Fimbrin Is a Homologue of the Cytoplasmic Phosphoprotein Plastin and Has Domains Homologous with Calmodulin and Actin Gelation Proteins

Monika V. de Arruda, Susan Watson, Ching-Shwun Lin,* John Leavitt,* and Paul Matsudaira

Whitehead Institute for Biomedical Research, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; *California Institute for Medical Research, San Jose, California 95128

Abstract. Fimbrin is an actin-bundling protein found in intestinal microvilli, hair cell stereocilia, and fibroblast filopodia. The complete protein sequence (630 residues) of chicken intestine fimbrin has been determined from two full-length cDNA clones. The sequence encodes a small amino-terminal domain (115 residues) that is homologous with two calcium-binding sites of calmodulin and a large carboxy-terminal domain (500 residues) consisting of a fourfold-repeated 125-residue sequence. This repeat is homologous with the actin-binding domain of alpha-actinin and the amino-terminal domains of dystrophin, actin-gelation protein, and beta-spectrin. The presence of this duplicated domain in fimbrin links actin bundling proteins and gelation proteins into a common family of actin cross-linking proteins. Fimbrin is also homologous in sequence with human L-plastin and T-plastin. L-plastin is found in only normal or transformed leukocytes where it becomes phosphorylated in response to IL 1 or phorbol myristate acetate. T-plastin is found in cells of solid tissues where it does not become phosphorylated. Neoplastic cells derived from solid tissues express both isoforms. The differences in expression, sequence, and phosphorylation suggest possible functional differences between fimbrin isoforms.

ACTIN filaments are commonly organized in the cytoplasm into bundles or gels. Actin bundles are closely spaced, parallel arrays of filaments that provide structural support for fingerlike extensions of the plasma membrane such as microvilli, stereocilia, and filopodia. This arrangement of filaments contrasts with the orthogonal lattice of filaments in an actin gel, which compose a subcortical scaffold associated with the plasma membrane. Actin bundles and gels are formed by separate classes of actin cross-linking proteins (Pollard and Cooper, 1986). Although these proteins must bind actin, it is unknown whether they share similar mechanisms of binding and cross-linking actin filaments.

Actin gelation proteins consist of a class of large, flexible multisubunit molecules that cross-link actin filaments into a loosely organized meshwork supporting the plasma membrane. This network is anchored to the membrane by integral and peripheral membrane proteins. Various actin gelation proteins, including alpha-actinin (Noegel et al., 1987; Blanchard et al., 1989), actin-binding protein (ABP)[1] (Hartwig and Stossel, 1975), filamin (Wang et al., 1975), spectrin (Morrow, 1989), and actin gelation protein (Condeelis et al., 1981; Noegel et al., 1989), have been identified based on their ability in vitro to increase the viscosity of actin filaments. Actin gelation proteins are either dimers or tetramers. This oligomerization is required for actin cross-linking activity because monomeric actin-binding domains can bind but not cross-link actin filaments (Mimura and Asano, 1986). Gelation proteins have globular amino- and carboxy-terminal domains that are separated by long stretches of repeated crossed-beta sheet or alpha-helical segments. Actin binding activity in alpha-actinin has been identified in the amino-terminal globular (250 residues) domain (Mimura and Asano, 1986). This domain has also been identified at the amino terminus of the gelation proteins and dystrophin (Hammonds, 1987; Davison and Critchley, 1988).

Various actin bundling proteins have been purified. They include sea urchin egg fascin (Bryan and Kane, 1978), human erythrocyte band 4.9 (Siegel and Branton, 1985), and rat cell culture 55 kD (Yamashiro-Matsumura and Matsumura, 1985). Intestinal brush border microvilli contain two actin-bundling proteins (Matsudaira and Burgess, 1979), fimbrin (Bretscher and Weber, 1980*a*; Bretscher, 1981; Glenney et al., 1981) and villin (Bretscher and Weber, 1980*b*; Matsudaira and Janmey, 1988). Fimbrin is distributed widely in nonintestinal cell microvilli and filopodia (Bretscher and Weber, 1980), whereas villin is restricted to the absorptive epithelia covering the surface of the gut, kidney proximal tubules, and embryonic visceral yolk sac (Louvard, 1989). Because fimbrin, villin, and fascin are monomeric bundling proteins, they must contain two actin binding sites; however,

1. *Abbreviations used in this paper:* ABP, actin-binding protein; PMA, phorbol myristate acetate.

little is known of the sequences or domains involved in forming a cross-link between actin filaments.

Because fimbrin and villin bundle actin in the same microvillus, we were interested in comparing fimbrin structure with villin. In this paper we describe the complete sequence of fimbrin deduced from full-length cDNA clones from chicken intestine. The sequence reveals that fimbrin is organized, like villin, into headpiece and core domains, but that the two proteins are unrelated in sequence. The fimbrin headpiece domain lies at the amino terminus and contains two calmodulin-like calcium-binding sites. The carboxy-terminal core domain consists entirely of a fourfold repeat. Each pair of these repeats is homologous to the actin-binding domain of alpha-actinin and the putative actin-binding domains of dystrophin, actin gelation protein, and beta-spectrin.

Fimbrin is also homologous to human L- and T-plastin, 68-kD polypeptides of unknown function. L-plastin is expressed in the cytoplasm of leukocytes (Goldstein et al., 1985; Lin et al., 1988, 1990) and in response to IL-1 and phorbol myristate acetate (PMA) activation, L-plastin is phosphorylated at serine residues (Goldstein et al., 1985; Anderson et al., 1985; Matsushima et al., 1988). T-plastin is constitutively expressed in epithelial and mesenchymal cells derived from solid tissues but neoplastic cells from these tissues express both plastin isoforms. These results identify possible regulation of the membrane-associated cytoskeleton by phosphorylation of fimbrin and possible functional differences between fimbrin isoforms.

Materials and Methods

Fimbrin Proteolysis

Fimbrin was purified from the supernatant of calcium extracted chicken intestine epithelial cells using methods modified from Glenney et al. (1981*b*). The supernatant was passed through a DNase column to remove villin and actin. Fimbrin does not bind to the column and the flow-through fractions containing fimbrin were pooled. Fimbrin precipitated in a 45–60% ammonium sulfate cut and was further purified by gel filtration thorough AcA 34 and ion exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) and AffiGel Blue Agarose (Bio-Rad Laboratories, Richmond, CA). The purified protein was desalted into 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 10 mM Pipes buffer (pH 7.25) at a concentration of 1 mg/ml and digested with papain (1:400 wt/wt) at room temperature. Aliquots were removed at t = 5, 15, 30, 45, 60, 90, and 120 min and quenched by addition of PMSF to 0.1 mM and then boiled in SDS sample buffer. The digest products were separated by PAGE.

Amino-Terminal Sequence Analysis

Purified fimbrin was digested to completion with trypsin. The peptides were separated by reverse-phase HPLC, and well-separated peaks were collected manually and sequenced using a gas-phase instrument (model 470; Applied Biosystems, Inc., Foster City, CA) equipped for on-line analysis of phenylthiohydantoin (PTH) derivatives of amino acids. Fimbrin core generated by mild V-8 proteolysis was electroblotted onto an Immobilon membrane (Millipore Corp., Bedford, MA) as described previously (Matsudaira, 1987) and the amino terminus was sequenced using a gas phase sequencer (model 2090E; Porton Instruments, Tarzana, CA) equipped for on-line PTH amino acid identification.

Construction of Chicken Intestine cDNA Library

RNA was isolated from chicken intestine epithelial cells by the guanidinium isothiocyanate method (Chirgwin et al., 1979), and poly(A)$^+$ RNA was isolated by oligo (dT)-cellulose column chromatography. cDNA was synthesized from 5 μg of poly(A)$^+$ RNA with Moloney marine leukemia virus reverse transcriptase (Pharmacia Fine Chemicals) and random primers. Eco RI/Not I linkers were attached and DNA was ligated into λgt10 and packaged in vitro (Gigapack II Plus; Strategene Corp., La Jolla, CA). The total library, unamplified, contained 1.9 × 10^6 recombinants.

Cloning and Sequencing of Fimbrin cDNA

A rabbit polyclonal antiserum (R163.3) raised against purified chicken intestine fimbrin was used to screen transformants (10^6 clones) in a λgt11 library constructed from chicken intestine poly(A)+ RNA (Bazari et al., 1988). A single clone (SW6) containing an internal Eco RI site was isolated, cloned into M13, and sequenced on both strands using dideoxy-chain termination methods. This clone (2.4 kb) contained sequences that matched seven peptide sequences derived from V-8 and trypsin digests of chicken intestine fimbrin. SW6 did not contain the complete fimbrin sequence because its 5′ coding region started in the middle of protein sequences corresponding to the NH_2 terminus of fimbrin core. An 0.3 kb Xba I/Eco RI restriction fragment of clone SW6 was randomly labeled with ^{32}P and used to screen 2× 10^5 recombinants plated from the λgt10 library. Nine positive clones were isolated. The clones were 1.0–3.7 kb in size and all contained an internal Eco RI site. Two clones (M38 and M48) were sequenced on both strands as described above and a 1.9-kb open reading frame corresponding to the fimbrin protein sequence was identified. The introns in clone M48 were sequenced on one strand. The fimbrin protein sequence was compared with the GenBank and NBRF data bases using TFASTA. Sequence homologies were further analyzed and aligned using BESTFIT (UWGCG, Devereux et al., 1984).

Northern Blot Analysis

Poly(A)$^+$ RNA (2 μg) isolated from chicken intestine epithelial cells was fractionated in formaldehyde-agarose gels and transferred to nylon membranes (Biodyne; Pall Corp., Glen Cove, NY). The membranes were hybridized with randomly labeled ^{32}P probes generated from restriction digests of M38, M48, or SW6, washed with 2× SSC and 0.1% SDS at room temperature, dried, and exposed to x-ray film. The size of the transcript was determined by comparison with ^{32}P-labeled RNA size markers that were loaded in adjacent lanes.

Bacterial Expression of Plastin Isoforms

The corrected, full-length coding sequence of human L- or T- plastin (Lin et al., 1988, 1990) was cloned into the recombinant plasmid pET-3C (Studier and Moffat, 1986) having the T7 RNA polymerase promoter and transfected into *Escherichia coli*. Expression was induced with isopropyl-β-D-thiogalactopyranoside. Lysates of uninduced and induced bacteria were solubilized with SDS sample buffer and subjected to one-dimensional gel electrophoresis and immunoblot analysis.

Two-dimensional Gel Immunoblots and Autoradiography

Human SCC cells (gift from George Milo, Ohio State University) were labeled for 6 h with [^{35}S]methionine and subjected to two-dimensional gel electrophoresis as previously described (Lin et al., 1988). Proteins were electroblotted to nitrocellulose using a semi-dry blotting apparatus (Electrobiotransfer, Sunnyvale, CA) at a constant current (1.0 mA/cm^2 of gel) for 1 h. The filters were stained with Amido black to determine the effectiveness of protein transfer, incubated with a 1:1,000 dilution of rabbit anti-chicken fimbrin antiserum for 17 h, and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (1:500 dilution; Bio-Rad Laboratories) for 2 h. The filters were developed to visualize the secondary antibody and exposed to x-ray film for 2 and 18 h to identify radioactive proteins.

Plastin Isoform Expression in Different Cell Lines

Various primary or secondary cell strains established from different tissues were assayed for L- and T-plastin expression by Northern blot analysis as described previously (Lin et al., 1988) or by two-dimensional PAGE as described previously (Goldstein et al., 1985).

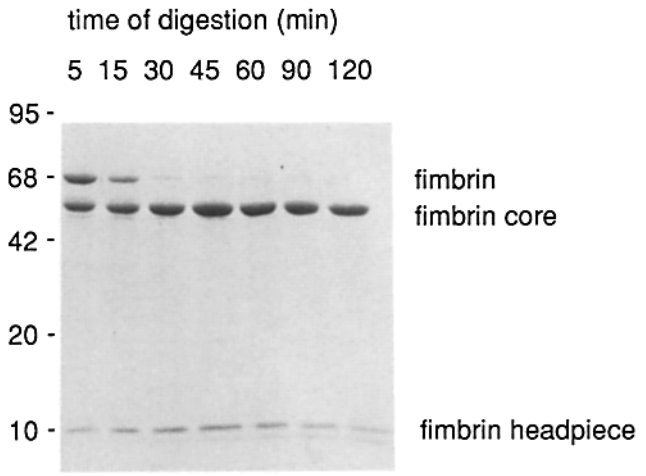

Figure 1. Proteolysis of fimbrin produces two fragments, fimbrin core (58 kD) and headpiece (10 kD). With longer times of digestion, fimbrin core remains stable but headpiece is degraded into smaller fragments. Numbers on the left indicate the positions of the molecular weight standards.

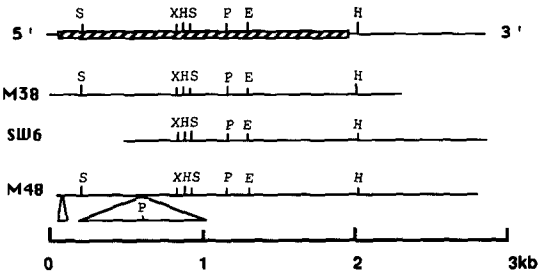

Figure 2. Restriction maps of the fimbrin cDNA clones. The upper diagram is assembled from the individual clones. The translated region is indicated by the striped box. Sites for Eco RI (*E*), Xba I (*X*), Sac I (*S*), Hind III (*H*), and Pst I (*P*) are indicated. The inserts marked in clone M48 indicate the size and position of the unspliced introns.

Results

Fimbrin Proteolysis

In nondenaturing conditions small amounts of papain (Fig. 1), trypsin, thermolysin, proteinase K, subtilisin, or V-8 protease (not shown) cleaved intact fimbrin with the simultaneous appearance of two fragments, termed core and headpiece (58 and 10 kD). With longer times of digestion, the fimbrin headpiece but not core was cleaved to smaller fragments. Neither intact fimbrin nor fimbrin headpiece polypeptides could be sequenced, suggesting that the amino terminus of each was blocked and that fimbrin headpiece corresponded to the amino-terminal fragment of fimbrin. The amino terminus of fimbrin core was sequenced (see Fig. 4). The presence of a sequenceable amino terminus, size, and time course of appearance during proteolysis suggested the core fragment was derived from the carboxy-terminal part of fimbrin.

Fimbrin cDNA and Protein Sequence

A fimbrin-specific antiserum identified a single cDNA clone (SW6) in a λgt11 library (Fig. 2). This clone (2.4 kb) hybridized with a 3.2-kb band in Northern blots of chicken intestine RNA (Fig. 3). The sequence of SW6 contained an open reading frame encoding a 498-amino acid polypeptide. Two other clones (M38, 2.3 kb; M48, 3.7 kb, Fig. 2), isolated from the λgt10 library by screening with SW6 as a probe, contained a 1.9-kb open reading frame encoding a 630-amino acid protein with a predicted molecular weight of 70,894 (Fig. 4). The coding regions of all three clones were identical. One clone (M48) lacked a poly(A) tail and contained additional sequences in the 5' untranslated region (90 bp) and in the coding region (830 bp) that were not in M38 or SW6. The 830 bp sequence lies in the codon for cys165 between nucleotides 496 and 497. Because there are splice-junction sequences at the borders of the additional sequences, this clone probably represents an incompletely processed transcript. The amino terminus of the polypeptide was assigned based on the calculated molecular weight of the encoded protein and the absence of other initiation codons in the region. Fimbrin headpiece corresponded to residues 1–114 and fimbrin core to residues 115–630.

Duplicated Domains in Fimbrin and Plastin

A search of sequence data bases (GenBank and NBRF) showed that fimbrin is the chicken homologue of human T- and L-plastin. L- and T-plastin were cloned from a human fibroblast library (Lin et al., 1988), and encode 630 and 627 amino acid proteins respectively. The sequences of both isoforms have been recently amended (Lin et al., 1990) to include the correct amino termini. Fimbrin was slightly more homologous with T-plastin (72.1% identity in 627 residues)

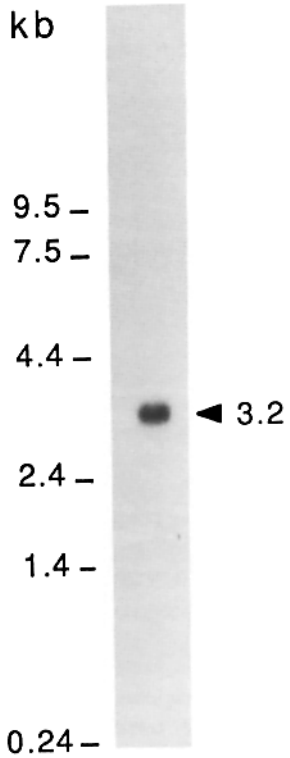

Figure 3. A Northern blot of total chicken intestine RNA was probed with an 0.3-kb fragment from clone M38. A single band of 3.2 kb is detected.

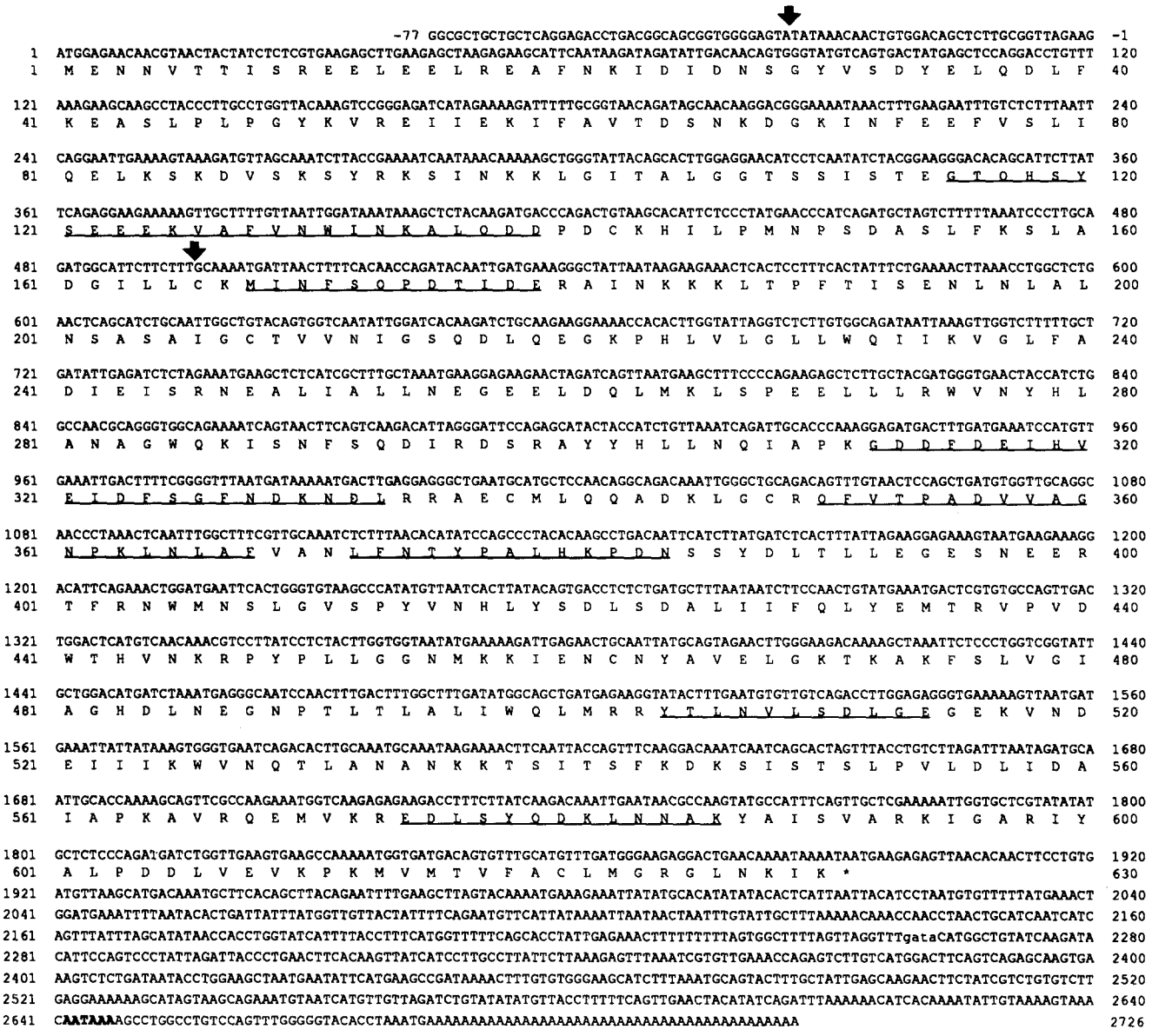

Figure 4. The DNA and predicted protein sequence of chicken intestine fimbrin. The underlined regions identify sequences obtained independently from protein sequence. The amino-terminal sequence of fimbrin core generated by V8 proteolysis begins at residue 115. Additional sequences in clone M48 (not shown) are indicated by arrows. The DNA sequence at positions 2258–2261 denoted in small letters identifies a noncoding sequence difference between clones SW6 and M38, probably an artifact generated during reverse transcription. The polyadenylation signal sequence is in bold letters. These sequence data are available from EMBL/GenBank/DDBJ under accession number X5262.

than with L-plastin (69.9% identity in 627 residues). Chicken intestinal epithelial cells probably contain a single fimbrin isoform because two-dimensional gels of isolated brush borders indicate a single fimbrin spot (Glenney et al., 1981*b*) and cDNA probes to fimbrin detected a single band on Northern blots of chicken intestinal cell RNA (Fig. 3). This finding is consistent with our observation that various human noncancer cells express a single plastin isoform distinguishable on Northern blots by a characteristic size for each isoform mRNA.

Matrix comparisons of fimbrin and plastin polypeptide sequences (Fig. 5) display four diagonal lines, indicating four homologous regions referred to as *A*, *B*, *A'*, and *B'*. These regions can be paired in two groups in which regions A and B (residues 100–350) comprise the amino-terminal half of fimbrin core and regions A' and B' (residues 350–630) comprise the carboxy-terminal half. More detailed analysis (Table I) shows that the AB regions of fimbrin and plastin are the most highly homologous (76–78% identical); their A'B' regions are also highly homologous (72–75% identical), but region AB is only weakly homologous with region A'B' (19–25% identical). A pairwise comparison of all A, A', B, and B' regions (Table II) shows that the A regions are more similar to A' regions than to B or B' regions. The percent identities between the regions are low (15–28%); however, each region contains a common pattern of conserved residues (boxed) within a 90-residue sequence (Fig. 6). This sequence is bounded by a conserved trp-x-asn sequence at the

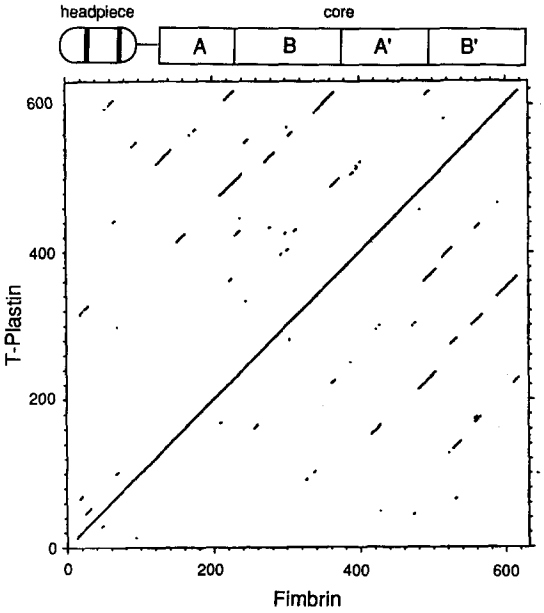

Figure 5. Matrix comparison between chicken fimbrin and human T-plastin shows a continuous diagnonal throughout their sequence. In addition, there are four off-diagonal lines that indicate internal regions of homology. The diagram above the matrix shows the positions of fimbrin headpiece and core domains and the positions of the repeated homologous sequences. (A, B, A', and B'). The sequences were analyzed using the program COMPARE (UWGCG) with stringency set at 25.

amino terminus and by a asp-ile/leu/val-val-x-x-pro sequence at the carboxy terminus. Most of the conserved residues are hydrophobic.

Homologies with Actin Gelation Proteins

The AB regions of fimbrin are homologous with the amino-terminal domains of chicken (Lemaire et al., 1988) and human (Koenig et al., 1988) dystrophin (18.7 and 19.8% identical) and chicken alpha-actinin (Baron et al., 1987) 17.6% identical) (Fig. 9 and Table I). Mimura and Asano (1986) demonstrated that the amino-terminal domain of alpha-actinin binds actin filaments. Other recent reports have shown that the actin-binding domain of alpha-actinin is homologous with the amino-terminal domains of *Drosophila* beta-spectrin (Byers et al., 1989) and *Dictyostelium* actin gelatin protein (Noegel et al., 1989). These domains were 20.7 and 17.2% identical with the AB region of fimbrin.

The amino-terminal domains of the actin gelation proteins also displayed the AB organization that we detected in fimbrin and plastin. Most of the boxed areas in Fig. 6 indicate the residues conserved not only between fimbrin and plastin but also with the amino terminal domains of dystrophin and homologues. Other conserved areas, marked by the consensus sequence, are unique for the A and A' domains or the B and B' domains. The consensus sequences shared by the A and B domains are primarily hydrophobic residues, clustered in two groups and separated by charged residues. The duplicated sequence within the amino-terminal domains of the actin gelation protein sequences have not been previously reported.

Table I. Amino Acid Sequence Homology between Fimbrin, Plastin, and Actin Gelation Proteins

	% Identity (% similarity) with fimbrin	
Domain	Region AB	Region A'B'
Fimbrin		
Region AB	100 (100)	23.4 (50.2)
Region A'B'	23.4 (50.2)	100 (100)
L-plastin		
Region AB	75.7 (88.0)	20.0 (48.3)
Region A'B'	18.5 (49.1)	74.6 (87.7)
T-plastin		
Region AB	78.2 (89.7)	25.3 (46.7)
Region A'B'	22.1 (49.7)	72.0 (85.7)
Chicken alpha-actinin	17.6 (46.8)	21.2 (49.8)
Human dystrophin	18.7 (40.8)	19.2 (45.6)
Chick dystrophin	19.8 (38.1)	19.0 (44.7)
Drosophila beta-spectrin	20.7 (41.6)	20.5 (44.3)
Dictyostelium actin gelation protein	17.2 (40.5)	14.5 (44.1)

* The percent identity and similarity were calculated from the alignment shown in Fig. 6.

Calmodulin-like Calcium-binding Sites

The sequence of fimbrin headpiece shows no similarity to fimbrin core or to villin headpiece. A database search revealed that fimbrin headpiece is homologous with vertebrate calmodulin (Simmen et al., 1985) (34.7% identity) and a pseudogene (Stein et al., 1983) for skeletal muscle calmodulin (37.5% identity). Although not expressed in muscle, the pseudogene sequence encodes a fully functional calmodulin, demonstrated by binding studies on protein expressed in bacteria (Putkey et al., 1985). Matrix comparisons between headpiece and calmodulin (not shown) indicated that two regions in headpiece are homologous to calcium-binding sites of calmodulin. Previous sequence analysis of plastin had described a single calcium binding site (Lin et al., 1990). Both of the identified regions in headpiece contained a consensus calcium-binding site flanked by predicted alpha-helix, forming a pair of EF hand or helix-loop-helix motifs (Fig. 7). In the three-dimensional structure of calmodulin, the calcium-binding domains I and II and domains III and IV of calmodulin are at opposite ends of a central alpha-helical linker region (reviewed by Strynadka and James, 1989). Fimbrin headpiece is more similar to calmodulin domains III and IV (37.5% identity) than to domains I and II (23% identity). Both sites in fimbrin headpiece are predicted to

Table II. Amino Acid Sequence Homology between Fimbrin Domains

	% Identical matches (% similarity)*			
Domain	A	B	A'	B'
A	100	16.8 (35.7)	23.6 (48.0)	20.4 (46.2)
B		100	19.5 (38.4)	27.6 (51.7)
A'			100	15.0 (39.6)
B'				100

* The percent identity and similarity were calculated using the alignment in Fig. 6.

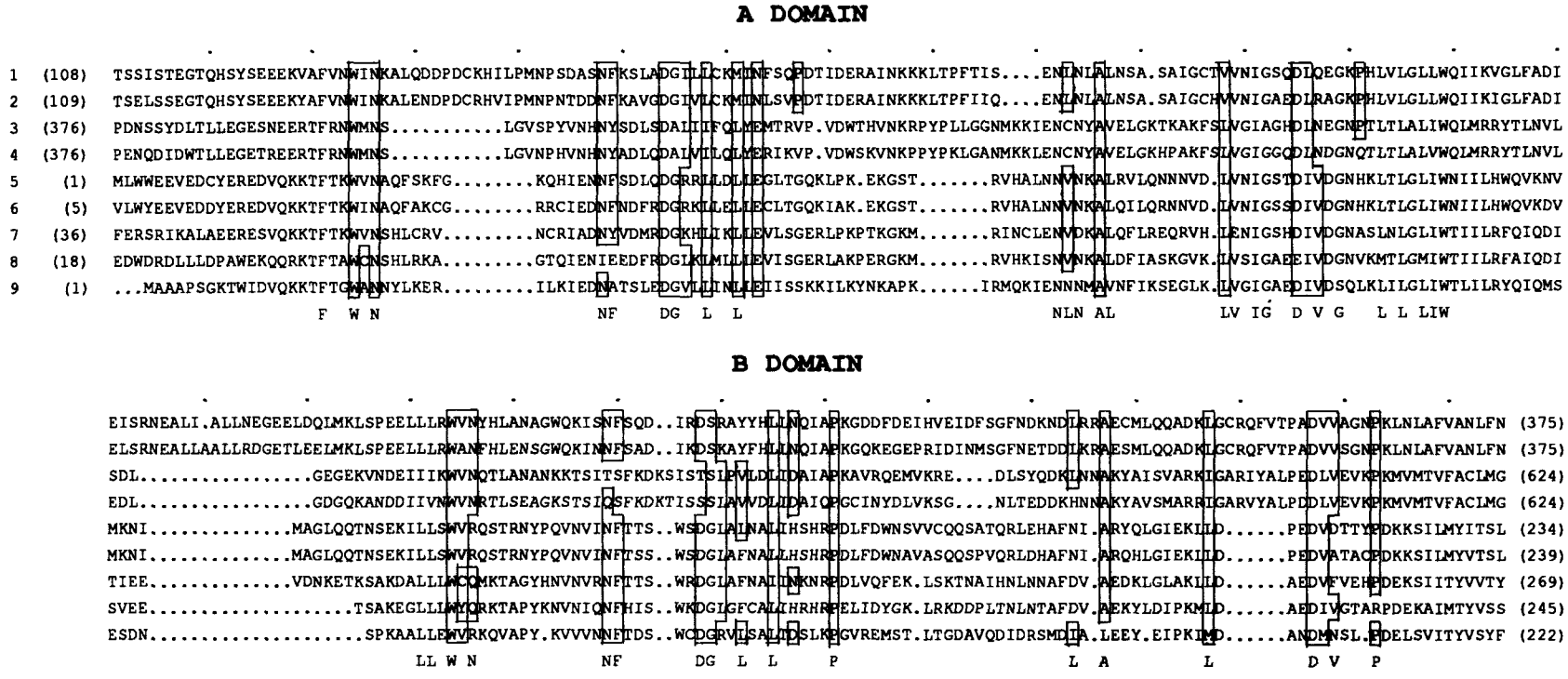

Figure 6. A comparison between fimbrin (lines *1* and *3*), T-plastin (lines *2* and *4*), human dystrophin (line *5*), chicken dystrophin (line *6*), chicken alpha actinin (*line 7*), *Drosophila* beta-spectrin (line *8*), and *Dictyostelium* actin gelation protein (line *9*) identify a repeated hydrophobic consensus sequence (*boxed residues*). The repeat indicates the sequence can be divided into an A and B domain. The amino acids noted beneath the sequences identify residues conserved within the A and B domains. Numbering indicates the sequence positions of the amino- and carboxy-terminal amino acids. These sequence data are available from EMBL/GenBank/DDBJ under accession number X5262.

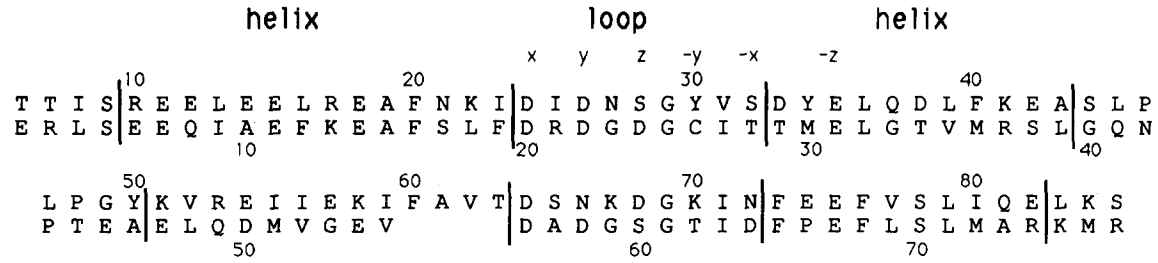

Figure 7. Alignment of fimbrin headpiece (*top line*) with a calmodulin pseudogene (*bottom line*) sequence. The calcium-binding loop and flanking alpha-helices of calmodulin are indicated. Following the convention for describing calcium-binding sites, the positions of the residues that coordinate with calcium are labeled *x*, *y*, and *z*.

bind calcium, but their specificities and affinities can not be predicted from the sequence.

Fimbrin/Plastin Homology Demonstrated by Antibody Cross-Reactivity

Isoforms of plastin, identified by their positions on two-dimensional gels, have been detected in a variety of human and mouse cells (Goldstein et al., 1985). Fimbrin has also been detected (using immunofluorescence) in a variety of cell types from different species (Bretscher and Weber, 1980). Fig. 8 *a* shows in an autoradiogram of [^{35}S]methionine-labeled proteins of the human squamous cell carcinoma cell line SCC separated by two-dimensional electrophoresis. L-plastin focused at a characteristic position (pI 5.9, 68 kD). When a blot of the two-dimensional gel (Fig. 8 *b*) was reacted with anti-fimbrin serum, L-plastin, and phospho-L-plastin were detected. T-plastin is less abundant in these cells and could not be detected on the two-dimensional gel or the

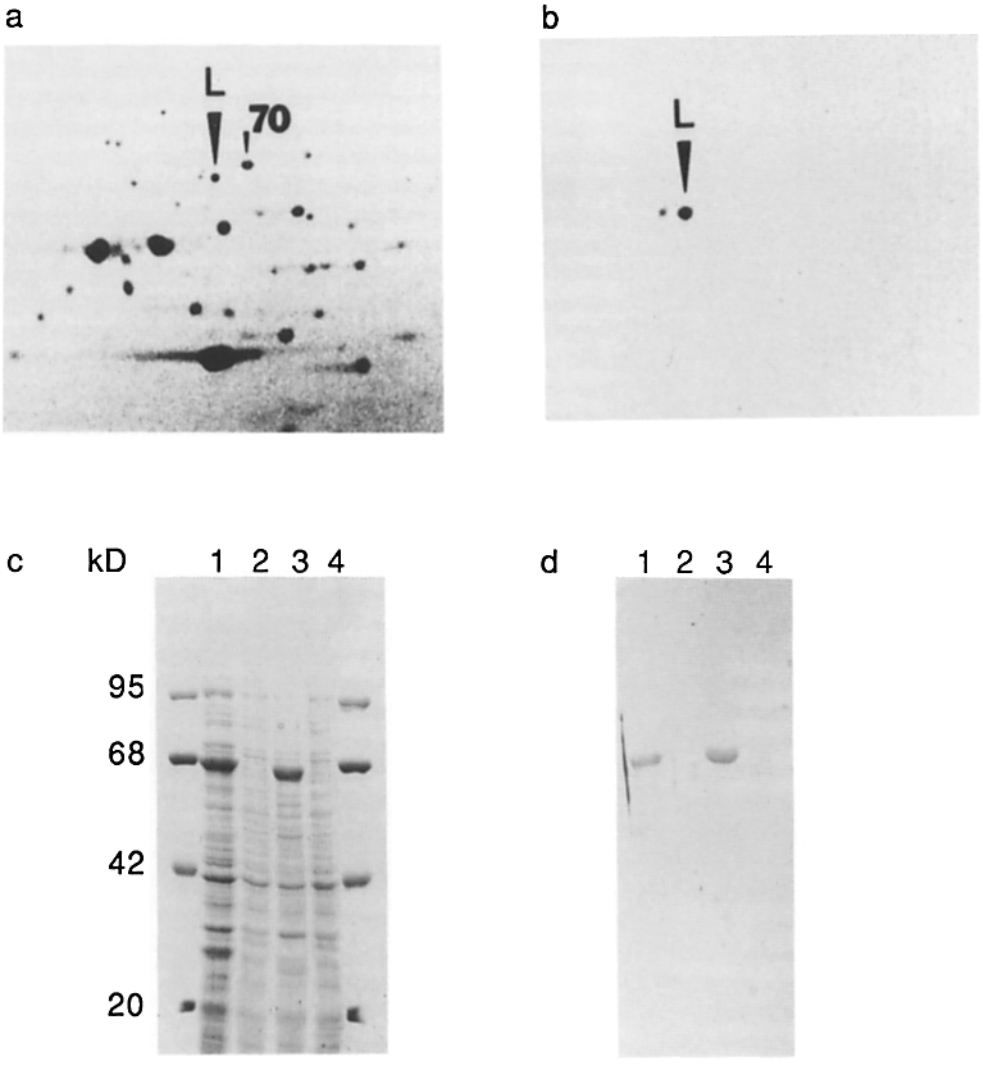

Figure 8. Reaction of plastin with fimbrin-specific antibodies. A two-dimensional gel of [^{35}S]methionine-labeled proteins from SCC cells (*a*), shows the position of L-plastin (*large arrowhead*) and a ubiquitous 70-kD polypeptide used as a marker to identify the positions of L- and T-plastin. The acidic portion of the gel is on left. The corresponding immunoblot (*b*) shows that L-plastin and its more acidic phosporylated derivative is detected by the fimbrin antiserum. The SDS-PAGE gel (*c*) of bacterial cultures transformed with expression plasmids containing human T-plastin (lanes *1* and *2*) and L-plastin (lanes *3* and *4*) before induction (lanes *2* and *4*) and after 1.8 h induction (lanes *1* and *3*) with 0.4 mM IPTG. The corresponding immunoblot (*d*) shows the fimbrin antiserum detects bacterially expressed T-plastin (lane *1*) and L-plastin (lane *3*) in the induced but not in uninduced (lanes *2* and *4*) bacteria. Molecular weight markers are in the flanking lanes.

Table III. Survey of Plastin Expression in Human Cell Lines

Cell type	L-plastin	T-plastin	Protein assay	mRNA assay
Hematopoietic cells				
B-lymphocytes	+	−	+	ND
T-lymphocytes	+	−	+	ND
Granulocytes	+	−	+	ND
Monocytes/macrophages	+	−	+	ND
Erythrocytes	−	−	+	ND
Platelets	−	−	+	ND
Solid tissue cells				
Skin keratinocytes	−	+	+	ND
Umbilical cord endothelium	−	+	+	ND
Ocular trabecular endothelium	−	+	+	ND
Skin melanocytes	−	+	+	ND
Fetal amniotic cells	−	+	+	ND
Foreskin fibroblast	−	+	+	ND
Embryonic lung fibroblast	−	+	+	ND
Brain fibroblast	−	+	+	ND
Gingiva fibroblast	−	+	+	+
Human cancer cells (hematopoietic origin)				
AG1484, GM6991 (B-leukemia)	+	−	+	+
CEM, Molt-4 (T-leukemia)	+	−	+	+
HL-60 (promyelocytic leukemia)	+	−	+	ND
Human cancer cells (solid tissue origin)				
HuT fibrosarcoma	+	+	+	+
8387 fibrosarcoma	+	+	+	+
HT1080 fibrosarcoma	+	+	+	+
HOS osteosarcoma	Trace	+	+	+
Sarcoma-2 leiomyosarcoma	+	+	+	+
Ovarian carcinoma	+	+	+	ND
BeWo choriocarcinoma	+	+	+	ND
MCF-7 mammary carcinoma	+	+	ND	+
HTB-130 mammary carcinoma	+	+	ND	+
HTB-132 mammary carcinoma	+	+	ND	+
SSC83-01-82 squamous cell carcinoma	+	+	+	ND
MeWo melanoma	Trace	+	+	+
GM1232C (Y79) retinoblastoma	−	−	+	+
MG63 osteosarcoma	−	+	+	ND
HeLa cervical carcinoma	−	+	+	ND
WiDr colon carcinoma	−	+	+	ND
HT-29 colon carcinoma	+	+	ND	+
RD rhabdomyosarcoma	−	+	+	ND
Wilm's tumor kidney carcinoma	−	+	+	ND

immunoblot. To show that our polyclonal antisera could detect T-plastin, we tested for cross-reactivity with recombinant T- and L-plastin (Fig. 8 *c*) on immunoblots. The fimbrin antisera crossreacted with recombinant L- and T-plastin polypeptides (Fig. 8 *d*) that were expressed in bacteria after induction with IPTG. Control lanes showed no crossreactivity with bacterial proteins.

Isoform-specific Expression

The presence of two fimbrin isoforms in mammalian cells may signify functional differences in actin-binding or cytoskeletal organization. To determine whether plastin isoform expression is restricted to particular cells, we surveyed a variety of human cell types (Table III) for expression of L- and T-plastin isoforms using Northern blot analysis or two-dimensional gel electrophoresis. In normal cell strains assayed, expression of L-plastin was restricted to replicating populations of hematopoietic cells: erythrocytes and platelets exhibited no detectable L-plastin (data not shown). Cell strains derived from normal solid tissues expressed only T-plastin.

Expression of the plastin isoforms in human cancer cells was studied using the same assays. Like their normal counterparts, T- and B-lymphoblastoid and myeloid cell lines expressed only L-plastin. However, in contrast with our findings for normal cells from solid tissues, 16 out of 22 cell lines derived from solid tumors or tumor-derived secondary cultures (not established as stable cell lines) expressed L-plastin in addition to T-plastin. In most cases, the L-plastin levels were several-fold higher than T-plastin although expression of L-plastin in individual tumor-derived cell lines varied greatly (data not shown) compared with the more constant level of T-plastin expression (Lin et al., 1988). The retinoblastoma cell line Y79, an anchorage-independent cell line, was the only human cell line tested that did not synthesize detectable levels of plastin messenger RNA or polypeptide (Lin et al., 1988).

Discussion

Our results provide the first evidence that links two classes of actin-binding proteins, actin bundling proteins and actin gelation factors, into a structurally related family of actin cross-linking proteins. A striking feature of these proteins is their modular organization (Fig. 9). A simple model of fimbrin depicts an amino-terminal calcium-binding domain and tandem arrangement of actin binding domains, AB and A'B'. Each actin-binding domain consists of a duplicated sequence, suggesting that fimbrin structure arose from two gene duplication events. The first duplication resulted in the A and B domains; a second duplication produced the A' and B' domains. The close spacing of actin filaments in a fimbrin bundle might result from the tandem arrangement of closely separated actin-binding domains.

Different arrangements of a single actin-binding domain (Fig. 9) can account for the variety of actin gels and bundles present in a cell. The actin gelation proteins generally consist of an actin-binding domain, a rod-like spacer domain, and a carboxy-terminal domain responsible for binding calcium, membranes, or other actin-binding proteins. Alpha-actinin for example, contains a single actin-binding domain per monomer, but when oligomerized into an antiparallel dimer the two actin-binding sites lie at opposite ends of the molecule. Proteins in which actin-binding domains are spaced increasingly apart from each other retain crosslinking activity but bundle actin filaments more loosely (Brotschi et al., 1978; Condeelis et al., 1984). The flexibility of a protein, increasing with length of the molecule, could allow the cross-linking of orthogonally arranged filaments into a three-dimensional meshwork.

Role of Calcium in Actin Bundle Formation

Fimbrin is composed of two structural domains (Fig. 9), headpiece and core, linked by a protease-sensitive region. Sequence homologies with other actin- or calcium-binding proteins suggest that these domains have separate functional roles: headpiece binds calcium and core binds actin. By equi-

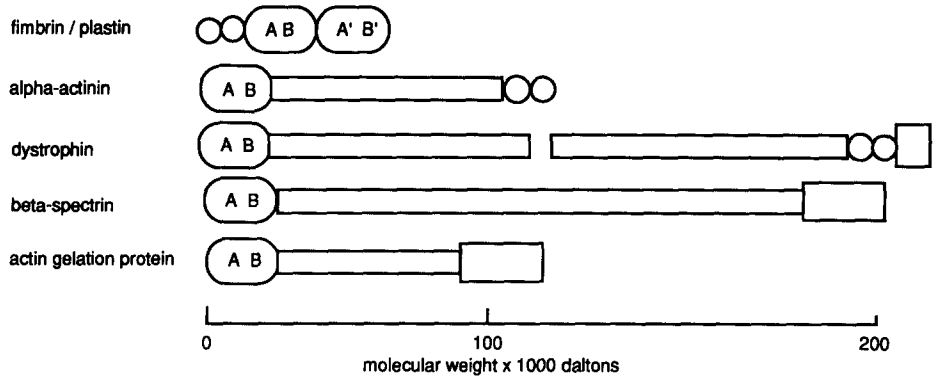

Figure 9. A diagram of the modular arrangement of domains in fimbrin, plastin, alpha-actinin, dystrophin, beta spectrin, and actin gelation protein. Domains homologous to the AB or A'B' regions of fimbrin and plastin are indicated. Circles identify calmodulin-like calcium-binding domains. The boxed domains at the carboxy terminus of the gelation protein are responsible for membrane-binding or oligomerization of the polypeptide.

librium dialysis, Glenney and collaborators (1981) identified in fimbrin a single calcium-binding site that is competed by Mg^{2+}. This site is probably one of the sites predicted in fimbrin headpiece; whether a second calcium-binding site in headpiece also binds calcium is not known. The functional role of calcium in fimbrin regulation is not understood because preliminary evidence (Glenney et al., 1981*b*; Matsudaira, P., manuscript in preparation) suggests that fimbrin binding to actin is independent of calcium. The homology between fimbrin and plastin is weakest in the headpiece domain raising the possibility that the isoforms may be differentially regulated by calcium. Alpha-actinin, which has actin- and calcium-binding domains (Noegel et al., 1987) similar to fimbrin, is inhibited by calcium from crosslinking actin filaments. Chemical cross-linking studies demonstrate that the calcium-binding domain interacts with the actin-binding domain (Imamura et al., 1988). Although fimbrin headpiece is more closely related to calmodulin (34.7% identify) than the calcium-binding domains of alpha-actinin (18.4% identity), a similar interaction of headpiece with core may influence actin-binding activity in a different fimbrin isoform.

The calcium-binding headpiece domains of fimbrin and villin are not homologous despite their similar sizes. In villin this domain binds calcium (Hesterberg et al., 1985) and lies at the carboxy terminus (Glenney et al., 1981*b*). The isolated villin headpiece domain binds but does not bundle actin filaments and binding is not affected by calcium. We do not know if fimbrin headpiece contains an actin-binding activity.

Do Conserved Sequences Represent Actin-binding Sites?

The presence of a consensus sequence in various actin binding proteins is often interpreted to indicate conservation of an actin-binding site, given the evolutionary conservation of actin (Blanchard et al., 1989). An alternative interpretation of a conserved sequence in actin-binding proteins is suggested by the fact that the most variable region of the actin sequence is at the acidic amino terminus, where basic residues in several actin-binding proteins can be chemically crosslinked (Sutoh and Mabuchi, 1986). The consensus sequence in the A and B domains of fimbrin and the larger actin binding proteins is largely hydrophobic and thus is likely to be buried in the protein, an unlikely location for an actin-binding site.

A possible function for the conserved hydrophobic sequence is suggested from studies on villin and other actin-severing proteins with a conserved hydrophobic repeated sequence. We have concluded that these sequences might be involved in maintaining a conformation or structure of the domains (Matsudaira and Janmey, 1988): the consensus sequence maps within protease-resistant regions of villin, and amino acids such as cysteine (Matsudaira et al., 1985) or methionine (unpublished data) within the conserved repeat cannot be chemically modified in the folded, native protein. These experiments directly support the prediction that conserved sequences in villin are buried in the protein. Although we have not mapped the residues that are accessible to solvent and the protease-resistant domains of fimbrin to the same resolution as villin, we suggest the conserved sequences in fimbrin may possibly play a similar structural role.

Possible Regulation of Actin Binding by Phosphorylation

The identification of fimbrin as plastin suggests that the actin- and/or calcium-binding activity of fimbrin may be modulated by phosphorylation. Two other actin bundling proteins, band 4.9 of the erythrocyte membrane (Husain-Chistri et al., 1988) and synapsin I of the nerve synapse (Bahler et al., 1986), also become phosphorylated. In both cases, phosphorylation inhibits actin bundle formation. L-plastin was first identified on the basis of its exclusive expression in transformed cells and was later detected in normal lymphocytes and macrophages. L-plastin becomes phosphorylated, presumably by protein kinase C, on unidentified serine residues when leukemia cells are activated by PMA or when leukocytes are activated by IL 1 (Goldstein et al., 1985; Anderson et al., 1985; Matsushima et al., 1988). The level of phosphorylation varies between cell types, but can approach as much as 30% of the total soluble L-plastin (Leavitt, J., unpublished data). Unlike L-plastin, T-plastin phosphorylation has not been detected in any cell line and thus T-plastin might not be a substrate for kinases. Fimbrin purified from chicken intestine epithelia is a single spot on a two-dimensional gel and phosphorylated intestinal fimbrin has not been detected.

Functional Differences between Fimbrin Isoforms

In addition to differences in sequence and phosphorylation, the differential expression of the L- and T-isoforms in hematopoietic and non-hematopoietic cells and the change in isoform expression in neoplastic cells suggest the isoforms are functionally different. This differential expression was not

detected in immunofluorescence studies of various cells (Bretscher and Weber, 1980*a*) because polyclonal antisera raised against chicken fimbrin cannot distinguish the two human fimbrin/plastin isoforms (Fig. 8 *d*). The pattern of isoform expression may be correlated with the motile or adhesive properties of the cell because antibodies specific for fimbrin have detected fimbrin in focal contacts and adhesion sites on the ventral surface of cultured cells, monocytes, and macrophages (Bretscher and Weber, 1980*b*; Carley et al., 1985). Recent identification of protein kinase C at sites of focal contact (Jaken et al., 1989) and our finding that fimbrin is a cytoplasmic phosphoprotein in leucocytes raise the tantalizing possibility that fimbrin phosphorylation is involved in cell–substratum or cell–cell interactions. Changes in expression and distribution of microfilament proteins often accompany neoplastic transformation in many cell systems. The discovery of phosphorylated isoforms of fimbrin provides additional molecular details of the possible mechanism of regulation of cellular actin bundles during the re-organization of the microfilament-based cytoskeleton.

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