BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **243,** 73–78 (1998)
ARTICLE NO. RC978064

Direct Binding of the Verprolin-Homology Domain in N-WASP to Actin Is Essential for Cytoskeletal Reorganization

Hiroaki Miki and Tadaomi Takenawa[1]

Department of Biochemistry, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Received December 16, 1997

Verprolin is a yeast protein whose inactivation leads to a cytoskeletal defect characterized by the abnormal organization of actin filaments. Recently, two mammalian proteins previously shown to regulate the actin cytoskeleton, Wiskott-Aldrich Syndrome Protein (WASP) and its homolog expressed in neurons (N-WASP), were found to possess short peptide motifs homologous to one part of verprolin. However, the physiological function of the homologous regions (verprolin-homology domain, VPH domain) remains unknown. Here we report the importance of the VPH domain as the direct actin binding region. In the case of N-WASP, the VPH domain co-acts with the cofilin-homologous region to sever actin filaments *in vitro*. Furthermore, the VPH domain is indispensable for the reorganization of the actin cytoskeleton by N-WASP downstream of tyrosine kinases in living cells. All data demonstrate that the VPH domain plays critical roles in the regulation of the actin cytoskeleton.

In searching for vinculin-related proteins in *Saccharomyces cerevisiae,* Donnelly et al. screened a *S. cerevisiae* library using vinculin cDNA as probe (1). As a result, an unidentified cDNA was isolated through homology between the poly-proline region-encoding nucleotide sequences, and the product was named verprolin. Disruption study of the gene (*VRP1*) revealed that verprolin plays important roles in the regulation and/or maintainance of actin cytoskeletal organization (1).

Recently, the gene whose mutation is responsible for the human hereditary disease Wiskott-Aldrich syndrome has been identified (2). The gene product, WASP, was shown to induce the granule-like assembly of actin filaments in a Cdc42- and/or tyrosine kinase-dependent manner (3, 4). The WASP sequence contains a short peptide motif homologous to one part of verprolin, and the authors named the sequence WASP homology 2 domain (3). However, it is still unclear how WASP regulates the actin cytoskeleton, and the function of the WASP homology 2 domain remains unknown.

We found a WASP homolog protein expressed in neurons (N-WASP) in the course of a study to examine the downstream signal transduction of the Ash/Grb2 adaptor protein (5-9). An ectopic expression study revealed that N-WASP also regulates the reorganization of the actin cytoskeleton downstream of tyrosine kinases and Cdc42 (9, 10). We have shown that the carboxyl-terminal region of N-WASP binds directly to actin and severs actin filaments *in vitro.* This region is composed of two domains homologous to known proteins, verprolin and cofilin. Cofilin is known to be an actin-binding protein and to sever actin filaments (11). Indeed, the deletion of the cofilin-homologous domain results in a severe reduction in actin-binding activity (9). On the other hand, the physiological function of the verprolin-homology (VPH) domain remains unclear.

In this paper, we re-analyze the carboxyl-terminal region of N-WASP to characterize the importance of the VPH domain, and report that it can bind directly to actin and is critical for the regulation of the actin cytoskeleton by N-WASP *in vivo.*

[1] To whom correspondence should be addressed. Fax: 81-3-5449-5417. e-mail: takenawa@hgc.ims.u-tokyo.ac.jp.

Abbreviations used: EGF, epidermal growth factor; G-actin, globular (monomeric) actin; GST, glutathione-S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; *S. cerevisiae, Saccharomyces cerevisiae;* SDS, sodium dodecyl sulphate; VPH domain, verprolin-homology domain; WASP, Wiskott-Aldrich Syndrome Protein.

0006-291X/98 $25.00

EXPERIMENTAL PROCEDURE

Antibodies. The anti-N-WASP antibody and anti-Ash/Grb2 antibody were prepared as described previously (9, 5). The anti-actin antibody was purchased from Chemicon International Inc. The anti-phosphotyrosine antibody, PY20, was from TAKARA. The anti-GST antibody was from Santa Cruz. The secondary antibodies linked to alkaline phosphatase were from Promega. Secondary antibody linked to fluorescein was from Cappel.

GST-fusion proteins. Glutathione-S-transferase (GST)-fusion proteins, such as G-VCA, G-VC, and G-V, were all prepared as described previously (9). The G-CA construct was generated as follows. The DNA encoding the CA region of N-WASP (amino acids 450-505 of N-WASP) was amplified by polymerase chain reaction (PCR) and the product was ligated into pGEX plasmid vector (Pharmacia Biotech.). The expression and purification of the recombinant proteins were performed as described previously (5).

Binding assay. GST-fusion proteins were immobilized on 20 μl Glutathione-Sepharose 4B beads (Pharmacia Biotech.) and then mixed with 20 μg globular actin (G-actin purified from chicken muscle as described previously (9)) in a final volume of 500 μl for 2 hours at 4 °C. After washing, the beads were suspended in sodium dodecyl sulphate (SDS) sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% skim milk-containing PBS and then incubated with anti-actin antibody. After washing, the membranes were incubated with a secondary antibody linked to alkaline phosphatase. Protein bands were detected with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

For binding assays with [^{35}S] methionine-labelled COS 7 cell lysates, the bound proteins were separated by SDS-PAGE and the gels were dried and subjected to autoradiography. Metabolic labelling of cultured cells with [^{35}S] methionine was carried out as described previously (9).

Actin depolymerization assay. 5 μM G-actin was first polymerized by the addition of 50 mM KCl and 1 mM MgCl2, and then 1, 2.5, or 5 μM GST-fusion protein (G-VCA, G-V, or G-CA,) or control GST was added to the solution. The mixtures were incubated for 10 min at room temperature and then centrifuged at 100,000 $\times$ g for 60 min. The resulting supernatants were subjected to SDS-PAGE and Coomassie Brilliant Blue staining.

Construction of ΔVPH mutant N-WASP. The CA region-encoding DNA was amplified by PCR (a *Pst I* recognition site was added to the forward primer at the 5′ terminus). The VCA-coding region was excised from the full length N-WASP by digestion with *Pst I* and *Eco RI*. Then, the PCR product (CA) was inserted into the *Pst I-Eco RI* site of the plasmid, replacing the VCA region with the CA region and deleting the VPH domain.

Ectopic expression of N-WASP in COS 7 cells. Plasmids containing the wild-type or ΔVPH mutant of N-WASP were transfected into COS 7 cells by electroporation as described previously (9). After culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24 hours, the culture medium was replaced with serum-free medium. After another 24 hours, cells were treated with 100 ng/ml human epidermal growth factor (EGF) (Gibco) for 10 min (control cells were untreated). The cells were then harvested and lysates were prepared for use in biochemical analyses such as Western blot and immunoprecipitation, or fixed in 3.7% formaldehyde for immunofluorescence microscopic analyses.

RESULTS AND DISCUSSION

The character of the VPH domain. The VPH domain consists of about 25 amino acid residues. Database search revealed the existence of several other proteins containing VPH domain sequences other than verprolin, WASP, and N-WASP. Sequence alignments of the VPH domains are shown in Fig. 1*A*. It is noteworthy that large hydrophobic residues (such as L and I) are perfectly conserved (LLxxIxxgxxL) in the central region of the VPH domains. Secondary structure prediction by the Chou-Fasman method (12) revealed that the VPH domain is likely to form an α-helix (data not shown). A wheel model of the central region of the VPH domain of verprolin is illustrated in Fig. 1*B*. As is clear from the figure, the conserved hydrophobic residues align to one side of the helix while the other side is rich in charged or polar residues. Thus, this is an amphipathic α-helix. This is also the case with the other VPH domains. Amphipathic α-helixes have been shown to be protein-protein interaction modules in several cases, as revealed by three dimensional protein structure analysis (13-16). This suggests the possibility that the VPH domain may also be a binding-interface to other proteins.

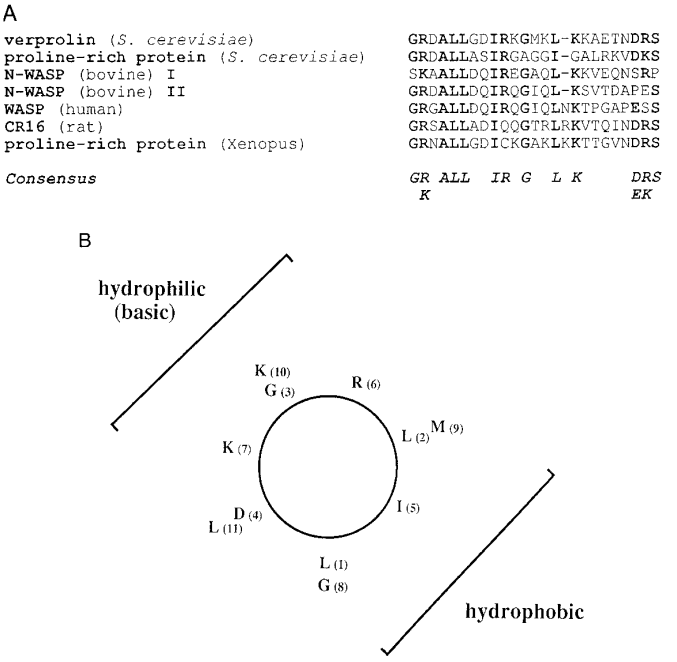

FIG. 1. Structure of VPH domains. A, amino acid sequence alignments of VPH domains. Database accession numbers (NCBI) are as follows: verprolin (*S. cerevisiae*), 439289; Bee1 (*S. cerevisiae*), 1101757; WASP (human), 695151; N-WASP (bovine), 1644232; CR16 (rat), 1185397; and proline-rich protein (Xenopus), 64956. Similar conserved residues are shown as bold letters. B, wheel model of the verprolin VPH domain. The central region (LLxxIxxGxxL) is shown. The order of the residues is also shown.

The VPH domain binds directly to actin. To examine the importance of the VPH domain in the actin-binding of N-WASP, the GST-fusion protein G-VCA (*v*erprolin-homology and *c*ofilin-homology domains,

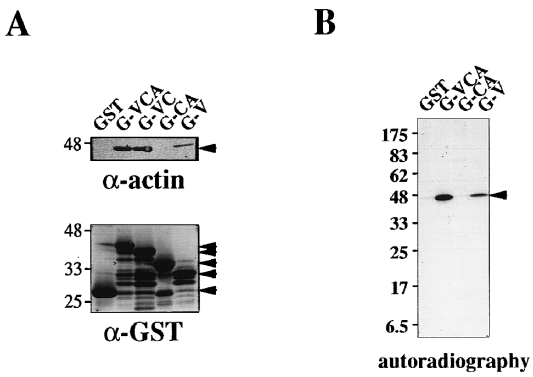

FIG. 2. Direct and specific binding of the VPH domain to actin. A, actin-binding assay against partial carboxyl-terminal fragments of N-WASP. GST-fusion proteins (G-VCA, G-VC, G-CA, and G-V) were immobilized on beads and then mixed with actin. The amount of bound actin was analyzed by Western blot with anti-actin antibody (upper figure). The amount of GST-fusion proteins was also checked with anti-GST antibody (lower figure). The GST-fusion proteins used are shown below the figure. Actin and GST-fusion proteins are indicated by arrowheads. B, binding assay using [^{35}S]-labelled lysates. Immobilized GST-fusion proteins were mixed with [^{35}S]-labelled lysates. The bound proteins were separated by SDS-PAGE and visualized by autoradiography. Actin is indicated by an arrowhead.

and highly *a*cidic region), G-VC, G-CA, and G-V were constructed, and actin binding assays were performed. As a result, it was found that G-VCA and G-VC bind to actin very effectively (Fig. 2*A*). G-V protein, which possesses only the VPH domain, still has actin-binding activity, although less than G-VCA or G-VC (Fig. 2*A*). However, no actin-binding was observed for G-CA protein (Fig. 2*A*). This clearly demonstrates that the isolated cofilin-homology domain does not bind to actin by itself, and that the VPH domain plays a central role in actin-binding and works cooperatively with the cofilin-homology domain.

To determine whether any other target molecule for the VPH domain exists, binding assays were performed using [^{35}S] methionine-labelled COS 7 cell lysates. As shown in Fig. 2*B*, G-V protein binds specifically to actin with no other specific positive signals observed, indicating that actin is the specific binding target of the VPH domain.

In addition, the actin-binding assay confirmed that the VCA region of WASP also binds directly to actin and that the VPH domain is required for binding (data not shown), suggesting that actin-binding is the universal role of VPH domains.

The VPH domain is required but not sufficient for actin-severing. The VCA region has previously been shown to have actin-severing (depolymerizing) activity (9). Thus, we examined whether it is required for this activity and whether the VPH domain can sever actin filaments by itself. The GST-fusion proteins G-VCA, G-V, and G-CA were used for the assay. As shown in Fig. 3*A*, incubation with G-VCA for only 10 min results in the depolymerization of significant amounts of actin. In contrast, G-V and G-CA have no marked effect on actin depolymerization in 10 min. Only a slight increase in G-actin is seen when G-V is used at the maximal concentration of 5 μM (the same concentration as actin).

Next we examined the effects of longer incubations with the recombinant proteins. As shown in Fig. 3*B*, 10 min incubation is sufficient for G-VCA to depolymerize actin filaments, indicating that this depolymerization is caused by filament severing, not the sequestration of G-actin. In contrast, longer incubations with G-V result in gradual actin depolymerization, although the amount of depolymerized actin is small compared to that resulting from incubation with G-VCA. This phenomenon can be explained as follows. As described above, G-V has weak binding activity to actin (Fig. 2*A* and *B*). Thus, G-V produces a change in the equilibrium of the actin polymerization state to change by binding and sequestering monomer pool actin. Indeed, a G-actin sequestering protein, profilin, has been shown to have similar effects on actin depolymerization (17). G-

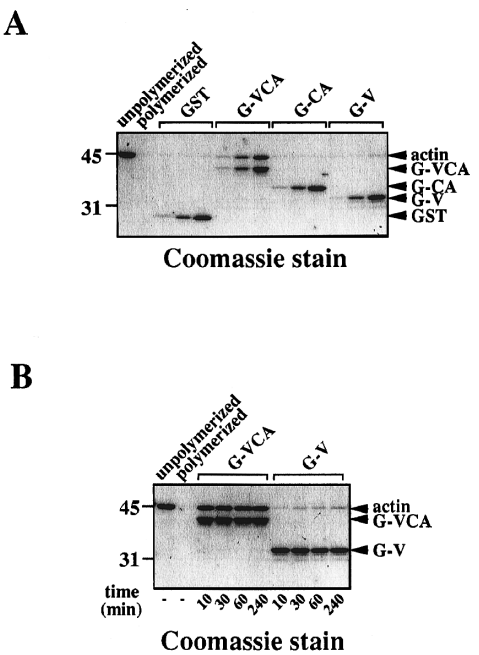

FIG. 3. Requirement of the VPH domain for actin severing. A, effect of various GST-fusion proteins on actin depolymerization. 5 μM actin was first polymerized and then 1, 2.5, or 5 μM GST-fusion proteins were added. After 10 min, the protein mixtures were centrifuged and the resulting supernatant fractions (containing G-actin) were subjected to SDS-PAGE. The gels were stained with Coomassie Brilliant Blue. Actin and GST-fusion proteins are indicated by arrowheads. B, time-course of actin depolymerization. 5 μM G-VCA and G-V proteins were used. Incubation times were varied as indicated in the figure. Other procedures were done as described in A. Actin and GST-fusion proteins are indicated by arrowheads.

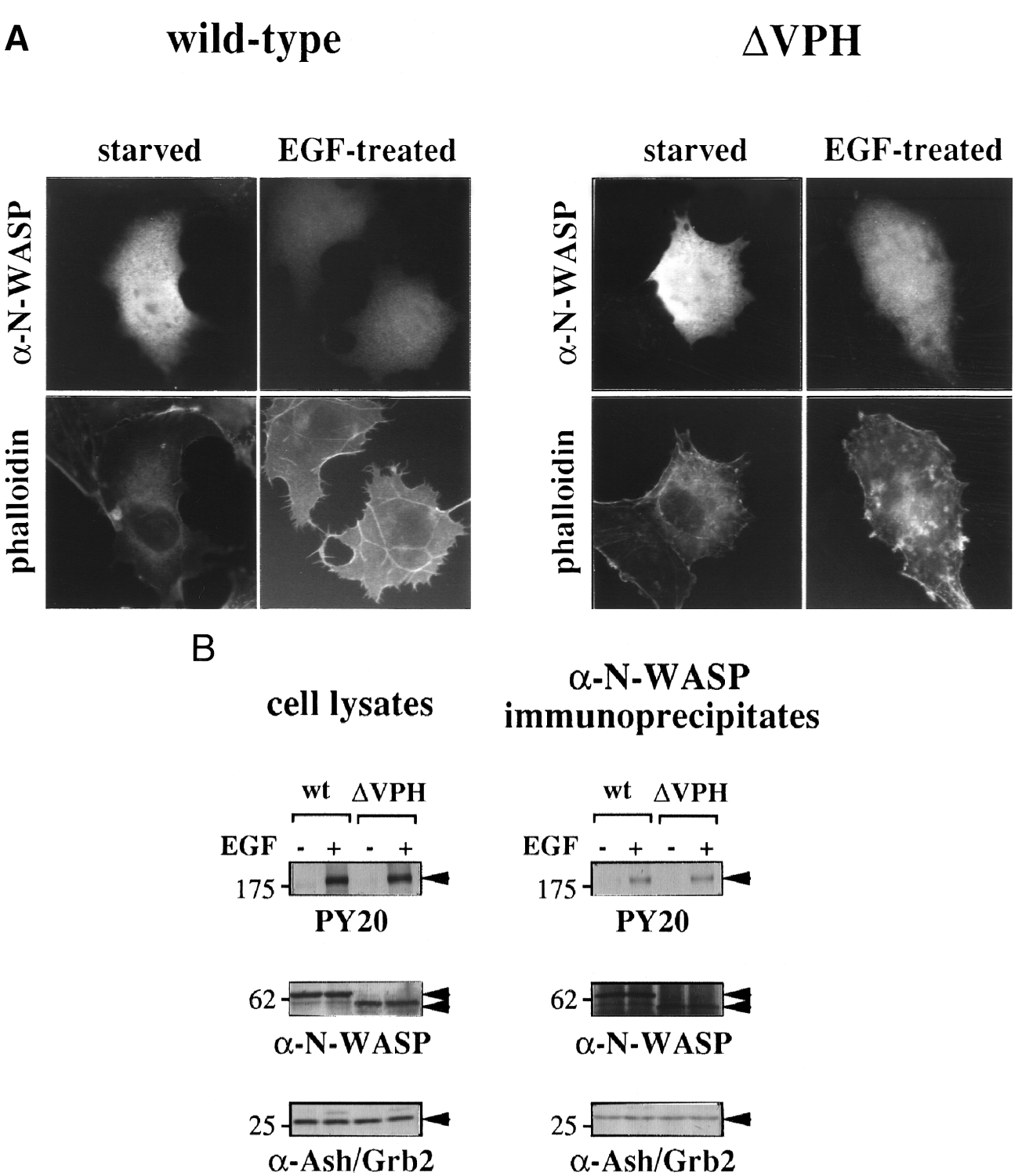

FIG. 4. Requirement of the VPH domain for actin cytoskeletal reorganization. A, Ectopic expression of wild-type or ΔVPH mutant N-WASP in COS 7 cells. Plasmids were transfected into COS 7 cells by electroporation. Cells were serum-starved and then stimulated with or without EGF for 10 min. After fixation, cells were stained with anti-N-WASP antibody and phalloidin (to visualize actin filaments). The plasmids used are shown at the top of the photographs. The left half of the photographs indicates cells not treated with EGF; the right indicates cells treated with EGF. B, complex formation of wild-type or ΔVPH mutant N-WASP with Ash/Grb2 and tyrosine-phosphorylated EGF receptor. Transfected cell lysates were immunoprecipitated with anti-N-WASP antibody and then subjected to Western blot analysis with anti-N-WASP, anti-Ash/Grb2, and PY20. Cell lysates were also directly subjected to Western blot analysis. Tyrosine-phosphorylated EGF receptor, wild-type (wt) and ΔVPH mutant (ΔVPH) N-WASP, and Ash/Grb2 are indicated by arrowheads.

CA does not cause any actin depolymerization even after 4 hours of incubation (data not shown), which is reasonable since G-CA has no actin binding activity (Fig. 2*A* and *B*).

The results presented here clearly indicate that the VPH domain is required for actin severing although the isolated VPH domain cannot sever actin filaments and only binds to G-actin.

The VPH domain is indispensable for the in vivo regulation of the actin cytoskeleton. To examine the functional importance of the VPH domain *in vivo*, we generated a N-WASP mutant that lacks the VPH domain (ΔVPH). As previously reported (9), the ectopic expression of wild-type N-WASP in COS 7 cells induces the formation of actin-microspikes in a process dependent on EGF treatment (Fig. 4*A*). In addition, N-WASP forms signalling complexes with tyrosine-phosphorylated EGF receptor, possibly through an adaptor protein Ash/Grb2 (Fig. 4*B*).

When the ΔVPH mutant is expressed, the localization of ΔVPH does not differ so markedly from wild-type N-WASP (Fig. 4*A*). However, the treatment of cells with EGF has no effect on the organization of the actin cytoskeleton, that is, they cannot form microspikes (Fig. 4*A*). This result suggests that the ΔVPH mutant does not reorganize the actin cytoskeleton because of its inability to sever actin filaments.

In support of this possibility, complex formation with Ash/Grb2 and tyrosine-phosphorylated EGF receptor occurred normally even in the case of the ΔVPH mutant (Fig. 4*B*). In control experiments using pre-immune serum, these proteins did not co-precipitate with wild-type and ΔVPH mutant N-WASP (data not shown). Taken together, we conclude that the ΔVPH mutant receives the input signal from tyrosine kinases, but cannot transmit the signal to the downstream target, that is, to the actin cytoskeleton.

Function of other VPH domain-containing proteins. In this report, we demonstrate the importance of the VPH domain *in vitro* and *in vivo.* Especially, we show that the VPH domain binds directly to actin. As described above, VPH domains are also found in several other proteins including verprolin, WASP, *S. cerevisiae* Bee1 (putative WASP/N-WASP homolog protein in *S. cerevisiae*), rat brain CR16, and *Xenopus laevis* proline-rich protein (shown in Fig. 1*A*). Thus, VPH domains are conserved from yeast to mammals. Among these proteins, verprolin, WASP, and Bee1 have already been shown to regulate the actin cytoskeleton (1, 3, 4, 18). The physiological function of the other proteins has not yet been studied; however, it is possible that they also participate in the regulation of the actin cytoskeleton.

Finally, there is one more important point. All the VPH domains thus far identified are preceded by long poly-proline stretches. A poly-proline motif is known to be a binding site to profilin (19), which participates in actin polymerization (20, 21). In fact, several poly-proline motif containing proteins such as VASP (22), Mena (23), and mDia (24) are shown to bind directly to profilin and induce actin polymerization *in vivo.* Furthermore, we also confirmed that N-WASP associates directly with profilin (data not shown). At present, we cannot tell clearly how VPH domains and poly-proline sequences work together. However, we think that this fact also supports the possibility that VPH domain-containing proteins participate in the reorganization of the actin cytoskeleton.

ACKNOWLEDGMENTS

We thank Drs. C. Sasakawa and T. Suzuki (Department of Bacteriology, Institute of Medical Science, University of Tokyo) for providing us with the oligonucleotide primers used for constructing the G-CA and ΔVPH expression plasmids.

REFERENCES

1. Donnelly, S. F., Pocklington, M. J., Pallotta, D., and Orr, E. (1993) *Mol. Microbiol.* **10,** 585–596.
2. Derry, J. M. J., Ochs, H. D., and Franke, U. (1994) *Cell* **78,** 635–644.
3. Symons, M., Derry, J. M. J., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Franke, U., and Abo, A. (1996) *Cell* **84,** 723–734.
4. Miki, H., Nonoyama, S., Zhu, Q., Aruffo, A., Ochs, H. D., and Takenawa, T. (1997) *Cell Growth. Differ.* **8,** 195–202.
5. Miki, H., Miura, K., Matuoka, K., Nakata, T., Hirokawa, N., Orita, S., Kaibuchi, K., Takai, Y., and Takenawa, T. (1994) *J. Biol. Chem.* **269,** 5489–5492.
6. Nakanishi, H., Orita, S., Kaibuchi, K., Miura, K., Miki, H., Takenawa, T., and Takai, Y. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1255–1261.
7. Miura, K., Miki, H., Shimazaki, K., Kawai, N., and Takenawa, T. (1996) *Biochem. J.* **316,** 639–645.
8. Itoh, T., Miura, K., Miki, H., and Takenawa, T. (1996) *J. Biol. Chem.* **271,** 27931–27935.
9. Miki, H., Miura, K., and Takenawa, T. (1996) *EMBO J.* **15,** 5326–5335.
10. Miki, H., Sasaki, T., Takai, Y., and Takenawa, T. (1998) *Nature* **391,** 93–96.
11. Nishida, E., Maekawa, S., and Sakai, H. (1984) *Biochemistry* **23,** 5307–5317.
12. Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13,** 222–245.
13. Knighton, D. R., Zheng, J. H., Ten Eyke, L. F., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) *Science* **253,** 414–420.
14. Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) *Nature* **376,** 191–194.
15. Bagby, S., Kim, S., Maldonado, E., Tong, K. I., Reinberg, D., and Ikura, M. (1995) *Cell* **82,** 857–867.
16. Li, T., Stark, M. R., Johnson, A. D., and Wolberger, C. (1995) *Science* **270,** 262–269.
17. Nishida, E., Maekawa, S., and Sakai, H. (1984) *J. Biochem.* **95,** 399–404.

18. Li, R. (1997) *J. Cell Biol.* **136,** 649–658.
19. Tanaka, M., and Shibata, H. (1985) *Eur. J. Biochem.* **151,** 291–297.
20. Pantaloni, D., and Carlier, M. F. (1993) *Cell* **75,** 1007–1014.
21. Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldscmidt-Clermont, P. J., and Mitchson, T. J. (1994) *Cell* **76,** 505–517.
22. Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., and Jockusch, B. M. (1995) *EMBO J.* **14,** 1583–1589.
23. Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano P. (1996) *Cell* **87,** 227–239.
24. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kaizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997) *EMBO J.* **16,** 3044–3056.