The control of cellular shape and motility

Mg²⁺ and tropomyosin regulate the formation and the dissociation of microfilament bundles

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At pH 7.14 and 37 °C, in 7.2 % (w/v) poly(ethylene glycol) 6000, tropomyosin-regulated actin filaments are converted into filament bundles by increasing the free Mg²⁺ concentration to 1.7–2.0 mM. When free Mg²⁺ concentration is decreased below 1.7 mM, bundles dissociate back into tropomyosin-regulated actin filaments. Pure actin filaments are insensitive to this mechanism of control and are found as filament bundles in all the range of free Mg²⁺ concentrations tested (1.37–2.2 mM). The mechanism of regulation described above is likely to operate in the cell, where the concentration of free Mg²⁺ is linked to the energy charge of the adenine nucleotide system.

INTRODUCTION

The control of cell shape and motility relies on a complex system of microtubules, intermediate filaments and microfilaments. The function of microfilaments is modulated by many actin bundling and cross-linking proteins (Pollard & Cooper, 1986). Furthermore, it was recently stressed that the high concentration of macromolecules in the cell is itself one of the main determinants of the function and of the architecture of the cytoskeleton (Suzuki *et al.*, 1989; Grazi *et al.*, 1990a,b, 1991; Grazi & Trombetta, 1991; Cuneo *et al.*, 1992). It was shown that the formation of microfilament bundles is favoured by the high concentration of protein in the cytosol (Suzuki *et al.*, 1989) and is modulated by the ancillary cytoskeletal proteins (Grazi *et al.*, 1990a; Cuneo *et al.*, 1992). It was proposed that the distribution of these proteins determines the regions of the cell where either bundles or microfilaments prevail, and that a change of the distribution of the ancillary proteins may promote the slow rearrangement of the bundles and microfilaments areas (Cuneo *et al.*, 1992). No mechanism was proposed, however, to explain the rapid dissociation of actin bundles that occurs in the cell.

We show here that free Mg²⁺, in the range of concentrations occurring in the cell, controls the formation (high Mg²⁺) and the dissociation (low Mg²⁺) of actin bundles. The mechanism operates on tropomyosin-decorated F-actin but not on pure F-actin.

MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as described by Spudich & Watt (1971) and further gel-filtered through Sephadex G-150 (McLean-Flechter & Pollard, 1980). Tropomyosin was prepared from chicken gizzard (Bretscher, 1984). The absorption coefficients used were as follows: actin, $A_{290}^{1\%} = 6.2$ (Gordon *et al.*, 1976); tropomyosin, $A_{276}^{1\%} = 3.85$ (Wegner, 1979). Molar concentrations were calculated on the basis of the following molecular masses: actin, 42 kDa (Collins & Elzinga, 1975); tropomyosin, 65 kDa (Weber & Osborn, 1969). [³H]N-Ethylmaleimide-labelled tropomyosin was prepared as described by Ishii & Lehrer (1985). Poly(ethylene glycol) 6000 was from BDH (Poole, Dorset, U.K.).

The incubation mixtures were prepared by mixing 19 vol. of the F-actin–tropomyosin/salt/buffer solutions with 6 vol. of 30 % (w/v) poly(ethylene glycol) 6000 solution in water. Final electrolyte concentrations were as follows: 70 mM-potassium propionate, 25 mM-KCl, 4.74–6.3 mM-MgCl₂ and either 5 mM-

potassium phosphate plus 4.07–6.29 mM-ATP or 9.29–11.29 mM-potassium phosphate plus 4.29–6.29 mM-ADP. The temperature was 37 °C. The pH of the complete incubation mixtures, including poly(ethylene glycol) and proteins, was measured at 37 °C and was 7.1–7.16.

MgCl₂ stock solutions were titrated by standard procedures. Stock solutions of ATP and ADP (as the disodium salts) and of KH₂PO₄ were adjusted to pH 7.1 by addition of KOH, and made up to 0.2 M. The concentration of nucleotides was determined spectrophotometrically at 259 nm on the basis of $\epsilon = 15.4$ (Pabst Laboratories Circular OR-10, 1956).

The conversion of actin filaments into bundles was studied by monitoring either protein sedimentation or light-scattering. Neither method is completely satisfactory.

Actin bundles were sedimented by centrifugation for 10 min at 9900 g in the TL100 rotor of a Beckman TL100 centrifuge. Under these conditions F-actin, when centrifuged alone, remains completely in the supernatant. However, as pointed out by Cuneo *et al.* (1992), bundles and filaments are not completely resolved when centrifuged together. The method thus provides a semi-quantitative estimate of the formation of actin bundles.

The increase in light-scattering, which occurs when F-actin is converted into bundles of filaments, is a convenient way to follow the transition qualitatively, provided that the solutions are continuously stirred. In resting solutions the measurements provide erratic results, because bundles distribute in irregular masses in the cuvette. Stirring, of course, influences the rate of bundle formation (Suzuki *et al.*, 1989) and the intensity of the light scattering. Care must also be taken to avoid trapping of air bubbles in the very viscous solutions.

Radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter with 5 ml of a Packard emulsifier liquid-scintillation cocktail. To optimize radioactivity determinations, the protein in the supernatants and in the dissolved pellets was hydrolysed for 4 h at 25 °C with Pronase (250 µg/ml) (Silverman *et al.*, 1985).

Protein was determined by the Coomassie Blue method (Bradford, 1976) as modified by Stoscheck (1990).

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

Free Mg²⁺ concentrations were calculated on a Tektronic 31 programmable calculator by using an iterative procedure and the values of the constants given by Alberty (1968).

RESULTS

Constant mixing is needed for the disassembly of F-actin bundles by tropomyosin in poly(ethylene glycol)

In resting solutions, F-actin is converted into bundles by 7.2% (w/v) poly(ethylene glycol), whereas tropomyosin-decorated F-actin remains in the filamentous form. Under the same conditions, however, tropomyosin fails to dissociate actin bundles. This behaviour was ascribed to kinetic constraints (the very slow diffusion of tropomyosin into the tight actin bundles) hindering the attainment of equilibrium (Grazi *et al.*, 1990a; Cuneo *et al.*, 1992).

We show here that the kinetic constraints are removed by

Fig. 1. F-actin bundles, formed in 7.2% poly(ethylene glycol), are dissociated by tropomyosin

The complete incubation mixture (2.6 ml) contained F-actin ($6.46 \mu\text{M}$ as monomer), $0.36 \mu\text{M}$ -tropomyosin, 5 mM-phosphate, 5.48 mM- MgCl_2 , 5.1 mM-ATP, 70 mM-potassium propionate, 25 mM-KCl and 7.2% (w/v) poly(ethylene glycol). pH was 7.16, and temperature was 37°C . First arrow, polyethylene glycol added; second arrow, tropomyosin added. The reaction was performed with stirring.

Fig. 2. F-actin bundles, formed in the presence of ADP and tropomyosin, are dissociated by addition of ATP

The complete incubation mixtures (2.6 ml) contained F-actin ($6.46 \mu\text{M}$ as monomer), $0.36 \mu\text{M}$ -tropomyosin, 9.7 mM-phosphate, 6.04 mM- MgCl_2 , 4.7 mM-ADP, 1.6 mM-ATP, 70 mM-potassium propionate, 25 mM-KCl and 7.2% (w/v) poly(ethylene glycol). pH was 7.16, and temperature was 37°C . First arrow, poly(ethylene glycol) added; third arrow, ATP added; second and fourth arrows, samples taken for protein assay. The reaction was performed with stirring.

constant mixing of the solution and that, as a consequence, the addition of tropomyosin promotes the dissociation of actin bundles.

As shown in Fig. 1, the addition, under stirring, of poly(ethylene glycol) to a F-actin-salt-buffer solution produces a rapid increase in the light-scattering of the mixture (in part owing to trapping of air in the mixture), which then decreases to a lower, stable, level. When tropomyosin is added, the light-scattering decays to the level found before the addition of poly(ethylene glycol), with a half-time of 90 s. The change in light-scattering is accompanied by a change in the sedimentation behaviour of F-actin. Two samples were taken before and 12 min after addition of tropomyosin, and were centrifuged. The two supernatants contained, respectively, 8% and 96% of total protein, as expected from the conversion of the easily sedimentable bundles into filaments.

Tropomyosin-decorated F-actin is converted into bundles by poly(ethylene glycol), even under constant mixing, when ADP replaces ATP in the incubation mixture

The effect of the replacement of ATP with an equimolar concentration of ADP and phosphate on the formation of bundles by tropomyosin-decorated F-actin was also studied. It was found that, in the presence of ADP, the light-scattering of a solution of F-actin and tropomyosin was increased by addition of 7.2% (w/v) poly(ethylene glycol). At this stage of the experiment, addition of 1.6 mM-ATP decreased the light-scattering of the solution to the level observed before addition of poly(ethylene glycol) (Fig. 2). This indicates that bundles had been dissociated into filaments. These conclusions were confirmed by protein sedimentation analysis. Two samples were taken immediately before and 15 min after the addition of ATP, and centrifuged. The amount of protein sedimented was respectively 97% and 2% of the total.

Supramolecular structures formed by F-actin and by tropomyosin-decorated F-actin in the presence of either ATP or ADP

The nature of the supramolecular structures formed by F-actin and by tropomyosin-decorated F-actin in the presence of either ATP or ADP was studied at the electron microscope. In 7.2% (w/v) poly(ethylene glycol), compact paracrystalline structures were formed by F-actin in ADP (Fig. 3a) and loose bundles were formed in ATP (Fig. 3b). With tropomyosin-decorated F-actin, loose bundles were formed in the presence of ADP (Fig. 3c) and filaments were observed in the presence of ATP (Fig. 3d). We recall here that the samples were taken while the solutions were under stirring, this may have contributed to the formation of loose bundles. The results of protein sedimentation analysis were in agreement with the electron-microscopic observations. More than 90% of total protein was sedimented by centrifugation of the samples in Figs. 3(a), 3(b) and 3(c), whereas only 6% of total protein was sedimented by centrifugation of the sample in Fig. 3(d).

In resting solutions, the formation of tropomyosin-decorated F-actin bundles is rapidly and reversibly regulated by changes in the ATP/(ATP+ADP) ratio and by the concomitant change in free Mg^{2+} concentration

The effect of addition of adenine nucleotides [at different ATP/(ATP+ADP) ratios] on the conversion of the tropomyosin-decorated F-actin into bundles of filaments was studied. Since the aim of these studies was to simulate the changes which occur *in vivo*, when ATP is hydrolysed to ADP and phosphate, whenever ADP was added the incubation mixture was supplemented with an equimolar concentration of phosphate,

Fig. 3. Supramolecular structures formed by F-actin and by tropomyosin-decorated F-actin in the presence of either ATP or ADP

The incubation mixtures (2.6 ml) contained F-actin (6.46 μ M as monomer), 6.29 mM-MgCl₂, 70 mM-potassium propionate, 25 mM-KCl, 7.2% (w/v) poly(ethylene glycol) and either 5.14 mM-ADP plus 9.14 mM-phosphate (a and c) or 5.14 mM-ATP plus 5 mM-phosphate (b and d). To mixtures (c) and (d) 0.36 μ M tropomyosin was also added. pH was 7.16. After 15 min incubation at 37 °C, samples were taken for protein sedimentation analysis and for electron-microscopic observation. Bar represents 333 nm.

over the basal phosphate concentration of 5 mM. Furthermore, the experiments were performed on resting solutions and analysed by the protein sedimentation method. The concentration of total adenine nucleotides was 4.29–6.29 mM, and total Mg²⁺ concentration was 6.29 mM.

When the concentration of adenine nucleotides was 4.29 mM,

95% of total protein was sedimented at all the ATP/(ATP + ADP) ratios.

When the concentration of adenine nucleotides was 5.14 mM, more than 90% of protein was sedimented at a ratio lower than 0.2, whereas protein was found essentially 100% in the supernatant at a ratio larger than 0.55. At ratios between 0.3 and 0.45,

Fig. 4. Effect of change in the ATP/(ATP+ADP) ratio and of the concomitant change in free Mg²⁺ concentration on formation of F-actin and of tropomyosin-decorated F-actin bundles

The incubation mixtures contained F-actin (6.76 μ M as monomer), 0.38 μ M-tropomyosin, 6.29 mM-MgCl₂, 70 mM-potassium propionate, 25 mM-KCl and 7.2% (w/v) poly(ethylene glycol). Total adenine nucleotide (ATP+ADP) concn. was 4.29 mM ($\blacktriangle$), 5.14 mM ($\circ$) or 6.29 mM ($\bullet$). The concentration of phosphate was equal to that of ADP plus 5 mM. In the experiment marked by crosses (x), tropomyosin was omitted and total adenine nucleotide concn. was 5.14 mM. pH was 7.14. The reaction was performed in resting solutions. After 15 min incubation at 37 °C, samples were taken for protein sedimentation analysis. Points represent means $\pm$ range for triplicate experiments.

Fig. 5. Effect of change in Mg²⁺ concentration on the formation of F-actin and of tropomyosin-decorated F-actin bundles

The incubation mixtures contained F-actin (6.8 μ M as monomer), 0.38 μ M-tropomyosin, 4.07 mM-ATP, 70 mM-potassium propionate, 25 mM-KCl and 7.2% (w/v) poly(ethylene glycol). Mg²⁺ concentration was as indicated in the Figure. In the experiment marked by crosses (+), tropomyosin was omitted. pH was 7.14. The reaction was performed in resting solutions. After 15 min incubation at 37 °C, samples were taken for protein sedimentation analysis.

34–80% of total protein was sedimented, with a large scatter of the data (such scatter occurs usually at the transition between bundles and filaments).

When the concentration of adenine nucleotides was 6.29 mM, the zone of transition between filaments and bundles was slightly displaced toward a lower ATP/(ATP+ADP) ratio; however, 93% of protein was sedimented when the ratio was zero.

When the concentration of adenine nucleotides was 5.14 mM but tropomyosin was absent, about 90% of total protein was

Table 1. Binding of [³H]N-ethylmaleimide-tropomyosin to F-actin in the presence of either ATP or ADP

The incubation mixtures contained 7.2 (w/v) poly(ethylene glycol), F-actin (6.76 μ M as monomer), 0.44 μ M-[³H]N-ethylmaleimide-tropomyosin (sp. radioactivity 16000 d.p.m./nmol), 70 mM-potassium propionate, 25 mM-KCl, 6.29 mM-MgCl₂ and either 5 mM-phosphate plus 5.15 mM-ATP (Expt. 1) or 10.15 mM-phosphate plus 5.15 mM-ADP (Expt. 2). pH was 7.14. After incubation for 15 min at 37 °C, samples were taken and centrifuged for 10 min at 37 °C at either 348000 g or 9900 g. The supernatants were assayed for protein and radioactivity content as indicated in the Materials and methods section. The reaction was performed in resting solutions.

Centrifugation at ...	Protein (%)		Tropomyosin (%)	
	348000 g (a)	9900 g (b)	348000 g (c)	9900 g (d)
Expt. 1				
Supernatant	4	89	41	100
Pellet	96	11	59	0
Expt. 2				
Supernatant	5	12	32	36
Pellet	95	88	68	64

sedimented at all ratios (Fig. 4a).

Calculation of the free Mg²⁺ concentration revealed that, independently of the total adenine nucleotide concentration, the transition between filaments and bundles took place between 1.7 and 2 mM free Mg²⁺ when tropomyosin-decorated F-actin was used. In contrast, only bundles were found between 1.37 mM and 2.2 mM free Mg²⁺ when pure F-actin was used (Fig. 4b).

The fact that free Mg²⁺ concentration is one of the critical parameters that regulate filament bundling was confirmed by varying the total Mg²⁺ concentration at fixed ATP concentration. As shown in Fig. 5, in the presence of 4.07 mM-ATP, filament bundling of tropomyosin-decorated F-actin occurs at 5.6–5.8 mM total Mg²⁺ concentration, i.e. between 1.5 and 1.7 mM free Mg²⁺ concentration.

Replacement of ATP by ADP is not accompanied by the dissociation of the F-actin-tropomyosin complex

In 7.2% poly(ethylene glycol), the replacement of ATP by ADP promotes the transition of tropomyosin-decorated F-actin into bundles of filaments. The possibility was thus considered that the phenomenon could be due to the dissociation of the tropomyosin-F-actin complex. Mixtures containing F-actin (6.76 μ M as monomer) and 0.44 μ M-[³H]N-ethylmaleimide-labelled tropomyosin were centrifuged for 10 min at 348000 *g*, and it was found that actin was sedimented together with 59% and 68% of tropomyosin in the presence of ATP and ADP respectively (columns *a* and *c* of Table 1). This shows that the replacement of ATP by ADP is not accompanied by the desorption of tropomyosin from F-actin. When the samples were centrifuged for 10 min at 9900 *g*, to sediment actin bundles, tropomyosin, in the presence of ATP, was found in the supernatant solutions together with 89% of protein. In contrast, in the presence of ADP, 64% of tropomyosin was sedimented together with 88% of protein (columns *b* and *d* of Table 1). This shows that, in the presence of ADP, tropomyosin is still bound to actin bundles.

DISCUSSION

Many proteins such as filamin, fimbrin, villin and synapsin are known to display actin-bundling activity *in vitro*. However, the problem is not to understand how, in the cell, actin bundles are formed, but how actin filaments are kept as such and how actin bundles are dissociated into filaments. In fact, in the cell, owing to the high concentration of protein, the formation of actin bundles is favoured (Suzuki *et al.*, 1989).

In intact cells the formation and the dissociation of actin bundles may take days, as in regenerating arterial endothelial cells (Gabbiani *et al.*, 1983), minutes to hours, as in motile cells (Izzard & Lochner, 1976, 1980), and from 10 to 60 s, as in the reversible aggregation of human platelets (Carlsson *et al.*, 1979; Pribluda *et al.*, 1981; Pribluda & Rotman, 1982).

In vitro, the formation of actin bundles requires increasing concentrations of poly(ethylene glycol) for filamin-decorated F-actin, for caldesmon-decorated F-actin, for pure F-actin and for tropomyosin-decorated F-actin (Grazi *et al.*, 1990a; Cuneo *et al.*, 1992). It is thus likely that, *in vivo*, tropomyosin may contribute to keep actin in the filamentous form, while caldesmon may balance the effect of tropomyosin. A change in the relative abundance of these proteins (and perhaps of other cytoskeletal proteins), possibly linked to the synthesis of new protein molecules, may represent a mechanism for the slow conversion of actin filaments into actin bundles in some areas of the cell.

The reverse process, i.e. the dissociation of actin bundles into actin filaments, may even be slower because tropomyosin diffuses very slowly into actin bundles, which are tightly packed at the high concentration of macromolecules in the cell. Tropomyosin, in resting solutions and in 3% poly(ethylene glycol), dissociates caldesmon-F-actin bundles, but is ineffective when the concentration of poly(ethylene glycol) is raised to 5% (Cuneo *et al.*, 1992). However, provided that the protein solutions are continuously stirred, tropomyosin dissociates F-actin bundles even in 7.2% poly(ethylene glycol) (the present paper).

Many actin-bundling proteins have been proposed to regulate the rapid formation and dissociation of actin bundles.

Human erythrocyte protein 4.9 is reported to display an actin-bundling activity which is abolished when the protein, in preformed actin bundles, is phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase (Husain-Chishti *et al.*, 1983).

Lipocortin 85 is reported to display a Ca²⁺-dependent actin-

bundling activity (Ikebuchi & Waisman, 1990).

The actin-bundling activity of synapsin I is reported to be abolished when the protein is phosphorylated by Ca²⁺-calmodulin-dependent protein kinase II (Bähler & Greengard, 1987).

The Ca²⁺-calmodulin-caldesmon complex is reported to dissociate at low Ca²⁺ concentration; in turn, the released caldesmon is reported to displace filamin from filamin-actin bundles, which then dissociate into filaments (Sobue *et al.*, 1981, 1982).

In no case has it been checked, however, whether preformed actin bundles actually dissociate when the concentration of macromolecules is close to that present in the cytosol. As an example, we have studied the behaviour of the Ca²⁺-calmodulin-caldesmon-F-actin system in 3% poly(ethylene glycol). We have found that Ca²⁺-calmodulin prevents the binding of caldesmon to F-actin, but fails to dissociate caldesmon-F-actin bundles, which are formed at that concentration of poly(ethylene glycol) (E. Grazi, P. Cuneo & A. Cataldi, unpublished work). This is at variance with the data obtained by Sobue *et al.* (1981) in solutions of small electrolytes.

We show here that, *in vitro*, the change of free Mg²⁺ concentration is an adequate signal to trigger the rapid and reversible interconversion of tropomyosin-decorated actin filaments into tropomyosin-decorated F-actin bundles. At 37 °C, pH 7.14, in 7.2% (w/v) poly(ethylene glycol), the transition between filaments and bundles occurs between 1.7 mM and 2 mM free Mg²⁺. At lower and higher concentrations of Mg²⁺, tropomyosin-decorated F-actin is present as filaments and as bundles, respectively. Interestingly, the regulation by Mg²⁺ operates also at sub-saturating concentrations of tropomyosin, whereas it is ineffective with pure actin, which is fully present as bundles of filaments even at a free Mg²⁺ concentration as low as 1.37 mM.

There is some uncertainty as to the level of free Mg²⁺ in the cell. Free Mg²⁺ concentration has been found to be 0.4 mM (null-point titration) in Ehrlich ascites-tumour cells (Cittadini & Scarpa, 1983); 0.48 mM (titration with 2-[2-(5-carboxy)oxazole]-(hydroxy-6-aminobenzofuran-*NNO*-triacetic acid)) in cultured chicken heart cells (Murphy *et al.*, 1989); 0.4 mM (Blatter & McGuigan, 1986) and 2.4 mM (Fry, 1986) (Mg²⁺-selective micro-electrodes) in heart muscle fibres; 0.8 mM (Gupta *et al.*, 1983) and 2.4 mM (Wu *et al.*, 1981) (³¹P n.m.r.) in perfused rat heart.

Under our experimental conditions the transition between tropomyosin-decorated actin filaments and tropomyosin-decorated actin bundles is set at 1.7–2.0 mM free Mg²⁺. The zone of transition is very probably displaced toward lower free Mg²⁺ concentrations by an increase in the concentration of the macromolecules of the cell (cell shrinkage) and by actin-bundling proteins (caldesmon).

In the cell, the actual concentration of free Mg²⁺ is mainly regulated by the energy charge of the ATP system (Cittadini & Scarpa, 1983). Free Mg²⁺ concentration may be increased by an altered intracellular Mg²⁺ binding, secondary to changes in Ca²⁺ influx or pH or to cell shrinkage (Murphy *et al.*, 1989). In our experimental conditions a shift from 2.0 mM to 1.7 mM free Mg²⁺, which produces a substantial increase in the amount of tropomyosin-decorated F-actin bundles over tropomyosin-decorated actin filaments, is brought about by a 0.5 mM decrease in ATP concentration. Stimulation by ADP of human platelets induces a similar decrease in 'metabolic' ATP concentration (Holmsen *et al.*, 1972). These changes in ATP concentration are accompanied by the formation of actin bundles in the course of the aggregation reaction (Pribluda *et al.*, 1981).

The hypothesis can thus be made that, *in vivo*, Mg²⁺ and tropomyosin contribute to the rapid conversion of actin bundles into microfilaments.

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