A Role for Wiskott-Aldrich Syndrome Protein in T-cell Receptor-mediated Transcriptional Activation Independent of Actin Polymerization*

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Wiskott-Aldrich syndrome protein (WASP) plays a key role in cytoskeletal rearrangement and transcriptional activation in T-cells. Recent evidence links WASP and related proteins to actin polymerization by the Arp2/3 complex. To study whether the role of WASP in actin polymerization is coupled to T-cell receptor (TCR)-mediated transcriptional activation, we made a series of WASP deletion mutants and tested them for actin colocalization, actin polymerization, and transcriptional activation of NFAT. A WASP mutant with a deletion in the C-terminal region (WASP2C) that is defective in actin polymerization potentiated NFAT transcription following TCR activation by anti-CD3 and anti-CD3/CD28 antibodies, but not by phorbol 12-myristate 13-acetate/ionomycin. Furthermore, cotransfection of a dominant-active mutant (WASP-WH2-C) for Arp2/3 polymerization did not inhibit NFAT activation. Finally, by analyzing a series of WASP double-domain deletion mutants, we determined that the WASP homology-1 domain is responsible for NFAT transcriptional activation. Our results suggest that WASP activates transcription following TCR stimulation in a manner that is independent of its role in Arp2/3-directed actin polymerization.

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† The abbreviations used are: WASP, Wiskott-Aldrich syndrome protein; GBD, GTPase-binding domain; SH, Src homology; WH, WASP homology; Arp, actin-related protein; TCR, T-cell receptor; IL-2, interleukin-2; NFAT, nuclear factor of activated T-cells; FITC, fluorescein isothiocyanate; ERK, extracellular signal-regulated kinase; WT, wild-type; PPr, polyproline-rich; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; WIP, WASP-interacting protein; PAE, porcine aortic endothelial.
Role for WASP in TCR-mediated Transcriptional Activation

Vector and Transient Transfection—Jurkat cells (1 × 10⁷) were electroporated (250 V, 960 microfarads) with FLAG-tagged WASP-WT, WASP mutants, or vector control (pEF vector, 20 μg) and a plasmid containing the luciferase reporter gene driven by the NFAT-responsive element (20 μg). At 24 h post-transfection, cells were treated with 500 ng/ml soluble anti-CD3 antibody (UCHT1), anti-CD3 plus 500 ng/ml anti-CD28 (CD28.2) antibodies, or 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 5 h in the presence or absence of 1 μM latrunculin A or 10 μM cytochalasin D (where indicated). Cell lysates were analyzed for luciferase activity. Each graph represents the mean of at least three independent experiments. Error bars represent the S.D. of these experiments. Equal expression levels of full-length FLAG-tagged WASP and WASP mutants were verified by immunoblotting with anti-FLAG epitope tag antibody. To derive WASPΔC cell lines, Jurkat cells were transfected with WASP and WASPΔC plasmids by electroporation as in the transient transfection procedure. Briefly, Jurkat cells were transfected by electroporation at 250 V and 960 microfarads in 0.4-cm cuvettes with mammalian expression constructs (20 μg) all in the pEF vector. Cells (1 × 10⁷) were transfected with WASP-WT, the WASPΔC mutant, or the pEF vector control (20 μg). Electroporated cells were transferred to a 10-cm dish containing 20 ml of prewarmed fresh RPMI 1640 medium. The cell suspension (100 μl) was transferred to a flat-bottom 96-well plate and incubated overnight. Transfected cells were then selected 15–24 h post-transfection with 1 mg/ml G418 for 2–3 weeks. The medium was changed every 3–4 days, and positive clones (those occupying >50% of the wells) were expanded into 12-well plates containing selection medium. Positive clones were then confirmed by Western blot analysis of cell lysates using anti-FLAG antibody. Each graph represents the average of at least three separate experiments. The relative luciferase activity described in Figs. 3–5 is expressed as fold increase in the NFAT luciferase activity of various WASP mutants (stimulated over unstimulated) over vector control.

Receptor Capping and Immunofluorescence Photomicroscopy—Jurkat T-cells were incubated at 37 °C for 30 min in the absence or presence of 10 μM cytochalasin D and stimulated with 500 ng/ml anti-CD3 antibody (UCHT1). Cells were cytospun onto poly-L-lysine-coated slides, fixed in 3% paraformaldehyde, and permeabilized in 0.1% Triton X-100. To visualize the CD3 complex or polymerized actin, the slides were incubated with anti-CD3 antibody or Texas Red-conjugated phallloidin (Molecular Probes), respectively. The anti-CD3 antibody was detected with FITC-conjugated anti-mouse immunoglobulin. Fluorescence photomicroscopy was carried out on a Zeiss Axiohot with appropriate filter sets for epifluorescence detection of FITC or Texas Red signals.

Flow Cytometry Analysis—Cells were resuspended in staining buffer (PBS containing 1% fetal calf serum and 0.05% NaN₃). WASPΔC and parent T-cell lines (2 × 10⁶ cells/ml) were incubated for 30 min on ice with anti-CD3 antibody (1 μg/ml) or isotype-matched control IgG antibody and then washed and incubated for an additional 30 min on ice with FITC-conjugated anti-mouse antibody (1 μg/ml). Cells were washed to 37 °C and incubated for 60 min, followed by fixation for 15 min in 4% paraformaldehyde. Stained cells were analyzed using a FACScalibur™ with CellQuest™ software (Becton Dickinson).

Phospho-ERK Western Blotting—Parent vector control and WASPΔC stable cells (1 × 10⁷) were stimulated with anti-CD3 antibody (1 μg/ml), pervanadate (0.02 mM), or PMA/ionomycin. Cells were lysed in 1% Nonidet P-40 lysis buffer, suspended in SDS sample buffer, separated on SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. The membranes were blocked in 5% skim milk containing PBS and then incubated with anti-phospho-ERK antibody (New England Biolabs Inc.). After washing, the membranes were incubated with a secondary mouse antibody linked to horseradish peroxidase. Protein bands were subsequently detected with the ECL chemiluminescence kit (Amersham Pharmacia Biotech).
RESULTS AND DISCUSSION

Actin Polymerization and Clustering in PAE Cells Require Discrete WASP Domains—Given the multifunctional domain structure of WASP and its role in both signaling and cytoskeletal reorganization, WASP likely represents a critical player that coordinates receptor-mediated signaling pathways with the actin cytoskeleton. Although WASP regulates both cytoskeletal reorganization and transcriptional activation, it is not clear whether TCR-mediated transcriptional activation by WASP is controlled by a pathway that is independent of WASP-Arp2/3-directed actin polymerization.

To determine whether the role of WASP in actin polymeri-
zation is directly coupled to TCR-mediated transcription, we generated a series of WASP deletion mutants and compared their functions in actin polymerization and transcriptional activation assays. We previously showed that overexpression of WASP-WT in PAE cells induces WASP clustering that co-localizes with polymerized actin (8). To map the region of

![Activation of NFAT by the WASPΔC mutant.](image)

A. Jurkat cells were cotransfected with WASP or WASP deletion mutants and a plasmid containing the luciferase reporter gene driven by the NFAT-responsive element. A, after 24 h, cells were treated with soluble anti-CD3 antibody, and the luciferase activity of WASPΔC and vector control was compared with that in unstimulated cells. B–D, cells transfected with the indicated WASP constructs were treated with anti-CD3 antibody, anti-CD3/CD28 antibodies, or PMA/ionomycin (P/I), respectively, for 5 h. Lysates were then analyzed for luciferase activity. The luciferase activity was normalized and is expressed as relative activity over vector control. E, shown are the results from Western blot analysis of WASP-WT and deletion mutants using anti-FLAG antibody. F, shown is a comparison of NFAT activation in stable Jurkat T-cell lines expressing WASP-WT, WASPΔC, or plasmid control. G, shown are the results from Western blot analysis of cell lysates from stable Jurkat T-cell lines expressing WASP-WT, WASPΔC, or vector control using anti-FLAG antibody. H, WASPΔC or vector control Jurkat cell lines were stimulated with the indicated stimulus, and cell lysates were prepared and immunoblotted with anti-phospho-ERK antibody. The membranes were reprobed with anti-Zap-70 antibody, indicating that equal amounts of proteins were loaded in each lane (lower panels). I, Ionomycin.
WASP that is essential for WASP clustering and association with F-actin (polymerized) or G-actin (monomeric), we microinjected epitope-tagged WASP DNA constructs into PAE cells and immunostained for WASP, G-actin, and F-actin (Figs. 1 and 2). WASP-WT-expressing cells had large extended cluster formations of both F-actin and G-actin that co-localized with WASP (Fig. 2A). Similar results were obtained for cells microinjected with the WASPAGBD or WASPAGWH2 mutant (Fig. 2A). This is in contrast to a recent report that described a role for the WH2 domain in actin polymerization and co-clustering with WASP (21). It is possible that at higher levels of WASP expression, the WH2 domain is not required for actin polymerization.

Cells expressing WASPΔC had punctate staining throughout the cytoplasm and co-localization of WASPΔC with G-actin clusters (Fig. 2A). No F-actin clustering or polymerization was observed in any of the cells expressing WASPΔC. Recombinant WASPΔC was also inactive in in vitro pyrene-actin polymerization assays (data not shown). These results suggest that the C-terminal 59 amino acids are essential for actin polymerization, whereas the remaining 443 amino acids retain the ability to co-localize with monomeric actin. Our results, like those of Macheky et al. (14, 15), indicate that the Arp2/3-interacting domain of WASP is critical for actin polymerization.

Cells microinjected with the WASPΔWH1 or WASPAPr DNA construct had a diffuse pattern of WASP staining (Fig. 2A). These two mutant proteins were detected throughout the cytoplasm and were not co-localized with G-actin or F-actin clusters. Although our data suggest that these regions are essential for WASP co-clustering and co-localization with actin, the microinjection method may not fully detect polymerized actin by a mutant that is defective in clustering. Therefore, co-clustering of WASP with actin may be required for polymerization.

The WH1 Domain of WASP Is Required for Co-clustering

with Actin—Since the WASPΔC mutant retains the ability to cluster G-actin, it is likely that the region(s) responsible for clustering actin monomers resides in one or more of the remaining domains. To further map the domain(s) on WASP required for G-actin co-localization and clustering, we constructed a series of WASP double-domain deletion mutants lacking the WH1, GBD, or PPr domain in a WASPΔC background. As shown in Fig. 2B, WASPWH1ΔC failed to cluster and co-localize with G-actin. In contrast, WASPAPrAC and WASPAGBDΔC were able to cluster or co-localize with G-actin like the WASPΔC mutant. These data indicate that the WH1 domain is necessary for G-actin co-localization and co-clustering with WASP. Our results also indicate that WASP clustering and G-actin clustering are linked to each other, in contrast to results reported by Kato et al. (21). The discrepancies between our findings and those of Kato et al. may be due either to different expression methods used (i.e. microinjection versus transient transfection) or to cell type variations.

WASPΔC Enhances TCR-mediated Transcriptional Activation—Recent studies with WASP-deficient T-cells suggest that WASP links T-cell receptor engagement to cytoskeletal reorganization, receptor clustering, and cap assembly (6, 7). Deletion of the WASP gene has been shown to impair T-cell proliferation, cytokine production, and IL-2 transcription (6, 7). Although numerous studies have suggested a role for WASP in actin reorganization and transcriptional activation, it is not clear whether these two functions are directly linked by WASP. To investigate the role of WASP in TCR-mediated transcriptional activation, Jurkat T-cells were cotransfected with either WASP or WASP deletion mutants and a reporter gene driven by the NFAT-responsive element. Transfected Jurkat cells stimulated with soluble anti-CD3 antibody revealed a selective 8–10-fold enhancement of activation of NFAT-dependent transcription by the C-terminally truncated WASPΔC mutant (Fig. 3, A and B), but not by any other mutants (Fig. 3B). Similar results were seen when cells were stimulated with antibodies cross-linking both CD3 and CD28 (Fig. 3C). No statistically significant differences between the various WASP mutants were observed in cells stimulated with soluble stimuli such as PMA and ionomycin (Fig. 3D). Enhancement of NFAT activity was also observed in stable Jurkat cell lines expressing the WASPΔC mutant (Fig. 3, F and G). Moreover, ERK phosphorylation was significantly increased in the WASPΔC cell line over vector control following stimulation with either anti-CD3 antibody or pervanadate, but not with PMA/ionomycin (Fig. 3H). ERK phosphorylation in the WASP-WT cell line was comparable to that in the vector control cell line (data not shown). These results indicate that enhancement of transcriptional activation by WASPΔC is dependent on proximal signals coming from the TCR, but independent of CD28 co-stimulation or downstream signaling events initiated by PMA and ionomycin. These data are consistent with previous reports describing T-cells from Wiskott-Aldrich syndrome patients and WASP-deficient mice that suggest the defect is downstream of early TCR activation in that all defined signaling pathways appear to be intact (5–7). T-cells from WASP−/− and Vav−/− mice exhibit normal tyrosine phosphorylation of Zap-70, TCR-ζ, and other major substrates following TCR engagement (7, 18). Furthermore, IκBα phosphorylation and the c-Jun NH2-terminal kinase/stress-activated protein kinase, p38 kinase, and mitogen-activated protein kinase pathways also appear intact in these cells (7, 18). These data suggest that WASP may function as a cytoskeletal scaffold, integrating one or more of these pathways and thereby overcoming an activation threshold by assembling receptors and signaling molecules at the cap.

To rule out the possibility that WASPΔC acts in a dominant-
negative way to block TCR- and WASP-mediated actin polymerization, we examined the induction of TCR capping and internalization in the WASPΔC stable cell line. It was previously shown that WASPΔC cells are defective in actin-mediated TCR capping and internalization (7). Normal ligand-induced capping was observed in our WASPΔC cell line as visualized by immunofluorescence using anti-CD3 antibody (Fig. 4A). Furthermore, TCR-mediated actin recruitment and polymerization at the cap were not affected in the WASPΔC cell line. In contrast, treating cells with the actin inhibitor cytochalasin D completely blocked receptor capping and actin polymerization. These data suggest that WASPΔC does not act in a dominant-negative way to block actin polymerization and receptor capping during T-cell activation.

To rule out the possibility that NFAT activation by WASPΔC may be a consequence of aberrant TCR internalization, we tested the rate of receptor endocytosis in WASPΔC and control cell lines. Comparable TCR internalization was detected in stable Jurkat T-cell lines expressing WASP-WT, WASPΔC, or vector control (Fig. 4B).

The potentiation of NFAT activation by WASPΔC may result from stimulation of a novel signal transduction pathway (19). It is possible that this mutant interacts with an unidentified protein(s) that normally associates with activated endogenous WASP. In this model, WASP may exist in one of two activation states (17, 22–24). It was recently proposed that in the inactive state, the acidic C terminus of WASP interacts with the basic region immediately upstream of the GBD (22–24). Binding of Cdc42-GTP therefore relieves this interaction, opening the molecule to allow multiple protein complexes to form.

The WH1 Domain of WASPΔC Is Required for TCR-mediated Potentiation of NFAT—It has recently been suggested that WASP normally exists in an autoinhibited state similar to that observed in the structurally related Cdc42 effector, PAK1 (23). In both cases, it has been shown that the N-terminal half of the molecule interacts with the C terminus, thereby sequestering protein activity. We hypothesized that the NFAT potentiation observed with the C-terminally truncated WASPΔC mutant
might be due to a loss of this autoinhibition. To determine which of the remaining regions of WASP is responsible for enhanced transcriptional activation, a series of WASP double-domain deletion mutants were constructed in a WASPΔC background (Fig. 1). Jurkat T-cells were then cotransfected with WASPΔC or WASPΔC double-domain deletion mutants and a reporter gene driven by the NFAT-responsive element. Cells transfected with WASPΔGBDΔC or WASPΔPPαΔC and stimulated with anti-CD3 antibody exhibited enhanced activation of the NFAT reporter by 8- and 7-fold, respectively (Fig. 5A). Cells transfected with the WASP3WH1ΔC mutant and stimulated with anti-CD3 antibody did not enhance NFAT activation, suggesting that the WH1 domain is required for activation (Fig. 5A). In agreement with these results, a recent study showed that WASP-interacting protein (WIP) and Vav synergize to enhance NFAT-dependent transcription (25). WIP was previously shown to interact with the WH1 domain of WASP, and WIP-dependent NFAT activation requires the WASP-interacting region on WIP. (25, 26). Although our result clearly suggests that the WH1 domain is required for transcriptional activation, we were unable to effect NFAT activation with the WH1 domain alone (data not shown). These results indicate that the WH1 domain is required but not sufficient for transcriptional activation. In addition, it is possible that the dominant-negative or dominant-positive effects could not be achieved due to misfolding or mislocalization of the expressed WH1 protein.

Numerous signaling proteins have been shown to interact both directly and indirectly with the WH1 domain, including WIP and the SH3 domain-containing proteins Nck, Fyn, Vav, and Grb2 (3, 26). The physiological relevance of these interactions remains unknown. The WH1-related domain EVH1 is structurally similar to the pleckstrin homology domain, containing a binding pocket for polyproline sequences and basic residues (27–30). WH1 and EVH1 domains derived from different proteins were found to bind proline-rich peptides with a specific sequence motif (27, 30). For instance, WASP was shown to bind the proline-rich peptide DPFPPTDEEL derived from ActA (30). The function of the proteins containing the WH1 domain implies that this region acts to couple signaling pathways to actin polymerization. Our results are consistent with such a hypothesis.

WASPΔC Potentiates Transcriptional Activation Independent of WASP-Arp2/3-directed Actin Polymerization—WASPΔC did not polymerize actin, but did activate NFAT. It is possible that endogenous levels of WASP effected polymerization and subsequent transcriptional activation. To determine whether activation of NFAT by WASPΔC is linked to WASP-directed actin polymerization, Jurkat T-cells were cotransfected with WASPΔC and WASP-WH2-C, a WASP mutant shown to be dominant-active for WASP-Arp2/3-directed polymerization (Fig. 6, A and B) (31). Coexpression of WASPΔC with WASP-WH2-C had little or no effect on NFAT activation (Fig. 6A). This suggests that the ability of WASP to regulate IL-2 transcription can be uncoupled from its role in actin polymerization.

Several recent studies have linked cytoskeletal rearrangement to signals originating from the TCR leading to downstream IL-2 transcription (3, 19). Our data suggest that the contribution of WASP-Arp2/3-directed actin polymerization to the process of transcriptional activation of T-cells is marginal. To further address whether the actin cytoskeleton plays a role in NFAT activation of transcription, Jurkat T-cells were cotransfected with WASPΔC and the reporter gene driven by the NFAT-responsive element, followed by treatment with an actin inhibitor (latrunculin A or cytochalasin D). Transcriptional activation of NFAT by WASPΔC following anti-CD3 or anti-CD3/CD28 antibody stimulation was reduced by ~50% with both agents (Fig. 6, C and D). In addition, no statistically significant changes were observed in latrunculin A- or cytochalasin D-treated cells stimulated with PMA/ionomycin (Fig. 6, C and D). This confirms that TCR-mediated transcriptional activation of NFAT by WASPΔC requires an intact actin cytoskeleton.

In summary, we have identified the WH1 domain of WASP as an important domain required for TCR-mediated NFAT transcriptional activation in T-cells. We demonstrated, by use of a dominant-active mutant for actin polymerization (WASP-WH2-C), that this potentiation is independent of WASP-Arp2/3-directed actin polymerization. Our data suggest that the regulation of IL-2 transcription by WASP can be uncoupled from its role in actin polymerization. The model in Fig. 7 describes a pivotal role for WASP in integrating signals to activate both transcription and actin polymerization. During TCR stimulation, activated Cdc42 binds to the GBD of WASP to relieve intramolecular autoinhibitory interactions. The active form of WASP can then deliver simultaneously at least two signals through two different domains. The C terminus interacts with the Arp2/3 complex to initiate actin polymerization, and the WH1 domain binds to unknown protein(s), possibly WIP, to initiate transcriptional activation.

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