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# Direct interaction of the Wiskott–Aldrich syndrome protein with the GTPase Cdc42

**(immunodeficiency/small guanosine 5′-triphosphate binding protein/Rho family/cytoskeleton/actin)**

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**ABSTRACT** **Wiskott–Aldrich syndrome (WAS) is an X-linked immunodeficiency disorder with the most severe pathology in the T lymphocytes and platelets. The disease arises from mutations in the gene encoding the WAS protein. T lymphocytes of affected males with WAS exhibit a severe disturbance of the actin cytoskeleton, suggesting that the WAS protein could regulate its organization. We show here that WAS protein interacts with a member of the Rho family of GTPases, Cdc42. This interaction, which is guanosine 5′-triphosphate (GTP)-dependent, was detected in cell lysates, in transient transfections and with purified recombinant proteins. A weaker interaction was also detected with Rac1 using WAS protein from cell lysates. It was also found that different mutant WAS proteins from three affected males retained their ability to interact with Cdc42 and that the level of expression of the WAS protein in these mutants was only 2–5% of normal. Taken together these data suggest that the WAS protein might function as a signal transduction adaptor downstream of Cdc42, and in affected males, the cytoskeletal abnormalities may result from a defect in Cdc42 signaling.**

The Wiskott–Aldrich syndrome (WAS) results from a monogenic defect that has been mapped to the short arm of the X chromosome at Xp11.23 (1–3). Affected males have severe thrombocytopenia and abnormally small platelets. They are susceptible to recurrent pyogenic and opportunistic infections and they develop eczema (4–9). Antibody responses to polysaccharide and, to a lesser extent, protein antigens are defective (4–9). The gene that is mutated in WAS was recently cloned (10). It encodes a 503-amino acid protein of unknown function. Clues to the function of the WAS protein (WASp) are the observations that T lymphocytes of affected males have a paucity of microvilli and that the cytoskeleton is in disarray (11, 12), which suggests that the WAS protein may regulate the actin cytoskeleton in T cells. Members of the Rho family of small guanosine 5′-triphosphate (GTP) binding proteins are involved in regulating the actin cytoskeleton (13–15), and Cdc42 appears to regulate the cytoskeleton in T cells (16). The cytoskeletal defects in the T cells of WAS patients may therefore be due to defective Cdc42 signaling. A comparison of WASp with proteins in the data base revealed homology with a domain that has been shown to bind Cdc42 and Rac1 in the serine/threonine protein kinase PAK65 and in the nonreceptor tyrosine kinase ACK (17, 18). We therefore investigated whether WASp associates with Cdc42 and related proteins in the Rho family.

## MATERIALS AND METHODS

**Antibodies.** Antiserum to the WASp was raised in rabbits by injection of synthetic peptides B4 (HDKKRSGKKKISKADI) and C6 (DEGEDQAGDEDEDDEWDD) and was affinity purified with the corresponding peptides conjugated to Affi-Gel 10 or 15 (Bio-Rad). Antibodies to Rac1 and Cdc42 were obtained from Santa Cruz Biotechnology. The monoclonal antibody, specific for the hemagglutinin epitope tag, was 12CA5.

**Expression Vectors.** Full-length human WAS cDNA was provided by Sau-Ping Kwan (Rush Medical School, Chicago). A truncated form of WASp, amino acid residues 1–321, was appended to glutathione *S*-transferase (GST-ΔWASp) by insertion of the *Eco*RI/*Xho*I fragment of WAS cDNA into the corresponding sites in the polylinker of the prokaryotic expression vector pGEX 4T3 (Pharmacia). A *Bam*HI/*Not*I fragment containing the complete open reading frame of WAS was also appended to GST of the eukaryotic expression vector PEBG under the transcriptional control of the elongation factor-1α promoter (gift of B. Meyers, Harvard Medical School). GST-fusion constructs of Rac1, RhoA, and Cdc42 were provided by Larry Feig (Tufts University). Expression of the GST fusion proteins in *Escherichia coli* and subsequent purification were performed as described (19).

The wild-type (wt) wtCdc42 and the mutant forms V12Cdc42 and N17Cdc42 (15, 20, 21) were expressed by using the eukaryotic expression vector PJ3Ω under the transcriptional control of the simian virus-40 promoter. These constructs were provided by J. Blenis and M. Chou (Harvard Medical School).

**Cells and Cell Lines.** Mononuclear cells were obtained from freshly drawn blood of controls and two male patients with WAS and subsequent separation on Hypaque-Ficoll. Epstein–Barr virus (EBV)-transformed B cells (EBV-B; R86C and R86H) from males with missense mutations in WAS have been described (22). These cells, as well as a human erythroleukemia (HEL) cell line and EBV-B from normal donors were maintained in RPMI 1640 medium. COS-7 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM). Both media contained 10% fetal calf serum, 100 μg of penicillin G per ml, and 100 μg of streptomycin per ml (GIBCO).

**Binding Assays.** Cells (≈5 × $10^6$) were washed with phosphate-buffered saline (PBS) and solubilized in lysis buffer (50 mM Tris, pH 7.5/150 mM NaCl/5 mM $MgCl_2$/1% Triton X-100/10 μg of leupeptin per ml/10 μg of aprotinin per ml/1 mM phenylmethylsulfonylfluoride), followed by gentle rotation for 20 minutes at 4°C. Lysates were clarified by centrifu-

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*Abbreviations*: EBV, Epstein Barr virus; EBV-B, EBV-transformed B cell; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTP[γS], guanosine 5-′-[γ-thio]triphosphate; GST, glutathione *S*-transferase; HA, hemagglutinin; HEL, human erythroleukemia; WAS, Wiskott–Aldrich syndrome; WASp, Wiskott–Aldrich syndrome protein.

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gation at 13,000 × *g* for 10 min at 4°C. GST or GST-fusion GTPase proteins (15 μl) bound to glutathione-Sepharose CL4B beads (Sigma) were loaded with guanosine 5′-diphosphate (GDP)[βS] or guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (23) and incubated with 2 mg of cell lysate in a volume of 0.5 ml for 2 h at 4°C with constant rocking. The beads were washed twice with 1 ml lysis buffer and an additional two times with lysis buffer without detergent. The bound proteins were fractionated by SDS/PAGE and analyzed by Western blotting with antibodies to WASp using enhanced chemiluminescence (Amersham).

GST-ΔWASp (10 μg) bound to glutathione-Sepharose beads was used in binding assays to study the direct interaction of WASp with Cdc42 and Rac1. The Rac1 and Cdc42 proteins used in these experiments were cleaved from GST with thrombin, loaded with GDP[βS] or GTP[γS] and subsequently separated from the components of the loading buffer by gel filtration (19). Western blotting was performed as above with antibodies to Cdc42 or Rac1.

**Transfections and Coimmunoprecipitations.** COS-7 cells were plated at a density of ≈$10^6$ cells per 100-mm dish and cotransfected the next day by the $CaPO_4$ precipitation method with 1 μg of PEBG-WASp and 5 μg of either V12Cdc42, N17Cdc42, or wild-type Cdc42, all tagged with the hemagglutinin (HA) epitope. Immunoprecipitates of the overexpressed proteins were obtained from the cells lysed 36 h after transfection. Aliquots (100 μl) of lysates (out of a total of 1 ml) were incubated with 50 μl of mAb 12CA5 to the HA epitope tag or with 20 μl of polyclonal antibodies B4 to WASp in a total volume of 500 μl of lysis buffer. After 2 h at 4°C, 10 μl of protein-A Sepharose beads (Pharmacia) was added for an additional 30 min. The beads were washed and processed for Western blot analysis as described above.

## RESULTS

**Cdc42 Associates with WASp.** The cytoskeletal abnormalities noted in T cells from males with WAS and the presence of a consensus binding site for Rac1 and Cdc42 in the WASp of human and mouse (17, 18) (Fig. 1) suggested that the WASp might bind to small G proteins. GST-fusion proteins of Rac1, Cdc42, and Rho, and GST alone as a control were bound to glutathione-Sepharose beads, loaded with GTP[βS] or with GTP[γS], and incubated with lysates of EBV-B or HEL cells. The beads were then washed and analyzed by SDS/PAGE and Western blotting. WASp associated significantly with GTP-Cdc42 (Fig. 2). In overexposed films, the association with GTP-Rac1 can also be faintly discerned, but this is not evident in the Western blot shown in Fig. 2. There was no detectable binding to GTP-Rho. The GDP form of Cdc42 bound little WASp and GST alone bound none. These results suggested that the WASp in the EBV-B and HEL cell lysates interacts with Cdc42 in a GTP-dependent manner.

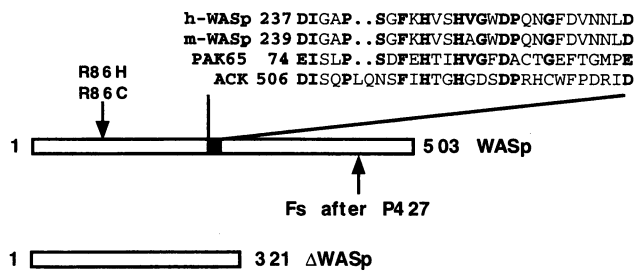


FIG. 1. Location of a putative Cdc42 binding domain in WASp. Schematic representation of human (h-WASp) and murine (m-WASp) open reading frames indicate the position of a region with significant sequence homology with the Cdc42 binding region of the serine/threonine kinase PAK65 and the nonreceptor tyrosine kinase ACK. The positions of WAS mutations in patient samples used in this study are shown. The part of WASp contained in GST-ΔWAS is also illustrated.

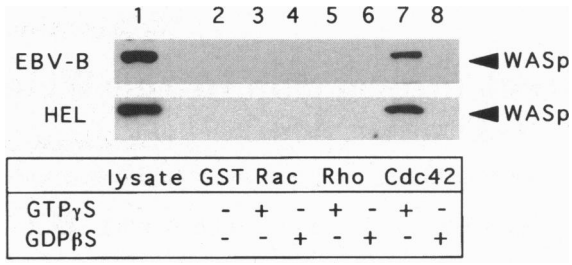


FIG. 2. Recruitment of Cdc42 by the WASp. Lysates (10 μg) of EBV-B and HELs were probed by Western blotting with a mixture of the B4 and C6 antibodies to the WASp (lane 1). After incubation of lysates (2 mg) with agarose beads containing GST-Rac, Rho, or Cdc42, the bound proteins were analyzed by Western blotting with the same antibodies. Only Cdc42, loaded with GTP[γS], visibly bound WASp in the lysates (lane 7).

**Direct Association of Cdc42 and WAS Protein.** The experiments using cell lysates just described did not distinguish a direct from an indirect association. We used recombinant proteins to determine whether the interaction of WASp with Cdc42 is direct. The amino-terminal 321-amino acid residues of WASp were expressed in *E. coli* as a GST-fusion protein (GST-ΔWASp). This portion of WASp contains the region spanning residues 237–264 that is homologous to putative binding sites for Cdc42 (17, 18) (Fig. 1). GST-ΔWASp, bound to glutathione-Sepharose beads, was incubated with Cdc42 or Rac1, which had been loaded with GDP[βS] or GTP[γS]. After incubation, the beads were washed, and the extent of Cdc42 or Rac1 binding to ΔWASp was determined by Western blot analysis with an antibody to Cdc42 or Rac1 (Fig. 3). Both Rac1 and Cdc42 bound directly in a GTP-dependent fashion.

**Cdc42 Associates with WASp in Transfected Cells.** To determine whether the interaction of WASp with Cdc42 occurs in intact cells, COS-7 cells were cotransfected with a plasmid containing GST-WASp and with plasmids containing HA-epitope tagged Cdc42. Three different Cdc42 plasmids were used in these experiments. The first contained wild-type Cdc42; the second a mutant N17Cdc42 that has a dominant negative effect, and the third a mutant V12Cdc42 that is GTPase-deficient and thus activated constitutively (15, 20, 21). Thirty-six hours after transfection, cells were lysed and immunoprecipitated with antibody to the HA epitope tag. As can be seen in Fig. 4, WASp was found in the immunoprecipitates from cells transfected with V12Cdc42 and to a lesser extent from cells transfected with wtCdc42 but not from cells trans-

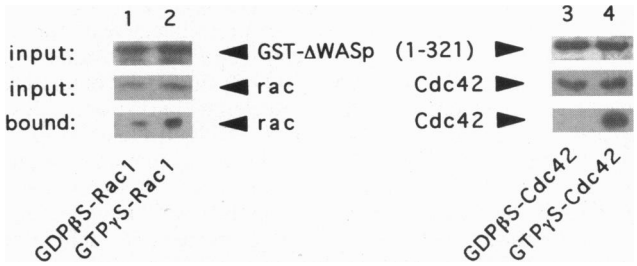


FIG. 3. Direct interaction between the amino-terminal portion of WASp and members of the Rho family of GTPases. (*Upper*) Relative amount of the recombinant fusion protein GST-ΔWASp containing amino acid residues 1–321 by Western blot analysis with antibody B4. (*Middle*) Relative amount of Rac1 and Cdc42 in the interaction mixture by Western blot analysis with antibodies to Rac1 and Cdc42. (*Bottom*) Preferential recruitment by GST-ΔWASp immobilized on the glutathione-Sepharose beads of the GTP-forms of Rac1 (lane 2) and Cdc42 (lane 4) compared to the corresponding GDP forms (lanes 1 and 3). These samples were probed with the antibodies to Rac1 and Cdc42. The molecular weights of Rac1 and Cdc42 are 25 kDa, which indicates complete cleavage from GST.

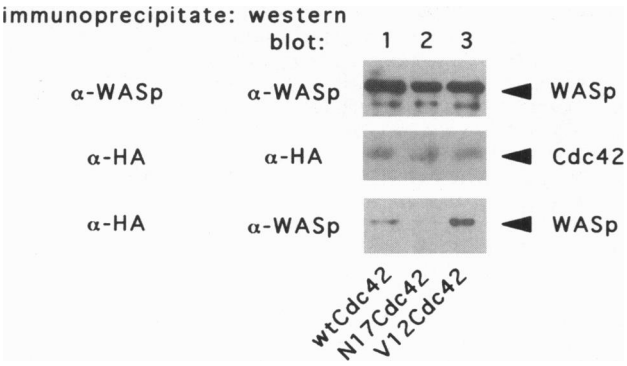


FIG. 4. Interaction of Cdc42 and WASp in transfected COS-7 cells. Wild-type Cdc42, a dominant-negative form of N17Cdc42, and the GTPase-deficient mutant V12Cdc42 were transiently expressed with GST-WASp and tested for their capacity to interact. (*Upper*) It is evident by immunoprecipitation followed by Western blot analysis that all three transfections express comparable amounts of WASp. (*Middle*) It can been seen that all three forms of HA epitope tagged Cdc42 are comparably expressed. (*Lower*) Only the dominant-negative mutant of Cdc42 (lane 2) fails to interact with WASp (probed with C6), whereas the GTPase-deficient mutant (lane 3) binds comparatively more WASp than the wild type (lane 1). WASp was immunoprecipitated with antibody B4 and probed for Western blot analysis with C6.

fected with N17Cdc42. These results confirm that a GTP-dependent association of Cdc42 with WASp can occur intracellularly.

**Mutant WASp Is Expressed at Low Levels But Binds Cdc42.** To ascertain whether mutant WASps could bind to Cdc42, EBV-B from two unrelated affected males (R86H and R86C) with missense mutations, resulting from single nucleotide changes, were lysed and probed with antibody to WASp by Western blot analysis (Fig. 5*A*). Only small amounts of mutant proteins, estimated to be 3–5% of normal, were expressed by these EBV-Bs (compare lane 5 with lanes 7 and 8, respectively). When lysates of these cells were incubated with GST-Cdc42, the mutant WASps were able to bind to Cdc42 in a GTP-dependent manner (Fig. 5*B*, lanes 5 and 7). When normalized for WASp content, the extent of binding of mutant WASp was the same as that of wild-type WASp from lysates of normal EBV-Bs. We observed similar results using patient cells directly rather than EBV-transformed lines. We isolated mononuclear cells from freshly drawn blood of a control and of an affected male. The latter had the nucleotide deletion $\Delta G_{1305}$ in exon 10 (24) and a consequent frame shift eliminating the C-terminal 76 amino acid residues of WASp. Although the expression level of mutant WASp was very low (≈2% of normal), the mutant protein retained its ability to bind to GTP-Cdc42 (Fig. 5*B*, lanes 12 and 13).

## DISCUSSION

We have shown that WASp binds to Cdc42 in a GTP-dependent manner. We obtain similar results using WASp derived from cell lysates, from transient transfections, as well as by using purified recombinant protein. Recombinant ΔWASp also binds to Rac1, although intact WASp from cells does so to a lesser extent. Members of the Rho family of GTPases are critically involved in the regulation of actin polymerization (13–15). Cdc42 regulates the formation of filopodia in fibroblasts (15, 21) and localizes actin to sites of cell–cell contact during antigen presentation (16). Lymphocytes from males affected with WAS have a severe disturbance in the organization of their actin cytoskeleton (25). This may be a consequence of the important role of the WASp if it coordinates the assembly of the actin cytoskeleton in resting cells as well as in cells following activation signals.

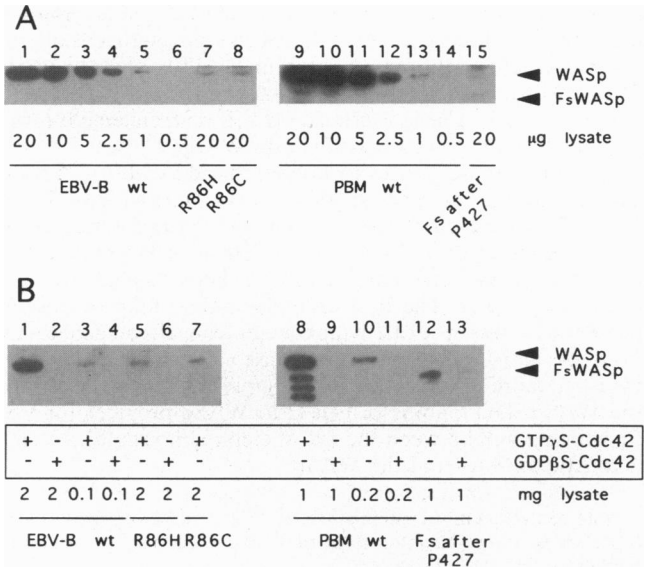


FIG. 5. Interaction of mutant WASp with Cdc42. (*A*) Comparison of the level of expression of WASp between cell lysates of EBV-Bs from normal control (lanes 1–6), EBV-Bs from males with WAS (lanes 7, 8), and peripheral blood mononuclear (PBM) cells from a normal control (lanes 9–14) and from an affected male (lane 15). Serial dilutions (protein amounts as indicated) of the normal controls were included to estimate the relative level of expression by Western blot analysis with antibody B4. Serial dilutions of recombinant WASp were similarly used to show that WASp constitutes about 0.07% of the total protein in lysates of normal EBV-Bs and freshly drawn platelets (data not shown). Normal WASp has an apparent molecular mass of 65–66 kDa. (*B*) Preferential association of mutant WASp with GTP-Cdc42. After incubation of the indicated amounts of lysates from EBV-Bs expressing wild-type (lanes 1–4) and mutant WASp (lanes 5–7) or from peripheral blood mononuclear cells expressing wild-type (lanes 8–11) and mutant WASp (lanes 12, 13) with Sepharose beads containing GST-Cdc42, the extent of WASp bound was analyzed by Western blot analysis with antibody B4. To control for the low level of expression of WASp in patient cells, the normal EBV-B lysates were diluted 1:20 with a lysate of HeLa cells (lane 3), which do not express WASp. Only Cdc42, loaded with GTP[γS], visibly bound WASp in the lysates. Bands in lane 8 that run below the intact WASp are proteolytic products.

WASp contains multiple polyproline sites in its carboxy-terminal region that could potentially interact with SH3 domains of other proteins (10). For example, the adaptor protein Nck binds to this part of the WASp (26). In contrast Cdc42 binds to a region within the first 321-amino acid residues of the WASp. These findings taken together suggest that the WASp has the potential to interact with multiple partners and may therefore form a scaffold that acts as a framework for bringing together interactive proteins in the cell. Cdc42 may be an important component of this complex.

Missense mutations in WAS do not significantly affect mRNA levels in EBV-transformed B lymphocytes derived from the patients studied here (22). The drastically diminished levels of WASp we have found suggest that the phenotype in some cases of WAS results from rapid intracellular degradation rather than from decreased translation. Low levels of WASp may lead to diminished signals through Cdc42 as well as through other proteins that interact with WASp. The mutant WASps we have examined bind Cdc42 and contain the putative interaction site. Thus, their phenotype appears to result from a lack of WASp rather than from a defect in its function. The spectrum of phenotypes in WAS may eventually be correlated with the amount of WASp that survives in the relevant cells.

The data presented here on the interaction of WASp with a GTPase, Cdc42, suggest a model that may be germane to the

pathogenesis of WAS. The collaboration of T and B lymphocytes involves direct contact of these cell types, with a resultant rearrangement of the cytoskeleton in T cells so that the actin filaments are concentrated and polarized toward the area of contact (27–29). Cdc42 is critical for this rearrangement (16), perhaps through the association with WASp.

The antibody responses to polysaccharide, and to a lesser extent, to protein antigens are defective in males with the WAS (7–9, 25, 30). Based on several immunological criteria, these faulty responses do not result from defects in B lymphocytes but rather from inadequate delivery of helper signals from T lymphocytes (25). The best example is the faulty or absent proliferative response of T lymphocytes from affected males to insoluble anti-CD3 (31). This response also requires extensive cytoskeletal re-organization (32, 33), presumably mediated by the WASp. The binding of Cdc42 to WASp provides for the first time a link between the cytoskeletal abnormalities characteristic of WAS and the WASp.

**Note.** Subsequent to submission of this manuscript, two other reports describing the interaction of WASp with Cdc42 have been published (34, 35).

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