A Re-evaluation of Cytoplasmic Gelsolin Localization

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Abstract. Gelsolin is a 90,000-mol-wt Ca^{2+}-binding, actin-associated protein that can nucleate actin filament growth, sever filaments, and cap barbed filament ends. Brevin is a closely related 92,000-mol-wt plasma protein with similar properties. Gelsolin has been reported to be localized on actin filaments in stress fibers, in cardiac and skeletal muscle I-bands, and in cellular regions where actin filaments are known to be concentrated. Previous localization studies have used sera or antibody preparations that contain brevin. Using purified brevin-free IgG and IgA monoclonal antibodies or affinity-purified polyclonal antibodies for gelsolin and brevin, we find no preferential stress fiber staining in cultured human fibroblasts or I-band staining in isolated rabbit skeletal muscle sarcomeres. Cardiac muscle frozen sections show no pronounced I-band staining, except in local areas where brevin may have penetrated from adjacent blood vessels. Spreading platelets show endogenous gelsolin localized at the cell periphery, in the central cytoplasmic mass and on thin fibers that radiate from the central cytoplasm. Addition of 3–30 μg/ml of brevin to the antibodies restores intense stress fiber and I-band staining. We see no evidence for large-scale severing and removal of filaments in stress fibers in formaldehyde-fixed, acetone-permeabilized cells even at brevin concentrations of 30 μg/ml. The added brevin or brevin antibody complex binds to actin filaments and is detected by the fluorescently tagged secondary antibody. Brevin binding occurs in either Ca^{2+} or EGTA, but is slightly more intense in EGTA suggesting some severing and filament removal may occur in Ca^{2+}. The I-band staining is limited to the region where actin and myosin do not overlap. In addition, brevin does not appear to bind at the Z-line. A comparison of cells double-labeled with fluorescein–phallotoxin, exogenous brevin, and a monoclonal antibody, detected with a rhodamine-labeled secondary antibody, shows almost complete co-localization of F-actin with the brevin–gelsolin-binding sites. A major exception is in the area of the adhesion plaque. A quantitative comparison of the fluorescein–rhodamine fluorescence intensities along a stress fiber and into the adhesion plaque shows that the fluorescein signal, associated with F-actin, increases while the rhodamine signal decreases. We infer that exogenous brevin or endogenous gelsolin can bind to and potentially sever most actin filaments, but that actin-associated proteins in the adhesion plaque can prevent binding and severing. In addition, we conclude that our original localizations were artifacts that resulted from brevin in the antibody preparations and suggest that other reports of gelsolin localization on actin filaments must be viewed with caution.

THE actin-based cytoskeleton in nonmuscle cells is believed to undergo dynamic assembly and disassembly as the physiologic state of the cell changes. Recent studies have described proteins that interact with actin in vitro and could regulate the gel-sol state of cytoplasm either by cross-linking, bundling, capping, or severing actin filaments (for recent reviews see references 12 and 23).

The activities of several of these proteins appear to be sensitive to calcium. Gelsolin, a 90,000-mol-wt calcium-binding, actin-associated protein (22, 26), has been identified in a wide variety of vertebrate tissues (18, 27). This protein was identified initially in rabbit pulmonary macrophages (26) and has been isolated and characterized from human platelets (22) and cardiac muscle (17). There is increasing evidence that brevin (6) or actin-depolymerizing protein (7) from plasma is a closely related molecule (25) having a similar amino acid sequence but with an additional 25 amino acids on the amino terminal (29). Both proteins have been reported to interact with Ca^{2+} ions, to nucleate actin filament assembly, to cap the barbed end of filaments thereby preventing monomer addition at the ends, and to sever actin filaments (2, 4, 6, 7, 13, 17, 22, 26, 28).

Several studies have dealt with the localization of gelsolin (11, 17, 21, 27). Yin et al. (27) have reported gelsolin localization in macrophages in the cytoplasmic region surrounding an ingested particle. These authors also noted that I-bands in striated muscle were stained. Wang et al. (21) reported that gelsolin was present in the F-actin–containing rosette struc-

tures present in Rous sarcoma virus–transformed normal rat kidney cells (3, 16). These structures also contained vinculin, alpha-actinin, and pp60^{src} (3, 15) and were identified as regions of close substrate approximation by interference–reflection microscopy. Rouayrenc et al. (17) have isolated a 92,000-mol-wt protein from cardiac muscle that has all the properties of gelsolin. These workers produced antibodies against this protein and reported its localization in the I-bands of cardiac muscle. They concluded that gelsolin was an integral myofibrillar protein. We have recently characterized two monoclonal antibodies that react with Ca^2-induced conformations of human brevin and gelsolin (10). These antibodies have been purified to homogeneity and appear to recognize two specific conformations of gelsolin or brevin that are induced when one or the other of the two Ca^{2+}-binding sites are occupied. We have used these antibodies to study gelsolin localization and originally reported, in abstract form (11), localization of gelsolin on stress fibers in cultured cells, confirmation of muscle I-band staining, and localization of gelsolin in platelets. We demonstrate here, using the monoclonal antibodies and affinity-purified polyclonal antibodies specific for brevin and gelsolin, that part of this preliminary report is an artifact. The present report suggests that the published work of others describing gelsolin localizations needs re-evaluation. We show here that our initial results represent the localization of unoccupied gelsolin-binding sites that are accessible to brevin which was present in the fetal calf serum used to culture the hybridomas (11). We have found that the use of highly purified brevin-free antibodies (10), either monoclonal IgG's or IgA's, or affinity-purified polyclonal antibodies with bovine serum albumin as a diluent gives no obvious stress fiber staining and eliminates I-band staining. The inclusion of human brevin in the staining protocol at 3–30 μg/ml, ~1–10% of the reported serum levels, completely restores stress fiber and I-band staining. We note that in most of the published staining procedures (17, 27) brevin is present in the heterologous sera routinely used as a diluent (17, 27) or is present in the immune sera used for staining (17, 27). The polyclonal antibodies, which show no marked Ca^{2+} sensitivity of binding, have been used to demonstrate that brevin, at the concentrations used here, binds in both Ca^{2+} and EGTA. We conclude that brevin and gelsolin can bind laterally to sites on actin filaments in stress fibers or I-bands, but that these sites are normally not occupied by cytoplasmic gelsolin which appears to be diffusely distributed in cultured cells and muscle.

Materials and Methods

Preparation of Monoclonal Antibodies to Platelet Gelsolin

The generation of hybridomas and the characterization and purification of the gelsolin antibodies has been described elsewhere (10). These antibodies recognize Ca^{2+}-induced conformations of human gelsolin and brevin and show limited cross-reaction with mouse, rat, or rabbit tissues. For staining experiments we have fixed cells with 0.1 mM Ca^{2+} or with 1–10 mM EGTA present. Incubations with both the 8G5 IgG and 4F8 IgA monoclonal antibodies were done with 0.1–0.5 mM Ca^{2+} present, conditions in which gelsolin would be liganded with Ca^{2+} and hence recognized by these antibodies.

Brevin was isolated from human sera by immunoaffinity chromatography as described previously (10).

Preparation and Purification of Polyclonal Antibodies to Gelsolin and Brevin

Goats were injected and boosted with 100 μg of immunopurified human brevin per immunization. Immune sera were passed over a brevin–agarose column prepared using immunopurified brevin and CNBr-activated agarose (Sigma Chemical Co., St. Louis, MO). The brevin–agarose column contained ~3 mg of brevin per ml of gel and retained 4 mg of antibody per 10 ml of immune serum. The retained antibodies were released using 0.1 M glycine-HCl, pH 2.8, and after neutralization with 1 M Tris were dialyzed versus Tris- or phosphate-buffered saline. Using immunoblots, the antibodies show a single band at the molecular weight of gelsolin in human platelet extracts and show cross-reactivity with rabbit brevin. The polyclonal antibodies show no obvious Ca^{2+} sensitivity using immunoblots or enzyme-linked immunosorbent assays (Lin and J. Bryan, unpublished data). For staining experiments, cells were fixed with 0.1 mM Ca^{2+} or 1 mM EGTA present, and all subsequent antibody binding and wash steps were carried out with either Ca^{2+} or EGTA present.

Platelet and Cell Culture

Human platelet concentrates, obtained from the Gulf Coast Region Blood Center (Houston, Texas) were used within 1–2 d after drawing from blood donors. Platelets were allowed to spread on glass coverslips for varying periods of time and then processed for immunofluorescence. Human foreskin fibroblasts were a generous gift of Dr. M. Dodson, Baylor College of Medicine. Cultures of Swiss 3T3 and WI-38 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in Hams F-10 medium containing 10% calf serum and used 24–36 h after passage. Cultures were grown on glass coverslips at 37°C in a humidified incubator with 5 or 7% CO$_2$ in air.

Indirect Immunofluorescence Microscopy

Cells grown on glass coverslips were rinsed with room temperature phosphate-buffered saline (PBS) containing either 1 or 10 mM EGTA or 0.1 mM CaCl$_2$, pH 7.4, and fixed with 3% formaldehyde in PBS plus 0.5% dimethyl sulfoxide in EGTA or CaCl$_2$ for 20 min at room temperature, washed with PBS, and permeabilized by immersion in acetone at −20°C for 6 min. After acetone treatment, the cells were rinsed with PBS plus 0.1 mM CaCl$_2$, when using the monoclonals or with either 0.1 mM CaCl$_2$ or 1 mM EGTA when using the polyclonals, then inverted on a drop of antibody solution for 45 min at 37°C. The usual antibody concentration was 10 μg/ml diluted with 3% bovine serum albumin in PBS. Where indicated immunoaffinity-purified human brevin (10) was added at either 3, 15, or 30 μg/ml. CaCl$_2$ was present with the monoclonal and either CaCl$_2$ or EGTA was used with the polyclonals. Coverslips were then washed for 30 min in PBS and incubated with tetramethyl rhodamine isothiocyanate (TRITC)-[1] or rhodamine isothiocyanate (RITC)–conjugated rabbit anti-mouse IgG, fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse antiglobulin, or FITC-conjugated rabbit anti-goat antiglobulin (Miles-Yeda Ltd., Rehovot, Israel).

To compare the localization of gelsolin with that of F-actin, coverslips were incubated with 10 μg/ml FITC–phallotoxin, the generous gift of Professor T. Wieland (Max-Planck-Institut fur Medizinische Forschung, Heidelberg, Federal Republic of Germany), in PBS for 30 min at 37°C during or after incubation with the second-step antibody. Coverslips were washed for 1 h with four changes of PBS and then mounted in 90% glycerol, 10% PBS, pH 8.5–9.0, and sealed with nail polish.

Coverslips were examined using phase-contrast and epifluorescence illumination with a Zeiss standard microscope equipped with an HBO 50 W mercury lamp. Photographs were taken on Kodak Tri-X using a 63X, N.A. 1.4 oil immersion phase objective then developed in Accufine or Kodak HC-110 at 1,000 ASA. The microscope was equipped with a 485-nm excitation filter and 520-nm barrier filter for FITC fluorescence and a 535-nm excitation filter and 590-nm barrier filter for TRITC or RITC fluorescence.

Frozen Sections

Frozen sections were prepared from cardiac muscle obtained at autopsy 12–18 h post mortem. Sections were air dried on glass microscope slides coated with 1% bovine serum albumin. Sections were fixed with 3% formaldehyde, 0.5% dimethyl sulfoxide, 1 mM EGTA in PBS for 30 min at room temperature, then washed three times with PBS plus 0.1 mM CaCl$_2$ or 1 mM EGTA and

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[1] *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

permeabilized for 6 min with acetone at −20°C. Antibody staining followed the protocol described above.

Quantitative Fluorescence Microscopy

Quantitative determinations of fluorescence intensity were made using a Hamamatsu C-1000-12 SIT camera on a Leitz inverted fluorescence microscope. The analog output of the camera was digitized using a Grinnel 274 image processor. The detailed description of this system is given in reference 1.

Results

Antibody Localization with and without Exogenous Brevin

The results of one staining experiment using human foreskin fibroblasts are shown in Fig. 1. The cells shown in the top panels were stained with 4F8 IgA at a concentration of 10 μg/ml. Bovine serum albumin at 3% in PBS was used as a diluent. Fig. 1A shows a cell stained with no added brevin. The level of fluorescence is low and we see little or no localization on stress fibers. The endogenous gelsolin appears diffusely distributed. The cells in B were stained with the same antibody plus brevin at 30 μg/ml. There is marked stress fiber labeling plus staining at the periphery of the cell in ruffled membranes. We see similar results using 8G5 IgG and also observe similar staining using hybridoma harvest fluid that contains 10% fetal calf serum. We have used several fixation conditions including 0.1 mM $CaCl_2$-, 1.0 mM EGTA-, and 10 mM EGTA–containing buffers. We see no obvious differences in the localization of the endogenous gelsolin or the added brevin under these conditions. In addition, we have stained human fibroblasts after fixation in the presence of either Ca^{2+}- or EGTA-containing buffers with affinity-purified polyclonal antibodies that do not shown Ca^{2+} sensitivity. Representative results are given in the bottom panels of Fig. 1. C shows a cell fixed and stained in EGTA with no added brevin. We see no preferential stress fiber localization. D shows fibroblasts stained in EGTA with brevin added at 3 μg/ml. The prominent fibrillar staining only occurs if brevin is added and must be the result of brevin binding to
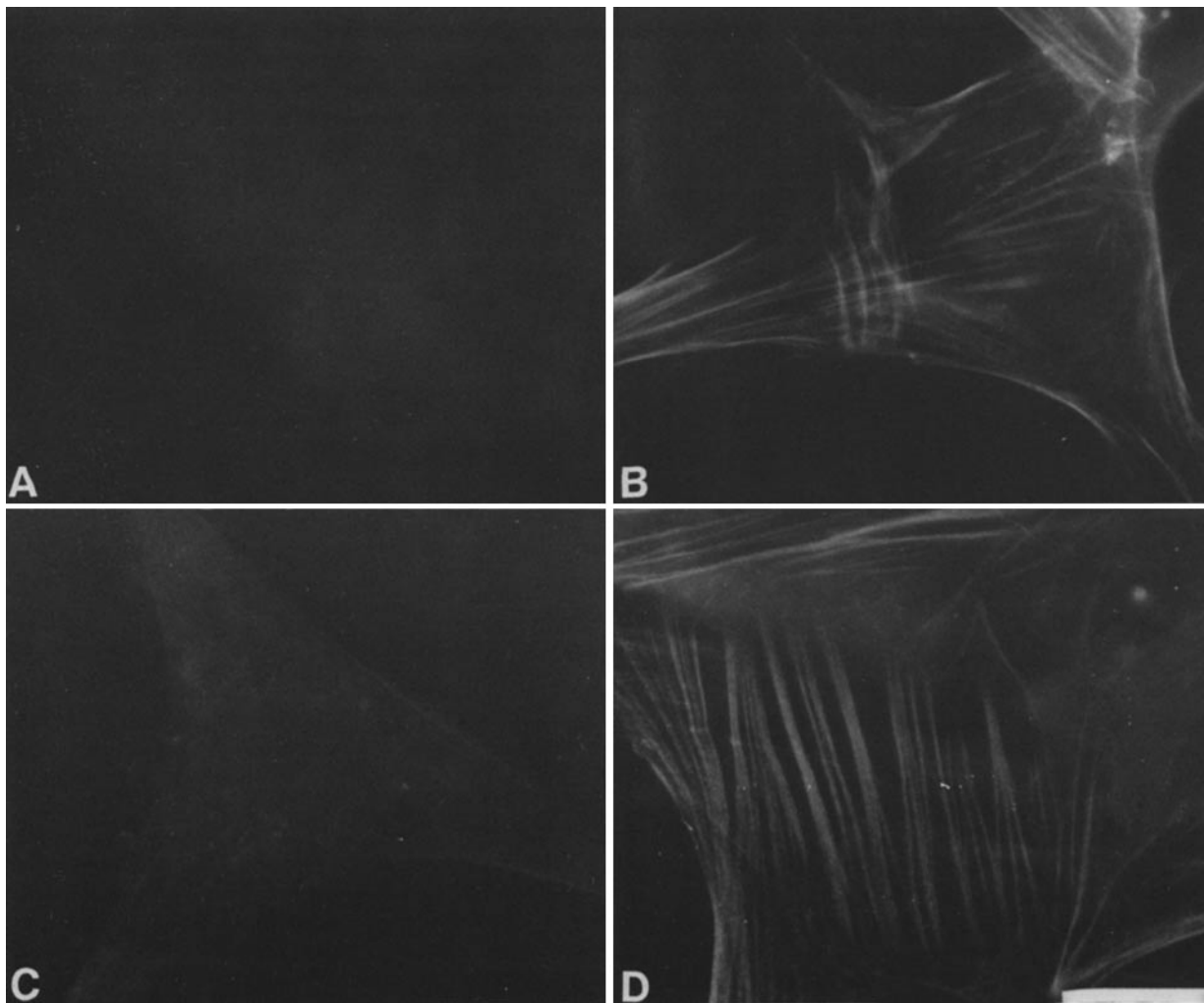

Figure 1. Comparison of immunofluorescence patterns from human fibroblasts stained with 4F8 IgA with and without added brevin. The upper panels show fluorescence micrographs of human foreskin fibroblasts stained with 4F8 IgA at 10 μg/ml; the antibody was diluted with 3% bovine serum albumin in PBS plus 0.1 mM $CaCl_2$. (A) No added brevin; (B) with brevin added at 30 μg/ml. The lower panels show fluorescence micrographs of human foreskin fibroblasts stained with goat polyclonal antibodies. All steps were done in the presence of 1 mM EGTA. The brevin and affinity-purified antibody were diluted with 3% bovine serum albumin in PBS. (C) No added brevin; (D) brevin added at 3 μg/ml. Qualitatively similar results were obtained with 0.1 mM $CaCl_2$ present. Bar, 13 μm.
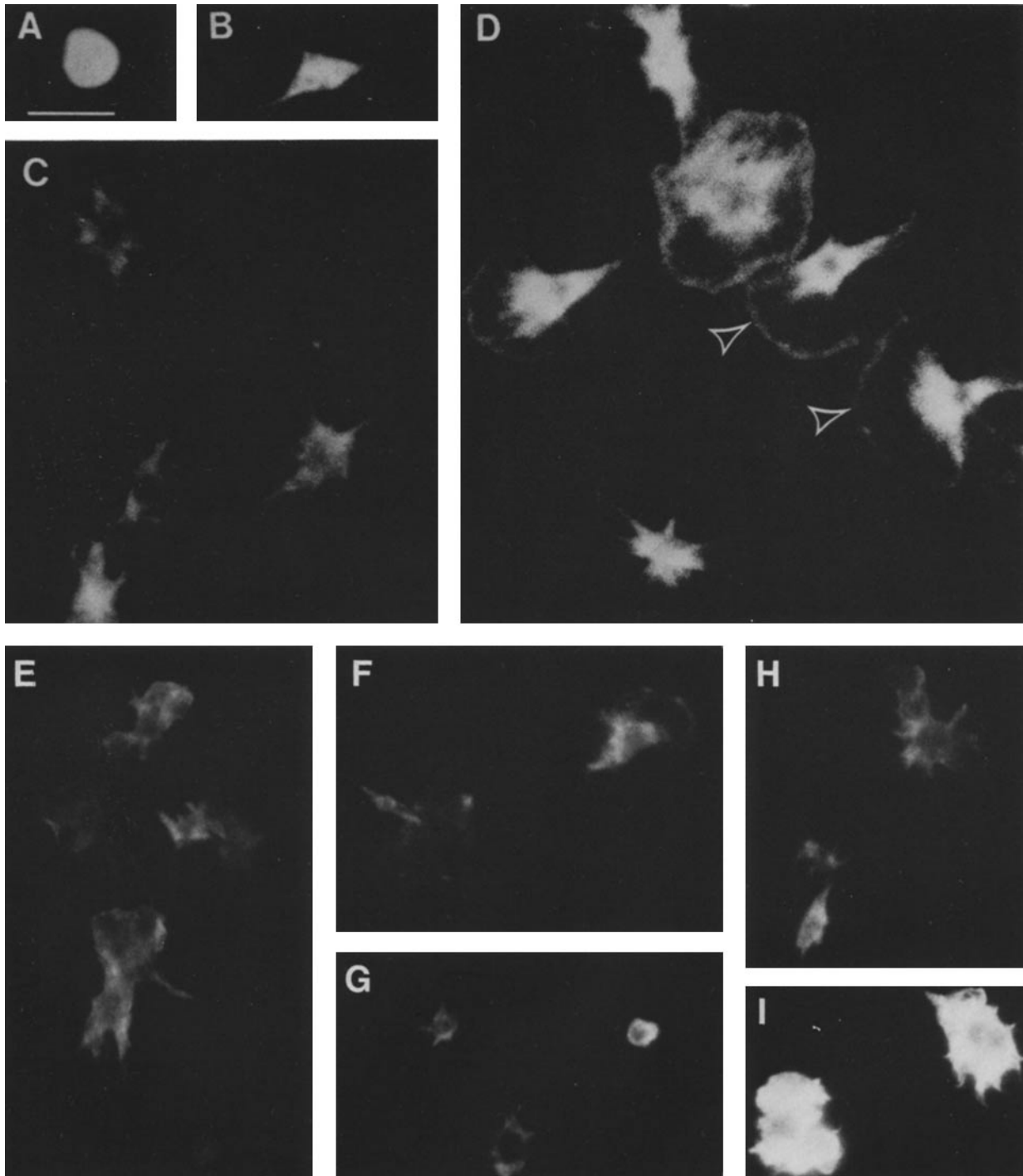
Figure 2. Immunofluorescence localization of gelsolin in human platelets. Platelets were allowed to spread on glass coverslips for varying periods of time then were fixed in formaldehyde and lysed in acetone. (*A–D*) Using 8G5 IgG diluted with 10% calf serum; (*E–H*) using 4F8 IgA diluted with 3% bovine serum albumin; (*I*) using 4F8 IgA diluted with 3% serum albumin plus brevin at 30 μg/ml. Staining of discoid platelets (*A*) and platelets exhibiting pseudopodia (*B*) show a diffuse pattern of immunofluorescence. In spreading platelets (*C*), the radial cytoplasmic projections are faintly fluorescent. Platelets in a more advanced state of spreading show a peripheral band of fluorescence (arrowheads in *D*) in addition to staining of the platelet body. Platelets stained in the absence of brevin show very similar images (*E–H*), but the staining is markedly less intense. The images in *I* with brevin are directly comparable with those in *E–H*, with no added brevin. Bar, 5 μm.

sites on the actin filaments in stress fibers. The exact nature of these sites is not clear, but co-localization studies show they are potential gelsolin severing sites on actin filaments. Brevin at this concentration labels these sites even in the presence of EGTA. We note that after formaldehyde fixation, severing appears to be markedly reduced and even added brevin at 30 μg/ml in Ca^{2+} does not effectively remove stress fibers. We do observe, however, that cells labeled with the polyclonal antibodies in EGTA are somewhat more intensely stained than those labeled in Ca^{2+}, indicating that there may be some

severing and release of gelsolin and/or brevin. The effect appears to be small and we have not made an attempt to quantitate this release.

Immunofluorescence Localization of Gelsolin–Brevin-binding Sites in Platelets and Cultured Cells

Cells were fixed in PBS plus 3% formaldehyde, 0.5% dimethyl sulfoxide, 1 mM EGTA, or 0.1 mM $CaCl_2$ for 30 min, then permeabilized in acetone for 6 min before staining with either 8G5 IgG or 4F8 IgA at 10 $\mu g/ml$ diluted either with 1–3% calf serum or with 3% bovine serum albumin. Fluorescently tagged secondary antibodies were then applied to visualize the distribution of either monoclonal antibody.

Human Platelets

Fig. 2 compares the distribution of antibody in human platelets stained with 8G5 IgG and 4F8 IgA with and without added brevin. Fig. 2, A–D, shows platelets stained with 8G5 IgG plus brevin at 30 $\mu g/ml$ at various stages of spreading. Similar images were obtained with 4F8 IgA. Fig. 2, E–H shows platelets stained with 4F8 IgA without added brevin. Similar images were obtained with 8g5 IgG without brevin. There is relatively little difference in the distribution of antibody under the two conditions; for example, compare Fig. 2, C and D (plus brevin) with 2, E–H (minus brevin). However, there is a marked difference in the staining intensity which can be appreciated at a qualitative level by comparing Fig. 2 I with the images in Fig. 2, E–H. The platelets in Fig. 2 I were processed, photographed, and the negatives printed in an identical manner to those in Fig. 2, E–H, but brevin, at 30 $\mu g/ml$, was present in the diluent used to stain the platelets in Fig. 2 I. We observe endogenous gelsolin in the periphery of the platelet, associated with the dense central cytoplasmic mass and on filamentous structures radiating from the central region.

Co-localization of Actin and Gelsolin in Cultured Cells

The distribution of actin- and brevin–gelsolin-binding sites was compared in WI-38 cells and human foreskin fibroblasts. The actin was identified with FITC–phallotoxin, and the added brevin with monoclonal antibodies. The results are shown in Fig. 3. The two labels are co-localized along stress fibers, radial fibers, and in the circumferential arcs of human foreskin fibroblasts and WI38 cells. The co-localization appears to be reasonably uniform except for two regions. The FITC–phallotoxin stains the nucleus somewhat more intensely than anti-gelsolin at the levels used here. A more interesting difference was observed at the ends or termini of stress fibers. A comparison of Fig. 3A with 3B, WI-38 cells
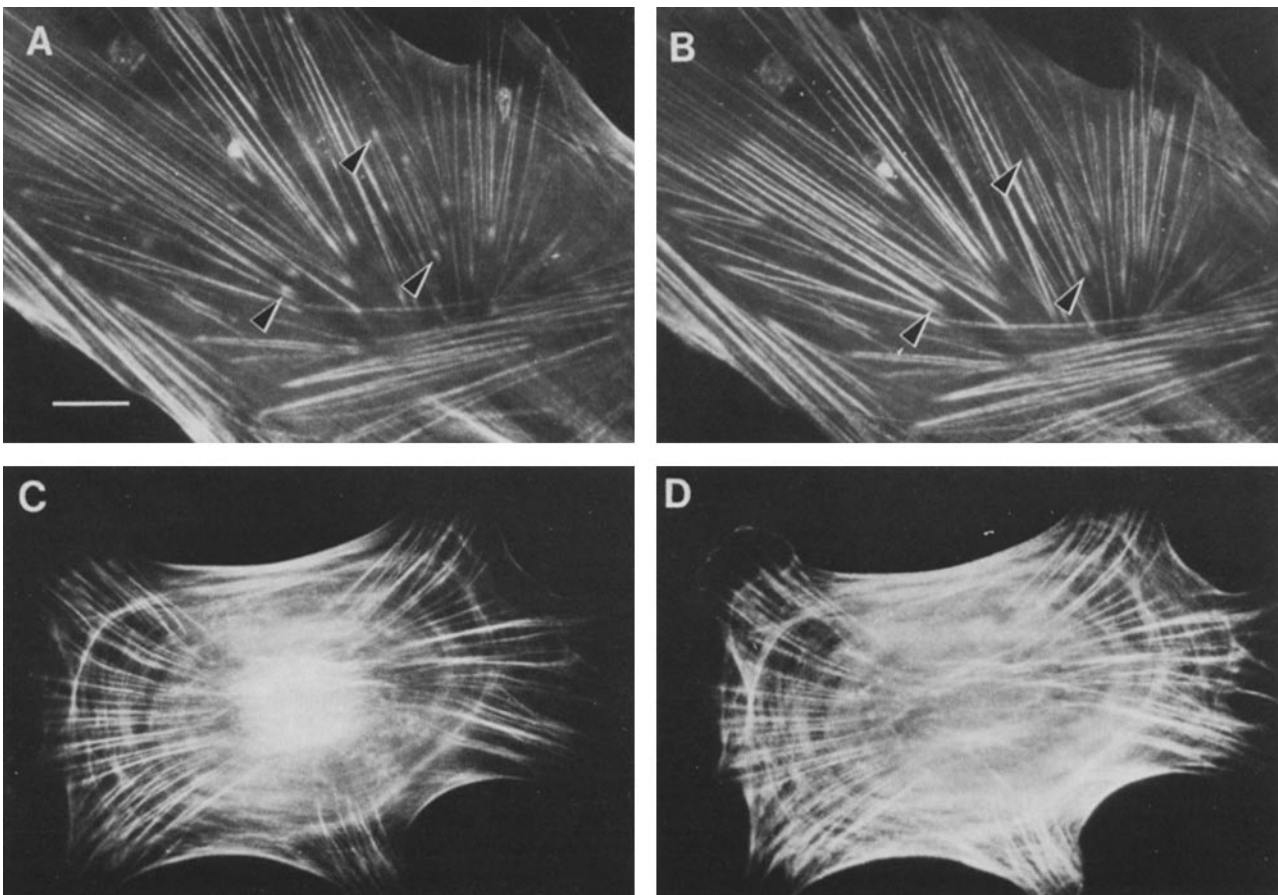

Figure 3. Co-localization of F-actin and brevin–gelsolin-binding sites. Double-labeling with 8G5 IgG (B and D) and FITC–phallotoxin (A and C) in 10% bovine serum shows that the brevin–gelsolin-binding sites and F-actin are co-localized in stress fibers, and the circumferential actin-containing fibers in WI-38 cells (*top panels*) and human foreskin fibroblasts (*lower panels*). The termini of stress fibers are stained brightly by FITC–phallotoxin but the number of brevin–gelsolin-binding sites is reduced. Compare the intensities at the arrow in A and B. Bar, 5 μm.

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stained both with FITC–phallotoxin and with anti-gelsolin visualized with a TRITC-labeled secondary antibody, shows that the actin signal is more prominent and that the added brevin is partially excluded from binding to actin in this area. Fig. 4 shows the results of a determination of the fluorescence intensities along the selected transits in Figs. 4, A and B. These run along stress fibers and through adhesion plaques. The results are summarized in the FITC/TRITC ratios plotted in Fig. 4 C. We see approximately a twofold increase in the ratio of phallotoxin to antibody that results from both an increased FITC signal and a decreased antibody signal.

Localization of Brevin–Gelsolin and Potential Binding Sites in Isolated Sarcomeres from Striated Muscle

Fig. 5 illustrates the localization of FITC–phallotoxin and added brevin in rabbit myofibrils along with the low level of endogenous staining. The top panel is a phase-contrast micrograph of an isolated myofibril; the arrows indicate the position of three consecutive Z-lines. The second panel shows the location of F-actin stained with FITC–phallotoxin; the Z-line is somewhat more heavily labeled. The third panel shows the

location of brevin–gelsolin-binding sites by staining with 8G5 IgG and a rhodamine-conjugated goat anti-mouse IgG antibody. Brevin is excluded from the Z-line, but binds to the I-bands in the region where there is no overlap with myosin thick filaments. In other experiments, we have extracted the sarcomeres with 0.3 M KCl plus 5 mM ATP to remove the thick filaments. In these preparations, staining with 8G5 IgG plus brevin at 30 μg/ml labels the entire I-band (data not shown). Staining with either antibody without added brevin gives no fluorescence localization on the I-band (fourth through seventh panels) which is expected since we do not detect cross-reactivity of these monoclonals with rabbit gelsolin or brevin (10 and J. Bryan, unpublished data). The final three panels show the results of staining with the affinity-purified polyclonal antibodies that do cross-react with rabbit gelsolin and brevin. Without added brevin, the eighth and ninth panels, there is no obvious localization of gelsolin on I-bands. At the highest antibody concentration used, 10 μg/ml, we see diffuse weak fluorescence over the entire sarcomere which does not photograph at these exposures. Addition of brevin gives the intense I-band staining shown in the final panel.
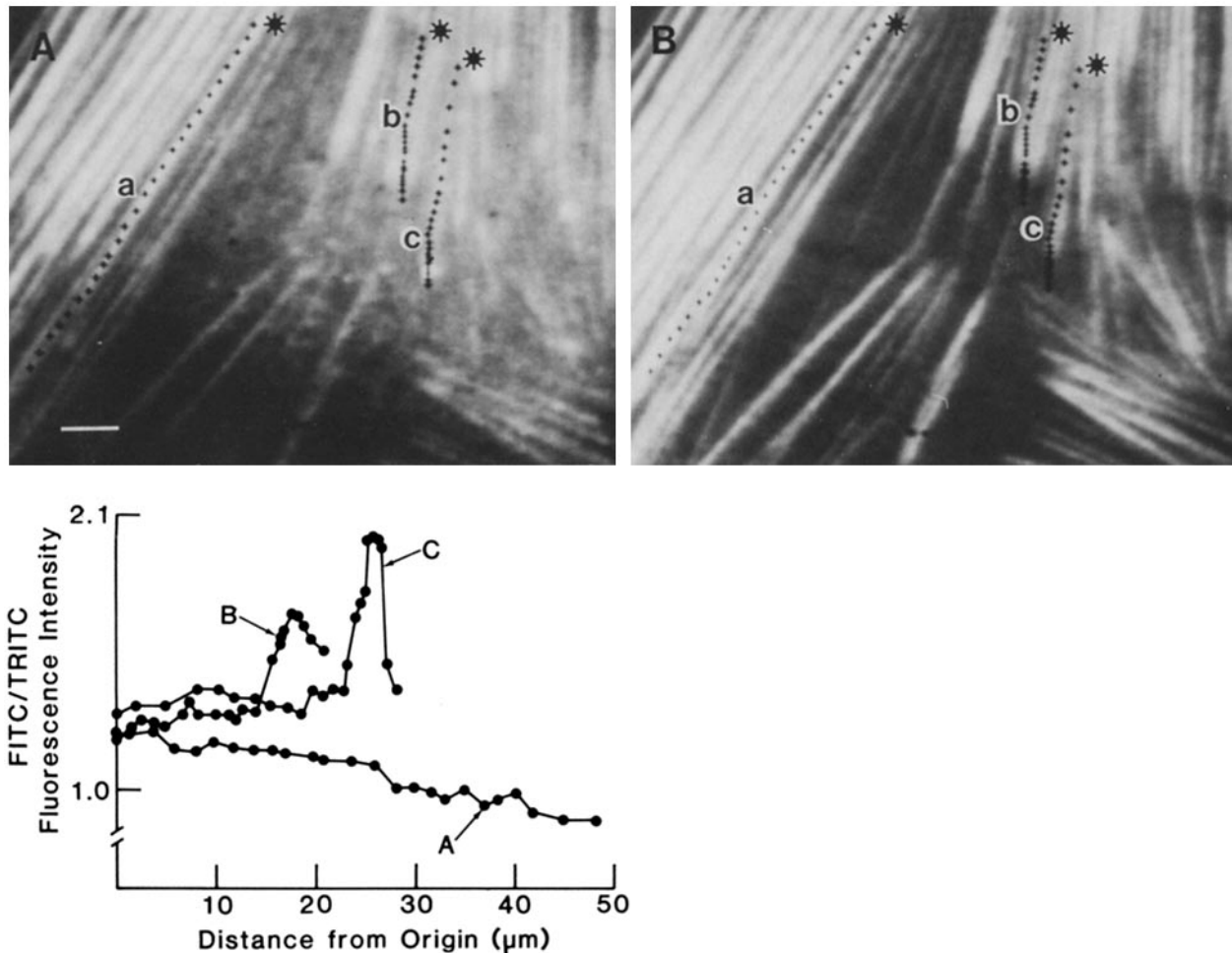

Figure 4. Transits for quantitation of fluorescein and rhodamine fluorescence intensities at the ends of stress fibers. Cells were stained as indicated in Fig. 3 and examined using the image processing equipment described in reference 1. * marks the start of a transit or trace whose path is delineated by the points. A is the image in the fluorescein channel; B is the image in the tetramethyl rhodamine channel. The graph (C) gives the actin–brevin fluorescence intensity ratios for all three fibers. Note the marked increase in the actin–gelsolin ratio at the ends of fibers b and c. Bar, 5 μm.
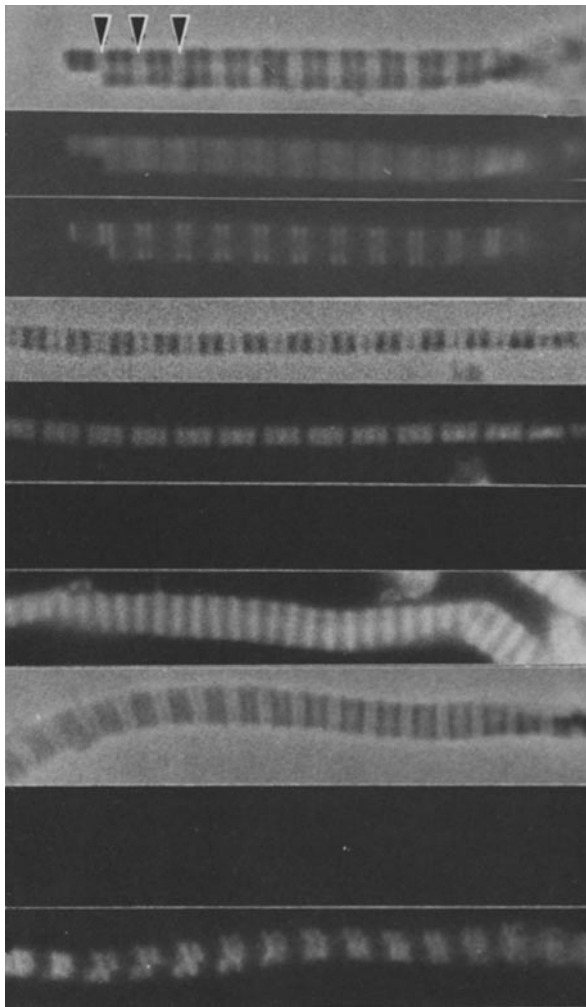
Figure 5. Localization of brevin–gelsolin sites and endogenous gelsolin in myofibrils. Myofibrils were prepared from glycerinated rabbit psoas muscle by homogenization in 0.1 M KCl, 2 mM MgCl$_2$, 1 mM EGTA, 10 mM Imidazole at pH 7.4 in a Waring blender. A drop of the suspended myofibrils was spread on a glass coverslip and allowed to attach for 5–10 min. The coverslips were then fixed with 3% formaldehyde in the same buffer, then washed three times with PBS plus 0.1 mM CaCl$_2$ and stained with FITC–phallotoxin and antibody diluted as indicated below. The top three panels are a phase-contrast micrograph, an FITC–phallotoxin image, and the 8G5 IgG staining pattern. The 8G5 IgG was diluted with 10% calf serum in PBS plus 0.1 mM CaCl$_2$. The arrows mark three consecutive Z-lines. The fourth through the sixth panels are a phase-contrast micrograph, an FITC–phallotoxin image, and the 4F8 IgA staining pattern. In this case the antibody was diluted with 3% bovine serum albumin in PBS plus 0.1 mM CaCl$_2$ with no added brevin. The panel 7 is directly comparable with panel 6, but was stained with 4F8 IgA diluted with 3% bovine serum albumin plus brevin at 30 µg/ml. Affinity-purified polyclonal antibodies were also used to look for endogenous gelsolin. Myofibrils were fixed and stained in either 0.1 mM CaCl$_2$ on 1 mM EGTA. The results were qualitatively the same. The eighth and ninth panels illustrate the low level of fluorescence observed with a first step antibody concentration of 10 µg/ml after fixation and staining in EGTA. The last panel shows similar myofibrils stained with antibody plus brevin at 15 µg/ml after fixation in EGTA.

Localization of Gelsolin and Unoccupied Brevin–Gelsolin-binding Sites in Cardiac Muscle

We have used the monoclonal antibodies to study the distribution of endogenous cardiac gelsolin in frozen sections from human autopsy material obtained 12–18 h post mortem. The results are shown in Fig. 6. In the absence of added brevin we commonly see the two patterns illustrated in panels *A* and *B*. In one of the sections we see diffuse fluorescence with little or no localization of antibody on the I-bands in the interior of the myofibrils. Occasionally we will see staining of sarcomeres at the periphery of a myofibril near a blood vessel (arrow in *A*). In other sections, we see very faint I-band staining throughout myofibrils that is visible in the microscope, but is barely detectable when printed at the same exposures as the brevin-containing samples (compare *B* with *D*). We also see staining of phase-dense structures that may be mitochondria. If brevin is added, we observe I-band staining that is most intense at the edges of a bundle of myofibrils and closely resembles the patterns seen with isolated sarcomeres when brevin is added. No staining in the actin–myosin overlap region is observed and the level of stain is reduced in the Z-line (Fig. 6, *C* and *D*).

Discussion

Several reports, including a preliminary study (11) from our lab, have described the localization of gelsolin on various cellular structures. Skeletal (11, 27) and cardiac (17) muscle I-band localizations have been reported. These studies used antibody samples that are "contaminated" with serum brevin. Using brevin-free monoclonal and polyclonal antibody preparations, we have been unable to detect significant levels of human gelsolin on cardiac myofibrils or isolated rabbit skeletal myofibrils. If brevin is included during staining we see intense I-band fluorescence. We interpret these results to mean that there is little or no gelsolin in the I-bands, but that added brevin or gelsolin can bind along these filaments. We suggest that the faint I-band staining we do observe in localized areas near blood vessels is the result of Ca^{2+} and brevin redistribution post mortem. A similar result holds for stress fibers. We see intense stress fiber staining only if brevin is present and able to bind to the actin filaments. Double staining with FITC–phallotoxin (24) and tetramethyl rhodamine–labeled second-step antibodies shows that added brevin can bind extensively to actin filaments. The only region where actin is clearly not accessible to brevin is in the adhesion plaques.

Clearly, we have not repeated all the gelsolin localization work. Wang et al. (21) have described the localization of gelsolin in the F-actin containing structures of Rous sarcoma virus–transformed normal rat kidney cells. We have confirmed this localization in the presence, but not absence of exogenous brevin. Brevin was present in the study by Wang et al. (21). Similarly, in a report on actin, actin-binding protein, and gelsolin localization in lymphoblastoid cells, Thorstensson et al. (20) note that gelsolin is found in filopodia in acetone-fixed cells, but can be removed by Ca^{2+} treatment. We do not see this type of redistribution of antigen in formaldehyde-fixed, acetone-permeabilized cells. However, in acetone fixed fibroblasts, addition of brevin gives a periodic pattern on the stress fibers suggesting severing may occur except in regions where filaments are stabilized by myosin and tropomyosin. The study by Thorstensson et al. used purified IgG's, but from the description in their Methods section it is not clear if heterologous serum was used as a diluent. Several figures in the paper by Yin et al. (27) were done with purified IgG's, but it is unclear whether they were
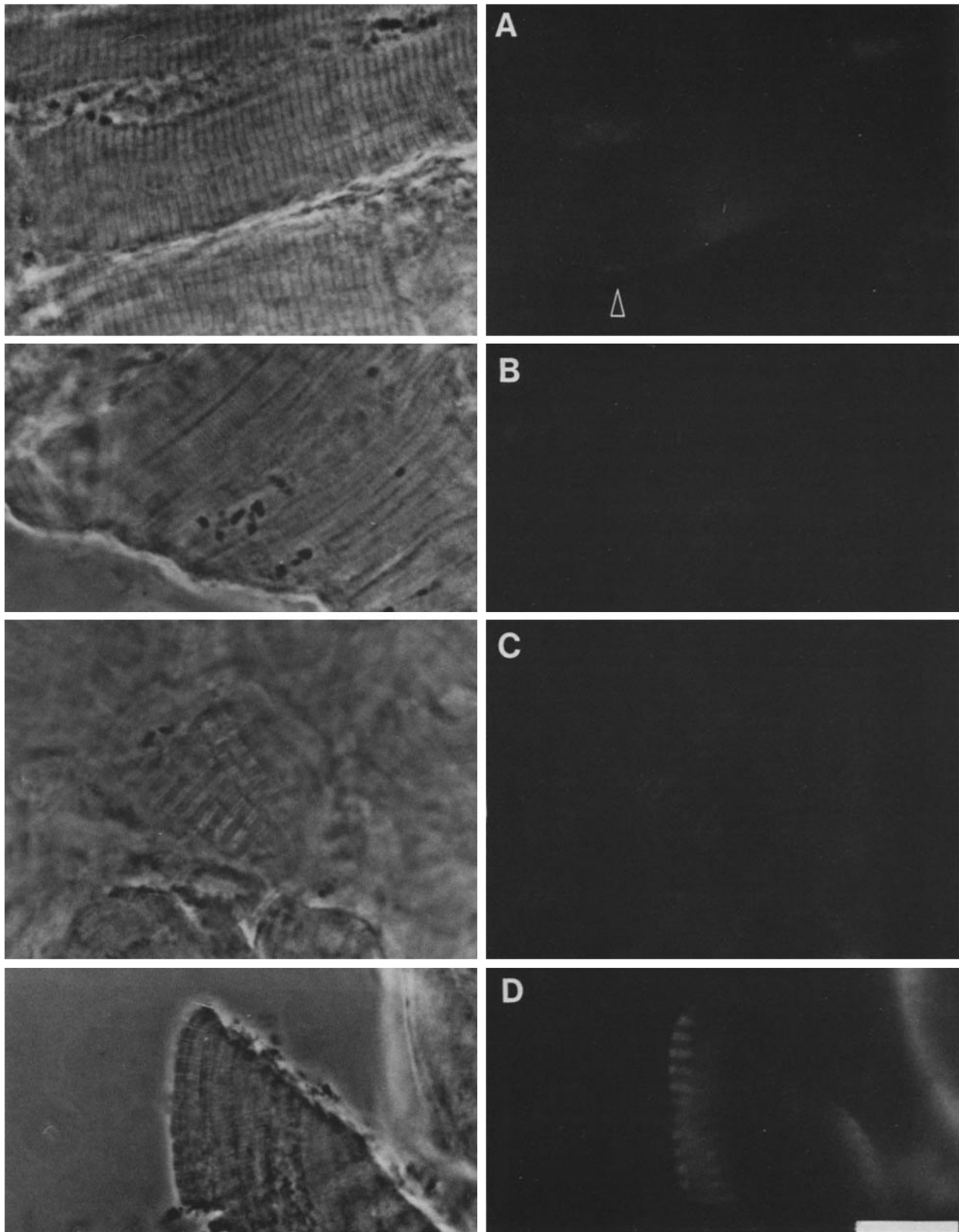
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Figure 6. Localization of gelsolin and brevin–gelsolin-binding sites in cardiac muscle. The top four panels show phase-contrast and immuno-fluorescence micrographs of cardiac muscle frozen sections stained with 4F8 IgA at 10 μg/ml diluted with 3% bovine serum albumin in PBS with no added brevin. We commonly see the two patterns of staining shown in *A* and *B*. In *A*, there is no clear I-band staining in the interior or at the periphery of the myofibrils. In *A*, we have included a region, at the arrow, where some peripheral staining is evident, perhaps in connection with a blood vessel. In *B*, there is very weak staining of the I-bands outside the region of myosin overlap. This can be detected in the microscope, but is not readily apparent when printed using the same exposures as the plus brevin samples (*C* and *D*). We have not definitively identified the granular structures. For the lower panels (*C* and *D*), the frozen sections were stained with 4F8 IgA plus brevin at 30 μg/ml diluted with 3% bovine serum albumin. The staining of I-bands outside the overlap region is easily detectable; the exclusion of brevin from the Z-line is readily apparent in *D*. Focus-through-series show that most of the added brevin is restricted to the periphery of the myofibrils and does not penetrate deeply under the conditions used. Bar, 16 μm.

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diluted with heterologous serum to block nonspecific binding. We think these localizations should be viewed with caution since in each case they mimic the distribution of actin and could result from the binding of exogenous brevin reacting with the localizing antibody.

The relatively low levels of fluorescence that are observed in cultured cells with either monoclonal or polyclonal antibodies make it difficult to establish the distribution of endogenous gelsolin in these cells. The images in Fig. 1 suggest a diffuse distribution of low intensity, but we have not done the necessary quantitative work to know whether these cells simply have low gelsolin concentrations or whether gelsolin is not completely retained after fixation.

These studies indicate that brevin–gelsolin-binding sites are found on "dynamic" structures which undergo marked changes. For example, in data not shown we have observed some preferential localization of added brevin in the cleavage furrow. Double staining of dividing chick embryo cells show that alpha-actinin, myosin, and filamin accumulate in the cleavage furrow and midbody region, and that at the completion of constriction, myosin decreases while alpha-actinin and filamin remain (5, 9). Nunnally et al. (14) have suggested that filamin and alpha-actinin may be concentrated in this region because they interact with actin to form the highly organized contractile ring. The presence of binding sites for brevin in the furrow may simply be an indication that more F-actin is present in this region. The arcs and circumferential fibers shown in Fig. 3 are also dynamic, motile structures (8, 19). Soranno and Bell (19) have shown that these organize at the cell periphery and move or contract toward the cell center. The exact role of gelsolin in the motility of these structures is not clear, but these binding studies show that there are no major areas where brevin–gelsolin-binding appears to be restricted. Therefore most of the cellular actin filaments, irrespective of the presence of other actin associated proteins, are potentially accessible to the severing action of gelsolin.

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