INTRODUCTION

The actin cytoskeleton is essential to eukaryotic cell movement. Fibroblastic cells attached to a substrate extend lamellipodia and filopodia in the direction of cell locomotion. Actin filaments are assembled into networks in lamellipodia and into radial bundles in filopodia (reviewed by Small et al., 1996). Polymerization of actin molecules near the cell membrane is thought to generate the force to move the membrane forward (Abraham et al., 1999; Bailly et al., 1999; reviewed by Borisy and Svitkina, 2000). Cdc42 and Rac, Rho-family small G-proteins, regulate the actin polymerization in filopodia and lamellipodia, respectively (Kozma et al., 1995; Nobes and Hall, 1995; Ridley et al., 1992; reviewed by Hall, 1998).

WASP, N-WASP and WAVE, of the Wiskott-Aldrich syndrome protein (WASP) family in mammalian organs, have been shown to be the downstream effectors of Cdc42 and Rac signal cascades (Aspenström et al., 1996; Kolluri et al., 1996; Miki et al., 1998a; Miki et al., 1998b; Syonyms et al., 1996). These proteins have common binding sites for actin, profilin and Arp2/3 complex (Egile et al., 1999; Machesky and Insall, 1998; Machesky et al., 1999; Miki et al., 1996; Miki et al., 1998b; Rohatgi et al., 1999; Suetsugu et al., 1998), but have unique amino acid sequences around their N-terminus. The C-terminal acidic domain of WASP family proteins stimulates the actin filament nucleation activity of Arp2/3 complex (Machesky and Insall, 1998; Rohatgi et al., 1999). WASP and N-WASP have a GBD/CRIB region for binding Cdc42, but WAVE does not (Miki et al., 1996; Miki et al., 1998b). The expression of WASP is restricted to hematopoietic cells (Derry et al., 1994), whereas N-WASP and WAVE are expressed ubiquitously in mammalian organs (Miki et al., 1996; Miki et al., 1998b). Recent studies have elucidated how Cdc42 induces filopodia to form in mammalian cells. The binding of active Cdc42 to the GBD/CRIB region of avian Ena, an avian homolog of Men, was localized to the lamellipodium edge and concentrated at the tip of microspikes. The SCAR homology domain (SHD) of human WAVE was distributed along the lamellipodium edge. These results indicate that N-WASP, WAVE and Men have different roles in the regulation of the cortical actin cytoskeleton in the protruding lamellipodium. WAVE and Men should be recruited to the lamellipodium through SHD and the EVH2 domain, respectively, to regulate the actin polymerization near the cell membrane. N-WASP should regulate the formation of the actin filament bundle in addition to activating Arp2/3 complex in lamellipodium under the control of Cdc42.

SUMMARY

WASP- and Ena/VASP-family proteins have been reported to regulate the cortical actin cytoskeleton as downstream effectors of the Rho-family small G-proteins Rac and Cdc42, but their functions are little understood. We observed the localization of the WASP family proteins, N-WASP and WAVE, and the Ena/VASP family protein, Men, in protruding lamellipodia. Rat fibroblast cell line 3Y1 protruded lamellipodia on poly-L-lysine-coated substrate without any trophic factor. N-WASP and Cdc42 were concentrated along the actin filament bundles of microspikes but not at the tips. By immunofluorescence and immuno-electron microscopy, both WAVE and Men were observed to localize at the lamellipodium edge. Interestingly, Men tended to concentrate at the microspike tips but WAVE did not. At the edge of the lamellipodium, the correlation between the fluorescence from Men and actin filaments stained with the specific antibody and rhodamine-phalloidin, respectively, was much higher than that between WAVE and actin filament. The Ena/VASP homology 2 (EVH2) domain of avian Ena, an avian homolog of Men, was localized to the lamellipodium edge and concentrated at the tip of microspikes. The SCAR homology domain (SHD) of human WAVE was distributed along the lamellipodium edge. These results indicate that N-WASP, WAVE and Men have different roles in the regulation of the cortical actin cytoskeleton in the protruding lamellipodium. WAVE and Men should be recruited to the lamellipodium through SHD and the EVH2 domain, respectively, to regulate the actin polymerization near the cell membrane. N-WASP should regulate the formation of the actin filament bundle in addition to activating Arp2/3 complex in lamellipodium under the control of Cdc42.

Key words: Actin cytoskeleton, WASP family, Ena/VASP family, Lamellipodia

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from yeast to humans, but also by the bacterial pathogen *Shigella flexneri* to produce an actin comet tail in the cytoplasm of infected cells (Égile et al., 1999). WAVE, a mammalian homolog of *Dictyostelium discoideum* Scar (Bear et al., 1998), has the same set of binding sites as other WASP family proteins, except for the small G-protein binding region (Miki et al., 1998b). The C-terminal acidic region of WAVE stimulates the actin filament nucleation activity of Arp2/3 complex in vitro but exogenous expression of this region disturbed Arp2/3 complex localization in cultured cells (Machesky and Insall, 1998; Machesky et al., 1999). Overexpression of WAVE induced ectopic actin polymerization and clustering (Miki et al., 1998b). The truncated form of WAVE inhibited Rac-mediated actin polymerization and membrane ruffling but not Cdc42-mediated filopodium extension (Miki et al., 1998b).

In addition, WAVE was colocalized with actin filaments to the lamellipodium (Miki et al., 1998b). These results suggest that WAVE regulates actin filament rearrangement downstream of Rac in the lamellipodium. The N-terminal SCAR homology domain (SHD) has been predicted to participate in the localization of WAVE (Miki et al., 1998b, Bear et al., 1998), but its function is still unclear.

Mammalian Ena/VASP family proteins, Evl, Mena and VASP (vasodilator-stimulated phosphoprotein), have been implicated in the temporal and spatial control of actin filament dynamics (Gertler et al., 1996; Reinhard et al., 1992; Rotnert et al., 1999). These proteins bind to actin, profilin and the proline-rich motif of ActA on the bacterial pathogen *Listeria monocytogenes*, and to the focal adhesion proteins vinculin and zyxin (Bachmann et al., 1999; Brindle et al., 1996; Chakraborty et al., 1995; Gertler et al., 1996; Reinhard et al., 1992; Reinhard et al., 1995a; Reinhard et al., 1995b; Reinhard et al., 1996). Ena/VASP family proteins play essential roles in the formation of the actin comet tail of *L. monocytogenes* in platelet and brain extracts (Laurent et al., 1999). A complex between ActA and Ena/VASP family protein regulates actin polymerization at the surface of the bacterium. The complex is considered to play the same role as N-WASP in *S. flexneri* (Laurent et al., 1999). Ena/VASP family proteins are expressed in various mammalian organs (Gertler et al., 1996; Lanier et al., 1999; Ohta et al., 1997; Reinhard et al., 1992). Mena and VASP are localized at focal adhesions and in cell surface protrusions that contain actin filaments (Gertler et al., 1996; Reinhard et al., 1992). Recently, Mena and VASP have been demonstrated to localize at the tips of protruding lamellipodia and filopodia (Rotnert et al., 1999). In addition, Mena has been observed to localize in filopodia of the neural growth cone and shown to play an important role in mouse neural development (Lanier et al., 1999). In Ena/VASP family proteins, three conserved regions are recognized, the Ena/VASP homology (EVH) 1 and 2 domains, and a central proline-rich region. All of these regions have been reported to participate in the subcellular localization of the protein. The EVH1 domain of Mena migrates to the focal adhesions and leading edges (Bear et al., 2000; Gertler et al., 1996). The EVH2 domain of VASP binds to actin filaments and mediates tetramerization of VASP molecules (Bachmann et al., 1999). In the neural tissue, the proline-rich region of Mena is linked to β-amylloid precursor protein through FE65 (Ermekova et al., 1997).

Previous results suggest that N-WASP, WAVE and Mena should be localized to the same area, such as the lamellipodium and filopodium, to regulate the dynamics of the actin cytoskeleton. Mena has been reported to localize at the tips, but the localization of WASP family proteins at that site has not been well documented. To elucidate the regulatory mechanism behind the actin dynamics, we compared their localization with various antibodies under a light and electron microscope. Then, to clarify the roles of WAVE and Mena in lamellipodia, we measured the correlation coefficient between actin filaments and these proteins. Finally, the domains of these proteins were expressed exogenously in mammalian cultured cells to determine which domains were required for the localization.

**MATERIALS AND METHODS**

**Antibodies and other materials**

Affinity purified polyclonal antibodies against N-WASP and WAVE were characterized as described previously (Miki et al., 1996; Miki et al., 1998b). Monoclonal antibodies against Mena (clone no. 21) and Rac1 (clone no. 102) were purchased from Transduction Laboratories Inc (KY, USA). Rabbit antiserum against green fluorescence protein was purchased from Molecular Probes (OR, USA). Monoclonal antibodies against vinculin (clone no. VIN-11-5) and talin (clone no. 84d4) and FITC-conjugated anti-mouse IgA goat IgG were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Rhodamine- or FITC-conjugated goat IgG fractions against mouse and rabbit IgG were obtained from Organon teknika Corp. (NC, USA). Gold particle-conjugated secondary antibodies against mouse IgA and rabbit IgG were provided by British BioCell International (Cardiff, UK). Dubelcco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Antibiotics, 0.01% poly-L-lysine solution and rhodamine-phalloidin were obtained from Sigma-Aldrich Japan K.K. A mammalian expression vector pEGFP-N1 was obtained from CLONETECH Laboratories Inc (CA, USA). GENETICIN was purchased from Life Technologies (MD, USA). TransIT-LT1 was provided by PanVera Corp. (WI, USA).

**Cell culture**

A rat embryonic fibroblast cell line 3Y1 was cultured as previously described (Nakagawa and Miyamoto, 1998). To induce lamellipodium, cells were cultured on poly-L-lysine-coated substrates. A glass coverslip and carbon-coated formvar film on a copper EM grid were incubated in a 0.01% poly-L-lysine solution for 30 minutes at 37°C and washed with PBS (137 mM NaCl, 2.7 mM Na₂HPO₄, 8.1 mM KCl and 1.5 mM KH₂PO₄) just before use. Cells were cultured on a glass coverslip from 1 to 15 hours for immunofluorescence microscopy, and on formvar film for 30 minutes for electron microscopy.

**Expression vectors and transfection**

The cDNAs encoding the Ena/VASP homology 1 (EVH1) domain (residues 1-113), EVH2 domain (residues 376-550) and proline-rich (PR) region (residues 294-358) of avian Ena (avEna) were amplified by PCR with avEna cDNA (accession no. AB017437). The cDNAs encoding the WAVE N-terminal (WNT) region (residues 1-225) and the SCAR homology domain (SHD) region (residues 1-171) were amplified by PCR with human WAVE1 cDNA (Miki et al., 1998). All 5’ primers contained a SacI restriction endonuclease site and all 3’ primers a BamHI endonuclease site. In the 5’ primers for the EVH2 domain and the proline-rich region, the ATG sequence for the first methionin was inserted between the SacI site and complementary sequences. Amplified products were subcloned into *SacI*-BamHI-
digested pEGFP-N1. In all constructs, the sequence of enhanced green fluorescence protein (EGFP) was fused to the 3′ terminus of the insert sequence.

For transfection experiments, 3Y1 cells were grown to 70% confluence in 35 mm dishes and transfected with 3 μg of each EGFP fused protein construct using Transit-LT1. Transfected cells were selected and maintained with 1 mg/ml Geneticine.

**SDS-PAGE and immunoblotting**

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

**Whole-mount electron microscopy**

Whole-mount cells were prepared according to a method previously described (Small and Sechi, 1998) and observed under a JEOL JEM-1200EX (JEOL, Tokyo, Japan).

**Immunofluorescence and immunoelectron microscopy**

The immunofluorescence microscopy was performed as previously described (Nakagawa et al., 2000). Cells on glass coverslips were fixed with 3% formaldehyde in PBS, permeabilized 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 immunofluorescence observations were carried out under the confocal laser scanning microscope, Zeiss LSM510 (Carl Zeiss Jena, GmbH., Jena, Germany).

Immunoelectron microscopy on whole-mount cells was performed according to a previously described method (Rottner et al., 1999). We could not use glutaraldehyde as a fixative, however, because it heavily reduced the antigenicity of each protein, even at 0.1% in solution. Cells were cultured on formvar film according to a previously described method (Small and Sechi, 1998), incubated in cytoskeleton buffer (CB; 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2 and 5 mM glucose, pH 6.1) containing 10 μg/ml phalloidin, 0.1% Triton X-100 and 3% formaldehyde for one minute, fixed with CB containing 10 μg/ml phalloidin and 3% formaldehyde for 30 minutes, washed with 5 mg/ml phalloidin in PBS, blocked at nonspecific binding sites with 10 mg/ml phalloidin, 1 mg/ml BSA and 100 mM glycine in PBS (blocking solution), reacted with primary antibody diluted in blocking solution, washed with 5 mg/ml phalloidin in PBS, reacted with 10 nm gold particle conjugated secondary antibody diluted in blocking solution, washed with 5 μg/ml phalloidin in PBS, and fixed with 2.5% glutaraldehyde and 5 μg/ml phalloidin in PBS. The fixed cells were washed with distilled water and negatively stained with 1% uranyl acetate solution. Using this protocol, we obtained a well-contrasted whole-mount specimen with uranyl acetate solution but not sodium phosphotungstic acid solution. Electron microscopic observation was carried out using a JEOL JEM-1200EX (JEOL, Tokyo, Japan).

**Measurement of fluorescence intensity**

We measured the fluorescence intensity (recorded as counts per pixel) at the tip of lamellipodia double-stained with rhodamine-phalloidin and primary antibody against WAVE or Mena followed by FITC conjugated secondary antibody. Measurements were taken from eight cells per immunostaining set. The number of data points was at least 587 per cell. The net fluorescence intensity of each pixel was obtained by subtracting the background fluorescence intensity from the raw data. A correlation coefficient of the fluorescence intensity between rhodamine and FITC was calculated for each cell.

**RESULTS**

**Protrusion of lamellipodia from 3Y1 cells**

We developed a simple method to induce lamellipodia in 3Y1 rat fibroblastic cells. Fifteen hours after plating on a plain glass surface, cells were well spread (Fig. 1a) and had developed many actin stress fibers of fibroblastic form (Fig. 1b). On the poly-L-lysine-coated glass, cells immediately attached to the surface and extended lamellipodia. Interestingly, almost all cells extended large lamellipodia during the 15 hour incubation (Fig. 1c). In these cells, actin filaments were distributed

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**Fig. 1. Lamellipodia formation was induced in 3Y1 cells on poly-L-lysine.** Cells adhered to a plain glass surface as a typical fibroblastic form within 15 hours of plating (a). Cells on the poly-L-lysine-coated glass surface extended large lamellipodia around their bodies (c). Cells in a and c observed in phase contrast are shown in b and d stained with rhodamine-phalloidin. Many stress fibers developed in cells on the plain glass (b). Most of the actin filaments were concentrated in lamellipodia and did not form stress fibers (d). Lamellipodia protruded from the cell body one hour after plating on poly-L-lysine (e), and the number of actin filament bundles increased during an incubation period of 15 hours (f). Both lamellipodia were stained with rhodamine-phalloidin. Under an electron microscope, an actin filament meshwork was observed over the lamellipodia (g). A high magnification view of the boxed area in g is shown in h. Actin filament bundles (arrows in h) extended from the microspike tip (h, arrow head) into the actin filament meshwork. Bar, 10 μm (d,f,g); Bar, 2 μm (h).
throughout the lamellipodium and many bundles of them, usually called microspikes, were observed but stress fibers were not (Fig. 1d). Within one hour of plating on the poly-L-lysine-coated surface, cells extended lamellipodia (Fig. 1e). Many thick microspikes developed in the lamellipodium during the 15 hour incubation (Fig. 1f). By electron microscopy, microspike bundles of actin filaments were observed that also extended as filopodia beyond the lamellipodium edge (Fig. 1h). When 3Y1 cells were cultured on poly-L-lysine for 15 hours, microspikes did not protrude as filopodia and lamellipodia were gradually retracted to cell bodies (data not shown). Swiss 3T3 fibroblasts have been reported to protrude large lamellipodia on a poly-L-lysine-coated glass surface (Machesky and Hall, 1997; reviewed by Small et al., 1999b). These results indicated that the lamellipodia of 3Y1 cells on poly-L-lysine were typical, even 15 hours after plating.

**Localization of N-WASP, WAVE and Mena in the protruding lamellipodia**

Using antibodies against N-WASP, WAVE and Mena, we identified their localization in lamellipodia. To clarify the specificity, we immunoblotted the SDS-whole extracts of 3Y1 cells with these antibodies. Antibodies against N-WASP reacted with a single band of 3Y1 cell SDS-whole extract (b). Anti-WAVE antibody was recognized as a doublet at around 78 kDa, as previously shown (Miki et al., 1999) (c). Anti-Mena antibody reacted with a single band at 80 kDa (d). Lane a shows a Commassie-stained polyvinylidene difluoride (PVDF) membrane to which the peptides separated in SDS-PAGE gel were transferred. On the left, the mobilities of relative molecular mass markers are listed (kDa).

whereas our method does not require this step (see the methods section). In the well-spread cells on the plain glass surface, vinculin was localized at focal adhesions but not along the stress fibers (Fig. 2a). By contrast, in the cells on the poly-L-lysine-coated glass, vinculin was distributed along microspikes (c) at microspike-associated contacts. Bar, 10 μm (b,d).

**Fig. 2.** Localization of cell-substrate adhesions. Cells were double-stained with anti-vinculin antibody (a,c) and rhodamine-phalloidin (b,d). Vinculin was localized to focal adhesions at both ends of stress fibers in the cells on plain glass (a). By contrast, in the cells on poly-L-lysine, vinculin was concentrated to the actin filament bundles of microspikes (c) at microspike-associated contacts. Bar, 10 μm (b,d).

**Fig. 3.** Immunoblotting analysis with anti-N-WASP, WAVE and Mena antibodies. Antibody against N-WASP reacted with a single band of 3Y1 cell SDS-whole extract (b). Anti-WAVE antibody was recognized as a doublet at around 78 kDa, as previously shown (Miki et al., 1999) (c). Anti-Mena antibody reacted with a single band at 80 kDa (d). Lane a shows a Commassie-stained polyvinylidene difluoride (PVDF) membrane to which the peptides separated in SDS-PAGE gel were transferred. On the left, the mobilities of relative molecular mass markers are listed (kDa).

**Fig. 4.** N-WASP and Cdc42 were localized to actin bundles in lamellipodia. Cells on poly-L-lysine were double-stained with rhodamine-phalloidin (b,d,f) and antibody against N-WASP (a,c) or Cdc42 (e). N-WASP was concentrated not only in the actin bundles of microspikes (c,d, large arrows), but also in thin bundles in lamellipodia (c,d, small arrows). Cdc42 showed a similar localization pattern to N-WASP. Bar, 5 μm (b,d,f).
These results suggest that N-WASP regulates actin filament bundling downstream of Cdc42 in lamellipodia. Both WAVE and Mena were localized along the edges of lamellipodia one hour after plating (Fig. 5a-d). Monoclonal antibody against Rac1 stained lamellipodia diffusely (Fig. 5e). Interestingly, in the cells plated for 15 hours, Mena seemed to concentrate at the tips of microspikes (Fig. 6a), whereas WAVE was localized along the edge of lamellipodia, as seen in the cells plated for one hour (Fig. 6e). In the polarized cells, antibodies against these proteins stained the lamellipodium edge but not the non-lamellipodium marginal regions (Fig. 6c,d,g,h, large arrows). Double-staining with antibodies against Mena and WAVE showed a clear difference in the localization of these proteins. Many fluorescence spots stained with anti-Mena antibody were

Fig. 5. Localization of Mena, WAVE and Rac in lamellipodia. Cells cultured on poly-L-lysine for one hour were double-stained with rhodamine-phalloidin (b,d,f) and antibody against Mena (a), WAVE (c) or Rac1(e). Mena and WAVE showed a similar localization along the edge of lamellipodia. Rac, however, distributed diffusely over lamellipodia. Bar, 10 μm (f).

Fig. 6. Localization of Mena and WAVE in lamellipodia. Cells were plated on poly-L-lysine for 15 hours and double-stained with rhodamine-phalloidin (b,d,f,h) and antibody against Mena (a,c) or WAVE (e,g). Both Mena and WAVE were distributed at the edges of lamellipodia (a,c,e,g). After 15 hours of plating, Mena tended to concentrate at the tip of microspikes (a,b, small arrows), but not WAVE. In polarized cells (c,d,g,h), both proteins were localized at the edge of lamellipodia but not non-lamellipodium cell membrane (large arrows). Double-staining with antibodies against Mena (i) and WAVE (j) showed a speckled distribution of Mena at the lamellipodium edge, where WAVE was localized continuously. Bar, 10 μm (h); Bar, 5 μm (j).
Fig. 7. Correlation coefficient between actin filaments and WAVE or Mena at the lamellipodium edge. To clarify the correlation of actin filaments with WAVE or Mena at the lamellipodium edge, using cells double-stained with rhodamine-phalloidin and antibody against WAVE or Mena, we measured the fluorescence intensities of rhodamine to actin filament and of FITC conjugated with secondary antibodies to each antigen. The values of the correlation coefficient between actin filament and WAVE or actin filament and Mena were $0.21 \pm 0.08$ and $0.42 \pm 0.08$ (mean ± s.e.m.), respectively. Scattered plots are shown (b,c) for the cell that shows the closest value to each mean correlation coefficient, these values are 0.22 and 0.45, respectively. The antigen of primary antibody used for immunostaining is indicated in the upper-left corner of the graph. Rhodamine and FITC show fluorescence for rhodamine-phalloidin and secondary antibody, respectively. All fluorescence intensities are normalized to the mean value.

Localized along the lamellipodium edge (Fig. 6i), whereas anti-WAVE antibody stained the same edge with a continuous fluorescence band (Fig. 6j). To elucidate the relation between actin filaments and WAVE or Mena, we measured the fluorescence intensity at the edge of the lamellipodia double-stained with rhodamine-phalloidin for actin filaments and antibody against WAVE or Mena, and calculated the correlation coefficient between the intensity of fluorescence from actin filaments and that from each antigen. The fluorescence intensities of the proteins stained with each antibody correlated with that of the actin filaments (Fig. 7). The correlation coefficient between Mena and actin filament was significantly higher, by about twofold, than that between actin filament and WAVE. This result suggests that these proteins play a different role in actin cytoskeleton dynamics at the edge of the lamellipodium.

Under an electron microscope, Mena has been observed to localize at the edge of the lamellipodium (Rottner et al., 1999). The localization of WASP family proteins, however, has not been observed at the electron microscopic level. To clarify the localization of N-WASP and WAVE in the lamellipodium, we observed whole mount cytoskeletons of 3Y1 cells by immunoelectron microscopy (Fig. 8). Cells adhered immediately to the poly-L-lysine-coated formvar film after plating, and lamellipodia protruded within 30 minutes (see Fig. 1g). In contrast to the cells on the poly-L-lysine-coated glass surface, cells maintained lamellipodia only for a few hours on the poly-L-lysine-coated formvar film. By the immunogold method with anti-N-WASP antibody, gold particles conjugated with a secondary antibody were found to be localized mainly to the actin filament antibody, gold particles conjugated with secondary antibody were found to be localized mainly to the actin filament bundles, although some gold particles were scattered in the actin filament meshwork (Fig. 8b,c). We confirmed that N-WASP was not concentrated at the tip of microspikes as observed by immunofluorescence microscopy (Fig. 4). We observed that WAVE was localized along the edge of the lamellipodium, and did not recognize a difference in the density of gold particles between the tips of microspikes and the edge of the lamellipodium (Fig. 8d). Previous reports showed Mena localized at the edge of the lamellipodium (Rottner et al., 1999) (Fig. 8e), whereas gold particles were clearly concentrated at the tip of microspikes (Fig. 8e, inset).

Localizations of EVH2 and SHD to the edge of the lamellipodium

To determine which domains of Mena and WAVE were required for the localization of these proteins to the edge of the lamellipodium, we expressed the three conserved domains of Ena/VASP family proteins and two N-terminal fragments of WAVE tagged with EGFP (Fig. 9). Because the fluorescence from these EGFP-tagged proteins was too weak to detect in living cells (data not shown), we observed the localizations of these proteins by immunofluorescence microscopy using anti-GFP antiserum. In the cells on a plane glass surface, EVH1- and EVH2-GFP were localized to the focal adhesions in the same manner as endogenous Mena (Fig. 10a,d,g). EVH1-GFP was distributed diffusely over the lamellipodium but EVH2-GFP was localized to the edge (Fig. 10e,h). EVH2-GFP was also concentrated at the tip of microspikes with thick actin bundles, similar to endogenous Mena (Fig. 10m). Essentially the same results were obtained with different antibodies (anti-Mena and anti-GFP). This indicates that the higher correlation between Mena and actin filament was not an artifact caused by the character of antibody. PR-GFP distributed mainly to nuclei and did not show any clear localization in the cytoplasm (Fig. 10j). In the cells on poly-L-lysine, PR-GFP seemed to be excluded from the lamellipodium (Fig. 10k). Interestingly, both WNT-GFP and SHD-GFP were concentrated at the cell membrane (Fig. 11d,g), whereas the endogenous WAVE was scattered over the cytoplasm of the cells on the plane glass surface (Fig. 11a). These N-terminal fragments were localized to the edge of the lamellipodium in the same manner as endogenous WAVE (Fig. 11b,e,h). The EVH2 domain and SHD were localized to a similar region of the lamellipodium, but no significant homology was found between their amino acid sequences using GENETYX-Mac software (data not shown).

DISCUSSION

The recent progress in the study of the motility machinery of parasitic bacteria, L. monocytogenes and S. flexneri, in the invaded cytoplasm has provided insight into eukaryotic cell movement. The bacterial cell body is propelled directly by the actin polymerization within the invaded cell cytoplasm (Tilney and Portnoy, 1989). The endogenous proteins of the invaded cell, such as N-WASP of the WASP family and several
Ena/VASP family proteins, play an essential role in these bacterial movements. The protrusion of lamellipodia and filopodia is thought to be driven by the same machinery responsible for these bacterial movements (reviewed in Borisy and Svitkina, 2000). Indeed, several proteins of these families, such as N-WASP, WAVE, Mena and VASP, have been reported to regulate the protrusion of filopodia and/or lamellipodia (Lanier et al., 1999; Miki et al., 1998a; Miki et al., 1998b;)

Fig. 8. Localization of N-WASP, WAVE and Mena in whole-mount lamellipodia. Cells on poly-L-lysine-coated formvar film were stained with various primary antibodies followed by 10 nm gold conjugated secondary antibody. At 30 minutes after plating, lamellipodia protruded from the cell body. In the control lamellipodium stained only with the secondary antibody mixture, a few gold particles were observed (a, arrows). Some gold particles were scattered in the lamellipodium stained with anti-N-WASP antibody (b, arrows), but most particles were localized to the actin filament bundles of microspikes (c, the enhanced image of the bracketed region in b; arrows indicate the gold particles on the actin filament bundle). In the lamellipodia stained with the antibodies against WAVE (d) or against Mena (e), gold particles were localized along the edges. The density of gold particles in the anti-WAVE-stained cells was indistinguishable between the tips of microspikes (d, inset, large arrow) and the edge of lamellipodia (d, inset, small arrows). In the anti-Mena stained cells, however, the gold particles were more concentrated at the tips of microspikes (e, inset, which shows an enhanced image of the microspike indicated by the large arrow) than the edge of lamellipodia (e, small arrows). Bars, 0.2 μm.

Fig. 9. A schematic representation of EGFP-tagged constructs of avian Ena (A) and WAVE1 (B). EVH, SHD, PR, B, V and A indicate Ena/VASP homology, SCAR homology domain, proline-rich, basic region, VPH domain, and acidic domain, respectively. WNT includes SHD and B of WAVE1. The identities of the amino acid sequence of EVH 1 and 2 domains, and the PR region of avian Ena against mouse Mena are shown in A.
However, it is still not clear how they function in these cellular processes. In this article, we present evidence that these proteins perform different functions in cell movement.

On a poly-L-lysine-coated substrate, serum-starved Swiss 3T3 cells protruded lamellipodia around the cell body following stimulation with PDGF (Machesky and Hall, 1997). Here, we described how large lamellipodia were induced from 3Y1 cells on poly-L-lysine, even when the cells were cultured in a serum-containing medium without any additional trophic factors. Our observation that both vinculin and talin were localized to the actin filament bundles of microspikes suggested that 3Y1 cells attached to the poly-L-lysine-coated substrate via the ventral side of the microspike. The activation of Rac was required for lamellipodia to protrude from Swiss 3T3 cells (Machesky and Hall, 1997), whereas Rac scattered over the lamellipodia of 3Y1 cells and did not concentrate at the edge. This result suggests two possibilities: first, that most of the Rac molecules remain inactive even in the protruding lamellipodium; and second, that the lamellipodia on poly-L-lysine are still in a premature stage. It is difficult to clarify these possibilities with the results of our present work. How the Rac cascade is activated by poly-L-lysine is still obscure. Our method to induce lamellipodia formation from 3Y1 cells is useful for uncovering this cascade. This is because the stability of the lamellipodia of 3Y1 cells favors cell biological manipulation, such as the microinjection of various proteins, cDNAs and drugs that induce rearrangement of the cytoskeleton.

Overexpression of N-WASP has been reported to enhance the Cdc42-induced formation of filopodia in COS-7 cells (Miki et al., 1996; Miki et al., 1998a). On the surface of S. flexneri, N-WASP binds the bacterial protein IcsA to promote the elongation of actin filaments (Egile et al., 1999). Consequently, N-WASP has been expected to localize at the tip of microspikes in lamellipodia (reviewed by Borisy and Svitkina, 2000). We previously showed that N-WASP was concentrated in the membrane ruffles but did not proceed further, because the
membrane ruffle is a highly complicated structure containing lamellipodia and filopodia (Miki et al., 1996). By the method described in this article, however, we observed that N-WASP was localized along the actin bundles of microspikes but was not concentrated at the microspike tip. Cdc42 was localized along microspikes in the same manner as N-WASP. This remarkable result suggests that N-WASP regulates the bundling of actin filaments, in addition to the activation of Arp2/3 complex, downstream of Cdc42 in the lamellipodium. N-WASP should bundle up actin filaments into microspikes through the F-actin binding site at its N-terminus (Egile et al., 1999) and elongate them so that they protrude as filopodia as previously reported (Miki et al., 1998a; Rohatgi et al., 1999).

We previously reported that WAVE was localized to lamellipodia induced to form by Rac activation (Miki et al., 1998b). Mena has been reported to localize in filopodia at the neural growth cone and at the edge of lamellipodia in addition to the focal adhesions of B16 melanoma cells (Lanier et al., 1999; Rottner et al., 1999). To clarify the function of these proteins in lamellipodium protrusion, we immunostained the protruding lamellipodia of 3Y1 cells with antibodies against these proteins. Both proteins showed a similar localization along the edge of the lamellipodium one hour after the plating. Interestingly, at 15 hours, Mena concentrated at the microspike tips but WAVE did not. These results suggested that the localization of each of these proteins was regulated by different molecular mechanisms. To confirm this, we expressed the EGFP-tagged constructs.

Only the EGFP-tagged EVH2 domain among the three conserved regions of Ena/VASP family proteins was localized along the lamellipodium edge and concentrated at the microspike tip similarly to endogenous Mena. This result suggests that, in the lamellipodium, the localization of Ena/VASP family proteins is regulated through the EVH2 domain. By contrast, it has been reported that the EVH1 domain migrated to the leading edge (Bear et al., 2000). Judging from the figures presented in their article, the lamellipodia were less developed and it was difficult to observe fine structures. The EVH1 domain might have been localized to focal complexes aligned near the lamellipodium edge, similar to EGFP-tagged VASP (Rottner et al., 1999). Under our experimental conditions, no focal adhesion or complex developed in the lamellipodium (Fig. 2). Because the EVH2 domain of VASP composes a homotetramer (Bachmann et al., 1999), we cannot rule out the possibility that EVH2-GFP is localized at these edges through the binding with the EVH2 domain of endogenous Mena. The finding that neither EVH1-GFP nor PR-GFP is localized to the lamellipodium edge strongly indicates the important role of the EVH2 domain in the regulation of the subcellular localization of Ena/VASP family proteins.

Interestingly, in the cells on the plane glass surface, the EGFP-tagged SHD of WAVE migrated to the cell membrane, in contrast to the endogenous WAVE, which showed a diffuse distribution. SHD-GFP was also localized to the edge of the lamellipodium. These results show that SHD has cell membrane localization activity and this activity of a whole WAVE molecule is suppressed in the non-lamellipodium region. When the Rac cascade is activated, SHD might be released from inhibition and the WAVE molecule might migrate to the edge of the lamellipodium through it. Because SHD contains the putative leucine zipper motif (Miki et al., 1998b), WAVE might bind to some unidentified membrane protein(s) with this motif. The low homology of the EVH2 domain and SHD amino acid sequences suggests that the localization of Mena and WAVE is regulated by different mechanisms. This possibility is confirmed by the result that only Mena is localized at focal adhesions.

What are the functions of Mena and WAVE in the protruding lamellipodium? The key here is the higher correlation between actin filaments and Mena, than between actin filaments and WAVE. The fast growing ends of actin filaments face the edge of the lamellipodium (Small et al., 1978), so we propose the hypothesis that Mena would modulate actin filament polymerization directly and WAVE functions indirectly. This hypothesis is strongly supported by the observation that the density of Mena at the tips of lamellipodia closely correlates with the rate of protrusion (Rottner et al., 1999). How do these proteins regulate actin filament polymerization? We present a tentative model of actin filament polymerization at the edge of the lamellipodium (Fig. 12). In the protruding lamellipodium, the GTP-bound form of Rac activates WAVE to migrate to the edge through SHD. Subsequently, WAVE stimulates Arp2/3 complex to promote actin filament nucleation (Machesky et al., 1999). Mena should be recruited around the fast growing ends of actin filaments through the EVH2 domain and supply actin molecules from profilin-actin complexes to the fast-growing ends of actin filaments branched by Arp2/3 complex. When Cdc42 is activated in the lamellipodium, N-WASP induces the bundling of actin filaments to form microspikes.

Fig. 12. Schematic model of actin polymerization in the lamellipodium. The GTP-bound form of Rac stimulates WAVE to localize at the lamellipodium edge through SHD; subsequently, WAVE induces Arp2/3 complex to promote actin filament nucleation near the plasma membrane. Mena and/or VASP are recruited to the cell membrane via their EVH2 domain and supply actin molecules from profilin-actin complexes to the fast-growing ends of actin filaments branched by Arp2/3 complex. When Cdc42 is activated in the lamellipodium, N-WASP induces the bundling of actin filaments to form microspikes.

Localization of WASP and Ena/VASP families

![Localization of WASP and Ena/VASP families](image)
activity might be regulated by Cdc42 in the protruding lamellipodia as reported (Nobes and Hall, 1995).

Recently, the movement of endocytic vesicles was shown to be associated with an actin comet tail similar to that of parasitic bacteria, and N-WASP was recruited to the surface of each moving vesicle (Rozelle et al., 2000; Taunton et al., 2000). These observations indicate that an actin comet tail is a necessary physiological structure for cytoplasmic vesicle movement not an aberrant one. Ena/VASP family proteins might regulate the formation of the actin comet tail in other vesicle species. An analysis of actin dynamics in the comet tail should provide important information on the functions of these proteins. This information, however, could not be applied directly to the lamellipodium protrusion, because the role of protein is greatly dependent on its subcellular localization. For instance, as described in this article, the localization of N-WASP along the actin filament bundles of microspikes is unexpected given S. flexneri’s comet tail. N-WASP should play a different role in the lamellipodium from that in the actin comet tail. This study is the first attempt to compare the localization of WASP and Ena/VASP family proteins in the lamellipodium, and our results shed light on the roles of these proteins in cell membrane protrusion.

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