

## Organization of Actin Meshworks in Cultured Cells: The Leading Edge

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Since the early studies of Buckley and Porter (1967), Spooner et al. (1971), and others (see Goldman et al. 1976), it has been generally recognized that F-actin in cultured cells occurs in at least two distinguishable states of structural organization: in linear fibrillar bundles, normally recognizable in the light microscope and commonly referred to as "stress fibers," and in meshworks or networks, confined to the motile lamella zones and ruffling membranes and thought to be generally present beneath the plasmalemma in a subcortical layer (Spooner et al. 1971; Goldman et al. 1976). In many subsequent studies the existence and general organization of the more obvious stress-fiber bundles, as well as the identification of microfilaments within them as F-actin, have been exhaustively documented by both electron microscopy (Ishikawa 1974; Goldman et al. 1976) and particularly by immunofluorescence microscopy (for first reports, see Lazarides and Weber 1974; Lazarides 1976a,b). In contrast, the more transitory and dynamic network regions have been generally overlooked or have received only passing attention. Rather than being deliberate, this neglect derived from the difficulties encountered in revealing any discernible order in these meshwork regions at the ultrastructural level.

In earlier studies (Small and Celis 1978a,b; Small et al. 1978, 1980) we developed procedures utilizing Triton X-100 extraction, glutaraldehyde fixation, and negative staining that allowed the direct visualization in the electron microscope of the different filament networks in cultured cells; similar procedures were also adopted independently by other investigators (Brown et al. 1976; Osborn et al. 1978). A particularly noteworthy feature of the cytoskeletons so obtained was the preservation of a broad and relatively well-ordered meshwork of actin filaments and actin-filament bundles in the peripheral, normally convex regions of cells, corresponding to the "leading edge" described by Abercrombie et al. (1970). Thus, under appropriate conditions (in the presence of Mg and EGTA and at a low pH of ~6.0), the removal of the cell membrane with detergent left the substrate-associated actin in these peripheral, mobile zones apparently intact. Moreover, the amenability of these thin lamellar regions to negative-staining methods allowed a more detailed analysis of their structural organizations than was previously possible (Höglund et al. 1980; Small et al. 1980).

From careful light microscopy studies (Abercrombie et al. 1970; Izzard and Lochner 1980), the characteristic phases of movement of the leading edge of living fibroblasts in vitro have already been established, but precious little is known about the basis of these movements at the molecular level. We have therefore pursued our analysis of these regions, using mainly chick heart fibroblasts, and here present a synopsis of our findings. In particular, we illustrate the complexity of filament interactions in the leading-edge network, the ease with which the filament order is disrupted by conventional electron microscopy procedures, and finally, the prospects of defining the nature of the various integral proteins and their interactions.

### MATERIALS AND METHODS

**Cells.** Primary cultures of fibroblasts were produced by trypsinization of hearts taken from 14–16-day chick embryos. The cells were maintained and subcultured in Eagle's minimum essential medium with 10% fetal calf serum added and in the presence of 5% CO<sub>2</sub>. In general, fibroblasts were taken for analysis within the first six replatings from the primary culture. For electron microscopy, cells were grown on composite parlodion/carbon films mounted on silver grids (hexagonal, 150 mesh; Tebra, Holland); for light microscopy, the cells were grown on glass coverslips.

**Extraction-fixation procedures.** The preparation procedures used were essentially those described previously by Small and Celis (1978a), with some modifications as introduced by Höglund et al. (1980). The basic buffer system (Small and Celis 1978a), which we refer to as cytoskeleton buffer, was used at a pH of about 6.1. In addition to Triton X-100 we found it advantageous to introduce glutaraldehyde into the cytoskeleton buffer for the initial extraction, as suggested by Höglund et al. (1980). By modifying the ratio of Triton X-100 to glutaraldehyde, different degrees of extraction may be effected; an appropriate ratio was chosen to give optimal preservation, on the one hand, and sufficient penetration of the negative stain, on the other. For heart fibroblasts we found that the most useful mixtures ranged from 0.25% Triton and 0.5% glutaraldehyde to 0.5% Triton and 0.25% glutaraldehyde.

Extractions were carried out in solutions at room

temperature as follows: The grids, or coverslips, were removed from the growth medium, rinsed briefly in two 5-ml reservoirs of cytoskeleton buffer, and then transferred for 1 minute to the Triton-glutaraldehyde mixture. They were then rinsed again in cytoskeleton buffer and transferred to glutaraldehyde in the same buffer (1%, 15 min for coverslips; 2.5%, 10–30 min for grids).

For electron microscopy, incubation for 1 minute in  $2 \times 10^{-5}$  M phalloidin (a gift from T. Wieland, Max-Planck-Institute, Heidelberg) generally preceded the final fixation in glutaraldehyde. Other modifications are indicated in the text.

**Antibody staining and fluorescent probes.** Antibodies to chicken gizzard filamin and  $\alpha$ -actinin (for purity of antigens, see Small and Sobieszek 1980) were raised in rabbits by J. De Mey (Janssen Pharmaceutica, Belgium) and affinity-purified before use. Following the second fixation in glutaraldehyde, the Triton-glutaraldehyde cytoskeletons (0.5% Triton and 0.25% glutaraldehyde) were treated for 5 minutes in  $\text{NaHBO}_4$  (Weber et al. 1978) in cytoskeleton buffer, and, after washing, they were treated for a further 30 minutes in 0.5% Triton X-100 before incubation with the first antibody. The antibodies were diluted in Tris-buffered saline with 1% normal goat serum added to avoid non-specific binding. The second antibody was a fluorescein-labeled goat anti-rabbit IgG obtained from Behring.

Staining for actin was carried out using an antibody to gizzard actin or using a fluorescent derivative of phalloidin (Wulf et al. 1979) obtained from T. Wieland. In the latter case, coverslips were incubated in  $1 \times 10^{-5}$  M to  $2 \times 10^{-5}$  M fluorescent phalloidin in cytoskeleton buffer for 2 minutes following the initial Triton-glutaraldehyde extraction.

After mounting in Gelvatol 20-30 (Monsanto Polymers and Petrochemical), the preparations were observed and photographed in a Zeiss Photomicroscope III equipped with epi-illumination.

**Electron microscopy.** Grids taken from the final glutaraldehyde fixation were rinsed twice in distilled water and negatively stained in aqueous 1% uranyl acetate, according to the method of Small and Celis (1978a), or in 3% sodium silicotungstate (Höglund et al. 1980; Small 1981).

To investigate the effects of  $\text{OsO}_4$  and dehydration on the integrity of the actin networks, grids were taken after glutaraldehyde fixation and either treated with  $\text{OsO}_4$  or dehydrated in an acetone or ethanol series before final negative staining. Negative staining was then done in aqueous uranyl acetate, as above, or in the latter case, in 1% uranyl acetate in methanol. Details of the procedures used are described elsewhere (Small 1981).

**Removal of actin using the actin-depolymerizing protein from slime mold.** For the selective removal of the leading-edge meshworks, unfixed cytoskeletons were

used. These cytoskeletons could be produced by extraction in 0.05% Triton in cytoskeleton buffer for 40–60 seconds at room temperature. We found, however, that the retention of the leading-edge regions was more reproducible when phalloidin ( $2 \times 10^{-5}$  M) was introduced into the extraction buffer. In this case a minute growth of actin filaments occurred at the periphery of the actin meshworks, but this may be disregarded for the purpose of the present report. Following a wash in cytoskeleton buffer, the cells were treated at room temperature for different times in 0.4 mg/ml of the actin-modulator protein (Hinssen 1981a,b) in cytoskeleton buffer with added 2 mM  $\text{CaCl}_2$  at pH 6.5. (Since the cytoskeleton buffer contained 2 mM EGTA, the final  $\text{Ca}^{++}$  concentration was in the micromolar range.) Control preparations were treated in the same buffer without the protein.

**Cinematography.** Time-lapse recordings of living fibroblasts were taken using a simple flowthrough chamber operating at 37°C and with a Bolex 16-mm camera operating at 2 frames/sec.

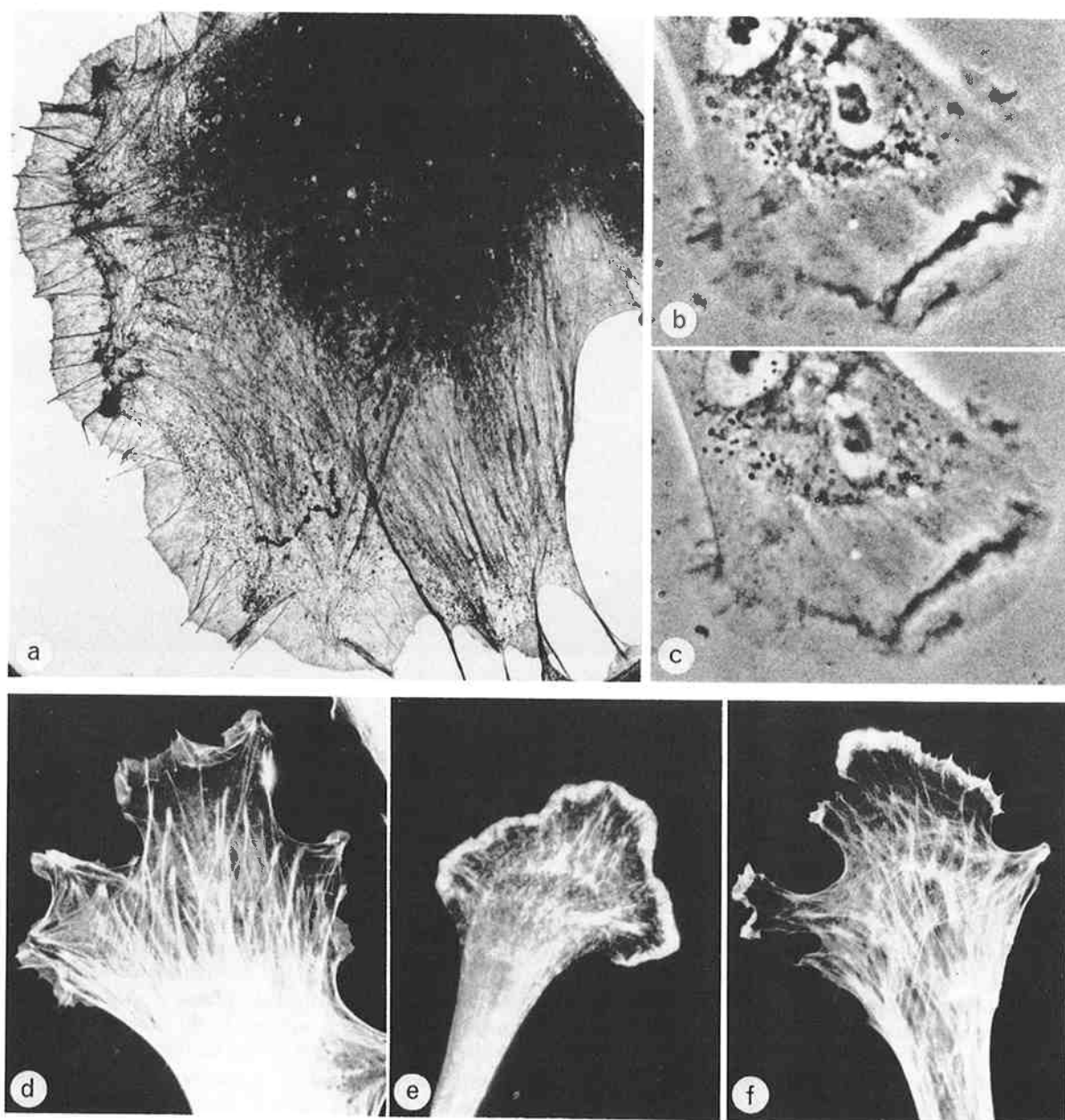
## RESULTS

### Evaluation of Extraction Procedures

The fixation of cells with glutaraldehyde alone, followed by negative staining or by critical-point drying (see Wolosewick and Porter 1979), reveals variable numbers of microvilli and membrane ruffles on the dorsal surfaces of cultured cells. Such structures, which have been well documented in earlier studies by light microscopy, are seen with particular clarity in the scanning electron microscope (see, e.g., Vasiliev and Gelfand 1977). After Triton extraction or Triton-glutaraldehyde treatment, these surface structures are lost and only the inner cytoskeleton and the peripheral, substrate-associated lamellae (Figs. 1a and 2), as well as the ruffles attached directly to them, remain. Studies by these methods are therefore restricted by necessity to the substrate-associated zones and to ruffles that, by chance, collapse onto the substrate during extraction.

Using cinematographic analysis we established that the Triton-glutaraldehyde mixtures were able to arrest cell movement without any recognizable morphological distortions at the level of resolution offered by the light microscope. This is shown in Figure 1 b and c in two consecutive frames of movie film taken at 0.5-second intervals before and after initiation of fixation. Thus, we could be confident that the structures observed in the electron microscope (see below) would reflect a phase in the motile process.

In a separate study using HeLa cells (Bravo et al. 1981), it was established that extraction with 0.1% Triton X-100 in our cytoskeleton buffer releases into the supernatant, among various other proteins, 50% total actin, 70% total tubulin, and 60%  $\alpha$ -actinin. For technical reasons it is not possible to quantitate the



**Figure 1.** General morphology and composition of Triton-glutaraldehyde cytoskeletons of chick heart fibroblasts. (a) Low-power electron micrograph of 0.5% Triton/0.25% glutaraldehyde cytoskeleton negatively stained with uranyl acetate. Magnification,  $5300\times$ . (b,c) Sequential frames in a movie sequence taken at 2 frames/sec and corresponding to the time of extraction-fixation. (b) Living cell; (c) extracted cell. Recording was done at  $37^{\circ}\text{C}$ , and the extraction mixture used was 0.3% Triton and 0.5% glutaraldehyde. Magnification,  $1140\times$ . (d-f) Triton-glutaraldehyde cytoskeletons labeled with fluorescent phalloidin (d), antibody to smooth-muscle  $\alpha$ -actinin (e), and antibody to smooth-muscle filamin (f). Magnifications: (d)  $750\times$ ; (e)  $1250\times$ ; (f)  $790\times$ .

loss of protein during extraction in the presence of glutaraldehyde; however, we may presume that less of the structural protein is lost under these conditions. Consistent with this was the presence of a generally more dense network of filaments in the leading-edge region of Triton-glutaraldehyde cytoskeletons than in those prepared with Triton alone. Moreover, it could be shown by procedures using antibody that indeed a considerable amount of the actin-associated proteins,

filamin, and  $\alpha$ -actinin remains in the Triton-glutaraldehyde cytoskeletons (Fig. 1d-f).

#### Ultrastructure of the Leading Edge

The general structural characteristics of the leading-edge region have been reported previously (Small and Celis 1978a; Small et al. 1978, 1980) and are illustrated in Figures 2 and 3. The leading edge is clearly delin-

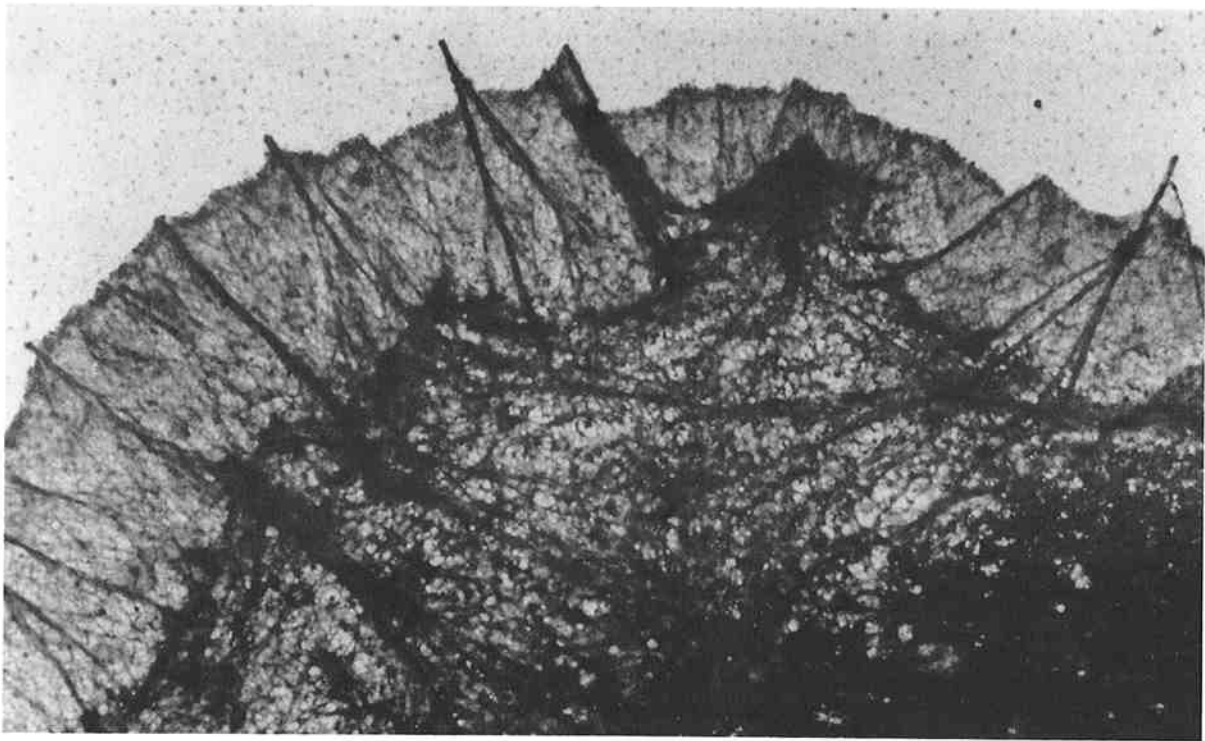


Figure 2. Survey view of the leading-edge region of a cell prepared as in Fig. 1. Magnification, 20,000 $\times$ .

eated from the inner cytoskeleton, both in electron micrographs and in immunofluorescence images of cells labeled for actin,  $\alpha$ -actinin, and filamin (Fig. 1d-f; see also Discussion). The transition from the main cytoskeleton to the leading edge is also very clear in living cells viewed under Nomarski interference optics (Izzard and Lochner 1980).

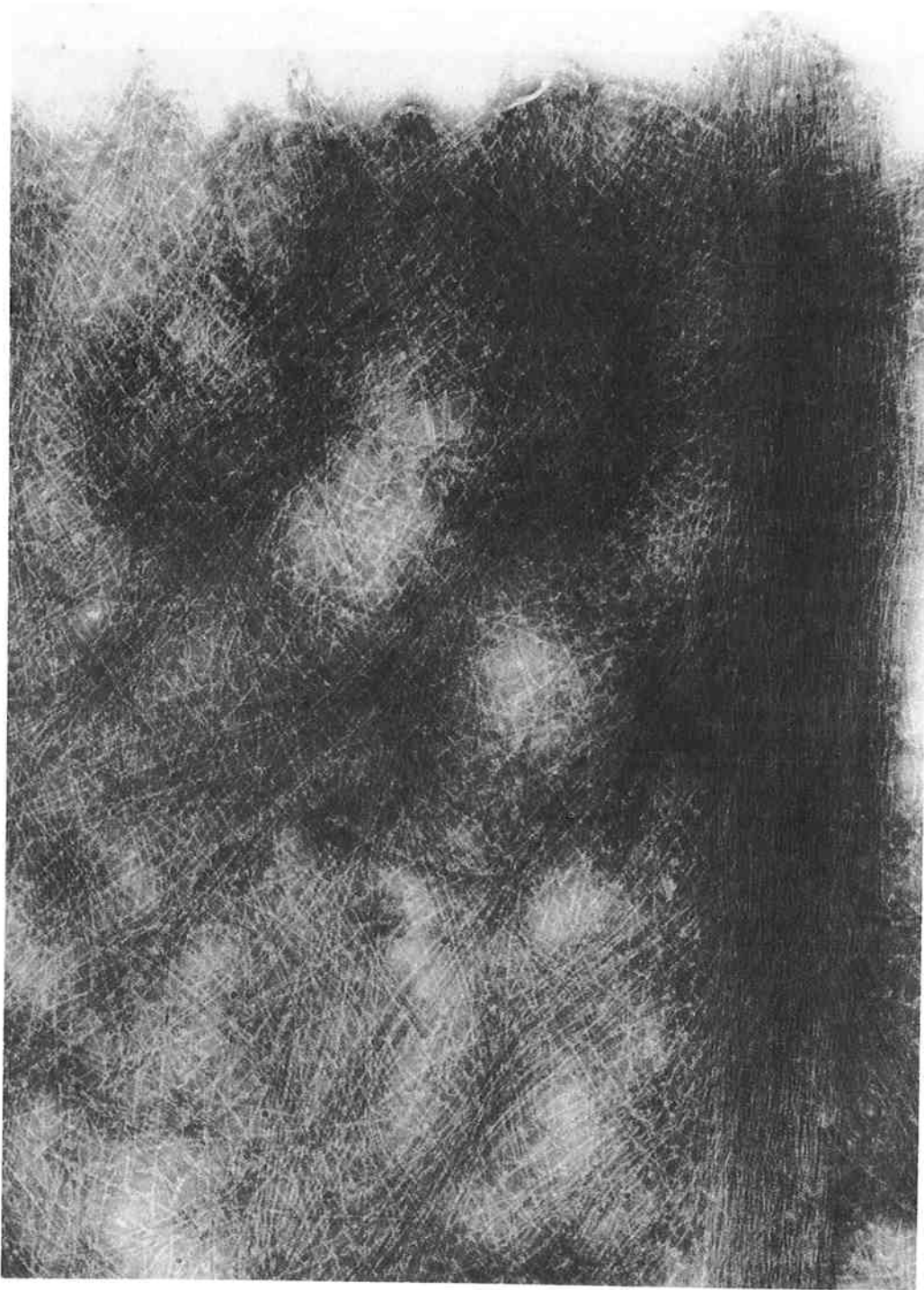
Within the leading edge itself, actin filaments predominate, intermediate filaments being totally excluded and microtubules occurring only rarely (see later). Two organizational forms of actin filaments occur: actin meshworks and actin-filament bundles of about 0.1  $\mu\text{m}$  to 0.2  $\mu\text{m}$  in diameter (Figs. 2 and 3). When they project from the cell boundary, the actin-filament bundles correspond to the filopodia seen in the light microscope (for references, see Albrecht-Buehler 1976). For convenience we shall include all the bundles seen in the leading edge under the category of microspikes (Weiss 1961) whether or not they project beyond the border of the meshwork.

After negative staining with sodium silicotungstate the organization of actin in the leading edge is particularly clear (Figs. 3-5) (Höglund et al. 1980; Small 1981) and can be shown with the use of stereo electron micrographs to preserve a certain degree of three-dimensional order (Fig. 4). We also found that the treatment of fixed cytoskeletons with phalloidin resulted in a marginally better preservation of the filament networks, and we now routinely use such a preparation step. That phalloidin binds to the glutaraldehyde-fixed cytoskeletons is shown by the use of the fluorescent

derivative (Wulf et al. 1979) with cells processed on glass coverslips (Fig. 1d). The reason for the slight improvement of order is not known, although in other situations, phalloidin has been shown to have a general stabilizing effect on F-actin (Wieland 1977).

From single micrographs, one has the impression that individual filaments in the meshworks extend from the cell edge across to the opposite side of the leading edge. However, because of the numerous junctions in the network, it is difficult to follow a single filament unambiguously from one end to the other. The study of stereo pairs (Fig. 4) brings to light an even more complex arrangement of filaments within the meshworks. From these views a three-dimensional cross-linking of filaments can be seen, although the precise details of the junctions, e.g., whether or not they involve filament ends, are unclear. It is apparent, however, that the extra particulate material present on the filaments and presumably representing actin-associated proteins is in some way involved in filament cross-linking. In addition, we have noted the common occurrence of actin filaments running in pairs or "doublets" with an interfilament separation of 75-80 Å; such doublets are particularly common around and within the microspikes.

A feature that is also evident from the micrographs is the origination of the microspikes from the meshworks (and perhaps also vice versa) and all transition stages between the two (Fig. 5a,b; Small et al. 1980). In this respect we may now draw attention to the anterior border of the leading edge, corresponding to the



**Figure 3.** Leading edge of 0.5% Triton/0.25% glutaraldehyde cytoskeleton postincubated in phalloidin and stained with sodium silicotungstate showing the details of the actin meshwork and a microspike. Magnification, 102,000 $\times$ .

position of the leading membrane prior to extraction. At this border the meshwork filaments do not terminate at random but converge at specific foci that invariably exhibit extra globular material (Fig. 5a). The same material is found at the tips of the microspikes (Fig. 4). From this and other considerations (see Discussion), we conclude that a collection of groups of the

meshwork filaments constitutes an initial step in the formation of the microspikes.

As already indicated, microtubules are normally absent from the leading edge; however, some likely significant exceptions are worth noting. First, in leading-edge regions for which the meshwork zones are large and uninterrupted by microspikes, microtubules may



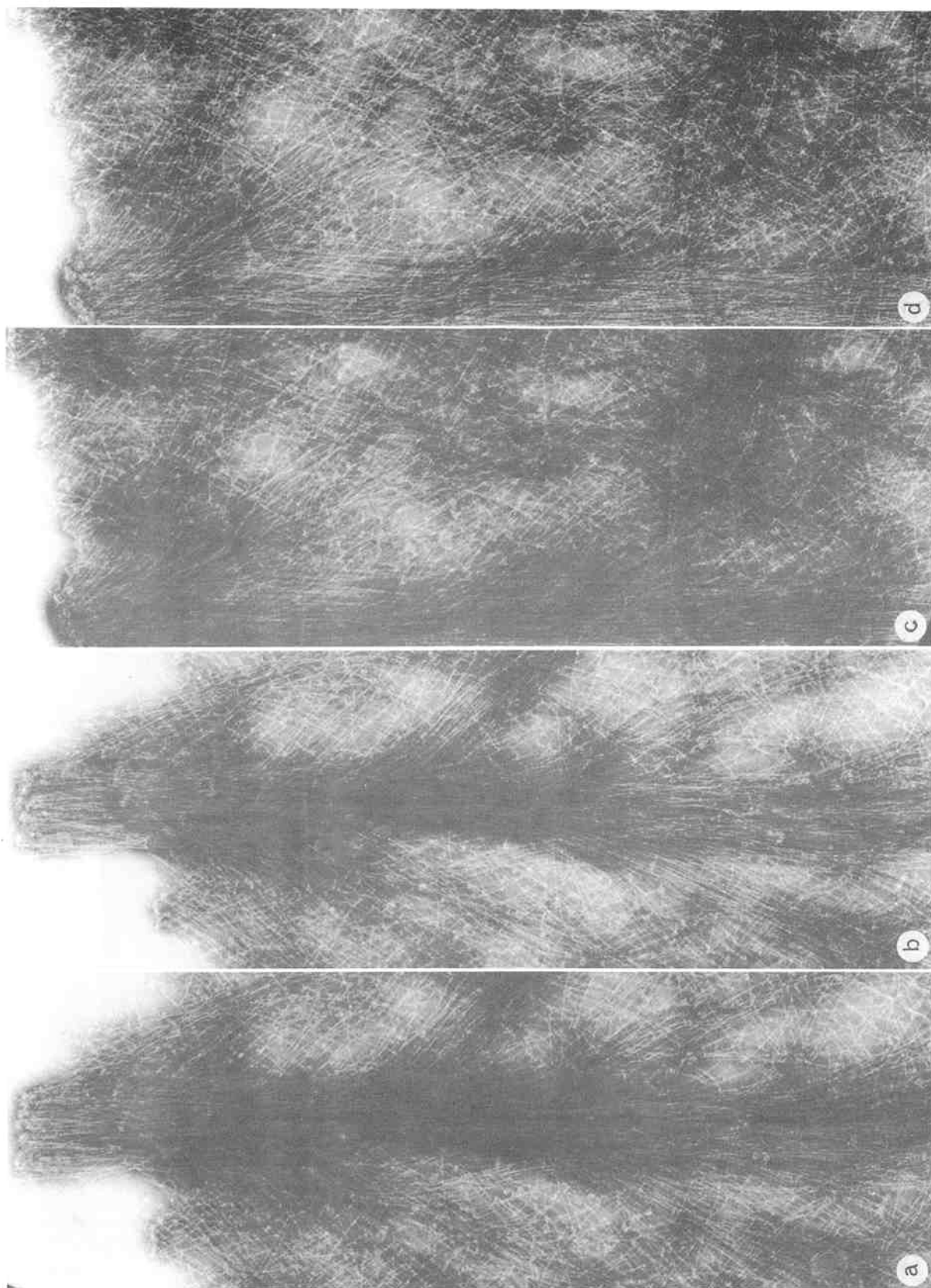
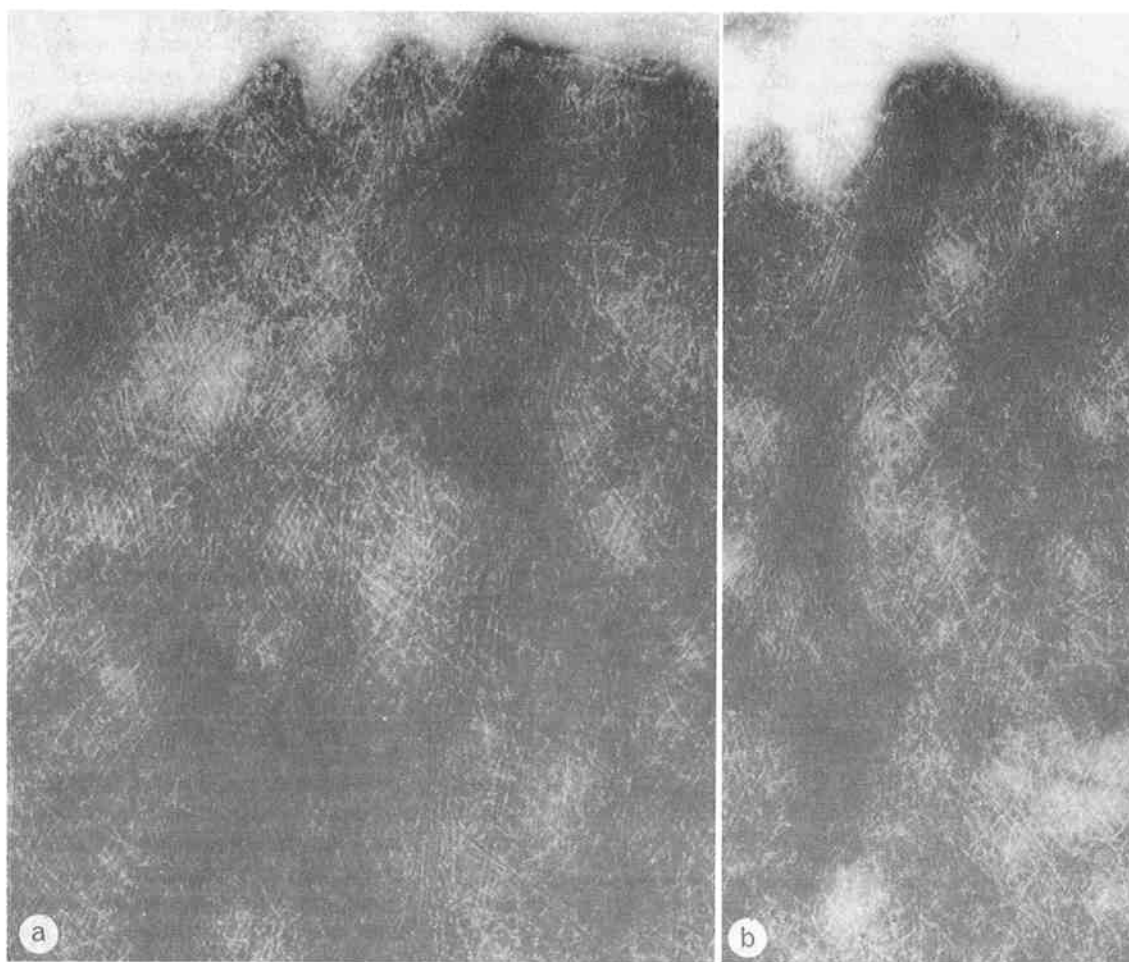


Figure 4. (See facing page for legend.)



**Figure 5.** Foci of actin-filament convergence at the leading edge (a) and a transition stage between converging filament groups and a microspike (b). Note the extra globular material at the focal points. Magnifications: (a) 99,000 $\times$ ; (b) 79,000 $\times$ .

often be found penetrating well into the leading edge and sometimes extending across more than half its width. Although no clear rearrangement of actin filaments around the microtubule ends has yet been detected, some interaction with actin seems likely to occur via extra flocculent material, presumably introduced, directly or indirectly, by the microtubule (Fig. 6). In other instances, microtubules may be found terminating at the base of microspikes or running along beside them to terminate at some point removed from the microspike tip.

#### **Sensitivity of Actin-meshwork Organization to Postfixation and Dehydration Procedures**

In another report we have considered in some detail the effects of  $\text{OsO}_4$  postfixation and dehydration in organic solvents on the integrity of actin meshworks

in the leading edge (Small 1981). In view of the relevance of the findings to the general problem of preserving actin meshworks for electron microscopy, the main findings are summarized here.

After postfixation of glutaraldehyde-fixed (non-phalloidin-treated) cytoskeletons in 1%  $\text{OsO}_4$  for 7 minutes at room temperature, the actin meshworks appear as shown in Figure 7a. The actin filaments both in the meshworks and in the microspikes are distorted and kinked in a fashion resembling that shown for muscle F-actin similarly treated (Maupin-Szamier and Pollard 1978). Thus, the presence of actin-associated proteins such as  $\alpha$ -actinin and filamin in the leading edge (Fig. 1e,f) does not offer any protection against  $\text{OsO}_4$  degradation. It is worth noting that the effect is specific for the meshwork regions and microspikes, the linearity of the filaments in the stress fibers being essentially unaffected.

**Figure 4.** Stereo electron micrograph pairs of leading-edge regions (prepared as in Fig. 3) demonstrating the three-dimensional nature of the filament arrangements. (a,b) Arrangement of filaments in the region of a microspike. Note the entry of filaments into the spike from different levels and neighboring regions of cross-linked filaments. (c,d) Degree of cross-linking evident in the meshwork regions. Magnifications: (a,b) 80,000 $\times$ ; (c,d) 90,000 $\times$ .



**Figure 6.** Microtubules entering into a meshwork region of the leading edge and showing peripheral flocculent material. Magnification, 75,000 $\times$ .

Dehydration of cytoskeletons in ethanol or acetone also has a destructive effect (Fig. 7b). In this case the filaments become rearranged and aggregated so that little of the original order remains. The final picture is similar whether one stains in methanol-uranyl acetate at the end of dehydration (Fig 7b) or whether one rehydrates and stains in aqueous solution (Small 1981).

For conventional embedding or critical-point-drying procedures, both  $\text{OsO}_4$  postfixation and ethanol or acetone dehydration are routinely employed. For cytoskeletons prepared by the critical-point-drying method (Small 1981), the leading-edge region bears a close resemblance to regions of the microtrabecular lattice described by Wolosewick and Porter (1979).

#### Selective Extraction of Actin-containing Structures Using an Actin-depolymerizing Protein

Although additional components of the actin-containing structures of cultured cells have been identified using antibodies to proteins taken from either muscle, normally smooth muscle, or other tissues, direct approaches to define the actin-associated proteins, for example, in the stress fibers and in the actin meshworks have been seldom undertaken (Schloss and Goldman 1980).

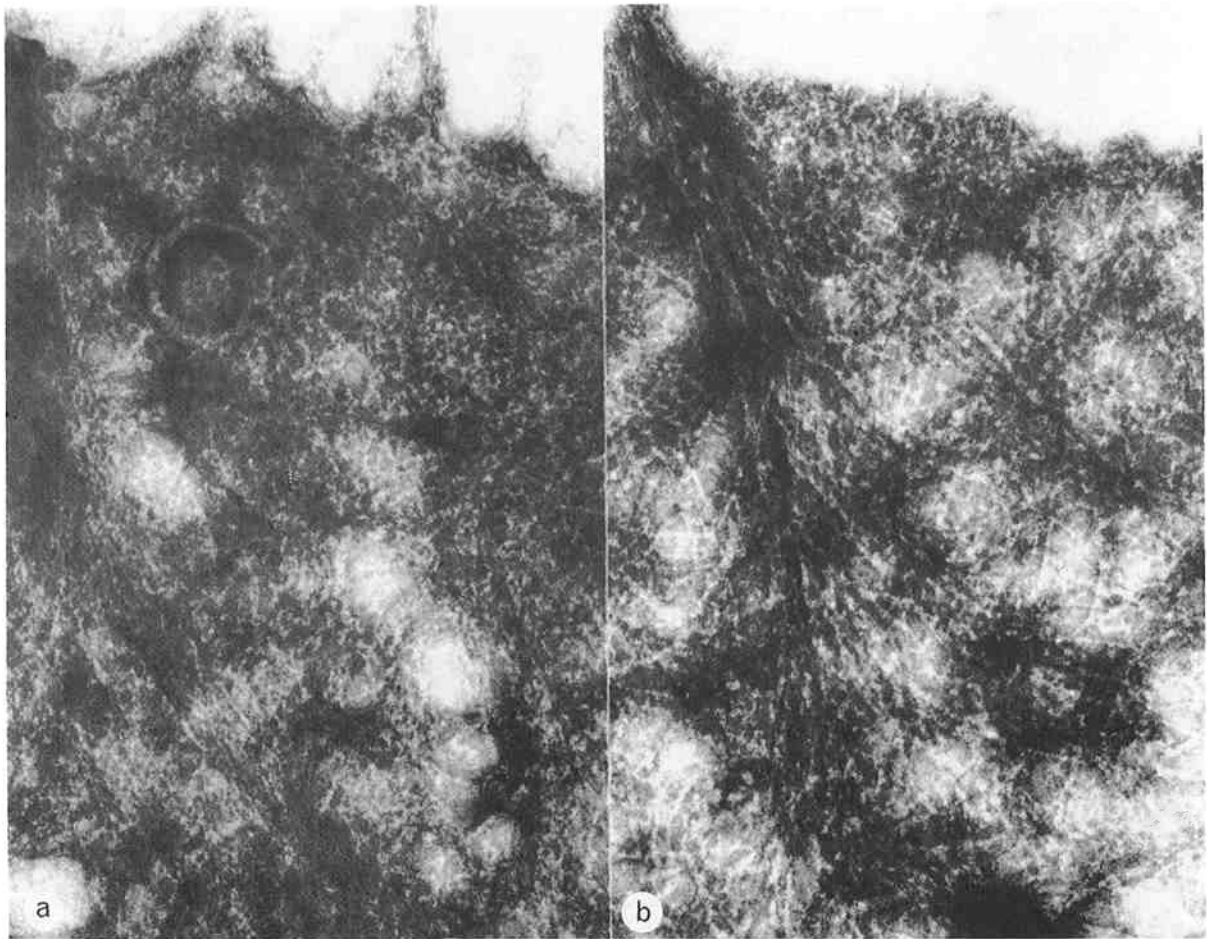
In a more recent study (Bravo et al. 1981) we were able to show that the residual actin remaining in cytoskeletons extracted in high salt could be efficiently removed using the actin-modulator protein (Hinssen 1981a,b) or fragmin (Hasegawa et al. 1980) from slime mold. We have therefore investigated the relative sensitivities of the actin meshworks (i.e., the leading edge) and the stress-fiber bundles to this depolymerizing protein. The first results are illustrated in Figure 8. Figure 8, a and b, shows cytoskeletons extracted with Triton X-100 (see Materials and Methods) and treated for 20 seconds in  $\text{Ca}^{++}$ -containing buffer or with the actin-modulator protein in the same buffer. Under the latter conditions the leading-edge meshwork and any loosely bound actin are removed, leaving the stress fibers mainly intact. Treatment of the remaining cytoskeletons for a further 4 minutes releases the stress fibers into the supernatant and leaves the intermediate-filament net attached to the substrate (Fig. 8c). Analysis of these supernatants by two-dimensional gel electrophoresis (Bravo et al. 1981) is in a preliminary stage but already indicates the feasibility of this approach to search for actin-associated proteins.

#### DISCUSSION

From the rapidity of arrest of fibroblasts, relative to the rate of movement, as well as the faithful retention of cell morphology with the glutaraldehyde-Triton mixtures, we conclude that the images obtained of the leading edge reflect the true ultrastructure of this region. In this context the effects of  $\text{OsO}_4$  postfixation and dehydration on actin networks are perhaps alarming. It could have been argued that the effects of  $\text{OsO}_4$  on muscle actin, reported by Maupin-Szamier and Pollard (1978), were not applicable to cellular actin where it is complexed with various other proteins. But this is not the case. Moreover, dehydration itself has as dramatic an effect as  $\text{OsO}_4$  on actin networks. Since it is the actin networks and not the stress fibers that suffer under these agents (Small 1981), the effect has escaped general attention. This is not the place to discuss the implications of these findings in detail; we shall only point out the striking difference in order between actin networks in the leading edge prepared by the present procedures and that observed in cells or cytoskeletons prepared by the critical-point-drying method (Wolosewick and Porter 1979; Small 1981).

The leading edge and membrane ruffles of cultured cells were previously shown to be rich in actin by immunofluorescence microscopy (Lazarides 1976a,b); they were also shown to react with antibodies to  $\alpha$ -actinin from smooth muscle (Geiger 1979), filamin from smooth muscle (Heggeness et al. 1977), and fimbrin from the intestinal brush border (Bretscher and Weber 1980a). With our filamin antibody we have noted a difference in the distribution of staining in the leading edge between cells fixed in glutaraldehyde and cells fixed in glutaraldehyde-Triton mixtures. In the former



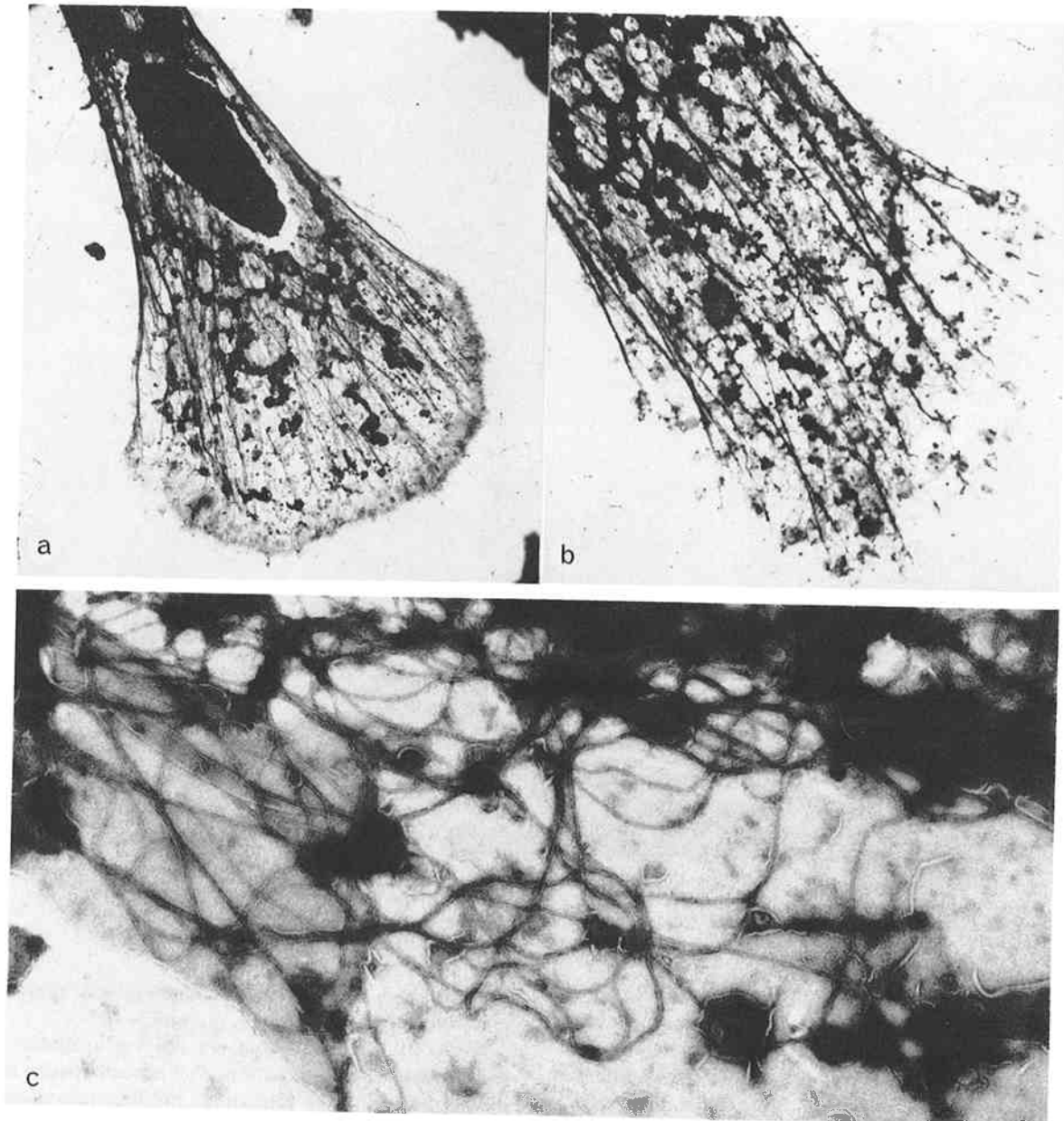


**Figure 7.** Effects of  $\text{OsO}_4$  (a) and dehydration (b) on the integrity of the actin meshworks of Triton-glutaraldehyde cytoskeletons. (a) After postfixation in 1%  $\text{OsO}_4$  for 7 min at room temperature and uranyl acetate staining; (b) after dehydration in ethanol and negative staining in methanol-uranyl acetate (see text). Magnifications: (a)  $104,000\times$ ; (b)  $79,000\times$ .

case the staining is restricted to the rear part of the leading edge, as described earlier by Heggeness et al. (1977), whereas Triton-treated cells show strong overall staining, coincident with that found for actin. We conclude that Triton makes the presumably less accessible antigen in the forward region of the leading edge and in the microspikes available to the antibody. Further comments on the composition of the leading-edge region should include a note on the absence of tropomyosin (Lazarides 1976b), as well as of myosin (Weber and Gröschel-Stewart 1974; Heggeness et al. 1977), and the presence, in discrete regions, of vinculin (Geiger 1979). We can expect further additions to this current list, especially among the class of proteins that exhibit a  $\text{Ca}^{++}$ -dependent effect on the polymer state of actin (Mimura and Asano 1979; Yin and Stossel 1979; Bretscher and Weber 1980b; Craig and Powell 1980; Harris and Gooch 1980; Hasegawa et al. 1980; Hinssen 1981a,b), and that likely play significant regulatory roles in motile processes. Indirect evidence for the presence of such factors in fibroblasts comes from the observed disruption of the leading edge of Triton

cytoskeletons after prolonged treatment with micromolar concentrations of  $\text{CaCl}_2$  (unpubl.).

Evidently, sufficient data are not yet available to piece together the interactions that occur between actin and its associated proteins to produce movement. However, from the absence of myosin (Heggeness et al. 1977) and from the single polarity of actin filaments in the leading edge (Small and Celis 1978a; Small et al. 1978), we have suggested (Small et al. 1978, 1980) that the controlled polymerization of actin, as in some other systems (Tilney 1975; Lindberg et al. 1978), forms the basis of the forward extension of the leading membrane of cultured cells. This is illustrated in a very schematic way in Figure 9. As shown most clearly by Izzard and Lochner (1980), the leading edge in its phase of forward growth is lifted slightly above the substrate. We propose that this growth results from the inward polymerization of actin filaments, initiated at the membrane sites or foci that we have described here and previously (Small et al. 1980). Höglund et al. (1980) have also drawn attention to the same sites. Concomitant with filament growth would be a cross-linking of actin fil-

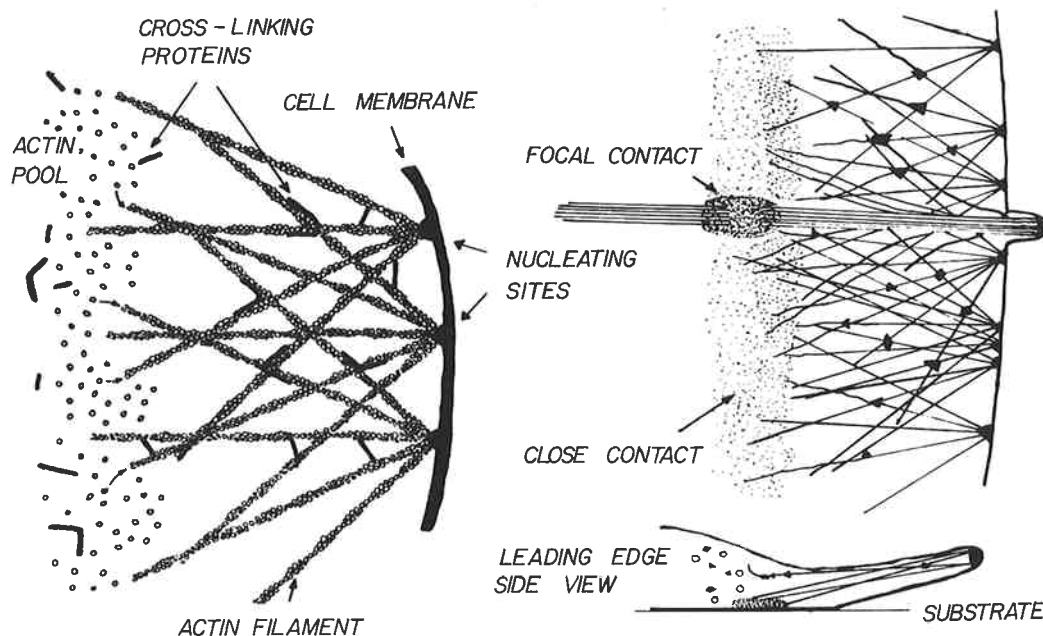


**Figure 8.** Dissection of actins in the cytoskeleton using the actin-modulator protein from slime mold. (a) Control Triton cytoskeleton treated for 20 sec in Ca-containing buffer. (b) Cytoskeleton treated as in a, but incubated for 20 sec in the same buffer containing 0.4 mg/ml of actin-modulator protein. The leading edge is removed, leaving the stress fibers intact. (c) Periphery of cell treated as in b, but for 4 min in the presence of the actin-modulator protein. Only the intermediate filaments remain. All stained with uranyl acetate. Magnifications: (a) 5000 $\times$ ; (b) 6300 $\times$ ; (c) 33,000 $\times$ .

aments by proteins like filamin and  $\alpha$ -actinin to form a continuous, temporarily stable network. To effect forward movement, this growing network could exert traction against the inner cytoskeleton or immobilized regions of substrate attachment, corresponding to the "close" or "focal" contacts seen by interference reflection microscopy (Abercrombie and Dunn 1975; Izard and Lochner 1980).

Can we draw any conclusions about the ends of the actin filaments at which polymerization may take

place? After decoration with myosin subfragment 1, the actin filaments in the leading edge show arrowhead complexes that point toward the cell center (Small and Celis 1978a; Small et al. 1978). By an extrapolation of the results obtained for F-actin in vitro (Woodrum et al. 1975; Hayashi and Wallace 1976), we might expect growth of actin to occur at the "fast-growing" end (see, e.g., Kirschner 1980) associated with the leading membrane. However, as emphasized by Tilney (1975), the in vitro system containing actin as the only protein



**Figure 9.** Schematic diagram indicating a possible mechanism for extension of the leading edge in the motile process. The total locomotion scheme, including the forward translocation of the trailing cell body by the stress fibers, is not shown. The inward growth of actin filaments from nucleating sites on the cell membrane is accompanied by cross-linking with actin-associated proteins ( $\Delta$ ,  $\diamond$ ). The inwardly growing filaments bear against stationary regions of the cytoskeleton, or substrate attachment zones (close or focal contacts), to produce forward movement. Lateral association of filament groups initiated at the cell membrane leads to the formation of microspikes. Such spikes may signal the laying down of focal contacts (Izzard and Lochner 1980).

component is far removed from the *in vivo* situation; recent studies have shown that actin-binding proteins occur that can readily block the addition of G-actin monomers to the fast-growing end (Isenberg et al. 1980). Since the addition of monomers to the free "slow-growing" end (away from the membrane) raises fewer questions as to how a diagonal net of filaments extends itself in two dimensions, we find this situation more plausible. Experiments are now being designed to answer this question.

The images of the actin-filament meshworks seen in the leading edge in the electron microscope fit well with the dynamic activity and rearrangements seen in this region in the living cell (Abercrombie et al. 1970; Izzard and Lochner 1980). The arrangement of the nets suggests a great flexibility in filament reorganization, consistent with the rapid and reversible formation of microspikes and upfolding or ruffling activities. The microspikes themselves are apparently not required for the forward extension of the leading edge but appear in some way to predetermine the position of close-contact sites behind it (Izzard and Lochner 1980). A similar rearrangement of filaments from networks into bundles has been described in the coelomocytes of the sea urchin (Edds 1977; Otto et al. 1979). As in these latter studies we conclude that microspikes form by a lateral collection of filaments that is initiated at the distal end associated with the membrane.

Earlier attempts to correlate the phases of movement

of the leading edge seen in the light microscope with the ultrastructure seen in the electron microscope have, in retrospect, been generally hindered by the poor preservation of actin networks achieved with plastic-embedded material (Buckley 1974; Heath and Dunn 1978). By using the types of methods described here, together with the further biochemical "dissection" of the leading edge, we can be optimistic that more clues about the basis of the locomotory process will emerge.

#### ACKNOWLEDGMENTS

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#### REFERENCES

- ABERCROMBIE, M. and G. A. DUNN. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp. Cell Res.* 92: 57.

- ABERCROMBIE, M., J. E. M. HEAYSAN, and S. M. PEGRUM. 1970. The locomotion of fibroblasts in culture. I. Movements of the leading edge. *Exp. Cell Res.* **59**: 393.
- ALBRECHT-BUEHLER, G. 1976. The function of Filopodia in spreading 3T3 mouse fibroblasts. *Cold Spring Harbor Conf. Cell Proliferation* **3**: 247.
- BRAVO, R., J. V. SMALL, S. J. FEY, P. MOSE LARSEN, and J. E. CELIS. 1981. Architecture and polypeptides of HeLa cell cytoskeletons. Modification of cytoarchitectural polypeptides during mitosis. *J. Mol. Biol.* (in press).
- BRETSCHER, A. and K. WEBER. 1980a. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* **86**: 335.
- . 1980b. Villin is a major protein of the microvillus cytoskeleton which binds both G and F-actin in a calcium-dependent manner. *Cell* **20**: 839.
- BROWN, S., W. LEVINSON, and J. A. SPUDICH. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* **5**: 119.
- BUCKLEY, I. K. 1974. Subcellular motility: A correlated light and electron microscopic study using cultured cells. *Tissue Cell* **6**: 1.
- BUCKLEY, I. K. and K. R. PORTER. 1967. Cytoplasmic fibrils in living cultured cells. *Protoplasma* **64**: 349.
- CRAIG, S. W. and L. D. POWELL. 1980. Regulation of actin polymerisation by villin, a 95,000 dalton cytoskeletal component of intestinal brush borders. *Cell* **22**: 739.
- EDDS, K. T. 1977. Dynamic aspects of filopodial formation by reorganisation of microfilaments. *J. Cell Biol.* **73**: 479.
- GEIGER, B. 1979. A 130K protein from chicken gizzard: Its localisation at the termini of microfilament bundles in cultured chicken cells. *Cell* **18**: 193.
- GOLDMAN, R. D., J. A. SCHLOSS, and J. M. STARGER. 1976. Organizational changes of actinlike microfilaments during animal cell movement. *Cold Spring Harbor Conf. Cell Proliferation* **3**: 217.
- HARRIS, H. E. and J. GOOCH. 1980. An actin depolymerizing protein from pig plasma. *FEBS Lett.* **123**: 49.
- HASEGAWA, T., S. TAKAHASHI, H. HAYASHI, and S. HATANO. 1980. Fragmin: A calcium ion sensitive regulatory factor on the formation of actin filaments. *Biochemistry* **19**: 2677.
- HAYASHI, T. and A. WALLACE. 1976. Polymerization of actin. *J. Mechanochem. Cell Motil.* **3**: 163.
- HEATH, J. P. and G. A. DUNN. 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron microscope study. *J. Cell Sci.* **29**: 197.
- HEGGENESS, M. H., K. WANG, and S. J. SINGER. 1977. Intracellular distributions of mechanochemical proteins in cultured fibroblasts. *Proc. Natl. Acad. Sci.* **74**: 3883.
- HINSSEN, H. 1981a. An actin-modulating protein from *Physarum polycephalum*. I. Isolation and purification. *Eur. J. Cell Biol.* **23**: 225.
- . 1981b. An actin-modulating protein from *Physarum polycephalum*. II.  $\text{Ca}^{2+}$ -dependence and other properties. *Eur. J. Cell Biol.* **23**: 234.
- HÖGLUND, A.-S., R. KARLSSON, E. ARRO, B.-E. FREDRIKSSON, and U. LINBERG. 1980. Visualisation of the peripheral weave of microfilaments in glia cells. *J. Muscle Res. Cell Motil.* **1**: 127.
- ISENBERG, G., U. AEBI, and T. D. POLLARD. 1980. An actin-binding protein from *Acanthamoeba* regulates actin filament polymerisation and interactions. *Nature* **288**: 455.
- ISHIKAWA, H. 1974. Arrowhead complexes in a variety of cell types. In *Exploratory concepts in muscular dystrophy* (ed. A. T. Milhoret), vol. 2, p. 37. Excerpta Medica, Amsterdam.
- IZZARD, C. I. and L. R. LOCHNER. 1980. Formation of cell-to-substrate contacts during fibroblast motility: An interference reflexion study. *J. Cell Sci.* **42**: 81.
- KIRSCHNER, M. W. 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo. *J. Cell Biol.* **86**: 330.
- LAZARIDES, E. 1976a. Aspects of the structural organization of actin filaments in tissue culture cells. *Cold Spring Harbor Conf. Cell Proliferation* **3**: 347.
- . 1976b. Two general classes of cytoplasmic actin filaments in tissue culture cells: The role of tropomyosin. *J. Supramol. Struct.* **5**: 531.
- LAZARIDES, E. and K. WEBER. 1974. Actin antibody: The specific visualisation of actin filaments in non-muscle cells. *Proc. Natl. Acad. Sci.* **71**: 2268.
- LINDBERG, U., L. CARLSSON, F. MARKEY, and L. E. NYSTRÖM. 1978. The unpolymerised form of actin in non-muscle cells. In *Methods and achievements in experimental pathology—The cytoskeleton in normal and pathologic processes* (ed. G. Gabbiani), vol. 8, p. 143. Karger, Basel.
- MAUPIN-SZAMIER, P. and T. D. POLLARD. 1978. Actin filament destruction by osmium tetroxide. *J. Cell Biol.* **77**: 837.
- MIMURA, N. and A. ASANO. 1979.  $\text{Ca}^{2+}$ -sensitive gelation of actin filaments by a new protein factor. *Nature* **282**: 44.
- OSBORN, M., R. E. WEBSTER, and K. WEBER. 1978. Individual microtubules viewed by immunofluorescence and electron microscopy in the same PtK2 cell. *J. Cell Biol.* **77**: R27.
- OTTO, J. J., R. E. KANE, and J. BRYAN. 1979. Formation of filopodia in coelomocytes: Localisation of fascin, a 58,000 dalton actin cross-linking protein. *Cell* **17**: 285.
- SCHLOSS, J. A. and R. D. GOLDMAN. 1980. Microfilaments and tropomyosin of cultured mammalian cells: Isolation and characterisation. *J. Cell Biol.* **87**: 633.
- SMALL, J. V. 1981. Organisation of actin in the leading edge of cultured cells. Influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J. Cell Biol.* (in press).
- SMALL, J. V. and J. E. CELIS. 1978a. Filament arrangement in negatively stained cultured cells: The organisation of actin. *Cytobiologie* **16**: 308.
- . 1978b. Direct visualisation of the 10 nm (100 Å)-filament network in whole and enucleated cultured cells. *J. Cell Sci.* **31**: 393.
- SMALL, J. V. and A. SOBIESZEK. 1980. The contractile apparatus of smooth muscle. *Int. Rev. Cytol.* **64**: 241.
- SMALL, J. V., J. E. CELIS, and G. ISENBERG. 1980. Aspects of cell architecture and locomotion. In *Transfer of cell constituents into eukaryotic cells* (ed. J. E. Celis et al.), p. 75. Plenum Press, New York.
- SMALL, J. V., G. ISENBERG, and J. E. CELIS. 1978. Polarity of actin at the leading edge of cultured cells. *Nature* **272**: 638.
- SPOONER, B. S., K. M. YAMADA, and N. K. WESSELS. 1971. Microfilaments and cell locomotion. *J. Cell Biol.* **49**: 595.
- TILNEY, L. G. 1975. The role of actin in non-muscle cell motility. In *Molecules and cell movement* (ed. S. Inoué and R. E. Stephens), p. 339. Raven Press, New York.
- VASILIEV, J. M. and I. M. GELFAND. 1977. Mechanisms of morphogenesis in cell cultures. *Int. Rev. Cytol.* **50**: 159.
- WEBER, K. and U. GRÖSCHEL-STEWART. 1974. Antibody to myosin: The specific visualisation of myosin-containing filaments in nonmuscle cells. *Proc. Natl. Acad. Sci.* **71**: 4561.
- WEBER, K., P. C. RATHKE, and M. OSBORN. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci.* **75**: 1820.
- WEISS, R. 1961. From cell to molecule. In *The molecular control of cellular activity* (ed. J. Allen), p. 1. McGraw-Hill, New York.
- WIELAND, T. 1977. Modification of actins by phallotoxins. *Naturwissenschaften* **64**: 303.
- WOLOSEWICK, J. J. and K. R. PORTER. 1979. Microtrabecular lattice of the cytoplasmic ground substance: Artefact or reality. *J. Cell Biol.* **82**: 114.

- WOODRUM, D. T., S. A. RICH, and T. D. POLLARD. 1975. Evidence for biased bidirectional polymerisation of actin filaments using heavy meromyosin prepared by an improved method. *J. Cell Biol.* **67**: 231.
- WULF, E., A. DEBOEN, F. A. BAUTZ, H. FAULSTICH, and T. WIELAND. 1979. Fluorescent phalloxin, a tool for the visualisation of cellular actin. *Proc. Natl. Acad. Sci.* **76**: 4498.
- YIN, H. L. and T. P. STOSSEL. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature* **281**: 583.



