Rapid WAVE dynamics in dendritic spines of cultured hippocampal neurons is mediated by actin polymerization

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Abstract

The Wiskott–Aldrich syndrome protein family Verprolin-homologous protein (WAVE) complex has been proposed to link Rho GTPase activity with actin polymerization but its role in neuronal plasticity has never been documented. We now examined the presence, distribution and dynamics of WAVE3 in cultured hippocampal neurons. WAVE3 was localized to dendritic spines via its N-terminal domain. Green fluorescent protein (GFP)-tagged WAVE3 clusters demonstrate an F-actin-dependent high rate of local motility. Constitutive Rac activation translocates WAVE3 (via the N-terminus), to the leading edge of lamellipodia. Also, spinogenesis is associated with actin-based motility of the WAVE3 protein. Brain specific WAVE1 showed similar localization and effects on spine density. Cytoplasmic fragile X mental retardation protein interacting protein (CYFIP) and non-catalytic region of tyrosine kinase adaptor protein 1 (NCK-1), proteins that are assumed to complex with WAVE, have a somewhat similar cellular distribution and motility. We propose that the WAVE complex is a downstream effector of the Rac signaling cascade, localized to sites of novel synaptic contacts by means of its N-terminal domain, to guide local actin polymerization needed for morphological plasticity of neurons.

Keywords: actin, cytoplasmic fragile X mental retardation protein interacting protein 1, dendritic spines, fragile X mental retardation protein, Wiskott–Aldrich syndrome protein family verprolin-homologous protein complex.

Recent years have seen a flurry of publications on the role of the small GTPases of the Rho family in development, signal transduction and synaptic plasticity. The three members of the Rho GTPase family include the Rho, Rac, and CDC42, all of which have putative roles in the development and function of the nervous system (reviewed in Govek et al. 2005; Van Galen and Ramakers 2005). Mutations in Rho GTPase family interactors such as Pak3, oligophrenin and alpha Pix are associated with mental retardation. The Rac protein has been shown to exert a strong influence on neuronal morphology when introduced either in its constitutively active or dominant negative form (Tashiro et al. 2000).

The recently described WAVE (Wiskott–Aldrich syndrome protein family verprolin-homologous protein) complex acts directly downstream to the Rac GTPase and the adaptor protein Nck1 (non-catalytic region of tyrosine kinase adaptor protein 1) (Eden et al. 2002). The WAVE protein is found in a complex together with several other proteins such as CYFIP (cytoplasmic fragile X mental retardation protein interacting protein), NAP125, Abi and HSPC300. In fact, this complex is so highly regulated that knocking out any one of the proteins in the complex has been shown to cause the degradation of its partners (Schenck et al. 2004).

The relevance of the WAVE complex to brain function and development is strongly indicated by several findings. First, both WAVE1 and WAVE3 are heavily expressed in the brain (Suetsugu et al. 1999). WAVE isoforms 1–3 have all been shown to interact with the same members of the protein complex (Stovold et al. 2005). Second, the WAVE complex has been recently shown to regulate several different aspects of neuronal function and connectivity, including nerve and synapse formation in Drosophila melanogaster (Schenck et al. 2004).
et al. 2004), and a sublocalization of the different WAVE isoforms in axonal growth cones (Nozumi et al. 2003). The WAVE1 knockout mouse displays several neuronal phenotypes such as reduced brain size, thinning and a decrease in the length of the cerebral cortex. Although WAVE1 knockout mice invariably die at the first postnatal month, besides the described differences, their brain morphologies are generally normal. This fact could indicate a late developmental role but could also be due to a compensation by the other brain-expressed WAVE protein, WAVE3 (Dahl et al. 2003). Also of interest is the fact that one of the members of the WAVE complex interacts with the fragile-X mental retardation protein (FMRP), and is indeed aptly named Cytoplasmic Fragile-X Interacting Protein (CYFIP, Schenck et al. 2003). In spite of all these suggestive data, no study of the WAVE complex in neuronal synaptic compartments has been reported.

Primary hippocampal neurons offer an attractive experimental model system for the investigation of effect of such candidate neuronal regulatory molecules. We have used transfection to introduce fluorescently labeled WAVE3 and other members of the WAVE complex into these cells. WAVE3 was found to preferentially localize to dendritic spines, and it is highly motile and can lead the formation of novel dendritic protrusions and spines. We propose that WAVE3 motility is induced via a mechanism involving actin polymerization by its verprolin homology/cofilin homology/acidic (VCA) domain and that it is likely to play a pivotal role in neuronal plasticity.

Materials and methods

Materials

Latrunculin B, 6,7-Dinitroquinoxaline-2,3-dione (DNQX), TRITC-conjugated Phalloidin, 2-amino-5-phosphonovalerate (APV) and nacodazole were obtained from Sigma (St Louis, MO, USA). Tetrodotoxin (TTX) was obtained from Alomone Laboratories (Jerusalem, Israel). Bisindolylmaleimide I (GF109203x) and Phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (San Diego, CA, USA).

Plasmids

A mammalian expression plasmid encoding green fluorescent protein (GFP)-fused CYFIP1 was the kind gift of Professor Barbara Bardon. Mouse brain was used to prepare cDNA from which WAVE1, WAVE3, and Nck1, were cloned in frame into GFP-C1 (Clontech, Palo Alto, CA, USA) by PCR amplification (using Takara LA-TAQ; Takara, Shiga, Japan) and subsequent restriction and ligation between the BglII and SalI restriction sites (using Fermentas BglII, SalI and T4-DNA ligase; Fermentas, Vilnuius, Lithuania). A WAVE3 mutant lacking the C-terminal verprolin homology/cofilin homology/acidic (VCA)-domain was prepared by amplifying the entire WAVE3 N-terminal to this domain, and reinserting it into GFP-C1 using the same restriction enzymes. Oligonucleotides used to amplify Nck1 were 5'-CCAAGATCTACTGAGAGAAGAAAGACACGTCGAATTTTGA and 5'-CCATGTCGACTTACTGCTTTAGTGAAGAGAAACATC and 5'-CCGAACACTGGAGTCGACTTACTCCAGTTACCCATGTTATCTTATC. To generate WAVE3-Delta-VCA the same N-terminal (sense) primer was used as for wild-type (WT) WAVE3, the C-terminal (reverse complementary) primer was 5'-CCATGTCGACTTTAATTCTATGTTGTTGGTGCGTG. WAVE1 was cloned using the oligonucleotides 5'-CCGAACACTGGAGATCTAGTGTGTTGGTGGTGG- and 5'-CCGAACACTGGAGTCGACTTTAATTCTATGTTGTTGGTGG-.

A mammalian expression plasmid encoding constitutively active Rac1 (RacV12) was a gift from Dr Sima Lev and Dr Alexander Bershadsky. A synaptophysin–GFP construct was the gift of Professor S. Vicini. GFP driven by the EF1alpha promoter was from Chaim Kahana.

Cell culture

Primary hippocampal neurons, taken from P1 pups and grown on a glia bed for 10–20 days were used (Goldin et al. 2001). Cultures were transfected at the age of 7–8 days in vitro (DIV) and visualized within the following week (11–14 DIV).

Transfection

A lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) mix was prepared at 1 μL/well with 50 μL/well optimem™ (Invitrogen), and incubated for 5 min at room temperature (25°C). This was mixed with 1.5 μg/well total DNA in 50 μL/well optimem™, and incubated for 15 min at 25°C. The mix was then added on the transfected culture wells, and allowed to incubate for 4–6 h until medium was changed. For WAVE3 and WAVE3-Delta-VCA a 3–4:1 ratio of WAVE to pDsRed2 encoding plasmid was used to compensate for lower WAVE3 protein levels. Also, because WAVE3 protein levels were seen to drastically drop from one day to the next, we visualized most cultures at 2–3 days post-transfection (DIV 10-12).

Confocal microscopy

Glass coverslips of the cultured neurons were placed in a chamber and bathed in recording medium [containing (in mM) 10 HEPES, 4 KCl, 2 CaCl2, 1 MgCl2, 139 NaCl, 10 glucose, adjusted with sucrose to an osmolarity of 320mOsm, and with HCl to a pH of 7.4]. The chamber was placed in an inverted Zeiss confocal laser-scanning microscope (LSM-510). High-resolution thin optical sections of the cells were obtained, and LSM image analysis software was used to create three-dimensional composite images.

Immunostaining

Coverslips bearing primary hippocampal cells were washed briefly with standard extracellular solution containing 2 mM Ca2+ and 1 mM MgCl2. The cells were then fixed with 4% paraformaldehyde/4% sucrose (w/v) in phosphate-buffered saline (pH 7.4) for 20 min, and washed with phosphate-buffered saline. For WAVE3 immunostaining, blocking was then performed for 1 h with 10% rabbit serum and 0.1% Triton X-100, after which primary antibody (Goat polyclonal anti-WAVE3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at 1:100 dilution in blocking serum overnight at 4°C. Slides were then washed with phosphate-buffered saline and incubated for 1 h with
Cy™3 conjugated donkey anti-goat antibody (Jackson Laboratories, West Grove, PA, USA; 1:200). Slides were washed with phosphate-buffered saline and mounted for visualization with the confocal microscope. Control cultures were incubated with secondary antibodies alone and imaged as above. For F-actin staining, coverslips were incubated with TRITC-conjugated phalloidin (1:750 in phosphate-buffered saline) for 1 h and then washed again with phosphate-buffered saline, and mounted on slides.

Analysis
To quantify motility of WAVE3 puncta, 10 images were taken sequentially every 30 s over 5 min. Total distance traversed by each puncta during this interval was normalized to units of μm/s. Spine counts were performed on several dendrites from each cell, and data normalized per micron. To quantify phalloidin-TRITC intensity in transfected cells, Image-J software was used.

Results

WAVE3 is ubiquitously expressed in primary hippocampal neurons with a preference towards dendritic processes and protrusions
Cultures were immunostained for WAVE3 in order to view the endogenous expression and distribution of the protein. WAVE3 immunoreactivity was found throughout the soma and dendritic processes. Distinct punctate staining was seen in the dendritic branches, and in many dendritic spines (Fig. 1). Control experiments showed that WAVE3 immunostaining was indeed specific. Thus, dense WAVE3 puncta were identified in select compartments of cultured hippocampal neurons, including dendritic spines.

Overexpressed WAVE complex proteins preferentially target dendritic protrusions
We chose overexpression of GFP-tagged proteins of the WAVE complex as a tool to visualize their localization. Overexpressed GFP-WAVE3 was distributed in a punctate manner, and localized preferentially to most dendritic protrusions, although distinct puncta also appeared in the dendritic shaft (Figs 2a and b). This corroborates the data acquired by immunostaining of the native WAVE3.

The C-terminal deletion mutant, WAVE3-Delta-VCA displayed a similar distribution as the WT WAVE3 (Figs 2c and d). This is perhaps not entirely surprising, as Oikawa et al. (2004) have recently shown that the N-terminal basic domain of WAVE2 (conserved through WAVE 1–3) is necessary and sufficient for phosphoinositide binding, a gradient of which leads the rearrangement of the actin cytoskeleton. The fact that the VCA domain is also not required for localization of WAVE3 in spines suggests a similar mechanism.

Fig. 1 Immunostaining against WAVE3. WAVE3 is abundant and punctate in primary hippocampal neurons. (a) WAVE3 immunostaining signal (Red) in a primary hippocampal neuron. (b) Brightfield image of the same neuron. (c) Magnified image of the bottom right section from (a). (d) High magnification of boxed area from (c). Arrows point towards dendritic spines and protrusions with WAVE3 puncta. Scale bar = 10, 5, and 1 μm for respective magnifications.
Fig. 2 WAVE complex localization in dendritic spines and protrusions. WAVE3 is localized to dendritic spines and protrusions. (a, b) A dendritic segment (a) and magnification thereof (b) of GFP-WAVE3/DsRed2 co-transfected cells (green channel = WAVE3, red channel = DsRed2). Newly synthesized WAVE3 protein targets mostly to dendritic spines and protrusions, but is also present along the dendrite. (c, d) Similar images of dendritic segments of WAVE3-Delta-VCA-transfected neurons, demonstrating a similar distribution. Arrows show puncta in dendritic spines. Square in (c) is magnified in (d). Scale bars = 2 μm for (a–d). Other complex members are also similarly localized. (e, f) A dendritic segment (e) and magnification thereof (f) of GFP-CYFIP1/DsRed2 co-transfected cells (green channel = CYFIP1, red channel = DsRed2). (g, h) Respective images of Nck1-transfected neurons. Overexpressed CYFIP1 and Nck1 both display punctate staining primarily in dendritic spines, and also to dendritic segments. Arrows show puncta in dendritic spines. Squares in (e) and (g) are magnified in (f) and (h), respectively. Scale bars = 5 μm for low, 2 μm for high magnifications. CYFIP1, cytoplasmic FMRP interacting protein; GFP, green fluorescent protein; Nck1, non-catalytic region of tyrosine kinase adaptor protein 1; VCA, verprolin homology/cofilin homology/acidic; WAVE3, Wiskott–Aldrich syndrome protein family verprolin-homologous protein 3.
We also studied the distribution of the CYFIP1 protein. CYFIP1 co-precipitates with WAVE, and reconstitution of the protein–protein interactions within the WAVE complex by in vitro translation shows that it is probably the site of Rac binding that initiates the release of the WAVE protein. It is also the protein member of the complex which directly interacts with fragile X mental retardation protein (FMRP) (Schenck et al. 2003; Gautreau et al. 2004). Indeed, CYFIP1 showed a similar distribution to WAVE3 (Figs 2e and f).

WAVE3 has been recently shown to concentrate in lamellipodia of the leading edge of migrating MDA-MB-231 cells (Sossey-Alaoui et al. 2005). We have previously shown that overexpression of active Rac (RacV12) promotes the formation of lamellipodia in primary neurons (Tashiro et al. 2000; Pilpel and Segal 2004). We therefore co-transfected RacV12 together with WAVE3. Strikingly, WAVE3 was distinctly localized to the leading edge of lamellar structures that are abundant in Rac-overexpressing cells (Figs 3a and b). Interestingly, WAVE3-Delta-VCA was also localized at the leading edge of lamellar structures (Figs 3c and d) as well as CYFIP1 (Figs 3e and f).

Nck1 is an adaptor protein with three SH3 domains and one SH2 domain. The first SH3 domain has been shown to be necessary and sufficient for complex formation and activation (Eden et al. 2002). Nck1 was localized to dendritic branches with a strong affinity for dendritic spines and protrusions (Figs 2g and h). Rac activation, however, caused Nck1 to diffuse together with the spine structure, and did not localize it to the leading edge of the lamellipodia (Figs 3g and h). Taken together, these data suggest that Nck1 does not co-localize with the WAVE complex in these cells.

WAVE expression and effects on morphology

WAVE3 overexpression had a pronounced effect on the morphology of the neurons. The density of dendritic spines was significantly lower (down to 57% of control) in WAVE3-transfected cells. Interestingly, WAVE3-Delta-VCA expressed similar spine density as controls (0.199 ± 0.0191, 0.114 ± 0.0188, 0.164 ± 0.0195 spines/μm for DsRed2 control, WAVE3, and WAVE3-Delta-VCA-transfected cells, respectively (Fig. 4). One-way ANOVA revealed a significant difference between groups \( F_{2,55} = 5.738, p < 0.005 \); post hoc Scheffe test revealed a significant difference between WAVE3 and the control group \( p < 0.005 \); n = 21, 25, and 12 cells from control, WAVE3, and WAVE3-Delta-VCA groups, respectively). We did not detect any morphological abnormalities with overexpression of Nck1 or CYFIP1 (Nck1: 0.230 ± 0.0222 spines/μm, CYFIP1: 0.193 ± 0.0208 spines/μm, not significantly different than controls; \( p > 0.28 \), \( p > 0.84 \), respectively; \( n = 18 \) and 16 cells, respectively).

Of the three WAVE isoforms, both WAVE1 and WAVE3 are brain specific, whereas WAVE2 is ubiquitous (Sossey-Alaoui et al. 2003). WAVE1 and 3 have also shown to associate to the same complex proteins (Stovold et al. 2005). They may therefore be redundant. We did not attempt to characterize WAVE1 in full; however, a GFP-WAVE1 construct was cloned and tested, and could be seen to localize in a similar manner to WAVE3 (Fig. 4e). We also counted spines in a small set of WAVE1-transfected cells, and spine densities were similarly reduced (0.146 ± 0.0187, \( n = 7 \) cells, \( p < 0.05 \), Student’s one-tailed t-test).

WAVE3 induces accumulation of F-actin

As the WAVE complex is involved in the initiation of actin polymerization, we used Phalloidin staining to assay for the presence of F-actin in transfected cells. Control cells displayed scattered punctate and fibrous staining of dendritic branches and protrusions, and weak, sometimes hardly visible staining in soma boundaries. WAVE3 positive cells, on the other hand, displayed a significant accumulation of Phalloidin staining in the cell soma and in and about WAVE3-GFP puncta (Fig. 5). This was only true for WAVE3, and required the C-terminal actin and ARP2/3 binding domain, as other members of the complex (CYFIP1, Nck1 and the mutant WAVE3-Delta-VCA) did not exhibit levels of F-actin staining above background (See Fig. 5j) for quantification. One-way ANOVA revealed a significant difference between groups \( F_{5,126} = 37.473, p < 0.0001 \); post hoc Scheffe test revealed a significant difference between the WAVE3 and all other groups \( p < 0.0001 \); \( n = 71 \), 11, 8, 15, 16 and 11 cells for control, GFP-control, CYFIP1, WAVE3, WAVE3-Delta-VCA, and Nck1 groups, respectively). This finding indicates that overexpression of the WAVE3 protein acts in a constitutively active fashion. Neither CYFIP1 nor Nck1 overexpression promoted this kind of F-actin formation.

WAVE3 displays high actin polymerization-dependent motility

Although WAVE3 is targeted to dendritic protrusions and spines, time-lapse images revealed that the WAVE3 protein was very motile (Fig. 6). GFP-WAVE3 puncta were translocated along the dendrite, in and out of spines, and, most strikingly, in and out of the dendrite, forming and collapsing new protrusions. Figure 6(g) shows a series of time-lapse images demonstrating this effect. We used glutamate to stimulate rapid morphological changes in our cells. On several occasions, we were able to capture a new dendritic spine form at the location of a pre-existing WAVE3 puncta. In these cases, WAVE3 led the formation of a filopodia or dendritic protrusion, eventually becoming a dendritic spine (Fig. 6).

Of all the overexpressed WAVE complex proteins, WAVE3 showed striking motility, although this is not to say that other proteins of the complex may not also be intrinsically motile. Overall motility of WAVE3 was very rapid. Average values were \( 0.0047 ± 0.00036 \) μm/s.
Fig. 3 Redistribution of WAVE complex proteins within Rac-induced lamellipodia. All WAVE complex proteins tested except Nck1 are localized to the leading edge of Rac-induced lamellae. (a, b) Low (a) and high (b) magnification images of triple-transfected GFP-WAVE3 (green channel), DsRed2 (red channel), and active RacV12. WAVE3 is strikingly localized at the leading edge of Rac-induced lamellar structures. The same is shown for Delta-VCA-WAVE3 (c, d) and CYFIP1 (e, f). Nck1 (g, h), on the other hand, remains diffuse throughout lamella, without advancing towards the leading edge. Boxed areas in (a), (c), (e) and (g) are magnified in (b), (d), (f) and (h), respectively. Scale bars = 5 μm for low, 2 μm for high magnifications. CYFIP1, cytoplasmic FMRP interacting protein; GFP, green fluorescent protein; Nck1, non-catalytic region of tyrosine kinase adaptor protein 1; VCA, verprolin homology/cofilin homology/acidic; WAVE, Wiskott–Aldrich syndrome protein family verprolin-homologous protein.
However, for many puncta motility was much faster, as punctal movement was not smooth but rather in quick jumps followed by intervals in which puncta jiggled in place. Fastest 'jumps' were > 0.1 μm/s (as calculated from distance traveled between two consecutive 30-s frames). This implies a mode of transport other than conventional cellular systems. We assayed the effect of several treatments on the rapid motility of WA VE3 in primary neurons (Fig. 6h).

WA VE3 can cause polymerization of actin in these cells. We therefore wanted to assay the effect of inhibiting WA VE3-mediated actin polymerization by using the WA VE3-Delta-VCA mutant, and depolymerizing F-actin by adding latrunculin B (2.5 μM). Both caused a nearly complete cessation of WA VE3 movement (WA VE3-Delta-VCA puncta moved at 0.0014 ± 0.0005 μm/s, and latrunculin addition lowered motility to 0.0006 ± 0.0001 μm/s). Remaining motility is most likely to be an artifact of the preparation (e.g. micromovements of the entire coverslide, movement of the entire cell and its processes, etc.). However, some motility could possibly be the effect of active transport of puncta by motor proteins or in vesicles.

If this is the case, inhibition of the microtubule cytoskeleton should interfere with this process and be reflected in motility. To assay for this possibility we added nacodazole (33 μM) for 1 h. Movement of puncta remained high (0.003421 ± 0.0006 μm/s after nacodazole addition, not significantly different). We therefore conclude that actin polymerization is necessary and sufficient for WA VE3 puncta movement. We studied the effects of activity blockade on WA VE3 motility. To this end 2-amino-5-phosphonovalerate (APV, 50 μM), 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM), and Tetrodotoxin (TTX, 0.5 μM) were added. This, however, did not affect WA VE3 motility (motility was 0.0038 ± 0.0007 μm/s after inhibitors’ addition, no significant difference). In control experiments, we assayed the motility of WA VE3 puncta, washed the cells with standard extracellular solution, and assayed their motility 1 h later (0.0057 ± 0.001 μm/s after passive medium change, no significant difference). Thus, WA VE3 motility seems to be controlled in primary neurons as well by its association to the complex, and its motility is dependent on self-induced actin polymerization. In one-way ANOVA, there is a significant difference between all six groups ($F_{5,374} = 9.39$, $p < 0.0001$). Post hoc Scheffe test revealed significant differences between WA VE3 WT controls and Delta-VCA mutants ($p < 0.001$), as well as after latrunculin B treatment ($p < 0.0001$). n = 160, 60, 30, 50, 50, and 30 puncta from 35, 10, 6, 10, 9, and 6 cells in WA VE3-control, WA VE3-Delta-VCA, latrunculin, nacodazole, activity inhibitors, and control (passive medium change) groups, respectively.

**Discussion**

The WA VE complex has emerged in recent years as a main and important downstream effector of the Rac GTPase and its upstream regulators. Other studies assign increasing significance to the function of the Rho GTPases in synaptic morphological and functional plasticity. To our knowledge, this study is the first to view the WA VE proteins in primary neurons.

Our data provide conclusive evidence that the WA VE complex is endogenously localized to dendritic protrusions and spines in primary hippocampal neurons. Each of the three members assayed here independently displayed a clear
Localization to spines, indicating the presence of endogenous complex to which overexpressed proteins can bind.

Both CYFIP1 and WAVE3 are localized to the leading edges of Rac-induced lamellipodia. Eden et al. (2002) demonstrated that in vitro activation of the complex by Rac causes the dissociation of these proteins (HSPC300 is the only protein that remains associated with WAVE1 in these experiments). Steffen et al. (2004), demonstrated a different possible mechanism in which CYFIP1 (also called Sra-1), Nap1 and Abi1, are recruited with WAVE to the leading edge of lamellipodia. In fact, these proteins are required in order to form lamellipodia downstream to Rac activation. Our data concur with those of Steffen et al. (2004) on this point. Nck1 did not redistribute in the leading edge of lamellipodia, but was rather spread on the surface of lamellipodia. This data is in agreement with both possible models. One possible explanation for these discrepancies is that activation of the complex by Rac (or Nck1) induces a conformational or other post-translational change that lowers the affinity by which these proteins are associated with each other.

Overexpression of WAVE3, but not CYFIP1, causes the accumulation of F-actin in transfected neurons. This is in good agreement with a regulatory role for the rest of the WAVE complex. However, Nck1 is both localized at the same subcellular level in our system and has been shown to activate the WAVE complex (Eden et al. 2002). Overexpression of Nck1 induces neither increased actin polymerization comparable to that driven by WAVE3 overexpression, nor any distinct morphological change in the transfected neurons. This may be explained by the presence of an upstream

Fig. 5 Phalloidin staining shows accumulation of F-actin in WAVE3 transfected cells. F-actin is highly increased in WAVE3, but not other complex members transfected cells, an effect dependent on the VCA domain. (a) A GFP-transfected control cell (green channel). (b) Phalloidin staining of the same cell (red channel). (c) Merge of the two channels. Note that the soma of the cell can hardly be distinguished using staining for F-actin. (d) A GFP-WAVE3-transfected neuron. (e) Phalloidin staining of the same cell shows intense staining correlating with GFP-WAVE3 expression. The soma, containing the highest levels of exogenous GFP-WAVE3 and also high levels of F-actin, is marked with an arrow. (f) Merge of green and red channels. (g–i) A GFP-tagged WAVE3-Delta-VCA transfected cell. Note that there is no actin accumulation in the soma in (h). Scale bars = 5 μm. (j) Phalloidin staining intensity in the soma of neurons was quantified and compared between control non-transfected cells (Con.), transfected (GFP) controls, and WAVE3 (WAVE3), WAVE3-Delta-VCA (Delta-VCA), CYFIP1 (CYFIP), Nck1 (Nck1) GFP fused constructs. Only full-length WAVE3 was able to induce a significant increase in F-actin levels. CYFIP1, cytoplasmic FMRP interacting protein; GFP, green fluorescent protein; Nck1, non-catalytic region of tyrosine kinase adaptor protein 1; VCA, verprolin homology/cofilin homology/acidic; WAVE, Wiskott–Aldrich syndrome protein family verprolin-homologous protein.
regulatory mechanism that may be necessary to activate Nck. A recent study suggests a functional mechanism for Nck-mediated actin rearrangements (Rivera et al. 2004). The researchers showed that only clustering of Nck1 in the membrane (using antibodies against extracellular epitopes fused to Nck1), but not overexpression per se, caused the appearance of actin puncta and tails. Oddly, there was no interaction detected between WA VE and Nck1 in this study. As most research into the WA VE complex has been performed in systems that utilize Rac for its activation, the significance and function of Nck1 in this context remains obscure.

WA VE3 puncta displayed a very high motility, with occasional bursts that translate to several microns per minute. WA VE3 did not show a dependence on microtubule-based transport for motility, and no evidence links it to any motor protein. The rapid motility was entirely dependent on actin polymerization initiated by the VCA domain of WA VE3, as could be seen by lack of motility of the C-terminal deletion mutant, although this mutant is properly localized by virtue of its N-terminal domain. This kind of motility is indicative of the mechanism by which Rickettsia type of intracellular parasites move, utilizing an Arp2/3 complex-dependent nucleation mechanism to generate comet tails consisting of Y-branched filament arrays. A recent study has found that a protein named RickA, with similarity to the WASP and also WA VE proteins in the VCA domain, activates the actin-nucleating and Y-branching activities of the Arp2/3 complex, and is sufficient to direct actin-based motility and the formation of Y-branched actin arrays (Jeng et al. 2004). Addition of human WASP or WA VE causes Y-branching in an in vitro actin polymerization assay (Blanchoin et al. 2000). We submit that such an actin-polymerization based mechanism drives WA VE3 motility in primary neurons.
tempting to speculate that, with the help of the N-terminal domain, the WAVE protein also subserves the formation of dendritic filopodia and spines. Research performed into the WAVE protein interaction with actin supports this hypothesis. Such a study showed that the N-terminal basic domain of the protein assists in the actin network branching activity (Suetsugu et al. 2001). Thus, there is little doubt of the capability of WAVE to perform this activity. Therefore, from previous data and from novel data presented here, a functional model of the WAVE protein can be inferred. The N-terminal domain, being led by a gradient of phosphatidylinositol 3,4,5-triphosphate produced in sites of filopodia, and even synapse formation, targets WAVE3 to the membrane, and the C-terminal VCA domain interacts with ARP2/3 and actin to bring about the formation of membrane protrusions in the dendritic nucleation model (Pollard and Borisy 2003).

One finding is that active WAVE3 (but not VCA-deleted), caused a reduction of the number of dendritic spines. A possible explanation for this activity, in light of the data brought here, would be that the actin polymerization function of the VCA domain tends to destabilize pre-existing actin-filament structures and form new ones, when overexpressed and underregulated. Although a higher steady state level of F-actin would be expected (and is indeed seen here), the mode by which this is achieved would mean a shorter life time for otherwise relatively stable actin-based structures such as dendritic spines. Indeed, Matus (2005) suggests that ‘the transition from filopodium to mature spine involves the down-regulation of actin dynamics’.

In conclusion, the actin based motility of WAVE3, and the presence of the WAVE complex in the synaptic compartment, promise a distinctive role for these proteins in dendritic spine development and synaptic plasticity. Further research is required to elucidate the exact function of WAVE in this context, the role and regulation of Nck, and the possible connection to Fragile-X type of mental retardation.

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References


