Vav1 Acidic Region Tyrosine 174 Is Required for the Formation of T Cell Receptor-induced Microclusters and Is Essential in T Cell Development and Activation

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Vav proteins are multidomain signaling molecules critical for mediating signals downstream of several surface receptors, including the antigen receptors of T and B lymphocytes. The catalytic guanine nucleotide exchange factor (GEF) activity of the Vav Dbl homology (DH) domain is thought to be controlled by an intramolecular autoinhibitory mechanism involving an N-terminal extension and phosphorylation of tyrosine residues in the acidic region (AC). Here, we report that the sequences surrounding the Vav1 AC: Tyr142, Tyr160, and Tyr174 are evolutionarily conserved, conform to consensus SH2 domain binding motifs, and bind several proteins implicated in TCR signaling, including Lck, PI3K p85α, and PLCγ1, through direct interactions with their SH2 domains. In addition, the AC tyrosines regulate tyrosine phosphorylation of Vav1. We also show that Tyr174 is required for the maintenance of TCR-signaling microclusters and for normal T cell development and activation. In this regard, our data demonstrate that while Vav1 Tyr174 is essential for maintaining the inhibitory constraint of the DH domain in both developing and mature T cells, constitutively activated Vav GEF disrupts TCR-signaling microclusters and leads to defective T cell development and proliferation.

The cells of the αβ T cell lineage progress through a developmental program that links ordered V(D)J gene rearrangement and antigen receptor protein expression to further developmental progression in which CD4+8− cells give rise to CD4+8− cells that are precursors to CD4+8− or CD4+8+ single positive thymocytes (reviewed in Ref. 1). In developing and mature T cells, the T cell receptor (TCR)2 activates Src family kinases that phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3/TCRζ proteins, providing docking sites for Syk/ZAP-70 family protein-tyrosine kinases. Subsequently, the recruitment of the adaptors LAT, GADS, and SLP-76, and enzymes such as Tec family kinases, phosphoinositols, and Akt, leads to generation of the secondary signaling intermediates, 1,4,5-inositol trisphosphate (IP3) and diacylglycerol (DAG), and activation of intracellular Ca2+, Rho GT Pases, and mitogen-activated protein kinases (MAPK) (reviewed in Ref. 2). Together, these events promote the transcription of genes involved in T cell proliferation, differentiation, and cytoskeletal reorganization. Live cell imaging studies using T cells stimulated on peptide:MHC-containing planar bilayers or on anti-CD3 antibodies immobilized on a planar surface revealed formation of microclusters of signaling proteins, including the TCR, ZAP-70, LAT, and SLP-76, within seconds of contact (3, 4), and recent reports suggested that TCR signal transduction may be initiated and sustained within these dynamically regulated microclusters (5, 6).

The Vav family of Rho GEFs consists of three members: Vav1, Vav2, and Vav3, which are expressed in both T and B lymphocytes. Vav proteins contain multiple domains, including a calponin homology (CH) domain, an acidic region (AC), a catalytic Dbl homology domain (DH), a pleckstrin homology domain (PH), a cysteine-rich region (CR), and a Src homology 2 (SH2) domain flanked by two SH3 domains. The importance of Vav1 in lymphocytes was first demonstrated in mice lacking Vav1 (Vav1−/−), which show activation defects in T and B lymphocytes (7–10), γδ T cells (11), and NK cells (12–16). However, Vav1−/− lymphocytes retain significant functional ability, 2

The abbreviations used are: TCR, T cell receptor; ITAM, immunoreceptor tyrosine-based activation motif; AC, acidic region; CH, calponin homology; DH, Dbl homology; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; PH, pleckstrin homology; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; SH2, Src homology 2; SH3, Src homology 3; TIRFM, total internal reflection fluorescence microscopy; GST, glutathione S-transferase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; IL, interleukin; WT, wild type.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

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presumably because of redundancy between Vav family members. Indeed, mice with combined Vav deficiencies show more severe lymphocyte defects (17–19), whereas mice lacking all three Vav proteins (VavNK1) lack functional T and B lymphocytes (17).

Control of Vav GEF activity is complex and involves multiple modes of regulation (reviewed in Ref. 20). Structural studies suggested that autoinhibitory intramolecular interactions between the CH domain and the CR region and the AC and the DH domain are responsible for maintaining the Vav DH domain in an inactive state (21, 22). One mechanism for relieving such an autoinhibitory constraint is via phosphorylation of three conserved AC tyrosines: Tyr142, Tyr160, and Tyr174, which results in unraveling of the autoinhibitory N-terminal extension and its release from the DH domain (21, 23). Given that tyrosine phosphorylation distinguishes Vav from the plethora of other Rho GEFs (24, 25), it is possible that Vav has evolved a complex mechanism for regulation of GEF activity in which tyrosine phosphorylation represents a specific adaptation of Vav proteins to function downstream of ITAM-containing antigen receptors in lymphocytes. It has also been suggested that binding of the Vav PH domain to phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3) binding to the PH domain of Vav may lead to activation of DH domain activity, whereas phosphatidylinositol 4,5-bisphosphate (PIP2) binding has been suggested to negatively regulate DH domain activity (26). A thermodynamic model has been proposed in which PIP3 binding to the PH domain may relax interactions between the Vav PH domain and the autoinhibited DH domain, thereby allowing access of protein-tyrosine kinases to the AC tyrosines, with the resulting phosphorylation of AC tyrosines and activation of GEF activity (21). In this regard, GEF activity is diminished in a mutant of Vav1 in which the PH domain is rendered incapable of interacting with phosphatidylinositol 4,5-bisphosphate (27). In addition, binding of proteins to the N-terminal CH domain of Vav may contribute to activation of the DH domain. For example, interaction of the adaptor protein APS with the CH domain of Vav3 has been implicated in activation of Vav3 (28).

In this report, we show that the tyrosine residues in the Vav1 AC are evolutionarily conserved and conform to consensus SH2 domain binding motifs for several proteins implicated in TCR signaling (including Lck, PI3K p85α, and PLCγ1). Indeed, Tyr142, Tyr160, and Tyr174 can bind these proteins through direct interactions with their SH2 domains. Moreover, the AC tyrosines are essential for total tyrosine phosphorylation of Vav1. Our data also indicate that Tyr174 is critical for TCR-induced Vav1 microcluster stability and for normal T cell development and activation, because the Y174F mutation disrupts TCR signaling.

**EXPERIMENTAL PROCEDURES**

**Generation of Reconstituted J.Vav Cell Lines, Stimulation, and Immunoblotting**—The Vav1-deficient J.Vav cell line was previously described (29). To generate J.Vav cell lines expressing Vav1WT, Vav1 AC Tyr→Phe mutants, and Vav1Y174F/GEF−, GFP-tagged Vav1 expression constructs were transduced into J.Vav cells via “spinfection” with retroviral particles at room temperature, 2000 rpm for 90 min. GFP+ cells were fluorescent-activated cell-sorted and subcloned. Vav1-GFP constructs were generated by ligation of an Xbal-BamHI Vav1-GFP cDNA fragment into IRES-GFP-RV digested with Xhol-BamHI replacing IRES-GFP. Mutagenesis was performed by PCR (Stratagene, La Jolla, CA) and confirmed by sequencing. Cells were stimulated with anti-CD3ε (clone HIT3a; 1 μg/ml, BD Biosciences, San Diego, CA) and anti-IgG2a cross-linker (1 μg/ml, Southern Biotechnology Assoc., Birmingham, AL), and lysed in radioimmune precipitation assay buffer supplemented with a protease inhibitor mixture (Boehringer, Ridgefield, CT), 10 mM NaF, and 1 mM Na3VO4. Western blotting was performed following standard procedures. Primary antibodies were developed with horseradish peroxidase-conjugated secondary antibodies (anti-mouse, Zymed Laboratories Inc., San Francisco, CA; anti-rabbit, Amersham Biosciences, Piscataway, NJ). Immune complexes were detected by enhanced chemiluminescence (Amersham Biosciences).

**Peptide Immunoprecipitation Assays**—For GST fusion protein immunoprecipitations, peptide-bead complexes were generated by mixing 30 nmol of biotinylated peptide (QCB, Hopkinton, MA) and 20– μl SA-conjugated beads (Sigma) in 500 μl of phosphate-buffered saline at 4 °C for 1 h. Complexes were washed four times by adding 500 μl of phosphate-buffered saline followed by centrifugation at 4 °C and 13,000 rpm for 1 min. To peptide-bead complex, 500 μl of Triton X-100 lysis buffer (% Triton X-100, 0.15 μM NaCl, 25 mM HEPES pH 7.5) supplemented with a protease inhibitor mixture (Boehringer), 10 mM NaF, and 1 mM Na3VO4, and 1 μg of GST fusion proteins were added followed by rotation at 4 °C for 1 h. Immunoprecipitates were washed four times with cold Triton X-100 lysis buffer, resuspended in SDS sample buffer, and analyzed by Western blotting with anti-GST antibodies (Upstate Biotechnology, Lake Placid, NY) following standard procedures.

For immunoprecipitation from cell lysates, 50 × 10⁶ Jurkat T cells were lysed in Triton X-100 lysis buffer for 20 min on ice. Postnuclear lysates were obtained by centrifugation at 4 °C and 13,000 rpm for 10 min. Clarified lysates were precleared by adding 20 μl of SA-conjugated beads followed by rotation at 4 °C for 1 h. During preclearance, peptide-bead complexes were generated by mixing 30 nmol of biotinylated peptide and 200 μl of beads with 500 μl of PBS with rotation at 4 °C for 1 h. Complexes were washed as described above. Precleared lysates were centrifuged at 4 °C and 13,000 rpm for 1 min and incubated with washed peptide-bead complexes at 4 °C for 1–2 h. Immunoprecipitates were washed and Western blotting performed as above.

**TIRFM Imaging**—Imaging of dynamic Vav1-GFP microclusters assembly and movement was performed using TIRF microscopy as described in Ref. 30. 1 × 10⁶ cells were resuspended in non-fluorescent medium, dropped onto glass bottom dish coverslips (MatTek, Ashland, MA) coated overnight at 4 °C with 1 μg/ml anti-CD3ε (clone HIT3a; BD Biosciences) as previously described (31, 32). A beam from a solid state laser (488 nm, 20 milliwatt, SAPHIRE 488-20-OPS, Coherent) was introduced into an inverted microscope (IX-81, Olympus) for illumination. Images were captured using an EB-CCD camera (C-7190-23,
alignment shows that amino acid sequences surrounding AC (Tyr142, Tyr160, and Tyr174; numbering relative to the human sequence) are conserved within several mammalian and non-mammalian species, including frog (Xenopus laevis), zebrafish (Danio rerio), fruit fly (Drosophila melanogaster), and nematode (Caenorhabditis elegans) (Fig. 1A). Moreover, AC tyrosines are found within consensus binding motifs for the SH2 domains of several proteins implicated in TCR signaling, including the Src family kinase Lck (YXXΦ), the p85α subunit of PI3K (YXXM/Φ), and PLCγ1 (YXXΦ) (X is any residue, Φ is Val, Ile, or Leu) (Fig. 1A and Refs. 33–35). These data suggest that the AC tyrosines may be involved in Vav interactions with these SH2 domain-containing proteins during TCR signaling.

**AC Tyrosines Are Critical for Inducible Tyrosine Phosphorylation of Vav1**—AC tyrosines have been implicated in regulation of Vav tyrosine phosphorylation and function in T lymphocytes (36–40), and as potential phosphorylation sites for Src and Syk family protein-tyrosine kinases (23, 26, 41). To determine whether the AC tyrosines can function in T cells as potential SH2 domain docking sites, we first tested whether these residues are phosphorylated during T cell activation using antibodies that specifically recognize phosphorylated-AC harboring tyrosine-to-phenylalanine (Tyr → Phe) substitutions of the AC tyrosines, we found that consistent with previously published studies (27, 36) Vav1 AC tyrosines were rapidly phosphorylated during T cell activation (Fig. 1B and supplemental Fig. S1A). To determine if the AC tyrosines are required for inducible tyrosine phosphorylation of Vav1 in activated T cells, J.Vav cell lines reconstituted with either Vav1WT or AC tyrosine-mutated Vav1 were stimulated with orthovanadate, and tyrosine phosphorylation of immunoprecipitated Vav1 was examined by immunoblotting with total anti-phosphotyrosine antibodies (Fig. 1C). These experiments reveal that while mutation of any single AC tyrosine has no discernible effect on Vav1 phosphorylation, mutation of Tyr174 in combination with Tyr142 and/or Tyr160, results in severely diminished phosphorylation of Vav1 (Fig. 1C). Strikingly, loss of all three AC tyrosines (Vav1Y3F) leads to a virtually complete disruption of Vav1 total tyrosine phosphorylation (Fig. 1C), even though in addition to the AC tyrosines, there are six tyrosine residues outside of the AC (Tyr267, Tyr482, Tyr603, Tyr604, Tyr826, Tyr841) that conform to consensus tyrosine phosphorylation motifs (35). Importantly, we observed similar results when cells were activated by TCR cross-linking with anti-CD3 antibodies (supplemental Fig. S1B), indicating that these results are not an artifact of orthovanadate treatment. Diminished Vav tyrosine phosphorylation is not caused by altered levels of Vav protein expression because the reconstituted J.Vav cell lines express recombinant Vav1-GFP at a level similar to endogenous Vav1 (Fig. 1 and data not shown). Together, these data suggest that the AC tyrosines are critical for TCR-induced phosphorylation of Vav1, suggesting that the AC tyrosines may function as docking sites and/or facilitate access for kinases such as Lck to phosphorylate additional Vav tyrosines. In this context, tyrosine residues outside of the AC may not be accessible to protein-
tyrosine kinases as they may not be surface-exposed, or they may be masked by intramolecular interactions. Thus, the AC tyrosines appear to control Vav1 tyrosine phosphorylation and may be required for protein-tyrosine kinase recruitment.

**Vav1 AC Tyrosines Bind SH2 Domains of TCR-signaling Proteins**—To examine AC tyrosine interactions with the SH2 domains of Lck, PI3K p85α, and PLCγ1, we first tested binding of biotinylated peptides derived from the AC of Vav1 containing either phosphorylated or unphosphorylated Tyr142, Tyr160, or Tyr174 (supplemental Table S1) to purified GST-SH2 domain(s) of Lck, PI3K p85α, PLCγ1, or Grb2. As a positive control, we used the previously characterized binding of CD22- or BTLA-derived peptides to the SH2 domain of Grb2 (supplemental Fig. S2A and Refs. 42 and 43). These experiments show that, as expected based on the predicted sequence motif binding (Fig. 1A), each of the phosphorylated but not unphosphorylated AC tyrosines can bind to the SH2 domain of Lck, the C-terminal SH2 domain of PLCγ1, or to either of the two SH2 domains of PI3K p85α (Fig. 2A). In contrast, the N-terminal SH2 domain of PLCγ1 did not associate with any of the AC tyrosines nor did the SH2 domain of Grb2 (Fig. 2A), consistent with the preference of the Grb2 SH2 domain for the YXXNM motif (Fig. 1 and Ref. 44). Taken together, these data indicate that phosphorylated Vav1 AC tyrosines can directly bind SH2 domains of several proteins implicated in TCR signaling, consistent with a recent report showing association of the Lck SH2 domain with AC tyrosines (23).

To determine whether the AC tyrosines could interact with SH2 domain-containing proteins in their native conformation, we performed immunoprecipitation experiments using the biotinylated AC tyrosine-containing phosphopeptides and cytoplasmic extracts generated from Jurkat cells. In these experiments, we used the previously characterized interaction between Grb2 and CD22 Tyr828 as a positive control (supplemental Fig. S2B and Ref. 42). These experiments confirmed binding of phosphorylated Tyr142, Tyr160, and Tyr174 AC tyrosine-containing peptides to native Lck protein (Fig. 2B). Moreover, we find that PI3K p85α and PLCγ1 bind specifically to phosphorylated but not unphosphorylated AC tyrosines (Fig. 2B). As expected, Grb2 fails to bind to any of the Vav1 AC tyrosines in these assays (Fig. 2B). Thus, while associations of Vav with Lck, PI3K p85α, and PLCγ1 have been previously shown by co-immunoprecipitation experiments (45, 46), these data indicate the direct binding of Vav with SH2 domain-containing proteins via phosphorylated AC tyrosines.

**Vav1 Tyrosine 174 Controls Dynamic Redistribution of Vav1 upon TCR Activation**—Recent imaging studies examining activation of T cells on stimulatory planar surfaces have identified microclusters of signaling proteins at the sites of TCR contacts...
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Tyr\textsuperscript{174} 4, indicating a specific requirement for Tyr\textsuperscript{174} for normal formation of Vav1 microclusters.

Vav1 Tyrosine 174 Is Essential for T Cell Development and TCR Function—Having established that Tyr\textsuperscript{174} is required for formation of TCR-induced Vav1 microclusters (Fig. 3), we decided to examine if this residue is required for T cell development and primary T cell proliferation. To this end, we generated T cells that express Vav1\textsuperscript{Y174F} in the absence of any other Vav proteins to avoid the complicating issue of compensatory effects of endogenous Vav proteins. We developed a Vav\textsuperscript{NULL}—hematopoietic stem cell complementation approach in which we expressed either Vav1\textsuperscript{WT} or Vav1 harboring the Y174F mutation (Vav1\textsuperscript{Y174F}) and found that Vav1\textsuperscript{WT} expression rescues Vav\textsuperscript{NULL} T cell development, and, as expected, a majority of thymocytes and peripheral T lymphocytes are GFP\textsuperscript{+} (Fig. 4A and data not shown). In contrast, thymocytes expressing Vav1\textsuperscript{Y174F} show significantly diminished total thymic cellularity (10-fold) and drastically decreased populations of DP cells (Fig. 4A). To determine if Tyr\textsuperscript{174} is required in TCR-induced responses of peripheral T cells, we examined proliferation of such Vav1\textsuperscript{Y174F} T cells and found that in contrast to wild-type T cells or Vav\textsuperscript{NULL} T cells reconstituted with Vav1\textsuperscript{WT}, these cells fail to proliferate in response to anti-CD3 stimulation (Fig. 4B). Importantly, Vav1\textsuperscript{Y174F}(-GFP) protein is expressed at essentially the same level as endogenous Vav1 in unmanipulated normal T cells (Fig. 4C), indicating that these developmental and functional defects are not caused by aberrant protein expression. Together, these results indicate that Tyr\textsuperscript{174} is essential for T cell development and for TCR-induced proliferative responses in primary T cells. Of note, our analyses with Vav1\textsuperscript{Y3F} mutant were inconclusive because of low expression levels of the protein in this system.

Vav1 Tyrosine 174 Controls Vav GEF Activity—Because Tyr\textsuperscript{174} is phosphorylated (Fig. 1) and can function as a docking site for signaling proteins (Fig. 2), loss of this residue could disrupt the ability of Vav1 to bind to TCR-signaling proteins, resulting in unstable microclusters and deregulated TCR signaling. Alternatively, defects observed in Vav1\textsuperscript{Y174F}-expressing T cells may be caused by deregulated activation of Vav1 DH domain GEF activity because Tyr\textsuperscript{174} has been implicated in inhibitory constraint of GEF activity (36–38). To test this possibility, we performed experiments to directly assay Rac1 activation in primary Vav1\textsuperscript{Y174F} T cells in the presence or absence of anti-CD3 stimulation. Indeed, we found that expression of Vav1\textsuperscript{Y174F} led to constitutive Rac1 activation, which is readily detectable in non-stimulated Vav1\textsuperscript{Y174F} T cells, in contrast to Vav1\textsuperscript{WT} T cells (Fig. 4D). These data suggest that the effects of the Y174F mutation may be caused by constitutive activation of Vav GEF activity. Therefore, we examined Vav1 microcluster
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Together, these data show the requirement for Tyr<sup>174</sup> in the restraint of Vav<sup>1</sup> DH domain activity (21, 23), suggesting a mechanistic basis for Tyr<sup>174</sup> function in TCR signaling.

DISCUSSION

Vav1 was among the first phosphotyrosine proteins identified in signaling pathway(s) downstream of the TCR (24, 25). In fact, all three Vav proteins can be inducibly tyrosine-phosphorylated and are recruited to the TCR following antigen receptor cross-linking (17). Although tyrosine phosphorylation distinguishes Vav family proteins from the plethora of other DH domain-containing Rho GEFs, the exact mechanism of Vav regulation by tyrosine phosphorylation is not known. It was reported that AC Tyr<sup>142</sup>, Tyr<sup>160</sup>, and Tyr<sup>174</sup> can bind to and become phosphorylated by, tyrosine kinases such as Lck and ZAP-70 (23, 27, 36, 41); however, it is not known whether the AC tyrosines (in particular Tyr<sup>174</sup>) are required for tyrosine phosphorylation of Vav as previously published reports reached differing conclusions (26, 36, 37). For example, transient transfection experiments showed that an N-terminally truncated Vav (lacking Tyr<sup>142</sup> and Tyr<sup>160</sup>) expressing the Y174F mutation could not be phosphorylated by Lck <i>in vitro</i>, suggesting that Tyr<sup>174</sup> is required for Vav phosphorylation (26), whereas other studies showed that Tyr<sup>174</sup> is not required for TCR-induced phosphorylation of Vav (36, 37, 40). In this report, using a system in which full-length Vav1 is stably expressed at physiological levels, we show that Tyr<sup>142</sup>, Tyr<sup>160</sup>, and Tyr<sup>174</sup> together are indeed critical for regulation of tyrosine phosphorylation of Vav1, because combined mutations of all three AC tyrosines led to a complete loss of phosphorylation.

Whereas Vav1 contains a total of 30 tyrosine residues, in addition to the AC tyrosines, six other tyrosines (Tyr<sup>267</sup>, Tyr<sup>482</sup>, Tyr<sup>603</sup>, Tyr<sup>604</sup>, Tyr<sup>826</sup>, Tyr<sup>841</sup>) are located within known consensus motifs for tyrosine phosphorylation (35). However, some of these residues may not be surface-exposed or may be involved in intramolecular interactions, thereby precluding formation using a Vav<sup>1 Y174F</sup> protein, which also harbors a GEF-inactivating mutation (Fig. 5, J.Vav<sup>1 Y174F/GEF−</sup> and Refs. 47 and 48). These experiments reveal that the introduction of the GEF-inactivating mutation completely alleviates the defects in microcluster formation of Vav<sup>1 Y174F</sup> (Fig. 5). Thus, similar to wild-type Vav1, Vav<sup>1 Y174F/GEF−</sup> microclusters form quickly and remain stable over time, in contrast to Vav<sup>1 Y174F</sup> (Fig. 5).

**FIGURE 3.** Tyrosine 174 is required for Vav1 microcluster maintenance. A, J.Vav<sup>1 WT</sup>, J.Vav<sup>1 Y142F</sup>, J.Vav<sup>1 Y160F</sup>, and J.Vav<sup>1 Y174F</sup> cells expressing GFP-tagged Vav proteins were plated on anti-CD3-coated coverslips. Images were obtained in real time using TIRFM (times, above images). Shown is representative of n > 10 cells. B, diagonal lines indicate sections of a representative J.Vav<sup>1 WT</sup> and J.Vav<sup>1 Y174F</sup> cells used for kymographic analysis. Fluorescence of individual Vav<sup>1 WT</sup> and Vav<sup>1 Y174F</sup> microclusters over time are presented as horizontal streaks in kymographs for sections shown. C, relative fluorescence intensity over time of individual Vav<sup>1 WT</sup> and Vav<sup>1 Y174F</sup> clusters. Shown are two representative of n = 5 clusters examined.
tyrosine phosphorylation. For example, an interaction between Tyr603 and proline 607 is important for the stability of the N-terminal SH3 domain of Vav1 (49, 50). Alternatively, phosphorylation of the acidic region tyrosines may allow Tyr142, Tyr160, and Tyr174 to become docking sites for kinases, such as Lck, which can then phosphorylate additional tyrosine residues in Vav (23). It is also possible that phosphorylation of Tyr142, Tyr160, and/or Tyr174 results in conformational changes of Vav1 that subsequently allow additional Vav1 tyrosine residues to become accessible to protein-tyrosine kinases; however this remains speculative at present.

Whereas tyrosine phosphorylation of the AC tyrosines has mainly been considered in the context of the regulation of the intrinsic GEF activity, studies in lymphoid and non-lymphoid cell lines suggested that phosphorylation of AC tyrosine residues may be required for regulation of Vav1-mediated biological activity independently of the intrinsic GEF activity (21, 23, 36–38). For example, whereas expression of Vav1Y174F leads to induction of antigen receptor-induced NFAT transcriptional activity, expression of a constitutively active N-terminally truncated Vav1 (lacking the CH domain) does not (36, 37). Thus, it is possible that the Vav CH domain binding to other proteins may enable Vav to mediate signals downstream of the TCR leading to NFAT activation independently of the intrinsic GEF activity.

Data presented in this report suggest that Tyr174 contributes to a function of Vav downstream of the TCR by keeping the catalytic activity of the DH domain in check, possibly through phosphorylation and/or association with SH2 domain-containing proteins. In addition to being required for inducible phosphorylation of Vav, we found that the AC tyrosines can function as docking sites for SH2 domain-containing proteins. In complex, DAP10-Grb2-Vav1 (16, 51), may conceivably require AC tyrosine interactions to engage the p85 subunit of PI3K; however, this remains to be experimentally tested. In this context, co-immunoprecipitation experiments in T cells indicate no absolute requirement for the AC tyrosines for binding to PI3K.
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p85α, Lck, or PLC-γ1 (data not shown), whereas previous studies indicated that Vav1 translocation to glycosphingolipid-enriched microdomains (GEM) is dependent upon the SH2 domain (52). Thus, it is possible that the AC tyrosines are necessary for formation of a stable TCR-signaling complex, but because the TCR signalosome is composed of numerous proteins that include several other linker proteins in addition to Vav1, any reduction in binding in the absence of Tyr$^{142}$, Tyr$^{160}$, and/or Tyr$^{174}$ may not be easily discernible in immunoprecipitation experiments.

Recent studies using live cell imaging of T cells activated on planar surfaces revealed the formation of dynamic microclusters of signaling proteins including the TCR, ZAP-70, LAT, SLP-76, Nck, and WASp, within seconds of contact (3–6,32). In this regard, it is thought that signal transduction may actually be initiated and sustained within such TCR-containing microclusters (5, 6). Data presented in this report show that while Vav1 forms TCR-induced microclusters similar to other TCR-signaling proteins, the Y174F mutation results in defects in microcluster formation. One possible reason for these results is that phosphorylated Tyr$^{174}$ is required for Vav1 microcluster maintenance by mediating association(s) of Vav with other SH2 domain-containing signaling molecules necessary for efficient TCR signaling. However, a second possibility, not mutually exclusive of the first, is that the mutation of Tyr$^{174}$ leads to deregulated GEF activity resulting in disrupted TCR signaling. Consistent with the latter scenario and with previously published reports (36–38), we found that the Y174F mutation leads to hyperactivation of Vav GEF activity and aberrant accumulation of activated Rac1. Surprisingly, these defects can be reversed by the introduction of a GEF-inactivating mutation, which completely alleviates TCR-induced microcluster instability in a Vav1$^{Y174F/GEF−}$ double mutant. Thus, while it still remains to be established if Vav GEF activity is required for its function during T cell development, our data indicate that Tyr$^{174}$ regulates Vav1 function by maintaining the inhibitory constraint of DH domain activity.

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