Filopodia Formation in the Absence of Functional WAVE- and Arp2/3-Complexes[□] [▽]

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Cell migration is initiated by plasma membrane protrusions, in the form of lamellipodia and filopodia. The latter rod-like projections may exert sensory functions and are found in organisms as distant in evolution as mammals and amoeba such as *Dictyostelium discoideum*. In mammals, lamellipodia protrusion downstream of the small GTPase Rac1 requires a multimeric protein assembly, the WAVE-complex, which activates Arp2/3-mediated actin filament nucleation and actin network assembly. A current model of filopodia formation postulates that these structures arise from a dendritic network of lamellipodial actin filaments by selective elongation and bundling. Here, we have analyzed filopodia formation in mammalian cells abrogated in expression of essential components of the lamellipodial actin polymerization machinery. Cells depleted of the WAVE-complex component Nck-associated protein 1 (Nap1), and, in consequence, of lamellipodia, exhibited normal filopodia protrusion. Likewise, the Arp2/3-complex, which is essential for lamellipodia protrusion, is dispensable for filopodia formation. Moreover, genetic disruption of *nap1* or the WAVE-orthologue suppressor of cAMP receptor (*scar*) in *Dictyostelium* was also ineffective in preventing filopodia protrusion. These data suggest that the molecular mechanism of filopodia formation is conserved throughout evolution from *Dictyostelium* to mammals and show that lamellipodia and filopodia formation are functionally separable.

INTRODUCTION

Filopodia and lamellipodia are prominent protrusive organelles generated by the polymerization of actin filaments. Filament nucleation and elongation are catalyzed in cells by molecular machines such as the Arp2/3-complex, which is stimulated, for instance, by WASP and WAVE proteins (Stradal *et al.*, 2004). The WASP/WAVE family of proteins has been implicated in various cellular functions including adhesion, endocytosis, trafficking, and migration, although a common consensus on the precise cellular functions of the different family members has yet to be reached. For example, N-WASP—ubiquitously expressed in mammals—was generally thought to stimulate filopodia formation, although subsequent analyses of N-WASP knockout cells demonstrated its dispensability for this process (Lommel *et al.*, 2001; Snapper *et al.*, 2001).

WASP and WAVE family proteins act downstream of Rho-family GTPases, the activation of which can trigger the

formation of lamellipodia and filopodia (Hall, 1998). In mammals, Rac subfamily GTPases (Rac1, -2, and -3) are thought to be essential for the protrusion of lamellipodia, whereas members of the Cdc42 family (Kozma *et al.*, 1995; Nobes and Hall, 1995) and Rif (Rho in filopodia) trigger filopodia formation (Aspenstrom *et al.*, 2004; Pellegrin and Mellor, 2005). Interestingly, WASP proteins can be activated by Rho-GTPases such as Cdc42 through direct protein–protein interaction, whereas WAVEs are linked to Rac proteins by intermediary factors such as Specifically Rac-associated protein 1 (Sra-1) or its isogene PIR121, Nck-associated protein 1 (Nap1), Abl interactor (Abi) proteins, and a peptide called HSPC300. Sra/Nap/Abi/WAVE and HSPC300 constitute a stable protein assembly now generally referred to as the WAVE-complex (Stradal *et al.*, 2004). In various vertebrate and nonvertebrate systems, WAVE-complex assembly and stability was demonstrated recently to be essential for the induction and maintenance of lamellipodia protrusion, i.e., downstream of Rac activation (Kunda *et al.*, 2003; Rogers *et al.*, 2003; Steffen *et al.*, 2004). In this scenario, WAVE-complex recruitment to and activation at the cell periphery lead to activation of Arp2/3-complex-mediated polymerization of actin filaments to drive lamellipodial protrusion. Arp2/3-complex, which is strongly enriched in lamellipodia is thought to induce de novo nucleation and/or branching of filaments generating the dense criss-cross arrangement of actin filaments observed by electron microscopy (Small *et al.*, 1999; Pollard and Borisy, 2003). Filopodia, in contrast, are composed of bundles of parallel actin filaments (Small and Celis, 1978; Small, 1988), whose generation may or may not

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Abbreviations used: AlF_4^- , aluminum fluoride; Nap1, Nck-associated protein 1; Sra-1, Specifically Rac-associated protein 1; WT, wild type.

depend on the presence of lamellipodial actin meshworks (Faix and Rottner, 2006).

Many early studies have documented the coexistence of lamellipodia and filopodia at the cell periphery and from recent studies on B16-F1 melanoma cells, Svitzkina *et al.* (2003) proposed a model for filopodia protrusion, coined “the convergent elongation model.” In this model, filopodia arise from the lamellipodial meshwork by filament elongation from specific precursor sites, delta-shaped structures, which are generated by lateral translocation and conversion of a dendritic array of actin filaments. Thus, a “cascade pathway” of filopodia formation is foreseen (Biyasheva *et al.*, 2004), with lamellipodia polymerizing “upstream” in the signaling cascade to filopodia protrusion, implicating the dependence of filopodia formation on preexisting lamellipodia. Here, we have tested these models in mammalian cells by abolishing lamellipodia formation through knockdown of WAVE- or the Arp2/3-complex and in *Dictyostelium* amoebae by disruption of the genes encoding *nap1* and *scar*.

MATERIALS AND METHODS

Mammalian Cell Culture and RNA Interference

B16-F1 mouse melanoma cells (ATCC CRL-6323) and VA-13 human lung fibroblasts (ATCC CCL-75.1) were grown and transfected as described previously (Steffen *et al.*, 2004). For microscopy, cells were plated on acid-washed glass coverslips coated with 50 $\mu\text{g ml}^{-1}$ fibronectin (Roche Diagnostics, Mannheim, Germany). For RNA interference, 64mer-oligonucleotides harboring 19-nucleotide (nt) target sequences specific for Arp3 (5'-AGGTTTATG-GAGCAAGTGA-3') or p21-Arc (5'-GAATGAAGCGGACAGGACA-3') were ligated into the pSUPER.retro.puro vector (OligoEngine, Seattle, WA) and sequence verified. Control and stable Nap1 knockdown cell lines were generated and maintained as described previously (Steffen *et al.*, 2004).

Dictyostelium discoideum Cells

Cultivation and transformation of *D. discoideum* AX2 wild-type (WT) strain was as described previously (Schirenbeck *et al.*, 2005). For construction of the *scar* (GenBank accession no. XM_633219) targeting vector, a 5' BamHI/PstI fragment and a 3' HindIII/SalI fragment were amplified from genomic AX2 WT DNA by PCR. The oligonucleotide primers used for the 5' fragment were 5'-CGCCG-GATCCGATGGTATTAATTACAAGATATTTACCA-3' and 5'-GCGGTG-CAGGACATCGTCCATGTATGGGTCCA-3' and the primers for the 3' fragment were 5'-GCGAAGCTTTCATTAATAAACTCTACACCAATCCAGAC-3' and 5'-CGCGTCGACGTTTGCAGCTCCACCATTTTGTGTCAT-3'. Both fragments were gel purified after cleavage with BamHI/PstI and HindIII/SalI, and cloned into the corresponding sites of pLPBLP containing the blasticidin S resistance cassette (Faix *et al.*, 2004). The resulting vector was cleaved with BamHI and SalI and used to disrupt the *scar* gene in WT cells. The *nap1* (GenBank accession no. XM_638991) targeting vector was generated using the same strategy. The oligonucleotide primers used for the 5' fragment were 5'-CGCGGGATCCGCATG-GCACATACAAATTTACCAGAAA-3' and 5'-CGCCTGCAGTTTCATTAT-GAATTGAAATGACTGTAA-3' and the primers for the 3' fragment were 5'-CGCAAGCTTGATGGTGCCCTCAATCTCATCCTTAAACCT-3' and 5'-CGCGTCGACACATGGTTTATCTCTAAACAAATTTCCA-3'. Null mutants were screened by PCR as described previously (Faix *et al.*, 2004). At least two independent clones were analyzed for each mutant. Scar and Nap1 null strains were transformed with green fluorescent protein (GFP)-dDla2 and fixed and stained as described previously (Schirenbeck *et al.*, 2005).

Antibodies and Immunoblotting

Polyclonal anti-Arp3 and monoclonal anti-Sra-1 antibodies were raised against synthetic peptides NH₂-EELSGGRLKPKPIDV-COOH and NH₂-CDWETGHEPFNDPALRGEKDPKSGFDIKVPRRAVGPSS-COOH, respectively, and affinity purified using the same peptides immobilized on CNBr-Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Specificity of Arp3 antibodies was confirmed as shown in Supplemental Figure S1. Anti- α -tubulin antibody was used as described previously (Steffen *et al.*, 2004). Monoclonal antibodies raised against p21-Arc (ArpC3; clone 24A6), p16A (ArpC5; clone 323H3) (Millard *et al.*, 2003), and Sra-1 (clone 30A4) are available from Synaptic Systems (Göttingen, Germany). The latter antibody was generated by immunization against peptide NH₂-CDWETGHEPFNDPALRGEKDPKSGFDIKVPRRAVGPSS-COOH. Polyclonal anti-WAVE2 and monoclonal anti-Abi-1 antibodies were kindly provided by Giorgio Scita (The FIRIC Institute for Molecular Oncology, European Institute of Oncology, Milan, Italy) (Innocenti *et al.*, 2004; Steffen *et al.*, 2004). Anti-myc

clone 9E10 was purchased from Abcam (Cambridge, United Kingdom). Polyclonal anti-GFP antibodies were as described previously (Faix *et al.*, 2001).

Immunoblotting was performed according to standard protocols. For the quantification of WAVE-complex subunits shown in Figure 1G, band intensities were measured by luminometry using a cooled charge-coupled device (CCD) camera (Luminescent image analyzer LAS-1000; Fujifilm, London, United Kingdom) and analyzed using AIDA software (Raytest, Straubenhardt, Germany). Data were expressed as percentage of control for each component as indicated.

Fluorescence and Time-Lapse Microscopy and Microinjection

B16-F1 and VA-13 cells were fixed with 4% formaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min, extracted with 0.1% Triton X-100 in 4% PFA for 50 s and stained with phalloidin and/or anti-myc as indicated. Alexa dye-labeled secondary reagents and phalloidin were from Invitrogen (Karlsruhe, Germany). L61Cdc42 was purified and injected at 1.5 $\mu\text{g ml}^{-1}$ essentially as described (Nobes and Hall, 1995). Mammalian cells were observed on an inverted microscope (Axiovert 100TV; Carl Zeiss, Jena, Germany) equipped for epifluorescence as described previously (Steffen *et al.*, 2004). *Dictyostelium* cells were fixed, stained with tetramethylrhodamine B isothiocyanate-phalloidin and subjected to confocal scanning microscopy as described previously (Schirenbeck *et al.*, 2005). Live mammalian cells were observed in an open chamber with a heater controller (Warner Instruments, Hamden, CT) at 37°C. Data were acquired with a back-illuminated, cooled CCD camera (TE-CCD 800PB; Princeton Scientific Instruments, Princeton, CT) driven by IPLab software (Scanalytics, Fairfax, VA). For time-lapse microscopy with *D. discoideum*, cells were plated into glass-bottomed dishes (Corning Glassworks, Corning, NY) in phosphate buffer containing 14.7 mM KH₂PO₄ and 2.5 mM Na₂HPO₄, pH 6.0. Images were acquired via a CCD camera (XC-75CE; Sony, Cologne, Germany) coupled to an Argus 20 image processor (Hamamatsu, Herrsching, Germany) and digitalized using a video grabber (VG-5) and Scion Image 1.62 software (Scion, Frederick, MD). Data were stored as 16-bit digital images and processed using IPLab, Scion Image 1.62, and Adobe Photoshop 5.0/CS software (Adobe Systems, Mountain View, CA).

Quantification of Filopodia Formation Upon Cdc42 Injection

Rates of filopodia initiation were assessed from randomly chosen cell peripheries with 30 μm in width and over a time period of ≈ 20 min after injection of constitutively active Cdc42. Seven independent movies were analyzed for each experimental condition, i.e., control and Nap1 knockdown cells. Newly formed filopodia were marked manually at the time point of initiation as indicated in Supplemental Video 2, and counted subsequently. In total, 357 and 422 filopodia were marked and counted for control and Nap1 knockdown cells, respectively. Extension rates were measured by tracking the tips of individual filopodia protruding parallel to the substrate over time periods of not <66 s using the Dynamic Imaging Analysis System (Solltech, Oakdale, IA). In total, 84 (from 6 videos) and 81 (from 4 videos) filopodia were analyzed for control and Nap1 knockdown, respectively. Kymographs (Hinz *et al.*, 1999) were made with a custom-written script in IPLab software (Scanalytics).

Statistical analyses were carried out using SigmaPlot, version 9.0 (SPSS, Chicago, IL), Microsoft Excel, version 9.0 (Redmond, WA), and MINITAB, version 10.5 (Minitab, State College, PA).

Morphological Assessments of B16-F1 Cells

Transient RNA interference was performed and evaluated as described previously (Steffen *et al.*, 2004). Arp3 and p21-Arc knockdown cells were analyzed on day 5 and Nap1 knockdown cells on day 4 after transfection of the respective knockdown plasmid. For quantification of lamellipodia, cells were treated with aluminum fluoride (AlF₄) for 20 min as described previously (Steffen *et al.*, 2004).

For filopodia quantification, cells were transfected with a mixture of pRK5-myc-L61Cdc42 and pRK5-myc-N17Rac in a ratio of 10:1 16 h before fixation and staining. EGFP-tagged versions of Scar-W or Scar-WA, which were generated by subcloning of respective fragments from pRK5-myc (Machesky and Insall, 1998) to EGFP-C1 (BD Biosciences, San Jose, CA), were cotransfected along with L61Cdc42 and N17Rac in a ratio of 10:10:1. Statistics were done as described above.

Negative Staining and Electron Microscopy

Cells were plated on fibronectin-coated Formvar electron microscopy (EM) support films on coverslips and prepared for electron microscopy essentially as described previously (Small *et al.*, 1999). Briefly, cells were washed with PBS and extracted for 1 min with prewarmed extraction/fixation buffer (0.25–0.5% Triton X-100, 0.25% glutaraldehyde in cytoskeleton buffer [CB]; Small *et al.*, 1999). Cells were fixed with 1.0% glutaraldehyde in CB for at least 3 h at room temperature and incubated in the same buffer supplemented with 10 $\mu\text{g/ml}$ phalloidin overnight at 4°C. After detaching the Formvar film from coverslips and mounting on 200 mesh Cu/Pd EM grids, the samples were rinsed with a few drops of SST/T (2–4% sodium silicotungstate, pH 7–8,

Figure 1. Microinjection of L61Cdc42 triggers filopodia formation in the absence of functional WAVE-complex. (A–B'') Mock (A–A'') or Nap1 (B–B'') RNAi-treated cells were microinjected with L61Cdc42 as indicated. Panels show video frames from representative movies before (A and B) and after (A', A'', B', and B'') injection (also see Supplemental Video 1). Time is given in minutes and seconds and is valid for both experiments. Cdc42 injection induced filopodia formation around the cell periphery (arrows) in both cell types. Bar, 20 μm . (C–E) Quantification of filopodia formation from time-lapse movies upon L61Cdc42 injection. (C) Kymography of parts of control and Nap1

0.125–0.25% trehalose) negative stain solution, and excess liquid was carefully removed. After air drying, the samples were analyzed using a LEO 910 transmission electron microscope at 80 kV and recorded on x-ray film.

RESULTS

Cdc42 Triggers Filopodia Formation in Nap1 Knockdown Cells

To test whether filopodia can form in the absence of detectable lamellipodia, we exploited fibroblast cell lines for which the expression of the WAVE-complex constituent Nap1 was stably suppressed (Figure 1). We had described previously that Nap1 knockdown in these fibroblasts abolishes lamellipodia protrusion and membrane ruffling upon growth factor treatment or microinjection of constitutively active Rac1 (Steffen *et al.*, 2004). As expected (Nobes and Hall, 1995), microinjection of constitutively active Cdc42 (L61Cdc42) into mock siRNA-treated VA-13 fibroblasts (Figure 1A) stimulated the protrusion of both filopodia (arrows in Figure 1, A' and A'') and lamellipodia/membrane ruffles (Supplemental Video 1), proving the functionality of the microinjected small G protein. Significantly, Nap1 knockdown cells (Figure 1B) protruded numerous filopodia upon Cdc42 injection as well (Figure 1, B' and B''). Lamellipodia formation was not observed under these conditions, corroborating the view that Cdc42-induced lamellipodia formation involves Rac signaling (Nobes and Hall, 1995). To quantify filopodia formation induced by microinjection of constitutively active Cdc42 in both cell types, we measured two independent parameters, rate of extension of individual filopodia (Figure 1, C and D) and frequency of filopodia initiation (Figure 1E; see *Materials and Methods*). Rate of extension was tracked from filopodia as indicated by the kymographs shown in Figure 1C. The rate of extension was 3.05 ± 1.09 and $3.32 \pm 1.11 \mu\text{m}/\text{min}$ for control and Nap1 knockdown cells, respectively. Rate of initiation was determined by marking newly formed filopodia after L61Cdc42 injection, as shown in Supplemental Video 2. Mock and Nap1 RNA interference

(RNAi)-treated cells formed 16.11 ± 6.67 and 19.04 ± 5.56 filopodia per $10 \mu\text{M}$ of cell periphery within 20 min. Thus, both rates of extension and the number of filopodia induced by Cdc42 injection were even slightly increased in Nap1 knockdown cells (Figure 1, D and E), although this difference was not statistically significant.

It had been reported earlier that knockdown of Sra-1, Nap1, or Abi-1 also causes significant reduction of expression of other WAVE-complex constituents (Kunda *et al.*, 2003; Rogers *et al.*, 2003; Innocenti *et al.*, 2005). To document Nap1 suppression in our stable knockdown fibroblast cell lines and concomitant reduction of the expression of the WAVE-complex components Sra-1, Abi-1, and WAVE2, we performed semiquantitative Western blotting using antibodies against WAVE-complex subunits and tubulin as loading control (Figure 1, F and G). Interestingly, quantification of WAVE-complex subunits revealed not only a strong reduction for Nap1 and WAVE2 but also for Sra-1 and Abi-1, although for the latter subunits suppression was less severe (Figure 1G). This might be explained by engagement of Sra-1 and Abi-1 in additional protein complexes (Scita *et al.*, 1999; Schenck *et al.*, 2001; Innocenti *et al.*, 2005). Importantly, Nap1 knockdown did not affect expression levels of Arp2/3-complex subunits (Figure 1F), confirming that lack of lamellipodia protrusion in these cells is due to the lack of WAVE-complex-mediated Arp2/3-complex activation (Steffen *et al.*, 2004), and not Arp2/3 subunit suppression.

Together, these data strongly suggest that filopodia formation induced by injection of L61Cdc42 requires neither functional WAVE-complex nor expression of prominent lamellipodia or membrane ruffles.

Arp2/3-Complex Knockdown Abrogates Lamellipodia Formation

Arp2/3-complex has frequently been implicated in triggering actin filament assembly in the formation of cellular projections, including filopodia (Svitkina *et al.*, 2003; Biyasheva *et al.*, 2004). To examine Arp2/3-complex function in the protrusion of both lamellipodia and filopodia, we suppressed constituents of this protein assembly by transient knockdown of ArpC3 (p21-Arc) and Arp3 in B16-F1 cells. To enable monitoring of Arp3 protein run-down after RNAi, we generated polyclonal anti-Arp3 antibodies (Supplemental Figure S1) for Western blot analyses. Both constituents of the Arp2/3-complex were virtually abolished on days 4 and 5 after transfection of respective knockdown plasmids (Figure 2A). As observed previously for Arp3 (Di Nardo *et al.*, 2005), knockdown of this subunit but not of the peripheral subunit p21-Arc resulted in reduced expression of other Arp2/3-complex constituents (Figure 2A), suggesting that Arp3 is required for complex stability in vivo. We then examined the morphology of mock and both Arp2/3-complex knockdown cell populations upon stimulation of lamellipodia formation by AIF₄⁻ (Figure 2, B–D). Lamellipodia formation triggered by this treatment was shown to be strongly abrogated in cells interfered for WAVE-complex function (Steffen *et al.*, 2004). Importantly, lamellipodia formation was also severely impaired upon knockdown of both p21-Arc and Arp3 compared with control. Quantification of the percentage of cells with lamellipodia revealed that <5 and 10% of cells displayed detectable lamellipodia for p21-Arc and Arp3 knockdown cells, respectively (Figure 2E), confirming that Arp2/3-complex is indeed crucial for lamellipodia formation (Machesky and Insall, 1998; Kunda *et al.*, 2003; Rogers *et al.*, 2003). These data unambiguously emphasize the relevance of Arp2/3-complex-mediated actin filament assembly, most likely activated by WAVE-complex, for the formation of

Figure 1 (cont). knockdown cells illustrating the selection of filopodia protruding parallel to the substrate for measuring rates of extension (see white arrows). Dashes in time line at the bottom indicate minutes. White asterisks mark time points of injection. Bar, $10 \mu\text{m}$. (D) Box plot summarizing the results from rate of extension measurements. The line within the box marks the median (3.07 and 3.18 for control and Nap1 knockdown, respectively), the boundaries of the box contain 50% (25–75%) and the whiskers 80% (10–90%) of all measurements. Outlying points are shown as dots; $n = 85$ and 82 for mock and Nap1 RNAi cells, respectively. Percentiles were computed using the “Cleveland” method. The nonparametric Mann–Whitney rank sum test ($\alpha = 0.05$) revealed no statistically significant difference between the two data sets ($p = 0.1194$). (E) Box plot summarizing rates of initiation displayed in number/ $10 \mu\text{m}/20 \text{ min}$ as indicated. The medians were 14.62 and 16.42 for control and Nap1 knockdown, respectively. $n = 8$ from seven independent movies for each cell type. The nonparametric Mann–Whitney rank sum test ($\alpha = 0.05$) revealed no statistically significant difference between the two data sets ($p = 0.248$). (F) Expression of the WAVE-complex constituents Nap1, Sra-1, WAVE2, Abi-1, and the Arp2/3-complex subunits Arp3 and p21 as assessed from extracts of stable Nap1 knockdown and mock-treated VA-13 clones as indicated. Tubulin was used as loading control. Note WAVE-complex but not Arp2/3-complex suppression in the Nap1 knockdown clone. (G) Quantification of suppression of WAVE-complex subunits in Nap1 knockdown cells as indicated. Data are pooled from two independent clones of control and Nap1 knockdown cells, respectively. Columns represent arithmetic means $\pm$ standard deviations. $n = 8$ for Nap1 and Abi1; $n = 6$ for Sra-1 and WAVE2.

Figure 2. Arp2/3-complex is essential for lamellipodia formation. (A) Samples from mock, p21, and Arp3 siRNA-treated B16-F1 cells collected at days after transfection (tfx) were analyzed for protein expression by Western blotting as indicated. Arp3 knockdown led to significantly reduced levels of p21 and p16A, whereas p21 knockdown did not affect expression of other Arp2/3-complex subunits. (B–D) Architecture of the actin cytoskeleton in mock (B), p21 (C), and Arp3 (D) siRNA-treated B16-F1 cells after AIF₄[−] stimulation. Bar, 20 μ m. (E) Lamellipodia quantification. Values are means $\pm$ SEMs from five independent experiments (at least 450 cells for each condition). Cells were classified according to the categories with or without lamellipodia as well as with ambiguous morphology as indicated. Lamellipodia formation is strongly suppressed upon p21 and Arp3 knockdown. Differences in lamellipodia formation between p21 or Arp3 knockdown and control populations were confirmed to be statistically significant using the nonparametric Mann–Whitney test at $\alpha = 0.05$ ($p = 0.0122$).

lamellipodia and membrane ruffles. In spite of the loss of prominent lamellipodia observed by phalloidin stainings of p21- and Arp3 knockdown cells, the cell peripheries of these cell populations still displayed actin filament bundles reminiscent of filopodia (Figure 2, C and D). To test for the subcellular distributions of distinct examples of both lamellipodial and filopodial markers upon interference with Arp2/3- or WAVE-complex, we stained mock and Nap1 or p21-Arc RNAi-treated B16-F1 cells with antibodies for cortactin, fascin, and the Arp2/3-complex subunit p16A as control. Because the actin filament and Arp2/3-complex-interacting protein cortactin was reported to be capable of Arp2/3-complex activation, at least in vitro (Urano *et al.*,

2001; Weaver *et al.*, 2003), we tested whether its localization would suggest a potential contribution to Arp2/3-complex positioning or activation at the cell periphery in cells interfered for WAVE-complex function, although both Arp2/3-complex and cortactin were reported to be excluded from filopodia (Svitkina and Borisy, 1999; Svitkina *et al.*, 2003). However, whereas cortactin accumulated in lamellipodia and membrane ruffles of mock RNAi cells, the cell peripheries of both Nap1 and p21-Arc knockdown cells were devoid of any specific cortactin enrichment (Supplemental Figure S2A). Accordingly, the Arp2/3-complex subunit p16A could not be detected at the peripheries of Nap1 or p21-Arc knockdown cells (Supplemental Figure S2B), suggesting that

Figure 3. Frequency of filopodia formation in the absence of lamellipodia. Mock (A and A'), Nap1 (B and B'), p21 (C and C'), and Arp3 (D and D') siRNA-treated cells were transfected with a mixture of myc-tagged L61Cdc42 and N17Rac and stained for the actin cytoskeleton (A–D); transfectants were identified by anti-myc staining (A', B', C', and D'). Control as well as knockdown cells formed numerous filopodia. Bar, 10 μ m. (E) Myc-L61Cdc42/N17Rac-transfected knockdown, control, and control cells expressing Scar-W and Scar-WA as indicated were classified according to the presence of filopodia. Differences in filopodia formation between p21, Arp3, Nap1 knockdown, Scar-W- or Scar-WA-overexpressing cells, and control cell populations were not statistically significant, as assessed by the nonparametric Mann-Whitney test at $\alpha = 0.05$ ($p = 0.2330$, $p = 0.2703$, $p = 0.5510$, $p = 0.7656$, and $p = 0.5510$, respectively). (F) The number of filopodia per cell counted for those cells that were evaluated to form filopodia depicted in E. Data represent means $\pm$ SEMs from three independent experiments (>100 cells analyzed for each condition).

the bundled arrays observed in B16-F1 cells upon transient Nap1 knockdown were indeed formed independently of Arp2/3-complex activity. In contrast, fascin, a marker of protrusive filopodia and microspikes (Kureishy *et al.*, 2002; Svitkina *et al.*, 2003), was enriched in peripheral actin filament bundles of both mock and Nap1 or p21-Arc RNAi-treated cells (Supplemental Figure S2C), indicating that the bundled peripheral structures formed in transient Nap1 and p21-Arc knockdown B16-F1 cells indeed represented canonical filopodia capable of protruding independently of both WAVE- and Arp2/3-complexes.

Interference with WAVE-Arp2/3-Complex Signaling Does Not Affect the Frequency of Filopodia Formation

To obtain quantitative information on the relevance of the presence of lamellipodia or constituents of the WAVE- and Arp2/3-complex pathway for filopodia protrusion in B16-F1 cells, we performed a comparative quantification of filopodia formation in cells suppressed for expression of Nap1, p21-Arc, or Arp3. In addition, mock-siRNA-treated cells expressing the Arp2/3-complex-sequestering fragment of Scar1 (Scar-WA) and the G-actin-binding fragment of Scar1 (Scar-W) as control (Machesky and Insall, 1998) were ana-

lyzed. Because of the difficulty of comparing filopodia numbers in the presence (control) and absence (Nap1 or Arp2/3-complex knockdown) of lamellipodia formation, measurements were made on cells coexpressing constitutively active Cdc42 (L61Cdc42) with dominant negative Rac1 (N17Rac1) (Nobes and Hall, 1995), to suppress lamellipodia under all experimental conditions. Bundles of actin filaments concluded to represent filopodia were readily observed in control and all knockdown populations (Figure 3, A–D) as well as upon Arp2/3-complex sequestration by Scar-WA (Figure 3E; data not shown). The identification of these structures as filopodia was further corroborated by the presence of the Ena/VASP family member VASP, a marker of filopodia initiation (Svitkina *et al.*, 2003) and of active actin polymerization in cellular protrusions (Rottner *et al.*, 1999), at their tips (Supplemental Figure S3).

In all experiments, the percentage of cells expressing filopodia exceeded 80% (Figure 3E). We then asked whether the number of filopodia per cell rather than the number of cells that form filopodia may be reduced. To answer this question, we quantified the number of filopodia induced by L61Cdc42/N17Rac expression after different RNAi treatments or after co-overexpression of the WA-domain of

Figure 4. Filopodia ultrastructure in control and p21 knockdown cells. Transmission electron micrographs of negatively stained whole mount cytoskeletons of mock (A–C and G) and p21 siRNA-treated (D–F and H) B16-F1 cells. (A) Representative overview of a control cell with several filopodia embedded into the lamellipodial mesh. Note the strikingly distinct structural organization of the cell periphery in p21 knockdown cells (D), with filopodia emanating from the ends of concave peripheral filament bundles. (B and E) High magnifications of the base of filopodia in control and p21 knockdown cells, which show actin filaments feeding into the filopodial shaft. Most lamellipodial filaments in mock siRNA-treated cells (C) approach the cell front at an angle of around 60°, which is contrasted by the parallel arrangement of actin filaments with respect to the cell periphery in p21 knockdown cells (F). Filopodia in both control and p21 knockdown cells are composed of bundles of densely packed, parallel actin filaments (G and H). Bars, 2 μ m (A and D), 200 nm (B, C, and E–H).

Scar1. Cells were grouped into three categories: few (up to 5), intermediate numbers of (5–10), or many (>10) filopodia and quantified accordingly. Clearly, the different populations of B16-F1 cells responding to L61Cdc42/N17Rac expression were unchanged under all conditions, and no bias for the formation of either more or less filopodia per cell after interference with Arp2/3- or WAVE-complex function was observed. Together, these data indicate that Cdc42-induced filopodia formation requires neither WAVE-complex-mediated Arp2/3-complex activation nor Arp2/3-complex-mediated actin polymerization (Figure 3F).

Ultrastructure of Filopodia Formed in the Absence of Functional Arp2/3-Complex

To assess the ultrastructure of filopodia formed in our experiments, we prepared negatively stained whole mount

cytoskeletons of mock-treated Nap1 (our unpublished data) or p21-Arc knockdown B16-F1 cells to examine the structure of the actin cytoskeleton in the presence and absence of functional Arp2/3-complex. In mock siRNA-treated cells (Figure 4, A–C), filopodia seemed embedded into the criss-cross arrangement of lamellipodial actin filaments as described previously (Small, 1981). As expected, lamellipodial actin filaments abutted the plasma membrane at slightly tilted angles (Figure 4C), which was in marked contrast to the organization of the cell periphery upon p21-Arc knockdown, characterized by concave bundles of filaments arranged in parallel to the cell periphery (Figure 4F). In these cells, filopodia mainly emanated from the edges of concave actin filament bundles (Figure 4D). Most notably, the ultrastructural organization of actin filaments in both base (Figure 4, B and E) and along shafts (Figure 4, G and H) of

Figure 5. Filopodia formation in *Dictyostelium* cells lacking Scar and Nap1. (A) Strategy for inactivation of the *scar* and *nap1* genes. Top shows constructs used to disrupt the *scar* or *nap1* genes. (B) Inactivation of *scar* and *nap1* was confirmed by two PCRs for each gene to screen for disruption (KO) or the presence of the WT allele using specific primer pairs as indicated by arrows in A. (C) Cell morphology and F-actin organization in WT, Scar, or Nap1-null cells. Three-dimensional (3D) reconstructions were computed from confocal sections. Both Scar and Nap1-null amoebae form multiple filopodia (for animated 3D reconstructions of these cells, see Supplemental Videos 2–4). Bar, 5 μ m. (D) Dynamic protrusion of filopodia in Scar and Nap1 knockout *Dictyostelium* cells. Time-lapse series of WT cells showing a mixture of both lamellipodia and filopodia protrusion (left and Supplemental Video 5), and of Scar null (middle) and Nap1 null (right) cells forming numerous protrusive filopodia (asterisks and Supplemental Videos 6 and 7). Elapsed time is valid for all panels. Bar, 2 μ m. (E) Scar-null and Nap1-null transformants expressing GFP-dDia2 (green) and counterstained with phalloidin (red). Asterisks indicate specific accumulation of dDia2 at filopodia tips.

filopodia was indistinguishable between control and p21-Arc knockdown cells, suggesting that interference with Arp2/3-complex function does not affect the molecular mechanism of actin filament polymerization into filopodia.

Dictyostelium Cells with Disrupted *nap1* or *scar* Genes Protrude Multiple Filopodia

Our data on RNAi-mediated knockdown of WAVE- and Arp2/3-complex constituents in mammalian cell types suggest that these regulators of the actin cytoskeleton are not required for filopodia formation, although residual protein activity may contribute—at least in part—to the formation of the structures observed. To test this hypothesis and to confirm our results in an alternative cell system, we examined

filopodia formation in WT and mutant *D. discoideum* strains. Vegetative *Dictyostelium* cells null for the single WAVE-orthologue Scar were reported previously to display numerous defects in actin cytoskeleton reorganization (Bear *et al.*, 1998), but filopodia formation was not studied. To address WAVE-complex function in filopodium protrusion in *Dictyostelium*, we generated strains with gene disruptions for either *scar* or *nap1* (the latter encoding the single orthologue of mammalian Nap1) (Figure 5, A and B). Vegetative WT and Scar or Nap1 null amoebae were allowed to spread on glass coverslips and first analyzed for actin cytoskeleton architecture by confocal microscopy. Importantly, similar to WT cells, both Scar and Nap1 null cells displayed numerous straight peripheral actin bundles, identical in appearance to

canonical filopodia (Figure 5C and Supplemental Videos 3–5). To confirm that these actin filament bundles were capable of active protrusion, we performed phase contrast time-lapse microscopy (Figure 5D and Supplemental Videos 6–8). These experiments revealed that both Scar and Nap1 null cells formed multiple protrusive filopodia, which were virtually identical in dynamics and overall behavior to the filopodia observed in WT cells. We then wondered whether the molecular mechanism of filopodia formation in Scar and Nap1 null amoebae is comparable with WT cells. To gain more insight into this question, we transformed Scar and Nap1 null amoeba with a GFP-tagged version of the diaphanous-related formin dDia2, which was shown recently to be critical for filopodia formation in this organism (Schirenbeck *et al.*, 2005; Faix and Rottner, 2006). Importantly, GFP-dDia2 prominently accumulated at the tips of filopodial bundles formed in amoebae disrupted for both Scar and Nap1 (Figure 1E), identical to previous observations with WT cells (Schirenbeck *et al.*, 2005), strongly suggesting that the molecular mechanism of filopodium formation is maintained in *Dictyostelium* cells lacking WAVE-complex subunits. Together, these data provide the first compelling evidence that WAVE-complex function is dispensable for the protrusion of filopodia in *D. discoideum*.

DISCUSSION

Filopodia are finger-like protrusions composed of bundles of parallel actin filaments (Small and Celis, 1978) polymerizing at their tips (Mallavarapu and Mitchison, 1999). Numerous studies have documented the protrusion of these structures triggered by activation of small GTPases of the Rho-family such as Cdc42 (Hall, 1998; Aspenstrom *et al.*, 2004), although the precise molecular mechanisms driving actin polymerization downstream of Rho-GTPase activation have remained controversial (Takenawa and Miki, 2001; Small *et al.*, 2002; Faix and Rottner, 2006). In contrast, lamellipodia protrusion is commonly assumed to require Scar/WAVE family proteins and associated factors (Stradal *et al.*, 2004). Frequent emergence of nascent filopodia from protruding lamellipodia in B16-F1 melanoma cells prompted the proposal of the convergent elongation model of filopodium protrusion (Svitkina *et al.*, 2003), which was supported by Scar knockdown experiments in *Drosophila* cell lines (Biyasheva *et al.*, 2004). The idea that filaments from lamellipodia contribute to filopodia formation already arose from the earliest electron microscope studies of actin networks in cytoskeletons, in which convergent “delta-like arrays” were also described (Small *et al.*, 1980; Small, 1988). Here, we directly tested whether lamellipodial filaments are an essential prerequisite of filopodium protrusion by triggering them in cells abrogated for lamellipodia.

Microinjection of constitutively active Rac1 into fibroblasts depleted by RNA interference of constituents of the WAVE-complex like Nap1 frequently caused membrane blebbing but no lamellipodium protrusion (Steffen *et al.*, 2004). As opposed to controls, Cdc42 injection failed to stimulate lamellipodium protrusion and membrane ruffling, indicating that Cdc42-induced formation of lamellipodia does indeed require Rac activation as suggested previously (Nobes and Hall, 1995). Surprisingly, Cdc42 injection effected protrusion of multiple filopodia in Nap1 knockdown cells at a frequency and rate of elongation equivalent to controls, indicating that the efficiency of filopodia formation is independent of both WAVE-complex function and detectable lamellipodia, at least in fibroblasts.

We also demonstrate the dispensability of Arp2/3-complex for filopodia but not lamellipodia formation. Notably,

lamellipodia protrusion was reported previously to be suppressed by knockdown of p20-Arc (Arp-C4) and p34-Arc (Arp-C2) subunits in *Drosophila* S2 cells (Kunda *et al.*, 2003; Rogers *et al.*, 2003). A recent provocative study failed to detect defects in filopodia formation in stable Arp3 knockdown fibroblasts (Di Nardo *et al.*, 2005). However, because these cells also showed unimpaired membrane ruffling and only partially blocked actin tail formation behind cytoplasmic *Listeria monocytogenes*, they apparently expressed residual levels of functional Arp2/3-complex. In our experiments, independent knockdowns of Arp3 and the peripheral p21-Arc subunit in B16-F1 mouse melanoma cells strongly abrogated lamellipodia protrusion, with p21-Arc being slightly more effective than Arp3 knockdown. Interestingly, in vitro reconstitution and examination of the actin nucleation activity of recombinant Arp2/3-complex demonstrated that Arp3 was indispensable for nucleation, whereas incomplete Arp2/3-assemblies lacking p21-Arc still showed some (but little) nucleation activity (Gournier *et al.*, 2001). The fact that lamellipodia formation upon p21-Arc knockdown was at least as strongly compromised as in Arp3 knockdown cells suggests that residual nucleation activity observed in the absence of p21-Arc in vitro is insufficient to trigger lamellipodia protrusion in vivo or alternatively that p21-Arc exerts additional functions essential for site-directed Arp2/3-complex-mediated filament assembly in lamellipodia. Notwithstanding this, our data strongly suggest that Arp2/3-complex, most likely activated by WAVE-complex, is essential for the formation of lamellipodia and membrane ruffles, but not filopodia. This was confirmed by overexpression of Scar-WA, which interferes with site-directed Arp2/3-complex-mediated actin filament nucleation in vivo, as exemplified by suppression of lamellipodia formation (Machesky and Insall, 1998), the intracellular motility of *Listeria* (May *et al.*, 1999), and of actin assembly accompanying the internalization of clathrin-coated pits (Benesch *et al.*, 2005).

All these data suggest that the WAVE/Arp2/3-complex pathway is dispensable for filopodia protrusion, at least in mammals, which is contrasted by reduced filopodia numbers recently observed in *Drosophila* Scar knockdown cells (Biyasheva *et al.*, 2004). The reason for this discrepancy is currently unclear. Notably, filopodia-like structures observed by these authors were concluded to represent narrow lamellipodia rather than filopodia, formed by residual Scar activity, because they seemed to be composed of branched arrays of short actin filaments (Biyasheva *et al.*, 2004) instead of long, parallel arrays of filaments typical for filopodia. Our ultrastructural analyses of whole mount cytoskeletons of both control and Arp2/3-complex knockdown cells confirmed the presence of canonical filopodia in both cell types and loss of lamellipodial filament arrays in Arp2/3 knockdown cells.

Together, although our data do not exclude a route of filopodia formation involving the recruitment and bundling of preexisting lamellipodial actin filaments, they clearly demonstrate that filopodia can form in their absence.

A distinct advantage of RNA interference is that the function of a given protein may be addressed by transient depletion of its expression, allowing analysis even of proteins or protein complexes essential for cell viability and growth (Harborth *et al.*, 2001). Our experiments in mammalian cells unambiguously show that cells in which lamellipodia protrusion is abolished fail to display defects in the formation of filopodia, suggesting that the molecular regulation of lamellipodia and filopodia formation is distinct and separable, at least in mammalian cell types. However, it is difficult to

formally exclude the presence of residual protein activity (for example, of WAVE-complex components), which may suffice for filopodia formation without the recognizable induction of lamellipodia. To address this issue directly, we turned to the evolutionary distant amoeba *D. discoideum*, which is a well proved model of cell motility (Affolter and Weijer, 2005). The herein demonstrated filopodia formation in cells genetically disrupted for Scar and Nap1 cannot be explained by residual protein activity.

In addition to confirming the dispensability of WAVE-complex function in the formation of filopodia, our observations indicate that the molecular hardware of filopodium protrusion is conserved through evolution from amoebae to mammals. This conclusion is in line with the recent discovery of the pivotal function in filopodium formation of the *Dictyostelium* formin dDia2 (Schirenbeck *et al.*, 2005), as evidenced by its genetic disruption and accumulation at filopodia tips. Similar to dDia2, this localization was also observed for a mammalian diaphanous-related formin, mDia2, also known as Drf3 (Peng *et al.*, 2003; Pellegrin and Mellor, 2005), although this protein is not detectable in certain fibroblast cell lines capable of filopodium formation. The latter observation may suggest a certain degree of redundancy in the molecular mechanism of filopodium protrusion in mammals, although the idea of a key function also for mammalian formin(s) in filopodial actin filament nucleation and/or elongation seems increasingly attractive.

In conclusion, a comparison of the common and distinct molecular mechanisms of actin reorganization in cellular model systems of evolutionary distant organisms continues to serve as a promising approach (Noegel and Schleicher, 2000; Van Haastert and Devreotes, 2004) to further our understanding of the regulation of actin-based motility in vivo.

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