The Rat Homologue of Wiskott-Aldrich Syndrome Protein (WASP)-interacting Protein (WIP) Associates with Actin Filaments, Recruits N-WASP from the Nucleus, and Mediates Mobilization of Actin from Stress Fibers in Favor of Filopodia Formation*

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We cloned and characterized the rat homologue of the Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP). Rat WASP shows 86% amino acid sequence identity to human WIP. Northern analyses revealed two major mRNA species of 5.0 and 3.8 kb, which were ubiquitously expressed, though predominantly in spleen and lung. Minor species of 2.4, 1.8, 1.4, and 1.1 kb were also detected in some tissues and cell lines. Thus, WASP is subject to tissue-specific alternative splicing. WIP bound to N-WASP in vivo, as revealed by co-immunoprecipitation. Expression of WIP in rat fibroblasts revealed a clear co-localization with actin stress fibers. However, expression in tumor cells lacking actin cables did not restore these structures. Interestingly, co-expression of WIP and N-WASP resulted in redistribution of N-WASP, abrogating its dominant nuclear expression and leading to co-localization with WIP in the perinuclear area and with actin in membrane protrusions. Moreover, stress fibers and, concomitantly, the associated WIP were largely dissolved. Very similar effects were seen upon epidermal growth factor stimulation of serum-starved cells. Our results suggest that WIP might be involved in transmitting mitogenic signals to cytoskeletal functions, perhaps by modulating the subcellular localization of N-WASP. Interaction of N-WASP with WIP may in turn lead to mobilization of actin from stress fibers and nucleation of new actin filaments in filopodia.

The actin cytoskeleton represents a complex and highly dynamic structure that plays a crucial role for the processes of cell motility, cytokinesis, and the formation of specialized structures (reviewed by Refs. 1–4). To fulfill these functions under appropriate situations, the cytoskeleton is linked to external as well as internal signals. Thus, mitogenic stimulation of cells may result in gross remodeling of the cytoarchitecture, governed by polymerization and depolymerization reactions, which in turn are mediated by associated factors such as gelsolin, myosin, actinin, etc.

Molecules involved in translating extracellular stimuli to remodel the actin cytoskeleton include members of the Rho family of small GTPases, including Cdc42, Rac, and Rho (5, 6). Of these, Cdc42 induces filopodia formation, Rac regulates membrane ruffling and lamellipodia formation, and Rho triggers the assembly of focal contacts and stress fibers (7). However, the precise mechanism at the molecular level by which these proteins induce assembly and disassembly of the actin filament system is not fully understood.

One class of adaptor molecules that bind multiple signaling and cytoskeletal proteins is the WASP (Wiskott-Aldrich syndrome protein) family. WASP was originally identified as a protein that was altered in patients with Wiskott-Aldrich syndrome, a genetic immunodeficiency disorder characterized by reduced mobility of lymphoid cells (8). The mammalian WASP family consists presently of five members, WASP itself, which is exclusively expressed in lymphoid tissues (9), neural WASP (N-WASP), which is predominantly expressed in brain but also in other tissues (10), and the related protein group SCAR/WAVE1 (suppressor of cAMP receptor/mutation/WASP family verprolin homologous protein 1) (11, 12), WAVE2, and WAVE3 (13). WASP proteins have been recognized as key molecules transducing Cdc42- and Rac-dependent signals to filopodia and lamellipodia formation, respectively (12, 14, 15).

Due to its lymphoid-specific expression, WASP may perform some specialized function, perhaps in immune receptor signaling, since T lymphocytes of Wiskott-Aldrich syndrome patients show diminished proliferative response to stimulation through the T cell receptor-CD3 complex (16). In contrast, N-WASP seems to be involved in general signal transduction, since it has been isolated by virtue of its interaction with Grb2, an SH2/SH3 domain-containing adapter that is directly linked to receptor tyrosin kinase (10). Furthermore, it plays an important role in neurite outgrowth (17). However, since the majority of N-WASP is located in the nucleus (10, 18), its biological role remains obscure.

Recently, a human WASP-interacting protein (WIP) was isolated in a two-hybrid screen (19). WIP is a proline-rich 503-amino acid protein. It binds to WASP within the NHL-terminal region, at a site distinct from the Cdc42-binding site. Interestingly, this region of WASP is frequently mutated in Wiskott-Aldrich syndrome patients (9, 20). Thus, impairment of WASP-
Cloning and Functional Characterization of Rat WIP

WIP interaction may contribute to the disease. Like WASP, WIP contains a number of putative SH3 domains, which might provide links to signaling molecules, and several domains implicated in binding, either directly or indirectly to actin (19). Overexpression of WIP in human lymphoma BJAB cells led to accumulation of F-actin and induced actin-containing projections beneath the cell membrane, suggesting a role for WIP in actin polymerization.

We isolated the rat homologue of the human WIP gene. Northern blotting analyses revealed extensive differential splicing in a tissue-specific manner. Expressing WIP as green fluorescence protein (GFP) or FLAG-tagged fusion protein in rat fibroblasts demonstrated a clear co-localization with the actin cytoskeleton, while N-WASP was predominantly in the nucleus. Interestingly, co-expression of WIP and N-WASP resulted in nuclear exclusion of N-WASP, partial dissolution of the actin cytoskeleton, and formation of filopodia. Similar effects were seen upon EGF stimulation. These data suggest that WIP might be a regulator of N-WASP and that both proteins contribute to the dynamic changes of the actin cytoskeleton in response to appropriate signals.

EXPERIMENTAL PROCEDURES

Cell Lines—Normal rat embryonic fibroblast (REF52.2) cells, the tumor cell lines TZ102, TZ103, and TZ107 derived from SV40 large T-antigen (LT)-induced rat brain tumors and the LT-immortalized E14-2 rat fetal brain cells (21) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 10% CO2. PC12 (pheochromocytoma) cells were grown on collagen-coated plates in RPMI (Invitrogen) containing 10% horse serum and 5% FCS.

Generation of the cDNA Library and Yeast Two-hybrid Screening—The cDNA library and the two-hybrid screening have been described previously (22). Briefly, poly(A)+ RNA was isolated from TZ103 cells, reverse-transcribed, and inserted into the HybriZAPTM vector (23) using the ZAPExpressTM cDNA synthesis kit (Stratagene, La Jolla, CA). Two-hybrid screening was performed with the large T-antigen cDNA fused to the Gal4 DNA binding domain as bait. His and LacZ-positive clones were isolated and the cDNA inserts were further analyzed by dideoxy sequencing (22).

Cloning and Expression of Full-length WIP cDNA—For the isolation of the full-length WIP cDNA, a 1100-bp cDNA fragment was labeled by random primer labeling with [α-32P]dCTP (Megaprome Labeling Kit, Amersham Biosciences Inc., Freiburg, Germany) and used as probe for screening the TZ103 cDNA library. One of the positive clones containing an insert of 3.8 kb was regarded full-length and completely sequenced (MWG Biotech, Munich, Germany). Sequence comparisons were performed with the EMBL Gene Data Base using the BLAST analysis program.

For further analyses, rat WIP was expressed as GFP or FLAG-tagged fusion protein in mammalian cells. To this end, a 1681-bp fragment, including the complete coding region of the WIP cDNA, was amplified by PCR with primers containing either EcoRI or XhoI restriction sites and cloned into pEGFP-C1 (CLONTECH Laboratories Inc., Heidelberg, Germany). The 3′-untranslated region of the rat WIP cDNA was cloned into pCMV-Tag 2B expression vector (Stratagene) to generate NH2-terminal fusion proteins with EGFP or FLAG (denoted GFP-WIP or FLAG-WIP, respectively). Likewise, a COOH-terminally truncated form of WIP coding for the NH2-terminal 400 amino acids was cloned into pCMV-Tag 2B to yield FLAG-WIP400, thus lacking the WASP binding region.

RNA Expression Analysis by Northern Blotting—Blotting membranes containing ~2 μg of poly(A)+ RNA per lane from different rat tissues (CLONTECH) or 26 μg of total RNA from various cell lines, respectively, were used for Northern analyses. Filters were prehybridized and hybridized in 50% deionized formamide, 2× Denhardt’s, 5× SSC, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 50 μg/ml RNA. Blots were hybridized overnight at 42 °C with different [α-32P]dCTP-labeled WIP cDNA fragments, as specified in the text, and processed according to standard procedures (24). After autoradiography, the membranes were dehybridized by immersion in boiling 0.1% SDS, and a further round of hybridization was performed with a [α-32P]dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase probe.

Transfection and Immunofluorescence Analyses—REF52.2 cells and COS-7 cells were seeded at a density of 3 × 104 cells onto coverslips and transiently transfected with 100 ng each of GFP-WIP, FLAG-WIP, or FLAG-WIP400 expression vectors or co-transfected with any of the former and the following N-WASP constructs, pcDL-SRαII-N-WASP, pCMV-GFP-N-WASP, pCMV-GFP-N-WASP II, pCMV-GFP-N-WASP WSH1, and pCMV-GFP-N-WASP WSH2 (N-WASP amino acids 146-501) (25), the latter two kindly provided by M. Way (EMBL, Heidelberg, Germany). Transfections were performed with LipofectAMINETM (Invitrogen) according to the manufacturer’s protocol.

42 h after transfection, the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min. The cells were treated with 5% nonfat dry milk for 1 h and stained with an affinity-purified polyclonal N-WASP antibody (10) at 1:500 dilution or with FLAG M2 monoclonal antibody (Stratagene) at 1:5000 dilution for 1 h at room temperature. As secondary antibodies, Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG (Dianova, Hamburg, Germany) were used at 1:1000 dilution and incubated for 30 min. Actin staining was performed either with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma, Deisenhofen, Germany) at room temperature for 15 min. Nuclei were stained with 4,6-diamidino-2-phenylindole for 15 min and subsequently washed, as described above. Cells were examined with an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) and a 63× oil immersion objective. For extraction experiments, WIP-transfected cells were treated with 0.1% to 1% Triton X-100 in microtubule-stabilizing buffer (80 mM PIPES, pH 6.9, 1 mM MgCl2, 1 mM EGTA, 0.1 mM polyethylene glycol) for 10 to 1 min immediately before fixation.

Labeling of Cells and Immunoprecipitation—PC12 cells were transiently transfected with 7.5 μg of FLAG vector, FLAG-WIP, FLAG-WIP400, and with the same amount of N-WASP expression vector as described above. 42 h post-transfection, the cells were metabolically labeled with 250 μCi of [35S]methionine (specific activity ~1000 Ci/mmol, Amersham Biosciences Inc.) per 9-cm culture dish in methionine-free Dulbecco’s modified Eagle’s medium supplemented with dialyzed fetal calf serum for 2 h. After labeling, cells were washed with ice-cold phosphate-buffered saline and lysed in isotonic lysis buffer (10 mM NaPO4, pH 8.0, 140 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol, 0.5% Nonidet-P40, 50 μM leupeptin). The lysates were cleared by centrifugation and subjected to immunoprecipitation with the FLAG M2 monoclonal antibody (Stratagene) or with the N-WASP polyclonal antibody (10) at 4 °C overnight. Protein A-Sepharose (Sigma) was added and incubated for 1 h. Immunoprecipitates were washed three times with lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis according to standard protocols. Proteins were visualized by autoradiography.

RESULTS

Molecular Cloning of Rat WIP and Sequence Comparison with Human WIP—The 3′-untranslated region of the rat WIP cDNA was originally identified as an apparently false positive clone in a yeast two-hybrid screen intended to search for novel interaction partners of SV40 LT (22). Sequence analysis of the 5′ portion of this 1.1-kb cDNA clone showed 83% sequence identity to a murine expressed sequence tag of 407 bp (data base number AA6234.5.mshet16). Northern analysis (see below) indicated that our clone was incomplete. Using the 1.1-kb cDNA fragment as probe, we screened a cDNA library from TZ103 cells and identified 10 positive clones out of 200,000, one of which, containing an insert of 3.8 kb, was further analyzed. Sequence analysis revealed 3797 nucleotides with an open reading frame for 483 amino acids. Searching the GenBankTM data base revealed that the isolated cDNA clone was 82% identical to human WIP cDNA (Ref. 19, accession number AF031588; Ref. 26, accession number NM_003387), and to Prp2, a partial human cDNA sequence isolated from tanssilar B cells (accession number X86019). Sequence alignment of our original two-hybrid cDNA clone revealed that it was derived from the 3′-untranslated region of the 3.8-kb cDNA, thus excluding the possibility that WIP does interact with LT.

Fig. 1 shows alignments of the deduced amino acid sequence of rat WIP with the human homologues Prp2 and WIP. The sequences of the human proteins are identical except for one...
additional valine residue at position 361 in Prpl2 and the COOH terminus, where the last 7 residues in Prpl2 are replaced by 17 different residues in WIP. The rat sequence is identical to human WIP at these positions. It is possible that the Prpl2 protein represents a splicing variant (see below). In the following text we will refer only to the sequence of Ramesh et al. (19).

The rat and human WIP proteins show an overall sequence identity of 86% with some gaps in the rat versus the human protein. Generally, the protein is extremely rich in proline and glycine. Forty-two glycine residues are clustered between amino acids 64 and 221, whereas the proline residues are distributed throughout the molecule. Furthermore, there are particular sequence modules that are completely identical between rat and human WIP, some of which occur several times. There are two so-called verprolin homology (VH) domains, also referred to as WASP homology (WH-2) domains (residues 32–60 and 92–108), that occur also in its binding partners WASP and N-WASP. The VH domain binds actin and, in cooperation with the cofilin homology and the acidic domains, participates in activation of the Arp2/3 complex (27, 28). Within the first VH domain, the WIP proteins share a KLKK motif, marked by asterisks, which has been implicated in actin binding (29). Furthermore, the proteins contain three copies of the unique proline-rich motif GRSGPXPPXP. Two copies of this motif are also present in WASP proteins and have been implicated in binding to SH3 domain-containing proteins, i.e. Src, Fyn, Fgr, and phospholipase Cγ1 (30, 31). Finally, the WIP proteins contain several identical APPPPP sequences, so-called actin-based motility sequences (ABM-2 motifs), required for binding to profilin, a protein that regulates actin polymerization (32).

Interestingly, the human and rat cDNAs differ greatly in their 3′-untranslated regions. In contrast to 82% identity in the coding region, they show only 72% sequence identity in the 3′-untranslated region. However, the human sequence extends only for 365 nucleotides, while our rat cDNA continues for additional 1810 nucleotides. The differences may reflect alternative splicing variants (see below). The complete sequence of the 3.8-kb rat WIP cDNA has been deposited in the GenBank database, accession number AJ303456.

WIP mRNA Expression Varies in Different Tissues and Is Subject to Alternative Splicing—When we probed a multiple tissue Northern blot with a probe derived from the 5′ end of the coding region of rat WIP cDNA (nucleotides 49–561), we detected two major WIP-related transcripts with estimated sizes of 5.0 and 3.8 kb, the 5.0-kb species being the most prominent one (Fig. 2). These presumably represent alternative splice forms as also seen in human tissues (19). Expression of these two transcripts was highest in spleen and lung, less prominent in heart, brain, liver, and kidney, and rather low in testis. Identical results were obtained with the complete coding sequence as probe. However, the precise relative abundance is difficult to judge, because glyceraldehyde-3-phosphate dehydrogenase, which is commonly used as reference has clearly different expression levels in the different tissues (see lower panel in Fig. 2). Since identical results were obtained with another blot, we assume that equal amounts of mRNA had been loaded on these blots.

Interestingly, a large transcript of 6.9 kb was specific for skeletal muscle. Additionally, we observed signals at 2.4 kb in liver, at 1.8 kb in testis, and at 1.4 kb in heart, liver, and kidney. They all may represent alternatively spliced transcripts of the WIP gene (see below and “Discussion”). Our data are in
general agreement with those of Ramesh et al. (19) on human WIP showing ubiquitous expression of three mRNA species of 5.0, 3.5, and 2.4 kb, the former with highest abundance. The 6.9-kb species in skeletal muscle and the smaller transcripts have not been mentioned by these authors.

**WIP Splice Variants Differ in Their Coding and Their 3'-Untranslated Regions**—We next wanted to see whether the pattern of WIP transcripts observed in rat tissues was resembled in cell line TZ103, the origin of the isolated cDNA clone. For comparison, we included an additional tumor cell line, TZ102, and two nontransformed cell lines, E14-2 and TZ107 (21) (Fig. 3). Using the same 5' probe as above (designated WIP1 in Fig. 3A), we detected five mRNA species, the two prominent species of 5.0 and 3.8 kb, as above, and at least three additional species of 2.4, 1.8, and 1.1 kb. The 1.4-kb transcript was not seen.

To investigate whether these minor transcripts indeed represent splicing variants or transcripts of related genes, we employed additional probes from the coding region as well as from the 3'-untranslated region that together span almost the entire sequence of our WIP cDNA clone (WIP1, nucleotides 49–561; WIP2, nucleotides 589–1059; WIP3, nucleotides 1059–1408; WIP4, nucleotides 1618–1972; and WIP5, nucleotides 2521–3620, respectively). The probes originating from the 3'-untranslated region (WIP4 and WIP5) hybridized only with the 5.0- and 3.8-kb mRNA species, indicating that the smaller transcripts lacked (most of) the 3'-untranslated regions (data not shown). Interestingly, hybridization with fragments from the coding region (WIP1–3) revealed distinct patterns for each probe. They all hybridized equally with the 5.0- and 3.8-kb transcripts (Fig. 3A), but differentially with the smaller transcripts. Thus, the WIP2 fragment hybridized only with the 1.8-kb transcript, present in all cell lines, while WIP3 hybridized only with the 1.4-kb transcript in addition to the 5.0- and 3.8-kb transcripts. Interestingly, the 1.4-kb transcript was the most abundant WIP transcript in E14-2 cells. These data are summarized in Fig. 3B. Included is a schematic representation of the putative exon composition of the coding region of rat WIP cDNA, as deduced from the genomic structure of the human WIP gene (compare accession numbers AF031588 and NT_005182). The positions of the three probes (WIP1, -2, and -3) used in this study are indicated below. The coding region of the human WIP gene includes exons 2–8 with the start and stop codons in exons 2 and 8, respectively. From this structure, we derived the putative exon composition of the different splicing variants. Thus, the 5.0- and the 3.8-kb transcripts include all exons, whereas the 2.4- and the 1.1-kb species apparently miss exons 5–7, the 1.8-kb species only exons 6 and 7. Moreover, the difference in size between the 1.1- and the 1.8-kb species corresponds exactly with the size of exon 5 (736 bp). The 1.4-kb transcript only consists of exons 6 and 7. Taken together, these results indicate that WIP is subject to extensive alternative splicing resulting in at least seven different species. Of these, the smaller species not only lack the 3'-untranslated region but differ in their coding region, too. The possible consequences of differential splicing are discussed below.

**Rat WIP Associates with Actin Fibers in Vivo**—To analyze the subcellular localization of WIP, we employed a GFP-WIP fusion construct. This construct was transfected into REF52.2 cells, and its localization was examined by fluorescence microscopy. As shown in Fig. 4A, GFP alone exhibited a diffuse distribution, filling the entire cell, as previously reported (33). In contrast, GFP-WIP (Fig. 4C) showed a diffuse enrichment in the perinuclear area, which was superimposed by a filamentous pattern, resembling actin fibers. Indeed, co-staining the transfected cells with TRITC-labeled phalloidin, which specifically binds to F-actin (34), revealed an identical pattern (Fig. 4D) indicating that rat WIP co-localized with actin filaments. Compared with neighboring untransfected cells, there was no...
under monoclonal antibody FLAG M2 and anti-mouse IgG-Cy3 as described aldehyde and examined directly by fluorescence microscopy (in the case of WIP400 (G), FLAG-WIP (H), encoding GFP alone (A) REF52.2 cells were transiently transfected with expression plasmids GFP or GFP-WIP (Fig. 4, B and D). Identical results were obtained with COS-7 cells, the only difference being that COS-7 cells contained fewer and shorter stress fibers (data not shown).

To ensure that the observed co-localization of GFP-WIP with actin filaments was not influenced by the GFP fusion partner, we employed a FLAG-tagged WIP fusion protein in our studies. As shown in Fig. 4E, the subcellular distribution of FLAG-WIP was identical to that of GFP-WIP, again without a noticeable effect on actin fibers (Fig. 4F). We further included a truncated WIP mutant (denoted FLAG-WIP400) containing the actin binding KLKK motif and two profilin binding ABM-2 sequences, but lacking the WASP binding region, which in human WIP was mapped to amino acids 417–503 (19). In this case, association with actin fibers was even more pronounced (Fig. 4, G and H) often accompanied with thickening of stress fibers (compare the transfected and surrounding cells in Fig. 4H). These results demonstrate a direct association of WIP with the actin cytoskeleton in vivo. Whether WIP binds directly to actin or merely to actin-associated proteins cannot be deduced from this study.

To address this issue, we mutated the putative actin interaction motif, KLKK in the NH₂-terminal region. Surprisingly, this mutant associated with actin fibers like wild-type WIP (data not shown). To investigate whether wild-type and mutant WIP differed in their affinities for actin filaments, we treated cells in situ with low concentrations (0.2%) of Triton X-100 for 10–60 s. Afterward, cells were fixed and examined by fluorescence microscopy. In either case, the majority of WIP was dissociated from actin fibers after 10 s (data not shown), indicating that association of WIP with the actin cytoskeleton is rather weak and that this interaction is not mediated by the KLKK motif.

Expression of WIP in Tumor Cells Does Not Restore Actin Cables—Tumor cells often show disruption of their actin cytoskeleton correlating with their migratory and metastatic potential (35). This is also the case with the brain tumor cell line TZ103 (22). Since human WIP has been shown to induce formation of actin filaments in lymphoid cells (19), we asked whether WIP would affect the organization of actin in tumor cells. TZ103 cells were transfected with the GFP-WIP construct and examined by fluorescence microscopy. As a control, we included the nontransformed E14-2 cells, which contain well organized actin filaments. In E14-2 cells, GFP-WIP was associated with actin fibers (Fig. 5, A and B) as seen before in fibroblasts (compare Fig. 4C). In contrast, in TZ103 cells WIP was diffusely distributed throughout the cells with some enrichment at plasmamembrane protrusions (Fig. 5C). Thus, WIP seemed to associate only with preformed actin fibers and was not able to restore the actin microfilament system of transformed cells (Fig. 5D).

Rat WIP Binds to N-WASP in Vivo—The human WIP protein has been identified by virtue of its interaction with WASP (19), and more recently it has been shown to interact with N-WASP too (25). In nonlymphoid cells, this latter interaction might be more relevant, because WASP expression is restricted to lymphoid cells. To investigate whether rat WIP binds to N-WASP, we performed co-immunoprecipitation experiments. PC12 cells were transfected with vectors encoding either FLAG-WIP, FLAG-WIP400 (lacking the putative N-WASP binding region), or N-WASP or combinations of these. At 42 h post-transfection, cells were metabolically labeled with [35S]methionine, harvested, and the cell lysates were subjected to immunoprecipitation with anti-FLAG or anti-N-WASP antibodies, respectively. The results of this experiment are shown in Fig. 6. The anti-FLAG antibody clearly precipitated a prominent protein of about 55 kDa from FLAG-WIP-transfected cells but not from FLAG-WIP400 or FLAG vector-transfected cells (Fig. 6, lanes 1, 4, and 7, respectively). In the case of the FLAG-WIP400 construct, a strong protein band of 46 kDa was seen instead (lane 4). Thus, the 55- and 46-kDa proteins represented the FLAG-tagged WIP or WIP400 proteins, respectively.

The 55-kDa protein was also precipitated from cells co-transfected with FLAG-WIP and N-WASP (lane 2). More importantly, this same protein was coprecipitated from double-transfected cells using N-WASP-specific antibodies (lane 3). N-WASP appeared as a relatively faint band of 68 kDa (compare with lanes 6 and 9). When we performed the same experiment with FLAG-WIP400, no coprecipitation was seen, as N-WASP-specific antibodies precipitated only N-WASP but no
other prominent protein (lane 6). Thus, the truncation mutant was not capable of interacting with N-WASP, as expected. In a further control experiment, cells were co-transfected with empty vector and N-WASP, and the same immunoprecipitations were carried out. In this case N-WASP was clearly detected in anti-N-WASP immunoprecipitates (lane 9), but no specific band was seen with anti-FLAG immunoprecipitates (lanes 7 and 8). Together, our results indicate that rat WIP interacts with N-WASP in vivo and that this interaction required the presence of the carboxyl terminus of WIP, as shown for human WIP and WASP (19).

Rat WIP Alters the Subcellular Distribution of N-WASP—To further investigate the interaction between WIP and N-WASP and the possible consequences, we performed immunofluorescence analyses upon co-expression of both proteins in rat fibroblasts. REF52.2 cells were transfected with plasmids encoding N-WASP or FLAG-WIP, immunostained with the respective antibodies, and inspected by immunofluorescence microscopy. As seen in Fig. 7A, N-WASP alone strongly accumulated in the nucleus and in the perinuclear region of transfected cells, but some fainter staining was also seen throughout the cytoplasm. Thus, the distribution of N-WASP was clearly distinct from that of WIP (see Fig. 4, C and E) and of actin (Fig. 7B). It should be noticed that the overall organization of the cytoskeleton did not change upon overexpression of N-WASP (see Fig. 7B) in agreement with previous reports (10).

Surprisingly, when we expressed N-WASP together with FLAG-WIP, the subcellular distribution of N-WASP changed completely from a predominant nuclear to an exclusive cytoplasmic localization with accumulation in the perinuclear area and in cortical areas, particularly in filopodia (Fig. 7C). WIP, on the other hand, was no longer associated with actin filaments (Fig. 7D). Rather, it also accumulated in perinuclear and in cortical areas, like N-WASP (see arrows in Fig. 7, C and D), suggesting that both proteins form complexes in the cytoplasm as already deduced from the co-precipitation experiments. Strikingly, relocation of N-WASP to the cytoplasm seemed to induce disassembly of stress fibers. This relocalization of N-WASP was not seen when N-WASP was co-expressed with WIP400. In this case, N-WASP remained in the nucleus, while WIP400 remained associated with actin fibers (Fig. 7, E and F).

Identical results were also obtained with COS-7 cells (data not shown).

To examine which domains of N-WASP are required for the nuclear localization as well as its relocalization upon co-expression of WIP, we included two N-WASP deletion mutants, N-WASP-WH1 and N-WASP-DWH1, expressed as GFP fusion proteins, in our studies. GFP-N-WASP-WH1 consists of the first 148 amino acids containing the WIP binding region, a VH and PH domain, while GFP-N-WASP-DWH1 lacks this region (25). Upon transfection into REF52.2 cells both mutants were accumulated in the nucleus (Fig. 7, G and J), although the N-WASP-DWH1 mutant (Fig. 7J) also displayed diffuse staining in the perinuclear region. However, co-expression of GFP-N-WASP-WH1 with FLAG-WIP resulted in clear nuclear exclusion of N-WASP-WH1 with prominent cytoplasmic fluorescence staining (Fig. 7H), as seen for co-expressed FLAG-WIP (Fig. 7I). In contrast, when we co-expressed mutant ΔWH1, lacking the WIP binding domain, with FLAG-WIP, the mutant protein still resided in the nucleus (Fig. 7K), whereas FLAG-WIP remained associated with actin fibers (Fig. 7L). These findings indicate that the relocation of N-WASP upon co-expression of WIP was dependent on their direct interaction.

To obtain further evidence that the co-expression of WIP was responsible for the altered subcellular localization of N-WASP, we transfected cells with N-WASP and WIP in different ratios (at 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4, based on the amount of expression plasmids used for transfection). Two days post-transfection, the subcellular distribution of N-WASP and WIP was analyzed by fluorescence microscopy. Quantitative data are shown in the graph in Fig. 7. Expression of N-WASP alone revealed an almost exclusive nuclear expression (Fig. 7, first column in the graph). Co-expression of N-WASP and WIP at a ratio of 3:1 resulted already in a marked redistribution of N-WASP in about 50% of REF52.2 cells, while with COS-7 cells the effect was only marginal (about 6%, Fig. 7, 2nd column of the graph). Further increasing the N-WASP:WIP ratios resulted in a pronounced abrogation of nuclear localization of N-WASP in both cell lines (see the third through seventh columns). These data indicate that the nuclear localization of N-WASP depends on the relative expression level of WIP.

Co-Expression of WIP and N-WASP Resembles the Effects of EGF Stimulation—N-WASP was originally isolated as an interaction partner of Grb2, an adapter protein that connects the epidermal growth factor receptor to the SOS-Ras signaling cascade (10), thus providing a link between mitogenic signals and components or regulators of the cytoskeleton. Indeed, treatment of serum-starved cells with EGF or platelet-derived growth factor was shown to cause accumulation of N-WASP and actin at membrane protrusions and microspikes (36, 37). To compare these effects with our findings upon co-expression of WIP and N-WASP, we transfected REF52 cells with N-WASP constructs, removed the serum for 20 h, and stimulated them with EGF. Serum starvation alone had little or no effects on the distribution of N-WASP or actin fibers (data not shown). In contrast, stimulation of cells with EGF lead to dissolution of stress fibers, accumulation of both N-WASP and actin in perinuclear and plasmamembrane regions, and to extensive microspike formation, as shown in Fig. 8, C (N-WASP) and D (actin staining). These effects were very similar to those observed upon WIP/N-WASP co-expression (compare Fig. 8, A and B), except for the nuclear exclusion of N-WASP, which was not observed upon EGF stimulation. Together, these results suggest that a high relative expression level of WIP mimics or mediates the effects of signal transduction. Interestingly, EGF treatment affected only the N-WASP-transfected but not the untransfected surrounding cells (Fig. 8D), suggesting that a
The relationship between WIP and N-WASP was explored in this study. We described the cloning and functional characterization of the rat WASP-interacting protein, WIP. The rat protein shows 86% sequence identity to human WIP (19). In particular, all presumptive interaction motifs are completely conserved. These include a KLKK motif and two VH domains, presumably involved in actin binding, and three so-called ABM motifs with the sequence APPPPP, which mediate profilin binding (19, 32). Additionally, WIP contains a number of putative SH3 domain-binding motifs, which might play a role for interaction with signaling or adapter molecules like Src kinases or Nck (25, 31, 38). Interestingly, WIP shares several of the interaction motifs with its interaction partners WASP and N-WASP, suggesting that they are capable of independently interacting with an overlapping set of proteins. The modular structure of WIP is also reflected by the different splice variants.

**Fig. 7. Co-expression of rat WIP and N-WASP alters the subcellular distribution of N-WASP.** REFS2.2 cells were transiently transfected with plasmids encoding either N-WASP (A and B), GFP-N-WASP-RH1 (G), or GFP-N-WASP-ΔWH1 (J) or co-transfected with N-WASP and FLAG-WIP (C and D), N-WASP and FLAG-WIP400 (E and F), GFP-N-WASP-RH1 and FLAG-WIP (H and I), or GFP-N-WASP-ΔWH1 and FLAG-WIP (K and L). At 42 h post-transfection, cells were fixed and stained for indirect immunofluorescence with polyclonal anti-N-WASP antibody (A, C, E, G, and I) or with monoclonal antibody FLAG M2 to stain WIP (D, F, I, and L) as outlined under "Experimental Procedures." Bar, 10 μm.

**Fig. 8. Comparison of WIP/N-WASP co-expression with EGF stimulation of N-WASP transfected cells.** REFS2.2 cells were transfected with N-WASP (C and D) or co-transfected with N-WASP and WIP encoding constructs (A and B) as indicated. The cells in C and D were serum-starved for 20 h and then stimulated with 100 ng/ml mouse EGF for 10 min. Cells were fixed and stained for N-WASP (A and C) or actin (B and D) as described under "Experimental Procedures." Bar, 10 μm.

**DISCUSSION**

In this study, we described the cloning and functional characterization of the rat WASP-interacting protein, WIP. The rat protein shows 86% sequence identity to human WIP (19). In particular, all presumptive interaction motifs are completely conserved. These include a KLKK motif and two VH domains, presumably involved in actin binding, and three so-called ABM motifs with the sequence APPPPP, which mediate profilin binding (19, 32). Additionally, WIP contains a number of putative SH3 domain-binding motifs, which might play a role for interaction with signaling or adapter molecules like Src kinases or Nck (25, 31, 38). Interestingly, WIP shares several of the interaction motifs with its interaction partners WASP and N-WASP, suggesting that they are capable of independently interacting with an overlapping set of proteins. The modular structure of WIP is also reflected by the different splice variants.

**Significance of Alternative Splicing**—Northern blot analyses revealed ubiquitous expression and, more interestingly, alternative splicing in a tissue-specific manner. The 5.0-kb and the 3.8-kb mRNA species were most abundant and ubiquitously expressed. In contrast, a 6.9-kb transcript was specifically expressed in skeletal muscle and the smaller transcripts of 2.4, 1.8, and 1.4 kb seemed to be specific for liver, heart, and/or tests, suggesting that the products they encode fulfill tissue-specific functions. In fact, the splicing variants differed not only in the 3' untranslated region but also in the coding region. Comparison of the transcripts with the exon composition deduced from the human WIP gene showed that the smaller transcripts lacked two or more exons. Consequently, the respective proteins must differ in their interaction profiles. Each of the splice variants would contain at least one ABM motif and be capable of interacting with profilin/actin. But only the 2.4-, 1.8-, and 1.1-kb products contain the NH₂-terminal VH domains, including the KLKK motif. Interestingly, the 1.4-kb product contains only the WASP interaction domain and thus could be a negative regulator of WASP proteins. On the contrary, the 2.4-, 1.8-, and 1.1-kb products lack the WASP interaction domain and thus must have functions independent of WASP proteins. It will be interesting to isolate and characterize the individual splicing variants and their products and...
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elucidate their functions. Of particular interest is the 6.9-kb message in skeletal muscle, which presumably has a special function in the muscle acto-myosin system.

Subcellular Localization of WIP and N-WASP—Overexpression of human WIP in lymphoid BJAB cells was reported to result in actin polymerization (19). When we ectopically expressed WIP in rat fibroblasts, we observed a clear co-localization with actin stress fibers which was not reported before. On the other hand, expression of WIP in tumor cells lacking stress fibers did not restore these structures. Thus, WIP expression per se does not seem to influence the state or formation of actin fibers, at least not in the fibroblast or the brain tumor cells used here. Rather, WIP seems to associate with preformed fibers. Indeed, during the completion of our manuscript Martinez-Quiles and co-worker (39) reported that WIP can bind directly to both F- as well as G-actin. Thus, the formation of stress fibers or other actin filaments like in filopodia appears to depend on other factors, such as thymosin β4 or gelsolin, both of which counteract actin polymerization. In fact, gelsolin is overexpressed in the tumor cell line TZ103, which might be related to the loss of stress fibers (22). However, there are probably tissue-specific differences in the interactions and functions of WIP. Additionally, the splicing variants might contribute to the diversity of cytoskeletal functions.

The subcellular localization of N-WASP was rather distinct from that of WIP. The major fraction of N-WASP was located in the nucleus and did not exhibit co-localization with stress fibers. The nuclear localization of N-WASP has been reported before (10, 18). Indeed, N-WASP contains three putative nuclear localization signals (two bipartite NLSs at positions 128 and 131 and a KKKR motif at position 194) and is predicted to be localized to 69% in the nucleus (PSORT II). As seen with WIP, overexpression of N-WASP alone did not significantly alter the organization of the actin cytoskeleton, although there was some enrichment of actin with N-WASP at the plasma membrane and at microspikes. Together, these observations suggest that N-WASP has functions independent of WIP.

Interaction of WIP and N-WASP—Co-immunoprecipitation and co-expression experiments demonstrated that rat WIP, but not the deletion mutant WIP400 binds to N-WASP, indicating that the interaction with N-WASP requires the COOH-terminal tail of WIP (i.e. residues 401–487) as published for the human homologues (19). Interestingly, co-expression of N-WASP with WIP resulted in nuclear exclusion and partial co-localization of N-WASP and WIP in the perinuclear and plasma membrane areas. This relocation provided further evidence for an interaction between the two proteins in vivo. Relocation of N-WASP could result from active export or from inhibition of nuclear import due to complex formation with WIP. Since the WIP interaction domain of N-WASP (25) overlaps with one of the putative NLSs, it is conceivable that WIP retains N-WASP in the cytoplasm by masking the putative nuclear localization signals.

Relocation of N-WASP by WIP had a profound effect on the actin cytoskeleton. In most cells the filamentous staining of both WIP and actin disappeared. Concomitantly, there was an increase of WIP, N-WASP, and actin at the plasma membrane and in newly formed filopodia. These findings can be interpreted as remodeling of the actin cytoskeleton involving disassembly of stress fibers in favor of filopodia formation. Thus, upon accumulation in the cytoplasm, N-WASP seems to affect WIP and its function at actin fibers as well. Obviously, each of these proteins can act upstream of the other and affect its interactions and functions. This interpretation is in agreement with other systems in which vaccinia virus or Shigella are used to visualize actin polymerization. In the case of vaccinia virus, WIP seems to associate with vaccinia virus protein and then recruits N-WASP to the complex, in Shigella, it is reverse (25).

What Is the Physiological Significance of Rat WIP/N-WASP Interaction?—N-WASP was originally isolated in a two-hybrid screen with the adaptor protein Grb2 as bait, implicating N-WASP in signal transduction. A number of recent investigations have pointed to a role of N-WASP in regulating cell motility, facilitated by actin polymerization, particularly in filopodia or microspikes. According to the present model, N-WASP is located in an inactive form by interaction between its amino and carboxyl termini. Signaling from growth factor receptors leads to an increase inPIP2 and activation of Cdc42, which binds to the NH2-terminal pleckstrin homology domain of N-WASP. This interaction releases the COOH terminus for interaction with the Arp2/3 complex and actin and perhaps other signaling molecules. This in turn will initiate actin polymerization at the leading edge of cells, thereby regulating cell migration (for review, see Ref. 27).

The role of WIP is less clear. It probably has its own activities, independent of WASP proteins. This is suggested (i) from the similar modular composition, enabling WIP protein to interact with a similar set of proteins as WASP, (ii) from the different subcellular distribution, and (iii) from the prediction of different splice variants that cannot interact with WASP. One function of WIP might be its association with stress fibers to facilitate actin mobilization under appropriate conditions.

Our investigation addressed the possible consequences of the WIP/N-WASP interaction. Most striking was the relocation of N-WASP from the nucleus to the cytoplasm and to the cell surface and, concomitantly, dissolution of WIP and actin fibers and extensive formation of filopodia. Very similar effects on N-WASP relocation and disassembly of actin fibers were observed upon EGF stimulation of serum-starved cells (Ref. 10 and this study). Thus, overexpression of WIP seems to mimic the effects of growth factor signaling. From these findings we propose that WIP and N-WASP have mutual effects on each other, the results depending on their relative expression levels. WIP may participate in regulating the subcellular localization of N-WASP. After relocation to the cytoplasm, N-WASP may have two functions. First, it seems to target WIP for mobilizing actin from stress fibers, thereby supplying actin for the polymerization activity at the cell surface, and second, it drives this polymerization process by recruiting the Arp2/3 complex and actin to the plasma membrane (for review, see Ref. 27). Under physiological conditions, these processes are probably regulated by interaction with signaling molecules such as SH3 domain-containing adaptor proteins (Grb-2, Nck) or GTPases of the Rac/Rho/Cdc42 family (reviewed in Ref. 40), by phosphorylation or by other interactions yet to be identified. Clearly, much more work needs to be done to get insight into the complex processes involved in remodeling of the cytoskeleton and to understand how extracellular signals are converted into cytoskeletal activities such as migration or vesicle transport.

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