Biochemical and Biophysical Research Communications **291,** 41–47 (2002)
doi:10.1006/bbrc.2002.6406, available online at http://www.idealibrary.com on IDEAL®

WICH, a Novel Verprolin Homology Domain-Containing Protein That Functions Cooperatively with N-WASP in Actin-Microspike Formation

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Received December 31, 2001

We describe a novel protein that contains a verprolin-homology (V) region, through which several actin-regulating proteins, including Wiskott–Aldrich syndrome protein (WASP) family members, bind directly to actin. The amino acid sequence is homologous to the sequences of WASP-interacting protein (WIP) and CR16, both of which associate with WASP and/or N-WASP, and thus these three proteins constitute a new protein family. We named the protein WICH (*WI*P- and *C*R16-*h*omologous protein). WICH associates strongly with N-WASP but only weakly with WASP via its C-terminal WASP-interacting (W) region. Ectopic expression of WICH induces actin-microspike formation through cooperation with N-WASP. In addition, expression of the W fragment of WICH suppresses microspike formation induced by N-WASP, indicating an essential role for WICH in N-WASP-induced microspike formation.

Key Words: **WASP; WIP; CR16; WICH; actin; microspike.**

The WASP family proteins, WASP, N-WASP, and WASP family verprolin homologous protein (WAVE)/Suppressor of cAMP receptor (Scar) subfamily proteins (WAVE 1, 2, and 3), regulate reorganization of the actin cytoskeleton through their C-terminal verprolin homology-cofilin homology-acidic (VCA) regions (1). It has been shown that the verprolin homology (V) domain binds directly to monomeric actin (G-actin) (2) and that the cofilin homology-acidic (CA) region interacts with Arp2/3 complex (3–5), leading to activation of the complex, which plays an important role in nucleating actin polymerization. *In vitro* reconstitution studies (5–8) revealed that N-WASP is activated via cooperative binding to phosphatidylinositol 4,5-bisphosphate (PIP2) and Cdc42, a Rho family small GTPase. Activated N-WASP then induces actin polymerization through the Arp2/3 complex, which is thought to be the basis for inducing filopodium formation by N-WASP (9, 10). Recently, our group and several others reported that N-WASP can be activated by various SH3 domain-containing proteins, including Grb2/Ash (11), WISH (12), and Nck (13). Although the precise molecular mechanism has yet to be determined, indications are that N-WASP functions downstream of various intracellular signaling cascades. Another WASP family member, WAVE, has been shown to participate in membrane ruffling induced by Rac, another Rho family protein (4, 14). In this case, Rac does not directly bind to WAVE but instead binds indirectly through IRSp53, which binds to both Rac and WAVE (15). Therefore, the WASP family proteins appear to be the integrating platform of various upstream signals for reorganization of the actin cytoskeleton. In either case, V domain-deleted mutants of N-WASP or WAVE are incapable of inducing filopodia formation or membrane ruffling (2, 14). The V domain, therefore, is thought to play an essential role in reorganization of the actin cytoskeleton by WASP family proteins.

There are several other proteins that contain a V domain. One such protein is WIP, which was originally discovered in a screen for WASP-interacting proteins (16). WIP has one V domain at the N-terminus, and it binds to actin via the V domain (17). Interestingly, WIP also contains a region at its C-terminus where binding

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0006-291X/02 $35.00

to WASP and N-WASP occurs (16, 17). A recent report indicated an essential role for WIP in filopodium formation via cooperation with N-WASP in fibroblasts (17). WIP rescues a cell polarity defect in verprolin-null mutant yeast, suggesting that WIP is the human homolog of verprolin (18). A V domain-modified mutant of WIP, which can no longer bind actin, fails to rescue verprolin-null mutant yeast, also suggesting the importance of the V domain in WIP. CR16, which is identified as a glucocorticoid-inducible gene product (19), has recently been shown to be a binding partner of N-WASP (20). CR16 also contains a V domain and an N-WASP-binding region at its N-terminus and C-terminus, respectively, and it shows approximately 40% amino acid identity with WIP over its entire length, suggesting the functional similarity between CR16 and WIP.

Here we report a novel V domain-containing protein, WICH, that shows significant homology to both WIP and CR16. Ectopic expression analyses revealed that WICH plays an important role in actin-microspike formation through cooperation with N-WASP.

MATERIALS AND METHODS

Cloning of WICH. Full-length WICH cDNA was isolated by screening a Jurkat T-cell cDNA library. EST clone (AA339495), which was isolated from the cDNA library by polymerase chain reaction (PCR), was used as a probe. The fragment was used to probe Human Multiple Tissue Northern Blots (CLONTECH) to examine expression of WICH mRNA.

Antibodies. Anti-WICH antibody was generated in rabbits immunized with a His-tagged fragment (amino acids 381-429) of WICH. For purification of the anti-WICH antibody, the antiserum was affinity-purified. Anti-c-Myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology, Inc. Anti-actin monoclonal antibody was from Chemicon International, Inc. The polyclonal antibody for N-WASP was prepared as described previously (21). The secondary antibody conjugated to alkaline phosphatase (used in Western blotting) was from Promega, and fluorescence (FITC and Cy5)-linked antibody (used in immunofluorescence microscopy) was from Capel and Amersham Pharmacia Biotech, respectively.

Recombinant proteins. His-tagged full-length WICH was expressed in Sf9 insect cells with the Bac-to-Bac Baculovirus Expression System (Gibco BRL). His-WICH was purified with Ni^{2+}–NTA–agarose beads (Qiagen). The purified His-WICH protein tended to form aggregates, and thus 0.5% octylglucoside was included in the buffer. The method of purification for N-WASP was described previously (10). Partial proteins of WICH (N, NΔV, W) were expressed in *E. coli* as a GST-fusion proteins and purified as described previously (22).

Binding assays with GST-fusion proteins. GST-fusion proteins were first immobilized on glutathione beads and then incubated with appropriate proteins or cell lysates for 2 h with continuous rotation. After beads were washed with buffer, they were suspended in SDS-sample buffer and subjected to SDS-PAGE and then Coomassie brilliant blue staining or Western blot analysis. Actin (G- and F-form) and cell lysates were prepared as follows: G-actin was prepared from rabbit skeletal muscle and purified by gel filtration on Sephadex 200 (Pharmacia Biotech) in G-buffer (2 mM Tris/HCl (pH 8.0), 0.1 mM $CaCl_2$, 0.1 mM ATP and 1 mM DTT). F-actin was prepared from purified G-actin by adding KCl, $MgCl_2$ and ATP (final concentrations of 100 mM, 1 mM and 0.2 mM, respectively). Cells transfected with indicated constructs were harvested with lysis buffer (50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100) and briefly sonicated. After centrifugation, the soluble fraction was collected as cell lysate.

Actin depolymerization assay. Actin was labeled with pyrene as previously described (5). Pyrene-labeled actin was first polymerized, and the resulting F-actin solution was mixed with the indicated proteins. The F-actin-containing mixture was diluted to 0.5 μM (calculated as the concentration of G-actin included), and then the fluorescence intensity was determined as an indicator of the polymerization state of actin as previously described (5).

Transient expression in mammalian cells and immunofluorescence microscopy. Myc-tagged WICH (WT and ΔV) was subcloned into pEF-BOS mammalian expression plasmid vectors. N-WASP was contained in pcDL-SRα plasmid (9). Cells were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's instructions. After transfection, cells were starved for 24 h in serum-free Dulbecco's modified Eagle medium. When needed, cells were treated with 100 ng/ml epidermal growth factor (EGF) (Gibco BRL) for 10 min and then fixed with formaldehyde. The fixed cells were subjected to immunofluorescence microscopy as previously described (9).

RESULTS AND DISCUSSION

Molecular Cloning of WICH

A search of the database for a novel V domain-containing protein yielded an EST clone, AA339495. We screened a Jurkat T-cell cDNA library with this EST as a probe and isolated a cDNA clone encoding the full length of a novel protein (WICH, for *Wi*P- and *C*R16-*h*omologous protein), which contains a V domain. WICH comprises 440 amino acids containing one V domain and many proline-rich regions (Fig. 1A). Across the entire protein, WICH showed approximately 30–40% identity to WIP and CR16 (Fig. 1B). Similarity was especially in the N-terminal V domain and the C-terminal putative WASP-binding region. This sequence similarity indicates that these three proteins constitute a protein family. As described above, both WIP and CR16 play important roles in regulation of the actin cytoskeleton through interactions with WASP and/or N-WASP (16, 17, 20). We hypothesized that WICH also plays an important role in regulation of the actin cytoskeleton through binding to actin and WASP family proteins.

Northern blot analysis indicates that mRNA of WICH is ubiquitously expressed, with high expression in brain, kidney, lung, and placenta (Fig. 2A). We also performed an Western blot analysis using anti-WICH antibody. The results indicated that the WICH protein exists mainly in brain, colon, lung, and stomach (Fig. 2B).

WICH Associates with Actin and N-WASP

To confirm that the V domain of WICH interacts with actin, we prepared constructs encoding an N-terminal partial protein of WICH that contains the V

A

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  1 MPIPPPPPPPPPGPPPPPPTFHQANTEQPKLSRDEQRGRGALLQDICKGTKLK
 51 KKVTNINDRSAPILEKPKGSSGGYGSGGAALQPKGGLFQGGVLKLRPVGA
101 KDGSENLAGKPALQIPSSRAAAPRPPVSAASGRPQDDTDSSRASLPELPR
151 MQRPSLPDLSRPNTTSSTGMKHSSSAPPPPPPGRRANAPPTPLPMHSSKA
201 PAYNREKPLPPTPGQRLHPGREGPPAPPPVKPPPSPVNIRTGPSGQSLAP
251 PPPPYRQPPGVPNGPSSPTNESAPELPQRHMSLHRKTPGPVRGLAPPPPT
301 SASPSLLSNKPPPPARDPPSRGAAPPPPPPVIRNGARDAPPPPPPYRMHG
351 SEPPSRGKPPPPPSRTPAGPPPPPPPPLRNGHRDSITTVRSFLDDFESKY
401 SFHPVEDFPAPEEYKHFQRIYPSKTNRAARGAPPLPPILR
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B

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wich    1 MPIPPPPPPPP-------G-PPPPPTFH-QANTEQPKLSRDEQRGRGALLQDICKGTKLK   51
CR16    1 MPVPPPPPPPLPPPPPPLGAPPPPPPSAPPVSTDTSSLRRADPKGRSALLADIQQGTRLR   60
WIP     1 MPVPPPP------------APPPPPTFA-LANTEKPTLNKTEQAGRNALLSDISKGKKLK   47

wich   52 KVTNINDRSAPILEKPKGS-SGGYGSGG--------------------AALQPKGGLFQG   90
CR16   61 KVTQINDRSAPQIESSKGTNKEGGGSANTRGA----------------STPPTLGDLFAG  104
WIP    48 KTV-TNDRSAPILDKPKGAGAGGGGGGFGGGGGFGGGGGGGGGGSFGGGGPPGLGGLFQA  106

wich   91 GVLKLRPVGAKDGSENLAG---KPALQIPSSRAAAPRPPVSAASGRPQDDTDSSRA-SLP  146
CR16  105 GFPVLRPAGQRDVAGGKTG--QGPGSRAPSPRLPN-----KTISGPLI-PPASPRLGNTS  156
WIP   107 GMPKLRSTANRDNDSGGSRPPLLPPGGRSTSAKPF-----SPPSGPGRFPVPSPGHRSGP  161

wich  147 ELPRMQRPSLPDLSRPNTTSSTGMKHSSSAPPPPPPGRRANAPPTPLPMHS-SKAPAYNR  205
CR16  157 EAHGAA---RTAPPRPNVP----------APPPPTPPPPPPPLPPPLPSSSPIKTPLVSP  203
WIP   162 PEP--QRN-RMPPPRPDVGSKP-----DSIPPPVPSTPRPIQSSLHNRGS---------P  204

wich  206 EKPLPPTPGQRLHPGREGPPAPPPVKPPPSPVNIRTGPSGQSLAPPPPPYRQPPGVPNGP  265
CR16  204 PGPL--TKGNLPVVAPPVPCAPPPPPPPPPPTPPPLPPASVLSDKAVKPQLAPLHLPPIP  261
WIP   205 PVPG-----------------GPRQPSPGPTPPPFPGNRGTALGGGSIRQSPLSSSSPF  246

wich  266 SSPTNESAPELPQRHNSLHRKTPGPVR------GLAPPPPTSAS----------------  303
CR16  262 PPLPLLPPCGYPGLKAEPASPAQDAQE------PPAPPPPLPPY---ASCSPRASLPAPP  312
WIP   247 SNRPPLPPTPSRALDDKPPPPPPPVGNRPSIHREAVPPPPPQNNKPPVPSTPRPSAPHRP  306

wich  304 ----------------------------------------------------PSLLSNRPP  312
CR16  313 -LPGVNSSSETPPPLPPKSPSF---QAPPQKAGAQALPAPPAPPGSQPFLQKKRHGRPGA  368
WIP   307 HLRPPPPSRPGPPPLPPSSSGNDETPRLPQRNLSLSSSTPPLPSPGRSGPLPPPPSERPP  366

wich  313 PPARDPPSRGAAPPPPPPVIRNGARDAPPPPPPYRMHGSEPPSRGKPPPPPSRTP---AG  369
CR16  369 G----------------------GGKLNPPPAPPARSPTTELSSKS--------QQATAWT  399
WIP   367 PPVRDPPGRSGPLPPPPPVSRNGSTSRALPATPQLPSRSGVDSPRSGPRPPLPPDRPSAG  426

wich  370 PPPPPPPP--LRNGHRDSITTVRSFLDDFESKYSFHPVEDFPAPEEYKHFQRIYPSKTNR  427
CR16  400 PTQQPGGQ--LRNGSLHII-------DDFESKFTFHSVEDFPPPDEYKPCQKIYPSKIPR  450
WIP   427 APPPPPPSTSIRNGFQDSPCE-----DEWESRFYFHPISDLPPPEPYVQTTKSYPSKLAR  481

wich  428 --------------------AARGAPPLPPILR                             440
CR16  451 SRTPGPWLQAEAVGQSSDDIKGRNSQLSLKTLR                             483
WIP   482 ----------NESRSGSNRRERGAPPLPPIPR                              503
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C

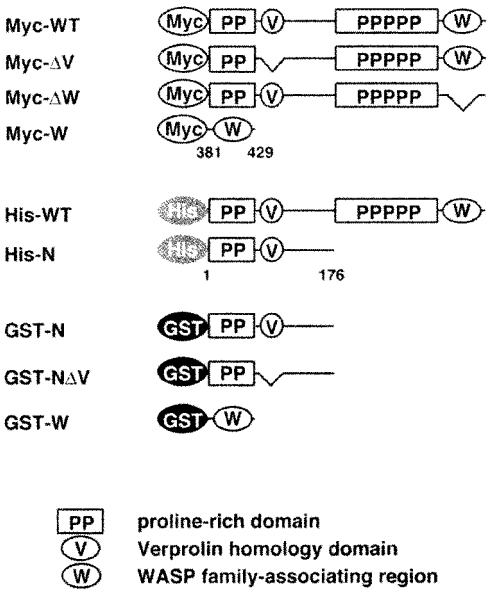

FIG. 1. WICH sequence. (A) Amino acid sequence of WICH. The V domain is shown in boldface. The WASP family-associating (W) region is boxed. (B) Comparison of amino acid sequences of WICH, WIP, and CR16. Residues identical between two or three members are boxed. (C) Schematic diagrams of the expression constructs used in this study.

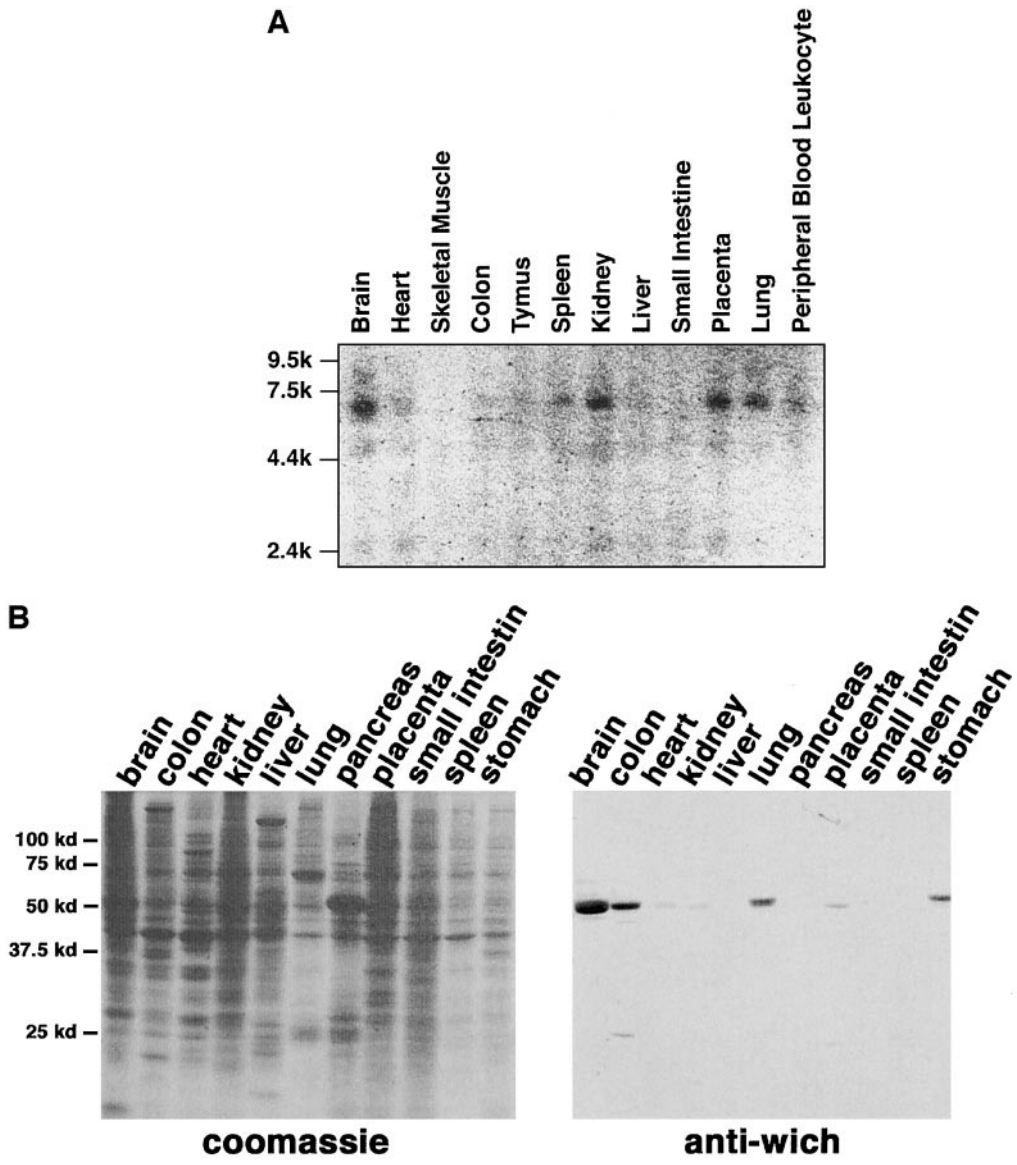

FIG. 2. Distribution of WICH. (A) Northern blot analysis of the expression of WICH. A Human Multiple Tissue Northern Blot (Clontech) was used. A cDNA encoding the V domain of WICH was labeled with [α-^{32}P]dCTP and used as the probe. (B) Western blot analysis. The indicated organs were taken from mice and homogenized in lysis buffer. The resulting lysates were subjected to SDS–PAGE and Western blot analysis with anti-WICH antibody. A Coomassie-stained image of the gel is also shown.

domain (GST-N) and a V domain deletion mutant (GST-NΔV) of WICH (shown schematically in Fig. 1C) and mixed the resulting proteins with monomeric actin (G-actin). As shown in Fig. 3A, GST-N interacted with G-actin, whereas GST-NΔV did not associate with G-actin, indicating that the V domain is essential for binding to G-actin. We also examined whether WICH binds to filamentous actin (F-actin). We expressed His-tagged full-length WICH with recombinant baculoviruses and subjected the His-WICH protein to ultracentrifugation in the presence or absence of F-actin. As shown in Fig. 3B, clear co-sedimentation of His-WICH was observed in the presence of F-actin but not in the absence of F-actin. This result indicates that WICH associates with F-actin as well as G-actin, which is consistent with previous finding for WIP and CR16 (17, 20). WIP was also shown to reduce the rate of spontaneous actin depolymerization and was thus suggested to stabilize F-actin (17). We therefore examined whether WICH also reduces the rate of actin depolymerization. When F-actin solution was diluted, spontaneous actin depolymerization occurred as reported previously (17). However, in the presence of WICH, the rate of actin depolymerization was reduced (Fig. 3C). This effect is similar to that of WIP, and therefore, WICH may be involved in stabilization of F-actin.

The C-terminal regions of WIP and CR16 have been reported to bind to WASP and/or N-WASP (16, 17, 20).

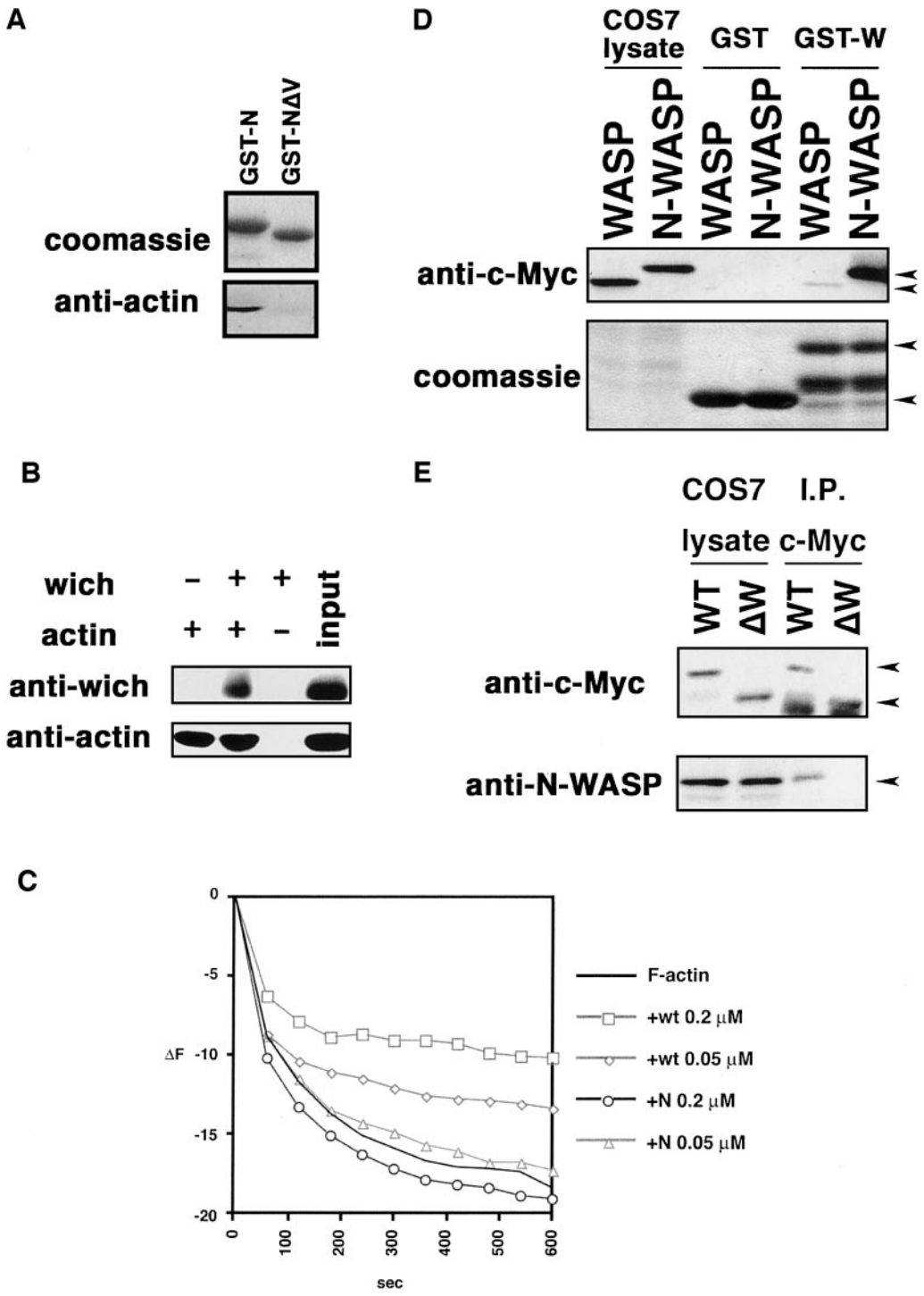

FIG. 3. WICH binding to actin and N-WASP. (A) Western blot showing binding of WICH to G-actin. GST-N or GST-NΔV immobilized on glutathione beads was incubated with 1 μM G-actin. Actin bound to GST-fusion proteins was detected by Western blotting with anti-actin monoclonal antibody. (B) Binding of WICH to F-actin. His-tagged full-length WICH was mixed with F-actin. The mixture was then ultracentrifuged, and the resulting supernatants and precipitates were examined by SDS-PAGE. (C) Stabilization of F-actin by WICH. 0.5 μM F-actin (pyrene-labeled) was incubated with indicated concentrations of His-tagged full-length or N-fragment of WICH. The fluorescence intensity was then determined as an indicator of the polymerization state of actin. (D) Western blot showing binding of WICH to N-WASP. GST-W immobilized on beads was incubated with lysates of COS7 cells expressing Myc-tagged WASP or N-WASP. Bound proteins were analyzed by Western blotting with anti-c-Myc monoclonal antibody. (E) The W region is required for interaction of WICH with N-WASP. Lysates derived from COS7 cells expressing both N-WASP (non-tagged form) and Myc-tagged WICH (WT or ΔW) were immunoprecipitated with anti-c-Myc monoclonal antibody. The precipitates were then subjected to Western blotting with anti-N-WASP antibody and anti-c-Myc antibody.

Because the C-terminal region of WICH is similar to those of WIP and CR16, WICH may also interact with WASP family proteins. To examine this possibility, we prepared a C-terminal partial protein of WICH (GST-W) (Fig. 1C) and performed a pull-down assay of lysates from cells expressing Myc-tagged WASP or N-WASP and found that N-WASP binds to GST-W (Fig. 3D). In contrast, we observed precipitation of a very small amount of WASP, suggesting that WICH binds preferentially to N-WASP. In addition, because WASP expression is restricted to hematopoietic cells and because both N-WASP and WICH are predominantly present in non-hematopoietic cells, N-WASP, rather than WASP, should be the functional partner of WICH. To confirm that the W region (WASP family-associating region) is required for binding, we prepared Myc-tagged ΔW WICH (Fig. 1C) and performed an immunoprecipitation assay to detect *in vivo* interaction between N-WASP and WICH (WT or ΔW). As shown in Fig. 3E, WT WICH was precipitated with N-WASP, whereas ΔW WICH was not. These results confirm that WICH associates with N-WASP via its C-terminal W region.

WICH Cooperates with N-WASP in Actin-Microspike Formation

To investigate the function of WICH *in vivo*, we expressed WICH (WT or ΔV) ectopically in COS7 cells. We found that expression of WT WICH greatly reduced the amount of actin filaments and the image of phalloidin-stained cells became "dark" (Fig. 4A). In contrast, ΔV WICH had no significant effect on the organization of the actin cytoskeleton (data not shown), indicating the importance of the V domain. We do not know the mechanism by which such a reduction of actin filaments occurs in WT WICH-expressing cells, but the actin binding V domain presumably sequesters G-actin and inhibits actin polymerization.

We then examined the effect of co-expression of N-WASP and WICH. As shown in Fig. 4A, expression of N-WASP alone did not alter the actin cytoskeleton in the absence of a stimulus such as EGF. However, when N-WASP was co-expressed with WICH, significant reorganization of the actin cytoskeleton occurred. First, several aggregates of actin filaments that overlapped with N-WASP and WICH formed in the cytoplasm. In addition, many actin-microspikes protruded from the plasma membrane. Because expression of N-WASP or WICH alone had no positive effect on actin polymerization *in vivo*, it is likely that these effects are caused by functional cooperation between N-WASP and WICH. We observed significant formation of actin-microspikes in COS7 cells expressing N-WASP, but this required EGF stimulation (9, 12, 23, 24). Therefore, it should be noted that microspike formation by co-expression of N-WASP and WICH occurs even in the absence of such stimulation, suggesting that WICH is an important regulatory protein of N-WASP.

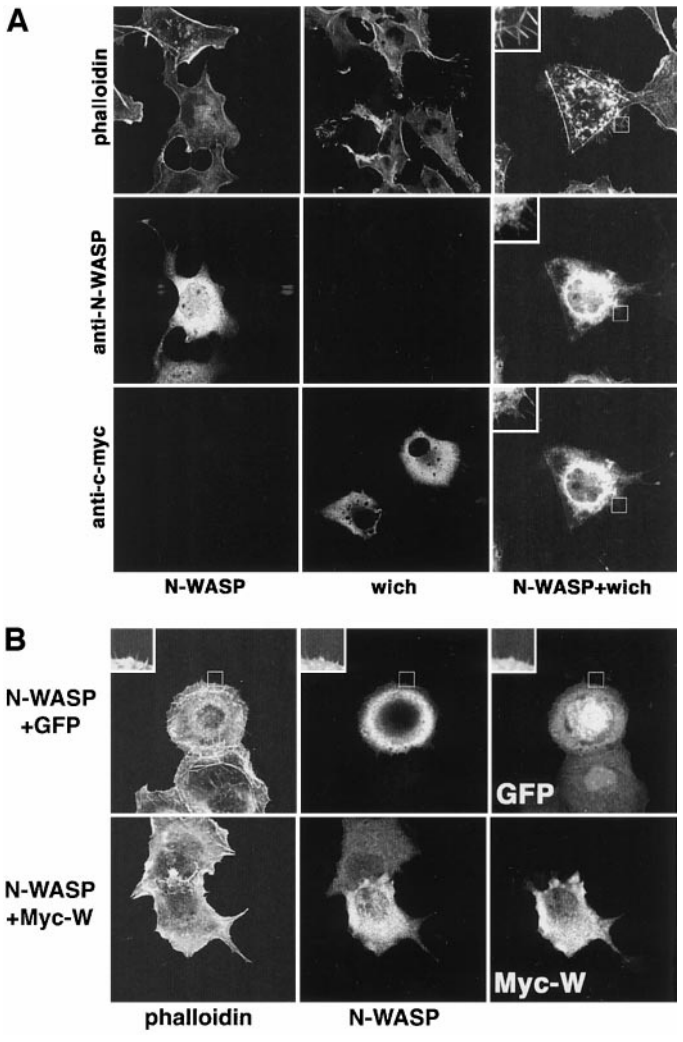

FIG. 4. Microspike formation induced by WICH and N-WASP. (A) Microspike formation is induced by WICH and N-WASP. COS7 cells were co-transfected with constructs encoding N-WASP and Myc-tagged WICH. After serum-starvation for 24 h, cells were fixed. F-actin, N-WASP, and WICH were visualized with phalloidin, anti-N-WASP polyclonal antibody, and anti-c-Myc monoclonal antibody, respectively. (B) WICH is required for microspike formation induced by N-WASP. COS7 cells were co-transfected with N-WASP and Myc-tagged W fragment of WICH or control GFP. After serum starvation for 24 h, transfected cells were stimulated with 100 ng/ml of EGF for 10 min. Superimposed images show microspikes.

The findings of the co-expression study described above strongly suggests the functional importance of WICH in microspike formation. To examine whether WICH is required for microspike formation by N-WASP, we used the W fragment of WICH, which is the smallest region for binding to N-WASP, as a possible dominant-negative fragment in microspike formation. When the W fragment was co-expressed with N-WASP, it significantly suppressed the microspike formation that occurs in an EGF-dependent manner,

whereas co-expression with the GFP control showed no inhibitory effect (Fig. 4B). This result clearly supports the functional requirement for WICH in microspike formation induced by N-WASP.

These findings are consistent with those of a report in which WIP was shown to be an essential functional partner of N-WASP in microspike formation (17). In addition, it was recently reported that endogenous CR16 localizes at the tips of microspikes extending from the growth cones of neurons (20), suggesting that CR16 is also involved in microspike formation. As for the function played by the WIP family proteins, Moreau *et al.* (25) showed that WIP plays an important role in the intracellular motility of vaccinia virus. In such a case, WIP functions as an adapter for recruiting N-WASP to vaccinia viruses, and through such a mechanism WICH may help recruit N-WASP to sites where microspike formation is occurring.

ACKNOWLEDGMENTS

We thank Hideki Yamaguchi, Shiro Suetsugu, Nobunari Sasaki, and Yoshikazu Nakamura in our laboratory for technical assistance. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan and by a Grant-in-Aid for Research for the Future Program from the Japan Society for the Promotion of Science.

REFERENCES

1. Takenawa, T., and Miki, H. (2001) WASP and WAVE family proteins: Key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* **114,** 1801–1809.
2. Miki, H., and Takenawa, T. (1998) Direct binding of the verprolin-homology domain in N-WASP to actin is essential for cytoskeletal reorganization. *Biochem. Biophys. Res. Commun.* **243,** 73–78.
3. Machesky, L. M., and Insall, R. H. (1998) Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8,** 1347–13564.
4. Machesky, L. M., Mullins, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E., and Pollard, T. D. (1999) Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc. Natl. Acad. Sci. USA* **96,** 3739–37445.
5. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97,** 221–231.
6. Rohatgi, R., Ho, H. Y., and Kirschner, M. W. (2000) Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J. Cell Biol.* **150,** 1299–1310.
7. Higgs, H. N., and Pollard, T. D. (2000) Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J. Cell Biol.* **150,** 1311–1320.
8. Prehoda, K. E., Scott, J. A., Mullins, R. D., and Lim, W. A. (2000) Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* **290,** 801–806.
9. Miki, H., Miura, K., and Takenawa, T. (1996) N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *EMBO J.* **15,** 5326–5335.
10. Miki, H., Sasaki, T., Takai, Y., and Takenawa, T. (1998) Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* **391,** 93–96.
11. Carlier, M. F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. (2000) GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. *J. Biol. Chem.* **275,** 21946–21952.
12. Fukuoka, M., Suetsugu, S., Miki, H., Fukami, K., Endo, T., and Takenawa, T. (2001) A novel neural Wiskott-Aldrich syndrome protein (N-WASP) binding protein, WISH, induces Arp2/3 complex activation independent of Cdc42. *J. Cell Biol.* **152,** 471–482.
13. Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. (2001) Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J. Biol. Chem.* **276,** 26448–26452.
14. Miki, H., Suetsugu, S., and Takenawa, T. (1998) WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO. J.* **17,** 6932–6941.
15. Miki, H., Yamaguchi, H., Suetsugu, S., and Takenawa, T. (2000) IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* **408,** 732–735.
16. Ramesh, N., Anton, I. M., Hartwig, J. H., and Geha, R. S. (1997) WIP, a protein associated with Wiskott-Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *Proc. Natl. Acad. Sci. USA* **94,** 14671–14676.
17. Martinez-Quiles, N., Rohatgi, R., Anton, I. M., Medina, M., Saville, S. P., Miki, H., Yamaguchi, H., Takenawa, T., Hartwig, J. H., Geha, R. S., and Ramesh, N. (2001) WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat. Cell Biol.* **3,** 484–491.
18. Vaduva, G., Martinez-Quiles, N., Anton, I. M., Martin, N. C., Geha, R. S., Hopper, A. K., and Ramesh, N. (1999) The human WASP-interacting protein, WIP, activates the cell polarity pathway in yeast. *J. Biol. Chem.* **274,** 17103–17108.
19. Masters, J. N., Cotman, S. L., Osterburg, H. H., Nichols, N. R., and Finch, C. E. (1996) Modulation of a novel RNA in brain neurons by glucocorticoid and mineralocorticoid receptors. *Neuroendocrinology* **63,** 28–3817.
20. Ho, H. Y., Rohatgi, R., Ma, L., and Kirschner, M. W. (2001) CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proc. Natl. Acad. Sci. USA* **98,** 11306–11311.
21. Fukuoka, M., Miki, H., and Takenawa, T. (1997) Identification of N-WASP homologs in human and rat brain. *Gene* **196,** 43–48.
22. Miki, H., Miura, K., Matuoka, K., Nakata, T., Hirokawa, N., Orita, S., Kaibuchi, K., Takai, Y., and Takenawa, T. (1994) Association of Ash/Grb-2 with dynamin through the Src homology 3 domain. *J. Biol. Chem.* **269,** 5489–5492.
23. Suetsugu, S., Miki, H., and Takenawa, T. (1998) The essential role of profilin in the assembly of actin for microspike formation. *EMBO J.* **17,** 6516–6526.
24. Yamaguchi, H., Miki, H., Suetsugu, S., Ma, L., Kirschner, M. W., and Takenawa, T. (2000) Two tandem verprolin homology domains are necessary for a strong activation of Arp2/3 complex-induced actin polymerization and induction of microspike formation by N-WASP. *Proc. Natl. Acad. Sci. USA* **97,** 12631–12636.
25. Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D., and Way, M. (2000) A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. *Nat. Cell Biol.* **2,** 441–448.