Specific Subdomains of Vav Differentially Affect T Cell and NK Cell Activation1

Daniel D. Billadeau, Stacy M. Mackie, Renee A. Schoon, and Paul J. Leibson2

The Vav protooncogene is a multidomain protein involved in the regulation of IL-2 gene transcription in T cells and the development of cell-mediated killing by cytotoxic lymphocytes. We have investigated the differential roles that specific protein subdomains within the Vav protooncogene have in the development of these two distinct cellular processes. Interestingly, a calponin homology (CH) domain mutant of Vav (CH−) fails to enhance NF-AT/AP-1-mediated gene transcription but is still able to regulate the development of cell-mediated killing. The inability of the CH− mutant to enhance NF-AT/AP-1-mediated transcription appears to be secondary to defective intracellular calcium, because 1) the CH− mutant has significantly reduced TCR-initiated calcium signaling, and 2) treatment with the calcium ionophore ionomycin or cotransfection with activated calcineurin restores NF-AT/AP-1-mediated gene transcription. The pleckstrin homology (PH) domain of Vav has also been implicated in regulating Vav activation. We found that deletion of the PH domain of Vav yields a protein that can neither enhance gene transcription from the NF-AT/AP-1 reporter nor enhance TCR- or FcγR-mediated killing. In contrast, the PH deletion mutant of Vav is able to regulate the development of natural cytotoxicity, indicating a functional dichotomy for the PH domain in the regulation of these two distinct forms of killing. Lastly, mutation of three tyrosines (Y142, Y160, and Y174) within the acidic domain of Vav has revealed a potential negative regulatory site. Replacement of all three tyrosines with phenylalanine results in a hyperactive protein that increases NF-AT/AP-1-mediated gene transcription and enhances cell-mediated cytotoxicity. Taken together, these data highlight the differential roles that specific subdomains of Vav have in controlling distinct cellular functions. More broadly, the data suggest that separate lymphocyte functions can potentially be modulated by domain-specific targeting of Vav and other critical intracellular signaling molecules. The Journal of Immunology, 2000, 164: 3971–3981.

A ctivation of cells within the immune system involves the interaction of various cell-surface receptors on the immune cells with their respective ligands. For instance, T cell activation requires the interaction of the TCR on the T cell with a foreign peptide Ag in the context of endogenous major histocompatibility complex on an APC (1). In contrast, NK cells can use their low-affinity FcγRIIIA receptor to bind Ab coated virus-infected cells and thereby mediate Ab-dependent cellular cytotoxicity (2). A common feature of these and other immune activating receptors is that they require the activation of proximal protein tyrosine kinases (PTK)3 to activate multiple intracellular biochemical signaling cascades (3, 4). These intracellular generated signals lead to functional outcomes as diverse as gene transcription, proliferation, differentiation, and cellular cytotoxicity. One intracellular signaling molecule involved in the regulation of both TCR-initiated gene transcription (5, 6) and the development of cellular cytotoxicity by NK cells and CTLs is the Vav protooncogene (7).

The Vav gene product was initially identified due to a mutation that allowed it to transform fibroblasts (8). Interestingly, Vav is primarily expressed in hemopoietic cells (8) and is tyrosine phosphorylated following cross-linking of many multisubunit immune recognition receptors, including the TCR (9, 10) and FcγRIIIA (11, 12). The requirement for Vav in TCR-mediated signaling has been demonstrated in mice carrying a disruption of the Vav gene. These mice have diminished T cell numbers, proliferate poorly, and secrete little or no IL-2 in response to TCR stimulation (13–15). In addition, more recent experiments using T cells from Vav-deficient mice have identified roles for Vav in mediating TCR capping in thymocytes, regulation of positive and negative selection, and regulation of intracellular Ca2+ fluxes in response to TCR stimulation (16–20). Furthermore, using the Jurkat T cell line, it was found that overexpression of Vav could enhance basal and TCR-mediated gene transcription of the IL-2 gene or a reporter construct containing multiple NF-AT binding sites (5, 6). Together, these data suggest a key role for Vav in mediating signals transduced from the TCR. More recently, we have demonstrated that Vav is activated during both natural cellular cytotoxicity and killing mediated through the FcγRIIIA receptor on the NK cell surface (7). Our data suggest a role for Vav in regulating the development of granule polarization, an event required for cell-mediated killing. Together, these data clearly demonstrate that Vav plays important roles in the activation of T cells and NK cells, resulting in two very distinct biological outcomes, namely gene transcription and cellular cytotoxicity.

Recent in vivo and in vitro studies have demonstrated that Vav tyrosine phosphorylation is required for its ability to act as a guanine nucleotide exchange factor (GEF) for the Rho family of low m.w. GTP-binding proteins, converting them from an inactive

1 This research was supported by the Mayo Foundation and by National Institutes of Health Grant CA-47752. D.D.B. is a Leukemia Society of America Special Fellow and is supported by a grant from the Levy Foundation.

2 Address correspondence and reprint requests to Dr. Paul J. Leibson, Department of Immunology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail address: leibson.paul@mayo.edu

3 Abbreviations used in this paper: PTK, protein tyrosine kinase; CH, calponin homology; PH, pleckstrin homology; AD, acidic domain; GEF, guanosine nucleotide exchange factor; SH, Src homology; ERK, extracellular regulated kinase; PI3-K, phosphotidylinositol 3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; MOI, multiplicity of infection; ADCC, Ab-dependent cellular cytotoxicity.

Department of Immunology, Mayo Graduate and Medical Schools, Mayo Clinic, Rochester, MN 55905
Received for publication November 5, 1999. Accepted for publication February 1, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1, 2000.

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00
VAP-DEPENDENT ACTIVATION OF T AND NK CELLS

GDP-bound state to an active GTP-bound state (21–23). The GEF activity of Vav is mediated by its Dbl domain, which is similar in structure to a domain found in the protooncogene dbl which is also a GEF for Rho family GTPases (24, 25). In addition to this domain, Vav contains a Src homology (SH) 2 domain, two SH3 domains, a cysteine-rich region, a pleckstrin homology (PH) domain, an acidic domain (AD), and a calponin homology (CH) domain (26, 27). The presence of so many domains within Vav sug-
gest that it may serve to interact with or bring together many signal transduction pathways (26, 27). Indeed, mutations within the SH2 domain were found to impair the ability of oncogenic Vav to transform fibroblasts (28), or to interact with the SLP-76 adaptor protein and thereby control NFAT-mediated gene transcription in Jurkat T cells (29). In addition, mutations within the cysteine-rich region of the dbl domain have been shown to block the intrinsic GEF activity of Vav (22). Although partial deletion of the CH domain results in the production of an oncogene when expressed in mouse fibroblasts (8, 30), this same deletion mutant is unable to enhance NFAT-mediated gene transcription in Jurkat T cells (6). The exact role that the CH domain has in Vav-mediated TCR signaling events is currently unclear. However, it has been sug-
gested that the CH domain may be important in binding to poly-
merizing actin and may thereby localize Vav and its associated molecules to zones of activation (31). Lastly, in vitro studies have suggested that the ability of Vav to become tyrosine phosphory-
lated by Lck, a Src family PTK, requires an intact PH domain, phosphotidylinositol 3-kinase (PI3-K)-generated products, and tyro-
sine 174 (Y174) in the AD (32, 33). These data suggest a role for P3-K in the activation of Vav. However, the exact role that the PH or the AD have on the ability of Vav to regulate gene transcription or cell-mediated killing has not been examined.

To determine the functional significance that the CH, PH, and AD have in mediating normal Vav activity, we have designed a series of mutations within these domains and measured their ability to regulate NF-AT/AP-1-mediated gene transcription, and the development of cell-mediated cytotoxicity. To this end, we have identified that the CH domain is important in allowing Vav to enhance the Ca2⁺ arm of the signaling pathway following TCR cross-linking. Deletion or specific mutation of the CH domain results in a protein that lacks the ability to enhance NF-AT/AP-1-mediated gene transcription compared with wild-type Vav. How-
ever, this defect can be overcome by addition of ionomycin or by cotransfection with activated calcineurin. Interestingly, these CH mutations are not defective in their ability to regulate cell-mediated killing or cell-mediated cytotoxicity, indicating a differential requirement for cellular cytotoxicity, enhancement of natural cytotoxicity by the PH domain, and AD have in mediating normal Vav activity, we have designed a series of mutations within these domains and measured their ability to regulate NF-AT/AP-1-mediated gene transcription, and the development of cell-mediated cytotoxicity. To this end, we have identified that the CH domain is important in allowing Vav to enhance the Ca2⁺ arm of the signaling pathway following TCR cross-linking. Deletion or specific mutation of the CH domain results in a protein that lacks the ability to enhance NF-AT/AP-1-mediated gene transcription compared with wild-type Vav. However, this defect can be overcome by addition of ionomycin or by cotransfection with activated calcineurin. Interestingly, these CH mutations are not defective in their ability to regulate cell-mediated killing, indicating a differential role for this domain in Vav function during these two separate biological responses. The PH domain has also been suggested to regulate Vav activation. However, whereas Vav mutants lacking their PH domain are defective in their ability to enhance NF-AT/AP-1 reporter activity and TCR- or FcR-mediated cellular cytotoxicity, enhancement of natural cytotoxicity by the PH deletion mutants is unaffected, indicating a differential requirement for the PH domain in the regulation of these two distinct forms of cell-mediated killing. Lastly, mutation of all three tyrosines within the AD results in a protein that is hyperactive in its capacity to mediate cellular activation. This hyperactivity is due in part to the ability of this mutant to maintain a high level of intracellular Ca2⁺. Together, these data highlight the differential roles that these three domains have on Vav-dependent activation of NF-AT/AP-1-mediated gene transcription and cellular cytotoxicity.

Materials and Methods

Reagents, cells, and Abs

Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO) The Jurkat T cell line, anti-CD3-producing hybridoma OKT3 (murine IgG2a), K562 erythroid leukemia cell line, and murine mastocytoma cell line P815 were obtained from American Type Culture Collection (Manassas, VA). Human NK cells and CD8⁺ T cells were cloned by limiting dilution as previously described (34). Abs included the anti-FLAG murine mAb FLAG-M2 (Sigma), anti-

DNA constructs and recombinant vaccinia virus generation

The recombinant FLAG.Vav and FLAG.Vav.C295S (C295S) recombinant vaccinia virus have been previously described (6). To obtain pCDNA3 expression constructs containing FLAG.Vav, the FLAG.Vav coding sequence was removed from the vaccinia cloning vector (pSHN11.FLAG.Vav) by double digestion with HindIII and Nol. The fragment was then subcloned using standard molecular biology techniques into similarly digested pCDNA3. All of the mutants described below were initially made in pSHN11.FLAG.Vav and then subcloned into the mammalian expression vector pCDNA3 as described above. All recombinant vaccinia virus were produced as previously described (36). Using the site-directed mutagenesis kit from Clontech (Palo Alto, CA), several of the mutations were obtained as previously described (6).

Briefly, the CH′ mutant of Vav was generated by introduction of an Nco site within the DNA sequence encoded by amino acid 115–117 of Vav using the mutagenic oligonucleotide 5′-CATCCTACCCCTGTGCTGAGCATG GACACCATTCGCCAG-3′ and the pSHN11 selection oligonucleotide 5′- GAGGACCTACCCGCACGTGC-3′. The resulting mutant was digested with Nco to remove amino acids 1–115 and tagged with the FLAG adaptor (6). Introduction of the Nco site at amino acid 115–117 introduces a Ser-Asp mutation at amino acid 117, which is now amino acid 2 of the CH′ mutant. Single amino acid point mutations within the CH domain were also generated using the pSHN11 selection oligonucleotide along with the specific mutagenic oligonucleotides, 5′-GGTGTTGCCCACGGTCTGAC-3′ and 5′- GACGTTGACCCTCCAGCAG-3′. Two additional mutations that were introduced into the mutagenic oligonucleotides were the pSHN11 mutant oligonucleotide containing an Nco site proximal to the ATG start codon for addition of the FLAG-Tag (5′-GGGACCATGCTGACGGACACAG-3′) and a 3′ oligonuclo
deotide containing an internal Nco site for subcloning into the pSHN11.FLAG.Vav.CH′ mutant (5′- TCGTCGTGACGAAGACACGCATA-3′). The amplified product was subcloned into the pCR2 TA cloning vector (Invitrogen, Carls-
bad, CA), recombinant plasmid was sequenced, and then the α-PIX CH′ vector was subcloned as an Nco fragment into similarly digested pSHN11.FLAG.Vav.CH′. The PH domain mutant was generated by introducing the pSHN11 mutagenic oligonucleotide along with two mutagenic oligonucleo-
tides that introduce Sau III sites at amino acid 397/398 (5′-GAGACACCTGGGACACCTGACTGAC-3′) and amino acid 506/ 507 (5′-GAAATGCCATCCTCAAGCGCCACGAG-3′). The resulting mutant was digested with Sau to remove the PH domain and following gel purification was religated. This produced a PH′ mutant lacking amino acids 398–506. The 3′SAF mutant and individual amino acid point mutants were generated using the pSHN11 selection oligo-

tides described above along with additional specific oligonucleotides, 5′-GACGTTGACATCCTCCAGCAG-3′ and amino acid 506/ 507 (5′-GAAATGCCATCCTCAAGCGCCACGAG-3′). The amplified product was subcloned into the pCR2 TA cloning vector (Invitrogen, Carlsbad, CA), recombinant plasmid was sequenced, and then the α-PIX CH′ vector was subcloned as an Nco fragment into similarly digested pSHN11.FLAG.Vav.CH′. The PH domain mutant was generated by introducing the pSHN11 mutagenic oligonucleotide along with two mutagenic oligonucleo-
tides that introduce Sau III sites at amino acid 397/398 (5′-GAGACACCTGGGACACCTGACTGAC-3′) and amino acid 506/ 507 (5′-GAAATGCCATCCTCAAGCGCCACGAG-3′). The resulting mutant was digested with Sau to remove the PH domain and following gel purification was religated. This produced a PH′ mutant lacking amino acids 398–506. The 3′SAF mutant and individual amino acid point mutants were generated using the pSHN11 selection oligo-

Reagents, cells, and Abs

Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO) The Jurkat T cell line, anti-CD3-producing hybridoma
Electroporation and luciferase assays

Using the BTX Electro Square Manipulator Model 600 (San Diego, CA), Jurkat T cells (10^6) in RPMI 1640 media containing 10% FCS (RPMI-10) were electroporated with 20 μg of pCDNA3 control vector, Vav-expressing vector, or mutant Vav-expressing vectors along with 10 μg of the previously described pNF-AT1-luciferase construct (39). This construct contains 5 tandem repeats of the NF-AT/AP-1-binding site located approximately at position –287 in the murine IL-2 promoter. In one set of experiments, 0.5 μg of a construct expressing an activated allele of calcineurin was also added to the electroporation. Following the electroporation, the cells were resuspended in 5 ml of RPMI-10, transferred to a six-well tissue culture plate, and left to recover overnight in a humidified 37°C incubator. After 18–24 h of incubation, the cells were aliquoted, 1 ml each of the subpopulations containing either 100 μl of RPMI-10 alone or 100 μl of RPMI-10 with anti-CD3 mAb mAb OKT3 (1 μg/ml), a combination of anti-CD3 (1 μg/ml) and PMA (20 ng/ml), anti-CD3 (1 μg/ml), and ionomycin (2 μM, Calbiochem-Novabiochem, La Jolla, CA), or a combination of PMA (20 ng/ml) and ionomycin (2 μM). After 6 h of incubation in a humidified 37°C incubator, the cells were harvested, lysed, and luciferase activity was assayed using the luciferase assay system from Promega (Madison, WI) on a luminometer model LB 9501/16 lumat (Berthold Systems, Alquiippa, PA). The percentage of maximal NF-AT activity was determined by dividing the luciferase activity obtained in the absence or presence of stimulation by that obtained with stimulation by PMA plus ionomycin. The maximum NF-AT/AP-1 responses did not differ significantly between transfection conditions. Protein expression of the electrroporated constructs was determined by immunoprecipitating FLAG-Vav-expressing proteins from 2 × 10^6 electroporated cells using the anti-FLAG mAb, followed by anti-FLAG immunoblotting.

Cytotoxicity assays

The 3Cr release assays were performed as described previously (34). In all cases, spontaneous release did not exceed 10% of maximum release. In redirected cytotoxicity assays, NK clones and CD8^+ T cell clones were only able to kill the P815 target cell in the presence of anti-FcR or anti-CD3 mAb, respectively. Lytic units were calculated based on 20% cytotoxicity (40).

Ca^{2+} mobilization assays

Changes in levels of intracellular Ca^{2+} of vaccinia infected, INO-1-loaded cells was assessed by flow cytometry using a FACSstar^+ (Becton Dickin-

son, San Jose, CA) as previously described (41). Briefly, Jurkat T cells were infected with the indicated nonrecombinant (WR) or recombinant vaccinia virus for 2 h in a humidified 37°C incubator at a multiplicity of infection (MOI) of 10:1. For the last 30 min of the infection, the cells were loaded with 5 μM Indo-1 (Calbiochem-Novabiochem). They were then washed in PBS containing 1% BSA and resuspended in RPMI-10 until analyzed. For analysis, the Indo-1-loaded Jurkat T cells were incubated with either goat anti-mouse IgG Fab( ') alone (as a baseline control) or a combination of anti-CD3 mAb OKT3 (2.5 μg/ml) and goat anti-mouse IgG F(ab')2. The sample was immediately analyzed by flow cytometry using a UV laser for excitation with violet (390 nm) and blue (500 nm) fluorescence emissions recorded. In some cases, 10 μM EGTA was added to the sample just before the addition of goat anti-mouse IgG Fab( ')2. The data plots were generated using the FlowJo software program (Tree Star, Palo Alto, CA).

Cytoplastic staining for recombinant protein

Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (PharMingen, San Diego, CA). The cells were then stained for 30 min on ice with 0.25 μg/ml of anti-FLAG mAb or an IgGl isotype-matched control, washed three times in permeabilization buffer, and then stained for 30 min on ice in 50 μl of permeabilization buffer containing a 1:1500 dilution of FITC-conjugated goat anti-mouse IgG (Calbiochem). The cells were then washed three times in permeabilization buffer and analyzed by flow cytometry.

Cell stimulation and immunoblot analysis

In all experiments, NK clones or Jurkat T cells were infected with the indicated recombinant vaccinia virus at an MOI of 20:1 for 5 or 10:1 for 2 h, respectively. For experiments where NK cells were activated by target cells, 5 × 10^5 cloned NK cells were briefly pelleted with 2.5 × 10^5 target cells and then incubated at 37°C for the indicated period of time. In experiments involving specific cell-surface receptor cross-linking, 5 × 10^5 Jurkat T cells were incubated for 3 min on ice with anti-CD3 mAb OKT3 (1 μg/ml). Washed cells were then incubated with goat anti-mouse IgG F(ab')2, at 37°C for the indicated period of time. Following stimulation, the cells were lysed on ice for 10 min in 1 ml of buffer containing 20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 50 mM NaPO_4_, 0.1% BSA, 1 mM NaVO_4, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. Cellular debris was removed by centrifuga-
tion at 14,000 rpm for 5 min at 4°C. FLAG-Vav was immunoprecipi-
tated from the lysate for 1–2 h at 4°C using 1 μg of anti-FLAG-M2 mAb bound to goat anti-mouse IgG-agarose beads. Protein complexes were washed four times in wash buffer (lysis buffer lacking BSA). Bound pro-
teins were then eluted in 40 μl of SDS sample buffer, resolved by SDS-
PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Tyrosine-phosphorylated proteins were detected using the 4G10 mAb, and anti-FLAG proteins were detected using anti-FLAG mAb followed by sheep anti-mouse IgG coupled to HRP (Amersham, Buckingham-
shire, U.K.) and the enhanced chemiluminescence detection system from Amersham.

Results

Mutation of the Vav CH domain affects Vav-dependent NF-AT/AP-1 transcription

Previous work demonstrated that Vav, but not the oncogenic ver-

sion of Vav that is lacking the first 67 amino acids of the CH domain, can enhance basal activation of the transcription factor NF-AT in a Jurkat T cell line (6). This result suggested that the CH domain plays an important role in the regulation of Vav-dependent NF-AT activation in T cells. To further characterize the function of this domain, we generated a CH deletion mutant of Vav (CH^-) that lacks the first 115 amino acids (Fig. 1, CH^- mutant). In agree-

ment with previous data, transfection of Jurkat T cells with a mam-

mal expression vector expressing Vav, results in enhanced NF-
AT/AP-1 activity as measured with a luciferase reporter construct con-
taining a tandem repeat of the NF-AT/AP-1-binding sites from the IL-2 promoter (Fig. 2A). The enhancement of NF-AT/AP-1 activity is most pronounced following anti-CD3 stimulation, or stimulation with a combination of anti-CD3 and PMA (Fig. 2A). In contrast, the CH^- mutant of Vav is unable to enhance tran-
scriptional activity from the NF-AT/AP-1-binding sites from the IL-2 promoter (Fig. 2A). Thus, this deletion mutant behaves similar to the previously de-

scribed oncogenic version (6). The inability of the CH^- mutant to enhance NF-AT/AP-1-mediated gene transcription is not due to its inability to undergo receptor-initiated tyrosine phosphorylation, as both Vav and the CH^- mutant demonstrate similar kinetics of

FIGURE 1. Structural features of Vav and mutants used in this study. The murine Vav protoonco

protein is an 845-aa protein that has a number of complex structural domains, including CH, AD, PH, cysteine-rich (CR), proline-rich (PR), SH3, and SH2 domains. Wild-type Vav is schematically depicted in the top line of this figure (Vav). The relative numeric positions of amino acids defining borders of certain subdomains of Vav are shown above the Vav structure. The mutants used in this study include the CH domain deletion mutant of Vav (CH^-) missing amino acids 1–115, the PH domain deletion mutant (PH^-) missing amino acids 398–506, and an AD mutant of Vav (3YF) in which tyrrosines 142, 160, and 174 have been mutated to phenylalanine.

3YF

FIGURE 1.
The data shown is representative of six separate experiments. The intact CH domain of Vav is required for enhanced NF-AT-mediated gene transcription. Jurkat T cells (10^7) were electroporated with 10 μg of an NF-AT/AP-1.luciferase reporter construct and 20 μg of the indicated regulatory plasmids (right insets). All Vav and Vav mutant constructs contain an amino-terminal 8-aa FLAG epitope. Following electroporation, the cells were left to recover overnight, and then they were left unstimulated or stimulated for 6 h with anti-CD3 mAb mAb OKT3 (1 μg/ml), a combination of anti-CD3 (1 μg/ml) and PMA (20 ng/ml), or a combination of PMA (20 ng/ml) and ionomycin (2 μM). Cells were then harvested and assayed for luciferase activity as described in Materials and Methods. The data are presented as the percentage of maximal NF-AT/AP-1 responses in any stimulation condition, reaching nearly 90% of the maximal NF-AT/AP-1 response with anti-CD3 and PMA stimulation (Fig. 2C; compare Vav to Vav.CAAX). In contrast, the CAAX sequence to the CH^-mutant (CH^-CAAX) did not overcome its inability to enhance transcription from the NF-AT/AP-1 reporter (Fig. 2C). These data suggest that the sole purpose of the CH domain is not the recruitment of activated Vav to the inner leaflet of the plasma membrane, and it must therefore be involved in some other process of Vav activation.

**The Vav CH domain is involved in the regulation of TCR-induced intracellular Ca^{2+} fluxes**

Recent studies have suggested that Vav may regulate Ca^{2+} influxes following TCR cross-linking (16, 20). Because the CH^-mutant fails to enhance NF-AT activity in the presence of anti-CD3 and PMA, which should pharmacologically promote a Ras/protein kinase C signal, we postulate that the defect of the CH^-mutant might be in its inability to activate intracellular Ca^{2+} fluxes following anti-CD3 cross-linking. To test this possibility, we infected Jurkat T cells with recombinant vaccinia virus expressing either Vav, the CH^-mutant, or a nonrecombinant control virus (WR), then loaded them with Indo-1 and measured TCR-induced tyrosine phosphorylation following anti-CD3 stimulation of Jurkat T cells (D.D.B. and P.J.L., unpublished observations).

The CH domain is found as a single domain in many signaling molecules, as well as in the actin-binding protein calponin. Therefore, we asked whether specific point mutations of highly conserved residues within the Vav CH domain would impact the ability of these mutants to regulate the NF-AT/AP-1 reporter. Among the specific point mutations are cysteine to serine mutations at positions 44 and 71 of Vav (C44S and C71S) and mutation of the conserved asparagines N48 and N74 to alanine (N48A and N74A). As shown in Fig. 2B, the Vav point mutants C44S, N48A, and C71S did not affect their ability to enhance NF-AT/AP-1-mediated gene transcription compared with wild-type Vav. In contrast, similar to that of the CH^-mutant, the N74A mutation was unable to enhance activity of the NF-AT/AP-1 reporter (Fig. 2B, compare CH^- to N74A). This discrepancy is not due to levels of expression of transfected protein as all of the point mutants are equivalently expressed (Fig. 2B, left inset). This suggests that N74 is an important residue within the CH domain of Vav.

It was recently suggested that the CH domains of Vav and the Rac-1 GEF, α-PIX, are highly conserved at the amino acid level and might therefore be functionally interchangeable (42). Therefore, we asked if the α-PIX CH domain could functionally compensate for the Vav CH domain in a chimeric protein. However, as shown in Fig. 2B, the chimera of Vav containing the α-PIX CH domain (PIX.CH.Vav) in place of the Vav CH domain was unable to enhance NF-AT/AP-1-mediated gene transcription (compare Vav to PIX.CH.Vav, Fig. 2B). Therefore, although these two CH domains contain the highly conserved asparagine at 74, they are not functionally equivalent. Moreover, this data suggests that the CH domain of Vav has other structural requirements aside from N74. Consistent with this idea, oncogenic-Vav, which contains N74, is unable to enhance basal NF-AT activation (6).

The function of the CH domain in Vav is unclear. One possibility is that it is involved in the localization of Vav upon stimulation by association with some other activated protein, a membrane-bound protein, or the intracellular membrane itself. To determine whether membrane localization could overcome the inability of the CH^-mutant to activate the NF-AT/AP-1 reporter, we generated a Vav and CH^-mutant containing a CAAX membrane localization signal from the RhoA protein. In contrast to wild-type Vav, cellular fractionation and immunoblot analysis demonstrated that the majority of Vav.CAAX and CH^-CAAX is membrane bound (data not shown). As shown in Fig. 2C, Vav.CAAX, which contains the CH domain, dramatically increases NF-AT/AP-1-mediated gene transcription following transfection in any stimulation condition, reaching nearly 90% of the maximal NF-AT/AP-1 response with anti-CD3 and PMA stimulation (Fig. 2C; compare Vav to Vav.CAAX). In contrast, addition of the CAAX sequence to the CH^-mutant (CH^-CAAX) did not overcome its inability to enhance transcription from the NF-AT/AP-1 reporter (Fig. 2C).
intracellular Ca\textsuperscript{2+} fluxes by flow cytometry. We have found that a suboptimal concentration of anti-CD3 mAb is required to observe differences in intracellular Ca\textsuperscript{2+} following TCR cross-linking (D.D.B. and P.J.L., unpublished observations). All three viral infected populations had similar baseline Ca\textsuperscript{2+} profiles in the absence of TCR stimulation (Fig. 3A). Jurkat T cells infected with WR demonstrate an increase in intracellular Ca\textsuperscript{2+} levels upon anti-CD3 stimulation. The duration of this response is enhanced in cells infected with recombinant Vav virus (Fig. 3A, compare Vav with WR). In contrast, we did not observe this extended Ca\textsuperscript{2+} influx in cells infected with the CH\textsuperscript{-} mutant (Fig. 3A, compare Vav with CH\textsuperscript{-}), suggesting the CH domain may be important for Vav regulation of TCR-induced Ca\textsuperscript{2+} fluxes. If regulation of Ca\textsuperscript{2+} levels is the function of the CH domain, then treatment of CH\textsuperscript{-} mutant-transfected Jurkat T cells with a combination of anti-CD3 plus the calcium ionophore, ionomycin, should lead to levels of NF-AT activation observed with Vav transfection. In fact, treatment of Vav and CH\textsuperscript{-} mutant transfected Jurkat T cells with anti-CD3 plus ionomycin yields comparable levels of NF-AT/AP-1 activity (Fig. 3B). Furthermore, if we bypass the calcium requirement for NF-AT activation by cotransfecting in a constitutively active calcineurin construct along with the Vav constructs, we see that there is no difference between Vav and the CH\textsuperscript{-} mutants ability to enhance transcription from the NF-AT/AP-1 reporter in the presence of anti-CD3 stimulation or a combination of anti-CD3 plus PMA (Fig. 3C). Together, these data suggest that the CH domain of Vav is important for its ability to regulate TCR-induced Ca\textsuperscript{2+} fluxes.

**The CH domain of Vav is not required for enhancement of cellular cytotoxicity**

We have previously demonstrated that overexpression of Vav in NK or CTLs can enhance the ability of these cells to mediate cellular cytotoxicity through its regulation of granule polarization, a critical event in the development of cell-mediated killing (7). To investigate the role of the Vav CH domain in the regulation of cell-mediated killing, we evaluated cytotoxicity using cloned human NK cells or CD8\textsuperscript{+} T cell lines that had been infected with recombinant vaccinia virus-expressing Vav, the CH\textsuperscript{-} mutant, a mutant that lacks GEF activity (C529S), or the WR control virus. As previously demonstrated, infection of NK cells with Vav increased their capacity to kill the K562 erythroid leukemia cell line by natural cytotoxicity, as well as their ability to mediate killing through the FcR in a reverse Ab-dependent cellular cytotoxicity (ADCC) assay using the murine mastocytoma cell line P815 as a target (Fig. 4A). Also, killing initiated through the TCR of a CD8\textsuperscript{+} T cell line is enhanced by overexpression of Vav, compared with WR infected cells (Fig. 4A). In contrast, overexpression of the GEF mutant C529S in either NK cells or CTLs does not enhance natural or Ab-dependent killing, indicating that intact GEF activity is required for normal Vav function (Fig. 4A; compare Vav with C529S). Surprisingly, although the CH\textsuperscript{-} mutant is unable to enhance NF-AT/AP-1-mediated gene transcription, it is fully competent in its ability to regulate NK cell-mediated natural cytotoxicity and ADCC and TCR-initiated killing by CTLs (Fig. 4A). This difference was not due to the levels of expression of the CH\textsuperscript{-} mutant protein because 1) similar levels of recombinant protein (range 35–80%) (Fig. 4A) and data not shown). 2) similar proportions of the cells expressed the recombinant protein (range 35–80%) (Fig. 4C and data not shown). Together these data indicate that the CH domain of Vav is required for Vav-dependent regulation of NF-AT/AP-1-mediated gene transcription but is dispensable when it comes to the regulation of cellular cytotoxicity.

**The PH domain of Vav is required for optimal NF-AT activation, ADCC, but not natural cytotoxicity**

A previous report has suggested that the PH domain of Vav is an important regulator of Vav GEF activity in vitro (32), and, in one
example in vivo, deletion of the PH domain resulted in the production of a protein with increased GEF activity (33). Together, these data suggest a critical regulatory role for the PH domain in normal Vav function. To determine the role of the Vav PH domain in immune cells, in particular its ability to regulate gene transcription and cell-mediated killing we generated a PH deletion mutant (PH$^2$) of Vav that is missing amino acids 398–506 (Fig. 1, PH$^2$ mutant). We first determined if deletion of this protein domain would affect the ability of the PH$^2$ mutant to undergo receptor-initiated tyrosine phosphorylation. In fact, TCR or FcR cross-linking or incubation of NK cells with susceptible targets resulted in receptor-mediated tyrosine phosphorylation of both Vav and the PH$^2$ mutant (data not shown). We next tested whether this mutant can enhance NF-AT/AP-1-mediated gene transcription. Jurkat T cells were transfected with the NF-AT/AP-1 luciferase reporter construct, with or without the Vav or the PH$^2$ mutant expression constructs. As previously demonstrated, transfection of Vav results in an increase in NF-AT/AP-1 reporter activity following anti-CD3 and anti-CD3 plus PMA stimulation (Fig. 5A). In contrast, although the PH$^2$ mutant of Vav was able to undergo tyrosine phosphorylation following anti-CD3 cross-linking, it could not enhance NF-AT/AP-1-mediated gene transcription under the stimulatory conditions.

**FIGURE 4.** The CH$^2$ mutant of Vav can regulate cellular cytotoxicity. A, NK clones (2 × 10$^6$) and CD8$^+$ T cell clones (1 × 10$^6$) were infected with the indicated recombinant vaccinia viruses (right inset). The infected NK clones were then incubated with either $^{51}$Cr-labeled K562 cells (NK/K562) or $^{51}$Cr-labeled P815 cells coated with 0.15 μg/ml of the anti-FcR mAb 3G8 (NK/P815 + anti-FcR). The infected CD8$^+$ T cell clones were incubated with $^{51}$Cr-labeled P815 cells coated with 0.15 μg/ml of anti-CD3 mAb OKT3 (CD8$^+$ T/P815 + anti-CD3). The data are expressed as lytic units. The data shown is representative of six separate experiments. B, Jurkat T cells (1 × 10$^6$) were electroporated with the indicated constructs as described in Fig. 2 and left to recover overnight. NK clones (5 × 10$^6$) were infected with the indicated virus at an MOI of 20:1 for 6 h. Whole-cell lysates were prepared, and the expression of endogenous Vav and transfected Vav (CH$^-$) or vaccinia-encoded Vav (CH$^-$) were analyzed by Western blotting. The lysates, 125 μg of protein per lane, were resolved by SDS-PAGE, transferred to a nylon membrane, and probed with anti-Vav polyclonal rabbit antisera. This is a representative example of three separate experiments. C, Jurkat T cells (1 × 10$^6$) were electroporated with the CH$^-$ construct as described in Fig. 2 and left to recover overnight. NK clones (3 × 10$^6$) were infected with the CH$^-$ mutant at an MOI of 20:1 for 6 h. The samples were then fixed, permeabilized, and stained with anti-FLAG mAb or an isotype-matched mAb, followed by goat anti-mouse FITC. The samples were then analyzed by FACS for fluorescence. Using the isotype-matched mAb as a control, separate gates were set up for cells expressing (filled histogram) or not expressing (open histogram) the recombinant protein. In this experiment, 48% of the Jurkat population was expressing the recombinant protein compared with 35% of the NK population. This is a representative example of three separate experiments.

**FIGURE 5.** The PH$^2$ deletion mutant of Vav fails to enhance NF-AT activity, TCR-initiated cytotoxicity, or FcR-initiated cytotoxicity, but does augment natural cytotoxicity. A, Jurkat T cells (10$^7$) were electroporated with the indicated constructs (right inset) and stimulated as described in Fig. 2. Data are expressed as the percentage of maximal NF-AT/AP-1 activity. The maximal NF-AT/AP-1 response did not vary significantly between electroporation conditions. Left inset, Protein expression of Vav and the PH$^2$ mutant were determined as described in Fig. 2. This is a representative example of four separate experiments. B, NK clones and CD8$^+$ T cell clones were infected with the indicated recombinant vaccinia viruses (right inset) and subjected to cell-mediated killing of $^{51}$Cr-labeled targets. This is a representative example of five separate experiments.
conditions tested (Fig. 5A). These data suggest that the PH domain of Vav is required for optimal Vav-dependent activation of NF-AT/AP-1-mediated gene transcription.

The role of the Vav PH domain in regulating cellular cytotoxicity was next examined in NK cells and CD8+ T cells infected with the recombinant vaccinia virus. Following infection, the cytotoxic potential of the infected cells was assessed by reverse ADCC or natural cytotoxicity (Fig. 5B). As previously observed, infection of a CD8+ T cell line with Vav enhanced its ability to kill following anti-CD3 cross-linking in a reverse ADCC (Fig. 5B). However, the PH− mutant was unable to couple Vav to the TCR-generated cytotoxic response in the CD8+ T cell line (Fig. 5B). The inability of the PH− mutant to enhance killing was not due to the level of expression, as Vav and the PH− mutant were expressed at similar levels (data not shown). Interestingly, NK cells expressing the PH− mutant showed a dichotomy in their ability to regulate ADCC vs natural cytotoxicity. Although Vav-expressing NK cells enhanced both forms of cellular cytotoxicity, the PH− mutant-expressing cells were defective in their ability to enhance FcR-initiated killing, but not natural cytotoxicity (Fig. 5B, compare PH− in NK/K562 with NK/P815 plus anti-FcR). These data delineate a differential requirement for the PH domain during these two distinct forms of NK cell-mediated killing.

The AD domain is a site of negative regulation in Vav

Tyrosine 174 (Y174) within the AD has been found to be a site of phosphorylation by both Lck and the Syk PTK, and evidence suggests that Y174 is required for regulating Vav GEF activity (32, 43). Two other tyrosines (Y142 and Y160) are also located within the AD and may be potential sites of tyrosine phosphorylation important for the activation of Vav. To determine the role that these three tyrosines have in regulating Vav activity, we mutated them to phenylalanine, creating a 3YF mutant (Fig. 1, 3YF mutant). We initially determined if mutation of these residues affected the ability of Vav to couple to TCR-initiated tyrosine phosphorylation. Therefore, we infected Jurkat T cells with recombinant FLAG.Vav- or FLAG.Vav.3YF-expressing vaccinia virus and stimulated them with cross-linked anti-CD3 for the indicated times. Recombinant FLAG.Vav or FLAG.Vav.3YF was specifically immunoprecipitated and then analyzed for tyrosine phosphorylation. As seen in Fig. 6A, the FLAG.Vav.3YF is still capable of undergoing TCR-induced tyrosine phosphorylation. However, the level of tyrosine phosphorylation of FLAG.Vav.3YF is somewhat decreased compared with FLAG.Vav. In addition, in repeated experiments we found that the phosphorylation of FLAG.Vav.3YF in contrast to FLAG.Vav rapidly decreased below basal level by 15 min and completely disappeared by 30 min (Fig. 6A). Interestingly, although this mutant shows a decrease in the extent and duration of tyrosine phosphorylation following TCR crosslinking when transfected into Jurkat T cells, expression of the 3YF mutant in Jurkat T cells leads to increased levels of NF-AT/AP-1 activity above
those observed with Vav (Fig. 6B). Furthermore, all three tyrosines need to be mutated for optimal stimulation, as individual tyrosine mutants fail to hyperactivate the NF-AT/AP-1 reporter to the same levels observed with the 3YF mutant (Fig. 6B). However, the Y174F mutant consistently demonstrated an increase in NF-AT/AP-1 transcriptional activity over and above that observed with any of the other individual tyrosine mutants or Vav itself. These data indicate a role for the three tyrosines within the AD in regulating Vav activity, in particular that activity resulting in activation of NF-AT/AP-1 gene transcription.

To identify the mechanism by which mutation of the AD can result in the production of a hyperactive Vav protein, we assayed the ability of the 3YF mutant to mobilize Ca\(^{2+}\) in response to anti-CD3 stimulation. Jurkat T cells were infected with WR, Vav, or the 3YF mutant expressing viruses, loaded with Indo-1, and anti-CD3-induced intracellular Ca\(^{2+}\) mobilization was assayed by flow cytometry. All three infected cell populations had similar initial basal levels of intracellular Ca\(^{2+}\) (Fig. 6C). Consistent with the ability of the 3YF to enhance NF-AT/AP-1 reporter activity above that observed with Vav expression, the 3YF mutant demonstrated a markedly sustained elevation of intracellular Ca\(^{2+}\) (Fig. 6C). The Ca\(^{2+}\) flux in the 3YF-infected cells was maintained throughout the duration of the assay, suggesting that at least one mechanism by which the 3YF mutant is hyperactive in its regulation of NF-AT is that it causes an extension in elevated levels of intracellular Ca\(^{2+}\) following anti-CD3 cross-linking. The sustained Ca\(^{2+}\) flux observed in the 3YF-infected cells and the increase observed in the Vav-expressing cells could be inhibited by chelating extracellular Ca\(^{2+}\) with EGTA before TCR cross-linking (Fig. 6C, +EGTA). This suggests that the sustained increase in intracellular Ca\(^{2+}\) regulated by Vav and the 3YF mutant is due to an influx of extracellular Ca\(^{2+}\) resulting in sustained levels of intracellular Ca\(^{2+}\) and therefore increased NF-AT/AP-1-mediated gene transcription.

Lastly, we tested the ability of the 3YF mutant to enhance cellular cytotoxicity. NK cells and a CD8\(^{+}\) T cell line were infected with the indicated recombinant vaccinia viruses shown in Fig. 6D and then assayed for their cytotoxic response. We found that overexpression of the 3YF mutant enhances killing to levels observed with Vav overexpression during natural cytotoxicity and ADCC in both cell types. Our inability to observe a substantial increase in the 3YF activity over Vav in this assay may be because the level of enhancement observed with Vav overexpression is close to the maximal response that can be obtained. Alternatively, Vav-dependent changes in intracellular calcium may be less critical for the development of cellular cytotoxicity, as was observed previously for the CH\(^{-}\) mutant of Vav (see Fig. 4A).

**Discussion**

The Vav protooncogene is a multidomain protein involved in the regulation of gene transcription and the development of cell-mediated cytotoxicity in T and NK cells. The presence of a protein domain within Vav that confers GEF activity to Rho family GTPases, along with protein-protein and protein-lipid interacting domains, suggest that Vav may interact with and influence multiple intracellular biochemical signaling cascades. However, the precise roles that specific domains within the Vav protein play in the regulation of Vav-dependent signaling events in T and NK cells are not known. Here, we show that the CH domain, PH domain, and AD of Vav can differentially affect the regulation of NF-AT/AP-1-mediated gene transcription and the development of cell-mediated killing. Although deletion of the CH domain has no effect on the ability of Vav to regulate the development of natural cytotoxicity or ADCC by cytotoxic lymphocytes, it severely impacts its ability to regulate NF-AT/AP-1-mediated gene transcription following TCR stimulation in Jurkat T cells. The difference may be due in part to the inability of the CH deletion mutant of Vav to functionally transduce TCR-mediated signals culminating in the production of sustained intracellular Ca\(^{2+}\) fluxes that are required for optimal NF-AT activation (44–47) but not the development of cellular cytotoxicity, which can occur within 15 min of target recognition (48). We also demonstrated that a PH deletion mutant of Vav cannot properly regulate TCR-induced NF-AT/AP-1-mediated gene transcription, nor enhance cytotoxicity initiated through either the TCR on CD8\(^{+}\) T cells or the FcR on NK cells. Interestingly, the ability of the PH deletion mutant of Vav to enhance natural cytotoxicity is unaffected, suggesting a differential requirement for this domain in the development of these separate forms of killing. Lastly, we show that mutation of three critical tyrosines within the Vav AD results in a mutant protein that mediates superactivation of NF-AT/AP-1-mediated gene transcription. The hyperactive quality of this Vav mutant can be partly explained by its ability to greatly sustain elevated intracellular Ca\(^{2+}\) levels following TCR cross-linking. Together, our data define novel requirements and differential roles for these domains in Vav-dependent generation of NF-AT/AP-1-mediated gene transcription and cellular cytotoxicity.

Although partial deletion of the Vav CH domain produces an oncogenic protein when expressed in mouse fibroblasts (8, 30), this mutant does not function normally in the regulation of NF-AT-mediated gene transcription in Jurkat T cells (6). This result suggests that at least in T cells, the CH domain is required for the normal regulation of Vav function. Optimal NF-AT activation requires a rise in intracellular Ca\(^{2+}\) levels to activate the Ca\(^{2+}\)-dependent phosphatase calcineurin (49, 50). This activation leads to NF-AT dephosphorylation and subsequent nuclear localization (45, 46, 51–53). In addition to NF-AT activation, a Ras/protein kinase C-generated signal is also required for activation of the AP-1 transcription factor that binds with NF-AT to mediate transcriptional activation of the reporter construct (50, 54–56). A recent study demonstrated that constitutively activated Rac1, a Rho family GTPase that is activated by Vav, can mediate both NF-AT dephosphorylation and nuclear localization and AP-1 activation when overexpressed in mast cells (57). In addition, it was observed that thymocytes from mice carrying a disruption of the Vav gene are defective in their ability to mobilize Ca\(^{2+}\) and in their activation of the extracellular-regulated kinase (ERK) pathway in response to TCR and CD28 stimulation (20). Together, these data suggest that Vav has the potential to regulate both pathways required for optimal NF-AT/AP-1-mediated gene transcription. However, consistent with a previous report (58), we have been unable to identify Vav activity toward an isolated AP-1 luciferase reporter construct following TCR or CD28 cross-linking (D.D.B. and P.J.L., unpublished observations), suggesting that Vav may not directly activate the AP-1 transcription factors, but may instead lead to signals required for modification of, or sustained nuclear localization of, NF-AT. Indeed, a recent study has suggested that both Raf- and Rac-mediated signals can strongly stimulate the CBP/p300-mediated activation of a putative transcriptional activation domain from NF-ATc (59). Interestingly, the CH\(^{-}\) mutant we generated was unable to enhance NF-AT/AP-1 reporter activity even in the presence of both anti-CD3 and PMA stimulation, which will pharmacologically activate the Ras/protein kinase C pathway leading to AP-1 activation (see Fig. 2A). This observation suggests that inactivation of the CH domain produces a Vav protein that can no longer lead to sustained intracellular Ca\(^{2+}\) fluxes following TCR stimulation to give enhanced levels of NF-AT activation. Consistent with this hypothesis is the observation that overexpression of the CH\(^{-}\) mutant in Indo-I-loaded Jurkat T cells...
fails to enhance intracellular Ca\textsuperscript{2+} fluxes to those levels observed by Vav overexpression (see Fig. 3A). Furthermore, the defect in enhanced NF-AT activation by the CH\textsuperscript{−} mutant could be rescued by either costimulation with anti-CD3 plus ionomycin or cotransfection with an activated calcineurin construct (see Fig. 3, B and C). However, the mechanism by which the CH domain influences the ability of Vav to regulate Ca\textsuperscript{2+} fluxes following TCR stimulation remains unclear.

It had been originally suggested that the CH domain of Vav might interact with F-actin and thereby localize Vav and its associated molecules to zones of activation (31). However, recent reports have found that the CH domain of the actin-binding protein calponin, after which the Vav CH domain was identified, does not use this domain to bind actin (60). Consistent with these data, we have been unable to detect the association of actin with endogenous Vav or overexpressed FLAG.Vav in communoprecipitation studies (D.D.B. and P.J.L., unpublished observations). In addition, it has been suggested that proteins that use the CH domain to bind actin contain two tandem repeats of the domain (42). Although these domains are found as single units on many signaling proteins involved in the regulation of low-m.w. GTPases (31, 42), they are not functionally redundant because replacement of the α-PIX CH domain with that of the Vav CH domain did not enhance its ability to activate the NF-AT/AP-1 reporter (see Fig. 2B). Together, these data suggest that there is a specific substrate for this domain that helps link Vav to Ca\textsuperscript{2+} mobilization. However, the functional target of the CH domain in proteins that contain only one unit remains elusive. Interestingly, phosphatidylinositol-4,5-biphosphate (PIP2) was found to bind to the first CH domain of the actin-binding protein α-actinin, suggesting that the CH domain may have a dual role in binding to actin and interacting with membrane phospholipids (61). It has been shown that members of the Rho family of GTPases can influence the production of PIP2 through their interaction with phosphatidylinositol-4-phosphate-5-kinase (62–65). Production of these phospholipids could lead to an increase in substrate for phospholipase-C\textgamma, followed by increases in inositol 1,4,5-trisphosphate and a subsequent rise in intracellular Ca\textsuperscript{2+} levels. In fact, CD19-mediated activation of phosphatidylinositol-4-phosphate-5-kinase in B lymphocytes from Vav-null mice is defective, as is the CD19-mediated Ca\textsuperscript{2+} response (66). Because downstream effectors of Vav have the ability to regulate PIP2 generation, it is conceivable that the CH domain of Vav interacts with PIP2 to localize Vav to areas where PIP2 is being generated. Such a mechanism might lead to increased levels of PIP2 as a result of a more sustained activation of phosphatidylinositol-4-phosphate-5-kinase by Vav-activated effector molecules. In fact, T cells from Vav-deficient mice produce far less inositol 1,4,5-trisphosphate upon anti-TCR plus anti-CD28 crosslinking than do their wild-type counterparts (20). The ability of the CH domain of Vav to interact with PIP2 is intriguing, but support for this idea will require further analysis.

It is clear that the CH domain is not involved simply in membrane localization because addition of a CAAX signal sequence to the end of the CH\textsuperscript{−} mutant did not restore its ability to enhance NF-AT/AP-1-mediated gene transcription (see Fig. 2C). Also, addition of the CAAX sequence to Vav led to a protein with the ability to enhance NF-AT/AP-1 gene transcription over and above that of wild-type Vav in the absence or presence of TCR stimulation (Fig. 2C, compare Vav with Vav.CAAX). A previous study assessed the role of a myristoylated form of Vav in its ability to activate an NF-AT reporter construct and found that, in contrast to Vav, it required addition of ionomycin for optimal activation (5). However, they acknowledge that expression of their myristoylated form of Vav was low compared with wild-type Vav. As shown in the inset in Fig. 2C, we observe comparable levels of Vav and Vav.CAAX expression, which may explain the fact that we get enhanced transcription from the NF-AT/AP-1 reporter even in the absence of ionomycin treatment. Another possible explanation is that attachment of the amino-terminal end of Vav into the membrane with the myristoylation signal sequence interfered with the normal function of the Vav CH domain.

Overexpression of the CH\textsuperscript{−} mutant was found to enhance cellular cytotoxicity by NK cells and CTLs, indicating that the mutant protein still has the capacity to regulate the development of this process (see Fig. 4A). Rises in intracellular Ca\textsuperscript{2+} are observed during the generation of cytotoxicity, and although an initial Ca\textsuperscript{2+} influx is observed, and is required for the development of cell-mediated killing (49, 67–70), killing may not require the sustained Ca\textsuperscript{2+} influx needed for optimal NF-AT activation. Indeed, cellular cytotoxicity can take place within minutes of the NK cell binding to a sensitive target (49). Moreover, we have recently determined that the linker for activation of T cells protein, which is required for Ca\textsuperscript{2+} mobilization during TCR stimulation (71), plays a significant role in the development of cell-mediated killing in human NK cells (72). Therefore, it is possible that the CH\textsuperscript{−} mutant of Vav can still regulate the development of cellular cytotoxicity because the linker for activation of T cells protein activation is resulting in the required initial increase in intracellular Ca\textsuperscript{2+} levels. In addition, the CH\textsuperscript{−} mutant may be able to couple to and activate other signaling pathways that are required for the development of cellular cytotoxicity. Our previous observation demonstrating that overexpression of the oncogenic version of Vav resulted in intermediate or no enhancement of cell-mediated killing may be because the CH domain of Vav contains different sites that can both positively and negatively influence the development of cell-mediated killing (6). In fact, smaller deletions within the Vav CH domain have been observed to have no affect on the ability of a particular mutant to enhance cellular cytotoxicity, whereas other deletions do affect this property (D.D.B. and P.J.L., unpublished observations).

Many intracellular signaling pathways are activated during the development of cell-mediated killing. In fact, recent data has identified a critical role for ERK2 in the regulation of cell-mediated killing (73, 74). Interestingly, it has been reported that Vav plays a critical role in the activation of ERK1 and 2 following a combination of anti-TCR and anti-CD28 stimulation (20). In addition, expression in Jurkat T cells of a dominant negative p21-activated kinase, a downstream target of the Vav effector molecules Rac-1 and CDC42, inhibited ERK2 activation in response to TCR crosslinking (75). Therefore, it is conceivable that the CH\textsuperscript{−} mutant of Vav can regulate the development of cell-mediated killing because it is still capable of activating the ERK pathway. It will be important to determine whether Vav and the CH\textsuperscript{−} mutant can similarly modulate the ERK pathway during the development of natural cytotoxicity and TCR- and FcR-initiated signals.

Members of the dbl family of GEFs have an interesting characteristic in that a PH domain is always found C-terminal to the Dbl domain (24, 26, 27). This observation suggests an evolutionarily preserved requirement for the PH domain in the regulation of this family of GEFs. Indeed, PI3-K-generated phospholipids have been shown to modulate both Vav tyrosine phosphorylation and activation in vitro (32). Although we found that Vav mutants containing a deleted PH subdomain underwent receptor-initiated tyrosine phosphorylation following TCR or FcR cross-linking, or during the development of natural killing (D.D.B. and P.J.L., data not shown), deletion of the Vav PH domain mutant creates a protein that can neither activate NF-AT/AP-1-mediated gene transcription in response to TCR stimulation (Fig. 5A) nor regulate the development of TCR- or FcR-initiated cellular cytotoxicity.
(Fig. 5B). This functional defect may reflect in part the inability of the PH element to interact with PI3-K-generated membrane phospholipids following TCR- or FcR-crosslinking and undergo phosphorylation at critical tyrosine residues required for optimal Vav activation. Interestingly, we found that Vav regulation of the development of natural cytotoxicity against the K562 target cell was unaffected by removal of the PH domain (Fig. 5B). This dichotomy in the requirement of the Vav PH domain in the regulation of these two distinct forms of killing may be explained by our previous finding that the specific PI3-K inhibitor wortmannin blocked FcR-induced granule release and the development of ADCC, but had no effect on natural cytotoxicity (76). Similarly, treatment of CD8+ T cell lines with concentrations of wortmannin that affect ADCC by NK cells can also inhibit the cellular cytotoxicity initiated through the TCR (D.D.B. and P.J.L., unpublished observations). These data suggest that compared with natural cytotoxicity, killing mediated through the FcR or TCR are more dependent on PI3-K, but can still couple Vav to the activation program whereby it can regulate killing.

The tyrosines within the AD of Vav have been thought to play a key role in Vav function, because Vav with a Y174F mutation fails to become tyrosine phosphorylated by the PTK Lck or Syk in vitro (32, 43) and lacks GEF activity (32, 33). Together these data suggest a key function of this tyrosine in the activation of Vav GEF activity. However, it is clear from our data that the 3YF mutant results in a hyperactive protein that has the ability to mobilize extended Ca2+ fluxes following TCR cross-linking and hyperactivate the NF-AT/AP-1 reporter (see Fig. 6, B and C). The observation that the 3YF mutant can increase NF-AT/AP-1 activity in the absence of TCR cross-linking suggests that it can influence both arms of the signaling pathways required for optimal NF-AT/AP-1-mediated gene transcription. Therefore, it might be expected that the 3YF mutant would increase NF-AT/AP-1 activity in the presence of PMA or ionomycin, which should pharmacologically stimulate AP-1 and NF-AT activity, respectively. However, in contrast to ionomycin, which results in an increase in NF-AT/AP-1 activity of 3YF transfected cells, addition of PMA to 3YF-expressing cells had no effect on NF-AT/AP-1 activity when compared with unstimulated cells (D.D.B. and P.J.L., unpublished observations). The 3YF mutant’s ability to hyperactivate the NF-AT/AP-1 reporter requires the presence of the CH domain because a dual mutant containing both the CH deletion and the 3YF mutation no longer leads to enhanced activation of the NF-AT/AP-1 reporter under any stimulation conditions (D.D.B. and P.J.L., unpublished observations). This double mutant may not be able to hyperactivate the NF-AT/AP-1 reporter because removal of the CH domain inhibits its ability to lead to increased levels of Ca2+ following TCR stimulation (D.D.B. and P.J.L., unpublished observations). However, it might be expected that this double mutant would still retain its ability to enhance cellular cytotoxicity. Indeed, the 3YF mutant enhances cell-mediated cytotoxicity to similar levels as wild-type Vav (see Fig. 6D, compare Vav with 3YF).

Although individual mutation of tyrosines in the AD did not result in the level of activation of the NF-AT/AP-1 reporter construct observed with the 3YF mutant, the Y174F mutant routinely led to higher levels of activation of the NF-AT/AP-1 reporter when compared with wild-type Vav or the Y142F and Y160F mutants (see Fig. 6D). Therefore, in contrast to the previous observations identifying Y174 as critical residue involved in the activation of Vav GEF activity (32, 33), our data suggest that Y174 has a role in negatively regulating Vav function. It is possible that following tyrosine phosphorylation of the tyrosines in the AD, in particular Y174, they recruit a negative regulator of Vav function that could either antagonize Vav GEF activity or exert negative effects on Rho family GTPase activation. Identification of such a protein, if it exists, will be important for further understanding how Vav is regulated.

Our findings show that specific protein domains within the Vav protooncogene can differentially regulate the development of Vav-dependent T cell and NK cell activation. It also raises the possibility that domain-specific therapeutic strategies can be designed to modulate specific aspects of the immune response. For instance, a drug that could interact with the CH domain of Vav could block the ability of Vav to activate T cells through its regulation of NF-AT/AP-1-mediated transcription of the IL-2 promoter, but would not affect the ability of CTLs and NK cells to mediate cellular cytotoxicity. Further detailed analysis of domains within Vav and other signaling molecules involved in cell activation will be important in defining domain-dependent requirements for the activation of distinct intracellular signaling pathways.

Acknowledgments

We thank Karen Hedin and Dave McKeon for their helpful discussions and review of the manuscript. We also thank J. D. McKeon and M. P. Bell for the generous gift of the NF-AT3.luciferase reporter construct and C. V. Paya for the gift of the activated calcineurin construct.

References

1. Cantrell, D. 1996. T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. 14:259.
2. Trinchieri, G. 1989. Biology of natural killer cells. Adv. Immunol. 47:187.
3. Alberola-Ila, J., S. Takaki, J. D. Kerner, and R. M. Perlmutter. 1997. Differential signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. 15:25.
4. Daeron, M. 1997. Fc receptor biology. Annu. Rev. Immunol. 15:203.
5. Holsinger, L. J., D. M. Spencer, D. J. Austin, S. L. Schreiber, and G. R. Crabtree. 1995. Signal transduction in T lymphocytes using a conditional allele of Sos. Proc. Natl. Acad. Sci. USA 92:9870.
6. Wu, J., S. Katzav, and A. Weiss. 1995. A functional T-cell receptor signaling pathway is required for p95Vav activity. Mol. Cell. Biol. 15:4337.
7. Billedeaux, D. D., K. M. Brumbaugh, C. J. Dick, R. A. Schoon, R. X. Bustelo, and P. J. Leibson. 1998. The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing. J. Exp. Med. 188:549.
8. Katzav, S., D. Martín-Zanca, and M. Barbacid. 1989. Vav, a novel human oncogene from a locus ubiquitously expressed in hematopoietic cells. EMBO J. 8:2263.
9. Bustelo, X. R., J. A. Leibetter, and M. Barbacid. 1992. Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates. Nature 356:58.
10. Margolis, B., P. Hu, S. Katzav, W. Li, J.M. Oliver, A. Ullrich, A. Weiss, and J. Schlessinger. 1992. Tyrosine phosphorylation of vav proto-oncogene product containing SH2 domain and transcription factor motifs. Nature 356:71.
11. Darby, C., R. L. Geha, and D. A. Schreiber. 1994. Stimulation of macrophage FcγRIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62GAP-Associated protein. J. Immunol. 152:5429.
12. Xu, X., and S.-F. Chong. 1996. Vav in natural killer cells is tyrosine phosphorylated upon cross-linking of FcγRIIA and is constitutively associated with a serine/threonine kinase. Biochem. J. 318:527.
13. Fischer, K.-D., A. Zunzunegui, S. Gardner, M. Barbacid, A. Bernstein, and C. Giddos. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient CD4+ CD8+ thymocytes. Nature 374:374-374.
14. Arakovsky, L., M. Turner, S. Schaal, P. J. Mee, L. P. Dukky, J. Rajewsky, and V. J. L. Tybulewicz. 1995. A defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature 374:467.
15. Zhang, R., F. W. Alt, L. Davidson, S. H. Orkin, and W. Swat. 1995. Defective signaling through the T- and B-cell antigen receptors in lymphoid cell lacking the vav proto-oncogene. Nature 374:470.
16. Turner, M., P. J. Mee, A. E. Walters, M. E. Quinn, J. M. Penninger, and V. J. L. Tybulewicz. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity 7:451.
17. Fischer, K.-D., Y. Y.-N. Kong, H. Nishina, K. Tedford, L. E. Marenzere, I. Kozieradzki, T. Sasaki, M. Starr, G. Chan, S. Gardener, et al. 1998. Vav is a regulator of TCR-mediated cytokine receptor reorganization required for proliferation, interleukin 2 production, and T cell maturation. Curr. Biol. 8:534.
18. Holsinger, L. J., L. A. Small, W. Swat, T. Chu, D. M. Baustista, L. Davidson, R. S. Lewis, F. W. Alt, and G. R. Crabtree. 1998. Defective in actin-cap formation in Vav-deficient mice implicates an actin requirement for lymphocyte signal transduction. Curr. Biol. 8:563.
19. Kong, Y.-Y., K.-D. Fischer, M. F. Bachmann, S. Mariahathan, I. Kozieradzki, M. P. Ngiem, D. Bouchard, A. Berstein, P. S. Ohashi, and J. M. Penninger. 1998. Vav regulates peptide-specific apoptosis in thymocytes. J. Exp. Med. 188:2099.
The Journal of Immunology 3981

20. Costello, P. S., A. E. Walters, P. J. Mee, M. Turner, L. F. Reynolds, A. Prisco, N. J. Mackay, R. R. Scherer, R. A. R. Aaro, and V. L. J. Tyndall. 1999. The Vh family of GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kB pathways. Proc. Natl. Acad. Sci. USA 96:3035.

21. Olson, M. F., N. G. Pasteris, J. L. Gorski, and A. Hall. 1996. Faciogenital dysplasia syndrome (FGD) and Vav, two related genes. X-linked and Xq25-q26. J. Biol. Chem. 261:1628.

22. Crespo, P., K. E. Schuebel, A. A. Ostrom, S. J. Gutkind, and X. R. Bustelo. 1997. Phosphotyrosine-dependent activation of Rac-1 GTP/GDP exchange by Vav protein-proto-oncogene product. Nature 385:169.

23. Katzav, S. 1995. Vav. Captain Hook for signal transduction? Crit. Rev. Oncog. 6:87.

24. Adams, J. M., H. Houston, J. Taint, and R. Harvey. 1992. The hemato- poietically expressed Vav protein-encoding homologue with the small GTP- GTP exchange factor, the ber gene and a yeast gene (CDC24) involved in cytoskeletal organization. Oncogene 7:611.

25. Hild, J. M., A. Eva, D. Zangrill, S. A. Arason, T. Evans, R. A. Cerione, and Y. Zheng. 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J. Biol. Chem. 269:62.

26. Ting, A. T., C. J. Dick, R. A. Schoon, L. M. Karnitz, R. T. Abraham, and L. Y. Djeu. 1998. Role of kinases and overlapping pathways in regulating nuclear factor of activated T cells. J. Exp. Med. 187:1753.

27. Bustelo, X. R. 1996. The Vav family of signal transduction molecules. Crit. Rev. Oncog. 7:65.

28. Katzav, S. 1993. Single point mutations in the SH2 domain impair the trans- lation of the protein kinase C regulatory subunit by protein kinase C activation. J. Cell Sci. 111:1813.

29. Leibson, P. J., D. E. Midthun, K. P. Windebank, and R. T. Abraham. 1990. Calcium-dependent natural killer and Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells. Scand. J. Immunol. 32:1410.

30. Katzav, S. 1993. Single point mutations in the SH2 domain impair the trans- lation of the protein kinase C regulatory subunit by protein kinase C activation. J. Cell Sci. 111:1813.

31. Castresana, J., and M. Saraste. 1995. Does Vav bind to F-actin through a common domain in the SH2 domain? FEBS Lett. 374:149.

32. Leibson, P. J., D. E. Midthun, K. P. Windebank, and R. T. Abraham. 1990. Calcium-dependent natural killer and Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells. Scand. J. Immunol. 32:1410.

33. Ting, A. T., C. J. Dick, R. A. Schoon, L. M. Karnitz, R. T. Abraham, and L. Y. Djeu. 1998. Role of kinases and overlapping pathways in regulating nuclear factor of activated T cells. J. Exp. Med. 187:1753.

34. Windlebank, K., P. T. Abraham, G. Potempa, R. A. Olsen, T. J. Barna, and P. J. Leibson. 1988. Transmembrane signaling via the human natural killer cell actin binding protein. J. Exp. Med. 168:1192.

35. Hild, J. M., A. Eva, D. Zangrill, S. A. Arason, T. Evans, R. A. Cerione, and Y. Zheng. 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J. Biol. Chem. 269:62.

36. Ting, A. T., C. J. Dick, R. A. Schoon, L. M. Karnitz, R. T. Abraham, and L. Y. Djeu. 1998. Role of kinases and overlapping pathways in regulating nuclear factor of activated T cells. J. Exp. Med. 187:1753.