Vav1 is a guanine nucleotide exchange factor that is expressed specifically in hematopoietic cells and plays important roles in T cell development and activation. Vav1 consists of multiple structural domains so as to facilitate both its guanine nucleotide exchange activity and scaffold function following T cell antigen receptor (TCR) engagement. Previous studies demonstrated that the calponin homology (CH) domain of Vav1 is required for TCR-stimulated calcium mobilization and thus downstream activation of nuclear factor of activated T cells. However, it remained obscure how Vav1 functions in regulating calcium flux. In an effort to explore molecules interacting with Vav1, we found that calmodulin bound to Vav1 in a calcium-dependent and TCR activation-independent manner. The binding site was mapped to the CH domain of Vav1. Reconstitution of vav1-null Jurkat T cells (J.Vav1) with CH-deleted Vav1 exhibited a severe deficiency in calcium release to the same extent as that of Jurkat cells treated with the calmodulin inhibitor or J.Vav1 cells. The defect persisted even when phospholipase-Cγ1 was fully activated, indicating a prerequisite role of Vav1 CH domain in calcium signaling. The results suggest that Vav1 and calmodulin function cooperatively to potentiate TCR-induced calcium release. This study unveiled a mechanism by which the Vav1 CH domain is involved in calcium signaling and provides insight into our understanding of the role of Vav1 in T cell activation.

Stimulation of the T cell antigen receptor (TCR) initiates a cascade of signaling events that lead to T cell activation. Calcium plays a central role in this process and has been studied intensively (1–4). Engagement of TCR triggers the activation and accumulation of enzymes and adapter molecules to the proximal membrane (5, 6), such as tyrosine phosphorylation and activation of phospholipase-Cγ1 (PLC-γ1), thereby increasing the production of inositol 1,4,5-trisphosphate (IP3). IP3 binds to and activates the inositol 1,4,5-trisphosphate receptor (IP3R), which results in Ca2+ release from the endoplasmic reticulum (ER) and the subsequent calcium influx from Ca2+ release-activated Ca2+ channel (CRAC) (1, 7). The elevated cytoplasmic [Ca2+] evokes a multitude of cellular responses, such as the NFAT-mediated gene expressions and the cell proliferation (8, 9).

Vav1 is expressed specifically in hematopoietic cells as a 95-kDa protein, which plays pivotal roles as a guanine exchange factor (GEF) for small GTPases as well as a scaffold protein in the activation of hematopoietic cells (10–12). The importance of Vav1 is because of its multiple structural elements, including a calponin homology (CH) domain, an acidic motif, a Dbl homology domain, a pleckstrin homology (PH) domain, a cysteine-rich motif, and one single SH2 domain flanked by two SH3 domains responsible for signaling protein assembly (12, 13). Upon TCR engagement, Vav1 is phosphorylated on the key tyrosine residues in the acidic motif, leading to the exposure of active Dbl homology domain for GDP/GTP exchange activity (14). Studies on vav1−/− T cells isolated from knock-out mice demonstrated that Vav1 is essential for normal T cell activation and proliferation (15–17). In addition, the vav1-null cell line, J.Vav1, derived from Jurkat cells by somatic gene targeting approach, also exhibits pleiotropic defects in TCR-mediated signaling pathways (18).

T cell stimulation evokes a biphasic calcium flux as follows: calcium release from intracellular stores followed by calcium influx across the plasma membrane (7, 19). IP3Rs dominantly control the initiation of IP3-induced calcium release, demonstrated by using antisense knockdown of IP3R to block calcium release from the ER (20). Jurkat T cells express three IP3R isomers, IP3R-1, IP3R-2, and IP3R-3 (21), which differ significantly in their sensitivity to IP3 (22, 23). A tyrosine kinase, Fyn, was suggested to modulate IP3R channel activities (24, 25). Interactions between IP3R and other proteins, such as calmodulin (CaM), were reported to control the channel opening. Although some observations viewed CaM as an inhibitory protein of IP3R (26–28), more recent study illustrated that the
**Vav1-CaM Association Regulates Calcium Release**

Ca\(^{2+}\)-dependent association of CaM to IP\(_3\)R is necessary for normal calcium release (29).

Analysis of Vav1-modulated calcium mobilization has been emphasized on its recruitment and activation of PLC-\(\gamma\)1 (10, 13, 30, 31). Vav1, together with SLP-76 and other adapter proteins, stabilizes PLC-\(\gamma\)1-linker for activation of the T cell complex necessary for tyrosine phosphorylation and activation of PLC-\(\gamma\)1 (30). On the other hand, Vav1 was reported to facilitate PLC-\(\gamma\)1 tyrosine phosphorylation via GEF-mediated phosphoinositide 3-kinase-dependent pathways (31). However, these models were challenged by the fact that oncVav1 (lacking 66 amino acids at the N terminus) or Vav1 bearing mutations in the CH domain failed to provoke calcium flux and NFAT(IL2) activity in T cells, although the domains necessary for GEF activity or complex formation remained intact (13, 18, 32). Therefore, the CH domain of Vav1 must play a GEF-independent role in TCR-mediated calcium response and NFAT activity. Sequence analysis predicted the CH domain to be involved in F-actin binding (33). Up to now, few proteins were reported to interact with the N terminus of Vav1, such as lymphoid-specific guanine dissociation inhibitor (LY-GDI) (34) and a polycomb family protein ENX-1 (35). However, the function of the N-terminal domain of Vav1 (CH) and the mechanism by which Vav1 participates in calcium flux are poorly understood.

In this study, we investigated the mechanism of Vav1 in regulating calcium signaling in T cells. We found that Vav1 associated with CaM. The binding region was mapped to the CH domain of Vav1, and the association was dependent on calcium. Tyrosine phosphorylation of Vav1 had no impact on the interaction. Importantly, we showed that Vav1, via its CH domain, predetermined calcium release from the intracellular store in cooperation with CaM. This study revealed a new binding partner of Vav1 CH domain and may help to understand the mechanisms of Vav1 in T cell calcium signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The anti-IP\(_3\)R-1 antibody was purchased from Calbiochem. The anti-CaM antibody was purchased from Upstate. The anti-PLC-\(\gamma\)1 Tyr(P)-783 antibody and anti-\(\alpha\)-tubulin antibody were purchased from Sigma. The anti-CD3 mAb OKT3, anti-CD28, anti-Vav1, anti-Zap70, and goat anti-mouse IgG were described previously (18). The Indo-1-AM, CaM-agarose beads, protein A-Sepharose 4 Fast Flow beads, Indo-1-AM, thapsigargin, ionomycin, and W-7 were purchased from Sigma. The 20 mM pervanadate solution was prepared by adding 2.3 \(\mu\)l of 30% \(\text{H}_2\text{O}_2\) to 1 ml of 20 mM \(\text{Na}_2\text{VO}_4\) and allowing the mixture to react for 5 min at room temperature.

**Plasmids**—The pcDNA3.FLAG.Vav1, pcDNA3.FLAG.CH, Vav1, and pEFAP4.HIV.Vav1 were described elsewhere (32, 36). The C-terminal SH3 domain (position 786 – 845) truncated Vav1 was obtained by PCR and cloned into pcDNA4/HisMax C (Invitrogen) by BamHI and XhoI. The mixture to react for 5 min at 25 °C. The mixtures were incubated at 37 °C for another 2 h. The EGFP fragment was being imaged simultaneously at emission wavelengths of 398 and 490 nm (10 nm slit). Cells were stimulated with the indicated agents, and the concentrations of the stimuli were 1

**Cell Culture, Transfection, and Stimulation**—Jurkat T leukemia cells and J.Vav1 cells were obtained as described previously (18). Primary lymphocytes were kindly provided by the Institute of Blood Diseases (Tianjin, China). Jurkat T leukemia cells were grown in RPMI 1640 medium at 37 °C containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. For transient transfections, 2 \(\times\) 10\(^7\) Jurkat cells were electroporated with a BTX Electro-square Porator model ECM830 (BTX Inc., San Diego) at 310 mV, 10 ms with 30 \(\mu\)g of total DNA. 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mM l-glutamine, and 1% (v/v) penicillin/streptomycin at 37 °C. 3 \(\times\) 10\(^6\) 293T cells were transfected with a total of 25 \(\mu\)g of plasmid by the calcium phosphate precipitation method. The concentrations of the stimuli used in both luciferase reporter assays and biochemical assays were 1 \(\mu\)g/ml OKT3 (cross-linked with 1 \(\mu\)g/ml goat anti-mouse IgG), 5 \(\mu\)g/ml CD28, and 1 \(\mu\)g ionomycin.

**Luciferase Reporter Assay**—The reporter constructs were described previously (18). The dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine the activity of firefly luciferase and Renilla luciferase according to the manufacturer’s instructions. Luciferase activity in cell extracts was measured in a TD20/20 luminometer (Turner Designs Inc, Sunnyvale, CA) by injecting 100 \(\mu\)l of assay buffer and measuring light emission for 10 s after injection. Normalized luciferase activity was obtained by dividing the firefly luciferase activity by the Renilla luciferase activity.

**Retroviral Transduction**—Recombinant retroviruses were generated as described previously (37). 293T cells were transfected with 40 \(\mu\)g of plasmids consisting of HIV trans, vesicular stomatitis virus G, and the HIV-Vav1/HIV-Vav1\(\Delta\)CH using the calcium phosphate precipitation method. 48 h after the transfection, supernatants were collected and centrifuged to remove cell debris. The processed supernatants were then mixed with J.Vav1 cells and centrifuged at the speed of 2000 \(\times\) g for 2 h at 25 °C. The mixtures were incubated at 37 °C for another 2 h. Supernatants were removed by centrifuge, and transduced cells were cultured in 37 °C incubator with RPMI 1640 supplemented with 10% FBS.

**Calcium Measurement**—1 \(\times\) 10\(^7\) cells were resuspended in 1 ml of HBSS supplemented with 5 mM dextrose and buffered to pH 7.0 with HEPES. Indo-1-AM was added to the cell suspension to a final concentration of 5 mM, and the mixture was incubated for 30 min at 37 °C. An equal volume of HBSS (pH 7.4) containing 5 mM dextrose was then added, and the mixture was incubated for another 30 min at 37 °C. The Indo-1-loaded cells were washed with HBSS (pH 7.5) supplemented with 5 mM dextrose and 0.05% bovine serum albumin. Samples were prewarmed for 5 min at 25 °C. To determine changes in \([\text{Ca}^{2+}]_i\), a cuvette filled with 1 ml of Indo-1 loaded cells was mounted to a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) and were excited at 352 nm (5 nm slit) while being imaged simultaneously at emission wavelengths of 398 and 490 nm (10 nm slit). Cells were stimulated with the indicated agents, and the concentrations of the stimuli were 1.
RESULTS

Vav1 Is Prerequisite to TCR-induced Intracellular Calcium Release—T cell stimulation evokes a biphasic calcium flux; the calcium release followed by calcium depletion-activated calcium entry (7, 19). We firstly recorded cellular [Ca\(^{2+}\)]\(_i\) profiles of Jurkat cells, vav1-null cells (J.Vav1), and wild type Vav1 reconstituted J.Vav1 cells (J.WT), respectively. Upon TCR activation by OKT3, J.Vav1 cells exhibited a slightly attenuated spike of [Ca\(^{2+}\)]\(_i\) elevation after the supplementation of 2 mM Ca\(^{2+}\) (Fig. 1A). The abnormal calcium flux of J. Vav1 cells may result from the combination of the following two processes: 1) defective Ca\(^{2+}\) release from intracellular store; and 2) impaired Ca\(^{2+}\) influx via CRAC. To dissect the two scenarios, we performed experiments in a Ca\(^{2+}\)-free solution prior to supplementation of 2 mM Ca\(^{2+}\) (Fig. 1B, top bar). Therefore, the initial Ca\(^{2+}\) spike represents the Ca\(^{2+}\) release from the intracellular store, and the subsequent [Ca\(^{2+}\)]\(_i\) elevation after the supplementation of 2 mM Ca\(^{2+}\) reflects the Ca\(^{2+}\) influx via CRAC. As seen in Fig. 1B, OKT3-stimulated J.Vav1 cells presented a delayed and attenuated spike of [Ca\(^{2+}\)]\(_i\), in comparison with that of Jurkat and J.WT cells. Upon addition of extracellular Ca\(^{2+}\) (at 400 s post-stimulation), all three kinds of cells displayed a rapid increase in intracellular calcium concentration, indicating comparable Ca\(^{2+}\) influx capabilities in cells with or without Vav1. Thus, the abnormal [Ca\(^{2+}\)]\(_i\) profile of J.Vav1 cells is largely because of the defect of initial calcium release, suggesting that Vav1 is essential for the TCR-mediated Ca\(^{2+}\) release from intracellular store.

The deficiency in TCR-stimulated Ca\(^{2+}\) release in J.Vav1 cells is thought to be the consequence of the impaired activation of PLC-γ1, represented by tyrosine phosphorylation on both Tyr-783 (38) and Tyr-775 residues (39). Indeed, the defective tyrosine phosphorylation of PLC-γ1 Tyr-783 was observed in J.Vav1 cells (30). However, when we treated J.Vav1 cells with pervanadate (PV), a potent protein-tyrosine phosphatase inhibitor, to induce PLC-γ1 phosphorylation (40), the calcium defects still remained in J.Vav1 cells (Fig. 2A). Meanwhile, Jurkat and J.WT presented similar intracellular calcium release in response to PV, demonstrating the indispensable role that Vav1 plays in calcium release. The activation of PLC-γ1 was moni-
and Western blot analysis using antibodies against IP3-R-1 and Zap-70, stored for 400 s in each sample and plotted as fluorescence emission ratio (FER)-time curve. A, cells as indicated were lysed and subjected to SDS-PAGE and Western blot analysis using antibodies against IP3-R-1 and Zap-70, respectively.

...tored by the phosphorylation of Tyr-783 (Fig. 2B, upper panel), and J.Vav1 displayed equal trends to that of wild type cells at 2, 5, and 10 min post-induction by PV (Fig. 2B, lower panel). Considering that both N- and C-SH2 domains of PLC-γ1 were shown to be dispensable for PV-induced phosphorylation (41), the remarkable defect of calcium release seen in J.Vav1 cells was independent of PLC-γ1 activation. The possible explanation is that Vav1 may bear other machineries to regulate Ca2+ mobilization in addition and in parallel to facilitating PLC-γ1 phosphorylation. This result was supported by our previous observation in which defect calcium profiles were seen in J.Vav1 despite normal IP3 production comparable with that of Jurkat cells (18). Therefore, Vav1 is prerequisite to ensure a normal calcium release upon TCR-induced PLC-γ1 activation and IP3 production.

vav1-null Cells Possess Intact Intracellular Calcium Store and IP3-R-1 Expression—Because J.Vav1 cells are derived from the Jurkat E6 cell line by the gene targeting approach, the possibility should be noted that J.Vav1 cells have disrupted calcium store or gene expression, which can lead to the abnormal calcium signals observed above. Thus, we inspected the intracellular calcium stores and calcium channel expression of ER. ER calcium pools were measured indirectly by treating cells with a known inhibitor of sarcoplasmic reticulum Ca2+-ATPase pumps, thapsigargin (TG), which causes passive calcium depletion from the ER lumen into the cytoplasm. As presented in Fig. 3A, the magnitude of TG-releasable calcium in J.Vav1 cells resembled that of Jurkat and J.WT cells. Moreover, the Ca2+ ionophore, ionomycin, evoked the same calcium release patterns in cells with or without Vav1 (data not shown), indicating the equal calcium stores in J.Vav1, Jurkat, and J.WT cells. Considering IP3-R-1 as a predominant player in IP3-induced calcium release in T lymphocytes (20), we also confirmed its expression level in J.Vav1 cells. As shown in the upper panel of Fig. 3B, the same IP3-R-1 expression levels were observed in all the three cell lines, with equal protein loading verified by Zap-70 (lower panel) and α-tubulin (not shown). Moreover, proteomics analysis showed no detectable differences in proteins that related to calcium signaling between J.Vav1 cells and Jurkat cells. Hence, the loss of Vav1 had no effect on ER calcium content nor on IP3-R-1 expression, pinpointing the function of Vav1 in the course of TCR-induced calcium release rather than interfering with the intracellular Ca2+ store or protein expression.

Vav1 Interacts with Calmodulin via CH Domain in a Ca2+-dependent and Activation-independent Manner—To explore the possible mechanisms that Vav1 modulates calcium release, we looked for Vav1 binding partners. CaM came to the scene as it was demonstrated to ubiquitously bind and regulate IP3-R (29, 42–44). CaM pulldown assay was performed by incubating CaM-conjugated agarose with cell lysates of Jurkat and J.Vav1, respectively, or using plain agarose beads as a negative control. The pulldown contents were resolved by SDS-PAGE and Western blot analysis. The amount of Vav1 in Jurkat cell lysate was used as protein input indicator (labeled as lysate) for the CaM pulldown assay. The precipitated products were analyzed by Western blot, using indicated antibodies. D, Jurkat cells were left unstimulated (None) or stimulated with OKT3 or OKT3 plus CD28 for 5 min and then lysed, and cell extracts were incubated with CaM-agarose for pulldown assay as described above. Immobilized fractions were subjected to Western blot with anti-Vav1 antibody (upper panel). The TCR activation of Jurkat cells was monitored by reprobing the same membrane with antibodies specific for phosphotyrosine residues (middle panel) or phospho-Tyr-783 of PLC-γ1 (lower panel).
pulldown mixture containing eukaryotically overexpressed Vav1 (Fig. 4B, left). In the competition experiment, decreased amounts of Vav1 were pulled down in the presence of 20 μg of CaM, and the Vav1 band turned undetectable when 50 μg of CaM was added to compete the binding. Meanwhile, Ca²⁺ dependence of Vav1-CaM association was also characterized. As shown in the right panel of Fig. 4B, Vav1 binds to CaM in the presence of external Ca²⁺, whereas chelating agent EGTA completely abolished the interaction, indicating that the association between Vav1 and CaM is Ca²⁺-dependent.

To examine if Vav1 endogenously interacts with calmodulin, we performed co-immunoprecipitation (co-IP) experiments. As shown in Fig. 4C, co-IP performed with anti-Vav1 antibody showed the co-precipitation with CaM from lysates of both Jurkat cells and primary lymphocytes (Fig. 4C, lower panel, 1st and 2nd lanes). Likewise, co-IP using anti-CaM antibody displayed Vav1 from Jurkat and primary lymphocytes (Fig. 4C, upper panel, 5th and 6th lanes). J.Vav1 cell lysate was used in both cases as a vav1-null control (Fig. 4C, 3rd and 7th lanes), and preimmune IgG was applied as negative control (lane labeled IgG). The total protein input was shown as indicated (Fig. 4C, lysate). These data show that the endogenous CaM and Vav1 associate in vivo.

Tyrosine phosphorylation and activation of Vav1 are early events in TCR engagement; therefore, we asked if Vav1-CaM interaction is modulated by the activation status of T cells. Jurkat cells were treated with solvent only, OKT3, or OKT3 plus anti-CD28 IgM as co-signal ligand, respectively, and then Vav1 was pulled down from the cell lysates by immobilized CaM. As shown in Fig. 4D (top row), the association of Vav1 and CaM appeared to be constant, regardless of tyrosine phosphorylation of Vav1 (Fig. 4D, middle row). Cell activation was also monitored by phosphorylation of PLC-γ1 Tyr-783 in whole-cell lysates (Fig. 4D, bottom row). Taken together, these results indicate that Vav1 interacts specifically with CaM. This interaction is dependent on Ca²⁺ but unrelated to the tyrosine phosphorylation of Vav1 during T cell activation.

To locate the CaM-binding site(s) of Vav1, we constructed a series of truncated Vav1 mutants. The selected mutants are depicted in Fig. 5A. These mutants include deletions of CH, PH, and C-SH3 domains, respectively. One mutant was designed with a deletion of C-SH3 plus a stretch of sequence containing a putative IQ motif (LQFPKFEPEKR) as the IQ motif of IQGAP1 was identified as a binding site for CaM (45). Among all the truncations of Vav1, only CH-deleted mutant failed to associate with CaM, whereas the others exhibited the same capabilities of binding as that of full-length Vav1, demonstrating that the CH domain of Vav1 is responsible for association with CaM (Fig. 5B).

Calmodulin and the CH Domain of Vav1 Interdependently Contribute to TCR-stimulated Calcium Release—To address the effect of Vav1-CaM interaction on calcium signaling, we reconstituted J.Vav1 cells with retroviruses encoding wild type Vav1 (Vav1) or CH-deleted Vav1 (Vav1CH) to obtain the homogenous expression. In comparison with mock-infected cells using viruses packed with the plasmid encoding EGFP, the expression of Vav1 and/or Vav1CH was verified to be even in J.Vav1 cells transduced with corresponding viruses (Fig. 6A, left panel). We also confirmed the binding of CaM by the pulldown procedure described above, and only cells harboring wild type Vav1, not Vav1ΔCH, showed positive interaction (Fig. 6A, right panel). As reported previously, CaM antagonists (such as W-7) or high affinity CaM-binding peptides strongly inhibited IP₃-induced Ca²⁺ release (29, 42). Therefore, by using intact cells and virally reconstituted cells, we tested the effects of Vav1 and Vav1CH on Ca²⁺ release by the inhibition of CaM. First, we validated that W-7 indeed led to a dose-dependent attenuation of OKT3-stimulated Ca²⁺ release (Fig. 6B) but not TG-elevated calcium in Jurkat cells (Fig. 6C). As similar doses of W-7 were administered to J.Vav1 cells, no additive impact was observed on OKT3-induced Ca²⁺ release (Fig. 6D). Second, cells reconstituted with wild type Vav1 showed a similar pattern of calcium release that was severely attenuated by W-7 (Fig. 6E), whereas in cells containing CH-deleted Vav1, the calcium release was inert to the addition of W-7 (Fig. 6F), similar to that of J.Vav1 or mock reconstituted cells (Fig. 6G). The above observation reveals a coordinate correlation between Vav1 and CaM in controlling the process of IP₃-dependent calcium release and suggests that the CH domain in Vav1 may provide a standing platform for CaM to exert its function in regulating calcium release.

CH Domain of Vav1 Potentiates Normal Calcium Flux and NFAT Transcriptional Activity Induced by TCR—Based on the mapping of the CaM binding region to the CH domain of Vav1, we subsequently examined the role of this domain in modulating intracellular calcium release. Cells expressing Vav1ΔCH showed severe defects in calcium release, whereas a quick
Vav1-CaM Association Regulates Calcium Release

FIGURE 6. Reconstitution of Vav1 or Vav1ΔCH and their cooperation with CaM on TCR-induced calcium release. A, cultured J.Vav1 cells were transduced with retrovirus particles engineered with Vav1 or Vav1ΔCH and harvested at the 4th day of post-infection, respectively. The expression of Vav1 and Vav1ΔCH was monitored by Western blot with anti-Vav1 antibody (left panel). CaM pulldown assay was performed as described, and CaM-bound fractions were subjected to SDS-PAGE and Western blotting analysis (right panel). Zap-70 was used as an internal control for even protein loading. B–D, intact cells were treated with increased concentrations of the CaM antagonist, W-7, for 5 min. In the absence of extracellular Ca²⁺, Jurkat cells were then stimulated with OKT3 (B) or TG (C), respectively. Similarly, J.Vav1 cells were pretreated with W-7 and then stimulated with OKT3 (D). The calcium profile was monitored for 300 s and plotted as described above. E and F, J.Vav1 cells were reconstituted with retroviruses harboring Vav1(J.Vav1(Vav1)) (E), Vav1ΔCH(J.Vav1(Vav1ΔCH)) (F), and EGFP fragment (Mock) (G), and treated with 30 μM of W-7 for 5 min, respectively. In the absence of extracellular Ca²⁺, cells were then stimulated with OKT3 at the indicated time. The calcium profile was monitored for 300 s.

FIGURE 7. Effect of PLC-γ1 activation on Vav1-mediated calcium release. In the absence of extracellular Ca²⁺, virally reconstituted cells, J.Vav1(Vav1), J.Vav1(Vav1ΔCH), and mock-transduced J.Vav1 (Mock) were treated with OKT3 (A) or with PV (B), and the released calcium was measured as described previously. The phosphorylation of PLC-γ1 at Y783 was monitored for cell activation stimulated by OKT3 (C) or PV (D) at the indicated time. α-Tubulin was determined as protein loading control. The activation of PLC-γ1 was presented by the ratio of Tyr(P)-783 to α-tubulin measured by densitometry.

DISCUSSION

Vav1 has been recognized to modulate TCR-dependent calcium signals by activating PLC-γ1 in virtue of its two characters in NFAT(IL2) (46, 47). As shown in Fig. 8A, NFAT(IFN) reporter activity was severely defective under OKT3 stimulation in J.Vav1 cells compared with the parental Jurkat cells. The NFAT activity was mostly restored by reconstituting J.Vav1 cells with wild type Vav1 but not Vav1ΔCH. The defect of NFAT response in Vav1ΔCH cells was rescued by the presence of ionomycin plus OKT3, indicating that Vav1ΔCH delivers an aberrant calcium signal upon OKT3 stimulation. The equivalent expression of Vav1 and Vav1ΔCH was monitored by Western blot (Fig. 8B). It is worth noting that treatment of vav1-null cells (J.Vav1) with OKT3 plus ionomycin forced an increase in NFAT(IFN) activity to an extent half that seen in cells expressing wild type Vav1 or Vav1ΔCH. This discounted NFAT activity is thought to result from the lack of functional domains other than the CH motif, demonstrating that the CH domain in coordination with other domains of Vav1 contributes to the activation of NFAT.
as follows: 1) as a scaffold to recruit PLC-γ1 in the complex of immunological synapse (30), and 2) as a guanine nucleotide exchange factor (GEF) to indirectly activate PLC-γ1 via the phosphoinositide 3-kinase pathway (31). Both of the above will lead to the production of IP3 and calcium release from intracellular stores, followed by calcium influx across plasma membrane via CRAC (1, 7). In this study, we illustrate that the overall calcium profiles of J.Vav1 exhibited most defects in the maintenance of [Ca2+]i, (Fig. 1A), consistent with those obtained from independent laboratories (18, 30, 32). However, we noticed more a obvious defect of J.Vav1 in the stage of calcium release from intracellular stores (Fig. 1B). Because our experimental procedures arbitrarily distinguish the calcium release from the whole calcium profile, we do not exclude that Vav1 may also be involved in calcium entry through its known GEF function to regulate cytoskeleton rearrangement. In fact, it was recently reported that Wave2, a Rac1-dependent cytoskeleton regulator, participates in CRAC-mediated calcium entry during T cell activation (48). The scope of this paper is focused on the role of Vav1 in the course of calcium release.

The role of Vav1 CH domain in regulating calcium signaling has received much attention (13, 18, 32). Previous studies showed that the CH domain may not be responsible for Vav1 membrane targeting or TCR-induced Vav1 phosphorylation, but it is involved in the regulation of calcium flux (32). As the CH domain is rich in α-helical content, it was predicted to be involved in protein-protein interactions (49). So far, only few proteins have been reported to interact with the CH domain of Vav1, and the only binding partner related to calcium signaling is Ly-GDI (34, 50), a GDP dissociation inhibitor protein. Synergistic activation of PLC-γ1 and NFAT was observed in Jurkat T cells overexpressing Ly-GDI and Vav1 but not oncVav1 that bears a truncation at the N terminus (50). The interaction between Ly-GDI and Vav1 was believed to keep Ly-GDI away from its target GTPase, allowing Vav1 to exert GEF function more efficiently and to promote PLC-γ1 activation and calcium mobilization (50). Here, from our data and previous observations, we deduced a model that the CH domain facilitates Vav1-CaM association and potentiates calcium flux via the PLC-γ1-uncoupled and GEF-independent mechanism. First, we showed that activation of TCR induced the phosphorylation of PLC-γ1 Tyr-783 to the same extent in cells expressing CH-deleted Vav1 as that of full-length Vav1. Second, complete activation of PLC-γ1 could not mask the deficient calcium release in cells reconstituted with CH-deleted Vav1. Third, as discussed previously, deletion of the CH domain does not impair but enhances the GEF activity of Vav1. Thus the role that the CH domain plays in calcium release is independent of its GEF activity. It is to be further scrutinized on the fine-tuning machineries in which Ly-GDI and CaM simultaneously bind to the CH domain of Vav1 to modulate calcium signaling.

Actin binding is one of the features of concatenated CH domains (49), yet it was reported that a single CH domain interacts with Ca2+/CaM (51–54). Interestingly, IQGAP1, which contains a single CH domain, was demonstrated to bind CaM via its IQ motif (45). This interaction between IQGAP1 and CaM attenuated the association of IQGAP1 with Cdc42 and thus linked the Ca2+/CaM signaling pathways to Cdc42-mediated cytoskeleton rearrangement. In the case of Vav1, although both the CH domain and a putative IQ motif were presented, the interaction with CaM was mapped to its CH domain (Fig. 5). Inhibition of CaM concomitantly affected calcium release similar to that seen in cells expressing the CH-deleted Vav1, indicating a functional coordination of Vav1 and CaM in calcium signaling. It is noteworthy that two other isoforms of Vav family proteins, Vav2 and Vav3, are both present in Jurkat cells. These isoforms, which undergo tyrosine phosphorylation and exhibit functional redundancy as Vav1 in response to TCR stimulation (18), appeared negative in CaM binding. Therefore, we inferred that the incompetence of Vav2 and/or Vav3 in controlling calcium release could be attributed to their incapability to associate with CaM. Therefore, the CH domain of Vav1 is not only a prerequisite but is also irreplaceable in controlling calcium release in T cells.

The involvement of CaM in calcium flux was well documented. Stripping of CaM from the IP3R in vivo strongly inhibited calcium release, leading to a model that endogenous CaM is a subunit of IP3R (29). Although not fully understood, observations supported that intramolecular interactions may deliver a conformational change of IP3R and thus operate the opening of the Ca2+ channel (55–57). Moreover, it was reported that

4 Z. Zhou and Y. Cao, unpublished data.
Vav1-CaM Association Regulates Calcium Release

CaM/CaM kinase II triggers IP$_3$R phosphorylation and thus modulates the channel activity (58). As far as biological significance is concerned, we introduce a model by which the association of Vav1 with CaM may modulate the channel activity of IP$_3$R by affecting the intramolecular interactions and/or phosphorylation. In this study, we present our new findings that Vav1 interacts with CaM via its CH domain and thus contributes to the modulation of calcium release upon TCR activation. This may shed light on the mechanism of Vav1 in T cell calcium signaling. It is to our interest to attempt to investigate the intermolecular assembly of Vav1-CaM-IP$_3$R and its functional correlation with T cell activation.

Acknowledgments—We thank Professors Mian Wu (University of Science and Technology of China) and Weiguo Zhang (Duke University) for providing suggestions and reagents. We also thank Professors Cuifeng Li (Nankai University) and Mingjie Zhang (Hongkong University) for providing purified calmodulin protein.

REFERENCES

1. Lewis, R. S. (2001) Annu. Rev. Immunol. 19, 497–521
2. Premack, B. A., and Gardner, P. (1992) Am. J. Physiol. 263, C1119–C1140
3. Wulfing, C., Rabinowitz, J. D., Beeson, C., Sjaastad, M. D., McConnell, K., and Tybulewicz, V. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6295–6299
4. Tan, L., Liu, Y., and Weiss, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13017–13022
5. Kane, L. P., Lin, J., and Weiss, A. (2000) J. Biol. Chem. 275, 6233–6237
6. Secrist, J., Purns, L. A., Kari, L., and Artzt, G. (2004) Immunity 5, 59–60
7. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11382–11388
8. Irvin, B. J., Williams, B. L., Nilson, A. E., Maynor, H. O., and Abraham, R. T. (2003) Mol. Cell. Biol. 20, 9149–9161
9. Bultynck, G., Szlufcik, K., Atia, N., Callewaert, G., and De, S. H. (1999) J. Biol. Chem. 274, 13748–13751
10. Yamada, M., Miyawaki, A., Saito, K., Nakajima, T., Yamamoto-Hino, M., Ryo, Y., Fujii, T., and Mikoshiba, K. (1995) Biochem. J. 308, 83–88
11. Li, Z., and Sacks, D. B. (2004) Am. J. Physiol. 289, C1119–C1140
12. Billadeau, D. D., Mackie, S. M., Schoon, R. A., and Leibson, P. J. (2000) J. Immunol. 164, 3971–3981
13. Castresana, J., and Saraste, M. (1995) FEBS Lett. 374, 149–151
14. Groysman, M., Russek, C. S., and Katzav, S. (2000) J. Biol. Chem. 275, 6233–6237
15. Secrist, J., Purns, L. A., Kari, L., and Artzt, G. (2004) Immunity 5, 59–60
16. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11382–11388
17. Hill, T. D., Campos-Gonzalez, R., Kindmark, H., and Boynton, A. L. (1988) J. Biol. Chem. 263, 16479–16484
18. Missiaen, L., Parys, J. B., Weidema, A. F., Sipma, H., Vanlingen, S., De, S. P., Callewaert, G., and De, S. H. (1999) J. Biol. Chem. 274, 13748–13751
19. Yamada, M., Miyawaki, A., Saito, K., Nakajima, T., Yamamoto-Hino, M., Ryo, Y., Fujii, T., and Mikoshiba, K. (1995) Biochem. J. 308, 83–88
20. Li, Z., and Sacks, D. B. (2003) J. Biol. Chem. 278, 4347–4352
21. Kaminou, F., Hove, M., Lil, J., and Artzt, G. (2001) Mol. Cell. Biol. 21, 3126–3136
22. Sweetser, T. M., Hoey, T., Sun, Y. L., Weaver, W. M., Price, G. A., and Wilson, C. B. (1998) J. Biol. Chem. 273, 34775–34783
23. Noz, I. C., Gomez, T. S., Zhu, P., Li, S., Medeiros, R. B., Shimizu, Y., Burkhardt, J. K., Friedman, B. D., and Billadeau, D. D. (2006) Curr. Biol. 16, 24–34
24. Banuelos, S., Saraste, M., and Carugo, K. D. (1998) Structure (Lond.) 6, 1419–1431
25. Groysman, M., Hornstein, I., Alcover, A., Callewaert, G., and Katzav, S. (2002) J. Biol. Chem. 277, 50121–50130
26. Medvedeva, M. V., Kolobova, E. A., Wang, P., and Gusev, N. B. (1996) Biochem. J. 315, 1021–1026
27. Winder, S. J., Walsh, M. P., Vazulla, C., and Johnson, J. D. (1993) Biochemistry 32, 13327–13333
28. Willis, L. F., McCubbin, W. D., and Kay, C. M. (1993) Biochemistry 32, 2321–2328
29. Willis, L. F., McCubbin, W. D., Gimm, M., Strasser, P., and Kay, C. M. (1994) Protein Sci. 3, 2311–2321
30. Boeringh, D., and Joseph, S. K. (2000) EMBO J. 19, 5450–5459
31. Uchida, K., Miyauchi, H., Furuichi, T., and Mikoshiba, K. (2003) J. Biol. Chem. 278, 16551–16560
32. Bultynck, G., Szlufcik, K., Kasri, N. N., Assenfa, Z., Callewaert, G., Missiaen, L., Parys, J. B., and De, S. H. (2004) Biochem. J. 381, 87–96
33. Bare, D. J., Kettlun, C. S., Liang, M., Bers, D. M., and Mignery, G. A. (2005) J. Biol. Chem. 280, 15912–15920