Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 3739–3744, March 1999
Cell Biology

Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex

(polymerization/cell motility)

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Contributed by Thomas D. Pollard, February 1, 1999

ABSTRACT **The Arp2/3 complex, a stable assembly of two actin-related proteins (Arp2 and Arp3) with five other subunits, caps the pointed end of actin filaments and nucleates actin polymerization with low efficiency. WASp and Scar are two similar proteins that bind the p21 subunit of the Arp2/3 complex, but their effect on the nucleation activity of the complex was not known. We report that full-length, recombinant human Scar protein, as well as N-terminally truncated Scar proteins, enhance nucleation by the Arp2/3 complex. By themselves, these proteins either have no effect or inhibit actin polymerization. The actin monomer-binding W domain and the p21-binding A domain from the C terminus of Scar are both required to activate Arp2/3 complex. A proline-rich domain in the middle of Scar enhances the activity of the W and A domains. Preincubating Scar and Arp2/3 complex with actin filaments overcomes the initial lag in polymerization, suggesting that efficient nucleation by the Arp2/3 complex requires assembly on the side of a preexisting filament—a dendritic nucleation mechanism. The Arp2/3 complex with full-length Scar, Scar containing P, W, and A domains, or Scar containing W and A domains overcomes inhibition of nucleation by the actin monomer-binding protein profilin, giving active nucleation over a low background of spontaneous nucleation. These results show that Scar and, likely, related proteins, such as the Cdc42 targets WASp and N-WASp, are endogenous activators of actin polymerization by the Arp2/3 complex.**

Eukaryotic cells move by coordinating membrane protrusion and substrate attachment at the leading edge with detachment from the substrate at the trailing edge. Either spontaneously or in response to chemotactic signals and growth factors, motile cells assemble actin filaments at the leading edge and crosslink them into a rigid, orthogonal network (1, 2). The growth of this network drives protrusion of the membrane, probably by a Brownian ratchet-type mechanism (3, 4). The initial step in the assembly of this network is the creation of free, fast-growing (barbed) filament ends (5). In some cases, uncapping (6) or severing existing actin filaments (John Condeelis, personal communication) contributes free barbed-ends, but *de novo* creation of barbed ends seems to be particularly important (7). In no case is the pathway from receptor to newly formed actin filaments established, and the situation is complicated by the branching of signals into multiple pathways downstream of both seven-helix G protein-coupled receptors and receptor tyrosine kinases. Some of these pathways depend on Rho-family GTPases, such as Rho, Rac, and Cdc42 (8, 9).

The Arp2/3 complex (10) is the prime candidate to create new actin filaments that grow at the barbed ends (9, 11, 12). This stable and abundant complex of two actin-related proteins (Arp2 and Arp3) with five other polypeptides nucleates actin filaments (11, 12), binds the pointed end of actin filaments with high affinity (11), and forms mechanically rigid networks of filaments (13) by crosslinking them end-to-side at a fixed angle (11). We named this combination of nucleation and lateral branching dendritic nucleation.

The basal nucleating activity of Arp2/3 complex is low, apparently because such activity depends on the spontaneous formation of actin dimers (11). The spontaneous self-assembly of actin proceeds stepwise: monomers associate to form a dimer that binds another monomer to form a trimer and so on, until a stable filament is formed (14). The first two steps are highly unfavorable and create a lag in polymerization that varies with the monomer concentration. The Arp2/3 complex seems to favor nucleation by stabilizing actin oligomers as they form spontaneously, rather than binding actin monomers directly (11).

However, Welch *et al.* (12) established that another protein, ActA, a cell-surface protein from *Listeria monocytogenes*, can stimulate nucleation by Arp2/3 complex. *Listeria* uses ActA and Arp2/3 complex to initiate actin polymerization (15), which propels the bacterium through cytoplasm (16). In contrast to pseudopod formation, the actin-driven motility of *Listeria* is apparently independent of endogenous signaling pathways that depend on kinases or GTP-binding proteins (17). GTP-γ-S-stimulated actin polymerization in cell extracts depends on activated Cdc42 (2), Arp2/3 complex (7, 18), and at least one other activating factor (18), which remains to be identified.

Machesky and Insall (19) discovered two potential endogenous regulators of the Arp2/3 complex: WASp, the protein responsible for Wiskott–Aldrich Syndrome, and a related protein, Scar. Both WASp and Scar bind the p21 subunit of the Arp2/3 complex. Wiskott–Aldrich Syndrome is a human genetic disease characterized by defects in platelet development and lymphocyte function (20, 21). Scar was identified originally in *Dictyostelium discoideum* as a suppressor of mutations in a cAMP receptor (22), but homologs have been identified in other organisms, including humans (22, 23). *Dictyostelium* Scar-deletion mutants have reduced actin-filament content, abnormal morphology, and aberrant cytoskeletal organization during chemotaxis, but they survive and undergo cAMP-mediated aggregation and tip formation (22). Consistent with the idea that Scar and WASp regulate actin assembly, transient overexpression of domains of Scar and WASp that bind p21 delocalizes Arp2/3 complex and suppresses lamellipod forma-

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tion in cultured vertebrate cells (19). However, this study did not determine whether Scar and WASp function as activators, inhibitors, or targeting signals for Arp2/3 complex.

We find that Scar enhances nucleation of purified actin by Arp2/3 complex. Without Arp2/3 complex, Scar or domains of Scar either have no effect or inhibit polymerization. The Scar construct most effective at stimulating Arp2/3 activity consists of the C-terminal half of the protein, including the proline-rich domain, the actin monomer-binding W domain, and the p21-binding A domain. Even with saturating concentrations of Scar, spontaneous polymerization lags initially, unless Scar and Arp2/3 complex are preincubated with actin filaments, suggesting that Arp2/3 complex and Scar nucleate more efficiently when they assemble on the side of an existing actin filament. Scar and Arp2/3 complex activate nucleation effectively even when the actin monomer pool is saturated with profilin, a strong inhibitor of spontaneous nucleation, but profilin binding to the proline-rich domain of Scar is not required and does not activate nucleation. We conclude that Scar and, likely, related proteins like WASp are endogenous activators of *de novo* actin filament assembly.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma unless indicated otherwise. Buffer G consists of 2 mM Tris (pH 8), 0.5 mM DTT, 0.2 mM ATP, 0.1 mM $CaCl_2$, and 3 mM sodium azide.

Protein Preparation. Proteins were purified according to the following methods: *Acanthamoeba* Ca-ATP-actin was gel-filtered as in ref. 24; rabbit-skeletal-muscle Ca-ATP-actin was gel-filtered as in ref. 25; actin was pyrene-labeled as in ref. 24; *Acanthamoeba* Arp2/3 complex A was purified by binding to a poly-L-proline affinity column as in ref. 26; *Acanthamoeba* profilins-I and -II were purified as in ref. 27; and recombinant human profilin-I was purified as in ref. 28. Just before the experiments, Ca-ATP-actins in buffer G were converted to Mg-ATP-actin by the addition of $MgCl_2$ to 50 μM and EGTA to 200 μM either for 2 min at 25°C or for up to 4 h on ice. *Acanthamoeba* Arp2/3 complex B, the fraction flowing through a poly-L-proline affinity column, was purified by column chromatography by using a method developed by Kelleher *et al.* with modifications (29). Human Arp2/3 complex was purified from neutrophils isolated from 6 units of fresh human blood (H.N.H. and T.D.P., unpublished work). Briefly, cells were washed in 120 mM NaCl/10 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/5 mM glucose/25 mM Hepes, pH 7.4, stimulated for 10 s at 37°C with 200 nM *N*-formyl-methionine-leucine-phenylalanine, and lysed with an equal volume of ice-cold 10 mM imidazole, pH 7.4/140 mM KCl/2 mM $MgCl_2$/2 mM EGTA/1 mM ATP/1 mM DTT/0.5% Thesit (Boehringer Mannheim)/20 μg/ml leupeptin/5 μg/ml pepstatin A. Arp2/3 complex was isolated from a high speed supernatant by chromatography on an Amersham Pharmacia Source Q15 column, followed by an Amersham Pharmacia MonoQ 10/10 column and by gel filtration on an Amersham Pharmacia Superdex 200 16/10 column. Arp2/3 complex was detected during the purification by Western blotting with a polyclonal antibody raised against *Escherichia coli*-expressed human p34 subunit. All three Arp2/3 fractions were free of major contaminants, as determined by SDS/PAGE (Fig. 1).

Recombinant Scar Preparations. Full-length human Scar (19) and the domains of Scar (Fig. 1) were produced as N-terminal GST-fusion proteins. Scar-PWA corresponds to amino acids 172–559; Scar-WA corresponds to amino acids 443–559; Scar-W corresponds to amino acids 443–547; Scar-A corresponds to amino acids 528–559. Overnight, 15-ml cultures of *E. coli* (DE-Lys strain) containing various fusion-protein constructs grown in LB medium with 50 μg/ml ampicillin/25 μg/ml chloramphenicol were added to 1 liter of prewarmed LB medium and shaken at 250 rpm at 37°C for 2 h alone and for 4 h with 0.2 mM isopropyl β-D-thiogalactoside (IPTG). For Scar-PWA or full-length Scar, proline was added to 1 mM at the same time as IPTG. Bacteria were pelleted and washed once in 20 ml of PBS at 4°C, and the pellets were frozen on dry ice for 10 min. Frozen pellets were resuspended in 8 ml of 50 mM Tris, pH 8.0/40 mM EDTA/2.5% (wt/vol) sucrose/0.02% NaN_3/1 mM PMSF/2.5 mg/ml lysozyme and shaken at room temperature for 20 min. Subsequent steps were at 0–4°C. Lysates were centrifuged for 30 min at 100,000 $\times$ *g*. The supernatant was incubated overnight with 0.5 ml of glutathione-agarose (Sigma) with gentle shaking. The beads were washed twice with 20 ml of PBS containing 0.2% Tween 20 and 1 mM DTT, then washed twice with TRB (50 mM Tris-Cl, pH 8.0/5 mM $MgCl_2$/150 mM NaCl/2.5 mM $CaCl_2$/1 mM DTT), and resuspended in TRB with 10 units of bovine thrombin. After overnight incubation at 4°C, the supernatant was collected, and thrombin was adsorbed to 50 μl of *p*-aminobenzamidine beads for 30 min on ice. Uncleaved GST-fusion proteins were eluted from the beads with 5 mM glutathione in TRB. Glutathione was removed by dialysis overnight against two changes of TRB. Proteins were concentrated by using Centricon spin filters (Amicon) and stored as aliquots frozen at −80°C.

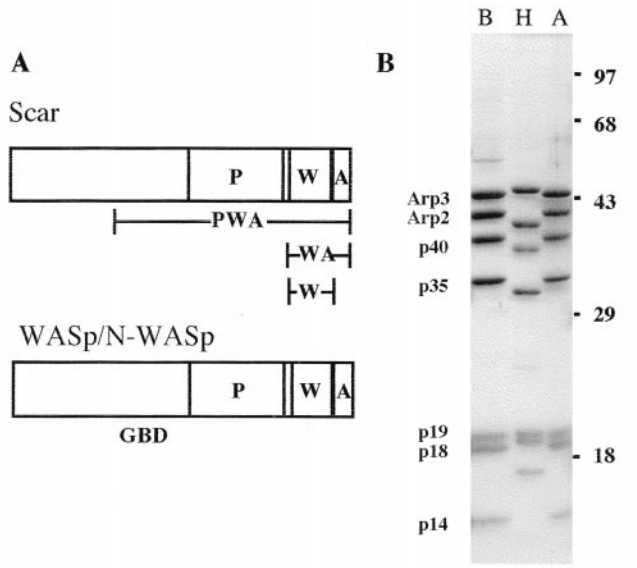

FIG. 1. Proteins used in this study. (*A*) Linear diagrams of Scar and WASp domains. (*B*) SDS/PAGE of 10 μg of purified Arp2/3 complexes. A, amoeba Arp2/3 complex A; B, amoeba Arp2/3 complex B. H, human Arp2/3 complex. The gel is stained with Coomassie blue

Polymerization Experiments. Polymer concentration was measured as a function of time by monitoring fluorescence intensity of 5–10% pyrenyl-actin included with 90–95% unlabeled actin by using either a PTI Alpha-scan (Photon Technology International, Princeton) or an SLM 8100 spectrafluorometer (Spectronic, Urbana, IL) with excitation at 366 nm and emission at 407 nm. Polymerization was carried out at room temperature (24°C) in buffer G with KME (50 mM KCl, 1 mM $MgCl_2$, and 1 mM EGTA, buffered with 10 mM imidazole or Tris). The details of the buffer composition and protein concentrations are given in the figure legends. We measured the concentration of ends from the rate of elongation (30), by using the following relationship: $R = k_+(A)(ends)$, where R is the rate of elongation, k_+ is the association rate constant (10 $\mu M^{-1} \cdot s^{-1}$), A is the concentration of actin monomer, and *ends* is the concentration of growing filament ends. Thus, one can calculate the concentration of ends from the elongation rate at any point in a polymerization experiment. Alternatively, we used the final products of a polymer-

ization experiment as the seeds for the elongation of monomeric actin. Preincubation experiments with Scar, Arp2/3 complex, and actin filaments were carried out in three steps. First, unlabeled actin filaments were polymerized from 10 μM unlabeled actin monomers in KME for 60 min at 25°C. Second, Arp2/3 complex and Scar-PWA were reacted with preformed 2 μM unlabeled actin filaments for a given time at 25°C in buffer G with KME. Third, the mixture was diluted 6.7-fold into buffer G with KME with a final concentration of 4 μM pyrene-actin monomers, and the time course of polymerization followed.

RESULTS

Scar Stimulates the Nucleation Activity of the Arp2/3 Complex. The combination of Scar and Arp2/3 complex strongly stimulates spontaneous assembly of monomeric actin (Fig. 2*A*). Together, they reduce the initial lag, during which nuclei form, and increase the maximum rate of elongation, which depends on the concentration of growing ends. Thus, we interpret the effect to be an enhancement of nucleation. Under the conditions in this experiment, pure actin monomers (●) assemble slowly, and neither 50 nM Scar-PWA (▽) nor 100 nM Arp2/3 complex (○) alone promote polymerization. The mixture of Scar and Arp2/3 complex (▲) shortens the half time for polymerization by more than 15-fold and increases the maximum rate by 50-fold. At higher (micromolar) concentrations, Arp2/3 complex alone stimulates spontaneous assembly of actin filaments (11), and Scar-PWA or Scar-WA augment this activity (Fig. 3 *A* and *B*). Human Scar stimulates the nucleation activity of both amoeba and human Arp2/3 complex. There were minor differences in the activities of these three preparations (data not shown).

Experiments with expressed fragments of Scar indicated the domains required to activate Arp2/3 complex (Fig. 2). Full-length Scar (Figs. 2 and 3, ▲) and Scar-PWA (Figs. 2 and 3, △) are the most effective enhancers of actin nucleation by Arp2/3 complex (Fig. 2). Scar-WA is active at 20-fold higher concentrations than Scar-PWA (Fig. 3). Scar-A (▼) and Scar-W (□) are not active (Fig. 2*A*). The effect depends on the concentrations of both the Scar construct and the Arp2/3 complex, but, even at high concentrations of both proteins, the initial lag persists, providing that the actin is highly purified and monomeric (Fig. 3).

Actin Filaments Stimulate Nucleation by the Arp2/3 Complex and Scar Constructs. Preincubation of actin filaments with both Arp2/3 complex and Scar-PWA allows nanomolar concentrations of these activators to overcome the initial lag at the outset of actin polymerization and to produce very high rates of elongation relative to either the filaments alone or filaments with either Arp2/3 complex or Scar (Fig. 4*A*). At a concentration of 4 μM, highly purified pyrenyl-actin monomers (Fig. 4, ×) have a long lag, with a half time of >2,000 s. A mixture of 15 nM Arp2/3 complex and 29 nM Scar-PWA (Fig. 4, ■) reduces the lag (half time of 135 s) and gives a maximum elongation rate of 42 nM·s^{-1}. The addition of 300 nM polymerized actin (Fig. 4, ▲) initiates elongation without a lag at a rate of 3 nM·s^{-1}, corresponding to 0.08 nM ends (4,000 subunits per filament). The inclusion of either 15 nM Arp2/3 complex (Fig. 4, ◆) or 29 nM Scar-PWA (Fig. 4, ●) has no effect on elongation from the filaments. However, mixing Arp2/3 complex and Scar-PWA with filaments for just 1 min before adding pyrenyl-actin monomers (Fig. 4, ○) nearly eliminates the initial lag (half time of 40 s) and gives rapid polymerization at 50 nM·s^{-1} (17 times faster than filaments alone, corresponding to 2.4 nM filaments).

The effect of preincubation of Scar and Arp2/3 complex with actin filaments depends on the duration of the incubation. The initial polymerization rate is enhanced in less than 30 s and is enhanced increasingly over 5 min. The initial polymerization rate also depends on the concentration of Scar. Although high concentrations of Scar and Arp2/3 complex cannot eliminate the initial lag in the polymerization of actin monomers alone (Fig. 3), low concentrations of Scar and Arp2/3 complex eliminate the lag when preincubated with filaments (Fig. 4). Increasing the concentration of Scar-PWA enhances the formation of nuclei, but even a 2-fold molar excess of Scar over 15 nM Arp2/3 complex has a powerful effect on nucleation. Preincubation of filaments, 58 nM Scar, and 15 nM Arp2/3 complex for 5 min (Fig. 4, △) produces 7 nM ends (one filament per two complexes), a result calculated from the elongation rate of 140 nM·s^{-1}.

The nucleating activity of Scar and Arp2/3 complex varies with pH (Fig. 4 *A* and *B*). Mixtures of Scar-PWA and Arp2/3 complex reduce the lag and increase the polymerization rate more effectively at pH 8 than at pH 7. Similarly, preincubation of Scar-PWA and Arp2/3 complex with filaments generates about four times as many ends at pH 8 than at pH 7.

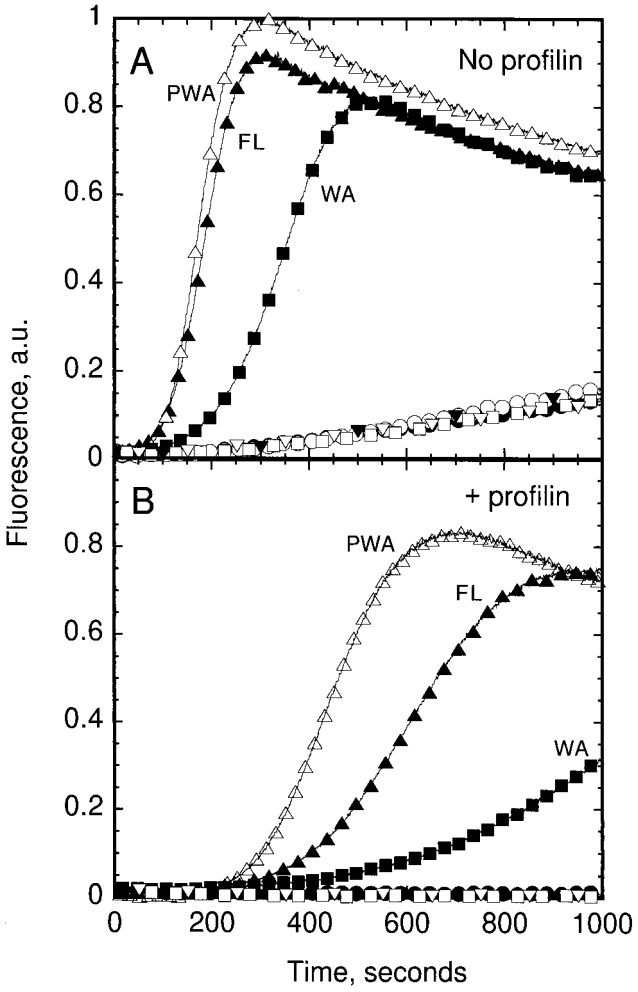

FIG. 2. Effects of Scar constructs, Arp2/3 complex, and profilin on the time course of actin polymerization. (*A*) No profilin. (*B*) *Acanthamoeba* profilin-II at 7.5 μM. (*A* and *B*) Conditions: 2.8 μM *Acanthamoeba* actin monomers, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM ATP, 3 mm NaN_3, and 10 mM imidazole (pH 7.0) at 22°C. The polymerization of actin was monitored by pyrene fluorescence. (●) No additions to actin; (○) 100 nM Arp2/3 complex; (▲) 50 nM full-length Scar/100 nM Arp2/3 complex; (△) 50 nM Scar-PWA/100 nM Arp2/3 complex; (■) 50 nM Scar-WA/100 nM Arp2/3 complex; (□) 50 nM Scar-W/100 nM Arp2/3 complex; (▼) 50 nM Scar-A/100 nM Arp2/3 complex; (▽) 50 nM Scar-PWA (*A*) or 50 nM full length Scar (*B*). Without Arp2/3 complex, these concentrations of Scar constructs have no effect on actin polymerization in either the presence or absence of profilin-I or profilin-II.

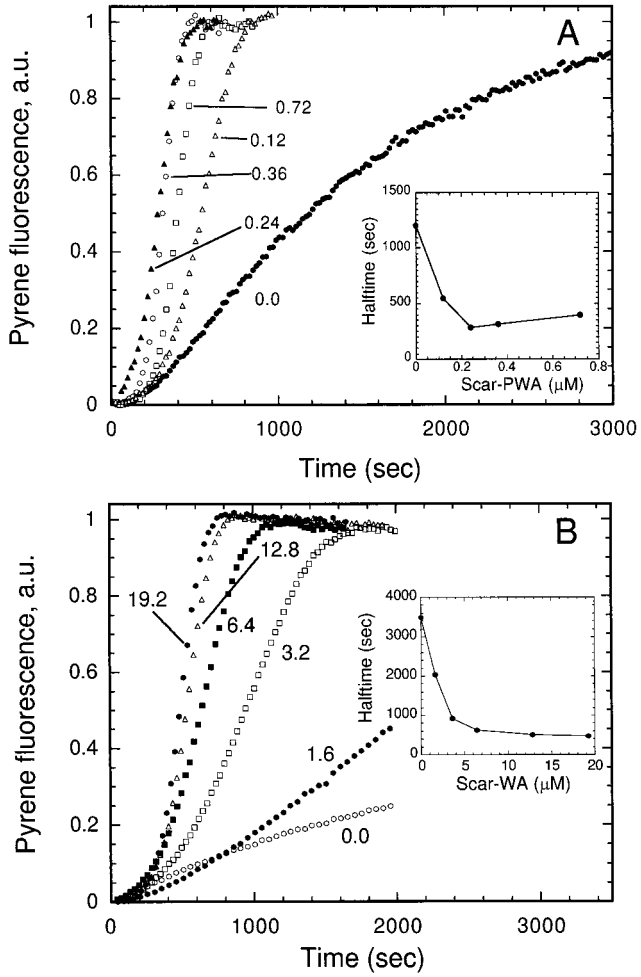

FIG. 3. Dependence of the activation of actin nucleation by Arp2/3 complex A on the concentrations of Scar-PWA (*A*) and Scar-WA (*B*). Conditions: 3.3 μM actin and 0.9 μM amoeba Arp2/3 complex A with Scar-WA or Scar-PWA as indicated by the micromolar concentrations beside each curve. The buffer included 50 mM KCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM imidazole (pH 7.5). *Insets* indicate the time (in seconds) to half-maximal polymerization vs. concentrations of Scar-WA or Scar-PWA.

Effect of Scar on Polymerization of Profilin-Actin Complexes. Because the cellular pool of unpolymerized actin monomers is bound to sequestering proteins like profilin, we studied the activity of Scar and Arp2/3 complex with an excess of profilin over actin (Fig. 2*B*). Profilin inhibits spontaneous polymerization of pure actin by inhibiting nucleation, but Scar and Arp2/3 overcome this inhibition. Nucleation by Arp2/3 complex and Scar in the presence of profilin requires the W and A domains of Scar. Nucleation is stronger with constructs containing the P domain but does not require the P domain (Fig. 2, △ and ■), a part of the WASp family of proteins postulated to interact with profilin (23). Thus, profilin reduces spontaneous nucleation *in vitro* and, likely, *in vivo*, making the effect of Scar and Arp2/3 complex on nucleation more dramatic. This property may allow cells to control background nucleation without eliminating the ability of activators such as Scar and Arp2/3 complex from activating nucleation in response to a signal.

DISCUSSION

Because of its biochemical activities, cellular localization, and mutant phenotypes, we postulated that the Arp2/3 complex regulates the cellular actin cytoskeleton by *de novo* formation of actin filaments by a dendritic nucleation mechanism (11). Proteins that interact with the Arp2/3 complex such as Scar or WASp might target the complex to zones of active filament assembly and/or activate or inhibit actin nucleation, pointed end capping, or binding to the side of actin filaments. Overexpression of Scar constructs that bind Arp2/3 complex alters the distribution of the complex and inhibits lamellipod formation in cultured cells (19). This result indicates that the Scar binding site on the p21 subunit of the Arp2/3 complex contributes to the localization of the complex *in vivo* but did not indicate whether Scar binding influences any activity of the Arp2/3 complex.

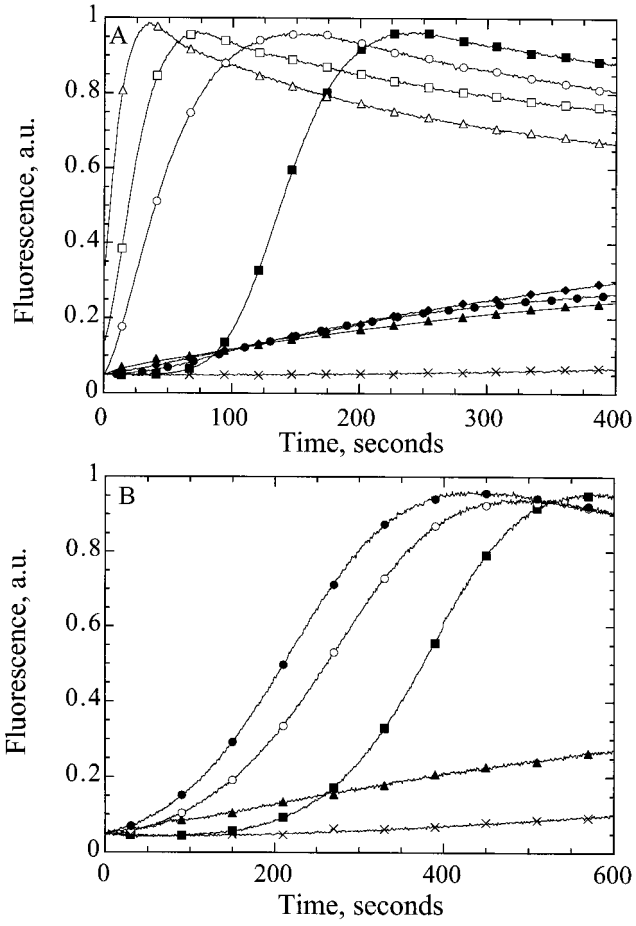

FIG. 4. The effect on the time course of polymerization of preincubating Arp2/3 complex and/or Scar constructs with actin filaments before the addition of actin monomers. The reaction was carried out in three steps as described in *Materials and Methods*. The concentrations given are those after a 6.7-fold dilution of the preincubated components into pyrenyl-actin monomers. Final conditions: 4 μM amoeba actin monomers, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM ATP, and 3 mM NaN_3. (*A*) Tris-Cl (pH 8.0) at 10 mM. (×) Actin monomers alone; (■) actin monomers with 29 nM Scar-PWA/15 nM amoeba Arp2/3 complex A; (▲) actin monomers with 300 nM actin filaments; (●) actin monomers with 300 nM actin filaments preincubated with 29 nM Scar-PWA; (◆) actin monomers with 300 nM actin filaments preincubated with 15 nM amoeba Arp2/3 complex A; (○) actin monomers with 300 nM actin filaments preincubated with 29 nM Scar-PWA/15 nM amoeba Arp2/3 complex B for 1 min; (□) actin monomers with 300 nM actin filaments preincubated with 58 nM Scar-PWA/15 nM amoeba Arp2/3 complex A for 3 min; (△) actin monomers with 300 nM actin filaments preincubated with 58 nM Scar-PWA/15 nM amoeba Arp2/3 complex A for 5 min. (*B*) Imidazole (pH 7.0) at 10 mM. The symbols used are defined in *A*, except that ○ indicates actin monomers with 300 nM actin filaments preincubated with 400 nM phalloidin/29 nM Scar-PWA/15 nM amoeba Arp2/3 complex A for 1 min.

Mechanism of Nucleation. In support of a role for Scar as a cellular activator of the Arp2/3 complex, we find that Scar and all constructs tested that contain both the actin-binding (W) and Arp2/3 complex binding (A) motifs dramatically stimulate actin nucleation by Arp2/3 complex. The most effective construct, Scar-PWA, also includes a proline-rich domain. Constructs containing only the W or A domains are inactive. Machesky and Insall (19) reported that Scar-W binds actin monomers but not filaments. No Scar construct initiates actin polymerization on its own, and some retard spontaneous actin polymerization modestly at high concentrations (data not shown).

Because the Arp2/3 complex caps the pointed end of actin filaments with high affinity, each Arp2/3 complex might generate a barbed end, but the concentration of ends produced during spontaneous polymerization by the combination of Arp2/3 complex and Scar constructs is lower than the concentrations of either Arp2/3 complex or Scar. These concentrations were quantitated by using the rate of polymerization. Elongation at the barbed ends dominates polymerization, and the barbed-end elongation rate is equal to the product of the association-rate constant, the concentration of actin monomer, and the concentration of growing ends [$R = k_+(A)(ends)$]. At the midpoint of polymerization, no mixture of Scar and Arp2/3 complex produced more than one end for every 6 complexes (pH 8) and, under many conditions, less than one end for every 50 complexes (pH 7). We expect that the number of ends formed before the midpoint of polymerization to exceed the number formed after the midpoint, because nucleation is very concentration-dependent and because the concentration of actin monomers falls rapidly late in the assembly reaction. An elongation assay for ends in the final products of polymerization of mixtures of actin, Arp2/3 complex, and Scar constructs confirmed that the concentration of ends is much lower than Arp2/3 complex, even when it is highly activated by Scar.

Even at maximally effective concentrations, Scar-PWA and Arp2/3 complex do not eliminate the lag at the outset of actin polymerization, even when they are preincubated together before adding actin monomers. This result and the substoichiometric production of ends show that Scar-PWA does not turn the whole population of Arp2/3 complex into stable nuclei comparable to the barbed end of actin filaments. Some Arp2/3 complexes might have been denatured, but the remarkable effect of preincubation with filaments argues against this result.

The activation of nucleation by preincubating Arp2/3 complex and Scar-PWA with actin filaments provides evidence that Scar activates spontaneous polymerization of monomeric actin by promoting the formation of Arp2/3 complex nuclei on the sides of actin filaments (Fig. 5). We postulate that Scar enhances the binding of Arp2/3 complex to the sides of actin filaments and that this binding promotes nucleation. An alternative explanation is that Arp2/3 complex creates new ends by severing actin filaments in the presence of Scar-PWA; however, we have no evidence for such severing, and phalloidin has little effect on the ability of actin filaments to activate nucleation (Fig. 4*B*). The time required for the assembly of a multicomponent structure on the side of a filament may explain why high concentrations of Arp2/3 complex and Scar do not eliminate the lag at the outset of polymerization and why preincubation with filaments is required to activate most of the Arp2/3 complex. *In vitro*, Arp2/3 complex and Scar assemble nuclei efficiently only after filaments form spontaneously. On the other hand, cells contain an abundance of filaments to act as a scaffold for nucleation of new filaments. The effects of Scar and Arp2/3 complex depend on their concentrations, owing to at least two second-order reactions necessary to assemble the nucleation structure. Much additional work is required to establish the details of this complicated multimolecular reaction.

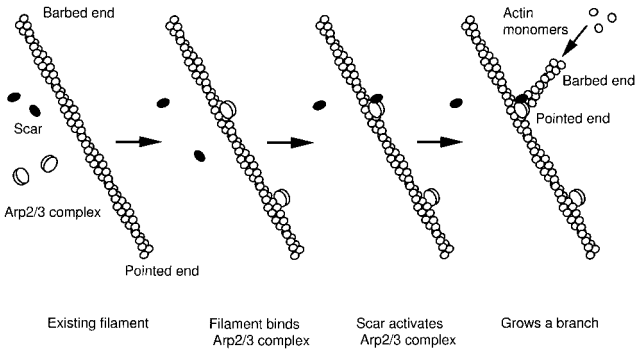

FIG. 5. Dendritic nucleation hypothesis. The association of Arp2/3 complex with Scar and the side of an actin filament activates nucleation of a new actin filament, which is capped at its pointed end by the Arp2/3 complex and which grows in the barbed direction as a branch of the older filament. The exact order of events is not yet known. Scar could bind to Arp2/3 complex before their association with the side of an actin filament.

Effect of Profilin. Scar and Arp2/3 complex overcome the inhibition of nucleation by profilin but are not activated by profilin. Rather, profilin accentuates the effect of Scar and Arp2/3 complex on nucleation by reducing the background level of spontaneous nucleation, creating a better on/off switch. Arp2/3 complex binds profilin, but the affinity is low such that most of the complex is free in our experiments. The proline-rich domain of N-WASp, comparable to the P domain of Scar, is thought to bind profilin (23, 31), but profilin inhibits rather than stimulates polymerization in the presence of Arp2/3 complex and Scar-PWA. As such, the proline-rich region does not seem to enhance nucleation by recruiting profilin-actin to the Arp2/3 complex.

Regulation of Scar Family Proteins. Scar is constitutively active in our experiments and in those of Machesky and Insall (19), although it seems likely that cells regulate its activity to prevent spurious nucleation of actin filaments, perhaps by an autoinhibitory mechanism (22). The Scar relatives WASp and N-WASp are regulated by the small GTPases Rac and Cdc42 (21, 32) and by tyrosine kinases (23, 33), although the nature of this regulation (positive or negative or localization) is unknown. No ligands are known for the part of Scar placed similarly on the Cdc42 binding site of WASp. Proteins with SH3 domains that bind the proline-rich regions of WASp and, possibly, Scar are good candidates to modulate the activity of these proteins. Future investigation must address whether Scar is regulated by activating proteins, by inhibitory proteins, by posttranslational modification, and/or by localization. We expect the P, W, and A domains of WASp and N-WASp to have effects on Arp2/3 complex similar to those of Scar constructs. The Cdc42 binding site of WASp may provide a pathway for small GTPases to regulate actin polymerization (5, 9, 7).

We thank Velia Fowler and Ryan Littlefield for many helpful suggestions about the presentation of this work. This work was supported by National Institutes of Health Research Grant GM-26338 to T.D.P. and a Medical Research Council Career Development Fellowship to L.M.M.

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