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Direct Stimulation of Vav Guanine Nucleotide Exchange Activity for Ras by Phorbol Esters and Diglycerides†

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We recently identified Vav as a Ras-activating guanine nucleotide exchange factor (GEF) stimulated by a T-cell antigen receptor-coupled protein tyrosine kinase (PTK). Here, we describe a novel, protein kinase-independent alternative pathway of Vav activation. Phorbol ester, 1,2-diacylglycerol, or ceramide treatment of intact T cells, Vav immunoprecipitates, or partially purified Vav generated by in vitro translation or COS-1 cell transfection stimulated the Ras exchange activity of Vav in the absence of detectable tyrosine phosphorylation. GEF activity of gel-purified Vav was similarly stimulated by phorbol myristate acetate (PMA). Stimulation was resistant to PTK and protein kinase C inhibitors but was blocked by calphostin, a PMA and diacylglycerol antagonist. In vitro-translated Vav lacking its cysteine-rich domain, or mutated at a single cysteine residue within this domain (C528A), was not stimulated by PMA but was fully activated by $p56^{lck}$. This correlated with increased binding of radiolabeled phorbol ester to COS-1 cells expressing wild-type, but not C528A-mutated, Vav. Thus, Vav itself is a PMA-binding and -activated Ras GEF. Recombinant interleukin-1α stimulated Vav via this pathway, suggesting that diglyceride-mediated Vav activation may couple PTK-independent receptors which stimulate production of lipid second messengers to Ras in hematopoietic cells.

The three Ras proteins (Ha, Ki, and N), members of the small GTP-binding protein superfamily (10, 12, 33), play an essential role in controlling cellular growth and differentiation. Biochemical and genetic evidence indicates that they couple signals initiated by membrane-associated receptor or nonreceptor protein tyrosine kinases (PTKs) to a downstream cascade of serine/threonine kinases that coordinate signal transduction to the nucleus (46, 61, 73). The rate-limiting step in Ras activation is the exchange of bound GDP for GTP, which is stimulated by various mitogenic or differentiation-inducing ligands, and leads to formation of an active Ras · GTP complex (23, 46, 65). This reaction is catalyzed by guanine nucleotide exchange factors (GEFs), and several Ras GEFs have recently been isolated by molecular cloning from yeast species, *Drosophila melanogaster*, and mammals (12, 22).

Ras also seems to play a role in the activation of T lymphocytes via their antigen-specific T-cell receptor (TCR)-CD3 complex, a process that involves the early and obligatory (41, 52) activation of nonreceptor PTKs of the Src and/or ZAP-70/Syk families (51, 71, 72). This notion is based on several findings: first, T-cell stimulation with mitogenic antireceptor antibodies or protein kinase C (PKC)-activating phorbol esters (PE) such as phorbol 12-myristate 13-acetate (PMA) stimulates Ras (24); second, the interleukin-2 (IL-2) gene promoter is activated in T cells by an oncogenic Ha-Ras protein in conjunction with Ca^{2+}-dependent signals (9, 60, 74, 75) and, conversely, its activation is inhibited by a transdominant inhibitory *ras* mutant (8, 60, 75); and finally, the same transdominant *ras* mutant also blocks TCR-CD3-stimulated activation of Erk2, a member of the mitogen-activated protein kinase family (40).

PTK-dependent Ras stimulation in T cells most likely reflects activation of GEFs. We recently identified Vav, the SH2/SH3 domain-containing 95-kDa product of the *vav* proto-oncogene, which is expressed specifically in hematopoietic cells (1, 20, 42, 43) and undergoes tyrosine phosphorylation in response to ligation of hematopoietic cell receptors (3, 15, 16, 47), as an antigen receptor-stimulated Ras GEF in T (29) and B (31) lymphocytes. The enzymatic activity of Vav is stimulated via its rapid and obligatory tyrosine phosphorylation, presumably by antigen receptor-coupled PTKs (29, 31). T cells also express another Ras GEF, the ubiquitous Sos protein (13, 14, 19), which was recently found to undergo activation-induced indirect association with the ζ subunit of TCR-CD3 as part of a complex that includes the Shc and Grb2 adaptor proteins (59). While the intrinsic enzymatic activity of Vav is stimulated by its direct tyrosine phosphorylation (29), Sos, which is constitutively bound to Grb2, acts by translocating from the cytosol to the membrane (where Ras is located), a process mediated by binding of the Shc-Grb2-Sos complex to activated receptor or nonreceptor PTKs (14, 59).

Vav also contains, downstream of its putative catalytic domain, a cysteine-rich region consisting of a $Cys\text{-}X_2\text{-}Cys\text{-}X_{13}\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_4\text{-}His\text{-}X_2\text{-}Cys\text{-}X_6\text{-}Cys$ motif (11, 20). This motif is conserved in several proteins, and in the case of PKC enzymes (56), *n*-chimerin (2), and the *unc*-13 gene product (53), it mediates PE-diacylglycerol (DAG) binding. This suggested

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that Vav may be regulated by diglycerides, a notion supported by our recent analysis using *vav*-transfected NIH 3T3 fibroblasts and partially purified Vav (30). We have analyzed, therefore, the structural basis of PMA- or DAG-mediated Vav activation and addressed its physiological significance. Here, we report that (i) Vav itself is a PMA-binding and -responsive Ras GEF; (ii) activation of its GEF activity depends on the cysteine-rich domain; and finally, (iii) a PTK-independent receptor, i.e., the interleukin 1 (IL-1) receptor, utilizes this diglyceride-mediated signaling pathway to activate Vav in intact T cells. Thus, the exchange activity of Vav is subject to dual, independent regulation by PTKs and lipid second messengers.

MATERIALS AND METHODS

Cell lines, stimulation, and lysis. Leukemic Jurkat (human) or EL4 (murine) T-cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (Irvine Scientific), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES [pH 7.3]), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM β-mercaptoethanol. Cells were washed twice, resuspended in a 10 mM HEPES–saline buffer (pH 7.3), and activated at 37°C with anti-human (OKT3) or -murine (2C11) CD3 monoclonal antibodies (MAbs) (10 μg/ml), PMA (10 ng/ml; Molecular Probes, Inc.), 1-oleyl-2-acetyl glycerol (OAG; 20 μg/ml; Calbiochem), recombinant human IL-1α (1 ng/ml; Dai-Nippon Pharmaceutical Co.), or type III ceramide (2 or 20 μM; Sigma) as indicated. In order to study the effect of kinase inhibitors, the cells were pretreated at 37°C for 5 min with staurosporine or calphostin (1 μM each; Kamiya Biomedical Co.), or for 12 h with herbimycin A (10 μM), prior to stimulation. Stimulation was terminated by lysis as described previously (30), and nuclei and cell debris were cleared by centrifugation at 16,500 × *g* for 5 min.

Immunoprecipitation and immunoblotting. Cleared lysates representing equal numbers of cell (verified independently by determining protein concentrations, which varied by less than ±10% among samples) were subjected to immunoprecipitation with a rabbit anti-Vav peptide (residues 576 to 589) serum or, as a control, normal rabbit immunoglobulin or preimmune serum and then with formalin-fixed *Staphylococcus aureus* as described previously (29, 30). Sos was similarly immunoprecipitated with a rabbit anti-human Sos1 serum (UBI). Immunoprecipitates (IPs) were washed five times in 10 mM HEPES (pH 7.5), 100 mM NaCl, 2% Nonidet P-40, 10 mM each NaF and Na_3VO_4, 12 mM $MgCl_2$, and protease inhibitors, resuspended in the same buffer containing 1% Nonidet P-40 (exchange buffer), and subjected to exchange assays. Where indicated, the IPs were incubated in vitro prior to the exchange assay in the absence or presence of inhibitors and then stimulated with potential Vav activators as indicated. For immunoblotting or renaturation (see the description below), Vav or control IPs, or partially purified preparations, were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%) gel electrophoresis (PAGE), transferred to Immobilon-P membranes, and immunoblotted with anti-Vav antibodies or with an antiphosphotyrosine MAb (4G10; UBI) as described previously (29–31).

Partial purification and assay of in vitro-translated or COS-transfected Vav. The human proto-*vav* cDNA was cloned into pTag/CMV-neo, a modified pRc/CMV (Invitrogen) expression vector that contains a cytomegalovirus promoter, a T7 promoter, and sequences encoding six histidine residues and a human immunodeficiency virus type 1-derived tag epitope (6). Correct orientation and in-frame cloning were confirmed by restriction enzyme digestion and nucleotide sequencing of the 5′ region, respectively. This vector was used for in vitro translation or transient COS-1 cell transfections as described previously (30). In order to generate cDNAs encoding full-length or differentially truncated Vav, the *vav* plasmid was linearized with *Ssp*I, *Sau*I, or *Bsm*I prior to in vitro transcription (see Fig. 4). The histidine-tagged Vav was partially purified by Ni^{2+}-agarose affinity chromatography, washing in Tris-buffered saline plus 20 mM imidazole, and elution with 500 mM imidazole (30). Vav expression was confirmed by immunoblotting with anti-Vav or antitag antibodies. Five-microliter (10% of the total) Vav aliquots (or control preparations) were added to 145 μl of exchange buffer and incubated (10 min, 37°C) in the absence or presence of the indicated combinations of PMA, staurosporine, and/or calphostin. Alternatively, Vav was activated by phosphorylation with 200 ng of recombinant $p56^{lck}$ (5) in the presence of 12 mM Mg^{2+} and 10 μM ATP. Vav or control samples were then assayed for Ras exchange activity.

GDP-GTP exchange assay. The assay for determining exchange activity has been described in detail earlier (29–31). Briefly, [^{3}H]GDP-loaded recombinant Ha-Ras (0.9 μM) was stabilized with $MgCl_2$ (12 mM), immobilized by filtration on 0.45 μm nitrocellulose membranes (Costar), and washed to remove excess free [^{3}H]GDP. The exchange reaction was initiated by adding membrane pieces to resuspended Vav IPs or partially purified Vav preparations in 150 μl of exchange buffer which was reconstituted with 20 μM cold GTP. Samples were incubated at 37°C, and 30-μl aliquots were removed at the indicated times for liquid scintillation counting to determine the amount of released [^{3}H]GDP. Counts for the 30-μl aliquot were multiplied by five to correct for the total reaction volume. Typically, 0.5×10^5 to 1×10^5 cpm of [^{3}H]GDP-Ras was bound to each membrane piece at time zero, and stimulated Vav caused a release of 10 to 20% [^{3}H]GDP from Ras during the initial 1 to 2 min of the assay (30). In addition, the binding of [α-^{32}P]GTP to soluble or filter-immobilized Ras · GDP complexes was also measured. Partially purified Vav (see the paragraph below) was added to exchange buffer supplemented with 0.5 μCi of [α-^{32}P]GTP (ICN; specific activity of 3,000 Ci/mmol). The reaction was initiated by adding soluble GDP-loaded recombinant Ras to a final concentration of 150 nM (solution phase assay), or by adding filter-immobilized and washed Ras · GDP complexes to Vav-plus-[α-^{32}P]GTP-containing exchange buffer in Eppendorf tubes (filter assay). In the first case, labeled soluble Ras · GTP complexes were filtered through nitrocellulose. Membranes were washed extensively, and exchanged, Ras-bound [α-^{32}P]GTP was quantitated by liquid scintillation counting at the indicated time points.

Vav renaturation. Jurkat cell lysates, labeled where indicated for 4 h with 1 mCi of [^{35}S]methionine plus [^{35}S]cysteine (Tran^{35}S-label; ICN; specific activity of 1,000 Ci/mmol) in methionine-free medium, were precleared by incubation for 8 h with preimmune serum plus 60 μl of Pansorbin before immunoprecipitation with preimmune or Vav-specific serum and 40 μl of Pansorbin for another 45 min. IPs were washed seven times in 500 mM NaCl, 50 mM HEPES (pH 7.4), 0.5% sodium deoxycholate, 0.005% SDS, and 1% Triton X-100. Five-microliter aliquots of unstimulated or PMA-stimulated Vav IPs were assayed for their exchange activity, and the remainder was subjected to SDS-PAGE. Following transfer to Immobilon-P membranes, an ~2-mm-long piece containing the Vav band (identified by immunoblotting a parallel sample

with Vav-specific antibodies) was cut, denatured for 1 h at room temperature in 7 M guanidine-HCl, and renatured overnight at 4°C in 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM each dithiothreitol and EDTA, 0.1% Nonidet P-40, 1 mM sodium orthovanadate, and 1% (wt/vol) bovine serum albumin (BSA [18]). Electrophoresed Sos IP was similarly renatured. Untreated or PMA-treated renatured membranes were added to 200 μl of incubation buffer (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.01% BSA, 0.2 mM dithiothreitol, 12 mM EDTA, and 22 mM $MgCl_2$) containing 180 pmol of Ras · GDP, and subjected to a solution phase [α-^{32}P]GTP binding exchange assay.

Site-directed mutagenesis of Vav. The recombinant pTag/Vav vector was subjected directly to mutagenesis by using the Transformer site-directed mutagenesis kit (Clontech) according to the manufacturer's protocol. Mutagenesis primers included a *vav*-specific primer, 5′-GACCACATCCGCCAAG GCCTGTC-3′, which replaces Cys-528 with alanine, and a selection primer, 5′-GACTGGTGAGTTCTCAACCAAG-3′, which destroys a unique *ScaI* site in the pTag/CMV-neo vector. The presence of the desired mutation was verified by limited sequencing, and the mutated cDNA was subjected to in vitro transcription and translation and partial affinity purification as described above.

[^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding. COS-1 cells (1×10^6 per well in a six-well tissue culture plate) were transfected in triplicate wells with 20 μg of control pTag, Vav (wild type or C528A), or PKCα (58) plasmid DNA as described earlier (30). Sixty hours later, the cells were washed in RPMI 1640 medium containing 10 mM HEPES and 0.1% (wt/vol) BSA and incubated for 30 min at 37°C in 1 ml of the same medium containing 10 nM [^{3}H]PDBu (NEN; specific activity of 18.6 Ci/mmol). The cell monolayers were washed three times, lysed in 100 mM NaCl, 50 mM HEPES (pH 7.4), 1% Triton X-100, and counted. Nonspecific [^{3}H]PDBu binding values in the presence of excess (2.5 μM) cold PDBu were determined in parallel and subtracted in order to determine specific binding.

RESULTS

Vav is activated in intact T cells by PE and diglycerides. In order to test whether PMA or synthetic DAG activates the exchange activity of Vav in intact T cells, the TCR-CD3$^+$ human leukemic T-cell line, Jurkat, was stimulated for different lengths of time with PMA, OAG (a synthetic DAG analog) or, as a positive control (29), with an anti-CD3 MAb. Exchange activity of immunoprecipitated Vav was determined by the release of labeled GDP from immobilized, [^{3}H]GDP-loaded recombinant Ha-Ras, or by [α-^{32}P]GTP binding to Ras · GDP complexes (see the description below). Treatment with PMA or OAG caused an approximate fivefold increase in the exchange activity of Vav, a level similar to that induced by anti-CD3 (Fig. 1A). Vav was stimulated by an active PE, 4-β-phorbol dibutyrate (4-βPDD), but not by its inactive analog, 4-αPDD (data not shown). Unlike the OKT3- or OAG-induced activity which peaked 1 min after stimulation and declined rapidly, the PMA-stimulated exchange activity peaked later and remained elevated even 10 min after stimulation. This is due, most likely, to the slow permeability and degradation of PMA in cells.

Since OKT3-induced Vav stimulation is associated with obligatory tyrosine phosphorylation (29) and PMA can cause phosphorylation of proteins, e.g., mitogen-activated protein kinase, on tyrosine (26), we determined whether PMA treatment also causes tyrosine phosphorylation of Vav. As reported

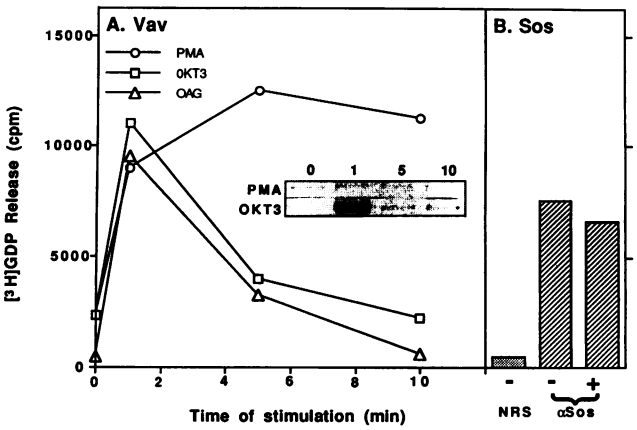

FIG. 1. (A) Anti-CD3- and diglyceride-induced activation and tyrosine phosphorylation of Vav. Lysates (20×10^6 cells per group) from unstimulated Jurkat cells or from cells stimulated for 1, 5, or 10 min with PMA (10 ng/ml), OAG (20 μg/ml) or OKT3 (10 μg/ml) were subjected to immunoprecipitation with a rabbit anti-Vav serum or, as a control, preimmune serum. Washed IPs were tested for Ras exchange activity or immunoblotted with anti-phosphotyrosine MAb 4G10 (inset). Counts in a 30-μl aliquot of each exchange reaction sample were multiplied by five to correct for the total reaction volume (150 μl). Control IPs did not contain detectable Vav and gave a corrected background [^{3}H]GDP release of 500 cpm. This experiment was repeated three times with similar results. (B) Ras GEF activity of control (NRS) or Sos IPs from unstimulated (−) or OKT3-stimulated (+) Jurkat cells. NRS, normal rabbit serum. [^{3}H]GDP binding to the filter at time zero was 52,000 cpm.

earlier (16, 29, 47), anti-CD3 stimulation induced a rapid and transient increase in Vav tyrosine phosphorylation. In contrast, PMA was negative in this regard (Fig. 1A, inset). Similar Vav activation by PMA was observed in fresh human peripheral blood T cells, or in a murine B-lymphoma cell line, WEHI-231 (data not shown). These results indicate that the Ras exchange activity of Vav IPs is stimulated by PE-DAG in the absence of tyrosine phosphorylation. IPs of another Ras GEF, i.e., Sos (14, 19), were also active in the same exchange assay and caused release of a similar fraction of [^{3}H]GDP from Ras (Fig. 1B). In contrast to Vav, however, anti-CD3 stimulation did not increase the activity of Sos. The possibility that GEF activity in Vav IPs is mediated by Sos (that could, e.g., bind to the two SH3 domains of Vav) was ruled out by our findings that an anti-Sos antibody failed to react with Vav IPs. Conversely, Sos or Shc IPs from activated T cells did not contain detectable Vav (data not shown).

Vav activation in intact T cells is independent of protein kinases. Since PMA and synthetic DAG are potent PKC activators, it was important to determine whether these agents activate Vav directly or indirectly, possibly via the activation of PKC and/or other, PKC-stimulated protein kinases. We evaluated, therefore, the effects of several pharmacological inhibitors on anti-CD3- or PMA-induced stimulation of the exchange activity of Vav in intact Jurkat cells. The drugs included staurosporine (an ATP analog that inhibits both PKC and tyrosine kinases), herbimycin A (a specific PTK inhibitor), and calphostin (an antagonist that competes with diglycerides for binding to the cysteine-rich, PE-DAG-binding C1 domain of PKC [17]).

Drug pretreatment per se had no effect on the basal exchange activity of Vav isolated from unstimulated cells, and anti-CD3 or PMA stimulation increased the exchange activity of Vav by four- to fivefold (Table 1). Staurosporine or herbi-

TABLE 1. Effect of pharmacologic inhibitors on OKT3- versus PMA-stimulated Ras exchange activity of Vav in intact cells[a]

Inhibitor	% [^{3}H]GDP release		
	No stimulus	OKT3	PMA
None	2.5	10.7	13.0
Herbimycin A	2.1	2.7 (94)[b]	11.1 (14)
Staurosporine	2.7	ND	16.1 (0)
Calphostin	2.1	15.8 (0)	5.9 (64)

[a] Jurkat cells (10×10^6 per group) were incubated in the absence or presence of staurosporine or calphostin (1 μM each) for 5 min or with herbimycin A (10 μM) for 12 h prior to stimulation with PMA or OKT3 for 1 min. The Ras exchange activity of Vav IPs was determined in a 1-min assay. Counts were corrected for the total exchange reaction volume (see Fig. 1A), and percent [^{3}H]GDP release was calculated. Approximately 1×10^5 cpm of [^{3}H]GDP was bound to the filter at time zero. Corrected background [^{3}H]GDP release in the presence of a control (preimmune serum) IP was 0.5%. The results represent three similar experiments. ND, not determined.

[b] The numbers in parentheses represent percent inhibition relative to the corresponding untreated group. Percent inhibition was calculated as follows:

$$1 - \frac{\text{cpm (OKT3 or PMA)} - \text{cpm (no stimulus) in drug-treated group}}{\text{cpm (OKT3 or PMA)} - \text{cpm (no stimulus) in control group}} \times 100$$

mycin A pretreatment had no appreciable effect on the PMA-stimulated exchange activity; in contrast, preincubation of the cells with calphostin reduced by ~70% the same activity (Table 1). Calphostin inhibited to about the same extent the activation of Vav by OAG (data not shown). Conversely, OKT3-mediated Vav activation, which is dependent on its tyrosine phosphorylation (29), was blocked by a similar herbimycin A concentration but was not affected by calphostin pretreatment. Simultaneous activation with anti-CD3 and PMA did not reveal an additive or synergistic effect on Vav activity (data not shown).

These findings obtained with intact cells were supported by in vitro experiments in which Vav IPs from unstimulated Jurkat cells were first pretreated in the absence or presence of calphostin or staurosporine, stimulated with PMA, and assayed for Ras exchange activity. PMA-induced Vav activation was inhibited by calphostin but not by 1 μM staurosporine, a concentration 50-fold higher than that required for complete inhibition of the enzymatic activity of a partially purified PKC preparation from Jurkat cells. Moreover, both immunoblots of Jurkat-derived Vav IPs with a mixture of anti-PKC antibodies specific for all T-cell-expressed PKC enzymes (7, 37) and kinase assays of such IPs in the presence of known PKC cofactors failed to detect the presence of PKC (data not shown). Collectively, these data suggest that PE-DAG directly stimulates Vav in a protein kinase-independent manner.

Next, we tested the ability of PMA to activate in vitro partially purified Vav generated by transient COS-1 cell transfection. The *vav* protooncogene cDNA was cloned into pTag/CMV-neo (6). Expression in this vector introduces a histidine tag that can be used for partial purification by Ni^{2+} agarose chromatography. The Ras exchange activity of the partially purified Vav was assayed in the [^{3}H]GDP filter release assay, as well as in another assay previously used by others to document the catalytic activity of several exchange proteins, i.e., the binding of [α-^{32}P]GTP to Ras · GDP complexes in solution (14, 19, 35, 69). The exchange activity of Vav was markedly stimulated when tested in either assay (Fig. 2A and B), and [^{3}H]GDP release from nitrocellulose-immobilized Ras was paralleled by increased [α-^{32}P]GTP binding to the filter (data not shown). Several 1,2-DAGs also stimulated Vav in vitro. In contrast, 1,3-DAG and monoacylglycerol were inactive (data not shown). The complete kinetics of PMA-stimulated Vav activity in the [^{3}H]GDP filter release assay and the fact that counts released from the filter-immobilized Ras · GDP complex represent free [^{3}H]GDP have been established (30). Taken together, these results demonstrate that the filter-bound [^{3}H]GDP release protocol represents a valid assay for measuring guanine nucleotide exchange activity.

These assays were then used to evaluate the effects of staurosporine or calphostin on PMA-stimulated exchange activity of partially purified Vav. Consistent with data from our previous studies utilizing intact cells (Table 1) or Vav IPs (data not shown), the PMA-stimulated exchange activity of partially purified Vav was markedly inhibited by calphostin but not by staurosporine (Fig. 2C and D). Thus, results of the use of partially purified Vav strongly suggest that PMA directly stimulates its exchange activity.

PMA directly activates, and binds to, Vav via its cysteine-rich domain. To unequivocally determine whether Vav itself (as opposed to some associated protein) mediates PMA-stimulated GEF activity, we fractionated precleared, rigorously washed Vav IPs from metabolically labeled Jurkat cells by SDS-PAGE and analyzed them by autoradiography, anti-Vav immunoblotting, and exchange assays (Fig. 3). Although Vav and control IPs contained several proteins, only the Vav IP displayed an ~95-kDa ^{35}S-labeled band that comigrated with a protein recognized by anti-Vav (Fig. 3A). A nitrocellulose piece containing the Vav band was subjected to a denaturation-renaturation protocol (18), separated into two halves which were either left untreated or stimulated with PMA, and assayed for GEF activity by determining the ability to promote [α-^{32}P]GTP binding to soluble Ras · GDP complexes (14, 19).

The untreated membrane contained low exchange activity which was markedly stimulated by PMA (Figs. 3B and C). The difference in fold stimulation by PMA between the two experiments may reflect differences in the initial amount of Vav or in the efficiency of Vav elution and/or renaturation. A corresponding, identically treated nitrocellulose membrane containing an electrophoresed control (preimmune serum) IP did not display significant exchange activity (Fig. 3C). The exchange activity of a similarly renatured, SDS-PAGE-separated Sos IP was also restored under similar conditions (Fig. 3C). The in situ renaturation of the low constitutive exchange activity of gel-purified Vav and the ability to stimulate it with PMA demonstrate that Vav itself is a PMA-stimulated Ras GEF.

A structure-function analysis was next undertaken in order to assess the role of the cysteine-rich Vav domain in its PMA-mediated stimulation and the relationship between the PTK- and diglyceride-mediated activation pathways. Truncated Vav proteins or a mutated Vav in which a single cysteine residue (Cys-528) has been replaced by alanine was generated by in vitro translation. To generate full-length proteins, the wild-type or C528A-mutated *vav* protooncogene cDNAs, cloned in the pTag/CMV-neo vector under control of the T7 promoter, were linearized with *Ssp*I. In addition, the wild-type *vav* plasmid was linearized with *Sau*I or *Bsm*I, which would generate, upon proper in vitro transcription and translation, fragments truncated immediately downstream of the proximal SH3 domain (residues 1 to 664) or downstream of the putative exchange domain but upstream of the cysteine-rich, potentially PMA-responsive domain (residues 1 to 510), respectively (Fig. 4A, bottom). The linearized vectors were transcribed by T7 RNA polymerase, and Vav was translated in vitro by a wheat germ extract translation system and partially purified by Ni^{2+} resin affinity chromatography. All translation reaction mixtures contained similar amounts of Vav, as revealed by immunoblotting with the antitag MAb (data not shown).

The full-length or truncated Vav proteins were tested for

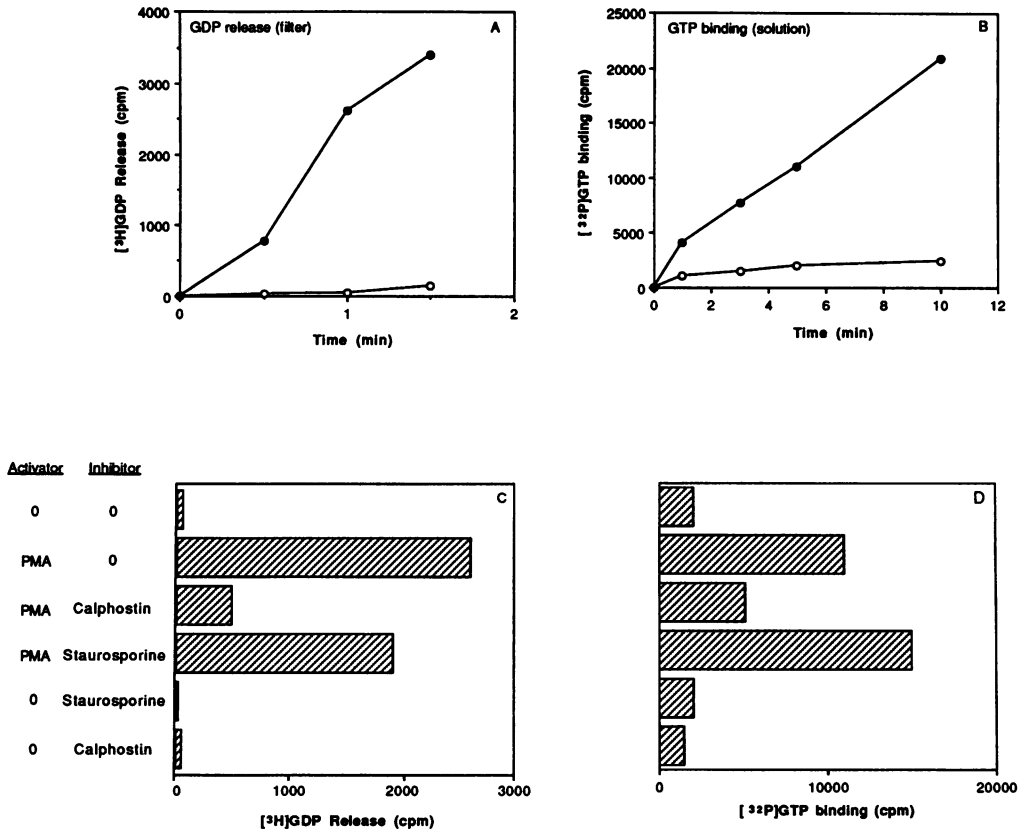

FIG. 2. Stimulation of the exchange activity of partially purified Vav by PMA and the influence of calphostin or staurosporine pretreatment. Exchange activity of 5-μl aliquots of partially purified unstimulated (○) or PMA-treated (10 min) (●) Vav, derived from transiently transfected COS-1 cells, was assayed by [^{3}H]GDP release from nitrocellulose-immobilized Ras · GDP complexes (shown are uncorrected counts in a 30-μl aliquot) (A) or [α-^{32}P]GTP binding (0.5 μCi per assay) to 150 nM GDP-loaded recombinant Ha-Ras in solution (B). Labeled soluble Ras · GTP complexes were filtered through nitrocellulose and washed. Values of unspecific release (determined in the presence of a similar preparation from COS-1 cells transfected with a control vector lacking *vav* cDNA) of [^{3}H]GDP (60 to 180 cpm at 0.5 to 1.5 min) or [α-^{32}P]GTP binding (1.7×10^3, 3×10^3, 8×10^3, and 10×10^3 cpm at 1, 3, 5, and 10 min, respectively) were subtracted from all datum points. The effects of a 5-min staurosporine or calphostin pretreatment on the exchange activity of Vav were determined in the same two assays (C and D, respectively). A single assay time point (1 or 5 min in C or D, respectively) is shown in each case. Similar results were obtained at the other time points examined (data not shown).

their basal, PMA-stimulated, or recombinant p56^{lck}-stimulated Ras exchange activity (Fig. 4A). All proteins possessed low levels of basal exchange activity, similar to that associated with Vav IPs from resting cells. The full-length wild-type protein and the larger of the two truncated Vav proteins containing the cysteine-rich domain were stimulated by PMA. In contrast, the C528A-mutated full-length protein and the nonmutated fragment lacking the PKC-homologous cysteine-rich domain were not stimulated by PMA. On the other hand, all proteins were activated by recombinant p56^{lck} (Fig. 4A). Vav activation by p56^{lck} correlated with increased tyrosine phosphorylation (data not shown). These results indicate, first, that truncation at the *Bsm*I site or mutation of Cys-528 per se did not abolish the intrinsic ability of Vav to become enzymatically activated. Second, and more important, they prove that the cysteine-rich domain of Vav and, more specifically, Cys-528, is required for the activation of Vav by PMA but not by PTK. Thus, the two activation mechanisms are independent.

Control or Vav-transfected COS-1 cells were next used in order to assess the binding of [^{3}H]PDBu to Vav, a method previously used to demonstrate PE binding to PKC (54, 56). COS-1 cells expressing wild-type, but not C528A-mutated, Vav (both of which were expressed at similar levels [Fig. 4C]) displayed a 2.5-fold increase in [^{3}H]PDBu binding by comparison with control, vector-transfected cells (Fig. 4B). As a positive control, cells transfected with a PKCα construct (58) also displayed increased (3.8-fold) [^{3}H]PDBu binding. Binding was blocked in each case by excess cold PDBu. Specific [^{3}H]PDBu binding to partially purified Vav or PKC preparations from lysates of the transfected cells was similarly demonstrated (data not shown). These data correlate with the functional effect of PMA and provide evidence for the binding of PE to Vav.

IL-1 and ceramide stimulate Vav via a PTK-independent pathway. The physiological relevance of the PTK-independent activation of Vav by PE-DAG is not clear, particularly since TCR-CD3-mediated PTK activation would cause DAG production via phospholipase C-γ1 activation and subsequent inositol phospholipid hydrolysis (4, 51, 71, 72). We examined the possibility that this alternative activation pathway might enable Vav to couple PTK-independent receptors that stimulate diglyceride production to Ras. This was addressed by assessing the effects of IL-1 on Vav activity in the murine TCR-CD3$^+$ EL4 T-cell lymphoma line which expresses functional IL-1 receptors (49). Although IL-1-mediated signaling mechanisms are controversial (32, 49, 57), the earliest signaling response appears to be hydrolysis of sphingomyelin, leading to the formation of ceramide that acts as a second messenger to

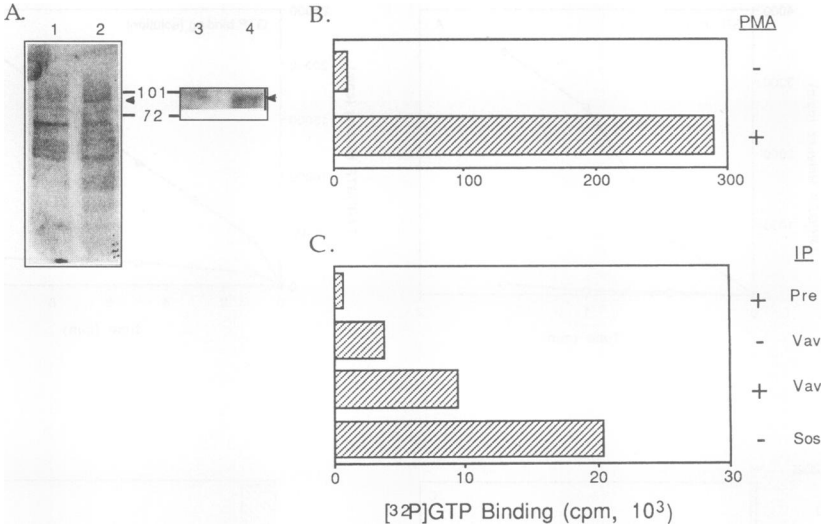

FIG. 3. Renaturation of the Ras exchange activity of gel-purified Vav. Control or Vav IPs were prepared from 4×10^7 [^{35}S]methionine- and [^{35}S]cysteine-labeled Jurkat cells as described in Materials and Methods. Five-microliter aliquots of unstimulated or PMA-stimulated Vav IP caused the release of 1,245 or 5,530 cpm of [^{3}H]GDP in a 1-min GEF assay. Control or Vav IPs were subjected to SDS-PAGE and transferred electrophoretically to Immobilon-P membranes. (A) Autoradiograms (lanes 1 and 2) or anti-Vav immunoblots (lanes 3 and 4) of control (odd-numbered) or Vav (even-numbered) IPs. The arrowheads indicate the position of Vav. Size markers (between lanes 2 and 3) are in kilodaltons. (B) A membrane piece containing the Vav band (panel A, lane 2) was subjected to denaturation and renaturation (18), cut into two halves which were either untreated (−) or treated (+) for 10 min with PMA (10 ng/ml) in exchange buffer, and assayed for exchange activity by measuring the binding of [α-^{32}P]GTP (20 μCi per assay) to 100 pmol of soluble Ras · GDP. An aliquot of 100 μl was removed from each reaction mixture after 2 min and filtered through nitrocellulose membrane before washing and counting. The bars show net [α-^{32}P]GTP counts bound to the membrane after subtracting nonspecific binding in the absence of gel-purified Vav (38,200 cpm). (C) Control, Vav, or Sos IPs from precleared Jurkat cell lysates were separated by SDS-PAGE, and the positions of Vav and Sos were identified by immunoblotting with the corresponding antibodies. Membrane strips containing the Vav or Sos band or, in the case of the control IP, a strip corresponding to the Vav-containing piece, were renatured and assayed for GEF activity against 25 pmol of soluble Ras · GDP in the presence of 5 μCi of [α-^{32}P]GTP. Renatured Vav was either left untreated (−) or stimulated with PMA (+) prior to the exchange assay. Background [α-^{32}P]GTP binding in the absence of GEF (1,780 cpm) was subtracted from all groups. Pre, preimmune serum control IP.

activate an Mg^{2+}-dependent serine/threonine kinase (49). Tyrosine phosphorylation appears to be a secondary response that occurs more slowly (32, 49).

Similarly to TCR-CD3 triggering by an anti-CD3 MAb (2C11), recombinant IL-1α treatment of EL4 cells stimulated the exchange activity of Vav IPs (Fig. 5A). Unlike anti-CD3 stimulation, however, IL-1 did not cause detectable tyrosine phosphorylation of Vav (data not shown), and the IL-1 effect was completely resistant to a herbimycin A concentration that blocked the anti-CD3-mediated increase in the exchange activity of Vav (Fig. 5A), implying that IL-1 stimulation did not activate PTK(s) capable of phosphorylating Vav under these conditions. Conversely, the IL-1, but not the anti-CD3, effect was blocked by calphostin, suggesting that diglycerides may be directly involved in Vav activation by IL-1.

Recent studies identified sphingomyelin-derived ceramide as the second messenger that mediates the action of IL-1 or tumor necrosis factor alpha (48, 49, 68). We evaluated, therefore, the in vitro effect of ceramide on the Ras exchange activity of immunoprecipitated Vav derived from resting Jurkat cells or in vitro-translated Vav. Ceramide concentrations similar to those previously shown to activate the IL-1- or tumor necrosis factor-stimulated protein kinase (48, 49) markedly stimulated the exchange activity of the Vav preparations (Fig. 5B). These findings support the notion that receptors not directly coupled to PTKs can also stimulate Vav, presumably via some Vav-activating second messenger that could be, e.g., phosphatidylcholine-derived DAG (62) or ceramide (48, 49), two structurally related lipids.

DISCUSSION

Our recent study (30) indicated, first, that the Ras exchange activity of partially purified Vav preparations can be stimulated by PMA and, second, that *vav*-transfected, but not control, NIH 3T3 fibroblasts respond to PMA stimulation with increases in their Ras exchange activity and the fraction of active, GTP-bound Ras. These results suggested that Vav is directly stimulated by PMA or diglycerides, most likely via its cysteine-rich, PKC-homologous domain (11, 20). In this study, we have used a combination of biochemical and genetic approaches to confirm this possibility and establish its structure-function relationship and potential physiological relevance. The results presented herein define an alternative pathway of Vav activation mediated by physiological phospholipid-derived second messengers, namely, DAG and ceramide, or by PEs, pharmacological agents that mimic the action of DAG in many cell types. The activation of gel-purified and renatured Vav by PMA and the increased [^{3}H]PDBu binding to COS-1 cells transiently transfected with wild-type, but not C528-mutated, Vav clearly demonstrate that Vav itself is a PE-binding and -stimulated Ras GEF. These findings, the inability of staurosporine to block Vav stimulation by PE-DAG, and the failure to detect PKC (or other serine/threonine phosphorylating activity) in Vav IPs, virtually rule out the possibility that these mediators stimulate Vav indirectly via the activation of some protein kinase(s).

Our findings underscore the importance of the cysteine-rich domain of Vav in PMA binding and activation and the

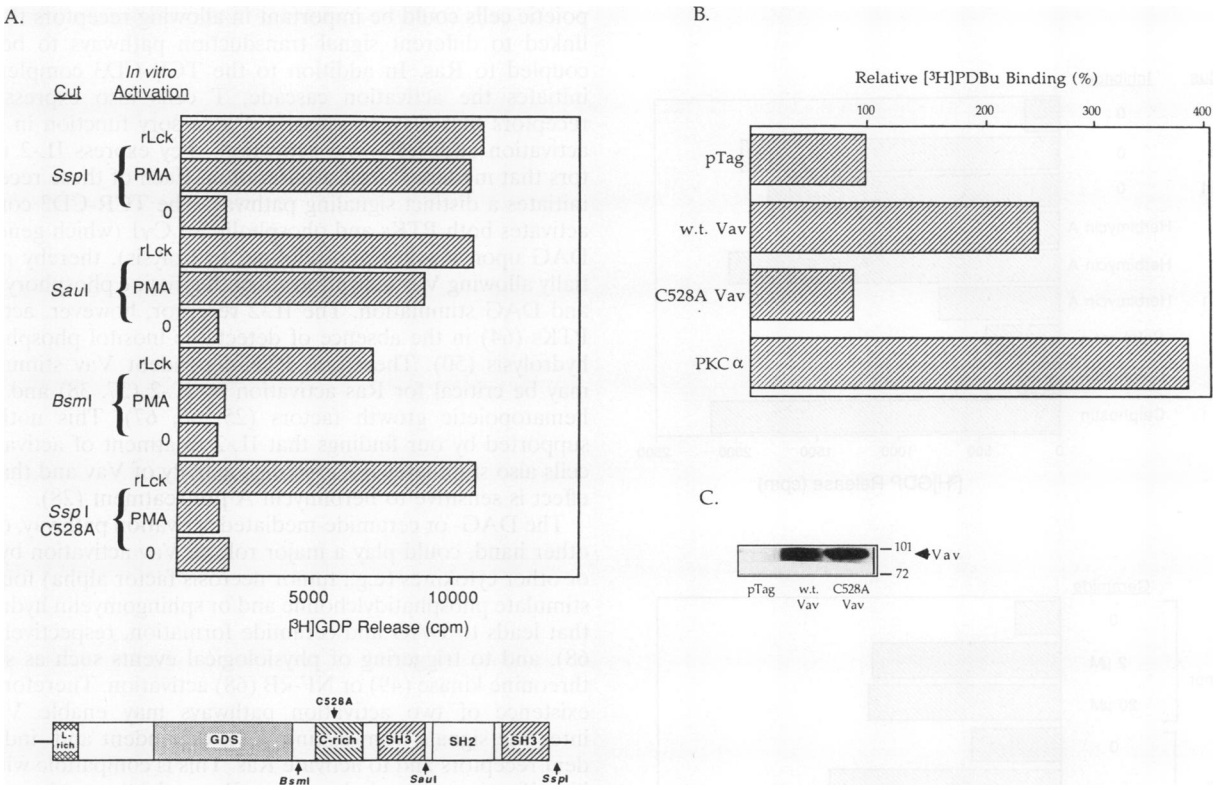

FIG. 4. (A) Recombinant p56^{lck} and PMA-induced exchange activity of partially purified wild-type or mutated Vav proteins. Full-length wild-type (w.t.) or Cys-528-mutated Vav proteins or truncated proteins (as shown at the bottom) were generated by in vitro transcription-translation and partially purified by Ni^{2+} resin affinity chromatography. All translation reaction mixtures contained similar amounts of Vav, as revealed by immunoblotting with the antitag MAb (data not shown). The bars show the Ras exchange activity (corrected for the total reaction volume; see Fig. 1A legend) in 5-μl Vav aliquots, either unstimulated (0) or following treatment with 200 ng of recombinant p56^{lck} (in the presence of 10 mM Mg^{2+} and 10 μM ATP) (rLck) or 10 ng of PMA. Unspecific [^{3}H]GDP release (≤1,000 cpm in the different groups) was subtracted. This experiment was repeated with essentially the same results. (B) [^{3}H]PDBu binding to transfected COS-1 cells (n = 3). Nonspecific [^{3}H]PDBu binding in the presence of 2.5 μM cold PDBu (≤240 cpm) represented <10% of the specific binding, and this value was subtracted for each group. Standard errors of each triplicate were ≤10%. Results represent the means of three similar experiments and are expressed as percent [^{3}H]PDBu binding normalized to binding by vector control-transfected cells (100%). (C) Vav immunoblot. Size markers on the right are in kilodaltons.

similarity of this mechanism to the activation of PKC, which is likewise dependent on its homologous cysteine-rich domain (56). Thus, direct stimulation of the enzymatic activity of Vav by diglycerides represents a novel mode of regulating a Ras exchange protein. The expression of this PMA-stimulated Ras GEF in hematopoietic cells may account for the observed Ras activation by PMA in T (24) and B (36) cells. This notion is reinforced by the findings that PMA-mediated Ras activation in B cells was PKC independent (36). Interestingly, α2-chimerin, a member of the chimerin family of PMA-activated Rac-specific GTPase-activating proteins (2, 21), expresses, like Vav, both a PMA-responsive cysteine-rich motif and an SH2 domain (34).

Several lines of evidence indicate that the PMA-diglyceride-mediated Vav activation pathway described here is independent from the PTK-mediated activation of Vav initiated by lymphocyte antigen receptors, as defined by our previous studies (29, 31): the OKT3- but not PE-DAG-induced Vav activation was accompanied by readily detectable tyrosine phosphorylation and was blocked by a specific PTK inhibitor; conversely, the PE-DAG effect, but not the anti-CD3-induced one, was blocked by calphostin, a known antagonist of PE-DAG binding to the regulatory C1 domain of PKC (17). In addition, Vav activation by PE-DAG (but not by p56^{lck}) required the presence of a region (residues 511 to 664) containing the Vav cysteine-rich domain and, more specifically, was abolished by mutating a single cysteine residue (Cys-528) within this domain.

The two Vav activation pathways described herein and elsewhere (29, 31) differ substantially from the mechanism that regulates the activity of another Ras exchange protein, i.e., the ubiquitously expressed mammalian (13, 14, 19, 44, 63) and *Drosophila* (55, 70) Sos proteins. Unlike Vav, whose enzymatic activity is directly regulated by tyrosine phosphorylation (or PE-DAG), Sos does not become phosphorylated on tyrosine upon epidermal growth factor stimulation, and its enzymatic activity is regulated at the level of intracellular localization rather than by changes in its intrinsic activity. Sos association with the activated, autophosphorylated epidermal growth factor receptor, mediated by the Grb2 adaptor protein (45), causes it to translocate to the membrane where it has access to its substrates, i.e., the Ras proteins that are located at the plasma membrane (14). Vav contains SH3-SH2-SH3 domains (16, 47) similar in their arrangement to Grb2. Therefore, Vav may have incorporated the properties of an adaptor and a Ras exchange factor in a single protein. The SH2 domain of Vav could play an important role in its enzymatic activation by mediating associations with TCR-CD3-coupled PTKs of the

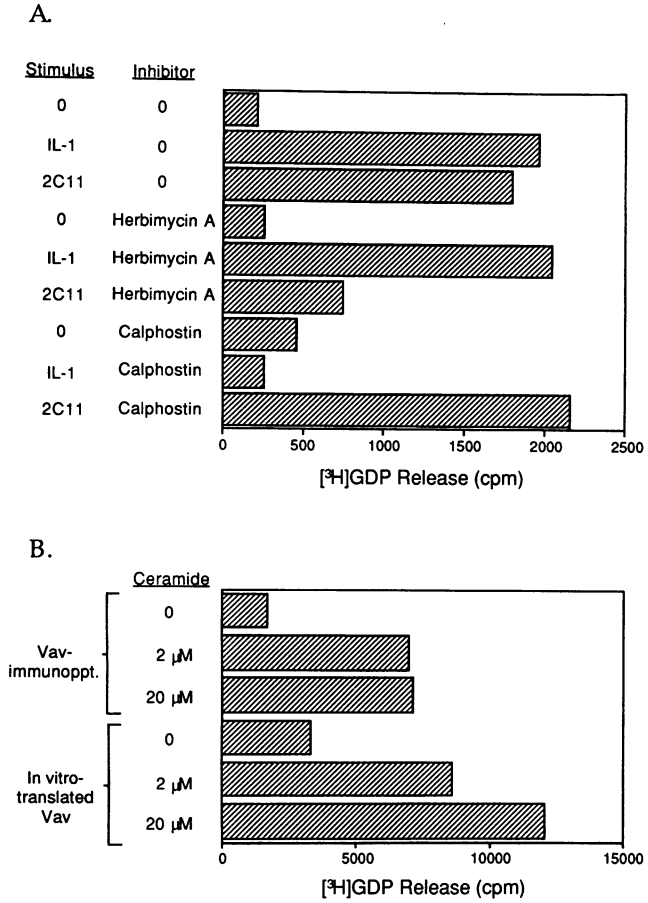

FIG. 5. Stimulation of the exchange activity of Vav by IL-1 or ceramide. (A) Aliquots of 4×10^6 EL4 cells, pretreated in the absence of an inhibitor (0) or the presence of herbimycin A (10 μM, overnight) or calphostin (1 μM, 5 min), were stimulated for 1 min with recombinant IL-1α (1 ng/ml) or 2C11 (10 μg/ml). Ras exchange activity of Vav IPs was determined in a 0.5-min assay. Counts were corrected for the total reaction volume. (B) Aliquots of in vitro-translated Vav or Jurkat-derived Vav IPs (Vav-immunoppt.) were treated for 10 min with ceramide and subjected to a Ras exchange assay. The presence of ceramide did not affect background [^{3}H]GDP release (~400 cpm) in the presence of a control IP (prepared with normal rabbit immunoglobulin) or translation reaction.

Src and/or ZAP-70/Syk (51, 71, 72) families during T-cell activation. This association could facilitate tyrosine phosphorylation and enzymatic activation of Vav. The resulting assembly of an active, Vav-containing signaling complex at or near the plasma membrane would enable Ras activation and stimulation of downstream intermediates, leading to activation of the IL-2, and other, genes. In this scheme, the SH3 domains of Vav could mediate the binding of Vav to other elements required for its optimal activity or localization.

The physiological relevance of the PE-DAG-mediated Vav activation pathway is supported by the finding that the pleiotropic cytokine, IL-1, also stimulated the enzymatic activity of Vav in intact T cells via a tyrosine phosphorylation-independent pathway. Moreover, ceramide, a second messenger induced by IL-1 stimulation (49), had a similar effect not only on immunoprecipitated, but also on in vitro-translated Vav (which cannot be associated with any other T-cell-derived proteins). The existence of distinct Vav activation pathways in hematopoietic cells could be important in allowing receptors that are linked to different signal transduction pathways to become coupled to Ras. In addition to the TCR-CD3 complex that initiates the activation cascade, T cells also express IL-1 receptors that play an important accessory function in T-cell activation and, following activation, they express IL-2 receptors that mediate T-cell proliferation. Each of these receptors initiates a distinct signaling pathway. The TCR-CD3 complex activates both PTKs and phospholipase Cγ1 (which generates DAG upon inositol phospholipid hydrolysis), thereby potentially allowing Vav activation via both tyrosine phosphorylation and DAG stimulation. The IL-2 receptor, however, activates PTKs (64) in the absence of detectable inositol phospholipid hydrolysis (50). Therefore, PTK-dependent Vav stimulation may be critical for Ras activation by IL-2 (27, 38) and other hematopoietic growth factors (25, 66, 67). This notion is supported by our findings that IL-2 treatment of activated T cells also stimulates the exchange activity of Vav and that this effect is sensitive to herbimycin A pretreatment (28).

The DAG- or ceramide-mediated activation pathway, on the other hand, could play a major role in Vav activation by IL-1 or other cytokines (e.g., tumor necrosis factor alpha) found to stimulate phosphatidylcholine and/or sphingomyelin hydrolysis that leads to DAG and ceramide formation, respectively (49, 68), and to triggering of physiological events such as serine/threonine kinase (49) or NF-κB (68) activation. Therefore, the existence of two activation pathways may enable Vav to integrate signals from distinct PTK-dependent and -independent receptors and to activate Ras. This is compatible with the identification of two independent Ras activation pathways in T cells, only one of which is PTK dependent (39). Additional studies on the mechanisms that regulate the catalytic activity of Vav will contribute, therefore, to a better understanding of the function and receptor coupling of Ras proteins in hematopoietic cell growth and differentiation pathways.

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