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RELATIVE ACTIN NUCLEATION PROMOTION EFFICIENCY BY WASP AND WAVE PROTEINS IN ENDOTHELIAL CELLS

Hyeran Kang^a, Jingjing Wang^b, Sarah J. Longley^c, Jay X. Tang^a, and Sunil K. Shaw^c

^aDepartment of Physics, Brown University, Providence, RI 02912, USA

^bGraduate Program of Biomedical Engineering, Brown University, Providence, RI 02912, USA

^cDepartment of Pediatrics, Kilguss Research Laboratories, Women and Infants Hospital and Brown University, Providence, RI 02905, USA

Abstract

The mammalian genome encodes multiple WASP1 (Wiskott-Aldrich Syndrome Protein)/WAVE (WASP-family Verprolin homologous) proteins. Members of this family interact with the Arp (actin related protein) 2/3 complex to promote growth of a branched actin network near the plasma membrane or the surface of moving cargos. Arp2/3 mediated branching can further lead to formation of comet tails (actin rockets). Despite their similar domain structure, different WASP/WAVE family members fulfill unique functions that depend on their subcellular location and activity levels. We measured the relative efficiency of actin nucleation promotion of full length WASP/WAVE proteins in a cytoplasmic extract from primary human umbilical vein endothelial cells (HUVEC). In this assay WAVE2 and WAVE3 complexes showed higher nucleation efficiency than WAVE1 and N-WASP, indicating distinct cellular controls for different family members. Previously, WASP and N-WASP were the only members that were known to stimulate comet formation. We observed that in addition to N-WASP, WAVE3 also induced short actin tails, and the other WAVES induced formation of asymmetric actin shells. Differences in shape and structure of actin-based growth may reflect varying ability of WASP/WAVE proteins to break symmetry of the actin shell, possibly by differential recruitment of actin bundling or severing (pruning or debranching) factors.

Keywords

polymerizing activity; actin based motility; actin comet tails; HUVEC

¹Abbreviations: WASP, Wiskott-Aldrich Syndrome Protein; WAVE, WASP-family Verprolin homologous protein; Arp, Actin related protein; HUVEC, human umbilical vein endothelial cells; VASP, vasodilator stimulated phosphoprotein; VCA Verprolin homology, Central and Acidic domain; GBD, GTPase binding domain

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Address correspondence to: Sunil Shaw, Dept of Pediatrics, Women and Infants Hospital of Rhode Island, Kilguss-210, 101 Dudley Street, Providence, RI 02905. Fax 401-277-3617; Sunil_Shaw@brown.edu; phone 401-274-1122 x8005.

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Research Highlights

- Nucleation promotion of GFP tagged WASP/WAVE proteins was measured in HUVEC extract
- WAVE2 and WAVE3 stimulated actin assembly more strongly than WAVE1 and N-WASP
- All 4 proteins induced asymmetric actin clouds and WAVE3 even short comet tails

Introduction

The actin cytoskeleton is essential for cellular locomotion, phagocytosis, intracellular transport and morphogenesis, and is comprised of dynamic networks of actin filaments that can undergo rapid assembly and disassembly. This process is tightly controlled by a variety of regulatory proteins including nucleation factors such as the Arp 2/3 complex, formins and Spire, and also stabilizing or filament severing agents such as ADF (actin depolymerizing factor)/cofilin, gelsolin, capping protein and VASP (vasodilator stimulated phosphoprotein)[1-4]. The Arp 2/3 complex stimulates actin nucleation by binding to a preexisting filament and inducing formation of branched daughter filaments at a characteristic 70° angle. The actin nucleating activity of Arp 2/3 is weak by itself, but it can be stimulated significantly with the help of nucleation promoting factors (NPFs) such as WASP and WAVE proteins [5,6].

Mammalian WASP family proteins include WASP and N- (neuronal-) WASP, and WAVE family proteins consist of WAVE1, WAVE2, and WAVE3. WASH (WASP and SCAR homolog) and WHAMM (WASP homolog associated with actin, Golgi membranes and microtubules) are more distantly related members that have been recently described, but currently relatively little is known about them [7-9]. Both WASP and WAVE family proteins share a common carboxy-terminal VCA (Verprolin-homology, central and acidic) domain, which directly binds and activates Arp 2/3 to stimulate branched actin nucleation. In the case of WASP family proteins, the VCA domain is masked by its interaction with the GTPase binding domain (GBD), resulting in an autoinhibited structure. WASP family proteins are activated by binding to signaling molecules like the acidic phospholipid PIP₂ and small Rho GTPase Cdc42 [10]. In contrast to the autoinhibited WASP family proteins, WAVE family proteins are not autoinhibited [11], but they normally exist as part of a pentameric complex making the VCA domain unavailable for binding Arp2/3. The WAVE pentamer needs activation by membrane bound Rac and acidic phospholipids [12-14] which frees the VCA domain. In addition, WASP and WAVE proteins are regulated by oligomerization, indicating yet another layer of control [15].

Listeria and *Shigella* bacteria can bypass mammalian NPFs and instead use their own proteins to directly stimulate Arp2/3, leading to formation of long actin tails (rockets or comets) which serve to propel the organism through the cytoplasm [16]. *In vitro*, comet activity can be reconstituted by N-WASP coated microspheres which result in actin comet tails in cell-free extracts and also in defined mixtures of purified proteins. Both WASP and N-WASP and their active VCA domains are known to stimulate growth of actin comets, but WAVE proteins have not been previously shown to induce this activity.

Previous studies have examined the relative ability of isolated VCA domains from mammalian WASP, N-WASP and WAVE1 to nucleate actin filament formation in *Acanthamoeba* extract [17]. However the nucleation ability of full length proteins was not addressed, and there was possibility for a mismatch between mammalian WASP/WAVE proteins and the *Acanthamoeba* actin machinery. In this report, we used full length GFP tagged N-WASP and WAVE family proteins captured on beads to quantify their efficiencies in actin nucleating activities as well as the actin comet tail formations in a mammalian cell extract from primary HUVEC (Human Umbilical Vein Endothelial Cells). From quantitative analyses, we show that in the same cytoplasmic milieu, WAVE1, 2, 3 and N-WASP have different nucleation abilities. In HUVEC extract WAVE2 and WAVE3 had higher nucleation efficiency, and WAVE1 and N-WASP were significantly lower. Moreover, we demonstrate that like WASP and N-WASP, WAVE family proteins can also induce actin tail formation in HUVEC extract under the right test conditions. The implications of these observations are discussed following the presentation of our experimental data.

Materials and Methods

HUVEC extract preparation

HUVEC (from Lonza, Gaithersburg, MD) were grown in EGM-2 medium for up to subculture 5. To prepare extracts, confluent HUVEC monolayers (grown on 100 mm petri plates) were washed twice with DPBS⁻ (Dulbecco's Phosphate Buffered Saline without Ca²⁺ or Mg²⁺, BioWhittaker, Walkersville, MD) and then incubated in 100 μ l cold lysis buffer (10 mM Tris-HCl, pH 7.5, 20 mM EGTA, and 2 mM MgCl₂) supplemented with 1mM PMSF and protease inhibitors cocktail (Sigma, Saint Louis, Missouri). Cells were scraped off the plate and collected in a microfuge tube, followed by sonication (with cooling on ice). Lysates were clarified by centrifugation at 14,000 rpm for 10 min at T = 4 °C. Supernatants were supplemented with 1 mM ATP, 1 mM DTT, and 150 mM sucrose.

Production of GFP-fusion proteins

cDNAs for full length GFP-N-WASP [18], GFP-WAVE1 and GFP-WAVE3 [19], GFP-WAVE2 [20] and WAVE2-GFP [21] were transferred to a recombinant adenovirus vector and used to infect HUVEC as previously described [22]. Monolayers were lysed by sonication as described above. Protein A/G beads (~40 μ m diameter, Thermo Scientific, Waltham, MA) were coated with Anti-GFP antibody (Neuromab, Davis, California) by incubation for 1 hr, and washed twice to remove unbound antibody with Hepes buffer (10 mM HEPES at pH 7.8, 0.1 M KCl, 1 mM MgCl₂, 1 mM ATP, 0.1 mM CaCl₂, and 1 mM DTT). The anti-GFP antibody-coated beads were put into HUVEC lysate containing GFP-WAVE proteins to adsorb fusion proteins onto their surface, and washed again to remove unbound proteins.

Actin nucleation assay

The GFP-WAVE coated beads were mixed with HUVEC extract supplemented with 2.5 μ M rhodamine-labeled G-actin (Cytoskeleton, Denver, CO) and ATP regenerating mix (containing 20 mM ATP, 100 mM creatine phosphate disodium salt, and 35 U/ml creatine kinase). A 6 μ l aliquot sample was sandwiched between a glass slide and coverslip and incubated for 30 min at room temperature (22 °C) before microscopy observation. A schematic diagram of the actin nucleation assay is shown in Figure 1b.

Actin comet assay

Carboxylated polystyrene beads (1 μ m diameter, Polysciences, Warrington, PA) were coated with Anti-GFP antibody followed by GFP-WAVE1, -2, -3 and N-WASP coating in the same way used for the actin nucleation assay. Briefly, 10 μ l HUVEC extract supplemented with 5 μ M rhodamine-labeled G-actin was mixed with 1 μ l of 10x ATP regenerating mix, and 2 μ l GFP-WAVE1, -2, -3 or GFP-N-WASP coated beads. A sample of 2 μ l aliquot was taken from the mixed motility assay and immediately sandwiched between a BSA-coated glass slide and coverslip, sealed with vacuum grease and incubated at room temperature overnight before microscopy observation.

Microscopy imaging and quantitative analysis

A Nikon TE2000E inverted microscope was used for fluorescence imaging with either a 20x Plan Apo lens (NA = 0.75) or a 100x Plan Apo oil lens (NA = 1.40) and a cooled CCD camera (Roper Scientific Coolsnap HQ). The integrated intensity was measured as the total gray scale value of green or red channel fluorescence collected after excluding background intensity using Metamorph (v6.0r1) software. A rectangular image was taken of the bead and its surroundings, and the integrated fluorescence intensity was calculated as the difference between the average gray value of the area and the average background gray value, multiplied by the number of pixels in the area.

RESULTS

Actin nucleation assay

We overexpressed GFP-tagged full-length N-WASP and WAVE proteins in HUVEC using an adenovirus vector (WASP was not included as this member is restricted to hematopoietic cells and is not expressed in endothelial cells [23]). In Figure 1A, the GFP-tagged full length proteins are identified by Western blot analysis. To immobilize the fusion proteins, anti-GFP antibody was coupled to 40 μ m diameter beads, and used to capture GFP-tagged WASP/WAVE proteins from HUVEC lysates. To initiate actin nucleation the beads were washed to remove non-bound proteins, and then incubated with HUVEC cytoplasmic extract with additional rhodamine labeled actin monomers for 30 minutes at room temperature as illustrated in the schematic diagram (Figure 1B). Beads were imaged using fluorescence microscopy, where captured GFP-tagged WASP/WAVE was visualized in the green channel, and polymerized actin filaments in the red channel. First, we compared the nucleation promotion ability of GFP-WAVE2 and WAVE2-GFP. In this pair of constructs GFP-WAVE2 stands for GFP tagged at the N-terminus of WAVE2, and WAVE2-GFP for GFP tagged after the C-terminal VCA domain (Figure 2A). We reasoned that the bulky GFP tag (or its attachment to the bead via an antibody linker) might inhibit WAVE2-GFP activation of Arp2/3 by steric hindrance of the VCA domain.

Representative fluorescence images of WAVE2-GFP beads and GFP-WAVE2 beads showed that both fusion proteins were captured onto the beads with roughly equal efficiency (Figure 2B, green channel). While GFP-WAVE2 beads showed strong actin polymerization around them (red channel), WAVE2-GFP beads were negative in this activity. This result demonstrates that full-length WAVE proteins fused to GFP can promote actin nucleation as long as the VCA domain is free to interact with Arp2/3. In contrast, WAVE2-GFP was inactive even with essentially the same molecular mass and composition as GFP-WAVE2, likely because of the non-availability of the VCA domain.

For quantitative analysis, we measured the integrated fluorescence intensity of both GFP and rhodamine-actin for multiple beads ($n=30$ for GFP-WAVE2, $n=27$ for WAVE2-GFP) as shown in Figure 2C. We observed a proportionality of signals between the green (GFP) channel and the red (actin) channel, indicating that beads with the highest level of captured GFP-WAVE2 protein consequently showed the highest levels of actin polymerization. The inset slope indicates the ratio between polymerized rhodamine-actin and GFP tagged WAVE proteins associated with the beads, and is a measure of the strength of actin nucleation per fluorescently labeled WAVE protein, enabling comparison of the actin nucleation of different WAVE fusion proteins. From the measured slopes (GFP-WAVE2 = 38.9; WAVE2-GFP = 2.0), we show the nucleation activity of GFP-WAVE2 beads is 20 times stronger than that of WAVE2-GFP beads. These results demonstrate specificity of actin nucleation induced by GFP-WAVE2 but not WAVE2-GFP.

Comparison of full-length proteins in actin-nucleating activity

We used the in vitro actin nucleation assay described above to compare the relative efficiency of different members of the WASP/WAVE family. In each case, we used full length proteins with a GFP tag at the N-terminus to leave the C-terminal VCA domain accessible to the Arp2/3 complex and actin. Comparing the slopes obtained from Figure 3, we observed significant differences in nucleation promotion activity among different GFP-WAVE proteins and GFP-N-WASP. GFP-WAVE2 and GFP-WAVE3 have relatively higher actin nucleation efficiency than GFP-WAVE1 and GFP-N-WASP in resting (unstimulated) HUVEC extract. We expect that the values we obtain here may change in different cell types or under different stimulatory conditions, although we have not tested this in the present study.

WAVE proteins can form actin comet tails

WASP and N-WASP have been shown to induce long actin comet tails *in vitro* and *in vivo* [24,25]. We sought to investigate whether WAVE proteins could do the same. To facilitate the formation of comet tails, we modified the actin nucleation assay as described above, using smaller beads (1 μ m diameter) to allow for easier breaking of symmetry of the resulting actin shells, as shown previously using VCA-coated beads [26]. We also allowed longer incubation times ($t = 16$ hrs) for greater actin polymerization. We observed that, like N-WASP, WAVE proteins also form asymmetric actin clusters and even short comet tails in HUVEC extract (Figure 4A). The average actin cluster length along the longest axis as well as the corresponding integrated fluorescence intensity of both GFP and rhodamine-actin varied between WAVE1, -2, -3 and N-WASP (Figure 4B and 4C).

WAVE2 was not an efficient activator as measured by tail length in these comet assays, in contrast to its potency demonstrated in the short term actin nucleation activity shown in Figure 3. We speculate that this may reflect instability of the WAVE2 complex due to proteolysis or changes in phosphorylation, or may be due to limiting amounts of a critical unique cofactor that is not replenished in the cytosolic extract. Instead, WAVE3 and N-WASP beads induced the longest actin tails among all the full length proteins and WAVE2 induced similar lengths of actin tails to that of WAVE1 in the actin motility assay (Figure 4B). This trend is consistent with that of average integrated fluorescence intensity measured for WAVE1, -2, -3 and N-WASP (Figure 4C).

DISCUSSION

Although WASP and WAVE proteins all possess a VCA domain for activating Arp2/3, these domains function with different efficiency. In the current study, we used the approach of overexpressing full length tagged WASP/WAVE proteins in endothelial cells, and purifying the complex by means of the attached GFP tag. We then observed their actin nucleation ability in a cytoplasmic extract where regulatory factors were freely available to stimulate or inhibit their activity. This approach allowed us to measure actin polymerization and relate it to the amount of GFP-tagged WASP/WAVE protein present.

We observed that WAVE2 and WAVE3 complexes led to greater actin nucleation than did WAVE1 (or N-WASP). The activity of full length N-WASP was comparable to that of full length WAVE1, with both proteins showing relatively weak nucleating activity. There are several potential biochemical explanations for these results. We speculate that the full length N-WASP may remain in an autoinhibited state [10], due to the lack of signaling molecules such as active cdc42 or PIP₂ in the unstimulated HUVEC extract. In contrast to N-WASP, WAVE proteins are known to exist in a pentamer with Abi, HSPC300, Nap and Sra. From biochemical analyses in this system (not shown), GFP-WAVE proteins are overexpressed by 5-10 fold as compared to endogenous levels. However, whether the overexpressed GFP-WAVE is fully complexed in WAVE pentamers is not known. It is possible that the different activities of GFP-WAVES may reflect differing degrees of WAVE pentamer formation. For instance, WAVE1 may have higher propensity towards forming pentamers, leading to its lower activity as compared to WAVE2 or WAVE3. As a second possibility, denaturation of the WAVE complex during isolation may lead to the exposure of the VCA domain and its subsequent activity of actin polymerization [14], and WAVE proteins are differently susceptible to denaturation. Alternatively as a third possibility, phosphorylation differences may account for the variability between WAVE proteins or that specific cofactors are required for activation of the different WAVE complexes. Lastly, it is possible that despite similar overall structure, different WAVE proteins may respond differently to N-terminal GFP tags. Since we used the GFP-tag to both capture target proteins and to measure their amount, this

represents a weakness inherent to our approach. Nevertheless, the present work is the first report to assess relative activity of multiple full-length WAVE proteins in a mammalian system.

Zalevsky *et al.* compared the nucleation promotion efficiency of isolated VCA domains from N-WASP, WASP and WAVE1 as Arp2/3 complex activators [17]. They reported that the activities of VCA domains of N-WASP and WASP are up to ~70 fold higher than that of WAVE1 in a protozoan extract. Besides variability in their VCA domains, WASP/WAVE proteins are also regulated by associated factors such as cdc42, Rac, WIP and PIP3 [6]. Further regulation of actin nucleation activity may also occur via post-translational modifications such as phosphorylation [6].

Numerous stimuli are known to induce actin polymerization in cultured endothelial cells, and to cause changes in endothelial barrier function *in vitro* and *in vivo* [27]. It will be of interest to test whether GFP-WASP/WAVE complexes isolated from stimulated HUVEC will result in altered actin nucleation efficiency in comparison with that from unstimulated HUVEC. Such a difference would indicate that the activities that we measured are not static but may be altered by factors such as activating molecules or differential post-translational modifications.

WAVE proteins are generally thought to be required for lamellipodium and ruffle formation in cells [28]. In contrast, WASP family proteins are crucial in filopodia formation. While it has also been shown that WASP and N-WASP produce bacterial motility by actin comet tail formation in the pathogen infection of gram-negative bacteria such as *Shigella* [29], evidence for WAVE proteins playing a potential role in this function is lacking. Here we demonstrate that the WAVE family proteins can generate actin comet tails *in vitro* like the WASP family proteins. Our results suggest that it may be generalized that intracellular aggregation of any WASP/WAVE family proteins can potentially lead to asymmetric actin shells or comet formation in cells, as has been previously demonstrated only for WASP and N-WASP, although *in vivo* examples of WAVE mediated comet formation have not been reported at this time.

CONCLUSIONS

We measured the relative activities of full length WAVE proteins in an endothelial cytosolic extract, and observed that WAVE2 and 3 had the highest activities, and WAVE1 and N-WASP the lowest, indicating specific cellular control mechanisms. Although N-WASP is well known to form actin tails *in vitro*, we also observed that WAVE proteins also had this ability to varying degrees, with WAVE3 inducing short tails. This method allows measurement of relative activity of WASP/WAVE complexes in a mammalian background, which may be useful in studying cell signaling pathways that regulate branching actin polymerization.

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Figure 1.

(A) Western blot results of GFP-WASP/WAVE proteins used in this study. HUVEC over-expressing GFP fusion proteins were lysed and resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-GFP antibody. Control lane (ctrl) shows the absence of endogenous GFP in HUVEC. GFP-N-WASP, GFP-WAVE1, GFP-WAVE2, WAVE2-GFP and GFP-WAVE3 all migrated at approximately 110 kDa. Densitometry indicated that approximately over 80% of each expression protein was full length, with minor proteolytic fragments present in some cases. Molecular mass markers are indicated on the right (kDa). (B) Schematic diagram of actin nucleation assay. Protein A/G Beads ($D = 25.9 \pm 11.01 \mu\text{m}$) functionalized with anti-GFP antibody were coupled to GFP tagged WAVE proteins. These beads were then placed in a cytoplasmic extract from HUVEC in the presence of rhodamine-actin to stimulate polymerization of actin shells around the beads. The amount of WAVE protein was monitored in the green (GFP) channel, and the actin polymerization in the red (rhodamine) channel.

Figure 2.

Specificity of actin nucleation induced by GFP-WAVE2 and WAVE2-GFP. **A.** Schematic diagram of GFP-WAVE2 and WAVE2-GFP. In WAVE2-GFP the C-terminal VCA domain that is responsible for the activation of Arp 2/3 complex is hindered from binding by the bulky GFP tag. However, GFP-WAVE2 is able to stimulate actin polymerization efficiently. **B.** Comparisons of actin nucleating activity of WAVE2-GFP coupled beads and GFP-WAVE2 coupled beads after 30 min incubation since the initiation of the assay, visualized by fluorescence imaging. Green and red channels were used for GFP and for rhodamine-labeled actin, respectively. (Scale bar = 20 μ m). **C.** Integrated fluorescence intensity of rhodamine actin (y-axis) is plotted versus that of GFP (x-axis) for multiple beads. The relative ratios between the two axes for the two cases are indicated as slopes in the inset showing that GFP-WAVE2 (slope=39) is more potent in actin polymerization than WAVE2-GFP (slope=2).

Figure 3.

Comparison of full-length WASP/WAVE family proteins in actin-nucleating activity. GFP-WAVE2 and WAVE3 showed greater efficiency in the actin nucleation *in vitro* than WAVE1 and N-WASP, at $t = 30$ min after the initiation of polymerization. Results are presented from 2 independent experiments. Number of beads tested=32 for WAVE1, 20 for WAVE2, 15 for WAVE3 and 26 for N-WASP.

Figure 4.

WAVE family proteins induce actin tail formation, like N-WASP, in HUVEC extract. **A.** Representative fluorescence images showing examples of asymmetric actin shells (or short tails) formed on GFP-WAVE1,2,3 and N-WASP coupled beads (1 μm diameter). (Scale bar = 5 μm). **B.** Average actin cluster lengths (in μm) plotted for different WAVE family proteins and N-WASP. The value of actin cluster length is measured along the longest axis from the bead surface to actin cloud edge, using Metamorph (v6.0r1) software. Beads without detectable actin clouds are counted as zero length for cluster length. Error bars represent standard deviation of the mean. **C.** Average integrated fluorescence intensity plotted for different WAVE family proteins and N-WASP. Error bars represent standard deviation of the mean. Number of beads tested=21 for WAVE1, 18 for WAVE2, 29 for WAVE3, and 33 for N-WASP.