JCS ePress online publication date 27 November 2002

Differential localization of WAVE isoforms in filopodia and lamellipodia of the neuronal growth cone

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Accepted 21 October 2002
Journal of Cell Science 116, 239-246 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00233

Summary

The formation and extension of filopodia in response to an extracellular stimulus by guidance cues determine the path of growth cone advance. Actin-filament bundling and actin polymerization at the tips supply the driving force behind the formation and elongation. We tried to clarify how signals in response to extracellular cues are transformed to induce filopodial generation and extension. Observations on the formation process of filopodia at growth cones in the neuroblastoma cell line NG108 showed that WAVE (WASP (Wiskott-Aldrich syndrome protein)-family verprolin homologous protein) isoforms played crucial and distinct roles in this process. WAVE1 was continuously distributed along the leading edge only and was not found in the filopodia. WAVE2 and WAVE3 discretely localized at the initiation sites of microspikes on the leading edge and also concentrated at the tips of protruding filopodia. We further found that WAVE isoforms localized at the filopodial tips through SHD (SCAR homology domain), next to its leucine zipper-like motif. Furthermore, time-lapse observations of filopodial formation in living cells showed that WAVE2 and WAVE3 were continuously expressed at the tips of filopodia during elongation. These results indicate that WAVE2 or WAVE3 may guide the actin bundles into the filopodia and promote actin assembly at the tips.

Movies available online

Key words: WAVE, WASP, NG108, Actin, Filopodia, Growth cone

Introduction

Functions of the nervous system are based on the correct formation of a network by neuronal cells, which is established by the path finding of extending lamellipodia and filopodia at the growth cone. Filopodia, which initiate and elongate from the growth cone lamellipodia, are thin protrusions with actin bundles at their core. Their extension and retraction are initial events in the steering of a growth cone (Lewis and Bridgman, 1992; Bentley and O'Connor, 1994; Nikolic, 2002). Clarifying the molecular mechanisms by which filopodia extend and retract, and the signal pathways that control these events, is an important step in understanding how extracellular cues guide growth cones. A typical neuronal filopodium comprises ~15-20 actin filaments oriented with their barbed ends toward the tip (Lewis and Bridgeman, 1992). The actin cytoskeleton has been shown to be assembled near the leading edge and transported backward by a retrograde flow (Theriot and Mitchson, 1991; Heath and Holifield, 1993; Watanabe and Mitchson, 2002). Correspondingly, attempts have been made to elucidate the signal pathways from guidance cues to lamellipodial extensions (Hall, 1998; Kim et al., 2002; Li et al., 2002). WASP (Wiskott-Aldrich syndrome protein) family proteins have been isolated as mediators that convert signals to actin polymerization downstream of Rho family proteins, Rac and Cdc42 (Pollard et al., 2000; Takenawa and Miki, 2001; Small et al., 2002). In principle, the C-terminal VCA (verprolin-homology and cofilin-like acidic) domain of the WASP proteins activates an Arp2/3 (Actin related protein 2 and 3) complex, whereas the other domain primarily regulates the activity of VCA (Takenawa and Miki, 2001). WAVE (WASP-family verprolin homologous protein) isoforms, WAVE1, WAVE2, and WAVE3, have an arrangement similar to that of WASP and N-WASP, including a C-terminal VCA domain and a proline-rich region in the middle. The VCA domains of the WAVE isoforms associate with the Arp2/3 complex near the actin filament and promote actin polymerization. By contrast, the N-terminal domain (SCAR homology domain; SHD) is specific to the WAVE proteins (Suetsugu et al., 1999), and is thought to determine their localization at the leading edge of lamellipodia of fibroblasts and melanoma cells (Nakagawa et al., 2001; Hahne et al., 2001).

To date, many aspects have been made clear about the extension of lamellipodia (Borisy and Svitkina, 2000; Small et al., 2002), but almost nothing is known about the way filopodia are assembled and disassembled in vivo. In this study, we aimed to clarify the localization and roles of WAVE isoforms in the formation and extension of lamellipodia and filopodia in the growth cones of cultured neuroblastoma NG108-15 cells. To confirm the endogenous expression of WAVE isoforms, we observed cells immunostained with an antibody reacting against all these isoforms. Additionally, we characterized the localization and roles of the WAVE isoforms in living cells transfected with fusion constructs of enhanced green fluorescent protein (EGFP) and each WAVE isoform, and subsequently observed a temporal change in their localization in the protruding lamellipodia and filopodia. Furthermore, to clarify which domain and region of WAVE isoforms control the localization, we observed the distribution of various

truncated fragments tagged with EGFP. To understand how actin bundles are involved in lamellipodial extension and filopodial elongation at the growth cone, we studied the actin bundles' localization and simultaneous correlation with WAVE isoforms.

Materials and Methods

Antibodies and other materials

Affinity purified polyclonal antibody against WAVE isoforms was characterized as described previously (Miki et al., 1998). FITC-conjugated anti-rabbit IgG goats were obtained from British BioCell International (Cardiff, UK). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Dainippon Pharmaceutical (Osaka, Japan). The nutrient mixture F-12 Ham, antibiotics, a 0.01% poly-L-lysine solution and rhodamine-phalloidin were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The TOPO TA cloning kit was purchased from Invitrogen (Groningen, Netherlands). The mammalian expression vector pEGFP-N1 was obtained from CLONTECH Laboratories (Palo Alto, CA). TransIt-LT1 was purchased from PanVera Corp. (Madison,WI).

Cell culture

NG108-15 cells (gift from Haruhiro Higashida, Kanazawa University Graduate School of Medicine, Japan) were cultured in DMEM supplemented with 10% FCS, and penicillin/streptomycin at 37.5°C in 5% CO_2. In preparation for immunofluorescence microscopy observation, cells were briefly trypsinized (0.05% in 1 mM EDTA) and replated on a glass coverslip or glass bottom dish. These coverslips were coated with poly-L-lysine. To induce the formation of axons and growth cones in cells, the medium was supplemented with 1 mM dibutyryl cyclic AMP (Bt_2cAMP) (Furuya and Furuya, 1983).

Expression vectors and transfection

WAVE1-, WAVE2- and WAVE3-EGFP were generated by polymerase chain reaction (PCR) with the corresponding human cDNA as template. WAVE1 SHD (amino acid residues 1-171) and SHD-basic region (SHD-BR) (residues 1-225) were amplified by PCR with human WAVE1 cDNA. WAVE2 N-terminal (NT) region, NT54 (residues 1-54) and NT83 (residues 1-83), and proline-rich (Pro-rich) region (residues 265-400) were amplified by PCR with human WAVE2 cDNA (Suetsugu et al., 1999). All 5′ and 3′ primers contained a *Xho*I restriction endnuclease site. In the 5′ primer for the WAVE2 Pro-rich region, the ATG sequence for the first methionine was inserted between the *Xho*I site and complementary sequence. Amplified fragments were subcloned using the TOPO TA cloning kit and were sequenced. The subcloned fragments were inserted into *Xho*I-digested pEGFP-N1. In all constructs, the sequence for EGFP was fused to the 3′ terminus of the insert. NG108-15 cells were transfected at 1×10^5 cells in 35 mm dishes, using 6 μl of TransIt-LT1 and 2 μg of DNA. After being transfected overnight, cells were replated on dishes with fresh medium.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were carried out as previously described by Nakagawa et al. (Nakagawa et al., 2000).

Immunofluorescence

Observation by immunofluorescence microscopy was performed as described by Nakagawa et al. (Nakagawa et al., 2000). Cells on glass coverslips were fixed with 3% formaldehyde in PBS (137 mM NaCl, 2.7 mM Na_2HPO_4, 8.1 mM KCl and 1.5 mM KH_2PO_4), permeablized with 0.1% Triton X-100 in PBS, and reacted with primary and secondary antibodies. Rhodamine-phalloidin was diluted in the secondary antibody solution. All observations were made with a confocal laser-scanning microscope, LSM510 (Carl Zeiss, Germany).

Living cell observation

A few hours before the observation, the medium of the cells attached to glass bottom dishes was changed to nutrient mixture F-12 Ham with 1 mM Bt_2cAMP. Under the microscope, the culture dishes were placed in an open heating chamber maintained at 37.5°C in air. Differential interference and fluorescence images were acquired through the confocal laser-scanning microscope, LSM510.

Results

Localization of endogenous WAVE isoforms and reorganization of actin bundles at the growth cone

Using the specific antibody, we identified the distributions of WAVE isoforms at growth cones of NG108 cells. To clarify the specificity, we immunoblotted SDS-whole extracts of NG108 cells with the antibody (Fig. 1). The antibody (anti-WAVEs) used in this study reacted to all WAVE isoforms, and to less- and hyper-phosphorylated forms of them as reported by Miki et al. (Miki et al., 1999). On the poly-L-lysine-coated dish, NG108 cells adhered to the substrate and extended typical large lamellipodia in 24 hours incubation (Fig. 2). To identify the localization of WAVEs, we immunostained the cells with anti-WAVEs and also simultaneously double stained them with rhodamine-phalloidin after fixation. WAVEs continuously localized along the leading edge of the growth cone lamellipodium. Actin bundles densely distributed throughout lamellipodia, many of which orthogonally encountered the leading edge. Some diagonally running actin bundles joined others at a point on the leading edge. These findings are consistent with those observed by birefringence microscope in the Aplysia growth cones (Katoh et al., 1999a) and are also described for melanoma lamellipodia by Small et al. (Small et al., 2002). To elucidate how WAVE participates in the reorganization of the actin cytoskeleton during filopodial extension, we immunostained the filopodia with anti-WAVEs and simultaneously labeled actin filaments with rhodamine-phalloidin after fixation. Interestingly, the WAVEs concentrated at the tips of pre-mature and mature filopodia (Fig. 3), and always existed at the tips of elongating filopodia, independent of their length. These findings show that WAVE participates in the generation and elongation of filopodia. The proximal portions of actin bundles constituting the pre-mature filopodia core extended into the cytoplasm and sometimes fused with other actin bundles. Around the filopodium's base, some actin bundles were partially separated and radially spread into the veil, which is an active lamellar protrusion that extends between filopodia in growth cones (Steketee and Tosney, 2001). Accordingly, at this stage pre-mature filopodia exhibited a nub-like shape. With the filopodia growing, actin bundles were organized to become the filopodial core.

Temporal change in the localization of WAVE isoforms

To date three isoforms, WAVE1, WAVE2 and WAVE3, have been identified in mammalian tissues (Suetsugu et al., 1999).

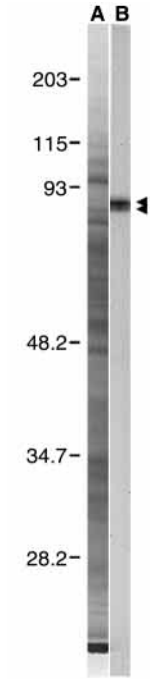

Fig. 1. Immunoblotting with anti-WAVEs. Anti-WAVE antibody (anti-WAVEs) reacting with all WAVE isoforms was recognized as a doublet at around 85 kDa of NG108 cell SDS-whole extract, corresponding to less- and hyper-phosphorylated forms of antigen as previously reported (Miki et al., 1999). Lane (A) shows the pattern of PAGE for SDS-whole extract and (B) shows immunoblotting pattern with anti-WAVEs.

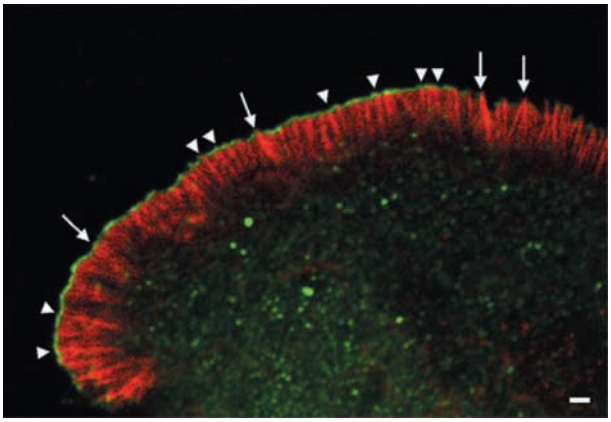

Fig. 2. Localization of WAVEs and reorganization of actin bundles. NG108 growth-cone lamellipodia of cells cultured for 24 hours in the presence of 1 mM Bt_2cAMP were double stained with rhodamine-phalloidin (red) and anti-WAVEs (green). WAVEs show a continuous localization along the leading edge of lamellipodium. Many actin bundles orthogonally encounter the leading edge (arrowhead) and some diagonally running ones fuse with others at the leading edge (arrows). Bar, 1 μm.

We prepared constructs in which the cDNA of these isoforms was tagged with EGFP at the C-terminus, and transfected each into NG108 cells by lipofection; we subsequently induced the formation of lamellipodia and filopodia at growth cones by treatment with Bt_2cAMP as shown in Figs 4 and 5. The expression of fusion proteins of WAVE tagged with EGFP in the C-terminus was very low, therefore the fluorescence signal from GFP was substantially weak. So, to clarify whether fluorescence for EGFP is really emitted only from the fusion protein of EGFP and WAVE isoform expressed in the cell, we immunostained these cells with antibody against EGFP to enhance the fluorescence signal, and simultaneously labeled

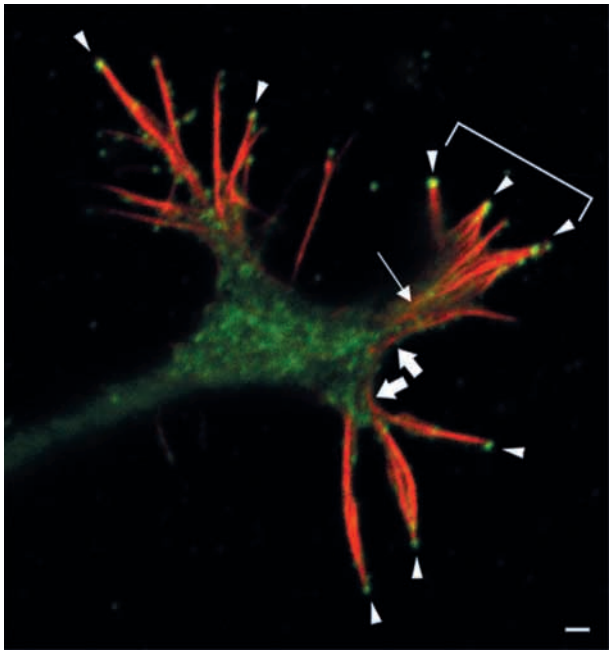

Fig. 3. Localization of WAVEs and reorganization of actin bundles. Growth cone was double stained with anti-WAVEs (green) and rhodamine-phalloidin (red) after 24 hours culture in the presence of 1 mM Bt_2cAMP. WAVEs clearly localize at the tips of filopodia (arrowheads). The proximal portions of actin bundles extend into the cytoplasm and sometimes associate with others (thin arrow). Some actin bundles separate around the filopodial base and radially spread into the veil (thick arrows). Premature filopodia show a nub-like shape (square bracket). Bar, 1 μm.

actin filaments fluorescently with rhodamine-phalloidin. We confirmed that the localization of WAVE isoforms detected by immunofluorescence with anti-GFP sufficiently coincided with that from EGFP fluorophores in both lamellipodia and filopodia (Fig. 4). We had already obtained the preparatory result that the functional domain to control the localization might exist in the N-terminus, SHD region, of each WAVE. Hence, in this study EGFP was consistently tagged with the C-terminus. In ruffled regions of lamellipodia, the continuous distribution of WAVE1 along the leading edge indicated no significant changes during observation (Fig. 5A,B). WAVE1-EGFPs became more concentrated with time, especially in the extending parts of lamellipodia. By contrast, in the retracting areas of lamellipodia, the fluorescence intensity from WAVE1-EGFPs gradually weakened. At the microspikes and filopodia, we failed to observe the localization of WAVE1 either at the tips or in the shafts. These findings show that WAVE1 is involved in lamellipodial extension but not filopodial formation and elongation. We paid special attention to the growth cones in which the microspikes were emerging and growing into filopodia. WAVE2-EGFPs concentrated as dot-like spots at the sites where the distal ends of actin bundles encountered the leading edge (Fig. 5C,D). With time, the microspikes appeared with ends at sites corresponding to WAVE2-EGFP-concentrated points. In addition, these WAVE2 spots moved along the leading edge with the lateral motion of microspikes throughout growth-cone lamellipodia. These results show that WAVE2 on the leading edge controls the microspike formation

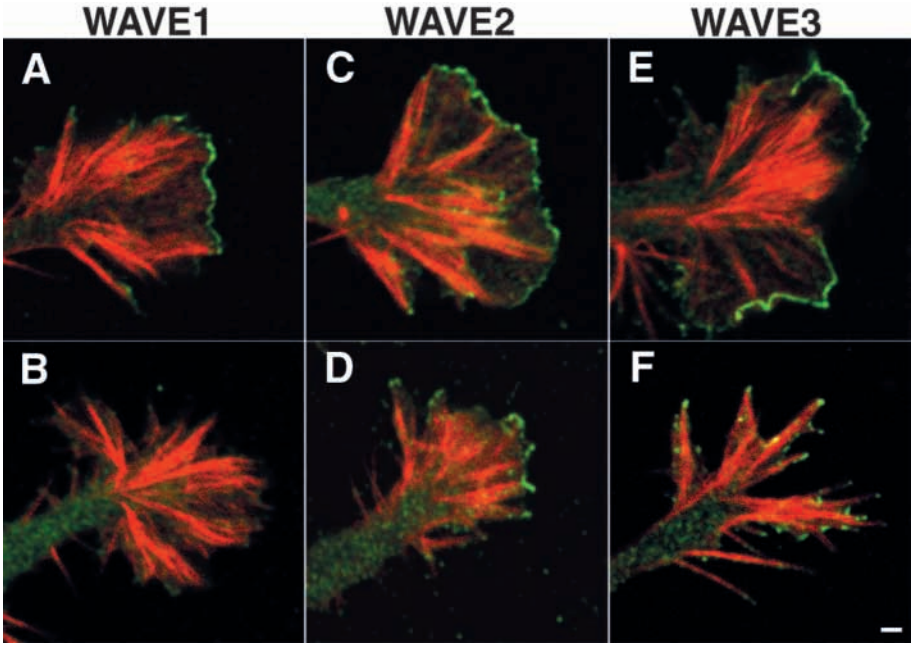

Fig. 4. Localization of WAVE1-EGFP, WAVE2-EGFP and WAVE3-EGFP along the leading edge of lamellipodia and at the tips of filopodia. The growth cones were double stained with anti-GFP antibody (green) and rhodamine-phalloidin (red). The upper panel shows the growth-cone lamellipodium (A,C,E) and the lower panel shows the growth-cone microspikes and filopodia (B,D,F). The fluorescence signal emitted from each WAVE-EGFP coincides with that from anti-GFP antibody labeled with fluorescence dye. (A,B) WAVE1-GFP. (C,D) WAVE2-EGFP. (E,F) WAVE3-EGFP. Bar, 1 μm.

and movement. By contrast, WAVE2 disappeared at the tips of retracting filopodia. WAVE3 also localized at the tips of microspikes and filopodia similarly to WAVE2 (Fig. 5E,F). These findings imply that WAVE2 and/or WAVE3 might regulate actin polymerization at the tips of filopodia.

Functional domain to control the localization of WAVE isoforms

We previously reported that WAVE should be recruited to the lamellipodial leading edge through the SHD region in the fibroblast cells (Nakagawa et al., 2001). To clarify the functional domain that regulates the concentration of WAVE isoforms at the filopodial tips, we made various truncated fragments of each WAVE and investigated their localization at the neuronal growth cones. WAVE1 SHD-EGFP concentrated at the filopodial tips (Fig. 6B). An N-terminal fragment of WAVE1 SHD plus basic region (SHD-BR-EGFP) similarly localized at the filopodial tips (Fig. 6C). Full-length WAVE1, however, failed to localize at the filopodial tips (Figs 4 and 5). This suggests that Pro-rich + VCA region of WAVE1 might influence an inhibitory effect to the localization. For another possibility, perhaps full length of WAVE1 might not localize at the filopodial tips because it usually exists in heterotetrameric complex, as reported recently (Eden et al., 2002). To clarify the detailed functions of SHD of WAVE2 that contributed mainly to the filopodia formation, we studied the localization of various N-terminal fragments. Among them, an N-terminal 54 amino-acid fragment of SHD tagged with EGFP (WAVE2 NT54-EGFP) migrated neither to the leading edge nor to filopodial tips (Fig. 6D), but an 83 amino-acid fragment (WAVE2 NT83-EGFP) localized to the filopodial tips (Fig. 6E). A proline-rich region of WAVE2 (Pro-rich-EGFP) distributed diffusely throughout the growth cone (Fig. 6F). These results indicate that 29-amino-acid residues from 54 to 83 of WAVE2 SHD are essential to the localization at the filopodial tips. Miki et al. reported that this region of SHD contains the putative leucine-zipper-like motif (Miki et al., 1998). The above results show that this motif might play a crucial role in the localization at the filopodial tips.

Discussion

The growth cones of neuronal axon and dendrite, responding to the extracellular stimuli via guidance cues, navigate themselves to the correct target by extension and retraction of filopodia. This process comprises two series of molecular events: (1) signal pathways that lead to the formation and elongation of filopodia after the growth cones receive signals from guidance cues, and (2) the molecular machinery that converts the signal to the mechanical force that drives the formation and elongation. Several working models have been proposed to explain these processes, but details remain obscure (Lanier and Gertler, 2000; Govind et al., 2001). Here we propose a new working model based on our findings.

Signal pathways from small G proteins to WAVE isoforms and their localization at the growth cone

The signal pathways that lead to the Rho family of small G-proteins, Cdc42 and Rac, via various intermediators such as tyrosine kinase and phosphatidylinositol 4,5-bisphosphate after accepting guidance cues have almost been elucidated (Nobes and Hall, 1995; Hall, 1998). However, these molecules cannot directly reorganize the actin cytoskeleton. Recently, WASP family proteins have received attention as mediators that intervene between these two steps (Pollard et al., 2000; Takenawa and Miki, 2001), whereas the details as to the localization and the roles of these proteins at neuronal growth cones remain vague. In our study, we found that WAVE1 distributed continuously along the leading edge alone, and WAVE2 and WAVE3 showed a discrete and dynamic localization at the initiation sites of microspikes on the leading edge. They also continued to remain at the tips of

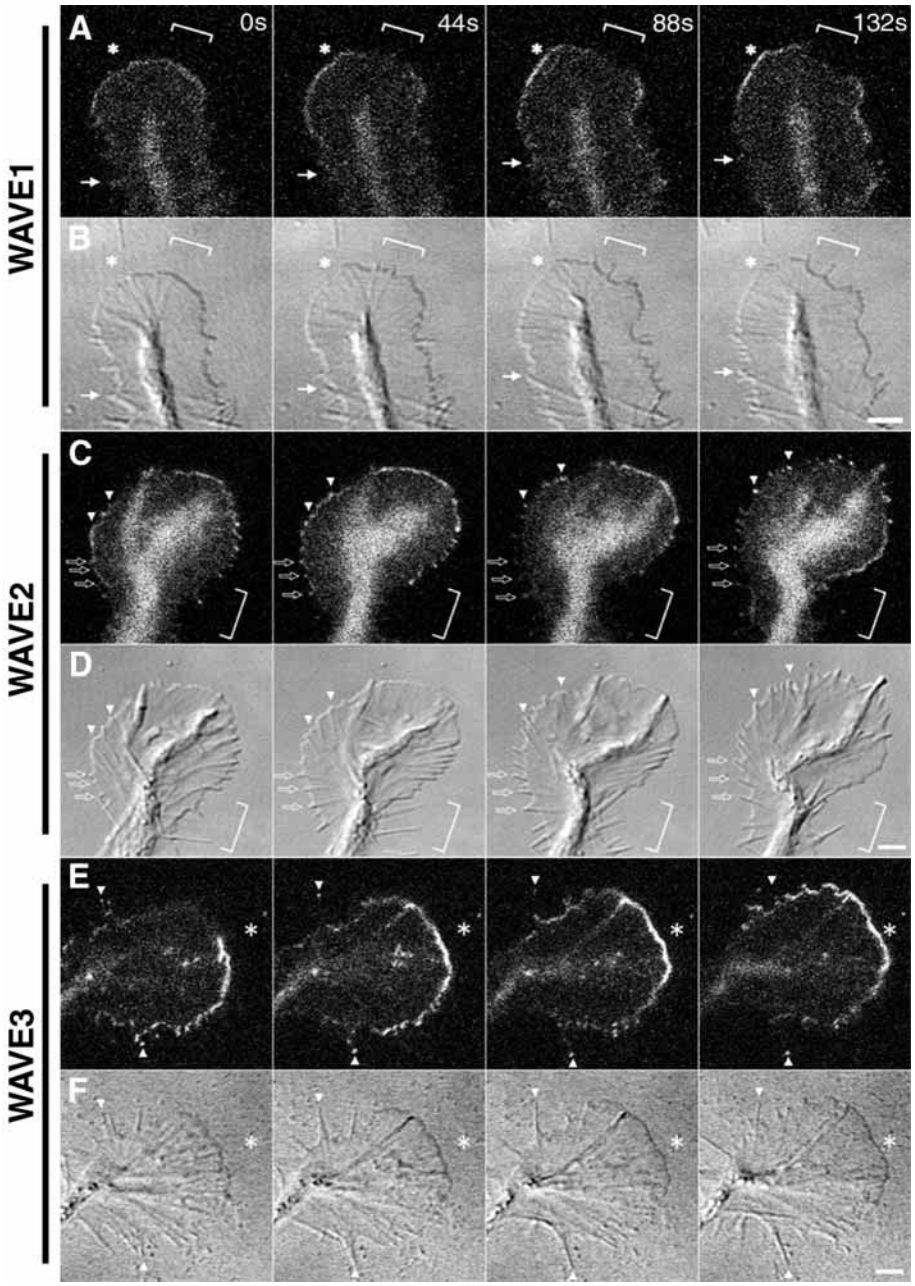

Fig. 5. Time-lapse images of localization of WAVE isoforms tagged with EGFP in the growth cone. (A,B) WAVE1-EGFP localized along the leading edge and concentrated in the extending region (asterisk) but disappeared in the retracting region (indicated by square bracket). Neither was found at the filopodial tips (arrows). The upper panel (A) shows fluorescence images of WAVE1-EGFP, whereas the lower panel (B) shows differential interference contrast (DIC) images corresponding to the upper images. The four columns show the image every 44 seconds. (C,D) WAVE2-EGFP condensed as dot-like spots at the sites where microspikes encounter the leading edge (arrowheads). They correspond to the tips of filopodia and project extracellularly with elongation (open arrows). WAVE2-EGFP remained at the tips of extending filopodia (open arrows) and sometimes moved with time laterally along the leading edge with the lateral motion of actin bundles through the lamellipodium (arrowheads). By contrast, WAVE2-EGFP spots disappeared at the tips of retracting filopodia in the region indicated by square brackets. The upper panel (C) shows fluorescence images from WAVE2-EGFP at the growth cone and the lower panel (D) indicates the corresponding DIC images. (E,F) WAVE3-EGFP localized at the tips of extending filopodia and remained there during elongation (arrowheads), and also distributed along the leading edge of extending lamellipodia (asterisks). The upper panel (E) shows fluorescence images of the WAVE3-EGFP distribution at the growth cone and the lower panel (F) shows the corresponding DIC images. Bar, 1 μm.

filopodia during elongation. These findings indicate that WAVE2 and WAVE3 are involved in the emerging site induction of the filopodia and subsequently promote their elongation. Additionally, the recent reports that filopodia can be formed even in fibroblasts isolated from N-WASP-deficient mice (Snapper et al., 2001; Lommel et al., 2001) indicate that N-WASP does not always participate in the formation and elongation of filopodia. However, WAVE isoforms have no GBD/CRIB (GTPase-binding domain/Cdc42 and Rac interactive binding) region different from WASP and N-WASP, and do not directly bind small G-proteins, Rac and Cdc42 as previously reported (Miki et al., 1998). WAVE isoforms commonly have a wave homology domain (WHD)/SHD region at the N-terminus. Previously, we confirmed that the localization of SHD-EGFP coincides with that of WAVE at fibroblast lamellipodia (Nakagawa et al., 2001). In the present study we showed that WAVE isoforms localized at the filopodial tips through the SHD region, exactly by its leucine-zipper-like motif. To clarify the more detailed function of WAVE isoforms we will continue to search for the protein interacting with this leucine-zipper motif at the growth cone. Recently, a scaffold protein IRSp53, which activates WAVE2 by binding Rac-GTP, was isolated (Yeh et al., 1996; Miki et al., 2000). IRSp53 transforms WAVE2 into an active form that subsequently mobilizes the Arp2/3 complex and supplies monomeric actins via its VCA region (Takenawa and Miki, 2001; Suetsugu et al., 2002). These results show that WAVE isoforms convert the signals to the reorganization of the actin cytoskeleton in the peripheral region of growth cones. However, Ena/VASP

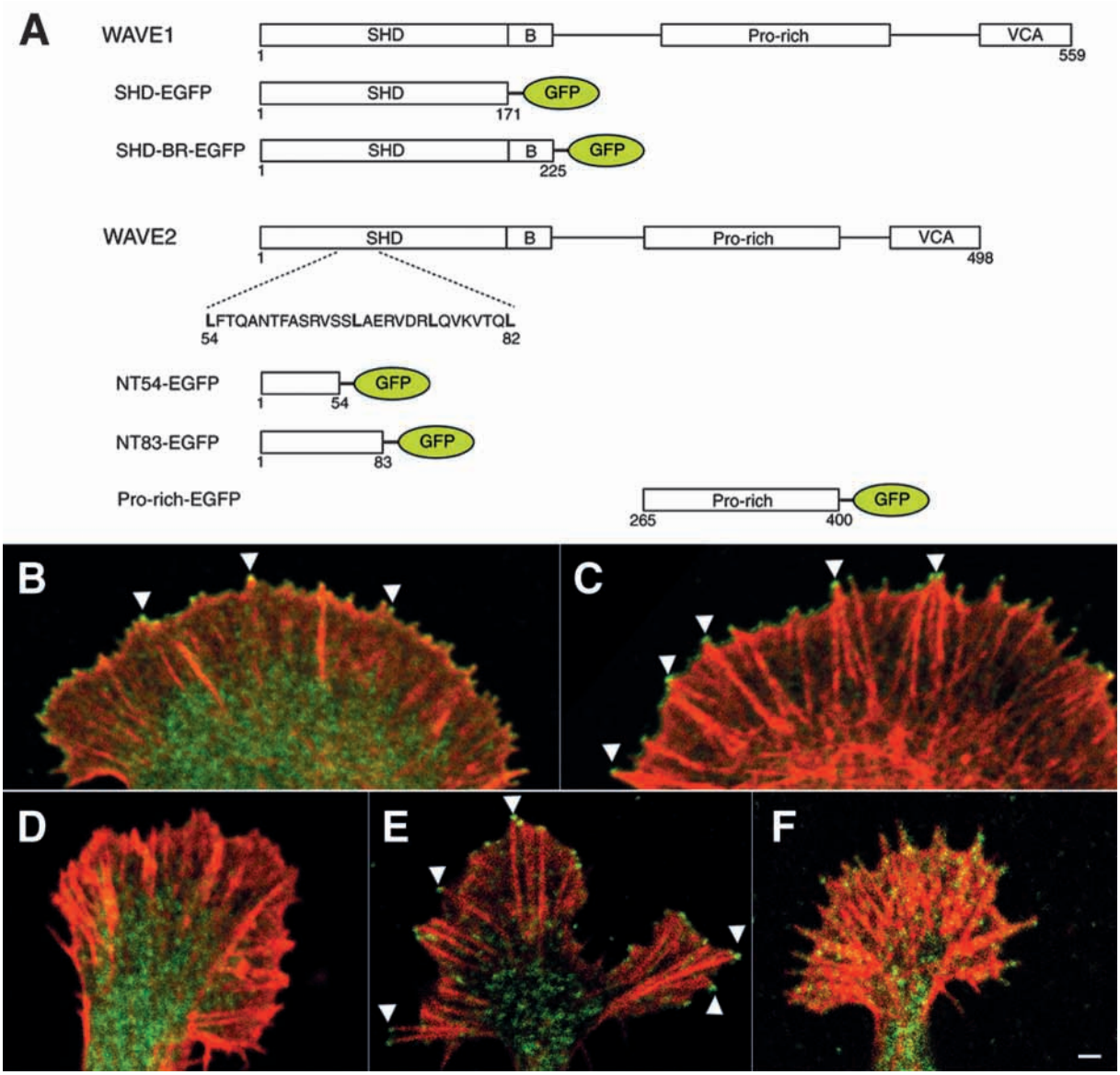

Fig. 6. Localization of the EGFP-tagged domain and region fragments of WAVE1 and WAVE2. (A) EGFP-tagged constructs of WAVE1 and WAVE2. SHD, B, NT54, NT83 and Pro-rich indicate SCAR homology domain, basic region, N-terminal 54-amino-acid fragment of WAVE2, N-terminal 83-amino-acid fragment of WAVE2 and proline-rich region, respectively. Numbers show the position of amino acids relative to the N-terminus. (B-F) Localization of the truncated fragments of WAVE1 and WAVE2 at neuronal growth cone. The growth cone was double stained with anti-GFP (green) and rhodamine-phalloidin (red). WAVE1 SHD (B) and SHD-BR (C) localized at the filopodial tips (arrowheads). NT83 fragments of WAVE2 SHD (E) localized at the filopodial tips (arrowheads), whereas NT54 fragments (D) did not. WAVE2 Pro-rich domain (F) distributed diffusely at growth cone. Bar, 1 μm.

(Enabled/vasodilator-stimulated phosphoprotein) family proteins have been reported to localize at the leading edge of lamellipodia and also at the filopodia tips (Nakagawa et al., 2001). VASP, localized to the leading edge of lamellipodia, correlates with actin filaments (Rottner et al., 1999). Additionally, Mena (mammalian Ena) has been reported to localize at filopodia tips (Lanier et al., 1999). However, more recently, Bear et al. have reported that Ena/VASP proteins are not always necessary for filopodia to protrude at the neuronal growth cone (Bear et al., 2002). Consequently, we think it is more reasonable that WAVE isoforms, whose localization at the growth cone was confirmed in this study, regulate the formation and extension of lamellipodia and filopodia. The evidence that WAVE2 is a ubiquitous protein and especially well expressed in neuronal cells (Suetsugu et al., 1999) also supports the hypothesis that WAVE2 is a major regulator of growth cone morphogenesis.

Formation and extension of filopodia by actin bundle reorganization

In the periphery of the growth cone, the distal ends of actin bundles (barbed ends) fuse with others at the site where WAVE2 is concentrated at the leading edge (Fig. 7A).

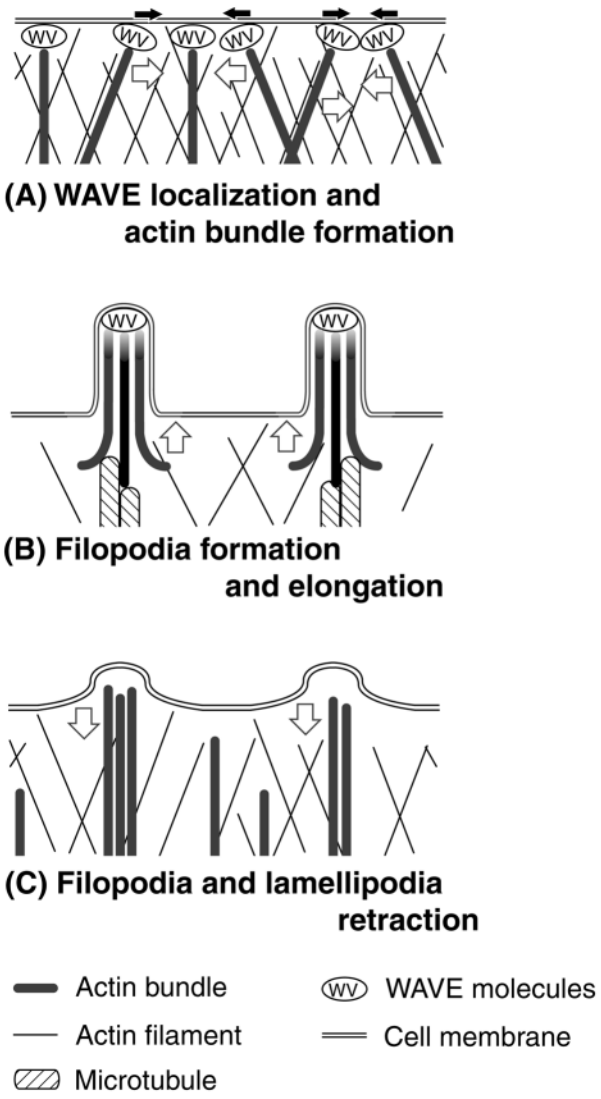

Fig. 7. Filopodial formation and elongation. (A) WAVE localizes at the distal ends of actin bundles. WAVE localized at the initiation site of filopodia and moved along the leading edge (small arrows) with the lateral motion of actin bundles through lamellipodium cytoplasm as indicated by large arrows. (B) Filopodia formation and elongation. Several actin bundles are fused at the WAVE-concentrated points and form the filopodium core. Actin polymerization via WAVE at the barbed ends elongates the microspikes and filopodia in the direction indicated by the arrows. Some proximal portions of actin bundle extend radially into the actin network and others reach the microtubule in the cytoplasm. (C) Filopodia and lamellipodia retraction. When WAVEs diffuse into the cytoplasm from the leading edge and filopodial tips, actin bundles are detached from the membrane, and then lamellipodia and filopodia retract as indicated by the arrows.

Therefore, the actin bundles with their tips at these sites are organized to constitute the core of microspikes. This is consistent with findings reported previously (Katoh et al., 1999b; Oldenbourg et al., 2000). Additionally, the WAVE-concentrated point becomes an emergent site of microspikes and protrudes outward nub-like (Fig. 7B). In the vicinity of the WAVE molecule, the actin-barbed ends interact loosely with the VCA region, and monomeric actins bind to the ends stochastically with Brownian motion of filaments. The filopodial portions of actin bundles are fastened by fascin (Cohan et al., 2001) and the proximal ends join the actin network and microtubule in the filopodial base in the lamellipodia cytoplasm (Schaefer et al., 2002). Therefore, they suppress the backward Brownian motion of the proximal portion of actin bundles and cause a ratchet effect on the whole bundle. Consequently, actin polymerization at the tips produces a mechanical force to drive extension in the forward direction as theoretically described (Mogilner and Oster, 1996; Theriot, 2000). G-actin molecules once polymerized in the bundle move towards the base by a retrograde flow (Kirschner, 1980; Watanabe and Mitchson, 2002). If the polymerization rate at the tip is faster than the velocity of retrograde flow, filopodia continue to elongate (Mallavarapu and Mitchson, 1999). Our observations show that clusters of WAVE isoforms remained at the filopodial tips during elongation. By contrast, when filopodia and lamellipodia retracted, the WAVE isoforms disappeared at the leading edge and tips (Fig. 7C). Recently, Steketee and Tosney proposed another working model of how filopodia emerge and elongate after the formation of a focal ring at the periphery of the veil (Steketee and Tosney, 2001; Steketee and Tosney, 2002). However, their model explains the emergence and extension of new filopodia only if mature filopodia already exist in the growth cone. By contrast, our model describes the case in which new filopodia simultaneously emerge and elongate from the growth cone of lamellipodia in response to guidance cues. In the latter case, our model is more reasonable. In the near future we will, hopefully, shed light on the detailed roles of profilin, Arp2/3 and WAVE isoforms at the filopodia tips.

We thank Haruhiro Higashida (Kanazawa University Graduate School of Medicine, Japan) for generously donating the NG108-15 cell line. This work was partly supported by the Japan Society for the Promotion of Science.

References

Bear, J. E., Svitkina, T. M., Krause, M., Schafer, D. A., Loureiro, J. J., Strasser, G. A., Maly, I. V., Chaga, O. Y., Cooper, J. A., Borisy, G. G. et al. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* **109**, 509-521.

Bentley, D. and O'Connor, T. P. (1994). Cytoskeletal events in growth cone steering. *Curr. Opin. Neurobiol.* **4**, 43-48.

Borisy, G. G. and Svitkina, T. M. (2000). Actin machinery: pushing the envelope. *Curr. Opin. Cell Biol.* **12**, 104-112.

Cohan, C. S., Welnhofer, E. A., Zhao, L., Matsumura, F. and Yamashiro, S. (2001). Role of the actin bundling protein fascin in growth cone morphogenesis: localization in filopodia and lamellipodia. *Cell Motil. Cytoskeleton* **48**, 109-120.

Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M. and Kirschner, M. W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* **418**, 790-793.

Furuya, S. and Furuya, K. (1983). Ultrastructural changes in differentiating neuroblastoma x glioma hybrid cells. *Tissue Cell* **15**, 903-919.

Govind, S., Kozma, R., Monfries, C., Lim, L. and Ahmed, S. (2001). Cdc42Hs facilitates cytoskeletal reorganization and neurite outgrowth by localizing the 58-kD insulin receptor substrate to filamentous actin. *J. Cell Biol.* **152**, 579-594.

Hahne, P., Sechi, A., Benesch, S. and Small, J. V. (2001). Scar/WAVE is localised at the tips of protruding lamellipodia in living cells. *FEBS Lett.* **492**, 215-220.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Heath, J. P. and Holifield, B. F. (1993). On the mechanisms of cortical actin

flow and its role in cytoskeletal organisation of fibroblasts. *Symp. Soc. Exp. Biol.* **47**, 35-56.

Katoh, K., Hammar, K., Smith, P. J. and Oldenbourg, R. (1999a). Birefringence imaging directly reveals architectural dynamics of filamentous actin in living growth cones. *Mol. Biol. Cell* **10**, 197-210.

Katoh, K., Hammar, K., Smith, P. J. and Oldenbourg, R. (1999b). Arrangement of radial actin bundles in the growth cone of Aplysia bag cell neurons shows the immediate past history of filopodial behavior. *Proc. Natl. Acad. Sci. USA* **96**, 7928-7931.

Kim, M. D., Kolodziej, P. and Chiba, A. (2002). Growth cone pathfinding and filopodial dynamics are mediated separately by Cdc42 activation. *J. Neurosci.* **22**, 1794-1806.

Kirschner, M. W. (1980). Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo. *J. Cell Biol.* **86**, 330-334.

Lanier, L. M. and Gertler, F. B. (2000). From Abl to actin: Abl tyrosine kinase and associated proteins in growth cone motility. *Curr. Opin. Neurobiol.* **10**, 80-87.

Lanier, L. M., Gates, M. A., Witke, W., Menzies, A. S., Wehman, A. M., Macklis, J. D., Kwiatkowski, D., Soriano, P. and Gertler, F. B. (1999). Mena is required for neurulation and commissure formation. *Neuron* **22**, 313-325.

Lewis, A. K. and Bridgman, P. C. (1992). Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. *J. Cell Biol.* **119**, 1219-1243.

Li, Z., Aizenman, C. D. and Cline, H. T. (2002). Regulation of rho GTPases by crosstalk and neuronal activity in vivo. *Neuron* **33**, 741-750.

Lommel, S., Benesch, S., Rottner, K., Franz, T., Wehland, J. and Kuhn, R. (2001). Actin pedestal formation by enteropathogenic *Escherichia coli* and intracellular motility of *Shigella flexneri* are abolished in N-WASP-defective cells. *EMBO Rep.* **2**, 850-870.

Mallavarapu, A. and Mitchison, T. (1999). Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. *J. Cell Biol.* **146**, 1097-1106.

Miki, H., Suetsugu, S. and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* **17**, 6932-6941.

Miki, H., Fukuda, M., Nishida, E. and Takenawa, T. (1999). Phosphorylation of WAVE downstream of mitogen-activated protein kinase signaling. *J. Biol. Chem.* **274**, 27605-27609.

Miki, H., Yamaguchi, H., Suetsugu, S. and Takenawa, T. (2000). IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* **408**, 732-735.

Mogilner, A. and Oster, G. (1996). Cell motility driven by actin polymerization. *Biophys. J.* **71**, 3030-3045.

Nakagawa, H., Yoshida, M. and Miyamoto, S. (2000). Nitric oxide underlies the differentiation of PC12 cells induced by depolarization with high KCl. *J. Biochem.* **127**, 113-119.

Nakagawa, H., Miki, H., Ito, M., Ohashi, K., Takenawa, T. and Miyamoto, S. (2001). N-WASP, WAVE and Mena play different roles in the organization of actin cytoskeleton in lamellipodia. *J. Cell Sci.* **114**, 1555-1565.

Nikolic, M. (2002). The role of Rho GTPases and associated kinases in regulating neurite outgrowth. *Int. J. Biochem. Cell Biol.* **34**, 731-745.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Oldenbourg, R., Katoh, K. and Danuser, G. (2000). Mechanism of lateral movement of filopodia and radial actin bundles across neuronal growth cones. *Biophys. J.* **78**, 1176-1182.

Pollard, T. D., Blanchoin, L. and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545-576.

Rottner, K., Behrendt, B., Small, J. V. and Wehland, J. (1999). VASP dynamics during lamellipodia protrusion. *Nat. Cell Biol.* **1**, 321-322.

Schaefer, A. W., Kabir, N. and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J. Cell Biol.* **158**, 139-152.

Small, J. V., Stradal, T., Vignal, E. and Rottner, K. (2002). The lamellipodium: where motility begins. *Trends Cell Biol.* **12**, 112-120.

Snapper, S. B., Takeshima, F., Anton, I., Liu, C. H., Thomas, S. M., Nguyen, D., Dudley, D., Fraser, H., Purich, D., Lopez-Ilasaca, M. et al. (2001). N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nat. Cell Biol.* **3**, 897-904.

Steketee, M. and Tosney, K. W. (2001). Filopodial initiation and a novel filament-organizing center, the focal ring. *Mol. Biol. Cell* **12**, 2378-2395.

Steketee, M. B. and Tosney, K. W. (2002). Three functionally distinct adhesions in filopodia: shaft adhesions control lamellar extension. *J. Neurosci.* **22**, 8071-8083.

Suetsugu, S., Miki, H. and Takenawa, T. (1999). Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem. Biophys. Res. Commun.* **260**, 296-302.

Suetsugu, S., Miki, H. and Takenawa, T. (2002). Spatial and temporal regulation of actin polymerization for cytoskeleton formation through Arp2/3 complex and WASP/WAVE proteins. *Cell Motil. Cytoskeleton* **51**, 113-122.

Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* **114**, 1801-1809.

Theriot, J. A. (2000). The polymerization motor. *Traffic* **1**, 19-28.

Theriot, J. A. and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature* **352**, 126-131.

Watanabe, N. and Mitchison, T. J. (2002). Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science* **295**, 1083-1086.

Yeh, T. C., Ogawa, W., Danielsen, A. G. and Roth, R. A. (1996). Characterization and cloning of a 58/53-kDa substrate of the insulin receptor tyrosine kinase. *J. Biol. Chem.* **271**, 2921-2928.