Filamin Concentration in Cleavage Furrow 
and Midbody Region: 
Frequency of Occurrence Compared with 
That of Alpha-Actinin and Myosin

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ABSTRACT Affinity-purified rabbit antibody to purified chicken gizzard filamin was used in 
indirect immunofluorescence to localize filamin in dividing chick embryo cells. The antibody 
was shown to bind only chick embryo cell filamin when whole cell extracts were analyzed by 
the sensitive sodium dodecyl sulfate-polyacrylamide gel electrophoresis overlay technique 
described by Adair et al. (1978, J. Cell Biol. 79:281-285). The results show that filamin is located 
in stress fibers and membrane ruffles during interphase. As cells enter prophase, the condensing 
chromosomes are surrounded by diffuse antifilamin staining. No stress fibers are apparent. 
During metaphase and anaphase, the staining is bright but diffuse. There is often peripheral 
membrane staining. Filamin is not concentrated in the spindle region but neither is it excluded 
from the spindle. During cytokinesis, filamin is found highly concentrated in the cleavage 
furrow in 16 out of 100 cells examined. This frequency of concentration in the furrow is 
comparable to that observed for alpha-actinin (14%). Myosin concentration in the furrow is 
more frequent; it is observed in 37% of the cells examined. Neither myosin, alpha-actinin, nor 
filamin is observed concentrated in the furrow 100% of the time. We conclude that the results 
are consistent with, but not sufficient to prove, the hypothesis that alpha-actinin and filamin 
are essential components of the mechanism of cytokinesis.

Filamin is a high molecular weight (250,000 mol wt) actin- 
binding protein that was first isolated from chicken smooth 
muscle (37). Filamin may be functionally homologous to the 
high-molecular-weight actin-binding protein isolated from rabbit 
alveolar macrophages as described by Hartwig and Stossel 
(18). A molecule that is antigenically as well as functionally 
homologous to filamin has since been found in mammalian 
smooth muscle, platelets, fibroblasts, macrophages, kidney, and 
liver and in avian skeletal muscle (3, 19, 35). The name filamin 
derives from the filamentous staining pattern given by indirect 
immunofluorescent staining of cultured cells with antifilamin 
antibodies (37). Because purified filamin does not form fila-
ments in vitro, its filamentous intracellular distribution is thought to be attributable to its association with actin-contain-
ing stress fibers (19).

The in vitro actin-binding properties of filamin have been 
partially characterized. Filamin cross-links F-actin filaments, 
causing the formation of a gel. Gelation requires F-actin (G-
actin will not substitute) (39). Filamin has also been shown to 
inhibit the actin-activation of myosin ATPase (9). Davies and 
co-workers (10, 36) have demonstrated that filamin is a phos-
phoprotein whose phosphorylation in cell extracts is stimulated 
by cyclic AMP. However, the significance of the phosphoryl-
ation of filamin with respect to its in vitro properties has not 
been demonstrated.

Although some details are known about the in vitro inter-
action of actin and filamin, there is far less information con-
cerning the possible interaction of these proteins in vivo. By 
immunofluorescent staining, filamin has been found in several 
cellular structures known to contain actin, i.e., stress fibers, 
microspikes, and membrane ruffles (19). Filamin has also been 
found in the terminal web of intestinal epithelial cells (4), a 
region shown to be enriched in contractile proteins such as 
actin (34), myosin (27), and alpha-actinin (4, 8, 16). The
localization of filamin in cellular structures known to contain actin suggests that filamin interacts with and possibly regulates the state of actin in vivo.

We decided to take these immunolocalization studies a step further by determining whether filamin redistributes during a contractile process thought to involve actin. Mitosis and cytokinesis are dynamic cellular processes in which actin has been postulated to play an important mechanistic role (12, 15, 32).

In this paper we report observations on the distribution of filamin during the cell cycle.

MATERIALS AND METHODS

Purification of Filamin

Filamin was purified from frozen (~20°C), glycercinated chicken gizzards (Pel-Freeze Farms, Inc., Rogers, Ark.) according to a modification of the method of Shizuta et al. (33). Modifications of the published procedure included dialysis of the high-salt extract, before (NH₄)₂SO₄ fractionation, against a no-salt buffer to precipitate myosin. Sepharose 4B chromatography was done in the presence of 0.6 M KCl to decrease protein aggregation. Purified filamin was stored at ~20°C in 50% glycerol.

Antibodies

**ANTIFILAMIN**: Antifilamin antibody was raised in rabbits and affinity purified on cyanogen bromide-activated Sepharose-alpha-actinin and characterized as to its reactivity against chick embryo cells (14). The other antibody and precipitating the immune complexes with heat- and formalin-fixed *S. aureus*. These gizzard extracts are identified to those used in the preparation of the filament antigen and therefore contain proteins that may have contaminated the original antigen. In Fig. 2, it can be seen that in both the low- (lane C) and high- (lane G) salt extracts, filamin is the only

**PURITY OF FILAMIN ANTIGEN**: The specificity of the affinity-purified antifilamin antibody was tested by incubating low- and high-salt extracts of chicken gizzard with antibody and precipitating the immune complexes with heat- and formalin-fixed *S. aureus*. These gizzard extracts are identical to those used in the preparation of the filament antigen and therefore contain proteins that may have contaminated the original antigen. In Fig. 2, it can be seen that in both the low- (lane C) and high- (lane G) salt extracts, filamin is the only
FIGURE 1 Coomassie Blue-stained SDS-polyacrylamide gels of purified chicken gizzard filamin. (a) Electrophoresis of filamin antigen (70 µg) on 4% acrylamide cylindrical gel. (b) Electrophoresis on 7.5% acrylamide slab gel of filamin (3 µg) that was coupled to Sepharose 2B and used to affinity-purify antifilamin. As little as 0.1 µg of protein can be detected by Coomassie Blue on the 7.5% acrylamide slab gel, indicating that the filamin in b is at least 95% pure. India ink marks dye front in b.

FIGURE 2 Immunoprecipitation. Polyacrylamide gel analysis of immunocomplexes formed between purified antifilamin antibody and gizzard extracts. Lane A, low-salt extract. Lane B, high-salt extract. Lane C, low-salt extract + antifilamin + S. aureus. Lane D, low-salt extract + nonadherent IgG + S. aureus. Lane E, low-salt extract + NRS + S. aureus. Lane F, low-salt extract + S. aureus. Lane G, high-salt extract + anti-filamin + S. aureus. Lane H, high-salt extract + nonadherent IgG + S. aureus. Lane I, high-salt extract + NRS + S. aureus. Lane J, high-salt extract + S. aureus. Lane K, NRS + S. aureus. The positions of the heavy and light chains of IgG are marked with the black h and f, respectively. The filamin position is marked with a small black arrow.

protein precipitated by the antifilamin S. aureus complex. Controls for this indirect immunoprecipitation included incubation of the low- and high-salt extract with S. aureus extract in the absence of antibody (Fig. 2, lanes F and J) and substitution of antifilamin with either normal rabbit serum (NRS) (Fig. 2, lanes E and I) or nonadherent IgG (the fraction of immune serum that passed directly through the filamin-Sepharose column; Fig. 2, lanes D and H). All controls were negative; that is, nonspecific precipitation of filamin did not occur. The indirect immunoprecipitation technique detects both precipitating and nonprecipitating antibodies.

ANTIBODY OVERLAY: When tested against total proteins of chick embryo cells, the purified antifilamin antibody recognized only a filamin-sized molecule (Fig. 3). The antibody overlay technique is so sensitive that protein bands not detected by Coomassie Blue (<1 µg of protein/band) can be detected by autoradiography. Fig. 3a is a Coomassie Blue-stained gel of purified filamin and varying concentrations of cell lysate subjected to the antibody overlay technique. Fig. 3b is the corresponding autoradiograph of that gel. The only protein in the chick embryo cell lysates that bound the purified antifilamin antibody comigrates with chicken gizzard filamin. If this technique is applied to chick embryo cells that are solubilized directly into SDS-electrophoresis sample buffer, identical results are obtained (data not shown).

Cell Fixation and Staining

Many different fixatives were tested in these immunofluorescence studies. The fixation procedures that gave the best preservation of cell morphology and staining intensity are: (a) 3.0–3.7% formaldehyde (10 min) followed by either −20°C acetone (5 min), 0.05% digitonin (2 min), or 0.1% Triton X-100 (2 min); (b) absolute methanol (−20°C) for 5 min.

Examples of the type of staining patterns observed with these two basic fixation methods are seen in Figs. 4–6. In Fig. 5, all of the cells were fixed with the cold methanol procedure. Fig. 6 shows cells fixed by formaldehyde followed by acetone (Fig. 6c, d, and k), Triton (Fig. 6e, f, and h), or digitonin (Fig. 6g, h, i, and j) and fixed by methanol (Fig. 6a and b). In general, the various fixation procedures gave comparable staining patterns. A qualitative difference with the methanol fixation was noticed, however. Cells fixed with methanol gave greater contrast between stained and unstained regions (note fine stress fiber detail in Fig. 4b). This difference in contrast may be attributable to the fact that methanol may not “fix” proteins that are...
Localization of Filamin by Indirect Immunofluorescence

INTERPHASE: There are two distinctive filamin staining patterns in interphase cells. Filamin is found in membrane ruffles (Fig. 4a) and stress fibers (Fig. 4b). Our data on filamin localization in interphase cells confirm the findings of Heggesness et al. (19).

MITOSIS: In prophase the cell rounds up and stress fibers are no longer present (data not shown). Filamin distribution during metaphase is shown in Fig. 5a. As in prophase, the filamin staining is bright, but diffuse. There is often a slight concentration of filamin around the periphery of the cell. Filamin is not concentrated in the spindle but neither is it excluded from the spindle region. In contrast to actin (6, 21), there is no indication that filamin is organized into spindle fibers. In anaphase (Fig. 5c), the filamin distribution is similar to that during metaphase. The staining is bright, but is not organized into any distinctive structure.

CYTOKINESIS: The cells shown in Fig. 5 e–n depict filamin distribution from early cleavage through the completion of cell division. The antifilamin staining shows that filamin is associated with the membrane in the region of the furrow (Fig. 5e and g), but also that filamin appears to be concentrated in the cytoplasm near the furrow. This staining pattern differs somewhat from that seen for alpha-actinin and myosin (14), in that membrane staining is not as intense. As furrowing continues (Fig. 5i), filamin remains associated with the cleavage furrow region. At the completion of cell division, filamin remains associated with the midbody region (Fig. 5k and m); however,
in the majority of cases the filamin is not concentrated in the midbody but rather in the adjacent cytoplasm (arrows in Fig. 5 k and m). This is particularly apparent in Fig. 5 m, where the cells are held together by a narrow intercellular bridge, which is devoid of detectable filamin.

Fig. 6 shows other examples of filamin concentration in the cleavage furrow and in the midbody region. These cells have been fixed in a variety of ways as indicated in the legend. These photographs show clearly that in these cells the cleavage furrow is the most intensely stained region of the cell. The poles of the cells before midbody formation are less intensely stained (Fig. 6 a, c, e, k, and l). The increased staining of the furrow is often diffuse (Fig. 6 a, c, and e). However, it has also been observed that the antifilamin appears to stain the membrane in the furrow region (Fig. 6 k and l).

Controls for Indirect Immunofluorescent Staining: Controls for the indirect immunofluorescent staining included substituting normal rabbit IgG for the purified antifilamin antibody (data not shown) and staining the cells with only rhodamine-labeled goat anti-rabbit IgG (Fig. 7). All controls were negative, i.e., there is no nonspecific staining of the cells. Therefore, antifilamin staining results from the specific interaction of the purified antifilamin antibody with filamin in situ.

Frequency of Filamin, Alpha-Actinin, and Myosin Association with the Cleavage Furrow and Midbody Regions

During the course of these studies we noticed that the intensity of filamin staining of the cleavage furrow varied from cell to cell. The range of staining intensity was from no apparent concentration of filamin in the furrow region to high concentration as depicted in Fig. 5 g and 6 a, c, k, and l. To further analyze the frequency of association of filamin with the cleavage furrow, we decided to compare it with the frequency of association of alpha-actinin and myosin with the cleavage furrow. We chose to look at alpha-actinin and myosin because Fujiwara and co-workers (13, 14) have previously reported that these two proteins are associated with the cleavage furrow.

Tables I and II list data obtained with chick embryo cells.
stained with either affinity-purified antifilamin (110 μg/ml), affinity-purified anti-alpha-actinin (110 μg/ml), whole rabbit serum (used at 1:100) directed against chick gizzard alpha-actinin, or whole goat serum (used at 1:25) directed against mouse L cell myosin. These cells were scored for the concentration of the particular protein in the cleavage furrow or midbody region. Assignment to the specific categories of "high contrast," "no contrast," or "marginal contrast" depended upon the intensity of staining of the furrow or midbody. Cells were classified as high contrast only if the cleavage furrow or midbody region was more intensely stained than surrounding regions of the cell. (Examples are shown in Figs. 5 and 6.) In cases where the furrow or midbody was stained to the same degree as the rest of the cell, the cell was classified as having no contrast in the furrow or midbody region. The category marginal contrast was used for those cells that showed only a slight increase in staining intensity of the cleavage furrow or midbody region over the rest of the cell. To simplify the analysis, all stages of cleavage furrow (Table I) or midbody region (Table II) were combined.

It was found (Table I) that filamin was concentrated in the cleavage furrow in 16% of the cells examined. There was no apparent concentration of filamin in 45% of the cells. These numbers are very similar to those observed for alpha-actinin. Alpha-actinin appeared to be concentrated in the cleavage furrow ~14% of the time; there was no apparent concentration in 55% of the cells. The alpha-actinin data include cells stained with both our anti-alpha-actinin antibody and an anti-alpha-actinin previously used by Fujiwara et al. (14). Comparable results were seen with both antibodies. The myosin data differ from both the filamin and alpha-actinin data. Myosin was found concentrated in the cleavage furrow 37% of the time; this frequency is 2–2.5 times that observed for filamin or alpha-actinin. Myosin did not appear to be specifically concentrated in the cleavage furrow in 34% of the cells we examined. We found some instances where myosin, alpha-actinin, or filamin appeared to be excluded from the furrow, but these instances occurred <5% of the time in all three cases.

The results presented in Table II show that alpha-actinin was found highly concentrated in the midbody region in half of the cells observed. There was no apparent concentration of alpha-actinin in the midbody region in 26% of the cells. Filamin was highly concentrated in the midbody region less frequently than alpha-actinin, at only 35% of the time. However, only 11% of the cells showed no apparent concentration of filamin. Myosin was found highly concentrated in the midbody region in only 19% of the cells; there was no apparent concentration in nearly 40% of the cells. Again, it is important to note that it is not the intercellular bridge that is stained in most cases, but rather the adjacent cytoplasm. Because the midbody stage is quite long, these results suggest that filamin and alpha-actinin may remain associated with the midbody region longer than myosin, an observation previously made for myosin and alpha-actinin by Fujiwara et al. (14).

DISCUSSION

Filamin Distribution during the Cell Cycle

The significance of the distribution of filamin during mitosis and cytokinesis can be better appreciated if compared to the distribution of other microfilament-associated proteins during these processes. Unlike myosin, which is concentrated in the mitotic spindle (13), and actin, which appears organized into distinct spindle fibers (6, 20, 21), filamin is not concentrated in the mitotic spindle. During mitosis the distribution more closely resembles that of alpha-actinin, although membrane staining is not so intense (14). Neither anti-alpha-actinin nor antifilamin staining of the spindle region indicates increased contrast of the spindle or distinct spindle fibers; however, the staining is not excluded from the spindle and, therefore, filamin and alpha-actinin may be associated with it.
In contrast to the diffuse antifilamin staining during mitosis, filamin is often seen to be nonuniformly distributed during cytokinesis. As seen in Figs. 5 and 6, filamin is concentrated in the cleavage furrow of dividing cells. This localization is similar to that of alpha-actinin (14) and myosin (13, 14). Toward the end of cytokinesis, filamin is very often concentrated in the cytoplasm adjacent to the intercellular bridge (Figs. 5k and m and 6g and i). This localization is again similar to that for alpha-actinin (14) and myosin (13, 14), although myosin seems to leave this area sooner than alpha-actinin or filamin.

Although the results are straightforward, the appropriate interpretation is much less evident. Authors of analogous studies with other microfilament-associated proteins have interpreted preferential concentration of a protein in a particular structure of the cell as evidence for participation of the protein in the architecture or function of the cell structure. For example, it has been proposed that the concentration of myosin in the cleavage furrow indicates that myosin interacts with the microfilaments of the contractile ring to provide the force required for cleavage (14). In fact, subsequent support for this proposal has come from studies in which microinjection of antimyosin into cells of starfish blastulae was found to prevent furrowing and subsequent cell cleavage (25). By analogy, we might suggest, on the basis of studies that show a concentration of filamin (this paper) or alpha-actinin (14) in the cleavage furrow, that these proteins are also involved in the cleavage process. However, the results of a frequency analysis of myosin, alpha-actinin, and filamin concentration in the cleavage furrow make it risky to draw such a conclusion from immunofluorescence data alone.

**Frequency Analysis of the Association of Filamin, Alpha-Actinin, and Myosin with the Cleavage Furrow and Midbody Regions**

Previous reports of immunofluorescence localization of microfilament-associated proteins in dividing cells did not include quantitation of the frequency of association between the various proteins and the cleavage furrow (2, 13, 14, 20, 21, 30). In the absence of such data, we assumed, as perhaps others did, that microfilament-associated proteins, such as myosin, alpha-actinin, and tropomyosin, are always concentrated in the cleavage furrow. We were concerned, therefore, when we observed that the degree of filamin concentration in the cleavage furrow was quite variable. At first, we thought that the increased association between filamin and the cleavage furrow that was observed in many cases might be an artifact. To explore this problem, we compared the frequency of filamin concentration in the cleavage furrow and midbody regions with that of alpha-actinin and myosin. Because independent evidence exists for the involvement of myosin in furrowing (25), we were particularly interested in determining the number of times that myosin was found to be concentrated in the cleavage furrow. The results demonstrated that filamin is concentrated in the cleavage furrow as often as alpha-actinin (~15% of the time), but only half as frequently as myosin. However, the most important point is that none of these proteins are found preferentially concentrated in the cleavage furrow 100% of the time.

It is important to distinguish between preferential concentration of a protein and presence of the protein. Preferential concentration means that the staining is brighter in a particular region, compared with surrounding regions. If the particular region is stained but is not brighter than the surrounding areas, it cannot be determined whether the protein is specifically associated with the structure in question. At the light microscope level, mere presence of a protein in a general region is not very informative. In almost all of the dividing cells we observed, the various proteins analyzed were detectable in the cleavage furrow, but they were preferentially concentrated there at a frequency of only 15-40%.

The implications of the frequency analysis are varied. First, it questions the validity of presenting immunofluorescence localization data as evidence for the participation of a protein in a specific cellular activity, if there is no analysis of either the time-course of association or the frequency of association to enable informed evaluation of the findings. Second, it shows that the interpretation of immunofluorescence localization is not trivial. For example, how cogent would the suggestion that myosin is required for cleavage have been, if it had been known that only 37% of cells in cleavage showed concentration of myosin in the furrow and if the subsequent corroborative evidence from microinjection of antimyosin (25) were not available? Third, quantitative analysis might help to explain some of the current controversies in the literature. For example, there is some disagreement at this time as to whether the concentration of actin in the cleavage furrow is significantly higher than in other parts of the cell cortex. An early report by Sanger (30), using fluorescent HMM labeling, indicated that actin was concentrated in the cleavage furrow, presumably in the contractile ring. These findings have been corroborated by Aubin et al. (2). However, reports by Herman and Pollard (20, 21), using both fluorescent antiaxin and HMM, have stressed that actin is not significantly concentrated in the cleavage furrow. Nevertheless, one of these papers (21) does present a figure indicating the range of actin concentration in the cleavage furrow. Herman and Pollard suggested that the cortical actin concentration is constant throughout the cell and that the contractile ring represents only a specialized realignment or repacking of preexisting filaments. Wang and Taylor (40) have reported on the distribution of fluorochrome-labeled skeletal muscle actin that was injected into sea urchin eggs. Their results show that, immediately after fertilization, the fluorochrome-labeled actin is concentrated in the membrane-cortical regions. However, during cell cleavage there is no distinctly fluorescent cleavage furrow. It is possible, in light of our findings with filamin, alpha-actinin, and myosin, that the different results obtained for actin localization may in part be attributable to a not fully recognized variability of actin concentration in the furrow from cell to cell and to differences in interpretation of its importance by the researchers.

**Why Is There Variation in the Apparent Concentration of Actin-binding Proteins in the Cleavage Furrow Region?**

There are at least two explanations for the variability in the concentration of filamin, alpha-actinin, and myosin in the cleavage furrow. There may be differential extraction of these proteins during the fixation and staining procedures. However, if there is differential extraction, it occurs under many fixation conditions and suggests that these proteins are often in a less extractable form when associated with the cleavage furrow, as they seem to be during interphase when they are associated with stress fibers. Unfortunately, our attempts to analyze the extraction of filamin during fixation and staining were not
successful, probably because filamin makes up a very small percentage of the total cellular protein and it is highly sensitive to proteolysis. Another possible explanation arises from the transient nature of the cleavage furrow. Actin-binding proteins may be needed and thus concentrated in the furrow for a very brief time; therefore, only a small percentage of dividing cells would be seen with good preferential localization. A detailed time-course analysis of localization in a highly synchronized population of cells would permit an investigation of this second possibility.

If Filamin Is Required for Cleavage, What Functions Might It Perform?

The in vitro properties of filamin, i.e., that it is a phosphoprotein that induces the gelation of F-actin and inhibits the actin-activation of myosin ATPase, are the only facts that we have with which to speculate on the role of filamin in cell cleavage. These properties indicate that filamin may be involved in the increase in cortical gel strength that has been reported to occur before cleavage (26). Alternatively, filamin may act to regulate the interaction of actin with myosin.

Another possible role for filamin is suggested by a recent report by Schollmeyer et al. (31), which indicates that actin-binding protein (ABP) and alpha-actinin together may be capable of orienting actin filaments into parallel arrays. These workers used purified human-pla-atelet actin filaments, stabilized by porcine skeletal muscle troponin (TN) and tropomyosin (TM), to analyze the types of macromolecular structures that are formed when these filaments are allowed to react with porcine muscle alpha-actinin or platelet ABP (a protein that appears to be homologous to filamin in several respects) (5, 35, 38). When added individually, alpha-actinin and ABP were both found to gel actin and, by electron microscope examination, the actin-TM/TN filaments were seen to be randomly cross-linked. However, when alpha-actinin and ABP were added sequentially to the actin-TM/TN filaments, gelation occurred and the actin filaments were organized into bundles of parallel arrays. The similarities in microfilament organization between the contractile ring (32) and the in vitro complex formed by actin, alpha-actinin, and ABP suggest that filamin (possibly analogous to ABP) and alpha-actinin may be concentrated in the cleavage furrow because they interact with actin to form the highly organized, parallel microfilaments of the contractile ring.

Although it is easy to imagine possible roles for alpha-actinin and filamin in cytokinesis, it is clear from this immunofluorescence study that the next objective must be to obtain more direct evidence that these proteins are actually required for cytokinesis.

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A preliminary account of some of the work presented in this paper has appeared previously (28).