Scar1 and the related Wiskott–Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex

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Background: The actin-related proteins Arp2 and Arp3 are part of a seven-protein complex which is localized in the lamellipodia of a variety of cell types, and in actin-rich spots of unknown function. The Arp2/3 complex enhances actin nucleation and causes branching and crosslinking of actin filaments *in vitro*; *in vivo* it is thought to drive the formation of lamellipodia and to be a control center for actin-based motility. The Wiskott–Aldrich syndrome protein, WASP, is an adaptor protein implicated in the transmission of signals from tyrosine kinase receptors and small GTPases to the actin cytoskeleton. Scar1 is a member of a new family of proteins related to WASP, and it may also have a role in regulating the actin cytoskeleton. Scar1 is the human homologue of *Dictyostelium* Scar1, which is thought to connect G-protein-coupled receptors to the actin cytoskeleton. The mammalian Scar family contains at least four members. We have examined the relationships between WASP, Scar1, and the Arp2/3 complex.

Results: We have identified WASP and its relative Scar1 as proteins that interact with the Arp2/3 complex. We have used deletion analysis to show that both WASP and Scar1 interact with the p21 subunit of the Arp2/3 complex through their carboxyl termini. Overexpression of carboxy-terminal fragments of Scar1 or WASP in cells caused a disruption in the localization of the Arp2/3 complex and, concomitantly, induced a complete loss of lamellipodia and actin spots. The induction of lamellipodia by platelet-derived growth factor was also suppressed by overexpression of the fragment of Scar1 that binds to the Arp2/3 complex.

Conclusions: We have identified a conserved sequence domain in proteins of the WASP family that binds to the Arp2/3 complex. Overexpression of this domain in cells disrupts the localization of the Arp2/3 complex and inhibits lamellipodia formation. Our data suggest that WASP-related proteins may regulate the actin cytoskeleton through the Arp2/3 complex.

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Background

Actin polymerization in lamellipodia is thought to be the driving force for much of cell motility. A complex of seven proteins, called the Arp2/3 complex, is thought to be an essential player in this process. The seven components of the Arp2/3 complex are Arp2, Arp3, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc [1,2]. The Arp2/3 complex is localized in the lamellipodia of crawling cells, and in actin-rich spots of unknown function [2,3]. *In vitro*, the Arp2/3 complex can enhance the nucleation of new actin filaments [4,5], and *in vivo* it can cause the formation of branched structures similar to those previously observed at the leading edges of moving cells [4,6]. Arp2/3 complexes can also cap the slow-growing ends of actin filaments, an observation which has implications for the turnover of actin filaments in cells [4].

The first evidence that the Arp2/3 complex could be important for actin-based motility *in vivo* came from a study showing that it is capable of initiating actin polymerization on the surface of the motile intracellular pathogen *Listeria monocytogenes* [7]. *Listeria* is used as a model for actin-based cellular motility because it recruits the cytoskeletal proteins of host cells to assemble actin tails, which in turn propel it through the cytoplasm. The Arp2/3 complex localizes along the length of these actin tails, where it may nucleate and crosslink actin filaments [7]. It is not, however, yet known if the Arp2/3 complex is essential for actin tail formation.

Genetic studies in yeast also provide an insight into the *in vivo* function of the Arp2/3 complex. The complex localizes to cortical actin patches and is required for their motility and their polarized organization in cells [8–10]. Yeast actin patches are thought to be important for membrane and cell-wall deposition during cytokinesis and cell growth [11]. Both Arp2, which is required for endocytosis [12], and Arp3 are essential in *Saccharomyces cerevisiae*

[10,13], and Arp3 is essential in *Schizosaccharomyces pombe* [14]. The function of the Arp2/3 complex is also connected to profilin function in *S. pombe*, as a mutation in the p41 subunit, named SOP2 in *S. pombe*, can suppress a loss-of-function mutation in the gene encoding profilin which causes a block in cytokinesis [15]. This fits with studies in amoebae showing that the Arp2 subunit of the Arp2/3 complex can bind to profilin [16,17].

Although many of the key players in actin cytoskeletal organization have been identified, it is less clear how these proteins respond to signals from outside of the cell that induce cytoskeletal reorganization and cell motility. The WASP family of proteins, including WASP, N-WASP, and recently Scar proteins, is most likely to be involved in transmitting signals from receptors to the cytoskeleton. All of these proteins are organized into several modular domains, characterized by sequence homology and binding interactions. The amino-terminal half of WASP-family proteins appears to be important for communication with receptors or small GTPases. Both WASP and N-WASP bind to activated Cdc42 and Rac through their amino-terminal CRIB domains (a Cdc42/Rac small GTPase binding motif) [18–20]. Both WASP and N-WASP also contain amino-terminal pleckstrin homology (PH) domains, and at least N-WASP binds to acidic phospholipids, which may confer a plasma membrane localization [20]. The amino termini of Scar proteins are homologous to each other, but not to WASPs, and their binding interactions are unknown. The middle portions of WASP and Scar proteins contain polyproline stretches. WASP binds to proteins that contain Src homology 3 (SH3) domains, such as the adaptor protein Nck, through its polyproline-containing domain [21]. Nck binds directly to the platelet-derived growth factor (PDGF) receptor and is thought to be an essential adaptor protein for relaying signals downstream of PDGF-receptor activation [22]. The carboxy-terminal portions of N-WASP, WASP and Scar also contain an actin-binding motif, known as the WH2 or verprolin homology domain. A conserved series of acidic residues lies carboxy-terminal to the WH2 domain.

WASP family proteins are required for normal cytoskeletal function in both humans and yeast. Wiskott–Aldrich syndrome, the genetic disease associated with mutations in the gene encoding WASP, results in a severe immune dysfunction and thrombocytopenia [23,24]. Macrophages from patients with Wiskott–Aldrich syndrome are defective in chemotaxis and show altered actin cytoskeletal morphology [25]. The *S. cerevisiae* WASP-related protein Las17p/Bee1p is required for cortical actin assembly [26]. Interestingly, overexpression of Las17p can compensate for some Arp2 and Arp3 mutations (B. Winsor, personal communication), indicating a connection with the Arp2/3 complex in yeast.

Scar is a *Dictyostelium* WASP-related protein that is connected with the cAMP signalling pathway that acts through the heterotrimeric G-protein-coupled receptor cAR2 [27]. Disruption of the gene encoding Scar allows *Dictyostelium* cells to overcome a block in development caused by a lack of cAR2. The cells lacking Scar are much smaller than wild-type cells and have an altered cortical actin structure. It is not clear how Scar functions downstream of cAMP receptors, but one idea is that it negatively regulates a signalling pathway downstream of cAR2. Four human homologues of Scar have been identified in Genbank and named Hs-Scar1 – Hs-Scar4 [27]. Unlike WASP and N-WASP, Scar proteins do not contain a CRIB domain so their regulation is probably not directly through small GTPases.

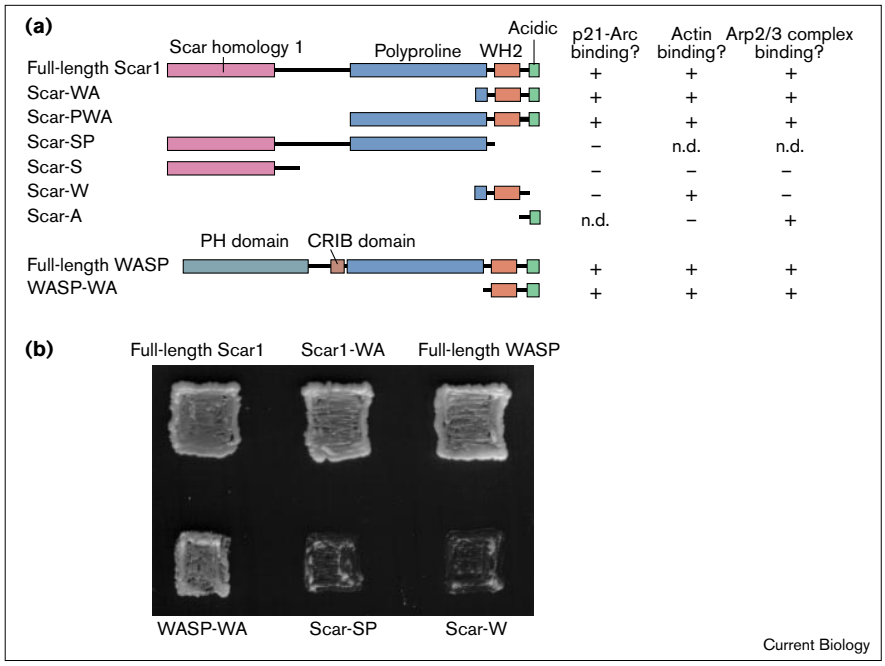
Results

Scar1 and WASP bind to p21-Arc

We have sought cellular binding targets of the Arp2/3 complex using the yeast two-hybrid protein interaction assay [28]. We used p21-Arc or p34-Arc (fused to the GAL4 DNA-binding domain) as the 'bait' to 'fish out' proteins (fused to the GAL4 transcription-activation domain) from a human brain cDNA library. All of the p34-Arc-interacting clones that we isolated encoded p20-Arc. We found eight p21-Arc-interacting clones, one of which encoded p20-Arc and the remainder novel proteins. One clone showed a particularly strong response in the β-galactosidase assay for activation of GAL4 transcription. This cDNA was sequenced and its sequence found to contain homology to WASP in the carboxy-terminal region, which includes the WH2 homology domain and a short acidic domain (Figure 1a). It was also found to contain a sequence identical to the 3′ end (nucleotides 1330–1741) of a cDNA encoding Scar1, a human homologue of *Dictyostelium* Scar1 (Hs-Scar1; Genbank accession number D87459). Neither the full-length sequence nor the p21-Arc-binding fragment (Scar-WA; Figure 1a) showed any interaction with the unfused pYTH6 GAL4 transcription-activation domain, p16-Arc or p34-Arc in the two-hybrid assay (data not shown), but both showed a strong interaction with p21-Arc, as measured by growth on 3AT plates and β-galactosidase expression (Figure 1b). Because the p21-Arc-binding region of Scar1 is homologous to WASP, we also assayed full-length WASP and an equivalent carboxy-terminal fragment (WASP-WA; Figure 1a) for interaction with p21-Arc. Both full-length WASP and the carboxy-terminal fragment of WASP bound tightly, as measured by growth on 3AT plates and expression of β-galactosidase (Figure 1b).

To analyze the interaction between p21-Arc and Scar1 in more detail, we made deletions in the cDNA encoding Scar1. We named these deletion constructs according to the domains they contained: S, Scar homology; P, polyproline; W, WH2 WASP homology; A, acidic. We found that the amino-terminal portion of Scar1, up to and including the polyproline region, did not interact with

Figure 1



The p21-Arc component of the Arp2/3 complex binds to the carboxy-terminal domains of Scar1 and WASP. **(a)** Comparison of WASP and Scar1 domain organization and yeast two-hybrid results. The right-hand three columns represent a summary of the two-hybrid and GST fusion-protein-binding experiments. For p21-Arc binding, + indicates a strong interaction in the two-hybrid assay, as measured by growth on 3AT Trp$^-$ Leu$^-$ His$^-$ plates and β-galactosidase expression. For actin binding and Arp2/3 complex binding, + indicates a strong signal in western blot analysis (data shown in Figure 2). In each column, n.d. indicates that the interaction was not tested. **(b)** Growth on 3AT Trp$^-$ Leu$^-$ His$^-$ plates of selected yeast two-hybrid strains. Y190 yeast were transformed with the integrating plasmid pYTH6 containing the cDNA encoding p21-Arc and pACT-II containing cDNA fragments encoding the construct indicated. A single colony was picked from each transformation and re-streaked on 3AT Trp$^-$ Leu$^-$ His$^-$ plates (as shown) to select for interaction.

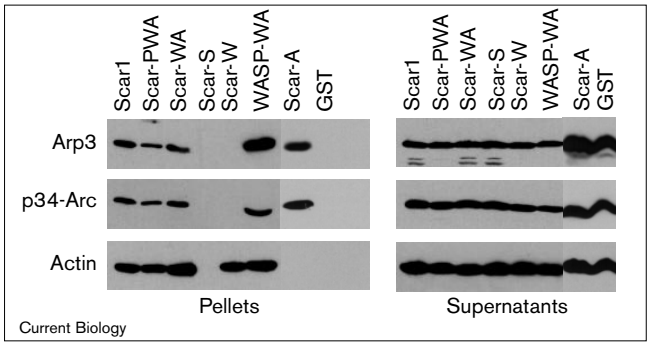
p21-Arc (Figure 1a,b). Deletion of the carboxy-terminal 15 amino acids to create Scar-W also resulted in a complete loss of interaction with p21-Arc (Figure 1b). We conclude that amino acids 445–559 of Scar1, containing the WH2 and acidic domains, bind specifically to p21-Arc, and that the carboxy-terminal 15 amino acids, comprising the acidic domain, are required for this interaction. The equivalent carboxy-terminal domain of WASP and full-length WASP also show a strong and specific interaction with p21-Arc in the two-hybrid assay (Figure 1b).

Scar1 and WASP bind to the Arp2/3 complex and to monomeric actin *in vitro*

We immobilized fragments of Scar1 and WASP fused to glutathione-S-transferase (GST) on glutathione–agarose and determined which of the constructs would bind to the Arp2/3 complex in porcine brain cytosol. All constructs containing the carboxy-terminal acidic domain bound to the complex (Figure 2). Scar-W, which contains the WH2 domain but not the acidic domain, bound to actin but not to the complex (Figure 2). Neither Scar-S, which contains the amino-terminal half of the molecule, nor GST alone, bound to either actin or the Arp2/3 complex (Figure 2). We conclude that the acidic domain of Scar1, containing residues 522–559, is sufficient for binding to the Arp2/3 complex but not to actin. Because Scar-A binds only to the Arp2/3 complex and not to actin, we conclude that actin is not required for the interaction between Scar1 and the Arp2/3 complex.

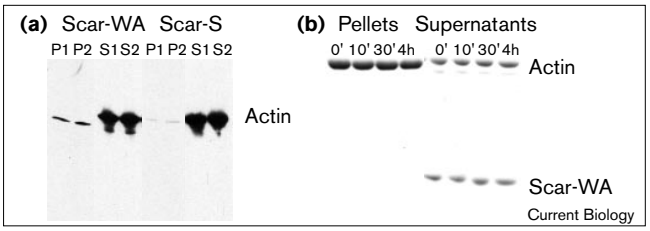
It has previously been reported that monomeric actin can bind to WH2 homology domains (also called verprolin

Figure 2



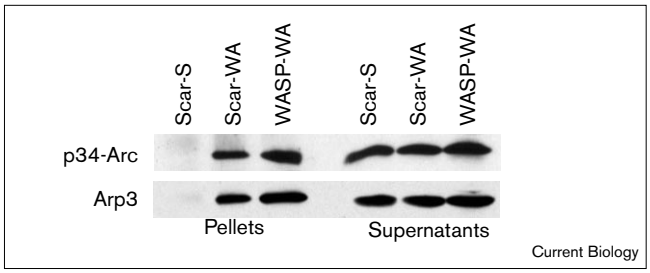
Scar1 and WASP fragments interact with the Arp2/3 complex and actin in cytosol. Porcine brain cytosol was incubated with approximately equal amounts of GST-fused Scar1 and WASP fragments immobilized on glutathione–agarose. Western blots of approximately equal percentages of the total of supernatant and pellet fractions of binding assays were probed with antibodies to Arp3, p34-Arc and actin. A representative experiment is shown here. We obtained a semi-quantitative estimate of the binding from these data, assuming that the cytosolic concentration of the Arp2/3 complex is similar to that published for *Acanthamoeba* [3]. Overall, the binding to actin was between 0.5 and 1 mole of actin per mole of Scar or WASP fragment, as estimated by scanning densitometry. Less than 1 mole of actin bound per 1000 moles of Scar-A. For the Arp2/3 complex, the binding was approximately 1 mole of complex per 70 moles of the Scar or WASP fragment. The estimated K_d for Scar or WASP-WA with Arp2/3 complex from these observations is around 150 nM.

Figure 3



Scar-WA interacts with actin monomers but not actin filaments. **(a)** Approximately equal amounts of Scar-WA and Scar-S fragments immobilized on glutathione–agarose were incubated with purified monomeric rabbit muscle actin. Western blots of approximately equal loads of the supernatant (S) and pellet (P) fractions from two representative parallel experiments were probed with an antibody to actin. **(b)** Rabbit muscle actin (7 μM) was polymerized for 30 min and incubated for the indicated time with Scar-WA (10 μM) prior to centrifugation to pellet the actin filaments. The proteins in the supernatant and pellet fractions were separated by polyacrylamide gel electrophoresis and stained with Coomassie blue.

Figure 4



The endogenous Arp2/3 complex co-immunoprecipitates with WASP-WA and Scar1-WA. Cos-7 cells were transfected with Scar-S, Scar-WA or WASP-WA and cytosol from each was incubated with agarose beads covalently linked to the anti-Myc monoclonal antibody 9E10. Supernatants and pellets from a representative immunoprecipitation are shown on a western blot probed with an anti-p34-Arc and an anti-Arp3 antibody. From scanning densitometry measurements, we estimate that 38 ± 4% of the soluble Arp2/3 complex co-precipitated with Scar-WA, and 44 ± 4% co-precipitated with WASP-WA.

homology domains) containing the sequence LLxxIxxGxxL (single-letter amino-acid code, where x is any amino acid), including those found in WASP and N-WASP [29]. All of our GST constructs containing a WH2 domain from Scar1 or from WASP bound to actin (Figure 2). We further explored this actin binding by testing whether purified monomeric and filamentous actin bound to Scar-WA. We found that GST–Scar-WA bound to monomeric actin but GST–Scar-S did not (Figure 3a). We also tested the ability of Scar-WA to bind to, or depolymerize, filamentous actin. Because it was previously reported that an N-WASP-WA fragment could depolymerize actin filaments [20], we thought that Scar-WA might also have this activity. We found that, even with incubation times of up to 4 hours, there was no detectable interaction between Scar-WA and filamentous actin and no detectable depolymerization (Figure 3b). If we added increasing concentrations of Scar-WA to polymerized actin, we could see a decrease in filamentous actin commensurate with a 1:1 binding of actin monomers to Scar-WA (data not shown). While we did not see actin depolymerization in our assay, we cannot rule out a depolymerizing activity for Scar proteins until we have further characterized them. We also used various drugs to try to disrupt the interaction of Scar-WA with actin in cytosol, in order to probe further the relationship between the Arp2/3 complex and actin binding. We found no effect from adding DNase I or latrunculin B, both of which can bind monomeric actin, but a relatively high concentration of cytochalasin D (50 mM), which prevents actin polymerization but can also bind to actin monomers at high concentrations, removed 90% of the bound actin without affecting the bound Arp2/3 complex (data not shown). We conclude that Scar-WA binds to monomeric but not to filamentous actin, and that this interaction is not dependent on the association with the Arp2/3 complex.

Scar1 and WASP carboxyl termini bind to the Arp2/3 complex *in vivo*

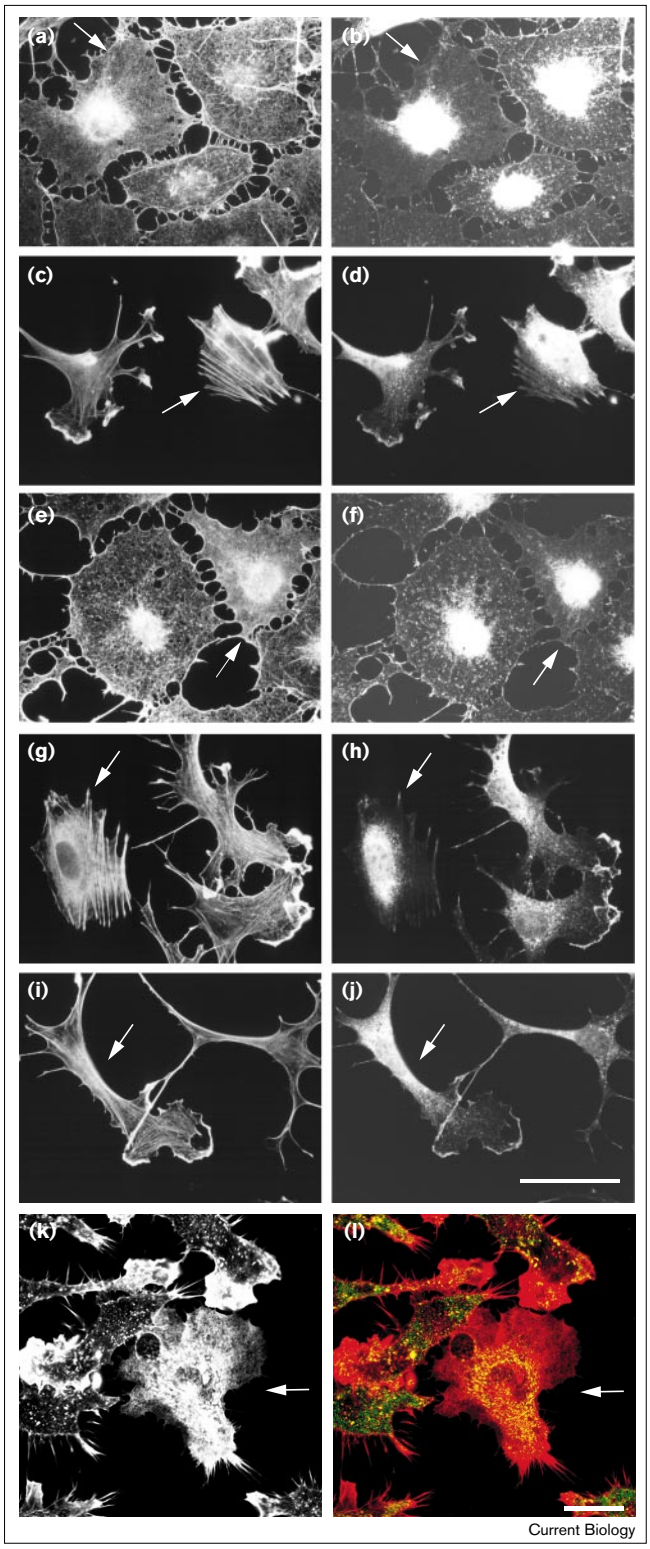
Because p21-Arc is an integral part of the Arp2/3 complex, we assayed its interaction with Scar1 or WASP *in vivo*. We transfected Cos-7 cells with DNA encoding Scar and WASP fragments tagged with a c-Myc epitope, and immunoprecipitated the constructs from cell lysates using an anti-Myc antibody (Figure 4). We found that the endogenous Arp2/3 complex interacts with Scar-WA or WASP-WA, but not with Scar-S (Figure 4). All constructs were expressed to similar levels and all were immunoprecipitated as assayed with the anti-Myc antibody (data not shown). All of the components of the Arp2/3 complex for which we have probes (Arp3, Arp2 and p34-Arc) co-precipitated in these assays. Thus, the carboxyl termini of Scar1 and WASP bind to endogenous Arp2/3 complex *in vivo*, presumably through a direct interaction with p21-Arc. We attempted, but were unable, to immunoprecipitate full-length WASP or Scar proteins. This may be due to folding or insolubility of the full-length constructs.

Overexpression of Scar and WASP disrupts the actin cytoskeleton

Given that Scar1 and WASP bound to the Arp2/3 complex *in vitro*, it seemed possible that their roles *in vivo* involve targeting the complex to regions of the cell where dynamic actin remodelling is required. We tested this hypothesis by microinjecting a mammalian expression vector containing Myc-tagged fragments of Scar1 and WASP into Swiss 3T3 fibroblasts and J774 macrophages. In serum-deprived Swiss 3T3 cells, which lack lamellipodia and stress fibers, the Arp2/3 complex was localized primarily to actin-containing spots (uninjected cells; Figure 5a,b), whereas it was localized primarily to lamellipodia and punctate structures in growing cells (uninjected cells; Figure 5c,d). When

Figure 5

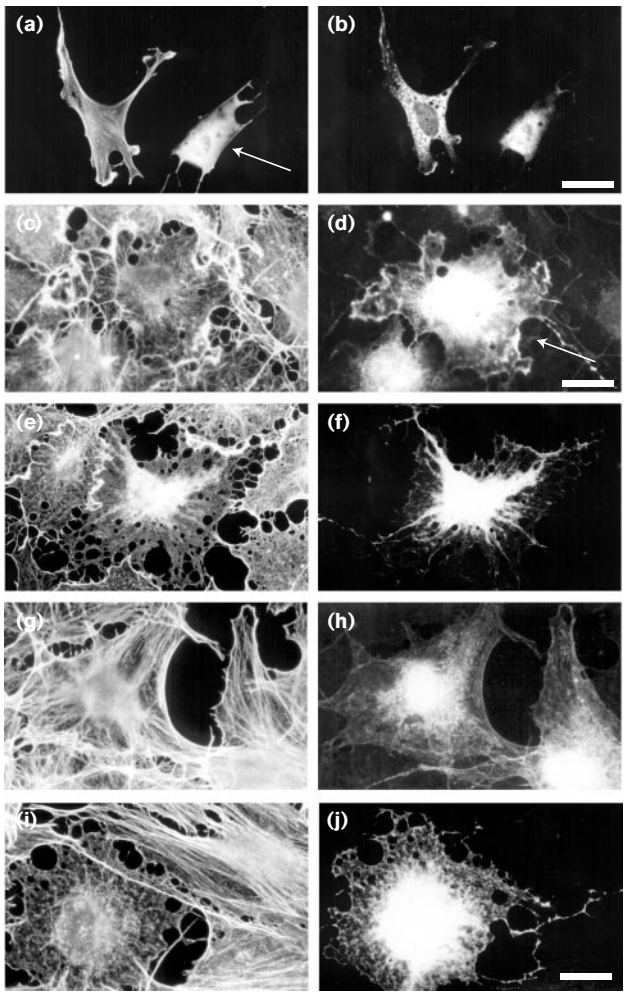
Localization of the Arp2/3 complex is disrupted upon overexpression of Scar-WA or WASP-WA constructs in cells. **(a,c,e,g,i,k)** Filamentous actin stained with rhodamine–phalloidin. **(b,d,f,h,j)** The localization of p34-Arc, as revealed using an anti-p34-Arc rabbit polyclonal antiserum and a fluorescein-conjugated anti-rabbit antiserum. Injected cells were determined by co-injection of biotinylated dextran and affinity staining with cascade blue avidin (data not shown). (a) Serum-deprived Swiss 3T3 fibroblasts; the arrow points to a cell expressing the Scar-WA domain. (b) Localization of p34-Arc in the same cells as shown in (a). (c) Growing Swiss 3T3 fibroblasts; the arrow points to a cell expressing the Scar-WA domain. (d) Localization of p34-Arc in the same cells as shown in (c). (e) Serum-deprived Swiss 3T3 fibroblasts; the arrow points to a cell expressing WASP-WA. (f) Localization of p34-Arc in the same cells as shown in (e). (g) Growing Swiss 3T3 cells; the arrow points to a cell expressing WASP-WA. (h) Localization of p34-Arc in the same cells as shown in (g). (i) Growing Swiss 3T3 cells; the arrow points to a cell expressing Scar-W. (j) Localization of p34-Arc in the same cells as shown in (i). The scale bar for (a–j) is 30 μm. (k) J774 macrophages stained with rhodamine–phalloidin; the arrow points to a cell expressing Scar-WA. (l) Overlay photograph of the same cells as shown in (k); p34-Arc staining is green and rhodamine–phalloidin labelling is red. The scale bar for (k,l) is 5 μm.



Scar-WA was expressed in serum-deprived cells, localization of the Arp2/3 complex was disrupted but there was minimal effect on the organization of filamentous actin, apart from a loss of actin-rich spots and a slight increase in phalloidin staining (Figure 5a,b). In growing cells, however, we observed a complete loss of lamellipodia, in addition to a disruption of the localization of the Arp2/3 complex (Figure 5c,d). Interestingly, we did not see any reduction in phalloidin staining and, in addition to prominent stress fibers, the cells showed an increased perinuclear diffuse phalloidin staining. Similarly, overexpression of WASP-WA also disrupted the localization of the Arp2/3 complex in serum-deprived cells with minimal effect on the actin cytoskeleton (Figure 5e,f). In growing cells it also abolished lamellipodia and displaced Arp2/3 complex from the periphery of the cell (Figure 5g,h). Cells overexpressing Scar-W, which binds to monomeric actin but not to the Arp2/3 complex, did not show any disruption of lamellipodia (Figure 5i) nor disruption of the Arp2/3 complex localization (Figure 5j). Overexpression of Scar-SP did not have any effect on filamentous actin staining, lamellipodia or Arp2/3 complex localization, whereas full-length Scar1 or Scar-PWA caused a similar phenotype to the Scar-WA fragment (data not shown). All constructs showed a similar expression level and an even cytoplasmic distribution, as shown by immunostaining for the Myc epitope (Figure 6, and data not shown). Overexpression of full-length WASP in fibroblasts caused accumulation of perinuclear filamentous actin structures similar to those previously reported [19], and concomitant disruption of Arp2/3 complex localization. The perinuclear filamentous actin structures also contained the Arp2/3 complex, as visualized with an anti-p34-Arc antibody, and WASP, as visualized with an anti-Myc epitope antibody.

The effect of Scar-WA expression was not cell-type specific, as we also observed a complete loss of lamellipodia from J774 macrophages which had been injected with Scar-WA (Figure 5k,l). Macrophages expressing Scar-WA did not round up, but lost surface protrusions (ruffles and filopodia) and showed a uniform filamentous actin

Figure 6



Scar-WA overexpression inhibits lamellipodia formation and stress fiber assembly. Filamentous actin is stained with rhodamine–phalloidin in **(a,c,e,g,i)**. The Arp2/3 complex localization is shown by staining with an anti-p34-Arc antibody **(b)**. Expression of Myc-tagged Scar-WA from an injected DNA construct is shown by staining with the 9E10 monoclonal anti-Myc antibody **(d,f,h,j)**. White arrows point to cells injected with Scar-WA DNA. (a,b) Cells in serum 6 h after injection of Scar-WA. (c,d) Cells stimulated for 10 min with PDGF 2 h after injection of Scar-W. (e,f) Cells stimulated for 10 min with PDGF 2 h after injection of Scar-WA. (g,h) Cells stimulated for 10 min with sphingosine 1-phosphate 2 h after injection of Scar-W. (i,j) Cells stimulated for 10 min with PDGF 2 h after injection of Scar-WA. The scale bar in (b) is 50 μm and applies to (a,b); the scale bar in (d) is 30 μm and applies to (c–h); the scale bar in j is 35 μm and applies to (i,j).

localization, rather than in spots or peripheral lamellipodia. The Arp2/3 complex staining pattern remained punctate, but was no longer associated with peripheral structures.

We found that the fragments of Scar and WASP that bind the Arp2/3 complex dramatically altered both actin localization and Arp2/3 complex localization in cells. The lack of inhibition of lamellipodia by the Scar-W construct suggests that the binding of actin by WASP or Scar1 does not mediate their effects on the actin cytoskeleton. Instead, it appears that a direct interaction between Scar1/WASP-related proteins and the Arp2/3 complex is essential for the assembly and maintenance of lamellipodia and actin spots under normal growth conditions.

Overexpression of Scar-WA prevents the growth-factor-induced assembly of actin structures

As the Arp2/3 complex has multiple activities *in vitro*, we investigated its role in the growth-factor-induced assembly of specific actin-cytoskeletal structures. We found that, in cells growing in serum, lamellipodia were completely suppressed within 10 minutes by injecting Scar-WA protein (data not shown), or within 2 hours for the Scar-WA DNA expression construct. But if cells injected with DNA expression constructs were left for longer times (4–6 hours), stress fibers also disassembled, leaving the cell full of diffuse filamentous actin (Figure 6a,b). As activation of the small GTPase Rac through the PDGF receptor induces lamellipodia in quiescent serum-starved Swiss 3T3 fibroblasts [30], we tested whether Scar-fragment expression could inhibit this response. Overexpression of the actin-binding fragment Scar-W had no effect on PDGF-induced ruffling (Figure 6c,d). In contrast, Scar-WA overexpression completely suppressed lamellipodia assembly in response to PDGF addition (Figure 6e,f). Of 66 injected cells, none ruffled in response to PDGF under conditions where more than 95% of uninjected cells or Scar-W-expressing cells were ruffling. Thus, the Arp2/3 complex appears to be important for lamellipodia assembly both in serum and in response to PDGF.

Given that activation of the small GTPase Rho through the sphingosine 1-phosphate (S-1-P) or lysophosphatidic acid receptors results in the assembly of actin stress fibers [31,32], we tested the effect of Scar-fragment overexpression on this response. Overexpression of the actin-binding fragment of Scar1, Scar-W, had no effect on the assembly of stress fibers in response to S-1-P (Figure 6g,h). In contrast, Scar-WA-overexpressing cells could only partially assemble actin bundles resembling stress fibers (Figure 6i,j). Of 84 injected cells, none showed parallel stress fibers, but all showed some weak bundling of actin filaments under conditions where 100% of Scar-W-expressing or uninjected cells formed stress fibers. Our data suggest that the Arp2/3 complex is important for the assembly of actin stress fibers in response to S-1-P.

As WASP interacts with Cdc42 [18,19], it would be desirable to ask whether the formation of filopodia is also inhibited by overexpression of fragments of WASP or Scar1. Although it has been reported that bradykinin

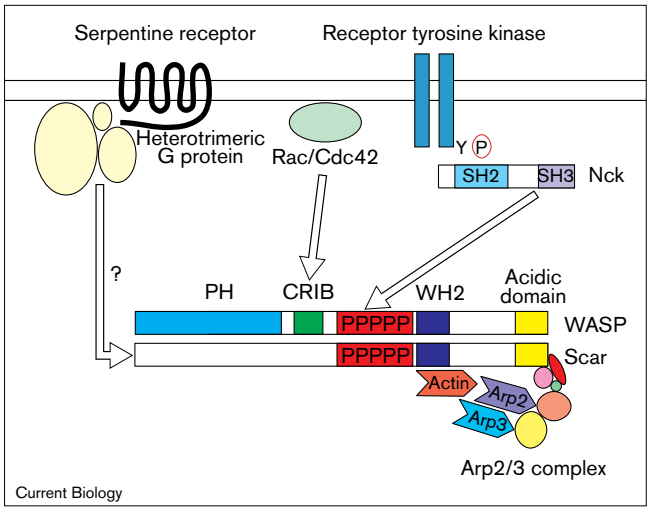
induces filopodia in Swiss 3T3 cells in a Cdc42-dependent manner [33], we found this effect to be too subtle to allow a reliable investigation of inhibition by Scar or WASP fragments. Overexpression of a constitutively active mutant Cdc42 [34] in serum-starved quiescent Swiss 3T3 cells induced filopodia which were not inhibited by co-expression of Scar-WA (data not shown). However, in J774 macrophages, endogenous filopodia were inhibited by overexpression of Scar-WA (Figure 5k,l). We conclude that disruption of the endogenous Arp2/3 complex–Scar1/WASP interaction by overexpression of Scar fragments has multiple effects on the actin cytoskeleton, the strongest of which is the suppression of lamellipodia. This indicates multiple roles for the Arp2/3 complex in cells, a suggestion which is supported by *in vitro* data [4].

Discussion

Much excitement has been generated by recent findings that the Arp2/3 complex can nucleate, cap and crosslink actin filaments [4,5]. Studies in *Listeria monocytogenes* showed that the purified Arp2/3 complex can initiate actin polymerization in conjunction with the bacterial protein ActA [5,7]. ActA amino-terminal fragments greatly enhance the nucleation activity of the Arp2/3 complex *in vitro* [5]. This finding suggests that a cellular factor might serve a similar purpose to regulate the assembly of dynamic actin structures such as lamellipodia. It is possible that WASP and Scar proteins could have this function in cells. Alternatively, WASP and Scar proteins may serve to concentrate or localize the Arp2/3 complex to sites of dynamic actin assembly. Although we cannot distinguish between these possibilities until we can determine the effects of WASP/Scar proteins on the various activities of the Arp2/3 complex *in vitro*, we favor a model in which WASP and Scar proteins serve to initiate actin nucleation in response to signals. This could be achieved by bringing actin monomers and the Arp2/3 complex into close proximity, perhaps in the correct orientation for filament-nucleus formation (Figure 7). This could explain the function of the actin-binding site on WASP and Scar proteins.

The disruption of the interaction of actin with Scar-WA by cytochalasin D also suggests that the interaction of Scar1 with actin may be conformationally sensitive. Cytochalasin D induces a change in the conformation of actin monomers that is thought to resemble the filamentous state and it also induces the formation of actin dimers [35,36]. Perhaps a conformational change or a dimerization causes actin monomers to dissociate from Scar or WASP and thus opens up the binding site for a new monomer to promote further polymerization in conjunction with the Arp2/3 complex. We postulate that Scar or WASP proteins could thus shuttle actin monomers to the Arp2/3 complex for addition into filaments (Figure 7).

Figure 7



A model for how signals from receptors could connect to the Arp2/3 complex to stimulate actin assembly and reorganization in cells. Signals from cytokine and growth-factor receptors communicate with the small GTPases Rac and Cdc42, which in turn could trigger WASP or N-WASP to activate or localize the Arp2/3 complex, resulting in actin assembly in lamellipodia or microspikes. Both Scar and WASP proteins could connect to receptor tyrosine kinases through an interaction with the polyproline-containing domains. Additionally, Scar1 could be connected to seven-transmembrane serpentine receptors through heterotrimeric G proteins and thus could mediate actin polymerization in response to chemoattractants. Arp2/3 complex bound to the carboxyl terminus of WASP or Scar might interact with monomeric actin bound to the WH2 domain and nucleate a new actin filament and/or become localized through the other interactions of WASP and Scar proteins.

WASP interacts with the small GTPases Rac and Cdc42 as well as with the adaptor protein Nck [18,19,21]. As overexpression of the carboxy-terminal Arp2/3-complex-binding fragment of Scar1 inhibits PDGF-stimulated formation of lamellipodia, we think it likely that there is a direct connection between WASP/Scar proteins, the Arp2/3 complex and actin assembly downstream of small GTPases and receptors.

Our study may have a direct bearing on the interpretation of phenotypes in Wiskott–Aldrich syndrome. Mutations occurring in patients with the most severe forms of Wiskott–Aldrich syndrome (as characterized by thrombocytopenia and defects of chemotaxis and cytoarchitecture in T and B cells) often occur as mis-sense or premature-termination signals in the last 59 amino acids of WASP [24]. This region lies further towards the carboxyl terminus than the WH2 domain, so we expect that mutations here may impair binding to the Arp2/3 complex. Most Wiskott–Aldrich patients do not express any WASP, but we are currently searching for any who might express a protein with a mutation in the carboxyl terminus.

Although Scar1 is much less well-characterized than WASP, data from *Dictyostelium* suggest that Scar1 lies in a signalling pathway connected to the cyclic AMP receptor cAR2 [27], which is a classical serpentine receptor coupled to a heterotrimeric G protein. Scar1 mutants also show a greatly diminished level of filamentous actin. This suggests a model (Figure 7) in which signals generated through different receptors and mediators — such as growth factors, small GTPases and G proteins — converge on the Arp2/3 complex to trigger or localize actin assembly.

It may seem unexpected that overexpression of Scar-WA inhibits the assembly of both stress fibers and lamellipodia. We think that the inhibition of stress fiber assembly could suggest a requirement for new actin polymerization in stress fiber formation or, alternatively, a requirement for crosslinking or pointed-end capping by the Arp2/3 complex. The Arp2/3 complex does not localize strongly to stress fibers or filopodia, both of which are composed of actin in parallel bundles with actin-binding proteins. The complex does, however, appear near the base of filopodia in macrophages and along the distal tips of some stress fibers (L.M.M., unpublished observations). Thus, we propose that there may be an organizational or initiating role for the Arp2/3 complex in some bundled actin structures. Equally, the Arp2/3 complex may be required to maintain the general integrity of the actin cortex, in a way similar to its role in yeast. Cortical integrity is probably important generally for the assembly of actin-based structures, and cortical actin depletion may explain the fenestrated appearance of some of the cells overexpressing Scar or WASP-WA (for example, Figure 6e).

Other proteins contain domains similar to Scar and WASP-WA. We predict that N-WASP, which contains similar WH2 and acidic domains, will also interact with the Arp2/3 complex. It will be interesting to see whether verprolin [37], Las17p/Bee1p [26], and WIP [38], which also contain WH2 domains, will bind to p21-Arc. One of the next challenges will be to understand how the various WASP-related proteins transmit signals to the Arp2/3 complex and how cells can use the Arp2/3 complex to help generate actin structures as diverse as lamellipodia, filopodia and actin bundles, structures that we observe in motile cells.

Materials and methods

Transfections and immunoprecipitations

Cos cells were transfected using Lipofectamine (GIBCO-BRL) according to the manufacturer's instructions. Plasmids, based on the pRK5 vector, were transfected into 10 cm dishes of Cos-7 cells for 24 h before lysis in 0.5 ml lysis buffer (10% glycerol, 50 mM Tris–Cl pH 7.5, 100 mM NaCl, 1% NP-40) on ice for 10 min. Lysed cells were scraped from the dish and centrifuged for 10 min at 10,000 × *g* at 4°C. Supernatants were incubated with protein-A-coupled Sepharose beads covalently linked to 9E10 anti-Myc epitope antibody (a kind gift from D. Drechsel) for 30 min on ice. The supernatant was removed and the beads were washed three times in lysis buffer. Sample buffer was added to the supernatants and pellets, and both were separated on 12% polyacrylamide gels. The proteins were transferred to nitrocellulose and probed with the following antibodies: 9E10 (to recognize the epitope-tagged Scar1 constructs), anti-p34-Arc and anti-Arp3 (to recognize endogenous Arp2/3 complex). At least four independent transfections were done for each experiment, with similar results. To estimate the amount of co-precipitated Arp2/3 complex, we scanned autoradiograms of our western blots and used the program NIH Image to quantitate the density in the region of either Arp3 or p34-Arc on the blot. The average of four measurements was reported in each case. For all lysis conditions that we tried, including standard RIPA buffer (containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 8.0), approximately half of the total Arp2/3 complex remained insoluble and therefore inaccessible to the experiment.

Microinjection and immunofluorescence

Swiss 3T3 cells were maintained in DMEM containing 10% FCS and prepared for serum starvation as previously described [31,32]. A 0.1 mg/ml preparation of DNA in CsCl was microinjected into the nucleus as previously described [34]. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 10 min and processed as previously described [34]. J774 cells were maintained in DMEM containing 10% FCS, serum-deprived for 1 h prior to microinjection to increase cell adhesion which facilitates microinjection (E. Caron, personal communication), and were viewed using a BioRad MRC1000 confocal microscope. We and others have shown previously that the mammalian Arp2/3 complex subunits Arp3, Arp2, p34-Arc and p21-Arc have the same localization in cells and by biochemical fractionation [1,2].

For our microinjection experiments, we observed the described effect in > 90% of at least 50 microinjected cells. For Scar-WA and Scar-W, we injected cells blind, so that the person doing the injections and looking at the cells did not know which DNA construct was being used. Under these conditions, 69/72 cells in serum were ruffling after injection of Scar-W, whereas 0/50 cells were ruffling after injection of Scar-WA. In starved cells, 62/66 cells injected with Scar-WA showed delocalization of the Arp2/3 complex and 5/74 cells showed a similar delocalized Arp2/3 complex after Scar-W injections. All injected cells were fixed and stained at approximately 2 h post-injection, unless otherwise indicated. This is the minimum time at which most cells express injected pRK5 constructs in our hands. Injection of Scar-WA protein was done using approximately 1 mg/ml thrombin-cleaved protein in PBS. As we do not yet have antibodies to Scar1, we cannot compare the levels of overexpression to levels of endogenous proteins. However, we estimate that expression levels are in the range of 200 nM to 1 μM based on the immunofluorescence staining intensity as compared to injected Myc-tagged proteins of known concentration.

GST fusion protein binding assays

Porcine brain cytosol was produced by homogenization of one porcine brain (80 g tissue) in 80 ml homogenization buffer (10 mM HEPES pH 7, 1 mM ATP, 0.5 mM dithiothreitol, 0.1 mM benzamidine, 1 mM PMSF and 0.1 mg/ml each chymostatin, leupeptin, antipain and pepstatin). The homogenate was centrifuged at 50,000 × *g* for 30 min and the supernatant was stored in 1 ml aliquots in liquid nitrogen. An aliquot was thawed before each experiment and centrifuged for 20 min at 100,000 × *g* to clarify. The supernatant contained approximately 16 mg/ml protein and was used on the day of thawing. Approximately 10 μg of each GST-fusion protein bound to 10 μl of agarose beads was mixed with 50 μl extract and 150 μl bead buffer (PBS + 0.2% Tween-20). Tubes were incubated for 30 min at 4°C on a rotating wheel, then beads were pelleted at 5,000 × *g* for 15 sec in a microfuge and washed twice in bead buffer. Supernatants and beads were boiled in sample buffer and separated on 12% polyacrylamide gels. Gels were stained with Coomassie blue and scanned using an Agfa scanner or blotted onto nitrocellulose and assayed by immunoblotting using the ECL chemiluminescence detection kit (Amersham). All experiments

were repeated at least twice with similar results. We used the program NIH Image to obtain semi-quantitative estimates of the binding of Arp2/3 complex to Scar and WASP fragments. We assumed that actin represented 1% of the total protein in our extracts and that Arp2/3 complex was present at a 30-fold lower concentration [3].

Actin-binding assays

Actin was purified from rabbit muscle as previously described [39] and stored in liquid nitrogen in aliquots in G-buffer (2 mM Tris-Cl pH 7.5, 0.2 mM ATP, 0.5 mM dithiothreitol, 2 mM $CaCl_2$). All actin-binding assays were performed in G-buffer or G-buffer + 0.1% Tween-20 for GST fusion-protein binding experiments. Scar-WA was cleaved from the glutathione–agarose by overnight incubation with 10 units bovine thrombin (Sigma). We used western blotting instead of Coomassie blue staining to detect the bound actin because it co-migrated with Scar-WA. Actin was polymerized for filamentous-actin binding experiments by adding 0.1 volume of KME salts (500 mM KCl, 20 mM $MgCl_2$, 1 mM EGTA) and was separated from unpolymerized actin by centrifugation at $100{,}000 \times g$ for 20 min in a Beckman tabletop ultracentrifuge. Pellets were washed twice in KME + G-buffer, dissolved in sample buffer, and separated on 12% polyacrylamide gels, which were then stained with Coomassie blue.

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