Myosin II Filament Assemblies in the Active Lamella of Fibroblasts: Their Morphogenesis and Role in the Formation of Actin Filament Bundles

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Abstract. The morphogenesis of myosin II structures in active lamella undergoing net protrusion was analyzed by correlative fluorescence and electron microscopy. In rat embryo fibroblasts (REF 52) microinjected with tetramethylrhodamine–myosin II, nascent myosin spots formed close to the active edge during periods of retraction and then elongated into wavy ribbons of uniform width. The spots and ribbons initially behaved as distinct structural entities but subsequently aligned with each other in a sarcomeric-like pattern. Electron microscopy established that the spots and ribbons consisted of bipolar minifilaments associated with each other at their head-containing ends and arranged in a single row in an “open” zig-zag conformation or as a “closed” parallel stack. Ribbons also contacted each other in a nonsarcomeric, network-like arrangement as described previously (Verkhovsky and Borisy, 1993. J. Cell Biol. 123:637–652). Myosin ribbons were particularly pronounced in REF 52 cells, but small ribbons and networks were found also in a range of other mammalian cells.

At the edge of the cell, individual spots and open ribbons were associated with relatively disordered actin filaments. Further from the edge, myosin filament alignment increased in parallel with the development of actin bundles. In actin bundles, the actin cross-linking protein, α-actinin, was excluded from sites of myosin localization but concentrated in paired sites flanking each myosin ribbon, suggesting that myosin filament association may initiate a pathway for the formation of actin filament bundles. We propose that zig-zag assemblies of myosin II filaments induce the formation of actin bundles by pulling on an actin filament network and that co-alignment of actin and myosin filaments proceeds via folding of myosin II filament assemblies in an accordion-like fashion.

Myosin II is an actin-dependent motor protein which is capable of self-assembly with the formation of filaments of various size and morphology (see reviews by Korn and Hammer, 1988; Trybus, 1991; Tan et al., 1992). In non-muscle cells, myosin II has been implicated in the generation of cytoskeletal tension which contributes to the maintenance of cell integrity, cell locomotion and division (Mabuchi and Okuno, 1977; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Höner et al., 1988; Wessels et al., 1988; Spudich, 1989; Sims et al., 1992; Conrad et al., 1993; Jay et al., 1995) and may also be important in the process of assembly of cytoskeletal structures (Lamb et al., 1988; Fernandez et al., 1990; Fukui et al., 1990).

To serve the needs of changing cell morphology and variable motile activities, the cytoskeleton of nonmuscle cells is dynamic and, hence, cell spreading and locomotion on the substratum may be predicted to be associated with the formation and reorganization of myosin-containing structures. Several studies have shown that newly formed cell protrusions are initially devoid of myosin II (Yumura and Fukui, 1985; Conrad et al., 1989, 1993; Kolega and Taylor, 1993). In older and more stable regions of the cytoplasm further away from the active edge of cultured mammalian cells, myosin II is distributed in both a punctate and continuous manner (Weber and Groeschel-Stewart, 1974; Fujikawa and Pollard, 1976; Zigmund et al., 1979; Herman and Pollard, 1981; McKenna et al., 1989; Conrad et al., 1993) along bundles of actin microfilaments (stress fibers) which are considered to be contractile (Isenberg et al., 1976; Kreis and Birchmeyer, 1980; Conrad et al., 1993). Sites of myosin localization along stress fibers have been shown to alternate with sites of concentration of another component of contractile machinery, α-actinin, indicating similarity to the organization of skeletal muscle (Gordon, 1978; Langanger et al., 1986).

The dynamics of a microinjected fluorescent analog of cellular myosin demonstrated formation and centripetal movement of myosin-containing spots in the lamellum (McKenna et al., 1989) and centripetal movement and con-
traction of myosin-containing fibers (Giuliano and Taylor, 1990; Conrad et al., 1993). However, it was not clear how individual myosin containing spots became organized into fibers and how it was related to the organization of actin and actin-associated proteins, e.g., α-actinin. Precise understanding of the exact stages of formation of the myosin II-containing structures and their relationship to the contractile machinery has been hampered by a lack of information at the electron microscopic level. Although immunogold labeling of stress fibers was suggestive of an ordered myosin arrangement similar to skeletal muscle sarcomeres (Langanger et al., 1986), the internal organization of individual myosin-containing spots remained unclear.

By first removing actin filaments through treatment of cytoskeletons with gelsolin, we directly visualized at the electron microscopic level individual myosin bipolar mini-filaments (Svitkina et al., 1989) and showed that they were organized as an irregular, nonsarcomeric network (Verkhovsky and Borisy, 1993). We suggested that the network might represent one possible mode of a dynamic myosin organization and be interconvertible with a sarcomeric mode and we speculated that the myosin II network might play a role in the organization of actin. However, for technical reasons, our previous study was restricted to stationary cells treated with nocodazole to deplete the lamellum of interferring microtubules and intermediate filaments. Clearly, evaluation of the possible functional roles of the myosin II structures in fibroblast motility required placing these supramolecular assemblies in the kinetic context of cell locomotion and the protrusion—withdrawal cycle.

In the present study, we have attempted to determine a morphogenetic pathway for the development of myosin II filament assemblies and their possible role in the organization of actin filament bundles in cells undergoing net protrusion. We have analyzed: (a) the formation of myosin II-containing structures in relation to the behavior of the active lamellar edge by time-lapse fluorescence microscopy; (b) their supramolecular organization by correlative electron microscopy of actin-depleted preparations; and (c) the mutual arrangement of myosin structures, actin filaments and sites of concentration of α-actinin by immunofluorescent and immunogold procedures. We have focussed on rat embryo fibroblasts (REF 52) which we found to be characterized by exceptionally distinct and clear myosin II features that we term “spots” and “ribbons,” the latter term designating elongated spots of uniform width. Our results suggest that these myosin features comprise independent structural entities and that myosin II self-organization in the vicinity of the leading edge may precede and induce the formation of actin filament bundles. We present a model for the morphogenesis of myosin II-containing entities and the mutual interactions between the myosin assemblies and actin filaments.

Materials and Methods

Cells

The REF 52 (p53 transformed rat embryo fibroblasts) cell line was a generous gift of Dr. Soo-Siang Lim (Indiana University Medical Center, Indianapolis, IN); TC-7 cells (African green monkey kidney cells) were generously provided by Dr. J. C. Bulinski (Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York); Human 356 foreskin fibroblasts were kindly provided by Dr. R. I. DeMars (Department of Genetics, University of Wisconsin, Madison, WI); Swiss 3T3 mouse fibroblasts, and SL-29 chicken embryo fibroblasts were obtained from American Type Culture Collection (Rockville, MD). All cell lines except chicken fibroblasts were cultured in HAM's F-10 medium supplemented with 10% fetal bovine serum and antibiotics. Chicken embryo fibroblasts were cultured in DMEM supplemented with 5% tryptophosphatase broth, 5% fetal bovine serum, and antibiotics. For microinjection, cells were cultured on cover slips coated with gold through a finder grid and mounted in 35 mm Petri dishes as described (Verkhovsky and Borisy, 1993).

Microinjection

Microinjection of REF 52 cells with derivatized turkey gizzard myosin was performed essentially as described (Verkhovsky and Borisy, 1993) except that a tetramethyl-rhodamine myosin derivative which had better solubility was used instead of X-rhodamine myosin. Myosin isolation and derivatization with tetramethylrhodamine maleimide was performed in the same way as described for X-rhodamine myosin (Verkhovsky and Borisy, 1993). Cells were microinjected 5–10 h after plating at low density, returned to culture for 2–4 h to allow for myosin incorporation, and then observed in a Peri dish on the microscope stage kept at 32–35°C with the aid of water circuit incubator.

Light Microscopy

A Zeiss IM 35 microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 100 W mercury arc lamp was used for fluorescence and phase contrast microscopy of both living and lysed cells. A SIT camera (Duge-MTI, Inc., Michigan City, IN) was used for searching and focusing, and digital images were acquired with a series 200 CCD camera (Photometrics Ltd., Tucson, AZ) containing either 384 × 576 (Thompson CSF TH7828CA) or 512 × 512 (Tektronix) pixel chips that were thermoelectrically cooled to −50°C to reduce the dark current noise. To follow changes in myosin distribution and to not overbleach the specimen, fluorescence time-lapse sequences were generally acquired at a rate of 1 image per 2 min. Images were digitized to 14-bit depth using Photometrics software or Universal Imaging (Westchester, PA) MetaMorph software and stored on WORM drive optical discs (model 3363, IBM Corp., Danbury, CT) and 3.5 in. rewritable magneto-optical discs (Verbatim Corp., Charlotte, NC). Image-1 and MetaMorph (Universal Imaging Corp., Westchester, PA) image processors and Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) software were used for processing of images and reconstructing time-lapse series. Prints of the images were obtained from the digital files using a Phaser II dye-sublimation color printer (Tektronix Inc., Wilsonville, OR).

Labeling of Cytoskeletal Structures

Affinity purified polyclonal antibody to nonmuscle myosin was prepared and used as described earlier (Verkhovsky et al., 1987). Monoclonal antibody against α-actinin, rhodamine-phalloidin, and 10 nm colloidal gold-conjugated anti-rabbit IgG were obtained from Sigma Chem. Co. (St. Louis, MO); other secondary antibodies used were fluorescein-conjugated anti-mouse IgM-specific IgG, and fluorescein or rhodamine conjugated anti-rabbit IgG. Before staining for actin microfilaments, myosin, and α-actinin cells were washed with PBS, lysed with 1% Triton X-100 in a cytoskel-eton-stabilizing solution containing 50 mM imidazole, 50 mM KCl, 0.5 mM MgCl₂, 0.1 EDTA, 1 mM EGTA and 4% polyethylene glycol, M₄, 40,000, as described (Svitkina et al., 1984) and briefly washed with the same solution without Triton X-100 and polyethylene glycol. Brightest staining and the best preservation of cytoskeletal structures were obtained when lysed and washed cells were first incubated for 20 min with primary antibodies to myosin or α-actinin and rhodamine-phalloidin in cytoskele-ton-stabilizing solution, washed and fixed with 2% glutaraldehyde in so- dium-cacodylate buffer, treated with sodium borohydride and then stained with secondary antibodies. However, similar patterns of the distribution of myosin, α-actinin and actin were also observed when lysed cells were fixed with 4% formaldehyde and then treated with primary and sec-ondary antibodies. Collodial gold labeling was performed after incubation with anti-myosin antibodies, glutaraldehyde fixation and borohydride treatment by incubation overnight with the anti-rabbit colloidal gold-conjugated antibody in solution containing 20 nM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05% Tween 20, and 1% bovine serum albumin. After washing with the same buffer but containing 0.1% bovine serum albumin, speci-
mens were additionally fixed with glutaraldehyde, and processed for electron microscopy.

**Extraction of Actin from Cytoskeletons with Gelsolin**

Recombinant gelsolin NH$_2$-terminal domain was a generous gift of Dr. M. L. Greaser (Department of Meat and Animal Science, University of Wisconsin, Madison, WI). Treatment with gelsolin was performed as described (Verkhovsky and Borisy, 1993) after lysis of the cells for 3 min at ambient temperature in lysis buffer containing 50 mM MES-KOH, pH 6.0, 5 mM MgCl$_2$, 3 mM EGTA, 1% Triton X-100, and 4% polyethylene glycol (modified after Avnur et al., 1983). In our previous study (Verkhovsky and Borisy, 1993), we demonstrated that gelsolin treatment did not induce changes in myosin distribution in stationary, nocodazole-treated Swiss 3T3 cells. The purpose of nocodazole treatment was to induce collapse of the intermediate filaments which otherwise interfered with visualization of myosin structures. However, because nocodazole inhibits fibroblast motility, in this study we did not treat cells with nocodazole. We found that in actively protruding lamella, intermediate filaments were sparse, and did not interfere with visualization.

To confirm that the extraction/gelsolin-treatment procedure did not alter myosin organization in protruding REF 52 cells not treated with nocodazole, fluorescent images taken before extraction were compared to the images after extraction and gelsolin treatment. For this purpose, extraction was performed several seconds after taking an image of a living cell in a Petri dish on a microscopic stage by rapid aspiration of culture media and pipetting the extraction media. The image of extracted cell was then taken, the dish was removed from the microscope and washed in gelsolin incubation buffer. Then, the cytoskeletons were treated with gelsolin, fixed, and another image was taken.

**Electron Microscopy**

Platinum replicas were prepared from critical-point dried cytoskeletons essentially as described by Svitkina et al. (1984) with modifications that will be described in detail elsewhere (Svitkina et al., 1993) that greatly improved the performance of the method and made possible correlative light and electron microscopy of a large number of cells. Briefly, these modifications include: fixation by sequential application of 2% glutaraldehyde, 0.1% aqueous tannic acid and uranyl acetate, horizontal positioning of specimens during dehydration and drying, and uranyl acetate treatment during dehydration. Specimens were coated with platinum and carbon, replicas were separated from the coverslips with the use of hydrofluoric acid, mounted on formvar-coated grids, and observed and photographed with a Philips 300 transmission electron microscope. Negatives were converted to digital files using a Nikon (Melville, NY) Scantouch digital scanner.

**Results**

**Dynamics and Organization of Myosin II in Protruding REF 52 Cells**

**Formation of Nascent Myosin Spots.** In the course of exploring a range of mammalian cell lines (Swiss 3T3 mouse fibroblasts, REF 52 rat embryo fibroblasts, human 356 foreskin fibroblasts, SL-29 chicken embryo fibroblasts, and TC-7 African green monkey kidney epithelial cells), we found that REF 52 cells were characterized by a particularly distinct distribution of myosin II “spots.” To determine where and when in the protrusion–withdrawal cycle the myosin spots arise, we carried out time-lapse fluorescence observation of actively advancing lamellar regions in cells microinjected with labeled myosin II. For our experiments we used freshly plated cells in a sparse culture which were radially spreading or randomly locomoting. As shown by the example of Fig. 1, cells would significantly expand their area during the period of observation, demonstrating that their lamella were actively protruding. Expansion proceeded through numerous cycles of protrusion and partial retraction and was accompanied by the appearance of many nascent myosin spots out of a region of weak diffuse fluorescence. This diffuse fluorescence was likely to be due to the presence of soluble fluorescent myosin because it was removable upon extraction of injected cells. The diffuse fluorescence in living cells was helpful because it served as an internal indicator for the position of the cell edge, as confirmed by comparison of fluorescent and phase contrast images of the same cell (not shown).

At our image acquisition rate of 1 frame per 2 min, apparent changes in the myosin distribution were slow; that is, most fluorescent myosin features in frame $n + 1$ could be identified with features in frame $n$. By definition, a nascent myosin spot was a clearly resolved fluorescent feature which could not be identified with any feature or a part of any feature in a previous frame. Frame-by-frame analysis indicated that most of the nascent myosin spots first appeared in close vicinity of the leading edge (see Figs. 1 b and 2 a). For the cell shown in Fig. 1, 39 of a total of 84 new spots were first detected on the very leading edge, 31 within 3 μm distance of the leading edge, and the rest at distances of up to 6 μm from the edge. Another criterion that we used was the position of the new spot relative both to the edge and to other spots. Most of the new spots were situated closer to the edge than preexisting spots. Only 9 new spots in the cell shown in Fig. 1 and only 27 of a total of 185 spots in 7 cells analyzed arose further away from the edge than preexisting spots.

In all cases when a new spot was detected on the edge, the edge occupied a more retracted position than in the previous and the subsequent frames (Fig. 2 a) and/or had a concave shape which is characteristic for lamella in the phase of retraction. Thus, our data are consistent with the hypothesis that myosin spots generally arise on the active edge during its retraction. Since the active edge advanced at a rate up to 2.5 μm/min, the myosin features that were first detected at a slight distance (≤ 5 μm) from the edge could have arisen at the edge at time points between frames (≤ 2 min) and then been left behind during the next cycle of protrusion. However, spots that were first observed further away from the edge than preexisting spots must have been indeed formed in the region behind the edge. Thus, although formation of nascent myosin spots was favored at the edge of the cell during its retraction, this was not a necessary condition for the spot formation.

Retraction of the active edge was also not a sufficient condition for the formation of myosin features. Sometimes, edge activity did not result in the deposition of new myosin spots or was only accompanied by an appearance of transient spots that were seen in only one frame and then disappeared (for the cell shown in Fig. 1, 8 transient spots were detected). In the cases where no stable myosin features were deposited, edge activity usually resulted in little or no net protrusion. However, if a spot persisted for more than one frame, it invariably became a stable feature that could be traced for the whole length of the observation period.

**Formation of Ribbons and Their Translocation.** After their formation, nascent myosin spots usually became brighter and grew one-dimensionally to become a “ribbon” (Fig. 1 b, long arrow) defined as elongated spot of uniform width. Alternatively, several spots merged with the result of rib-
Figure 1. Dynamics of microinjected tetramethylrhodamine-myosin in the lamellum of a rat embryo fibroblast (REF 52 cell line). (a) An overview of the lamellum at start of observation. Dotted line shows position of the cell edge at time zero and broken line shows the position of cell edge 26 min later. Box shows the cell region for which dynamics are shown in detail in b. Numbers indicate time in minutes past start of observation. (b) Myosin ribbon that elongated dramatically is shown by long arrow; ribbon that broke into two pieces...
Figure 2. Formation of new myosin features on the edge of retracting lamellipodia. (a) Spots are first seen on the edge (arrowheads) during the retraction phase of the lamellipodia; then protrusion of the lamellipodia resumes and myosin features are left behind. (b) In a cell with sparser myosin organization and less activity at the edge, new myosin spots are formed both near the edge (arrowheads) and more proximally (long arrows), in between preexisting spots. Short arrows indicate identical features in two frames showing the extent of retrograde myosin flow. Numbers indicate time in minutes past start of observation; dotted line shows the position of cell margin. Bars, 2 μm.

In accordance with results reported by others (McKenna et al., 1989; Giuliano and Taylor, 1990; Conrad et al., 1993), our time-lapse sequences showed that myosin-containing spots and ribbons usually moved away from the edge, although the exact direction and speed of the motion varied. For the newest, most peripheral spots, the major vector of movement was away from the edge while older spots could also move parallel to the edge. In polarized cells, ribbons usually translocated perpendicular to their long axis. Thus, ribbons perpendicular to the edge translocated mostly parallel to it, while ribbons parallel to the edge moved away from it (Figs. 1 b and 2 b). In many cases, distances between ribbons shortened (perpendicular to their axis) and ribbons sometimes fused together forming regions of continuous myosin fluorescence. The overall process could be described as a uniform contraction to some focus of myosin condensation (perinuclear region, or, in other cases, some region in the lamellum) and was probably analogous to the previously reported contraction of transverse fibers in protruding cells (Conrad et al., 1993).

In summary, the dominant myosin features in the vicinity of the active edge of freshly plated REF 52 fibroblasts were distinct, small spots scattered in the lamellum that grew and fused to form ribbons which subsequently became aligned in a sarcomeric-like pattern. Similar patterns were observed in both injected and noninjected cells after extraction and staining for myosin with polyclonal antibody.
Ultrastructural Identity of Myosin Spots and Ribbons

To reveal the myosin ultrastructural organization, we used the strategy described previously (Verkhovsky et al., 1987; Svitkina et al., 1989; Verkhovsky and Borisy, 1993). We extracted the injected cells, treated them with recombinant gelsolin to remove actin, and compared platinum replicas of the treated cytoskeletons to their fluorescent images. Removal of actin, which comprises most of the cytoskeletal mass, allows small myosin filaments to be directly visualized in EM preparations. We have confirmed that similar to what was found in our previous study on nonmotile, nocodazole-treated Swiss 3T3 cells, the extraction/gelsolin-treatment procedure did not alter myosin organization in protruding REF 52 cells.

After gelsolin treatment, myosin minifilaments were easily identifiable in platinum replicas by their characteristic dumbbell shape and size. Single myosin filaments were infrequently observed; most of them contacted each other to form aggregates of various size and morphology (see below). Other structural features apparent in peripheral regions of gelsolin-treated cytoskeletons were some very thin filaments of unknown nature, some amorphous material, and a few intermediate filaments. This is in accord with the report that the active edge is mostly devoid of intermediate filaments (Hynes and Destree, 1978).

Figure 3. Ultrastructural identity of individual myosin features and modes of myosin filament arrangement visualized in cytoskeletons treated with gelsolin to remove actin. (a) Fluorescence micrograph of tetramethylrhodamine-myosin-injected and gelsolin-extracted cell. (b and c) Arrangement of myosin filaments in spot (b) and ribbon (c) shown in a by arrowhead and arrow, respectively. (d) Arrangement of myosin filaments in open ribbon and (e) in sets of tightly packed parallel ribbons. Bars: (a) 2 μm; (b-e) 0.2 μm.
All myosin features observed by fluorescence microscopy could be accounted for by structures seen in replicas of the same cells. Each spot or ribbon corresponded to a cluster of myosin bipolar minifilaments apparently contacting each other at their head-containing ends (Fig. 3, a–c). The faintest detectable spots corresponded to clusters of about five myosin filaments. In both myosin spots and ribbons, individual bipolar minifilaments were arranged mostly side by side in one row. Thus, there was no fundamental structural difference between a spot and a short ribbon. All ribbons were similar in width (the length of one bipolar minifilament), while they varied considerably in length depending on the number of filaments and the density of their packing. In the vicinity of the edge, ribbons were separated from spots and each other, whereas further from the cell periphery, ribbons aligned in parallel and could make contact with each other (Fig. 3 e). Similar arrangements of myosin bipolar filaments in the form of spot-size clusters and long ribbons were also observed in replicas of REF 52 cells that were not injected with exogenous myosin.

**Variability of Filament Packing in Spots and Ribbons**

Individual myosin spots and ribbons showed considerable variability in the density of filament packing. Two extreme conformations were identified: one loose with contacting filaments oriented at various angles to each other which we term “open” or “zig-zag” conformation (Fig. 3 d), and the other with filaments tightly packed parallel to each other termed “closed” or “stack” conformation (Fig. 3 c). The “open” conformation was more frequent in small spots and single ribbons localized close to the cell edge. In sets of several parallel ribbons, individual ribbons usually had a “stack” conformation with the degree of filament alignment apparently increasing towards the cell center. Another variable in ribbon organization was the frequency of contacts between neighboring ribbons. The closer to the perinuclear region, the more abundant were contacts between ribbons until ribbons sometimes become completely fused together. In some cell regions, individual ribbons were hard to trace because filaments in them were not perfectly aligned in a single row and often contacted neighboring ribbons. The overall myosin organization in such cases appeared similar to the irregular two-dimensional network described in our previous study (Verkhovsky and Borisy, 1993).

To estimate changes in filament packing with distance from the edge, we measured the angle between contacting filaments in three cellular domains: the vicinity of the cell edge (mostly spots and nonaligned ribbons); the transition zone (ribbons beginning to align); and an inner zone (parallel ribbons but myosin filaments still packed sparse enough to allow interfilament angles to be measured). All the filaments in apparent direct contact were scored irrespective of whether they belonged to one ribbon or different ribbons. If several filaments were apparently radiating from one point, all the angles between nearest neighbors were scored such that the sum of angles did not exceed 360°. As apparent from Fig. 4, the fraction of angles close to 0 (near-parallel packing of the filaments in ribbon) and to 180° (end to end contacts of filaments belonging to different ribbons) progressively increased with the distance from the cell edge. Thus, measurements confirmed our visual impression that the degree of myosin filament alignment and the frequency of contacts between ribbons increased towards the cell center.

In a protruding cell, regions closest to the edge obviously are the youngest. Even in the absence of net protrusion, myosin features were found to arise at the edge and then move centripetally. A reasonable inference was that the spots and open ribbons seen by electron microscopy close to the edge were the recently formed, nascent structures. To directly evaluate the correlation between age of the ribbon and its conformation, we examined replicas of cells with a known history. The dynamics of myosin spots were first monitored in the living cells shortly after they arose (Fig. 5, a–c). Comparison of fluorescence in the living cell to that after extraction/gelsolin treatment and fixation (Fig. 5, c and d) demonstrated that the procedure did not alter myosin organization at the light microscopic level. As shown in Fig. 5 e, two nascent ribbons and a spot showed an open conformation (arrowheads and a small arrow). This was the general rule. However, the packing of nascent myosin structures was variable. For example, a recently formed bigger spot (long arrow) had very dense filaments.
Figure 5. Arrangement of myosin filaments in newly formed myosin structures. (a–c) Dynamics of tetramethylrhodamine-myosin near the active edge of a living cell. Numbers indicate time in minutes past start of observation. (d) The same region of the cell after extraction and gelsolin treatment. Myosin distribution has not changed but the image looks crisper due to longer exposure time which was possible to use on fixed cell to obtain image of better clarity. (e) Platinum replica of the cell region indicated by box in d. Newly formed myosin ribbons showing open conformation and their predecessor spots in previous frames are shown by arrowheads; newly formed spots with loose and tight packing of filaments are shown by small and large arrows, respectively. Bars: (a) 2 μm; (e) 0.2 μm.

To discern the common patterns of myosin filament arrangement, we examined myosin organization in several other types of cells: mouse 3T3 fibroblasts, human and chicken fibroblasts, and monkey kidney epithelial cells. By immunofluorescence microscopy of these cells stained with antibodies to myosin, no recurrent ribbon pattern was evident although elongated myosin spots were quite frequent and sometimes aligned in parallel arrays. However, electron microscopy of actin-depleted cytoskeletons characteristically showed stack-like or zig-zag-like arrays of myosin filaments at the cell periphery (Fig. 6), although these arrays were not as large and distinct as in REF 52 cells. These mini-ribbons usually were easily recognizable only in a narrow region along the edge of the cell. Closer to the cell center, they appeared to fuse to each other forming an irregular network as described in a previous paper (Verkhovsky and Borisy, 1993). Thus, myosin ribbons characteristic of REF 52 cells represent a principle of myosin filament packing common for all the cell lines studied.

Mutual Arrangement of Actin Bundles, Sites of α-Actinin Distribution and Myosin Spots and Ribbons

To determine how myosin spots and ribbons were related to actin filament bundles or stress fibers, we performed double immunofluorescence staining for myosin and actin and also for myosin and another component of actin filament bundles, α-actinin (Lazarides and Burridge, 1975; Kreis and Birchmeier, 1980; Sanger et al., 1983; Byers et al., 1984; Langanger et al., 1986). Myosin and actin were also visualized in parallel at the level of electron microscopy.

Light microscopic images obtained with the use of rhodamine-phalloidin and a polyclonal antibody against myosin (Fig. 7, a–e) suggested that the relationship between myosin and actin distribution was complex. Actin filament bundles were seen close to the base of lamellipodia and were oriented parallel to the cell edge in radially spreading cells and both parallel and perpendicular to the edge in polarized cells. Between the bright actin bundles and throughout the lamellipodia, phalloidin stained the cells in a continuous and uniform manner which was suggestive of the presence of loosely organized actin filaments.

In contrast, antibody to myosin revealed very distinct spots and ribbons with little fluorescence in between. Some myosin spots and ribbons were found in the zone of diffuse actin distribution, suggesting that the formation of myosin spots may precede the organization of actin filament bundles. Other individual spots and ribbons in the vicinity of the cell edge were localized on small actin fibers (often only one spot or ribbon per fiber), but colocaliza-
tion was not perfect and some ribbons were significantly wider than the actin fiber with which they were associated. Often a single, seemingly continuous myosin ribbon crossed several distinct actin fibers which could be parallel to each other or spread out like a fan from the site of myosin localization. These ribbons were sometimes seen bent sharply at sites of crossover with individual actin bundles as if different regions of one ribbon were pulled in different directions. Further from the cell edge, sets of parallel myosin ribbons usually crossed well developed actin bundles, creating a sarcomeric-like pattern.

Staining for α-actinin, similar to staining for actin, showed bright fibers at the base of lamellipodia with a rather continuous and uniform distribution between these fibers (Fig. 7, f-j). Small actin bundles that were associated with individual myosin spots or ribbons showed an almost continuous distribution of α-actinin, with the exception of the very sites of myosin localization from which α-actinin was clearly excluded. In most cases, on both sides of the exclusion zone an enhanced staining for α-actinin was observed; that is, each myosin spot or ribbon was flanked by two regions of increased α-actinin concentration. Such a pattern suggested that the myosin spots were the organizing centers determining the sites where α-actinin was concentrated and from where it was excluded. In well-developed stress fibers, closely spaced myosin ribbons alternated with sites of α-actinin concentration creating the previously described striped pattern (Gordon, 1978; Langanger et al., 1986) similar to distribution of A- and I-bands of muscle sarcomeres. Thus, correlation of the myosin staining pattern to that of both actin and α-actinin was suggestive of a role of myosin spots and ribbons in organizing actin filament bundles.

To investigate the manner of actin–myosin association on the ultrastructural level, we performed immunogold localization of myosin in EM preparations of cytoskeletons from which actin was not removed. After gold labeling, myosin features were seen among actin filaments in replicas as distinct sites with a high density of bright (in reverse contrast) gold particles (Fig. 8). Single myosin filaments which sometimes were present at the cell periphery appeared as thick sticks of 0.3–0.4 μm length uniformly decorated with gold particles (Fig. 8, arrowheads). Because of the dense decoration by the polyclonal antibody against myosin and a secondary antibody conjugated to gold, it was neither possible to discriminate middle rod portions and head-containing ends of filaments nor to resolve individual filaments in dense aggregates. Nevertheless, with our knowledge obtained by direct imaging of undecorated myosin filaments in actin-depleted preparations, it was possible to recognize myosin spots and ribbons in gold-labeled specimens and extract information on filament arrangement. Spots were seen as small gold-labeled regions of irregular shape and ribbons as long bands about 0.3 μm in width. Some of these bands had a festooned profile reminiscent of the zig-zag conformation described above for the myosin filament ribbons in actin-depleted cytoskeletons; others were characterized by uniform dense packing of gold particles and smooth outlines, which is how one would expect stacks of labeled myosin filaments to look. In favorable cases, we observed clear zig-zag patterns made of gold-decorated sticks of characteristic length (0.3–0.4 μm).

Small spots and zig-zag ribbons were usually seen in cell regions close to the active edge identified by its characteristic convex shape and the presence of a dense rim of actin meshwork. These myosin features were associated with the actin network with no discernible preferential orientation of actin filaments (Fig. 8 a) or, more often, with small and rather loose bundles of actin filaments (Fig. 8 b). Similar to the light microscopic images, a single zig-zag ribbon frequently was seen to be associated with several actin filament bundles oriented in slightly different directions.

In contrast, myosin ribbons in stack conformation were seen to be associated with tight bundles of closely aligned actin filaments. Long ribbons in mixed conformation were generally associated with loose actin bundles in their open part near the cell edge, but further from the edge the same ribbon was seen to have stack conformation and be associated with more developed actin bundle (Fig. 8 c). Deeper in the cytoplasm, sets of parallel bands decorated with gold particles were found running across wide bundles of actin filaments or arrays of several parallel bundles resulting in a periodic pattern (Fig. 8 d). Thus, electron microscopy showed that small myosin ribbons (especially the “open” ones) were associated with random networks or loose bundles of actin filaments and that the “closing” of

Figure 6. Ribbon-like aggregates of myosin filaments in gelsolin-treated cytoskeletons of various cells. In chicken embryo fibroblasts (a) myosin ribbons are often localized close to intermediate filaments which are particularly abundant in these cells; in Swiss 3T3 fibroblasts (b) filaments in ribbon-like aggregates are typically not in perfect register. Bar, 0.2 μm.
Figure 7. Mutual arrangement of myosin to actin and α-actinin. Myosin (red), (a, b, d, f, g, and i) and α-actinin (blue) (f, h, and j) distributions are revealed by immunofluorescence staining; actin distribution, rhodamine phalloidin staining, is shown in blue (a, c, and e). Regions shown by boxes in a and f are also reproduced in b–e and g–j, respectively, to show distribution of actin (c and e), α-actinin (h and j), and myosin (b, d, g, and i) separately. Arrows indicate: bends of myosin ribbons at crossover points with actin filament bundles (b and c); myosin ribbons co-localized with amorphous foci of actin distribution (d and e); myosin ribbons coinciding with gaps in α-actinin banded pattern (g and h); and myosin spots and ribbons coinciding with gaps in α-actinin distribution flanked by paired sites of actinin concentration (i and j). Bars, 2 μm.
Figure 8. Immunogold staining of myosin filament aggregates in cytoskeletons from which actin has not been removed. (a) Open ribbon of myosin filaments is associated with randomly oriented actin filaments in a cell region close to the active edge (identified in a low magnification view by its overall convex outline). (b) Open myosin ribbon associated with loosely aligned actin filaments. Gold-decorated sticks that are likely to be individual myosin filaments or their small clusters are shown in a and b by arrowheads. (c) Myosin ribbon part of which is closed while the other part has open conformation. Tightly packed part of the ribbon is associated with well developed bundle of actin filaments and the open part (arrow) is associated with several small clusters of filaments. (d) Regularly spaced parallel myosin bands (closed ribbons) are associated with broad bundle of mostly well aligned actin filaments. Bars, 0.2 μm.
Discussion

Assembly and Organization of Myosin Structures

The principal objectives of this study were to determine how myosin II structures arise in the course of cell protrusion and what their role might be in the assembly of the actomyosin contractile machinery. By in vivo fluorescence microscopy, nascent myosin features had the appearance of small spots which grew and gradually assumed a characteristic ribbon-like morphology. By electron microscopy, the spots and ribbons were visualized as stacks or zig-zag arrays of bipolar minifilaments. The reality of these structures is supported by the fact that they were observed in noninjected as well as myosin-injected cells, by direct fluorescence as well as by immunofluorescence, in actin-depleted as well as intact cytoskeletons, and that individual features seen by fluorescence in living cells could be correlated with characteristic structures seen by electron microscopy of the same cells. Although the spot and ribbon features were particularly striking in REF 52 cells, our observations do not contradict previous reports about myosin distribution. Elongated myosin spots have been observed by many authors in a variety of cell types (Fujiwara and Pollard, 1976; Langanger et al., 1986; McKenna et al., 1989) and recently, images similar to ours were published by Maupin et al. (1994). However, the elongated spots described in these reports were considered an attribute of a punctate myosin distribution along stress fibers; that is, each spot was deemed analogous to the A-band of a muscle sarcomere. The new aspect which emerges from our study is that nascent myosin spots preferentially grew in one dimension, forming arrays of bipolar minifilaments. The reality of this type of assembly was supported by the observation that each visible myosin spot was in close vicinity of or directly at the edge of the cell during the retraction phase of its protrusion-withdrawal cycle. Then the distance between the newly formed spots and the edge rapidly increased due to the advance of the edge and to retrograde movement of the spot away from the edge. In this way, although many new spots first appeared at the edge, most spots were seen just behind the edge and no spots were seen at the edge during its protrusion phase. Growth of spots and formation of ribbons were subsequent events, occurring at some distance from the edge. These findings are consistent with reports that cytoskeletal myosin is essentially absent from new cell protrusions, but appears in "established" protrusions (DeBiasio et al., 1988; Conrad et al., 1989, 1993) and that the preferential site for myosin assembly is the region immediately behind the leading edge (Kolega and Taylor, 1993). By indicating more specifically that the site for formation of nascent myosin structures is retracting lamellipodia, our study raises the possibility that myosin may be involved in the process of its retraction. Alternatively, retraction of the lamellipodia may be a myosin-independent process which creates favorable conditions for aggregation of previously randomly dispersed myosin.

Our study also provides new information on the supramolecular structure of the myosin features and suggests a pathway for their assembly. Since each visible myosin spot consists of several minifilaments, the process of spot formation may be considered as equivalent to the aggregation of bipolar minifilaments. The source for this aggregation is presumably some soluble pool of myosin which is visible in the lamellipodia as a diffuse fluorescence removable upon extraction, but the resolution at the light microscopic level is not sufficient to distinguish whether the pool consists primarily of myosin molecules or minifilaments. Estimation of the diffusion coefficient of the myosin pool suggests that myosin is in an unpolymerized state (Kolega and Taylor, 1993). On the assumption that this conclusion is correct, single bipolar minifilaments would be predicted to be transient intermediates in the assembly of spots and ribbons. Consistent with this interpretation, single bipolar minifilaments were only rarely observed in our preparations.

After the initial aggregation of filaments which makes a spot visible, myosin spots dramatically elongate, while new separate spots rarely appear away from the active edge. This suggests that the assembly of myosin-containing structures preferentially occurs by addition of new filaments onto preexisting seeds, while formation of seeds is restricted primarily to the region of the newly spread lamellipodia. Such nucleated assembly may be indicative of a cooperative interaction between minifilaments. Nascent myosin spots preferentially grew in one dimension, forming ribbons characterized by a side-to-side association of filaments into a stack. Why is growth restricted to the side addition of bipolar filaments? Although we have no definitive answer to this question, the assembly pathway appears to be dependent on the cell type. In an exploration of a variety of cell lines including mouse, rat, human and chicken fibroblasts and monkey kidney epithelial cells, we found a spectrum of bipolar minifilament structures ranging from the ribbons prominent in REF 52 cells to the 2-dimensional network described previously (Verkhovsky and Borisy, 1993). Some regulatory factors may play a role in the restriction of the dimensionality of association and this restriction may be more stringent in REF 52 cells than in other cell lines. However, our data do not discriminate whether the dimensionally restricted interaction between myosin filaments is a direct myosin–myosin interaction or is mediated by some other protein(s). If other proteins are involved, nonmuscle titin, which has been reported to colocalize with myosin in cells and to induce myosin aggregation in vitro (Eilertsen et al., 1994) would be a likely candidate.

Relation to the Formation of Actin Filament Bundles

It has been proposed that tension developed in the course of actin–myosin interaction induces the alignment of actin filaments into bundles (Fleischer and Wohlfarth-Bottermann, 1975; Burridge, 1981; Ingber, 1993) and that myosin plays a role in organizing actin–filament arrays of opposite polarity (Rhee et al., 1994). Several studies showing disassembly of stress fibers and focal contacts upon inactivation of myosin by different treatments have also indicated an important role for myosin in actin organization (Honer et
Our structural data suggest that myosin aggregation may precede and induce formation of actin bundles. Aggregates of bipolar myosin filaments in the form of independent spots or open ribbons initially formed in the midst of or in association with randomly oriented actin filaments close to the active edge. Subsequently, individual spots and open ribbons became associated with small bundles of loosely aligned actin filaments while closed ribbons and parallel rows of ribbons were coincident with well developed actin bundles. The close correlation between the degree of organization of actin filaments and myosin structures implies a cause-effect or mutually interactive relationship.

The primary role of myosin aggregates in organizing actin bundles is also supported by our observations on the distribution of α-actinin, a component of Z-lines and dense bodies in striated and smooth muscle cells. If myosin ribbon assembly is induced along I-Z-I-like assemblies (actin filaments - Z-line analog - actin filaments), one would predict formation of paired myosin ribbons on both sides of presumptive Z-lines and, therefore, that myosin staining would flank α-actinin staining in paired densities. We did not observe pairs of myosin aggregates. In contrast, we observed paired densities of α-actinin flanking individual myosin spots. This result indicates that in the vicinity of the active edge, myosin aggregates rather than Z-lines or dense bodies appear to be starting points for the organization of actin and actin-binding proteins. It cannot be excluded, however, that similar to what has been proposed for skeletal muscle, the distribution of both myosin and of Z-line analogs is controlled by some template protein, such as titin (Fulton and Isaacs, 1991). Titin was reported to interact directly with myosin (Eilertsen et al., 1994) and was also proposed to specify positioning of CapZ, an actin-capping protein that is essential for development of striated patterns of actin and α-actinin in myofibrillogenesis (Schafer et al., 1995). From the template perspective, to explain the appearance of double sites of α-actinin concentration flanking a single myosin ribbon, it is necessary to postulate that the template carries a single site for myosin assembly and a pair of sites to specify the organization of actin.

Although it cannot be excluded that the organization of actin may proceed independently of or in parallel to organization of myosin, our kinetic and structural data is indicative of a pathway where myosin assemblies contribute to and control formation of actin bundles in the fibroblast lamellae. Based on our data, we have developed a hypothesis on how myosin aggregation may induce actin bundle formation and how reciprocal influences of actin bundles may channel myosin aggregation. In this model (Fig. 9), the driving force for the formation of small myosin aggregates is the capacity of myosin bipolar filaments to self-associate (either directly or through some association factor), not its interaction with actin. The myosin aggregate would be predicted to interact with the large number of actin filaments in which it is immersed and experience pulling forces from a variety of directions which are likely to equilibrate. Such an aggregate will be relatively stationary and may start to exert counterforces on actin filaments inducing them to align and organize into bundle(s), provided that actin filaments are anchored to some outside sites, e.g. nascent substrate contact sites. The pulling action of individual myosin aggregates could be transmitted over long distances and possibly in different directions by acting through long and interconnected actin filaments. This would account for how even single myosin ribbon may induce formation of one or several long actin bundles which could extend distally to the ribbon. Sharp bends of myosin ribbons at sites of crossover with actin bundles may be indicative of multidirectional pulling forces acting on the ribbon and of an ability of myosin aggregates to withstand these forces by virtue of the binding interaction between myosin filaments.

Simultaneously with alignment of actin filaments, components of the pulling forces acting on myosin will cause myosin filaments also to align with respect to each other and with respect to actin as discussed in our previous paper (Verkhovsky and Borisy, 1993). The axis of initial alignment of myosin and actin filaments may be determined by some anisotropic factor in the environment, such as the position of the cell edge or the orientation of preexisting actin bundles. As shown in the diagram in Fig. 9, initially filaments may align parallel to the cell edge and then realign parallel to the preexisting filament bundle if its local orientation is different. As alignment progresses, "open" spots and ribbons will become closed in an accordion-like fashion to form tight stacks of minifilaments. This idea is supported by the results of measurement of angle between contacting myosin filaments indicating progressive increase in the degree of filament alignment with the distance from the cell edge. Since actin bundle formation and myosin align-
ment are predicted to start shortly after formation of myosin aggregates, this is consistent with our observations that even nascent spots were sometimes in a "closed" configuration. The relatively rare examples of "open" myosin filament aggregates in an as yet completely disorganized actin environment are interpreted as transient intermediates.

Because the whole myosin ribbon/actin bundle system is inherently under tension, it will contract if actin attachment sites are broken or weakened. Myosin ribbons will come closer together in the process. In all events discussed above, tension developed due to actin–myosin interaction and resistance caused by association of myosin filaments with each other are likely to be primary driving forces. The force generating interaction of actin and myosin has long been a subject of study, but the capacity and possible physiological role of myosin filament self-interaction has only recently been emphasized (Verkhovsky and Borisy, 1993).

To further investigate the role of myosin filament self-association, it will be necessary to determine its molecular mechanism, to find a specific way to block it and to study the effect of this block on cytoskeletal assembly and cell behavior.

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