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Analysis of a cortical cytoskeletal structure: a role for ezrin-radixin-moesin (ERM proteins) in the marginal band of chicken erythrocytes

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SUMMARY

We are studying how the cytoskeleton determines cell shape, using a simple model system, the marginal band of chicken erythrocytes. We previously identified a minor component of the marginal band by a monoclonal antibody, called 13H9 (Birgbauer and Solomon (1989). *J. Cell Biol.* 109, 1609-1620; Goslin et al. (1989). *J. Cell Biol.* 109, 1621-1631). mAb 13H9 also binds to the leading edges of fibroblasts and to neuronal growth cones and recognizes the cytoskeletal protein ezrin. In recent years, two proteins with a high degree of homology to ezrin were identified: moesin and radixin, together comprising the ERM protein family. We now show that the contiguous epitope sufficient for mAb 13H9 binding is a sequence present in each of the ERM proteins, as well as the product of the gene associated with neurofibromatosis 2, merlin or schwannomin. We used biochemical and immunological techniques, as well as PCR to characterize the expression and localization of the ERM proteins in chicken erythrocytes. The results demonstrate that radixin is the major ERM protein associated with the cytoskeleton. Both ezrin and radixin localize to the position of the marginal band. Our results suggest that the ERM proteins play functionally conserved roles in quite diverse organelles.

Key words: cytoskeleton-membrane connection, ERM protein, marginal band

INTRODUCTION

Differentiated cells express a wide variety of asymmetric morphologies that are often intimately linked to the cells' functions. Drug interference experiments and morphological correlations indicate that the cytoskeleton is involved in establishing and maintaining these asymmetric cell shapes. The cytoskeletons in these various cell types are composed of essentially the same major building blocks (tubulin, actin and intermediate filament subunits). We are studying how a particular cell shape is specified. The activity of minor cytoskeletal components might account for the establishment of different shapes by differentially regulating the assembly and interactions of these major structures. Proteins that interact with microtubules (Matus, 1988; Tucker, 1990; Wiche, 1989; Wiche et al., 1991) or microfilaments (Pollard and Cooper, 1986; Matsudaira, 1991; Stossel, 1993; Luna, 1991) in vitro have been described in many systems.

As a model system for studying how the cytoskeleton specifies asymmetric cell shape, we have tried to understand a relatively simple asymmetric cell shape, that of the chicken erythrocyte, and its relatively simple cytoskeletal structure, the marginal band. The chicken erythrocyte is shaped like a flattened ellipsoid. At the equator of this ellipsoid lies a bundle of microtubules, the marginal band. The marginal band is thought to play a role in establishing the asymmetric shape of the mature cell (Behnke, 1970). The marginal band is the only microtubule-containing element of the chicken red blood cell, and it is precisely conserved qualitatively and quantitatively in each cell: both the position of the marginal band in the cell and the number of microtubule profiles in each marginal band are highly conserved in each individual cell. Some other proteins besides tubulin have also been found in the position of the marginal band: a significant fraction of the cell's F-actin (Kim et al., 1987) and several proteins that interact with microtubules in vitro (Murphy and Wallis, 1985; Feick et al., 1991; Stetzkowski-Marden et al., 1991).

In the marginal band, microtubules, F-actin and a domain of the plasma membrane are in close apposition to one another and there is little apparent heterogeneity of microtubule and microfilament organization in the cell. For these reasons, the marginal band may serve as a valuable model system for identifying proteins that modulate interactions between cytoskeletal elements and the cell membrane. Functional interactions between the cell membrane and the cytoskeleton are crucial for many cellular events, including cell motility and the establishment of cell shape. As part of an analysis to understand the marginal band, we screened for monoclonal antibodies that decorate it. We identified one monoclonal antibody, 13H9, that binds to the marginal band and to an 80 kDa protein (Birgbauer and Solomon, 1989). The 13H9 antigen behaves as if it were a stable component of the marginal band. It is not restricted to chicken erythrocytes. Rather, it is present in actin-rich structures such as growth cones of neurons in culture (Goslin et al., 1989; Birgbauer et al., 1991) and leading edges of fibroblasts (Birgbauer, 1991). In neurons, it has properties indicating a

possible association with both microtubules and microfilaments (Birgbauer and Solomon, 1989; Goslin et al., 1989). Because of its interesting characteristics and distribution, we have studied the 13H9 antigen further.

We showed previously that 13H9 binds to purified intestinal ezrin (Birgbauer and Solomon, 1989). Two more recently identified proteins, moesin and radixin, are highly related to ezrin (75% amino acid identity; Funayama et al., 1991; Lankes and Furthmayr, 1991). Ezrin was initially identified as a minor component of brush border microvilli and is found in actin-rich surface structures of many cell types (Bretscher, 1989; Franck et al., 1993; Turunen et al., 1989). Moesin (Lankes and Furthmayr, 1991) was cloned from HL-60 cells and shows a distribution, by immunofluorescence, similar to that of ezrin (Franck et al., 1993). In a single cell type in culture, A431 cells, moesin and ezrin in fact co-localize to the same structure, microvilli (Franck et al., 1993). Radixin was identified as a component of liver cell-cell adherens junctions and may bind to F-actin in vitro (Funayama et al., 1991; Sato et al., 1992). Antibodies that bind to radixin stain both adherens junctions and cleavage furrows (Sato et al., 1991), but these antibodies also recognize ezrin and moesin (Sato et al., 1992). Amieva et al. (1994) could not confirm the adherens junction localization of radixin using specific anti-radixin antibodies raised to a fusion protein directed by part of a human radixin cDNA (corresponding to amino acid residues 194-477). Rather, they report staining with the anti-radixin antibody of microvilli in hepatocytes of rat liver sections (Amieva et al., 1994). It is unclear at this point in time whether radixin in fact gives rise to the adherens junction staining of the antibodies used by the Tsukita group (Tsukita et al., 1989).

Each of the ERM proteins shows about 30% sequence similarity in the amino-terminal 260 amino acids with the amino-terminal domain of band 4.1 (Gould et al., 1989) and talin (Rees et al., 1990). This region of band 4.1 has been implicated in polyphosphoinositide-sensitive binding to glycophorin (Anderson and Marchesi, 1985). In addition, the predicted protein product of the neurofibromatosis 2 gene, called merlin or schwannomin (Rouleau et al., 1993; Trofatter et al., 1993), displays 45% homology with the ERM proteins. On the basis of, mostly, the homology to the membrane-binding regions of band 4.1 and talin and on the immunolocalization, several groups have suggested that the ERM proteins might serve as linkers between the membrane and the cytoskeleton (Gould et al., 1989; Rees et al., 1990; Algrain et al., 1993; Franck et al., 1993; Sato et al., 1992).

One important question is: why are there three highly related proteins (ezrin, radixin and moesin)? Using immunocytochemistry on tissue sections, Berryman et al. (1993) showed that ezrin and moesin are expressed in different cell types. An attractive hypothesis is that each member of the ERM family is found in different subcellular structures and interacts with different membrane and cytoskeletal proteins. The ERM proteins might then perform different functions in different structures in the cell, as has been suggested by Sato et al. (1992). The available evidence to date suggests that all three ERM proteins localize to actin-rich surface structures (such as microvilli). In cultured cells, more than one ERM protein can be found in the same place (Franck et al., 1993), but in vivo they might show cell-type-specific expression (Berryman et al., 1993).

In this report, we identify the precise amino acid sequence recognized by 13H9 and show that its epitope sequence is present in all members of the ERM family and merlin/schwannomin. We provide evidence that more than one member of this family is expressed in a simple non-cultured cell: the chicken red blood cell, which is postmitotic (no cleavage furrow), non-motile (no leading edges) and exists in suspension (no adhesion). Radixin is the major ERM component in the cytoskeleton. Using antipeptide antibodies that distinguish between the three proteins, we show that both radixin and ezrin are found in the same subcellular structure, the marginal band. We follow the behavior of radixin during erythropoietic development and show that radixin co-localizes with F-actin throughout development. In addition, radixin becomes increasingly associated with the cytoskeleton over the course of development. This time course of increasing cytoskeletal association of radixin parallels that of increasing stability of the cells to the microfilament-disrupting drug cytochalasin D (Winckler and Solomon, 1991). This raises the possibility that radixin plays a role in stabilizing the shape of the mature erythrocyte.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies 13H9 and 6-1D, both of the IgM class, have been previously described (Birgbauer et al., 1991; Birgbauer and Solomon, 1989; Goslin et al., 1989; Bond et al., 1986). To raise antisera specific to moesin, ezrin and radixin, the following acetylated peptides corresponding to mouse sequences were synthesized onto a lysine core (Tam, 1988) by Research Genetics, Huntsville, Alabama: moesin 466-477 AMSTPHVAEPA; radixin 400-409 KSA-IAKQAAD; ezrin 480-489 PVNYHVQEGL.

Rabbits were injected subcutaneously with 500-750 μg of peptide in Freund's complete adjuvant, followed by three boosts, at two-week intervals, with 500-750 μg of peptide in Freund's incomplete adjuvant.

Mapping the 13H9 ezrin epitope

TrpE fusion proteins were produced using the pATH11 vector (Koerner et al., 1991) in *Escherichia coli* HB101 by induction with 20 mg/ml indoleacrylic acid in the absence of tryptophan. Bacterial extracts were prepared for SDS-PAGE by lysing cells in gel loading buffer.

The 13H9 epitope was mapped using a 815 bp *Sac*I-*Sac*I fragment and a 1053 bp *Sac*I-*Sac*I fragment from the human ezrin pGEM-4Z/p81 plasmid (kindly provided by T. Hunter, Salk Institute, San Diego, CA; Gould et al., 1989) in pATH11 (shown in Fig. 1A). Restriction sites within the ezrin *Sac*I-*Sac*I 815bp/pATH11 plasmid were used to map the 13H9 epitope to a region containing the ezrin amino acids 170 to 281. The epitope mapping procedures of Mehra et al. (1986) and Kosik et al. (1988a,b) using DNase I were used to construct λgt11 libraries containing fragments of the 324 bp *Bam*HI-*Bam*HI ezrin pATH11 plasmid. Unamplified λgt11 libraries were screened following the methods of Young and Davis (1985), using 13H9 as the primary antibody and biotinylated goat anti-mouse Ig (Vector Labs) as secondary antibody followed by the Vectastain avidin/biotin/horseradish peroxidase complex reagent (Vector Labs). The 13H9 positive plaques were amplified by PCR (Perkin Elmer Gene AMP PCR DNA Kit) and subcloned into M13mp18 phage for sequencing with Sequenase (United States Biochemical Co.) using the dideoxy chain termination method of Sanger and the M13/pUC sequencing primer (−20) (New England Biolabs). The shortest DNA sequence common to the 26 sequenced clones defined a 13H9 epitope

comprising the 15 ezrin amino acids, 192-206. The 13H9 epitope was further delimited to the 13 amino acids, 194-206, by cloning a PCR product coding for the 13 amino acids and with 5′ and 3′ *Eco*RI sites into pATH11. The correct clone was confirmed by sequencing.

Cell isolation and immunofluorescence

Pure preparations of mature erythrocytes and embryonic red blood cells were obtained as previously described (Miller and Solomon, 1984; Kim et al., 1987). The cells were washed twice in phosphate buffered saline (PBS) plus 0.025 M glucose prior to use. For the disruption experiments, washed cells were incubated either at 4°C for 90 minutes or at 37°C in the presence of 0.1 g/ml cytochalasin D (from a 1000× stock in DMSO) or DMSO for control populations. Mature red blood cells were allowed to settle onto glass coverslips, washed, and extracted at 37°C for 5 minutes with 0.1% NP-40 in PM2G (Solomon et al., 1979; 0.1 M Pipes, 2 mM EGTA, 1 mM magnesium sulfate, 2 M glycerol, pH 6.9). They were then fixed for 1 minute with 0.25% glutaraldehyde in PM2G, followed by 30 minutes with 4% paraformaldehyde in PM2G. Immature red blood cells were extracted for 10 seconds in 0.5% NP-40 in PM2G, then fixed in 0.25% glutaraldehyde/PM2G for 1 minute followed by 4% paraformaldehyde/PM2G for 30 minutes. After several washes in PBS, coverslips were quenched in 5 mg/ml sodium borohydride in PBS three times for 5 minutes each time.

Affinity-purified antibodies were prepared by slight modifications of established procedures (Olmsted, 1981) from proteins transferred onto nitrocellulose. Anti-radixin 457 was affinity-eluted from hydroxyapatite-purified radixin or from the radixin-immunoreactive band of NIH-3T3 cells; anti-moesin 454 was affinity-eluted from the moesin-immunoreactive band of NIH-3T3 or of P19 mouse teratocarcinoma cells; and anti-ezrin 464 was affinity-eluted from the ezrin-immunoreactive band of P19 mouse teratocarcinoma cells.

Immunofluorescence was performed with anti-tubulin mAb 6-1D, anti-radixin pAb 457, anti-moesin pAb 454 and anti-ezrin pAb 464, and FITC-conjugated goat anti-mouse and FITC- or Texas Red-conjugated goat anti-rabbit secondary antibodies (Organon Teknika-Cappel Malvern, PA). To visualize F-actin, we used rhodamine-coupled phalloidin (Molecular Probes, Eugene OR) simultaneously with the FITC-conjugated goat anti-rabbit secondary antibodies. Prior to the primary antibody incubations, coverslips were blocked for 20 minutes at 37°C with 10% normal goat serum and 1% bovine serum albumin (BSA) in PBS. The antibody incubations were carried out for 30 minutes each time at 37°C in a moist chamber in the presence of 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS. Coverslips were mounted onto slides using Gelvatol mounting medium containing 15 mg/ml 1,4-diazabicyclo[2.2.2]octane, an anti-fade agent obtained from Aldrich Chemical Co., Milwaukee, WI. They were examined on an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) using a ×100, 1.4 NA, objective.

Protein extracts and protein blotting

Red blood cell extractions were performed similarly to those previously described (Birgbauer and Solomon, 1989). Briefly, red blood cells were extracted for 5 minutes with 0.1% NP-40 in PM2G in the presence of protease inhibitors (aprotinin, leupeptin, PMSF or Pefabloc; Boehringer Mannheim) to obtain the 'soluble' fraction. After one wash with the extraction buffer, the pellet was further extracted with 8 M urea in phosphate-buffered saline (PBS) with protease inhibitors to obtain the 'cytoskeletal' fraction. Unless otherwise noted, the cytoskeletal fraction was dialyzed into PBS + 1 mM dithiothreitol prior to SDS-PAGE. To determine the extent of cytoskeletal association at different stages in development, the extraction volumes were normalized to cell number and equal volumes were loaded onto gels. Normalization to total protein is less meaningful because of the large changes in protein production that occur during hemopoiesis.

For total cell extracts from 3T3 cells, nine confluent 100 mm tissue culture dishes, grown in DME with 10% bovine calf serum, were washed once in PBS, cells were scraped into PBS and spun down at 115 ***g*** in an IEC model HN-S centrifuge. The cell pellet was resuspended in 400 μl PBS with protease inhibitors, then 3× sample buffer was added to a final concentration of 1×. The extract was passed repeatedly through a 26 G needle to shear the DNA.

For immunoblotting, samples separated on a 7.5% polyacrylamide gel with a 3% 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 stained for total protein with 0.2% Ponceau S (Sigma Chemical Co., St Louis, MO) in 3% trichloroacetic acid, photographed, and then the Ponceau S was washed away. The nitrocellulose filters were blocked with 3% BSA in PBS with 0.05% sodium azide for several hours. Antibody incubations (overnight for first antibody, one hour for second antibody) were done in the same buffer. Following the primary and secondary antibody incubations, blots were 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 Dupont New England Nuclear, Boston, MA. Alternatively, all steps were performed in TNT (25 mM Tris-HCl, 170 mM NaCl, 0.05% Tween-20, pH 7.5).

Binding of 13H9 to synthetic peptides

The peptide IAQDLEMYGINYF was synthesized by the M.I.T. Biopolymers Facility with an unblocked amino terminus. This peptide was completely insoluble in aqueous solutions over a range of pH values and was dissolved in DMSO. Two unrelated peptides were used as controls : CQQLRELQRH and CADSYAEEEE. The control peptides dissolved in water. A 1 mg/ml solution of the peptides was prepared and 1, 2, 4 and 7.5 μl were applied onto PVDF membrane using a Bio-Rad slot blot apparatus. One row of slots was filled with DMSO alone. The membrane was processed as described above for Western blots except that TNT was used instead of 3% BSA/PBS.

Purification of ERM members from adult chicken erythrocytes

A cytoskeletal fraction was prepared from the blood from 4-12 chickens as described above except that 4 washes were performed before solubilization in 8 M urea. The urea extract was dialyzed against PBS + 1 mM DTT overnight at 4°C with 3 changes of buffer. After addition of protease inhibitors the dialysate was centrifuged for 10 minutes at 27,000 ***g*** at 4°C. Further steps followed the published protocol for the purification of ezrin from chicken intestine (Bretscher, 1983, 1986). The supernatant was adjusted to 40% ammonium sulfate and stirred for 30 minutes at 4°C. After centrifugation the supernatant was adjusted to 65% ammonium sulfate and stirred for 30 minutes. The pellet was recovered and dissolved in a minimal volume of 20 mM NaCl, 1 mM DTT and 10 mM imidazole-HCl, pH 6.7, and dialyzed against this buffer overnight. After centrifugation the supernatant was made 0.1 M in KH_2PO_4/K_2HPO_4, pH 7.0, by addition of a 0.8 M stock. It was then loaded onto a 12 ml hydroxyapatite column pre-equilibrated with 0.1 M KH_2PO_4/K_2HPO_4 + 1 mM DTT, pH 7.0. The column was developed with a 40 ml linear gradient of 0.1 M to 0.8 M KH_2PO_4/K_2HPO_4 + 1 mM DTT. The elution was monitored by absorbance at 280 nm and all protein fractions were dialyzed against PBS + 1 mM DTT.

Protein sequencing

The appropriate hydroxyapatite fractions containing the 80 kDa protein were separated on a 7.5% SDS-PAGE and transferred to nitrocellulose. After staining with Ponceau S, the 80 kDa band was excised and frozen in water. Trypsin digestion was performed from the nitrocellulose pieces and the resulting peptides separated by HPLC (Stone et al., 1990). Twelve of the peptides were sequenced at the M.I.T.

Biopolymers Facility using an Applied Biosystems model 477 Protein Sequencer with on-line model 120 PTH Amino Acid Analyzer.

Polymerase chain reaction

Poly(A)$^+$ RNA was prepared from 9-day embryonic chicken erythrocytes (see Materials and Methods: **Cell isolation and immunofluorescence**) by lysis of 10^9 cells in 0.2 M NaCl, 0.2 M Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 2% SDS and 200 μg/ml proteinase K at 45°C and elution from oligo(dT)-cellulose. To obtain cDNA, we used the First-Strand cDNA Synthesis Kit (Pharmacia). This cDNA prepared from 5 μg poly(A)$^+$ RNA was used as a template for PCR experiments. Two sets of fully degenerate primers were chosen from conserved regions in all ERM proteins (Funayama et al., 1991; Gould et al., 1989; Lankes and Furthmayr, 1991; Turunen et al., 1989). The first set of primers corresponded to $_{78}$FKFRAK$_{83}$ and $_{266}$DFVFYA$_{271}$, the second set corresponded to $_{118}$PPETAV$_{123}$ and $_{235}$TPKIGF$_{240}$. The sense 5′ primers contained an *Eco*RI site at the 5′ end and the anti-sense 3′ primers contained an *Xba*I site at the 3′ end. The primer sequences were:

```
5′ primers: GGAATTCTT(TC)AA(GA)TT(TC)(CA)GIGCIAA
            GGAATTCCCNCCIGA(GA)ACIGCNGT
3′ primers: GCTCTAGA(AG)AAICC(AGT)AT(CT)TTIGGNGT
            GCTCTAGAIGC(AG)TA(AG)AAIAC(AG)AA(AG)TC
```

PCR amplification using reagents from Perkin Elmer Cetus Corp. were performed according to the following schedule: 94°C for 2 minutes, 45°C for 2 minutes, 72°C for 4 minutes per cycle for 40 cycles. The fragments were cloned into M13/mp18 and the correctly oriented clones were sequenced using the Sequenase Version 2.0 Kit (United Stated Biochemical Corp.).

RESULTS

The 13H9 antibody recognizes an epitope common to all members of the ERM family

Previous data demonstrated that 13H9 binds to ezrin purified from chick intestinal epithelia (Birgbauer and Solomon, 1989). We have determined the protein sequence from ezrin that is sufficient to bind 13H9. Fig. 1A depicts a simplified map of human ezrin cDNA. The two *Sac*I-*Sac*I fragments representing most of the coding region were expressed separately as TrpE fusion proteins and bacterial lysates were probed with 13H9. Fig. 1B, lane 1, contains the 5′ *Sac*I-*Sac*I fusion protein, lane 2 contains the 3′ *Sac*I-*Sac*I fusion protein and lane 3 contains TrpE protein alone. 13H9 recognized an epitope in the 5′ (lane 1), but not the 3′ (lane 2) *Sac*I-*Sac*I fusion protein. It also did not recognize TrpE by itself (lane 3) (see arrows). The 3′ *Sac*I-*Sac*I fusion protein migrates more slowly on SDS-PAGE than predicted by its molecular mass. Gould et al. (1989) made a similar observation for full-length ezrin. Beginning with the 5′ *Sac*I-*Sac*I fragment, we mapped the 13H9 epitope by successive deletions (see Materials and Methods). A fusion protein containing a 13 amino acid region of human ezrin is still positive with 13H9 (Fig. 1A).

To confirm the epitope mapping on fusion proteins, we synthesized a peptide of the sequence IAQDLEMYGINYF. 13H9 recognized this peptide, but not two unrelated control peptides, on slot blots (Fig. 1C). We note that the presumptive 13H9 epitope is highly conserved in the amino acid sequences predicted from the cDNA sequences of radixin, moesin and merlin/schwannomin. There is a single amino acid substitution from isoleucine in ezrin to valine in moesin, radixin and merlin/schwannomin. The sequence QDLEMY, completely conserved in all of these proteins, is sufficient for binding of 13H9 (data not shown).

Radixin is the major cytoskeleton-associated ERM family member in chicken erythrocytes

We showed previously that the 13H9 antigen is stably associated with the detergent-extracted cytoskeletal fraction, but can be solubilized readily with 8 M urea (Birgbauer and Solomon, 1989). In order to determine which ERM family members were present in the cytoskeleton of adult chicken red blood cells, we purified the ERM family members from the cytoskeletal fraction of adult chicken erythrocytes (see Materials and Methods). The urea-soluble cytoskeletal proteins were dialyzed into PBS. Western blots using 13H9 showed that more than 90% of the ERM family remained soluble after dialysis out of urea into PBS

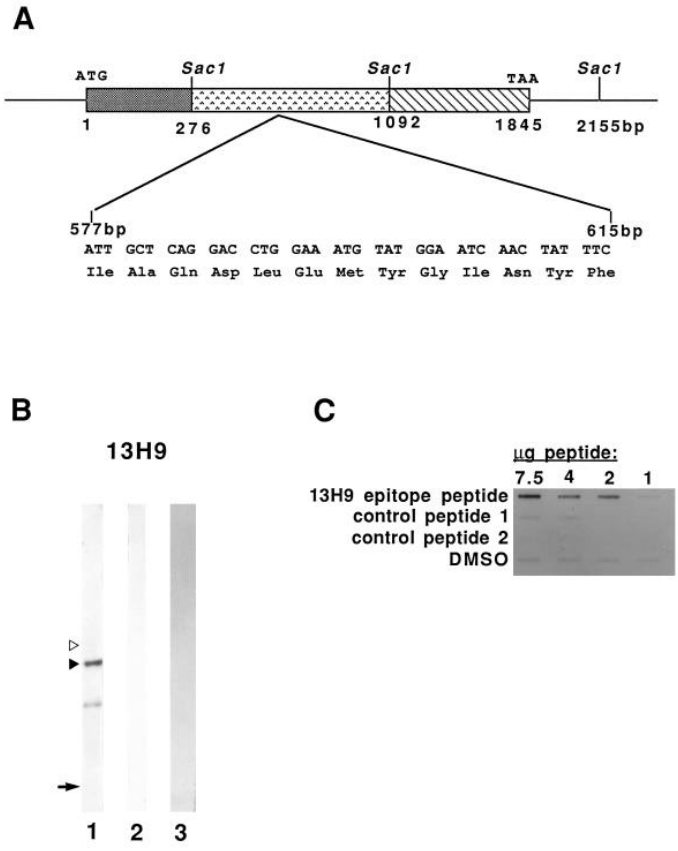
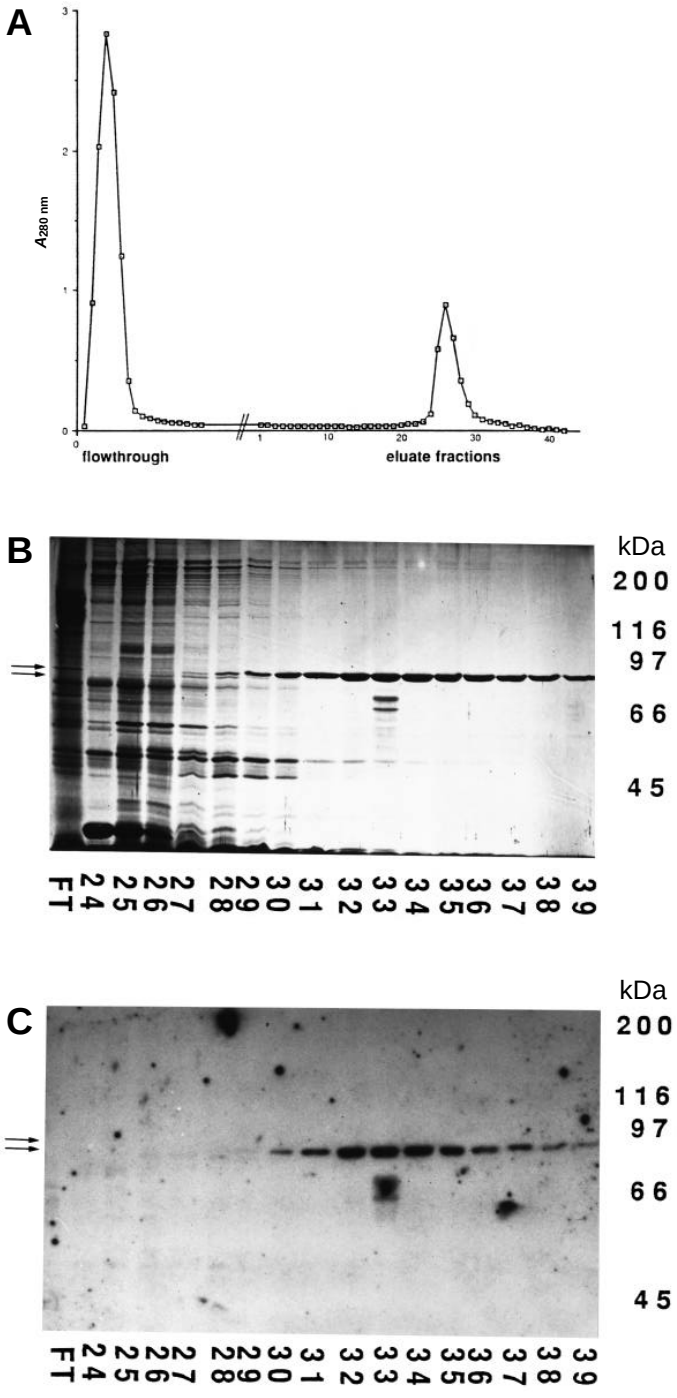


Fig. 1. Mapping of the 13H9 epitope. (A) Simplified restriction map of human ezrin showing the position of the *Sac*I (*Sac1*) restriction sites used for making the TrpE fusion proteins shown in B. The position and sequence of the 13 amino acid 13H9 epitope are indicated. (B) Western blot using 13H9 on human ezrin TrpE fusion proteins. Lane 1 contains the 5′ *Sac*I-*Sac*I fusion protein; lane 2 the 3′ *Sac*I-*Sac*I fusion protein; and lane 3 the TrpE protein without insert. The black arrowhead indicates the position of the 5′ *Sac*I-*Sac*I fusion protein that is recognized by 13H9. The white arrowhead points at the position of the 3′ *Sac*I-*Sac*I fusion protein that is not recognized by 13H9. The black arrow indicates the position of TrpE alone. (C) Peptide slot blot using 13H9. 13H9 binds to the epitope peptide (IAQDLEMYGINYF) but not to control peptide 1 (CQQLRELQRH), control peptide 2 (CADSYAEEEE) or DMSO. The amount of peptide spotted is indicated across the top.

(data not shown). The observed failure to solubilize in NP-40 extraction buffer, therefore, is not due to an intrinsic insolubility of the polypeptide, but likely reflects the interaction with other components of the detergent-insoluble cytoskeleton.

Fig. 2A shows the elution profile from the hydroxyapatite column monitored by absorbance at 280 nm. The peak fractions from the eluate were separated on 7.5% SDS-PAGE and the proteins visualized by silver stain (Fig. 2B) or blotting with 13H9 (Fig. 2C). The fractions corresponding to the eluting protein peak (fraction nos 23-28) contained many polypeptides, but a protein of <30 kDa contributed the bulk of the protein mass. The trailing edge at the later eluting fractions (fraction nos 29-39) contained an 80 kDa protein as the major protein and only relatively small amounts of a few other proteins. It is this 80 kDa band that is recognized by 13H9 (compare Fig. 2B and 2C). Fraction 33 contains 13H9-positive bands of faster mobility that are probably proteolytic breakdown products.



To identify this protein, we excised the 80 kDa band after electrophoresis and transfer to nitrocellulose and sequenced the major tryptic peptides. We obtained sequence information from 12 peptides. Of those, eight absolutely corresponded to the mouse sequences of radixin, but not of moesin or ezrin (Fig. 3). One of those eight peptides ('pep3') consists of 27 amino acids that include the entire 13H9 epitope region. The amino acid sequence of chicken ezrin is available in this region (Gould et al., 1989) and is distinct from the sequence of 'pep3' (valine instead of isoleucine at residue 203). In addition, the entire sequence of 'pep3' is identical to that predicted for this region from a PCR product identified as radixin (see below). The remaining four peptides correspond to regions of complete conservation between the ERM members, and therefore gave no clues as to the identity of the chicken red blood cell protein. We conclude that radixin is the major cytoskeletal ERM member in adult chicken erythrocytes.

We also note that after long exposures of 13H9 blots, we detect a second, faster mobility band that elutes in the earlier fractions (see arrows in Fig. 2C). This lower band appears as a very minor component by silver staining (see arrows in Fig. 2B). Due to its low abundance, we were unable to obtain peptide sequence from this band, but it may represent a second cytoskeletal ERM protein.

Specific anti-peptide antibodies against individual family members

To obtain antisera specific for individual proteins in the ERM family, we raised polyclonal antibodies to peptides that occur in divergent regions of the ERM family (Funayama et al., 1991; Gould et al., 1989; Lankes and Furthmayr, 1991; Turunen et al., 1989) and which showed no significant identity to any non-ERM sequences in the database. We used synthetic peptides corresponding to mouse radixin 400-409 KSA-IAKQAAD, mouse moesin 466-477 AMSTPHVAEPA, and mouse ezrin 480-489 PVNYHVQEGL.

To test whether these polyclonal antibodies were in fact specific for individual family members, we chose a source of protein in which the ERM members showed distinct mobilities on SDS-PAGE, i.e. the mouse cell line, NIH-3T3. As shown

Fig. 2. Purification of ERM proteins from chicken erythrocyte cytoskeletons. (A) Elution profile from the hydroxyapatite column monitored by absorbance at 280 nm. A single protein peak elutes in a linear gradient of KH_2PO_4/K_2HPO_4. Eluate fractions 24 through 39 were used for the analysis shown in B and C. (B) Silver-stained gel of the hydroxyapatite column fractions. The flowthrough fraction 4 and eluate fractions 24 through 39 were separated on a 7.5% SDS-PAGE and silver-stained. The trailing edge of the protein peak (fractions 29-39) contains an 80 kDa band as the major species. Fraction 33 suffered some proteolysis (lower bands), which is also recognized by 13H9 (see Fig. 2C). (C) Western blot using 13H9 on the hydroxyapatite column fractions. On a blot containing the same fractions as in B, 13H9 recognizes the 80 kDa band in the trailing edge fractions and a minor species of faster mobility in fractions 25 through 30. The arrows point to the position of the doublet recognized by 13H9.

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M          - - A - - - R
E     (65) - S A - E - R
R          V T Q Q D V K

pep1       V T Q Q D V K


M          - - - - - - -
E    (157) - M D - - -
R          V L E Q H K

pep2       V L E Q H K


M          - - - - - - - - - - - F - S - - - - - - S - - - - - -
E    (194) - - - - - - - - - - I - F - - - - - - - - - D - - - - -
R          I A Q D L E M Y G V N Y F E I K N K K G T E L W L G V

pep 3      I A Q D L E M Y G V N Y F E I K ? ? K G T E L ? L G V


M          - - - - - - - K
E    (343) - - - - - - L -
R          E K E E L M E R

pep 4      E K E E L M E R


M          T - - - - - S - M - - L - - R
E    (412) S - - - - - - - - - - Y - - -
R          N Q E Q L A A E L A E F T A K

pep 5      N Q E Q L A A E L A E F T A K


M          - - S - - V -
E    (448) - - D - V - -
R          K E E E A T E

pep 6      K E E E A S E
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Fig. 3. Comparison of mouse ERM proteins with peptide sequences obtained from the 80 kDa band purified on the hydroxyapatite column. The sequence of 6 tryptic peptides is given in comparison to the corresponding mouse ERM protein sequences (M, moesin; E, ezrin; R, radixin). Conserved residues are indicated by dashes. The numbers refer to the residue in the ezrin sequence. Question marks indicate positions where the amino acid analysis failed. Five out of six tryptic peptide sequences absolutely match the sequence of mouse radixin. Peptide 6 (pep 6) shows a conserved substitution of serine by threonine in radixin. This is likely to be an interspecies difference.

in Fig. 4 (lanes 1-3), each of our anti-peptide antibodies recognized a single major band in the region of 70-80 kDa. The bands recognized by the antisera have different mobilities and show no discernible crossreactivity. Anti-ezrin serum 464 recognized the band of slowest mobility, anti-radixin serum 457 the band of intermediate mobility and anti-moesin serum 454 the band of fastest mobility. This order of mobility agreed with data published by Sato et al. (1992). They compared the electrophoretic mobility of in vitro translated mRNA from the cloned sequences to the three bands seen with antibodies in various tissue culture cells and tissues.

Using the affinity-purified antipeptide polyclonal antibodies on chicken red blood cell cytoskeletal fractions, we saw reactivity with bands of 80 kDa (see Fig. 4, lanes 4-6), which were not present in the preimmune sera (data not shown). On 7.5% SDS-PAGE, the anti-radixin and anti-ezrin sera gave bands of identical mobility on chicken red blood cell material, which could not be separated from one another. We could not detect a band with the anti-moesin serum 454 (Fig. 4, lane 4). We cannot completely rule out the possibility that the avian ERM sequences are sufficiently different from the mouse sequences that the anti-peptide antibodies crossreact on chicken protein.

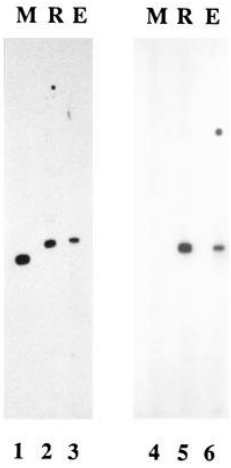

Fig. 4. Western blots using specific ERM anti-peptide antibodies. Strips of total 3T3 protein extracts (lanes 1-3) or chicken red blood cell cytoskeletons (lanes 4-6) were probed with anti-moesin antibody 454 (lanes 1,4; M), anti-radixin antibody 457 (lanes 2,5; R) and anti-ezrin antibody 464 (lanes 3,6; E) after affinity purification. Antibodies 454, 457 and 464 recognize three bands with different electrophoretic mobility in the 70-80 kDa region in 3T3 cells. Each antibody recognizes only one of the three bands and there is no detectable crossreactivity. Each antibody recognizes a single major band in the 80 kDa region of chicken red blood cell cytoskeletons. None of the preimmune sera recognizes any band in this region of either cell type.

We consider this possibility remote, since a stretch of amino acids that was chosen especially because it was characteristic and unique for a particular ERM protein from the published sequences of several species would have to be present in more than one ERM protein in chicken. The definitive test of this possibility, though, awaits the cloning of the chicken sequences.

Expression of ERM proteins in chicken erythrocytes

To confirm the biochemical and immunological results, we looked at the expression of the ERM family in chicken red blood cells with PCR using primers from regions conserved among the three proteins (see Materials and Methods). Since erythrocytes are not transcriptionally active after they mature, we used mRNA from embryonic chicken red blood cells. We obtained 14 independent sequences from the resulting products. Fig. 5 shows a comparison of the amino acid sequences predicted from the PCR products and the published mouse cDNA sequences. The predicted amino acid sequence of 12 of the clones corresponded precisely to the mouse radixin sequence (sequence A in Fig. 5). It also matches the sequence of ‘pep3’ obtained from the peptide sequencing (see earlier). This result, therefore, supports our biochemical identification of radixin as the major cytoskeletal ERM protein. The amino acid sequence of the two other clones was 96% identical to mouse moesin and 84% identical to mouse ezrin and radixin (sequence B in Fig. 5). It is therefore likely that sequence B represents part of the chicken moesin sequence. This result is consistent with our immunological data showing that more than one ERM member is expressed in chicken red blood cells. These cell preparations are very pure and we have never observed cells that did not contain marginal bands, by immunostaining with anti-tubulin antibodies. Nevertheless, it is possible that a contaminating cell might have contributed to

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               I                      II    III        IV                           V

A:        REDSMM                     FEIK   GTEL       EHDDK                        KAP

R:        REDSMM                     FEIK   GTEL       EHDDK                        KAP
E:   GMLKDSAMLEYLKIAQDLEMYGVNYFEIKNKKGTDLWLGVDALGLNIYEKDDKLTPKIGFPWSEIRNISFNDKKFVIKPIDKKAP
M:        REDAVL                     FSIK   GSEL       EQNDR                        KSP

B:         GDAVL                     FNIK   GSEL       EQNDR
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Fig. 5. Comparison of the sequences from the PCR products using conserved ERM primers with the mouse ERM sequences. The sequence of the mouse ERM proteins is shown in the region spanned by the primers. The full sequence is given for ezrin (E); for radixin (R) and moesin (M) only the divergent sequences in five blocks (I-V) are indicated. The sequences of the two PCR products are shown as A (100% identical to radixin) and B (96% identical to moesin).

the PCR products obtained. The failure to detect any other transcript, for example ezrin, by PCR might arise because of its low abundance or because of differences between the chosen primers and the unknown chicken sequence. A single codon change might suffice to obliterate the amplification reaction below detectable levels for such a message. Given our failure to detect a Western blot signal with the anti-moesin serum 454, moesin might be expressed at very low levels.

Radixin and ezrin are components of the marginal band

To localize radixin in chicken erythrocyte cytoskeletons, we performed immunofluorescence using anti-radixin antibody 457 on NP40-extracted cytoskeletons. Affinity-purified anti-radixin serum 457 crisply stains the marginal band with little staining elsewhere in the cell (Fig. 6A). Affinity-eluted anti-ezrin antiserum 464 also stains the marginal band of adult erythrocytes (Fig. 6B), albeit with lower intensity than the anti-radixin antiserum. A control staining without first antibody, but only FITC-goat anti-rabbit is shown in Fig. 6C. Affinity-eluted anti-moesin antibody 454 gives no staining over background (data not shown), in agreement with our blotting data.

Radixin distribution and fractionation changes during development

Since one can obtain fairly uniform populations of early erythropoietic precursors from early chick embryos, we were able to study the changes in behavior of the ERM proteins during the maturation of the red blood cell. In early red cells (day 1 and 2), microtubules are often found in diffuse arrays coursing throughout the cytoplasm. Most cells obtained from the circulation of day 2-3 embryos contain bundles of microtubules that criss-cross through the cytoplasm frequently, near the periphery. Starting at day 4, the majority of cells contain a single dominant bundle of microtubules near the periphery reminiscent of the mature marginal band, but some microtubules are still found in off-band positions. By day 5, virtually all cells contain marginal bands (Kim et al., 1987).

Using affinity-purified anti-radixin 457, we followed the distribution of radixin during the formation of the marginal band in early erythropoietic precursors. Fig. 7 shows immunofluorescence staining using affinity-purified anti-radixin 457. We find that in the earliest cells anti-radixin 457 gives diffuse and patchy staining. The staining appears membrane-associated, most conspicuously in blebs. In day 3 and 4 cells, anti-radixin 457 staining is less diffuse and instead concentrated in brightly staining patches. In day 5 cells, anti-radixin 457 staining is largely found in the position of the marginal band, although some cytoplasmic staining persists.

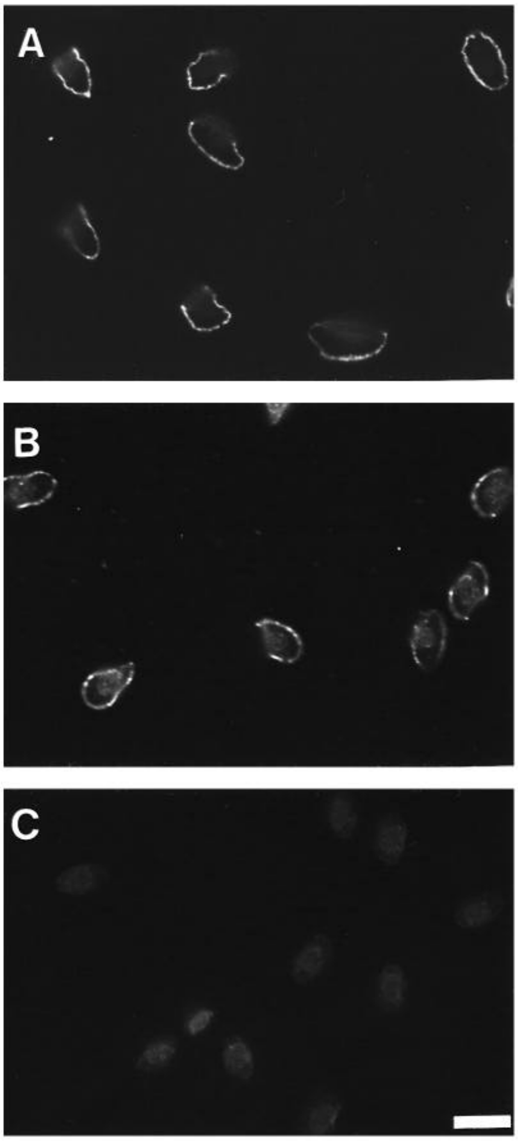

Fig. 6. Localization of radixin and ezrin in adult chicken erythrocytes. Adult chicken erythrocytes were stained with: (A) anti-radixin 457 affinity-purified antibody; (B) anti-ezrin 464 affinity-purified antibody; and (C) FITC-goat anti-rabbit secondary antibody alone. Radixin and ezrin localize to the marginal band. Bar, 10 μm.

To determine the extent of cytoskeletal association of

radixin at various stages during development, we prepared soluble and cytoskeletal fractions (see Materials and Methods) from embryonic red blood cells and measured the proportion of anti-radixin 457 signal in the cytoskeletal fraction. The extraction volumes were normalized to cell number and equal volumes loaded onto gels. Normalization to total protein is less meaningful because of the large changes in protein production that occur during hemopoiesis. As shown in Table 1, the proportion of radixin in the cytoskeleton increases over the course of development from 5% to 40% in adult cells.

Radixin co-localizes with F-actin, but not with microtubules during development of the red blood cell

Since radixin co-localizes with both microtubules and F-actin in the adult red blood cell, a cytoskeletal association of radixin could be mediated by an interaction with microtubules or F-actin. To address this possibility, we simultaneously visualized radixin and F-actin or radixin and tubulin. Fig. 8A,B shows double staining of day 3 cells with anti-radixin 457 (A) and rhodamine-phalloidin (B). Fig. 8C,D shows double staining with anti-radixin 457 (C) and the monoclonal anti-tubulin antibody 6-1D (D). Radixin distribution largely overlaps that of F-actin. Radixin and tubulin staining are frequently found in similar regions of the cell, but radixin is not co-linear with microtubules until later in development. At no developmental stage do we detect radixin at the equator before microtubules appear there. These results show that radixin follows microtubules to the position of the marginal band, but arrives there concurrently with F-actin.

Since radixin is found in the position of the marginal band, it might interact with microtubules. To test whether radixin localization to the position of the marginal band was dependent on intact microtubules, we disrupted microtubules in day 5 cells. Cold treatment leads to virtually complete disappearance of the tubulin staining. The anti-radixin 457 staining on the other hand is entirely unchanged and still found in the position of the marginal band (data not shown). The same result had been obtained with 13H9 (Birgbauer and Solomon, 1989). We conclude that the localization of radixin to the position of the marginal band is not dependent on intact microtubules.

Given the colocalization of radixin and F-actin in early embryonic cells and the reported barbed end capping activity of radixin (Tsukita et al., 1989), it is possible that radixin binds to F-actin directly or indirectly in the marginal band as well. We wanted to test this possibility. Cytochalasin D treatment leads to disruption of a substantial proportion of the cell's F-actin in day 5 red blood cells. Some F-actin is still found in the position of the marginal band, but there are aberrant brightly staining bars and patches throughout the cell (Fig. 9D). Anti-radixin 457 staining is identical to that of control cells (Fig. 9A,B) and is not found in the aberrant F-actin bars (Fig. 9C).

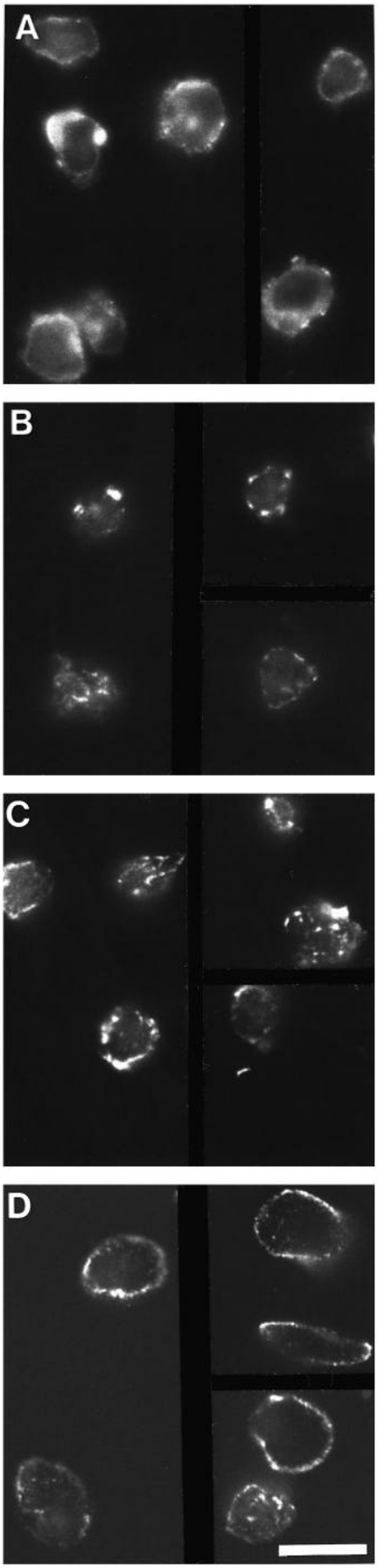

Fig. 7. Localization of radixin in developing chicken red blood cells. Anti-radixin 457 affinity-purified antibodies were used for staining of chicken red blood cells obtained from day 2 (A), day 3 (B), day 4 (C), and day 5 (D) embryos. Initially, 457 gives diffuse and patchy staining that appears to be near the membrane. Later 457 stains predominantly bright patches and by day 5 the majority of the 457 staining is in the position of the marginal band. Bar, 10 μm.

Table 1. Subcellular fractionation of radixin in embryonic chicken red blood cells

Source of RBCs age of embryos (in days)	3	4	5	7	10	Adult
Radixin in cytoskeletal fraction (%)	5	10	15	34	37	40

Red blood cells (RBCs) were isolated from chick embryos or adult chickens. Cells were fractionated into soluble and cytoskeletal fractions as described in Materials and Methods. Western blots were performed using anti-radixin antibody 457 and the signal from multiple loadings of each sample quantitated. The loadings were normalized for cell number.

This result raises the possibility that radixin additionally interacts with a detergent-insoluble component other than F-actin. Alternatively, radixin might bind to the subset of F-actin in the marginal band that is refractory to cytochalasin D treatment.

DISCUSSION

We are interested in studying the molecular structure of the cytoskeleton and its links to the membrane in a simple model system, the marginal band of chicken erythrocytes, in order to elucidate how the cytoskeleton is put together to support a particular cell shape. We have previously shown that one antibody, 13H9, binds to ezrin, and binds to a protein that co-assembles with tubulin (Birgbauer and Solomon, 1989; Goslin et al., 1989; Birgbauer et al., 1991). 13H9 intensely stains both the non-motile marginal band in chicken erythrocytes and the motile domains of fibroblasts and neurons. We show here that the sequence of the 13H9 epitope is present in at least four related proteins (ezrin, radixin, moesin and merlin/schwannomin). The expression pattern and localization of the ERM family in chicken erythrocytes raise possibilities for their role in erythrocyte morphogenesis.

A postmitotic non-motile cell in suspension, the chicken erythrocyte, expresses multiple ERM proteins

We present evidence that suggests that at least two ERM proteins are expressed in a single cell type, the chicken erythrocyte, from three distinct lines of experimentation: (1) purification of the cytoskeletal ERM proteins from mature erythrocytes and microsequencing of the major protein showed that radixin is the major ERM protein, in particular in the cytoskeletal fraction of this cell; (2) anti-peptide antibodies to radixin and ezrin blot specific bands for each of these proteins. The availability of these specific reagents overcomes a major problem arising in many previous studies of the ERM proteins using cross-reacting reagents, and allows us to track all three

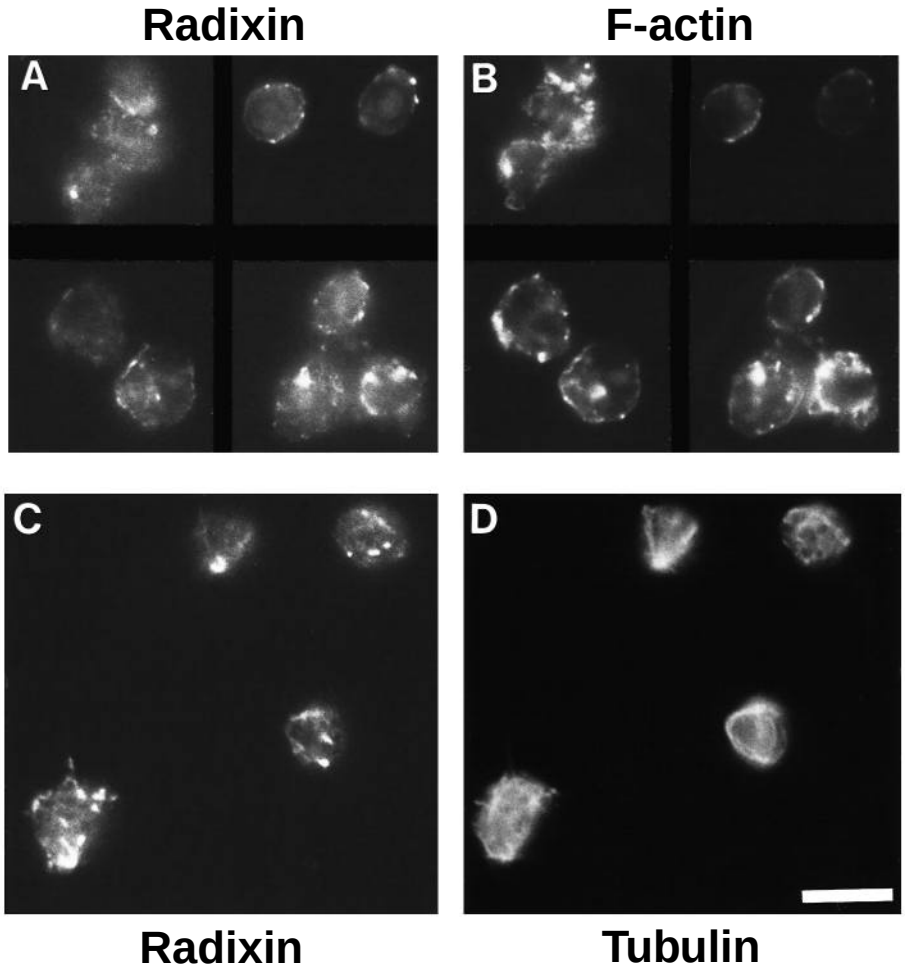

Fig. 8. Comparison of the localization of radixin with the localization of F-actin and microtubules in day 3 embryonic red blood cells. (A,B) Double immunofluorescence of day 3 red blood cells using anti-radixin 457 affinity-purified antibody (A) and phalloidin to visualize F-actin (B). There is substantial overlap between the two staining patterns. (C,D) Double immunofluorescence of day 3 red blood cells using anti-radixin 457 affinity-purified antibody (C) and anti-tubulin monoclonal antibody 6-1D (D). Radixin does not stain microtubules along their lengths. Bar, 10 μm.

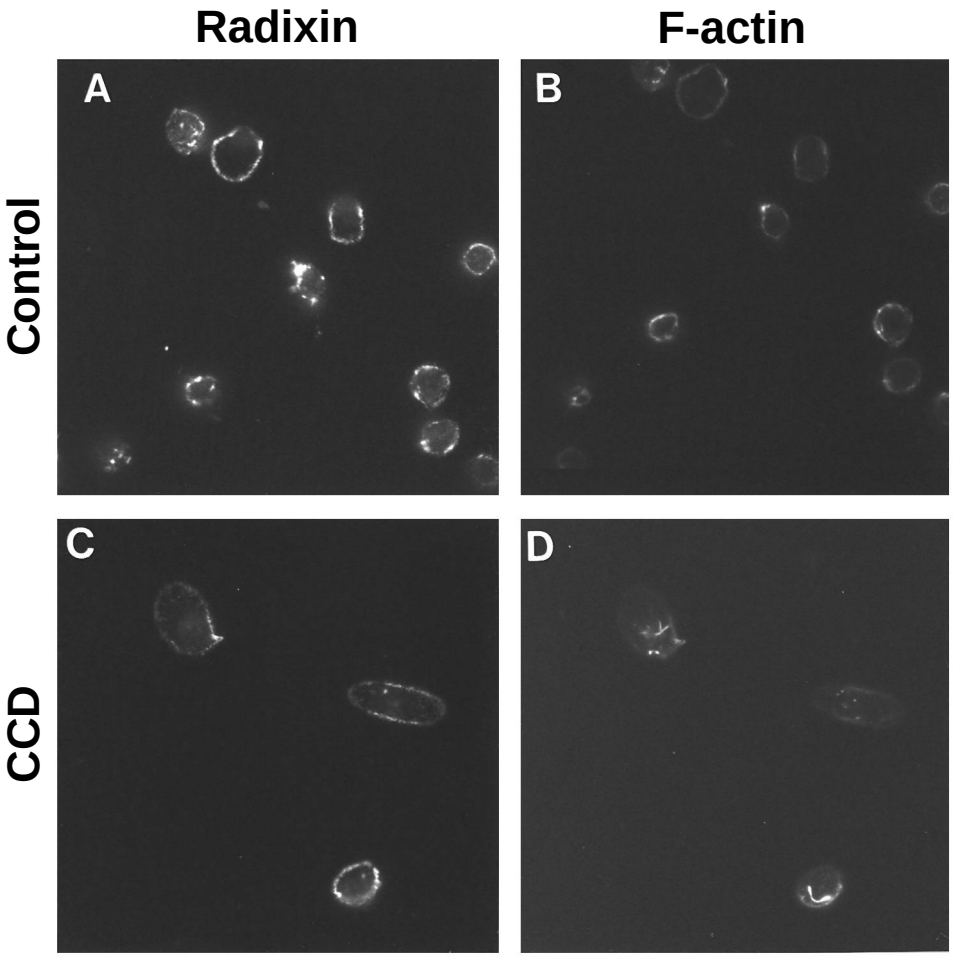

Fig. 9. Radixin staining is not disrupted by cytochalasin D. Double immunofluorescence of day 5 red blood cells pre-incubated in DMSO as a control (A,B) or 0.1 μg/ml cytochalasin D (C,D) using rhodamine-coupled phalloidin to visualize F-actin (B,D) or affinity-eluted anti-radixin antibody 457 (A,C).

proteins individually in the same cell type; and (3) using PCR to look at expressed transcripts, we were able to detect the expression of radixin and possibly moesin in day 9 embryonic red blood cells. The ERM proteins have been previously localized to cell-cell and cell-substratum adhesion points, cleavage furrows, microvilli and leading edges (Bretscher, 1983; Tsukita et al., 1989; Sato et al., 1991, 1992; Amieva et al., 1994). Chicken erythrocytes are post-mitotic (no cleavage furrow), non-motile (no leading edges), and develop and function in suspension (no adhesion), but apparently express several ERM proteins.

Radixin and ezrin localize to the same subcellular structure, the marginal band

Using the specific antibody reagents, we were able to show that at least two of these proteins (radixin and ezrin) co-localize to the same subcellular structure, the marginal band. All of the cell's microtubules and the majority of the cell's F-actin are found in this position. Therefore, in the avian erythrocyte, there are not many functionally and structurally distinct populations of F-actin (i.e. ruffling edges vs adherens junctions vs microvilli vs focal contacts etc.) that might require different members of the ERM family as Sato et al. (1992) suggested. Radixin has so far been identified as a component of two structures: cell-cell adherens junctions and cleavage furrows (Sato et al., 1991; Tsukita et al., 1989), although the antibodies used for the localizations did show crossreactivity with ezrin and moesin (Sato et al., 1992). Amieva et al. (1994), on the other hand, showed no staining of adherens junctions in liver sections, but microvillar staining with specific anti-radixin antibodies. We show here that members of the ERM family are not only found in actin-rich surface structures like microvilli and filopodia, but also in a different structure, the marginal band. These structures perform quite distinct functions in cells, but share structural features, namely a close apposition of F-actin and the cell membrane. The ERM proteins could, be among the universal cytoskeleton-membrane linkers whose activities are modified by other associated proteins specific to a given structure.

A role for radixin in the formation of the marginal band

During the development of the chicken red blood cell, the post-mitotic spherical precursor cell undergoes a series of morphological changes that are accompanied or even driven by cytoskeletal rearrangements (Kim et al., 1987; Winckler and Solomon, 1991). In the spherical cell microtubules are in diffuse arrays. Later they form several bundles near the periphery. By day 4-5 a single microtubule bundle encircling the cell's periphery is found. At this stage, the cell is disc-shaped, and the microtubule bundle is at the equator.

Here, we show that radixin co-localizes with F-actin in

brightly stained patches in early erythropoietic cells and does not localize to the cell's equator until microtubules have already arrived in this position. We have argued previously that the structural properties of microtubule bundles alone could be responsible for driving all the microtubules into a single bundle and flattening the cell to a disc shape (Birgbauer and Solomon, 1989; Winckler and Solomon, 1991). This microtubule-autonomous model of erythrocyte morphogenesis was based on the observation that experimental manipulations, especially disrupting the F-actin network with cytochalasin D, lead to dramatic microtubule-dependent shape changes in embryonic chicken red blood cells. The susceptibility to undergo cytochalasin D-induced shape changes is restricted to a narrow early window in development (day 2-3), and disappears with increasing developmental age (Winckler and Solomon, 1991). The radixin localization studies presented here are in agreement with a microtubule-autonomous model of marginal band formation and suggest that radixin is not involved in directing the formation of the marginal band.

The flattened shape of the mature erythrocyte is not dependent on intact microtubules while that of the immature red blood cell is (Behnke, 1970). This suggests that components of the non-tubulin cytoskeleton that assemble over the course of development are sufficient to maintain the flattened cell shape. Therefore, radixin might play a role in stabilizing the cytoskeleton at later developmental stages by providing membrane-cytoskeletal linkages. This suggestion is supported by the observation that the time course of the association of radixin with the cytoskeleton and the time course of the cell's loss of cytochalasin D susceptibility are co-incident (Winckler and Solomon, 1991).

Binding partners of radixin and ezrin in the marginal band

We show here that the fractionation of radixin changes over the course of development: it is increasingly associated with the cytoskeleton as assayed by insolubility in nonionic detergents. We are interested in determining the molecular basis for this association and for the change in association in development. We demonstrate here that the cytoskeletal fractionation of radixin is not due to inherent insolubility of the polypeptide, but likely reflects the association with other cytoskeletal or membrane components. Both radixin and ezrin can be phosphorylated (Bretscher, 1989; Fazioli et al., 1993). It is possible that the presence of a post-translational modification determines the association of radixin with the cytoskeleton. This possibility needs to be addressed directly. In A431 cells, no correlation exists between the phosphorylation state of ezrin and its fractionation properties (Bretscher, 1989).

It remains to be determined whether ezrin and radixin have identical binding partners in the marginal band or they might bind a common membrane protein through the highly conserved amino terminus (>80% identity), but different cytoskeletal elements through the divergent carboxy termini. Members of this family might also form dimers with one another as has been suggested in preliminary reports (Andreoli et al., 1992; Gary et al., 1992). In thinking about the molecular roles of ezrin and radixin in the marginal band, we have to keep in mind, though, that the stoichiometries of radixin and ezrin in the marginal band are far from equal. Given the high degree of similarity among the ERM proteins and their colocalization to the same structures (in microvilli and in the marginal band), the possibility that their functions are redundant appears high.

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