Evidence that intermediate filament reorganization is induced by ATP-dependent contraction of the actomyosin cortex in permeabilized fibroblasts

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Summary

Intermediate filaments (IFs) undergo specific re-arrangements in cells, some aspects of which can be induced experimentally. Centripetal aggregation of the IF network, for example, can be produced by a variety of perturbations. However, the source of motive force is clear for neither in vivo nor experimentally generated IF movements, since, unlike microtubules and actin filaments, IFs have no known force-generating system directly associated with them. We recently obtained evidence that the drug-induced aggregation of vimentin IFs in fibroblasts is an active event, which requires ATP and involves the actin cytoskeleton. In the present study, we sought to test the hypothesis that IF aggregation is driven by a centripetally directed contraction of the actomyosin cortex. To that end, we have permeabilized fibroblasts with Triton X-100 in a stabilizing buffer and reactivated cytoskeletal movements in vitro, under defined solution conditions. Upon nucleotide treatment, these permeabilized cells undergo a nucleotide-dependent centripetal aggregation of vimentin IFs similar in appearance and time course to that induced in intact cells by drug treatment. During in vitro IF aggregation, the permeabilized cells remain fully spread and adherent to the substratum, and the distal ends of the microtubules and actin microfilaments retain their positions in the cell periphery. IF aggregation is accompanied by a contraction of F-actin and myosin into focal aggregates in the same perinuclear region in which the IFs accumulate. If permeabilized cells are treated with the actin-severing protein gelsolin prior to the reactivation of EF movement, the actin cytoskeleton is eliminated and IF aggregation fails to occur when ATP is added. These results strongly support a model in which the motive force for IF movement is supplied indirectly by association with a contracting actomyosin network.

Key words: intermediate filaments, F-actin, myosin, permeabilized cells.

Introduction

Vimentin-containing intermediate filaments (IFs), like microtubules and actin filaments, undergo redistribution and rearrangement during the cell cycle. During mitosis, for example, many cell types rearrange their vimentin IFs from an extended radial array into a central cagé of filaments surrounding the mitotic spindle (Aubin et al. 1980; Zieve et al. 1980), while in some cell types they are transiently disassembled (Franke et al. 1984). IF redistribution can also be induced by experimental perturbation: drug-induced destruction of microtubules causes the formation of thick vimentin cables, which accumulate around the nucleus (Goldman and Knipe, 1972; Croop and Holtzer, 1975; Blose and Chako, 1976; Franke et al. 1978; Hynes and Destree, 1978; Osborn et al. 1980), and some treatments can cause IF aggregation while leaving the microtubules intact (e.g. neurotoxins: Durham et al. 1983; Eckert, 1985; vanadate ion: Wang and Choppin, 1981; heat shock: Thomas et al. 1981; anti-vimentin or anti-tubulin injection: Klymkowsky, 1981; Gawlitta et al. 1981; Blose et al. 1984; cyclic AMP-dependent protein kinase injection: Lamb et al. 1989). IF aggregation also occurs naturally in skin fibroblasts from patients with giant axonal neuropathy (Pena, 1981; Pena, 1982; Klymkowsky and Plummer, 1985; Manetty et al. 1987). However, the basis of these rearrangements is unclear, since IFs, unlike microtubules and actin filaments, have no known force-generating system associated directly with them. One possibility is that they are attached to another cellular structure that is undergoing movement (Hollenbeck et al. 1989).

There are several candidates for this structure, since vimentin IFs are associated with a number of different cellular components. In interphase cells, IFs radiate from the perinuclear area, where they form connections with the nuclear surface (Goldman et al. 1985; Georgatos and Blobel, 1987), to the cell periphery, where they associate...
with the cytoplasmic side of the plasma membrane (Green and Goldman, 1986; Georgatos and Blobel, 1987), with vinculin-containing adhesion plaques (Bershadsky et al., 1987), and with the actin cortex (Green et al. 1986; Hollenbeck et al. 1989). Throughout much of their path from the nucleus to the cortex, IFs associate closely with microtubules (Goldman and Follett, 1969; Geiger and Singer, 1980; Geuens et al. 1983; Traub, 1985).

Recently we demonstrated that the vimentin IF aggregation caused by microtubule depolymerization in fibroblasts was ATP-dependent; moreover, we showed that the coating of vimentin bundles into the perinuclear region was both inhibited and reversed by cytochalasin D, suggesting that the actin cortex or cytoskeleton may be involved in driving this process (Hollenbeck et al. 1989). In the present study, we have permeabilized fibroblasts with detergent and reproduced both the centrietal aggregation of IFs and actomyosin contraction under defined solution conditions. Our data indicate that IF movement is dependent upon contraction of the actomyosin network.

**Materials and methods**

**Materials**

ATP, TRITC-labeled phalloidin, vinblastine and leupeptin were obtained from Sigma (St Louis, MO). ATP, ITP, UTP and GTP were obtained from Serva (Heidelberg, FRG). Unlabelled phalloidin was a generous gift from Dr Matthew Stiffness (NCI, Bethesda, MD).

**Cell culture and permeabilization**

Secondary cultures of mouse embryo fibroblasts were grown on coverslips for 24 h prior to fixation as previously described (Hollenbeck et al. 1989). Cells were washed with PBS and then permeabilized with buffer M (0.1% Triton X-100 in 50 mM imidazole, 50 mM KCl, 0.5 mM MgCl₂, 1 mM EGTA, 1 mM 2-mercaptoethanol, pH 6.8) containing protease inhibitors (50 μM N-acetyl-L-lysine-chloromethylketone, 50 μM L-1-tosylamide-2-phenylethylchloromethylketone, 5 μM leupeptin) for 10 min at room temperature. To stabilize the cytoskeleton, the permeabilization solution was supplemented with 1–4% polyethylene glycol (PEG) with a relative molecular mass of 40 000. After permeabilization, cells were washed with buffer M and incubated in the same buffer in the presence or absence of 2 mM ATP for 45–60 min at 37 °C. During this incubation, the buffer was supplemented with 10 μM taxol to stabilize microtubules (Schiff and Horwitz, 1980; Manfredi et al. 1982) and 0.1 mg ml⁻¹ phalloidin to stabilize actin microfilaments. Cells were fixed with 0.1% glutaraldehyde in PBS.

**Extraction of actin from the cytoskeleton with gelsolin**

Cytoskeletons without actin microfilaments, but retaining microtubules and IFs, were generated by treatment of permeabilized cells with the actin-severing protein gelsolin. The procedure of extraction of gelsolin from bovine brain (Verkhovsky et al. 1984) and extraction of actin from the cytoskeleton (Avner et al. 1983; Verkhovsky et al. 1987) have been described. Cells were permeabilized with Triton X-100, washed with 50 mM Mes–KOH, pH 6.3, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM DTT (dithiothreitol) (buffer A), and incubated in the same buffer containing 1 mg ml⁻¹ gelsolin for 60 min at room temperature. Control cells were incubated in buffer A without gelsolin. Cells were then washed with buffer M and incubated with ATP as described above.

**Immunofluorescent staining**

To visualize the components of the cytoskeleton, we used double indirect immunofluorescent staining with antibodies to vimentin, tubulin and myosin. F-actin was revealed by staining with rhodamine–phalloidin. Monoclonal antibody to vimentin was kindly provided by Drs N. Romanova, L. Yakubov, O. Rokhlin (Cardiology Research Center, Moscow) and has been characterized elsewhere (Bershadsky et al. 1987). Polyclonal antibody to tubulin from bovine brain was affinity purified (Fuller et al. 1975). The polyclonal antibody to nonmuscle myosin (bovine spleen myosin) has been described (Verkhovsky et al. 1987). Details of immunostaining with these antibodies have been provided elsewhere (Bershadsky et al. 1987; Verkhovsky et al. 1987).

**Results**

**Distribution of cytoskeletal structures in permeabilized cells**

Immunofluorescent staining of cells fixed after Triton permeabilization revealed dense radial networks of vimentin filaments that were markedly codistributed with microtubules (compare Fig. 1A and B). F-actin was found concentrated in numerous straight bundles spanning the cell, in ruffles at the cell edges, and more diffusely throughout the cell (Fig. 1C). The intensity of the diffuse F-actin staining was greatest in the perinuclear region. Myosin was present in numerous punctate spots (about 0.2 μm in diameter) both along the actin bundles and also between them (Fig. 1D). The latter myosin staining was largely restricted to the central part of the cell, and coincided with areas of diffuse F-actin staining. As previously demonstrated by Zigmund et al. (1979) and Svitkina et al. (1984), these diffuse actomyosin-positive regions correspond to the submembranous sheath of actin microfilaments covering the dorsal side of the cell subjacent to the plasma membrane. Ruffling cell edges and the distal ends of microfilament bundles did not stain for myosin. The inclusion of taxol and phalloidin at low levels in the permeabilization buffer improved the preservation of cytoskeletal structures during subsequent incubations, and did not produce any qualitative changes in the organization of the cytoskeleton when compared to taxol- and phalloidin-free buffers.

**ATP induces centrietal movement of vimentin IFs**

Incubation of permeabilized cells in buffer M for 45–60 min at 37 °C without ATP produced little or no apparent change in the arrangement of the vimentin or actomyosin systems. Microtubules, which can undergo gradual depolymerization after permeabilization in buffer M (Bershadsky et al. 1978; Bershadsky and Gelfand, 1981), were completely stabilized by including 10 μM taxol in the buffer. In contrast, the addition of 2 mM ATP to permeabilized cells induced a profound redistribution of vimentin IFs. The normal extended array of IFs became condensed and underwent a centrietal shift into the perinuclear area, leaving much of the cell devoid of IFs. The fraction of the IF array that remained extended took the form of thick bundles rather than disperse filaments (Fig. 2B). This is very similar to the centrietal aggregation of vimentin IFs that occurs in unpermeabilized cells after microtubule depolymerization (Hollenbeck et al. 1989). During ATP-induced IF aggregation, the microtubule array of the permeabilized cells remained radially extended (Fig. 2A).

Scoring of cells double-stained for tubulin and vimentin indicated that only 18% of cells showed a divergence between the distribution of MTs and IFs in the absence of...
Fig. 1. Double immunostaining of cytoskeletal structures in permeabilized mouse fibroblasts with antibodies against tubulin (A) and vimentin (B) demonstrates the colocalization of microtubules and IFs. Double staining with rhodamine-phalloidin for F-actin (C) and anti-myosin (D) reveals the typical disposition of the actomyosin system in these cells: actin bundles fill the cytoplasm, and myosin is localized periodically along the bundles and more diffusely between them. The distal regions of actin bundles are myosin-negative. Bar, 20 μm.
ATP treatment, compared to 71% after ATP treatment. This, along with the retention of fully spread cell areas, indicated that the IF redistribution was a discrete event, and not the result of a general collapse of the cytoskeleton or the cell as a whole.

**ATP-induced reorganization of actomyosin accompanies IF rearrangement**

The addition of ATP to permeabilized cells also induced a redistribution of actin and myosin. The density of the array of parallel actin filament bundles was reduced, diffuse actin staining disappeared, and local nodes or aggregates of F-actin appeared. These aggregates tended to be located in the perinuclear region (Fig. 3A). The extent and nature of this change showed cell-to-cell variation, with some cells displaying a more extreme loss of actin bundles and greater development of F-actin aggregates than others (compare Figs 3, 4 and 5), but in general the tips of the remaining actin bundles extended to the cell periphery. Myosin was also redistributed during ATP treatment, colocalizing mainly with the actin aggregates rather than along the length of the remaining actin bundles (Fig. 3B). These changes in actin and myosin distribution after ATP treatment occurred in the absence of overall contraction of the cells (that is, the cell areas did not decrease significantly), indicating that local contraction of the actomyosin cytoskeleton can occur in the absence of cell rounding.

Comparison of the distribution of vimentin with those of F-actin and myosin after ATP treatment demonstrated similarity in the rearrangements undergone by these proteins. The regions of vimentin accumulation in the perinuclear area were colocalized with actin aggregates (Fig. 4). Myosin, located mainly in the region of actin aggregates, was also relocated to the regions of vimentin aggregation. In contrast to actin, myosin accumulations did not exceed the boundaries of the IF mass (Fig. 5). N-ethylmaleimide (Karlsson and Lindberg, 1985), applied at 1 mM either before or during ATP treatment of permeabilized cells, inhibited the ATP-induced redistribution of IFs, actin, and myosin (not shown).

**Selective elimination of F-actin by gelsolin treatment prevents ATP-induced IF rearrangement**

To determine whether an intact actin cytoskeleton was necessary for ATP-induced rearrangement of IFs we sought to disrupt F-actin prior to ATP treatment. We have previously demonstrated that gelsolin treatment of permeabilized fibroblasts leads to nearly complete removal of actin without influencing the organization of vimentin filaments (Verkhovsky et al. 1987) (Fig. 6A). Here we incubated permeabilized cells with gelsolin prior to ATP treatment, and found that the network of vimentin IFs failed to undergo the ATP-induced centripetal aggregation described above (Fig. 6B). Cells permeabilized and incubated in parallel without gelsolin retained the ability to rearrange IFs upon subsequent ATP treatment.

**Effects of nucleotides and calcium on actomyosin rearrangement**

We also examined the influence of different nucleotides...
Fig. 3. The ATP-induced actomyosin contraction in permeabilized fibroblasts is revealed by double staining with rhodamine-phalloidin (A) and anti-myosin (B). The ends of microfilament bundles are preserved, but much of the total actin stain is concentrated in focal aggregates, which colocalize with sites of myosin aggregation (arrowheads). Myosin is no longer found along the length of the actin bundles, but is largely limited to the foci. Bar, 20 μm.

and calcium on the reorganization of actomyosin system. ATP (2 mM) in the incubation buffer could be replaced by GTP while similar concentrations of ITP, UTP, CTP and ATPγS failed to cause a marked reorganization of actomyosin. Cyclic AMP and its analogues (8 Br-cyclic AMP and dibutyryl-cyclic AMP) had no obvious effect on the nature or degree of actomyosin reorganization when they were added to the incubation solution with ATP at the same concentration. In contrast to several reports (Cande et al. 1983; Holzapfel et al. 1983; Masuda et al. 1983; Masuda et al. 1984), we observed neither calcium-dependent rounding nor a calcium requirement for actomyosin reorganization in permeabilized cells. Actomyosin redistribution occurred in the presence of 1 mM EGTA to the same extent or even more effectively than in buffer containing Ca^{2+} in excess over EGTA. It is notable that reducing the PEG concentration and eliminating protease inhibitors from our system did lead to significant cell rounding during ATP treatment, probably due to the degradation of cell-substratum contacts that are stabilized by these agents.

Discussion

Cells reorganize their microtubules and actin filaments in several well-characterized ways. Polymerization and depolymerization – controlled by small molecules, associated proteins, covalent modification, and the activity of organizing centres – permit precise, rapid regulation of the form of microtubules and actin filament arrays. In addition, both filament systems can interact their respective protein motors (Porter and Johnson, 1989; Vale, 1987; Vallee and Shpetner, 1990; Spudich, 1989) to undergo contraction or sliding displacement. The nature and mechanism of IF deployment are less clear, although some evidence suggests that the actin cytoskeleton is involved. Morphological studies of fibroblasts have provided evidence for interactions between IFs and the actin cytoskeleton in the region of the cortex (Green and Goldman, 1986), at adhesion plaques (Bershadsky et al. 1987) or other foci (Green et al. 1986). In addition, we recently demonstrated that the ATP-dependent IF aggregation induced in normal fibroblasts by microtubule depolymerization occurred only in the presence of an intact actin cytoskeleton: disruption of the F-actin system by cytochalasin treatment prevented IF rearrangement, suggesting a role for actin in IF movement (Hollenbeck et al. 1989).

Here we sought to test directly the hypothesis, advanced in the latter study, that IF movements can be produced by interaction with a contracting actomyosin system. Many other workers have produced general actomyosin contractions in permeabilized cell models, but these have involved the contraction of the entire cell (Hoffman-Berling, 1964; Vasiliev et al. 1975; Kreis and Berchmeier, 1980; Masuda et al. 1983; Masuda et al. 1984; Cande and Ezzell, 1986). In the detergent permeabilization protocol that we developed here, fibroblasts lysed in a stabilizing buffer including protease inhibitors retained the integrity of their microtubules, actin filaments and IFs, and also remained adherent to the substratum. This allowed us to reactivate ATP-dependent actomyosin contraction in the absence of
Fig. 4. After ATP-induced actomyosin contraction and IF aggregation, double staining with rhodamine–phalloidin and anti-vimentin reveals that the actin foci (A,C) show similar localization to the vimentin aggregates (B,D), mainly in the perinuclear region of the cell. Bar, 20 μm.
the whole cell rounding observed in previous studies. In addition, the permeabilized cells in this study displayed an ATP-dependent centripetal aggregation of IFs very similar to that which we described previously in intact cells (Hollenbeck et al. 1989). This allowed us to analyze the relation between actomyosin contraction and IF movements under defined solution conditions.

Our principal results from this system are: (1) neither actomyosin contraction nor IF aggregation occurred in the absence of ATP or GTP; non-hydrolyzable analogues or other nucleotides did not support aggregation. (2) IF aggregation and actomyosin contraction occurred in concert, and the positions of the IF and actomyosin aggregates coincided. Thus, the process that reorganized
Fig. 6. Rhodamine-phalloidin (A,C) and anti-vimentin (B,D) double staining show that gelsolin treatment of permeabilized cells removes the F-actin (A) without disrupting the vimentin IFs (B). Subsequent ATP treatment fails to cause IF aggregation (D); the F-actin array is still absent (C). Bar, 20 μm.
they delivered them to the same region. In addition, it was the diffuse, cortical actomyosin staining that disappeared, to be replaced by focal staining, while much of the actin stress fiber mass remained intact and extended throughout the cell. This suggests a contraction of the cortical cytoskeleton. (3) IF aggregation did not occur in the absence of actomyosin contraction. When actomyosin contraction was prevented by treating permeabilized cells with N-ethylmaleimide (Karlsson and Lindberg, 1985), or by dissolving the actin cytoskeleton with gelsolin, then subsequent ATP treatment did not result in IF aggregation. On the basis of these results, we propose that the ATP-dependent centripetal contraction of actin and myosin seen in permeabilized cells is necessary to induce IF aggregation, and that this in vitro phenomenon reflects a physiologically significant mechanism for controlling the position of IFs. The actomyosin system seems most likely to exert force on the IF array in the cortical region, where the two systems come into contact (Green et al. 1986; Green and Goldman, 1986; Georgatos and Blobel, 1987; Bereshady et al. 1987), and where the actomyosin contraction occurs.

In some of the studies of permeabilized cells a calcium dependence of contraction has been observed (Cande et al. 1983; Holzapfel et al. 1983; Masuda et al. 1983; Masuda et al. 1984), which was interpreted to reflect the calcium sensitivity of myosin light-chain phosphorylation. The calcium dependence of actomyosin and IF reorganization in our system may mean that dephosphorylation does not take place under our extraction conditions, or that the contraction we obtain is qualitatively different from whole cell contraction. Another possible role for phosphorylation in IF rearrangement has been suggested by recent work of Lamb et al. (1989), in which microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase into REF-52 cells was shown to produce both dephosphorylation and redistribution of vimentin IFs. This may indicate that IF phosphorylation is required for IF movement, or at least for IF detachment from supporting structures such as microtubules. However, since introduction of this kinase into cells has also been shown to cause actin reorganization (Lamb et al. 1988), the resulting IF movement may reflect an interaction with a moving actomyosin system similar to that which we propose for fibroblasts.

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