

Contact Formation During Fibroblast Locomotion: Involvement of Membrane Ruffles and Microtubules

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Abstract. We have correlated the motility of the leading edge of fibroblasts, monitored by phase-contrast cinematography, with the relative distributions of several cytoskeletal elements (vinculin, tubulin, and actin) as well as with the contact patterns determined by interference reflection microscopy. This analysis has revealed the involvement of both ruffles and microspikes, as well as microtubules in the initiation of focal contact formation. Nascent vinculin sites within the leading edge or at its base, taken as primordial cell-substrate contacts, were invariably colocalized with sites that showed a history of transient, prolonged, or cyclic ruffling activity. Extended microspike structures, often preceded the formation of ruffles. Immunofluorescent labeling indicated that some of these primordial contacts were in close apposition to the

ends of microtubules that penetrated into the leading edge. By fluorescence and electron microscopy short bundles of actin filaments found at the base of the leading edge were identified as presumptive, primordial contacts. It is concluded that ruffles and microspikes, either independently or in combination, initiate and mark the sites for future contact. Plaque proteins then accumulate (within 10–30 s) at the contact site and, beneath ruffles, induce localized bundling of actin filaments. We propose that all primordial contacts support traction for leading edge protrusion but that only some persist long enough to nucleate stress fiber assembly. Microtubules are postulated as the elements that select, stabilize, and potentiate the formation of these latter, long-lived contacts.

OUTGOING from the pioneer studies of Abercrombie and others the translocation of fibroblastic cells over a planar substratum has been widely accepted as a useful model of metazoan cell movement (for reviews see Wessells et al., 1973; Vasiliev and Gelfand, 1976; Abercrombie, 1980; Buckley, 1981; Trinkaus, 1984). From these detailed, but mainly descriptive studies, it has been shown that despite its morphological variability and irregularity the locomotory process of fibroblastic cells may be divided into essentially three interdependent, sequential phases: the polar extension of a leading lamella via the active motility of the anterior, leading edge; the formation of anterior contacts with the underlying substrate; and, subsequently, the retraction of the trailing tail into the advancing cell body (Abercrombie et al., 1970a; Abercrombie et al., 1977; Harris, 1973; Goldman et al., 1976; Chen, 1981).

Morphological and immunocytochemical studies have suggested that actin and its various associated proteins play major roles in all these processes. The leading edge of motile cells comprises a dense meshwork and fine bundles of actin-containing microfilaments (Spooner et al., 1971; Lazarides, 1976; Small and Celis, 1978; Höglund et al., 1980; Small, 1981) complexed with various actin-associated proteins such as alpha-actinin, filamin, fimbrin, talin, and others (Heggeness et al., 1977; Geiger, 1979; Bretscher and Weber, 1980;

Small et al., 1982; Burridge and Connell, 1983; Yamashiro-Matsumura and Matsumura, 1986) and the active movements of this region are readily inhibited by the microfilament-disrupting drug cytochalasin B (Carter, 1967; Wessells et al., 1971; Goldman and Knipe, 1973; Ohnishi, 1981; Domnina et al., 1982). More recently, the results from microinjection studies (Kreis et al., 1982; Wang, 1984) have lent credence to the idea (Small et al., 1978) that leading edge protrusion may be largely mediated via the polymerization of actin filaments.

Other studies have shown that actin is also involved in the formation of contact with the substrate. Examination of cell-substrate contacts formed along the ventral aspects of the leading lamellae by electron microscopy or immunocytochemical labeling showed that bundles of actin filaments are attached to the endofacial surfaces of the membrane in defined areas of closest cell-substrate contact, namely focal contacts or focal adhesion plaques (Heaysman, 1973; Heath and Dunn, 1978; Wehland et al., 1979; Izzard and Lochner, 1980). Immunocytochemical labeling with antibodies specific for various actin-related proteins have further indicated that vinculin (Geiger, 1979) and talin (Burridge and Connell, 1983) are specifically localized at the cytoplasmic aspects of focal contacts and it was thus proposed that they and probably other yet unidentified proteins are involved in the attach-

ment of actin to the membrane in these sites (Geiger, 1983; Mangeat and Burridge, 1984).

The third phase of the locomotory cycle, namely the retraction of the trailing tail, studied in some detail by Chen (1981), is likewise actin-dependent and can be attributed to the contractile activity of the large stress fiber bundles (Isenberg et al., 1976) that span the cell between contact foci at the rear and similar, well established contacts at the base of and behind the leading edge. The involvement of other cytoskeletal systems (intermediate filaments and microtubules) in the various phases of the locomotory cycle has not been elucidated so far. It has nevertheless been demonstrated that microtubule-disrupting drugs largely abolish cell polarization and directional locomotion (Vasiliev et al., 1970; De Brabander et al., 1977) and that the centrosome may have a fixed orientation relative to the general direction of movement (Albrecht-Buehler, 1977; Singer and Kupfer, 1986).

Despite the accumulation of much data on the phenomenology of the locomotion process and on the static organization of various cytoskeletal elements, the molecular mechanisms underlying involvement of these elements in the motility of the leading edge and in the directional establishment of new substrate contacts remain elusive. In an attempt to gain insight into the molecular dynamics and mode of involvement of cytoskeletal elements in contact formation we have undertaken studies to correlate the localization of cytoskeletal components in cells whose history of locomotion up to the time of fixation was known. In essence, similar approaches have been attempted in the past (see for example, Buckley, 1974; Heath and Dunn, 1978; Herman et al., 1981; Izzard et al., 1985). However, by combining time lapse cinematography with triple immunofluorescent labeling and by adopting new fixation procedures which optimally preserve a wide range of cytoskeletal structures, new information has emerged. In the present report we demonstrate that ruffling of the leading edge as well as microspike protrusion are associated with the presence of nascent vinculin-containing contact sites, taken as precursors of focal contacts. Further, the oft and apparently delayed association of microtubule ends with these vinculin loci reveals a possible route by which microtubules, known to be required for cell polarization and translocation (Vasiliev et al., 1970; Vasiliev and Gelfand, 1976), may regulate directional locomotion. Preliminary results relating to this work have appeared in earlier reports (Geiger et al., 1984; Small and Rinnerthaler, 1985).

Materials and Methods

Cells

Chick fibroblasts obtained by trypsinization of 9–11 d embryo hearts were used from the 1st to the 5th passage after primary culture. The cells were grown at 37°C in the absence of CO₂, compatible with conditions in the filming chamber (see below). For this purpose either of two media were used, L15 or BME with Hanks' salts both supplemented with 10% FCS (all from Flow Laboratories, Vienna, Austria or Gibco, Vienna, Austria). Cells were plated onto round 12-mm diameter coverslips that were etched with a finder grid pattern for cell relocation (Small, 1985), and used 2–5 h after plating.

Perfusion Chamber

The filming chamber used in this study was of the Dvorak-Stotler design (Dvorak and Stotler, 1971), but modified to give a rectangular chamber area

of 4 × 8 mm with the long axis in line with the inlet and outlet syringe needles (Rinnerthaler, 1982). The latter needles (Nr. 20) had small, Record-type heads to allow faster drainage of fluid when exchanging solutions (see fixation).

The chamber was supplied with solution by a simple drop feed from a filled syringe, with needle, that was hung freely in a loop (and therefore quickly exchangeable) so that the needle tip was just above the cone of the chamber inlet needle. The outlet needle cone was connected to a long tube whose end could be placed at different positions below the plane of the chamber to control the flow rate by siphon action. To ensure that no air entered the chamber the inflow had to exceed the outflow. Overflow of liquid at the inflow cone was drained away by filter paper. With this arrangement any number of liquids could be exchanged at the inflow by changing the freely hanging syringe.

Immediately before filming, the chamber was assembled together with the coverslips carrying the cells, filled with medium (L15 or BME, see above) and mounted on the microscope. The temperature of the chamber was controlled by a thin, home-made resistor-type heating stage (Rinnerthaler, 1982) mounted between the microscope stage and the chamber that allowed temperature maintenance within less than 0.5°C. Filming was carried out at 37°C.

Fixation

Fixation was carried out using Triton X-100 glutaraldehyde mixtures in a "cytoskeleton buffer" (137 mM NaCl; 5 mM KCl; 1 mM Na₂HPO₄·2H₂O; 0.4 mM KH₂PO₄; 5.5 mM glucose; 4 mM NaHCO₃; 2 mM MgCl₂; 2 mM EGTA; 10 mM MES; pH 6.1) essentially as described previously (Small, 1981), with only minor modifications indicated below. For cells not used for filming, coverslips were briefly rinsed in medium without serum, followed by a rinse in cytoskeleton buffer and then by fixation in cytoskeleton buffer supplemented with 0.5% Triton X-100 (Sigma Chemical Co., Munich, FRG) and 0.25% glutaraldehyde (Polaron, Austria; E. M. grade) for 2 min at R. T. This was followed by a buffer rinse and then by a 10-min post fixation in 1% glutaraldehyde in cytoskeleton buffer at R. T. The coverslips were subsequently rinsed and stored in cytoskeleton buffer until immunolabeling.

Cells whose locomotion was monitored by cinematography were fixed during filming in the perfusion chamber. At the appropriate time the inflow of medium was stopped by removing the feed syringe containing medium and changed, after drainage of medium in the needle head had occurred, to one containing cytoskeleton buffer at pH 6.5. Approximately 5–10 s thereafter the buffer syringe was exchanged for one containing the glutaraldehyde-Triton mixture (0.5% Triton X-100, 0.25% glutaraldehyde in cytoskeleton buffer). After a 2-min fixation in this mixture the fixative syringe was changed again for a buffer syringe and the interference reflection microscopy (IRM)¹ image (see below) of the fixed cell recorded. The chamber was then opened, the coverslip rinsed in cytoskeleton buffer, and post-fixed in 1% glutaraldehyde in the same buffer for 10 min.

Immunolabeling

Before immunolabeling, free aldehyde groups were reduced by treatment of the coverslips with sodium borohydride (0.5 mg/ml), in ice cold cytoskeleton buffer; three changes, 5 min each. After 2 rinses in cytoskeleton buffer (5 min each) the coverslips were incubated for 40–60 min on 20 µl of a first antibody mixture containing rabbit anti-vinculin and rat anti-tubulin (a gift from Dr. J. Kilmartin, Cambridge, U.K.) made up in cytoskeleton buffer (pH of 6.5–7.0). Antibody incubations were carried out at R. T. on a sheet of parafilm that was overlaid on a glass plate mounted on a moist filter paper in a 15-cm diameter petri dish. After washing (2 changes, 10 min each) the coverslips were transferred to the second antibody mixture consisting of goat anti-rabbit rhodamine (see Geiger, 1979) and goat anti-rat fluorescein (Nordic, Vienna, Austria) for 40–60 min. After the final wash, as above, the coverslips were mounted into the filming chamber in a 1:3 mixture of Gelvatol mounting medium (see below) and 50% Glycerol in cytoskeleton buffer, with added 3 mg/ml n-propyl gallate (Sigma Chemical Co.) as anti-bleach agent to record the first two fluorescent images (see light microscopy). The coverslips were then removed, thoroughly washed and over-stained with fluorescein-labeled phalloidin (a gift from Prof. H. Faulstich, Heidelberg, FRG) in cytoskeleton buffer. After washing they were mounted in Gelvatol (Monsanto Corp., St. Louis, MO) to record the final actin image. Under the labeling conditions used here the actin label was sufficiently

1. *Abbreviation used in this paper:* IRM, interference reflection microscopy.

strong as to effectively obscure the microtubule pattern labeled in the first step.

Light Microscopy, Cinematography, and Projection

Light microscopy was carried out on a Zeiss photomicroscope III equipped with epi-illumination (50 W HBO lamp).

Fluorescence patterns of cells were recorded using a Planapochromat 63 \times 1.4 objective on Agfapan professional 400 film (27 Din) with a DIN setting of 36 for rhodamine and 30 for fluorescein. IRM images were obtained with the Zeiss antifix system using Kodak technical pan film (18 Din: 21 Din setting on microscope). Leading edge movement was recorded in phase contrast (Neofluar 40 \times 0.75) on 16-mm Kodak technical pan film using a 16-mm Bolex camera at a speed of 2 frames/s. Cells were usually filmed for 2–3 min before fixation. The movie films were processed by reversal development in a JOBO (FRG) 16-mm developing system.

The film projector used in this work was built up from a normal 16-mm projector (EIKI, Japan) into an analytical projector. Via mechanical modifications and the construction of an electronic control system, single frame analysis, frame counting and variable speed control were made possible (Rinnerthaler, 1982).

Photographic and Graphic Analysis

Multiple images of cells were analyzed for overlapping structures by a combination of photographic and graphic procedures. Initially a photographic procedure was employed as follows: Each of the five patterns of the cell (actin, vinculin, microtubules, phase contrast, and IRM) were transferred at the same magnification onto 13 \times 18-cm sheet film (Agfa) either as a positive or negative copy so that any combination of superimposition was possible. Films with the protein patterns were stained either blue-green or purple (according to the methods described in Krug and Weide, 1976) to facilitate recognition of each pattern in superimposed film combinations. To investigate the frequency of colocalizations in the leading edge the five patterns were matched on a light box. In practice it was difficult to establish by this means the overlapping structures in more than three films at once. Hence it was necessary to make a drawing on transparent overhead foil of the leading edge characteristics summarized from two or three patterns and then to overlay the remaining images on this. The specific structures of interest were: membrane ruffles, microspikes, vinculin foci, free microtubule ends and cell-substrate contacts as shown by IRM. This type of analysis was useful for comparison of the cytoskeletal and contact patterns but was less applicable for the analysis of movie sequences.

Analysis of the patterns together with locomotion sequences involved projection of the original 16-mm images onto a translucent screen as follows. The film projector was mounted on a table fitted with a double mirror system and a translucent projection screen. With the use of a zoom objective on the projector the projected image could be readily adjusted in size to match the cytoskeleton patterns on 13 \times 18 cm sheet film. In an initial analysis the patterns on the sheet films were overlaid on and compared with the locomotion sequences. In this way, however, the accumulated activities of the leading edge (ruffling patterns, microspikes, protrusion, and retraction) over any period of interest was too complex to be condensed on a single image, necessitating a further refinement, as follows.

Graphic Analysis. For the above reason it was found necessary to make a graphic recording of the movie sequences. This was done by drawing, for each time point (intervals of down to 15 s real time) the outline of the cell, the ruffling pattern and the visible microspikes, on three separate transparent foils. In this case the final magnification on the projection screen was fixed at 3,000. The single drawings were additionally marked with the same fixed coordinate points, taken from the coverslip. The sequences of drawings were then overlaid and taped at one edge on millimeter paper, one above the other in the appropriate time sequence, in three separate sequence sets. The millimeter paper had squares with 3-mm sides giving a corresponding linear scale of 1 μ m per square side. Each of the sequences was then copied on transparent foil in a photocopy machine and these copies overlaid in any combination, as desired, to establish the temporal and spatial relations between different structures.

Using a photographic enlarger, the 35-mm negatives of the fluorescent patterns of the fixed cell were also projected, via a mirror onto a transparent screen in a second set up, to give, likewise, a final magnification of 3,000 \times . The details in the leading edge region were again transferred onto transparent overhead foils using colored fine-tipped felt pens. These images were again copied in multiple copies (in black) that could be overlaid on the three time sequences of the leading edge described above. The original colored

drawings themselves were overlaid directly for analysis of the colocalizations of actin, vinculin, and microtubules and for comparison with the photographic transparency (magnification: 3,000) of the IRM image.

Although the application of this graphic method was somewhat laborious we found it to be the only way to relate the complex movements of the leading edge at different time points with each other and with the cytoskeleton and contact patterns.

Composite Photographic Patterns

The reproduction of more than one pattern on a single print was done according to the following scheme: The individual patterns were first photographically enlarged as positives onto either sheet film or print paper. These were then transferred (for black and white) via a double exposure over a light box (film) or via flash exposure (prints) onto Agfapan 25 professional roll-film in a Mamiya 6 \times 7-cm camera. For color combinations, the individual fluorescent patterns (in black and white) were transferred onto a single color negative (Agfacolor roll-film N 80L) in the same way, but with different color filters (green, red, and blue) interposed for each exposure. By this means an additive color mixing was produced, e.g., green plus red giving yellow, all three colors giving white, and so on. Color prints were made on Agfa Color paper 310 using a Linhof (Munich FRG) enlarger and an Agfa color processing machine (Agfaprint C 66 TR). All the photographic processing required for such compositions is too involved to be described here in detail.

Negatively Stained Whole Mounts for Electron Microscopy

Whole mount cytoskeletons for electron microscopy were prepared essentially as described previously (Small, 1981) using a mixture of 0.25% glutaraldehyde and 0.5% Triton X-100 as primary fixative (2 min, R. T.) and 1% glutaraldehyde as secondary fixative (15–30 min at R. T.); both in cytoskeleton buffer. In some cases actin was additionally stabilized after fixation by treatment with phalloidin (2.10^{-5} M in cytoskeleton buffer; 20 min at R. T.). Negative staining was carried out as earlier (Small, 1981) but with 2% phosphotungstic acid as the stain (Pagh et al., 1985).

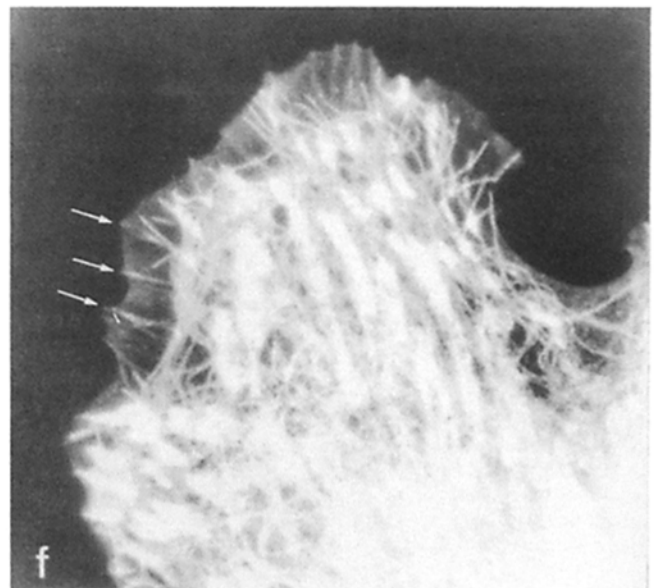
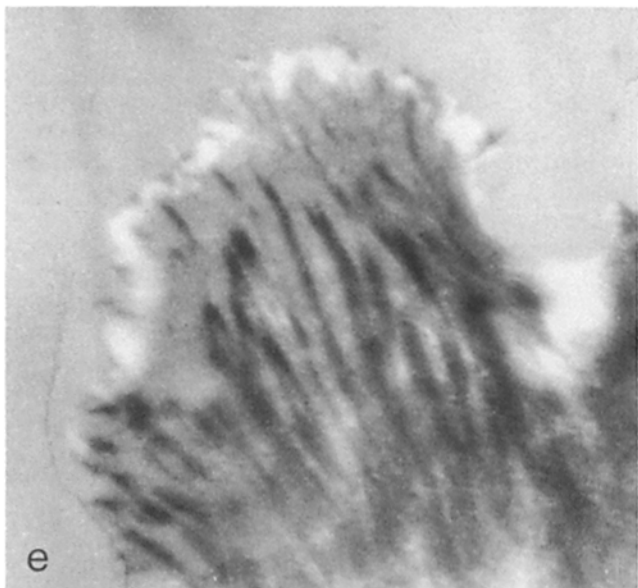
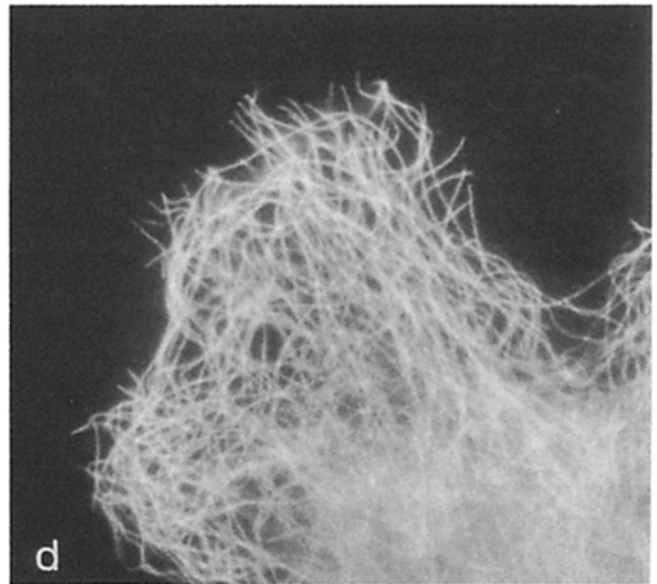
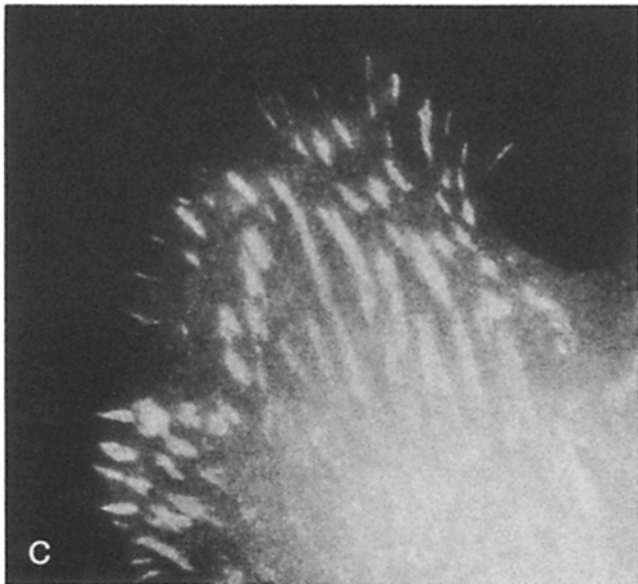
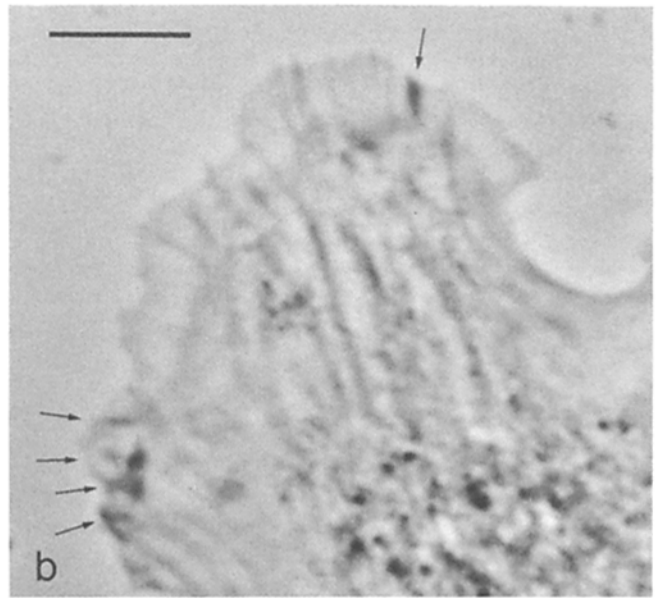
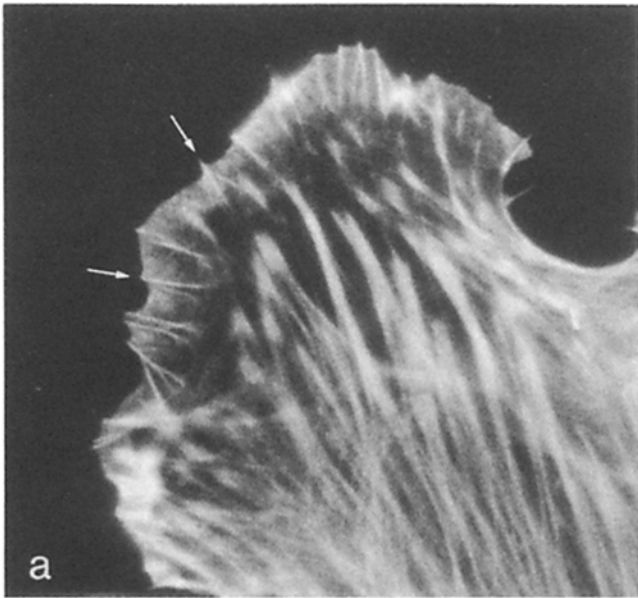
Electron microscopy was carried out using a Zeiss EM 10A operating at 80 kV and with a 50- μ m objective aperture.

Results

The results presented in this paper and their analysis rely, to a large extent, on time-lapse cinematographic recording of locomotory cells (in phase contrast) correlated with the superimposed images of actin, vinculin, and microtubules as well as the contact patterns revealed by IRM after fixation. Before describing the results with filmed cells it is necessary to briefly draw attention to the various morphological aspects of fibroblasts that are relevant to this study.

Morphology and Cytoskeletal Organization of the Leading Edge: Spatial Interrelationships

Fig. 1 gives an example of a chick heart fibroblast that was subjected to the multiple imaging methods used and serves to introduce the methodology as well as the structural aspects that were investigated. The different organizational forms of actin are clearly shown in Fig. 1 *a*: here the prominent stress fibers dominate the main part of the cell while the delicate and motile leading edge is clearly defined by a band of diffuse actin at the cell periphery spanned by numerous fine radial fibres, or microspikes (e.g., arrows). In the phase contrast image (Fig. 1 *b*) the leading edge region is recognized as a thin anterior lamella, within which some of the microspikes seen in the actin pattern may be identified. Additionally, any upward protrusive activity, or ruffling (see also below), is evident from localized increases in phase density (arrows). The interference reflection image (Fig. 1 *e*) provides infor-



mation about cell contacts and compares very closely to the pattern of vinculin distribution (Fig. 1 *c*). The distribution of microtubules is illustrated in Fig. 1 *d*. Superimposition of any of the patterns in combination allows a precise evaluation of their structural interrelationships (Fig. 1 *f*). For example, a small but prominent group of microtubules is seen to penetrate into the leading edge and their frequency of coincidence with microspikes and vinculin-containing contact sites is revealed. Likewise, two classes of vinculin spots are recognized, the larger ones associated with stress fiber bundles and smaller foci situated anterior to the stress fiber ends and present at the base of and within the leading edge, sometimes in association with the microspike bundles.

Significantly we observed in many cells the common penetration of some microtubules into parts of the leading edge region as well as the presence of fine vinculin containing sites, sometimes colocalized with microtubule ends and or microspikes. Semiquantitative analysis (not shown) of the colocalization of these structures, together with ruffles and contacts in or at the base of the leading edge, revealed some consistent correlations. In particular, ruffles were invariably associated with microtubules or vinculin or both. Likewise, we noted a high frequency of colocalization of microspikes with radial contact streaks (intermediate in density in IRM between focal and close contacts) and of microtubules and ruffles with close contacts.

Spatio-Temporal Interrelationships Revealed by Correlated Cinematography

While the above data revealed some interesting relationships between the different structures, their bearing on cell motility was difficult to deduce in the absence of information on the locomotion history of the cells. For this reason correlated cinematography was performed. This was carried out under phase contrast optics and thus allowed the monitoring of protrusion and withdrawal of the leading edge as well as the changing pattern of ruffling activity and microspike extensions. In the following presentation the term ruffling is used to describe any activity of the leading edge causing a localized increase in phase density (see also Discussion). This includes not only the macroscopic backfolding of the lamellipodium but radial upfoldings (radial ruffles) and foci of apparent increased thickness (mini ruffles) in the leading edge (see Fig. 2 *a* for some examples).

Spike Formation and Ruffling Activity Mark Sites of Subsequent Contact. In a graphic analysis (see Materials and Methods) of the locomotion sequences and the cytoskeletal and contact patterns a consistent relationship emerged between a history of ruffling activity and/or microspike formation at a particular site and the development of contact. An example of this relationship is illustrated in Fig. 2. The leading edge of this cell showed active ruffling and protrusive activity over the recorded period of 3 min (only terminal 135 s shown). Of particular interest were the three contacts

(Fig. 2 *b*, arrows) that formed in the medial region of the leading edge which protruded forward in the last 90 s before fixation. At the sites of these contacts, microspikes were recognized at around 135 s before cell arrest (Fig. 2 *a*, 135 arrows); subsequently they disappeared and were then replaced by ruffles in the same positions at different times during the last 80 s (see legend to Fig. 2 *a*). In the adjacent regions that showed more or less continuous ruffling activity at the base of the leading edge, much larger and more prominent contacts were formed (Fig. 2, *b* and *c*).

Fig. 2, *d-f* illustrates part of the graphic analysis of this cell and show the relationship between the leading edge profiles at different time points and the vinculin and microtubule patterns in the fixed cell. The 1 μm square orthogonal scale allows direct measurement of movement: for this cell the rate of expansion of the medial leading edge was 2.2–3.5 $\mu\text{m}/\text{min}$ in the last 105 s. As observed in Fig. 2 *d* (arrows), there is an apparent relationship between the location of the spike structures seen at 135 s before fixation with the anterior vinculin sites which are established later. Since these sites lie out of the cell boundary seen at 105 s (arrows Fig. 2 *e*) they must have formed at a later time point; likewise, one microtubule end (arrowhead in Fig. 2 *e*) must have penetrated into the leading edge some time during the last 105 s before fixation. Note that the new vinculin sites are located beyond the microtubule ends.

Microtubules Show Colocalization with Contact Sites Along the Leading Edge. The sites of substrate contact in the leading edge were often found to be associated not only with ruffling activity but also with the apparently free ends of microtubules. An example of this type of association is shown in Figs. 3 and 4. Prominent in this cell were radially oriented ruffles (arrows in Fig. 3 *a*, 0 s) that existed for different times up to the point of fixation. Superimposition of the phase contrast and vinculin and microtubule images (Fig. 4, *a* and *b*) clearly reveals the correlation between these latter cytoskeletal elements and the radial ruffles. The vinculin sites in the leading edge on the right half of the cell correlate with distinct streaks of low intensity in the IRM image (Fig. 4 *c*, arrows) that are less dark than the more posterior focal contacts. Comparison of the vinculin (Fig. 4 *a*), actin (Fig. 4 *d*) and interference reflection images (Fig. 4 *c*) reveals that only a minority of the microspike bundles are associated with contact regions. As can be seen there is a general correspondence between the larger vinculin containing sites and the focal contacts in the IRM image (Fig. 4, *a* and *c*). The precise relationship between the three cytoskeletal patterns is shown in Fig. 4 *e*.

As illustrated in Fig. 3 *b*, which shows a superimposition of the last time lapse frame (0 s) and the cell outline 129 s beforehand the leading edge of this cell showed a very low speed of protrusion, ranging from 0.5–1.0 $\mu\text{m}/\text{min}$. Of further interest in this cell was the formation of a fine radial ruffle around 10 s before fixation (arrowhead in Fig. 3 *a*; 0 s) that showed neither association with vinculin nor with

Figure 1. Multiple imaging of fibroblasts. This figure shows the standard images obtained for the present analysis. The phase contrast (*b*) and IRM image (*e*) were obtained directly after fixation and the cytoskeleton images (*a*, actin; *c*, vinculin; and *d*, tubulin) after immunolabeling (see Materials and Methods). (*f*) Shows the composite picture of the cytoskeletal components. Note in *f* the penetration of microtubules into the leading edge, as well as the fine vinculin foci, some of which are colocalized with microspikes and microtubules. Arrows in *a* and *b* point to examples of microspike bundles and ruffling sites, respectively. Bar, 10 μm .

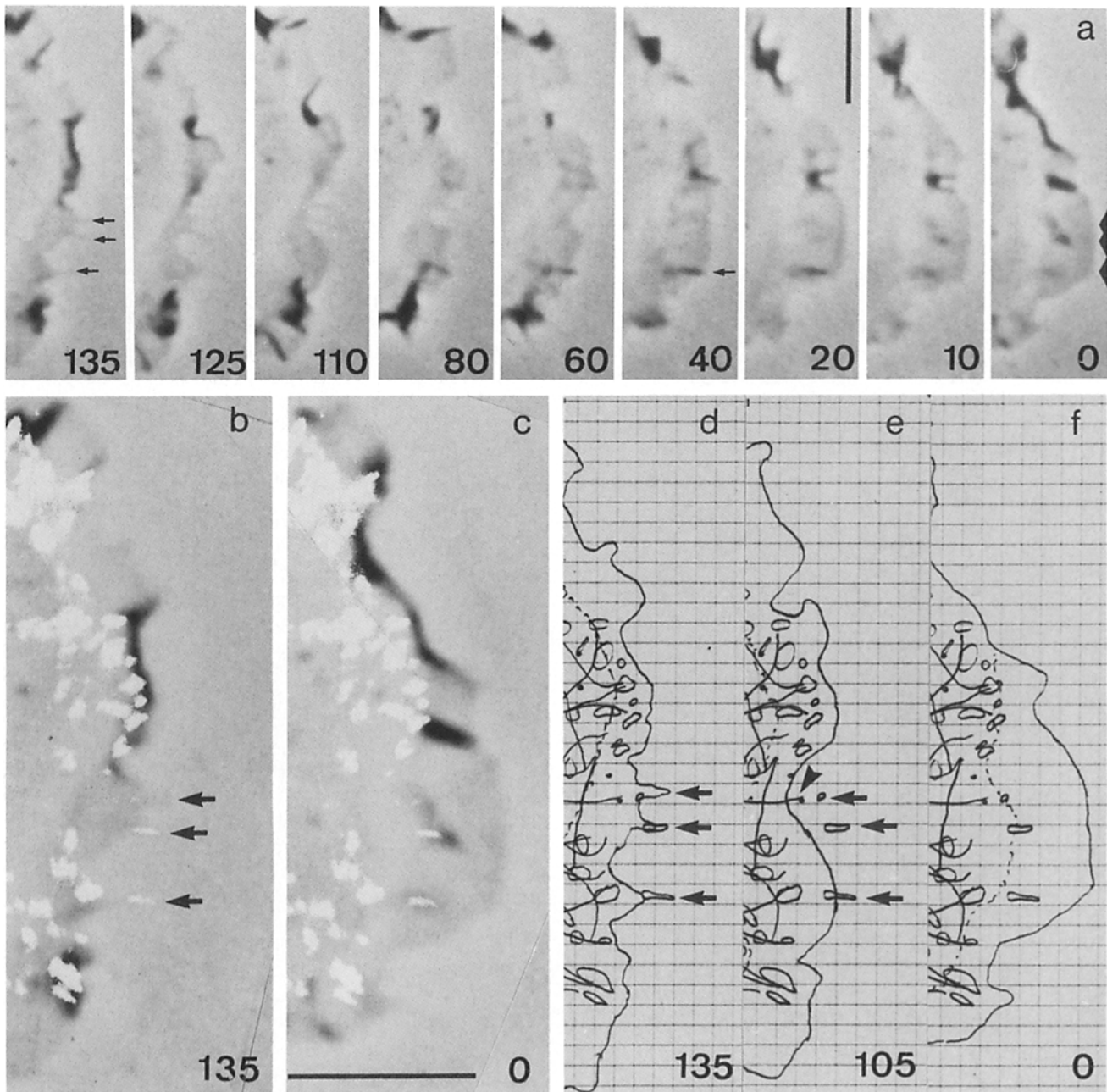
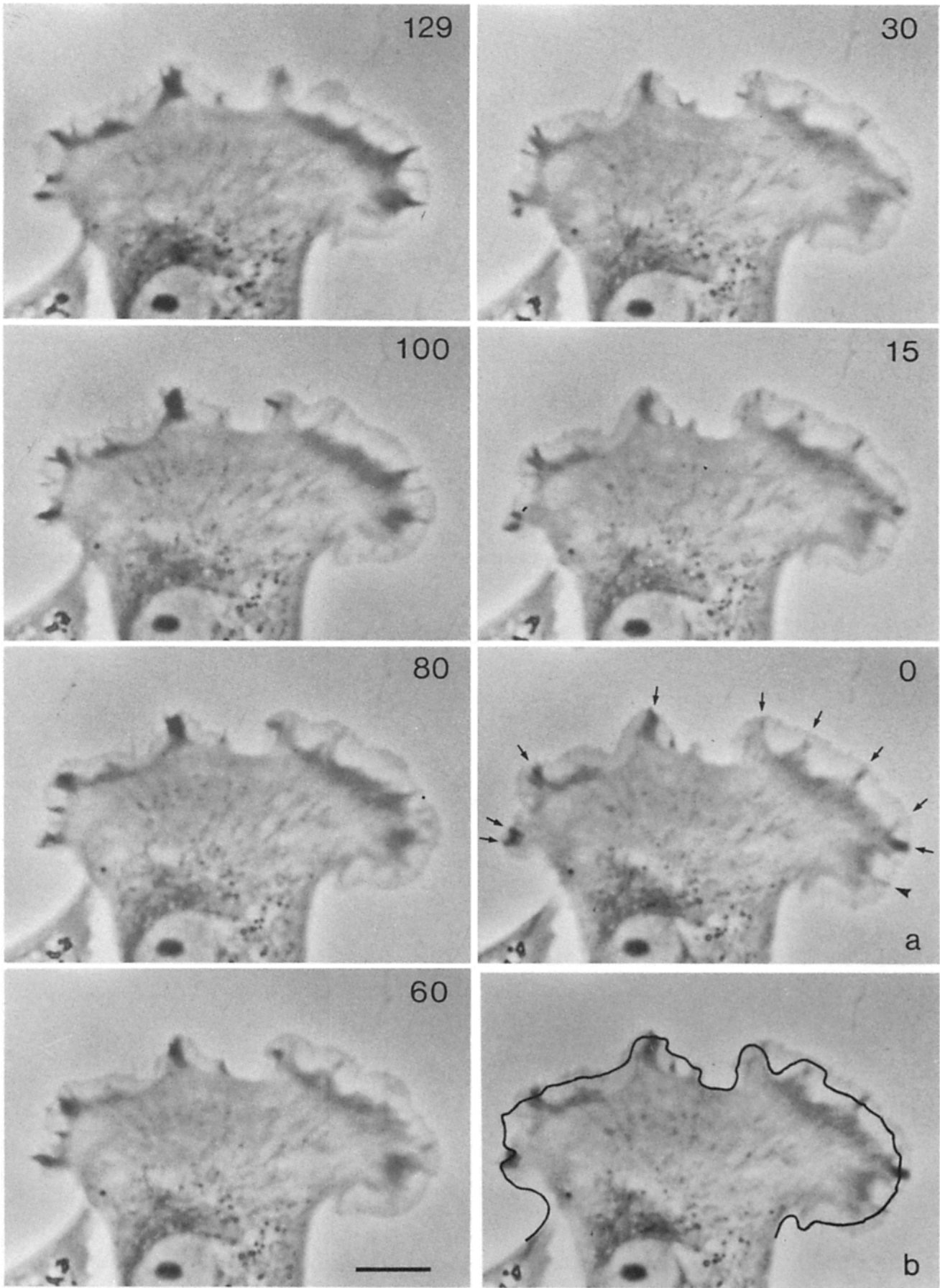


Figure 2. Microspikes, ruffling, and contact. (a) Time lapse sequence of a chick fibroblast leading edge, showing extension of microspikes (135–110 s; a, arrows in 135) followed by rapid lamellipodium protrusion (80–0), coincident with the appearance of a radial ruffle between 80 and 20 s (arrow, 40) and three “mini ruffles” between 20 and 0 s (arrowheads, 0). Note decrease in phase density of radial ruffle from 20–0 and the coincidence of the same ruffle with a microspike extension at –135 s (arrows in 135). b and c Show the superimposition of the vinculin pattern with the time lapse frames at 135 and 0 and illustrate the coincidence of the microspike and ruffling sites with vinculin foci in the newly protruded leading edge (arrows in b). (Note, the finest vinculin spot under the smallest ruffle indicated by top arrow was difficult to demonstrate in the double exposure combination, but was clear in the original negative, used for graphic analysis in d–f.) The same and additional features are revealed in the graphic analysis shown in part in d–f. Here the short lines correspond to the microtubule ends and closed shapes to vinculin spots. The rate of protrusion of the leading edge (continuous line) can be directly ascertained from the 1 μm square scale (2.2–3.5 $\mu\text{m}/\text{min}$ over the last 105 s). From these three composite drawings selected from the graphic time sequences it is clear that vinculin accumulation at the upper two sites must have begun after 105 s, since the cell outline at this time lay behind them. In fact, this was also true at –60 s, by this criterion. Note also that microtubules extend towards the vinculin sites but lie behind them (also see text). Bars, 10 μm .

Figure 3. Coincidence of vinculin, microtubule ends and ruffling loci. (a) Time lapse sequence of cell with slowly protruding leading edge. b Shows superimposition of cell edge at –129 s on the time lapse frame at 0 (leading edge protrusion, 0.5–1.0 $\mu\text{m}/\text{min}$). Note, predominance of radial ruffles in this cell (arrows in a, 0). Bar, 10 μm .



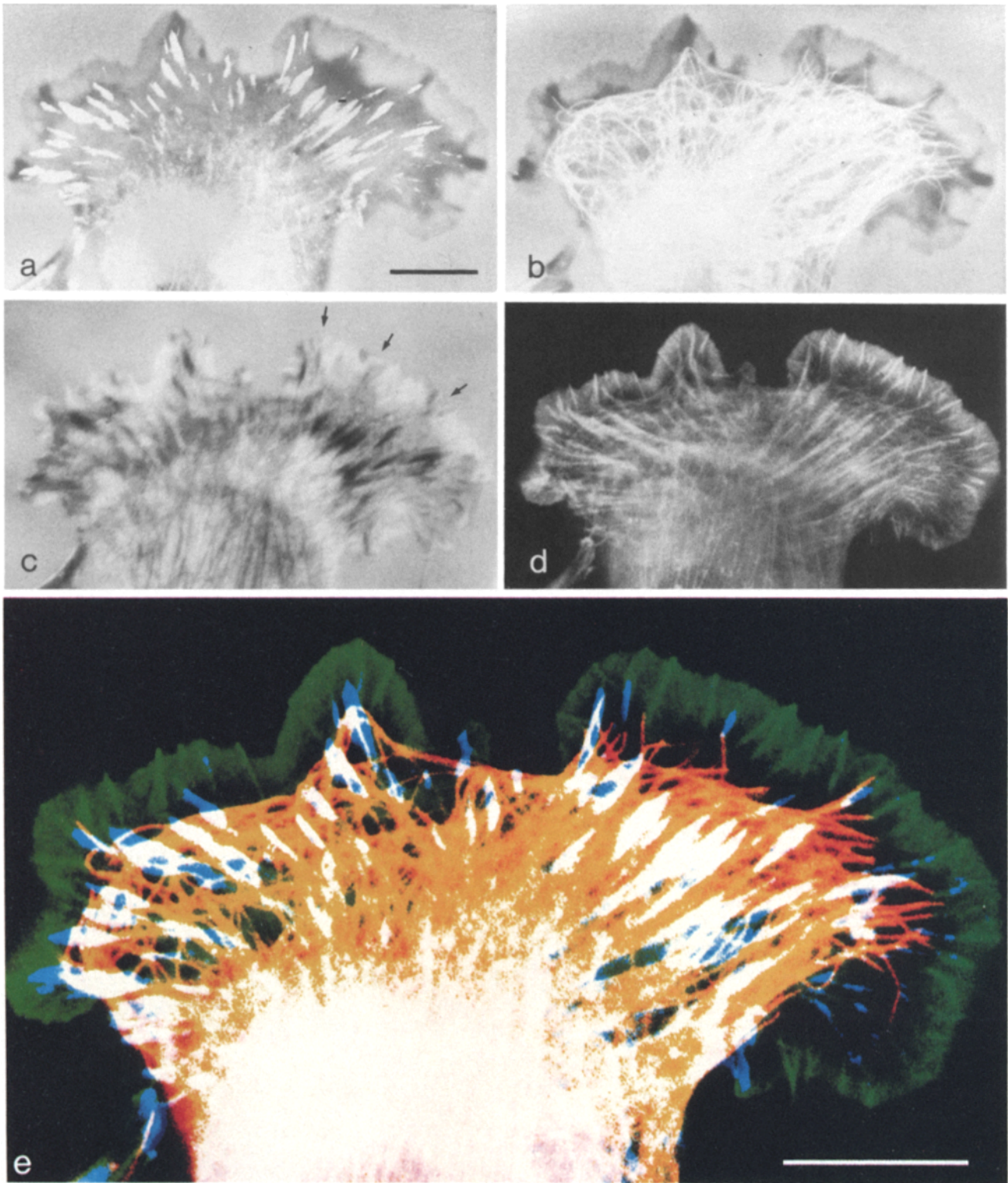
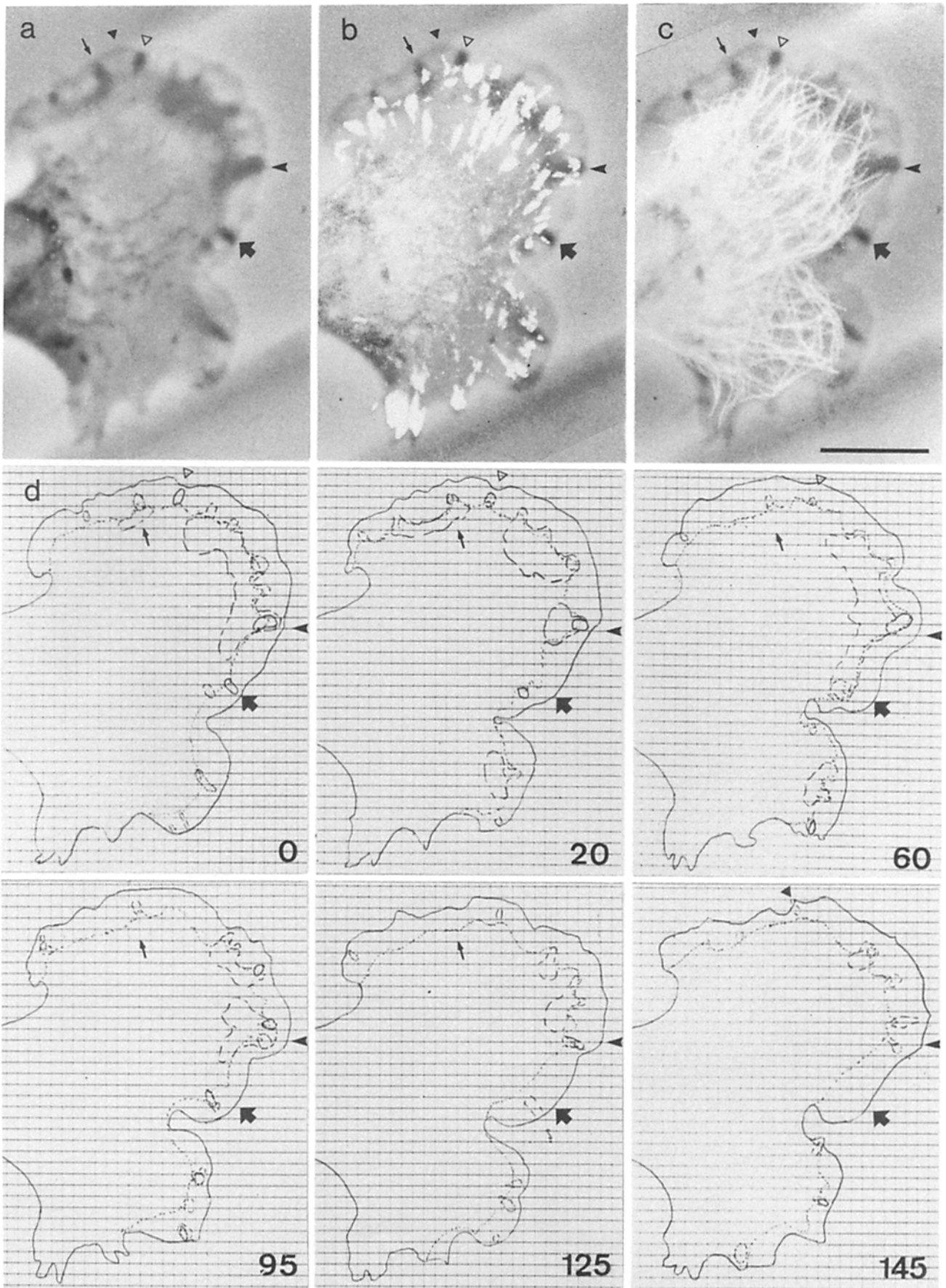
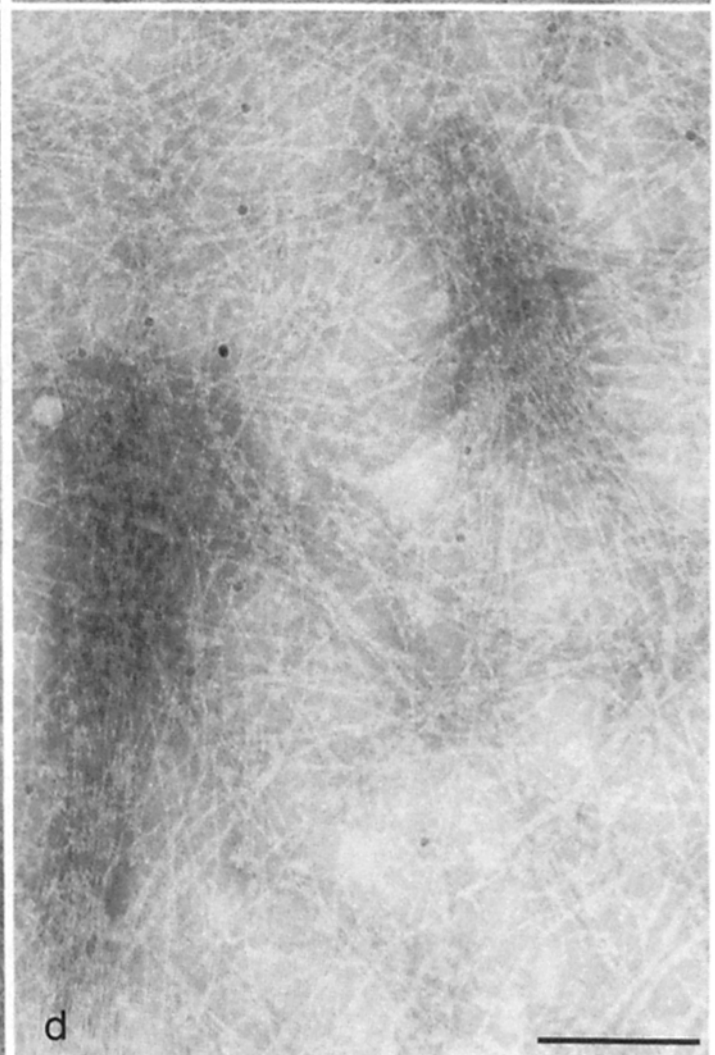
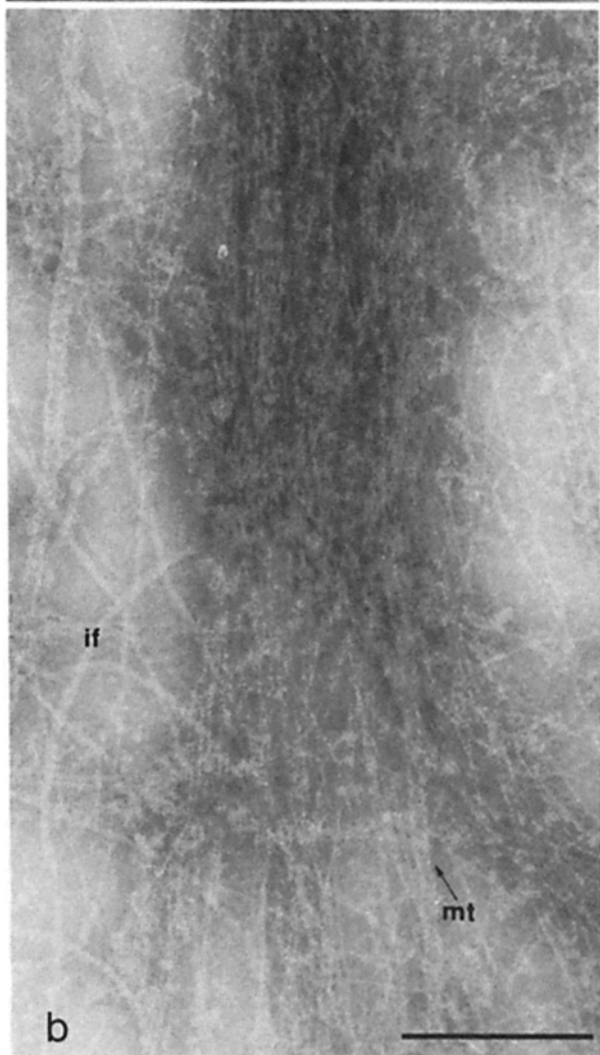
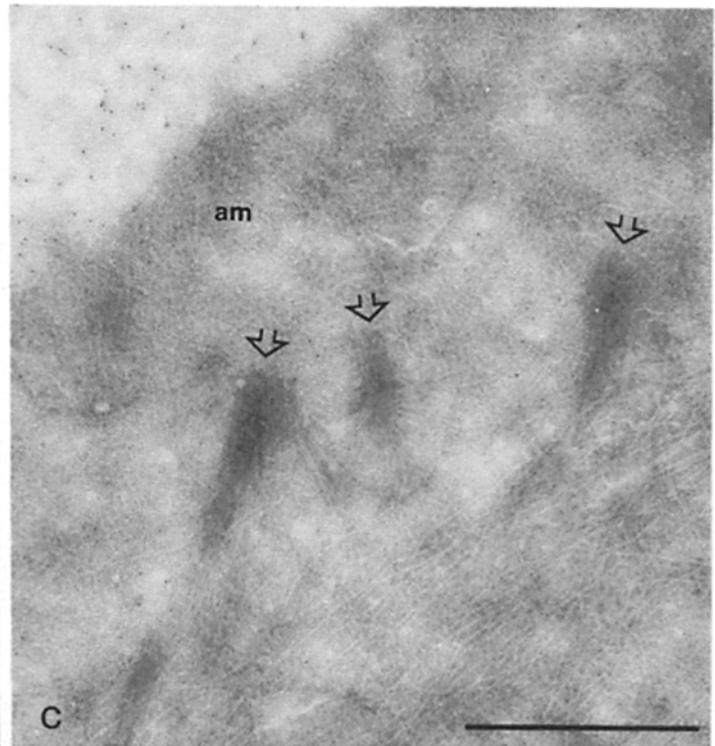
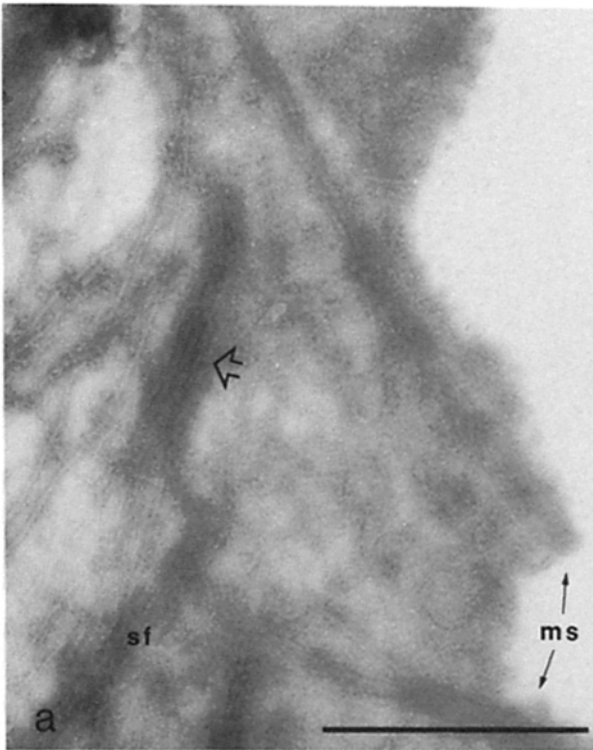


Figure 4. Coincidence of vinculin, microtubule ends and ruffling loci. Cytoskeletal patterns and IRM images of cell from Fig. 4. (*a* and *b*) show superimpositions of the vinculin and tubulin patterns on the 0 time lapse frame, respectively: these illustrate the coincidence of microtubule ends and vinculin foci with the ruffles in the leading edge. The vinculin sites in the leading edge on the right half of the cell correspond to contact streaks in the IRM image (*arrows* in *c*). The actin pattern in *d* reveals the abundance of microspike bundles in the leading edge. (*e*) Composite cytoskeletal image (tubulin, red; vinculin blue; actin, green) showing coincidence of microtubule termini in the leading edge with primordial vinculin foci. Bars, 10 μm .

Figure 5. Temporal relationships between ruffling, microtubule ends, and vinculin colocalizations. For this cell the graphic analysis (*d*) of the locomotion sequences is presented (time sequences 145 s to 0 s before fixation) where the contour patterns correspond to sites of ruffling activity. In addition, the time lapse image at 0 in phase contrast (*a*) is shown as well as this image superimposed on the vinculin (*b*) and microtubule (*c*) patterns. Sites of interests are marked by the different arrows and arrowheads. The appearance of ruffles R (with



approximate starting times) and presence (+) or absence (-) of vinculin (V) and microtubule ends (MT) at time 0 at these five sites was as follows: (†) R from 125-0 (+ V - MT); (▲) R before 125 (+ V + MT); (Δ) R from 95-0 (+ V + MT; base only); (▲) R from 145-0 (+ V + MT); (▲) R from 20-0 (+ V - MT). Bar, 10 μm.



microtubules. The same ruffle also lacked visible contact with the substrate as judged from the interference reflection image.

Other examples of ruffling, vinculin, and tubulin localizations in the leading edge are visible in the last cell shown in Fig. 5. Here the phase contrast picture of the cell at the time of fixation is shown alone (Fig. 5 *a*) and with superimposition of either the vinculin (Fig. 5 *b*) or microtubule patterns (Fig. 5 *c*). The graphic analysis (Fig. 5 *d*) below shows the leading edge outline and the ruffling patterns (contoured zones) at different times before fixation, as indicated. The different arrows and arrowheads in Fig. 5, *a-d* point to selected sites of interest. In the legend to Fig. 5 the lifetime of ruffles at these sites and the colocalizations of vinculin, microtubule ends, or both have been indicated.

With the exception of one site (open triangle) there was a direct correlation between ruffling activity ranging from less than 20 s to more than 145 s before fixation and the localization of vinculin within the leading edge. Only two of these sites showed microtubule colocalization (solid triangle and solid arrowhead), in which case the ruffles concerned persisted throughout the entire time sequence (145 s; solid arrowhead) or only transitorily, around 145 s (solid triangle). At the fifth site with a prominent ruffle at 0 time, cited above (open triangle) both vinculin and a microtubule end were colocalized at the base of the ruffle: this ruffle persisted for ~ 1 min before fixation.

In the absence of space to present more data brief mention will be made of a further relevant feature of leading edge protrusion. In the case of lamellipodia that protruded more rapidly than around 4 $\mu\text{m}/\text{min}$ and which normally lacked microspike bundles, neither microtubule ends nor vinculin loci were found beyond the base of the leading edge: in other words the protrusion of the actin rich network was clearly microtubule and vinculin independent.

Actin-Bundling Sites at the Base of the Leading Edge

With the aim of establishing the ultrastructure of the primordial vinculin-containing contacts we carried out immunoelectron microscopy on whole-mount cytoskeletons using the anti-vinculin antibody. Two problems were however encountered. First, the immunogold conjugates used as secondary probes failed to label with convincing intensity, even the large contact sites at the termini of the stress fibers, known to be rich in vinculin. And second, the multiple incubation and washing steps required for immunolabeling caused a structural deterioration of the actin meshworks in the leading edge.

Despite these setbacks it was possible to recognize structural differentiations in the leading edge actin system of unlabeled cells whose distribution and general morphology correlated precisely with that of the vinculin-containing foci seen in this region by immunofluorescence microscopy. Examples of these sites in negatively stained cytoskeletons are shown in Fig. 6. At low magnification such sites characteristically showed increased electron density, presumably

due to their greater thickness as compared with the surrounding filament mat, and often an arrowhead-like morphology, a common feature of vinculin foci. Closer inspection of these sites showed them to be short bundles of actin filaments clearly distinct from the microspike bundles and from which filaments characteristically splayed out from the posterior ends. The smallest recognizable sites of this type were $\sim 0.5 \mu\text{m}$ long by 0.1–0.2 $\mu\text{m}/\text{min}$ width (e.g., Fig. 6 *d*). In some cases, microtubules were observed to enter these sites (Fig. 6, *a* and *b*) again identifying them with loci of the type already described in the foregoing sections.

Discussion

We draw attention first, in this report, to the striking relationship between sites of ruffling activity in the leading edge and sites of substrate contact formation. Here the term “ruffling” as it is generally understood needs some qualification (see also Small and Rinnerthaler, 1985). We use this term not just for the large veil-like ruffles described in much detail by Abercrombie and colleagues (Abercrombie et al., 1970*b*; Ingram, 1969; Harris, 1973) but also for other smaller perturbations of the leading edge that lead to localized increases in phase density. Abercrombie and Ambrose (1958) were well aware of this multiplicity of “ruffle” forms but subsequent attention was restricted mainly to the large membrane folds for which the name is most appropriate. And since ruffling of this latter type may be inhibited without inhibiting locomotion (Heaysman and Pegrum, 1982; Trinkaus, 1984) ruffling per se has been generally considered an “unimportant epiphenomenon” (Trinkaus, 1984), as far as movement is concerned. This contention has been further reinforced by the example of epidermal keratinocytes that locomote very rapidly in culture and show no macroscopic ruffling activity (Cooper and Schliwa, 1986; Kolega, 1986). However, close inspection of these latter cells revealed earlier the presence of small perturbations or “microcolliculi” (Bereiter-Hahn et al., 1981) in the leading edge. Since these microcolliculi resemble the smallest ruffling sites, or mini ruffles, seen in chick fibroblasts we propose that ruffling on this scale may indeed be a necessary part of the locomotory process and is linked to the kind of contact formation required for leading edge protrusion.

Our observations reveal, in general, a clear correlation between the amount and extent of ruffling activity taking place at a particular site and the size of the contact foci seen after fixation. We conclude therefore that ruffling, taken here in its broader sense as localized increases in vertical thickness of the leading edge is an expression of a specialized activity concerned with the accumulation and laying down of components necessary for making contact. It is interesting to note that a correlation between ruffling and contact development is also seen in spreading epithelial cells: active ruffling takes place during the spreading process and subsides once extensive peripheral contacts have been established (Domnina et al., 1985).

Figure 6. Presumptive, early contact foci in the leading edge, observed in negatively-stained whole mount cytoskeletons (phosphotungstic acid staining). (*a* and *c*) Intermediate magnification views of leading edge regions of chick heart fibroblasts. Open unmarked arrows point to presumptive, early contacts. (*b* and *d*) Higher magnification views of sites in *a* and *c*. In *b*, *mt* indicates microtubule entering actin bundle. Preparation in *c* and *d* was additionally stabilized with phalloidin after fixation. *sf*, Small stress fiber; *ms*, microspike; *am*, actin meshwork; *if*, intermediate filament. Bars (*a* and *c*) 1 μm ; (*b* and *d*) 0.2 μm .

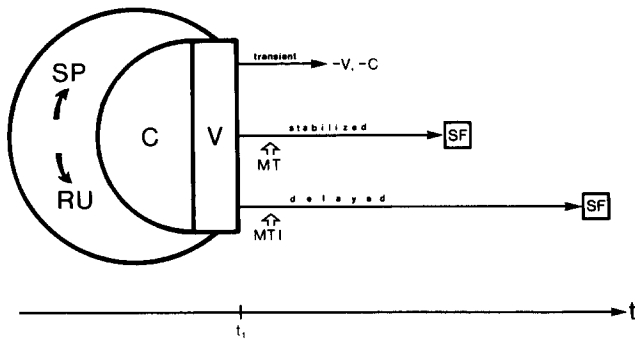


Figure 7. Schematic representation of sequence of events leading to contact formation. The contact-forming unit comprises ruffles or microspikes (or both), vinculin and other plaque proteins, and microtubules, appearing in the following sequence: Initially leading edge protrusion takes place and the protruding leading edge then undergoes structural reorganization, seen as the expression of ruffles (RU) and microspike bundles (SP). The single or combined formation of these structures, often in a cyclic fashion, leads to contact formation (C). Contact is followed by vinculin accumulation (V) at the contact site. Thereafter, at t_1 , the contact may exist only transiently (-V and -C) or persist, enlarge and nucleate stress fiber (SF) formation. Under normal conditions (middle horizontal line) microtubules (MT) are proposed to potentiate and stabilize stress fiber contacts. In their absence (with microtubule inhibitors: MTI, lower line) stable contact development is delayed and is no longer coordinated with regional leading edge movements (see text for further discussion).

In concert with the conclusions of Izzard and Lochner, 1980; Izzard et al., 1985) contact was also observed to be mediated via the extension of a microspike from the periphery of the leading edge, often in combination with ruffling activity at or behind the same site. And some vinculin foci were located directly beneath microspike bundles. However, a large complement of the microspikes, revealed in the actin pattern, were not associated with contacts. Moreover, we have identified vinculin-containing contact loci at the base of the leading edge, aside from microspikes and that clearly correspond to the actin-bundling sites seen in negatively-stained cytoskeletons in the electron microscope. Thus, in general terms the conclusion of Izzard and coworkers that contact is associated with actin bundles appears to be correct, but these bundles take on forms that are also distinct from microspikes. Also, while we agree in general that pre-existing actin bundles (as microspikes within or separate from ruffles) can mark the sites of future contacts, these bundles do not have to be directly associated with the membrane. They may disappear concomitantly with the de novo assembly of actin bundles in the new contact.

Before discussing further aspects of contact formation that arise out of this work some additional comment on previous studies on cell-to-substrate contacts is appropriate. According to the criteria of the interference reflection method, forward protrusion of the leading lamella of chick fibroblasts (Izzard and Lochner, 1976; 1980; Couchman and Rees, 1979) and other cells (Haemmerli et al., 1980; Bereiter-Hahn et al., 1981) requires only close contact formation, predominantly at the base of the leading edge. More firm focal contacts can develop within close-contact zones and elsewhere within the leading edge and these act to nucleate and anchor the stress fiber bundles (Izzard and Lochner, 1980) that

characteristically span the cytoplasm of most cells in culture. The stress fibers have been attributed a contractile function (Isenberg et al., 1976; Izzard and Lochner, 1980) necessary for the maintenance of the spread state (Vasiliev, 1985) and clearly expressed during the retraction of the trailing tail of moving fibroblasts (Chen, 1981). Close contacts have thus been considered as specialized for spreading and locomotion whereas focal contacts have the opposite function of mediating firm adhesion and immobility (see also Trinkaus, 1984). This generalization only applies, however, in the context of the limitations of the interference reflection method itself. By this technique the smallest recognizable focal contacts fall in the size range of 0.25 by 1–2 μm (Izzard and Lochner, 1980). Vinculin foci in the leading edge can be much smaller than this and from the results of the antibody exclusion method of Neyfakh and coworkers (Neyfakh et al., 1983) these very small sites appear to be close enough to the substrate to be regarded as focal contacts of a primordial type.

With the foregoing considerations in mind and the demonstrated association of microtubules with some of the new contact loci in the leading edge we propose here a general scheme for contact formation in chick fibroblasts. This scheme incorporates the concept of a "contact forming unit" comprising ruffling and spike activity, contact proteins (including vinculin) and microtubules (Fig. 7). After the first event of leading edge protrusion, mediated presumably by actin polymerisation at the tip of the leading edge (Small et al., 1978; Wang, 1984). The leading edge meshwork (Small, 1981) undergoes structural reorganisations leading to the formation of microspike bundles (SP) and ruffles (RU). (Depending on the cell type the initial protrusion may also involve extension of pre-existing microspike bundles e.g. Wessels et al., 1973). Contact (C) at specific sites is then made, possibly by accumulated interactions with the substrate (beneath ruffles and or spikes), during which cellular material may or may not be deposited on the substrate. In any case an extracellular structure located by or deposited by the cell is presumed to trigger contact (Geiger et al., 1984). Membrane receptors (e.g., members of the integrin family: Hynes, 1987) then accumulating at these sites signal the deposition of the cascade of proteins (including vinculin, V) that finally bind actin to the membrane (see Geiger, 1982; Geiger et al., 1984; Horwitz et al., 1986). The specific bundling of actin at the membrane is presumably effected by an actin-binding protein within or associated with the cascade. Ruffling appears to be necessary for the accumulation of the peripheral cascade of proteins at the contact site. We further propose that the primordial contacts formed may follow either of two paths: they may be short lived, existing only within the leading edge (Fig. 7 "transient"), or long lived and committed to enlarge and act as nuclei for stress fiber bundles (SF). The traction for leading edge protrusion is provided by the primordial contacts.

How do microtubules fit into this scheme? The evidence we have obtained suggests that microtubules can enter the cycle of contact formation and influence contact development. Clearly, microtubules are not essential for contact formation since contacts, as well as stress fibers associated with them, can form in the presence of microtubule-disrupting drugs (Lloyd et al., 1977). However, the orientation of the stress fibers and the direction of the protruding leading edges are then uncoordinated. This lack of coordination gives rise to

the random protrusion of lamellipodia and the inability to undergo net locomotion (e.g., Vasiliev and Gelfand, 1981). Significantly, cells without microtubules take 5–10 times longer to spread than those with their normal complement of microtubules (Vasiliev and Gelfand, 1981). Taking this observation together with our own data we suggest the following: that microtubules stabilize and thus accelerate the development of contacts destined to nucleate stress fiber bundles for anchorage (Fig. 7 “stabilized”). Via this potentiation of contact development, a stress fiber system can be laid down rapidly enough that has a polarity compatible with locomotion in the direction of the preceding leading edge protrusion. Without this potentiation, in the presence of microtubule inhibitors (MTI) stress fiber development lags too far behind lamellipodia protrusion for coordination to be possible (Fig. 7 “delayed”).

In more general terms Lloyd et al. (1977) indeed concluded that microtubules influence the directionality of microfilament bundle formation, in line with the above arguments. What we are proposing here is that microtubules exert their influence through their ability to stabilize and thus potentiate the growth of cell-substrate contacts. In this context it is noteworthy that the measured growth rate for microtubules in a cultured cell line *in vivo* (3–4 $\mu\text{m}/\text{min}$: Schulze and Kirschner, 1987) closely matches the speed of protrusion of the leading edge (Abercrombie et al., 1970a, see also this paper), fully consistent with microtubules (presumably under the direction of perinuclear signals) being able to follow and target contact sites.

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