The Bulk of Unpolymerized Actin in *Xenopus* Egg Extracts Is ATP-bound

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Non-muscle cells contain 15–500 μM actin, a large fraction of which is unpolymerized. Thus, the concentration of unpolymerized actin is well above the critical concentration for polymerization in vitro (0.2 μM). This fraction of actin could be prevented from polymerization by being ADP bound (therefore less favored to polymerize) or by being ATP bound and sequestered by a protein such as thymosin β_4, or both. We isolated the unpolymerized actin from *Xenopus* egg extracts using immobilized DNase 1 and assayed the bound nucleotide. High-pressure liquid chromatography analysis showed that the bulk of soluble actin is ATP bound. Analysis of actin-bound nucleotide exchange rates suggested the existence of two pools of unpolymerized actin, one of which exchanges nucleotide relatively rapidly and another that apparently does not exchange. Native gel electrophoresis of *Xenopus* egg extracts demonstrated that most of the soluble actin exists in complexes with other proteins, one of which might be thymosin β_4. These results are consistent with actin polymerization being controlled by the sequestration and release of ATP-bound actin, and argue against nucleotide exchange playing a major role in regulating actin polymerization.

INTRODUCTION

Actin-dependent cell movements, such as cytokinesis or locomotion toward a chemoattractant, require precise temporal and spatial regulation of actin polymerization (Korn, 1982; Pollard, 1986a,b; Cooper, 1991). Although several proteins have been identified that might be important for this regulation, the exact mechanisms for control of actin polymerization at specific sites in cells have remained elusive. In vitro, actin polymerization is regulated by monomer concentration and the bound nucleotide. Under conditions considered physiological, the critical concentration for polymerization of ATP-actin is 0.2 μM whereas that of ADP-actin is 2 μM. (Pollard, 1986a). Because the concentration of unpolymerized actin in most cells (8–250 μM) is well above the critical concentration for actin polymerization, it has long been realized that cells must possess a mechanism for stabilizing unpolymerized actin (Korn, 1982).

A number of actin monomer binding proteins have been implicated in actin polymerization regulation. Recent studies have highlighted two proteins that may be important for actin polymerization control, thymosin β_4 and profilin. Thymosin β_4 is an abundant, 5-kDa polypeptide that is thought to be the major actin-sequestering protein in many higher eukaryotic cells (Safer *et al.*, 1990; Cassimeris *et al.*, 1992; Yu *et al.*, 1993). Thymosin β_4 binds ATP-actin with a 50-fold higher affinity than ADP-actin (Carlier *et al.*, 1993), inhibits actin nucleotide exchange when bound (Goldschmidt *et al.*, 1992), and inhibits actin polymerization in vitro. Microinjection or overproduction of thymosin β_4 in fibroblasts can induce extensive loss of actin stress fibers (Sanders *et al.*, 1992; Yu *et al.*, 1994) supporting its role as a negative regulator of actin filament formation. Profilin is a 15-kDa protein that binds actin monomer (Carlsson *et al.*, 1977) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) in a mutually exclusive manner and is thought to be regulated by multiple cellular signal transduction pathways (Vojtek *et al.*, 1991; Sohn, 1994). This link between profilin and signal transduction at the plasma membrane makes pro-

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filin an attractive candidate for a key regulator of actin polymerization. Certain studies show that profilin can act as an actin-sequestering protein and inhibit actin polymerization (Carlsson et al., 1977; Ozaki and Hatano, 1984; Haugwitz, 1994;). However, other work contends that profilin can also be a promoter of actin filament formation. Genetic studies have shown that profilin is important for actin filament assembly and stability in a variety of organisms (Haarer et al., 1990; Cooley et al., 1992; Magdolen et al., 1993; Balasubramanian et al., 1994; Finkel et al., 1994). In addition, profilin is localized at the cell membrane (Carlsson et al., 1977; Buss et al., 1992) and at the end of Listeria (Theriot et al., 1994) where actin polymerization occurs. In vitro studies have described two activities that could explain how profilin could act as a promoter of polymerization in vivo. One activity of profilin is its ability to act as a nucleotide exchange protein for actin (Korn, 1982; Goldschmidt et al., 1992) and the other is its ability to release actin from sequestration by thymosin β_4 and to lower the critical concentration for ATP-actin at the barbed ends of actin filaments (Pantaloni and Carlier, 1993). To date, the role profilin plays in controlling actin polymerization in vivo is unclear and the balance between its role in sequestering actin and promoting polymerization may vary in different cells.

To understand the key regulatory step in actin polymerization, we must examine how unpolymerized actin can exist at levels well above the critical concentration for polymerization in vivo. Specifically, the high monomer concentration requires either that unpolymerized actin is sequestered by monomer binding proteins and/or that the bound nucleotide is ADP. To examine the potential roles of these two mechanisms for actin sequestration in vivo, we have developed a protocol for isolating unpolymerized actin from cell cytoplasm and analyzing its bound nucleotide.

Isolation of actin from cells usually requires extensive dilution of the cytoplasm with buffer, which can cause depolymerization of actin filaments and thus potentially generate artificially high levels of ADP-actin. We have chosen Xenopus laevis egg extracts (Murray, 1991) to perform these experiments because these appear to recapitulate cytoplasmic regulation of actin polymerization and have the advantages of a cell-free system. Specifically, Xenopus egg extracts maintain a high level of unpolymerized actin, and this actin can be induced to polymerize by addition of a physiological desequestration/nucleating site, the surface of a Listeria monocytogenes bacterium (Theriot et al., 1994). Furthermore, Xenopus egg extracts must be capable of rapidly depolymerizing actin filaments because the Listeria tail actin turns over with normal kinetics (Theriot and Mitchison, 1992). In this paper we quantitate the level of unpolymerized actin in these extracts and further document their filament depolymerizing activity. We then investigate the mechanism that maintains this large concentration of unpolymerized actin by analyzing the actin's bound nucleotide and potential sequestering proteins.

To isolate actin with its bound nucleotide we used immobilized DNase 1 because this protein not only binds specifically to actin, but also blocks the nucleotide on actin from exchanging once bound (Hitchcock, 1980; Mannherz et al., 1980). This reagent allowed us to analyze the nucleotide content of unpolymerized actin as well as to study the extent and rate of actin nucleotide exchange in a model system for cellular actin dynamics. In addition, we used a native gel system (Safer, 1989) to determine whether the unpolymerized actin is complexed with any monomer binding proteins. Our results are inconsistent with nucleotide exchange playing a key regulatory role for actin polymerization and are more consistent with release of ATP-actin by a sequestering protein such as thymosin β_4.

MATERIALS AND METHODS

Materials

Frozen rabbit skeletal muscle was obtained from Pelfreeze (Rogers, AR). ATP and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Biogel P-6, Affigel-10, and low molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Ten-kilodalton cut-off filters were obtained from Millipore Co. (Bedford, MA). DNase 1 was obtained from Boehringer Mannheim Biolabs (Indianapolis, IN). DNase 1 beads were made by coupling DNase 1 to Affigel-10 using 50 mM HEPES, pH 7.7, and 80 mM $CaCl_2$ as the coupling buffer. Typically 20 mg of DNase 1 was bound per milliliter of Affigel. ^{32}P-α ATP was from NEN (Boston, MA). One milliliter of Mono-Q HR 5–5 column was obtained from Pharmacia (Piscataway, NJ). Mouse anti-actin monoclonal antibody was obtained from Amersham (Arlington Heights, IL). Alkaline phosphatase-conjugated rabbit anti-mouse antibody was obtained from Promega (Madison, WI). N-hydroxysuccinimidyl 5-carboxytetramethyl rhodamine was obtained from Molecular Probes Inc. (Junction City, OR)

Protein and Extract Preparation

Rabbit skeletal muscle globular actin (G-actin) was prepared as described (Spudich and Watt, 1971) and centrifuged at 436 000 × g for 15 min at 4°C. The supernatant was stored at 4°C and used within 10 days.

Concentrated meiotically arrested cytoplasmic extracts from Xenopus laevis eggs were prepared as described (Theriot et al., 1994). A partially purified fraction of thymosin β_4 was made by boiling Xenopus egg extracts for 10 min, centrifuging 10,000 × g for 10 min, and centrifuging the 10,000 g supernatant at 436,000 × g for 15 min. A purer thymosin β_4 fraction was made by concentrating the boiled supernatant using a 3000 molecular weight cut-off Centricon unit, desalting with a biogel P-6 column, and filtering through a 10,000 molecular weight cut-off Centricon unit.

Fluorescent Microscopy of Actin Filaments

Polymerized rabbit skeletal muscle actin at 30 mg/ml was mixed with a twofold molar excess of N-hydroxysuccinimidyl 5-carboxytetramethyl rhodamine (Molecular Probes Inc.) as previously described (Kellogg et al., 1988). The final G-actin was dialyzed

against G-buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP), clarified by centrifugation at 100,000 $\times$ g in a TLA Beckman centrifuge (Palo Alto, CA) for 15 min., frozen in aliquot in liquid nitrogen, and stored at $-80°C$. The stoichiometry of labeling was ≈ 0.125 rhodamine molecules per actin monomer as determined by absorption spectrophotometry using an extinction coefficient $e_{280} = 49,000$ M^{-1} cm^{-1} for actin and $e_{560} = 50,000$ M^{-1} cm^{-1} for tetramethyl rhodamine (Molecular Probes).

Pure rhodamine actin filaments were made by polymerizing 5 μg of rhodamine-labeled actin in 20 μl of F-buffer (50 mM KCl, 50 mM Tris-HCl, pH 8.0, 1 mM ATP, 0.2 mM DTT) and 1 μl of a 20× stock solution of oxygen scavengers (2 mg/ml catalase, 0.6 mg/ml glucose oxidase, 200 mM glucose, 20 μm DTT, 40 mg/ml hemoglobin) to prevent photo-damage and bleaching. This actin could be depolymerized by mixing with a 1:1 vol of Xenopus egg extract. Rhodamine-labeled actin was polymerized in Xenopus laevis egg extracts by mixing 1 μl of 5 mg/ml rhodamine actin with 10 μl of the extract and 0.5 μl of a 20× stock solution of oxygen scavengers. These rhodamine actin filaments could be removed by centrifugation at 436,000 $\times$ g in a TLA 100 rotor for 15 min.

ATP- or ADP-bound Actin

The free ATP was removed from 60 μg of rabbit muscle actin in 80 μl of G-buffer by spinning 3000 g for 2 min through a 0.7 ml Biogel P-6 column pre-equilibrated in G-buffer without ATP. Either ATP or ADP was added to a final concentration of 0.2 mM, and incubated for 15' at 4°C followed by 15' at 20°C. High-pressure liquid chromatography (HPLC) analysis revealed that the actin incubated in ATP was 100% ATP bound, and the actin incubated in ADP was 50% ADP bound and 50% ATP bound.

Isolation of the Actin-bound Nucleotide from Xenopus Egg Extracts

Actin-bound nucleotide was isolated as described in Table 1. High speed supernatants (h.s.s.) of either 100 μl rabbit muscle actin or Xenopus extracts were spun through a 0.7 ml Biogel P-6 column pre-equilibrated in G-buffer without ATP to remove the majority of unbound nucleotides. The flow through (≈ 120 μl) was then added to 25 μl of DNase 1 beads, and incubated at 4°C for 1 h with frequent vortexing. Nonspecific proteins were removed with two 0.5 ml washes of wash buffer (0.4 M NH_4Cl_2, 10 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT) for 1 min and the washed beads were recovered by centrifuging for 1 min at 10 000 $\times$ g in an Eppendorf microfuge. In a typical experiment, only ≈ 23 pmol of actin or 2–3% of the total actin was lost during the washes. The actin was denatured and eluted with 8 M urea, 10 mM Tris, pH 7.4, 0.2 mM $CaCl_2$ at 100°C for 5 min. Ten percent of the eluate was removed for

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the remainder was diluted threefold with H_2O and filtered through a 10,000 kDa cut-off spin filter unit. The isolated nucleotides in the filtrate were then analyzed by HPLC. Total free nucleotides from Xenopus extracts were isolated for HPLC analysis by filtering crude extract through a 10-kDa cut-off spin filter unit.

HPLC Analysis of the Actin-bound Nucleotide

The filtrate from the last step in Table 1 was analyzed on a 1 ml Mono-Q column equilibrated in 100 mM NH_4HCO_3 and eluted in a 100–500 mM NH_4HCO_3 gradient over 30 min at a flow rate of 1 ml/min. Peak areas were analyzed and recorded at OD_{254} using Gilson software. The entire DNase 1 filtrate or 5 μl of crude extract filtrate was loaded for each HPLC run.

Quantitation of Actin by SDS-PAGE

The concentration of rabbit skeletal muscle actin, the actin standard, was determined by absorption spectrophotometry using an extinction coefficient $e_{280} = 49,000$ M^{-1} cm^{-1}. Because the absorbance of ATP in the G-buffer may interfere with the absorbance of actin at OD_{280}, the free and bound ATP were removed before spectrophotometry by denaturating an aliquot of muscle actin with 6 M guanidine HCl and passing over a 0.7-ml Biogel P-6 column pre-equilibrated in G-buffer without ATP. DNase 1 bead eluates and a series of the above actin standards were analyzed by 10% SDS-PAGE followed by Coomassie blue staining. Actin was quantified by cutting the the DNase 1-derived actin and actin standard bands out of the stained gel and eluting the dye in 0.4 ml of 50% methanol, 7% acetic acid at 100°C. The eluted dye was quantitated by spectophotometrically at OD_{595}.

Nucleotide Exchange Analysis

The percentage of nucleotide exchange was determined by dividing the specific activity of actin-bound nucleotide by the specific activity of free nucleotide. To determine the specific activity of bound ATP and ADP from actin, ^{32}P-α ATP was incubated with crude extracts for either 0' or 30' at room temperature, then the actin was isolated as described in Table 1 and the released nucleotides were analyzed by HPLC. Each 1-ml fraction was collected and counted with 5 ml of scintillate on a Beckman Scintillation Counter. From the cpm counted per fraction, we determined the number of moles of ^{32}P-α ATP per moles of ATP in each peak. The specific activity of free nucleotides were determined in a similar manner using total nucleotide that passed through a 10-kDa cut-off filter.

Native Gel Electrophoresis

Proteins or extract h.s.s were analyzed on 7.5% native polyacrylamide gels at 4°C as described (Safer et al., 1990) except that 0.2 mM $MgCl_2$ was added to the gel buffer. Gels were transferred electrophoretically to nitrocellulose in 20 mM Tris, 25 mM glycine, 20% methanol, 0.1% SDS. After blocking in 5% milk in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), the blot was incubated with a 1:1000 dilution of mouse anti-actin monoclonal antibody (Amersham) in TBST + 0.02% NaN_3. Immunoreactive bands were detected using an alkaline phosphatase-conjugated rabbit anti-mouse antibody.

RESULTS

The Use of Xenopus Egg Extracts

We first sought to determine whether active turnover of actin filaments occurs in Xenopus egg extracts by assaying their effect on preformed filaments of rabbit muscle actin. When rhodamine-labeled actin filaments

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Table 1. Isolation and analysis of nucleotide from actin monomer

| Step	Method
Make concentrated Xenopus egg extract	Crush CSF-arrested X.1. eggs @ 21,000 $\times$ g 10'
Spin out F-actin	436,000 $\times$ g 15' @ 4°C
Remove unbound nucleotides	Pass over a Bio-gel P-6 column
Purify actin and bound nucleotide	Bind to DNase 1 beads at 4°C 1 h, wash
Isolate nucleotides from actin	Elute 8 M urea, 100°C; filter through a 10-kDa cut-off filter
Analyze nucleotides	HPLC analysis using a MONO-Q column
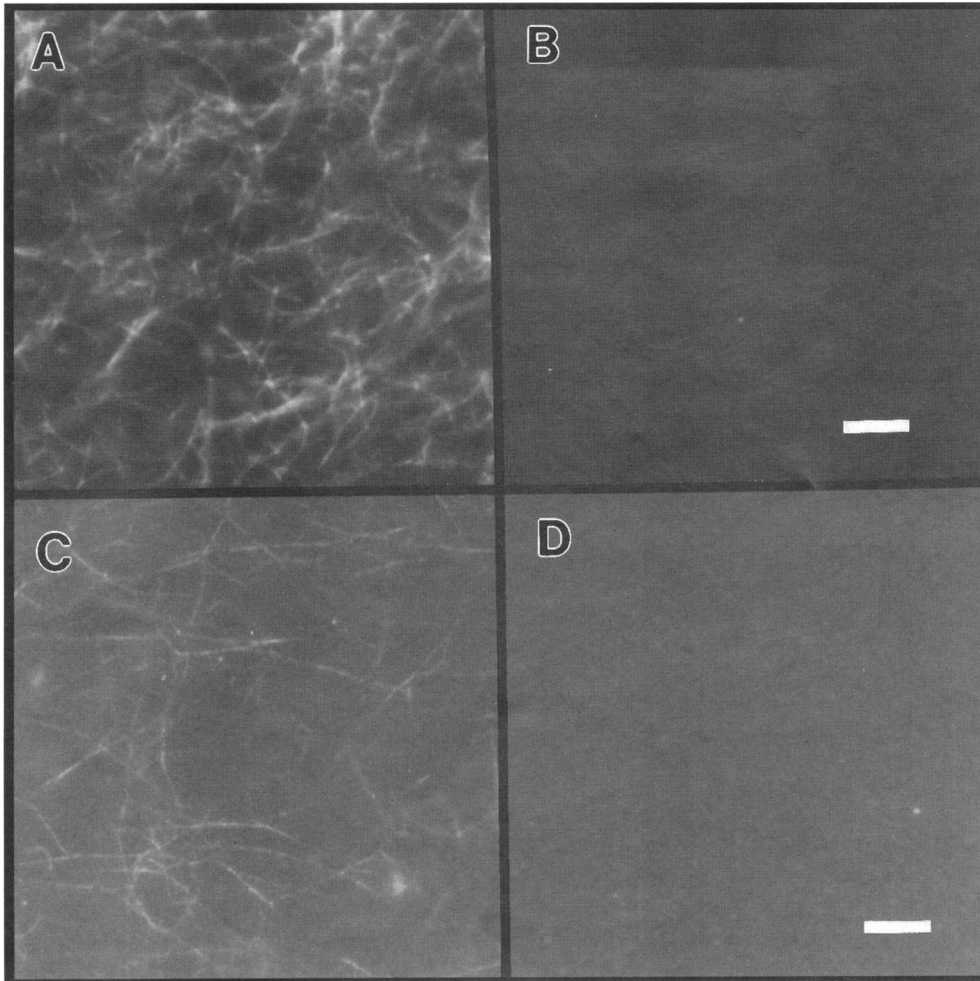
Figure 1. Micrographs of rhodamine-labeled actin filaments. Rhodamine-labeled rabbit muscle actin (0.25 mg/ml) polymerized in F-buffer alone (A) or mixed 1:1 with crude *Xenopus laevis* egg extracts (B). Rhodamine-labeled actin (5 μg) polymerized in 10 μl *Xenopus laevis* egg extracts before (C) and after (D) removal by high speed (436,000 × *g*) centrifugation. Bar represents 5 μm for (A) and (B) and 2.5 μm for (C) and (D).

(Figure 1A) were added to an equal vol of *Xenopus* extract they depolymerize within ≈1–2 min (Figure 1B). Dilution into buffer had no effect on these filaments. In fact, part of the released, labeled actin reincorporates into endogenous filaments in the extract, but these are not visible with the low amount of labeled actin in this experiment. In addition, higher concentrations of rhodamine-G-actin added to *Xenopus* egg extracts incorporated into endogenous filaments within ≈5 min (Figure 1C). By eye, these filaments could be seen to depolymerize and repolymerize over time suggesting that the actin in these extracts is not polymerized to equilibrium, but is fluxing rapidly between monomer and polymer to create a steady state, like actin in motile cells. Detailed imaging experiments are presently being done to more thoroughly analyze these actin dynamics. Although we could not see repolymerization of actin filaments in Figure 1B, we believe that the actin is being reassembled but that the rhodamine signal is too dilute to visualize these filaments, compared with that

in Figure 1C where the actin is more concentrated. A possible caveat with these experiments is that the behavior of rhodamine-labeled rabbit muscle actin may not accurately report the behavior of endogenous *Xenopus* egg actin. Holliday *et al.* (1993) have reported that pyrene-labeled muscle actin is not an accurate reporter of endogenous actin behavior in *Acanthamoeba* extracts. Resolving this issue will require more work. However, we feel that the results of Figure 1 combined with the *Listeria* data (Theriot *et al.*, 1994) suggest that *Xenopus* egg extracts mimic cytoplasmic actin dynamics. Therefore, we used these extracts as a model for cellular actin dynamics from which we could isolate the unpolymerized actin and analyze its nucleotide content.

Isolation of Actin-Bound Nucleotide

To analyze the actin-bound nucleotide in *Xenopus* egg extract we developed the procedure shown in Table 1, which depends on the high affinity of DNase 1 for

actin, and the fact that DNase 1 binding blocks nucleotide exchange. Approximately half of the actin in the *Xenopus* egg extracts is sedimentable, presumably corresponding to F-actin. It was necessary to remove this F-actin from the extracts before isolating the G-actin. Although DNase 1 beads should preferentially bind G-actin, we found in test experiments that a significant amount of actin derived from filaments also bound to DNase 1 beads. This binding may result from DNase 1 binding to the pointed end of the actin filament or the induction of F-actin depolymerization by DNase 1 and subsequent binding of the G-actin (Hitchcock, 1980). Because the bulk of F-actin is ADP-bound, binding of either F-actin or recently depolymerized G-actin caused artificially high levels of ADP to appear in the DNase 1-derived actin in test experiments. To eliminate this potential artifact, high speed centrifugation was used to remove the F-actin. Comparison of extracts with added rhodamine-labeled actin before (Figure 1C) and after centrifugation (Figure 1D) by fluorescent microscopy confirmed that centrifugation was sufficient to remove detectable F-actin from the extracts. Quantitative Western blot analysis showed that the total actin concentration in *Xenopus* egg extracts varied between 15–25 μM and approximately 50% of the total actin remained soluble after centrifugation.

A P-6 desalting column was used to remove the majority of free nucleotide from the h.s.s. before actin isolation and to equilibrate the actin into a buffer in which it would not polymerize during the isolation step. Although DNase 1 binding inhibits actin nucleotide exchange, free nucleotide was removed to try to prevent exchange from occurring during the relatively slow binding step. By HPLC analysis of soluble components derived from the 10-kDa filtrate of a P-6 flow through, we estimate that >99.8% of the unbound nucleotide from *Xenopus* egg extracts is removed by the P-6 desalting column. However, after desalting, only 5% of the remaining ATP bound to DNase 1 beads under conditions where >90% of the actin bound (estimated by using radioactive ATP as a tracer). Thus, the desalted extracts contain an excess of ATP presumably bound to other nucleotide-binding proteins. This ATP can still potentially exchange onto actin during the DNase 1 binding step. In test experiments, we found that actin-bound nucleotide profiles were in fact similar with or without the desalting step. We retained this step to decrease the amount of exchange during nucleotide isolation and to ensure reproducibility.

DNase 1 beads proved effective in isolating actin and its bound nucleotide from *Xenopus* h.s.s (Figure 2, lane 3), and no detectable actin bound to the unconjugated beads (lane 4). By quantitating the supernatant and pellet of the *Xenopus* extract DNase 1 precipitation using Coomassie blue binding and Western blot analysis, we determined that 90–95% of the actin from
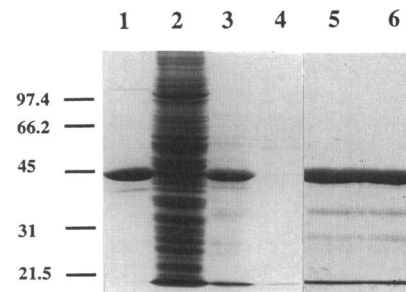

Figure 2. Coomassie blue stained SDS-PAGE of 5 μg rabbit skeletal muscle actin (lane 1), crude *Xenopus laevis* egg extract (lane 2), *Xenopus laevis* actin purified from DNase 1 beads (lane 3), and proteins bound to unconjugated Affigel-10 beads (lane 4). DNase 1 beads bind ATP-actin (lane 5) and ADP-actin (lane 6) equally well.

h.s.s. was recovered on the DNase 1 beads. In control experiments with pure actin we found that DNase 1 beads bind ATP- and ADP-actin equally well (Figure 2, lanes 5 and 6). Incubation of DNase 1 beads with ^{32}P-α ATP and excess cold ATP demonstrated that <2–4 pmol of the free nucleotide (<0.6% of the nucleotide bound to actin) bound to the DNase 1 beads in the absence of actin.

HPLC Analysis of Actin-bound Nucleotide

Actin and its bound nucleotide were released from the DNase 1 beads by denaturing with urea and heat treatment. The released nucleotides were analyzed on a Mono-Q HR 5–5 column (Figure 3, A–C). Two peaks were observed from nucleotide released from either pure actin or actin isolated from *Xenopus* egg extracts (Figure 3, A and B). These two peaks comigrate with ATP and ADP, and were quantitated using ATP and ADP standards. The total released nucleotide was compared with the amount of actin bound to the DNase beads quantitated by Coomassie blue binding. In a typical run, 0.95 nmol of nucleotide was released from 0.93 nmol of actin purified from 100 μl of crude *Xenopus* extract. The range over 22 experiments was between 0.7–1.1 nmol per 100 μl for both actin and released nucleotide. This corresponds to the soluble actin concentration of 7–11 μM, in agreement with our Western blot analysis. These values, together with our control experiments, make us confident that the majority of the nucleotide we were analyzing was derived from the bound actin.

As expected, greater than 99% of the nucleotide released from pure G-actin was ATP. Thus, no significant ATP hydrolysis occurs during the isolation steps. Nucleotide released from *Xenopus* extract actin (Figure 3B) had slightly more ADP, but the ratio of ATP to ADP was consistently high ($\approx$9:1). This ratio was similar to that for free nucleotide in the same extracts (Figure 3C). The mean ATP:ADP ratio for actin-bound
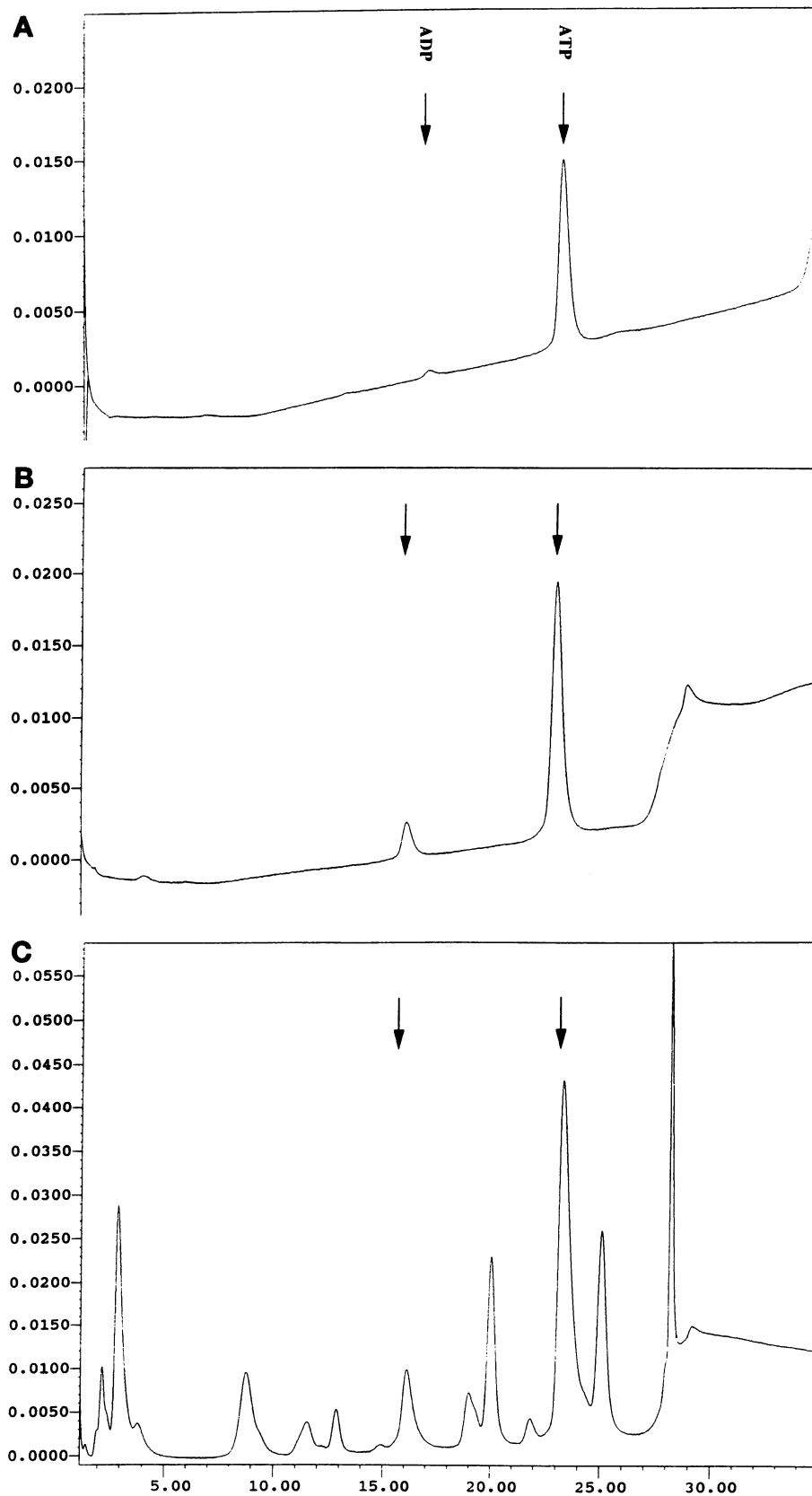
Figure 3. High pressure liquid chromatographs of nucleotides released from rabbit muscle actin DNase 1 bead eluate (A), *Xenopus* egg extract DNase 1 bead eluate (B), and nucleotides from 10-kDa cut-off filtrate of crude *Xenopus* egg extracts (C). The x-axis is elution time in minutes, and the *y*-axis is OD_{254}.

Table 2. Percentage of actin-bound nucleotide that exchanges with free nucleotide in *Xenopus* egg extracts

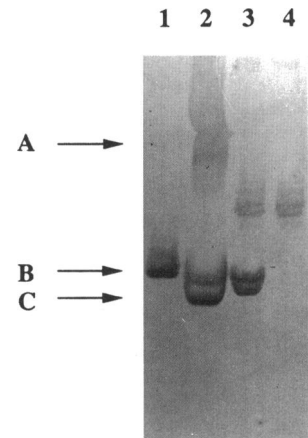| Incubation time before analysis[a]	% ATP exchanged	% ADP exchanged	Number of experiments
0' at 25°C	47 ± 3	30 ± 2.5	3
30' at 25°C	42 ± 7.5	30 ± 7	5

[a]Incubation time before analysis represents time that the $^{32\alpha}$P-ATP is incubated with crude extracts before isolating the actin and its bound nucleotide. Therefore, 0' at 25°C represents the dead-time for nucleotide isolation.

Figure 4. Anti-actin immunoblot of a nondenaturing gel of 0.9 μg rabbit muscle actin (lane 1), 85 μg *Xenopus laevis* extract h.s.s. (lane 2), 0.9 μg rabbit muscle actin mixed with ≈1.6 μg *Xenopus laevis* extract boiled supernatant (lane 3), and ≈1.6 μg boiled supernatant alone (lane 4). Arrow A indicates migration of unidentified actin complex and/or denatured actin, arrow B the migration of pure muscle actin, and arrow C the migration of actin complexed with a thymosin β₄-like protein.

nucleotide is 8.4 ± 4.2 over 22 experiments and the mean ratio for free nucleotides in *Xenopus* extracts is 7.6 ± 3.1 over nine experiments. Because >90% of the soluble actin was recovered in these experiments, and 1 mol of nucleotide was released per 1 mol of actin, these data indicate the absence of a large pool of ADP-actin.

Nucleotide Exchange in Soluble Actin

The similar ATP:ADP ratios for actin-bound and free nucleotide might reflect rapid nucleotide exchange in the soluble actin pool. If this were true, it would further suggest that control of nucleotide exchange is not the key regulator of actin polymerization. To test this possibility, we used ^{32}P-α ATP to estimate the rate and extent of nucleotide exchange occurring in the unpolymerized actin pool. ^{32}P-α ATP was added to crude extracts and allowed to incubate for either 0 or 30 min at 25°C. Actin was then isolated as outlined in Table 1, its nucleotide content analyzed by HPLC, and the specific activity determined. The specific activity of total unbound ATP and ADP was then determined by fractionating total unbound nucleotide, obtained as an ultrafiltrate, on HPLC. The percentage exchanged was obtained as the ratio of the specific activity of actin-bound nucleotide to the specific activity of free nucleotide in the same extract (Table 2). We found that the actin nucleotide exchange rate was rapid because the amount of exchange occurring within the dead-time of the isolation procedure (0 min at 25°C) was the same as that within 30 min at 25°C. We should note that this dead-time is difficult to accurately estimate but is potentially quite long. As discussed above, considerable nonactin bound ATP remains after the desalting step. Although this ATP is presumably bound to other proteins, it may still be able to exchange with actin-bound nucleotides during the DNase 1 binding step. Thus, the dead-time of the experiment could be as high as 60' at 4°C. Interestingly, only 30–50% of the actin exchanged with ^{32}P-α ATP at both time points. Thus, there appear to be two separate populations of actin in *Xenopus* extracts, one that exchanges nucle-

otide rapidly, and another which apparently does not exchange.

The Bulk of Soluble Actin Is in a Protein Complex

Analysis of the soluble actin pool in a h.s.s. of *Xenopus* egg extract by the native gel electrophoresis method of Safer (Safer, 1989) demonstrated the existence of three distinct actin species (Figure 4, lane 2). The first is a faint streak of slowly migrating actin (band A) in the extract lane, which could represent denatured actin or large, poorly resolved actin complexes. The more striking species are the two bands that migrate near pure actin (bands B and C). The faster migrating band (C) could represent a complex of actin and thymosin β4 (T β4), or a T β4-like protein (Safer *et al.*, 1990). T β4 is heat stable, so we tested boiled extract supernatant for its ability to cause this same shift in actin mobility. When boiled extract supernatant was added to rabbit muscle actin it increased the mobility of rabbit muscle actin (lane 3) as expected from a T β4-like activity. This result suggests that *Xenopus* extracts contain a T β4-like activity that may account for band C in extracts. On average, native gels of *Xenopus* extracts showed that between 50% to 80% of the actin is in a complex with the T β4-like activity.

DISCUSSION

Actin in Xenopus Egg Extracts

We used *Xenopus* egg extracts to analyze the state of unpolymerized actin because they are made with little dilution of the cytoplasm. *Xenopus* egg extracts can support cycles of actin filament assembly/disassembly (Figure 1), although caution is required in interpreting the behavior of labeled muscle actin in extracts. A stronger argument that *Xenopus* extracts support normal actin dynamics comes from analysis of *Listeria* behavior. *Listeria* can rapidly induce polymerization from the soluble actin pool, and the fil-

aments in the resulting *Listeria* tail are rapidly de-polymerized. Both events occur with kinetics similar to that seen in the cytoplasm of tissue culture cells (Theriot and Mitchison, 1992). It is formally possible that the actin in extracts is nondynamic, and *Listeria* induces dynamics, but we favor the simpler interpretation that the extracts mimic normal cytoplasmic regulation. Because *Listeria* motility does not depend on exogenously added actin, it reports on endogenous dynamics. Taken together, our data strongly suggest that actin polymerization in *Xenopus* egg extracts is subject to a similar regulation as that in somatic cells.

Analysis of the Actin-bound Nucleotide

In developing the protocol shown in Table 1, we sought to isolate the soluble actin pool using a rapid, nonperturbing method. However, some perturbations were inevitable for this isolation. We found it necessary to remove F-actin by high speed centrifugation before isolating the G-actin on DNase 1 beads because, in pilot experiments, a substantial amount of actin derived from filaments bound to DNase 1 beads. The centrifugation step could affect our nucleotide analysis because it disturbs the normal polymerization-depolymerization cycle that occurs within the extract by removing filaments. This perturbation could potentially alter the balance between nucleotide exchange and hydrolysis as compared with that in the cell. In addition, the centrifugation step could remove other unpolymerized actin populations in high molecular weight complexes that are important for polymerization (Hashimoto and Tatsumi, 1988). Thus, although we believe our results are relevant to the bulk pool of unpolymerized actin in cells, they may not be relevant to local regions such as the leading edge where rapid turnover of filaments is occurring.

Our analysis of actin-bound nucleotide in the soluble actin pool in *Xenopus* egg extracts showed that more than 80% of the unpolymerized actin contains bound ATP. Thus, when *Listeria* is recruiting soluble actin to rapidly assemble filaments in extracts, it must be recruiting actin that already contains ATP. This in turn means that the exchange of ADP for ATP is probably not a rate-limiting step for polymerization. The release of ATP-actin from the inhibitory effect of a sequestering protein is more likely to be a limiting step, or it may be that in fact filament elongation or nucleation are themselves limiting. Our results do not imply that the exchange reaction is not important because in the absence of profilin or some other exchange activity it is possible that ADP-actin would accumulate. For example, ADP-actin might accumulate as a kinetic intermediate in a region of the cell where the actin depolymerization rate is high. In such regions, profilin may be an

important catalyst of nucleotide exchange (Goldschmidt *et al.*, 1992).

Nucleotide Exchange on Actin

Our experiments testing the rate of actin nucleotide exchange showed that the extent of exchange was independent of incubation time. In test experiments where we incubated extracts with ^{32}P-α ATP at 25°C over of a time course from 0 to 120 min before analysis, we found that the amount of actin nucleotide exchange had plateaued by 0 min, i.e., the dead-time of the assay. We had concerns with taking time points much longer than 2 h because the ATP becomes consumed and the extracts begin to die at such long time points. For these reasons, we performed careful analysis for just two time points, 0 and 30 min at 25°C. The time resolution of the experiment is severely limited by technical constraints discussed in the MATERIALS AND METHODS section. Given the long dead-time of our assay, it is clearly unsuited for determining accurate exchange kinetics in cytoplasm.

Although nucleotide exchange on actin appears to be fairly rapid, it occurs in less than half of the actin population. As yet, we have not been able to determine how the exchanging and nonexchanging actin populations relate to the different species resolved by native gel electrophoresis. T β_4 blocks actin nucleotide exchange, but because T β_4 itself exchanges with actin this molecule alone may not account for our nonexchangeable fraction. So far we have been unable to alter the extent of exchange by adding either *Listeria* or boiled supernatants to the extracts. The fact that nucleotide exchange on actin in these extracts is not complete may also be reflected by the fact that the ratio of ATP-actin:ADP-actin is similar to the ratio of free ATP:ADP within the extracts. Given that the affinity of actin for ATP is $\approx$fourfold greater than for ADP (Kinosian *et al.*, 1993), we might expect that the ratio of ATP-actin:ADP-actin would be higher that the free ATP:free ADP if all of the actin was freely exchanging.

Given our exchange data, how confidently can we assert that unpolymerized actin is mostly ATP-bound in vivo? For the nonexchanging $\approx$60% of the actin, the ATP we analyzed must be the same ATP that was bound in vivo. For the exchanging $\approx$40%, the ATP we analyzed was effectively introduced during isolation, and could potentially have been ADP in vivo. However, this fraction would tend to rapidly equilibrate with unbound nucleotide, and is thus presumably mostly ATP-bound in vivo. Thus we are confident that no large pool of ADP-actin exists in *Xenopus* egg extracts.

Monomer Binding Proteins

Band C from *Xenopus* egg extracts (Figure 4, lane 2) most likely represents an actin complex with T β_4 or a T β_4-like protein because boiled *Xenopus* extract supernatants (Figure 4, lane 3, band C) and 10-kDa filtrates of these supernatants (unpublished data), fractions that contain T β_4 in extracts of mammalian cells, can produce a similar band when bound to rabbit muscle actin. Although this band does not exactly comigrate with band C in the *Xenopus* extracts lane (lane 2), this difference in shift could be due to the use of a different species of actin. A similar slight difference in mobility has been seen with actin complexes found from supernatants from resting polymorphonuclear leukocyte extracts versus muscle actin complexed with a T β_4-containing fraction (Cassimeris *et al.*, 1992).

If band B in the *Xenopus* h.s.s. lane (lane 2) represents uncomplexed ATP-actin, its concentration (≈ 2 μM-6 μM) appears to be quite high considering that it is unpolymerized and the critical concentration is 0.2 μM. When native gels were run without ATP in the buffer, pure rabbit muscle actin ran as a smear, presumably due to denaturation. However, omission of ATP did not affect the mobility of band B from *Xenopus* extracts. Thus, band B could represent an altered form of actin or uncharacterized actin complex that is sequestered from polymerization.

In summary, we have found that the majority of soluble actin from *Xenopus* extracts is ATP-bound. One population of the soluble actin exchanges with cytoplasmic nucleotide whereas the other does not. In addition, most of the soluble actin is in a complex with what appears to be the sequestering protein, thymosin β_4. Our results are not consistent with the existence of a large pool of ADP-actin in vivo, at least in resting cytoplasm. However, we could not rule out build up of significant levels of ADP-actin in areas of rapid filament depolymerization. Notwithstanding this possibility, our results on the bulk of cellular actin suggest that nucleotide exchange is probably not the key regulatory step for polymerization in vivo. More likely, actin assembly is regulated by release of ATP-actin from a complex with a sequestering protein. Our work shows considerable heterogeneity in the unpolymerized actin pool. Future work will need to examine how these different populations relate to each other, and which population of soluble actin is used when actin polymerization is induced.

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