Expression of a Nonpolymerizable Actin Mutant in Sf9 Cells†

Peteranne B. Joel, Patricia M. Fagnant, and Kathleen M. Trybus*

Department of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, Vermont 05405-0068

Received May 28, 2004; Revised Manuscript Received July 2, 2004

ABSTRACT: We have succeeded in expressing actin in the baculovirus/Sf9 cell system in high yield. The wild-type (WT) actin is functionally indistinguishable from tissue-purified actin in its ability to activate ATPase activity and to support movement in an in vitro motility assay. Having achieved this feat, we used a mutational strategy to express a monomeric actin that is incapable of polymerization. Native actin requires actin binding proteins or chemical modification to maintain it in a monomeric state. The mutant actin sediments in the analytical ultracentrifuge as a homogeneous monomeric species of 3.2 S in 100 mM KCl and 2 mM $MgCl_2$, conditions that cause WT actin to polymerize. The two point mutations that render actin nonpolymerizable are in subdomain 4 (A204E/P243K; "AP-actin"), distant from the myosin binding site. AP-Actin binds to skeletal myosin subfragment 1 (S1) and forms a homogeneous complex as demonstrated by analytical ultracentrifugation. The ATPase activity of a cross-linked AP-actin·S1 complex is higher than that of S1 alone, although less than that supported by filamentous actin (F-actin). AP-Actin is an excellent candidate for structural studies of complexes of actin with motor proteins and other actin-binding proteins.

A complex between native actin and myosin that is amenable to X-ray crystallography has been impossible to achieve because native actin monomers (G-actin) in the presence of even low concentrations of salt rapidly polymerize into long filaments (F-actin) of varying lengths. The creation of a nonpolymerizable actin monomer that is able to bind to myosin has been a long-sought-after goal, with many different strategies employed over the years. A number of cytoskeletal proteins that bind actin such as DNase I, gelsolin, profilin, and vitamin D-binding protein (DBP)[1] prevent its polymerization. However, the presence of gelsolin or DBP precludes myosin subfragment 1 (S1) binding, and DNase I allows only some myosin complexes to form (*1*).

Another approach to prevent polymerization is to perturb actin's structure by cross-linking or chemical modification. A disulfide cross-link between Cys 10 and Cys 374 in subdomain 1 of actin abolished polymerization while retaining the ability to bind S1, but no further work has been published with this modified actin (*2*). More recently, chemical modification of Cys 374 with tetramethylrhodamine (TMR) was shown to suppress polymerization to the extent that this monomeric actin was crystallized with ADP (*3*) or AMP-PNP at the active site (*4*). The S1 binding properties of this modified actin have not been reported, but they would presumably be impaired since the modification resides in subdomain 1 where S1 binds. The presence of either actin binding proteins or chemical modification potentially introduces constraints on the actin structure, thus limiting these approaches.

A mutational strategy is clearly desirable, but this requires an expression system. Attempts to produce actin mutants in *Escherichia coli* have failed (*5*), because bacteria lack the required chaperonin called CCT (cytosolic chaperonin containing TCP-1) (reviewed in ref *6*). The primary organism for producing specific mutants of actin has been yeast (*Saccharomyces cerevisiae*) (reviewed in ref *7*). Because yeast has only a single actin isoform that is essential for cell viability, actin mutants that are polymerization-defective or nonfunctional in other ways can only be coexpressed with endogenous actin as an epitope-tagged species for subsequent isolation (*8*). The Act88F gene specific for *Drosophila* indirect flight muscle (*9*) has been mutated for structure−function studies (*10*), but low protein yields preclude in-depth biochemical studies.

Here we establish that functional WT actin can be prepared in high yield in the baculovirus/Sf9 expression system. Having demonstrated feasibility, we next expressed a mutant actin (AP-actin) that does not polymerize but retains the ability to bind S1 and activate ATPase activity. The AP-actin·S1 complex is the smallest homogeneous, functional complex amenable for future structural studies.

MATERIALS AND METHODS

Plasmid Construction. A modified plasmid pAcUW2B containing the coding sequence for the *Drosophila* 5C actin

† This work was supported by National Institutes of Health Grant HL38113.

* To whom correspondence should be addressed at the Health Sciences Research Facility 130, University of Vermont, Burlington, VT 05405. Tel. 802-656-8750; fax 802-656-0747; e-mail trybus@physiology.med.uvm.edu.

[1] Abbreviations: AP-actin, expressed actin with the point mutations A204E and P243K; Arp, actin-related protein; CCT, cytosolic chaperonin containing TCP-1; DBP, vitamin D binding protein; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; G_0 buffer, G buffer lacking ATP; S1, myosin subfragment 1; S1(A1), myosin subfragment 1 with the A1 isoform of the essential light chain; S1(A2), myosin subfragment 1 with the A2 isoform of the essential light chain; smHMM, smooth muscle heavy meromyosin; TMR, tetramethylrhodamine; WT, wild type.

10.1021/bi048899a CCC: $27.50
Published on Web 08/20/2004

gene was a generous gift from Loy Volkman (*11*). 5C Actin is a cytoplasmic actin gene, with transcripts distributed in all cells and tissues in most stages of development (*12*). Its sequence is 94% identical to that of human striated muscle actin. Wild-type human α-cardiac actin was also cloned into this vector. Site-directed mutagenesis was used to create a mutant construct of the 5C actin in which Ala204 was replaced by Glu and Pro243 was replaced by Lys (AP-actin). The numbering system used here corresponds to that of skeletal muscle actin.

Expression and Purification of Actin. Recombinant baculovirus encoding the actin constructs was prepared by conventional protocols (*13*). Infected Sf9 cells were harvested 3 days after infection and lysed in 1 M Tris-HCl, pH 7.5 (at 4 °C), 0.6 M KCl, 0.5 mM $MgCl_2$, 0.5 mM Na_2ATP, 1 mM dithiothreitol (DTT), 4% Triton X-100, 1 mg/mL Tween-20, and protease inhibitors (*14*, *15*). The cell lysate was clarified, dialyzed versus buffer A (0.01 M imidazole, pH 7.5, 0.1 mM $CaCl_2$, 0.1 M KCl, 0.1 mM DTT, 0.5 mM Na_2-ATP, and 1 μg/mL leupeptin) and fractionated on a Whatman DE53 column (2.5 × 10 cm for a 750 mL culture) which was eluted with a linear gradient of 0.1−0.5 M KCl in buffer A (*16*).

For preparations of WT actin (*Drosophila* 5C or human α-cardiac), 2 mM $MgCl_2$ was added to the pooled DE53 fractions, and the solution was incubated for 1 h at room temperature and then overnight on ice. F-Actin was pelleted at 178000*g* for 3.5 h. F-Actin was depolymerized by extensive dialysis into G buffer (5 mM Tris-HCl, pH 8.2 at 4 °C, 0.2 mM $CaCl_2$, 0.1 mM sodium azide, 0.5 mM DTT, 0.2 mM Na_2ATP, and 1 μg/mL leupeptin) followed by clarification at 372000*g* for 20 min. A second round of polymerization was initiated by addition of 2 mM $MgCl_2$ and 100 mM KCl. The solution was incubated at room temperature for 1.5 h and then spun at 372000*g* for 30 min. The F-actin pellet was resuspended and dialyzed into 5 mM imidazole, pH 7.5, 5 mM KCl, 2 mM $MgCl_2$, and 3 mM sodium azide.

For the mutant AP-actin, the pooled fractions from the DE53 column were concentrated with an Amicon Ultra centrifugal filter device (Millipore Corp.) and then applied to a Sephacryl S300 column equilibrated with buffer A containing 0.3 M KCl. The purified protein was concentrated and dialyzed into G buffer. AP-Actin that was not used immediately was stored in liquid nitrogen. Actin concentration was determined from the absorbance at 290 nm by use of an extinction coefficient of 0.63 mL mg^{-1} cm^{-1}.

Preparation of Other Proteins. WT phosphorylated smooth muscle HMM was prepared as described (*17*). Chymotryptic S1 was prepared from chicken pectoralis myosin, and the essential light-chain isoforms of S1 [S1(A1) and S1(A2)] were separated by ion-exchange chromatography on DEAE-Sephacel (Pharmacia) as described (*18*). Chicken skeletal muscle actin was prepared as described (*19*).

ATPase and in Vitro Motility Assays. Actin-activated ATPase assays were performed in 10 mM imidazole, pH 7.0, 8 mM KCl, 1 mM DTT, 1 mM NaN_3, 1 mM $MgCl_2$, and 1 mM EGTA, at 25 or 37 °C as indicated. The reaction was initated by the addition of 2 mM MgATP and stopped with SDS at six time points per actin concentration, and inorganic phosphate was determined colorimetrically (*17*, *20*). In vitro motility assays were performed in 25 mM imidazole, pH 7.5, 25 or 60 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 0.5% or 0.7% methylcellulose, 1 mM MgATP, 10 mM DTT, 3 mg/mL glucose, 0.1 mg/mL glucose oxidase (Sigma−Aldrich), and 0.018 mg/mL catalase (Sigma−Aldrich) at 30 °C. Expressed phosphorylated smooth muscle HMM was used as the motor. Prior to use, the HMM (0.1 mg/mL) was mixed with 0.04 mg/mL actin and 1 mM MgATP and spun for 20 min at 350000*g* to remove HMM that was unable to dissociate from actin in the presence of ATP. An anti-rod monoclonal antibody (*21*) was used to bind the HMM to the nitrocellulose-coated coverslip (*17*, *20*).

Sedimentation Velocity Analysis. Sedimentation velocity data were obtained from absorbance scans at 280 or 290 nm at 20 °C with a rotor speed of 50 000 rpm in a Beckman Optima XL-1 analytical ultracentrifuge. The program DCDT+ was used to derive the apparent sedimentation coefficient distribution function $g(s^*)$ versus s^* from the sedimentation data (*22*).

Cross-Linking Reactions. Cross-linking between mutant AP-actin and S1 by EDC [1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide] (Sigma Chemical Co.) was carried out in a two-step reaction as described in refs *23* and *24*. Cross-linked products were analyzed on 4−15% gradient polyacrylamide gels. The AP-actin·S1 cross-linked complex was purified on a DE53 column equilibrated in 20 mM imidazole, pH 7.0, 0.1 mM $CaCl_2$, 0.2 mM Na_2ATP, 0.2 mM DTT, and 1 μg/mL leupeptin. The complex was eluted with a gradient of 20−250 mM NaCl. Complexes with S1(A1) and S1(A2) were pooled separately.

RESULTS

Functional WT Actin Can Be Expressed in the Baculovirus/SF9 System. A cytoplasmic actin from *Drosophila* (5C actin) was expressed in substantial amounts in the baculovirus/Sf9 insect cell system (Figure 1A, lane 3). WT actin was purified by ion-exchange chromatography followed by one or two cycles of polymerization (see Materials and Methods). SDS−PAGE of the purified actin showed very little contamination by other proteins (Figure 1A, lane 4). Yields were typically 4 mg/250-mL culture (1 billion cells) with one cycle of polymerization. Electron microscopy showed typical actin filament morphology (Figure 1B), and when visualized by light microscopy with rhodamine-phalloidin, the average lengths of the filaments were indistinguishable from tissue-purified actin.

To show that the expressed WT actin was functional, its ability to activate the ATPase activity of phosphorylated smooth muscle HMM (smHMM) was determined. The expressed 5C actin and tissue-extracted skeletal actin had comparable V_{max} and K_m values (Table 1). It should be noted that since we are comparing two different actin isoforms, there is no a priori reason that the values obtained in this assay should be identical. The in vitro motility assay was used as a second measure of functionality. The expressed and tissue-purified actins were moved by smHMM at the same rate within experimental error (Table 1).

Functional WT human α-cardiac actin was also successfully expressed (Table 1). Yields were similar to that of the 5C actin (3 mg/250-mL culture). These results establish that the baculovirus/insect cell system is the system of choice for actin expression.

Table 1: Actin-Activated ATPase and in Vitro Motility Assays of Expressed WT and Tissue-Purified Actin[a]

actin	actin-activated ATPase		in vitro motility (μm/s)
	V_{max} (s^{-1})	K_m (μM)	
tissue-purified from skeletal muscle	6.5 ± 0.4	48 ± 5	0.95 ± 0.04 (25 mM KCl); 1.76 ± 0.12 (60 mM KCl)
expressed WT 5C actin	6.8 ± 0.1	31 ± 1	0.91 ± 0.07 (25 mM KCl)
expressed WT human α-cardiac actin	6.2 ± 0.6	49 ± 9	1.69 ± 0.12 (60 mM KCl)

[a] Assays were carried out with phosphorylated smooth muscle HMM. The actin-activated ATPase assay was performed at 37 °C. The in vitro motility assay was performed at 30 °C with 25 or 60 mM KCl.

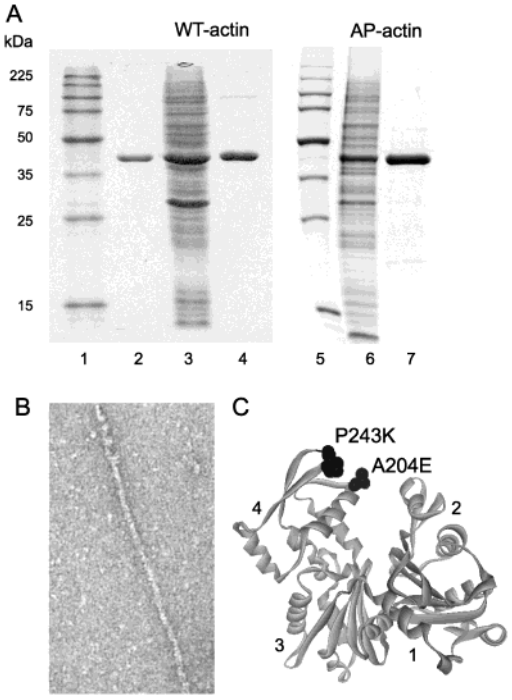

FIGURE 1: Expression and purification of WT actin or mutant AP-actin. (A) SDS−12% polyacrylamide gels. Lanes 1 and 5 are molecular weight markers. L*ane 2* is actin extracted from skeletal muscle. Lane 3 shows total expression of WT *Drosophila* 5C actin in Sf9 cells, and lane 4 shows the purified WT actin. Lane 6 shows Sf9 cells expressing mutant AP-actin, and lane 7 shows the purified mutant protein. (B) Negatively stained filaments of expressed actin show typical actin morphology. (C) Ribbon diagram of the three-dimensional structure of actin (coordinates 1NWK). The four subdomains are indicated. The two residues that were mutated to produce a nonpolymerizable mutant are indicated (A204E and P243K).

Two Point Mutations in Actin Create a Nonpolymerizable Monomer. To create a nonpolymerizable actin mutant, two mutations were made in subdomain 4 of the cytoplasmic 5C actin backbone (A204E/P243K) (Figure 1C). The mutant actin, called AP-actin, was expressed in the baculovirus/Sf9 system. Although the expression of AP-actin in Sf9 cells was somewhat lower than the WT actin, the final yield of highly purified product was higher (Figure 1A, lane 7). Approximately 10 mg of purified AP-actin were obtained per 250-mL culture. Analytical ultracentrifugation showed that AP-actin remains monomeric under conditions that cause WT actin to polymerize. In the presence of 100 mM KCl and 2 mM $MgCl_2$, the critical concentration for WT actin assembly decreases to $\approx$0.1 μM from $\approx$100 μM in G buffer (5 mM Tris-HCl, pH 8.2 at 4 °C, 0.2 mM $CaCl_2$, 0.1 mM sodium azide, 0.5 mM DTT, 0.2 mM Na_2ATP, and 1 μg/mL leupeptin) (reviewed in ref *7*). AP-Actin was incubated overnight at 19 μM under nonpolymerizing (G buffer) and polymerizing conditions (G buffer with 2 mM $MgCl_2$ and 100 mM KCl). AP-Actin sedimented as a homogeneous monomer in both solvents with sedimentation coefficients of 3.14S and 3.18S, respectively (Figure 2A−D). Tissue-extracted skeletal actin in G buffer also sedimented as a 3.2S homogeneous monomer. When 100 mM KCl and 2 mM $MgCl_2$ were added to 19 μM tissue-purified skeletal actin in G buffer, 75% of the actin formed large polymers that rapidly sedimented, while the remaining actin sedimented as smaller polymers ($\approx$17S and 29S).

AP-Actin Binds to Myosin Subfragment 1. Analytical ultracentrifugation was used to show that AP-actin forms a complex with S1. AP-Actin and the S1(A1) isoform of S1 were dialyzed separately into G buffer without ATP (G_0 buffer) and then combined (6 μM S1, 9 μM AP-actin). S1(A1) alone sedimented as a homogeneous 5.9S monomer (Figure 2 E and Table 2), while the complex of S1(A1) and mutant AP-actin sedimented as a homogeneous 6.8S species (Figure 2F and Table 2). These results establish that AP-actin binds to S1 and that S1 does not induce polymerization of AP-actin.

AP-Actin Can Be Cross-linked to S1. Cross-linking of AP-actin to S1 with EDC generated products with apparent molecular masses of 160 and 190 kDa (Figure 3, lanes 2, 5, and 8). The same doublet was seen when tissue-extracted skeletal G-actin was cross-linked with S1 (Figure 3, lane 9). Similar cross-linked bands (165, 175, and 200 kDa) have been shown to be one actin cross-linked to one S1 (reviewed in ref *25*). Addition of MgATP to the cross-linking reaction prevented the formation of the cross-linked products (Figure 3, lane 3), while MgADP greatly reduced their formation (Figure 3, lane 7). Increasing the molar ratio of actin to S1 increased the fraction of cross-linked product (Figure 3, lanes 2 and 5). A 265 kDa product, which has been reported to contain two actin monomers bound to one S1 (*25*), was never observed when AP-actin was cross-linked to S1, although this product was obtained when skeletal F-actin was cross-linked to S1 (data not shown). This result implies that AP-actin is not capable of dimerization even in the presence of S1.

The purified cross-linked products retained the same doublet seen before separation on the column. In G_0 buffer with 100 mM KCl, the cross-linked AP-actin·S1(A2) complex sedimented as a homogeneous 6.7S species, while the AP-actin·S1(A1) complex sedimented as a homogeneous 6.6S species (Table 2). Thus the sedimentation coefficients of the cross-linked and un-cross-linked complexes are similar (Figure 2F and Table 2).

S1 Cross-Linked to AP-Actin Has Higher MgATPase Activity than S1 Alone, and Addition of G-Actin Further Stimulates This Activity. The purified cross-linked AP-actin·

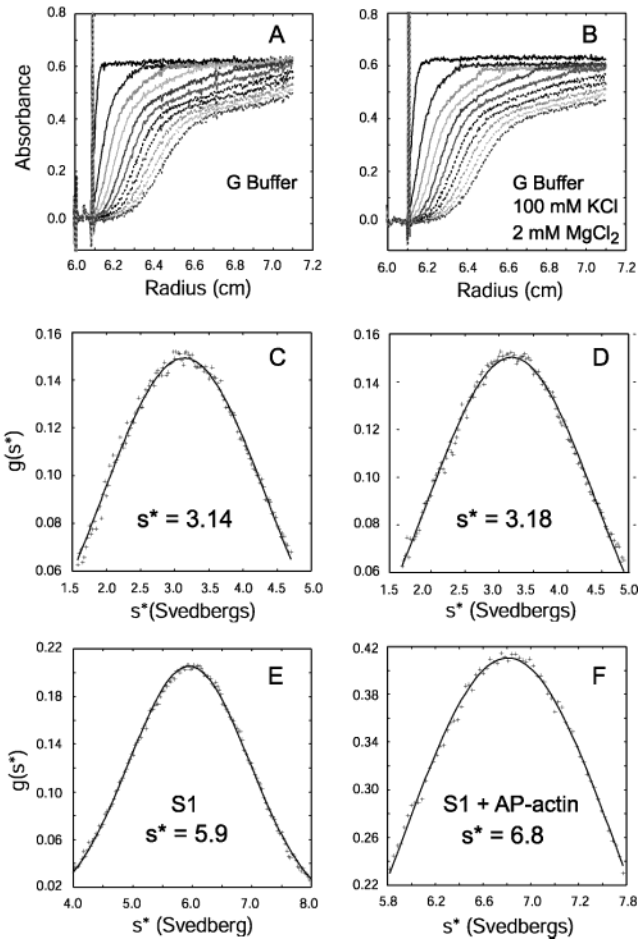

FIGURE 2: Sedimentation velocity analysis shows that AP-actin does not polymerize and forms a complex with S1. Mutant AP-actin (19 μM) was incubated 17 h at 4 °C in G buffer (nonpolymerizing conditions) or in G buffer with 100 mM KCl and 2 mM $MgCl_2$ (polymerizing conditions) and subsequently analyzed in the analytical ultracentrifuge. Data were acquired at 20 °C by use of absorbance optics at 290 nm and a rotor speed of 50 000 rpm. (A, B) Every fourth scan of absorbance versus distance. (C, D) Sedimentation velocity profiles of the data shown in panels A and B. The solid line indicates the theoretical fit of the data (+) to a single species. The sedimentation coefficients of AP-actin in the nonpolymerizing and polymerizing solutions are the same. (E, F) Sedimentation velocity profiles of S1 alone (E) and the complex of S1 and AP-actin (F) in G_0 buffer. The higher sedimentation coefficient is evidence for complex formation. Data were acquired at 20 °C by use of absorbance optics at 280 nm and a rotor speed of 50 000 rpm. The solid line indicates the theoretical fit of the data (+) to a single species.

Table 2: Hydrodynamic Data Showing Complex Formation between S1 and AP-Actin[a]

myosin subfragment	actin	X-linked	buffer	sedimentation coefficient (S)
S1(A1)			G_0	5.9
S1(A1)			G_0,100 mM KCl	5.5
	AP-actin		G_0, 100 mM KCl	3.2
S1(A1)	AP-actin	no	G_0	6.8
S1(A1)	AP-actin	yes	G_0,100 mM KCl	6.6
S1(A2)	AP-actin	yes	G_0,100 mM KCl	6.7

[a] Centrifugation conditions are described under Materials and Methods. G_0 buffer is 5 mM Tris-HCl, pH 8.2 at 4 °C, 0.2 mM $CaCl_2$, 0.1 mM sodium azide, 0.5 mM DTT, and 1 μg/mL leupeptin.

S1(A2) complex had a 5-fold greater MgATPase rate than that of S1(A2) alone (0.66 ± 0.10 vs 0.13 ± 0.01 s^{-1}) (Table 3), showing that a single monomer of actin can stimulate ATPase activity to some extent. MgATPase assays were also carried out after addition of 20 μM skeletal G-actin. The G-actin would be expected to polymerize given that the assay buffer contains KCl and $MgCl_2$. Addition of skeletal G-actin further stimulated the MgATPase activity of the cross-linked complex by 2.6-fold (1.7 ± 0.1 s^{-1}) (Table 3), suggesting that WT actin monomers can grow from the unmutated barbed end of AP-actin. For comparison, the actin-activated MgATPase activity of S1(A2) with 20 μM skeletal G-actin was 8.9 ± 0.5 s^{-1}. A monomer of actin can therefore stimulate ATPase activity but not to the same extent as F-actin.

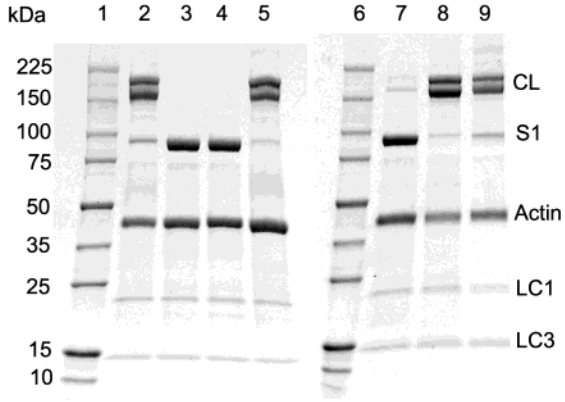

FIGURE 3: EDC cross-links AP-actin to S1 in a MgATP-dependent manner. AP-actin was cross-linked to S1 (see Materials and Methods). Aliquots were analyzed by SDS−PAGE on a 4−15% acrylamide gel. Lanes 1 and 6 are molecular weight markers. Lanes 2 and 8 show the complete reaction mixture. Inclusion of 2 mM MgATP results in no cross-linked product (lane 3), while addition of 2 mM MgADP greatly reduces the yield of cross-linked product (lane 7). Lane 4 is a control that lacked EDC in the reaction mixture. Lane 5 has twice the concentration of AP-actin as lane 2 in the cross-linking reaction (molar ratio of actin/S1 of 5/1 versus 2.5/1). Lane 9 shows that tissue-purified skeletal G-actin produces the same cross-linked products as AP-actin. CL, cross-linked.

Table 3: S1 Cross-Linked to Mutant AP-Actin Exhibits Higher MgATPase Activity than S1 Alone, and Addition of G-Actin to the Cross-Linked AP-Actin·S1 Complex Further Stimulates MgATPase Activity[a]

protein	ATPase rate (s^{-1})
S1	0.13 ± 0.01 (n = 6)
cross-linked AP-actin·S1	0.66 ± 0.10 (n = 10)
cross-linked AP-actin·S1 + 20 μM skeletal G-actin	1.71 ± 0.11 (n = 7)
S1 + 20 μM skeletal G-actin	8.9 ± 0.5 (n = 3)

[a] MgATPase assays were carried out at 25 °C in 8 mM KCl, 10 mM imidazole, pH 7.0, 1 mM DTT, 1 mM NaN_3, 1 mM $MgCl_2$, and 1 mM EGTA. n, the number of assays averaged to obtain the value (±SD), is indicated in parentheses. Two preparations of S1(A2) and of S1(A2) cross-linked to mutant AP actin were used in the assays.

DISCUSSION

Expression Systems. Here we show that the baculovirus/insect cell expression system is the method of choice for expressing WT and mutant actin. Sf9 cells were able to express actins from different sources (an invertebrate cytoplasmic actin or a vertebrate muscle actin) as well as both polymerization-competent (WT) and polymerization-defective actins. The folding pathway of actin is well-established

and is known to require the eukaryotic chaperonin CCT, which is a multimeric ringed structure (reviewed in ref *6*). Sf9 cells must therefore contain this complex or a close homologue that is able to interact with exogenously expressed actin.

The levels of overexpression are sufficiently high that epitope-tagging of the protein to separate it from endogenous actin is not required (12−40 mg/L of culture). The yield of the purified nonpolymerizable actin mutant was ≈2.5-fold higher than that of WT actin. One other group has recently reported expression of skeletal α-actin in the baculovirus/Sf9 expression system, but their yield of purified protein was estimated to be significantly lower (≈2 mg/L of culture) (*26*). Since qualitatively their SDS gels show similar substantial total expression, we can only speculate that their extraction and purification procedure resulted in more losses than ours. One key feature of our procedure is extraction with a high concentration of Tris, which is more effective than G buffer; this method was originally developed for purification of actin from nonmuscle tissue (*14*, *15*).

Until now, *S. cerevisiae* has been the expression system of choice, but its disadvantages are apparent. Yeast contains a single actin gene that is essential for growth. Actin overexpression is lethal and thus large volumes of cells must be grown. Expression and purification of some nonfunctional actin mutants can be achieved but only when they are coexpressed with the endogenous yeast gene and when the mutant is epitope-tagged for its subsequent purification (*8*). Moreover, yeast does not have N-terminal processing of actin (*27*) nor the methyltransferase necessary to methylate His73 (*28*), which forms a hydrogen bond that contributes to forces closing the interdomain cleft (*29*−*31*). Sf9 cells are known to be capable of a number of posttranslational modifications of eukaryotic proteins (*13*), but it remains to be established if the expressed actin is methylated.

WT-Actin Function. As judged by in vitro motility and actin-activated ATPase activity, expressed cytoplasmic invertebrate actin and tissue-purified skeletal actin interact similarly with smHMM. Invertebrate cytoplasmic actin, as well as human cytoplasmic actins, have one less acidic residue at the N-terminus compared with the striated muscle isoforms (3 versus 4). It is likely that if more stringent comparisons between the isoforms were performed, some differences would emerge. The overall identity between *Drosophila* 5C actin and striated muscle actin isoforms is >93%, with the remainder being predominantly conservative substitutions.

Design of a Nonpolymerizable Mutant. Actin-related proteins 2 and 3 (Arp2 and Arp3) are structurally related to actin but have distinctive features in loop regions. They are part of a larger protein complex that is involved in nucleating branches off an existing actin filament. The sequence of these related proteins provided the rationale for our mutational strategy. Even prior to the crystal structure of the Arp2/3 complex (*32*), it was noted that the subunit−subunit contacts at the pointed end of Arp3 differed from actin in regions that are believed to form the interface between subunits in the native actin polymer (*33*, *34*). We mutated two residues in surface loops at the pointed end of actin to the corresponding sequence found in *Acanthamoeba* Arp3. These residues should and did create significant steric clashes with a native barbed end (*33*), thus abolishing polymerization.

Properties of AP-Actin. The inability of AP-actin to polymerize under conditions that would lead to polymerization of WT actin was confirmed in two ways. First, high concentrations of AP-actin (19 μM) sedimented as a monomer in the analytical ultracentrifuge after incubation with 100 mM KCl and 2 mM $MgCl_2$, conditions that cause 0.1 μM WT actin to spontaneously assemble into filaments. Second, in the presence of S1, AP-actin formed a single homogeneous species with a sedimentation coefficient indicative of a 1:1 complex of the two proteins. In contrast, S1 induces the polymerization of low concentrations of WT-actin under buffer conditions where actin alone would remain monomeric (*35*).

The AP-actin·S1 complex, when cross-linked with the zero-length cross-linker EDC, formed products that migrated on SDS−PAGE with the same apparent molecular masses as EDC cross-linked complexes between tissue-purified skeletal muscle G-actin and S1. It has been shown that these complexes contain only one actin bound to one S1. The faster migrating band (165 kDa) is believed to involve the negatively charged N-terminal segment (1−12) of actin and positively charged residues in loop 2 of myosin (reviewed in ref *25*). These residues are the same as those involved in the initial, weak electrostatic binding between actin and S1. Since the point mutations in AP-actin are in subdomain 4, whereas binding to myosin occurs predominantly in subdomain 1, a direct interference with myosin binding would not be expected and was not seen. The folding pattern of actin is intricate, which can allow changes to be propagated through the molecule. Nonetheless, subdomain 4 is the only domain that is connected to only one other subdomain and thus is least likely to propagate changes.

A monomeric actin should also be able to engage in interactions with the so-called "cardiomyopathy" loop on myosin via actin residues 332−336 as well as the helix−turn−helix motif of myosin via C-terminal actin residues 349−351 (*36*). An interaction that will not occur between myosin and AP actin is binding of the "secondary actin binding site" on myosin to the actin that would be below it in filamentous actin, because two AP-actins cannot form a stable dimeric interface. Nonetheless, a cross-linked AP-actin·S1 complex, which would be expected to be operating at its maximal velocity (i.e., at "infinite" actin), can complete the entire ATPase cycle without this interaction, albeit at a reduced rate compared with F-actin. A similar degree of activation of S1, by an actin rendered monomeric by a cross-link between Cys10 and Cys 374, was previously observed (*2*). A study in which cross-linked dimers, trimers, and tetramers were isolated showed at most a 2-fold further stimulation of ATPase activity over that obtained with G-actin (*37*). When the oligomers were polymerized, they showed full activation, suggesting either that the K_m with small oligomers is very high (i.e., V_{max} was not achieved) or that only when polymerized into a filament does actin's structure change so as to present the optimal actomyosin interface.

Future Perspectives. The nonpolymerizable actin mutant described here allows one to examine the structure of monomeric actin that has not been chemically modified nor complexed with other proteins. We have crystallized AP-actin and are investigating nucleotide-dependent conformational changes in actin (unpublished data). Moreover, AP-

actin is ideally suited for biochemical, biophysical, and structural studies of actin with its binding partners, including myosin and other actins, as well as the vast superfamily of actin-binding proteins.

ACKNOWLEDGMENT

We thank Susan Lowey for helpful comments regarding the manuscript, Dorit Hanein and Larnele Hazelwood for the negatively stained image of expressed F-actin, and Loy Volkmann for his gift of the *Drosophila* 5C actin clone that started us on this project.

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BI048899A