

Intracellular Localization of the 55-kD Actin-bundling Protein in Cultured Cells: Spatial Relationships with Actin, Alpha-Actinin, Tropomyosin, and Fimbrin

Shigeko Yamashiro-Matsumura and Fumio Matsumura

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, and Department of Biochemistry, Rutgers University, Piscataway, New Jersey 08854. Correspondence should be addressed to the authors at Rutgers University.

Abstract. The 55-kD protein is a new actin-bundling protein purified from HeLa cells (Yamashiro-Matsumura, S., and F. Matsumura, 1985, *J. Biol. Chem.*, 260:5087-5097). We have prepared monoclonal antibodies against the 55-kD protein and examined its intracellular localization, as well as its spatial relationships with other components of microfilaments in cultured cells by double-label immunofluorescence. The localization of the 55-kD protein is similar to that of actin. The antibody to the 55-kD protein stained strongly both microspikes and stress fibers. The 55-kD protein was found from the basal portions to the ex-

termities of microspikes while alpha-actinin was localized only in the basal portions. In stress fibers, the 55-kD protein was found rather continuously in comparison to the periodic localizations of alpha-actinin and tropomyosin. Although fimbrin is located in microspikes and ruffling membranes, fimbrin is hardly found in stress fibers unlike the 55-kD protein. These observations coupled with the actin-bundling activity of the 55-kD protein imply that the 55-kD protein is involved in the formation of microfilament bundles in both microspikes and stress fibers.

ACTIN-BINDING proteins are responsible for the regulation of the assembly dynamics of microfilaments in nonmuscle cells through cross-linking filaments into networks or bundles, capping ends of filaments, severing pre-formed filaments, and inhibiting polymerization by binding monomeric actins (see for review, Craig and Pollard, 1982; Weeds, 1982). In the previous paper, we have reported the purification and characterization of a new actin-binding protein of 55-kD from HeLa cells (Yamashiro-Matsumura and Matsumura, 1985). The protein cross-links F-actin into bundles like other actin-bundling proteins such as fimbrin from brush borders (Bretscher and Weber, 1980; Bretscher, 1981; Glenney et al., 1981), fascin from sea urchin eggs (Bryan and Kane, 1978), 55-kD protein from brain (Maekawa et al., 1983) and 50-kD protein from *Dictyostelium discoideum* (Hock and Condeelis, 1985). Although it is not clear whether the HeLa 55-kD protein is related to brain 55-kD protein or amoeba 50 kD protein, HeLa 55-kD protein is biochemically and immunologically different from fascin or fimbrin (Yamashiro-Matsumura and Matsumura, 1985).

We have studied the localization of 55-kD protein in cultured cells to explore its functions in the microfilament organization. We have raised monoclonal antibodies against the 55-kD protein for double-label immunofluorescence to examine its spatial relationships with other components of microfilaments including actin, tropomyosin, alpha-actinin, and fimbrin. We find that 55-kD protein is localized in microspikes as well as in stress fibers. These results together

with the actin bundling activity of 55-kD protein suggest that the 55-kD protein organizes microfilaments into bundles in microspikes and stress fibers.

Materials and Methods

Cell Culture

Cultured cells were gerbil fibroma cells (CCL-146; American Type Culture Collection, Rockville, MD), L6 myoblasts and myotubes, REF-52 cells (an established rat embryo line), normal rat kidney (NRK)¹ cells (ATCC CRL-1570), Rous sarcoma virus-transformed NRK cells (4/435), chemically transformed human fibroblasts (Hut-11), and HeLa cells. All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in an atmosphere of 5% CO₂ and 95% air at 37°C.

Polyclonal Antibodies

Antibodies against alpha-actinin were raised by the subcutaneous immunization of rabbits with protein purified from HeLa cells. As described in the previous paper, actin gels made of HeLa cell extracts contain many actin-binding proteins including alpha-actinin, filamin, and 55-kD protein (see Fig. 1 in Yamashiro-Matsumura and Matsumura, 1985). Alpha-actinin was separated from these proteins by ion-exchange chromatography over DEAE-cellulose. Final purification for immunization was performed by preparative electrophoresis as described (Yamashiro-Matsumura and Matsumura, 1985). For the first injection, 200 µg (1 ml of PBS) of alpha-actinin was emulsified in an equal volume of Freund's complete adjuvant, and four subsequent boosts were done with 150 µg of the protein emulsified in Freund's incomplete adjuvant over a 4-mo period. 7 d after the last injection,

1. *Abbreviation used in this paper:* NRK, normal rat kidney.

serum was collected. The antibody was affinity-purified using purified alpha-actinin by the methods of Olmsted (1981) and of Smith and Fisher (1984) with slight modification (Yamashiro-Matsumura and Matsumura, 1985). The specificity was tested by Western blot by the method of Towbin et al. (1979). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) was used for the second antibody, and the immunoreaction was detected with 4-chloro-1-naphthol (Bio-Rad Laboratories).

The antibody to chicken smooth muscle tropomyosin was prepared and affinity purified as described (Matsumura and Yamashiro-Matsumura, 1985). The antibody to fimbrin (kindly provided by P. T. Matsumura, Whitehead Institute, Massachusetts Institute of Technology) was also affinity purified as described (Yamashiro-Matsumura and Matsumura, 1985). The purified antibody reacted with fimbrin when total cell lysates from a variety of cells including rat and human cells were examined. The antiserum against actin was described (Owaribe et al., 1979) and kindly provided by K. Owaribe (Nagoya University, Japan).

Monoclonal Antibody to 55-kD Protein

Female BALB/57 mice were immunized intraperitoneally with 20 µg of purified 55-kD protein homogenized in an equal volume of complete Freund's adjuvant. After 1 mo, the mice were boosted intraperitoneally with 20 µg of 55-kD protein homogenized in an equal volume of incomplete Freund's adjuvant. Twelve subsequent boosts were done over 6 mo. 7 d after the last boost, 100 µg of protein in PBS was given intravenously. 3 d later, the spleen was removed and used for fusion with the mouse myeloma cell line NS-1 according to the protocol of Kennett (1979). The enzyme-linked immunosorbent assay was used to screen for positive clones, and agarose cloning (Sato et al., 1972) was performed three times to obtain stable monoclonal clones using gerbil fibroma cells as a feeder layer. We have obtained three hybridoma clones which were found to react with purified 55-kD protein on immunoblots and by an enzyme-linked immunosorbent assay. All three clones secreted IgG₁, and the staining patterns of cultured cells with these antibodies are very similar. Ascites fluid with high titer was obtained by the intraperitoneal injection of ~10⁶ hybridoma cells into BALB/57 mice that had been previously (~1 wk) primed with 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane; Aldrich Chemical Co., Milwaukee, WI). The specificity of the antibodies was checked by Western blot (Towbin et al., 1979).

Indirect Double-label Immunofluorescence

Cells grown on glass coverslips were fixed with absolute methanol for 5 min at -10°C except for the staining with anti-tropomyosin antibody. After washing in PBS, cells were first reacted for 30 min at 37°C with mouse monoclonal anti-55-kD protein antibody (ascites fluids diluted ~1:500-1:1,000 with PBS containing 0.1 mg/ml BSA) together with rabbit polyclonal antibodies such as anti-actin, anti-alpha-actinin, and anti-fimbrin antibodies. After washing in PBS for 1 h, cells were reacted with a mixture of second antibodies, fluorescein-conjugated goat anti-rabbit IgG (1:25 dilution, affinity purified; CooperBiomedical Inc., Malvern, PA) and rhodamine-conjugated goat anti-mouse IgG (1:25 dilution, affinity purified; CooperBiomedical). After a further thorough washing, the coverslips were mounted in Gelvatol (Monsanto brand of polyvinyl alcohol; Monsanto, St. Louis, MO).

The above fixation method is not suitable for the double-label immunofluorescent localization of 55-kD protein and tropomyosin. The localization of tropomyosin needs formaldehyde fixation. In contrast, neither polyclonal nor monoclonal anti-55-kD protein antibody gave any immunofluorescence more than background when cells were first fixed with formaldehyde. Thus we have searched appropriate fixation method and found that brief treatment with methanol followed by formaldehyde fixation allowed simultaneous and adequate preservation of the antigenicity of both 55-kD protein and tropomyosin. Briefly, cells were first treated for 30 s in absolute methanol (-10°C) and transferred directly in 3.7% formaldehyde solution in PBS. After incubation for 5 min, coverslips were washed twice with PBS (15 min each), permeabilized with absolute acetone (-10°C) for 5 min. After a washing in PBS, the coverslips were processed for double-label immunofluorescence as described above. Immunofluorescent patterns of tropomyosin and 55-kD protein obtained by this method were indistinguishable from those of tropomyosin and 55-kD protein fixed separately by their conventional methods (formaldehyde for tropomyosin and methanol for 55-kD protein).

Cells were photographed on a Zeiss epifluorescence photomicroscope III with a Zeiss x63 oil phase 3 lens (numerical aperture [N. A.] 1.4). Fluores-

cein isothiocyanate was analyzed with a Zeiss dichroic excitation filter BP485/20 and barrier filter P520, and tetramethylrhodamine isothiocyanate with a narrow band pass interference excitation filter 546+2 nm and LP590 barrier filter. An Olympus BH-2 microscope with reflected light fluorescence attachment and a Olympus x60 oil phase lens (N.A. 1.4) was also used for some experiments.

Results

Monoclonal Antibody to 55-kD Protein

The specificity of the mouse monoclonal antibody against 55-kD protein was examined by immunoblotting (Fig. 1 A). When total cell lysates of a variety of cultured cells including HeLa cells (lanes 2 and 6), NRK cells (lanes 3 and 7), and gerbil fibroma cells (lanes 4 and 8) were examined, the monoclonal antibody reacted with one band which corresponded to the purified 55-kD protein (lanes 5 and 10). These results suggest that 55-kD protein is present in both human and rodent cells.

We have also examined the specificity of rabbit polyclonal antibody to alpha-actinin. As Fig. 1 B shows, the reaction with the antibody gave a doublet band in total cell lysates (lanes 3 and 5), which is consistent with the report that alpha-actinin is present as a doublet in nonmuscle cultured cells (Burridge and Feramisco, 1981). Because the antibody reacted with authentic alpha-actinin purified from chicken gizzard smooth muscle (lanes 2 and 4), and because the antibody gave a typical staining pattern of alpha-actinin in cultured cells (see Fig. 4 B and C), we have concluded that the antibody is specific to alpha-actinin.

Comparison of the Localization of 55-kD Protein with Actin

The localization was first compared with that of actin by double-label immunofluorescence using polyclonal antibody to actin. As Fig. 2 shows, monoclonal antibody to 55-kD protein decorated stress fibers (Fig. 2 B), as well as microspikes (Fig. 2 E). Simultaneous staining with anti-actin antibody showed that most of the localization of 55-kD protein (Fig. 2 B and E) could be superimposed with the actin localization (Fig. 2, C and F).

Comparison with the Localization of Tropomyosin and Alpha-Actinin

Tropomyosin has been found to be periodically distributed along the stress fibers between periodicities of alpha-actinin (Lazarides and Burridge, 1975; Lazarides, 1976a; Zigmond et al., 1979; Rathke et al., 1979). As Fig. 3 shows, 55-kD protein appeared to be localized along stress fibers in a rather continuous fashion (Fig. 3, B and E) in contrast to the marked periodic distribution of tropomyosin (Fig. 3, C and F). Furthermore, foci of polygonal networks were decorated with the antibody to 55-kD protein (as shown in Fig. 3 E) but not with anti-tropomyosin antibody (Fig. 3 F). In addition, the 55-kD protein but not tropomyosin was found in the periphery of cells including microspikes (Lazarides, 1976b).

Figs. 4 and 5 show the comparison in the localization between 55-kD protein and alpha-actinin by double-label immunofluorescence. 55-kD protein again showed rather continuous distribution along stress fibers in contrast to the periodic localization of alpha-actinin. Further, anti-55-kD protein antibody weakly decorated the terminal regions of

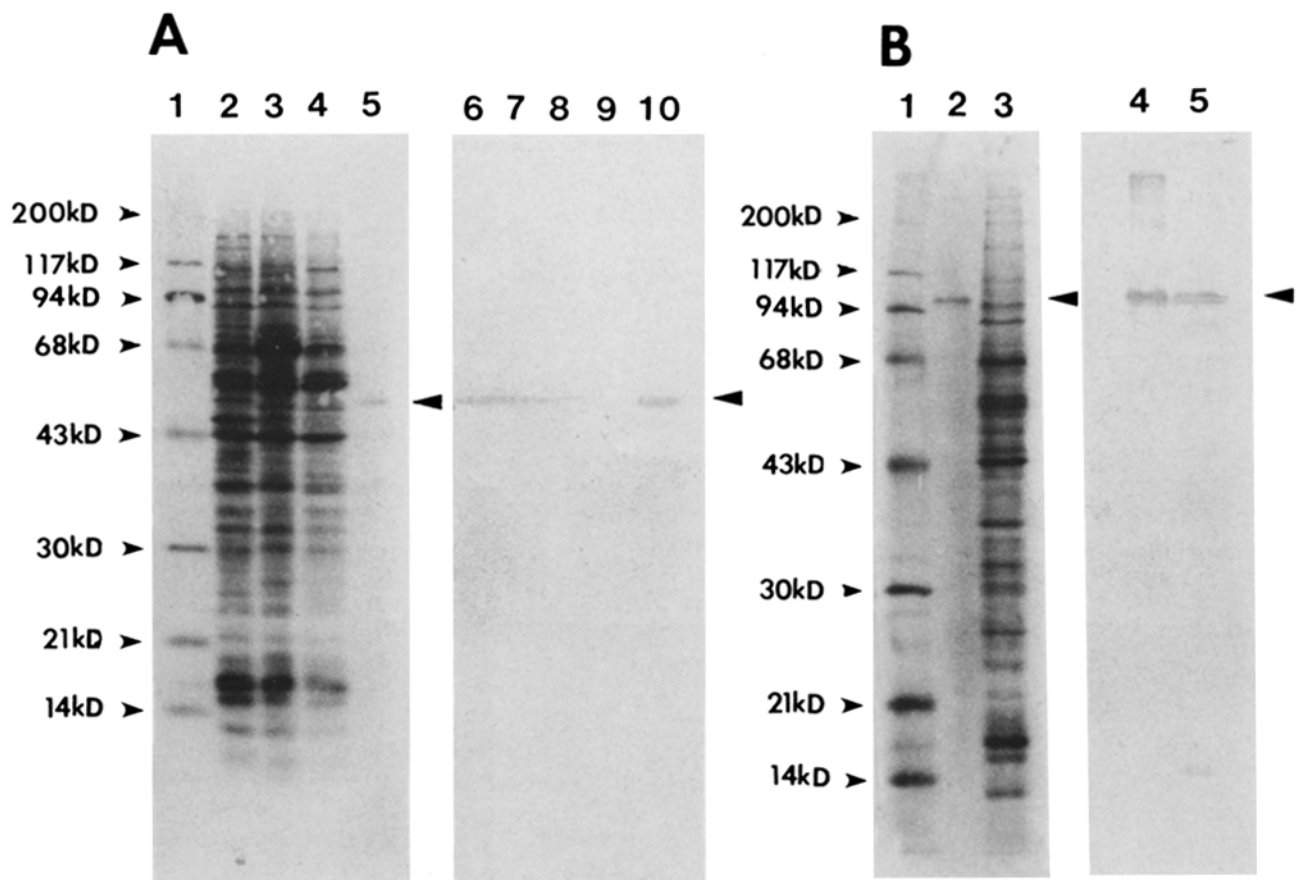


Figure 1. Immunoblots with the monoclonal antibody against 55-kD protein (**A**) and with the polyclonal antibody against alpha-actinin (**B**). (**A**) Purified 55-kD protein as well as total cell lysates were electrophoresed, transferred to nitrocellulose paper, and stained with Amido Black (lanes 1–5). Equivalent blots (lanes 6–10) were reacted with the monoclonal antibody to 55-kD protein (1:1,000 dilution). (Lane 1) Molecular mass markers; (lanes 2 and 6) HeLa cell lysates; (lanes 3 and 7) NRK cell lysates; (lanes 4 and 8) gerbil fibroma cell lysates; (lanes 5 and 10) purified 55-kD protein. No samples were loaded in lane 9 in order to avoid cross-contamination of the purified 55-kD protein to the total cell lysates during electrophoresis. (**B**) Blots stained with Amido Black (lanes 1–3) and immunoblots reacted with the polyclonal antibody to alpha-actinin (lanes 4 and 5). (Lanes 2 and 4) Alpha-actinin purified from chicken gizzard smooth muscle; (lanes 3 and 5) HeLa cell lysates; (lane 1) molecular mass markers. The antibody reacted with alpha-actinin either from chicken gizzard or from the total cell lysates.

stress fibers whereas anti-alpha-actinin antibody stained these regions strongly (compare Fig. 4, *B* and *C*). The 55-kD protein was distributed continuously in the polygonal networks of spreading cells (Fig. 5 *A*) while alpha-actinin was found at the foci, and in the connecting fibrous bundles of nets in a striated fashion.

It was reported that alpha-actinin is also present in peripheral regions of cells such as ruffling membranes (Lazarides and Burridge, 1975; Schollmeyer et al., 1976). Double-label immunofluorescence showed that 55-kD protein was found in microspikes along the entire lengths and at the ends of ruffling membranes while alpha-actinin was found in the basal regions of ruffles and microspikes (compare Fig. 4, *E* and *F*).

Comparison with the Localization of Fimbrin

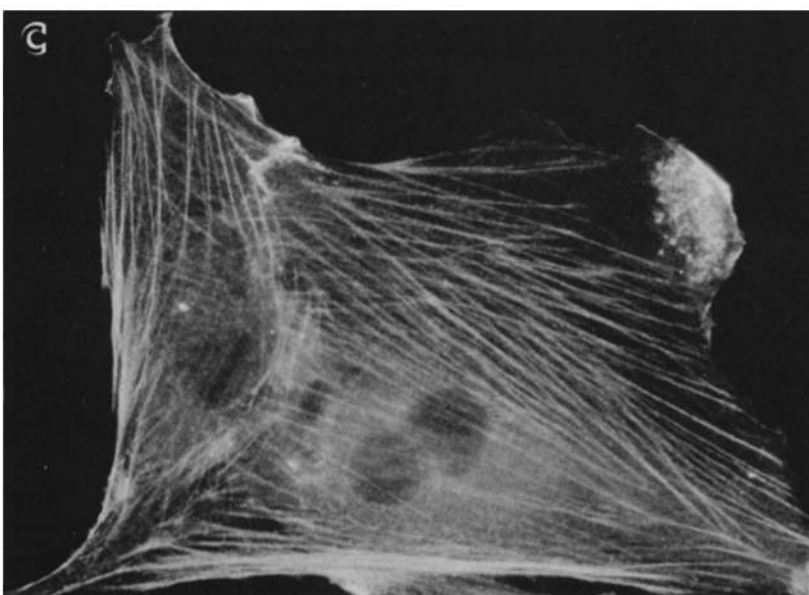
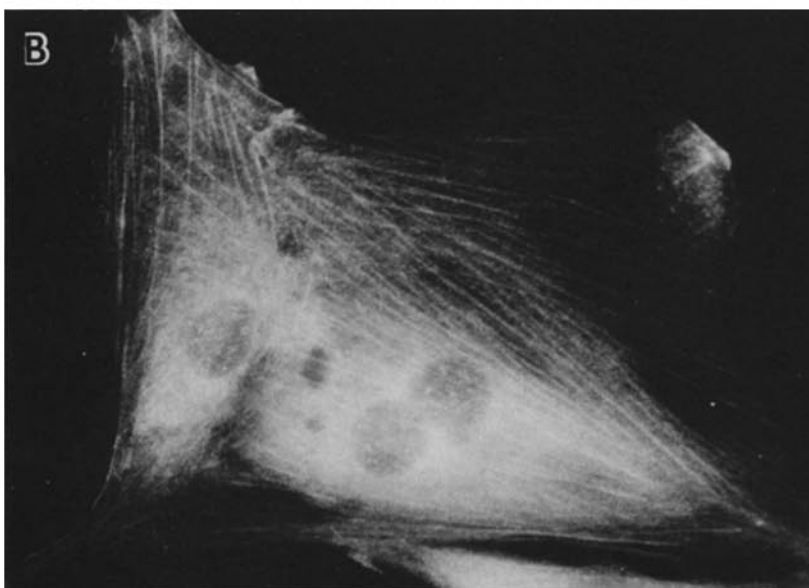
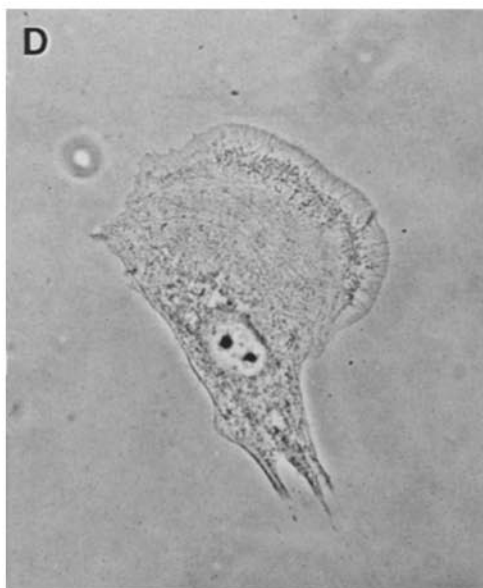
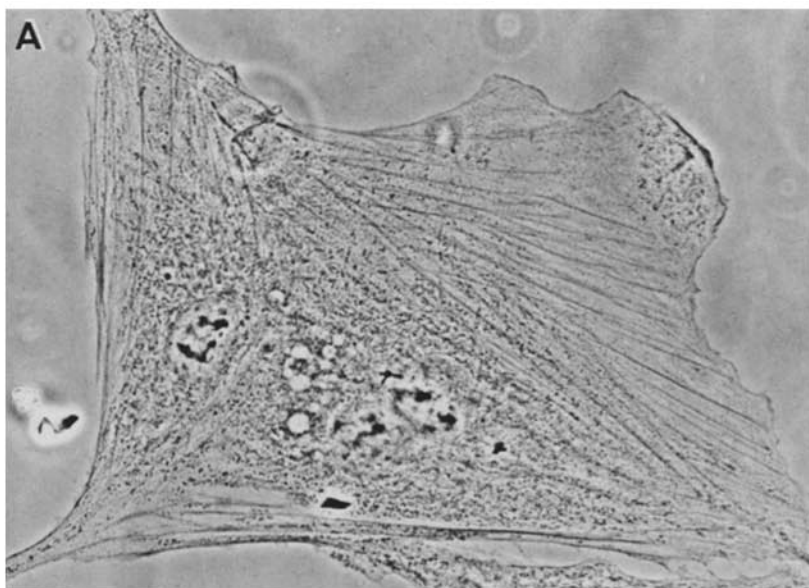
Fimbrin is an actin-bundling protein purified from brush borders of chicken intestinal epithelium (Bretscher, 1981; Glenney et al., 1981). In tissue culture cells, the protein is found in microspikes and membrane ruffles but very little is found in stress fibers (Bretscher and Weber, 1980). Because

55-kD protein was also localized in microspikes as described above, the distributions between these two proteins were compared by double-label immunofluorescence. As Fig. 6 shows, both antibodies stained membrane ruffles and microspikes simultaneously. However, the 55-kD protein was more prominently localized in microspikes than in membrane ruffles whereas fimbrin was predominant in ruffling membranes. In addition, the antibody to 55-kD protein stained stress fibers while fimbrin is hardly found in these structures. It is also interesting to note that some microspikes were stained with anti-55-kD protein antibody but not with fimbrin antibody (see arrows in Fig. 6).

Discussion

This paper has shown that 55-kD protein is localized in microspikes as well as in stress fibers of cultured cells. Because both structures are known to have bundles of microfilaments, this localization is consistent with the *in vitro* actin-bundling activity of 55-kD protein.

Double-label immunofluorescence has shown that micro-



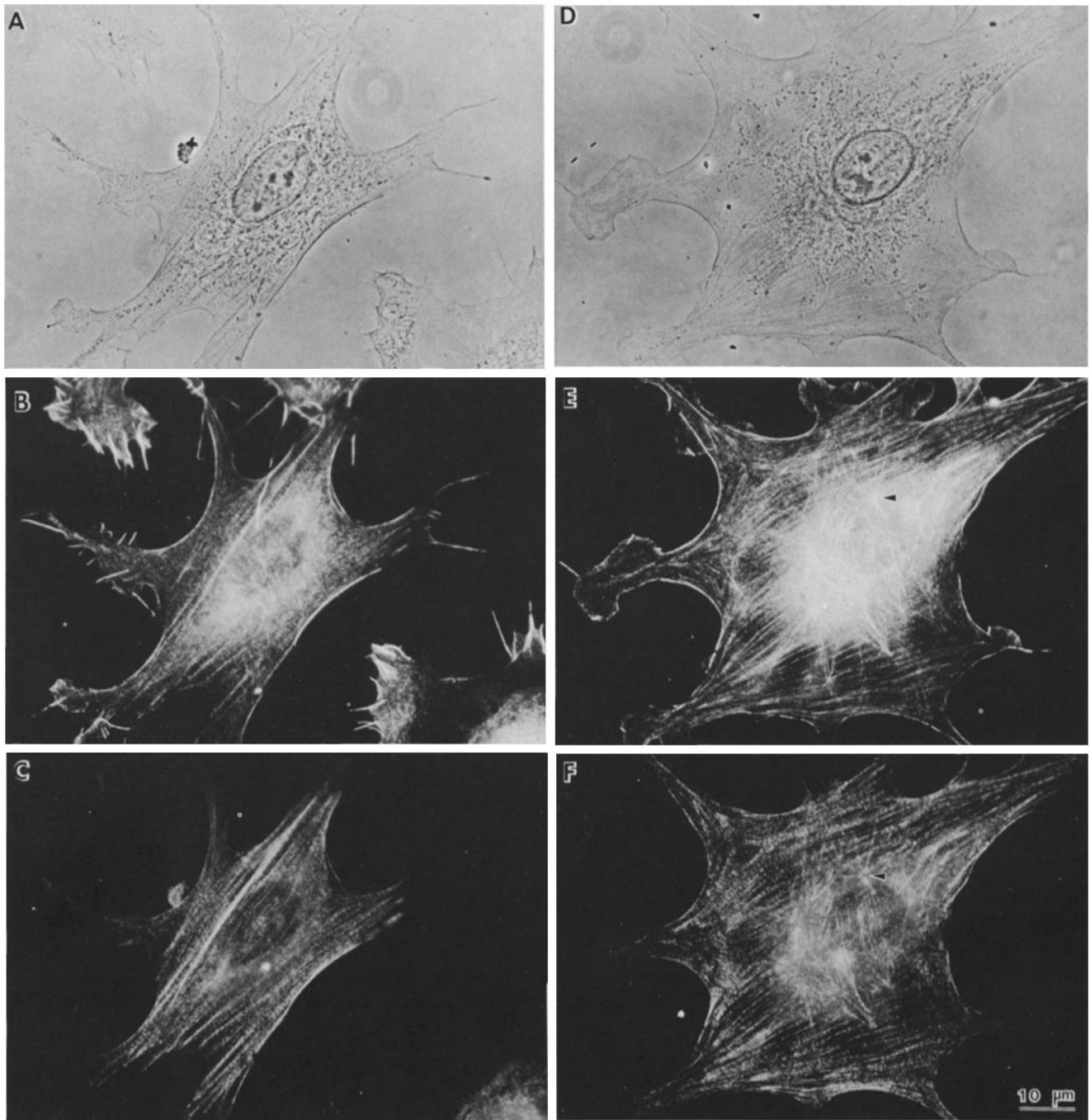


Figure 3. Comparison of the localization of 55-kD protein with tropomyosin in gerbil fibroblasts. Phase-contrast micrographs (*A* and *D*) and immunofluorescent micrographs double-labeled with anti-55-kD protein antibody (*B* and *E*) and anti-tropomyosin antibody (*C* and *F*). Note that microspikes are not stained with the antibody to tropomyosin. Arrows in *E* and *F* show foci of polygonal nets where 55-kD protein but not tropomyosin is localized.

filament bundles of stress fibers contain tropomyosin but those of microspikes do not. This observation has led us to examine the effects of 55-kD protein on the actin binding of tropomyosin (Matsumura and Yamashiro-Matsumura, 1986*a, b*). We have found that the effects of 55-kD protein varied with the isoforms of tropomyosin. Among five iso-

forms of tropomyosin (with apparent molecular weights of 40,000, 36,500, 35,000, 32,400 and 32,000) identified in cultured rat cells (Matsumura et al., 1983*a, b*), tropomyosin isoforms with high M_r (40,000 and 36,500) were able to co-bind to actin with 55-kD protein, thus forming tropomyosin-containing actin bundles. Tropomyosin isoforms with low

Figure 2. Comparison of the localization of 55-kD protein with actin in NRK cells. Phase-contrast micrographs (*A* and *C*) and immunofluorescent micrographs double labeled with anti-55-kD protein antibody (*B* and *E*) and with anti-actin antibody (*C* and *F*). 55-kD protein is present in microspikes (arrows in *E*) as well as stress fibers (*B*). Note that the 55-kD protein and actin co-localize.

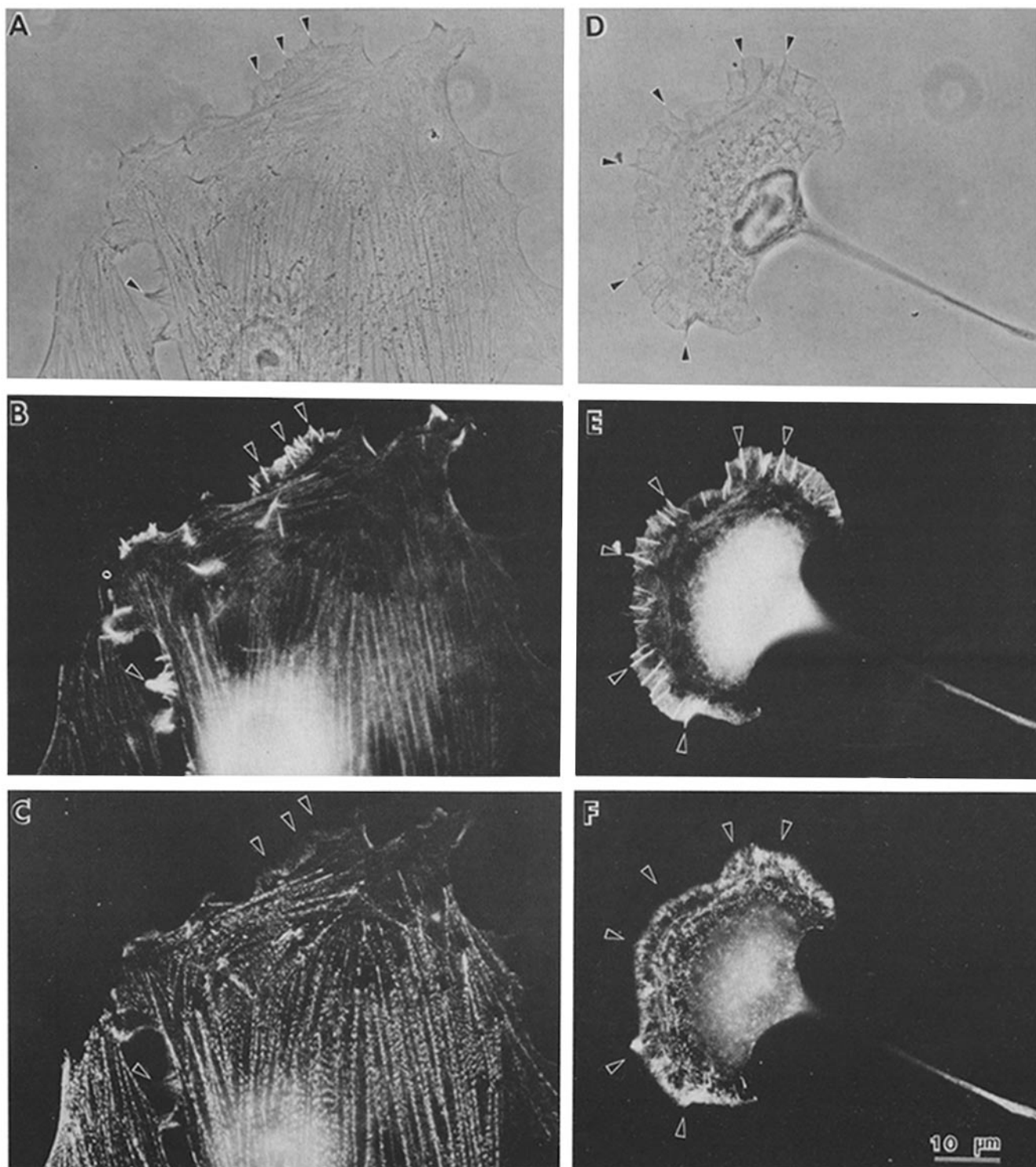


Figure 4. Comparison of the localization of the 55-kD protein with alpha-actinin in gerbil fibroma cells. Phase-contrast micrographs (*A* and *D*) and immunofluorescent micrographs double stained with anti-55-kD protein antibody (*B* and *E*) and with anti-alpha-actinin (*C* and *F*). 55-kD protein is present to the extremities of microspikes (shown by arrows) while alpha-actinin is localized in the basal portion of microspikes.

M_r (32,400 and 32,000) were, however, dissociated from actin by 55-kD protein, which resulted in the formation of bundles without tropomyosin. The tropomyosin-containing and tropomyosin-free actin bundles may, therefore, correspond to the microfilament bundles in stress fibers and to those in microspikes, respectively.

As reported by our laboratory (Matsumura et al., 1983*a*, *b*; Lin et al., 1984) and other laboratories (Hendricks and Weitraub, 1981, 1984; Leonardi et al., 1982; Lin et al., 1985; Cooper et al., 1985), cell transformation induced the changes in the expression of tropomyosin isoforms. Generally, tropomyosin isoforms with high M_r are decreased or missing

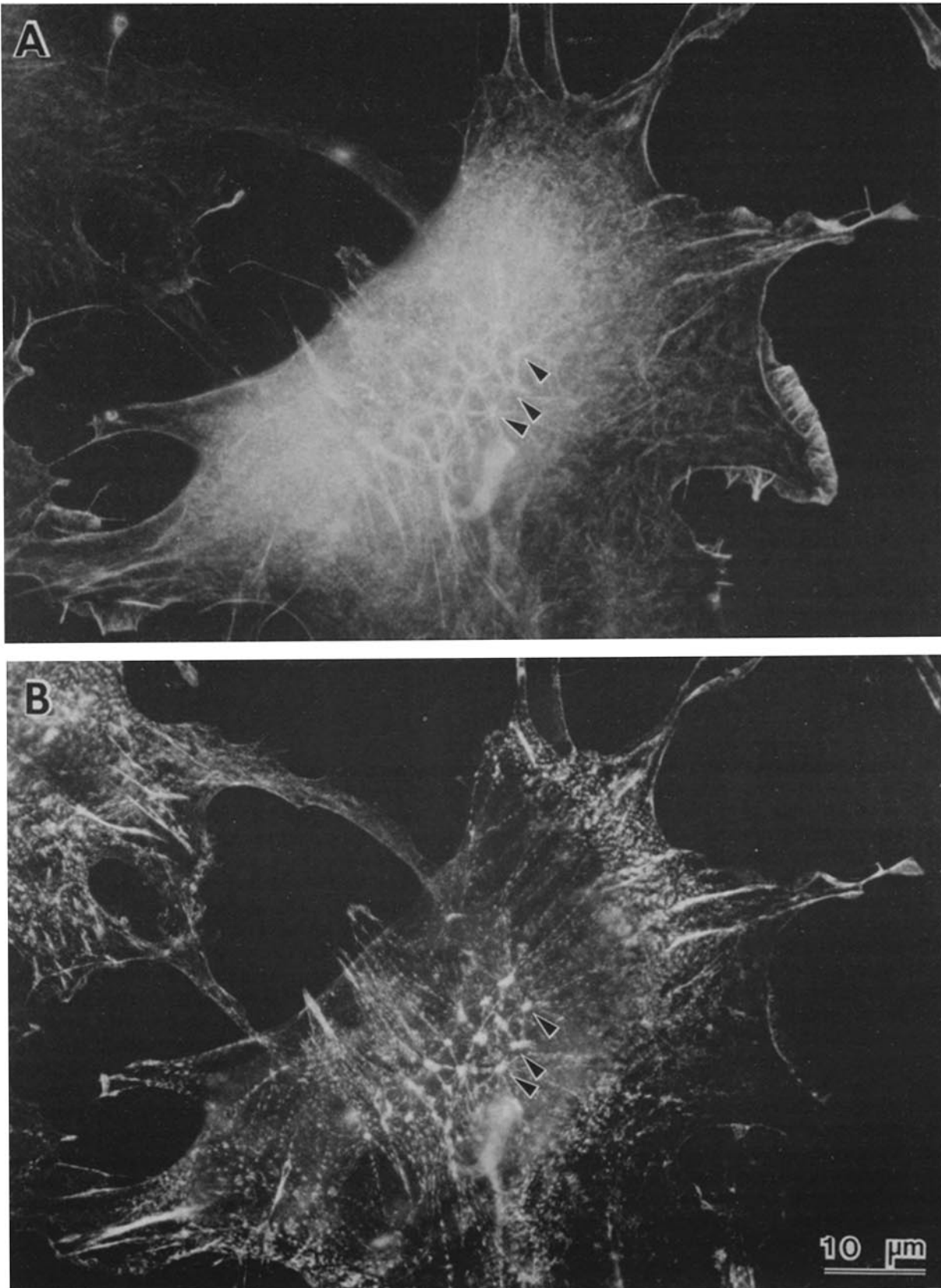


Figure 5. Comparison of the localization of 55-kD protein (*A*) with alpha-actinin (*B*) in foci (shown by arrows) of polygonal nets of gerbil fibroma cells.

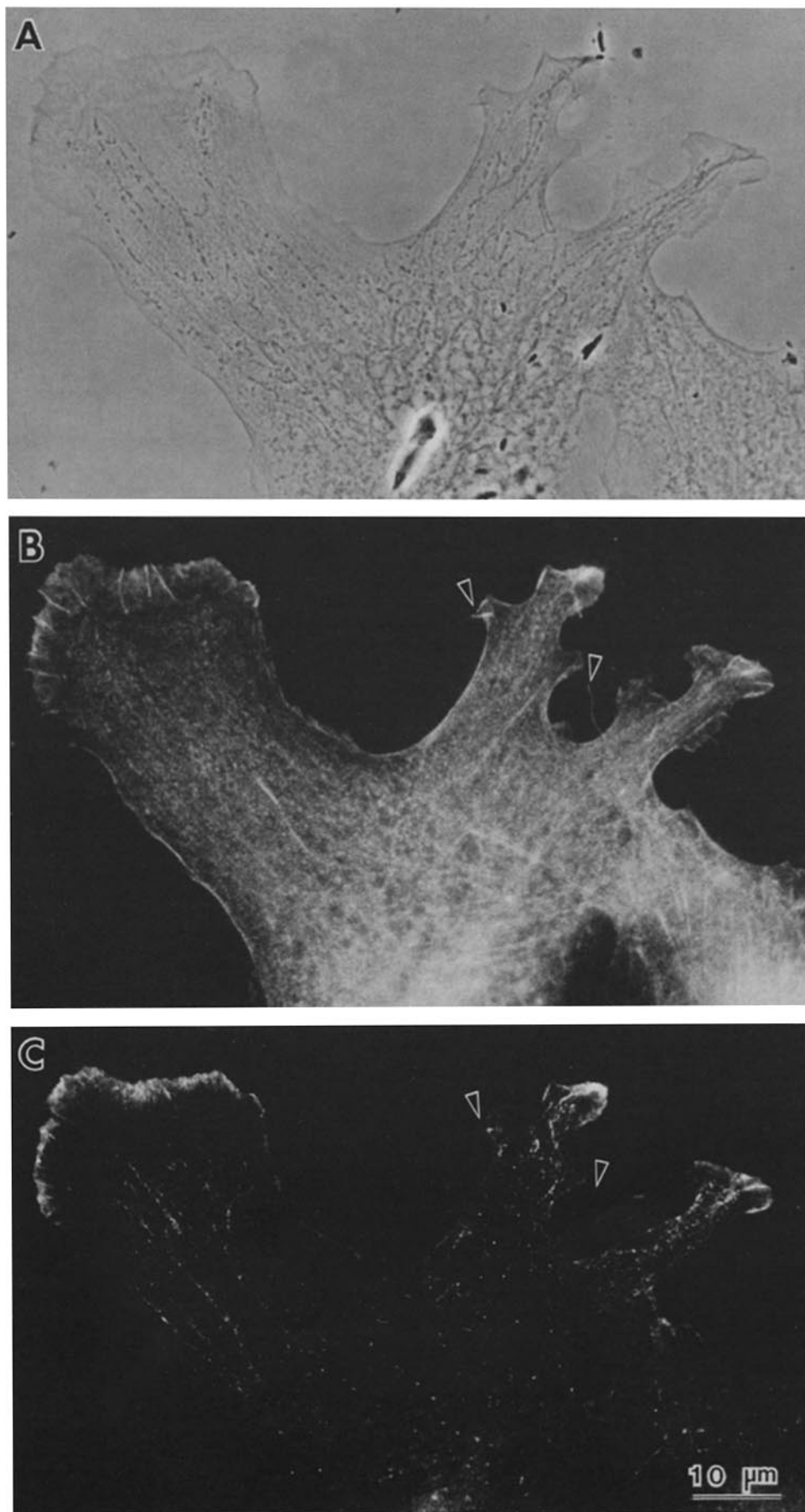


Figure 6. Comparison of the localization of 55-kD protein with fimbrin in Hut-11 cells. Phase-contrast micrograph (A) and immunofluorescent micrographs double-labeled with antibody to 55-kD protein (B) and antibody to fimbrin (C). Although both antibodies decorate cell periphery, 55-kD protein is more prominent in microspikes.

and tropomyosin with low M_r are increased in transformed cells. Furthermore, our preliminary study has shown that increased amounts of 55-kD protein were found in microfilaments isolated from Kirsten or Rous sarcoma virus-transformed NRK cells. These changes in the expressions of tropomyosin and 55-kD protein may result in the decrease of tropomyosin-containing actin bundles and increase of tropomyosin-free bundles. This may explain at least in part the disorganization of stress fibers upon cell transformation. We are currently studying the detailed localization as well as biochemical analyses of tropomyosin, 55-kD protein, and other actin-binding proteins in a variety of transformed cells.

Actin-binding proteins can be classified into three groups: (a) actin cross-linking proteins, (b) actin filament length regulators, and (c) actin-depolymerizing proteins (see reviews by Craig and Pollard, 1982; Weeds, 1982). 55-kD protein together with alpha-actinin (Podlubnaya et al., 1975; Suzuki et al., 1976; Burridge and Feramisco, 1981), filamin (Wang et al., 1975; Wallach et al., 1978) or actin-binding protein (Hartwig and Stossel, 1975 and 1981), spectrin (Tyler et al., 1980; Branton et al., 1981; Levine and Willard, 1981; Burridge et al., 1982; Glenney et al., 1982), fimbrin (Bretscher and Weber, 1980; Bretscher, 1981; Glenney et al., 1981) and fascin (Bryan and Kane, 1978) belongs to the first class. It seems that these actin cross-linking proteins can be further divided into two subgroups by both their functions and their molecular shapes. Filamin, alpha-actinin, and spectrin are gelation proteins whose shapes are long flexible rods. This molecular shape probably makes these proteins easier to cross-link two actin filaments at an angle with considerable flexibility, thus facilitating extensive cross-linking and development of actin gel (Hartwig and Stossel, 1975; see also review by Craig and Pollard, 1982). In contrast, 55-kD protein, fimbrin, and fascin are all globular and monomeric proteins and make bundles rather than rigid gel structure. The way of binding of these globular proteins to actin is probably fixed in such a way that two actin filaments are cross-linked in a parallel fashion, thereby forming bundles.

It is interesting to note that these structural and functional differences between two subgroups of actin cross-linking proteins reflect the difference in the localization of these proteins in microspikes. In cultured cells, both 55-kD protein and fimbrin are present in microspikes from the basal portions to the extremities where actin staining persisted. On the contrary, both alpha-actinin and filamin are found only in the basal portions of microspikes but not to the extremities (Heggeness et al., 1977). Furthermore, in intestinal brush borders, both alpha-actinin and filamin are localized in terminal webs but not in microvilli; however, fimbrin is found in microvilli (Bretscher and Weber, 1978). Fascin, an actin-bundling protein purified from sea urchin eggs (Bryan and Kane, 1978), is also localized in the microvillar cores which form after fertilization of sea urchin eggs (Otto et al., 1980). Thus these two subgroups of actin cross-linking proteins play different roles in the organization of microspike structures. The globular actin-bundling proteins are responsible for the formation of microfilament bundles in microspikes while linear rod-like actin-gelation proteins are involved in the formation of microfilament meshworks in the basal portions of microspikes.

Some microspike structures are highly motile. Filopodia of cultured cells and the growth cones of neurites are such

examples. They extend and retract at different sites on cell surface (Albrecht-Buehler, 1976). Thus, the organization and disorganization of microfilament bundles should be dynamically regulated in microspikes. As judged from the bundling activity and the localization in such structures, 55-kD protein is most likely involved in the dynamic formation of microfilament bundles. However, in vitro biochemical studies have shown that 55-kD protein/actin filament bundles appear rather stable and not to be regulated, for example, by Ca^{2+} (Yamashiro-Matsumura and Matsumura, 1985). Consequently we are currently examining whether other actin-binding proteins regulate the bundling activity of 55-kD protein.

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