The N-terminal 20-Amino Acid Region of Guanine Nucleotide Exchange Factor Vav1 Plays a Distinguished Role in T Cell Receptor-mediated Calcium Signaling*

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**Significance:** Vav1 is indispensable for T cell calcium mobilization despite of its co-existing isoforms.

**Results:** The N-terminal 20-amino acid region of Vav1 was identified to be essential for calmodulin-binding and TCR-induced calcium signaling.

**Conclusion:** The interaction with calmodulin distinguishes Vav1 from other Vav family members in TCR-mediated calcium release.

**Significance:** This study highlights the irreplaceable role of Vav1 in T cell calcium signaling.

Vav1 is a guanine nucleotide exchange factor (GEF) specifically expressed in hematopoietic cells. It consists of multiple structural domains and plays important roles in T cell activation. The other highly conserved isoforms of Vav family, Vav2 and Vav3, are ubiquitously expressed in human tissues including lymphocytes. All three Vav proteins activate Rho family small GTPases, which are involved in a variety of biological processes during T cell activation. Intensive studies have demonstrated that Vav1 is indispensable for T cell receptor (TCR)-mediated signal transduction, whereas Vav2 and Vav3 function as GEFs that overlap with Vav1 on TCR-induced cytoskeleton reorganization. T cells lacking Vav1 exhibited severe defect in TCR-mediated calcium elevation, indicating that the co-existing Vav2 and Vav3 did not compensate Vav1 in calcium signaling. What is the functional particularity of Vav1 in lymphocytes? In this study, we identified the N-terminal 20 amino acids of Vav1 in the calponin homology (CH) domain to be essential for its interaction with calmodulin (CaM) that leads to TCR-induced calcium mobilization. Substitution of the 1–20 amino acids of Vav1 with those of Vav2 or Vav3 abolished the association with CaM, and the N-terminal mutations of Vav1 failed to potentiate normal TCR-induced calcium mobilization, that in turn, suspended nuclear factor of activated T cells (NFAT) activation and IL-2 production. This study highlights the importance of the N-terminal 20 aa of Vav1 for CaM binding, and provides new insights into the distinguished and irreplaceable role of Vav1 in T cell activation and signal transduction.

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2 The abbreviations used are: GEF, guanine nucleotide exchange factor; CaM, calmodulin; NFAT, nuclear factor of activated T cells; SH, Src homology; aa, amino acids; IL-2, interleukin-2; BiFC, bimolecular fluorescence complementation; TG, thapsigargin; CH, calponin homology; DH, Dbl homology.
domain was indispensable for Vav1-CaM interaction, and that the complex potentiated calcium release in a GEF-independent manner (11). However, the precise role of Vav1 that is different from other Vav proteins still remains to be investigated.

In this study, we identified the N-terminal 1–20 amino acids (aa) of Vav1 to be an essential region for its interaction with CaM and function in calcium flux upon TCR engagement. Mutations of Vav1 with a substitution of the 1–20 aa fragment by that of Vav2 or Vav3 failed in binding to CaM and potentiating TCR-induced calcium mobilization. These data highlight a key region within the CH domain of Vav1 that endows Vav1 with a distinguished function from the other coexisting Vav family members. This study provides new insights into the irreplaceable role of Vav1 in T cell activation and signal transduction.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies against Vav1 and CaM were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Vav2 antibody and anti-Vav3 antibody were described previously (5). The anti-α-tubulin antibody, CaM-agarose beads, and thapsigargin (TG) were purchased from Sigma. The anti-CD3 monoclonal antibody OKT3 and goat anti-mouse IgG were purchased from Biolegend (San Diego, CA). Fura-2-AM was purchased from Invitrogen.

Plasmid DNA Constructs—The plasmids encoding Vav1 and the CH-truncated mutation, Vav1ΔCH, were prepared in our laboratory as described (11). Other constructs for the expression of N-terminal-truncated mutants of Vav1 were derived from plasmid encoding wild type Vav1 by polymerase chain reactions (PCR) with the corresponding oligonucleotides, and the mutant fragments were subcloned into pcDNA4/HisMax.C vector at BamHI and XhoI restriction sites. The cDNA of N-terminal substituted Vav1 mutants were amplified by PCR and subcloned into pcDNA4/HisMax at BamHI and XhoI. FLAG-tagged Vav2 and Vav3 or their N-terminal-substituted mutants were constructed by subcloning the PCR-amplified cDNA into the pcMV-Tag2B vector at EcoRI and HindIII restriction sites. For the lentiviral transduction of Jurkat or J.Vav1 cells, the lentiviral-based particles were made with 293T cells. Basically, 293T cells were transfected with the coding DNA of Vav1 mutants constructed in pCDH-MCS1-EF1-Puro or shRNA in pLKO.1, respectively, together with 4 μg of pCMV-HSV-G, 4 μg of pMDL/g/pRRE, and 4 μg of pRSV-REV to produce pseudoviral particles. At 48 h post-transfection, the supernatants were collected and viruses were harvested by centrifugation at 20,000 × g for 1.5 h. The virus-containing pellets were resuspended in RPMI 1640 supplemented with 10% FBS and incubated with J.Vav1 cells at 37 °C for 24 h. To obtain cells stably expressing variant mutants or shRNA, the transduced cells were selected with 0.25 μg/ml of puromycin for 7 days.

Intracellular Calcium Measurement—Cells were loaded with 5 μM Fura-2 AM in RPMI 1640 medium for 30 min at 37 °C, then washed with 2 mM Ca2+ Ringer’s solution containing 145 mM NaCl, 1 mM MgCl2, 4.5 mM KCl, 10 mM glucose, 10 mM Heps, pH 7.4, and 2 mM CaCl2, and kept at room temperature for 20 min. Cells were subjected to spectrophotometer analyzes with excitation wavelengths of 340 and 380 nm, and an emission intensity at wavelength of 508 nm was simultaneously recorded by Synergy4 spectrometer (BioTek). For each measurement, the cells were pre-warmed for 5 min at 37 °C and a baseline was recorded before stimulation by OKT3 as described above. The intracellular free Ca2+ concentration was presented as the ratio of firefly luciferase activity to Renilla luciferase activity.

Lentivirus-based Transduction—For reconstitution of J.Vav1 cells with Vav1 and its variants, or knockdown of the Vav proteins in Jurkat cells, the lentiviral-based particles were made with 293T cells. Basically, 293T cells were transfected with the coding DNA of Vav1 mutants constructed in pCDH-MCS1-EF1-Puro or shRNA in pLKO.1, respectively, together with 4 μg of pCMV-HSV-G, 4 μg of pMDL/g/pRRE, and 4 μg of pRSV-REV to produce pseudoviral particles. At 48 h post-transfection, the supernatants were collected and viruses were harvested by centrifugation at 20,000 × g for 1.5 h. The virus-containing pellets were resuspended in RPMI 1640 supplemented with 10% FBS and incubated with J.Vav1 cells at 37 °C for 24 h. To obtain cells stably expressing variant mutants or shRNA, the transduced cells were selected with 0.25 μg/ml of puromycin for 7 days.

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Luciferase Reporter Assay—J.Vav1 T cells were transfected with reporter constructs, pNFluc and TK-Renilla as described (26, 27), together with plasmids encoding Vav1 or mutants, respectively. At 24 h post-transfection, cells were harvested and stimulated with or without 1 μg/ml of OKT3 for 6 h. Cell lysates were prepared and Luciferase activity was measured with Dual Luciferase assay kit (Promega, Madison, WI) by TD20/20 luminometer (Turner Designs Inc, Sunnyvale, CA) as instructed by the manufacturer. Normalized luciferase activity was presented as the ratio of firefly luciferase activity to Renilla luciferase activity.

CaM-Agarose Pulldown Assay—The cells were lysed in ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μg/ml of pepstatin, 1 μg/ml of leupeptin) for 30 min. After centrifugation at 12,000 × g for 10 min, the supernatants were collected and incubated with CaM-agarose or nonconjugated agarose as control, and agitated for 3 h at 4 °C. The bound fractions were eluted and resolved by Western blot analysis.

Bioluminescence Complementation (BiFC) Assay—The BiFC method was adopted to visualize protein interactions in live cells (28–30). CaM was fused to the carboxyl-terminal fragment (YC) of YFP. The wild type Vav1 or Vav1 mutations were fused to the N-terminal fragment (YN) of YFP. YC-CaM and the YN-Vav1 variants were co-transfected into individual
HeLa cells. At 24 h post-transfection, the transfected cells were incubated for another 4 h at room temperature for the maturation of the fluorophore, and then subjected to fluorescence microscopy and FACS analysis. The mean fluorescence intensity was determined by FACS analysis.

Measurement of IL-2 Production—Cells were cultured in 24-well tissue culture plates at 1 × 10^6 per ml and remained unstimulated or stimulated with anti-CD3 antibody for 24 h. The cultured media were then collected by centrifugation and the secreted IL-2 was determined by ELISA using human IL-2 ELISA kit (NeoBioscience, China). The data from three independent experiments were subject to statistical analysis.

RESULTS

TCR-mediated Calcium Mobilization Requires Vav1, but Not Vav2 or Vav3—There are three Vav family proteins, Vav1 is specific for hematopoietic cells, whereas Vav2 and Vav3 are also expressed. All three Vav proteins share high homology in structure and base functional redundancy as guanine nucleotide exchange factors (6, 7). However, it has been reported that T cells and NK cells lacking Vav1, even though Vav2 and Vav3 were present, exhibited severe defect in receptor-induced cell signaling, especially the calcium related events, such as the activation of NFAT (5, 6, 10). Thus the defective calcium profiles in T cells and NK cells lacking Vav1, even though Vav2 and Vav3 were present, exhibited severe defect in receptor-induced cell signaling, especially the calcium related events, such as the activation of NFAT (5, 6, 10). We therefore compared the amino acid sequences of CH domains as aligned in Fig. 2A. The CH domain similarity of Vav1 versus Vav2 is 81%, and Vav1 versus Vav3 is 88%. The secondary structure prediction was performed using PRALINE multiple sequence alignment software, and no difference between Vav1 and Vav2/3 was revealed. Concurrently, our work reported that Vav1 binds to CaM via the CH domain to potentiate calcium signaling upon TCR stimulation (11). We thus examined the interaction between individual Vav proteins and CaM by CaM-agarose pulldown assay. As shown in Fig. 2B, three Vav members were detected in Jurkat cell lysates (Fig. 2B, left lanes). Only Vav1 was immobilized by CaM-agarose, whereas Vav2 and Vav3 appeared negative in CaM binding (Fig. 2B, right lanes), regardless of the high similarities in their CH domain. These data suggest that the CH domain provides a unique structural basis of Vav1 to interact with CaM, and thereby renders Vav1 a distinguished role in TCR-activated calcium mobilization.

The N-terminal 20 Amino Acids of Vav1 Are Identified for CaM Binding—As the CH domain of Vav1 contains a stretch of 116 amino acids, we aim to identify the region responsible for its interaction with CaM. A series of Vav1 mutations at its CH domain were constructed, and the representative mutations by shRNAs (Fig. 1B, right lanes) in contrast to that in control cells (Fig. 1B, left lanes). The tubulin was monitored as loading controls. We also verified that the suppression effects of shRNAs on Vav proteins could be maintained for at least 20 days (data not shown). The above data demonstrate an irreplaceable role of Vav1 in TCR-mediated calcium mobilization.

It was reported previously that TCR-stimulated calcium flux was defective in cells expressing CH-deleted Vav1, and the CH domain featured Vav1, a GEF-independent function in T cell calcium signaling (5, 10). We therefore compared the amino acid sequences of CH domains as aligned in Fig. 2A. The CH domain similarity of Vav1 versus Vav2 is 81%, and Vav1 versus Vav3 is 88%. The secondary structure prediction was performed using PRALINE multiple sequence alignment software, and no difference between Vav1 and Vav2/3 was revealed. Concurrently, our work reported that Vav1 binds to CaM via the CH domain to potentiate calcium signaling upon TCR stimulation (11). We thus examined the interaction between individual Vav proteins and CaM by CaM-agarose pulldown assay. As shown in Fig. 2B, three Vav members were detected in Jurkat cell lysates (Fig. 2B, left lanes). Only Vav1 was immobilized by CaM-agarose, whereas Vav2 and Vav3 appeared negative in CaM binding (Fig. 2B, right lanes), regardless of the high similarities in their CH domain. These data suggest that the CH domain provides a unique structural basis of Vav1 to interact with CaM, and thereby renders Vav1 a distinguished role in TCR-activated calcium mobilization.

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**N Terminus of Vav1 Plays a Role in Calcium Signaling**

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**FIGURE 3. Identification of the CaM-binding region within the CH domain of Vav1.** A, schematic depiction of Vav1 and its truncated mutations at CH domain. The numbers of deleted amino acids are listed in the *right column*, and the designated names on the *left column*. The CH domain was boxed in black. B, the indicated Vav1 proteins were eukaryotically expressed in 293T cells. Cell lysates were incubated with CaM-conjugated agarose at 4 °C for 3 h in the presence of 4 mM Ca\textsuperscript{2+}. After washing, the immobilized contents were analyzed by SDS-PAGE and Western blot with anti-Vav1 antibody (Pulldown panel). The expressions of Vav1 and its mutants in whole cell lysates (WCL) were resolved by Western blot (WCL panel). C, BiFC assay. HeLa cells were cotransfected with the indicated combination of plasmids, YN-Vav1 + YC, YN-Vav1 + YC-CaM, YN-Vav1ΔN20 + YC, and YN-Vav1ΔN20 + YC-CaM, respectively. Then the cells were subjected to fluorescence microscopy (*left column*) and FACS analysis (*center column*), with the mean fluorescence intensity (*MFI*) listed in the *right column*. D, protein expression of each transfected component was verified by Western blot with the indicated antibodies.

depicted in Fig. 3A, ΔN20 (a deletion of amino acids 1–20), ΔN70 (a deletion of amino acids 1–70), and CH domain-deleted mutant (Vav1ΔCH). CaM-agarose pulldown assays were performed and the immobilized contents were resolved by Western blot analysis. As shown in Fig. 3B (*upper panel*), wild type Vav1 was immobilized by CaM-agarose (*left lane*), whereas the truncated variants showed no association with CaM. The expressions of the Vav1 variants were monitored and comparable amounts of cell lysates were loaded in the pulldown assay (Fig. 3B, *lower panel*). As the minimum truncation of 20 aa abrogated Vav1-CaM association, we concluded that the stretch of 20 aa of Vav1 was essential for the interaction between Vav1 and CaM.

To verify the association of Vav1 and CaM in living cells, we adopted a BiFC assay, which has been widely applied to examine intracellular protein interactions (28–30). The wild type Vav1 and Vav1ΔN20 were expressed as fusion proteins with the N-fragment of YFP (YN), respectively, and CaM was fused with YC (YC-CaM). The cells transfected with the indicated constructs were visualized by fluorescence microscopy and complex formation was analyzed by flow cytometry. The mean fluorescence intensity reflected the relative binding efficiency of the two fusion proteins (29). As shown in Fig. 3C, fluorescence in the control samples was undetectable (Fig. 3C, *first and third top panels*), indicating no association between YN and YC fragments. In contrast, intense fluorescence was observed in cells co-transfected with YN-Vav1 and YC-CaM (Fig. 3C, *second top panel*), with a mean fluorescence intensity of 512, manifesting the complexation of Vav1 and CaM in the cytoplasm. Meanwhile, barely detectable fluorescence was seen in cells expressing YN-Vav1ΔN20 and YC-CaM (Fig. 3C, *bottom panel*), reflecting a defect of Vav1ΔN20 in binding to CaM. The comparable expression levels of the fusion proteins were analyzed by Western blot (Fig. 3D). The above analyses by the CaM-agarose pulldown assay and BiFC approaches suggest that the N-terminal 20 aa of Vav1 are indispensable for the interaction with CaM.

**The Segment of 1–20 aa Is Required for TCR-stimulated Calcium Flux**—We next aimed to see whether the N-terminal 20 aa of Vav1 functionally affected calcium signaling during T cell stimulation. To obtain homogeneous expressions of Vav1 and mutations for monitoring cellular calcium fluctuation, we constructed lentivirus-based expression vectors encoding wild type Vav1, Vav1ΔCH, Vav1ΔN20, or vector alone as a control, respectively. The vav1-null cell line, J, Vav1, was used for transduction, and expression of the Vav1 variants was verified by Western blot (Fig. 4A). The intracellular Ca\textsuperscript{2+} profiles were recorded upon stimulation with OKT3 (Fig. 4B). Control cells lacking Vav1 or cells with CH-truncated Vav1 showed a limited increase (Fig. 4B, *solid or dotted line*), and cells containing the full-length Vav1 presented a rapid elevation of intracellular calcium upon TCR activation (Fig. 4B, *dashed line*). In comparison, cells with Vav1ΔN20 exhibited attenuated elevation (Fig. 4B, *dash-dotted line*), which resembled the control group or the cells with Vav1ΔCH, indicating that the N-terminal 20 aa were the determinant region for the TCR-mediated calcium response.

Calcium profile is shaped by a combination of calcium release from intracellular stores, such as endoplasmic reticulum, and the extracellular calcium influx through the calcium release-activated calcium channel (31). We further investigated the effect of 1–20 aa of Vav1 on calcium release. T cell stimulation was carried out in buffer containing EGTA to deplete the extracellular Ca\textsuperscript{2+}. The Ca\textsuperscript{2+} spike in this condition would
present only the Ca$^{2+}$/H$^{11001}$/ release from intracellular stores. As seen in Fig. 4C, J.Vav1 cells reconstituted with Vav1/N20 and Vav1/CH presented severe defects in OKT3-stimulated calcium release (dash-dotted line), whereas a dramatic peak was observed in cells with wild type Vav1 (dashed line). To rule out the possibility that reconstituted cell lines bared different levels of calcium stores, we treated cells with TG, a chemical that leads to passive calcium depletion from the endoplasmic reticulum lumen into the cytoplasm (32). As shown in Fig. 4D, in the absence of extracellular Ca$^{2+}$/H$^{11001}$/, the magnitude of calcium elevation in TG-treated Vav1/N20 or Vav1/CH-reconstituted cells resembled that of cells bearing wild type Vav1 (Fig. 4D), indicating that the deletion of 1–20 aa or CH domain of Vav1 had no effect on the intracellular Ca$^{2+}$/H$^{11001}$/ stores. The above data suggest that the N-terminal 20 amino acids of Vav1 are essential for TCR-induced calcium release.

The N-terminal 20 Amino Acids of Vav1 Are Indispensable for TCR-induced NFAT Activation—The activation of NFAT is a major event following Ca$^{2+}$/H$^{11001}$/ elevation during T cell stimulation, and has been used as a functional indicator for calcium signaling (33). We therefore tested the effect of Vav1 and its N-terminal-truncated variants on the NFAT activity by luciferase reporter assays. As shown in Fig. 5, the normalized NFAT activity was induced by OKT3 (closed bars) to 6-fold higher than the basal level (open bars) in cells transfected with vector (pcDNA4), whereas nearly 25-fold above the basal level in cells transfected with wild type Vav1. In comparison, TCR-stimu-

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**FIGURE 4.** Identification of the amino acids of Vav1 responsible for TCR-induced calcium mobilization. A, lentivirus particles were produced with lentiviral vectors encoding Vav1, Vav1(ΔCH), or Vav1(ΔN20), and vector backbone as control. J.Vav1 cells were transduced and selected with antibiotic markers. The expressions of Vav1 and Vav1 mutations were verified by Western blot with anti-Vav1 antibody. For calcium profile analyses, the reconstituted J.Vav1 cells with the indicated Vav1 or Vav1 variants (labeled as the inlets) were loaded with Fura-2 AM and stimulated with OKT3 in the presence of extracellular Ca$^{2+}$/ (B) or chelating agent EGTA (C); or treated with TG in the presence of chelating agent EGTA (D). The intracellular calcium were monitored for 5 min post-stimulation and plotted with time (horizontal axis) versus the fluorescence emission ratio of the Ca$^{2+}$/H$^{11001}$/ bounded (340 nm) to free form (380 nm) of Fura-2.

**FIGURE 5.** The requirement of the N-terminal 20 aa of Vav1 for TCR-induced NFAT transcriptional activity. A, J.Vav1 cells were transfected with reporter plasmids, pNFAT-Luc and TK-Renilla, together with plasmid backbone (pcDNA4), or plasmids encoding Vav1, Vav1(ΔCH), or Vav1(ΔN20) as indicated. At 24 h post-transfection, the cells were left unstimulated (NS, open bars), or stimulated with OKT3 (dark bars) for 6 h. The luciferase reporter assay was performed, and NFAT activity was presented as the firefly luciferase activity normalized to Renilla luciferase activity in each sample. Histograms represent the mean values of three independent experiments with S.D. B, cell lysates were subjected to Western blot analysis to verify the protein expression. The displayed Western blot is from one of the three experiments.
N Terminus of Vav1 Plays a Role in Calcium Signaling

The N-terminal 20 Amino Acids of Vav1, but Not Other Vav Members, Determine the Association with CaM—As mentioned, there are three Vav family members in humans, Vav1, -2, and -3. They shared high similarity in primary structures, and their GEF functions for small GTPases exhibited redundancy to a certain extent (5–7). However, TCR-mediated calcium elevation was independent of GEF, and the existing Vav2 and Vav3 were incompetent to rescue calcium signaling in vav1-null cells (5, 11). As 1–20 aa of Vav1 were involved in CaM binding, we next asked whether the stretches of the N-terminal 20 aa from the three Vav proteins were interchangeable. A variety of substitutions were designed to swap the N-terminal 20 aa of Vav proteins as depicted in Fig. 6, A and C. In Fig. 6A, the N-terminal 20 aa of Vav1 were replaced by those from Vav2 and Vav3, and designated as Vav1(Vav2-N20) and Vav1(Vav3-N20), respectively. These mutants, together with wild type Vav1, were expressed in 293T cells and the lysates were pulled-down by CaM, whereas wild type Vav1 was immobilized by CaM-agarose at 4 °C for 3 h in the presence of 4 mM Ca2+. The immobilized fractions were analyzed by SDS-PAGE and Western blot with anti-Vav1 antibody (upper panel), and the expressions of Vav1 and its mutants in the whole cell lysates (WCL) were resolved by Western blot (WCL panel). The pulldown analysis for proteins listed in A are presented in panel B; and for mutations listed in panel C are presented in D.

Meanwhile, we wondered if Vav2 or Vav3 with the N-terminal 20 aa from Vav1 would gain the capability of CaM binding. The swapped mutations were constructed as shown in Fig. 6C, in which the N-terminal 20 aa of Vav2 and Vav3 were replaced by 1–20 aa of Vav1, and designated Vav2(Vav1-N20) and Vav3(Vav1-N20), respectively. The CaM pulldown assay was performed as described above. As seen in Fig. 6D, Vav2 and Vav3 were negative in CaM binding as expected (second and fourth lanes from the left, upper panel), whereas the mutations of Vav2/3 with the N-terminal 20 aa from Vav1 (third and fifth lanes from left, upper panel) exhibited efficient binding as the wild type Vav1 (first lane from left, upper panel). Thereby the N-terminal 20 aa of Vav1 endows Vav2/3 with the character of interacting with CaM, and this region indeed distinguishes Vav1 from the co-existing Vav2 and Vav3 in respect of CaM binding.

The N Terminus of Vav1 Is Essential for the Calcium Signal Lineage upon TCR Stimulation—Previous study demonstrated that the association of Vav1 with CaM is a prerequisite for TCR-stimulated Ca2+ elevation. It was of interest to examine the roles of the two Vav1 N-terminal-substituted mutants in calcium signaling. First, we reconstituted J.Vav1 cells with lentiviruses carrying wild type Vav1, Vav1(Vav2-N20), Vav1(Vav3-N20), Vav1ΔN20, or vector to obtain homogenous expressions of the variants in J.Vav1 cells. The expressions of the indicated proteins were verified by Western blot (Fig. 7A). The calcium profiles of the reconstituted cells were recorded during T cell activation. As seen in Fig. 7B, compared with cells lacking Vav1 (vector, solid line), the wild type Vav1-reconstituted cells exhibited a significant elevation of intracellular Ca2+ under OKT3 treatment (dashed line), whereas those cells harboring Vav1(Vav2-N20), Vav1(Vav3-N20), or Vav1ΔN20 exhibited similar Ca2+ patterns as the cells with vector.

We further analyzed the NFAT activation by luciferase reporter assay in cells transfected by plasmids encoding wild type Vav1 or its N-terminal-substituted mutations as indicated (Fig. 7C). Following OKT3 treatment or no stimulation (NS), the NFAT luciferase activities of the transfected cells were measured. As shown in Fig. 7C, upon TCR stimulation, cells with wild type Vav1 presented 4-fold higher NFAT activity than cells with vector (pcDNA4) or Vav1ΔN20. The substituted mutation by either Vav2-N20 or Vav3-N20 had no significant restoration of NFAT as the wild type Vav1, even though the expressions of Vav1 derivatives were comparable as that of full-length Vav1 as monitored by the Western blot analysis (Fig. 7D).
The CaM Binding Region of Vav1(N-20) Is Indispensable for the Production of IL-2 in T Cell Activation—The IL-2 secretion is a critical landmark following T cell activation (34). We therefore measured the secreted IL-2 by J.Vav1 cells reconstituted with wild type Vav1 or the mutations, which lack the functional N termini. As shown in Fig. 7E, all samples exhibited equivalent basal levels of IL-2 (open bars). Upon T cell activation, J.Vav1 cells expressing wild type Vav1 displayed at least 5-fold higher secreted IL-2 than those with the N-terminal 20 aa mutations or the control vector (dark bars). These data reveal that the N-terminal 20 aa of Vav1, rather than those of Vav2 or Vav3, are essential for TCR-induced IL-2 secretion.

Together, we identified the N-terminal 20 amino acids of Vav1 to be essential for its interaction with calmodulin and potentiation of TCR-induced calcium mobilization. Deletion or substitution of this 20-aa stretch of Vav1 abolished its functions in TCR-mediated transcriptional activity of NFAT and IL-2 production. The deficiencies of TCR-induced calcium mobilization and NFAT activation by the N-terminal mutants of Vav1 strongly suggest that the N-terminal 20 aa of Vav1 reckon for not only the unique feature among Vav proteins, but also the irreplaceable role of Vav1 in T lymphocytes.

DISCUSSION

It has been illustrated that the hematopoietic specific Vav1 plays a pivotal role in T cell activation by acting as a GDP-GTP exchange factor and a scaffold to recruit signal molecules to the immune synapse via its multiple structure domains (3, 12). The CH domain of Vav1 was originally found to be related to transformation of fibroblasts (35). As the confined expression of Vav1 in lymphocytes, the indispensable role of the CH domain of Vav1 in intracellular calcium signaling has been suggested in NK and T cells (5, 10). Several binding partners of the CH domain have been revealed, such as lymphoid-specific guanine nucleotide exchange factors (GEFs) and calcium-modulated proteins.
dissociation inhibitor (20, 21), phospholipase Cγ1 (22), and CaM (11), and they were involved in TCR-induced calcium signaling. However, a line of evidence proposed that the Vav1 that participated in calcium signaling is GEF-independent (8, 9, 12). In addition, the CH-deleted Vav1, which possesses hyperactive GEF activity, could not potentiate calcium flux in response to T cell activation (10, 11, 36). Here, we identify the N-terminal 20-amino acid stretch of the CH domain to be responsible for Vav1 in the modulation of calcium flux via binding to CaM.

Previous work (11) demonstrated that the interaction of Vav1 with CaM did not require its GEF activity. Recently, the high resolution structure by xtallography have revealed that the CH domain is a trans-inhibitory domain for Vav1 GEF activity, and amino acid residues in the interface of the CH and DH domains are key factors in autoinhibition (13). Nevertheless, the N-terminal 20 aa is located beyond the autoinhibitory region for the GEF, further supporting that CH binding to CaM is independent of its GEF activity.

Besides Vav1, two other Vav family members, Vav2 and Vav3, are expressed in all human cell types including lymphocytes, and their functions as GEF overlap with Vav1 in activating Rho/Rac GTPases, like Rac1 or Rac2 (37). It remains unsolved why Vav1 is particularly required in T cell activation. We herein addressed that Jurkat T cells with reduced expression of Vav2 or Vav3 by shRNA underwent normal calcium flux upon T cell activation, whereas knocking down the expression of Vav1 resulted in a defective calcium response (Fig. 1). Consistently, Vav2 was reported to have no function in controlling calcium signals in T cells (18), and Vav3 was demonstrated to play an unnecessary role on TCR-induced NFAT activation (7). Therefore the calcium signals evoked by TCR engagement are mainly contributed by Vav1, but not Vav2 or Vav3.

Although more than 80% similarity was revealed among the CH domains of Vav proteins, we only found Vav1 CH association with CaM. Nonetheless, as Vav proteins contain multiple functional domains, we cannot rule out the involvement of other regions or domains to contribute the binding to CaM. It was suggested that multiple domains of Vav1 synergize the formation and stabilization of the microclusters to promote T cell activation (12). Indeed, other motifs, such as the IQ consensus sequence for calmodulin interaction (38), does exist in Vav1. However, mutations of the IQ motif did not affect the association of Vav1 with CaM, nor the calcium profile following TCR ligation (data not shown). We speculate that the CH domain, especially the first 20-aa stretch of peptide, is essential for CaM binding, whereas the other domains of Vav1 may be required to cooperate with the CH domain for normal calcium flux. Further studies by crystallography of the Vav1-CaM complex would help to define the interaction sites between Vav1 and CaM.

In summary, we highlight the N-terminal 20 aa as a key region of Vav1 for association with CaM in T lymphocytes. As a result, this region endows Vav1 with a distinct function among the Vav proteins in antigen-induced T cell activation, including calcium flux, NFAT activation, and IL-2 production. Recently, the aberrant expressions of Vav1 were observed in several cancerous cells and associated with malignancy (39). The exploration for the distinct mechanisms of Vav1 may also contribute to the understanding of its pathological relevance.

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