Identification of Another Actin-related Protein (Arp) 2/3 Complex Binding Site in Neural Wiskott-Aldrich Syndrome Protein (N-WASP) That Complements Actin Polymerization Induced by the Arp2/3 Complex Activating (VCA) Domain of N-WASP*

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Neural Wiskott-Aldrich syndrome protein (N-WASP) is an essential regulator of actin cytoskeleton formation via its association with the actin-related protein (Arp) 2/3 complex. It is believed that the C-terminal Arp2/3 complex-activating domain (verprolin homology, cofilin homology, and acidic (VCA) or C-terminal region of WASP family proteins domain) of N-WASP is usually kept masked (autoinhibition) but is opened upon cooperative binding of upstream regulators such as Cdc42 and phosphatidylinositol 4,5-bisphosphate (PIP2). However, the mechanisms of autoinhibition and association with Arp2/3 complex are still unclear. We focused on the acidic region of N-WASP because it is thought to interact with Arp2/3 complex and may be involved in autoinhibition. Partial deletion of acidic residues from the VCA portion alone greatly reduced actin polymerization activity, demonstrating that the acidic region contributes to Arp2/3 complex-mediated actin polymerization. Surprisingly, the same partial deletion of the acidic region in full-length N-WASP led to constitutive activity comparable with the activity seen with the VCA portion. Therefore, the acidic region in full-length N-WASP plays an indispensable role in the formation of the autoinhibited structure. This mutant contains WASP-homology (WH) 1 domain with weak affinity to the Arp2/3 complex, leading to activity in the absence of part of the acidic region. Furthermore, the actin comet formed by the ΔWH1 mutant of N-WASP was much smaller than that of wild-type N-WASP. Partial deletion of acidic residues did not affect actin comet size, indicating the importance of the WH1 domain in actin structure formation. Collectively, the acidic region of N-WASP plays an essential role in Arp2/3 complex activation as well as in the formation of the autoinhibited structure, whereas the WH1 domain complements the activation of the Arp2/3 complex achieved through the VCA portion.

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† The abbreviations used are: WASP, Wiskott-Aldrich syndrome protein; N-WASP, neural Wiskott-Aldrich syndrome protein; WAVEs, WASP family verprolin homologous proteins; Arp2/3, actin-related protein 2/3; VCA, verprolin homology, cofilin homology, and acidic or C-terminal region of WASP family proteins; CRIB, Cdc42/Rac interactive binding region; WH1, WASP homology 1; G-actin, globular actin, monomer actin; GST, glutathione-S-transferase; RUs, resonance units; MES, 4-morpholineethanesulfonic acid; WIP, WASP-interacting protein; PIP2, phosphatidylinositol 4,5-bisphosphate.
G-actin or weakens their interaction, which subsequently inhibits the initiation of actin polymerization.

The formation of the autoinhibited structure is not well understood. The VCA region is composed of three distinct segments: verprolin homology, cofilin homology, and acidic regions. The middle region also has distinct segments such as the basic region and the CRIB motif (2). In the three-dimensional structure of truncated WASP, the CRIB motif and the cofilin homology region interact to form the folded structure (17). This structure is one of several possible associations resulting in autoinhibition of the full-length protein. Another candidate is an interaction between acidic and basic regions since opposite charges attract.

In this report, we show that partial deletion of the acidic region of N-WASP leads to constitutive activation of N-WASP in vitro. This suggests that the acidic region contributes to the formation of the autoinhibited structure in addition to being essential for activating Arp2/3 complex-mediated actin polymerization. The high activity generated by the mutant containing deletions in the acidic region is due to the presence of the WH1 domain. This domain has weak affinity for Arp2/3 complex, thereby providing another means of activating the Arp2/3 complex.

EXPERIMENTAL PROCEDURES

Proteins—Various mutant N-WASPs were expressed in SF9 cells by a bac-to-bac baculovirus expression system (Life Technologies, Inc.) with either a GST tag or a His tag. Recombinant virus-infected SF9 cells were lysed, clarified, and purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) or Ni-NTA agarose (Qiagen). GST fusion VCA, VCa, and VC were purified as described previously (2, 18). Arp2/3 complex was purified as described (19). Actin was purified from rabbit skeletal muscle, and monomeric actin (G-actin) was isolated by gel filtration on Superdex 200 (Amersham Pharmacia Biotech) in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.5 mM dithiothreitol, 0.2 mM ATP).

Pyrene-actin Assay—Pyrene-actin assay was performed as described previously (5). Final concentrations of Arp2/3 complex, G-actin, and pyrenyl-actin were 60 nM, 2 μM, and 0.2 μM, respectively. Concentrations of wild-type or mutant N-WASPs were 100 nM. Phosphatidylinosito-
Actin polymerization in bovine brain extract was monitored by pyrene fluorescence. The reaction mixture was prepared by mixing 10 μl of bovine brain extract, 20 μl of the ATP-regenerating mix, 70 μl of 1 × XB, 1 μl of GST-Cdc42 (final, 125 μM), 1 μl of 10 mM GTPγS, and finally 0.4 μM of pyrene-labeled actin. The reaction mixture was preincubated for 10 min. Wild-type or mutant N-WASP was added at a final concentration of 2 μM (9% pyrene-labeled) after a 5-min preincubation. Western blot analysis of the association between N-WASP domains and the Arp2/3 complex was performed essentially as described previously (20). Carboxylated polystyrene beads (Polysciences), 0.5 μm in size, were coated with His-tagged N-WASPs as described previously (21). Beads (2 μl) were incubated in 10 μl of protein solution containing 2 mg/ml bovine serum albumin and 1.6 mg/ml His-tagged N-WASP (45% of total protein or as otherwise indicated) for 1 h at room temperature. Then the beads were pelleted, washed, and resuspended in 30 μl of XB. For visualizing bead motility, protein-coated beads (0.5 μl) were added to 13.25 μl of motility solution consisting of 2.5 μl of bovine brain extract, 5 μl of tetramethylrhodamine dodecaacetylated actin (1 mg/ml) (prepared as described previously (22), 2.5 μl of ATP-regenerating mix with protease inhibitor mixture, 2.5 μl of 5 × XB (500 mM KCl, 0.5 mM CaCl2, 10 mM MgCl2, 25 mM EGTA, 50 mM K-Hepes, pH 7.7), 0.25 μl of 10 mM GTPγS, and 0.5 μl of 13 μM GST-Cdc42. The mix was allowed to incubate on ice for 1 h. A 0.5-μl sample was removed and squashed between a microscope slide and an 11 mm × 22 mm-glass coverslip, sealed with vaseline, and incubated at room temperature for 1 h in the dark before observation under a fluorescent microscope.

RESULTS AND DISCUSSION

Acidic Residues in N-WASP Are Essential for Autoinhibition of N-WASP and Activation of Arp2/3 Complex—To elucidate the regions responsible for Arp2/3 complex activation and formation of the autoinhibited structure, we made several C-terminal deletion mutants, as shown in Fig. 1. The N-VCa (Δa) mutant has an intact cofilin homology region (2) but lacks most of the acidic region. The N-VCa (Δa) mutant contains only three acidic amino acids at the C terminus. The N-VC (Δa) mutant lacks all C-terminal acidic residues.

We measured the ability of these mutants to activate Arp2/3 complex-mediated actin polymerization using a pyrene actin assay. The N-VCa (Δa) mutant was able to induce actin polymerization as well as was the VCA region or the activated wild-type N-WASP (Fig. 2). In contrast to wild-type N-WASP, initiation of polymerization did not require activators such as Cdc42 and PIP2 (Fig. 2B). Therefore, an association with C-terminal acidic residues is another possible way, like the proposed CRIB motif-cofilin homology interaction, to create the autoinhibited structure (17).

We next examined the mutant that lacks the acidic region (N-VC (Δa)). This mutant is unable to induce or activate Arp2/3 complex-mediated actin polymerization, suggesting that acidic residues are essential for this process (Fig. 3A). Our data show that the cofilin homology region, which contains three acidic amino acids (2), is essential for activating Arp2/3 complex in full-length N-WASP, whereas acidic residues at the C terminus are responsible for autoinhibition of N-WASP.

**WH1 Domain Is Responsible for Arp2/3 Complex Activation**—To examine whether the cofilin homology region in combination with the verprolin homology region is sufficient to initiate Arp2/3 complex-mediated actin polymerization, we cre-
Acidic Residues and WH1 Domain Cooperate in Their Association with Arp2/3 Complex—In the assays described above, the acidic residues and WH1 domain are shown to be involved in activating Arp2/3 complex-mediated actin polymerization. To understand the Arp2/3 complex-N-WASP interaction in detail, we used surface plasmon resonance measurements to directly observe the association and dissociation of the Arp2/3 complex. The surface of the sensor tip was coated with protein fragments such as VCA, VC, VCa, and N-VCa N-WASPs. Then, Arp2/3 complex was injected onto the surface of the sensor tip to monitor the association between Arp2/3 and the immobilized N-WASP fragment followed by the washing out with the running buffer for monitoring the dissociation. To estimate the association of N-WASP fragments and Arp2/3, the values measured were correlated with subtracting background signals (Fig. 4A).

The apparent $k_a$ and $k_d$ values were determined from linear regression analysis (by plotting $k_{obs}$ (slope of the plot of $dR/dt$ versus $R$) against Arp2/3 concentration (see “Experimental Procedures”). The $k_{obs}$ values were obtained by selecting the region that was linear with respect to the plot of $dR/dt$ versus $R$. Apparent association ($k_a$) and dissociation ($k_d$) rate constants are determined from slope and intercept ($k_{obs} = k_aC + k_d$), respectively. Box below indicates $k_a$, $k_d$, and $K_D (= k_d/k_a)$ values for each N-WASP fragments. $r$ represents correlation co-efficient for $k_{obs} = k_aC + k_d$. C, Western blot analysis of the association between various N-WASP mutants and the Arp2/3 complex. Equivalent amounts of GST-fusion proteins of various N-WASP mutants were immobilized and incubated with 0.1 $\mu$m of Arp2/3 complex. After incubation, the bound proteins were analyzed by Western blotting.

The VCa mutant was able to activate Arp2/3 complex, its activity was very weak compared with that of the N-VCa mutant. Complete deletion of the acidic region from the VCA fragment (VC mutant) resulted in complete loss of activity as reported previously (5). The data show that the acidic residues in the cofillin homology region are required for activation of the Arp2/3 complex but are not sufficient (Fig. 3).

We next determined which region of N-WASP is responsible for the high activity of the N-VCa mutant. A mutant containing the VCa region and proline-rich region (pro-VCa) showed weak activation for the Arp2/3 complex, similar to the VCa mutant. A mutant including the VCa region plus the IQ motif, the basic region, the CRIB motif and the proline-rich region (IQ-VCa) also showed activity similar to that of the VCa mutant. Therefore, WH1 domain appears to be responsible for potentiating Arp2/3 complex activation (Fig. 3A). The association between Arp2/3 complex and WH1 domain alone was seen in a pull down assay with GST fusion proteins (Fig. 3B), although the association was very weak compared with the interaction between Arp2/3 and VCa. We also detected an association between Arp2/3 complex and the region between the IQ and CRIB motifs as reported previously (15). However, this binding did not increase actin polymerization (Fig. 3). No association between the Arp2/3 complex and the proline-rich region was observed (data not shown). These findings suggest that the WH1 domain is responsible for achieving the greater activation of Arp2/3 complex by the N-VCa mutant, presumably by stabilizing the Arp2/3 complex-N-WASP interaction in its correct orientation.

Acidic residues of N-WASP—In the assays described above, the acidic residues and WH1 domain are shown to be involved in activating Arp2/3 complex-mediated actin polymerization. To understand the Arp2/3 complex-N-WASP interaction in detail, we used surface plasmon resonance measurements to directly observe the association and dissociation of the Arp2/3 complex. The surface of the sensor tip was coated with protein fragments such as VCA, VC, VCa, and N-VCa N-WASPs. Then, Arp2/3 complex was injected onto the surface of the sensor tip to monitor the association between Arp2/3 and the immobilized N-WASP fragment followed by the washing out with the running buffer for monitoring the dissociation. To estimate the association of N-WASP fragments and Arp2/3, the values measured were correlated with subtracting background signals (Fig. 4A).

The apparent $k_a$ and $k_d$ values were determined from linear regression analysis (by plotting $k_{obs}$ (slope of the plot of $dR/dt$ versus $R$) against Arp2/3 concentration (see “Experimental Procedures”). For the VCA fragment, $k_a$ and $k_d$ values are $1.2 \times 10^4$ M$^{-1}$ s$^{-1}$ and $2.7 \times 10^{-4}$ s$^{-1}$, respectively (Fig. 4B). This gives an equilibrium dissociation constant ($K_D = k_d/k_a$) of 23 nM. This value is roughly correlated with but is smaller than that reported previously, which gives the value of $10^{-7}$ mM (13, 19). This difference is probably from the different methods used (the plot of polymerization rate versus Arp2/3 concentration, thermodynamic analysis between labeled VCA and Arp2/3, and surface plasmon resonance measurement).

Association between VC fragment and Arp2/3 was very weak; with $k_a$, $k_d$, and $K_D$ values are $7.0 \times 10^2$ M$^{-1}$ s$^{-1}$, $1.2 \times 10^{-2}$ s$^{-1}$, and 17 mM, respectively (Fig. 4). With the VCa mutant, the graph clearly shows some affinity for Arp2/3 complex; with $k_a$, $k_d$, and $K_D$ values are $3.0 \times 10^3$ M$^{-1}$ s$^{-1}$, $8.2 \times 10^{-3}$ s$^{-1}$, and 2.7 mM, respectively (Fig. 4). This difference in $K_D$ values between VC and VCa suggests that acidic residues contribute to the association between Arp2/3 complex and N-
For the N-VCa mutant, the association rate was greater than and the dissociation rate was less than those seen with the VCa mutant ($ka = 5.8 \times 10^3 M^{-1}s^{-1}$ and $kd = 3.4 \times 10^3 s^{-1}$, respectively). This result indicates that the WH1 domain strengthens the binding of the Arp2/3 complex to the cofilin-homology region (Fig. 4, A and B). The $KD$ for the N-VCa mutant was about 590 nM, a value 10 times lower than that of the VCa mutant and 10 times higher than that of the VCA fragment. The difference in the $KD$ values of N-VCa and VCa mutants against Arp2/3 complex indicates that WH1 domain should provide the binding energy of −1 kcal/mol, which corresponds to about a 10-fold difference of the $KD$ values.

In the pull-down assay, we detected a strong association only between Arp2/3 complex and VCA. In the case of the N-VCa mutant, the association was very weak (Fig. 4 C), presumably due to the rapid dissociation of Arp2/3 from N-VCa ($kd = 3.4 \times 10^3 s^{-1}$) than from VCA ($kd = 2.7 \times 10^4 s^{-1}$). Both VCA and VCa mutants lacked affinity for Arp2/3 complex in the pull-down assay. These findings suggest that the dissociation of Arp2/3 complex from protein fragments missing the acidic region is very rapid. Therefore, acidic residues, which include conserved tryptophan, are presumably essential for retaining the Arp2/3 complex on N-WASP. Because VCa, which does not contain WH1, did not associate with Arp2/3 complex, but N-VCa mutant, which contains WH1, does associate with Arp2/3 in pull-down assay, WH1 domain certainly contributes to the association with Arp2/3 complex.

It should be noted that the N-VCa mutant and the VCA fragment were similar in their abilities to activate Arp2/3 complex despite a 10-fold difference in the affinities for the Arp2/3 complex (Figs. 2 and 4). This observation corroborates a previous report that the chimeric VCA fragment of N-WASP and WAVE1 were able to activate the Arp2/3 complex to the same extent as the VCA fragment of N-WASP although they possess different affinities for the Arp2/3 complex (23).
the Arp2/3 complex or the orientation between the Arp2/3 complex and N-WASP into that for higher affinity against G-actin, which might result in the high activity for inducing actin polymerization.

**Involvement of the WH1 Domain in Efficient Actin Polymerization and Actin Comet Formation**—We investigated the contribution of the WH1 domain in actin polymerization and actin comet formation in the presence of an intact VCA portion. First, we tested the ability of the IQ-VCA (ΔWH1) mutant of N-WASP to induce actin polymerization in a purified system and in bovine brain cytosol extract (Fig. 5). The purified system contained only pure actin and Arp2/3 complex. When the IQ-VCA (ΔWH1) mutant of N-WASP was added along with Cdc42 and PIP2 activators, it induced actin polymerization as reported previously (14). The rate of actin polymerization with wild-type N-WASP is faster than that of IQ-VCA (ΔWH1) mutant of N-WASP regardless of whether Cdc42 and PIP2 were included. The WH1 domain seems to enhance activation of Arp2/3 complex-mediated actin polymerization in proteins with an intact acidic region as well as in mutants with deletions in the acidic region (Fig. 5A).

In bovine brain cytosol stimulated with Cdc42, actin polymerization with the IQ-VCA (ΔWH1) mutant of N-WASP was weaker than that of wild-type N-WASP in both the rate and the amount of polymerization (Fig. 5B). Previous reports of actin polymerization by a ΔWH1 mutant in Xenopus extract also showed an instability in polymerized actin compared with that of wild type (14). The N-VCA mutant, which has a WH1 domain but lacks part of the acidic region, induced actin polymerization at a level similar to that induced by wild-type N-WASP. These results show that the WH1 domain contributes to the polymerization in bovine brain cytosol, possibly via interaction with the Arp2/3 complex.

We next examined the effect of the WH1 domain in actin comet formation, which is a model system for actin cytoskeletal structure formation. When exposed to cytosol, N-WASP-coated microspheres are able to form actin comets as WASP-coated beads (20, 24). In this system, N-VCA (Δa)-coated beads formed actin comets comparable with those of wild-type N-WASP. Comets generated from beads coated with the IQ-VCA (ΔWH1) mutant were apparently smaller than those from wild-type or N-VCA (Δa) N-WASP-coated beads (Fig. 5, C and D). Therefore, actin comet formation appears to require the WH1 domain. Collectively, the WH1 domain contributes to efficient actin polymerization as well as to actin comet formation in cytosolic extract, possibly by interacting with Arp2/3 complex.

In addition to the Arp2/3 complex, the WH1 domain is able to associate with WASP-interacting protein (WIP) and PIP2 (2, 25, 26). We do not know whether PIP2 and WIP contribute to actin comet formation. However, WIP seems to act as a scaffold for the assembly of actin comet-forming machinery such as N-WASP (26). The significance of WH1 binding to PIP2 is unclear (2) because deletion of the WH1 domain does not prevent PIP2-aided activation (Fig. 5A) (14–16). N-WASP-driven actin comet formation is able to be reconstituted without PIP2 or PIP2 or other factors with speed comparable with that in cytosolic extracts (27). From these findings, we conclude that an association between Arp2/3 complex and the WH1 domain is important for actin comet formation.

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