The Human WASP-interacting Protein, WIP, Activates the Cell Polarity Pathway in Yeast

(Received for publication, December 17, 1998, and in revised form, March 3, 1999)

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WASP, the Wiskott-Aldrich syndrome protein-interacting protein, is a human protein involved in actin polymerization and redistribution in lymphoid cells. The mechanism by which WIP reorganizes actin cytoskeleton is unknown. WIP is similar to yeast verprolin, an actin- and myosin-interacting protein required for polarized morphogenesis. To determine whether WIP and verprolin are functional homologues, we analyzed the function of WIP in yeast. WIP suppresses the growth defects of VRP1 missense and null mutations as well as the defects in cytoskeletal organization and endocytosis observed in vrp1–1 cells. The ability of WIP to replace verprolin is dependent on its WH2 actin binding domain and a putative profilin binding domain. Immunofluorescence localization of WIP in yeast cells reveals a pattern consistent with its function at the cortical sites of growth. Thus, like verprolin, WIP functions in yeast to link the polarity development pathway and the actin cytoskeleton to generate cytoskeletal asymmetry. A role for WIP in cell polarity provides a framework for unifying, under a common paradigm, distinct molecular defects associated with immunodeficiencies like Wiskott-Aldrich syndrome.

Wiskott-Aldrich syndrome (WAS) is an inherited immune deficiency characterized by eczema, bleeding, and recurrent infections. A deficiency in both cellular and humoral immunity is common among all WAS patients (1–3). Lymphocytes and platelets from WAS patients show cytoskeletal abnormalities, and T lymphocytes of WAS patients show diminished proliferative response to stimulation through the T cell receptor-CD3 complex (4).

Molecular analyses of the WAS gene (5) have provided insights into the roles of Wiskott-Aldrich syndrome protein (WASP) in the actin cytoskeleton function and in cell proliferation. WASP binds via its GTPase binding domain to CDC42Hs and weakly to Rac but not to Rho (6). Overexpression of WASP induces formation of actin-containing clusters, indicating a role for WASP in actin polymerization (6). These findings suggest that WASP may provide a connection between CDC42Hs, Rac, and the actin cytoskeleton. One possible link between the actin cytoskeleton and WASP is the recently identified WASP-interacting protein WIP (7). Transfection of WIP into BJAB cells induced actin-containing cerebriform projections beneath the cell membrane, suggesting that WIP may be involved in regulating the dynamics of the actin cytoskeleton.

WIP has sequence similarity to Saccharomyces cerevisiae verprolin, encoded by the VRP1 gene (8). Verprolin interacts directly with the yeast WASP, Las17p (9). This prompted us to determine whether WIP and verprolin are functional homologues. If the human protein is a homologue of the yeast protein, then the genetic and cell biology data available for verprolin should prove useful for learning more about the function of WIP in human cells.

In wild-type yeast cells, the actin cytoskeleton is polarized along the mother-daughter axis (10). When the function of verprolin is impaired (in vrp1–1) or absent (in vrp1 null), the asymmetry of actin cytoskeleton along the mother-bud axis is lost, actin cables are faint or absent, and cytoskeleton-associated functions like endocytosis are compromised (8, 11–13). In addition, vrp1–1 or vrp1 null cells cannot grow at 37 °C (11). WIP suppresses the growth defects of VRP1 missense and null mutations as well as the cytoskeletal and endocytosis defects of vrp1–1 cells. Mutations in conserved domains of WIP impair its ability to suppress vrp1 mutations. Furthermore, WIP has a polarized intracellular localization that often coincides to that of actin. The data support the hypothesis that WIP and verprolin are functional homologues and provide new ways to understand the molecular defects associated with the Wiskott-Aldrich syndrome.

MATERIALS AND METHODS

Strains and General Techniques—Yeast media was prepared using standard methods (14). Yeast strains were transformed by the one step method (15). Yeast strains used were TZ33 (MATa, SUP11 ade2–1 mods1–1 ura3–1 lys2–1 leu2–1, 112 his–519 vrp1–1) and T65–1D (MATa, ade1, ura3–52 leu2–3, 112 ile MEL1 vrp1::LEU2) (11). Standard recombinant DNA techniques (16) and DH5α and RR1 bacterial strains were used. Restriction endonucleases and modifying enzymes were obtained from New England Biolabs (Beverly, MA) or from Promega (Madison, WI).

Location of the vrp1–1 Mutation—Genomic DNA from TZ33 was prepared (17), and the vrp1–1 gene was polymerase chain reaction-amplified using the following primers: 5’-caatgttcggtttgcctcattgctg-3’ and 5’-gctaatcttgctgacttggcg-3’. The polymerase chain reaction products from several independent reactions were cloned into pGEM-T vector and subjected to automated sequencing.

Plasmid Constructions—WIP cDNA or WIP 2 (amino-terminal 116-amino acid truncation of WIP, (7)) cloned in pUC18 were digested with EcoRI, filled in, and redigested with PstI, and the insert was then ligated to SmaI-PstI-digested pEMBLyex4, a pEMBLy (18)-based vector (the vector was a kind gift of Dr. E. Orr, University of Leicester, UK).
Role of WIP in Cell Polarity

WIP Is Likely the Human Homologue of Verprolin—Sequence conservation predicts WIP might be a functional homologue of verprolin. vprp1::LEU2 is a null allele (11), and the vprp1–1 allele contains a Leu → Pro mutation at amino acid 425, which is part of a proline-rich region homologous to the Nck binding domain (21) of WIP (Fig. 1). Both mutations result in cells unable to grow at 37 °C. WIP cloned in the pEMBLyex4 vector was transfected in vprp1–1 and vprp1 null cells, and its effect on the temperature-sensitive growth defects of these cells was determined. Genes cloned in pEMBLyex4 are expressed from a CYC1 promoter preceded by GAL1–10 upstream-activating sequences, resulting in galactose-inducible gene expression. Cells were grown on glucose-selective media, transferred to media and, more importantly, among defined domains involved in actin, profilin, or WASP interaction (Fig. 1). The WH2 (WASP homology 2) domain of WIP is 44% identical to the actin-interacting WH2 domain of verprolin (Fig. 1). The next highest segment of sequence similarity between WIP and verprolin (47% identity) spans the first 116 amino acids of WIP (Fig. 1).

An artificial initiation codon (ATG) was introduced in WIP2 as follows: 5′-TTGACATCATGGACAGTAGACAAAGAGAATGAGCTTCTGGAGGAGGCGA and 5′-GCCGATCTACATGGACAGTAGACAAAGAGAATGAGCTTCTGGAGGAGGCGA and 5′-GCCGATCTACATGGACAGTAGACAAAGAGAATGAGCTTCTGGAGGAGGCGA and 5′-GCCGATCTACATGGACAGTAGACAAAGAGAATGAGCTTCTGGAGGAGGCGA and 5′-GCCGATCTACATGGACAGTAGACAAAGAGAATGAGCTTCTGGAGGAGGCGA. The region containing the WIP-Lys mutation was exchanged with EcoRI-BstEII digestion and ligated to WIP cDNA digested with EcoRI-BstEII to generate WIP-Lys. The WIP-Lys-Pro double mutant was constructed by exchanging a SfiI-BamHI fragment of WIP containing the WASP-binding domain of WIP, binds to yeast WASP, Las17p (9). Although verprolin interacts with Las17p (9), verprolin failed to bind human WASP by two-hybrid analysis (data not shown), suggesting that the conservation of the WASP binding region alone may not be sufficient for human WASP-Vrp1 interaction.

WIP also interacts with profilin (7) and contains two putative profilin binding sequences between amino acids 8–13 and 427–433. The latter is conserved in verprolin, but interaction between verprolin and profilin was not detected by two-hybrid analysis (8).

The middle portions of WIP and verprolin contain 10 and 11, respectively, potential SH3 binding domains. In WIP, amino acids 321–415 of this region interact with Nck (21). In verprolin, multiple sites in the SH3 binding domains region interact with the SH3 domain of Myo5p (22).

At the carboxyl terminus, WIP contains a 72-amino acid-long WASP-interacting domain (21) (Fig. 1). The carboxyl-terminal 337 amino acids of verprolin, containing a conserved version of the WASP-binding domain of WIP, binds to yeast WASP, Las17p (9). Although verprolin interacts with Las17p (9), verprolin failed to bind human WASP by two-hybrid analysis (data not shown), suggesting that the conservation of the WASP binding region alone may not be sufficient for human WASP-Vrp1 interaction.

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RESULTS

The WH2 domain of both proteins is underlined with the stippled blue bar. The amino-terminal 116 amino acids of WIP missing in WIP2 are boxed in blue. Two conserved lysines (17KK18) mutated in WIP-Lys are marked by underlined black bars. The Pro mutation at amino acid 425, which is part of a proline-rich region homologous to the Nck binding domain (21) of WIP (Fig. 1). Both mutations result in cells unable to grow at 37 °C. WIP cloned in the pEMBLyex4 vector was transfected in vprp1–1 and vprp1 null cells, and its effect on the temperature-sensitive growth defects of these cells was determined. Genes cloned in pEMBLyex4 are expressed from a CYC1 promoter preceded by GAL1–10 upstream-activating sequences, resulting in galactose-inducible gene expression. Cells were grown on glucose-selective media, transferred to media and, more importantly, among defined domains involved in actin, profilin, or WASP interaction (Fig. 1). The WH2 (WASP homology 2) domain of WIP is 44% identical to the actin-interacting WH2 domain of verprolin (Fig. 1). The next highest segment of sequence similarity between WIP and verprolin (47% identity) spans the first 116 amino acids of WIP (Fig. 1). The middle portions of WIP and verprolin contain 10 and 11, respectively, potential SH3 binding domains. In WIP, amino acids 321–415 of this region interact with Nck (21). In verprolin, multiple sites in the SH3 binding domains region interact with the SH3 domain of Myo5p (22).

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FIG. 1. Sequence and domain comparison between verprolin and WIP. The WH2 domain of both proteins is underlined with the stippled blue bar. The amino-terminal 116 amino acids of WIP missing in WIP2 are boxed in blue. Two conserved lysines (17KK18) mutated in WIP-Lys are marked by underlined black bars. The Pro mutation at amino acid 425, which is part of a proline-rich region homologous to the Nck binding domain (21) of WIP (Fig. 1). Both mutations result in cells unable to grow at 37 °C. WIP cloned in the pEMBLyex4 vector was transfected in vprp1–1 and vprp1 null cells, and its effect on the temperature-sensitive growth defects of these cells was determined. Genes cloned in pEMBLyex4 are expressed from a CYC1 promoter preceded by GAL1–10 upstream-activating sequences, resulting in galactose-inducible gene expression. Cells were grown on glucose-selective media, transferred to media and, more importantly, among defined domains involved in actin, profilin, or WASP interaction (Fig. 1). The WH2 (WASP homology 2) domain of WIP is 44% identical to the actin-interacting WH2 domain of verprolin (Fig. 1). The next highest segment of sequence similarity between WIP and verprolin (47% identity) spans the first 116 amino acids of WIP (Fig. 1).
Approximately 20% of small- and medium-budded cells have polarized actin patches in endocytosis (Fig. 5A). Expression of WIP in *vrp1–1* leads to partial suppression of the defect in endocytosis (Fig. 5C).

The WH2 and the Putative Profilin Binding Domains Are Critical for WIP Function—To analyze the requirement for the WH2 domain in WIP function, we generated a truncated version of WIP, WIP2, lacking the first 116 amino acids (Fig. 2B). WIP2 was cloned in pEMBLex4 downstream of a translation initiation codon and tested for its ability to rescue the temperature sensitivity of *vrp1* cells. WIP2 is unable to restore endocytosis (Fig. 5D) nor to complement the temperature-sensitive growth defect of either *vrp1–1* or the *vrp1* null cells on galactose at 37 °C (Fig. 3, compare A with C). However, because Western analysis revealed poor WIP2 expression in yeast (Fig. 2A), no conclusions can be inferred from this lack of complementation.

To address the role of the WH2 domain for WIP function in another way, we generated point mutations in this domain. The sequence KLKK has been shown to be critical for actin binding among several proteins (24). Mutation of **45KK46** abolishes the interaction of the WH2 domain of verprolin with actin (8). A mutant WIP, WIP-Lys, was generated that has the two homologous lysines, **47KK48**, replaced by alanines. WIP-Lys is expressed at levels comparable with wild-type WIP (Fig. 2A) but is unable to complement the null mutation (Fig. 6D) and poorly suppresses the *vrp1–1* mutation (Fig. 6B). Even at the permissive temperature the generation time of *vrp1–1* cells producing WIP-Lys is about 4 times longer than that of *vrp1–1* cells producing WIP (Table I). Therefore, lysines 47 and 48 are important for the biological function of WIP.

WIP interacts with profilin (7) and has two putative APPPPP profilin binding motifs, homologous to those found in profilin-interacting proteins Mena and VASP (24). The second APPPPP motif of WIP is conserved in verprolin (Fig. 1). To test the role of this motif for WIP function, we generated the WIP-Pro mutant (Fig. 2B) in which three proline residues are mutated to alanine, *i.e.* **427APPPPPP433** to APAPAPA. The expression of WIP-Pro is comparable with that of wild-type WIP (Fig. 2A). The generation time of *vrp1–1* cells producing WIP-Pro is 3-fold longer than that of WIP producing *vrp1–1* cells (Table I); the growth of *vrp1* null cells producing WIP-Pro, although not abolished as for WIP-Lys, is much reduced on galactose at 37 °C in comparison with the same cells producing WIP (Fig. 6D). Furthermore, a double mutant, WIP-Lys,Pro, containing both **47KK → AA48** and **427APPPPPP → APAPAPA433** mutations (Fig. 2B), leads to a further increase in generation time as compared with each mutant alone (Table I). Therefore, the two conserved lysines, **47KK48**, and the APPPPP motif contribute to the full activity of WIP in vivo.

Localization of WIP in Yeast—If WIP performs a conserved function in cell polarity, it should serve as a polarity marker at sites of active cell growth, as does verprolin (8). To test this hypothesis, we determined the intracellular localization of WIP in yeast cells by immunofluorescence using anti-WIP antibody (21). Like verprolin, WIP localizes to regions of active growth in yeast cells in a cell cycle-dependent manner. Although there is WIP staining throughout the cytosol, there are high local concentrations in a punctated pattern at the site of bud emergence, the tip of the bud, or throughout most of the bud (Fig. 7A, left). The WIP patches often colocalize (Fig. 7A, left, arrows) with actin patches (Fig. 7A, right). The patch-like staining is not present in control cells lacking WIP; a very small percentage of these control cells show diffuse cytoplasmic staining, perhaps because of weakly cross-reacting cytoplasmic protein(s) (Fig. 7B, left). Therefore, it appears that WIP can serve as a polarity marker in yeast, suggesting that the sequence containing either glycerol or galactose, and incubated at various temperatures. As expected, induction by galactose leads to elevated expression of WIP (Fig. 2A).

*vrp1–1* or *vrp1* null cells producing WIP are unable to grow at 37 °C on media containing glycerol as the carbon source. These same cells grow on media containing galactose at 37 °C (Table I; Fig. 3, compare A with C). Under the same conditions, *vrp1–1* or *vrp1* null cells with vector alone fail to grow (Fig. 3C). Thus, as with other human cytoskeletal proteins, high levels of protein are necessary for the complementation (23). The suppression by WIP of the growth defects of *vrp1* cells, together with the sequence similarity and domain conservation between the two proteins, indicate that WIP is likely the human homologue of verprolin.

**WIP Restores Actin Cytoskeleton Polarization and Endocytosis in *vrp1–1* Yeast Cells**—In a population of *vrp1–1* cells, only about 20% of small- and medium-budded cells have polarized cytoskeletons (Fig. 4A). In wild-type cells, under similar conditions, about 80% of cells are polarized (Fig. 4B). WIP expression in *vrp1–1* cell increases the proportion of polarized cells to 65% (Fig. 4C). Thus, like verprolin, WIP functionally participates in the mechanisms(s) that regulates the polarized distribution of actin patches between the mother and the bud.

Defects in fluid-phase endocytosis can be detected in yeast by monitoring the uptake of a fluorescent marker such as lucifer yellow into the vacuole, the equivalent of mammalian lysosomes. Although *vrp1–1* cells bearing VRP1 on a plasmid internalize lucifer yellow (Fig. 5B), *vrp1–1* cells transformed with vector alone are unable to internalize the dye (Fig. 5A). Expressions of WIP in *vrp1–1* leads to partial suppression of the defect in endocytosis (Fig. 5C).

**FIG. 2.** A, Western blot analysis of wild-type and mutant WIP. Approximately 20 μg of total protein extract were loaded in each lane and probed with anti-WIP rabbit antiserum. The blot was reprobed with an anti-Nsp1p monoclonal antibody to confirm equal loading in each lane. B, diagram of WIP mutants. Construct names are listed to the left of each construct. The size of each construct is indicated by numbers. Letters above each construct represent the amino acid replacements. CTR, control, vector alone.
determinants which asymmetrically localize verprolin along the mother-daughter cell axis are conserved in WIP. 

**FIG. 3.** WIP, but not amino-terminal-truncated WIP2, complements the temperature sensitivity of *vrp1–1* (strain TZ33) and *vrp1* null (strain T65–1D) cells. Cells were grown on glucose-containing media then replica-plated on galactose-containing media and incubated for 3 days at 23 °C (A), 34 °C (B), and 37 °C (C). The right half of the plate is strain T65–1D. The left half of the plate is strain TZ33. Genes on plasmids are as follows: 1, vector; 2, VRP1; 3, WIP; 4, WIP2; 5, WIP2; 6, WIP; 7, VRP1; 8, vector.

**FIG. 4.** WIP restores the polarized morphology of actin cytoskeleton in *vrp1–1* cells (strain TZ33). Logarithmic cultures of TZ33 transformed with vector alone (A), VRP1 (B), WIP (C), or WIP2 (D) were grown on galactose-containing media at 23 °C, fixed, and then stained with Oregon green phalloidin. Approximately 200 small- and medium-budded cells were scored to quantify cytoskeletal polarization for each strain. Bar, 4 μm.

**FIG. 5.** WIP restores endocytosis in *vrp1–1* cells (strain TZ33). Logarithmic cultures were grown on galactose-containing media at 23 °C, incubated with lucifer yellow for 30 min, washed, and visualized. A, vector; B, VRP1 on a centromeric plasmid; C, WIP; D, WIP2. In E, F, G, and H, the differential interference contrast (DIC) images corresponding to A, B, C, and D, respectively, are shown. Bar, 4 μm.

**FIG. 6.** Mutations in WH2 or the putative profilin binding domain of WIP impairs its ability to complement the temperature sensitivity of *vrp1–1* cells (A and B) or *vrp1* null strain (C and D). Cells were grown on glucose-containing media and then replica plated on galactose-containing media and incubated for 3 days at 23 °C (A and C) or 37 °C (B and D).
cause T cells transfected with CDC42G12V (a mutant locked in the GDP-bound conformation) or CDC42G12V (a mutant locked in the GTP-bound conformation) are unable to polarize their cytoskeletons toward the antigen (28). In yeast, Cdc42p is a major cell polarity establishment protein (29) required for actin nucleation (30). Thus both the yeast and human CDC42 proteins function in cell polarity.

A CDC42Hs effector, human WASP, has profound effects on actin polymerization (6). The yeast homologue of WASP, encoded by LAS17/BEE1, binds actin (30) and activates the actin assembly sites in the cortical cytoskeleton (31).

Like verprolin, WASP-interacting WIP may perform its function in cytoskeletal organization by localizing to specialized regions of the cell and recruiting additional proteins such as actin to initiate morphogenic changes. Thus, the CDC42Hs-WASP-WIP pathway possibly fulfills the critical function of generating and enforcing cytoskeletal polarity.

Other components in the pathway may exist and CDC42Hs, WASP, and WIP may also be involved in other pathways. CDC42Hs is also required for the induction of DNA synthesis upon mitogen activation of the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) mitogen-activated protein kinase cascade (32). WASP may act as a molecular switch, because it interacts with a multitude of proteins, including phospholipase C, Tec family members Btk, Itk, Tec, Grb, as well as p59vrp and Nck (33–35). WIP also interacts with Nck (21). Further work is required to fully delineate all pathways in which these proteins are involved.

Is Wiskott-Aldrich Syndrome a Cell Polarity Disease?—The clinical features of WAS suggest that cytoskeletal polarity defects may be the cause for an altered immune response. Morphological and biochemical studies show that the cytoskeletons of lymphocytes affected by WAS are aberrant (36, 37) and unable to polarize toward polysaccharide antigen-presenting cells (38). Recognition of mitogen stimuli requires cell polarization mediated by cytoskeletal rearrangements (28, 39). WAS lymphocytes are not able to respond to immobilized anti-CD3 antibodies (37) and have diminished response to chemottractants and a profound decrease in polarization (40, 41). When monocytes are stimulated with chemoattractants, rapid rearrangements of F-actin toward the poles occur. In contrast, actin distribution is uniform in monocytes derived from WAS patients (40). Because both WIP (7) and WASP (6) are involved in the redistribution of F-actin and because CDC42Hs is involved in macrophage chemotaxis (42), these three interacting proteins may act in a hierarchical order to trigger cell polarity. That WIP is able to restore polarity to vrp1–1 cells supports this contention.

Ligand engagement of the CD3 T cell receptor promotes its endocytosis (43), a function dependent on the actin cytoskeleton. Restoration by WIP of the endocytosis defects of vrp1 cells may reflect its ability to perform an analogous function in human cells. Further work is necessary to understand the possible role of WASP and WIP in endocytosis and their relevance in WAS.

In conclusion, although different levels of complexity operate in yeast and humans, a model emerges in which the polarity pathway described here has been adapted by various cells to specifically serve their cytoskeletal functions. Further dissection of this pathway in both yeast and metazoans will increase our understanding of its function and its connections with other signaling pathways.

Acknowledgments—We thank David Stanford, James Stanford, Simonit Sarkar, and Ann Benko for comments on the manuscript and stimulating discussions.

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