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'Macromolecular crowding' is a primary factor in the organization of the cytoskeleton

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We propose that, in the cell, the reversible conversion of actin filaments into actin bundles is controlled by the concentration of the macromolecules [we have employed poly(ethylene glycol) 6000 to mimic the macromolecules of the cell] as well as by the nature of the ancillary cytoskeletal proteins that decorate actin filaments. The proposal is based on the following evidence. (1) Under our experimental conditions the transition from filaments into bundles occurs at increasing concentrations of poly(ethylene glycol), with the following sequence: caldesmon–actin, 3%; filamin–actin, 4–5%; caldesmon–tropomyosin–actin, 5–7%; actin, 6–7%; tropomyosin–actin, 9–10%. (2) Under conditions of low osmoelastic stress [3% poly(ethylene glycol)], preformed caldesmon–actin bundles are dissociated by the addition of either tropomyosin or tropomyosin-decorated actin. The dissociation of the bundles promoted by the addition of tropomyosin-decorated actin is faster than that promoted by the addition of tropomyosin.

INTRODUCTION

The behaviour of macromolecules in the cell deviates from ideality because of the large concentration of high-molecular-mass solutes. This was pointed out for the first time by Ogston & Phelps [1] and by Laurent & Ogston [2]. They found that hyaluronic acid affects the partition of serum albumin between solutions of the polysaccharide and buffer and that solutions containing both hyaluronic acid and serum albumin have osmotic pressures in excess of the sum of osmotic pressures of solutions containing hyaluronic acid and serum albumin separately at the same concentration. These phenomena were regarded as resulting from the 'exclusion' of albumin from part of the solution occupied by hyaluronic acid.

The thermodynamic properties of these ternary systems were discussed by Ogston [3], Timasheff & Kronman [4], Kuntz & Kautzmann [5], Arakawa & Timasheff [6,7]. As a result of these studies it became clear that phenomena similar to those described by Ogston & Phelps [1] were of general occurrence in the cytoplasmic matrix, where the association and dissociation of macromolecules is influenced by other macromolecules present in solution. Accordingly, from time to time, reports appeared stressing the importance of 'macromolecular crowding' in the regulation of many reactions.

'Macromolecular crowding' has been shown to shift the monomer–tetramer equilibrium of glyceraldehyde phosphate dehydrogenase in favour of tetramer formation [8]; to allow blunt-end ligation by DNA ligase from rat liver and *Escherichia coli* [9]; to drive catenation of supercoiled and gapped DNA circles by topoisomerase I [10]; to influence the binding of gycolytic enzymes to cytoskeletal structures [11] and myofibrils [12]; to accelerate the rate and increase the extent of actin polymerization [13]; to favour the formation of bundles of actin filaments [14].

On these premises we decided to investigate the influence of 'macromolecular crowding' [mimicked by poly(ethylene glycol) 6000] on microfilament bundle formation and on the interaction of these structures with the ancillary proteins of the cytoskeleton. We studied (1) the effect of ancillary cytoskeletal proteins on actin bundle formation and (2) the conversion of actin bundles into actin filaments.

MATERIALS AND METHODS

G-actin from rabbit liver was prepared as described by Spudich & Watt [15] and further gel-filtered through Sephadex G-150 [16]. Caldesmon and tropomyosin were prepared from chicken gizzard [17]. The absorption coefficients used were the following: actin, $A_{290}^{1\%} = 6.2$ [18]; caldesmon, $A_{276}^{1\%} = 3.0$ [17]; tropomyosin, $A_{276}^{1\%} = 3.85$ [19]. Molar concentrations were calculated on the basis of the following molecular masses: actin, 42 kDa [20]; caldesmon, 93 kDa [21]; tropomyosin, 65 kDa [22].

Poly(ethylene glycol) 6000 was from BDH.

The incubation mixtures were prepared by gently mixing 6 vol. of the F-actin–ancillary cytoskeletal protein–salt–buffer solutions with 4 vol. of poly(ethylene glycol)/water solutions at the appropriate concentrations. Final electrolyte concentrations were as follows: 107 mM-K^+, 13 mM-Na^+, 6 mM-Mg^{2+}, 5 mM-orthophosphate, 4 mM-ATP, 37 mM-Cl^- and 70 mM-propionate. pH was 7.0, ionic strength 0.145 and temperature 37 °C. Immediately after mixing, 0.2 ml of the incubation mixtures was delivered, by a SMI digital adjust micropipette, into the centrifuge tubes and incubated at 37 °C for the times indicated in each experiment. After incubation the tubes were centrifuged at 37 °C for 10 min at 9900 *g* in the TL100 rotor of the TL100 Beckman centrifuge to sediment actin bundles. Under these conditions F-actin, tropomyosin and caldesmon, when centrifuged separately and in the absence of poly(ethylene glycol), were fully recovered in the supernatants.

Mixtures containing F-actin (11 μM as monomer), 1.1 μM-tropomyosin and 0.8 μM-caldesmon were also centrifuged for 10 min at 366000 *g* (under these conditions actin filaments are sedimented) and less than 4% of total protein was found in the supernatant. This shows that most of the tropomyosin and caldesmon was pelletted with the actin.

Protein was determined by the Coomassie Blue method [23] as modified by Stoscheck [24].

For electron-microscopic observations the samples were applied to carbon-coated 400-mesh grids, washed once with one drop of water and stained with five drops of 1% (w/v) uranyl acetate, pH 4.25. Electron microscopy was performed on an Hitachi H-800 electron microscope.

RESULTS

Effect of ancillary cytoskeletal proteins on the formation of actin bundles

Association of caldesmon in poly(ethylene glycol) solutions. The effect of poly(ethylene glycol 6000) on the association of caldesmon was first studied. It was found that the light scattering

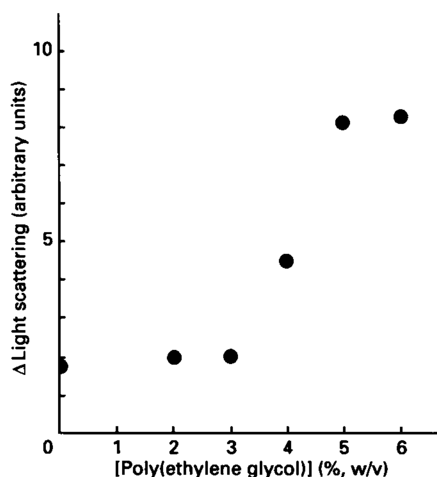

Fig. 1. Light scattering of caldesmon in poly(ethylene glycol) solutions

The mixtures contained poly(ethylene glycol) at the concentration indicated in the Figure, 0.23 μM-caldesmon and electrolytes at the concentrations indicated in the Materials and methods section. Temperature was 25 °C, pH was 7.0. Light-scattering measurements were performed at 500 nm with a Perkin–Elmer MPF3 spectrofluorimeter. ΔLight scattering in arbitrary units represents the difference between the light scattering of the caldesmon/poly(ethylene glycol) solutions and that of the poly(ethylene glycol) solutions alone.

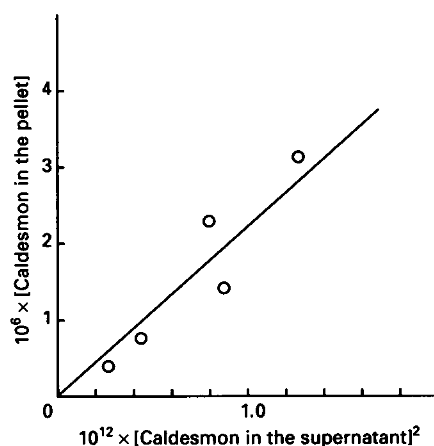

Fig. 2. Sedimentation of caldesmon in 6% poly(ethylene glycol) as a function of caldesmon concentration

The mixtures contained 6% (w/v) poly(ethylene glycol) 0.9–4.2 μM-caldesmon and electrolytes at the concentrations indicated in the Materials and methods section. pH was 7.0, temperature 25 °C. After 30 min of incubation, the samples were centrifuged for 10 min at 366000 *g*. Protein was determined before and after centrifugation. The amount of protein sedimented was calculated by difference.

of 0.23 μM-caldesmon increased abruptly when the concentration of poly(ethylene glycol) was increased above 3% (w/v). Light scattering reached a plateau above 5% poly(ethylene glycol) (Fig. 1).

The association of caldesmon was then studied in the presence of 6% (w/v) poly(ethylene glycol), by measuring the amount of protein sedimented by centrifugation. A linear relationship was found between the concentration of caldesmon sedimented and the square of concentration of caldesmon remaining in solution (Fig. 2). This relationship is expressed formally by the equilibrium constant:

$$K_{\text{ass.}} = \frac{\text{Caldesmon sedimented}}{\text{Caldesmon in solution}} = 2.15 \times 10^6\ \text{M}^{-1}$$

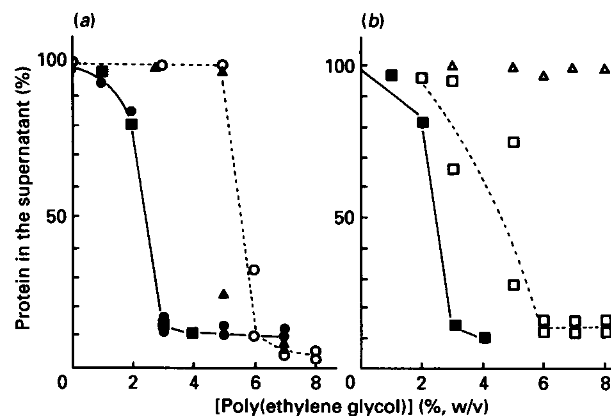

Fig. 3. Effects of caldesmon and tropomyosin on the formation of actin bundles

(*a*) The incubation mixtures contained poly(ethylene glycol) at the concentrations indicated in the Figure, F-actin (11 μM as monomer) and 0.8 μM- (●), 0.5 μM- (■) or 0.32 μM- (▲) caldesmon. A control sample with F-actin alone was also performed (○). Electrolyte concentrations were as indicated in the Materials and methods section. (*b*) The incubation mixtures contained poly(ethylene glycol) at the concentrations indicated in the Figure, F-actin (11 μM as a monomer) and 0.5 μM-caldesmon (■), 0.5 μM-caldesmon plus 0.96 μM-tropomyosin (□) or 0.96 μM-tropomyosin (△). Electrolyte concentrations were as indicated in the Materials and methods section. After 60 min of incubation at 37 °C in the centrifuge tubes, the samples were centrifuged and protein was determined as described in the Materials and methods section.

The aggregates formed by caldesmon in 6% poly(ethylene glycol) were observed in the electron microscope but they appeared amorphous (results not shown).

Effect of caldesmon and the tropomyosin on the formation of bundles of actin in the presence of poly(ethylene glycol). Actin filaments undergo massive conversion into actin bundles at poly(ethylene glycol) concentrations ranging from 6 to 7% (w/v). Furthermore, the transition from filaments into bundles is usually induced by a small increment in the concentration of poly(ethylene glycol) (0.5%, w/v) [14,25].

We have now observed that in a system containing F-actin (11 μM as a monomer) and caldesmon (0.8–0.5 μM), massive actin bundle formation occurs even at 3% (w/v) poly(ethylene glycol) (Figs. 3*a* and 4*b*). The effect of caldesmon, however, becomes undetectable at 0.32 μM (Fig. 3*a*).

F-actin (11 μM as a monomer), decorated with both 0.5 μM-caldesmon and 0.96 μM-tropomyosin, forms bundles at 6% (w/v) poly(ethylene glycol) (Figs. 3*b* and 4*c*), thus approaching the behaviour of F-actin alone. As reported previously [25], F-actin, decorated with tropomyosin, does not form bundles even at 8% poly(ethylene glycol) (Fig. 3*b*).

A common feature of the experiments presented above is the large scatter of the data at the concentrations of poly(ethylene glycol) at which the transition of filaments into bundles occurs. This scatter is due not to experimental error but to the intrinsic instability of the system under these conditions.

Conversion of actin bundles into actin filaments

Dissociation by tropomyosin of the bundles formed by caldesmon–decorated F-actin. As described by Suzuki *et al.* [14], the dissociation of the bundles of actin into filaments of actin is obtained easily by decreasing the concentration of poly(ethylene glycol). We show here that actin bundles can also be dissociated by the suitable addition of ancillary cytoskeletal proteins.

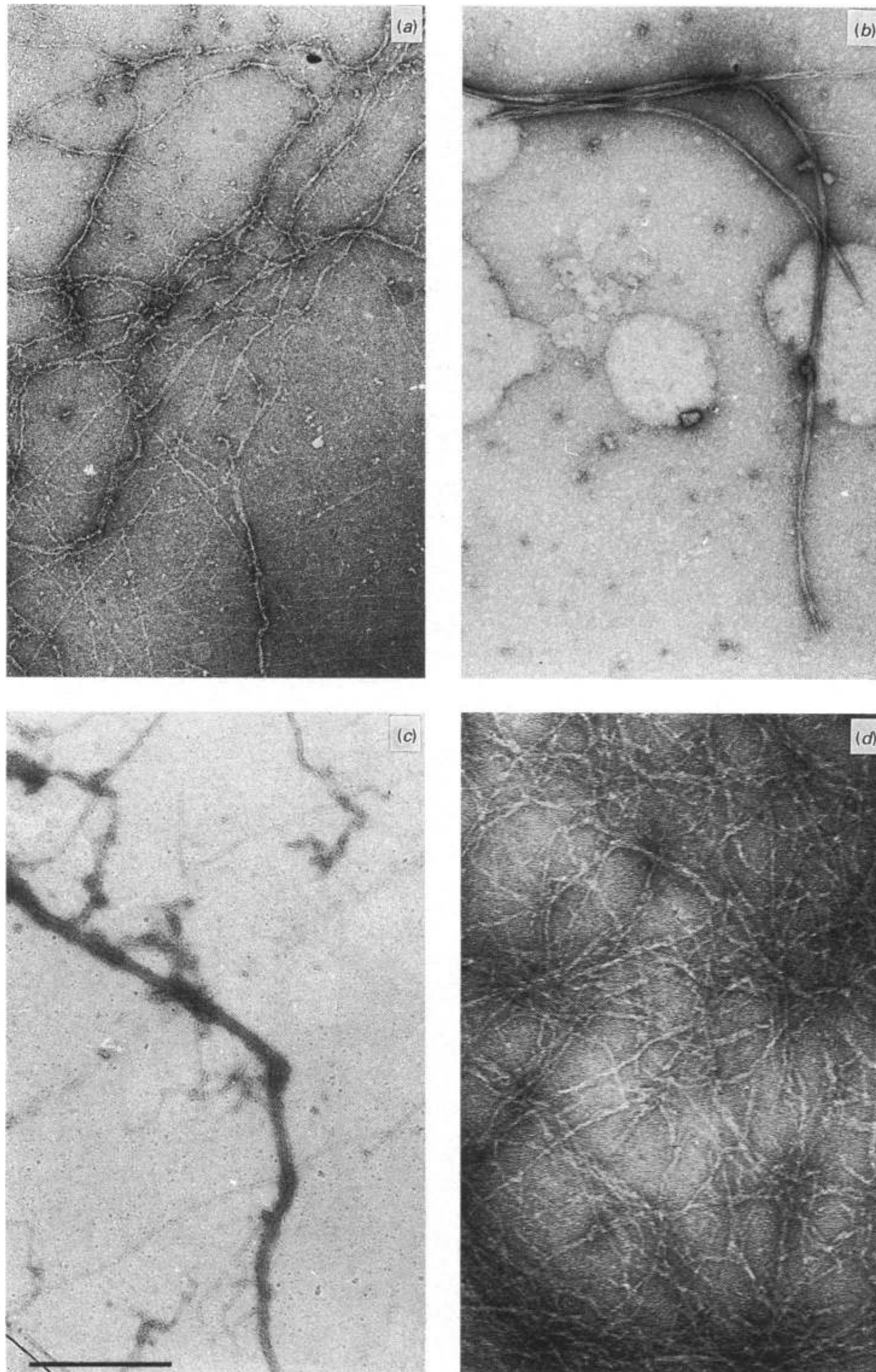

Fig. 4. Electron micrographs of F-actin (*a*), F-actin (11 μM as a monomer) plus caldesmon (0.5 μM) in 3% poly(ethylene glycol) (*b*), F-actin (11 μM as a monomer) plus caldesmon (0.5 μM) plus tropomyosin (0.96 μM) in 6% poly(ethylene glycol) (*c*) and F-actin (11 μM as a monomer) plus tropomyosin (0.96 μM) in 6% poly(ethylene glycol) (*d*)

Other conditions were as described in Fig. 3. Samples were diluted fivefold with the appropriate buffer before being applied to the grid. The bar represents 500 nm in (*a*), 430 nm in (*b*), 416 nm in (*c*) and 250 nm in (*d*).

We reported previously that, in 7% poly(ethylene glycol), tropomyosin failed to dissociate bundles formed by pure actin, even though at that concentration of poly(ethylene glycol) tropomyosin-decorated F-actin was in the filamentous form [25]. Since, besides the order of the additions, the system in the two cases was identical, the final state of the system should also have been identical. The difference found in the two cases could therefore ony be due to kinetic constraints hindering the attainment of the equilibrium.

It was thought that a 'spacer' between the actin filaments

might favour the penetration of tropomyosin into F-actin bundles. We thus tried to dissociate the bundles formed by F-actin decorated with caldesmon by suitable addition of tropomyosin.

F-actin–caldesmon bundles were, in fact, dissociated by tropomyosin in 3% poly(ethylene glycol). The process took about 80 min (Fig. 5*a*). In the reverse experiment, in 7% poly(ethylene glycol), caldesmon induced the formation of bundles from F-actin decorated with tropomyosin (Fig. 5*b*).

It is relevant to notice that in 5% poly(ethylene glycol) the system tropomyosin–caldesmon–F-actin is in the transition between filaments and bundles. Nevertheless, there was no sign of dissociation when tropomyosin was added to preformed actin–caldesmon bundles (Fig. 5). Apparently, even in the presence of the 'spacer' caldesmon, dissociation of bundles did not occur during the time of the experiment (up to 4 h) because of the large osmotic stress still present under those conditions.

Since it was shown previously [14] that mixing increases the rate of bundle formation, we tested whether mixing could promote and speed of bundle dissociation. The experiment was performed as described in Fig. 5(*a*) except that, immediately after the addition of tropomyosin to the caldesmon–F-actin bundles, the mixtures were forced twice through the narrow tip of a capillary micropipette. This operation fragments actin filaments and actin bundles, which, however, re-anneal in a few minutes. The treatment neither accelerated the dissociation of bundles in 3% poly(ethylene glycol) significantly nor promoted the dissociation of bundles in 5% poly(ethylene glycol).

The bundles formed by caldesmon-decorated F-actin are dissociated faster by tropomyosin-decorated F-actin than by tropomyosin alone. When two solutions containing tropomyosin-decorated F-actin or caldesmon-decorated F-actin are mixed, caldesmon and tropomyosin exchange between the two populations. Thus two new populations are formed: undecorated F-actin and tropomyosin–caldesmon-decorated F-actin. If the experiment is performed in 3% poly(ethylene glycol), the exchange must lead to dissociation of the bundles, since the two starting populations, actin–caldesmon bundles and actin–tropomyosin filaments, are converted into F-actin filaments and caldesmon–tropomyosin–F-actin filaments. This expectation was confirmed.

A solution containing F-actin (11 μM as a monomer) and 0.8 μM-caldesmon was mixed with an equal volume of a solution containing F-actin (11 μM as a monomer) and 1.1 μM-tropomyosin. Partial dissociation of the bundles was observed, which reached an equilibrium in 15 min. In a parallel experiment, in which 0.55 μM-tropomyosin was added to a solution containing F-actin (11 μM as a monomer) and 0.4 μM-caldesmon, the process of dissociation of the bundles was slower (Fig. 6).

Limits of the centrifugation method. The experiment described in Fig. 6 deserves a comment. The amount of protein in the supernatants obtained by centrifugation of the starting solutions, separately, was about 15% for actin–caldesmon and 97% for actin–tropomyosin. These values are in keeping with the expectations. However, after mixing and immediate centrifugation (zero time of Fig. 6), the supernatant contained 15% of total protein instead of the expected value calculated by averaging the behaviour of the two starting solutions. This anomaly was explained by the experiment illustrated in Table 1.

Two solutions containing either caldesmon-decorated F-actin or tropomyosin-decorated F-actin were prepared. Then, in one set of centrifuge tubes, caldesmon-decorated F-actin was delivered to the bottom of the tubes and tropomyosin-decorated F-actin was layered on top of the first solution. Care was taken to avoid mixing of the two solutions. In a second set of tubes, tropomyosin-decorated F-actin was delivered to the bottom and

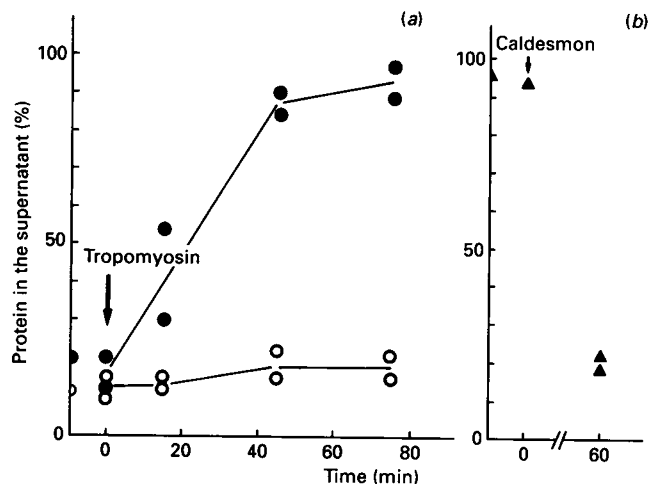

Fig. 5. Dissociation of the caldesmon–actin bundles by tropomyosin

(*a*) The mixtures contained poly(ethylene glycol) at a concentration of either 5% (○) or 3% (●), F-actin (11 μM as a monomer), 0.4 μM-caldesmon and electrolytes at the concentrations indicated in the Materials and methods section. At the beginning of the experiment (arrow) 1.1 μM-tropomyosin was added. (*b*) The mixtures contained 7% poly(ethylene glycol), F-actin (11 μM as a monomer), 0.96 μM-tropomyosin and electrolytes at the concentrations indicated in the Materials and methods section. At the beginning of the experiment (arrow) 0.5 μM-caldesmon was added. The mixtures were incubated in the centrifuge tubes for the times indicated in the Figure. At the end of the incubation the tubes were centrifuged and the protein was assayed as described in the Materials and methods section.

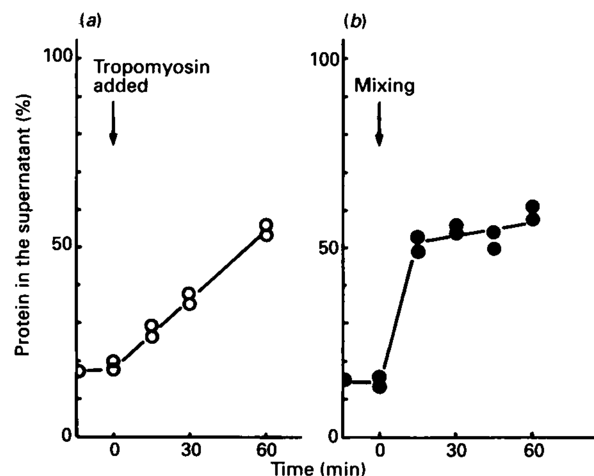

Fig. 6. Comparison of the kinetics of dissociation of the actin–caldesmon bundles by either tropomyosin or actin–tropomyosin

(*a*) The mixture contained 3% poly(ethylene glycol), F-actin (11 μM as a monomer), 0.41 μM-caldesmon and electrolytes at the concentrations indicated in the Materials and methods section. At the beginning of the experiment (arrow) 0.55 μM-tropomyosin was added. (*b*) Two mixtures were prepared containing electrolytes as indicated in the Materials and methods section, 3% poly(ethylene glycol) and either F-actin (11 μM as a monomer) plus 0.82 μM-caldesmon or F-actin (11 μM as a monomer) plus 1.1 μM-tropomyosin. At the beginning of the experiment equal volumes of the two solutions were mixed and samples were delivered into the centrifuge tubes. At time intervals the tubes were centrifuged and protein in the supernatants was determined as described in the Materials and methods section. The starting solutions before mixing or addition of tropomyosin were also submitted to the same procedure. Actin bundles dissociated by the addition of tropomyosin (○) and by F-actin–tropomyosin (●).

Table 1. The dragging of actin filaments by actin bundles limiting the usefulness of the centrifugation method

The incubation mixtures contained the electrolytes at the concentrations indicated in the Materials and methods section, 5% poly(ethylene glycol) and either F-actin (11 μM as a monomer) plus tropomyosin (1.05 μM) (solution A) or F-actin (11 μM as a monomer) plus caldesmon (0.41 μM) (solution B). Samples containing either solution A layered on an equal volume of solution B (Expt. 3) or solution B layered on an equal volume of solution A (Expt. 4) were prepared in the centrifugation tubes and immediately centrifuged. Solutions A (Expt. 1) and B (Expt. 2) were also centrifuged. The amount of protein in the supernatant was then determined.

		Protein in the supernatant (%)
Expt. 1	F-actin + tropomyosin	95
Expt. 2	F-actin + caldesmon	17
Expt. 3	F-actin + tropomyosin (top) F-actin + caldesmon (bottom)	74
Expt. 4	F-actin + caldesmon (top) F-actin + tropomyosin (bottom)	24

caldesmon-decorated F-actin was layered on the top of the first solution.

As shown in Table 1, when filaments of actin (tropomyosin-decorated actin) were layered on the top of the centrifuge tubes, the amount of protein found in the supernatants after centrifugation was much larger than that found when bundles of actin (caldesmon-decorated actin) were layered on the top of the tubes. Certainly the procedure selected in this experiment magnifies the dragging of filaments by the bundles; however, when filaments and bundles are mixed before centrifugation (as is usually done), the influence of dragging must also be present.

In conclusion, centrifugation provides only a rough estimate of the proportion of actin present as filaments and bundles. Apparently, when the proportion of the bundles is low, the estimate is quite correct but when the mass of actin present in the form of bundles is about 50%, the formation of the bundles is overestimated since as much as 76% of the protein can be found in the sediment. In spite of this limitation, centrifugation remains a very useful method for following the reversible conversion of filaments into bundles.

DISCUSSION

The reversible conversion of actin filaments into actin bundles plays an important role in motile cells. Unfortunately, so far, the factors that regulate this process are not completely understood. It is known that, by increasing the concentration of the macromolecules in the medium, it is possible to drive the conversion of actin filaments into actin bundles. It is also assumed that, at the concentration of the macromolecules present in the cell, actin is in the form of bundles of filaments [14].

We have now observed that actin filaments may undergo transition into bundles at a set of different macromolecular concentrations, depending on the nature of the ancillary cytoskeletal proteins bound to the filaments. When poly(ethylene glycol 6000) is employed to mimic the macromolecules of the cell, the formation of bundles of actin filaments occurs at 3% poly(ethylene glycol) for caldesmon-decorated actin, at 4–5% poly(ethylene glycol) for filamin-decorated actin [25], at 5–7% poly(ethylene glycol) for caldesmon–tropomyosin-decorated actin, at 6–7% poly(ethylene glycol) for F-actin and at 9–10% poly(ethylene glycol) for tropomyosin-decorated actin [25]. Of course this rank order is restricted to our experimental conditions. The concentration of poly(ethylene glycol) that induces the transition of filaments into bundles can be shifted by changing the ratios of the ancillary proteins with respect to actin. This is particularly true for caldesmon-decorated actin and filamin-decorated actin. This latter system, in fact, undergoes bundling, even in the absence of poly(ethylene glycol), when the filamin to actin molar ratio is increased to 1:8 [26]. On the contrary, caldesmon, even at a caldesmon to actin molar ratio of 1:3, does not induce actin bundling in the absence of poly(ethylene glycol). This is in agreement with the report of Riseman & Bretscher [27].

From these studies, which have been performed with a few cytoskeletal proteins, emerge two points. (1) Local gradients of the macromolecular concentration may explain why in some regions of the cell (the cortical region) the filamentous structure prevails. (2) The intracellular distribution of the ancillary cytoskeletal proteins may determine the state of aggregation of actin. This leads to the question of how the intracellular distribution of the proteins of the cytoskeleton is regulated. In some cases the binding of a first protein could promote the binding of a second one. As an example, caldesmon is reported to bind tighter to tropomyosin-decorated F-actin than to F-actin [28].

This implies that a system composed of tropomyosin-decorated actin and caldesmon-decorated actin evolves toward a system composed of F-actin and tropomyosin–caldesmon-decorated actin. In 3% poly(ethylene glycol) solutions, the progression of the system from the first to the second state increases the proportion of actin filaments over actin bundles. In more concentrated poly(ethylene glycol) solutions (6–7%), the same progression of the system on the contrary increases the proportion of actin bundles over actin filaments.

The sudden release of actin from precursors, such as profilactin, can also modify the stationary state of the filament–bundle system because of the subsequent redistribution of the ancillary cytoskeletal proteins among the whole population of the actin filaments.

A slower modification of the stationary state of the filament–bundle system can also be provided by synthesis *de novo* of one of the ancillary proteins. A feature complicating the kinetics of the process will certainly be the aggregation of the newly synthesized protein, as was shown in the case of caldesmon.

Regulatory effects can also be produced by the glycolytic enzymes. Aldolase and glyceraldehyde phosphate dehydrogenase undergo adventitious binding to microfilaments [29] and act as actin-bundling proteins [30,31]. Furthermore, their interaction with F-actin is regulated by physiological concentrations of their substrates [29,32].

The dissolution of bundles of pure actin filaments by cytoskeletal proteins *in vitro* seems to be hindered by the difficulty of penetration of the ancillary proteins into the actin bundles. The introduction of a 'spacer' (caldesmon) into the bundles provided a means of overcoming this difficulty. It is also possible, however, that the dissociation of the actin–caldesmon bundles, promoted by tropomyosin, is not due to the introduction of a 'spacer' but to the fact that, with the actin–caldesmon system, it is possible to operate under conditions of low osmoelastic stress [3% poly(ethylene glycol)].

Of interest also is the observation that tropomyosin-decorated actin induces a faster dissociation of the actin–caldesmon bundles than does tropomyosin. Many explanations may be found for this phenomenon. However, the selection of a mechanism requires study with a more accurate and faster method than centrifugation. In any case, we are inclined to think that, owing to the exceedingly low rate of diffusion of the actin filaments, the

dissociation of the bundles also occurs through the exchange of tropomyosin and caldesmon when tropomyosin-decorated actin filaments are employed to dissociate the bundles.

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REFERENCES

1. Ogston, A. G. & Phelps, C. F. (1961) Biochem. J. **78**, 827–831
2. Laurent, T. C. & Ogston, A. G. (1963) Biochem. J. **89**, 249–253
3. Ogston, A. G. (1962) Arch. Biochem. Biophys. Suppl. **1**, 39
4. Timasheff, S. N. & Kronman, M. J. (1969) Arch. Biochem. Biophys. **83**, 60–75
5. Kuntz, I. D. & Kautzmann, W. (1974) Adv. Protein Chem. **28**, 239–245
6. Arakawa, T. & Timasheff, S. N. (1985) Biochemistry **24**, 6756–6762
7. Arakawa, T. & Timasheff, S. N. (1985) Methods Enzymol. **114**, 49–77
8. Minton, A. P. & Wolff, J. (1981) Biochemistry **20**, 4821–4826
9. Zimmerman, S. B. & Pfeiffer, B. H. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5852–5856
10. Low, R. L., Kaguni, J. M. & Kornberg, A. (1984) J. Biol. Chem. **259**, 4576–4581
11. Shearwin, K., Nanhua, C. & Masters, C. (1989) Biochem. Int. **19**, 723–729
12. Harris, S. J. & Winzor, D. J. (1985) Arch. Biochem. Biophys. **243**, 598–604
13. Tellam, R. L., Sculley, M. J., Nichol, L. W. & Wills, P. R. (1983) Biochem. J. **213**, 651–659
14. Suzuki, A., Yamazaki, M. & Ito, T. (1989) Biochemistry **28**, 6513–6518
15. Spudich, J. A. & Watt, S. (1971) J. Biol. Chem. **246**, 4866–4871
16. McLean-Flechter, S. & Pollard, T. D. (1980) Biochem. Biophys. Res. Commun. **96**, 18–27
17. Bretscher, A. (1984) J. Biol. Chem. **259**, 12873–12880
18. Gordon, D. J., Yang, Y. Z. & Korn, E. D. (1976) J. Biol. Chem. **251**, 7474–7479
19. Wegner, A. (1979) J. Mol. Biol. **131**, 839–853
20. Collins, J. H. & Elzinga, M. (1975) J. Biol. Chem. **250**, 5915–5920
21. Graceffa, P., Wang, A. & Stafford, W. F. (1988) J. Biol. Chem. **263**, 14196–14202
22. Weber, K. & Osborn, M. (1969) J. Biol. Chem. **244**, 4406–4412
23. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
24. Stoscheck, C. M. (1990) Anal. Biochem. **184**, 111–116
25. Grazi, E., Trombetta, G. & Guidoboni, M. (1990) Biochem. Biophys. Res. Commun. **167**, 1109–1114
26. Wang, K. & Singer, S. J. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2021–2025
27. Riseman, V. M. & Bretscher, A. (1986) J. Cell Biol. **103**, 536a
28. Moody, C. J., Martson, S. B. & Smith, C. W. J. (1985) FEBS Lett. **191**, 107–112
29. Arnold, H. & Pette, D. (1970) Eur. J. Biochem. **15**, 360–369
30. Clarke, F. M. & Morton, D. J. (1976) Biochem. J. **159**, 797–804
31. Morton, D. J., Clarke, F. M. & Masters, C. J. (1977) J. Cell Biol. **74**, 1016–1024
32. Clarke, F. M. & Masters, C. J. (1975) Biochim. Biophys. Acta **381**, 37–42

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