Acceleration of Yeast Actin Polymerization by Yeast Arp2/3 Complex Does Not Require an Arp2/3-activating Protein*

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The Arp2/3 complex creates filament branches leading to an enhancement in the rate of actin polymerization. Work with Arp complexes from different sources indicated that it was inactive by itself, required an activating factor such as the Wiskott-Aldrich syndrome protein (WASP), and might exhibit a preference for ATP or ADP-Pi actin. However, with yeast actin, Pi release is almost concurrent with polymerization, eliminating the presence of an ADP-Pi cap. We thus investigated the ability of the yeast Arp2/3 complex (yArp2/3) to facilitate yeast actin polymerization in the presence and absence of the Arp2/3-activating factor Las17p WA. yArp2/3 significantly accelerates yeast actin but not muscle actin polymerization in the absence of Las17p WA. The addition of Las17p WA further enhances yeast actin polymerization by yArp2/3 and allows the complex to now assist muscle actin polymerization. This actin isoform difference is not observed with bovine Arp2/3 complex, because the neural WASP VCA fragment is required for polymerization of both actins. Observation of individual branching filaments showed that Las17p WA increased the persistence of filament branches. Compared with wild type actin, the V159N mutant actin, proposed to be more ATP-like in behavior, exhibited an enhanced rate of polymerization in the presence of the yArp2/3 complex. yArp2/3 caused a significant rate of P_i release prior to observation of an increase in filament mass but while branched structures were present. Thus, yeast F-actin can serve as a primary yArp2/3-activating factor, indicating that a newly formed yeast actin filament has a topology, unlike that of muscle actin, that is recognized specifically by yArp2/3.

The proper functioning of the actin cytoskeleton in processes such as cytokinesis (1), cell motility (2, 3), endocytosis and exocytosis (4, 5), and lamellipodial extension (6, 7) requires that the system be dynamic both temporally and spatially. That is, the actin microfilament system must be capable of being rapidly assembled at a certain place in the cell at a certain time in response to a stimulatory signal and then be able to be disassembled once the particular task is completed. Recent works have demonstrated that an important factor in the nucleation and deposition of new filaments is the Arp2/3 complex (8).

This complex, found throughout evolution from yeast to mammals, is composed of seven protein subunits, two of which are the actin-related proteins Arp2 and Arp3 (9). Like actin, each is an adenine nucleotide-binding protein, and nucleotide hydrolysis is needed for proper complex function (10). Filament nucleation occurs by the complex preferentially binding to the side of a pre-formed filament, creating a site for a branch of newly polymerized actin that extends from the mother filament at an angle of ~70° (11–14). Based on studies carried out with Arp2/3 complex from mammalian and amoeboid cells (15), the complex alone appears to be in an inactive conformation that requires prior activation by another protein factor before filament nucleation can occur. A number of such activating factors, known as nucleation-promoting factors (NPFs),1 have been identified, and one of the most well documented is the WASP family of proteins. These proteins contain, at the least, the WASP homology 2 and acidic (WA) domains that are required for Arp2/3 activation and actin binding. In most cases, these elements are also in an inactive state until activated by interaction with an element of a signal transduction pathway. However, recent work with the WASP analogue from Saccharomyces cerevisiae, Las17p, indicates that this family member may be constitutively active (16).

Initial experiments with the Arp2/3 system, using rabbit muscle actin, also indicated that the nucleotide state of the mother actin filament might be an important determinant in Arp2/3-dependent filament nucleation (17). With preformed filaments, Amann and Pollard observed that although branching occurred along the length of the filament, there seemed to be a preference for branch formation in the barbed end half of the filament (11). Ichetovkin et al. (13) studied this nucleotide dependence in more detail. They showed that filaments formed with AMPPNP-actin seemed to have at least a 2-fold higher propensity to form branches than those formed with either ADP or ADP-Pi, actin, and they further demonstrated that branching was most effective with newly formed F-actin. These studies led to the hypothesis that Arp2/3 complex has a preference for either the ATP- or ADP-Pi forms of F-actin or an immature, perhaps more stable form of the filament seen predominantly at the barbed end of the mother filament (9).

In vivo studies involving the role of Arp2/3 utilize complexes from eukaryocytes ranging from mammalian non-muscle cells of various types to yeast. Yeast in particular has been a very valuable system because it has allowed a genetic dissection of the Arp2/3 complex and its interacting proteins (18–20). However, most in vitro studies involving the mechanism of Arp2/3-

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1 The abbreviations used are: NPF, nucleation-promoting factor; AMPPNP, adenosine 5'-[β,γ-imino]triphosphate; bArp2/3, bovine Arp2/3; EM, electron microscopy; WASP, Wiskott-Aldrich syndrome protein; WA, WASP homology 2 and acidic (domains); N-WASP, neuronal WASP; WT, wild type; yArp2/3, yeast Arp2/3 complex.
dependent enhancement of actin polymerization have utilized muscle actin, despite the fact that a number of studies have shown distinct behavioral differences between yeast and muscle actins. Yeast actin polymerizes more rapidly than muscle actin (21, 22), and EM studies have implied that the yeast actin filament is more open and flexible than its muscle actin counterpart (23). Yeast actin exchanges its bound adenine nucleotide much more rapidly than muscle actin, and, more importantly, yeast actin releases its bound Pi following nucleotide hydrolysis much more rapidly than does muscle actin (24–27). At low actin concentrations where most of the in vitro actin work is performed, a lengthy lag exists in muscle actin between the time the actin polymerizes and the time the Pi is released, whereas in yeast actin, under these same conditions, Pi release and polymerization are almost simultaneous. This difference is particularly significant in light of the discussion regarding the state of actin preferred by Arp2/3.

Because of the use of the yeast cytoskeleton as a model system for studying Arp2/3 function, coupled with the differences between yeast and muscle actins described above, we felt it was important to determine whether the particular actin used influenced the behavior of the Arp2/3 complex, and we also wished to compare the effectiveness of the mammalian and yeast Arp2/3 complexes with these two actins in the presence and absence of an NPF. In this paper we report the results of experiments examining these parameters in terms of actin polymerization in solution, and we also examine them by looking at the effect of Arp2/3 on branching and debranching of individual actin filaments.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Preparation—**Yeast WT and V159N actins were purified by DNase I affinity chromatography as described by Cook et al. (28). Muscle actin was isolated from rabbit muscle acetone powder (29). A mixture of β and γ non-muscle actins was obtained from Cytoskeleton, Inc.

The actin was labeled on C374 with pyrene-maleimide (Sigma) as described previously (30) and with a labeling efficiency between 80 and 95%. Actin was stored in G buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl2, 0.1 mM EDTA, and 0.1 mM dithiothreitol) at 4 °C and used within 3 days. Protein A-tagged yeast Arp2/3 was expressed and prepared from the RLY1945 yeast strain (a generous gift from Dr. R. Li, Stowers Institute) as described by Pan et al. (31) with modifications. Briefly, the cell lysates were prepared from the cells in early log phase by a glass bead beater (BioSpec). Protein A-tagged yeast Arp2/3 in the cell lysate was affinity-purified on G-Sepharose affinity chromatography (Amersham Biosciences) and digested with recombinant tobacco etch virus to remove the protein A tag. Finally, yeast Arp2/3 was further purified on a Source fast flow anionic exchange column (Amersham Biosciences). The preparations were analyzed by SDS-PAGE and visualized by Coomassie Blue staining to assess the purity. The preparations were then aliquoted and stored at −80 °C. Each aliquot was used within 2 days after thawing.

**Results**

**Visualization of Actin Filaments with Fluorescence Microscopy—**The procedure was modified from that of Boujemaa-Paterski et al. (14). Yeast or muscle actin with yeast Arp2/3 in the presence or absence of Las17p WA was induced to polymerize as described above. Aliquots of the polymerization sample at different time points were incubated for 1 min with rhodamine phalloidin (Molecular Probes) at a 1:1 mol ratio to actin. The sample was then diluted 20–100-fold into F buffer containing 0.1% 1,4-diazabicyclo[2.2.2]octan (Sigma), 10 mM dithiothreitol, and 0.1% methylcellulose. An aliquot of the solution (1.6 μl) was applied to a poly-L-lysine-coated (average molecular mass 70 kDa at 2 μg/ml; Sigma) glass coverslip, which is able to stabilize yet does not fragment filaments, and the sample was sandwiched into a microscope slide. The sample slide was further sealed with nail polish to prevent evaporation. The filaments were observed with a Zeiss 200 m fluorescence microscope and a Hamamatsu ER ORCA camera. Branched and unbranched filaments were recorded and analyzed by the OPEN LAB software package (Improvision) and Image J (National Institutes of Health) respectively. At least 700 filaments were examined from each sample.

To monitor the location of branch formation on pre-formed yeast filaments, 4 μM pre-formed Alexa 488-labeled yeast actin filaments were stabilized by an equal concentration of phalloidin. This fluorescent labeled F-actin was fragmented by passage through a syringe with a 30-gauge needle five times before use in experiments. Yeast G-actin, 5 μM, with 25 nm Arp2/3 and 0.5 μM Las17p WA, was induced to polymerize as described above, following the addition of an equal volume of 4 μM fragmented fluorescent F-actin. After 3 min, rhodamine phalloidin (Molecular Probes) was added at a mole ratio at 1:3 with respect to G-actin to stabilize actin filaments. The sample was diluted and monitored by the same fluorescence microscope assay as described above. Image processing was performed using Adobe Photoshop version 7.0 software.

**RESULTS**

**Yeast Actin Polymerization in the Presence of γArp2/3 and Las17p WA—**To begin to characterize the biochemical behavior of the yeast actin/yeast Arp2/3 complex (γArp2/3) system, we assessed the relative ability of γArp2/3 alone to promote the polymerization of yeast versus muscle actin. Fig. 1 demonstrates a lack of ability of γArp2/3 to promote the polymerization of muscle actin, and it also depicts the faster polymerization of yeast versus muscle actin in the absence of exogenous factors, as has been shown previously. Surprising, however, was the significant enhancement of yeast actin polymerization by γArp2/3 in the absence of exogenous NPF. This result is...
Arp2/3 complex requires the Las17p WA. Fig. 3 demonstrates that the addition of Las17p WA enhances the presence of Las17p WA alone. The reaction demonstrated that this polymerization was not due to the ability of yArp2/3 to affect actin polymerization. A control of polymerization increased in the presence of Las17p WA. Fig. 4 shows that increasing amounts of yArp2/3 leads to increasing of yeast actin polymerization. The results, shown in Fig. 4, reveal three different types of structures, namely single filaments, filaments with a single branch point, and filaments with multiple branch points, regardless of whether yeast or muscle actin was used. With muscle actin, however, Las17p WA was included. With yeast actin, all three types of structures were observed whether or not the Las17p WA was included. The multiply branched structures were similar to those reported by others (11, 13) using muscle actin, bArp2/3, and the N-WASP VCA domain.

The density of branches on the multiply branched filaments varied over time; high branching density was observed predominantly during the nucleation phase. For example, the highly branched structure shown in Fig. 4 e derives from a sample deposited on the slide within the first 30 seconds following initiation of polymerization. Medium density branching was predominant during the early elongation phase, and low density branching was present during the late elongation phase. Furthermore, in these reactions the branches occurred randomly along the entire length of the filament. We were able to fit the decreasing portions of the curves in Fig. 5, A–C to a first order equation. The analysis showed that the apparent first order rate constants for the disappearance of branched yeast actin filaments were 0.25 and 0.10 min⁻¹ in the absence and presence of Las17p WA, respectively. The rate constant for muscle actin in the presence of WA was 0.14 min⁻¹, near that observed with the yeast actin under the same conditions. Overall, the presence of Las17p WA increased the persistence of branched structures in the sample.
either ActA (14) or WASP (11, 13) as an NPF showed that when pre-formed actin filaments were combined with the Arp2/3, branching was rare, and, when it did occur, it was mainly observed in either filaments nucleated de novo or on those few filaments that had branched from a preformed filament. We thus assessed the ability of the yArp2/3 complex to polymerize yeast actin off of pre-formed F-actin in the presence of Las17p WA. Inclusion of the WA was to maximize the time of existence of multiply branched filaments. Fig. 6 shows that for the yeast proteins also, branching from pre-formed filaments was rare and that most of the branching observed was either off of filaments initiated de novo or off of the barbed end of pre-formed filaments. Very few branches were observed attached to the middle of pre-formed filaments. It is possible that some of the discrimination between a branching preference for new versus old actin results from the use of phalloidin-stabilized mature filaments, because the phalloidin may have altered the topology of the yeast F-actin. Two observations argue against this view, however. Ichetovkin et al. (13) reported that phalloidin-stabilized filaments actually lead to more efficient branching than filaments not stabilized with phalloidin, and Orlova et al. (34), based on EM reconstruction data, demonstrated that phalloidin F-actin produces a filament that is more like that caused by AMPPNP-actin rather than by ADP-actin.

**Effect of yArp2/3 and Las17p WA on P release from polymerizing actin**—It has previously been established that during the polymerization of yeast actin at low concentrations, both hydrolysis of the bound ATP and the subsequent release of P, occurs concomitantly with polymerization. There was no lag in P release as is observed with muscle actin. We wished to know how Arp2/3-dependent branching of newly polymerized yeast actin affected P release relative to polymerization in the presence and absence of Las17p WA. The results are shown in Fig. 7. P release can be divided into three stages relative to polymerization, namely nucleation, early elongation, and steady state. Whether in the absence or presence of Las17p WA, there was an initial burst of P release that precedes any detectable increase in light scattering that would signify polymerization. In the presence of Las17p WA, elongation and its concomitant P release occurred more rapidly than in the absence of Las17p WA, presumably reflecting Las17p WA-dependent stabilization of Arp2/3-actin complexes and the related increased opportunity for subsequent actin polymerization. The continued rise in P release following completion of polymerization was due to...
monomer treadmilling in the filaments. The break in the $P_i$ release curve between the treadmilling and polymerization phases occurred at a $P_i$ amount equal to the amount of $P_i$ expected to be released from a single turnover of each polymerizing monomer; considering the actin concentration used and the critical concentration for the protein. This result would seem to preclude pre-polymerization monomer cycling as a major cause of the initial increase in $P_i$ observed prior to the detection of an increase in filament mass.

Effect of the V159N Actin Mutation on $yArp2/3$-dependent F-actin Formation—The V159N mutation in yeast actin has been reported to produce a hyper-stable filament (35), and subsequent EM characterization of these filaments show them to be in more like an ADP-Pi conformation than an ADP-actin conformation (23). If Arp2/3 complex had an enhanced affinity for ATP actin, ADP-actin (11, 13, 14, 17), or immature ADP-actin topology over mature ADP-actin filaments, there might be an increased efficiency of branching with this mutant actin in the presence of $yArp2/3$. Fig. 8 shows that although V159N and WT actin polymerize approximately at the same rate under the conditions of our experiment, the introduction of $yArp2/3$ substantially accelerates its rate of polymerization compared with that observed with WT actin. Observation of individual filaments formed with V159N actin revealed the same range of branch morphologies we observed with WT and muscle actins.

We next assessed the effect of the actin mutation on branch persistence as shown in Fig. 9. Analysis of the curve yielded a first order rate constant 0.09 min$^{-1}$, approximately the same as that obtained with WT actin in the presence of Las17p WA. These results correlate with the enhanced filament stability of V159N actin reported previously.

Bovine Arp2/3 Complex Enhances Yeast Actin Polymerization Only in the Presence of Activated N-WASP—Rodal et al. (36) showed that Arp2/3 complex could exist in three identifiable conformations, namely an open, an intermediate, or a closed conformation, and that there was approximately twice as much of the yeast complex in the closed conformation as there was of the bovine complex. We demonstrated above that yeast actin was much better able to be polymerized in the presence of $yArp2/3$ than was muscle actin. This result may have been due to an enhanced ability of yeast actin to pull the Arp2/3 complex into the more closed, active conformation. If our hypothesis was correct, yeast WT and V159N actins might be more effective than muscle actin in leading to an activated form of bArp2/3 as well. Fig. 10, however, shows that this was not the case. The only way in which bArp2/3 promoted polymerization of yeast actin was in the presence of an activated form of N-WASP.

**DISCUSSION**

Despite the apparent degree of conservation among Arp2/3 complexes from different organisms, the behavioral differences that we and others (21, 22, 26, 27, 37) have documented in yeast actin as compared with muscle actin suggested the possibility that there was co-evolution of these two proteins to maximize the efficiency of some physiological function in yeast. The focus of this paper was to test such a possibility. Our results show for the first time that, with yeast actin, $yArp2/3$ is capable of substantially promoting actin polymerization in the absence of an NFP. This characteristic derives from the differential ability of yeast actin versus muscle or higher eukaryotic nonmuscle actins to interact with the complex because, under the same conditions, $yArp2/3$ required an activating protein to promote muscle and nonmuscle actin polymerization. Although yeast actin polymerization could be promoted by $yArp2/3$ alone,
The Fig. 5 legend was repeated with 2 actin structures as a function of time. The experiment described in the Fig. 5 legend was repeated with 2 μM V159N actin and 25 nM yArp2/3 in the absence of Las17p WA.

Our results suggested that a yArp2/3-specific conformation facilitates the preferred interaction with yeast as compared with muscle actin. X-ray crystallography demonstrated that the Arp2/3 complex exists in an open form, which, the authors hypothesized, closes upon activation, thereby allowing the Arp2 and Arp3 subunits to interact (38, 39). This model was supported by work from Goley et al. (40) who used fluorescence resonance energy transfer to document closure of the complex upon activation by an activator protein. Based on the EM analysis of Rodal et al. (16) as stated above, the ratio of closed states for yeast versus the bArp2/3 complex is 2:1. If one groups the intermediate and closed states together, this ratio for yeast versus the bArp2/3 complex is 4:1. These structural differences indicate a greater propensity of yArp2/3 for a conformation that would lead to its activation. However, in neither case is the ratio large enough to account for the all-or-none behavior we observed with yeast versus muscle actin in the presence of yArp2/3.

Because the major function of the Arp2/3 complex is apparently to facilitate localized actin polymerization by initiating branches, assessment of bulk actin polymerization in solution is not the optimal way to assess Arp2/3 function. To form a filament branch, the Arp2/3 complex must interact with actin in two different ways. It must bind to the side of a pre-existing filament, and the Arp2 and 3 subunits must be in a conformation to allow nucleation and filament elongation of the branch. Either means of interaction represents a potential source of Arp2/3 activation brought about by yeast actin. The filament topology specific to yeast F-actin, following attachment of the complex, might lead to the closed activated state needed for new filament nucleation. Another origin of the difference could be caused by something as simple as a difference in surface-charged residues between the yeast and no-yeast actins. We cannot discern between these possibilities. In other words, yeast F-actin is functioning as an NPF. A similar role for F-actin, that of a secondary activator of Arp2/3 complex, had been proposed previously by Volkmann et al. (41) on the basis of their EM reconstruction work.

Alternatively, differences in the behavior of the yeast versus that of the muscle actin monomers could promote formation of an intermediate conformation of the complex that would facilitate side binding to an actin filament. This interaction would then promote the final closing of a complex already prone to closure. We cannot differentiate between these possibilities at this time. What does seem apparent from our results is that the ability of yeast actin to work alone with yArp2/3 does not stem from its ability to cause the complex to initiate filament formation de novo, because isolated filaments are not observed to any extent during the early phases of the polymerization process.

The additional activation of Arp2/3-dependent branching we observed in the presence of Las17p WA might result in part from a more efficient closure of the complex to the active state and, in part, stabilization of the branched complex once it forms. Visualization of individual filaments formed by yeast actin and yArp2/3 in the presence or absence of Las17p showed virtually the same branching behavior. In the presence of Las17p WA, however, the lifetime of the branched structures appeared to be lengthened; that is, Las17p appeared to stabilize the branch junction formed by Arp2/3 with the mother filament. This stabilization would result in a more persistent presence of a pointed end filament cap that could translate into the increased rate of polymerization we observed in solution assays in the presence of Las17p WA. It is unlikely that the Las17p is working by affecting the nucleotide state of the filament because, as we have shown previously with yeast actin, P_1 release is almost concomitant with polymerization and ATP hydrolysis (27). Interestingly, when we repeated the experiment in the presence of pre-formed F-actin, branches from the middle of old filaments were rare. This result is similar to
that observed by others with Arp2/3 complexes from different sources (11, 13, 14).

This difference in branching preference for new versus preformed filaments has led to the proposition that the Arp2/3 complex might have a preference for binding to ATP or to the ADP-P$_i$-F-actin formed during the initial stages of polymerization rather than the mature ADP-F-actin conformation that characterizes mature filaments as discussed above. Our previous work showing the absence of such a lag in P$_i$ release under these conditions (27) would seem to eliminate this possibility. We have recently demonstrated using yeast actin that, subsequent to P$_i$ release, there is a slow change to the mature ADP-F-actin conformation based on the change in intrinsic tryptophan fluorescence of the protein, and it may be that this immature ADP-F-actin conformation is what is being recognized by the yeast Arp2/3 complex (37).

Belmont et al. (23) had originally proposed that the hyperstability of the V159N mutant yeast actin they constructed resulted from the F-actin remaining in a more stable ATP-like conformation, even though during the polymerization of the protein ATP hydrolysis and P$_i$ release were normal. Subsequently we showed that, at least in terms of subdomain 1 of actin, this mutant underwent the same post-Pi release conformational change as did WT yeast actin (37). However, during the initial stages of polymerization it is possible that the mutant filament remained in a more ATP-like conformation that might be reflected by enhanced Arp2/3-dependent actin polymerization as compared with that observed with WT actin. Our results are consistent with this hypothesis, even though the polymerization rates for the two actins in the absence of Arp2/3 complex are essentially the same.

Finally, our assessment of P$_i$ release kinetics during Arp2/3-dependent yeast actin polymerization led to a surprising observation. There was an Arp2/3-dependent stimulation of P$_i$ release prior to our detection of filament formation by an increase in light scattering. The observation is perplexing in that during the polymerization of the immature ADP-F-actin conformation based on the change in intrinsic tryptophan fluorescence of the protein, and it may be that this immature ADP-F-actin conformation is what is being recognized by the yeast Arp2/3 complex (37).

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