Proc. Natl. Acad. Sci. USA
Vol. 74, No. 9, pp. 3883–3887, September 1977
Cell Biology

Intracellular distributions of mechanochemical proteins in cultured fibroblasts

(microfilament organization/cell–cell contact/cell motility/filamin)

MICHAEL H. HEGGENESS, KUAN WANG*, AND S. J. SINGER

Department of Biology, University of California at San Diego, La Jolla, California 92093

Contributed by S. J. Singer, June 24, 1977

ABSTRACT **We have used methods that have allowed simultaneous fluorescent staining of intracellular actin together with either myosin, filamin, or tubulin in normal rat kidney fibroblasts in monolayer culture. In the main portions of the cell body, the actin, myosin, and filamin are all present in two structures: in one, the three proteins are present in the same fiber bundles (stress fibers); in the other, there is a diffuse distribution of the three proteins. On portions of the cell periphery however—in the basal regions of microspikes, in ruffles, and in regions of cell–cell contact—actin and filamin are present, but myosin is severely depleted or absent. Microtubules are present in the cell body in a distribution independent of the stress fibers and are mostly absent from the cell periphery. Microspikes and ruffles are highly dynamic structures on the cell surface, and regions of cell–cell contact generally result from the association of ruffles on the two contacting cells. Therefore, the presence of filamin and actin but not myosin in these specialized regions on the cell surface, together with the recent demonstration [Wang, K. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2021–2025)] that pure filamin interacts with individual F-actin filaments in solution to form fiber bundles and sheet-like structures, suggest that *in vivo* filamin–actin interactions play an important role in the control of actin filament structure, in cell motility, and in the stabilization of cell–cell contacts.**

It is well known that eukaryotic nonmuscle cells contain contractile proteins such as actin and myosin similar to those found in muscle cells (for review, see ref. 1). With fibroblasts in monolayer culture, it has been observed that these proteins are, in part at least, organized into extended filaments inside the cell (2, 3). At present, however, detailed interactions among these proteins in forming such filaments, and the relationship of these structures to phenomena such as cell motility and cell–cell contact, are not understood. As a step towards the elucidation of such problems, we have carried out experiments to localize two specific mechanochemical proteins simultaneously in the same normal rat kidney (NRK) fibroblast. Our first studies have been done at the light microscopic level of resolution, by using specific fluorescence staining techniques for actin, myosin, filamin (3, 4), and tubulin. The actin was stained by a modification (5) of the fluorescein-labeled heavy meromyosin technique (6), whereas the other proteins were stained one at a time by specific rhodamine immunofluorescence methods. These experiments have revealed some interesting differential distributions of the four proteins within fibroblasts which are presented and discussed in this paper.

MATERIALS AND METHODS

Cell Culture. The cell line NRK (7) was maintained at 37° in Coons' modified F-12 medium supplemented with 10% fetal calf serum and antibiotics in an atmosphere of 90% air/10% CO_2. Cells to be stained were plated on glass coverslips at densities of 1 to 2 × 10^3 cells per cm^2 and allowed to grow for from 24 to 72 hr before fixation and staining, at which time the cell density was between 2 and 10 × 10^3 cells per cm^2.

Antibodies and Staining Reagents. Rabbit antibodies were used as primary reagents. Rabbit antibodies specific for human uterine myosin (which crossreacts with NRK myosin) (3, 8) and chicken gizzard filamin (ref. 3; K. Wang and S. J. Singer, unpublished data), have been described. Rabbit antibodies prepared against highly purified tubulin from 12-day-old chick embryo brains (9) were the gift of Melvin Simon. Goat antibodies against rabbit IgG were used for the indirect staining of the rabbit antibodies. The IgG fraction of the goat antiserum was derivatized with Lissamine rhodamine B sulfonyl chloride and was fractionated by ion exchange chromatography on DE-52 cellulose (10). The conjugates used in this study had rhodamine/IgG molar ratios of between 1.5 and 2.7.

Actin was localized in these cells using the reagents biotin-conjugated heavy meromyosin and fluorescein-conjugated avidin as described (5).

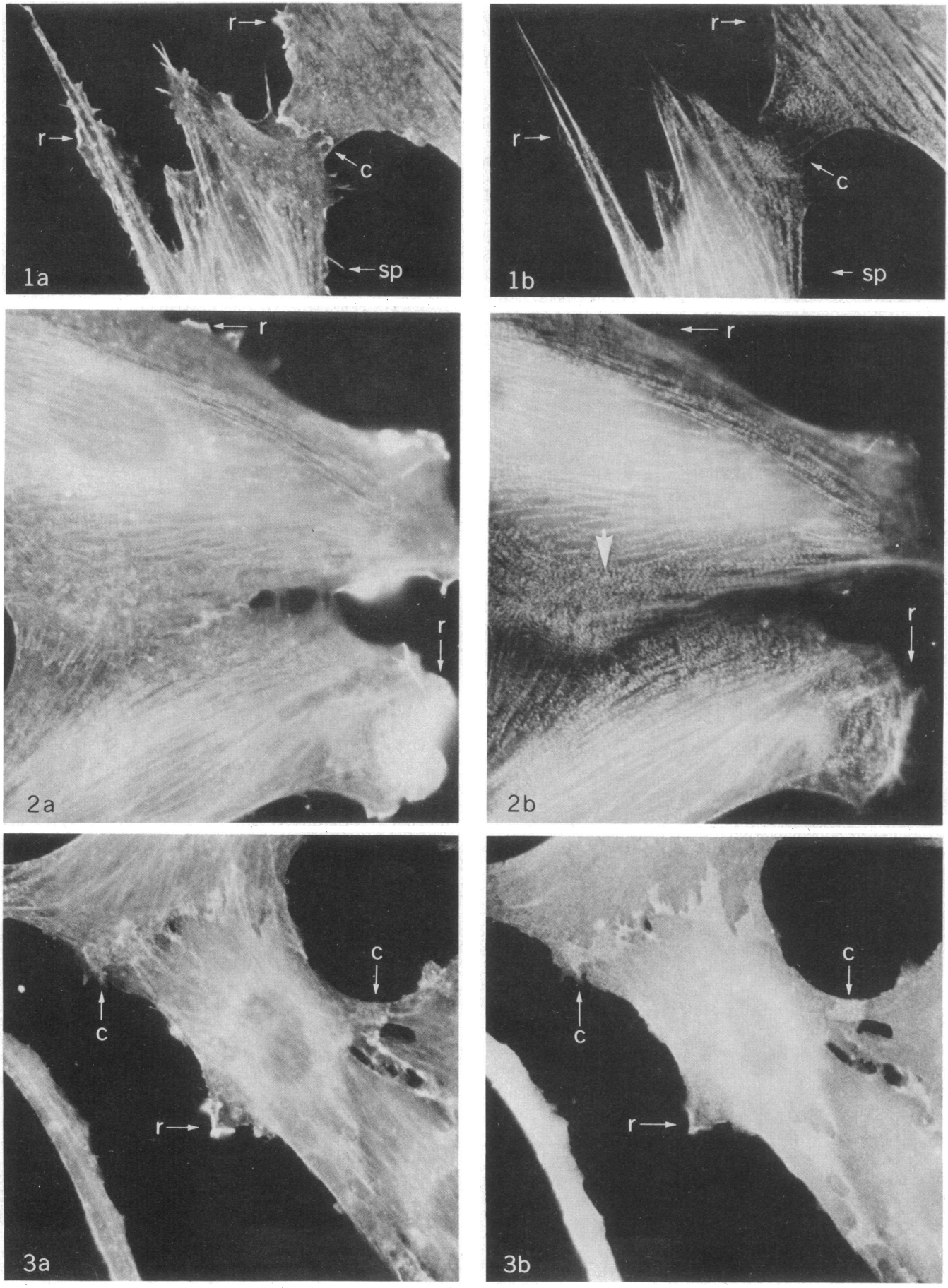
Staining of Cells. Formaldehyde (3%) in phosphate-buffered saline (PBS), pH 7.4, was warmed to 37° and applied to the cells on coverslips for 20–45 min at room temperature. The cells were then rinsed with PBS, incubated for 10 min in PBS containing 0.1 M glycine or 0.05 M NH_4Cl to quench any remaining aldehyde functions, and rinsed again in PBS. The fixed cells were then rendered permeable to protein reagents by a 2 min exposure to 0.1% Triton X-100 in PBS. This treatment resulted in better structural preservation than either acetone treatment or freezing–thawing. The cells were then washed thoroughly in PBS and were treated with a mixture of biotin-conjugated heavy meromyosin (0.2–0.7 mg/ml) and a rabbit antibody IgG (0.1–0.5 mg/ml) to either myosin, filamin, or tubulin for 20 min at room temperature. Purified IgG was always used in this step, as serum interfered with actin staining by this method. After thorough washing in PBS, the cells were then treated with a mixture of fluorescein-conjugated avidin (0.05 mg/ml) and rhodamine-conjugated goat anti-rabbit-IgG (0.05–0.2 mg/ml) in PBS for 20 min. Following a further thorough washing, the cover slip was inverted on a drop of 90% glycerol/10% PBS and the cells were observed with a Zeiss Photoscope III using epi-illumination. The filter combinations used were CZ 487710 and CZ 487714 for fluorescein and rhodamine observation, respectively. The two fluorescences were always photographed without changing focus. Specimens were photographed using Kodak Plus X or Panatomic X film.

As controls for the specificity of the staining reactions, it was

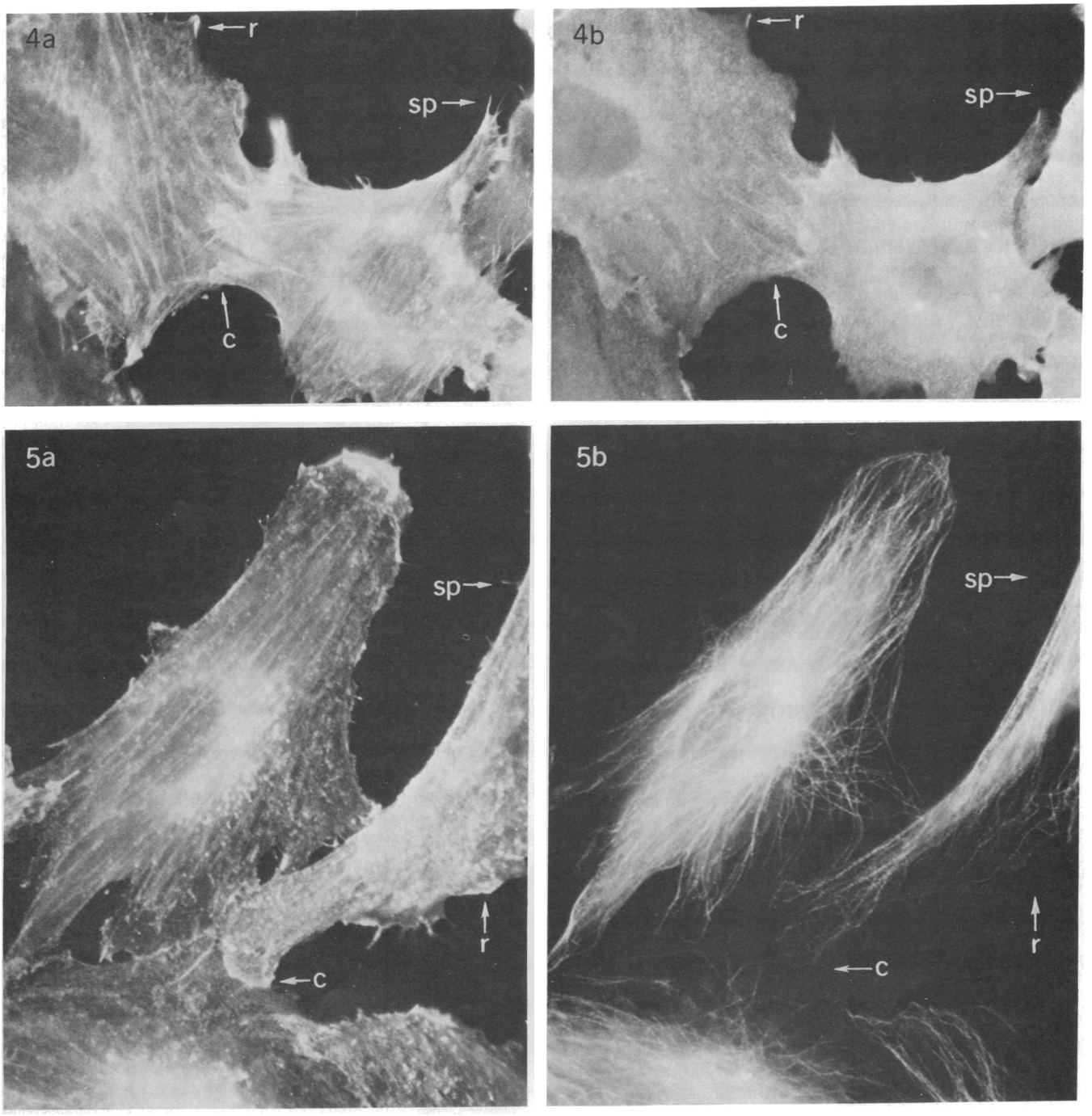
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: NRK, normal rat kidney; PBS, phosphate-buffered saline.

* Present address: Department of Chemistry and Clayton Foundation Biochemical Institute, University of Texas at Austin, Austin, TX 78712.



Legend to Figs. 1–5 on following page.



Figs. 1–3 on preceding page.

FIGS. 1–5. Double fluorescence staining pictures of mechanochemical proteins inside NRK cells in monolayer culture. Each figure shows the same cell stained in *a* for actin and in *b* for either myosin (1*b* and 2*b*), filamin (3*b* and 4*b*), or tubulin (5*b*). The symbols represent: *sp*, microspike; *r*, ruffle; *c*, region of cell–cell contact. Note the staining for actin in *sp*, *r*, and *c* (1*a*–5*a*), and the absence of staining for myosin (1*b* and 2*b*) in these same structures. Note also the staining for filamin in *r* and *c* (3*b* and 4*b*), and in the basal portion of *sp* (4*b*). The large arrow in 2*b* points to a reticular pattern of myosin staining. (×950.)

found that myosin, filamin, and tubulin staining could be eliminated by preabsorption of the respective rabbit antibodies by the appropriate antigen. The specificity of actin staining was established by its elimination by Mg pyrophosphate (5 mM) or by free biotin (0.1 mg/ml) (5).

RESULTS

Double staining for the intracellular actin and myosin components of NRK cells gave results such as those shown in Figs. 1 and 2. The staining observed was specific, because the controls were negative. All the staining was intracellular, as demonstrated by the fact that cells were not stained if the Triton X-100 treatment was omitted. The actin and myosin were found in part to be organized into extended filaments, the so-called stress fibers that have been observed previously (2, 3). As is particularly clear in Fig. 1, the same filaments were stained with both actin and myosin reagents, and hence both proteins were present in the same fiber bundles. In addition to the stress fibers, however, there was a more diffuse staining of actin in the cell

body, as is especially evident in Figs. 2*a* and 3*a*. The myosin present outside of the stress fibers, by contrast, often exhibited a more reticular pattern than the actin (compare Fig. 2 *a* and *b*, large arrow).

As illustrated by Figs. 1 and 2, marked differences between the extents of actin and myosin staining were always observed in certain regions of the cell periphery, at microspikes (*sp*) and at ruffles (*r*) on isolated cell surfaces, as well as at regions of contact between two cells (*c*). In each of these regions, actin staining was quite intense, often more intense than in the interior of the cell, but little or no myosin staining was visible.

When double staining for actin and filamin was performed, results such as those shown in Figs. 3 and 4 were obtained. In the interior of the cell, filamin was organized on stress fibers (Fig. 4*b*, upper cell) (see also ref. 3), as well as more diffusely spread. Especially interesting was the coincident staining of filamin and actin in ruffles and in regions of cell–cell contact. Filamin staining was also always observed in the basal portions of microspikes (Fig. 4*b*; contrast with Fig. 1*b*) but not in the extremities where actin staining persisted.

Double staining for actin and tubulin (Fig. 5) showed that the stress fibers and microtubules in the cell interior, although distinctly separate structures, were often in roughly parallel alignment along the long axis of a cell. Microtubules were present in much lower density near the cell periphery than in the interior and were generally depleted or absent from microspikes and ruffles. Whereas some microtubules extended into the regions of cell–cell contact, there was no regularity to their distribution in these regions.

DISCUSSION

The intracellular distributions of the mechanochemical proteins studied in this paper, and of others as well, have previously been individually investigated with fluorescence staining methods by several investigators (2, 3, 11–13). Such single staining experiments have yielded important results, but some of the conclusions drawn from our experiments could not have been derived in the absence of double staining. The double staining was facilitated by the development of a sensitive nonantibody method for the specific staining of actin (5), which allowed us to use an indirect immunofluorescence technique for the second stain.

Considering the three proteins, actin, myosin, and filamin, in NRK fibroblasts, we have observed several different kinds of distributions, which will be discussed in more detail elsewhere. For the purposes of the present paper, however, we wish only to distinguish between the distributions found in certain specialized regions of the cell periphery and those found in the rest of the cell body. Whereas all three proteins are present in most of the cell body (in part, on the same stress fibers), by contrast in basal regions of microspikes, in ruffles, and in regions of cell–cell contact, substantial amounts of actin and filamin are present, but myosin is greatly diminished or absent. We have obtained very similar results with human WI-38 fibroblasts. Consistent with the absence of myosin from these specialized peripheral regions, Lazarides (13) has recently shown that tropomyosin is also diminished or absent in the cell ruffles of cultured myoblasts and fibroblasts. Microtubules do not appear to play any role in these specialized peripheral regions.

These results are particularly interesting because of the functional relationships that have been proposed between microspikes and ruffles and between ruffles and regions of cell–cell contact.

Microspikes and ruffles are both highly dynamic structures, forming and retracting at different sites on the cell surface. Time-lapse photography of living fibroblasts (14) has suggested that the rapid extension and retraction of several microspikes generally precede the formation of a ruffle on the same region of the cell surface. Ruffles are pancake-like structures that lift off the surface of the substrate to which the cell is attached and appear to be the principal motile apparatus of the cell. When a cell is isolated from contact with other cells, such ruffles perform oscillatory motions until they retract or become anchored to the substrate. But when two cells make contact by their respective ruffles, ruffling stops at the contact region (contact inhibition of motility) (15) and the cells remain so attached for some time. These considerations suggest therefore that microspikes, ruffles, and regions of cell–cell contact all arise sequentially at the same areas on the cell surface. The absence of myosin from all three may therefore reflect this spatial and functional relationship among them.

When the ruffles of two normal cells make contact, leading to an inhibition of motility, it has been shown by electron microscopy (16, 17) that fiber bundles rapidly appear within the two ruffle regions. Our studies suggest that these fiber bundles differ from the stress fibers found in the cell interior in that they do not contain myosin. The presence of actin and filamin in ruffles, however, is especially interesting in view of our recent demonstration (18) that filamin and F-actin interact specifically in solution. Mixtures of the two pure proteins rapidly form aggregates that contain bundles of fibers and sheet-like structures made up of F-actin filaments crosslinked by filamin molecules. The interaction of these two proteins may therefore be responsible for the formation of fiber bundles *in vivo* in ruffles that have made contact (and perhaps also in other regions of the cell body). If such were the case, then the contact between ruffles must rapidly convey a signal for the filamin and actin present within the ruffles to interact with one another. This is of interest in connection with malignant transformation, because cancer cells that make contact via their ruffles do not show contact inhibition of motility nor do they form any fiber bundles within the contacting ruffles (19).

If indeed filamin–F-actin interactions are implicated in these cell contact phenomena, the detailed molecular mechanism of the interactions and the signals required to initiate them remain to be discovered.

We are grateful to Dr. J. F. Ash for substantial contributions to the development of these techniques and to Dr. Immo Scheffler for providing cell culture facilities. We thank Ms. Donna Luong and Mr. George Anders for excellent technical assistance. S.J.S. is an American Cancer Society Research Professor, and K.W. was a U.S. Public Health Service Postdoctoral Fellow, 1974–1976. This work was supported by U.S. Public Health Service Grant GM-15971 to S.J.S.

1. Pollard, T. D. & Weihing, R. R. (1974) *C.R.C. Crit. Rev. Biochem.* **2**, 1–65.
2. Lazarides, E. & Weber, K. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2268–2272.
3. Wang, K., Ash, J. F. & Singer, S. J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4483–4486.
4. Wang, K. (1977) *Biochemistry* **16**, 1857–1865.
5. Heggeness, M. H. & Ash, J. F. (1977) *J. Cell Biol.* **73**, 783–788.
6. Sanger, J. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1913–1916.
7. Duc-Nguyen, H., Rosenblum, E. N. & Zeigel, R. F. (1966) *J. Bacteriol.* **92**, 1133–1140.
8. Sheetz, M., Painter, R. G. & Singer, S. J. (1976) *Biochemistry* **15**, 4486–4492.

9. Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 765–768.
10. Brandtzaeg, P. (1973) *Scand. J. Immunol.* **2**, 273–279.
11. Brinkley, B. R., Fuller, G. M. & Highfield, D. P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4981–4985.
12. Pollard, T. D., Fujiwara, K., Niederman, R. & Maupin-Szamier, P. (1976) in *Cell Motility*, eds. Goldman, R. D., Pollard, T. & Rosenbaum, J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. B, pp. 689–724.
13. Lazarides, E. (1977) *J. Supramol. Struct.*, **5**, 531–563.
14. Albrecht-Buehler, G. (1976) in *Cell Motility*, eds. Goldman, R. D., Pollard, T. & Rosenbaum, J. (Cold Spring Harbor Laboratory, Cold Spring , Harbor, NY), Vol. A, pp. 247–264.
15. Abercrombie, M. (1961) *Exp. Cell Res. Suppl.* **8**, 188–198.
16. Heaysman, J. E. M. & Pegrum, S. M. (1973) *Exp. Cell Res.* **78**, 71–78.
17. Goldman, R. D., Schloss, J. A. & Starger, J. M. (1976) in *Cell Motility*, eds. Goldman, R. D., Pollard, T. & Rosenbaum, J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. A, pp. 217–245.
18. Wang, K. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2021–2025.
19. Heaysman, J. E. M. & Pegrum, S. M. (1973) *Exp. Cell Res.* **78**, 479–481.