Wiskott-Aldrich Syndrome Protein Induces Actin Clustering without Direct Binding to Cdc42*

(Received for publication, March 15, 1999, and in revised form, June 24, 1999)

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WASP (Wiskott-Aldrich syndrome protein) was originally identified as the gene product whose mutation causes the human hereditary disease Wiskott-Aldrich syndrome. WASP contains many functional domains and has been shown to induce the formation of clusters of actin filaments in a manner dependent on Cdc42. However, there has been no report investigating what domain(s) is(are) important for the function. Here we present for the first time the results of detailed analyses on the domain-function relationship of WASP. First, the C-terminal verprolin-cofilin-acidic domain was shown to be essential for the regulation of actin cytoskeleton. In addition, we found that the clustering of WASP itself is distinct from actin clustering. The partial protein containing the region from the N-terminal pleckstrin homology domain to the basic residue-rich region also clustered especially around the nucleus as wild type WASP without inducing actin clustering. Finally, we obtained the quite unexpected result that a WASP mutant deficient in binding to Cdc42 still induced actin cluster formation, indicating that direct interaction between Cdc42 and WASP is not required for the regulation of actin cytoskeleton. This result may explain why no Wiskott-Aldrich syndrome patients have been identified with a missense mutation in the Cdc42-binding site.

WASP (Wiskott-Aldrich syndrome protein) was originally identified as the gene product whose mutation causes the human hereditary disease Wiskott-Aldrich syndrome (1). WASP is composed of 502 amino acid residues and contains many functional domains such as the pleckstrin homology (PH)1 domain, GTPase binding domain (GBD)/Cdc42 and Rac interactive binding (CRIB) motif, proline-rich (PR) domain, verprolin homology (VPH) domain, cofilin homology domain, and C-terminal highly acidic region. Accumulating evidence has shown that WASP directly interacts with many signaling and/or cytoskeletal proteins such as Cdc42 (2–4), WASP-interacting proline-rich (PR), basic-rich region (BR), and Rac interactive binding (CRIB) motif, among others. WASP contains many functional domains and has been identified as the gene product whose mutation causes the human hereditary disease Wiskott-Aldrich syndrome. However, it still remains unknown how WASP is regulated by Cdc42 and how WASP induces actin clustering. We here report the results of expression analyses of various WASP mutants. We identified the regions that are responsible for the regulation of actin cytoskeleton and the intracellular localization of WASP itself. The most unexpected and interesting result is that the direct binding between WASP and Cdc42 has no physiological function in the actin cluster formation induced by WASP, at least in these expression systems.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal anti-WASP antibody was prepared in rabbits immunized with bacterially expressed recombinant protein (amino acids 149–310). Antiserum was purified with protein A gel (Pierce). The monoclonal antibody specific for the c-Myc epitope tag was purchased from Santa Cruz Biotechnology, Inc. The secondary antibodies linked to alkaline phosphatase (used in Western blotting) and fluorescein (used in immunofluorescence microscopy) were from Promega and Capel, respectively. The polyclonal antibody specific for the Arp3 was prepared in rabbits according to the method of Welch et al. (18).

WASP Mutagenesis—Construction of mutants of ΔPHWI (deletion of amino acids 1–193), ΔWI (deletion of amino acids 155–193), ΔPRVCA (deletion of amino acids 311–502), and ΔPR (deletion of amino acids 311–413) was done by using the polymerase chain reaction. ΔPH (107–502) was obtained by AccI digestion and excision of the cDNA fragment.

*This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan and a Grant-in-Aid for Research for the Future Program from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†The abbreviations used are: PH, pleckstrin homology; CRIB, Cdc42 and Rac interactive binding; GBD, GTPase binding domain; GST, glutathione S-transferase; PR, proline-rich; VCA, verprolin-cofilin-acidic; WT, wild type; GTPyS, guanosine 5′-3-O-(thiotriphosphate); WI, WASP insert; BR, basic-rich region; VPH, verprolin homology.
coding for amino acids 1–106. PHWI (amino acids 1–222) and PHBR (amino acids 1–240) were obtained as cDNA truncated at the PvuII site and ApaLI site, respectively. H246D was prepared as follows. The polymerase chain reaction fragment (1–247; H246D) obtained with mutagenized primer was ligated to the PmaCI site of the wild-type cDNA fragment coding for amino acids 250–502.

Recombinant Proteins—WASP full-length (WT and H246D), CRIB (131–309; WT and H246D), and VCA (414–502) were obtained as glutathione S-transferase (GST) fusion proteins or Histidine (His)-tagged proteins. GST-full length, -CRIB, and -VCA were constructed by inserting the cDNA fragments into pGEX-4T-1 (Amersham Pharmacia Biotech). The His-CRIB construction was obtained by subcloning the cDNA fragments into pqE 30 (Qiagen). Cdc42 and profilin I were also obtained as GST fusion proteins (14, 15). GST fusion and His-tagged proteins were expressed and purified as described previously (14, 16).

Transient Expression in COS7 Cells and Immunofluorescence Microscopy—WASP (WT and mutants) and Cdc42 (G12V and T17N) were subcloned into pEF-BOS mammalian expression vectors (14). COS7 cells were transfected with these plasmids by electroporation and subjected to immunofluorescence microscopy as described previously (14). About 70% of transfected cells indicated each phenotype.

GST Fusion Protein-WASP Binding Assay—WASP (WT, H246D, or ΔPR)-expressing COS7 cell lysates were incubated with GST or GST-profilin I-immobilized glutathione beads. The bound proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-WASP antibody. GST or GST-profilin I was visualized by Coomassie Brilliant Blue staining of the gels.
For 2 h with rotation. Then they were washed, and Western blotting was performed. The Arp2/3 complex was purified from bovine brain by the method described previously (19).

**RESULTS**

Verprolin-Cofilin-Acidic Domain Is Essential for the Actin Clustering Induced by WASP—We first tried to determine which region of WASP is essential for the regulation of actin cytoskeleton. As described above, WASP is composed of many functional domains. In the case of N-WASP, another WASP family protein, both the VCA domain and the PR domain have been shown to be important for the formation of actin microspikes (14, 15, 17, 20). The VCA domain and the PR domain of N-WASP directly bind to actin and profilin, respectively (15, 17). In reference to these results, to examine which domain of WASP is important for the actin clustering, we prepared two deletion constructs ΔPRVCA (lacking both the PR and VCA domains) and ΔPR (lacking only the PR domain) schematically shown in Fig. 1A, and transfected COS7 cells with them. We checked the expression of each protein by Western blotting and confirmed that the two proteins are expressed at similar levels (Fig. 1B). We next immunostained the cells with anti-WASP antibody and phalloidin to visualize the expressed WASP (including mutants) and actin filaments (F-actin), respectively. As a result, ΔPR was found to induce actin clustering as well as did the wild type (WT), whereas ΔPRVCA did not (Fig. 2A), suggesting that the VCA domain is essential for the formation of actin clustering. This conclusion is consistent with a previous report in which the deletion of the VCA domain was shown to abolish the actin clustering activity (3).

The results presented above also suggest that direct binding to profilin is not required for the actin clustering, because the PR domain in N-WASP is the site for direct binding to profilin. To confirm this hypothesis, we examined the possible interaction between WASP and profilin. WASP (WT or ΔPR)-expressing COS7 cell lysate was incubated with GST or GST-profilin I.
Functional Analysis of WASP

that the unidentified signal that determines the intracellular localization of WASP exists within the 310 residues. The N terminus of WASP contains many functional domains such as the PH domain, WASP insert (WI) domain, which is WASP-unique proline-rich domain compared with N-WASP, basic-rich region (BR), and GBD/CRIB motif (Fig. 1A). To determine which domains are important for the clustering of WASP, we transfected COS7 cells with various N-terminal deletion constructs such as ΔPHWI, ΔWI, and ΔPH (Fig. 1A). As shown in Fig. 3A, ΔWI was shown to cluster around the nuclei with F-actin as well as did the WT, indicating that the WI domain is not required for the clustering. In contrast, ΔPHWI and ΔPH did not cluster around the nuclei but instead induced marked retraction of the plasma membrane (Fig. 3A). Most of the expressed ΔPHWI and ΔPH proteins seemed to accumulate at the plasma membrane. These results suggest that the PH domain is important for WASP clustering. We next examined whether the PH domain is sufficient for the clustering by expressing other N-terminus-modified constructs such as PH, PHWI, and PHBR (Fig. 1A). Although PH (data not shown) and PHWI were dispersed throughout the cytoplasm, PHBR clustered around the nuclei as did wild type WASP (Fig. 3B). These results indicate that the region from the PH domain to the basic-rich region is essential for WASP clustering.

WASP Induces Actin Clusters without Direct Cdc42 Interaction—A previous study by Symons et al. (3) showed that the expression of dominant-negative Cdc42 suppresses WASP-induced actin clustering. Indeed, we also confirmed that this inhibition occurs in COS7 cells (described later). To examine whether direct binding is required for actin cluster formation, we prepared a GBD/CRIB motif-modified construct (H246ΔASH; Fig. 1A).

We first checked whether this mutation really abolishes binding to Cdc42. First, we investigated the binding by using GST-Cdc42-immobilized beads. WASP (WT or H246ΔASH)-expressing COS7 cell lysates were incubated with GST or GST-Cdc42 (pre-loaded with GDP or GTPγS) immobilized on beads. Then Western blotting with anti-WASP antibody was performed. H246ΔASH failed to bind to GTPγS-loaded GST-Cdc42, whereas WT did bind (Fig. 4A). Second, we performed immunoprecipitation. WASP (WT or H246ΔASH) and c-Myc-tagged Cdc42G12V were co-expressed in COS7 cells, and the cell lysates were subjected to immunoprecipitation with anti-c-Myc antibody. Fig. 4B shows that Cdc42 did not co-precipitate with H246ΔASH at all. Third, we examined the direct association between GBD/CRIB motif in WASP and Cdc42. GST and GST-CRIB (WT and H246ΔASH) blotted on membranes were incubated with Cdc42 (loaded with 32P-labeled GTP), and a positive signal was detected by autoradiography. As shown in Fig. 4C, Cdc42 bound to only wild type GST-CRIB. The results of these three experiments clearly indicate that H246ΔASH does not bind to Cdc42.

We next examined the effect of H246ΔASH expression by immunofluorescence microscopy. Unexpectedly, this Cdc42-binding defective mutant also induced actin clusters like wild type WASP (Fig. 5A). In the case of N-WASP, the actin-regulating VCA domain is thought to be masked at the resting state by the intra- and/or intermolecular interaction with the region containing the GBD/CRIB motif (14). Another effector of Cdc42, p21-activated kinase is also thought to be kept inactive at the resting state, and its GBD/CRIB motif-modified mutant that does not bind to Cdc42 becomes constitutively active (21, 22). Because there is a possibility that the GBD/CRIB motif-modified mutant, H246ΔASH, is also constitutively active, we checked for interaction between WASP (WT and H246ΔASH) and its downstream effector, the Arp2/3 complex (23). For this

immobilized on beads, and then the bound protein was examined by Western blotting with anti-WASP antibody. Fig. 2B shows that ΔPR failed to bind to profilin, whereas WT did bind. Taken together, these results indicate that WASP induces actin clustering in a manner independent of direct binding with profilin via the PR domain.

Determination of the Region Essential for the Clustering of WASP Itself—It was shown that ΔPRVCA composed only of the N-terminal 310 residues of WASP clusters especially around the nucleus like the wild type WASP (Fig. 2A). This indicates
purpose, we first prepared anti-Arp3 antibody (Fig. 5B). Using this antibody, it was found that full-length WASP (WT and H246DΔSH) weakly binds to the Arp2/3 complex compared with the VCA protein and that there is no significant difference in binding to the Arp2/3 complex between WT and H246DΔSH. GST, GST-full length (WT or H246DΔSH), or GST-VCA immobilized on beads was incubated with purified Arp2/3 complex. The amount of bound Arp2/3 complex was examined by Western blotting with anti-Arp3 antibody. D, comparison of VCA-binding affinity between WT and H246DΔSH. GST or GST-VCA immobilized on beads was incubated with purified His-CRIB (WT or H246DΔSH) solutions. The bound proteins were subjected to Western blotting with anti-WASP antibody.

with H246DΔSH and Cdc42T17N. As shown in Fig. 6, the co-expression of dominant-negative Cdc42 also suppressed the formation of actin clusters induced by H246DΔSH. Thus, we conclude that WASP has some functional interaction with Cdc42, but direct physical interaction between them is not required for actin cluster formation.

DISCUSSION

It has been suggested that WASP plays a key role in regulating cytoskeletal reorganization. However, little is known about the mechanism by which it does so. WASP has many functional domains through which it interacts with various signaling and/or cytoskeletal proteins. Ectopic expression of WASP has been shown to induce the clustering of the expressed WASP itself (3). In addition, actin filaments are also accumulated at the clusters of WASP. Although the physiological relevance of this WASP/actin cluster is unknown, the existence of similar vesicle-like structures is reported in phorbol ester-treated MEG-01 magakaryoblastic cell line (11), suggesting the importance of the clusters in vivo. It has been reported that Cdc42 regulates the formation of these WASP/actin clusters (3), because activated Cdc42 directly binds to the GBD/CRIB motif of WASP and the expression of its dominant-negative mutant completely inhibits the cluster formation (3). Thus, we started to investigate which domains are required for the actin clustering and the accumulation of WASP itself and the role of Cdc42 in the process.

First, the VCA domain was shown to be essential for the accumulation of actin filaments. This result is consistent with a previous study in which the deletion of the C-terminal 59 amino acid residues resulted in the inability to induce the actin clustering (3). The VCA domain consists of a verprolin homology (VPH) domain, a cofilin homology domain, and a highly acidic region. We reported that direct interaction between the VPH domain and actin is essential for cytoskeletal reorganization in the case of N-WASP, suggesting that the VPH domain of WASP might also be required for actin clustering (20). Recently, it has been reported that the Arp2/3 complex, which plays an important role in nucleating actin filaments, binds to
the C terminus of WASP (23). The binding region was narrowed down to the C-terminal 38 residues in the case of WAVE/Scar1, a WASP/N-WASP-related protein that also possess a similar VCA domain. Moreover, it was demonstrated that the region plays a critical role in Rac-induced reorganization of the actin cytoskeleton (23, 24). The C-terminal Arp2/3 binding region is corresponds to the coflin homology domain and the acidic region. The deletion of eight residues in the coflin homology domain (amino acids 473–480), as expected, resulted in the inhibition of the accumulation of actin filaments (data not shown). Thus, both the actin binding to the VPH domain and the Arp2/3-binding to the region from coflin homology domain to the acidic region are important in actin clustering. This may trigger the actin nucleation by the Arp2/3 complex and induce the subsequent actin polymerization on newly formed actin filaments.

The region important for the clustering of WASP itself was narrowed down to one covering from the N-terminal PH domain to the basic residue-rich region. The PH domain seemed to be essential, because the deletion mutants of the PH domain showed a different intracellular localization from the wild type WASP. It has been reported that the PH domain may be responsible for cellular localization via interactions with specific lipids and/or proteins (25–31). In the case of phospholipase C-β, the PH domain specifically interacts with phosphatidylinositol 4,5-bisphosphate and localizes to plasma membrane (28). In contrast, the PH domain of β-adrenergic receptor kinase specifically recognizes the β subunit of the G-protein and, through the interaction with Gβγ, inactivates G-protein-coupled receptor (29–31). Therefore, it is quite probable and reasonable that the PH domain of WASP also recognizes some specific lipids and/or proteins and locates WASP to the special structures seen in the photographs shown in the figures.

Lastly, we found that WASP does not need to associate directly with Cdc42 to induce the actin cluster formation. The GBD/CRIB motif-containing region was shown to bind to the actin-regulating VCA domain (Fig. 5D), suggesting that WASP also takes some folded inactive structure as N-WASP. Thus, some other proteins and/or specific lipids play a role as an activator of WASP in cells. However, the results shown in Fig. 6 indicate that Cdc42 affects the actin cluster formation induced by WASP, even in the case of Cdc42-binding defective mutant. Some other factors link Cdc42 and WASP, signal transduction pathways of WASP and Cdc42 are both required for actin cluster formation, or Cdc42 functions downstream of WASP. Although we cannot answer here which possibility is true, this is a new concept and gives us a novel insight into the regulation and/or function of WASP.

Acknowledgment—We thank Shiro Suetsugu in our laboratory for profilin constructs.

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