

The distribution of cofilin and DNase I in vivo

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ABSTRACT

Actin is the principal component of the cytoskeleton, a structure that can be disassembled and reassembled in a matter of seconds in vivo. The state of assembly of actin in vivo is primarily regulated by one or more actin binding proteins (ABPs). Typically, the actions of ABPs have been studied one by one, however, we propose that multiple ABPs, acting cooperatively, may be involved in the control of actin filament length. Cofilin and DNase I are two ABPs that have previously been demonstrated to form a ternary complex with actin in vitro. This is the first report to demonstrate their co-localisation in vivo, and differences in their distributions. Our observations strongly suggest a physiological role for higher order complexes of actin in regulation of cytoskeletal assembly during processes such as cell division.

Key words: *actin, cofilin, DNase I, actin-binding protein, ternary complex.*

INTRODUCTION

Actin (42 kDa) is the principal component of the cytoskeleton. It consists of globular monomers (G-actin) that polymerise to form helically symmetrical filaments (F-actin). A dynamic cytoskeleton is important in numerous cellular activities ranging from maintenance of cell shape to phagocytosis and cytokinesis. This reversible assembly of actin is regulated by a large number of actin-binding proteins (ABPs) and with few exceptions, these have been studied individually rather than in combinations. ABPs influence the state of actin assembly and/or maintain the monomer pool above its critical concentration[1].

Binding an ABP may influence the interactions of actin with other ligands. For example the exchange

of the bound nucleotide of actin is promoted by profilin[2] and inhibited by cofilin[3] despite the fact that these ABPs bind to a nearly identical loci on actin[4]. Furthermore, cofilin allosterically inhibits the binding of phalloidin[5] and tropomyosin[6] suggesting that ABPs may also modulate the binding of other ABPs. This would allow finer control of actin assembly and facilitate subtle remodelling of the cytoskeleton.

Cofilin (19 kDa) is a principal player in regulating the dynamics of actin. Like most ABPs, cofilin binds to the barbed end of actin (subdomains 1 and 3). It is capable of regulating the average length of actin filaments by severing[7] or depolymerising in a pH sensitive manner[8]. The biological action of cofilin is regulated via phosphorylation[9] by LIM-kinases[10] and by the binding of phosphoinositide lipids[11]. In vivo cofilin is involved in cell division, actin treadmilling and recycling of older actin filaments.

DNase I (31 kDa) is a secreted enzyme involved in digestion of DNA and has been implicated in apoptosis[12]. Although not widely recognised as an

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ABP, Lazarides and Lindberg [13] were the first to suggest that DNase I may be a cytoskeletal protein. They proposed three possible biological roles: (1) actin controls nucleotide hydrolysis of DNase I during the cell cycle; (2) the actin-DNase I complex has a specific function in DNA metabolism; and (3) the primary function of DNase I is related to the formation and function of actin filaments rather than the degradation of DNA. The fact that phosphoinositides can dissociate DNase I from its complex with monomeric actin[11] also suggests this interaction may be biologically relevant.

DNase I binds stronger to monomeric actin (K_d in the nM range) than to F-actin ($K_d = 100 \mu M$) where it acts as a capping protein and increases the dissociation of subunits from the pointed ends of filaments[14]. Thus, unless the concentration of DNase I is high, it is unlikely to bind to actin filaments. *In*

in vivo, the enzymatic activity of DNase I is inhibited by the binding of G-actin. However unlike most ABPs, including cofilin, it binds to the region of actin oriented towards the pointed end of F-actin, specifically to subdomain 2 (the DNase I binding loop) and to subdomain 4 (Fig 1)[15].

We have previously reported that it is possible for both cofilin and DNase I to bind simultaneously to G-actin *in vitro* forming a cofilin-actin-DNase I ternary complex[16]. Furthermore, we have demonstrated that the binding between cofilin, actin and DNase I in the ternary complex *in vitro* is essentially (Ca^{2+} or Mg^{2+})[17]. Conversely, the cofilin-actin and actin-DNase I binary complexes are strongly influenced by buffer nucleotides and cations. The formation of the cofilin-actin-DNase I ternary complex has since been shown to be a cooperative process [18].

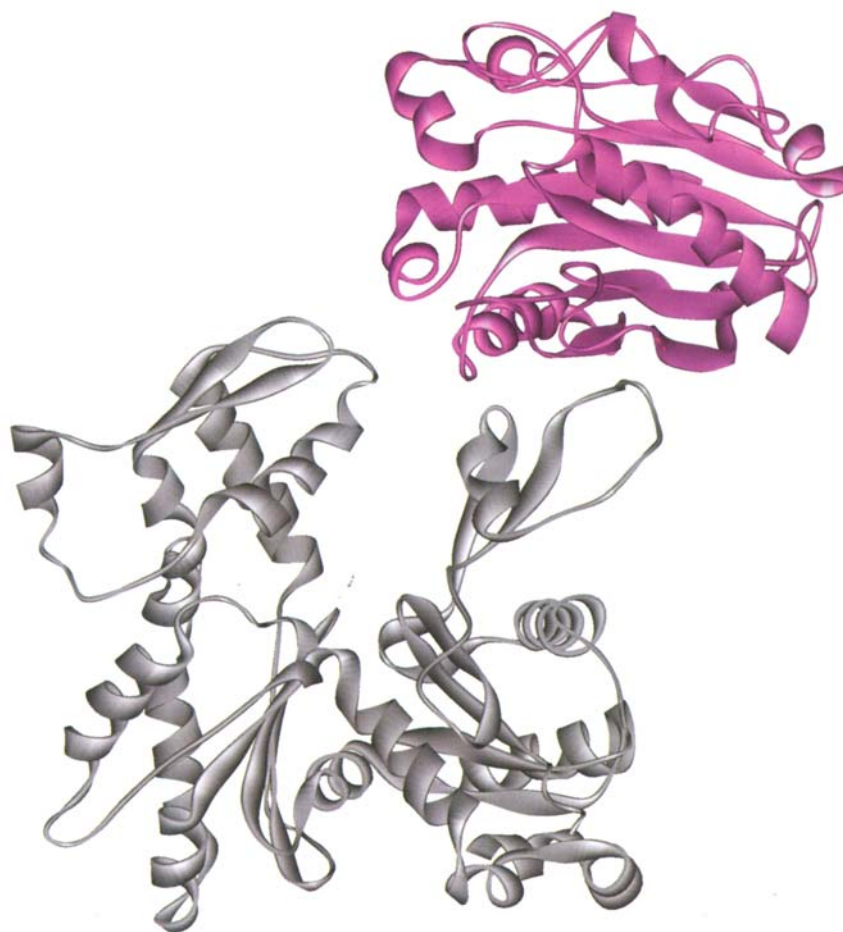


Fig 1. Crystal structure of actin-DNase I complex (PDB accession number, 1ATN)

DNase I (purple) interacts with loop on subdomain 2 of actin (grey)[15]. The underside of actin in this figure contains the binding site of a number of ABPs including cofilin.

A crucial factor in establishing the biological relevance of DNase I is the development of proof that the ternary complex actually occurs *in vivo*.

MATERIALS AND METHODS

Actin preparation

Actin was prepared from an acetone-dried powder of rabbit skeletal muscle according to the method of Spudich and Watt[19] with slight modifications as described in Barden and dos Remedios [20]. G-actin was snap frozen in liquid nitrogen in G buffer (2 mM Tris pH 8.0, 0.2 mM ATP, 0.1 mM CaCl₂) then freeze-dried and stored at -20°C. Monomeric actin concentration was determined from its OD₂₉₀, where $E^{0.1\%} = 0.63 \text{ cm}^{-1}$ [21].

Expression and purification of chicken cofilin

Recombinant chick embryonic skeletal muscle cofilin was expressed as a glutathione- S-transferase (GST) fusion protein. The clone for this fusion protein was a gift from Dr Takashi Obinata. Briefly, *E. Coli* transformants were grown at 37°C in LB medium (Gibco) containing 60 mg/mL ampicillin and induced with 1 mM isopropyl- β -D-thiogalactopyranoside when the cells were in log phase of growth (OD₆₀₀ \approx 0.6/cm). After 3 h growth, the cells were harvested by centrifugation and disrupted using a French press. After centrifugation, the supernatant was applied to a glutathione Sepharose 4B column (Pharmacia, Piscataway, NJ, USA) equilibrated with phosphate buffered saline (PBS, 0.35 M NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) containing 0.5 mM PMSF and 1 mM EDTA. The column was washed with 2 vol of thrombin buffer (50 mM Tris pH 8.0, 2.5 mM CaCl₂ and 50 mM NaCl). The matrix was suspended in 100 U of thrombin/L of culture and incubated at 37°C for 1 h with the matrix being mixed at 15 min intervals. Cofilin was eluted with 2-3 vol of thrombin buffer. The purified cofilin was dialysed overnight against 10 mM Pipes (pH 6.8) and further purified by application to a Mono-S-cation exchange column and eluted with a 0-0.5 M gradient of NaCl. The protein concentration was determined from the OD₂₈₀, where $E^{0.1\%} = 0.93 \text{ cm}^{-1}$ [22].

DNase I

DNase I (DPRF grade) was purchased from Worthington Biochemicals (Freehold, NJ, USA) and used without further purification. DNase I protein concentrations were determined from the OD₂₈₀, where $E^{0.1\%} = 1.1 \text{ cm}^{-1}$ [23].

Native polyacrylamide gel electrophoresis

An actin-DNase I complex was formed by incubation of DNase I (2.5 μ M) with excess actin (5.0 μ M). Increasing concentrations of cofilin (0.7, 1.4, 2.1, 2.8, 4.2 and 7.0 μ M) were then titrated into this actin-DNase I-actin solution. Samples were taken and analysed using native (non-denaturing) polyacrylamide gel electrophoresis (PAGE).

Native PAGE of samples was performed using a BioRad Mini-PROTEAN[®] II system (Richmond, CA, USA) and a discontinuous

Tris-glycine buffer system[24] with omission of SDS from all solutions. The stacking gel comprised 4% acrylamide in 80 mM Tris-glycine (pH 6.8) and the separating gel comprised 10% acrylamide in the same buffer adjusted to pH 8.8. Running buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.2 mM ATP, 0.2 mM CaCl₂). Samples were run at 120 V for 90 min at room temperature.

Since these gels were performed under non-denaturing conditions, proteins migrate according to their charge-density ratio rather than their apparent molecular weights as they do in SDS-PAGE gels. Non-covalent interactions between proteins are not disrupted under these conditions.

Gels were stained with 1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) ethanol, 7% (v/v) acetic acid and destained in 40% (v/v) ethanol and 7% (v/v) acetic acid.

Gels were scanned using a Molecular Dynamics scanning densitometer (Sunnyvale, CA, USA.) and Coomassie-stained protein bands were quantified by Image Quant 5.1 software (Molecular Dynamics Inc; Sunnyvale, CA, USA).

Distribution of cofilin and DNase I in vivo

The localisation of DNase I and cofilin proteins was examined by immunofluorescent labelling and confocal microscopy. Virulent monkey kidney epithelial cells (ACTC no. CRL1586) were suspended in 10% foetal calf serum (FCS) in RPMI-1640 medium (Sigma-Aldrich; St. Louis, MO, USA) and incubated overnight in a 35 ml culture flask under standard culture conditions (SCC, 37 °C in a humidified chamber atmosphere of 5% CO₂ and air). Following incubation, the supernatant was removed and the cells were washed three times in PBS and resuspended in T/E solution (0.5% w/v trypsin, 1 mM EDTA). The cells were centrifuged at 4000 \times g for 5 min and the pellet was washed in PBS and resuspended in 10% FCS in RPMI-1640 to a final density of 10⁶ cells/ml. Cells were subsequently seeded on 18 mm glass coverslips and incubated overnight under SCC. Cells were then washed three times in PBS, fixed in 3% paraformaldehyde in PBS for 20 min at room temperature and permeabilised in cold acetone at -20°C for 5 min. Cells were then washed and incubated in 10% FCS in RPMI-1640 for 45 minutes for blocking.

DNase I was labelled by incubating with a 1 in 250 dilution of rabbit anti-DNase I antibody (Sigma-Aldrich, St. Louis, MO, USA) for 45 min proceeded by a 1 in 200 dilution of a sheep anti-rabbit antibody conjugated with FITC (SAR-FITC) for 1 h. Cofilin was labelled by incubating with a 1 in 200 dilution of rabbit anti-cofilin antibody (Cytoskeleton, Denver, CO, USA) for 45 min proceeded by a 1 in 200 dilution of a donkey anti-rabbit antibody-biotin conjugate (DAR-B) for 30 min and a 1 in 200 dilution of streptavidin-Cy 3 conjugate for 30 min. Cells were washed and blocked in RPMI-1640 supplemented with 10 % FCS for 45 min between each step. To eliminate cross-reactivity between the secondary antibodies, excess SAR-FITC was incubated for 1 h at 37 °C to saturate all the binding sites on the DNase I primary antibody. Subsequently, rabbit anti-cofilin antibody was incubated and DAR-B labelling was applied. Omission of either primary antibody or replacement with an isotype control antibody resulted in abolishment of yellow co-localisation regions (data not shown) support-

ing the assumption that our antibody staining is specific for the corresponding antigen. The specificity of the antibody labelling is also consistent with the different distributions of the two ABPs.

The cells were viewed under a Leica TCS NT confocal microscope (Heidelberg, Germany) with bandpass filters centred at 525 nm and 590 nm in order to spectrally isolate the FITC and Cy3 probes respectively. Approximately 1 mm optical sections were taken in the vertical axis. Layered images were analysed using Adobe Photoshop 6.0 (Adobe Systems Japan; Tokyo, Japan) to demonstrate the simultaneous distribution of cofilin and DNase I and the distribution of co-localised cofilin and DNase I. The amount of labelled protein and co-localised molecules was quantified by Image Pro-Plus 4.5 (Scitech, Vic, Australia).

RESULTS

Native polyacrylamide gel electrophoresis

Fig 2 shows a Coomassie-stained native polyacrylamide gel of cofilin titrated into a mixture of actin-DNase I (2.5 μM) and free actin (2.5 μM) annotated by the volume densities of each band below. The concentration of cofilin in each lane (numbered 1-7 below the gel) is indicated above the respective lane. The identities of protein bands are shown adjacent to the relevant band.

Lanes 2-4 demonstrates the preferential binding of cofilin to actin-DNase complex rather than to free actin. Addition of increasing concentrations of cofilin to a mixture of actin-DNase I complex and

free actin results in increasing quantities of cofilin-actin-DNase I ternary complex coupled to decrease in the volume density of the actin-DNase I band. Conversely, the volume density of actin remains approximately constant. In lanes 5-7 no actin-DNase I complex is present because it has been converted to cofilin-actin-DNase I ternary complex. At this point cofilin binds to free actin, with increased volume density of cofilin-actin band coupled to a decreased volume density of the free actin.

Distribution of cofilin and DNase I in vivo

Fig 3A shows a phase-contrast presentation of a representative quiescent (above) and dividing cell (below). There is a prominent cleavage furrow within the dividing cell. The nuclei are identified as “empty” regions within the cells (upper border of the quiescent cell; binucleated nucleus in the dividing cell).

Fig 3B illustrates the distribution of the FITC probe, thereby demonstrating the distribution of intracellular DNase I (green). DNase I is seen diffusely throughout the cells but is greatly reduced or absent from the regions corresponding to the nuclei. The distribution in the cytoplasm is discrete rather than evenly dispersed and there appears to be no preferential localisation of DNase I in any specific region of the cytoplasm.

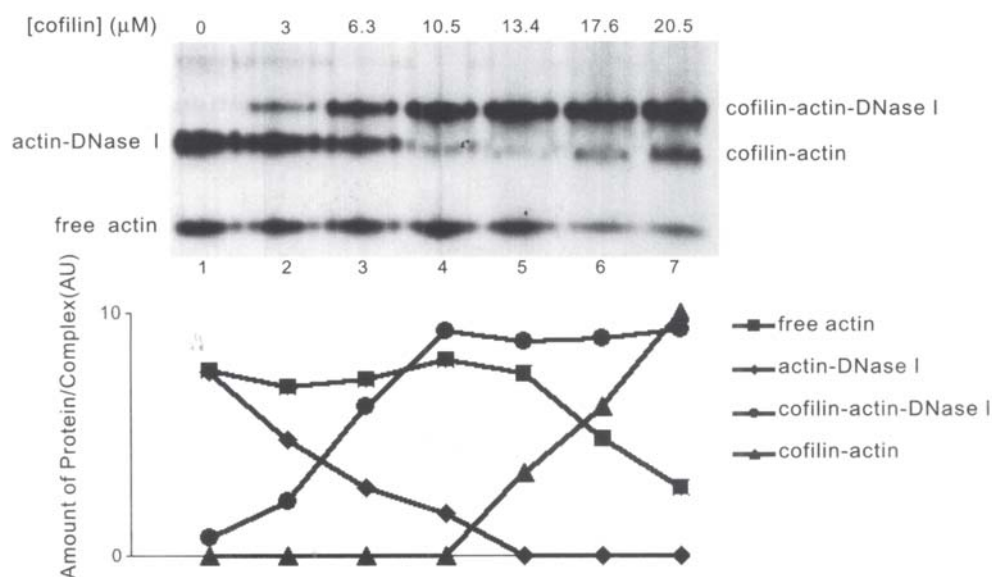


Fig 2. A native polyacrylamide gel shows the effect of titrating cofilin into a mixture of actin-DNase I complex and free actin. Lanes 1-7 contain a mixture of 8 μM DNase I and 14 μM actin and increasing concentrations of cofilin (0, 0.7, 1.4, 2.1, 2.8, 4.2 and 7.0 μM respectively).

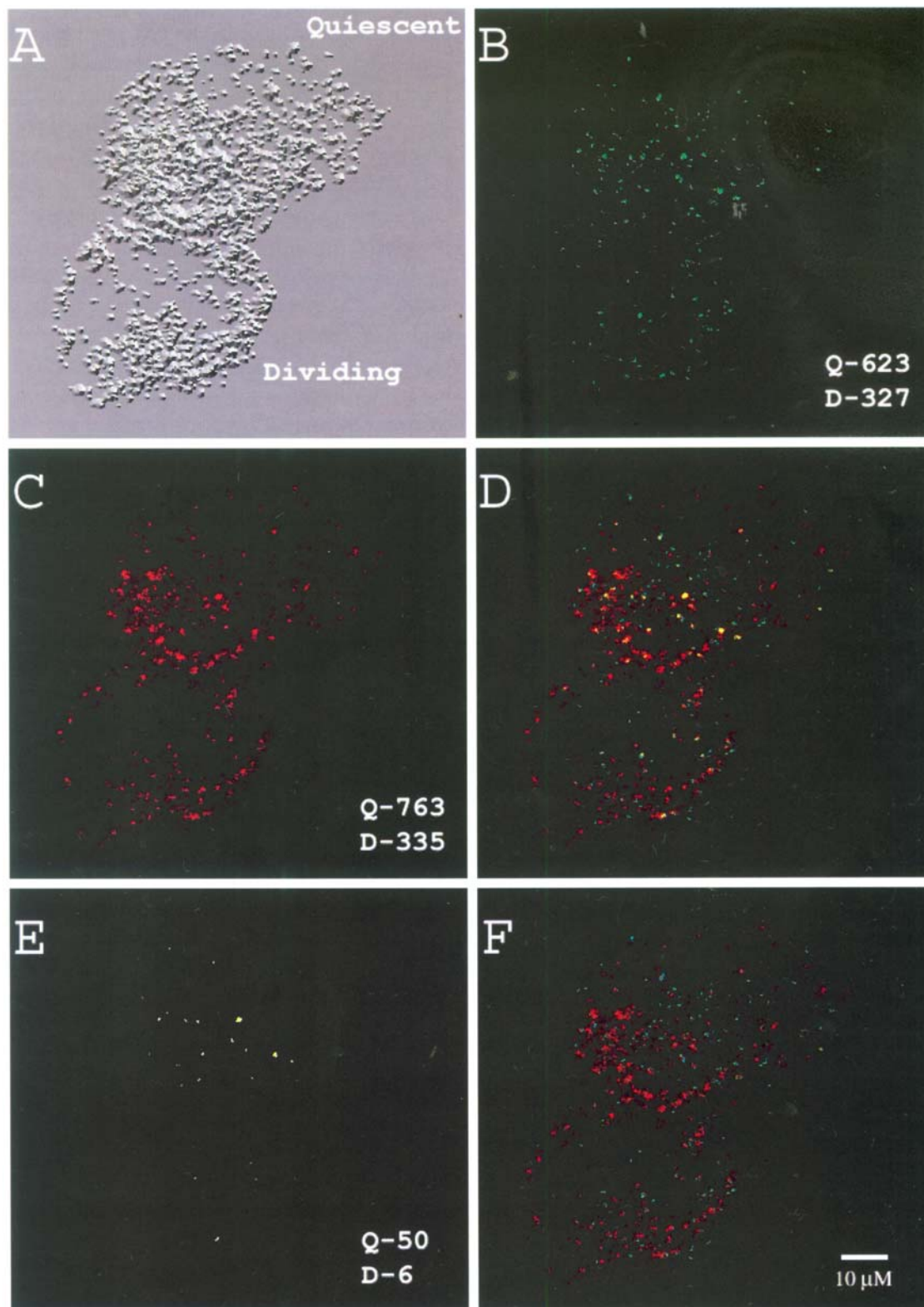


Fig 3. Distributions of intracellular DNase I (green) and cofilin (red) seen under a confocal microscope. The FITC and Cy3 probes were spectrally isolated by setting bandpass filters at 525 nm and 590 nm respectively. (A) Phase contrast image of representative cells (B) distribution of DNase I only; (C) distribution of cofilin only; (D) simultaneous visualisation of cofilin and DNase I; (E) distribution of co-localised cofilin and DNase I, and; (F) distribution of cofilin and DNase I not associated with each other. The number of labelled protein is quantified in image units for the quiescent (Q) and dividing cell (D).

Fig 3C illustrates the distribution of the Cy3 probe, thereby demonstrating the distribution of intracellular cofilin (red). It appears to preferentially aggregate at the periphery of the dividing cell. It also appears to be concentrated at the junction between the upper cell and lower dividing cell. There is a marked decrease in its nuclear localisation.

Fig 3D shows the overlapped signals from the FITC and Cy 3. Regions of co-localisation of these two probes appear as yellow spots. The co-localised signal has been optically isolated and are displayed in Figure 3E. This suggests there is a co-distribution of a cofilin-actin-DNase I ternary complex (yellow). The yellow spots appear to be co-incident with the largest cofilin aggregates. No overlap is seen in the regions corresponding to the nuclei, nor is there significant overlap in the cortical regions corresponding to high concentrations of cofilin. For comparison, the signals from probes that do not overlap are displayed in Fig 3F.

DISCUSSION

Regulation of the cytoskeleton is complex and it is likely that it requires more than one ABP to regulate actin assembly. Formation of a cofilin-actin-DNase I ternary complex has been demonstrated *in vitro* using both native (non-denaturing) PAGE gels [17] and Phast gels[18]. These reports determined that cofilin and DNase I bind cooperatively to actin forming a stable complex that is fairly insensitive to buffer conditions. The cooperative binding of cofilin to actin-DNase I demonstrated by Nosworthy et al has been confirmed quantitatively in this report. However this is the first report to provide evidence for a simultaneous distribution of cofilin and DNase I with actin *in vivo*. It is interesting to note that although the ratio of labelled cofilin to DNase I is approximately 1, their distributions differ markedly.

Cofilin has previously been reported as being diffusely distributed throughout the cell, with increased localisation at regions where the cytoskeleton is highly dynamic. This includes the leading edge of Lamellipodia[25] and the cleavage furrow in dividing cells[26], [27]. These previous findings are consistent with our results. We found cofilin localised near the cell periphery in the dividing cell and scattered throughout the cytoplasm but excluded from the nucleus. *In vivo*, cofilin functions to rapidly dis-

assemble actin near the cell periphery facilitating changes in cell shape during cell division. Cofilin binds to ADP-actin subunits in F-actin and promotes dissociation at the pointed ends of these filaments deeper in the cortex. They remain complexed to ADP-actin monomers until the actin-bound nucleotide is exchanged for ATP under the influence of profilin.

The presence of cofilin near the border between associated cells may be explained by its role in cytokinesis from which these two cells formed. The cleavage furrow appears within a dividing cell during cytokinesis as a result of the constriction of the contractile ring. This contractile ring is formed by a bundle of approximately 20 actin filaments, which gradually constrict by up to 90%[28]. Although the actin filaments at the cleavage furrow gradually shorten, the total number of filaments remains constant. Cofilin severs actin and is involved in bringing about the gradual constriction of the F-actin contractile ring at the cleavage furrow.

DNase I appears to be expressed in low concentrations and distributed mainly throughout the cytoplasm. It is principally known for its ability to hydrolyse DNA and is particularly active during the execution phase of apoptosis. It has previously been reported that the expression of DNase I is increased prior to the induction of apoptosis, with higher concentrations in the perinuclear space[29]. This is particularly true for cell types displaying rapid turnover rates, and ensures that the actual apoptotic event is rapid and efficient. Rapidly dividing cells exhibit low rates of apoptosis and so the virulent nature of these cells may explain the low DNase I staining with no particular areas of localisation.

In this report, the presence of an overlapped signal from the FITC and Cy3 probes indicates the co-localisation of DNase I and cofilin. Since we have previously reported that cofilin and DNase I do not bind to each other, we conclude that where ever they co-localise, they must be linked via actin. This can occur *in vivo* either due to the coincidence of a cofilin-actin and actin-DNase I and/or the existence of cofilin-actin-DNase I ternary complex. Similar techniques have been used previously to demonstrate interactions between actin and myosin[30] and the formation of complexes between the ABPs a-actinin and Nsp11[31] as well as between insulin and its associated receptor[32].

Our native-PAGE data suggest that cofilin and DNase I bind more tightly to actin- DNase I and cofilin-actin binary complexes respectively than for actin alone. In other words, the ternary complex is more stable than either of the binary complexes. It is therefore noteworthy that in the dividing cell, there is relatively little co-localised cofilin and DNase I. Conversely the quiescent cell has a higher proportion of DNase I sequestered in a ternary complex. It is likely that the remainder of the labelled protein is associated with actin suggesting the action of an intracellular mechanism that allows the binary and ternary complexes to co-exist within the cell. The incidence of co-localisation is 6-fold higher in the non-dividing cell than in the dividing cell. The differences in co-localisation between the quiescent and dividing cells implies that DNase I may modulate the regulatory activity of cofilin during cell division.

Not only may cofilin and DNase I influence the binding to actin of each other, they may influence the binding of other ABPs, such as thymosin β 4. We know that cofilin and thymosin β 4 compete for binding to actin (Irina Dedova, personal communication). DNase I binding to actin inhibits the binding of thymosin β 4 [33] and in this case DNase I may be a mediator in the exchange of bound thymosin β 4 on actin for cofilin. This process is outlined in Fig 4. We have demonstrated that the cofilin-actin-DNase I ternary complex is more stable than either binary complex. The mechanism of dissociation of DNase I from the ternary complex has not yet been determined, but probably involves phosphorylation or the binding of phosphoinositides [11].

DNase I affinity columns are widely used in the preparation of a number of muscle proteins including villin, actolinkin, destrin, actoseverin and radixin, which implies that these proteins also form ternary complexes involving actin-DNase I. Cofilin has also been shown to bind actin simultaneously with actin-interacting protein (Aip1). This coordinated binding of multiple ABPs to actin would allow finer control of the state of actin assembly.

In this report we have presented the first evidence supporting the co-existence of a cofilin-actin-DNase I ternary complex with the cofilin-actin and actin-DNase I binary complexes *in vivo*. We propose that DNase I may be important in modulating the binding of other ABPs, e.g. cofilin and thymosin β 4.

This hypothesis is currently under investigation *in vivo*. Our observations introduce further regulatory aspects of ABP cascades in the control of cytoskeletal dynamics.

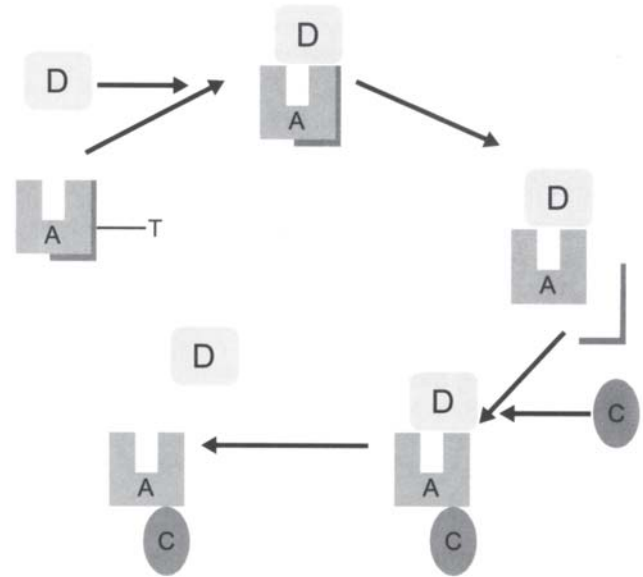


Fig 4. Illustration of the DNase I mediated exchange of thymosin β 4 for cofilin on actin. A-actin; T-thymosin β ; D-DNase I; C-cofilin.

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