Membrane Localization and Function of Vav3 in T Cells Depend on Its Association with the Adapter SLP-76* 

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The Vav family of guanine exchange factors plays a critical role in lymphocyte proliferation, cytoskeletal reorganization, and gene transcription upon immunoreceptor engagement. Although the role of Vav1 in T cells is well documented, the role of Vav3 is less clear. We investigated the subcellular localization of Vav3 during T cell activation. We report here that phosphorylation of Vav3 on tyrosine residue Tyr137 is not required for T cell receptor (TCR)-induced Vav3 membrane translocation or immunological synapse (IS) recruitment, but mutation of this residue enhanced TCR-induced nuclear factor of activated T cells (NFAT) activation. However, Vav3 mutants either containing an Src homology 2 (SH2)-disabled point mutation (R697L) or lacking its SH3-SH2-SH3 domains were unable to bind SLP-76 did not translocate to the membrane or to the IS and furthermore failed to activate NFAT. Importantly, the membrane translocation of Vav3 was abrogated in Lck, ZAP-70, LAT, and SLP-76-deficient T cells, where Vav3 binding to SLP-76 was disrupted. Finally, we confirmed and underlined the critical role of Vav3 in NFAT activation by knocking down Vav3 expression in Vav1-deficient T cells. Altogether, our data show that TCR-induced association of Vav3 with SLP-76 is required for its membrane/IS localization and function.

The T cell receptor (TCR) recognizes specific peptides presented by major histocompatibility complex molecules on the surface of antigen-presenting cells (APCs). The contact site formed during this recognition process has been termed the supramolecular activation complex or the immunological synapse (IS). This engagement initiates a series of intracellular biochemical events that result in biological responses, such as T cell activation, proliferation, and differentiation. One of the earliest events after T cell ligation is the activation of protein-tyrosine kinases (PTKs), including members of the Src (Lck and Fyn) and Syk (ZAP-70 and Syk) families. Once activated, these PTKs can phosphorylate many downstream substrates on tyrosine and induce the formation of multisubunit signaling complexes and signaling cascades that ultimately lead to the activation of transcription factors and cytokine production. This de novo gene expression mediates differentiation of the cells into effector cells capable of mediating adaptive immune responses.

One critical mediator of TCR signaling is the Vav family of guanine exchange factors for the Rho family of small GTPases. Vav proteins represent a key link between antigen receptor-coupled PTKs and signaling pathways controlled by these small GTPases (4–6). Following the discovery and characterization of the first member, Vav1 (7), two other mammalian members, Vav2 and Vav3, were identified (8, 9). Whereas Vav1 is expressed primarily in cells of the hematopoietic lineage, Vav2 and Vav3 display a much broader expression profile (10).

One major characteristic of Vav proteins is their rapid tyrosine phosphorylation mediated by PTKs of the Src and/or Syk families upon engagement of several receptors, including the B cell receptor and TCR (11). This process liberates the catalytic Dbl homology domain and causes Vav to promote GDP-GTP exchange on its small GTPase targets (12). Another specific structural feature of mammalian Vav proteins is the presence of adapter domains, SH3-SH2-SH3, in their C-terminal region. In T cells, these domains allow Vav proteins to associate with PTKs of the Syk family (13), adapters such as SLP-76 (14–15) or Grb2 (via a proline-rich motif in the N-terminal SH3 domain) (17). Association with SLP-76 is critical for the membrane recruitment of Vav1 in T cells (18) or in mast cells (19) following TCR or FcgRI engagement, respectively. This association depends on PTKs of the Syk family (20), and a Vav1 mutant incapable of binding SLP-76 cannot undergo TCR-induced tyrosine phosphorylation, confirming the critical role of SLP-76 in the regulation of Vav1 function (21). The SH2 domain of Vav2 also seems to be essential for its membrane targeting following epidermal growth factor receptor stimulation in COS7 cells (22).

In T cells, Vav proteins transcriptionally regulate IL-2 promoter activity by acting on several transcription factors, including nuclear factor of activated T cells (NFAT) (13, 23), NF-xB (24), activator protein-1 (25), and serum response factor (15, 26). Interestingly, Vav1 synergizes with SLP-76 to increase both TCR-induced NFAT and IL-2 promoter activation (14, 27). However, a physical Vav1/SLP-76 interaction seems to be dispensable, since SLP-76 mutants incapable of binding Vav1 can still synergize with the latter (28). Despite the high degree of homology among the three mammalian Vav family members, their effects on NFAT or NF-xB can differ according to the cell type. For example, Vav2 activates NFAT in B cells but not in T cells (29). However, in T cells, only Vav1 activates NFAT.

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¶The abbreviations used are: TCR, T cell receptor; APC, antigen-presenting cells; IS, immunological synapse; NFAT, nuclear factor of activated T cell; PH, pleckstrin homology; FMA, phorbol 12-myristate 13-acetate; PTK, protein-tyrosine kinase; SEE, staphylococcal enterotoxin E; SH2 and SH3, Src homology 2 and 3, respectively; shRNA, short hairpin RNA; mAb, monoclonal antibody; PBS, phosphate-buffered saline; IP, immunoprecipitate; CMTMR, Cell tracker® Orange CMTMR (5-(and-6)-[[[4-chloromethyl]benzoylamino]tetramethylrhodamine).
whereas both Vav1 and Vav3, but not Vav2, can enhance NF-κB-dependent transcription (30).

The role of Vav3 in T cells has recently been highlighted by analysis of gene knock-out mice lacking one, two, or all three Vav genes. Vav1−/−vav2−/−vav3−/− triple knock-out mice show a profound block in thymocyte development and deficiency in functionally mature T and B cells, calcium mobilization, and TCR-induced or B cell receptor-induced signaling. On the other hand, vav2−/−vav3−/− double knock-out thymocytes and mature T lymphocytes exhibited normal development and activation, implicating Vav2 and Vav3 as Vav1- auxiliary proteins (31). Additional studies in Jurkat T cells using a short hairpin RNA (shRNA) interference gene silencing approach suggested that Vav3 participates in TCR signaling by promoting serum response element-mediated gene transcription but is not required for NFAT, NF-κB, activator protein-1, and IL-2 promoter activation (16). Finally, in B cells, Vav3 recruitment into membrane lipid rafts depends on the adapters BLNK and Grb2 (32), and Vav3 regulates phosphoinositide 3-kinase activation upon B cell receptor stimulation (33).

In this report, we show that Vav3 translocates to the plasma membrane and is recruited into the IS upon TCR triggering independently of its phosphorylation on Tyr173. However, Vav3 membrane translocation and IS targeting depend on its TCR- inducible association with SLP-76. Consistently, Vav3 mutants with a disabled SH2 domain (R697L) or lacking their three SH domains were incapable of interacting with SLP-76 and, thus, could not undergo membrane and IS translocation or mediate NFAT activation. Moreover, the Vav3-SLP-76 complex was disrupted in Jurkat T cell lines deficient in either Lck, ZAP-70, LAT, or SLP-76, where the membrane recruitment of Vav3 was aborted. Finally, shRNA-mediated knock-down of Vav3 expression further inhibited the residual TCR/CD28-induced NFAT activation in Vav1-deficient T cells. Together, our results strongly support a dependence of Vav3 on SLP-76 for its membrane and IS recruitment and ability to mediate gene transcription upon T cell activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Wild-type (clone JEB.1) and Lck-deficient (JCam1.6) Jurkat T cells were obtained from the ATCC and were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1 mM minimal essential medium nonessential amino acid solution (Invitrogen), and 100 units/ml each penicillin G and streptomycin. Raji B cells were a gift of D. R. Green. RPMI 76-20, JZM, and SLP-76, where the membrane recruitment of Vav3 was aborted. Finally, shRNA-mediated knock-down of Vav3 expression further inhibited the residual TCR/CD28-induced NFAT activation in Vav1-deficient T cells. Together, our results strongly support a dependence of Vav3 on SLP-76 for its membrane and IS recruitment and ability to mediate gene transcription upon T cell activation.

**Antibodies and Reagents—**Monoclonal antibodies (mAbs) specific for human CD3 (OKT3), mouse CD3 (2C11), mouse CD28 (37.51), and phosphotyrosine (4G10) were affinity-purified from culture supernatants of the respective hybridomas as described (35). The anti-human CD28 mAb was from Pharmingen (San Diego, CA). The anti-yttrium (Y173F) and R697L mutants of Vav3 were obtained by point mutagenesis using a mutagenesis kit from GE Healthcare (Piscataway, NJ) and subcloned in frame with the c-Myc tag in the pEF4 vector (Clontech, Palo Alto, CA) using the restriction sites EcoRI and NotI. The Y173F and R697L mutants of Vav3 were obtained by point mutagenesis using a mutagenesis kit from Invitrogen. Generation of the single mutation was verified by sequencing. The c-Myc-tagged Vav1 and the NFAT-luciferase reporter gene have been previously described (37). The Vav3-specific short hairpin RNA interference (shVav3) and the mutated version (shVav3mt) have been previously described (16).

**Subcellular Fractionation—**Subcellular fractionation of Jurkat T cells was performed as described (38) with some modifications. Briefly, T cells were resuspended in ice-cold hypotonic lysis buffer and incubated on ice for 15 min. The cells were then washed with a 1% M/200 solution and sheared by passing them five times through a 27-gauge needle. The lysates were centrifuged at 200 × g for 10 min to remove nuclei and cell debris. The supernatant was transferred to ultra-centrifuge Eppendorf tubes and centrifuged at 45,000 × g for 30 min at 4 °C. The supernatant (cytosol) was collected, and the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 5 mM N-ethylmaleimide, 1 mM sodium orthovanadate (Na3VO4), 20 mM NaF, 0.5% Triton X-100, and 10 μg/ml each aprotinin and leupeptin), vortexed for 20 min at 4 °C, and centrifuged again at 45,000 × g for 30 min. The supernatant representing the membrane fraction was saved, and the detergent-insoluble fraction (cytoskeleton) was resuspended in 1% SDS in water. Protein concentrations in the cytosolic and the membrane fraction were measured by a Bio-Rad protein assay, and each fraction was then diluted with anti-CD3 mAb (10 μg/ml) plus soluble anti-CD28 mAb (5 μg/ml). The activated T cells were then cultured for 36 h without anti-CD3/CD28 mAbs but with recombinant human IL-2 (20 units/ml), washed, and restimulated with anti-CD3/CD28 mAbs (20 μg/ml) for the indicated times. The cells were then incubated in hypotonic buffer as described above, and 2 drops of polystyrene beads were added. Subcellular fractionation was performed as described above.

**Is Formation and Confocal Microscopy—**For conjugation of Jurkat T cells, Raji B cells were used as APCs and stained with CMTMR (4 μM) in serum-free medium for 30 min at 37 °C, washed in Hanks’ balanced salt solution, and incubated with or without SEE (10 μg/ml) for 30 min at 37 °C. After washings, these APCs were mixed with transfected Jurkat T cells at a 1:1 ratio in cell culture medium. B cells were activated for 48 h in the presence of anti-CD3 mAb (10 μg/ml) plus soluble anti-CD28 mAb (5 μg/ml). The activated T cells were then cultured for 36 h without anti-CD3/CD28 mAbs but with recombinant human IL-2 (20 units/ml), washed, and restimulated with anti-CD3/CD28 mAbs (20 μg/ml) for the indicated times. The cells were then incubated in hypotonic buffer as described above, and 2 drops of polystyrene beads were added. Subcellular fractionation was performed as described above.

**Immunoprecipitation—**Jurkat T cells were incubated in serum-free medium for 30 min on ice with anti-CD3 mAb in the presence or the absence of anti-CD28 (20 μg/ml) each mAb and cross-linked with goat-antimouse IgG (Pierce) for 1 min at 37 °C with gentle shaking. The cells were washed in ice-cold PBS and lysed as described above, and the lysates were collected after centrifugation at 13,000 × g for 10 min. Immunoprecipitation was performed by adding the anti-

**Inhibitory mAb and cross-linked goat antimouse IgG secondary antibody (Molecular Probes) for 30 min. After washing five times with PBS, the cells were mounted on glass slides using a drop of mounting medium (Vector Laboratories, Inc.). The immunofluorescence images were recorded by a Maranas digital fluorescence microscopy system (Intelligent Imaging Innovations, Denver, CO). The imaging system included a Zeiss Axioscam MRm charge-coupled device camera (Carl Zeiss Micro Imaging Inc., Thornwood, NY), a Zeiss Axiosvert 200M microscope (Carl Zeiss Micro Imaging, Inc.), a Zeiss Plan-Apochromat 63 × 1.4 numerical aperture objective, and excitation and emission filter wheels (Sutter Instrument Co., Novato, CA) with narrow band optical filters (Chroma Technology Corp., Rockingham, VT). Slidebook software (Intelligent Imaging Innovations) controlled all of these components and was used for image capture and analysis.

**Analysis—**
cated antibodies plus 30 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) and incubating the lysates overnight at 4 °C with gentle shaking. Samples were washed three times with lysis buffer, and the immunoprecipitates (IPs) were dissolved in 1× Laemmli buffer subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the indicated antibodies.

Reporter Gene Assays—Jurkat T cells were transfected with an NFAT-luciferase reporter plasmid and a β-galactosidase reporter plasmid as described (37). Transfected cells were stimulated for the last 6 h of culture, washed with PBS, and lysed, and luciferase or β-galactosidase activities were determined as described (37). The results are expressed in arbitrary luciferase units normalized to β-galactosidase activities.

RESULTS

Vav3 Membrane Translocation Is Dependent on CD3 Stimulation—Vav1 and Vav3 are highly homologous proteins, but their respective roles during T cell activation have not been fully examined. We initially compared the tyrosine phosphorylation pattern of both proteins in T cells. The phosphorylation of Vav3 on Tyr173 and Vav1 on Tyr174 was maximal after 1 min of anti-CD3/CD28 stimulation and gradually decreased at later time points (Fig. 1, A and B). However, basal phosphorylation of Tyr174 in Vav1 was clearly detectable (Fig. 1B). The overall tyrosine phosphorylation of Vav3 and Vav1 followed a similar pattern, with a substantial basal phosphorylation and, therefore, less pronounced inducible phosphorylation of Vav1. We also analyzed the subcellular distribution of both proteins in wild-type Jurkat T cells (JE6.1). To this end, we stimulated JE6.1 cells with anti-CD3 plus anti-CD28 mAbs or, as a control, with PMA and analyzed Vav expression in purified cytosolic (c in Fig. 1), membrane (m), and detergent-insoluble (i) fractions. We observed a transient anti-CD3/CD28-induced translocation of Vav3, which peaked after 1 min of stimulation (Fig. 1C). However, a substantial fraction of Vav1 was constitutively present in the membrane of unstimulated cells, and this level remained unchanged following anti-CD3/CD28 stimulation (Fig. 1C). PMA stimulation did not increase the membrane translocation of either Vav3 or Vav1. Indicative of proper activation, stimulation with either anti-CD3/CD28 mAbs or PMA induced strong membrane translocation of PKCθ. p38 was present only in the cytoplasm, whereas LAT was present in both membrane and insoluble fractions (Fig. 1C, lower panels), demonstrating a proper purification of the different fractions. Next, we stimulated Jurkat T cells with anti-CD3 and/or CD28 antibodies and observed that membrane translocation of Vav3 was induced by anti-CD3 stimulation but not by anti-CD3 ligation (Fig. 1D). Furthermore, anti-CD28 stimulation did not increase the membrane translocation of Vav3 induced by the anti-CD3 mAb. In contrast, and as reported earlier (39), combined anti-CD3/CD28 costimulation enhanced the membrane translocation of PKCθ relative to the translocation induced by anti-CD3 alone. As before (Fig. 1C), the membrane localization of Vav1 remained unchanged in unstimulated versus stimulated cells (Fig. 1D). We also analyzed the subcellular distribution of Vav proteins in primary murine T cells and found that they displayed a similar behavior to that of Jurkat T cells. Thus, anti-CD3/CD28 costimulation induced membrane translocation of Vav3, which peaked 5 min post-stimulation and was still observed after 10 min (Fig. 1E). Costimulation of the same cells also induced a strong membrane translocation of PKCθ, but not of Vav1. Similar to Jurkat cells, PMA stimulation did not induce detectable membrane translocation of Vav3 or Vav1 (data not shown).

To examine the membrane translocation of Vav proteins in the context of the IS, we stimulated Vav1- or Vav3-transfected Jurkat-TAg cells with Raji B lymphoma cells, which were either pulsed or not with a superantigen, staphylococcal enterotoxin E (SEE), and determined the localization of Vav proteins at the contact area (IS) formed between the T and B cells. Both Vav3 and Vav1 were concentrated in the IS in a stimulation-dependent manner (Fig. 1F). Taken together, our results suggest that Vav3 translocates to the plasma membrane and localizes to the IS upon T cell activation.

Defective Vav3 Membrane Translocation in Jurkat Cells Lacking Lck, ZAP-70, SLP-76, or LAT—PTKs of the Src and Syk families regulate the localization and function of Vav proteins by inducing their tyrosine phosphorylation and association with other signaling enzymes and/or adapter proteins (13, 30, 40). Initially, we examined the effect of a specific Src family kinase inhibitor, protein phosphatase 2, on the membrane translocation of Vav3. Protein phosphatase 2 pretreatment completely reversed the anti-CD3/CD28-induced membrane translocation of Vav3 and, as a positive control (39), also that of PKCθ (Fig. 2A). As a control, treatment with the phosphoinositide 3-kinase inhibitors, LY 294002 or wortmannin, had no effect on Vav3 membrane translocation (data not shown), indicating that the phosphoinositide 3-kinase pathway was not involved in this process. Next, we took advantage of an Lck-deficient Jurkat cell line (JCam1.6) and confirmed that in the absence of Lck, anti-CD3/CD28 stimulation failed to induce membrane translocation of Vav3 translocation (Fig. 2B). As shown previously (39), PKCθ also failed to undergo membrane translocation in JCam1.6 cells. We then examined the membrane translocation of Vav3 in a ZAP-70-deficient cell line (P116) and observed that ZAP-70 was also required for the translocation of Vav3 induced by TCR/CD28 costimulation; however, as we previously reported (39), the membrane translocation of PKCθ was independent of ZAP-70 (Fig. 2C).

Since all three Vav family members have been reported to associate with SLP-76 (14–16), we also investigated the role of SLP-76 in Vav3 membrane translocation. Anti-CD3/CD28-induced membrane translocation of Vav3 did not occur in SLP-76-deficient Jurkat T cells (J14) as compared with wild-type (JE6.1) T cells (Fig. 2D). However, the absence of SLP-76 had no effect on anti-CD3/CD28-induced PKCθ membrane translocation (Fig. 2D). Early after T cell activation, SLP-76 indirectly binds LAT via Gads to form a signaling complex that also contains Vav proteins (41). We thus questioned whether LAT plays a role in Vav3 translocation. As shown in Fig. 2E, Vav3 did not translocate to the membrane in stimulated LAT-deficient (JCam2.5) Jurkat T cells. Interestingly, TCR/CD28-induced PKCθ membrane translocation was also reduced, albeit not completely ablated, in JCam2.5 cells (Fig. 2E). Altogether, our results indicate that the PTKs of the Src (Lck) and Syk (ZAP-70) families and the adapters SLP-76 and LAT are required for TCR/CD28-mediated membrane translocation of Vav3.

Membrane and IS Recruitment of Vav3 Does Not Require Tyr173 Phosphorylation—The activity of Vav proteins is regulated by their tyrosine phosphorylation, and phosphorylation of Vav1 on Tyr174 in its acidic domain modulates its function (42). In order to analyze in more detail the mechanism of Vav3 membrane recruitment and the role of tyrosine phosphorylation in this event, we initially determined the tyrosine phosphorylation status of Vav3 in all four Jurkat mutant T cell lines, in which the anti-CD3/CD28-induced membrane translocation of Vav3 was inhibited (Fig. 2). The basal and inducible tyrosine phosphorylation of Vav3 on Tyr173 (the homologous residue to Tyr174 in Vav1) was completely suppressed in JCam1.6 cells and significantly reduced in P116 cells (Fig. 3A). We similarly investigated the role of the adapters, LAT and SLP-76, in Vav3 tyrosine phosphorylation. Phosphorylation of Vav3 on Tyr173 was not affected in J14 cells but was severely impaired in JCam2.5 (Fig. 3B). The
profile of overall tyrosine phosphorylation of Vav3 generally followed the same pattern as that observed for Tyr173. Next, we transfected Jurkat-TAg cells with a Vav3 point mutant, in which Tyr173 was replaced by a nonphosphorylatable alanine residue (Y173F), and analyzed the subcellular localization of this mutant. Following anti-CD3 stimulation, Vav3-Y173F was capable of undergoing membrane translocation to the same extent as wild-type Vav3 (Fig. 3C). Moreover, we observed intact recruitment of Vav3-Y173F to the IS in SEE-stimulated Jurkat T cells (Fig. 3, D and E). Of note, the mutant Vav3Y173F was phosphorylated on tyrosine to the same extent as wild-type Vav3 following TCR cross-linking (data not shown), indicating the existence of additional tyrosine phosphorylation sites. Finally, we also compared the

**Fig. 1. Anti-CD3/CD28-induced Vav3 membrane translocation.** A, Jurkat T cells (10 × 10⁶) were stimulated with anti-CD3 plus anti-CD28 mAbs for the indicated times. Vav3 IPs were prepared, washed, separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. B, Vav1 IPs were prepared and processed as in A. C, cytosol (c), membrane (m), and detergent-insoluble (i) fractions were prepared from Jurkat T cells stimulated for 1 or 5 min (or unstimulated; 0) with anti-CD3 plus anti-CD28 mAbs or with PMA (100 ng/ml) for 1 min. Equivalent protein amounts from each group were resolved by SDS-PAGE, and the expression of Vav3, PKCδ, or Vav1 in each fraction was determined by immunoblotting with specific antibodies. Anti-p38 and anti-LAT immunoblotting was included as a control of purity of the respective fractions. D, Jurkat T cells stimulated (+) or not (−) with anti-CD3 and/or anti-CD28 mAbs for 1 min were lysed, fractionated, and analyzed as in C. E, preactivated and rested primary T cells (10 × 10⁶) were restimulated with anti-CD3/CD28 mAbs (20 g/ml) for the indicated times. Subcellular fractions were prepared and analyzed as in C. F, Jurkat-TAg cells (5 × 10⁶) were transfected with empty vector, Myc-tagged Vav3, or Myc-tagged Vav1 (10 μg each). After 24 h, the cells were mixed for 10 min at a 1:1 ratio with CMTMR-labeled Raji B cells, which were pulsed or not pulsed with SEE. The cells were then plated on polylysine-coated slides, and localization of Myc-Vav proteins was analyzed using secondary Alexa 488-coupled anti-mouse Ig antibody and a Marianas digital fluorescence microscopy system. DIC, differential interference contrast. The right column panels in the resting or stimulated groups represent a 3-fold enlargement of a single cell marked with an arrow in the middle column panels. The bars in the lower right micrograph correspond to 15 μm. P-Tyrosine, phosphotyrosine.
ability of wild-type and Y173F Vav3 to stimulate the activity of a luciferase reporter gene driven by NFAT in cotransfected Jurkat cells. Wild-type Vav3 cooperated with anti-CD3 stimulation to further increase NFAT activation by ~3-fold, and the Vav3-Y173F mutant was even more active in this regard (~5-fold enhancement compared with anti-CD3 stimulation alone), despite the fact that both Vav3 proteins were expressed to the same extent (Fig. 3F). Altogether, these results indicate that phosphorylation on Tyr173 is not required for Vav3 membrane and IS recruitment or for its ability to activate NFAT.

Vav3 Mediates Residual TCR-induced NFAT Activation in Vav1-deficient T Cells—Several studies have recently shown that Vav3 does not play a role in NFAT activation upon TCR stimulation (16, 30). However, we could show that human Vav3 strongly increased TCR-induced NFAT activation (Fig. 3F). By comparing NFAT activation in wild type Jurkat T cells (JE6.1) and Vav1-deficient Jurkat T cells (J.Vav1.null), we observed that TCR-induced NFAT activation was drastically decreased in Vav1-deficient Jurkat T cells (Fig. 4A). However, anti-CD3 stimulation still increased NFAT activation in the absence of Vav1 (~4-fold enhancement) (Fig. 4A). In order to evaluate the role of Vav3 in this residual NFAT activation, we transfected Vav1-deficient T cells with a Vav3-specific shRNA to knock down endogenous Vav3 expression or with a mutated shRNA as a negative control. Interestingly, anti-CD3 with or without anti-CD28-induced NFAT activation in Vav1-deficient T cells was not affected by the control shRNA but was strongly decreased by expression of a Vav3-specific shRNA (Fig. 4B). We confirmed the specificity and effectiveness of the shRNA by assessing the expression of Vav3 (Fig. 4B, inset). Our results indicate that Vav3, along with Vav1, plays a role in residual NFAT activation in Vav1-deficient T cells.

Vav3 Association with SLP-76 Depends on TCR-proximal Signaling Proteins—Similar to Vav1 and Vav2 (14, 15), Vav3 was also recently found to form a signaling complex with the adapter SLP-76 via its SH2 domain following T cell activation (16). In view of the requirement of SLP-76 for Vav3 membrane translocation (Fig. 2D), we examined in more detail the association of Vav3 with SLP-76. Jurkat-TAg cells were transfected with Myc-tagged Vav1, Vav3, or the corresponding empty vector and stimulated with SEE-pulsed (or nonpulsed control) Raji B lymphoma cells. When IPs of the transfected Vav proteins were immunoblotted with an anti-phosphotyrosine antibody, we observed that both Vav3 and Vav1 were phosphorylated on tyrosine, and the phosphorylation level increased after stimulation with SEE-pulsed B cells (Fig. 5A). Similar results were obtained when Jurkat cells were stimulated with an anti-CD3 antibody (Fig. 5B). Next, we assessed the importance of Lck or ZAP-70 in mediating tyrosine phosphorylation of Vav3 and its association with SLP-76 by analyzing these events in Jurkat cells deficient in these PTKs. Following anti-CD3/CD28 stimulation, the associ-
ation of Vav3 with SLP-76 was dramatically reduced in Lck-deficient JCam1.6 cells and abrogated in ZAP-70-deficient P116 cells by comparison with JE6.1 cells, and this was also reflected by undetectable tyrosine-phosphorylated SLP-76 (Fig. 5C). The absence of LAT in JCam2.5 cells also resulted in the failure of SLP-76 to inducibly associate with Vav3, although basal, stimulation-independent association could still be detected (Fig. 5D). Analysis of Vav3 expression in the same IPs confirmed that the alteration in Vav3/SLP-76 association was not due to differences in the amount of Vav3 immunoprecipitated in each cell line (Fig. 5, C and D). Last, we transfected Jurkat-TAg cells with Myc-tagged Vav3 and/or FLAG-tagged SLP-76 and assessed the activation of a cotransfected NFAT-luciferase reporter gene. Vav3 and SLP-76 cooperated to increase the TCR-induced NFAT activation to a higher level than each protein alone (Fig. 5E). These results indicate that Vav3 can physically and functionally interact with SLP-76 following TCR stimulation.

The Vav3 SH2 Domain Is Required for Vav3 Membrane/IS Localization and Function—

FIG. 3. Tyr173 phosphorylation (P-Tyrosine) and its role in Vav3 membrane and IS translocation and NFAT activation. A, JE6.1, JCam1.6, or P116 cells were stimulated or not with anti-CD3/CD28 for 1 min, and IPs of endogenous Vav3 were analyzed by immunoblotting with the indicated antibodies (top three panels). Aliquots of cell lysates were also analyzed for expression of Lck or ZAP-70 by immunoblotting (two bottom panels). B, a similar analysis was conducted on JE6.1, J14, or JCam2.5 cells. Aliquots of cell lysates were analyzed for SLP-76 or LAT expression. C, Jurkat-TAg cells transfected with wild-type Vav3 or Y173F (10 μg each) were left unstimulated (0) or stimulated for 1 min with an anti-CD3 mAb. Subcellular fractions were prepared as in Fig. 1C, and expression of Myc-tagged Vav proteins was detected by immunoblot with an anti-c-Myc mAb. D, Jurkat-TAg cells transfected with either empty vector or Myc-tagged wild-type or Y173F-mutated Vav3. Twenty-four hours later, the transfected cells were stimulated with SEE-pulsed Raji cells and analyzed as in Fig. 1F. DIC, differential interference contrast. E, quantitative analysis of the results shown in D. The localization of Vav3 in the IS was analyzed in ~100 T-APC conjugates/group. Vav3 recruitment to the IS was scored and classified in three categories: partial recruitment (gray bars), complete recruitment (black bars), and no recruitment (white bars). F, Jurkat-TAg cells were cotransfected with Myc-tagged wild-type or Y173F Vav3 (10 μg each) plus NFAT (10 μg) and β-galactosidase (5 μg) reporter genes. Cells were either left unstimulated (white bars) or stimulated (black bars) for the final 6 h of a 24-h culture with an anti-CD3 mAb (5 μg/ml). Luciferase activity normalized to β-galactosidase activity was determined in duplicate samples. Expression of the transfected proteins was detected by immunoblotting with an anti-c-Myc mAb (inset).
with SLP-76 is mediated by their SH2 domains (14–16). In order to define more precisely the importance of the interaction between Vav3 and SLP-76 for the proper localization and function of Vav3, we generated two Vav3 mutants: an SH2 domain point mutation, which replaced arginine 697 for leucine (R697L), resulting in inactivation of the SH2 domain, and a construct lacking the adapter SH2-SH2-SH3 domains of Vav3 (1-PH) (Fig. 6A). We determined the interaction of these Vav3 mutants with SLP-76 by analyzing anti-c-Myc IPs from unstimulated or anti-CD33-stimulated Jurkat-TAg cells transfected with the relevant Vav3 expression vectors. As shown in Fig. 6B, both Vav3 mutants, R697L and 1-PH, did not interact with SLP-76 after anti-CD3 stimulation, in contrast to wild-type Vav3. Both Vav3-R697L and 1-PH showed a markedly reduced TCR-induced tyrosine phosphorylation, as compared with wild-type Vav3. Furthermore, anti-CD3 stimulation failed to induce detectable phosphorylation of Tyr173, and overall tyrosine phosphorylation of the mutated Vav3 proteins was also greatly reduced (R697L) or ablated (1-PH) (Fig. 6B, two top panels). Using subcellular fractionation of the transfected cells, we found that, unlike wild-type Vav3, the R697L and 1-PH mutants also failed to undergo membrane translocation in response to anti-CD3 stimulation (Fig. 6C). Moreover, The R697L and 1-PH mutants of Vav3 were incapable of activating an NFAT reporter gene in anti-CD3-stimulated cells (Fig. 6D).

We also determined whether these Vav3 mutants can be recruited to the IS by transfecting Jurkat-TAg cells with wild-type or mutated Vav3 and using confocal microscopy to analyze the localization of Myc-tagged Vav3 vis-à-vis the IS following stimulation with SEE-pulsed Raji cells. Fig. 7A shows that whereas stimulation induced a recruitment of wild-type Vav3 to the IS, which was manifested by tight clustering of the protein at the contact area between Jurkat and Raji cells, the two Vav3 mutants (R697L and 1-PH) showed a diffuse localization in Jurkat T cells and were not as tightly recruited to the IS. This result is underscored by quantitative analysis of fluorescence intensity, expressed as relative fluorescence units, along an imaginary line crossing the Jurkat-Raji cell conjugate through the contact zone. Thus, the green (Alexa-488) fluorescence curve for wild-type Vav3 displayed a sharp fluorescence peak at the contact area with the CMTMR (red)-stained Raji B cell (red curve) and disappeared at the rear of the T cell opposite the contact area (Fig. 7B, left). However, the distribution of the green fluorescence for both Vav3 mutants was diffused all along the T cell axis, and no clear focusing was observed at the T cell-B cell contact area (Fig. 7B, middle and right). Quantitative analysis of the green fluorescence in ~100 conjugates from each group also revealed that ~80% of wild-type Vav3 was recruited to the IS, whereas only ~10 and ~2% of Vav-R697L or Vav-1-PH, respectively, were fully recruited at the same site (Fig. 7C).

**DISCUSSION**

Formation of the IS and translocation to the plasma membrane of several signaling proteins known to play a pivotal role in T cell activation represent important aspects of T cell activation. The Vav family plays a critical role in T cell development and activation as highlighted by the analysis of different combinations of Vav-deficient mice. Although the role of Vav1 in T cells has been extensively studied in the past decade, the role of Vav3, more recently identified, is less clear. In this study, we investigated the mechanism of recruitment of Vav3 from the cytoplasm to the plasma membrane and the IS upon T cell activation. We found that Vav3 membrane recruitment is transient and depends on anti-CD3 stimulation but is not enhanced by costimulation with CD28. Moreover, PMA stimulation, known to act by mimicry of the physiological second messenger, diacylglycerol, did not enrich Vav3 in the membrane fraction (Fig. 1) despite the fact that Vav3 contains a cysteine-rich domain similar to the phorbol ester-binding C1 domain found in members of the PKC family. This dependence of Vav3 membrane translocation on TCR-CD3 signals indicates that the mechanism of Vav3 membrane translocation might be different from the one described for PKCθ (38), which is enhanced by CD28 cosimulation. Importantly, Vav3 membrane translocation also occurred in anti-CD3-stimulated primary mouse T cells (Fig. 1E), attesting to the biological relevance of this event. We also observed that, like Vav1, Vav3 was localized to the IS in SEE-stimulated T cells. However, although some studies reported that Vav1 can be recruited to the membrane upon T cell activation (43, 44), we could not detect any stimulus-induced enrichment of Vav1 in the plasma membrane in either Jurkat T cells or primary T cells, which can probably be explained by the use of different subcellular fractionation techniques.

The stimulus-induced membrane translocation of Vav3 did not occur in Jurkat T cells deficient in either the PTKs Lck and ZAP-70 or the adapters SLP-76 and LAT (Fig. 2). Similarly, the tyrosine phosphorylation of Vav3, including on Tyr173, was also impaired in the absence of Lck, ZAP-70, or LAT (Fig. 3, A and B), suggesting that the mechanism, which regulates Vav3 tyrosine phosphorylation, is similar to the one described for Vav1 (13, 45). Nevertheless, mutation of Tyr173 to phenylalanine did not inhibit the TCR-induced membrane and IS recruitment of Vav3 (Fig. 3C), indicating that localization of Vav3 in the membrane and, more specifically, in the IS, is largely inde-
dependent of its tyrosine phosphorylation, at least on Tyr173. This conclusion is supported by the additional findings that phosphorylation of Vav3 on Tyr173 was observed both in the cytoplasm and in the membrane fractions (data not shown) and that TCR-induced Vav3 membrane recruitment, but not its tyrosine phosphorylation, was abolished in the absence of SLP-76 (Fig. 2D). Again, those observations underscore the fact that Vav3 membrane translocation and IS localization occur without the need to phosphorylate Tyr173. Further experiments will be required to determine whether other tyrosine residues may be implicated. Although it is not required for Vav3 localization, the Tyr173 might play a negative regulatory role in Vav3 function, as described for Tyr174 in Vav1 (42), since a Y173F-Vav3 mutant activated NFAT more efficiently than wild-type Vav3 (Fig. 3). Interestingly, we could not observe a significant increase of the basal activation of NFAT in the presence of Vav3 as has been reported for Vav1. This difference could be attributed to the fact that Vav1 displays a considerably higher degree of basal tyrosine phosphorylation and membrane localization than Vav3, and, in contrast to Vav1, TCR

![Figure 5](https://example.com/figure5.png)

**Fig. 5. Physical and functional interaction of Vav3 with SLP-76.** A. Jurkat-TAg cells were transfected with Myc-tagged Vav1 or Vav3 or with the corresponding empty vector. After 24 h, the cells were stimulated with SEE-pulsed Raji B cells as in Fig. 3D. Transfected Vav proteins were immunoprecipitated using an anti-Myc mAb, and tyrosine phosphorylation (P-Tyrosine) of Vav proteins or expression of associated proteins was analyzed by immunoblotting with the indicated antibodies. Actin expression in cell lysates is shown as a control for sample loading (lower panel). B, a similar analysis was conducted on Jurkat-TAg stimulated for 1 min (or not) with an anti-CD3 mAb. C and D, JE6.1, JCam1.6, Pl16, J.14, and JCam2.5 cells were stimulated with anti-CD3/CD28 antibodies for 1 min. Vav3 IPs were separated by SDS-PAGE, and tyrosine phosphorylation of Vav proteins or expression of associated proteins was analyzed by immunoblotting with the indicated antibodies. E, Jurkat-TAg cells were transfected with Myc-tagged Vav3 (3 μg) and/or FLAG-tagged SLP-76 (10 μg) together with NFAT and β-galactosidase reporter plasmid. Stimulation and determination of luciferase activity were performed as in Fig. 3F. Expression of the transfected proteins was detected by immunoblotting with anti-Myc (for Vav) or anti-FLAG (for SLP-76) mAbs (inset).
cross-linking substantially increases the membrane localization of Vav3 (Fig. 1, C–E). Recent studies using overexpression (30) or shRNA techniques (16) reported that Vav1, but not Vav3, plays a critical role in NFAT activation. The reason for this discrepancy might be explained in part by the fact that we used a human Vav3, in contrast to a murine Vav3 used previously (30). Moreover, we showed that residual NFAT activation was present in Vav1-deficient T cells upon TCR stimulation (Fig. 4), which suggested that Vav3 could have a compensatory role in NFAT activation in Vav1-deficient cells. Consistent with this idea, both Vav3 mutants (R697L and 1-PH) failed to undergo substantial tyrosine phosphorylation, including on the residue Tyr173.

Whereas we clearly showed that binding to SLP-76 is required for Vav3 membrane localization, accumulating evidence indicates that the PH domain of Vav3 does not play a major role in its membrane and IS translocation. Indeed, pretreatment with the phosphoinositide 3-kinase inhibitors LY294002 or wortmannin had no effect on TCR-induced membrane recruitment of Vav3 (data not shown), and the 1-PH mutant of Vav3, which contains the PH domain, did not translocate to the membrane. We also considered the possibility that Grb2, which is known to associate via its SH3 domain with a proline-rich

FIG. 6. SH2-dependent Vav3 association with SLP-76 is required for its membrane translocation and NFAT activation. A, schematic representation of Vav3 mutants with an SH2-inactivating point mutation (R697L) or deletion of the SH3-SH2-SH3 domains (1-PH). Calponin homology (CH), acidic domain (AD), Dbl homology (DH), pleckstrin homology (PH), cysteine-rich domain (CRD), and Src homology (SH) are shown. B, Jurkat-TAg cells were transfected with the indicated Myc-tagged Vav3 plasmids (10 μg each). Cells were stimulated as in Fig. 4B. Myc-tagged proteins were immunoprecipitated and analyzed by immunoblotting with the indicated antibodies (top four panels). An aliquot of the cell lysate was analyzed by immunoblotting for SLP-76 expression (bottom panel). C, subcellular fractions were prepared from Jurkat-TAg cells transfected for 24 h with the indicated Vav3 plasmids. Stimulation and expression of Vav3 in different fractions were determined as in Fig. 2. D, Jurkat-TAg cells were cotransfected with the indicated Vav3 plasmids plus NFAT-luciferase and β-galactosidase reporter genes. Stimulation and determination of normalized luciferase activity were performed as in Fig. 4E. Expression of Vav3 proteins was analyzed by immunoblotting with an anti-Myc mAb (inset). P-Tyrosine, phosphotyrosine.
motif in the N-terminal SH3 of Vav1 (17), recruits Vav3 to the membrane. However, although this hypothesis remains to be tested, it is interesting to note that whereas all Vav proteins display a substantial homology, the proline-rich motif of Vav3 does not seem to fit a consensus SH3-binding site for Grb2 (30), making it unlikely that Grb2 could directly recruit Vav3 to the membrane/IS in a SLP-76-independent manner.

In summary, our study describes for the first time the recruitment of Vav3 to the membrane and the IS, as well as its role in NFAT activation upon T cell activation. We show that TCR-induced Vav3/SLP-76 association is crucial for these processes, whereas phosphorylation of Vav3 on Tyr173 regulates its transcriptional activity but not its membrane or IS localization. In addition, we clearly demonstrate that suppression of Vav3 expression by a Vav3-specific shRNA strongly decreases the residual TCR/CD28-induced NFAT activation in Vav1-deficient T cells. Considering the expression of Vav3 in various hematopoietic cells, our study addresses an interesting mechanism that regulates Vav3 localization and function, which may be extended to signaling by other immunoreceptors.

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REFERENCES

1. Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L. (1999) Science 285, 221–227
2. Glimcher, L. H., and Singh, H. (1999) Cell 96, 13–23
3. Lin, J., and Weiss, A. (2001) J. Cell Sci. 114, 243–244
4. Altman, A., and Deckert, M. (1999) Adv. Immunol. 72, 1–101
5. Bustelo, X. R. (2001) Oncogene 20, 6372–6381
6. Tybulewicz, V. L., Ardouin, L., Prisco, A., and Reynolds, L. F. (2003) Immunol. Rev. 192, 42–52
7. Katzav, S., Martin-Zanca, D., and Barbacid, M. (1989) EMBO J. 8, 2283–2290
8. Schubel, K. E., Bustelo, X. R., Nielsen, D. A., Song, H. J., Barbacid, M., Goldman, D., and Lee, I. J. (1996) Oncogene 13, 363–371
9. Movilla, N., and Bustelo, X. R. (1999) Mol. Cell. Biol. 19, 7870–7885

**Figure 7.** Vav3/SLP-76 association is required for Vav3 recruitment to the IS. A, Jurkat-TAg cells transfected with the indicated Myc-tagged Vav3 plasmids (or empty vector) were stimulated for 10 min with CMTMR-labeled SEE-pulsed (or control) Raji B cells, and Vav3 localization was analyzed as in Fig. 3D. B, analysis of green (Vav3) versus red (CMTMR, Raji B cell) fluorescence intensity (in relative units) as a function of the distance (pixel number) along a line (indicated by the white arrows in A) dissecting a T-APC conjugate through the IS. This curves are representative of 20 conjugates analyzed in each group. C, quantitative analysis of Vav3 localization in the IS was performed as in Fig. 3E. DIC, differential interference contrast.
