Radixin Is a Novel Member of the Band 4.1 Family

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Abstract. Radixin is an actin barbed-end capping protein which is highly concentrated in the undercoat of the cell-to-cell adherens junction and the cleavage furrow in the interphase and mitotic phase, respectively (Tsukita, Sa., Y. Hieda, and Sh. Tsukita. 1989*a*. *J. Cell Biol.* 108:2369–2382; Sato, N., S. Yonemura, T. Obinata, Sa. Tsukita, and Sh. Tsukita. 1991. *J. Cell Biol.* 113:321–330). To further understand the structure and functions of the radixin molecule, we isolated and sequenced the cDNA clones encoding mouse radixin. Direct peptide sequencing of radixin and immunological analyses with antiserum to a fusion protein were performed to confirm that the protein encoded by these clones is identical to radixin. The composite cDNA is 4,241 nucleotides long and codes for a 583–amino acid polypeptide with a calculated molecular mass of 68.5 kD. Sequence analysis has demonstrated that mouse radixin shares 75.3% identity with human ezrin, which was reported to be a member of the band 4.1 family. We then isolated the cDNA encoding mouse ezrin. Sequence analysis and Northern blot analysis revealed that radixin and ezrin are similar but distinct (74.9% identity), leading us to conclude that radixin is a novel member of the band 4.1 family. In erythrocytes the band 4.1 protein acts as a key protein in the association of short actin filaments with a plasma membrane protein (glycophorin), together with spectrin. Therefore, the sequence similarity between radixin and band 4.1 protein described in this study favors the idea that radixin plays a crucial role in the association of the barbed ends of actin filaments with the plasma membrane in the cell-to-cell adherens junction and the cleavage furrow.

THE cell-to-cell adherens junction (AJ)[1] is a specialized region of the plasma membrane, where cadherin molecules (uvomorulin, L-CAM, A-CAM, etc.) work as adhesion molecules, and actin filaments are densely associated with plasma membrane through a well-developed plasmalemmal undercoat (Farquhar and Palade, 1963; Gallin et al., 1987; Geiger, 1983; Ringwald et al., 1987; Staehelin, 1974; Takeichi, 1988, 1990; Volk and Geiger, 1984). It is now widely accepted that this type of junction is important for the formation and maintenance of tissues as well as for the transformation and metastasis of cancer cells. Recently, the cell adhesion function of cadherin molecules was reported to be regulated through the association of some cytoplasmic proteins with the cytoplasmic domain of cadherin molecules (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1989). Therefore, to understand the functions of AJ in molecular terms, it is prerequisite to clarify the molecular architecture of the undercoat of AJ, i.e., the molecular linkage from cadherin molecules to actin filaments. Recent studies have revealed that the molecular organization of the undercoat of AJ is not as simple as had been expected (Tsukita et al., 1990). Through immunohistochemical studies many AJ undercoat-constitutive proteins such as α-actinin, vinculin, filamin, and placoglobin have been identified (Cowin et al., 1986; Geiger, 1979; Geiger et al., 1984; Lazarides and Burridge, 1975). Recently, we developed an isolation method for AJ from rat liver to open the way for the systematic analysis of the molecular organization of its undercoat (Tsukita and Tsukita, 1989). From the isolated AJ, three novel undercoat-constitutive proteins—tenuin, radixin, and the 102-kD cadherin-associated protein (CAP102)—have been purified and characterized (Nagafuchi et al., 1991; Tsukita et al., 1989*a*,*b*). Most recently, taking advantage of the isolated AJ, it was shown that specific protooncogenic tyrosine kinases of src family are highly enriched in AJ (Tsukita et al., 1991).

The manner of association of actin filaments with the plasma membrane in AJ in general (end-to-membrane or side-to-membrane fashion) remains to be clarified. However, it is clear that, at least in AJ of cardiac muscle cells, all actin filaments are bound to the undercoat in an end-to-membrane fashion by their barbed ends. Therefore, one of the interesting approaches to the molecular linkage between cadherin and actin filaments from the actin side appears to be to identify a barbed-end capping protein in the AJ undercoat-constitutive proteins. Among the constituents of the AJ undercoat so far reported, only radixin shows the ability to cap the barbed ends of actin filaments (Tsukita et al., 1989*a*). Most recently, radixin was reported to be highly concentrated at the cleavage furrow during cytokinesis (Sato

1. *Abbreviation used in this paper*: AJ, adherens junction.

et al., 1991). Considering that the most prominent feature shared by AJ and the cleavage furrow is the tight association of actin filaments with plasma membranes, it is tempting to speculate that radixin may play a crucial role in directly or indirectly binding the barbed end of actin filaments to the plasma membrane.

In the present study, to clarify the structure and functions of radixin molecules in more detail, we have attempted to isolate and sequence cDNA encoding mouse radixin. The deduced amino acid sequence of radixin has led us to conclude that radixin is homologous with but distinct from ezrin which is reported to be localized immediately under the membrane of the microvilli (Bretscher, 1983; Gould et al., 1986; Gould et al., 1989; Pakkanen et al., 1987; Turunen et al., 1989), and that radixin is a novel member of a band 4.1 family. We believe this study will give us a clue to a better understanding of the functions of radixin at AJ and at the cleavage furrow.

Materials and Methods

Purification of Radixin and Its Direct Peptide Sequencing

The AJ was prepared from mouse liver by a combination of homogenization, sucrose density centrifugation, and NP-40 treatment as previously described (Tsukita and Tsukita, 1989). A low salt extract containing undercoat-constitutive proteins was obtained by dialysis of the AJ fraction against the extraction solution (1 mM EGTA, 0.5 mM PMSF, 1 μg/ml leupeptin, 2 mM Tris-HCl [pH 9.2]), followed by centrifugation at 100,000 *g* for 60 min. After electrophoresis of the extract obtained from 73 mice, the band of radixin was removed with a razor blade. Such bands were pooled, and radixin was eluted electrophoretically. The eluted protein was precipitated by adding acetone, and the pellet was redissolved in 10 mM Tris-HCl (pH 9.5). The lysyl endopeptidase (Wako Pure Chemical Industries, LTD., Osaka, Japan) was added in an enzyme-to-substrate ratio of 1:100 (wt/wt). A second aliquot of enzyme was added after digestion for 20 h at 37°C, and cleavage was continued for an additional 20 h. The enzyme digest was subjected directly to reverse-phase HPLC (Wakosil 5c18-200 column), using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at 1% acetonitrile per min. Selected peptide fragments were sequenced using a protein sequencer (model 477A, Applied Biosystems, Foster City, CA).

cDNA Library Screening

Two different λgt11 expression libraries made from mouse F9 poly(A)$^+$ RNAs were used in the following procedures (Nagafuchi et al., 1987). In preparing these libraries, either a random mixture of hexanucleotides or oligo(dT) was used as primer for the first-strand synthesis. The initial cDNA clones, R2 and R3 (see Fig. 1 *a*), were isolated from a randomly primed library using a polyclonal antiradixin antibody (see Tsukita et al., 1989*a*) according to the method described by Huynh et al. (1985), with the exception that 5% skim milk in TBS, was used as a blocking solution, instead of 20% FCS in TBS. Then the 320-bp Sma I-Pvu II fragment of R2 was radiolabeled with [α-^{32}P]dCTP. Using this fragment as a probe, the RT1 clone (Fig. 1 *a*) was isolated from the oligo(dT)-primed library. To obtain cDNA encoding mouse ezrin, we rescreened the oligo(dT)-primed library by DNA hybridization at low stringency with R2 as a probe. Two cDNA clones, T6 and T9, showing the same restriction map were isolated (see Fig. 4).

DNA Sequencing

All clones to be analyzed were subcloned into pBluescript SK(-) and sequenced with the 7-deaza Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). Long inserts were sequenced from nested deletion subclones created using the Deletion Kit for Kilo-Sequence (Takara Shuzo Co., LTD., Kyoto, Japan). Both strands of all clones were sequenced.

Isolation of RNA and Northern Blot Hybridization

Total RNAs from cultured F9 cells were isolated according to the method for rapid isolation of total RNA from mammalian cells described by Maniatis et al. (1989). Approximately 8 μg per well of RNA was subjected to electrophoresis and blotted onto nitrocellulose membrane. RNA ladder (Bethesda Research Laboratories, Bethesda, MD) was used as size marker. Mouse radixin cDNA R2 (1.2 kb) and mouse ezrin cDNA (2.5 kb) were labeled with [α-^{32}P]dCTP using the Random Primer DNA Labeling Kit (Takara Shuzo Co., LTD., Kyoto, Japan), and were used as probes. Hybridization was carried out at high stringency (50% formamide/10× Denhart's solution without BSA/5 × SSC/50 mM phosphate buffer [pH 6.5]/100 μg/μl boiled salmon sperm DNA).

Production of Antiserum against Radixin Fusion Protein

The 765-bp Pst fragment of R2 (see Fig. 1 *a*) was subcloned in pBluescript, and the Bam HI-Eco RV fragment was then inserted into the Bam HI site of the pGEMEX-1 (Promega Biotech, Madison, WI) in frame, the expression vector based on the T7 expression system for high level gene expression in vivo. Fusion proteins were expressed in JM109 (DE3) synthesized according to the Promega Biotec (Madison, WI) procedure, and *Escherichia coli* lysate was separated by SDS-PAGE. The fusion protein was purified electrophoretically, and the antiserum to this purified protein was elicited in a rabbit. The affinity purification of the antibody was performed using fusion proteins bound to nitrocellulose sheets.

Sequence Analysis

Nucleotide and amino acid sequences were analyzed with the program from GENETYX software package (Software Development Co., Japan).

Immunoblotting and Immunofluorescence Microscopy

SDS-PAGE was based on the discontinuous Tris-glycine system of Laemmli (1970). Immunoblotting was performed by one-dimensional electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets. Nitrocellulose transfers were incubated with affinity-purified antiradixin fusion protein antibody. For antibody detection, a blotting detection kit (Amersham International, Amersham UK) was used.

Indirect immunofluorescence microscopy of frozen sections of mouse liver was performed as described previously (Tsukita et al., 1989*a*). The frozen sections were air dried and fixed in 95% ethanol at 4°C for 30 min and in 100% acetone at room temperature for 1 min.

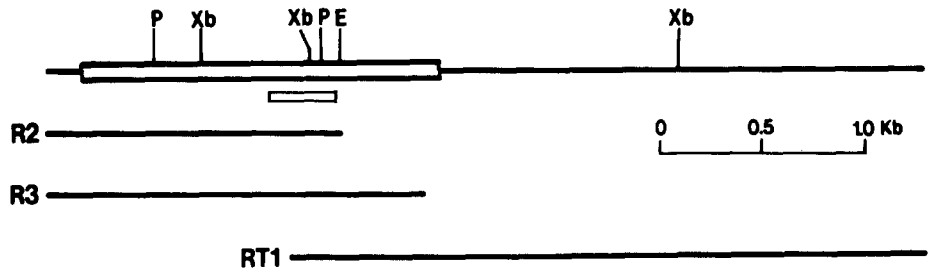
Results

Isolation and Sequencing of cDNA Encoding Mouse Radixin

We have previously obtained a polyclonal antibody specific for radixin (Tsukita et al., 1989*a*). Using this polyclonal antibody we screened ~2.4 × 10^6 plaques from a random primed λgt11 cDNA library made from mouse teratocarcinoma cells (F9 cells) as described in Materials and Methods, and cloned two positive phage recombinants: R2 (1.2 kb) and R3 (1.85 kb) (Fig. 1 *a*). Judging from their restriction maps, it was clear that these clones overlapped extensively, so both clones were sequenced.

To isolate the rest of the radixin gene, ~2 × 10^5 colonies of the oligo(dT) primed λgt11 cDNA library were screened by DNA hybridization with the radiolabeled Sma I-Pvu II fragment (320 bp) of R2 at high stringency. One positive clone (RT1) was isolated and then sequenced. Fig. 1 *a* shows the overall relationship of R2, R3, and RT1 and the restriction sites relevant for subcloning and sequencing. The complete nucleotide sequence and deduced amino acid sequences of the cloned molecule are shown in Fig. 1 *b*. The

a



b

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                                                    M  P  K  P  I  N  V  R  V  T  T  M  D  A  E  L  E  F  A  I  Q  P  N  T   24

ACTGGCAAACAACTTTTTGACCAGGTTGTAAAAACAGTTGGCTTACGTGAGGTCTGGTTTTTTGGGCTGCAATATGTAGACAGCAAAGGATATTCTACATGGCTTAAATTGAATAAAAAG  360
T  G  K  Q  L  F  D  Q  V  V  K  T  V  G  L  R  E  V  W  F  F  G  L  Q  Y  V  D  S  K  G  Y  S  T  W  L  K  L  N  K  K   64

GTAACACAGCAAGATGTTAAAAAAGAAAATCCTTTACAGTTCAAGTTTAGAGCTAAATTCTTTCCTGAAGATGTTTCTGAGGAATTGATTCAAGAAATAACACAGAGACTTTTCTTCTTG  480
V  T  Q  Q  D  V  K  K  E  N  P  L  Q  F  K  F  R  A  K  F  F  P  E  D  V  S  E  E  L  I  Q  E  I  T  Q  R  L  F  F  L  104

CAAGTTAAAGAAGCCATCTTAAATGATGAGATATATTGCCCACCAGAAACTGCAGTTCTTTTGGCTTCGTATGCTGTCCAAGCCAAGTATGGAGATTATAATAAAGAGATTCACAAACCA  600
Q  V  K  E  A  I  L  N  D  E  I  Y  C  P  P  E  T  A  V  L  L  A  S  Y  A  V  Q  A  K  Y  G  D  Y  N  K  E  I  H  K  P  144

GGCTATCTGGCTAATGACAGACTCTTACCACAGCGTGTTTTGGAACAACACAAACTGACCAAAGAGCAGTGGGAGGAGAGAATACAGAACTGGCATGAGGAACACAGAGGGATGCTAAGG  720
G  Y  L  A  N  D  R  L  L  P  Q  R  V  L  E  Q  H  K  L  T  K  E  Q  W  E  E  R  I  Q  N  W  H  E  E  H  R  G  M  L  R  184

GAAGATTCGATGATGGAATACTTGAAGATTGCGCAAGATCTAGAGATGTATGGTGTCAACTACTTTGAAATCAAGAATAAGAAGGGTACTGAGCTGTGGCTAGGTGTTGATGCCTTAGGC  840
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CTGAATATTTATGAACATGATGACAAGTTAACACCTAAAATTGGTTTTCCCTGGAGTGAAATCAGAAATATTTCATTTAATGACAAAAAATTCGTTATAAAGCCAATTGACAAAAAGGCA  960
L  N  I  Y  E  H  D  D  K  L  T  P  K  I  G  F  P  W  S  E  I  R  N  I  S  F  N  D  K  K  F  V  I  K  P  I  D  K  K  A  264

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V  M  N  H  R  S  E  E  E  W  V  T  E  T  Q  K  N  E  R  V  K  K  Q  L  Q  A  L  S  S  E  L  A  Q  A  R  D  E  T  K  K  544

ACACAGAACGATGTTCTTCACGCTGAGAATGTCAAGGCCGGCCGTGATAAGTACAAGACCCTTCGGCAGATTCGACAAGGCAATACGAAGCACGCGATCGATGAGTTTGAAGCCATGTGA  1920
T  Q  N  D  V  L  H  A  E  N  V  K  A  G  R  D  K  Y  K  T  L  R  Q  I  R  Q  G  N  T  K  H  A  I  D  E  F  E  A  M  •  583

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CCTTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                                              4241
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Figure 1. Mouse radixin cDNA sequence. (*a*) Restriction map and cDNA fragments of mouse radixin. Large and small open boxes indicate the coding region and the Sma I–Pvu II fragment used for the isolation of RT1, respectively. *E*, Eco RI; *P*, Pst I; *Xb*, Xba I. (*b*) Nucleotide sequence and deduced amino acid sequence of mouse radixin. The underlined amino acid sequences perfectly match the sequences obtained from amino-terminal sequencing of lysyl-endopeptidase fragments of mouse liver radixin. The complete nucleotide sequence determined is 4,241 bases long including a poly(A)$^+$ tail. The coding region is preceded by 168 nucleotides of 5′-untranslated sequence and followed by a 2,388-nucleotide-long 3′-untranslated region. These sequence data are available from EMBL/GenBank/DDBJ under accession number x60672.

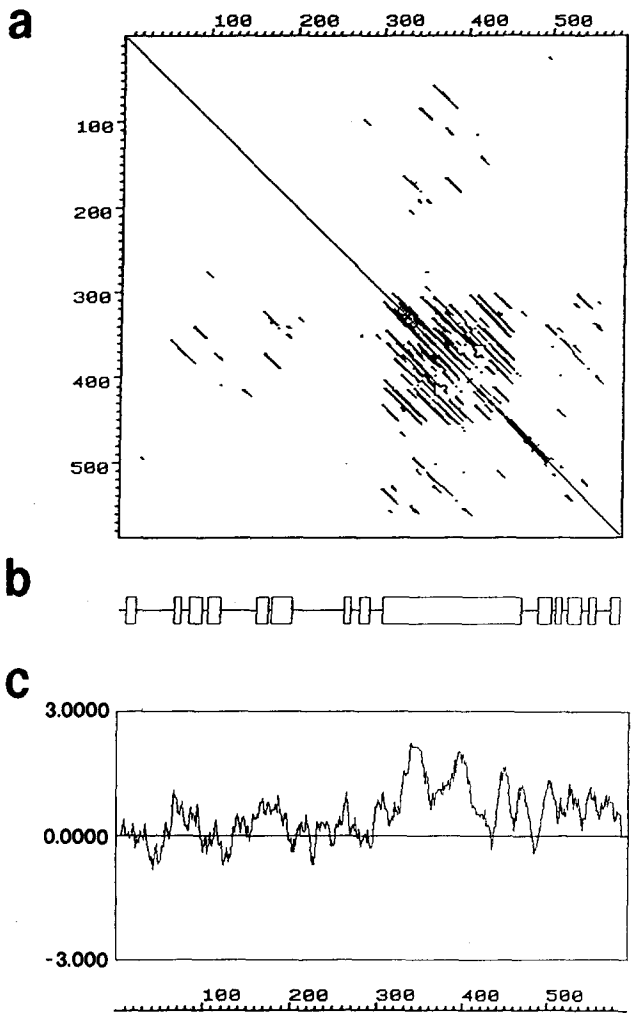


Figure 2. Internal similarities of radixin and secondary structure predictions. (*a*) A diagonal plot of amino acid sequence of mouse radixin compared with itself. Comparison was made using the HARPLT2 program. The axes are labeled in amino acid residue numbers. The dots correspond to midpoints of 50-residue spans with at least 10 identical residues between two proteins. The lines parallel to the central diagonal represent internal similarities. Note that there is a region (residues 304–468) within the protein containing short repeated similarities. (*b*) Open boxes indicate the regions which are predicted to form α helices. This prediction was obtained by the Robson method. Note that residues 304–468 are predicted to form an α helix. (*c*) Hydrophilicity plot for radixin by GENETYX software. The plot records the average hydrophilicity along the sequence over a window of 15 residues. Hydrophylic and hydrophobic residues are in the upper and lower part of the frame, respectively. The axis is labeled in amino acid residue numbers.

composite cDNA is 4,241 nucleotides long: 168 bp of the 5′-untranslated region, an ORF of 1,749 bp encoding 583 amino acids, and 2,324 bp of the 3′-untranslated region with a poly(A) tail. The calculated molecular mass of the protein encoded by this cloned cDNA is 68.5 kD. This cDNA has no long hydrophobic stretches which could encode a signal peptide or a transmembrane domain (Fig. 2 *c*), confirming that radixin is neither a secretory protein nor a transmembrane protein (Tsukita et al., 1989*a*). A plot of the deduced amino acid sequence of radixin against itself reveals that there is a region (residues 304–468) within the protein containing short repeated similarities (Fig. 2 *a*). This is consistent with the result of the secondary structure analysis of radixin, which predicts that residues 304–468 form an α helix (Fig. 2 *b*).

To confirm that the protein encoded by the above, cloned cDNA is identical to radixin, direct peptide sequencing of radixin purified from mouse liver was carried out. After lysyl endopeptidase digestion, three fragments were isolated. Sequencing of all of these fragments resulted in perfect matches with the amino acid sequence deduced from the cDNA sequence (Fig. 1 *b*). Furthermore, we generated fusion proteins encoded by the 765-bp Pst fragment of R2 and elicited a polyclonal antibody against this fusion protein in a rabbit. As shown in Fig. 3, this polyclonal antibody specifically recognized the 82-kD radixin band of F9 cells, mouse liver, and AJ fractions in immunoblot analysis, and clearly stained cell-to-cell AJ in the frozen sections of mouse liver by immunofluorescence microscopy. Taken all together, we can conclude that the cDNA encoding mouse radixin is successfully isolated and sequenced.

Isolation and Sequencing of cDNA Encoding Mouse Ezrin

Sequence analysis with the above, cloned cDNA demonstrates that mouse radixin shares 75.3% identity with human ezrin (cytovillin) (Gould et al., 1989; Turunen et al., 1989). Since ezrin is reported to be localized in microvilli of intestinal epithelial cells and not in the cell-to-cell adherens junctions, it is likely that radixin is not identical to ezrin (Bretscher, 1983). To verify this, we have isolated cDNA encoding "mouse" ezrin. For this purpose, R2 was used to screen $\sim$3.2 $\times$ 10^5 colonies of an oligo(dT)-primed λgt11 cDNA library made from mouse F9 cells by DNA hybridization at low stringency, and eight positive clones were obtained. Judging from restriction maps of the inserts of positive clones, a single class of clones (e.g., T6 and T9 in Fig. 4 *a*) was identified, which was distinct from cDNA encoding radixin. Then, the longest of these cDNA clones (T9) was sequenced. This cDNA is 2,701 nucleotides long, has a 95-nucleotide-long 5′-untranslated sequence, codes for a 586-amino acid polypeptide with a calculated molecular mass of 69.3 kD, and has an 848-nucleotide-long 3′-untranslated sequence (Fig. 4 *b*). The putative protein encoded by this cDNA clone is almost identical to human ezrin (96.2% identity). Furthermore, the 3′-untranslated sequences of this cDNA were also highly homologous to those of human ezrin cDNA (Fig. 5). These data conclusively show that cDNA clone T9 (and also T6) encodes "mouse" ezrin.

Comparison of Radixin with Ezrin

Now that both cDNA clones encoding mouse radixin and mouse ezrin have been obtained, the primary structure of the radixin molecule can be directly compared with that of ezrin. Based on the alignment shown in Fig. 6 *a*, mouse radixin shares 74.9% identity with mouse ezrin. As compared to the carboxyl-terminal half of these molecules, amino acid substitutions in the amino-terminal half are highly conservative; 85.6% identity for the residues 1–311, and 43% identity for the residues 312–COOH terminus (Fig. 6 *b*).

To detect mRNA encoding radixin and ezrin, Northern blot analysis with total mRNA obtained from mouse F9 cells

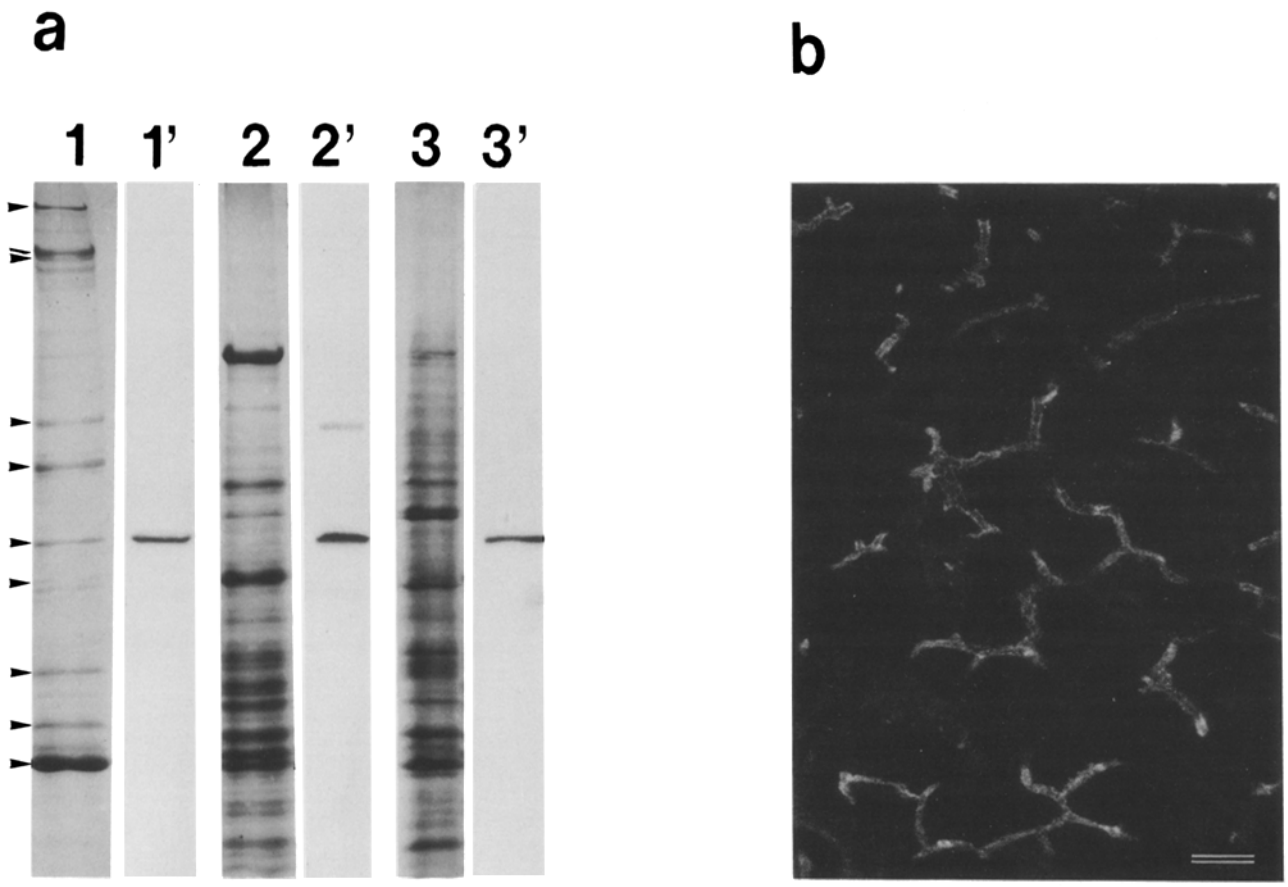


Figure 3. Immunoblotting analysis (*a*) and indirect immunofluorescence staining (*b*) with the polyclonal antibody raised against the radixin fusion protein. (*a*) Coomassie blue-stained gels (lanes *1–3*) and the corresponding immunoblot profiles with the antiradixin fusion protein antibody (lanes *1'–3'*). (Lanes *1* and *1'*) Low salt extract of isolated mouse AJ; (lanes *2* and *2'*) F9 cells; (lanes *3* and *3'*) mouse liver. Arrowheads indicate the major constituents of the undercoat of adherens AJ (400 [tenuin], 240, 235, 130 [vinculin], 100 [α-actinin], 82 [radixin], 70, 55, 50, and 43 kD [actin] from the top). Only a single band corresponding to radixin (82 kD) is detected in each sample. A faint staining around 130 kD in lane 2' appears to correspond to a dimeric form of radixin, since radixin occasionally forms a dimer even in SDS-PAGE sample buffer (our unpublished data). (*b*) Immunofluorescence staining of frozen sections of mouse liver cells. Intense fluorescence appears on both sides of the bile canaliculi, resulting in a pair of parallel lines. Bar, 10 μm.

was performed using R2 (see Fig. 1 *a*) and T9 (see Fig. 4 *a*) as probes for radixin and ezrin, respectively. As shown in Fig. 7, the band at 4.2 kb was detected by R2 (radixin), while the band at 2.7 kb was hybridized to T9 (ezrin). Taking the distribution of the nonidentical residues between radixin and ezrin into consideration, we are led to conclude that these mRNAs are not derived from a single gene by an alternative splicing mechanism.

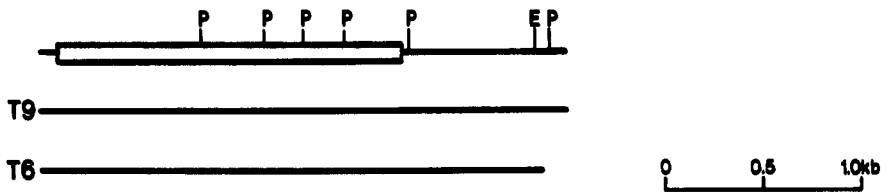
Comparison of Radixin with the Other Members of the Band 4.1 Family

Recently the existence of a novel family of submembranous cytoskeleton-associated proteins has been recognized. These include band 4.1 protein from red blood cells, ezrin (also called cytovillin or p81), and talin (Conboy et al., 1986; Gould et al., 1989; Rees et al., 1990; Turunen et al., 1989). Each member of this band 4.1 family was reported to have a homologous NH_2-terminal domain followed by a domain rich in α helix and a highly charged COOH-terminal segment (Rees et al., 1990). Radixin shares these structural features with these band 4.1–like proteins. Fig. 8 clearly reveals the existence of the homologous NH_2-terminal domain in radixin molecule. Taking all these findings together, we can conclude that radixin is a novel member of the band 4.1 family.

Discussion

We have isolated cDNA encoding mouse radixin. The sequence of radixin was determined from three overlapping clones encoding a protein of 583 amino acids. Identification of these clones as radixin came from two pieces of evidence. First, the direct peptide sequencing of three lysyl endopeptidase–digested fragments of radixin resulted in perfect matches with the amino acid sequence deduced from the cDNA sequence. Second, antiserum to a radixin fusion protein specifically recognized the mouse radixin molecule in immunoblotting analysis and clearly stained the cell-to-cell AJ by immunofluorescence microscopy. Interestingly, sequence analysis with this radixin cDNA has revealed that mouse radixin shows a similarity to human ezrin. In addition, we have cloned cDNA encoding mouse ezrin and

a



b

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GGTCCCGACGCGTCTCCGCCGCCCTCGCCCTCGCCCTCGCCTCCGCCGTACAGCCGAATAGCCGAGGACCCACGCAGAGCCACCAACCAGCCAAGATGCCCAAGCCAATCAACGTCCGGG  120
                                                                                                  M  P  K  P  I  N  V  R  V

TGACCACCATGGATGCCGAGCTGGAGTTTGCCATCCAGCCAAACACCACCGGGAAGCAGCTCTTTGATCAGGTGGTAAAGACGATTGGCCTCCGGGAAGTGTGGTACTTCGGCCTGCCGT  240
  T  T  M  D  A  E  L  E  F  A  I  Q  P  N  T  T  G  K  Q  L  F  D  Q  V  V  K  T  I  G  L  R  E  V  W  Y  F  G  L  P  Y

ATGTAGACAATAAAGGATTTCCTACCTGGCTGAAACTTGATAAAAAGGTCTCCGCACAGGAGGTCCGAAAGGAGAACCCTGTCCAGTTTAAATTCCGGGCCAAGTTCTACCCCGAAGACG  360
  V  D  N  K  G  F  P  T  W  L  K  L  D  K  K  V  S  A  Q  E  V  R  K  E  N  P  V  Q  F  K  F  R  A  K  F  Y  P  E  D  V

TGGCCGAGGAACTCATCCAGGACATCACACAGAAGCTCTTCTTCCTGCAAGTCAAGGACGGGATCCTCAGCGACGAGATCTACTGCCCCCCAGAGACAGCCGTGCTCCTGGGCTCCTATG  480
  A  E  E  L  I  Q  D  I  T  Q  K  L  F  F  L  Q  V  K  D  G  I  L  S  D  E  I  Y  C  P  P  E  T  A  V  L  L  G  S  Y  A

CCGTTCAGGCCAAGTTCGGAGATTATAACAAGGAAATGCACAAGTCTGGGTACCTCAGCTCGGAGCGGCTGATCCCCCAGAGAGTCATGGACCAACACAAGCTCAGCAGGGACCAGTGGG  600
  V  Q  A  K  F  G  D  Y  N  K  E  M  H  K  S  G  Y  L  S  S  E  R  L  I  P  Q  R  V  M  D  Q  H  K  L  S  R  D  Q  W  E

AGGACCGGATCCAGGTGTGGCACGCGGAACACCGAGGGATGCTCAAGGACAGTGCTATGCTGGAATACCTGAAGATTGCCCAGGACCTGGAAATGTATGGGATCAACTATTTCGAGATCA  720
  D  R  I  Q  V  W  H  A  E  H  R  G  M  L  K  D  S  A  M  L  E  Y  L  K  I  A  Q  D  L  E  M  Y  G  I  N  Y  F  E  I  K

AAAACAAGAAAGGAACAGACCTTTGGCTTGGAGTCGATGCCCTTGGACTTAACATTTATGAGAAAGATGACAAGTTGACCCCAAAGATCGGCTTCCCTTGGAGTGAGATCAGGAACATCT  840
  N  K  K  G  T  D  L  W  L  G  V  D  A  L  G  L  N  I  Y  E  K  D  D  K  L  T  P  K  I  G  F  P  W  S  E  I  R  N  I  S

CTTTCAACGACAAGAAGTTTGTCATTAAGCCCATCGACAAGAAGGCACCTGACTTTGTGTTCTACGCCCCGCGCCTGAGAATTAACAAGCGGATCCTGCAGCTCTGCATGGGGAACCATG  960
  F  N  D  K  K  F  V  I  K  P  I  D  K  K  A  P  D  F  V  F  Y  A  P  R  L  R  I  N  K  R  I  L  Q  L  C  M  G  N  H  E

AGCTGTACATGCGCCGCAGGAAGCCCGACACCATCGAGGTGCAGCAGATGAAGGCCCAGGCTCGGGAGGAGAAGCACCAGAAGCAGCTAGAGCGACAGCAGTTGGAAGCCGAGAAGAAGA 1080
  L  Y  M  R  R  R  K  P  D  T  I  E  V  Q  Q  M  K  A  Q  A  R  E  E  K  H  Q  K  Q  L  E  R  Q  Q  L  E  A  E  K  K  R

GGCGAGAGACGGTGGAGAGAGAAAAGGAGCAGATGCTCCGGGAGAAGGAGGAGCTGATGCTTCGGCTGCAGGACTACGAGCAGAAGACCAAGAGGGCGGAGAAAGAGCTCTCCGAGCAGA 1200
  R  E  T  V  E  R  E  K  E  Q  M  L  R  E  K  E  E  L  M  L  R  L  Q  D  Y  E  Q  K  T  K  R  A  E  K  E  L  S  E  Q  I

TTGAGAAGGCCCTCCAACTGGAGGAAGAGAGGAGGCGAGCCCAGGAGGAGGCTGAACGTCTGGAGGCCGACCGCATGGCCGCCCTGCGGGCCAAGGAAGAACTCGAGAGACAGGCGCAGG 1320
  E  K  A  L  Q  L  E  E  E  R  R  R  A  Q  E  E  A  E  R  L  E  A  D  R  M  A  A  L  R  A  K  E  E  L  E  R  Q  A  Q  D

ATCAGATAAAGAGCCAGGAGCAGCTGGCTGCAGAGCTGGCAGAGTACACGGCCAAGATCGCACTGCTGGAGGAGGCGCGGAGGCGCAAGGAGGACGAGGTAGAAGAGTGGCAGCACCGGG 1440
  Q  I  K  S  Q  E  Q  L  A  A  E  L  A  E  Y  T  A  K  I  A  L  L  E  E  A  R  R  R  K  E  D  E  V  E  E  W  Q  H  R  A

CTAAAGAAGCCCAGGACGACCTGGTGAAGACCAAAGAGGAGCTGCACCTGGTGATGACGGCCCCACCGCCCCCACCGCCCCCAGTGTATGAGCCTGTGAATTACCACGTGCAGGAGGGAC 1560
  K  E  A  Q  D  D  L  V  K  T  K  E  E  L  H  L  V  M  T  A  P  P  P  P  P  P  P  V  Y  E  P  V  N  Y  H  V  Q  E  G  L

TGCAGGACGAGGGAGCAGAGCCTATGGGCTACAGTGCCGAGCTCTCCAGTGAGGGCATCCTGGATGACCGCAACGAGGAGAAGCGGATCACAGAGGCAGAGAAGAATGAGCGCGTGCAGC 1680
  Q  D  E  G  A  E  P  M  G  Y  S  A  E  L  S  S  E  G  I  L  D  D  R  N  E  E  K  R  I  T  E  A  E  K  N  E  R  V  Q  R

GGCAGCTGCTGACCCTGAGCAATGAGTTGTCCCAGGCCCGGGATGAGAACAAGAGGACCCACAATGACATCATCCACAACGAGAACATGCGGCAAGGCAGGGACAAGTATAAGACGCTGC 1800
  Q  L  L  T  L  S  N  E  L  S  Q  A  R  D  E  N  K  R  T  H  N  D  I  I  H  N  E  N  M  R  Q  G  R  D  K  Y  K  T  L  R

GGCAAATCAGGCAGGGCAACACCAAGCAACGCATTGACGAGTTCGAGGCCATGTAGAGGCCAGGCTGGGACCAAGGGCAGAGGGCACCTCACTGCAGGCAGGTGTCACACTTGGCTCTTT 1920
  Q  I  R  Q  G  N  T  K  Q  R  I  D  E  F  E  A  M  •

AGTTCTCTTAAGTTTAGACACCCCCTTGCTGTGTTCCAGTCCCTTAAAGAGCAGTTACGGGGCCTGCATTCTGCCCCGAGACCCAGTGGGCTCCTCCTTGGTTCCTTCTAATTGTATCAC 2040
ATAGTGCCAAACAGGTCAGATTTAATGACAGTTACCGAATCACTTCCTGTTTGGAGCAGGGATTCGGAGGGCTGGCCCTCATGAAGCAAGATTCATTGTCACTGGGACAGCACTGTGGCT 2160
CACGGGTGCCATACTTTTCTCTAGTTTTACAATGAGCTCAAATCGATTTTGTTCTTGATTTTTATGAAGGATCCATCTCTGTGTATTGAGGGGTAAAAATGATTTTGAAATTTGAGTCTA 2280
AAGCATGCCCCCACAGAGTCTCCTTCCTCCAGACCGCTGGCAGAGTCTCCAGGGGTCCTTCAGAGTGTACGGGTACAGGACTCTCCGATACAAAATTCTCATGCTTATCTGTTAGCATAC 2400
ATTGTTGGACTTATATATCTAATGATCTGCTATAGAGGATAGTATTTATATATAAGCCGATAATATGGGTTTGTAACATTAGTTTTAAAAAAGGGAAAGTTTTGTTCTGTATATTTTGTT 2520
ACCTTTTACAGAATAAAAGAATTCAACACCAAGAACCATGTAACCGAGACACTTGATCTGACACAGGGGCAGTCGGGAAACCGATGACTGCAGTAATCACCACTGTACAAAAATGTTAGT 2640
GGGTTTTGTGCACGTAAAATGCACACTTCCGATTTCCTGTCAGTTTCTTATTTGAAAACCC                                                              2701
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Figure 4. Mouse ezrin cDNA sequence. (*a*) Restriction map and cDNA fragments of mouse ezrin. Open box indicates the coding region. *E*, Eco RI; *P*, Pst I. (*b*) Nucleotide sequence and deduced amino acid sequence of mouse radixin. The complete nucleotide sequence determined is 2,701 bases long. The coding region is preceded by 95 nucleotides of 5′-untranslated sequence and followed by a 848-nucleotide-long 3′-untranslated region. These sequence data are available from EMBL/GenBank/DDBJ under accession number x60671.

confirmed that radixin is distinct from ezrin although they share 74.9% amino acid sequence identity. Ezrin (also called cytovillin or p81) was originally identified as an undercoat-constitutive protein of the microvilli plasma membrane (Bretscher, 1983; Pakkanen et al., 1987), and was reported to be a good substrate in vivo for some tyrosine kinases (Bretscher, 1989; Gould et al., 1986; Hunter and Cooper, 1981, 1983). Recent sequence analysis of ezrin has revealed that ezrin shows homology to the band 4.1 protein of erythrocyte membranes (Conboy et al., 1986; Gould et al., 1989; Turunen et al., 1989). Most recently, talin, a major undercoat-constitutive protein at the cell-to-substrate AJ, was also reported to be a member of this band 4.1 family (Burridge and Connell, 1983; Burridge and Mangeat, 1984; Rees et al., 1990). Our present data add radixin as a new member to the band 4.1 family.

In various types of cells, there are some specialized regions of the cell surface which are tightly associated with actin filaments: the cleavage furrow during cytokinesis, cell-to-cell AJ, cell-to-substrate AJ (focal contacts), ruffling membrane, and microvilli. According to the previous immunolocalization results, it may be safe to say that the first

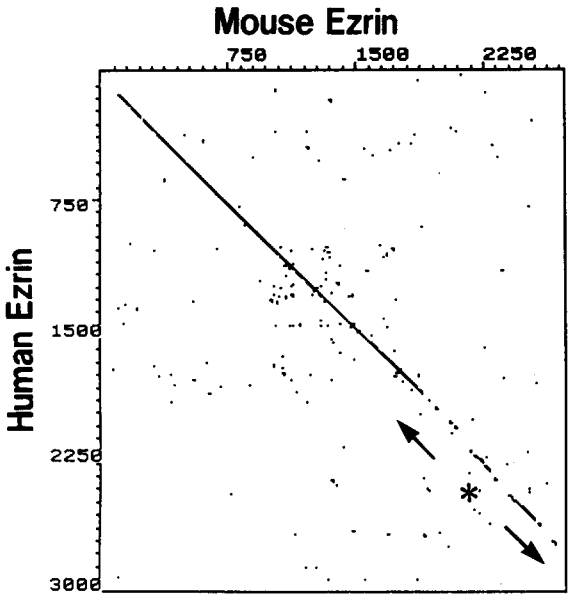

Figure 5. Comparison of nucleotide sequence of human ezrin cDNA with that of mouse ezrin cDNA. Diagonal plots were made using the HARPLT2 program. The dots correspond to midpoint of 15-nucleotide spans with at least 12 identical nucleotides between two cDNAs. The axes are labeled in nucleotide numbers. Note that they are highly homologous not only within the coding regions but also in 3′-noncoding regions (*).

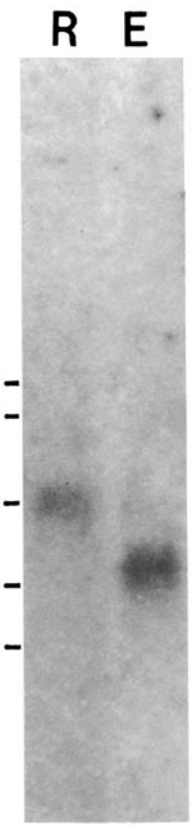

Figure 7. Northern blot analysis of total RNA isolated from mouse F9 cells. Total RNA was probed at high stringency with radixin R2 cDNA (*R*) and mouse ezrin cDNA (*E*) that covered almost the whole length of coding region of each protein. The bars on the left indicate the position of RNA markers of 9.5, 7.5, 4.4, 2.4, and 1.4 kb (from the top).

two of these regions contain high concentrations of radixin; the third and fourth, talin; and the fifth, ezrin (Bretscher, 1983; Burridge and Connell, 1983; Sato et al., 1991; Tsukita et al., 1989*a*). However, taking into consideration that the amino acid sequence of radixin is highly homologous to that of ezrin, all of the previously published immunolocalization work on ezrin and radixin should be reevaluated carefully because of the likelihood that antiezrin antibodies cross-react with radixin and vice versa. This is an important issue to re-

a

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radixin MPKPINVRVTTMDAELEFAIQPNTTGKQLFDQVVKTVGLREVWFFGLQYVDSKGYSTWLK  60
m.ezrin ------------------------------------I------Y---P---N--FP----  60

radixin LNKKVTQQDVKKENPLQFKFRAKFFPEDVSEELIQEITQRLFFLQVKEAILNDEIYCPPE 120
m.ezrin -D---SA-E-R----V--------Y----A-----D---K-------DG--S-------- 120

radixin TAVLLASYAVQAKYGDYNKEIHKPGYLANDRLLPQRVLEQHKLTKEQWEERIQNWHEEHR 180
m.ezrin -----G-------F------M--S---SSE--I----MD----SRD---D---V--A--- 180

radixin GMLREDSMMEYLKIAQDLEMYGVNYFEIKNKKGTELWLGVDALGLNIYEHDDKLTPKIGF 240
m.ezrin ---KDSA-L-------------I-----------D--------------K---------- 240

radixin PWSEIRNISFNDKKFVIKPIDKKAPDFVFYAPRLRINKRILALCMGNHELYMRRRKPDTI 300
m.ezrin ----------------------------------------Q------------------ 300

radixin EVQQMKAQAREVLHQKQLERAQLENEKKKREIAEKEKERIEREKEELMERLRQIEEQTVK 360
m.ezrin -----------EK-------Q---A---R--TV-R---QML-------L--QDY-QK-KR 360

radixin AQKELEEQTRKALELEQERQRAKEEAERLDRERRAAEEAKSAIAKQAADQMKNQEQLAAE 420
m.ezrin -E---S--IE---Q--E--R--Q------EAD-M--LR--EELER--Q--I-S------- 420

radixin LAEFTAKIALLEEAKKKKEEEATEWQHKAFAAQEDLEKTKEELKTVMSAPPPPPPPPVIP 480
m.ezrin ---Y----------RRR--D-VE----R-KE--D--V------HL--T--------VYE- 480

radixin PTENEHDEQDENSAEA   SAELSSEGVMNHRSEEEWVTETQKNERVKKQLQALSSELAQ 537
m.ezrin VNYHVQEGLQDEG--PMGY--------ILDD-N--KRI--AE-----QR--LT--N--S- 540

radixin ARDETKKTQNDVLHAENVKAGRDKYKTLRQIRQGNTKHAIDEFEAM               583
m.ezrin ----N-R-H--II-N--MRQ----------------QR-------               586
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b

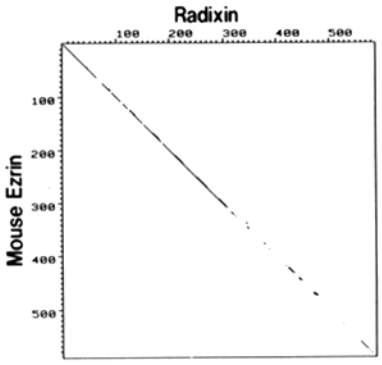

Figure 6. Comparison of amino acid sequence of mouse radixin with that of mouse ezrin. (*a*) The sequences were aligned by the GENETYX program. Identities are indicated by dashes; gaps, introduced to maximize alignment, are indicated by spaces. The mouse radixin and ezrin proteins are identical in 74.9% of their residues. The shadowed box indicates one of three regions whose amino acid sequences were directly determined from the purified radixin (see Fig. 1 *b*). In this boxed region, the amino acid sequence in radixin differs from that in ezrin. (*b*) Diagonal plots at the amino acid sequence level. As compared to the COOH-terminal half of these molecules, the NH_2-terminal half is highly conservative in its amino acid sequence.

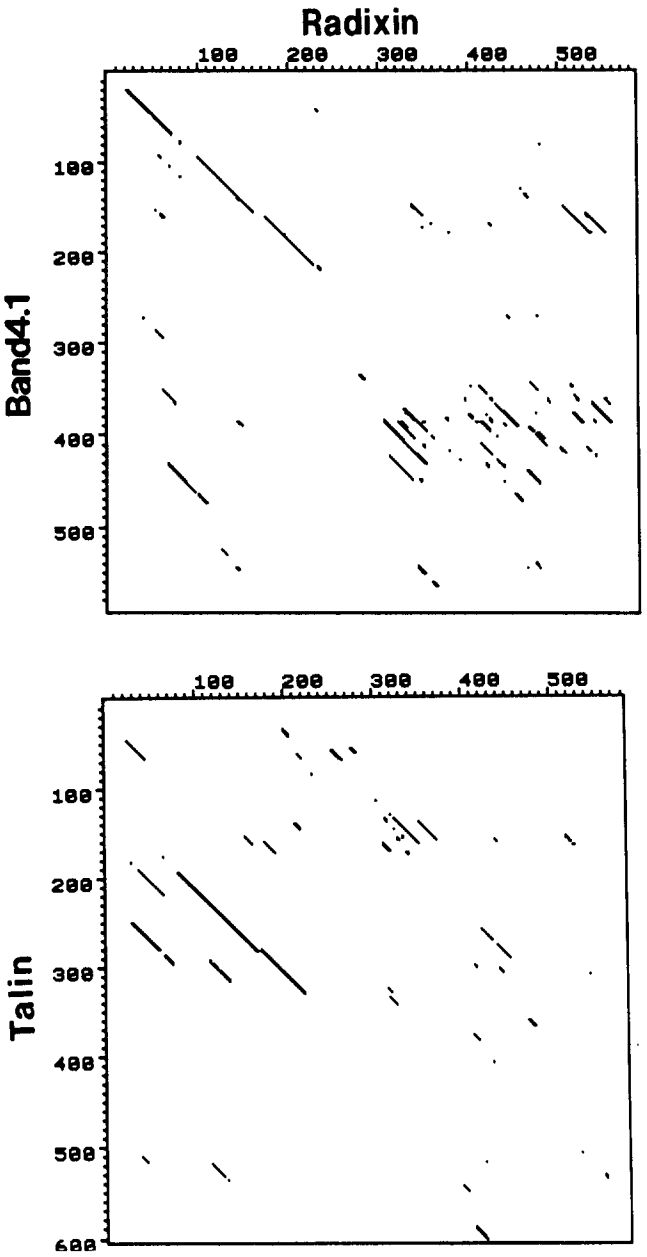

Figure 8. Comparison of amino acid sequence of mouse radixin with that of the other members of the band 4.1 family. Diagonal plots of mouse radixin compared with human erythrocyte membrane protein 4.1 (band 4.1 protein) and the amino-terminal region of mouse talin. Comparison was made using the HARPLT2 program. The dots correspond to midpoints of 50-residue spans with at least 10 identical residues between two proteins. The axes are labeled in amino acid residue numbers. Note that the homologous NH_2-terminal domain exists in these members of the band 4.1 family.

solve in near future. What we can say at present is that the band 4.1-like proteins play a pivotal role in cytokinesis, cell adhesion, and cell motility by directly or indirectly linking actin filaments to plasma membranes.

To understand the physiological functions of these band 4.1-like proteins in molecular terms, the following question must be answered in future: How do these proteins interact with actin filaments? Band 4.1 protein is one of the major accessory proteins of human erythrocyte membranes, and associated with spectrin and actin in membrane skeletons (Bennett, 1989). In vitro assays so far clarified the following properties of band 4.1 protein. (*a*) It participates in a ternary or higher order complex together with spectrin and actin. (*b*) It binds directly to spectrin and promotes spectrin binding to actin. (*c*) It does not interact directly with actin (Bennett, 1989; Correas et al., 1986; Fowler and Taylor, 1980; Ohanian et al., 1984; Ungewickell et al., 1979). In sharp contrast, radixin specifically binds to the barbed ends of actin filaments (Tsukita et al., 1989*a*). As shown in Figs. 8 and 9, the amino acid sequence of an NH_2-terminal domain of band 4.1 protein is homologous to that of radixin, and the binding site for a plasma membrane protein (glycophorin) is mapped to this domain of band 4.1 protein (Leto et al., 1986). Therefore, the rest part of each molecule (COOH-terminal half) seems likely to have a bearing on the interaction of each with cytoskeletal proteins. Actually, the COOH-terminal half of band 4.1 protein is reported to contain the spectrin-binding region (Correas et al., 1986). The amino acid sequence of this COOH-terminal half of band 4.1 protein is not homologous to that of radixin. This suggests that the COOH-terminal half of radixin is responsible for its barbed-end capping activity, although there are no obvious homologies between radixin and other barbed-end capping proteins such as gelsolin (Dieffenbach et al., 1989), fragmin (Ampe and Vandekerckhove, 1987), and Cap-Z protein (Casella et al., 1989; Caldwell et al., 1989). Considering that the other members of the band 4.1 family such as ezrin and talin have their respective COOH-terminal domains, it is likely that these proteins interact with cytoskeletons in distinct manners. No evidence has yet been obtained for any direct interaction between actin filaments and ezrin. Talin was believed not to directly interact with actin filaments, but recently the direct interaction between talin and actin was reported (Muguruma et al., 1990). The interaction of these band 4.1-like proteins with actin filaments should be examined under various conditions, for example, in the presence of phospholipids and/or other protein factors.

The existence of an homologous NH_2-terminal domain in proteins of the band 4.1 family will give us a clue toward understanding their physiological functions. In band 4.1 protein, this domain contains a binding site for glycophorin A or glycophorin C (also called glycoconnectin), one of the major membrane proteins in erythrocytes (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Bennett, 1989; Leto et al., 1986; Mueller and Morrison, 1981). As shown in Fig. 6, this domain is highly homologous between radixin and ezrin, leading us to speculate that a single class of membrane protein may be present in cell-to-cell AJ, cleavage furrows, and microvilli as membrane-attachment sites for radixin and ezrin and that this membrane protein may have a similarity to glycophorin A or glycophorin C (glycoconnectin).

In this study, we have shown that radixin is a new member of the band 4.1 family. Recent studies have suggested the possibility that in addition to band 4.1 protein, ezrin, talin, and radixin, many other proteins may be included in the band 4.1 family (Spiegel et al., 1984; Bretscher, 1989; Birgbauer and Solomon, 1989). For example, a nonerythroid 4.1 variant was identified in lymphocytes and its cDNA was cloned and sequenced, indicating that mRNAs of both erythrocyte and

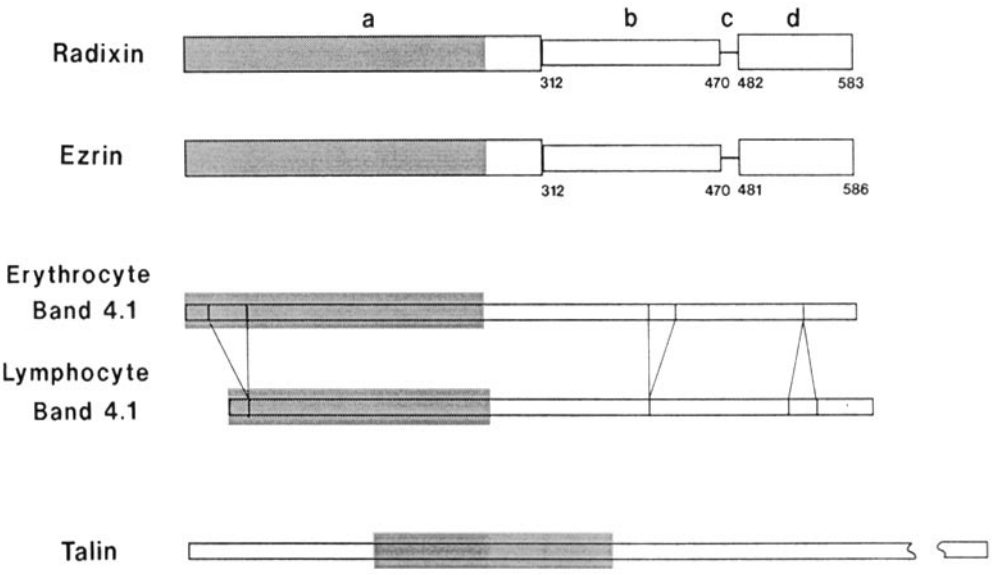

Figure 9. Models of the structure of radixin, ezrin, erythrocyte band 4.1, lymphocyte band 4.1, and talin. The domain structure of radixin and ezrin is schematically drawn in detail. Both proteins are divided into four domains; (*a*) a homologous domain between radixin and ezrin, (*b*) a domain rich in α-helix, (*c*) a proline-rich domain, (*d*) a highly charged domain. Boundaries between each domain of radixin and ezrin are shown by the number of boundary residues. Nonerythroid band 4.1 variant was identified in lymphocytes, and both erythrocyte and lymphocyte band 4.1 proteins are known to be produced from a single gene by alternative splicing. Each protein has a homologous domain (*shaded box*) in its NH_2-terminal region. In erythrocytes, this domain is responsible for the direct interaction of band 4.1 protein with glycophorin A or C (also called glycoconnectin).

lymphocyte band 4.1 proteins are produced from a single gene by alternative splicing (Tang et al., 1988) (Fig. 9). The relationship of radixin and ezrin to this variant in nonerythroid cells remains to be clarified. The expression experiments using cDNAs encoding the members of band 4.1 family will lead us to a better understanding of the physiological roles of these band 4.1–like proteins.

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