Polarity sorting of actin filaments in cytochalasin-treated fibroblasts

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SUMMARY

The polarity of actin filaments is fundamental for the subcellular mechanics of actin-myosin interaction; however, little is known about how actin filaments are oriented with respect to myosin in non-muscle cells and how actin polarity organization is established and maintained. Here we approach these questions by investigating changes in the organization and polarity of actin relative to myosin II during actin filament translocation. Actin and myosin II reorganization was followed both kinetically, using microinjected fluorescent analogs of actin and myosin, and ultrastructurally, using myosin S1 decoration and immunogold labelling, in cultured fibroblasts that were induced to contract by treatment with cytochalasin D. We observed rapid (within 15 minutes) formation of ordered actin filament arrays: short tapered bundles and aster-like assemblies, in which filaments had uniform polarity with their barbed ends oriented toward the aggregate of myosin II at the base of a bundle or in the center of an aster. The resulting asters further interacted with each other and aggregated into bigger asters. The arrangement of actin in asters was in sharp contrast to the mixed polarity of actin filaments relative to myosin in non-treated cells. At the edge of the cell, actin filaments became oriented with their barbed ends toward the cell center; that is, the orientation was opposite to what was observed at the edge of non-treated cells. This rearrangement is indicative of relative translocation of actin and myosin II and of the ability of myosin II to sort actin filaments with respect to their polarity during translocation. The results suggest that the myosin II-actin system of non-muscle cells is organized as a dynamic network where actin filament arrangement is defined in the course of its interaction with myosin II.

Key words: Actin, Myosin II, Polarity

INTRODUCTION

Cell contraction and translocation are believed to involve interaction of myosin II and actin (reviewed by Maciver, 1996; Mitchison and Cramer, 1996; Warrick and Spudich, 1987). Myosin II is a species unique among the myosin superfamily for its ability to self-assemble into bipolar filaments (Cheney et al., 1993). Bipolar myosin filaments have been directly observed in situ in a variety of mammalian cells in culture and shown to assemble into ordered assemblies in conjunction with actin filaments at the leading edge of the cell (Verkhovsky et al., 1995). Although myosin II filaments possess an intrinsic bipolarity, it is the polarity of actin filaments that determines the direction of relative translocation of actin and myosin; myosin is always transported toward the barbed end of an actin filament (Toyoshima et al., 1989; Yamada and Wakabayashi, 1993). Thus, the cellular processes that result from the relative translocation of actin and myosin are predicted to be dependent on how actin filaments are oriented with respect to myosin and to each other. The classic example of the fit of actin polarity organization to the ‘purpose’ of motility is striated muscle. Actin filaments within muscle sarcomeres are arranged in arrays of uniform polarity, which alternate periodically along the length of the fiber. Barbed ends of actin filaments are oriented away from the myosin-containing A-bands and are embedded in Z-line structures. When muscle is activated, myosin approaches the barbed ends of actin filaments which, given the above arrangement of actin relative to myosin, results in tension development and sarcomere shortening.

In contrast to skeletal muscle, the relationship of actin polarity organization to the mechanism of cell motility in non-muscle cells is not clear. Several basic questions remain to be answered: What is the polarity organization of actin involved in interaction with myosin II? Is this organization changed in the course of actin-myosin interaction? If so, what is the pathway of the reorganization? Several non-mutually exclusive models have been proposed as answers to these questions. We refer to them as the sarcomeric, transport and dynamic network models.

According to the sarcomeric model (Langanger et al., 1986; Sanger and Sanger, 1980), stress fibers of non-muscle cells are contractile structures, in design and function, essentially similar to the sarcomeres of striated muscle. Consistent with this view, stress fibers have been shown to contract under various conditions (Giuliano and Taylor, 1990; Isenberg et al., 1976; Kreis and Birchmeyer, 1980). A problem, however, is that the alternating polarity of actin filaments predicted by the sarcomeric model was not readily apparent in stress fibers; instead, mixed polarity was reported (Ishikawa et al., 1969; Sanger and Sanger, 1980). To resolve this disparity, it has been proposed that stress fibers in fact consist of alternating uniformly polarized arrays of filaments attached at their barbed ends to some Z-line-like structures, and mixed polarity results from overlapping of neighboring arrays (Sanger and Sanger,
However, barbed end-specific connections of actin filaments to specific sites within stress fibers have not been demonstrated. Further, mixed polarity may be functionally problematic. In the case of a Drosophila mutant where actin filaments in the flight muscle exhibited mixed polarity within a single half-sarcomere (Reedy et al., 1989), the muscles were non-functional. Recently, alternating polarity of actin filaments was described for a subset of actin filament bundles (Cramer et al., 1997); however, orientation of these filaments relative to myosin structures was not determined.

The transport model explains some of the phenomena of cell motility by the interaction of myosin II with uniformly, not alternately, polarized arrays of actin filaments (Maciver, 1996; Mitchison and Cramer, 1996). According to this idea, the postmitotic spreading of the cell body along retraction fibers, advance of the central domain of the axon growth cone and, more generally, pulling of the cell body behind the leading edge during locomotion, are powered by myosin II-mediated transport along tracks of uniformly polarized actin filaments. It is noteworthy that the sarcomeric and transport mechanisms have features in common: striated muscle contraction, in fact, consists of multiple events of transport along alternately polarized but uniform actin arrays. The differences between the two mechanisms are in the dimensions of the uniform actin arrays relative to the total amplitude of translocation. The sarcomeric mechanism allows the amplitude of contraction to exceed the dimensions of an individual actin array, whereas the transport mechanism implies that the amplitude of translocation is equal or less than the length of the array. The generality of the transport mechanism depends on the finding of sufficiently long actin arrays of uniform polarity. To date, arrays of uniform polarity have been demonstrated only in limited areas of the cell, namely, near the leading edge and in retraction fibers of postmitotic cells (Small et al., 1978; Cramer and Mitchison, 1995). Possibly supporting a transport mechanism, long actin bundles of graded polarity (polarity gradually changing from uniform to mixed) were recently described in primary heart fibroblasts (Cramer et al., 1997). However, it is not clear whether such bundles function as transport tracks or contractile structures. Another implication of the transport hypothesis is that myosin II is expected to be translocated significant distances relative to actin filaments in the direction of cell locomotion. Such translocation of myosin has so far not been documented in locomoting cells. On the contrary, myosin structures have been found to be transported away from the active edge (McKenna et al., 1989; Kolega and Taylor, 1993; Verkhovsky et al., 1995).

An alternative possibility is that the acto-myosin system of a non-muscle cell is a dynamic network of myosin II and actin filaments. Both the sarcomeric and transport models share the postulate that myosin II interacts with actin, which is organized in some ordered way independently of, and prior to, the interaction with myosin. Myosin is supposed to approach the locations of barbed ends of actin filaments, which are specified prior to the onset of translocation. In the dynamic network model, we speculate that the organization arises in the process of actin-myosin interaction. Using a correlative dynamic and ultrastructural approach, we have recently demonstrated that myosin II bipolar filaments in cultured cells associate with each other to produce assemblies, and that an accordion-like folding of zig-zag myosin assemblies is accompanied by formation of bundles of actin filaments (Verkhovsky and Borisy, 1993; Verkhovsky et al., 1995). Actin bundle formation was explained in terms of an interaction of myosin clusters with surrounding actin filaments of mixed polarity, restricted in their translocation due to association with each other and/or with substrate-contact sites. Under such conditions, tension is expected to be generated and to result in the self-organization of the actomyosin network.

It is not immediately evident how a myosin II-actin network could be capable of contraction. In order for contraction to occur, the network filaments need to be allowed to translocate and, thus, inter-filament associations in the network have to be disrupted or weakened, as proposed previously by the solution-contraction coupling hypothesis (Taylor and Fechheimer, 1982). However, randomly polarized actin filaments may be expected to move in every possible direction, which seems to fit better with an expansion rather than contraction of a network. Nevertheless, actomyosin gels (Stossel and Hartwig, 1976; Pollard, 1976; Janson et al., 1991) and acto-heavy meromyosin bundles (Takiguchi, 1991), reconstituted in vitro and presumably consisting of actin filaments of random polarity, were in fact shown to contract upon addition of ATP. Moreover, in vitro contraction of acto-heavy meromyosin bundles was shown to be accompanied by formation of aster-like structures containing uniformly polarized actin filaments. This phenomenon was explained in terms of actin filament polarity sorting by myosin and stabilization of bundles of uniform polarity (Takiguchi, 1991; Nakazawa and Sekimoto, 1996). Similar processes could conceivably take place in cells, but as yet have not been documented.

In this paper, we attempt to elucidate the mechanics of myosin II-actin interaction in fibroblasts by following changes in the organization and polarity of actin relative to myosin II in the process of actin filament translocation. To induce filament translocation, we used the tool previously employed by Kolega et al. (1991), namely, treatment of fibroblasts with cytochalasin D, which is a drug that disrupts actin filaments and brings about cell contraction without an apparent increase in the level of myosin activity (Kolega et al., 1991; Giuliano et al., 1992). Our data are consistent with a network model of local myosin II-actin organization. The network becomes transformed upon cytochalasin D treatment into actin asters, that is, radial arrays of actin filaments uniformly oriented with their barbed ends to foci of myosin II at the aster centers. These results suggest that actin arrays of ordered polarity can arise dynamically in the process of interaction with myosin.

**MATERIALS AND METHODS**

**Cell culture and labeling for actin and myosin**

Rat embryo fibroblast (REF-52) cell culture, microinjection with tetramethylrhodamine-myosin II, extraction, fluorescence labeling for actin and myosin and light microscopy (Verkhovsky et al., 1995) and myosin S1 decoration of actin, immunogold labeling of myosin and electron microscopy (Svitkina et al., 1995) were performed as described. Cellular F-actin content was quantified by rhodamine-phalloidin staining of detergent-extracted cells. Total fluorescence intensity (digitized to 16 bit depth) was integrated within the cell area as described (Zhai and Borisy, 1994). Actin dynamics were recorded following injection of rabbit muscle actin labeled with 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester, according to the
procedure of Isambert et al. (1995) or actin labeled with tetramethylrhodamineiodoacetamide (Wang, 1985), which was kindly donated by Dr D. J. Fishkind (University of Notre Dame, IN). To reduce photo-bleaching and photodamage, fluorescence observations of actin and myosin were made in oxynase-containing media (Mikhailov and Gundersen, 1995) in Petri dishes overlaid with mineral oil. Observation by phase and immunofluorescence microscopy confirmed that the effects of cytochalasin D in oxynase-containing media were similar to its effects in normal media. For combined S1 actin decoration and myosin immunogold labeling, extracted cells were first incubated for 15 minutes with antibodies against non-muscle myosin, then for 30 minutes with muscle myosin S1, then fixed and treated as described for immunogold labeling (Svitkina et al., 1995).

### Determination of actin filament polarity

The metal replica technique was employed for determination of actin filament polarity. In metal replicas, only the top surface of the biological material is contrasted, whereas the classic arrowhead or chevron pattern of S1-decorated actin filaments seen by negative or positive staining is generated by contrast at both top and bottom surfaces of the specimen, as well as in its internal structure. Because the polarity cues present in replica images of decorated actin filaments are less familiar than the chevron pattern, our rationale for assessing polarity is described in detail.

S1-decorated actin filaments visualized by the replica technique presented polar structures that were evident at low magnification (Fig. 1a), which could be characterized at high magnification as ‘double ropes’ (Fig. 1c) or ‘chains of potatoes’ (Fig. 1d) (see also Heuser and Cooke, 1983). Both individual turns of rope and potatoes exhibited asymmetry, which allowed for the determination of filament polarity. Occasionally the classic chevron pattern was also observed on short portions of actin filaments (Fig. 1e). A model of a decorated actin filament (Fig. 1b) shows in the outlined region how a half turn of rope or potato relates to myosin S1 bound to individual actin subunits. The end of the potato pointing toward the barbed end looks thinner and projects more to the side of the filament than the opposite part. To evaluate whether this interpretation of image asymmetry was correct, we simulated the combined view of top and bottom surfaces of the specimen by flipping a filament image about its long axis and superimposing it on the original image. This procedure yielded the classic arrowhead pattern of correct polarity, both for the diagram (Fig. 1f) and for an actual filament image (Fig. 1g).

For actin filaments in complex cytoskeletal arrangements, the regular rope or potato patterns were not always unambiguous. We considered filament polarity to be determined if a portion of a filament exhibited three or more consecutive asymmetric elements (asymmetric half turns of rope, potatoes or arrowheads) pointing in the same direction and no elements pointing in the opposite direction. In most cases, our improved critical point drying/platinum replication procedure (Svitkina et al., 1995) resulted in preparations where actin polarity could be clearly assigned for a significant fraction (30-70%) of visible filaments, although this fraction varied in different regions of the cell and in different experiments.

### RESULTS

#### Redistribution of actin and myosin II

Following the rationale of Kolega et al. (1991), we used cytochalasin D as a tool to disrupt the cytoplasmic actin network and allow for relative translocation of actin and myosin filaments. To analyze the pathway of filament translocation, conditions needed to be determined in which intermediates could be observed. Consistent with previous reports (Schliwa, 1982; Kolega et al., 1991), treatment of REF-52 cells with 0.2-2.0 μM cytochalasin D induced a dose- and time-dependent retraction of peripheral cytoplasm, while sites at the cell perimeter retained their original positions of attachment to the substratum. The combination of cytoplasmic retraction and retention of substratum attachment led ultimately to an arborization of the cell. Significant retraction, but not complete arborization, was achieved by treatment with 2 μM cytochalasin D for 15 minutes, which were the conditions in all experiments detailed below unless otherwise indicated.

The organization of actin and myosin II revealed by flu-
or escence microscopy was dramatically different in control and
cytochalasin-treated cells. In control cells, actin was distributed
in a faintly fibrous pattern in lamellipodia, in the form of bright
linear arrays in stress fibers and filopodia and in a uniform
diffuse manner between the stress-fibers. Myosin II was con-
centrated in numerous small spots in the lamellum and in
arrays of parallel periodic ribbons across stress fibers (see
Verkhovsky et al., 1995, for more detail). After treatment with
cytochalasin D, some remnants of stress fibers could be seen
in the central region of the cell, but most of the actin became
aggregated in a coarse network-like pattern with bright vertices
and short bundles, mostly of a characteristic tapered shape
(thicker ends towards the vertices), emanating from these
vertices. At the cell periphery, tapered bundles were usually
oriented with their thin ends pointed toward the outside. Indi-
vidual vertices with their associated bundles radiating in
various directions were reminiscent of microtubule asters, and
we termed these structures actin asters (Fig. 2).

Despite the dramatic difference in distribution, the average
total amount of F-actin per cell was not significantly different
in control and cytochalasin-treated cells. As assessed by
binding of rhodamine-phalloidin, cells varied considerably in
their F-actin content. However, the average integrated intensity
of phalloidin staining per cell (in arbitrary units) was similar
for control (92±49) and treated cells (95±58) (mean ± s.d.,
n=30 in each case). In accordance with previous studies
(Heacock et al., 1984; Verschueren et al., 1995), this result
suggests that cytochalasin-induced changes are due to actin
redistribution rather than to its depolymerization.

Myosin also exhibited a dramatically altered distribution
after cytochalasin treatment. Unlike actin, myosin II did not
show a network pattern, but mostly became aggregated in rel-
atively few large bright foci with virtually no detectible fluo-
rescence in-between these foci. Myosin foci coincided with the
thick ends of tapered actin bundles and the centers of actin
asters (Fig. 2).

Fig. 2. Actin asters are induced by
treatment of fibroblasts with cytochalasin.
Spreading REF-52 cells (6 hours after
plating) were double-stained for actin and
myosin II without cytochalasin treatment
(a) or after treatment with 2 μM
cytochalasin D for 15 minutes in culture
medium (b). Actin is shown in cyan and
myosin in red. Areas of superimposition
of high intensities of staining for both
actin and myosin are represented in
white. Inset in b shows a gallery of actin
asters with the myosin II spot in the
center and tapered actin bundles with the
myosin II spot at the base. Gallery
images were cut out from different
regions of the cell. Bars, 2 μm.
To understand how actin and myosin II became reorganized, we analyzed their dynamics during cytochalasin D treatment. In cells injected with rhodamine-actin, we observed non-uniform contraction of stress fibers, which eventually resulted in formation of asters containing bright foci (Fig. 3). Actin asters arose not only through contraction of stress fibers, but also through condensation of actin previously distributed in a diffuse manner, e.g. in lamellipodia. Actin asters and their constituent filament bundles exhibited irregular translocations, with smaller asters tending to aggregate into bigger asters (Fig. 3, arrows).

Changes in myosin distribution were of special interest because of the possibility that interactions with myosin II might drive actin reorganization. Consistent with the description of Kolega et al. (1991), breaking of stress fibers with the rapid shortening of their pieces was observed in tetramethylrhodamine-myosin II injected cells (Fig. 4). However, higher resolution imaging and the particularly favorable characteristics of the REF-52 cells allowed additional details of the process to be discerned. As the contraction progressed, myosin ribbons in linear arrays in stress fibers, as well as myosin spots distributed throughout the lamella, fused with their neighbors to result in foci, which became tighter and more symmetrical with prolonged treatment (Fig. 4). Remarkably, myosin foci formed not only by aggregation of preexisting myosin spots and ribbons but were also observed to form de novo in the lamellipodia and in gaps between contracted stress fibers (Fig. 4), presumably from the soluble myosin pool or small myosin aggregates, which were not discernible at the light microscopic level. This observation was consistent with the formation of actin asters in areas of diffuse actin distribution. Irrespective of their pathway of formation, myosin foci exhibited complex motility, with the overall trend being the fusion of foci with each other to form larger foci (Fig. 4, arrows), a process that was presumably analogous to the fusion of actin asters observed in cells injected with rhodamine-actin. Although we did not attempt simultaneous visualization of actin and myosin in the course of rearrangement, the patterns of changes observed separately displayed the same basic characteristics and were consistent with the pattern of relative distribution of actin and myosin revealed by double fluorescence staining in fixed cells. These data suggest that actin asters with myosin at their centers form spontaneously in cytochalasin D-treated cells. The asters arise both by rearrangement of stress fibers...
and by condensation of actin and myosin in areas of diffuse distribution.

**Actin filament polarity**

The reorganization of actin and myosin II into actin asters with myosin II foci raised the question of the polarity of the actin filaments in these structures. The polarity of actin filaments can be visualized in metal replica preparations of cytoskeletons after decoration with myosin S1, as recently described (Svitkina et al., 1995). Here we analyze cytochalasin-induced changes in actin filament polarity, with respect to the cell periphery and to sites of myosin concentration. In control cells, filament polarity was assessed with respect to the cell margin in characteristic actin-rich formations of the edge: filopodia, identified in electron microscopic images as thin actin bundles projecting outward from the cell edge but disappearing towards the cell center (Fig. 5a), distal ends of stress fibers, identified as actin filament bundles continuing over significant distances through the central region of the cell (Fig. 5b), and lamellipodia, identified as a dense actin network with a convex outer margin (Fig. 5c). In filopodia and in stress fiber termini, actin filaments exhibited a striking bias in polarity. As shown in Table 1, 98% and 91% of filaments with determined polarity in filopodia and stress fiber termini, respectively, were oriented with barbed ends outward, pointed ends inward. The few filaments oriented with pointed ends outward looked as if they might have been folded back and broken in the process of motility or during specimen preparation. In lamellipodia, actin filaments crossed each other at various angles and were oriented at various angles to the cell edge. Because of complex interweaving of filaments, any overall preferential orientation of filaments was not immediately obvious. However, close investigation again revealed a strong bias in polarity. Often, polarity could be determined on a segment of a filament at

![Fig. 4. Dynamics of myosin II in cytochalasin D-treated REF-52 cell. Time after addition of cytochalasin (minutes) is indicated by numbers. Contraction of stress fibers containing parallel myosin ribbons (0-6 minutes) was followed by formation of tight myosin foci (10-18 minutes). Myosin foci that appeared de novo during cytochalasin treatment and then fused together are indicated by arrows. Bars, 5 μm. Video sequence can be seen at http://www.cityscape.co.uk/users/ag64/](http://www.cityscape.co.uk/users/ag64/)

| Table 1. Polarity of actin filaments in untreated and cytochalasin D-treated cells |
|---------------------------------------------|---------|---------|---------|---------|---------|---------|
| Condition*                                  | Untreated | Cytochalasin D treated |
| Type of structure                           | Filopodia | Ends of stress fibers | Lamellipodia | Myosin spots | Tapered bundles and asters of filaments |
| EM fields (n)                               | 8        | 5        | 5        | 25        | 10 |
| Actin filaments scored (n)                  | 139      | 187      | 499      | 2,547      | 439 |
| Filaments with determined polarity (n and %)*| 90       | 79       | 155      | 1,033      | 287 |
|                                          | (65%)    | (42%)    | (31%)    | (41%)      | (65%) |
| Filaments with barbed ends outward or away from myosin spots (n and %)**| 88       | 72       | 149      | 604        | 13 |
|                                          | (98%)    | (91%)    | (96%)    | (58%)      | (5%) |
| Filaments with pointed ends outward or away from myosin spots (n and %)**| 2        | 7        | 6        | 429        | 274 |
|                                          | (2%)     | (9%)     | (4%)     | (42%)      | (95%) |

*REF-52 cells were untreated or treated with 2 μM cytochalasin D for 15 minutes.
†Polarity determination was made by S1 decoration and an EM replica procedure as described in Materials and Methods.
‡Filaments with specified orientation are given as % of filaments with determined polarity.
some distance from the cell edge but it was not possible to trace the filament to its termination. The orientation of such segments with respect to the edge was assigned by continuing them as straight lines and scoring whether the continuation of a barbed or pointed end crossed the edge. With such a definition, 96% of filaments were oriented with their barbed ends toward the edge. Therefore, in agreement with previous reports (Begg et al., 1978; Small et al., 1978; Lewis and Bridgman, 1992), we find that actin filaments at the periphery of untreated cells are oriented predominantly with their barbed ends outward, pointed ends inward.

Having confirmed the basic features of actin filament polarity in untreated cells, we were then prepared to determine the polarity of actin filaments with respect to myosin II features. Significant accumulations of myosin II (detected by immunogold localization) were generally not found in regions of uniform actin orientation with the exception of stress fiber termini, where distinct myosin spots were sometimes observed in the midst of uniformly polarized actin filaments. Most myosin spots were found in association with actin bundles and networks farther away from the cell edge, where actin filaments appeared to have mixed polarity. We analysed actin filament polarity, both with respect to myosin spots and to the actin bundle axis. If a sarcomere-like organization was present, one would expect actin filaments to be locally oriented preferentially with their barbed ends away from the sites of myosin concentration. Actin polarity along the bundle axis would alternate. In contrast, if actin polarity along the bundle axis was uniform, then polarity with respect to myosin would alternate on either side of a spot. The polarity of actin filaments was
determined with respect to 60 individual myosin spots in 25 electron microscopic fields (Fig. 5d; Table 1). Overall, filament polarity was mixed and only a slight bias was detected: 42% of filaments with assignable polarity were oriented with their barbed ends towards myosin and 58% away. Many filaments could indeed be directly traced to go in and come out of a myosin site without interruption, while only rarely was it possible to see a clear filament termination in the vicinity of a myosin site. Both barbed and pointed end terminations were observed. Still, most of the filaments could not be traced through myosin sites and it was not possible to estimate how many of them terminated at these sites and how many continued through. Determination of actin polarity along the bundle axis confirmed that most actin bundles were of mixed orientation. However, some bundles showed preferential polarity. In approximately 20% of myosin spots analyzed, the bundles in which they were contained showed >75% of the actin filaments polarized in the same direction. Thus, neither a clear sarcomeric nor a clear uniformly polarized arrangement of actin filaments was observed. Although actin filaments seemed to show a marginal preferential orientation of barbed ends away from myosin, and although some actin bundles were preferentially polarized, the more significant conclusion from these polarity determinations is that most of the myosin spots were surrounded by actin filaments of mixed polarity.

Actin filament organization was remarkably altered by treatment with cytochalasin D. Consistent with light microscopic data, electron microscopic images (Fig. 6) showed actin filaments to be organized mostly in tight, tapered bundles, 1 to 5 μm in length. The thicker ends of these bundles often converged with thicker ends of other bundles to form aster-like structures, consistent with the previous ultrastructural study of
In this study, we report a dramatic rearrangement of actin filaments in cultured fibroblasts treated with cytochalasin D. Actin filaments, which initially exhibited mixed polarity relative to myosin, were found to reorganize into tapered bundles of uniform polarity and asters in which barbed filament ends were uniformly oriented to the myosin aggregate at the center of the aster. In agreement with previous studies (Heacock et al., 1984; Verschueren et al., 1995), our quantitation of actin filament content showed that cytochalasin-induced rearrangement is not accompanied by a significant net depolymerization of actin in the cell. What is the mechanism of this rearrangement? We propose that the mechanism is a self-sorting of actin filaments, based on their intrinsic polarity and transport relative to myosin II.

Transport of actin filaments was made possible by a weakening of the actin network by cytochalasin D (Kolega et al., 1991). Although the weakening of the actin network structure by cytochalasins is not completely understood, several mechanisms have been proposed. These include: reduction of average filament length in terms of Flory’s network theory (Hartwig and Stossel, 1979), inhibition of filament-filament interaction (MacLean-Fletcher and Pollard, 1980), and competition between cytochalasins and some cellular components normally anchoring barbed ends of filaments (Cooper, 1987). Irrespective of the precise mechanism of action of cytochalasin D, disruption of the actin filament network is expected to facilitate the transport of actin filaments by myosin II. Transport of actin filaments, in turn, is expected to result in a change in actin polarity organization.

Several lines of evidence suggest that the observed rearrangement of actin is indeed a result of actin filament translocation. First, by following the kinetics of actin and myosin II rearrangement, we found that asters formed as a result of either stress fiber contraction or recruitment of loosely organized actin in the lamella to the sites of aster formation. Translocation of actin and myosin features was also directly observed during aggregation of small asters into bigger asters. De novo formation of myosin features was also observed, creating new foci for possible actin transport. Second, the co-localization of myosin II and barbed ends of actin filaments at the centers of asters and thicker ends of tapered actin bundles is consistent with relative translocation of actin and myosin that has proceeded to completion, that is, when myosin has reached its destination at the barbed ends of actin filaments. Third, transport of actin filaments by myosin in vitro has been shown to result in rearrangements similar to what we observed in living cells after cytochalasin treatment. In particular, formation of actin bundles of uniform polarity was observed in solutions of F-actin and heavy meromyosin in the presence of ATP. Multi-axial actin aggregates with myosin at the center were also reported in permeabilized cells treated with ATP to promote contraction (Tint et al., 1991), although the polarity organization of actin was not determined in that study. In our study, we document rearrangement of actin associated with transport in the living cell. Dramatic reorganization of the bulk of cellular actin indicates that most of actin filaments are involved in interaction with myosin and could be transported.

Formation of structures of uniform polarity can be explained by polarity sorting in the process of translocation. We have recently shown that each site of myosin concentration in the fibroblast cytoskeleton represents a cluster of several bipolar minifilaments (Verkhovsky et al., 1995). Myosin clusters would be expected to sort actin filaments according to their polarity. Each actin filament would be translocated by myosin in the direction of its pointed end. As a result of translocation, all filaments of a particular orientation will form a group of uniformly polarized filaments projecting from a myosin cluster in the direction of their pointed ends. In general, the consequence of unrestricted polarity sorting would be an aster of actin filaments, pointed ends outward, with myosin localized at the aster center (Fig. 7). If myosin clusters are located closer together than the length of the average actin filament, they are predicted to interact and fuse together, because the myosin core of each cluster will encounter pointed ends of actin filaments emanating from neighboring clusters and will be transported in the direction of barbed ends. Thus, larger asters will tend to form and absorb smaller ones.

The size of asters and the shape of their constituent actin filament bundles can be interpreted in terms of the length distribution of individual actin filaments. The assumption that barbed ends of actin filaments are co-localized with myosin in the center of the aster provides a natural explanation for the tapered shape of actin bundles emanating from the center: since filaments should vary in length, the portion of the bundle distal from the common location of barbed ends is predicted to contain fewer filaments than the proximal portion. If such an interpretation is correct, the length of a tapered bundle would be equal to the length of the longest actin filaments. The observed dimensions of asters and bundles provide an upper limit of about 5 μm for the length of actin filaments in the cytochalasin-treated cell. Since cytochalasin may reduce the length of actin filaments, the filaments in non-treated cells
As mentioned in the Introduction, three models have been treated cells could be similar to that in untreated ones. A model of uniformly polarized transport arrays and a dynamic network model, in which actin organization is not predefined but develops in the course of actin-myosin interaction.

Implications of actin polarity and aster formation

As mentioned in the Introduction, three models have been proposed for the organization of the actin-myosin II system of non-muscle cells: a sarcomeric-like alternating polarity model, a model of uniformly polarized transport arrays and a dynamic network model, in which actin organization is not predefined but develops in the course of actin-myosin interaction.

Two kinds of evidence are critical for the evaluation of these models: actin polarity with respect to myosin, and the pattern of motion of actin and myosin features. Although actin polarity in non-muscle cells was the focus of several studies (Begg et al., 1978; Lewis and Bridgman, 1992; Sanger and Sanger, 1980; Small et al., 1978; Cramer et al., 1997), the inability to clearly recognize myosin II structures prevented the determination of actin polarity with respect to them. Our previous work (Verkhovsky and Borisy, 1993; Verkhovsky et al., 1995) established procedures for identifying supramolecular clusters of myosin II, thus permitting determination of the relative actin polarity. The sarcomeric model predicts a preferential orientation of actin barbed ends away from myosin. The transport model predicts a preferential uniform polarity of actin. Both models predict that the polarity relationships would be conserved upon contraction. In contrast, with the exception of stress fiber termini, we found predominantly mixed polarity of actin with respect to myosin clusters. Further, the polarity relationship was not conserved upon contraction, but dramatically changed. Thus, our structural results do not support either the sarcomeric or the transport model. Rather, the dramatic change in actin polarity with respect to myosin during aster formation suggests that the actin polarity pattern is determined in the course of actin-myosin interaction.

Still, our polarity findings do not permit us to exclude sarcomeric and transport models: overlapping alternating polarity arrays, as suggested by Sanger and Sanger (1980), or uniform arrays at the bottom of the central part of the cell, could have escaped identification by the replica technique. In addition, it could not be excluded that the sarcomeric-like organization (if and where present) was disrupted by cytochalasin treatment, thus allowing for the change in actin polarity. Regarding transport mechanism and the recent finding of graded polarity bundles (uniform at the ends with gradual change to mixed polarity towards the middle) (Cramer et al., 1997), we should note that the mixed polarity of actin relative to myosin is not inconsistent with polarity in graded polarity bundles. That is, for myosin clusters located in the middle portion of a ‘graded polarity bundle’ one would expect to find mixed polarity of actin with respect to myosin. Thus, graded polarity bundles may be organized in a similar way to actin filament bundles in REF-52 cells used in our study, although graded polarity bundles are defined by Cramer et al. (1997) as distinct actin structures specific for highly locomotory primary fibroblasts, whereas the actin bundles in REF-52 cells represent typical stress-fibers with frequently periodic myosin arrangement. Study of myosin dynamics in graded polarity bundles would be necessary to determine whether they are transport arrays as proposed by Cramer et al. (1997), or contractile structures similar to stress fibers.

In our study, we used analysis of the pattern of actin and myosin II dynamics after rendering actin filaments free to be transported as a second approach for evaluation of the models. Each model predicts distinctive consequences. The sarcomeric model predicts contraction of stress fibers but not aster formation (unless asters form as a result of sarcomeres being disrupted during contraction). The uniform transport model predicts movement of myosin features over significant distances. The patterns of motility observed did not fit with either model. The initial contraction of stress fibers was consistent with the sarcomeric model, but the subsequent formation of asters was not. Contraction also cannot be considered conclusive evidence, since other types of organization may also support contraction. Actin-heavy meromyosin bundles lacking sarcomeric organization reportedly contract in vitro (Takiguchi, 1991), although the mechanism is not clear (Nakazawa and Sekimoto, 1996). Regarding the transport model, the concerted translocation of actin or myosin features over significant distances that might be expected based on this model and which actually was observed in cytochalasin-treated growth cones of Aplysia (Forscher and Smith, 1988), did not occur in the case of fibroblasts. Instead, actin and myosin features exhibited irregular translocations, with the net result being aster formation and fusion of asters with each other. This pattern of motion is suggestive of an initially mixed polarity of actin filaments and of a dynamic process of polarity sorting. The formation of myosin foci and actin asters in cell regions...
initially exhibiting diffuse organization is also indicative of self-sorting. Thus, the dynamic network model is supported by both polarity determination and the pattern of actin and myosin motion. Although other means of controlling actin organization cannot be excluded, our results show a potential for formation of ordered actin-myosin II assemblies by self-sorting in the process of transport.

Recently, the possibility that complex cytoskeletal organization could arise as a result of interaction of motor proteins with their targets has attracted attention in the microtubule field. Microtubule asters and spindle-like assemblies were reported to form as a consequence of the interaction of individual microtubules with molecular motors (Hyman and Karsenti, 1996; Heald et al., 1996; Rodionov and Borisy, 1997). This study is, to our knowledge, the first indication that a similar organizing principle could be applicable to the actin-myosin II system in living cells.

What might be the physiological role, if any, of polarity sorting and actin aster formation? In this study, asters were observed under non-physiological conditions, i.e. the unleashing of actin filament transport by cytochalasin treatment. One could speculate that polarity sorting and formation and fusion of asters may also contribute to the physiological processes of contraction that are associated with disruption of the cytoskeletal system, e.g. rounding up in mitosis, or upon detachment from the substratum. Actin-myosin interaction could also be predicted to result in limited polarity sorting even in the absence of free translocation of filaments. The pattern of filament attachment might provide a basis for sorting. If an actin filament interacting with a myosin cluster is tethered in the network at its pointed end, it will be expected to be pushed away from myosin, possibly resulting in buckling. On the contrary, a filament attached at the barbed end could be predicted to be constantly pulled by myosin and to transmit tension between a myosin cluster and the site of network attachment. Overall, filaments with barbed end attachments would participate in the formation of actin-myosin II bundles, while filaments with pointed end attachments would be excluded from the bundles. Such a selection process could account for the formation of contraction-competent filament bundles from the dynamic actin-myosin network.

One can also speculate that self-sorting mechanisms different from myosin-dependent transport may participate in the control of actin organization. One possibility is preferential elongation of barbed actin filament ends. During protrusion, even if initial filament orientation is random, barbed filament ends are predicted to outgrow pointed ends and consequently to be enriched in the nascent lamellipodium. Thus, self-sorting in the process of elongation could explain the experimentally observed uniform polarity of actin filaments at the leading edge. Self-sorting by transport and self-sorting by polymerization may represent emergent properties of polar cytoskeletal filaments, which contribute to spatial control over organization and motility of non-muscle cells.

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