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SLP-76 Coordinates Nck-Dependent Wiskott-Aldrich Syndrome Protein Recruitment with Vav-1/Cdc42-Dependent Wiskott-Aldrich Syndrome Protein Activation at the T Cell-APC Contact Site

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We have shown previously that Wiskott-Aldrich syndrome protein (WASP) activation at the site of T cell-APC interaction is a two-step process, with recruitment dependent on the proline-rich domain and activation dependent on binding of Cdc42-GTP to the GTPase binding domain. Here, we show that WASP recruitment occurs through binding to the C-terminal Src homology 3 domain of Nck. In contrast, WASP activation requires Vav-1. In Vav-1-deficient T cells, WASP recruitment proceeds normally, but localized activation of Cdc42 and WASP is disrupted. The recruitment and activation of WASP are coordinated by tyrosine-phosphorylated Src homology 2 domain-containing leukocyte protein of 76 kDa, which functions as a scaffold, bringing Nck and WASP into proximity with Vav-1 and Cdc42-GTP. Taken together, these findings reconstruct the signaling pathway leading from TCR ligation to localized WASP activation. The Journal of Immunology, 2003, 171: 1360–1368.

The interaction of a T cell with an APC induces the polymerization of F-actin at the cell-cell contact site. This process is important to facilitate integrin-dependent adhesion between the T cell and the APC. Moreover, it is thought to be involved in assembling the ordered array of proteins within the immunological synapse. The mechanisms that control actin remodeling are still not understood in detail. Although engagement of T cell integrins and costimulatory molecules clearly contributes to the overall regulation of cortical cytoskeletal remodeling during T cell responses to APCs (1–3), we and others have shown that TCR engagement alone can initiate actin polymerization (1, 4, 5). Downstream of the TCR, normal actin rearrangement requires signaling through the tyrosine kinases Lck and ζ-associated protein of 70 kDa (ZAP-70) (5). Beyond these earliest players, several important regulatory molecules have been identified using various experimental approaches. Studies using knockout mice point to roles for Vav-1, Wiskott-Aldrich syndrome protein (WASP), and Itk. T cells from Vav-1−/− mice are defective in TCR-capping, actin polymerization, and conjugate formation (6–8). We and others have recently shown that T cells from Itk−/− mice have similar defects (73).4 WASP−/− T cells also show defects in TCR-induced actin responses (9, 10), though surprisingly these T cells form conjugates with normal efficiency (8, 11). Other proteins likely to play an important role have been identified based upon mutant cell lines or overexpression of dominant mutants. For example, murine T hybridoma cells transfected with mutants of Cdc42 show defects in actin responses to APCs (12), Jurkat T cells expressing a phosphorylation mutant of Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) show defects in actin capping (13), and linker for activation of T cells-deficient Jurkat T cells exhibit abnormal actin dynamics when plated on anti-TCR coated coverslips (14). Finally, biochemical analysis of T cell lysates has shown that many of these proteins are present in large complexes (15, 16). Thus, numerous studies repeatedly point to the same set of proteins as key players in actin remodeling during T cell signaling. However, it has been difficult to develop a coherent model for how these proteins actually function together in situ based upon studies analyzing individual protein components in distinct experimental systems.

In our laboratory, we have focused on analyzing the function of the Rho family GTPase Cdc42 and its effector, WASP. Studies in other systems show that binding to Cdc42-GTP induces a conformational change in WASP, which allows the molecule to work in concert with the Arp2/3 complex to induce actin polymerization (reviewed in Ref. 17). Several pieces of evidence point to an important role for Cdc42 and WASP in regulating actin responses in T cells. Overexpression in T cells of Cdc42 mutants locked in either the GDP-bound (inactive) or GTP-bound (active) forms has been shown to lead to defects in actin polarization and decreased ability to form T cell-B cell (T-B) conjugates (11, 12). Naturally...
occurring mutations in WASP result in Wiskott-Aldrich syndrome, an X-linked immunodeficiency syndrome characterized by a clinical triad of eczema, decreased cellular and humoral responses, and thrombocytopenia (reviewed in Ref. 18). T cells from Wiskott-Aldrich syndrome patients and WASP−/− mice exhibit an abnormally smooth morphology and defects in TCR-cross-linking-induced F-actin polymerization and cap formation (10, 19–21).

WASP activation during T cell-APC interactions requires the convergence of two pathways, one leading to recruitment of WASP and the other to WASP activation by Cdc42-GTP. We found that Cdc42-GTP accumulates at the site of T cell-APC interactions (22). Like Cdc42-GTP, WASP accumulates at the T cell-APC contact site (16, 22). Surprisingly, however, we found that WASP recruitment is independent of its binding to Cdc42-GTP. Instead, the proline-rich region of WASP is necessary and sufficient to mediate recruitment. Many Src homology 3 (SH3) domain-containing proteins including Lck, Fyn, Btk, Itk, Nck, Grb2, Prolin, and serine, threonine phosphatase interacting protein (PSTPIP) have been shown to interact with the proline-rich domain of WASP in vitro (23–29). However, the protein responsible for WASP recruitment during T cell signaling is not known.

In the pathway leading to WASP activation, we have shown that TCR ligation is sufficient to induce localized activation of Cdc42 and that signaling through Lck and ZAP-70 is required (11). However, the proteins linking these early tyrosine kinases to the activation of Cdc42 and WASP have not been defined. As with other small GTPases, the activation of Cdc42 requires interaction with a guanine nucleotide exchange factor (GEF), which catalyzes the exchange of GTP for GDP. Of the proteins known to play an important role in actin remodeling in T cells, Vav-1 is a likely candidate because it has been shown to have GEF activity for Rho family GTPases (30–34). It is currently unclear whether Vav-1 or another protein functions to activate Cdc42 during T cell-APC interactions.

In the present study, we have investigated the components of the signaling pathway linking TCR engagement to WASP recruitment and localized activation by Cdc42. One important goal of this study was to define mechanistic relationships between individual actin-regulatory components in a single experimental system. Our results show that WASP recruitment occurs through binding to Nck, whereas WASP activation requires Vav-1-dependent activation of Cdc42. Coordination of these two pathways occurs via phosphorylation of SLP-76, which by mutual binding to Nck and Grb2 forms a SH3-Nck, whereas WASP activation requires Vav-1-dependent activation of Cdc42. Coordination of these two pathways occurs via phosphorylation of SLP-76, which by mutual binding to Nck and Grb2 forms a SH3

Materials and Methods

Cell lines

Nalm-6 B cells, E6 Jurkat T cells, and the Vav-1-deficient cell line J.Vav1 (35) were cultured in RPMI 1640 supplemented with 10% FCS (Invitrogen, Carlsbad, CA) in 5% CO2 at 37°C. A J.Vav1 derivative line stably expressing Vav1 was cultured similarly, with the addition of 0.5 mg/ml G418 (Invitrogen). Jurkat cells stably expressing wild-type SLP-76 or the Y3F3 SLP-76 mutant (a gift from Dr. A. Chan, Washington University, St. Louis, MO) were grown similarly, with the addition of 0.5 mg/ml G418 (Invitrogen). Jurkat cells stably expressing wild-type SLP-76 or the Y3F3 SLP-76 mutant (a gift from Dr. A. Chan, Washington University, St. Louis, MO) were grown similarly, with the addition of 0.5 mg/ml G418 (Invitrogen).

Plasmids

FLAG-tagged WASP cDNA was described previously (22). TAGF-tagged clones expressing wild-type and mutant Nck and Grb2 were described elsewhere (39). FLAG-tagged Vav-1 was generated by performing RT-PCR on NK cell total RNA using the forward (5′-GGCGAGGCGTTA AAGCTTGACCCCGCAAACTGACCCCA-3′) and reverse (5′-CG TCCTCTYCGGCYGCYGCGYGCTCAGTAATGCTAATGCTAATGCAGTAATTGCTA-3′) oligonucleotide primers. The resulting PCR product was subcloned into a pcDNA3 vector containing an amino-terminal FLAG epitope coding sequence (pCDNA3-F) and was sequenced. Vav-1C (starting at amino acid 598) was generated from the full-length Vav-1 clone using the same reverse primer in combination with a new forward primer (5′-CTGGGCTGCGCAAGCT TGAGGCTTTCAGGAATCTAAGGC3′). The resulting PCR product was subcloned into pCDNA3-F and sequenced.

Transfection and flow cytometric analysis of protein expression

Transient transfection was performed as described previously (22). Briefly, 20 × 10^6 T cells in log-phase were combined with 20 μg of DNA and electroporated using a Gene pulser II (Bio-Rad, Hercules, CA) at 0.24 kV and 1500 μF. Dead cells were removed by passage over Ficol-Haque (Amersham Pharmacia, Piscataway, NJ). Most studies were performed at 4 h after transfection. Transfected cells were analyzed for GFP expression using a FACSciCalibur Plus flow cytometer (BD Biosciences, San Jose, CA).

Conjugation and immunofluorescence microscopy

Cell conjugations and microscopy were performed as described previously (22). Briefly, Nalm-6 B cells were dyed with 7-amino-4-cholormethylcoumarin and incubated with or without 2 μg/ml SEE. B cells were centrifuged together with the same number of T cells, incubated at room temperature for 15 min, plated onto poly-L-lysine-coated coverslips, and fixed with 3% paraformaldehyde/PBS for 20 min at room temperature. For analysis of murine T cells, A20 B cells were pulsed for 1–2 h with 2 μg/ml OVA123–139 and washed, and 2 × 10^6 cells were combined with 2 × 10^7 T cells, placed on poly-L-lysine-coated coverslips for 7 min, and fixed. Fixed cells were quenched with 50 mM NH4Cl and permeabilized in 0.3% Triton X-100. Blocking and Ab incubations were performed in PBS/0.05% saponin/0.25% fish skin gelatin. Coverslips were mounted in Mowiol 4-88 (Hoechst Celanese, Charlotte, NC) containing 10% 1,4-diazobicyclo [2.2.2] octane. Samples were analyzed using an Axioskop microscope (Zeiss, Oberkochen, Germany) and a CoolSnap F/XHQ camera (Photometrics, Tucson, AZ). No-neighbors deconvolution was performed using Openlab v2.0.6 software (Improvision, Boston, MA). Quantitation of all protein polarization was determined by choosing 50 T-B conjugates at random. Those conjugates showing a distinct band of labeling at the T-B cell contact site were counted as polarized. Statistical significance was determined using the paired Student t test.

Immunoblotting

Immunoblotting of Jurkat cell lysates was performed essentially as described previously (22). Mouse anti-Flag was purchased from Chemicon (Temecula, CA). Mouse anti-TCR was from Sigma-Aldrich (St. Louis, MO). Rabbit anti-SLP-76 was described previously (38). Rabbit anti-green fluorescent protein (GFP) and rhodamine phalloidin were obtained from Molecular Probes (Eugene, OR). Secondary Abs conjugated to fluorochromes were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunoblotting of Jurkat cell lysates was performed essentially as described previously (22). Mouse anti-Flag was purchased from Chemicon (Temecula, CA). Mouse anti-TCR was from Sigma-Aldrich (St. Louis, MO). Rabbit anti-SLP-76 was described previously (38). Rabbit anti-green fluorescent protein (GFP) and rhodamine phalloidin were obtained from Molecular Probes (Eugene, OR). Secondary Abs conjugated to fluorochromes were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). GFP-WASP-GTase binding protein (GDB) was prepared and used as described previously (22). WASP mAb 26E6, which specifically recognizes the open active conformation of WASP, will be described elsewhere (S. D. Leung, C. Labno, D. You, J. Burkhardt, and M. Rosen, manuscript in preparation). Staphylococcal enterotoxin E (SEE) was obtained from Toxin Technologies (Sarasota, FL). 7-Amino-4-cholormethylcoumarin was obtained from Molecular Probes. Poly-L-lysine was obtained from Sigma-Aldrich.

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Results

The C-terminal SH3 domain of Nck is required for WASP recruitment

Previous work from our laboratory has shown that WASP recruitment to the site of TCR engagement is independent of interaction with Cdc42 and depends instead on binding through the proline-rich domain of WASP (22). Although this domain of WASP has been shown to interact in vitro with the SH3 domains of numerous proteins (23–29), we focused on Nck and Grb2, which have been shown to recruit WASP or N-WASP to the comet tails of vaccinia virus and the pedestals formed by enteropathogenic Escherichia coli (EPEC) (39–43). To test the role of Nck and Grb2 in recruiting WASP in T cells, we used a panel of point mutants that perturb protein interactions with the SH3 domains of these proteins (39, 44, 45). Wild-type Jurkat T cells were transiently transfected with clones expressing wild-type or mutant versions of Grb2 or Nck tagged with GFP, and relative levels of expression were assessed by flow cytometry. As shown in Fig. 1A, the mean fluorescence intensity of GFP-positive cells transfected with each set of mutants (Nck or Grb2) differed by less than twofold. T cells were then cotransfected with the mutant Nck or Grb2 constructs together with FLAG-WASP and allowed to form conjugates with SEElabeled Nalm-6 B cells, and conjugates formed with GFP-positive T cells were analyzed for the presence of FLAG-WASP at the T-B contact site by immunofluorescence microscopy. Although the signaling by superantigen engagement differs somewhat from signaling by peptide-MHC complexes, this model system closely mimics peptide-Ag stimulation (46) and has proven useful in analyzing molecular events during conjugate formation (5, 22, 47, 48).

WASP formed a bright band at the cell-cell interface in cells expressing FLAG-WASP alone or together with wild-type Nck (Fig. 1B, left, and data not shown). In contrast, WASP was distributed throughout the cytoplasm in T cells coexpressing Nck W229K, which disrupts the C-terminal SH3 domain (Fig. 1B, right). Quantitative analysis of the frequency of conjugates showing WASP polarization toward the T cell-APC contact site revealed that in the presence of Nck W229K, recruitment of FLAG-WASP was reduced to background levels, defined by conjugates formed in the absence of superantigen (Fig. 1C). Expression of wild-type Nck or a control Nck construct mutated in the second SH3 domain (W143K) had no effect on WASP recruitment. Unlike Nck, neither of the Grb2 mutants tested perturbed WASP localization (Fig. 1F). The specific effects of Nck W229K were not attributable to higher expression levels of this mutant because this mutant was expressed at similar or somewhat lower levels than the other Nck or Grb2 constructs (Fig. 1A). Importantly, the effects of Nck W229K on WASP localization were specific: recruitment of SLP-76 and Vav to the T-B interface was intact in cells expressing this Nck mutant (Fig. 1, D and E). These results show that the C-terminal SH3 domain of Nck is responsible for WASP recruitment. This finding thus extends previous in vitro studies showing that the C-terminal SH3 domain of Nck binds directly to the GRSGPXPPXP motif within the proline-rich domain of WASP (26) by demonstrating that this interaction is required for WASP recruitment in intact T cells responding to APCs (26).

To rule out the converse possibility, that WASP recruits Nck to the T-B contact site, we analyzed the distribution of Nck in conjugates formed using T cells from WASP-deficient mice (WASP−/−) and their heterozygous littermate controls. As shown in Fig. 2, Nck was recruited to the T-B contact site whether or not the T cells expressed WASP. Similar results were obtained using T cells from a Wiskott-Aldrich syndrome patient lacking expression of the WASP protein, in conjugates with superantigen-pulsed human B cells (data not shown). Thus, Nck recruitment is independent of WASP, in keeping with the finding that Nck acts immediately upstream of WASP in the pathway leading to WASP recruitment.

Tyrosine phosphorylation of SLP-76 is required for recruitment of Nck and WASP

Previous studies have shown that Nck interacts through its SH2 domain with the tyrosine-phosphorylated adaptor protein SLP-76 (13) upon TCR stimulation (13, 36). Jurkat cells stably expressing SLP-76 wild type or a mutant (Y3F3), in which tyrosines 112, 128, and 145 are mutated to phenylalanine, have been used previously to show that tyrosine phosphorylation by ZAP-70 at these sites is required for SLP-76 adaptor function (13, 36). Immunoblot analysis of these cells showed that the expression level of wild-type SLP-76 was approximately twofold higher than that of the Y3F3 mutant (Fig. 3A), which was expressed at approximately twofold higher levels than that of endogenous SLP-76 (data not shown). To test the hypothesis that SLP-76 functions to recruit Nck and WASP to the site of T-B contact, localization of these proteins was analyzed in the SLP-76 Y3F3 expressing cells. As shown in Fig. 3, B and D, expression of SLP-76 Y3F3 blocked Nck polarization. The SLP-76 Y3F3 mutant also effectively blocked the polarization of WASP (Fig. 3, C and E). These findings show that tyrosine phosphorylation of SLP-76 is required for targeting of Nck, and consequently WASP, to the site of APC binding.

SLP-76 also functions in the pathway leading to WASP activation

In addition to Nck, SLP-76 interacts with the SH2 domain of Vav-1 (49–51), an adaptor protein with GEF activity for Rho GT-Pases. One of these, Cdc42-GTP, is a key activator of WASP. It has been shown that SLP-76 can bind simultaneously with Vav and Nck (13). This raised the interesting possibility that SLP-76 acts as a scaffold to colocalize Cdc42-GTP and WASP via their respective interactions with Vav and Nck, thus coordinating WASP recruitment and activation. To test this idea, Vav recruitment was analyzed in T cells expressing wild-type SLP-76 and the Y3F3 mutant. As shown in Fig. 4, A and C, the frequency of conjugates showing Vav recruitment to the T-B contact site was significantly diminished in cells expressing the Y3F3 mutant, indicating that SLP-76 phosphorylation is required for efficient targeting of Vav. To assess whether the defect in Vav recruitment results in a defect in localized Cdc42 activation, the conjugates were labeled with recombinant GFP-WASP-GBD. We have previously shown that this reagent specifically detects Cdc42-GTP at the T cell-APC contact site in fixed specimens (22). As shown in Fig. 4, B and D, T cells overexpressing wild-type SLP-76 accumulated Cdc42-GTP normally at the T-B interface, but this accumulation was blocked by the Y3F3 mutant. Indeed, a higher percentage of conjugates showed defects in Cdc42-GTP recruitment than in Vav recruitment. Because our scoring is on an all-or-none basis, this result may reflect the reduced accumulation of Vav in some conjugates, such that the threshold required for localized Cdc42 activation is not reached. Alternatively, it may simply reflect technical differences in the sensitivity of labeling for Vav vs Cdc42-GTP. In either case, our results show that in addition to recruiting Nck and WASP to the T-B contact site, SLP-76 is required for the efficient recruitment of Vav-1 and the local accumulation of Cdc42-GTP, the key activator of WASP.
Vav-1 is required for localization of Cdc42-GTP at the contact site

To confirm that the failure to locally activate Cdc42 in the SLP-76 Y3F3-expressing cells is attributable to the diminished recruitment of Vav, we used a newly described Jurkat cell line, J.Vav1, in which the Vav-1 gene was deleted by homologous recombination (35). When conjugates were formed using these Vav-1-deficient cells, Cdc42-GTP was not detectable at the T-B contact site (Fig. 5A). Quantitative analysis showed that the frequency of Cdc42-GTP polarization was diminished by 75% in the absence of Vav-1 and approached the background frequency obtained in the absence of Ag (Fig. 5B and data not shown). Thus, Vav-1 is required either directly or indirectly for the localized activation of Cdc42-GTP. Reintroduction of Vav-1 into the J.Vav1 cells restored Cdc42-GTP localization (Fig. 5A and B), confirming that the defects in localized Cdc42 activation are attributable to the absence of Vav-1.

In light of our previous studies showing that WASP activation and recruitment proceed through independent pathways (22), we tested the effects of Vav-1 deficiency on the pathway leading to WASP recruitment. We predicted that in these Vav-1-deficient cells, WASP would be recruited normally but would fail to become activated. As shown in Fig. 5C, we found that SLP-76 recruitment to the T-B interface was normal in the J.Vav1 cells, as expected if SLP-76 lies upstream of Vav-1. In contrast, Nck recruitment to the T-B contact site was partially decreased (Fig. 5D). One possible explanation for this result is that binding of Vav-1 and Nck to SLP-76 may be cooperative, such that in the absence of Vav-1, Nck recruitment is less efficient. When we evaluated WASP localization at the T-B contact site, we found that overall WASP recruitment occurred efficiently even in the absence of Vav-1 (Fig. 5E). The normal recruitment of WASP under conditions where Nck recruitment is partially inhibited may be due to the ability of small amounts of Nck to recruit WASP efficiently, or to the ability of other proteins to participate in WASP recruitment, in parallel with Nck (48, 52). In either case, our findings show that WASP recruitment proceeds normally in Vav-1-deficient Jurkat cells, whereas Cdc42 activation is perturbed. To permit direct assessment of the activation state of the WASP, we have recently generated a mAb that specifically recognizes the “open” active form of WASP. Intact conjugates were analyzed in each of three independent experiments. Data represent means ± SD. Statistical difference from Nck wild-type was determined by Student’s t test (**, p < 0.001). D and E, Jurkat cells were transfected with Nck wild-type or W229K and conjugates were analyzed for accumulation of endogenous SLP-76 (D) or Vav (E) at the interface after Ab labeling. In each case, 50 randomly selected conjugates were analyzed. F, Jurkat T cells were cotransfected with the indicated Grb2 constructs together with FLAG-WASP and were analyzed for FLAG-WASP accumulation at the T-B interface as in C.
conformation of WASP (S. D. Leung, C. Labno, D. You, J. Burkhardt, and M. Rosen, manuscript in preparation). Using this reagent, we found that the frequency of conjugates showing active WASP at the T-B contact site was significantly reduced in the absence of Vav-1 (Fig. 5F). Thus, unlike SLP-76, which participates in both WASP recruitment and activation, Vav-1 functions only in the pathway leading to WASP activation by Cdc42-GTP.

**Vav family members play partially redundant roles in actin remodeling**

Recent work shows that although T cells from WASP−/− mice have defects in cytokine production, they show no obvious defects in actin-dependent conjugate formation (8, 11). In contrast, T cells from Vav-1−/− mice are grossly defective in actin-dependent conjugate formation (8). Therefore, we tested the ability of J.Vav1 T cells to form conjugates and polymerize actin in response to interaction with APCs. Surprisingly, these cells formed conjugates with SEE-pulsed B cells as efficiently as wild-type Jurkat controls (data not shown). Moreover, although the morphology of conjugates formed using the J.Vav1 cells was not as consistent as in wild-type controls (data not shown), conjugates showed a normal frequency of F-actin accumulation at the cell-cell interface (Fig. 6A). To test the possibility that the lack of a detectable actin phenotype in the J.Vav1 cells stems from redundancy among Vav family members, Jurkat cells were transfected with a C-terminal domain mutant of Vav-1 that competes with endogenous Vav proteins for binding to upstream phosphotyrosine motifs. This mutant dominantly interferes with TCR-induced transcriptional activity in untransfected cells or in cells overexpressing Vav 1, 2, or 3.
Wild-type Jurkat cells expressing this mutant showed significant defects in actin polymerization at the T-B contact site (Fig. 6B). Similar defects were observed in J.Vav1 cells expressing this mutant, indicating that Vav-2 and/or Vav-3 are involved in controlling actin responses in these cells. Interestingly, the actin responses in these cells were still above background levels as defined by the absence of SEE, suggesting that Vav-independent pathways are also involved.

**Discussion**

Our studies in T cells represent one of the first cases in which the proteins linking an exogenous signal to localized Cdc42-WASP activation have been defined in mammalian cells. Our results reveal the individual molecular components involved in the coordinate recruitment of WASP to the T cell-APC interface and its local activation at that site. In the cascade that we have defined, TCR signaling through Lck and ZAP-70 leads to the phosphorylation of SLP-76 at tyrosines 112, 128, and 145, creating binding sites for both Nck and Vav-1. Nck then functions to recruit WASP to the contact site via interactions of the C-terminal SH3 domain of Nck with the proline-rich domain of WASP. In parallel, Vav-1 functions to induce the localized activation of Cdc42. Finally, Cdc42-GTP interacts locally with WASP, activating Arp2/3-dependent actin polymerization at the T cell-APC contact site. In a diverse set of studies involving mutant cell lines, transfection studies, and knockout mice, most of the components in the cascade we have defined have been previously implicated in actin remodeling in T cells (4–10, 12, 13). We have now analyzed the interactions of these proteins in situ, in a single experimental system. Our results show that these proteins function in two convergent pathways leading on one hand to WASP recruitment and on the other to WASP activation by Cdc42-GTP.

Our results point to a trimolecular complex among SLP-76, Nck, and Vav-1 as a critical element of the WASP regulatory
mechanism. Phosphorylation of SLP-76 by ZAP-70 has been shown to facilitate its binding to both Nck and Vav-1 (13, 49, 50, 53, 54). Importantly, SLP-76 can bind simultaneously to Nck and Vav (13) and thus can function to bring Cdc42-GTP into molecular proximity with WASP. Our finding that Nck recruitment is reduced in Vav-1-deficient T cells suggests that the binding of these two proteins to SLP-76 may be cooperative. The failure of the Nck W229K mutant to perturb Vav recruitment does not argue against this possibility, because only the Nck domain predicted to affect WASP binding is mutated in those cells. A recent study shows that Nck can also interact directly with the TCR-CD3 complex (55). However, T cells expressing the SLP-76 mutant fail to recruit detectable levels of Nck, indicating that direct binding to CD3 is not sufficient for Nck recruitment to the T-B contact site. In addition to WASP, Nck has been shown to interact with other key actin regulatory proteins, including p21-activated kinase and WASP-interacting protein (WIP) (56–58). Thus, Nck may function as part of a larger actin regulatory network. In contrast with Nck, Vav recruitment in the SLP-76 Y3F3 cells is significantly diminished, but not eliminated, suggesting that other pathways do contribute to Vav recruitment. In keeping with this finding, a recent study shows that Vav-1 interacts with linker for activation of T cells in a fashion that depends on phosphorylation by Itk (59), and we have recently found that Itk-deficient cells, like the SLP-76 mutants, are defective in Vav recruitment and localized Cdc42 activation.

Although SLP-76 has been shown to bind both Grb2 and Nck (13, 60, 61), we find that only Nck participates in WASP recruitment. Our results showing that the third SH3 domain of Nck is required for WASP recruitment are consistent with a previous study showing that this SH3 domain binds to the proline-rich domain of WASP in vitro (26). Though our results show that over-expression of the Nck W229K mutant reduces WASP recruitment to levels observed in the absence of Ag, it remains possible that other proteins such as WIP and PST-PIP also participate in WASP recruitment. Indeed, this may explain why WASP recruitment is normal in the J.Vav1 cells, where Nck recruitment is partially reduced. Recent evidence shows that overexpression of a mutant of PST-PIP can also block WASP recruitment (52). However, this mutant severely perturbs conjugate formation, leaving the possibility that the effects on WASP recruitment are secondary to other signaling defects (55–58).

The pathway involved in WASP recruitment in T cells bears interesting similarities and differences to those used by intracellular pathogens such as shigella and vaccinia virus to form comet tails for movement within host cells and by EPEC to form actin-rich pedestals (reviewed in Refs. 62 and 63). We show here that in T cells, as in shigella, vaccinia, and EPEC (41, 42, 64), Nck is a component of the WASP/N-WASP signaling complex. The recruitment of Nck to tyrosine phosphorylation sites in SLP-76 parallels the recruitment of Nck to tyrosine phosphorylated sites in the Tir protein of EPEC (42, 65) and in the A36R protein of vaccinia (41). In T cells, we find that Nck functions to recruit WASP, just as Nck is required to recruit N-WASP to the vaccinia virus particle (39, 41) and to the EPEC pedestal (42). However, in the case of vaccinia, Grb2 has been shown to cooperate with Nck in recruiting N-WASP (39), whereas we have found no evidence for Grb2 participation in the T cell system. One common feature of the pathogen systems, which we have not yet explored in T cells, is the involvement of the WASP-interacting protein WIP. In the case of both vaccinia and shigellosis, it appears that N-WASP and WIP are recruited together as part of an obligate complex (64). WIP has been shown to play an important role in T cell activation (66, 67). Recent data have shown that WIP/WASP/CT10 regulator of kinase-like interactions are regulated upon T cell activation, and that these interactions function in targeting WASP to the lipid raft compartment (48). Although our studies on WASP targeting indicate that interactions with WIP are not required to recruit WASP (22), it seems clear that WIP will prove to be another component of this actin regulatory complex.

Our finding that Vav-1 null Jurkat cells fail to accumulate Cdc42-GTP at the site of APC binding is consistent with biochemical studies with T cells from Vav-1−/− mice (68). Though it is clear from both systems that the presence of Vav-1 is required for Cdc42 activation, it is not possible to conclude from these experiments that Vav-1 functions as the GEF for Cdc42. Indeed, the role of Vav-1 as a direct GEF for Cdc42 is controversial. Although some studies indicate that Vav-1 can catalyze GTP exchange for both Cdc42 and Rac (30, 31), other studies show that Vav-1 has little or no GEF activity for Cdc42 (32, 69, 70). The simplest interpretation of our findings is that Vav-1 functions as the direct GEF for Cdc42 under the conditions present in intact cells, which may not be duplicated in in vitro studies. Alternatively, because Rho family GTPases frequently function in complex regulatory cascades, it is also possible that Vav-1 functions upstream of Rac or other molecules, leading to Cdc42 activation. So far, however, we have been unable using pull-down assays to detect defects in Rac activation in the J.Vav1 cells (R.T.A., unpublished data). Additional studies will be required to resolve this issue.

Consistent with the critical role of Cdc42 as a key activator of WASP, we find that although WASP is recruited normally to the T-B interface in J.Vav1 cells, it does not undergo the conformational change required for activation of Arp2/3-induced actin polymerization. Although it has been reported that N-WASP can be directly activated by its interaction with Nck in vitro (71), we find in the J.Vav1 cells, where WASP-Nck interactions are intact, WASP is not activated. Thus, we conclude that binding to Nck is insufficient to activate WASP in our system. We have obtained similar results in T cells lacking the Tec family kinase Itk, where WASP recruitment occurs normally but activation is defective.

In these cells, as in the J.Vav1 cells, Cdc42 activation is defective, consistent with the view that binding to Cdc42-GTP, rather than Nck, is of primary importance for WASP activation in response to TCR ligation.

Surprisingly, we found that in the J.Vav1 cells, where activation of Cdc42 and WASP was at background levels, conjugate formation and polymerization of actin at the T-B interface was grossly normal. It has been widely assumed that the role of WASP in T cell activation is dependent on its actin regulatory function. However, we and others have recently shown that WASP−/− T cells conjugate normally and polymerize actin normally in response to APC binding (8, 11). Moreover, we have found that these cells organize marker proteins normally at the immune synapse.

Nonetheless, both the WASP−/− T cells and the J.Vav1 cells show significant defects in IL-2 production (9, 10, 35), suggesting that other aspects of WASP function are required. The lack of a detectable actin defect in the J.Vav1 cells is consistent with previous work showing that cholera toxin-induced raft clustering, an actin-dependent process, proceeds normally in these cells (35). However, the phenotype of these cells differs strikingly from that of T cells from Vav-1−/− mice, which shows numerous actin defects (6–8). The reason for this difference is currently unclear; it may be attributable to properties of the Jurkat cell lines, to defects in murine T cells that have developed in the absence of Vav-1, or to differences in the extent to which multiple Vav isoforms play a redundant role in mouse vs human T cells. Our results provide evidence that Vav-2...
and/or Vav-3 can participate in actin remodeling, because T cells expressing a mutant of Vav-1 that is predicted to interfere with the function of all Vav isoforms showed significantly impaired actin responses, even when expressed in J.VAv1 cells. It is interesting to note, however, that the actin responses in these cells are not completely abolished. Although this could be attributable to incomplete effects of the dominant negative mutant, it could also point to Vav-independent actin regulatory pathways. For example, Ku et al. (72) have defined a p21-activated kinase activation pathway that operates independently of the SLP-76/Nck complex defined here.

Though recent work has led to new questions about the extent to which WASP functions in T cells as a regulator of actin remodeling vs other processes, it is clear that normal WASP function is required for T cell activation. Taken together, our studies now permit the reconstruction of the molecular events leading from APC binding to the localized activation of WASP by Cdc42-GTP. Similar pathways are likely to control localized WASP activation during chemotaxis, phagocytosis, etc. In each case, we anticipate that localized WASP activity will be carefully regulated through the coordination of independent pathways for recruitment and activation.

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