Control of the Ability of Profilin to Bind and Facilitate Nucleotide Exchange from G-actin*

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A major factor in profilin regulation of actin cytoskeletal dynamics is its facilitation of G-actin nucleotide exchange. However, the mechanism of this facilitation is unknown. We studied the interaction of yeast (YPF) and human profilin 1 (HPF1) with yeast and mammalian skeletal muscle actins. Homologous pairs (YPF and yeast actin, HPF1 and muscle actin) bound more tightly to one another than heterologous pairs. However, with saturating profilin, HPF1 caused a faster etheno-ATP exchange with both yeast and muscle actins than did YPF. Based on the k-fold change in ATP exchange rate/Kd for ATP binding, the homologous pairs are more efficient than the heterologous pairs. Thus, strength of binding of profilin to actin and nucleotide exchange rate are not tightly coupled. Actin/HPF interactions were entropically driven, whereas YPF interactions were enthalpically driven. Hybrid yeast actins containing subdomain 1 (sub1) or subdomain 1 and 2 (sub12) muscle actin residues bound more weakly to YPF than did yeast actin (Kd = 2 μM versus 0.6 μM). These hybrids bound even more weakly to HPF than did yeast actin (Kd = 5 μM versus 3.2 μM). sub1/YPF interactions were entropically driven, whereas the sub12/YPF binding was enthalpically driven. Compared with WT yeast actin, YPF binding to sub1 occurred with a 5 times faster koff and a 2 times faster kon, sub12 bound with a 3 times faster koff and a 1.5 times slower kon. Profilin controls the energetics of its interaction with nonhybrid actin, but interactions between actin subdomains 1 and 2 affect the topography of the profilin binding site.

The ability of profilin to preferentially sequester ATP G-actin and to facilitate adenine nucleotide exchange from the actin is important, considering the role that an actin-dependent ATP hydrolysis cycle plays in actin dynamics. G-actin is a poor ATPase whose activity is stimulated by polymerization. Subsequent discharge of the P from ADP-Pi, F-actin occurs at different rates, depending on the particular actin isoform involved, and results in the generation of ADP-F-actin (11, 12). In terms of filament stability, ATP and ADP-Pi actin are more stable than ADP-F-actin (13). After release of ADP-monomers from the end of the actin filament, the ADP exchanges for ATP, and the polymerization cycle starts again. Profilin may have a role in facilitating this exchange and thereby help to regulate the dynamics of actin filament formation (13). However, the necessity for this rate enhancement may not be universally applicable. For example, plant profilin does not catalyze nucleotide exchange from actin (14). However, it could complement a profilin-deficient strain of Dictyostelium discoideum with little if any loss of normal cell behavior (15).

Although profilin generally is not thought of as an enzyme, it actually acts as one in its facilitation of the actin nucleotide exchange reaction. It must first reversibly bind to the actin and then cause a conformational change that results in enhanced rates of release of the bound adenine nucleotide. Initial studies revealed that the extent of the enhancement is highly dependent on the type of profilin used. For example, under saturating conditions, with muscle actin, mammalian profilin enhances the rate of exchange 30–1000-fold (16), yeast profilin enhances it 3-fold (17), and profilin from the plant Arabidopsis shows no enhancement of exchange rate (14).

Initial studies also suggest that the nature of the binding of profilin to actin seems to depend on both the profilin and the actin involved. For example, human platelet profilin binds muscle actin about 50-fold more tightly than it binds yeast actin (17). The energetics that define the actinprofilin interaction can be very different depending on the particular actinprofilin pair involved. Based on incomplete data obtained so far, change in enthalpy seems to control the interaction of yeast profilin with muscle actin, whereas change in entropy seems to drive the interaction of human profilin with muscle actin (17). Whether this difference applies only to these two actin-profilin pairs or is more general is not known. Insight into the molecular basis of the profilinactin interaction came from the crystallographic studies of the profilinactin complex carried out by Schutt and co-workers (18, 19). Profilin binds to actin across actin subdomains 1 and 3 at the barbed end of the actin monomer, and this interaction seems to result in an opening of the cleft separating the two domains of the actin molecule in which

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the ATP resides. This opens occurring by a pivoting motion of the two domains around a hinge region involving a helix containing residues 137–144 in the bridge between subdomains 1 and 3 (20). However, the manner in which this movement is brought about is not understood.

To gain more insight into the mechanism governing the profilin-dependent acceleration of the release of actin-bound nucleotide, we have carried out a detailed study of both the binding and exchange reactions involving the interaction of both yeast and human profilins with both muscle and yeast actins. We were especially interested in the yeast/muscle actin comparison, because yeast actin inherently exchanges its nucleotide 30 times faster than does muscle actin despite the 87% homology between the two proteins (17, 21). We also present work with a hybrid actin we have constructed in which subdomains 1 and 2 are from muscle actin and subdomains 3 and 4 are from yeast actin to better understand the relative importance of subdomains 1 and 3 in its interaction with profilin.

MATERIALS AND METHODS

Protein Preparations—Yeast hybrid and H372R mutant actins were generated as described previously (22, 23). Yeast wild type (WT)2 and mutant actins were purified by a combination of DNase I affinity chromatography and DEAE-cellulose chromatography as described by Cook et al. (24). Globular actins (G-actins) were stored in Globular buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl₂, 0.2 mM ATP, and 0.1 mM dithiothreitol) at 4 °C and used within 5 days. The yeast profilin and human profilin I Escherichia coli expression plasmids were kindly provided by S. Almo (Albert Einstein College of Medicine) and D. Schafer (University of Virginia), respectively. The mutant human profilin I molecules were engineered using the QuikChange directed mutagenesis kit from Stratagene (La Jolla, CA) and 50 μg/ml DNase I (Worthington) from yeast actin to better understand the relative importance of subdomains 1 and 2 in its interaction with profilin.

VOLUME 283 • NUMBER 14

For assessing the effects of profilin on the nucleotide exchange rate, the data at different profilin concentrations were analyzed by Excel and fitted to Equation 1,

\[ k_{\text{obs},[A]} = k_{[A]} + (k_{\text{eq}} - k_{\text{eq}}) \]

\[ \cdot \frac{(k_{\text{eq}} + [P] + [A])^2 - \sqrt{(k_{\text{eq}} + [P] + [A])^2 - 4([P][A])}}{2} \]  

\[ (\text{Eq. 1}) \]

which is derived from Equation 2,

\[ k_{\text{obs}} = k_{[A]} + k_{[PA]} \]  

\[ (\text{Eq. 2}) \]

where \( k_{\text{obs}} \) is the observed dissociation rate constant, \( k_{[A]} \) is the dissociation rate constant of G-actin in the absence of profilin, \( k_{[PA]} \) is the theoretical dissociation rate constant of the profilin-G-actin complex, \( [A] \) is the concentration of total actin, \( [A] \) is the concentration of free G-actin, \([P]\) is the concentration of free profilin, and \( K_d \) is the dissociation equilibrium constant of the complex. To obtain the best fit, \( k_{[PA]} \) was subjected to the constraint that it is larger than \( k_{[A]} \).

Isothermal Titration Calorimetry (ITC)—ITC measurements were performed using a VP-ITC calorimeter (MicroCal, Northampton, MA). The concentrations of the profilin and actin were measured by UV absorption at 280 or 290 nm as described above, and the proteins were degassed before each experiment. Titrations were performed in 20 mM PIPES, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, and 1 mM dithiothreitol. The concentrations of profilin and the actin mutants varied among experiments, and all interactions were repeated two times. Heats of dilution were calculated by averaging the last 3–5 injections and then were subtracted from the raw data. The data sets were then analyzed individually using a single-site binding model from the ORIGIN ITC analysis software package provided by the VP-ITC calorimeter manufacturer. In this analysis, the values for stoichiometry (n), change in enthalpy (\( \Delta H \)), and the affinity constant (\( K_d \)) were fit using nonlinear least squares

2 The abbreviations used are: WT, wild type; ITC, isothermal titration calorimetry; εATP, etheno-ATP.
analysis. The reported values for $n$, $\Delta H$, and $K_a$ are the average and S.D. of all injections for an individual interaction.

**Kinetics of Profilin Binding to G-actin**—The kinetics of the binding of profilin to G-actin was monitored over time by the decrease in intrinsic tryptophan fluorescence caused by the interaction using a BioLogic SFM3 stopped-flow instrument (BioLogic). The data were further analyzed with Kin-sim/Fitsim software (available on the World Wide Web) and fitted to the following model (Reaction 1),

$$A + P \rightleftharpoons AP$$

**REACTION 1**

where $A$ represents G-actin, $P$ is profilin, and AP is the actin-profilin complex. The kinetic rate constants ($k_{on}$ and $k_{off}$) of profilin binding to actin were obtained from the average of fitting data from at least three sets of experiments with different profilin concentrations. The $K_d$ is calculated from the results of $k_{off}/k_{on}$.

**RESULTS**

Our goal was to determine the molecular basis underlying the ability of profilin to facilitate the exchange of nucleotide from actin. Toward this end, we analyzed the interaction of yeast and human profilins with yeast and muscle actin. These two actin isoforms are 87% identical in sequence (21), and they only vary in three residues in what is thought to constitute the profilin binding surface (17). Residues Glu$^{167}$, Tyr$^{169}$, and Arg$^{372}$ in muscle actin are replaced by Ala$^{167}$, Phe$^{169}$, and His$^{372}$ in yeast actin, respectively.

We first assessed the rate of nucleotide exchange brought about by increasing concentrations of profilin in the presence of a constant amount of actin. In this analysis, the $K_d$ for the actin-profilin interaction can be derived from the first-order rate constants for the decrease in fluorescence resulting from the exchange of bound eATP from the actin surface. This analysis has been used previously (5) and is described under “Materials and Methods.” Fig. 1A shows the increase in nucleotide exchange rate from yeast actin caused by saturating concentrations of yeast profilin (YPF) and human profilin 1 (HPF1). HPF1 produces a 10-fold increase in this rate, whereas YPF produces only a 4-fold acceleration (Table 1). For muscle actin with YPF, there was 3-fold activation (17), and with HPF1 and muscle actin, there was a 35-fold enhancement (data not shown).

Clearly, the small enhancement of nucleotide exchange previously observed with yeast actin and yeast profilin does not result from some maximal rate at which a yeast actin can exchange nucleotide due to its inherently more open conformation (17, 26). HPF1, at saturating conditions, is simply a better catalyst of nucleotide exchange than YPF, whether yeast or muscle actin is utilized.

Fig. 1B shows the rate constants calculated from curves similar to those described in Fig. 1A for the interaction of different concentrations of YPF or HPF1 with yeast actin. Table 1 shows that like the case involving the two profilins with muscle actin (17), the homologous pair had a $K_d$ about 5 times stronger than that exhibited by the heterologous pair. However, it is apparent
from the data that $K_d$ and rate catalytic ability do not strictly correlate.

A better measure of catalytic activity with respect to enzyme function is the catalytic efficiency or $k_{cat}/K_d$, since most enzymes do not work at saturating conditions. This may very well be the case in vivo for profilins (16, 27). To apply this analysis to the profilin/actin system, for a given actin, we divided the $K_d$ of the particular actin-profilin pair. With yeast actin, this efficiency measure was $6.7 \mu M^{-1}$ for YPF and only $3 \mu M^{-1}$ for HPF1 (Table 1). Based on the data referred to above, the catalytic efficiency for the HPF1-muscle actin pair is $350 \mu M^{-1}$. From the work of Eads et al. (17), the catalytic efficiency of the YPF-muscle actin pair is about $1 \mu M^{-1}$. In summary, the homologous set of proteins was always more efficient than the heterologous pair.

We next used ITC to examine the energetics that characterized the interaction of yeast actin with yeast and human profilins. Fig. 2A shows the experimental data for the repeated injection of YPF into a solution of yeast actin, and Fig. 2B presents the corrected integrated data with a curve fit for the data in Fig. 2A. Values for $\Delta H$ and $T\Delta S$ were then extracted from these data as described under “Materials and Methods.” Table 2 shows these values along with the corresponding values for $K_d$ and $K_d$ for the interaction of yeast and human profilins with yeast actin. For the sake of comparison, values obtained previously for the interaction of these two profilins with muscle actin are shown (17). With both actins, the interaction with YPF is strongly enthalpically driven. The change in entropy is actually unfavorable. Conversely, for HPF1, although the change in enthalpy is favorable, it is much less so than for yeast actin. This lower $\Delta H$, however, is offset by a favorable change in entropy. The data demonstrate that the nature of the profilin isoform and not the actin isoform appears to dictate the energetics that characterize the interaction of these two proteins. It is interesting that the nature of the interactions is so different, considering that the profilin binding surface on actin is very much alike for the different isoforms involved.

**Role of Human Profilin Residue Glu82 in the Actin-bound Nucleotide Exchange—**We wished to gain insight into the reason that HPF1 produced a faster nucleotide exchange rate than did YPF. In comparison with YPF, mammalian profilins contain an extra loop between Leu78 and Asp86. Based on the β-actin-bovine profilin co-crystal (18), Glu82 in this loop might form a hydrogen bond with actin Lys113 in subdomain 1, which is conserved in both yeast and muscle actins, as shown in Fig. 3. Lys113 is located on the back face of the actin monomer close to His73 and the hinge region. This extra actin Lys113-profilin Glu82 hydrogen bond interaction might enhance the ability of profilin to open the actin cleft, leading to the greater rate of exchange that is observed. To test this hypothesis, we mutated HPF1 Glu82 to Lys, Ser, or Ala and assessed the actin-bound eATP release rate in the presence of the mutant profilins. Fig. 4 demonstrates that at saturating concentrations, all three mutant profilins facilitate the yeast actin nucleotide exchange 9–13-fold, respectively, similar to the value obtained with WT HPF1. The mutations also cause little if any effect on the $K_d$ for the actin-profilin interactions (data not shown). A similar enhancement for muscle actin-bound ATP was also observed (data not shown). Thus, the ability to form this postulated hydrogen bond is not critical for catalysis of nucleotide exchange or for the affinity of the interaction (data not shown).

**Role of Actin Residue 372 in the Actin-Profilin Interaction—**It had been suggested that Arg77 in subdomain 1 of muscle actin, via its ability to hydrogen-bond with residue Tyr79 in Schizosaccharomyces pombe profilin (Tyr78 in Saccharomyces cerevisiae profilin; Asp86 in mammalian profilin), played a major role in profilin-dependent catalysis of actin nucleotide exchange (28). Yeast actin contains a His at this position. To address the importance of Arg772 in the actin-profilin inter-

![TABLE 2](image)

Thermodynamic parameters obtained from ITC for the interaction between different profilins and actins

|       | Yeast actin | Muscle actin | HPF1-yeast actin | Human platelet profilin-muscle actin |
|-------|------------|--------------|------------------|-------------------------------------|
| $\Delta H$ (kcal/mol) | $-10 \pm 0.5$ | $-12.1$ | $-4.4 \pm 0.2$ | $-3.4$ |
| $T\Delta S^0$ (kcal/mol) | $-1.3$ | $-4.5$ | $2.74$ | $6.2$ |
| $K_d$ (μM) | $2.6 \pm 0.3$ | $0.2 \pm 0.5$ | $0.1$ | $0.2$ |
| $K_d'$ (μM$^{-1}$) | $0.4$ | $2.9$ | $5.5$ | $0.1$ |

$^a$ The data for the binding of profilins with muscle actin as determined by Eads et al. (17).
$^b$ The value is indirectly obtained for the computer simulation as described under "Materials and Methods."
action, we assessed the ability of YPF to catalyze nucleotide exchange from H372R yeast actin. As with WT actin, the profilin-catalyzed rate was 2.5 times that observed for actin alone. However, the binding affinity of this mutant actin ($K_d = 0.06 \, \mu M$) was about 10-fold greater than observed with WT actin, resulting in a large increase in catalytic efficiency for the YPF-dependent nucleotide exchange from 6.7 to 42 $\mu M^{-1}$. Again, these observations demonstrate a lack of correlation between tightness of the binding and catalytic ability at saturating profilin concentrations.

Characterization of Profilin Interaction with Yeast/Muscle Hybrid Actins—To gain greater insight into the relative contribution of the two actin domains to actin-actin-binding protein interactions, we had previously constructed two yeast/muscle hybrid actins in which either yeast actin subdomain 1 (sub1) or both subdomain 1 and 2 (sub12) had been converted to its muscle counterparts (22). In the most complete hybrid, sub12, 21 yeast residues had been substituted with their corresponding muscle residues. As the extent of muscle character of the actin increased, the nucleotide exchange rate slowed until in sub12, it was equal to that observed with muscle actin (22). Evidently, the structure of subdomain 1 of actin plays a major role in dictating the nucleotide exchange properties of the protein.

**FIGURE 3.** Location of actin Lys$^{113}$ and profilin Glu$^{82}$ in the actin-profilin complex. Actin Lys$^{113}$ (green) and bovine profilin Glu$^{82}$ (blue) are highlighted in the co-crystal structure of β-actin (light red) and bovine profilin (light blue) (Protein Data Bank code 2BTF). The ribbon structure of the extra loop from Leu$^{78}$ to Asp$^{86}$ in bovine profilin, absent in YPF, is in red. His$^{73}$ (purple) and the hinge region (brown ribbon) are highlighted.

**FIGURE 4.** Time course of $\alpha$ATP exchange of yeast actin with mutant human profilin I. The release of actin-bound $\alpha$ATP bound from 1 $\mu M$ yeast actin in G buffer without free ATP in the presence of 15 $\mu M$ HPF1 E82S (), E82A (○), or E82K (□) was triggered by the addition of 250 $\mu M$ ATP. The decrease in fluorescence caused by $\alpha$ATP exchange was followed over time, and data were fit to a first order reaction mechanism (solid lines) as described under “Materials and Methods.” The experiment was repeated with essentially the same results, and one data set is shown here. a.u., arbitrary units.

**FIGURE 5.** Dependence of hybrid actin $\alpha$ATP exchange rates on profilin concentration. The observed dissociation rates ($K_{obs}$) obtained from the actin-bound $\alpha$ATP exchange of 1 $\mu M$ sub1 actin (A) and sub12 actin (B) in the presence of either yeast (○) or muscle profilin (□) were plotted against varying profilin concentrations. Each experimental data set was simulated with Equation 1 and depicted with solid symbols and lines. One of two virtually identical data sets is shown here.
Since these hybrid actins would contain a hybrid profilin binding site (yeast subdomain 3 and muscle subdomain 1), they presented an opportunity to determine the relative roles played by each domain in both the binding of profilin to actin and the ability of profilin to accelerate nucleotide exchange. Fig. 5 and Table 3 show that for both sub1 and sub12 actins, both profilins at saturating conditions resulted in about a 16–20 times acceleration of eATP exchange. Note that the starting rates in the absence of profilin for sub1 and sub12 are 0.007 and 0.003 s\(^{-1}\), respectively. The \(K_a\) values for the interaction of YPF were about 2 \(\mu M\) for each of the hybrid actins. This value is higher than that for yeast actin and somewhat lower than that for muscle actin, as might be expected for a hybrid molecule. However, the analysis involving HPF1 produced unexpected results; The \(K_a\) values for sub1 (5.6 \(\mu M\)) and sub12 actins (8.1 \(\mu M\)) were 70–80-fold higher than with muscle actin, which has a \(K_a\) of 0.1 \(\mu M\) (data not shown), and 3–4-fold larger than with yeast actin. Meanwhile, the catalytic efficiencies for the YPF/sub1 and YPF/sub12 are 7.6 and 11 \(\mu M^{-1}\), and the catalytic efficiencies for the HPF1 complexes are 3 and 2 \(\mu M^{-1}\). These efficiencies are similar to those seen with YPF/HPF1 binding to yeast actin, far different from the efficiency of the HPF1-muscle actin complex based on the data from Eads et al. (17). This result suggests that actin subdomain 3 is the major determinant in the regulation of the catalytic efficiency of the actin-profilin complex.

To explore further the reason for the greater effect of the hybrid nature of the actin on HPF1 versus YPF binding, we repeated the ITC analysis with the two profilins using the two hybrid actins. As seen in Fig. 6 and Table 4, for both profilins, the enthalpic and entropic contributions with sub12 actin were similar to those observed with the WT actins. The YPF interaction is completely enthalpically driven, whereas the HPF1 interaction is both enthalpically and entropically driven. Surprisingly, for sub1 actin missing the three sub2 muscle residues, there is a sharp decrease in enthalpic change and a marked increase in entropic change for both profilins. This result suggests that either the interaction of actin subdomains 1 and 2 with each other or interactions between the two domains of actin across the nucleotide cleft plays a major role in dictating the topography of the surface at the barbed end of the protein where the profilin binds.

### Contribution of Three Muscle Actin-specific Subdomain 2 Residues to the Profilin-Actin Interaction

—Yeast and muscle actin subdomain 2 differ from one another by only three residues. The yeast to muscle changes, all very similar in nature to the original residues, are I43V, R68K, and V76I. However, when added to the sub1 hybrid actin, these residues together cause a 2-fold retardation in the rate of nucleotide exchange to near that seen with muscle actin, and they drastically alter the energetics of binding for the actin to profilin as shown above.

To determine the relative contributions of each of these three residues to these differences in actin behavior, each was introduced independently into sub1 actin. Compared with the exchange rate for sub1 actin alone (0.007 s\(^{-1}\)), I43V + sub1 or R68K + sub1 actins showed a mildly retarded exchange rate (0.005 s\(^{-1}\)) (Fig. 7). The addition of the V76I mutation, the residue nearest the binding site for the nucleotide phosphate, caused the greatest retardation in rate (0.003 s\(^{-1}\)), a value near that observed with muscle actin and sub12 actin (Fig. 7). All of these experiments were repeated twice with essentially the

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**TABLE 3**

| Actin          | YPF |          |          |
|----------------|-----|----------|----------|
|                | -Fold| \(K_a\) | Efficiency | -Fold| \(K_a\) | Efficiency |
| sub1 hybrid    | 16   | 2.1      | 7.6       | 17   | 5.6      | 3         |
| sub12 hybrid   | 22   | 1.9      | 11        | 18   | 8.1      | 2         |

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**FIGURE 6.** ITC analysis of the interaction of hybrid actins with yeast profilin. A and C present the raw data for a total of 290 \(\mu L\) of 300 \(\mu M\) yeast profilin injected in 20 aliquots into 2 ml of 20 \(\mu M\) sub1 and sub12 hybrid actins in ITC G buffer. B and D present the corrected integrated data with a curve fit for the data from A and C, respectively.
same results. Thus, of the three substitutions, the residue at position 76 seems to exert the greatest influence on the dynamics of the nucleotide binding site.

To determine if position 76 also exhibited the greatest influence on the profilin-binding properties of actin, we repeated the ITC profilin binding assay with each of these three new mutant actins. Table 5 shows that each of these mutant actins had nearly the same $K_d$ as sub1 hybrid actin, with the exception of R68K, which had a lower $K_d$ compared to sub1 sub12 hybrid actin. The enthalpy and entropy changes for these mutant actins were also similar, with the exception of R68K and I43V, which had smaller changes in entropy compared to sub1 and sub12 actins.

**Kinetics of Binding of Yeast Profilin to WT, sub1, and sub12 Yeast Actins**—The differences in thermodynamics describing the interaction of YPF with WT, sub1, and sub12 actins might be reflected in differences in $k_{on}$ and $k_{off}$ from complex formation that could affect the lifetime of the profilin complex stability, thereby, affecting the catalysis of nucleotide exchange. To determine these constants, we followed the change in intrinsic tryptophan fluorescence that occurs when profilin binds to actin, using a stopped-flow apparatus. Modeling of the decay curves (Fig. 8) as described under “Materials and Methods” allowed an extraction of the $k_{on}$ and $k_{off}$ for each of these interactions (Table 6). The $K_d$ values obtained from the ratio of $k_{on}/k_{off}$ are consistent with those obtained by the exchange and ITC assays. Although there appear to be relatively small differences in $k_{on}$ for the three actins tested, these differences were not statistically significant by $t$ test.
DISCUSSION

Our primary focus was to determine the factors that regulate the ability of profilin to bind to actin and then to promote the exchange of nucleotide from actin. This knowledge is necessary to appreciate the molecular basis that governs profilin regulation of actin filament dynamics within the cell. Our basic approach was to use two different profilins, YPF and HPF1, along with muscle, yeast, and yeast/muscle hybrid actins to try to gain insight into the control of these two processes at the molecular level. The results we have obtained have provided new insight into the manner in which profilin binds to actin and influences actin conformation, important for both the role of profilin as an actin buffer and as a facilitator for formin-dependent filament elongation. They also provide insight into how this binding reaction can be translated into facilitation of actin-dependent nucleotide exchange.

Catalysis of Nucleotide Exchange—We first addressed the ability of profilin to facilitate actin nucleotide exchange under saturating profilin concentrations. Our results clearly demonstrate that, independent of the actin being worked on, HPF1 is better able to cause nucleotide exchange than is YPF. One might imagine that two proteins from the same cell co-evolve to produce the most effectively acting system. Since yeast actin assumes a more open conformation compared with muscle actin (26), one could hypothesize that the actin cleft can only be opened so far to promote nucleotide exchange before the protein denatures. Consequently, the small degree of enhancement of nucleotide exchange associated with the YPF-yeast actin interaction would reflect this limiting rate. What appeared to be this limit based on YPF experiments can clearly be exceeded by HPF1. However, the inherently increased flexibility of yeast versus muscle actin may still be a significant factor in the differences in -fold increase in profilin-dependent release rate we observed using these two actins.

Minehardt et al. (29), based on molecular dynamics simulations, proposed that the open cleft state seen in the profilin-actin crystal complex by itself is very unstable and rapidly collapses to the closed state. In essence, there is an unfavorable equilibrium between the closed and open cleft states, and the role of profilin is to stabilize the small amount of open state that would exist, thereby promoting nucleotide exchange. Thus, the rate enhancement is passively rather than actively brought about. Our results show that either with yeast or muscle actin, two different profilins cause different degrees of enhancement of exchange rate. It had also been reported that plant profilin, which, through residue Glu82, might form a hydrogen bond with Lys113 on actin. This extra attachment between the two proteins might generate a lever arm that the human profilin could utilize to more easily alter the nucleotide cleft on actin leading to enhanced exchange. However, when we tested this theory by eliminating the Asp from position 82, no deleterious effect was produced on the nucleotide exchange caused by HPF1. Thus, the basis for this difference in catalytic ability between HPF1 and YPF must not involve formation of this interprotein bond.

Analysis of the Binding of Profilin to Actin—Our results provide new insight into the factors that regulate the binding of profilin to actin. In all cases we examined, the homologous pair of proteins (e.g. YPF and yeast actin) exhibited a tighter interaction than did a heterologous pair, and both homologous pairs had about the same $K_d$. What was interesting is that tight binding did not necessarily correlate with increased ability to alter the conformation of the actin around the bound nucleotide, leading to exchange. Our data also showed that, using a biologically more meaningful comparison, catalytic efficiency (-fold increase in exchange rate/$K_d$), for yeast actin-YPF and muscle actin-HPF1, the efficiencies are clearly higher than for the heterologous pairs.

Studies with Yeast/Muscle Hybrid Actins—Based on crystallographic evidence (18, 19), the profilin binding site on actin, at the barbed end of the protein, spans actin subdomains 1 and 3 across the interdomain hinge helix. It has been estimated that between the two proteins, about 70% of the contacts involve subdomain 3 (17, 30). Furthermore, across the entire profilin binding surface on actin, there are only three differences between the yeast and muscle actins. However, the binding parameters that characterize the interaction of the same profilin with different actins are strikingly different. This result strongly suggests that despite the high homology among different actins in terms of primary and tertiary structure, the few differences must alter the topology of the profilin binding site on the actin surface enough to affect the manner in which the pair of proteins interacts to form the complex.

The use of our yeast muscle hybrid actins allowed us to gain insight into the contributions of actin subdomains 1 and 3 to this difference. For both hybrid actins, which contain muscle subdomain 1, YPF binds about 3–4 times more tightly than does HPF1, consistent with subdomain 3 (yeast in this case) being the predominant binding surface. Although the data suggest that subdomain 3 is a major regulator of binding tightness, the contribution of actin subdomain 1 to binding is not inconsequential. In mammalian profilin, there is a potential hydrogen bond between profilin Asp86 (Tyr79 and Tyr78 in S. pombe and S. cerevisiae profilins, respectively) and actin Arg372 in subdomain 1. In plant profilin, the residue in this position is replaced by an Arg, which would cause a repulsive interaction. Lu and Pollard (28) altered the Tyr79 in S. pombe profilin to Arg, resulting in a significant weakening of binding and a loss of ability to catalyze nucleotide exchange. We examined the importance of this hydrogen bond from the perspective of actin. Yeast actin has His372 instead of Arg and should make a much weaker hydrogen bond. In our hands, conversion of His372 to Arg, which would strengthen the interprotein interaction, caused
Actin-Profilin Interaction

an about 8-fold tighter binding with profilin but no change in exchange activity, again consistent with lack of an obligatory coupling between binding affinity and catalytic capability.

Our binding data concentrating the importance of actin subdomain 3 are consistent with a proposal recently made by Zheng et al. (31) regarding the basis of the preference of profilin for ATP over ADP actin. They predicted a nucleotide-dependent change in conformation of a loop involving actin residues 165–175, which might contribute to the selectivity observed with profilin. Interestingly, two of the three divergent residues between yeast and muscle actin in the profilin binding site, residues 167 and 169, fall in this loop in actin subdomain 3, consistent with our results.

Profilin/Actin Binding Energetics—Our ITC experiments allowed us to examine separately the enthalpic and entropic contributions to the actin-profilin interaction. Using the muscle and yeast WT actins, we showed that the profilin, not the actin, seems to dictate the energetics of binding. For both actins, the binding of YPF was largely enthalpically driven, whereas that of HPF1 was much less enthalpically and much more entropically dependent. We observed an intriguing difference between the interaction of sub1 and sub12 hybrid actins with YPF; sub1 actin produced an entropically driven interaction, as seen with HPF1, whereas sub12 yielded an enthalpically driven interaction, typical of YPF. These observations suggest that subdomains 1 and 2 co-evolved in order to maintain the allosteric connections that would allow the entire domain containing these subdomains to function as an integrated unit. If this unit is intact, as is the case for the sub12 actin, our results suggest that actin subdomain 3 exerts the most influence over the manner in which actin and profilin interact. Creation of a subdomain 1/subdomain 2 mismatch, as in the case of sub1 actin, would destroy this integration, leading to a situation where subdomain 1 was now acting in an uncoupled fashion. The result of this creation would be an altered binding surface that results in a switch from enthalpically to entropically dominant binding of profilin.

To gain more insight into the basis of the alteration in the nucleotide exchange and profilin binding behavior caused by the introduction of the three actin subdomain 2 muscle residues, we examined the results of the individual subdomain 2 mutations against an otherwise muscle subdomain 1 background. For both nucleotide exchange and profilin binding, there was a predominant residue responsible for the switch. Surprisingly, however, the predominantly responsible residue was different for each process. For nucleotide exchange, the biggest influence was exerted by the V76I mutation. Residue 76 lies in a group of five tightly packed residues extending from residue 118 on the outer surface of actin through His73 to the nucleotide binding site. If the V76I conversion results in tighter packing with a resulting closure of the interdomain cleft, the result might very well be the retarded rate of nucleotide exchange activity, again consistent with lack of an obligatory control that regulates profilin binding and other actin-dependent processes is a major challenge for the future. The approach we have taken and the results we have obtained provide a foundation for this type of investigation and insight into how answering this question might be best achieved.

References

1. Southwick, F. S., and Young, C. L. (1990) J. Cell Biol. 110, 1965–1973
2. Babcock, G., and Rubenstein, P. A. (1993) Cell Motil. Cytoskeleton 24, 179–188
3. Romero, S., Didry, D., Larquet, E., Boisset, N., Pantaloni, D., and Carlier, M. F. (2007) J. Biol. Chem. 282, 8435–8445
4. Pantaloni, D., and Carlier, M. F. (1993) Cell 75, 1007–1014
5. Vinson, V. K., De La Cruz, E. M., Higgs, H. N., and Pollard, T. D. (1998) Biochemistry 37, 10871–10880
6. Kinosian, H. J., Selden, L. A., Gershman, L. C., and Estes, J. E. (2002) Biochemistry 41, 6734–6743
7. Selden, L. A., Kinosian, H. J., Estes, J. E., and Gershman, L. C. (1999) Biochemistry 38, 2769–2778
8. Vayylonis, D., Kovar, D. R., O’Shaughnessy, B., and Pollard, T. D. (2006) Mol. Cell 21, 455–466
9. Kovar, D. R., Harris, E. S., Mahaffy, R., Higgs, H. N., and Pollard, T. D. (2006) Cell 124, 423–435
10. Romero, S., Le Clainche, C., Didry, D., Egile, C., Pantaloni, D., and Carlier, M. F. (2004) Cell 119, 419–429
11. Yao, X., and Rubenstein, P. A. (2001) J. Biol. Chem. 276, 25598–25604
12. Carlier, M. F. (1990) Adv. Biophys. 26, 51–73
13. Carlier, M. F. (1991) J. Biol. Chem. 266, 1–4
14. Perelroizen, I., Didry, D., Christensen, H., Chua, N. H., and Carlier, M. F. (1996) J. Biol. Chem. 271, 12302–12309
15. Arasada, R., Gloss, A., Tunggal, B., Joseph, J. M., Rieger, D., Mondal, S., Faix, J., Schleicher, M., and Noegel, A. A. (2007) Biochim. Biophys. Acta 1773, 631–641
16. Goldschmidt-Clermont, P. J., Machesky, L. M., Dobert, S. K., and Pollard, T. D. (1991) J. Cell Biol. 113, 1081–1089
17. Eads, J. C., Mahoney, N. M., Vorobiev, S., Bresnick, A. R., Wen, K. K., Rubenstein, P. A., Haarer, B. K., and Almo, S. C. (1998) Biochemistry 37, 11171–11181
18. Schutt, C. E., Myslik, J. C., Rozycki, M. D., Goonesekere, N. C., and Lindberg, U. (1993) Nature 365, 810–816
19. Chak, J. K., Lindberg, U., and Schutt, C. E. (1996) J. Mol. Biol. 263, 607–623
20. Page, R., Lindberg, U., and Schutt, C. E. (1998) J. Mol. Biol. 280, 463–474
21. Rubenstein, P. A. (1990) BioEssays 12, 309–315
22. McKane, M., Wen, K. K., Meyer, A., and Rubenstein, P. A. (2006) J. Biol. Chem. 281, 29916–29928
23. McKane, M., Wen, K. K., Boldog, I. R., Ramcharan, S., Pon, L. A., and Rubenstein, P. A. (2005) J. Biol. Chem. 280, 36494–36501
24. Cook, R. K., Sheff, D. R., and Rubenstein, P. A. (1991) J. Biol. Chem. 266, 16825–16833
25. Wen, K. K., Yao, X., and Rubenstein, P. A. (2002) J. Biol. Chem. 277, 41101–41109
26. Belmont, L. D., Orlova, A., Drubin, D. G., and Egelman, E. H. (1999) Proc.
27. Magdolen, V., Drubin, D. G., Mages, G., and Bandlow, W. (1993) *FEBS Lett.* **316**, 41–47
28. Lu, J., and Pollard, T. D. (2001) *Mol. Biol. Cell* **12**, 1161–1175
29. Minehardt, T. J., Kollman, P. A., Cooke, R., and Pate, E. (2006) *Biophys. J.* **90**, 2445–2449
30. Cedergren-Zeppezauer, E. S., Goonesekere, N. C., Rozycki, M. D., Myslik, J. C., Dauter, Z., Lindberg, U., and Schutt, C. E. (1994) *J. Mol. Biol.* **240**, 459–475
31. Zheng, X., Diraviyam, K., and Sept, D. (2007) *Biophys. J.* **93**, 1277–1283
32. Crosbie, R. H., Miller, C., Cheung, P., Goodnight, T., Muhlrad, A., and Reisler, E. (1994) *Biophys. J.* **67**, 1957–1964
33. Khaitlina, S. Y., Moraczewska, J., and Strzelecka-Golaszewska, H. (1993) *Eur. J. Biochem.* **218**, 911–920