Structure-Function Analysis of the WIP Role in T Cell Receptor-stimulated NFAT Activation

EVIDENCE THAT WIP-WASP DISSOCIATION IS NOT REQUIRED AND THAT THE WIP NH\textsubscript{2} TERMINUS IS INHIBITORY\textsuperscript{a}\textsuperscript{,b}

Received for publication, June 15, 2007, and in revised form, July 31, 2007 Published, JBC Papers in Press, August 20, 2007, DOI 10.1074/jbc.M704972200

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WASP and its binding partner WIP play important roles in T cells both in actin polymerization and in interleukin-2 transcription. Aberrations thereof contribute to the pathology of Wiskott-Aldrich syndrome (WAS). To directly evaluate the cooperativity of WIP and WASP in interleukin-2 transcription, we investigated how the WIP-WASP complex regulates NF-AT-mediated gene transcription. We developed an improved model system for analysis, using WIP and WASP cotransfection into Jurkat cells, in which strong induction of NFAT reporter activation is observed with anti-T cell receptor (TCR) antibody without the phorbol 12-myristate 13-acetate usually used previously. Using this system, our findings contradict a prevailing conceptual model of TCR-induced WIP-WASP dissociation by showing in three ways that the WIP-WASP complex mediates TCR-induced NFAT activation without dissociation. First, phosphorylation of WIP Ser\textsuperscript{488} does not cause dissociation of the WIP-WASP complex. Second, WIP-WASP complexes do not dissociate demonstrably after TCR stimulation. Third, a fusion protein of WIP to WASP efficiently mediates NFAT activation. Next, our studies clarify that WIP stabilization of WASP explains otherwise unexpected results in TCR-induced NFAT activation. Finally, we find that the NH\textsubscript{2} terminus of WIP is a highly inhibitory region for TCR-mediated transcriptional activation in which at least two elements contribute: the NH\textsubscript{2}-terminal proline and the NH\textsubscript{2}-terminal actin-binding WH2 domain. This suggests that WIP, like WASP, is subject to autoinhibition. Our data indicate that the WIP-WASP complex plays an important role in WASP stabilization and NFAT activation.

Wiskott-Aldrich syndrome (WAS)\textsuperscript{2} is a human immunodeficiency with abnormalities in multiple cell types, including T cells, B cells, and platelets (1, 2). Two aspects of the T cell defect are an abnormal cytokines (especially actin polymerization) and defects in T cell proliferation (especially IL-2 production). Mutations in the WASP protein account for WAS (3, 4). Discovery and characterization of WASP showed it to be a critical link between activation of small G-proteins (Rac and Cdc42) and actin polymerization (5). Indeed, WASP is the prototype of a family of related proteins that are central to initiation of actin polymerization. WASP encodes a 502-amino acid protein that contains a GTPase-binding domain and a COOH-terminal verprolin/cofilin/acidic domain. The intramolecular interaction between the GTPase-binding domain and the verprolin/cofilin/acidic domain inhibits WASP activity in regulating Arp2/3-mediated actin polymerization. Cdc42 binding to the GTPase-binding domain results in release of the verprolin/cofilin/acidic domain, which then can interact with the Arp2/3 actin nucleating complex and activate actin polymerization (6).

Cloning of WIP (WASP-interacting protein) (7) expanded the functional connections between WASP and actin. 1) The WIP COOH terminus binds to a WH1 domain at the NH\textsubscript{2} terminus of WASP. 2) The WIP NH\textsubscript{2} terminus binds actin. 3) WIP knock-out causes defects in actin polymerization and IL-2 production. Recent work has suggested that WIP plays an important role in regulating actin polymerization by WASP by regulating its release from autoinhibition (8, 9). Of particular importance, during the last 18 months, six reports have demonstrated that WIP mediates stabilization of WASP protein, which is degraded rapidly in the absence of WIP (10–15).

A reasonable simplifying assumption in understanding the T cell pathology in WAS was that the defect in IL-2 was secondary to defects in actin polymerization. However, a growing body of evidence indicates that actin polymerization and promotion of IL-2 transcription are largely distinct functions of WASP. 1) Abo and co-workers (16) demonstrated that deletion of the verprolin/cofilin/acidic domain COOH terminus of WASP destroyed its function in actin polymerization but facilitated its role in IL-2 transcription as assessed by NFAT reporter assays. 2) Studies with a transgenic mouse strain expressing only the NH\textsubscript{2} terminus of WASP indicated that it functioned as a dominant negative for some but not other functions. Those mice demonstrated a defect in proliferative responses and cytokine production induced by TCR stimulation, but actin polymerization was normal (17). 3) Analysis in WASP-deficient T cells showed a major effect of WASP knock-out on IL-2 production...
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but only a subtle effect on synapse formation (18). 4) Anti-WH1 scFvs intrabodies (in transgenic mice) caused impairment of the proliferative response and IL-2 production induced by TCR stimulation but not TCR capping (19). 5) In NK cells, the Wiskott-Aldrich syndrome protein regulated nuclear translocation of NFAT2 and NF-κB (RelA) independently of its role in filamentous actin polymerization and actin cytoskeletal rearrangement (20).

Since the foregoing data showed that WIP-WASP facilitation of IL-2 production is largely distinct from its already defined role in actin polymerization, it is important to understand the structural basis for WIP-WASP function in IL-2 transcription. Two previous studies have contributed most to defining those structural requirements (16, 21). Given the known binding of WIP to WASP, we have explored an approach not previously exploited, namely analysis of NFAT transcription after co-transfection of WIP and WASP. This system proves to be a highly informative one, which has given rise to findings in three areas. First, WIP and WASP do not need to dissociate to facilitate TCR-induced NFAT-mediated transcription, in contrast to a recent study indicating that dissociation was involved in TCR-induced actin polymerization (8). Second, we have clarified the relationship between WIP stabilization of WASP and the TCR-induced NFAT-mediated transcription. Third, we have found that the highly conserved NH$_2$ terminus of WIP contains a region that is strongly inhibitory for NFAT activation with contributions from both an NH$_2$-terminal proline region and the actin binding NH$_2$-terminal WH2 domain.

MATERIALS AND METHODS

Antibodies, Reagents, and Cells—Antibodies and their sources were as follows. CD3 mAb 38.1 was provided by Dr. Carl June (University of Pennsylvania Cancer Center, Philadelphia, PA); WASP mAb (22), rabbit anti-WIP Ab (23), and rabbit anti-hemagglutinin (HA)-tagged Ab were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); p-WIP mAb was from BD Biosciences; and actin mAb was from Chemicon (Temecula, CA). Calyculin A was from Calbiochem. WAS-T cells (carrying frameshift mutation G57deletion) and normal human T cells (24) were maintained in RPMI 1640 (Invitrogen) with 10% fetal calf serum RPMI 1640 (Invitrogen). For transfection, 1 × 10$^7$ Jurkat-T antigen cells in 0.2 ml of RPMI 1640 with 20 mM HEPES were mixed with the indicated quantities of plasmids, 2.0 μg of NFAT-Luc reporter plasmid, 2.0 μg of pKLTK-Luc plasmid for normalization, and pDEST1732 (containing GFP to ensure equal DNA content) and electropolated at 310 V and 950 microfarads. 10 h after transfection, the cells were stimulated or not with CD3 mAb (clone 38.1 ascites; 1:1000 dilution) and cultured for an additional 8 h. Then cells were lysed using lysis buffer, and 50 μl of lysates was used for the Dual Luciferase Assay System according to the manufacturer’s instructions (Promega). Part of the transfected cells were used for Western blotting for analysis of expression of the constructs, WIP, WASP, and actin.

Cell Stimulation, Immunoprecipitation, and Western Blotting—Jurkat T cells were incubated with 10 μg/ml anti-CD3 mAb for the indicated time periods; cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM Na$_2$VO$_4$, 5 mM Na$_3$P$_2$O$_7$, protease inhibitor mixture tablet, and calyculin A; and lysates were centrifuged at 13,000 rpm for 15 min at 4 °C and preclarified for 1 h at 4 °C with recombinant protein G-agarose (Invitrogen). Immunoprecipitation was performed overnight at 4 °C with antibody (4 μg) adsorbed onto recombinant protein G-agarose. Beads were washed five times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on NuPAGE 4–12% bis-Tris gel, and analyzed by Western blotting with the indicated antibodies followed by goat anti-mouse or rabbit antibodies conjugated to horseradish peroxidase and ECL (Amersham Biosciences) using the protocol recommended by the manufacturer. Chemiluminescence was also recorded on the Fuji LAS-3000 imaging system, analyzed using MultiGauge software (Stamford, CT). To optimize quantification by Western-blot analysis, various volumes of sample were loaded to assure that all were within the linear range of the Western blot quantification. Results shown are representative of at least three experiments.

RESULTS

Ser$^{488}$ Phosphorylation Does Not Induce WIP-WASP Dissociation—Geha and co-workers (8) proposed that dissociation of WASP from WIP was important for CD3-induced WASP activation and that this dissociation was mediated by protein kinase C phosphorylation of WIP. The site of phosphorylation which they identified, Ser$^{488}$, is within the WIP COOH terminus close to the region critical for interaction with WASP (Fig. 1A). They assessed that phosphorylation indirectly by measuring the decrease in binding of a polyclonal antibody raised against the nonphosphorylated peptide. To facilitate direct analysis of phosphorylation of this site, we generated a

bovine serum (Gemini Bio-products, Woodland, CA), 100 mM (24) were maintained in RPMI 1640 (Invitrogen) with 10% fetal serum RPMI 1640 (Invitrogen). For transfection, 1 × 10$^7$ Jurkat-T antigen cells in 0.2 ml of RPMI 1640 with 20 mM HEPES were mixed with the indicated quantities of plasmids, 2.0 μg of NFAT-Luc reporter plasmid, 2.0 μg of pKLTK-Luc plasmid for normalization, and pDEST1732 (containing GFP to ensure equal DNA content) and electropolated at 310 V and 950 microfarads. 10 h after transfection, the cells were stimulated or not with CD3 mAb (clone 38.1 ascites; 1:1000 dilution) and cultured for an additional 8 h. Then cells were lysed using lysis buffer, and 50 μl of lysates was used for the Dual Luciferase Assay System according to the manufacturer’s instructions (Promega). Part of the transfected cells were used for Western blotting for analysis of expression of the constructs, WIP, WASP, and actin.

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monoclonal antibody specific for phospho-WIP (pS488). Using that antibody, we first investigated the kinetics of CD3-induced WIP phosphorylation (Fig. 1B). There was detectable basal phosphorylation of WIP Ser$^{488}$ in Jurkat cells, which increased markedly after a 1-min stimulation with anti-CD3 mAb and remained high for at least 30 min. These results confirm by a more direct readout what Geha and co-workers (8) first showed, namely that WIP undergoes CD3-induced phosphorylation.

The previous study suggested that WIP phosphorylation resulted in inhibition of WASP association with WIP and attributed dissociation of WIP-WASP after TCR stimulation to this phosphorylation (8). Since this is an important hypothesis, we sought to confirm and extend the findings. We first investigated Ser$^{488}$ phosphorylation site mutants of WIP for their association with WASP in transfected cells. Jurkat cells were transfected with HA-tagged WT WIP or WIP constructs in which the phosphorylation site was mutated to mimic the unphosphorylated and phosphorylated states (HA-WIP S488A and HA-WIP S488D, respectively). After 20 h, the transected WIP protein was immunoprecipitated with anti-HA antibody. Western blot for WIP confirmed good immunoprecipitation of WIP (Fig. 2A). WB with the phosphospecific antibody confirmed that there was some basal phosphorylation of WIP, which was lost on the S488A mutant WIP protein, confirming the phospho-specificity of the antibody. Of particular importance, WASP was brought down by the S488D mutant WIP as efficiently as by WT WIP (and by S488A WIP); this indicates that in vivo pseudophosphorylated WIP binds WASP as well as unphosphorylated WIP.

We also investigated the second element of the previously published model, the effect of CD3/TCR stimulation on WIP-WASP association. In the same experiment, WIP was immunoprecipitated from parallel samples that had been CD3-stimulated (Fig. 2B). As expected (e.g. see Fig. 1B), there was a marked CD3-induced increase in phosphorylation of WT WIP (but not of WIP-S488A or S488D). Western blot showed that there was no change of associated WASP before and after anti-CD3 antibody stimulation (and the resulting WIP phosphorylation). The specificity of immunoprecipitation was confirmed by a demonstration that no WASP was pulled down by anti-HA antibody in GFP-transfected cells or cells transfected by a WIP construct lacking the WASP-binding region (WIP 1–416), and no WIP was precipitated by anti-HA in GFP-transfected cells (Fig. 2B). Thus, our evidence does not confirm the previously described WIP-WASP dissociation induced by CD3 or association inhibition by a pseudophosphorylation mutation of Ser$^{488}$.

The foregoing findings reinforce the importance of the question of whether Ser$^{488}$ phosphorylation or WIP-WASP dissociation is important for downstream functional
affects of TCR stimulation. We therefore investigated whether WIP Ser^{488} phosphorylation was important for NFAT transcriptional activation. To analyze structure/function questions in WIP-WASP-facilitated transcription, we tried an experimental approach not previously used, namely co-transfection of both WIP and WASP together with an NFAT reporter into Jurkat cells. This choice was based on emerging evidence that WIP-WASP association is important for their expression (see Refs. 10–15 and Figs. 3 and 4). Jurkat cells were co-transfected with HA-WASP and either HA-WIP or phosphorylation site mutants thereof (Fig. 2A). Cotransfection of Jurkat cells with HA-tagged WIP plus HA-tagged WASP resulted in expression of transfected WIP and WASP together with a ~10-fold increase in NFAT reporter activity (Fig. 2C). This response was compared with similar transfections in which the HA-WIP was replaced with HA-WIP S488A and HA-WIP S488D, which resulted in equivalent levels of expressed protein. After anti-CD3 antibody stimulation, there was no difference found on the NFAT transcriptional activation among WIP wild type, WIP S488A, and WIP S488D in reporter assays (Fig. 2C). Thus, WIP Ser^{488} phosphorylation is not important for NFAT transcriptional activation.

**WIP Stabilization of WASP Is Required for NFAT Activation**—To better understand the individual roles of WIP-WASP in TCR-induced NFAT activation, we investigated the ability of transfection with WIP or WASP alone to promote TCR-induced NFAT activation. The results (Fig. 3) demonstrate that transfection of WIP alone augments TCR-induced NFAT activation, but WASP alone does not. Both of these results are initially surprising. The finding regarding WIP suggests that it is acting independently of WASP in this process. Further analysis (Fig. 3, C and D), however, shows that this interpretation is incorrect and instead indicates that the role of WIP in this context is WASP stabilization. WIP transfection alone could dramatically augment NFAT transcription (Fig. 3C), but under those conditions, it also markedly augmented expression of endogenous WASP (Fig. 3D). Based on recent evidence that WIP stabilizes WASP (10–15), our findings suggest that more WASP is synthesized in Jurkat than can be stabilized by endogenous WIP. Transfection of exogenous WIP stabilizes the excess endogenous WASP, resulting in more total WIP-WASP complex and thereby enhancing NFAT activation.

The second finding, that transfection of WASP alone cannot facilitate NFAT activation, was investigated further and found to also be explained by the underlying issue of WIP-WASP stabilization (Fig. 4, A and B). Following transfection with graded amounts of WASP DNA alone (up to 10 μg), no expression of exogenous WASP protein could be detected. Moreover, WASP transfection did not augment CD3-induced NFAT transcription. To test whether providing excess exogenous WIP could “rescue” expression of exogenous WASP, graded amounts of WIP were co-transfected with a constant amount of WASP DNA (5 μg, which by itself resulted in no WASP expression). The results show that exogenous WIP enables expression of exogenous WASP. These results are con-
sistent with recent findings (10–15) that 1) there is rapid degradation of WASP unless it is stabilized by interaction with WIP and 2) WIP synthesis is limiting in Jurkat cells.

The recently published studies on WIP stabilization of WASP are quite comprehensive, but one important clinically relevant prediction has been incompletely tested; some WASP mutations (especially missense mutations located in the WIP-binding first 151 amino acids) found to cause WAS have been shown to have defective interaction with WIP (23). The principle of WIP stabilization of WASP predicts that those interaction-defective WASP mutants would be easily degraded in the co-transfection system. The results (Fig. 5) confirm this prediction. Like WT WASP, each of the three point mutants tested (R86H, Y107C, and A134T) was expressed at very low levels without WIP, but unlike WT WASP, none of the point mutants were expressed at high levels when co-transfected with WIP. These results support the conclusion that WIP-WASP interaction is important for WASP stability and that impairment of WIP-WASP interaction results in WASP degradation that leads to WAS. Studying NFAT activation with these constructs was not informative, because the stabilization of excess endogenous WASP production (Fig. 3) substitutes for the transfected WASP.

WIP-WASP Dissociation Is Not Required for NFAT Activation—Although the majority of WIP and WASP have been reported to be associated (8), the biological effects may be mediated by a small fraction of WIP or WASP that is not associated. Free WIP or WASP may be formed by limited dissociation (which may be hard to detect) during CD3 stimulation by a mechanism other than Ser488 phosphorylation (Fig. 2) or may exist even when the majority of WIP and WASP are associated (Figs. 3 and 4). To explicitly address this important question, we made a fusion protein in which WIP and WASP were fused into a single protein. A short flexible region, GSGSG, was inserted between them in the fusion protein. The ability of this fusion protein to promote NFAT activation was compared with co-transfected WIP + WASP. The result showed that the WIP-WASP fusion protein was highly functional in increasing NFAT activation after anti-CD3 antibody stimulation (Fig. 6). Note that this marked increase cannot be attributed to an increase in endogenous WASP (supplemental Fig. 1). These results argue strongly that WIP-WASP dissociation is not required for NFAT activation.

The NH2 Terminus of WIP Mediates Inhibition—Regions of WIP that are important for NFAT/AP-1 activation have been studied previously by Savoy and co-workers in their investigation of VAV/WIP facilitated activation by CD3 and phorbol 12-myristate 13-acetate (21). They concluded that the first 111 residues were not essential, since truncation of that region did not influence the transcriptional response. Our analysis of evolutionary sequence conservation (supplemental Fig. 2) indicated that the NH2-terminal WH2 domain (residues 32–49) is the single most conserved element. Another distinct feature of the extreme NH2 terminus is a putative profilin-binding polyproline (residues 2–14). We therefore performed structure-function analysis of WIP in our system with special emphasis on a more detailed analysis of the NH2 terminus.

Among the constructs, the NH2-terminal 60-amino acid deletion (WIP 61–503) was the most active, with more than 8 times the activity of WT WIP (Fig. 7). The 12-amino acid deletion of the profilin-binding polyproline was also increased, but only ~2-fold. Similar
about 3-fold more CD3-induced NFAT transcription than the wild type construct (Fig. 8, A and B). Comparison of these point mutant constructs with the WIP 61–503 construct (Fig. 8, C and D) demonstrated that their abrogation of WH2 actin-binding function contributed part but not the entire enhancement observed with the deletion of amino acids 1–60. As shown in supplemental Fig. 3, WASP was brought down by WIP with WH2 domain deletion or WIP with mutations of the actin-binding sites as efficiently as by wild type WIP; this indicates that WH2 domain deletion or mutations of the WIP actin-binding sites had no effect on WIP-WASP association.

**DISCUSSION**

As outlined in the Introduction, multiple lines of evidence demonstrate that the role of WIP-WASP in actin polymerization is distinct from its role in IL-2 transcription. The present study has been able to address important questions about the WIP-WASP role in IL-2 transcription by taking advantage of the known binding of WIP to WASP and exploiting that in studies of protein expression and CD3-induced NFAT-mediated transcription during WIP and WASP co-transfection in Jurkat T cells. The choices of this approach in our model may have several advantages over other model systems reported. First, cotransfection of WIP and WASP is conceptually appealing, given the emerging evidence of WIP stabilization of WASP. Second, we use the physiologically relevant CD3 stimulus rather than CD3/H11001 phorbol 12-myristate 13-acetate, which is often used in Jurkat cells in order to obtain a sufficient signal (21). Third, we use an NFAT reporter (25), which we find has a better dynamic range than an NFAT/AP-1 reporter (21). As a result, we always observe more than a 3-fold augmentation of basal levels with WIP + WASP and often much higher enhancement. Discussion will focus on three areas in which new observations arise from this study. 1) WIP dissociation from WASP is not important for CD3-induced NFAT activation. 2) WIP stabilizes WASP and thereby controls the level of WIP-WASP complex that regulates CD3-induced NFAT activation. 3) The NH2 terminus of WIP is an inhibitory region.

**WIP-WASP Association**—Some molecular complexes persist independent of cell stimulation, and others are transient, either formed in response to stimulation or disassembled in response to stimulation. It has previously been reported that TCR stimulation induces WIP phosphorylation by protein kinase C on Ser488 and that this phosphorylation causes dissociation of WASP from WIP (8). Using a phosphospecific antibody for that site, our results confirm TCR-induced phosphorylation. This phosphorylation is not essential to NFAT transcription, since a normal response is observed with the S488A construct. Moreover, in our analysis, this phosphorylation does not result in dissociation of WASP from WIP, as evidenced by association of WASP with pseudophosphorylated mutant S488D (Fig. 2). Although this contrasts with the previous report that S488D disrupted the association (8), it should be noted that the previous analysis was done in vitro with only a fragment of WIP. Our results also do not confirm the reported dissociation (8) of 3 X. Dong, G. Patino-Lopez, F. Candotti, and S. Shaw, unpublished observations.
Instead, our findings match an amended interpretation that dissociation does not occur (but instead was attributable to the particular antibodies used for immunoprecipitation) (27). Because it is an important issue whether WIP-WASP function depends on their dissociation, we addressed it in an entirely different way. We generated a fusion construct containing both WIP and WASP in which their orientation is approximately physiological, since the COOH-terminal WASP-binding region of WIP is adjacent to the NH2-terminal WIP-binding domain of WASP (28). Transfection of this construct is as efficient in promoting CD3-induced NFAT transcription as the two constructs co-

**WASP from WIP after CD3 stimulation** (Fig. 2). Instead, our findings match an amended interpretation that dissociation does not occur (but instead was attributable to the particular antibodies used for immunoprecipitation) (27). Because it is an important issue whether WIP-WASP function depends on their dissociation, we addressed it in an entirely different way. We generated a fusion construct containing both WIP and WASP in which their orientation is approximately physiological, since the COOH-terminal WASP-binding region of WIP is adjacent to the NH2-terminal WIP-binding domain of WASP (28). Transfection of this construct is as efficient in promoting CD3-induced NFAT transcription as the two constructs co-

**WIP Stabilization of WASP**—In the last year, several groups have demonstrated that WIP binding to WASP stabilizes WASP that would otherwise be degraded (10–15). Our results confirm and extend these findings, especially as they relate to NFAT activation. First, our results demonstrate that WIP stabilization of WASP explains otherwise surprising findings that transfection WIP alone augments CD3-induced NFAT activation, whereas WASP transfection alone does not. Second we find that WASP point mutations (R86H, Y107C, and A134T) originating from WAS patients that are defective in their binding to WIP lack the capacity to be well expressed in Jurkat when transfected with WIP. This complements and extends analysis of cells derived from T45M and R86H WAS patients by de la Fuente (15). The mechanism for this stabilization of WASP by WIP is incompletely understood but may involve calpain and/or proteasome-mediated degradation (15). This kind of “chaperone” role for WIP may be a general one, since WIP also protects Syk from degradation in mast cells after FcRI ligation (29).

**WIP NH2 Terminus Is Inhibitory**—Proteins are complex computational machines, in which discrete regions contribute distinct functions. Inhibitory regions are elements that often contribute by mediating basal autoinhibition, as is the case for regions of WASP that autoinhibit its actin polymerization function. The present study identifies a strongly inhibitory region at the NH2-terminus of WIP (amino acids 1–60). Notably, truncation of an additional 51 residues (in the 112–503 construct) largely abolishes the augmentation (Fig. 7), indicating that residues 61–111 (the second WH2 domain and linker) play a strong augmenting role in transcription. This would explain why Savoy et al. (21) did not see changes from deleting the entire region 1–111. Our studies identify two distinct subregions within the 60-amino acid NH2-terminal region that contribute to the inhibitory effect: the 12-amino acid amino-terminal putative profilin-binding polyproline sequence and the key residues within the actin-binding NH2-terminal WH2 domain.

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![FIGURE 8. Mutations of the NH2-terminal actin-binding WH2 domain of WIP reduce inhibition.](image-url) Jurkat cells were transfected with 10 μg of the indicated constructs, and both TCR-induced NFAT activation (A and C) and WB for protein expression (anti-HA, anti-actin) (B and D) were performed as described under “Materials and Methods.” The bars represent the mean ± S.D. of data from three independent experiments.

![FIGURE 9. Models of autoinhibition involving the WIP NH2 terminus.](image-url) Shown is a schematic representation of two distinct ways in which the NH2 terminus could function in inhibition: autoinhibition involving WIP, actin, and profilin (A) and autoinhibition involving WIP, WASP, actin, and profilin (B). B, basic region; GBD, GTPase-binding domain; VCA, verprolin-cofilin-acidic motif; WB, WASP-binding domain; WH1, WH1 domain; WH2, WH2 domain.
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(Fig 9A). Alternatively, binding of actin profilin to the WIP NH2 terminus could cause conformational changes that create a binding surface of WIP NH2 terminus that interacts with other regions of WIP. Such intramolecular interactions are often difficult to demonstrate directly, since intramolecular binding is facilitated by high local concentration and can be functionally important without being strong enough to mediate intermolecular binding (30). As expected (from the understanding of the weak interactions involved in intramolecular associations), we found no evidence of WIP-WIP intramolecular complexes by pull-down. 3 Given the association of WIP with intermolecular binding (30). As expected (from the under-

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Acknowledgments—We are grateful to Natasha Belkina, Khadija Ben-Aissa, Jin-fjiang Hao, Tian Lan, David Nelson, Yin Liu, and Donn Stewart for critical reading of the manuscript and/or scientific discussion and to Gottfried Baier and Gerald Crabtree for the NFAT reporter construct.

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