Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14671–14676, December 1997
Immunology

WIP, a protein associated with Wiskott–Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells

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Communicated by Robert A. Good, University of South Florida, St. Petersburg, FL, October 28, 1997 (received for review September 2, 1997)

ABSTRACT **Wiskott–Aldrich syndrome (WAS) is an X-linked immunodeficiency caused by mutations that affect the WAS protein (WASP) and characterized by cytoskeletal abnormalities in hematopoietic cells. By using the yeast two-hybrid system we have identified a proline-rich WASP-interacting protein (WIP), which coimmunoprecipitated with WASP from lymphocytes. WIP binds to WASP at a site distinct from the Cdc42 binding site and has actin as well as profilin binding motifs. Expression of WIP in human B cells, but not of a WIP truncation mutant that lacks the actin binding motif, increased polymerized actin content and induced the appearance of actin-containing cerebriform projections on the cell surface. These results suggest that WIP plays a role in cortical actin assembly that may be important for lymphocyte function.**

Wiskott–Aldrich syndrome (WAS) is characterized by thrombocytopenia, eczema, impaired immunity, and a predisposition to develop lymphomas and leukemias (1). The size of platelets and lymphocytes is reduced in WAS, and scanning electron microscopy of T lymphocytes shows a relatively smooth surface with decrease in the number and size of microvilli, suggesting a defect in cytoskeletal architecture (2). The WAS gene is located on Xp11.22–Xp11.23 and encodes a 502-aa-long proline-rich protein (WASP) (3). WASP contains an N-terminal pleckstrin homology domain, which partially overlaps with a WASP homology (WH) domain, WH1, found in several proteins involved in the maintenance of cytoskeletal integrity that include Ena, Mena, Evl, and VASP (4). The WH1 domain in WASP is followed by a GTPase binding domain (GBD/CRIB) (5), a number of proline-rich stretches, a second WH domain (WH2), a short verprolin homology sequence, a cofilin homology sequence, and an acidic C-terminal region. Recently, a protein homologous to WASP was cloned from bovine brain and was termed N-WASP (6). N-WASP has a domain organization similar to that of WASP, and is widely expressed, in contrast to WASP, which is expressed only in hematopoietic cells.

WASP binds via its GBD to the small molecular weight GTPase Cdc42 and weakly to Rac but not to Rho (7–9). Cdc42, Rac, and Rho regulate cytoskeletal organization (10). Overexpression of WASP induces the formation of actin-containing clusters (9). This formation is inhibited by dominant negative mutants of Cdc42, but not of Rac or Rho (9). These findings suggest that WASP may provide a link between Cdc42, Rac, and the cytoskeleton.

WASP interacts with components of signal transduction pathways via their Src homology 3 (SH3) domains, which recognize the proline-rich domain in WASP (11). WASP associates with the adaptor protein Nck (12). Nck can be recruited via its SH2 domain to tyrosine phosphorylated receptors (13). WASP also binds *in vivo* to fyn (12, 14) and *in vitro* to the src kinase fgr, to the tyrosine kinases btk, itk, and Abl and to the p85 subunit of PLC-γ (14–16).

In an attempt to better understand the function of WASP, we cloned, by using the yeast two-hybrid system, a novel human gene whose 503-aa product interacts with WASP. We named the protein WIP for WASP-interacting protein. Overexpression of WIP increases F-actin content and induces actin containing structures in the human B cell line BJAB, suggesting an important role for WIP in the organization of the actin cytoskeleton.

MATERIALS AND METHODS

Molecular Cloning of WIP by Using Yeast Two-Hybrid System. Full-length WASP cDNA, obtained by reverse transcription–PCR from peripheral blood T cells, sequence verified, and cloned into the bait vector pGBT9 (CLONTECH) was used to screen a cDNA library constructed from the human lymphoma T cell line KT3 in the activation domain vector pGAD424 (17). Double transformants were selected on Leu^-, Trp^-, His^- plates containing 20 mM aminotriazole to suppress nonspecific background.

Rapid Amplification of cDNA Ends (RACE). The 5′ end of WIP cDNA was obtained by RACE. Two nested antisense primers corresponding to nucleotides 487–510 (5′-GCGGCATTCGGTTCCTCTGAGGCT, WIP-out) and 452–476 (5′-ACTTCTGTGGCCTGGAGAAGGCACA, WIP-in) of the WIP cDNA were constructed and used to PCR a RACE-ready library from human peripheral blood mononuclear cells (Marathon library, CLONTECH) along with the anchor primers supplied by the vendor and LA *Taq* enzyme (Panvera, Madison, WI). The PCR parameters were as follows: denaturation at 94°C for 30 sec, annealing at 65°C for 1 min, and extension at 68°C for 4 min. Five independent clones derived from three independent PCRs were sequenced to verify the sequence of WIP cDNA. Sequence analysis was performed by using the GCG version 8.1 package (Genetics Computer Group, Madison, WI). The BLAST and the FASTA programs were used to search the GenBank databases at the National Center for Biotechnology Information.

Northern Blot Analysis of WIP mRNA Expression. Human multiple tissue Northern blots were purchased from CLONTECH. After overnight hybridization with radiolabeled full-length WIP cDNA, the blots were washed with 0.5× SSC

Abbreviations: WAS, Wiskott–Aldrich syndrome; WASP, WAS protein; WIP, WASP-interacting protein; WH, WASP homology; GBD, GTPase binding domain; SH3, Src homology 3; GST, glutathione *S*-transferase; MBP, maltose binding protein; FITC, fluorescein isothiocyanate.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF031588).

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containing 0.1% SDS at 65°C for 1 hr with two changes of buffer, dried, and autoradiographed.

Glutathione *S*-Transferase (GST) and Maltose Binding Protein (MBP) Fusion Proteins. To obtain the GST-$WASP_{1–270}$ construct, cDNA coding for amino acids 1–270 of WASP was amplified by PCR using Pfu polymerase (Stratagene) and oligonucleotides with *Eco*RI (5′ end) or *Sal*I (3′ end) recognition sequence and cloned into *Eco*RI–*Sal*I-digested pGEX4T1 (Pharmacia). MBP fusion construct of WIP 4 (MBP-WIP4) was made by ligating WIP4 (a clone of WIP obtained by two-hybrid screen, see *Results*) cDNA excised from pGAD424 by digestion with *Eco*RI and *Bgl*II to *Eco*RI–*Bam*HI-digested pMAL-c2 expression vector (New England Biolabs). GST-WIP2 construct was made by ligating WIP2 cDNA excised from the yeast vector pGAD424 by *Eco*RI–*Bgl*II digestion to *Eco*RI–*Bam*HI-digested pGEX4T1. All expression constructs were verified by DNA sequence analysis and transformed into *Escherichia coli* BL21 for the expression of the fusion proteins.

Expression of fusion proteins was induced as follows: GST-$WASP_{1–270}$ expression was induced for 2 hr with 0.075 mM isopropyl β-D-thiogalactoside (IPTG). After induction, the bacterial cells were collected by centrifugation, suspended in GST-lysis buffer [20 mM Tris, pH 8.0/150 mM NaCl/1 mM EDTA/1 mM DTT/0.5% Nonidet P-40/protease inhibitor mixture (Complete, Boehringer Mannheim/0.1% lysozyme] and lysed by sonication (3 × 15 sec). The lysate was clarified by centrifugation at 10,000 × *g* for 5 min at 4°C. GST expression was induced by addition of 0.1 mM IPTG, and the cells were processed as above.

MBP-WIP4 was induced with 0.3 mM isopropyl β-D-thiogalactoside (IPTG) for 16 hr at 30°C. MBP-M was induced with 0.3 mM IPTG for 2 hr at 37°C. Bacteria were collected by centrifugation and suspended in 1/20 growth volume in *E. coli* lysis buffer (20 mM Tris, pH 7.4/200 mM NaCl/1 mM EDTA with protease inhibitors) and frozen at −20°C for 2 hr. The suspensions were thawed, sonicated (3 × 15 sec), and centrifuged for 20 min at 10,000 × *g* and 4°C. The supernatants were used in affinity precipitation experiments.

***In Vitro* Binding Assay Using GST and MBP Fusion Proteins.** Supernatant of bacterial cell lysates containing GST or GST-$WASP_{1–270}$ fusion protein was mixed with supernatant of bacterial cell lysates containing MBP-WIP4 or MBP-M for 20 min. at 4°C. One half of the mixture was tumbled with glutathione beads and the other with amylose resin at 4°C for 40 min. The beads were washed thrice with tris-buffered saline containing 0.5% Tween 20, and the beads were suspended in 1× Laemmli PAGE buffer and subjected to Western blot analysis.

Generation of WIP Expressing BJAB Cells. WIP and WIP4 cDNAs were cloned into modified pcDNA3 vector that expresses cloned cDNA as a N-terminal FLAG fusion protein (gift of V. Ramesh, Massachusetts General Hospital, Boston, MA). Twenty micrograms of plasmid were used to transfect 30×10^6 BJAB cells by electroporation (1,600 μF, 200 V), and cells were selected in medium containing 1.5 mg/ml G418 (GIBCO/BRL). The surviving cells were cloned by plating at 0.3 cells/well in a 96-well plate. The clones were checked for WIP4 expression by Western blotting with anti-FLAG. Because full-length WIP-FLAG did not transfer for immunoblotting, expression of WIP-FLAG was ascertained by PCR and by the presence of WASP in anti-FLAG immunoprecipitates.

Affinity Precipitation of WASP and Profilin from Cell Lysates by WIP. BJAB cells were washed twice with serum-free RPMI 1640 medium and suspended on ice for 30 min in lysis buffer (50 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/30% glycerol/0.4 mM sodium orthovanadate/10 mM NaF/10 mM sodium pyrophosphate/protease inhibitor mixture/1% Brij 96, Sigma) at 40×10^6 cells/ml. Lysates were centrifuged at 16,000 × *g* for 15 min at 4°C and precleared for 1 hr with 25 μl of GSH-Sepharose (Pharmacia). Supernatants were tumbled for 16 hr with ≈1 μg of GST-WIP2 immobilized on glutathione beads. The beads were washed three times with modified lysis buffer containing 10% glycerol and 0.2% Brij 96 (wash buffer), suspended in Laemmli loading buffer, and subjected to PAGE on 10% or 4–15% gradient gels and Western blotting. The blots were developed with rabbit anti-WASP peptide antiserum and protein A-HRP or with anti-profilin rabbit antibody.

Immunoprecipitation of FLAG-WIP from BJAB Cells. BJAB cells transfected with WIP or with pcDNA3 vector were washed twice with serum-free RPMI 1640 medium and lysed (40×10^6 cells/ml) in ice-cold lysis buffer (10 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/1 mM sodium orthovanadate/50 mM NaF/1 mM phenylmethylsulfonylfluoride). The lysates were incubated at 4°C overnight with 7 μg of anti-FLAG M2 antibody (Kodak-IBI) preadsorbed onto 40 μl of protein G Sepharose (Pharmacia). The precipitates were washed four times with wash buffer, eluted in Laemmli loading buffer, and subjected to SDS/PAGE on 4–15% gradient gels and Western blot analysis with anti-FLAG mAb or anti-WASP antiserum.

Determination of Polymerized Actin Content. F-actin content was estimated by flow cytometry using fluorescein isothiocyanate (FITC)-phalloidin (18). The fluorescence of FITC-phalloidin in this assay is proportional to the amount of F-actin. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 ,and labeled with FITC-phalloidin for 30 min at room temperature. The samples were immediately read in a Becton-Dickinson Excalibur flow cytometer. The samples were gated for live lymphocytes according to forward and side scatter profiles.

Immunofluorescence Microscopy. Cells were fixed with 3.7% paraformaldehyde in PBS at room temperature for 30 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min. The cells were incubated with 2 μM tetramethylrhodamine B isothiocyanate-conjugated phalloidin (Sigma) for 1 hr at 37°C. The cells were washed twice with PBS, examined, and photographed in a fluorescent microscope (Olympus).

RESULTS

Molecular Cloning of WIP. We used the yeast two-hybrid system to search for novel WASP interacting protein(s). A cDNA library from the human lymphoma T cell line KT3 was constructed in the activation domain vector pGAD424 (17) and was screened in the yeast two-hybrid system by using full-length WASP cDNA. Six clones that grew on Leu^- Trp^- His^- plates also tested positive for β-galactosidase with blue color developing in less than 30 min. The two largest cDNAs, designated WIP2 (1.7 kb) and WIP4 (1.6 kb), were chosen for further detailed characterization. DNA sequence analysis revealed that WIP2 and WIP4 cDNAs are products of the same gene because the WIP4 protein coding sequence was completely contained within WIP2 (Fig. 1*A*). The 3′-untranslated region of WIP4 was 0.5 kb larger than that of WIP2. Genbank database search revealed that WIP is virtually identical to Prpl2, a partial human cDNA isolated from tonsillar B cells whose function is unknown (accession number X86019). The last seven amino acids of the predicted Prpl2 protein are replaced in WIP by 17 amino acids of a different sequence (Fig. 1*A*). In addition, there is a deletion of one amino acid (aa 270 of the Prpl2 sequence). Both of these differences are unlikely to be cloning artifacts because they are found in both WIP2 and WIP4, which were isolated during independent screenings. The 5′ end of the WIP mRNA was obtained by rapid amplification of cDNA ends, by using a peripheral blood leukocyte cDNA library. Fig. 1*B* shows the deduced amino acid sequence

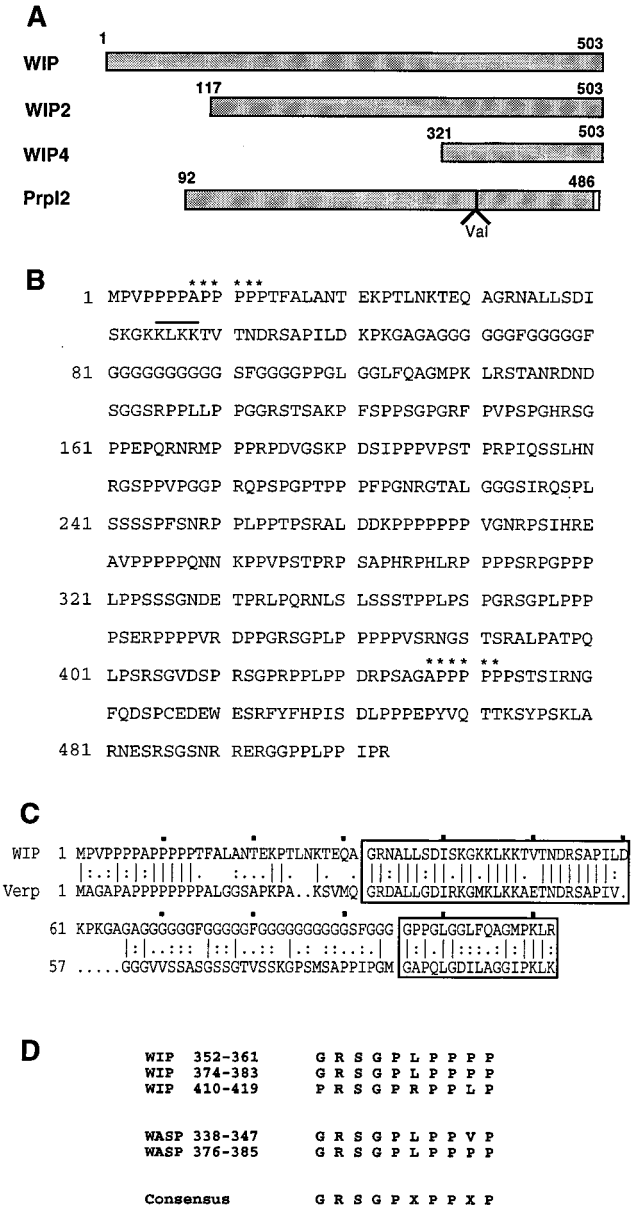

FIG. 1. WIP cDNAs, deduced amino acid sequence of WIP and its alignments. (*A*) Schematic representation of full-length WIP, WIP2, WIP4, and Prpl2 cDNAs. The open box in Prpl2 represents the seven amino acids that replace the C-terminal 17 amino acids in WIP. (*B*) Deduced amino acid sequence of WIP. The two APPPPP motifs implicated in profilin binding are denoted by ∗. A line is drawn over the KLKK motif implicated in acting binding. (*C*) Sequence alignment of the N-terminal regions of WIP and verprolin. The two verprolin homology regions are boxed. (*D*) Sequence alignment of GRSGPXPPXP motifs in WIP and WASP. Numbers refer to amino acid positions.

of WIP. The predicted protein product is a 503-aa-long proline-rich protein with a calculated molecular mass of ≈52 kDa. The N-terminal region contains two stretches, 28 and 17 aa residues in length, that are highly homologous to corresponding amino acid stretches in the N-terminal region of the yeast protein verprolin (Fig. 1*C*). Verprolin is involved in cytoskeletal organization because vrp1⁻ conditional mutants fail to grow at the restrictive temperature of 37°C, have abnormal actin formation and chitin deposition, and are defective in bud formation (19). Of note is the presence of the KLKK motif in the first WIP verprolin homology domain. This motif originally was identified in thymosin-β4 as a motif that

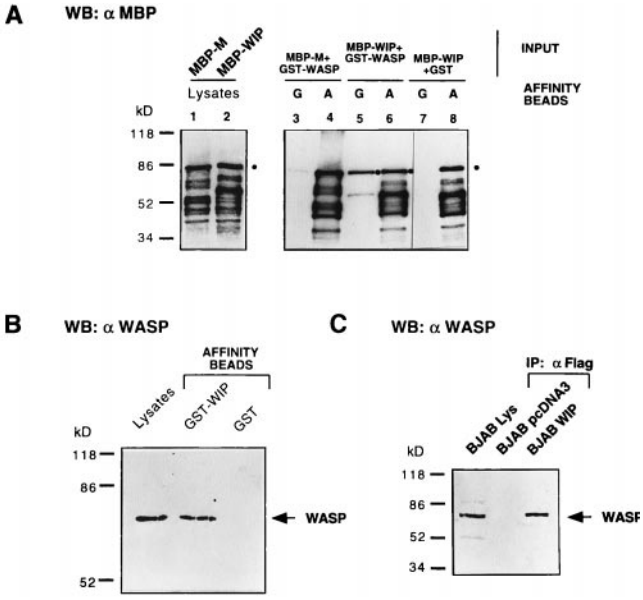

FIG. 2. *In vitro* binding and coimmunoprecipitation of WIP and WASP. (*A*) *In vitro* binding of WIP and WASP fusion proteins. $WASP_{1–270}$, WIP4, and the control protein M were expressed in *E. coli* as fusion proteins with GST or MBP. Soluble extracts from induced bacteria were mixed, and the fusion proteins were affinity-precipitated by using glutathione beads, or amylose resin, run on 4–15% gradient SDS/PAGE, and examined for the presence of MBP fusion proteins by Western blotting with rabbit anti-MBP antiserum. The blots were developed by using protein A conjugated to horseradish peroxidase followed by ECL. The position of MBP-WIP is indicated by a dot. Note that the molecular masses of MBP-WIP and the control MBP-M proteins are similar. The lower molecular bands represent degradation products of the fusion proteins. The presence of GST-$WASP_{1–270}$ in lanes 3 and 5 and GST in lane 7 was confirmed by Western blotting with anti-GST mAb (data not shown). (*B*) Affinity precipitation of endogenous WASP by GST-WIP. Lysates of BJAB cells were incubated with GSH-Sepharose beads coupled to GST-WIP2 or GST. Bound proteins were eluted, run on 10% SDS/PAGE, and examined for the presence of WASP by immunoblotting with rabbit anti-WASP peptide antibody. The blots were developed as described in *A*. (*C*) *In vivo* association of WIP and WASP. FLAG-tagged full-length WIP cDNA was transfected into BJAB cells, and the cell lysates were immunoprecipitated with M2 anti-FLAG mAb, blotted, and probed with anti-WASP peptide antibody.

is critical for actin binding (20). Identical, or similar (KLRK, KLRR) motifs, are present in the putative actin binding protein members of the Ena/VASP family (4) raising the possibility that WIP may interact directly with actin. In addition, WIP contains two APPPPP sequences (denoted by ∗ in Fig. 1*B*), which have been shown to bind profilin (21), a protein that regulates actin polymerization.

WIP contains a number of putative SH3 binding motifs with the sequence PPPΨXP. A unique proline-rich motif, GRSGPXPPXP, is repeated three times in WIP (Fig. 1*D*). This motif recurs twice in WASP and has been implicated in the binding of WASP to the SH3 domain containing proteins fyn, fgr, and phospholipase Cγ1 (16). The presence of conserved SH3 binding motifs in WIP suggests that WIP may link the signal transduction machinery to the cytoskeleton.

WIP Associates with WASP Both *in Vitro* and *in Vivo*. To confirm the association of WASP and WIP, we first examined the interaction of WASP and WIP expressed as recombinant bacterial fusion proteins with GST and MBP, respectively. GSH-Sepharose retained MBP-WIP after incubation with a mixture of MBP-WIP and GST-WASP, indicating binding of WIP to WASP (Fig. 2*A*) and suggesting that posttranslational modification of these proteins is not necessary for their interaction. We next showed that endogenous WASP from the

human B cell line BJAB binds to purified recombinant GST-WIP2 (Fig. 2*B*). More importantly, WIP and WASP were shown to associate in cells. Fig. 2*C* shows that a protein band corresponding to WASP was detected in anti-FLAG immunoprecipitates from BJAB cells transfected with full-length FLAG-WIP cDNA, but not in anti-FLAG immunoprecipitates from cells transfected with empty vector. WASP was not detected in control MOPC21 (mouse IgG1) immunoprecipitates from FLAG-WIP transfected BJAB cells (data not shown).

WASP binds to activated Cdc42 at a conserved GBD (amino acids 238–257) (7–9). To map the WIP binding site, truncations of WASP were examined for WIP binding in the yeast two-hybrid system. The results show that the WIP binding region lies within the N-terminal 170 amino acids of WASP (Fig. 3*A*). Because this region lacks the GBD (amino acids 238–257), the WIP binding site on WASP is distinct from the Cdc42 binding site. Neither the WASP 1–137 truncation mutant, which contains the WH1 domain, nor the proline-rich amino acid 139–270 region were able to bind WIP. Thus, both of these regions are necessary but not sufficient for WIP binding. Deletion of the N-terminal 46 amino acids, which disrupt the pleckstrin homology domain, but not the WH1 domain, sharply reduced the affinity of WASP for WIP, suggesting that amino acids 1–46 are required for optimal binding to WIP. Curiously, the majority of point mutations in patients with WAS are located in the WH1 domain, although this domain makes up only 18% of the WASP sequence (22). Mutations in the WH1 domain potentially could disrupt the association of WASP with WIP, raising the possibility that interaction of WASP with WIP may be critical for WASP function. The carboxy terminal 183 amino acid of WIP, i.e., WIP4 (aa 321–503) are sufficient for WASP binding. To further localize the binding site of WASP on WIP, truncation mutants of WIP4 were constructed and examined for binding to WASP in the yeast two-hybrid system (Fig. 3*B*). The results show that the WASP binding region lies within aa 377–503 of WIP.

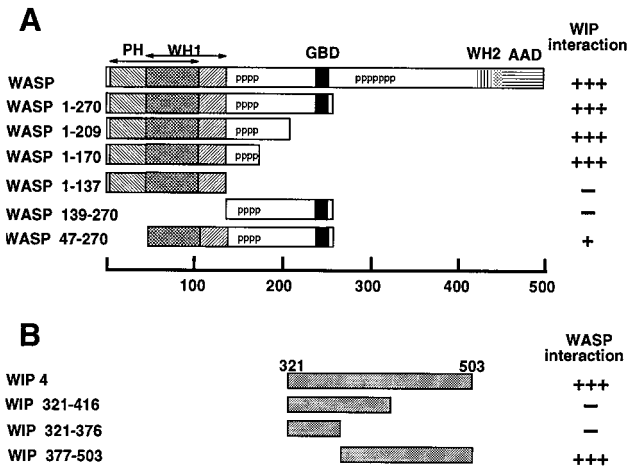

FIG. 3. Mapping of the binding domains of WIP and WASP. (*A*) Mapping of the WIP binding site of WASP. The domains of WASP are indicated. PH, Pleckstrin homology domain (amino acids 6–105); WH1, WH1 domain (amino acids 47–137); pppppp, proline-rich region; GBD, GTPase binding domain (amino acids 238–257); WH2, WH2 domain (amino acids 423–449); AAD, actin association domain (amino acids 443–502). The numbers under the bar at the bottom of the diagram represent the amino acids of WASP. Truncation mutants of WASP, generated either by PCR or cleavage with appropriate restriction enzymes, were cloned into the pGBT9 vector and examined for WIP binding in the yeast two-hybrid system. Blue color development by β-galactosidase activity was used to score the interaction of WIP with WASP truncations. +++ represents color change in 30 min or less, + represents color change in 3 hr, and − indicates no color change and lack of growth in His^- medium. For each mutant at least three independent colonies were tested in the β-galactosidase assay. (*B*) Mapping of the WASP binding site of WIP. Truncation mutants of WIP4 (amino acids 321–503), generated by cleavage with appropriate restriction enzymes, were cloned into the pGAD424 vector and examined for WASP binding in the yeast two-hybrid system. Interactions were scored as in *A*.

WIP Is Expressed Hematopoietic and Nonhematopoietic Tissues. The expression of WIP transcripts in human tissues was analyzed by Northern blot analysis of poly(A)$^+$ RNA from a panel of tissues that used full-length WIP cDNA as probe. Fig. 4 shows that three species of mRNA with estimated sizes of 2.4 kb, 3.5 kb, and 5 kb are present in all tissues tested. The different RNA species could represent a family of proteins or differently spliced/polyadenylated mRNA. Because the RNA bands are evident even when washed at relatively stringent condition (0.5× SSC, 0.5% SDS), it suggests that the different bands are probably splice variants. The level of expression of the WIP transcripts is highest in peripheral blood mononuclear cells with the 2.4-kb species expressed at a higher level than the other two species. Expression of WIP in nonhematopoietic tissues, which do not express WASP, suggests that WIP may have other interaction partners than WASP.

Expression of WIP Causes Actin Polymerization. WIP contains the highly charged KLKK sequence (amino acids 45–48) in its N-terminal verprolin homology domain, immediately preceded by a region that could fold as an α-helix. This motif mediates contact between G-actin and thymosin β4 (20). WIP also contains two copies of the actin- based motility sequence ABM-2, APPPPP, which has been implicated in binding to profilin in VASP and Mena (4, 23). One of the two profilin binding motifs in WIP (amino acids 8–13) is located in relative proximity to the G-actin binding motif as found in the Ena/VASP family of proteins. Simultaneous binding of G-actin and profilin by Ena/VASP is thought to promote actin polymerization (4). To examine the role of WIP on actin polymerization, the effect of overexpression of WIP on F-actin content was examined in B lymphocytes. FLAG-tagged full-length WIP and FLAG-tagged WIP4 (amino acids 321–503) were cloned into pcDNA3 and transfected into BJAB cells. Transfected cells were selected by using the antibiotic G418 and cloned. To ascertain the expression of FLAG-WIP proteins, cell lysates were immunoblotted with anti-FLAG mAb M2.

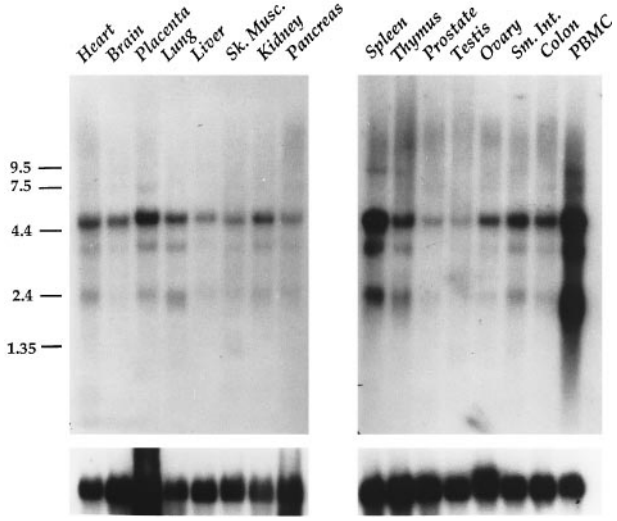

FIG. 4. Expression of WIP transcripts in human tissues. Multiple tissue Northern blots containing 2 μg of human mRNA per lane were hybridized with ^{32}P-labeled full-length WIP cDNA probe (*Upper*). The filter was exposed for 12 hr. As a control for loading, the blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase probe (*Lower*). PBMC, peripheral blood mononuclear cells.

The FLAG-WIP4 product was readily detectable in cell lysates (data not shown) and in immunoprecipitates with anti-FLAG M2 mAb as shown for the representative clone I in Fig. 5*A* (*Left*). Despite multiple attempts using a number of immunoblotting conditions, the full-length FLAG-WIP product could not be immunoblotted (Fig. 5*A*, *Center*), possibly because of its very high proline content. However, as illustrated in Fig. 5*A* (*Right*) for the representative clone 3, we were able to demonstrate the presence of WASP in anti-FLAG immunoprecipitates from FLAG-WIP-transfected cells, providing evidence for expression of tagged WIP protein that interacted with WASP. Expression of FLAG-WIP mRNA also was ascertained in several clones by reverse transcription–PCR.

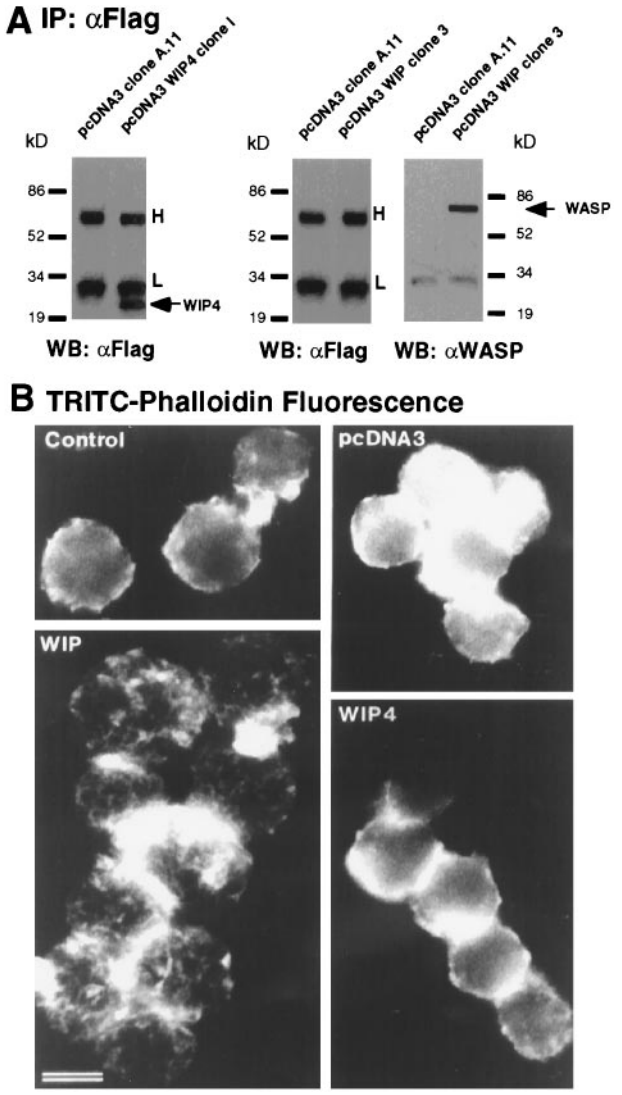

FIG. 5. Effect of WIP overexpression on F-actin distribution in BJAB cells. (*A*) Expression of WIP4 and WIP in BJAB clones. (*Left*) Expression of FLAG-WIP4 protein in clone I is demonstrated by the presence of a specific band corresponding to FLAG-WIP4 in immunoblots of anti-FLAG immunoprecipitates developed with anti-FLAG mAb followed by peroxidase labeled goat anti-mouse Ig and ECL. This band is absent in the control clone A.11 transfected with pcDNA3 alone. The heavy (H) and light (L) chains of the immunoprecipitating mAb are visualized. (*Center*) Full-length FLAG-WIP did not transfer in immunoblotting. (*Right*) Expression of FLAG-WIP protein in clone 3 transfected with FLAG-WIP is inferred by the presence of a specific band corresponding to WASP in immunoblots of anti-FLAG immunoprecipitate developed with rabbit anti-WASP followed by protein A and ECL. This band is absent in the control clone A.11. (*B*) F-actin distribution in BJAB cells. Untransfected cells (control), a pcDNA3 transfected clone (A.11), and representative clones expressing WIP (clone 3) and WIP4 (clone I) were permeabilized, stained with rhodamine-conjugated phalloidin, and examined by immunofluorescence microscopy. (Bar, 5 μm.)

F-actin content was assessed by staining permeabilized cells with FITC-conjugated phalloidin followed by flow cytometry analysis. Table 1 shows that there was an increase in baseline F-actin content in BJAB cell clones that overexpressed full-length WIP, but not in control transfected cells. The effect of WIP overexpression on F-actin content was dependent on the N-terminal region of WIP. Overexpression of WIP4, which lacks the first 320 amino acids and thus lacks the actin binding KLKK motif and one of two profilin binding ABM-2 sequences, did not cause an increase in F-actin content.

It previously was shown that overexpression of WASP causes the formation of cytosolic aggregates containing F-actin and WASP (9). The effect of WIP overexpression on F-actin distribution was assessed by immunofluorescence microscopy of permeabilized BJAB cells stained with tetramethylrhodamine B isothiocyanate-phalloidin. Fig. 5*B* shows that F-actin is uniformly distributed around the cortex in untransfected cells (control) and in cells transfected with pcDNA3. In contrast, in clone 3, which overexpresses full-length FLAG-tagged WIP, the cell surface was covered with cerebriform projections containing actin. The cerebriform nature of the projections is reflected in the lacey staining pattern. Formation of surface projections and the increase in F-actin content were dependent on the N-terminal region of WIP, because F-actin staining in clones that overexpress WIP4 was indistinguishable from that of control cells.

GST-WIP Affinity Precipitates Endogenous Profilin. In light of the capacity of WIP to increase F-actin content, the critical role of profilin in actin polymerization (24, 25), and the presence of profilin binding motifs in WIP, we examined the capacity of WIP to bind profilin. Fig. 6 shows that profilin is readily detected in lysates of BJAB cells by immunoblotting with rabbit anti-profilin antibody. GST-WIP2, but not GST, retained endogenous profilin after incubation with BJAB cell lysate.

DISCUSSION

We have identified a novel gene whose product, WIP, associates with WASP. WIP mRNA is expressed in many tissues although its level of expression varies between tissues. The finding that WASP is expressed only in hematopoietic cells suggests that WIP may interact with partners other than WASP. e.g., N-WASP, which we recently showed to interact with WIP (data not shown). Overexpression of WIP exerts powerful effects on the actin cytoskeleton, which include increase in the cellular content of polymerized actin and appearance of cerebriform projections on the cell surface containing F-actin. The effects of WIP overexpression on the

Table 1. Effect of overexpression of WIP on F-actin content in BJAB cells

Transfection	Cells	Relative F-actin content
Untransfected	BJAB	1.0
pcDNA3	Clone A.11	0.96 ± 0.25
FLAG-WIP	Clone 2	1.40 ± 0.25
	Clone 3	1.79 ± 0.11
	Clone 4	1.55 ± 0.01
FLAG-WIP4	Clone I	1.10 ± 0.17

F-actin content was determined by measuring mean fluorescence intensity in permeabilized cells stained with FITC-phalloidin. The F-actin content represents the mean ratio of the F-actin content in transfected cells to that in untransfected BJAB cell in four experiments. *t* tests revealed that FLAG-WIP clones 2, 3, and 4 were significantly higher ($P \leq 0.001$). There was no significant difference between clone A.11 and clone I ($P = 0.20$).

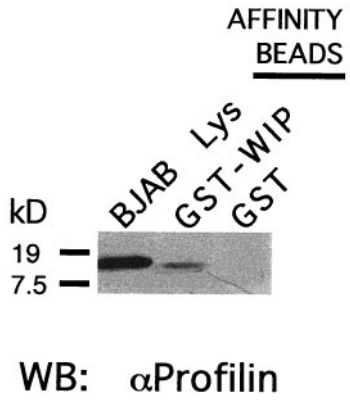

FIG. 6. Affinity precipitation of endogenous profilin by GST-WIP2. Lysates of BJAB cells were incubated with GSH-Sepharose beads coupled to GST-WIP2 or GST. Bound proteins were eluted, run on 4–15% gradient SDS/PAGE, and examined for the presence of profilin by immunoblotting with rabbit anti-profilin antiserum. The blots were developed as described in Fig. 2*A*.

actin cytoskeleton required the N-terminal end of the molecule (amino acids 1–320), which contains the actin binding KLKK motif. A number of proteins have been shown to assemble into a spatial actin monomer delivery system. These proteins, which include Mena and VASP, bind profilin via a proline-rich ABM-2 motif, XPPPPP, where X denotes G, A, L, or S (4, 21, 23). The sequence APPPPP is represented twice in WIP, once at the N-terminal end and once in the C-terminal region (Fig. 1*B*). Immobilized WIP bound profilin from cell lysates (Fig. 6), suggesting a direct interaction of WIP with profilin. Recombinant profilin was affinity-precipitated from bacterial cell lysates by MBP-WIP, further indicating that WIP interacts directly with profilin (data not shown). Thus, WIP may modulate actin dynamics by direct interaction with actin, via the KLKK motif, as well as with profilin, and possibly with other proteins that regulate actin polymerization, such as WASP.

The platelet and lymphocytes structural abnormalities in WAS support a functional link between WASP and the actin cytoskeleton. In addition, T lymphocytes from WAS patients fail to proliferate to immobilized anti-CD3 (15), a response that depends on actin cytoskeleton rearrangement (26, 27). The mechanism by which WASP modulates the actin cytoskeleton is unclear. Although WASP overexpression induces the actin clusters that contain WASP (9), no evidence exists for direct interaction between WASP and actin; however, N-WASP has been shown to interact with actin *in vitro* (9). Because WIP binds WASP and overexpression of WIP induces actin cytoskeletal changes, WIP may play an important role in linking WASP to the actin cytoskeleton. The actin-binding KLKK motif and one of the two profilin-binding ABM-2 motifs in WIP are located in proximity to each other, just as they are in members of the Ena/VASP family. Simultaneous binding of profilactin by WIP may promote actin polymerization by increasing the local concentration of actin monomers. Actin polymerization by a WASP-WIP complex may be further enhanced by the recruitment of profilin to the ABM-2 motifs in WASP.

Cytoskeletal rearrangement is triggered by a variety of signaling pathways induced by external stimuli such as growth factors, stress, and adhesion through integrins (28), and is mediated by small GTPases. WIP does not contain any discernible GBD; thus direct interaction of WIP with GTPases is unlikely. However, WASP may bridge Cdc42 to WIP, allowing Cdc42 to regulate WIP function. The WASP-WIP complex may get targeted by stimuli that activate Cdc42 to locate to the actin cytoskeleton via interactions between the WH1 domain of WASP and the proline-rich ABM-1 motifs of structural proteins such as zyxin and vinculin (21). The presence of SH3 binding motifs in both WIP and WASP suggests that the WASP-WIP complex couples additional signaling pathways to the actin cytoskeleton.

We thank Paul Swinton and Richard Dapoza for technical assistance, Dr. T. Kirchhausen for the WASP anti-peptide antibody (C6), Dr. D. Kwiatkowski for anti-profilin antibody, and Dr. L. Enjuanes for MBP-M construct. We also thank Drs. Tom Stossel and Fred Rosen for critical reading of the manuscript and Dr. David Zurakowski for help with statistical analysis. This work was supported by National Institutes of Health Grants AI-37130 and AI-35714 and grants from Baxter Healthcare, Olsten Healthcare, and Alpha Therapeutics Co. I.M.A. is a postdoctoral fellow of Fundación Ramón Areces.

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