Activation of Arp2/3 Complex by Wiskott-Aldrich Syndrome Protein Is Linked to Enhanced Binding of ATP to Arp2*

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In response to signaling, the Arp2/3 complex (actin-related proteins 2 and 3 complex) is activated by binding the C-terminal (WA) domain of proteins of the Wiskott-Aldrich Syndrome family to promote the formation of a branched actin filament array, responsible for cell protrusion. The Arp2/3 complex exists in different structural/functional states: the inactive Arp2/3, the activated WA-Arp2/3 complex, the ternary G-actin-WA-Arp2/3 complex, which branches the filaments. This work addresses the role of ATP binding in Arp2/3 function. Using photo-cross-linking, hydrodynamic, and fluorescence techniques, we show that in the inactive Arp2/3 complex only one rapidly exchangeable ATP is tightly bound to Arp3 with an affinity of 10^7 M^-1. Upon activation of the Arp2/3 complex by WA, ATP binds to Arp2 with high affinity (10^7 M^-1), implying that a large structural change of Arp2 is linked to Arp2/3 activation. ATP is rapidly exchangeable on Arp2 and Arp3 in WA-Arp2/3 and G-actin-WA-Arp2/3 complexes. ATP is not hydrolyzed in inactive Arp2/3, in WA-Arp2/3, nor in G-actin-WA-Arp2/3. Arp2 has a greater specificity than Arp3 for ATP versus ATP analogs. Using functional assays of actin polymerization in branched filaments, we show that binding of ATP to Arp2 is required for filament branching.

Motile cells respond to environmental signals by adopting a polarized shape and extending pseudopodia that result from site-directed barbed end assembly of actin filaments at the leading edge of the cell (1–3). The Arp2/3 complex, which contains seven polyepipeptides including actin-related proteins Arp2 and Arp3, is downstream target of a variety of signaling pathways, integrated by Wiskott-Aldrich Syndrome family proteins. Signaling leads to the recruitment and activation of WASP1 proteins at the membrane. Activation of WASP proteins unmasks binding sites for Arp2/3 complex in the C-terminal domain (WA) (see Refs. 4 and 5 for review). Upon interacting with WA, the Arp2/3 complex locally stimulates polymerization of actin into an arborescent array of branched filaments, with the Arp2/3 complex sitting at the branches (6). In vitro polymerization and microscopy studies show that Arp2/3 complex多多ivalent actin by branching, in an autocatalytic process involving interaction of the ternary G-actin-WA-Arp2/3 complex with a filament (see Refs. 7 and 8 for reviews).

The molecular mechanism for the generation of force by building a branched filament array and the nature of the elementary reactions that take place in Arp2/3 during this complex process are not known. Structural modeling indicates that Arp2 and Arp3 share the general fold of actin and must bind ATP or ADP (9). As in actin and Arp1 (centractin), ATP binding and hydrolysis on Arp2 and/or Arp3 may play an important regulatory role in the reactions that lead to Arp2/3 activation, filament branching, debranching, and recycling of Arp2/3 complex. Here we quantitate the binding and hydrolysis of ATP on Arp2 and Arp3 in the inactive and activated Arp2/3 complex, and we determine the specificity of different nucleotides for Arp2 and Arp3 and their ability to support filament branching.

EXPERIMENTAL PROCEDURES

Proteins—Actin was purified from rabbit muscle and labeled with pyrenylidooacetamido (10). Arp2/3 complex from bovine brain and the C-terminal WA fragment of N-WASp were purified (11). Protein concentrations were measured spectrophotometrically using values of extinction coefficients derived from their amino acid sequence (10, 11). Hence the molar ratios of bound ATP given in this work are underestimated by 3% (when one ATP is bound) and 6% (when two ATPs are bound).

ATP Binding Measurements—The binding of ATP to high affinity sites on Arp2/3 complex was measured using Dowex 1 to remove all the free nucleotides. Arp2/3 complex (3–4 μM) was equilibrated in G0 buffer (5 mM Tris, pH 7.5, 1 mM dithiothreitol, 0.1 mM CaCl2) containing 10 μM ATP by gel filtration (NAP-5, Amersham Pharmacia Biotech) and treated twice for 30 s at 0 °C with 15% (v/v) of a 50% Dowex 1 suspension in G0 buffer. The concentration of Arp2/3 in the Dowex supernatant was determined spectrophotometrically. A control sample of 10 μM [3H]ATP in G0 buffer was treated identically. Only 0.2 μM free ATP was found in the Dowex 1 supernatant. Therefore only ATP that binds with an equilibrium dissociation constant of 20 nM or less, like actin (12), remains bound to the protein following Dowex 1 treatment. Following perchloric acid extraction of the bound nucleotides, the amount of total bound nucleotides was determined spectrophotometrically, and the amount of bound ATP was measured using the luciferin-luciferase assay (13), using the kit CLS II (Roche Molecular Biochemicals) and a Berthold luminometer (LB 9501). Standards were run in a range of 0–0.6 μM ATP, within which the samples were measured.

The Hummel and Dreyer method (14) was used to measure binding of ATP at different concentrations of free ATP. Arp2/3 complex was gel-filtered on Sephadex G-25 columns pre-equilibrated in F0 buffer (G0 supplemented with 1 mM MgCl2, 0.1 M KCl) containing [3H]labeled ATP in the range 0.5–2.0 μM. The molar ratio of ATP bound to eluted Arp2/3 at saturation by ATP was derived.

The change in fluorescence of etheno-ATP (ε-ATP) upon binding to Arp2/3 complex was used to quantitate binding of ATP, at 20 °C in a Spex spectrofluorimeter, using excitation and emission wavelengths of 350 and 410 nm, respectively. Arp2/3 complex (1 μM) was equilibrated in either G0 or F0 buffer containing 0.25 μM ATP by gel filtration (NAP-5 column) and was titrated by ε-ATP. Data were analyzed in terms of competitive binding of ε-ATP (X) and ATP (Y) to two independent sites on Arp2/3 (P). The equilibrium dissociation constants were K and J for binding of ε-ATP to Arp3 and Arp2. They were noted K/m and J/m for binding of ATP. The fluorescence enhancements for binding of ε-ATP to Arp3 and Arp2 were F1 and F2, respectively. The following
equations were used to describe the system, in which PX describes the complex of X with Arp3, and XP describes the complex of X with Arp2.

\[
[P_0] = ([P]/K_J) \cdot ([X] + m[Y]) \cdot (J + K + [X] + m[Y]) \quad (\text{Eq. 1})
\]

\[
X_1 = ([P] + [XP] + [YPX] + [P_0]) = ([X] \cdot [J] + m[Y])/(K_J + [X] + m[Y])
\]

\[
X_2 = ([XP] + [YPX]) = ([X] \cdot [J] + m[Y])/(K_J + [X] + m[Y]) \quad (\text{Eq. 2})
\]

\[
\Delta F = F_1 \cdot X_1 + F_2 \cdot X_2 \quad (\text{Eq. 4})
\]

An iterative computer method (15) was used to calculate \(\Delta F\) as a function of \([X]\) at constant \([Y]\) and to derive the best fit values of \(K\) and \(J\). Hydrolysis of ATP by Arp2/3 complex was monitored using the acid extraction of \(^{32}\)P-labeled phosphomolybdate complex formed following hydrolysis of \(^{32}\)P-labeled ATP, at room temperature. Data were collected for up to 3 h (only 20 min if ATP-G-actin was present). Identical results were obtained in G0 or Fe buffer. Samples contained 1 \(\mu\)M Arp2/3 complex, 2.5 \(\mu\)M \(^{32}\)P-labeled ATP, with or without 15 \(\mu\)M WA or 15 \(\mu\)M WA and 1 \(\mu\)M ATP-G-actin 1:1 complex.

**RESULTS AND DISCUSSION**

To determine the number and the location of potential high affinity binding sites, the Arp2/3 complex was treated by Dowex 1 to remove free and low affinity bound ATP. Subsequent perchloric acid extraction of bound nucleotides showed that 0.7–0.9 mol of adenine nucleotide was bound per mol of Arp2/3 complex (four independent measurements). The same experiment was then done preincubating Arp2/3 complex for periods of 10 s up to 1 min with \(^{32}\)P-labeled ATP in the presence or absence of 10 \(\mu\)M WA before Dowex treatment. Again 0.8 mol of \(^{32}\)H-ATP, which had the same specific radioactivity as the ATP in the preincubation solution, was found bound with high affinity per mol of Arp2/3 or WA-Arpa2/3 complex. This result indicates that a single rapidly exchangeable high affinity site for ATP exists both on Arp2/3 and Arp2/3-WA complex, and that there is not a non-exchangeable site.

The binding of ATP to Arp2/3 complex in the presence of controlled concentrations of free nucleotides was then examined. The Hummel and Dreyer method was first used (14). When Arp2/3 alone was loaded on the column, a single high affinity ATP binding site (0.8 mol of ATP per mol of Arp2/3 at saturation) was titrated. This site is identical to the one found after Dowex 1 treatment. When Arp2/3 in the presence of WA (15 \(\mu\)M) was loaded on the column, the molar ratio of bound ATP to Arp2/3 was twice higher, indicating that two binding sites were titrated. Saturation was reached at 1.0 \(\mu\)M free ATP (Table I). WA itself did not bind ATP. The bound nucleotides found in the absence as well as in the presence of WA were identified and quantified as 100% ATP using the luciferin-luciferase assay (Table I).

To determine to which of Arp2 or Arp3 ATP is bound in the absence and in the presence of WA, Arp2/3 complex was equilibrated with 1 \(\mu\)M \([\alpha-^{32}\text{P}]\)ATP, with or without 10 \(\mu\)M WA, and irradiated at 254 nm. Only the Arp3 subunit was covalently labeled by \([\alpha-^{32}\text{P}]\)ATP in the absence of WA. Both Arp2 and Arp3 subunits were labeled in the presence of WA (Fig. 1A). The efficiency of photo-cross-linking was more pronounced on Arp3 than on Arp2. The extent of Arp3 labeling was not affected by WA, suggesting that Arp3, in contrast to Arp2, binds ATP in a WA-independent fashion. In conclusion, activation of Arp2/3 complex by WA is linked to a structural change of the ATP site in Arp2 (Fig. 1B).

To understand whether ATP hydrolysis takes place in different structural states of Arp2/3 complex, we measured the hydrolysis of ATP in solutions of Arp2/3 complex alone, in the presence of WA, or in the presence of both WA and ATP-G-actin 1:1 complex, in G0 or Fe buffer. No hydrolysis was detected. After 3 h identical very low amounts of hydrolyzed ATP (30 nM) were found in samples and in controls without Arp2/3. No hydrolysis of ATP was detected at a 10-fold higher concentration of ATP.

The change in fluorescence of e-ATP upon binding to Arp2/3 complex was used as another probe of ATP binding (Fig. 2A). In the absence of WA, exchange of e-ATP for Arp3-bound ATP was rapid (reaction is over in 10 s at 20 °C) and led to a 5-fold enhancement of fluorescence. The data are consistent with binding of e-ATP to one site with an equilibrium dissociation constant \(K = 0.01 \pm 0.005 \text{\mu M}\). This site is located on Arp3 according to the photo-cross-linking data. Binding to a second low affinity site (J = 40 ± 10 \mu M) was detected from a slight drift of fluorescence at the plateau. In the presence of WA, the change in fluorescence upon addition of e-ATP to Arp2/3 was
FIG. 2. Fluorescence measurements of ATP binding to Arp2/3 complex. A, titration of Arp2/3 by ε-ATP in the absence and presence of WA. Arp2/3 complex (1 μM) pre-equilibrated in F₀ buffer containing 0.25 μM ATP was supplemented with ε-ATP as indicated. The fluorescence of free ε-ATP is subtracted from the data. Open circles, no WA; closed circles, +10 μM WA. The curves are calculated using $K_{\text{WA}} = 0.01$ μM, $J = 40$ μM (no WA), and $J = 0.1$ μM (+ WA); $m = 2.5$ (from data in frame C); $F₀ = 260$. Values of $K$ lower than 0.01 or higher than 0.02 μM did not fit the data at any value of $F₀$ and $F₀$. B, binding of WA to Arp2/3. The change in ε-ATP fluorescence upon addition of WA to Arp2/3 complex (1 μM) supplemented with 3 μM ε-ATP reflects WA binding. The curve is calculated using a value of $K_{\text{WA}} = 1$ μM. C, displacement of ε-ATP bound to Arp3 by different adenine nucleotides. Arp2/3 complex (0.8 μM) in $F₀$ buffer containing 0.25 μM ATP was supplemented with 5 ε-ATP in the absence of WA. Bound ε-ATP was displaced by ATP (closed circles), ADP (open circles), or AMPPNP (open squares). Identical binding data were obtained in a low ionic strength, CsCl-containing buffer. Curves are calculated as simple isotherms and adjusted using $K_{\text{app}}$ values of 2.0 μM ATP, 4 μM ADP, and 10 μM for AMPPNP in the presence of 5 μM ε-ATP. D, displacement of ε-ATP bound to Arp2 and Arp3 by different adenine nucleotides. Same conditions and symbols as under panel C except for the presence of 10 μM WA. The fluorescence decrease at saturation by AMPPNP is the same as in the absence of WA (panel C) and corresponds to the displacement of ε-ATP from Arp3 only, with $K_{\text{app}} = 11$ μM (calculated curve).

more than 2-fold greater. The fluorescence binding curves were analyzed in terms of binding of ε-ATP to two sites. The second site was attributed to Arp2, as indicated by the Hummel-Dreyer (14) and photo-cross-linking experiments. The data were satisfactorily accounted for assuming that the extent of fluorescence change (5-fold increase) and the binding constant of ε-ATP to Arp3 was not changed by WA, and the fluorescence of ε-ATP was enhanced 8-fold upon binding to Arp2. Hence in the presence of WA, 35% of the overall fluorescence change is linked to the binding of ε-ATP to Arp3 and 65% to its binding to Arp2. The value of $J$ for ε-ATP binding to Arp2 in the presence of WA was 0.1 ± 0.05 μM. This value is high enough for the site to be emptied by Dowex treatment. Similar data (not shown) were obtained with ε-ADP instead of ε-ATP. Hence Arp3 and Arp2, in a WA-dependent fashion, can bind ADP. The addition of BeF₅ did not increase the apparent affinity of ε-ADP, indicating that as for ADP-G-actin (18), BeF₅ did not bind to Arp2 or Arp3 in the ADP-bound form (data not shown).

Overall, all data convey the view that in inactive Arp2/3 complex, ATP is bound to Arp3 with high affinity and the ATP binding site on Arp2 is empty in the presence of micromolar amounts of free ATP. Binding of WA to Arp2/3 complex causes a structural change that enhances the affinity of ATP (or ADP) for Arp2 by about 400-fold. In contrast, the binding of ATP to Arp3 is not affected by WA.

At saturating amounts of ε-ATP, binding of WA to Arp2/3 can be monitored by the increase in fluorescence of ε-ATP (Fig. 2B). A value of 1.0 ± 0.2 μM was found for $K_{\text{WA}}$, in agreement with Ref. 16. The interaction of WA with Arp2/3 involves contacts with other subunits of the complex, in particular Arp3 and ARC41p (17), which are independent of ATP. Detailed balance, however, implies that if the affinity of ATP for Arp2 is enhanced by WA, a structural change of the WA-Arp2 complex, linked to enhanced interaction of WA with Arp2, must accompany ATP binding to Arp2.

Arp2/3 and G-actin associate with WA in a tight ternary complex that interacts with the filaments to make a branch (16). Measuring the binding of ε-ATP to Arp2/3 in the ternary complex was feasible, because in our hands nucleotide exchange on G-actin is slowed down by WA, and nucleotide exchanges at a higher rate on Arp2 and Arp3 than on actin. Upon addition of ATP-G-actin to WA-Arp2/3 complex, the fluorescence increase linked to binding of ε-ATP was not affected. In conclusion, the enhanced binding of ATP to Arp2 depends on WA and is independent of G-actin binding.

The relative affinities of ATP, ADP, and AMPPNP (a non-hydrolyzable analog of ATP) for Arp3 and Arp2 subunits in the complex were determined by monitoring the decrease in fluorescence linked to their exchange for bound ε-ATP (Fig. 2, C and D). In the absence of WA, the competition curves show that ATP binds Arp3 with a 2.5-fold higher affinity than ε-ATP, and with a 2-fold and 6-fold higher affinity than ADP and AMPPNP, respectively.

In the presence of WA, ATP or ADP competed with ε-ATP bound to both Arp3 and Arp2. The fluorescence measured at saturation by these nucleotides was equal to the fluorescence of free ε-ATP. In contrast, in the range 0–50 μM, AMPPNP failed to completely displace ε-ATP. The fluorescence reached a plateau corresponding to a 35% decrease. Both the change in fluorescence and the binding constant of AMPPNP are identical to those observed in the absence of WA (compare open squares and calculated curves in Fig. 2, C and D). We conclude that it is with Arp3-bound ε-ATP that AMPPNP is competing in the presence as efficiently as in the absence of WA. In a one order of magnitude higher range of AMPPNP concentrations, however, AMPPNP could displace ε-ATP from Arp2, with an affinity two orders of magnitude lower than ATP (Table II). Other non-hydrolyzable analogs of ATP, like AMPPCP and AMPPCP, behaved like AMPPNP, i.e. displaced ε-ATP bound to Arp2 and Arp3 in WA-Arp2/3 complex within a biphasic competition curve, indicating that the Arp2 subunit displays a stronger specificity than Arp3 for ATP versus non-hydrolyzable analogs of ATP. Assuming that the binding of ATP and ADP to Arp3 is independent of WA, their binding to Arp2 in the presence of WA was derived by simple subtraction. Finally GTP and CTP bound weakly to Arp2/3 without WA. All binding data are summarized in Table II.

Two conclusions emerge from the binding studies. First, Arp2 and Arp3 have a lower affinity for ATP than G-actin ($K = 10^9$ M⁻¹ in $F₀$ buffer). Second, Arp2 is more similar to G-actin than Arp3 regarding its high specificity for ATP as compared with ATP analogs, which bind poorly. As a result, in solutions containing AMPPNP and ATP in a 25:1 molar ratio, ATP is bound to Arp2 whereas essentially AMPPNP is bound to Arp3.

Using the information derived from the above binding studies, we examined the stimulation of actin assembly by Arp2/3 complex and WA in solutions containing ATP, ADP, ADP + BeF₅, AMPPNP, or mixed ATP and AMPPNP (or ATP and AMPPCP) (Fig. 3). When Dowex-treated Arp2/3 (containing

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2 C. Le Clainche, D. Didry, M.-F. Carlier, and D. Pantaloni, unpublished data.
Equilibrium dissociation constants for binding of different nucleotides to Arp3 and Arp2 in Arp2/3 complex

Values of $K_d$ were derived from fluorescence data (four independent experiments) as in Fig. 2D. Arp2/3 complex (1 µM) was supplemented with ε-ATP (5 µM) in F₁ buffer, in the absence or presence of 10 µM WA. Bound ε-ATP was displaced by different nucleotides. The fluorescence curve obtained in the absence of WA representing displacement of ε-ATP from Arp3 was subtracted from the curve obtained in the presence of WA to obtain a curve representing displacement of ε-ATP from Arp2. The concentration of nucleotide ([N₅₀]) required for 50% displacement of bound ε-ATP from Arp3 and from Arp2 was determined. The value of the intrinsic dissociation constant $K_d$ was calculated as follows. $K_d = K_e \cdot [N₅₀]/[e₉₀]$, where $[e₉₀] = 4.6 \mu M$ and $K_e = 2.5 K_{ATP}$.

| Nucleotide | Arp3 $K_d$ | Arp2 $K_d$ |
|------------|------------|------------|
| ATP        | 0.01 ± 0.005 | 0.1 ± 0.02 |
| ADP        | 0.02 ± 0.005 | 0.6 ± 0.2  |
| AMPPNP     | 0.05 ± 0.02  | 15 ± 5     |
| AMPPCP     | 0.10 ± 0.03  | 25 ± 5     |
| AMPPCP     | 0.10 ± 0.03  | 25 ± 5     |
| ATP        | 0.3 ± 0.1    | 6 ± 2      |
| CTP        | 0.8 ± 0.2    | 20 ± 5     |

one ATP bound to Arp3, no ATP bound to Arp2) was added to ATP-G-actin 1:1 complex in the absence of free ATP, no stimulation of actin polymerization was observed. Stimulation resumed upon addition of ATP (Fig. 3A). At variance with Higgs et al. (19), Arp2/3 did not stimulate polymerization, hence did not branch filaments, in the presence of ADP (Fig. 3B). In a medium containing ADP + BeF₂⁻, ADP-BeF₂⁻ is bound to actin filaments, and ADP is bound to G-actin, Arp2, and Arp3. In these conditions actin filaments are substrates for Arp2/3-induced branching because BeF₂⁻ did not affect the branching when actin was polymerized in ATP (10, 19); however Arp2/3 did not stimulate actin polymerization suggesting that the ADP-bound form of Arp2/3 is inactive. No stimulation of actin assembly was detected when ATP-G-actin 1:1 complex was polymerized in the presence of Arp2/3 with AMPPCP bound to both Arp2 and Arp3. Stimulation of actin assembly appeared in an ATP-dependent fashion consistent with saturation of a site that had a 30-fold higher affinity for ATP than for AMPPCP (Fig. 3D). The binding data (Table II and Fig. 2) indicate that Arp3 has only a 5- to 10-fold higher affinity for ATP than for analogs. Hence this last result indicates that it is the binding of ATP to Arp2 that is required for filament branching. Similarly, no stimulation of polymerization of ATP-G-actin 1:1 complex was observed by Arp2/3 in the presence of 0.2 mM AMPPCP. Addition of only 10 µM ATP restored 80% maximum stimulation. Under these conditions, essentially AMPPNP is bound to Arp3 and ATP to Arp2 (Fig. 3C).

In summary, despite the similarity between actin, Arp2, and Arp3, ATP is bound less tightly to Arp2 and even to Arp3 than to G-actin. In the inactive state of the Arp2/3 complex, the affinity of ATP for Arp2 is low suggesting that the cleft separating the two domains that contains the ATP site must be largely open. However, even when the ATP site is empty, Arp2 does not denature, as actin would do under such conditions, suggesting that molecular interactions between Arp2 and neighboring subunits in Arp2/3 complex prevent denaturation of Arp2. The high affinity binding of ATP to Arp2 that is linked to activation of Arp2/3 by WASP proteins is expected to cause a large structural change of the complex. Branching of filaments occurs by association of the branching complex (G-actin-WA-Arp2/3) with a filament (8, 10, 20). Polymerization studies in the presence of ATP and analogs show that branching can occur if a non-hydrolyzable analog of ATP is bound to Arp3, but ATP binding to Arp2 is required for branching. This result suggests, but does not prove, that ATP hydrolysis on Arp2 is required for filament branching. The low affinity of the non-hydrolyzable analogs of ATP for Arp2 may also indicate that they do not induce the functional structural state of Arp2/3 complex. Whether ATP hydrolysis on Arp2 is required for branching remains an open issue.

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![Fig. 2. Stimulation of actin polymerization by Arp2/3 complex in the presence of different nucleotides.](http://www.jbc.org/content/190/40/11404.full)

**Fig. 2.** Stimulation of actin polymerization by Arp2/3 complex in the presence of different nucleotides. A, ATP-G-actin 1:1 complex (Dowex I-treated, 2.2 µM, 10% pyrenyl-labeled) was polymerized in the presence of 0.5 µM WA and the following additions: 25 mM Dowex-treated Arp2/3 complex and 10 µM ATP (a), 25 mM Dowex-treated Arp2/3 complex, no free ATP (b), none (c). B, ADP-actin (4.4 µM) was polymerized in 0.2 mM ADP or 0.2 mM ATP and 1 mM BeF₂⁻ as indicated; thick and thin lines correspond to the presence or absence of 50 nM Arp2/3 complex equilibrated in ADP. All solutions contained 20 units/ml hexokinase and 2 mM glucose to avoid contaminating ATP. C, ATP-G-actin 1:1 complex, 0.5 µM WA, 0.2 mM AMPPNP, 20 nM Arp2/3 (equilibrated in AMPPCP and the following additions: a, none; b, 10 µM ATP; c, 0.2 mM ATP, no AMPPNP; thin lines, controls (no Arp2/3) with or without 10 µM ATP. Gel filtration assays showed that at least 95% of actin is ATP-bound in the presence of a 100-fold excess of AMPPNP or AMP-PCP. D, ATP-G-actin 1:1 complex (5 µM) was polymerized in the presence of 0.5 mM AMPPCP, 0.5 µM WA, 60 nM Arp2/3 complex, and ATP as indicated (in µM). Bottom curve, control without Arp2/3, with or without ATP.
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