Control of Actin Dynamics by Proteins Made of β-Thymosin Repeats

THE ACTOBINDIN FAMILY*§

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Actobindin is an actin-binding protein from amoeba, which consists of two β-thymosin repeats and has been shown to inhibit actin polymerization by sequestering G-actin and by stabilizing actin dimers. Here we show that actobindin has the same biochemical properties as the Drosophila or Caenorhabditis elegans homologous protein that consists of three β-thymosin repeats. These proteins define a new family of actin-binding proteins. They bind G-actin in a 1:1 complex with thermodynamic and kinetic parameters similar to β-thymosins. Like β-thymosins, they slow down nucleotide exchange on G-actin and make a ternary complex with G-actin and Latrunculin A. On the other hand, they behave as functional homologs of profilin because their complex with MgATP-G-actin, unlike β-thymosin-actin, participates in filament barbed end growth, like profilin-actin complex. Therefore these proteins play an active role in actin-based motility processes. In addition, proteins of the actobindin family interact with the pointed end of actin filaments and inhibit pointed end growth, maybe via the interaction of the β-thymosin repeats with two terminal subunits.

Proteins of the β-thymosin family are small (5 kDa) peptides that act as G-actin sequestering factors, because they bind ATP-G-actin in a 1:1 complex that is unable to polymerize into filaments (1–5). In vivo, these proteins build a reservoir of unassembled (monomeric) actin (6–8). The interaction of thymosin β4, the major variant, and thymosin β10, a minor variant, with actin has been thoroughly analyzed (3, 9–14). Because β-thymosins are simple passive sequesters in rapid equilibrium with ATP-G-actin, the balance between polymerized (F-actin) and nonassembled actin in living cells is in fact controlled by regulatory proteins that affect the steady-state concentration of free ATP-G-actin by modulating the dynamic parameters of filament assembly-disassembly at the barbed and pointed ends. The amount of sequestered actin is increased by proteins that cause an increase in steady-state ATP-G-actin and vice versa. For instance, profilin makes a complex with G-actin that energetically contributes to monomer-polymer exchanges at the barbed ends as well as ATP-G-actin itself. As a result, profilin causes a decrease in the partial critical concentration of ATP-G-actin. Therefore, upon addition of profilin to a solution of F-actin in the presence of Tβ4, the amount of Tβ4-actin complex decreases, i.e. F-actin increases (15). Conversely, addition of actin depolymerizing factor (ADF), which increases the concentration of ATP-G-actin at steady state, leads to increased sequestration of G-actin by Tβ4, i.e. causes F-actin disassembly (16).

The structure of the Tβ4-actin complex is not known at atomic resolution; however, NMR and biochemical studies consistently show that Tβ4 binds actin in an extended configuration, the N-terminal segment interacting with the barbed end of the actin monomer, while the C-terminal region binds subdomain 2 at the pointed end of the actin monomer (13, 14). This view satisfactorily accounts for the fact that Tβ4 inhibits G-actin association with both the barbed and the pointed ends of actin filaments.

β-Thymosins have been found in all vertebrates and in echinoderms and mollusks (17, 18). Recently, a cDNA clone encoding for a 41-amino acid β-thymosin has been identified in the calcareous sponge Scyon raphanus (19), indicating the ancient character of β-thymosin among metazoa. On the other hand, β-thymosins are absent in yeast, amoeba, Drosophila, and plants. In Acanthamoeba castellanii, a protein called actobindin consists of two β-thymosin repeats and has been identified as a G-actin-binding protein that may also sequester actin dimers (20–24). A BLAST search on the complete genomic sequences of Drosophila melanogaster and Caenorhabditis elegans identified a triplicate β-thymosin sequence (19). Independently, this 3-β-thymosin repeat protein, called ciboulot (Cib), has been identified in Drosophila as being involved in the control of brain development during metamorphosis and characterized as a G-actin-binding protein (25). Amazingly, although ciboulot shares sequence homology with Tβ4, the Cib-actin complex participates in filament barbed end assembly like profilin-actin (25). The profilin-like property of Cib accounts for its function in actin-based motility and axonal growth. These observations raise an issue of structural and functional relevance about the evolution of the actin binding domain of β-thymosins. Are some biochemical properties of β-thymosins found unaltered in actobindin and Cib? What are the structural features at the origin of the functional difference between Tβ4 and Cib? Is actobindin, the amoeba β-thymosin two-repeat protein, functionally similar to Tβ4 or to Cib?

Here we compare Cib and actobindin regarding their binding to G-actin, their effects on nucleotide exchange on G-actin, and their effects on actin assembly at the two ends of filaments, with either MgATP-actin or CaATP-actin. We show that actobindin is functionally similar to Cib or profilin, and differs from β-thymosins, regarding the control of filament dynamics. On the other hand, actobindin and Cib both slow down nucleotide...
exchange on G-actin like Tβ4. We conclude that proteins that consist of β-thymosin repeats like actobindin in Acanthamoeba or ciboulot in Drosophila and probably the C. elegans homolog must play a positive role in motile properties of living organisms. The pure G-actin sequestering function of regular β-thy-
mosins may result from divergent evolution.

**MATERIALS AND METHODS**

**Proteins—**Actin was purified from rabbit muscle, isolated as CaATP-
G-actin by Sephadex G 200 chromatography in G buffer (5 mM Tris-Cl, pH 7.8, 0.2 mM ATP, 1 mM diethiothreitol, 0.01% NaN₃). Actin was pyrenyl labeled on cysteine 374 (29) and NBD-labeled on lysine 373 (27). Gelsolin was a kind gift from Dr. Yukio Doi (University of Kyoto, Japan) and actobindin was purified from *Acanthamoeba castel-
lanii* (20). Thymosin β4 and profilin were prepared as described previ-
ously (15). The fusion protein GST-Cib cloned in the expression vector p-GEX2T* (Amersham Bioscience) was induced in *Escherichia coli* strain BL21 and purified (15). The Cib protein was then cleaved off the GST moiety with thrombin, dialyzed against 20 mM Tris-Cl, 1 mM diethiothreitol, pH 7.5, and stored at −80 °C. The concentration of Cib was derived from amino acid analysis, from which a standardized bicinechonic acid assay was developed.

**Actin Polymerization Measurements—**Steady-state measurements of F-actin were derived from fluorescence measurements of pyrene-labeled actin. Actin (10% pyrenyl-labeled) was polymerized under physiological ionic conditions (0.1 mM KCl, 1 mM MgCl₂) in the absence or presence of gelsolin (at 1:300 molar ratio to actin) and in the presence or absence of Cib or actobindin at the indicated concentrations. The value of the equilibrium dissociation constant *Kc* for the Cib-actin (or actobindin-actin) complex [CA] was derived from measurements of the amount of assembled actin at steady state (after 18-h incubation) (see the following equations),

\[
\frac{[A]}{[CA]} = \frac{[A]}{[A]_0} + \frac{1}{[CA]_0} [CA] + \frac{1}{[CA]_0} [C] + \frac{[C]}{[CA]_0} [CA]
\]

where 

\[
[A]_0 \text{ and } [C]_0 \text{ are the concentrations of unassembled actin and free G-actin at steady state and } [C]_0 \text{ is the total concentration of acto-
bindin or Cib.}
\]

Initial rates of filament growth from the barbed and the pointed ends were measured spectrofluorometrically using either spectrin-actin or actobindin shuttle from one molecule of G-actin to the other at a faster rate than nucleotide dissociates from G-actin or from Cib-actin or acto-
bindin-actin complexes. The equilibrium dissociation constant for the

Cib-actin or actobindin-actin complex was derived from the dependence of the apparent exchange rate constant on the concentration of Cib or actobindin, as follows,

\[
[A] + [CA] = [A]_0 \\
[C] + [CA] = [C]_0 \\
(k_{\text{obs}} = (k_1[A] + k_2[CA][A]_0) + [A]/[K_c] \text{ (Eq. 2)}
\]

with

\[
[A] = (1/2)[[A]_0 - [C]_0 + K_c \pm \sqrt{([A]_0 - [C]_0 - K_c)^2 + 4K_c[A]_0}/2K_c
\]

\[
(C_0 + [C]_0) \text{ (Eq. 3)}
\]

\[
(C_0 + [C]_0) \text{ (Eq. 3)}
\]

\[
(C_0 + [C]_0) \text{ (Eq. 4)}
\]

where \([A], [A]_0, [C], [C]_0\) are the free and total concentrations of G-actin and Cib or actobindin and [CA] is the concentration of the complex; \(k_1\) and \(k_2\) are the rate constants for nucleotide dissociation from G-actin and from the CA complex, respectively; \(K_c\) is the equilibrium dissociation constant of the CA complex. The values of \(k_1\) and \(k_2\) were deter-
mined experimentally in the absence and in the presence of saturating amounts of Cib. The value of \(K_c\) was derived from the adjustment of the calculated curves \(k_{\text{obs}}(IC_{50})\) to the data.

**Equilibrium and Kinetic Measurements of the Interaction of Cib with G-actin—**The change in fluorescence of NBD-labeled G-actin was used as a probe for the formation of the complexes of G-actin with Tβ4, actobindin, or Cib. Static fluorescence measurements were carried out in a Spex Fluorolog 2 spectrofluorimeter in G buffer for CaATP-G-actin and in G buffer supplemented with 10 μM MgCl₂ and 0.2 mM EGTA for MgATP-G-actin. Samples contained 1.5 μM NBD-G-actin and different amounts of Cib. Excitation and emission wavelengths were 475 nm and 525 nm, respectively. The equilibrium dissociation constant for the complex was derived from the dependence of the fluorescence change on the total concentration of Tβ4, actobindin, or Cib, analyzed using Equa-

**RESULTS**

**Direct Binding and Kinetics of Interaction of Cib and Acto-
bindin with G-actin—**To evaluate the thermodynamic and rate parame-
ters of the interaction of Cib and of actobindin with G-actin, we sought suitable spectroscopic probes. While binding of Tβ4 to G-actin is accompanied by a 20% increase (11), and binding of profilin by a 25% decrease (30) in tryptophane fluo-
rescence of actin, no change was observed with Cib. Tβ4 also causes a large change in the fluorescence of AEDANS-labeled actin (14). Binding of Cib to AEDANS-G-actin caused a 7-nm blue shift in the excitation and emission spectra and a 5% increase in fluorescence (λem = 430 nm, λex = 480 nm), sug-
gest a less polar environment of the AEDANS fluorophore in the Cib-actin complex than in G-actin (an effect conspicuously opposite to the one observed (14) with Tβ4), but the signal was too small to be useful in kinetic experiments. Like Tβ4, neither actobindin nor Cib affected the fluorescence of pyrene-labeled G-actin, in agreement with previous reports (22, 25). On the other hand, the fluorescence of actin in which cysteine 374 was NBD-labeled was increased by 25% upon binding of Cib, acto-
bindin, or Tβ4 (Fig. 1). A similar change has been reported when actobindin bound to actin to which cysteine 374 was labeled by IANBD (22). The equilibrium dissociation constants \(K_c\) of the 1:1 complexes of G-actin with Tβ4, Cib, and acto-
bindin were derived from the analysis of the dependence of the fluorescence change on protein concentration according to Equation 4 (Fig. 1). Similar values of \(K_c\) were obtained for all

1 The abbreviations used are: NBD, 12-(N-methyl-N-(7-nitrobenz-2-
oxo-1,3-diazole-4-yl)-1-acetamido)ethylamino)naphthalene-1-sulfonic acid; IANBD, N-(2-iodoacetotio
ethyl)-N-methylamino-7-nitrobenz-2-oxo-1,3-diazole.
Depolymerization at the presence of latrunculin A. The change in fluorescence $\Delta F$ of NBD-G-actin (1.5 $\mu M$, in G buffer) was measured at the indicated concentrations of T4 or Cib, in the absence (closed circles) and in the presence (open circles) of 20 $\mu M$ latrunculin A. The fluorescence of NBD-G-actin was taken as 1 arbitrarily. It was increased to a value of 1.04 in the presence of latrunculin A.

**TABLE I**

**Compared equilibrium parameters for binding of T4, actobindin, and ciboulot to G-actin using different methods**

Values of $K_c$ derived from the change in NBD-actin fluorescence and from the decrease in the rate of nucleotide exchange were obtained in low ionic strength G buffer. Values derived from steady-state F-actin measurements were obtained in F buffer (0.1 M KCl). ND, not determined.

| Method                        | G-actin species          | $K_c$ (uM) |
|-------------------------------|--------------------------|------------|
| Change in NBD-actin fluorescence | CaATP-G-actin            | 2.5        |
|                               | LatA-CaATP-G-actin       | 44         |
|                               | MgATP-G-actin            | 0.65       |
|                               | LatA-MgATP-G-actin       | 1.2        |
| Nucleotide exchange           | CaATP-G-actin            | 1          |
| Depolymerization at barbed ends | CaATP-G-actin            | 9          |
|                               | CaATP-G-actin            | 9          |
|                               | MgATP-G-actin            | 2.0        |

proteins, typically 2–4 $\mu M$ for CaATP-G-actin and 0.7–2 $\mu M$ for MgATP-G-actin (Table I).

Latrunculin A is a drug that interacts with G-actin with high affinity ($5 \mu M^{-1}$) and prevents polymerization (31). Latrunculin A has been shown to noncompetitively inhibit T4 binding to G-actin, decreasing the affinity of actin for T4 by approximately an order of magnitude in the ternary complex (32). The present data show that in the presence of saturating (20 $\mu M$) concentrations of latrunculin A, the change in fluorescence of NBD-actin (1.5 $\mu M$) upon binding Cib or actobindin or T4 was lower. The value of $K_c$ for binding T4 or Cib or actobindin to NBD-G-actin was increased about 2-fold by latrunculin A (Table I). The data are consistent with the formation of a ternary complex between G-actin, latrunculin A, and either Cib or actobindin or T4.

No binding of Cib or actobindin to ADP-actin was detected using the change in NBD-fluorescence (data not shown). It is known that the fluorescence of NBD-actin, in contrast to pyrenyl-actin, is not affected by the bound nucleotide (33). In conclusion, like T4 and profilin, Cib has a high specificity for binding ATP-G-actin.

The change in NBD fluorescence was used to monitor the kinetics of Cib-actin complex formation. Cib bound to NBD-G-actin within a single exponential process. The apparent first order rate constant $k_{obs}$ increased practically linearly with Cib concentration in the range 0–40 $\mu M$ Cib (Fig. 1). The apparent association rate constant ($k_a = 1.6 \times 10^3 s^{-1}$) was derived from the slope, and the apparent dissociation rate constant ($k_d = 14 s^{-1}$) was derived from the lower limit of $k_{obs}$ at low Cib concentration. The value of the ratio $k_a/k_d$ (3 $\mu M$) was in good agreement with the equilibrium dissociation constant derived from the dependence of the fluorescence change on Cib concentration. The value of 14 $s^{-1}$ for $k_d$ was then confirmed by a competition experiment in which Cib was displaced from the Cib-NBD-actin complex by a 10-fold excess of unlabeled actin.

To verify that the affinity of Cib or actobindin is not affected by NBD labeling, the following experiment was carried out. Increasing amounts of actobindin or Cib were added to two parallel samples of actin, containing either 10 or 50% NBD-actin, polymerized at 1.6 $\mu M$ in the presence of 4 nM gelsolin. The linear decrease in the amount of F-actin was consistent with the same affinity of Cib or actobindin for G-actin independently of the proportion of labeled actin (supplementary data, Fig. 1).

**Actobindin and Cib Slow Down Nucleotide Exchange on G-actin**—Thymosin $\beta 4$ is known to slow down nucleotide dissociation from G-actin, while profilin accelerates it. It has often been proposed that the effect of profilin on nucleotide exchange supports its effect on motility in vivo. Both actobindin and Cib, which enhance actin-based motility like profilin in an in vitro reconstituted motility assay (25), slow down nucleotide dissociation from G-actin, like T4 (Fig. 3). The exchange rate was decreased 12- and 9-fold by actobindin and by Cib, respectively, while it was decreased 20-fold by T4 under the same conditions. Analysis of the data using Equation 2 yielded values of 2.0 $\mu M$ and 1.7 $\mu M$ for the equilibrium dissociation constants for binding of actobindin and Cib, respectively, to CaATP-G-actin in G buffer.

This result indicates that Cib and actobindin share some of the binding features of T4. Their association with G-actin is linked to the slower dissociation of ATP. However, the effects of these two proteins on the dynamics of actin filaments are independent of their effects on nucleotide exchange on G-actin.

**The Actobindin-Actin Complex Participates in Barbed End Assembly of MgATP-actin, Like Cib-Actin and Profilin-Actin**—The effect of actobindin on the steady-state amount of 10% pyrenyl-labeled F-actin was measured under physiological conditions (0.1 M KCl, 1 mM MgCl$_2$) in the absence and in the presence of gelsolin (Fig. 4). When the barbed ends of filaments...
were capped, actobindin caused depolymerization of F-actin. The amount of depolymerized actin increased linearly with the concentration of actobindin, consistent with sequestration of MgATP-G-actin by actobindin in a 1:1 complex, with an equilibrium dissociation constant \( K_C \) of 6 \( \mu \)M. This value is in good agreement with previous measurements (22–24), as well as with the value derived from nucleotide exchange kinetics (Fig. 3). A similar value (\( K_C = 2.5 \mu \)M) had been found for the Cib-actin complex using the same assay (25). In contrast, actobindin did not depolymerize F-actin when barbed ends were free. The very slow decrease in F-actin versus actobindin concentration is consistent with the lowering of the steady-state concentration of G-actin upon addition of actobindin. As an example, at 20 \( \mu \)M actobindin, the measured concentration of unassembled actin was 0.15 \( \mu \)M (Fig. 4). Using Equation 1 with \( [C_0] = 20 \mu \)M, \( [A_0] = 0.15 \mu \)M, and \( K_C = 5 \mu \)M, the value of \( C_C \) (free G-actin) is 0.03 \( \mu \)M, much lower than the value of 0.1 \( \mu \)M measured in the absence of actobindin. This result implies that actin-actobindin complex, like profilin-actin complex, participates in barbed end assembly as well as actin itself, hence it lowers the energetic contribution of G-actin to monomer-polymer exchanges at steady state. In conclusion, actobindin-actin complex, like Cib-actin and profilin-actin, can stabilize the barbed ends, via monomer-polymer exchange reactions, as efficiently as G-actin.

The validity of the above conclusions relies on the assumption that the affinity of actobindin for actin is not affected by pyrenyl labeling. This was first established by Lambooy and Korn (20). We have confirmed this conclusion both for Cib and actobindin by checking that the value of \( K_C \) derived from assays shown in Fig. 4 was independent of the proportion of pyrenyl-actin.

In agreement with the above data, actobindin failed to com-

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**Fig. 2.** Kinetics of interaction of NBD-labeled actin with Cib. A, stopped-flow fluorescence traces recorded upon mixing 1.5 \( \mu \)M NBD-G-actin with Cib at the following concentrations: 3, 10, and 38 \( \mu \)M (bottom to top curves). Time is expressed in milliseconds (ms). Noisy curves, experimental data; smooth curves, monoexponential best fits. B, replot of the extent of fluorescence change at different Cib concentrations. The curve is calculated using Equation 1. C, change in \( k_{obs} \) versus total concentration of Cib.

**Fig. 3.** Actobindin and Cib slow down nucleotide exchange on G-actin. ATP-G-actin 1:1 complex (1 \( \mu \)M, in G_0 buffer containing 20 \( \mu \)M CaCl_2) was supplemented with Cib (A) or actobindin (B) at the indicated concentrations. The dissociation of bound ATP was monitored by adding 5 \( \mu \)M e-ATP at time 0 and recording the subsequent increase in fluorescence of e-ATP. The pseudo-first order exchange rate constant is plotted versus the total concentration of actobindin or Cib. The curves are calculated using Equation 2 and values of 2 and 1.7 \( \mu \)M for the equilibrium dissociation constants for the actobindin-actin complex and Cib-actin complex, respectively.

Actobindin sequesters MgATP-G-actin only when barbed ends are capped. Actobindin was added at the indicated concentrations to F-actin (1.6 μM, 10% pyrenyl-labeled), assembled in the absence (open circles) and in the presence (closed circles) of gelsolin at 1:300 molar ratio to actin. The data obtained in the presence of gelsolin are consistent with the formation of a nonpolymerizable actin-actobindin 1:1 complex with an equilibrium dissociation constant $K_0$ of 5 μM. The dashed line is calculated assuming sequestration at the barbed ends with $K_0 = 5$ μM and a steady-state concentration of ATP-G-actin of 0.1 μM. Inset, the actin-actobindin complex productively associates with barbed ends. The rate of barbed end growth was measured using spectrin-actin seeds (see “Materials and Methods”) and 2.5 μM pyrenyl-actin. Values of the rate were identical at 2.5, 10, and 15 μM actobindin.

Actobindin and Cib with Filament Pointed Ends Blocks Pointed End Growth but Not Depolymerization—The steady-state measurements displayed in Fig. 1 show that actobindin and Cib form a 1:1 complex with MgATP-G-actin that does not participate in pointed end filament assembly. Formation of this complex is expected to cause inhibition of filament pointed end elongation from G-actin subunits. Seeded growth assays using gelsolin-actin seeds were carried out at two different concentrations of G-actin. The decrease in the initial rate of elongation off gelsolin-actin seeds upon addition of actobindin should reflect saturation of G-actin by actobindin. Hence more actobindin should be needed to saturate a higher amount of G-actin. Typically, since the pointed end critical concentration is 0.5 μM, the rate of growth is expected to reach zero when 1.5 μM complex is formed at 2 μM actin and when 4.5 μM complex is formed at 5 μM actin. 50% inhibition of elongation should therefore be reached at an actobindin concentration $A_{50}\% = 3.75$ μM at 2 μM actin and $A_{50}\% = 6.34$ μM at 5 μM actin (using the law of mass action with actin-actobindin complex = 1/2(total actin - 0.5 μM) and $K_0 = 5$ μM). In the case of Cib, 50% inhibition of filament growth should be achieved by addition of 2.25 μM Cib at 2 μM actin and 4.3 μM Cib at 5 μM actin. The experimental data differed from the expected behavior. Elongation of filaments off gelsolin-actin seeds was inhibited by actobindin and Cib, but the rate of growth at pointed ends displayed superimposable concentration dependencies (within experimental error) at 2 μM and at 5 μM G-actin and could not be accounted for by the calculated curves within a sequestration model (Fig. 5). Half-inhibition was observed at a lower concentration of actobindin or Cib than expected within the sequestration activity. The superimposable curves at two different actin concentrations suggest that actobindin or Cib bind to pointed ends with high affinity, preventing their growth. Analysis within pointed end capping with a $K_p$ value of 0.6 μM and a sequestration constant $K_c$ of 5 μM for actobindin and 2.5 μM for Cib satisfactorily accounted for the data (Fig. 5). The fact that a higher affinity is observed for binding to the pointed end than for binding to monomeric actin suggests, in agreement with previous results (23), that actobindin and Cib may interact with two actins at the pointed end, via their Tβ4 repeats. Similar data were obtained using CaATP-actin instead of MgATP-actin and a polymerization buffer that contained 0.1 M KCl.

To determine whether actobindin and Cib prevent pointed end disassembly, dilution-induced depolymerization of gelsolin-capped filaments was performed. The initial rate of depolymerization from the pointed ends was not affected by either actobindin or Cib up to 10 μM (see supplementary data Fig. 2). In conclusion, association of actobindin or Cib with pointed ends prevents growth but not depolymerization. The behavior of Cib and actobindin has some similarity here with DNase I, which also prevents pointed end growth but does not prevent depolymerization (34). The failure of Cib and actobindin to prevent pointed end depolymerization may be in relation with the poor binding of these proteins to ADP-actin, which is exposed at depolymerizing pointed ends, while ATP-actin is at the end of growing filaments. Therefore at the steady state of assembly of gelsolin-capped filaments, the relevant reaction is essentially the sequestration of monomeric actin by actobindin or Cib.

Actobindin and Cib Act as Purely G-actin Sequestering Proteins When CaATP Is Bound to Actin—Polymerization of CaATP-actin is quasi-reversible due to the slow hydrolysis of ATP on CaATP-P-actin. Consistently, the critical concentrations are practically identical (0.6 μM) at the two ends. Profilin-CaATP-actin failed to polymerize at the barbed ends (35). Both actobindin and Cib displayed the same behavior as profilin regarding interaction with CaATP-actin and caused depolymerization of F-actin (i.e. sequestration of G-actin) at the barbed and at the pointed end (Fig. 6). Values of 8 ± 1 μM were derived from the data for the equilibrium dissociation constants of the complexes of CaATP-G-actin with either acto-
Actobindin and Cib act as pure G-actin sequestering proteins with CaATP-G-actin. CaATP-actin (1.6 μM, 10% pyrenyl-labeled) was polymerized in the presence of 0.1 M KCl, in the absence (closed symbols) or presence (open symbols) of gelsolin (5 nM), and supplemented with actobindin (triangles) or Cib (circles) as indicated. The steady-state amount of F-actin was measured following 18-h incubation at room temperature.

In conclusion, the general features of the different interactions of profilin with CaATP-actin and MgATP-actin are displayed by actobindin and Cib.

**DISCUSSION**

We have shown that regarding its biochemical properties and biological function, the amoeba protein actobindin is a member of a new family of actin-binding proteins. These proteins consist of two or three β-thymosin repeats and share some biochemical properties with β-thymosins. For instance they bind ATP-G-actin specifically with thermodynamic and rate parameters very similar to Tβ4 and slow down nucleotide dissociation from G-actin-like β-thymosins, indicating that their binding sites appreciably overlap the Tβ4-actin complex with actobindin (13) and lysine 16 of actobindin (36) make contacts with the 4 N-terminal acidic residues of actin. The rate constant for association to G-actin is lower than the expected diffusion-limited rate constant, suggesting, as proposed for Tβ4 (14), that the formation of a low affinity rapid equilibrium collision complex is followed by a reversible isomerization step leading to a tighter interaction; alternatively, the protein might be in rapid equilibrium between several conformational states, one of which only binds G-actin.

On the other hand, unlike β-thymosins, these proteins are not pure G-actin sequesterers but actually regulate the dynamics of filament assembly using the same mechanism as profilin, i.e. their complex with MgATP-G-actin participates in barbed end growth exclusively. This property is observed in kinetic assays of seeded filament growth. Its consequence at steady state is the lowering of the steady-state concentration of free G-actin, consistent with copolymerization of actin and actobindin-actin or Cib-actin complexes. The failure of actobindin to depolymerize actin at steady state when barbed ends are free or inhibited barbed end growth had been noticed previously but had been explained differently (24). Due to the ability of their complex with actin to participate in barbed end growth, proteins of the actobindin family play an active role, like profilin, in actin-based motility processes. We have shown that the Drosophila homolog, ciboutil, is required for axonal growth during central brain development in metamorphosis of the fly and that either Cib or actobindin can replace profilin in a reconstituted motility assay (25). The present work indicates that actobindin likewise must be required for some motile processes of the amoeba. Like for profilin, the participation of actobindin-actin or Cib-actin to barbed end growth is observed with MgATP-actin only, and a pure G-actin sequestering function is observed with CaATP-actin. These results point to a possible role of ATP hydrolysis associated with actin polymerization in this function. Actobindin consists of two imperfect β-thymosin repeats, while the Drosophila and C. elegans proteins harbor three β-thymosin repeats, suggesting that their functional difference with Tβ4 might correlate with the fact that the β-thymosin motif is repeated. This view is not supported, however, by the following observations. Similar G-actin binding motifs showing similarity with Tβ4, called “verprolin homology region” or WH2 domain, are found in WASp family proteins, and in the ActA protein of Listeria, where they have been demonstrated to share the same functional homology with profilin (Refs. 37–39 and see Ref. 40 for a recent review). In these proteins, the G-actin binding module is generally not repeated, except in the case of N-WASp (the neural form of WASp), which contains a tandem of two verprolin homology regions. Hence it seems likely that a subtle difference in sequence in the G-actin binding motif, rather than the repeat of the motif, is responsible for the functional difference between β-thymosins and these proteins. The repeated sequences would then simply help to enhance the affinity of these proteins for G-actin. Further studies of the biochemical properties of the isolated repeats are required to challenge this view. A mutagenetic analysis of these proteins should also help to elucidate the structural basis for the change in function of the β-thymosin motif. In this respect, previous studies have shown that changing the sequence 1LKKTET24 in the actin binding motif of Tβ4 into LKETET caused Tβ4-induced actin aggregation in low ionic strength buffer (9). In addition to this segment, Tβ4 interacts with G-actin via helix 1 (residues 5–16) and helix 2 (residues 31–39) (42). According to Safer and co-workers (13, 14), C-terminal helix 2 interacts with subdomain 2 at the pointed end of G-actin. This contact may prevent association of the Tβ4-actin complex to the barbed end of an actin filament. In binding to G-actin, Cib and actobindin do not sterically interfere with subdomain 2 association to a barbed end.

Finally, β-thymosin repeat proteins display the original property, not shown by either profilin or β-thymosins, to prevent pointed end growth by a capping effect. This result has no physiological significance since in vivo pointed ends only depolymerize, but it is interesting from a structural point of view. The affinity for pointed ends is about 5-fold higher than for monomeric actin. This result is surprising and paradoxical. Actually, the view that actobindin-actin and Cib-actin participate in barbed end growth intuitively suggests that these proteins are transiently bound to the terminal subunit at the barbed end of the filament, but cannot be bound to the pointed end. To accommodate the unexpected pointed end capping we propose that two β-thymosin repeats of a single protein may interact with two terminal subunits at the pointed end. The structure of actobindin or Cib bound to the pointed end may be similar to the structure of the reported high affinity complex of actobindin with covalently cross-linked actin dimers obtained by reacting F-actin with para-phenylene-bis-maleimide (43). The covalent bond connects lysine 191 to cysteine 374 of a laterally adjacent subunit along the genetic helix (41), i.e. may reconstitute the pointed end structure of a filament. Resolution of the three-dimensional structure of the complex of actobindin or Cib with G-actin is required to challenge this hypothesis.

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