in Pallas et al. (1982).

Results

Generation and Screening of mAbs

We chose to search for marginal band associated proteins from among the set of proteins that interact with chicken microtubules in vitro, and to use antibodies to identify them. Relevant monoclonal antibodies were generated and characterized by the following scheme (Fig. 1). Chicken brain homogenates, rich sources of microtubule proteins, were carried through two cycles of temperature-dependent assembly and disassembly by standard techniques (Borisy et al., 1975). The microtubule proteins, containing ~85% tubulin, were chromatographed on phosphocellulose under conditions where the tubulin did not bind (Weingarten et al., 1975). The proteins that remain bound were eluted from the

1. *Abbreviation used in this paper*: PM2G, 0.1 M Pipes, 2 mM EGTA, 1 mM magnesium sulfate, 2 M glycerol, pH 6.9.

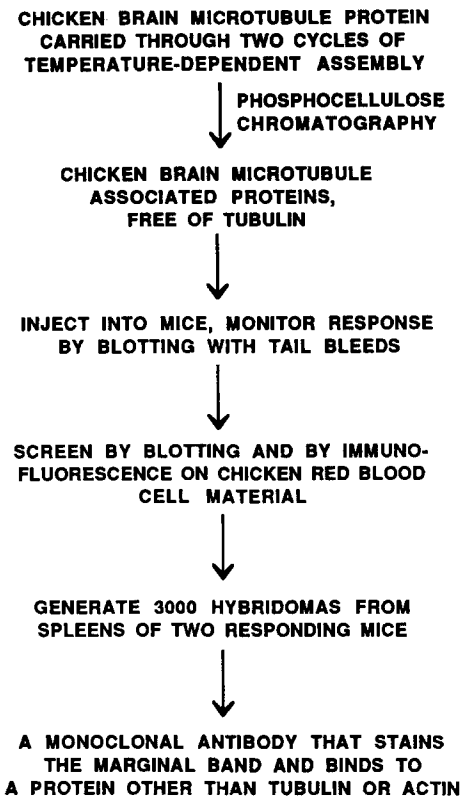


Figure 1. Scheme for generating mAbs. Chicken brain microtubule proteins were prepared by carrying homogenates through two cycles of temperature-dependent assembly, and then separated from tubulin by phosphocellulose chromatography. The high-salt eluate from this column was injected into mice. The immune response was followed by blotting of tail bleeds. The 3,000 hybridomas generated from two independent fusions were screened by blotting and immunofluorescence with chicken erythrocytes, rather than the original antigen. This screen identified antibodies that stained the marginal band and that were not against tubulin.

column with salt, then concentrated by acetone precipitation, and injected into mice.

The immune responses of four mice were monitored by blotting of tail bleeds against the brain antigen. Sera from two of these mice bound to many proteins of the antigen preparations, producing blotting patterns that were essentially the same as the stained protein pattern (data not shown). The spleens from these two mice were used for two independent fusions. The products of these fusions were screened not with the brain antigen but instead with chicken erythrocyte proteins, to search for antibodies that stained the marginal band region and did not bind to tubulin. The screen consisted of two parts. The first was blots onto proteins of detergent-extracted preparations of chicken erythrocytes containing assembled microtubules in an intact marginal band. Those hybridomas that gave unambiguous blotting patterns were screened again by immunofluorescence on cells extracted in the same way before fixation. Candidates were subcloned and retested by both assays.

Of ~3,000 hybridomas screened, two stained a single polypeptide on Western blots, and gave a staining pattern that was restricted to the marginal band. One of them probably recognized tubulin, judging from the mobility of the band to which it bound on blots. The second hybridoma, called 13H9, was not directed against tubulin, according to the several criteria described below. That hybridoma was subcloned twice. Among the multiple subclones, the blotting activity and the immunofluorescence staining pattern of the original hybridoma were not separable.

The Staining of Antibody 13H9 Colocalizes with the Marginal Band in Adult Erythrocytes

Fig. 2 (*a–d*) shows the double immunofluorescence staining of adult chicken erythrocytes with antibody 13H9 and antitubulin. Cells were preincubated either at 39°C to preserve microtubules (Fig. 2, *a* and *b*), or at 0°C to depolymerize microtubules (Fig. 2, *c* and *d*), then extracted with nonionic detergent in microtubule stabilization buffer before fixation in the same buffer. In cells containing assembled microtubules, the patterns of 13H9 (Fig. 2 *a*) and antitubulin (Fig. 2 *b*) staining are coincident. Both patterns go out of the plane of focus in some places. The relationship between the two patterns is presented more clearly at higher magnification (Fig. 3). All of the detectable 13H9 staining is in the same position as the antitubulin staining. The 13H9 staining appears more punctate than does the antitubulin staining, although this can be abolished at higher concentrations of antibody. The antitubulin staining is completely abolished in cells preincubated at 0°C before extraction (Fig. 2 *d*), but the pattern of 13H9 staining in the same cells (Fig. 2 *c*) is indistinguishable from that of cells preincubated at 39°C. Preincubation at 0°C produces no detectable alteration in the staining pattern of 13H9. In addition, the absence of signal from these cells in the antitubulin channel (Fig. 2 *d*) demonstrates the lack of crossover from the 13H9 staining into the channel used to detect tubulin staining. The control for crossover in the opposite direction is shown in Fig. 2 *e* (cells prepared and stained as in Fig. 2 *a*, except antibody 13H9 was omitted).

The behavior of the 13H9 staining can be compared to actin localization in chicken erythrocytes. Phalloidin staining demonstrates that polymerized actin is highly concentrated in the position of the marginal band (Kim et al., 1987; Fig. 2 *f*), but there is also considerable phalloidin staining throughout the periphery of the cell. Its pattern is consistent with the localization of actin to the cortical cytoskeleton of mammalian red blood cells (Bennett, 1985) and with the presence of a similar membrane skeleton in avian erythrocytes (Lazarides and Moon, 1984). The phalloidin staining is insensitive to preincubation at 0°C. We have been unable to detect either antitubulin or 13H9 staining at any position other than at the marginal band.

The Erythrocyte Protein Recognized by 13H9 Is Related to Ezrin

Immunoblot analysis shows that 13H9 recognizes an 80-kD protein in several protein preparations. In particular, in chicken erythrocytes extracted as for the immunofluorescence experiments in Fig. 2, 13H9 binds to a single protein of that size (Fig. 4 *a*, lane *l*). Frequently, the signal appears as a closely spaced doublet. Blots of two-dimensional sepa-

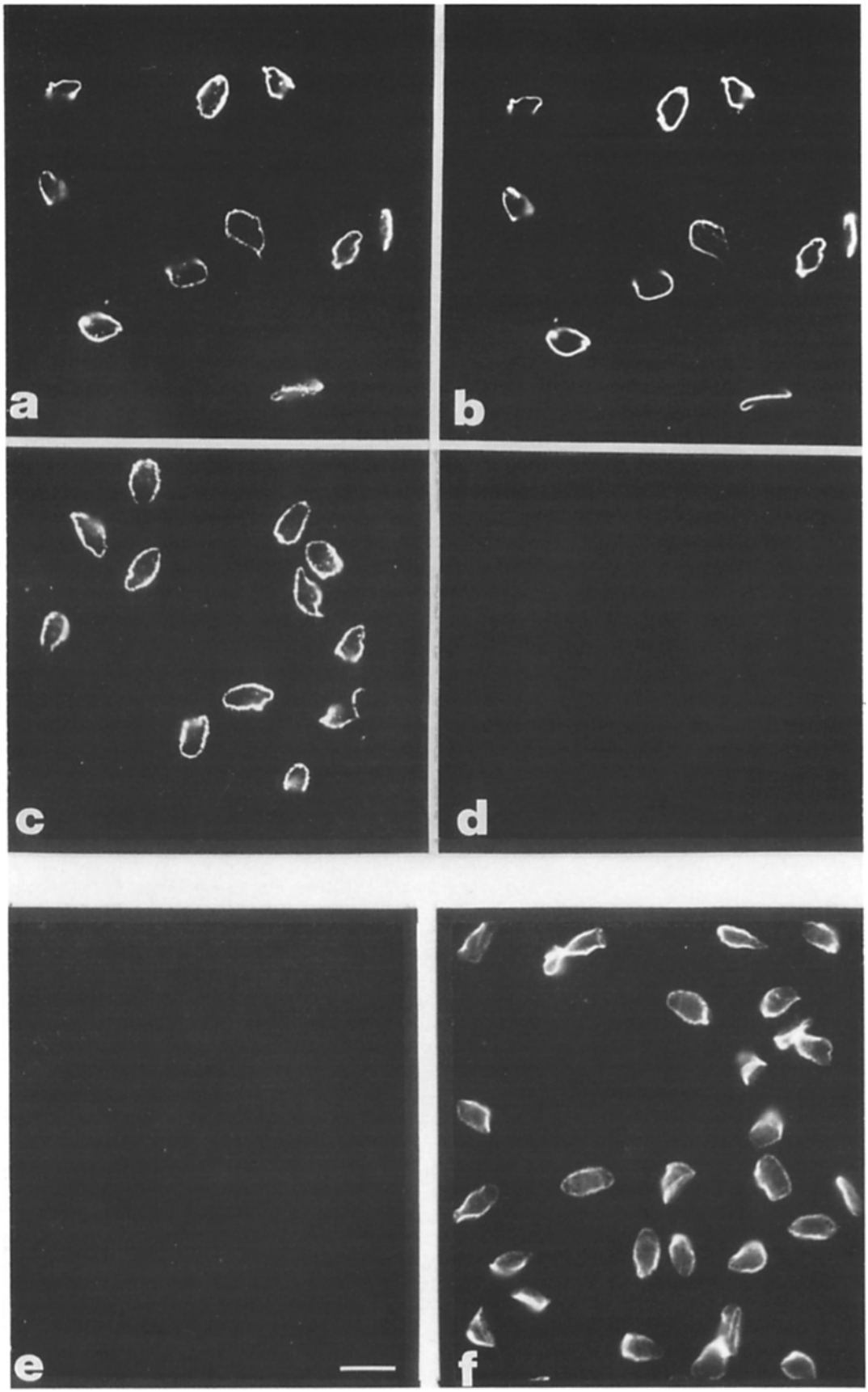


Figure 2. Staining of 13H9, antitubulin, and phalloidin in adult erythrocytes. (*a–d*) Double staining of adult erythrocytes with 13H9 (detected with fluoresceinated goat anti-mouse Ig) and antitubulin (detected with Texas red-labeled goat anti-rabbit IgG). Cells were preincubated at 39°C (*a* and *b*) or 0°C (*c* and *d*) then extracted with NP-40 in microtubule stabilizing buffer and fixed. In cells containing assembled

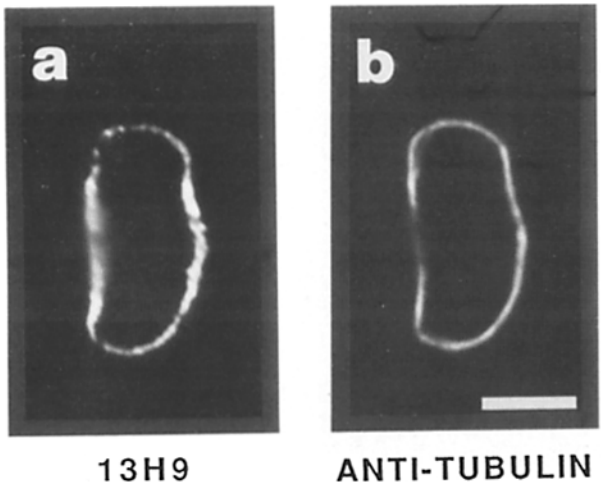


Figure 3. Double immunofluorescence of an adult erythrocyte with 13H9 (*a*) and antitubulin (*b*). Cell was preincubated at 39°C before extraction and fixation. Bar, 5 μm.

rations reveal that the staining can be resolved into one major spot of 80 kD, one minor spot of the same isoelectric point but slightly slower mobility, and at least four minor spots with more acidic isoelectric points (Fig. 4 *b*). The data suggest that 13H9 binds to a protein that is present in covalently modified forms.

The molecular properties of the protein that 13H9 binds suggested that it might be ezrin (Bretscher, 1983; Gould et al., 1986). Ezrin was originally isolated from brush border of intestinal epithelial cells by Bretscher (1983). Two-dimensional analyses of immunoprecipitates with antiezrin suggest that it, like the 13H9 antigen, is present in several covalently modified forms. The relationship of the 13H9 protein to ezrin is supported by further experiments. A polyclonal antibody against ezrin (supplied by A. Bretscher, Cornell University, and characterized in Bretscher, 1983) binds to a protein of the same mobility in chicken red blood cell preparations (Fig. 4 *a*, lane 2). In addition, 13H9 binds to ezrin in purified preparations from chicken intestine (Fig. 4

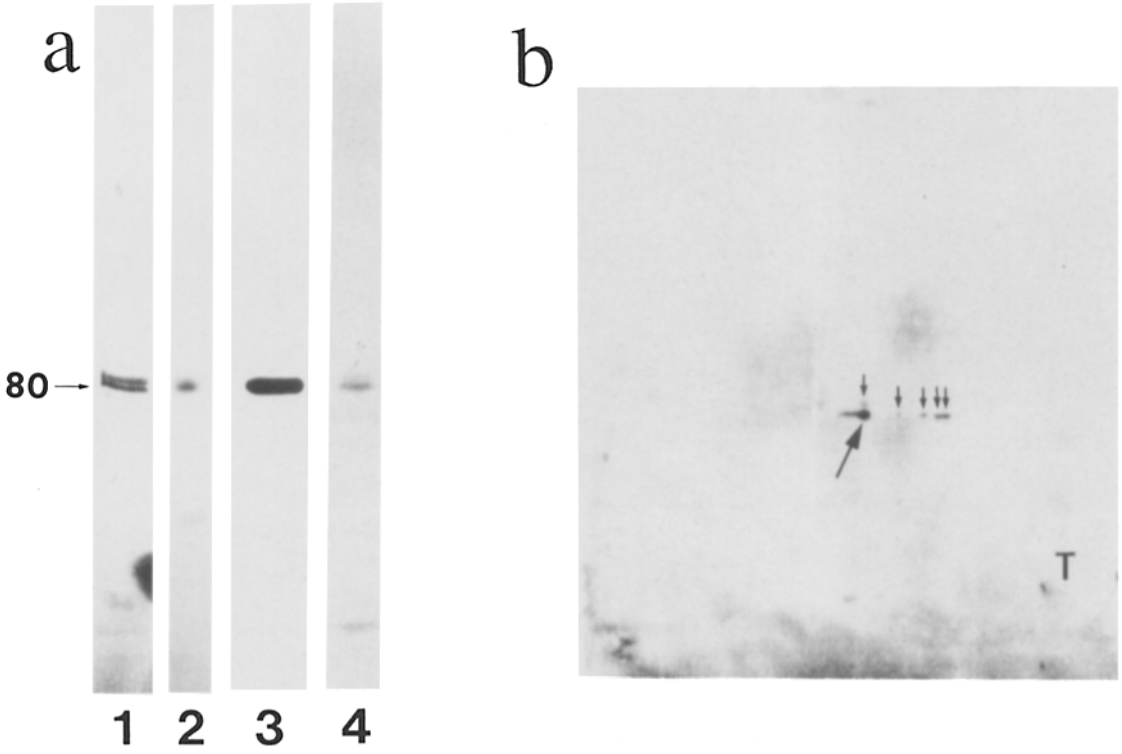


Figure 4. Immunoblotting of 13H9 against erythrocyte and microtubule proteins. (*a*) Western blots of one-dimensional gels. Each of the lanes in this panel is excised from separate slab gels. Each gel was standardized with molecular mass markers as indicated, and with the inclusion of chicken erythrocyte material stained with 13H9. Lane *1*, proteins from detergent-extracted adult erythrocytes, probed with 13H9. The mobility of the band stained corresponds to a molecular mass of 80 kD. Lane *2*, proteins from detergent-extracted adult erythrocytes, probed with polyclonal antiserum 507 against ezrin (Bretscher, 1983). Lane *3*, purified ezrin from chicken intestinal epithelia, probed with 13H9. Lane *4*, phosphocellulose-purified chicken microtubule-associated proteins, probed with 13H9. (*b*) Western blot of a two-dimensional gel, on adult erythrocyte proteins (as in lanes *1* and *2*, *above*). The large arrow marks the position of the major spot and the small arrows mark the position of the minor spots. *T* marks the position of tubulin. The acid end of the gel is to the right.

tubulin, the 13H9 staining (*a*) and antitubulin staining (*b*) are coincident. In cells preincubated in the cold, and so lacking assembled microtubules, the 13H9 staining (*c*) is localized to the same region of the cell while no antitubulin staining (*d*) is detected. The absence of signal in *d* demonstrates that there is no crossover from 13H9 staining into the Texas red channel used to visualize antitubulin staining. (*e*) Crossover control. Staining of cells with antitubulin alone. Cells prepared and stained as in *a* and *b* above, except that 13H9 was omitted. The absence of signal illustrates that there is no crossover from antitubulin staining into the fluorescein channel used to visualize 13H9 staining. (*f*) Staining of cells with rhodamine-phalloidin. Cells preincubated at 0°C, and stained with phalloidin after extraction and fixation. The staining is indistinguishable from that found in cells preincubated at 39°C (data not shown). Bar, 10 μm.

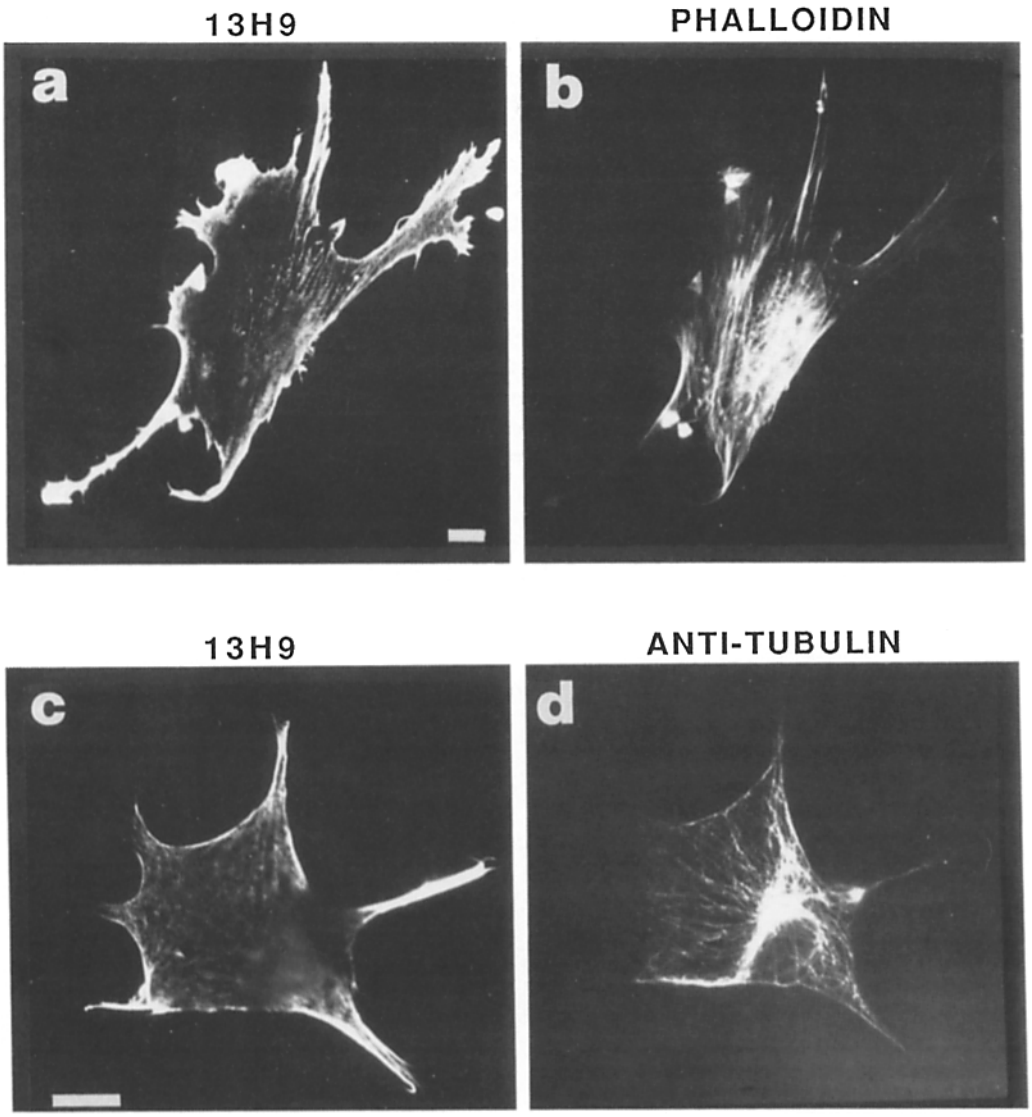


Figure 5. Double staining of chicken embryo fibroblasts with 13H9 (*a* and *c*) and either phalloidin (*b*) or antitubulin (*d*). Bar, 10 μm.

a, lane *3*). Antibody 13H9 also binds to a polypeptide of 80 kD in the protein preparation used to elicit it, the phosphocellulose-purified fraction of chicken brain proteins coassembling with microtubules (Fig. 4 *a*, lane *4*).

The presence of ezrin in various cultured cells, especially in motile surface extensions, was demonstrated by immunofluorescence (Bretscher, 1983, 1989). Staining of fibroblastic cells with 13H9 gives a similar, but not identical, pattern (Fig. 5, *a* and *c*). The antibody binds primarily to edges of these cells; it also binds strongly to the leading edge of migrating fibroblasts (data not shown). In double immunofluorescence experiments, the pattern of 13H9 staining is distinct from that of phalloidin (Fig. 5, *a* and *b*) and antitubulin (Fig. 5, *c* and *d*). Agents that disrupt microtubules or microfilaments change the morphology of these cells, but do not dramatically disrupt the organization of the 13H9 staining (data not shown). Therefore, if the 13H9 antigen interacts with microtubules or microfilaments in these cells, it must do so only in restricted domains of the cytoskeletal network.

Taken together, the results are consistent with the conclusion that 13H9 binds to chicken erythrocyte ezrin, but definitive identification requires more direct structural tests.

The Association of the 13H9 Antigen with the Erythrocyte Cytoskeleton Is Independent of the State of the Marginal Band Microtubules

To assay the state of the 13H9 antigen and tubulin in chicken erythrocytes, we used a previously described fractionation technique based on extraction with nonionic detergents in microtubule stabilizing and destabilizing buffers (Solomon et al., 1979). Before extraction, cells were preincubated either at 39°C (to preserve the marginal band microtubules) or 0°C (to depolymerize them). The fractions were separated by SDS-PAGE, transferred to nitrocellulose and probed with 13H9 (Fig. 6 *a*) or antitubulin (Fig. 6 *b*). The results show that little protein recognized by 13H9 is released by nonionic detergent extraction in either microtubule stabilizing or destabilizing buffers (Fig. 6 *a*, lanes *1–4*, *UNASS* and *ASSEM*). Instead, most of the antigen is present in the fraction dissolved in 8 M urea (Fig. 5, lanes *5* and *6*, *CYTOS*). This

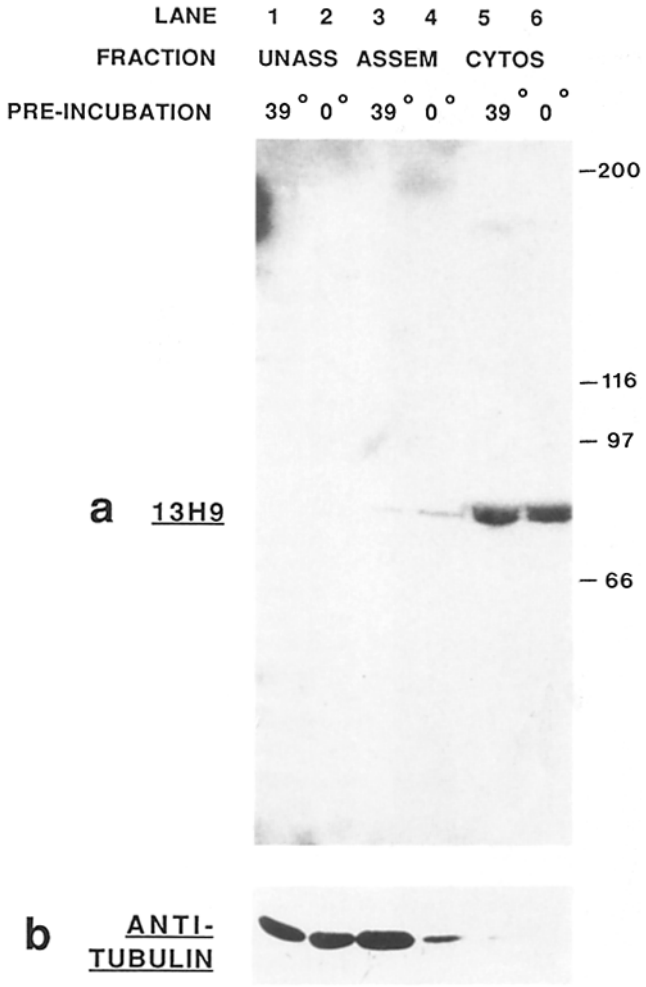


Figure 6. Fractionation of the 13H9 antigen in adult erythrocytes. Cells were preincubated at 39°C to preserve microtubules (*39°*, lanes *1*, *3*, and *5*) or preincubated at 0°C to depolymerize microtubules (*0°C*, lanes *2*, *4*, and *6*). They were then extracted sequentially as described in Materials and Methods, identical gels run and transferred, and the nitrocellulose filters probed with 13H9 (*a*) and antitubulin (*b*). Lanes *1* and *2* (*UNASS*) proteins released by microtubule stabilization buffer, to give the unassembled fraction of tubulin and other soluble proteins; lanes *3* and *4* (*ASSEM*) proteins released by microtubule depolymerizing buffer, to give the assembled fraction of tubulin; and lanes *5* and *6* (*CYTOS*), proteins released by 8 M urea, to give the cytoskeletal fraction. The CYTOS fractions, which contain the 13H9 antigen, are seven times more concentrated (with respect to cell equivalents) than the UNASS fractions, and four times less concentrated than the ASSEM fractions.

fractionation is not affected by preincubation in the cold to depolymerize microtubules, quantitatively supporting the immunofluorescence results shown in Fig. 2. In other experiments, in which different loads of these fractions were analyzed by the same procedure, and the autoradiograms exposed for varying times, we can detect some 13H9 antigen in the UNASS and ASSEM fractions. However, significantly >50% of the total cellular antigen is stably associated with the cytoskeleton by this assay. That result is similar to that reported for the compartmentalization of ezrin in other cells (Gould et al., 1986).

The fractionation of tubulin in adult erythrocytes (Fig. 6 *b*) is significantly different from that of 13H9. As is the case for several other cell types, most cell tubulin is released by extraction in microtubule stabilization buffer even in cells that contain intact microtubules (Fig. 6, lanes *1* and *2*; *UNASS*). The remainder of the tubulin in cells containing microtubules is released by the second extraction in microtubule destabilizing buffer including a trace of tubulin remaining in cells preincubated in the cold before extraction (Fig. 6, lanes *3* and *4*; *ASSEM*). There is little or no detectable tubulin remaining after these two extractions (Fig. 6, lanes *5* and *6*; *CYTOS*).

We conclude that the association of the 13H9 antigen with the erythrocyte cytoskeleton is not dependent upon the presence of assembled microtubules, irrespective of whether the microtubules were depolymerized in vivo or by extraction in a depolymerizing buffer. This fractionation behavior differs from that shown by several microtubule-associated proteins in cultured cells, which are quantitatively released from extracted cells when the microtubules are depolymerized (Solomon et al., 1979; Magendantz and Solomon, 1985; Peng et al., 1985).

Localization of 13H9 Staining during Development of the Marginal Band

The development of the primitive series of chicken erythrocytes occurs in readily isolated and roughly synchronous populations, from embryos between days 2 and 5 of development. The stages of microtubule band formation can be followed in these cells (Kim et al., 1987). Briefly, in early, spherical cells, individual microtubules appear to radiate from a centrally located microtubule organizing center. Subsequently, the microtubules gather in bundles that course through the cytoplasm in many planes and directions. Then, the microtubules of the embryonic erythrocyte are localized to a marginal band, thicker than that found in mature cells. After all of the microtubules are in the band as assayed by immunofluorescence, the cell displays its mature, lentiform morphology.

The 13H9 staining in embryonic cells shows a pattern of development that is distinct from that of the microtubules. Fig. 7, *a–d* show galleries of embryonic red blood cells, double stained with 13H9 and antitubulin and arranged to represent stages in the development of 13H9 staining. At early times, when the tubulin staining pattern emanates from a microtubule organizing center and when the microtubules bundle, the 13H9 staining is diffuse throughout the cytoplasm (Fig. 7 *a*). Even as the marginal band forms (Fig. 7 *b*), the 13H9 staining still fills the entire cytoplasm, frequently in a punctate pattern; however, there begins to be an increased signal in the vicinity and in the same plane as the developing marginal band. Subsequently (Fig. 7 *c*), much of the 13H9 staining does localize to the region of the band, although considerable punctate staining persists throughout the cell. Also, the staining at the marginal band does not display the smooth pattern of adult cells (compare Fig. 2 *a*). The appearance of this 13H9 staining correlates with the point in development when the cells elongate their spherical morphology. In the most developed embryonic cells studied, the 13H9 staining pattern is much smoother with very little off-band staining; however, a few patches of staining not on the band are still detected in these cells (Fig. 7 *d*). The morphol-

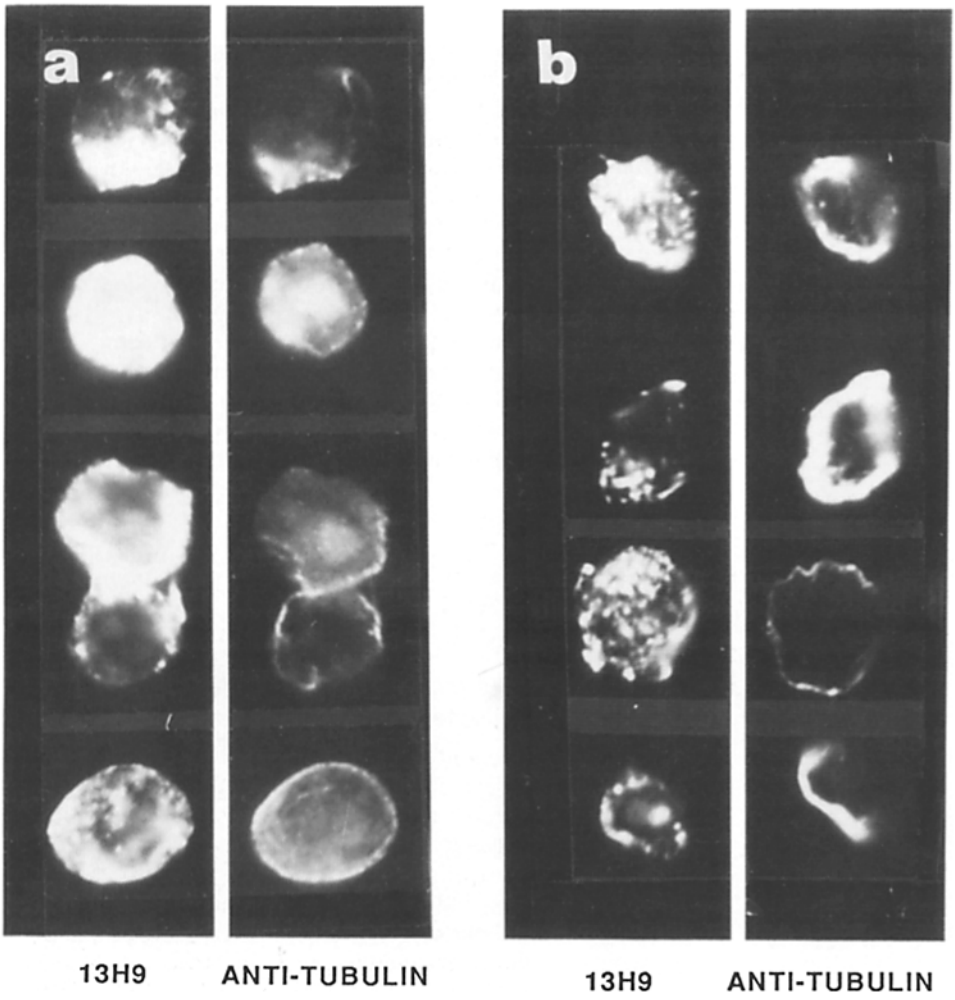


Figure 7. The staining pattern of 13H9 during erythrocyte development. Immunofluorescence of red blood cells from chicken embryos of 2 to 5 d after fertilization, double stained with 13H9 (*left*) and antitubulin (on the right). The cells have been grouped and arranged to represent stages in the development of the 13H9 staining pattern. (*a*) At early stages, when the microtubules are diffusely arrayed throughout the cytoplasm or in bundles, 13H9 staining is diffuse throughout the cytoplasm. (*b*) As the microtubules form a marginal band, 13H9 staining still fills the entire cytoplasm, frequently in a punctate pattern. Even at this stage, however, there is an increased concentration of staining in the vicinity of the marginal band. (*c*) Subsequently, 13H9 binds primarily at the marginal band, but with much punctate 13H9 staining throughout the cytoplasm. At this point, the shape of the cells is clearly no longer spherical, and the profile of the marginal band has begun to elongate. (*d*) Late embryonic erythrocytes, showing cells have become nearly lentiform. The 13H9 staining is almost completely localized to the marginal band in a continuous pattern. Bar, 5 μm.

ogy of these cells is very similar to that of adult cells. The data suggest that the restriction of cytoskeletal 13H9 antigen to the marginal band follows rather than precedes the organization of tubulin.

Fig. 8, *a–c* show galleries of embryonic red blood cells, double stained with 13H9 and phalloidin. The earliest stage detected (Fig. 8 *a*) corresponds to Fig. 7, *a* and *b*, which are distinguishable by differences in the tubulin patterns. The phalloidin pattern remains diffuse throughout the cell, although the 13H9 staining has begun to concentrate as punctate elements. Eventually, the 13H9 staining becomes largely restricted to the position of the marginal band (Fig. 8 *b*); at that time, the phalloidin stain largely but not precisely colocalizes with it. The phalloidin pattern appears smoother than the 13H9 pattern. In these cells, there is punctate 13H9 staining distinct both from the position of the marginal band and any detectable phalloidin staining. There is also diffuse phalloidin staining throughout the cell cortex. In older embryonic cells, the phalloidin and 13H9 staining are both concentrated at the band in quite coincident patterns, although again there is diffuse staining of phalloidin elsewhere in the cell (Fig. 8 *c*).

Discussion

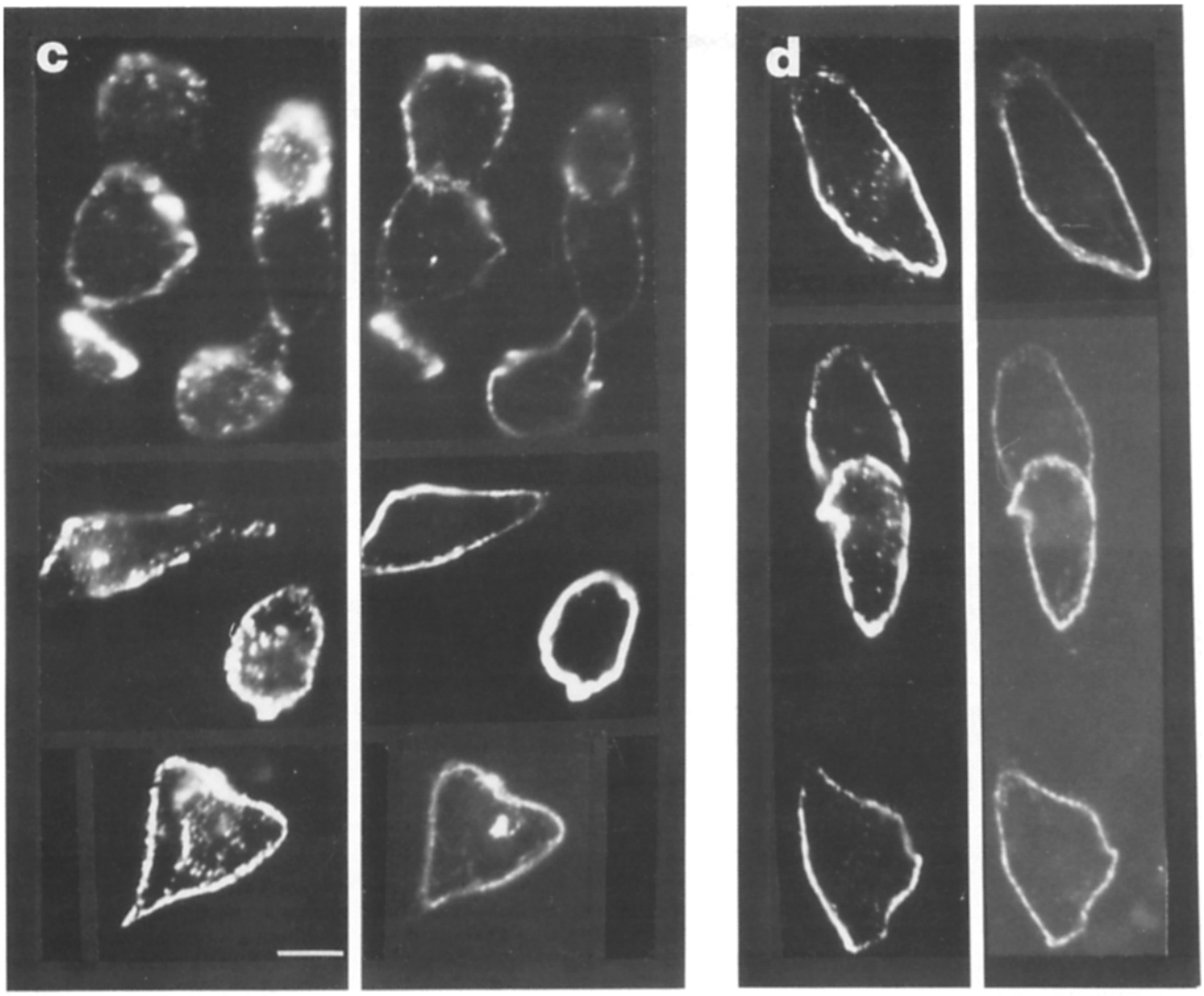
Identification and Properties of a Marginal Band–associated Protein

Two sorts of proteins might be associated with the assembled tubulin of marginal bands. One sort might be expected to bind to microtubules only, affecting their assembly or mediating interactions between them. The other sort might bind to both microtubules and other components of the cytoskeleton. Previous experiments suggest that this second sort of protein exists, and may be important in determining the properties of the marginal band. Particularly relevant is an in vitro reconstruction of the marginal band from detergent-extracted chicken erythrocytes depleted of assembled tubulin and incubated with purified calf brain tubulin lacking coassembling associated proteins (Swan and Solomon, 1984). In chicken red blood cells, such a protein would not be soluble under normal conditions, precluding its identification by previously devised approaches for analyzing microtubule components (Solomon, 1986). For that reason, we chose to prepare a battery of monoclonal antibodies against a set of chicken brain proteins that can interact with microtubules under in vitro conditions, and then determine if any of them are associated with the marginal band.

We obtained one such antibody, 13H9. The antigen it recognizes in chicken erythrocytes is largely insoluble under normal extraction conditions. All detectable 13H9 staining aligns with the marginal band. However, the pattern of 13H9 staining, and the amounts of it that behave as if associated with the cytoskeleton, are independent of the state of microtubule assembly. Thus, the 13H9 antigen is a candidate for a marginal band-associated protein of the second type, a protein that interacts both with microtubules and with other elements of the cytoskeleton.

It should be noted that this antibody screen detected no

Figure 7 (Continued)



marginal band–associated proteins of the first type, proteins associated exclusively with microtubules that might be expected to fractionate with assembled tubulin. The presence of such proteins among coassembling brain associated proteins, such as MAP 2, chartins, and tau (Peng et al., 1985; Magendantz and Solomon, 1985), is established in several systems, and therefore antibodies against them might have been represented in the monoclonal library generated from such proteins. There have been reports of such proteins in the marginal bands of other species (Nachmias et al., 1979; Sloboda and Dickersin, 1980; Tablin et al., 1988), and the existence of proteins mediating microtubule–microtubule interactions is suggested by the fact that marginal bands can be isolated from some nucleated erythrocytes (Cohen, 1978; Cohen et al., 1982). However, these proteins may not be expressed in the same covalent form in brain and erythrocytes, so an antibody against one form might not interact with the other.

The 13H9 Antigen May Interact with Both Microtubules and Microfilaments

The 13H9 antigen displays some properties of a microtubule-associated protein. First, in adult erythrocytes, it colocalizes with the marginal band microtubules. Colocalization with microtubules in cells is a hallmark of several minor microtubule components in other cell types (Connolly et al., 1977; Connolly et al., 1978; Bulinski and Borisy, 1980; Bloom et al., 1984; Parysek et al., 1984; Magendantz and Solomon, 1985; Huber et al., 1985). Second, it is among the proteins that coassemble with tubulin in vitro from brain homogenates. We and others have shown that coassembling proteins need not interact with microtubules in vivo (Lee et al., 1978; Solomon et al., 1979) although several proteins that do interact with microtubules in vivo also coassemble in vitro. We also do not know if the 80-kD antigen interacts directly with microtubules; it could interact with other elements of these complex homogenates.

Other properties of the 13H9 antigen indicate that it is clearly unlike previously characterized microtubule-associated proteins. It does not localize along the length of microtubules of cultured cells. Also, its association with detergent extracted cytoskeletons of adult erythrocytes is clearly independent of microtubules, as seen by immunofluorescence and protein blotting in erythrocytes with or without microtubules.

The 80-kD protein may interact with microfilaments. It colocalizes with most of the phalloidin staining in erythrocytes, and with a subset of that staining in fibroblasts. Its be-

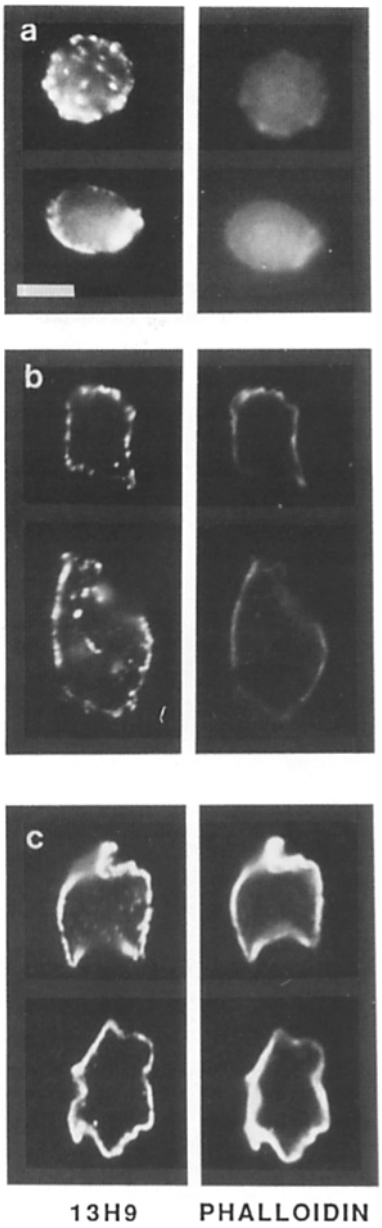


Figure 8. Comparison of 13H9 staining with phalloidin staining during embryonic development. Immunofluorescence of red blood cells from chicken embryos of 2 to 5 d after fertilization, double stained with 13H9 (*left*) and phalloidin (*right*). The cells have been grouped and arranged to represent stages in the development of the 13H9 pattern, as in Fig. 7. (*a*) At early stages, when 13H9 staining is distributed throughout the cytoplasm, either as punctate elements or more diffusely, the phalloidin staining is found throughout the cell. This stage corresponds to Fig. 7, *a* and *b*, which are distinguished by different tubulin patterns. (*b*) The 13H9 staining is primarily punctate, and is largely localized to the position of the marginal band. In the same cells, the phalloidin staining is present at the marginal band, in a smoother pattern. There is also phalloidin staining elsewhere in the cortex of the cell, which is faint and difficult to see in these micrographs. (*c*) At later stages, both the 13H9 and phalloidin staining are strongly localized at the position of the marginal band, and are closely coincident. Again, there is diffuse staining of phalloidin elsewhere in the cell. Bar, 5 μm.

havior during extraction is similar to that of a microfilament associated protein. But it does not colocalize with all microfilaments. Moreover, if it is ezrin, as the evidence suggests, then it does not bind to actin filaments in vitro (Bretscher, 1983). During erythrocyte development, the pattern of 13H9 staining is not identical to either that of antitubulin or phalloidin.

Clearly, then, if the 13H9 antigen does interact with microtubules or microfilaments, it does so only under certain circumstances, or only with particular domains of those structures. One possibility, that some proteins may mediate interactions between microtubules and microfilaments, is suggested by in vitro experiments (Griffith and Pollard, 1978). Functional connections between the two filament systems are implied by the ability of cytochalasin to block neurite retraction in neuroblastoma cells in which the microtubules are completely disassembled (Solomon and Magendantz, 1981; Joshi et al., 1985). Finally, in the accompanying paper (Goslin et al., 1989), we show that an antigen recognized by 13H9 localizes almost exclusively to the growth cones of primary neurons, in a pattern very similar to that of F-actin but nearly complementary to that of microtubules. However, it seems likely that in those cells the 13H9 antigen must interact with assembled tubulin, either directly or indirectly, since its localization is disrupted by the microtubule depolymerizing drug nocodazole.

The Marginal Band–associated Protein May Be Ezrin

The data presented here are consistent with the identification of the 13H9 antigen as ezrin, although this point is not established definitively. The interactions of ezrin are not fully understood, nor are its functions. It was originally identified in intestinal epithelia, and in particular has been localized to the microvilli at the apical surface in these cells (Bretscher, 1983); however, its precise position with respect to other structures in those cells has not been defined. The surface projections and motile elements with which it is associated in cultured cells also contain assembled actin, but ezrin clearly does not colocalize with the bulk of actin in these cells (see Bretscher, 1983, 1989; and Fig. 5). Also, it does not appear to bind to actin in vitro (Bretscher, 1983). Therefore, ezrin may interact with actin, but not under all circumstances. Ezrin is phosphorylated in vivo, in response to exposure to epidermal growth factor, and is concomitantly recruited into membrane ruffles and surface projections, suggesting that it may play a role in organizing such structures (Bretscher, 1989). It is important to point out that in the position of the marginal band, where all of the microtubules and 13H9 antigen of nucleated erythrocytes are localized, there is a significant concentration of assembled actin. It is possible that all three proteins interact in a complex, perhaps with other components as well.

Roles for a Marginal Band–associated Protein

The 13H9 antigen is unlikely to specify the position where the marginal band forms. The staining of 13H9 in embryonic erythrocytes demonstrates that it is originally dispersed throughout the cytoplasm. It does not colocalize with microtubules at any of the stages preceding the band, when they are in radial arrays or when they are in cytoplasmic bundles. Even when the band begins to form, there is significant 13H9

staining elsewhere in the cell. That the 13H9 antigen does not localize to microtubules until after the marginal band is formed places limitations on its role. It suggests that the positioning of the antigen is unlikely to be responsible for the positioning of the marginal band during development, and that it is unlikely to be involved in the intermediates of marginal band formation, such as bundle formation. Such a role is not strictly excluded, however, since there is some 13H9 staining in the region of the band as it forms.

What functions could a protein like the 13H9 antigen contribute? It is clear that several properties of the microtubules are altered during erythrocyte development. For example, some time after the formation of the band, the microtubules become resistant to microtubule depolymerizing drugs (Kim et al., 1987). Also, the microtubules of mature cells, depolymerized by incubation in the cold, will reassemble in precisely the position, shape, and numbers of the original band (Miller and Solomon, 1984) but the microtubules of immature cells reassemble with less fidelity (Kim et al., 1987). These results suggest that as the cells develop, the microtubules become more stable and the position of the marginal band becomes more rigidly specified, perhaps by interactions with associated proteins. The elliptical shape of marginal bands also may depend upon such interactions, since when isolated they can revert from elliptical to circular (Cohen, 1978; Cohen et al., 1982). Therefore, it seems possible that a protein like the 13H9 antigen, and other proteins that might connect microtubules to the cortex, may be important for these changes.

A Model for Morphogenesis of the Marginal Band

The intermediates in marginal band formation suggested by the immunofluorescence images of developing erythrocytes (Kim et al., 1985; Figs. 7 and 8) raise the possibility that important elements of marginal band morphogenesis can be specified by the properties of the microtubules themselves, independent of a marginal band–associated protein. A crucial and perhaps unique stage in this process is the formation of several bundles of microtubules in a cell. Perhaps the formation of the band is required by the presence of a few such assemblies attaining significant length and having a strong tendency to be straight. The straightest path will be the longest path, and inside a sphere that means a circumference. If more microtubules are recruited to these bundles, resulting in an increasing tendency to run straight, the force of such bundles could dictate the formation of a single circumferential path. To become longer, the bundle could distort a spherical cell to produce an equator that bulges, flattening the cell. Therefore, simply the accumulation of the assembled tubulin into a sufficiently long bundle could produce some of the features of the marginal band. However, this model does not account for the elongation along one of the equatorial axes, producing lentiform cells. Testing this model, and explaining the stages in marginal band morphogenesis, are some of the issues that remain.

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