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A Functional T-Cell Receptor Signaling Pathway Is Required for p95^{vav} Activity

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Stimulation of the T-cell antigen receptor (TCR) induces activation of multiple tyrosine kinases, resulting in phosphorylation of numerous intracellular substrates. One substrate is p95^{vav}, which is expressed exclusively in hematopoietic and trophoblast cells. It contains a number of structural motifs, including Src homology 2, Src homology 3, and pleckstrin homology domains and a putative guanine nucleotide exchange domain. The role of p95^{vav} in TCR-mediated signaling processes is unclear. Here, we show that overexpression of p95^{vav} alone in Jurkat T cells leads to activation of the nuclear factors, including NFAT, involved in interleukin-2 expression. Furthermore, p95^{vav} synergizes with TCR stimulation in inducing NFAT- and interleukin-2-dependent transcription. In contrast, NFAT activation by a G-protein-coupled receptor is not modulated by p95^{vav} overexpression, suggesting that the effect is specific to the TCR signaling pathways. Although removal of the first 67 amino acids of p95^{vav} activates its transforming potential in NIH 3T3 cells, this region appears to be required for its function in T cells. We further demonstrate that the p95^{vav}-induced NFAT activation is not mimicked by Ras activation, though its function is dependent upon Ras and Raf. Furthermore, the activating function of p95^{vav} is blocked by FK506, suggesting that its activity also depends on calcineurin. To further dissect p95^{vav} involvement in TCR signaling, we analyzed various Jurkat mutants deficient in TCR signaling function or TCR expression and showed that an intact TCR signaling pathway is required for p95^{vav} to function. However, overexpression of p95^{vav} does not appear to influence TCR-induced protein tyrosine phosphorylation or increases in cytoplasmic free calcium. Taken together, our data suggest that p95^{vav} plays an important role at an yet unidentified proximal position in the TCR signaling cascade.

Engagement of the T-cell antigen receptor (TCR) by a peptide-bound major histocompatibility complex molecule initiates a biochemical cascade involving activation of protein tyrosine kinases (PTKs), resulting in phosphorylation of multiple intracellular proteins, including the TCR ζ and CD3 chains, ZAP-70, and phospholipase C-γ1 (PLC-γ1) (61). Tyrosine phosphorylation of PLC-γ1 induces its enzymatic activity (45), leading to the generation of the two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, which are responsible for the rapid and sustained intracellular calcium increase and activation of protein kinase C (PKC), respectively. These early biochemical events, through poorly understood processes, lead to a variety of T-cell functions, including transcriptional activation of interleukin-2 (IL-2) gene.

Tyrosine phosphorylation of cellular proteins has been shown to be an early and obligatory step in TCR signal transduction (33). Therefore, the identification and understanding the function of these molecules is of considerable interest. One of these TCR-induced tyrosine-phosphorylated proteins is p95^{vav} (6, 42), the product of a proto-oncogene expressed exclusively in hematopoietic and trophoblast cells (35, 65). It was first identified as a result of its malignant activation during the course of a gene transfer assay (35). Truncation of the amino-terminal leucine-rich region activates its transforming potential when it is expressed in NIH 3T3 cells (11, 34, 35). p95^{vav} contains a putative guanine nucleotide exchange factor (GEF) domain for small GTPases of the Rho/Rac family. A homologous domain is also present in CDC24 and Dbl, two known GEFs for Rho and Rac proteins (1, 2, 18). Moreover, p95^{vav} also contains a number of structural motifs shared by other signaling molecules, including one Src homology 2 (SH2) domain, two SH3 domains, a pleckstrin homology domain, and a cysteine-rich domain (39, 44). Recent studies show that p95^{vav} becomes tyrosine phosphorylated following a variety of stimuli, including TCR, B-cell antigen receptor, and IL-2 receptor stimulation (5, 6, 16, 42). When p95^{vav} is expressed ectopically in fibroblasts, epidermal or platelet-derived growth factor will stimulate its rapid tyrosine phosphorylation. Activation of epidermal or platelet-derived growth factor receptors also results in association of the receptors with p95^{vav} via its SH2 domain (6, 42). Although p95^{vav} so far has not been found to associate with any receptors upon ligand stimulation in hematopoietic cells, its SH2 domain has been shown to interact with a number of signaling molecules, including ZAP-70 and an uncharacterized tyrosine-phosphorylated protein, Vap-1 (5, 36). Taken together, these findings suggest that p95^{vav} may play an important role in the receptor-mediated signaling processes in hematopoietic cells.

TCR activation leads to cell progression from resting G_0 to G_1 and the production of IL-2. IL-2 is then available for stimulation of the IL-2 receptor in an autocrine or paracrine fashion, causing T cells to enter S phase, which ultimately results in cell proliferation. Therefore, regulation of IL-2 gene transcription is a key step in T-cell activation. Two critical requirements for IL-2 gene activation upon TCR stimulation are the activation of Ras and calcineurin, a calcium/calmodulin-dependent protein phosphatase (10, 13, 47, 51). It is believed that calcineurin is activated by the increase of intracellular calcium

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caused by activation of PLC-γ1 (61). Two pharmacological agents, FK506 and cyclosporin A, can block TCR-mediated IL-2 gene activation by inhibiting calcineurin function (15, 17). Ras can be activated by PKC-dependent and PKC-independent pathways (32). Expression of an activated form of Ras leads to IL-2 gene activation in conjunction with a calcium ionophore or an activated form of calcineurin, whereas a dominant negative form of Ras inhibits TCR signaling (51, 63). These studies emphasize the critical role of calcineurin and Ras in TCR-mediated signals that regulate IL-2 gene expression.

Although p95^{vav} shares homology with the GEFs for Rho/Rac but not Ras family members, it has been reported to account for most of the receptor-stimulated Ras GDP-GTP exchange activity in both T and B cells (23, 26). In addition, tyrosine phosphorylation of p95^{vav} or binding of DAG to its cysteine-rich domain was shown to be required for this exchange activity (23–25). However, these findings have not been confirmed by other investigators, who demonstrated that neither p95^{vav} nor oncogenic vav has any detectable exchange activity for Ras and that the morphology of vav-transformed NIH 3T3 cells is distinct that of from Ras-transformed cells (7, 38). The DAG or phorbol ester binding activity of the cysteine-rich region in p95^{vav} also appears to be controversial, since in vitro assays failed to show any binding of p95^{vav} to DAG or phorbol ester (37). Therefore, the functional significance of p95^{vav} as a Ras exchange factor in lymphocytes remains unclear.

To explore the potential role of p95^{vav} in TCR-mediated signaling leading to IL-2 gene activation, we overexpressed p95^{vav} and oncogenic vav in Jurkat T cells and examined their effects on IL-2 promoter elements. Here, we find that overexpression of p95^{vav} alone can activate the IL-2 promoter through mechanisms which depend on both Ras and calcineurin function. By using various Jurkat mutants deficient in TCR signaling, we demonstrate that this p95^{vav} activity requires a functional TCR signaling pathway.

MATERIALS AND METHODS

Cells and reagents. Jurkat, JCaM1.6 (19, 59), J45.01 (40), and J.RT3-T3.5 (46) cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (medium). Simian virus 40 (SV40) T-antigen (TAg)-transfected Jurkat (TAg Jurkat; a kind gift from G. Crabtree), J.HM1.2.1 (20), J.HM1.2.2 (20), PF-2.4, and PF-2.8 (46) cells were maintained in medium with 2.0 mg of geneticin (Gibco) per ml and transferred to geneticin-free medium 48 h before experiments to prevent aminoglycoside-mediated inhibition of phosphoinositide hydrolysis. JCaM1.6/Lck cells (59) were maintained in medium containing 250 μg of hygromycin per ml.

Plasmids. The p95^{vav} and oncogenic vav expression plasmids (pSV115 and pSV67, respectively) were constructed by subcloning the *Eco*RI fragments of *vav* cDNAs from pSK115 and pSK67 into pSV7d, an SV40 origin-containing mammalian expression vector (34, 35). The same cDNA inserts were also subcloned into pEF-BOS, another mammalian expression vector (43), to generate pEF115 and pEF67. An oligonucleotide fragment comprising both a Kozak translational initiation and the myc epitope tag sequences was linked to the 5′ end of the p95^{vav} cDNA to generate both pSV115myc and pEF115myc. Plasmid pCD8T, expressing only the extracellular and transmembrane portion of the human CD8α molecule, has been described elsewhere (30). The N17ras and DN-Raf plasmids were kind gifts from D. Cantrell. The NFAT, NFIL-2A, and IL-2 luciferase reporter constructs (NFAT-Luc, NFIL2A-Luc, and IL2-Luc) were generous gifts from G. Crabtree. The v-H-ras expression plasmid was kindly provided by H. Bourne.

Abs. The monoclonal antibody (MAb) used for the stimulation of the TCR was C305, which recognizes the Jurkat Ti β chain (62). The anti-vav polyclonal antibody (Ab) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antiphosphotyrosine MAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). A MAb, 9E10, for the detection of the myc epitope was kindly provided by J. M. Bishop. A fluorescein isothiocyanate (FITC)-conjugated anti-CD8α (FITC-CD8) Ab was purchased from Becton Dickinson (Mountain View, Calif.).

Cell transfections, stimulations, and luciferase assays. TAg Jurkat cells (10^7) were transiently transfected by electroporation, as previously described (10), with 20 μg of the reporter plasmids indicated and 50 μg of a vector containing either no insert (empty vector) or a p95^{vav}, N-terminally truncated vav, or v-H-ras cDNA insert. Forty hours after transfection, 10^5 cells were aliquoted into a 96-well plate (Corning) and cultured in a final volume of 100 μl of RPMI 1640 growth medium. Cells were unstimulated or stimulated at 37°C in growth medium containing either 1.0 μM ionomycin, a 1:1,000 dilution of C305 ascites, 500 μM carbachol, or 50 ng of phorbol myristate acetate per ml and 1.0 μM ionomycin. After an 8-h stimulation period, cells were lysed in harvest buffer (100 mM KPO_4 [pH 7.8], 1.0 mM dithiothreitol, 1% Triton X-100), and 100 μl of lysate was mixed with 100 μl of assay buffer (200 mM KPO_4 [pH 7.8], 10 mM ATP, 20 mM $MgCl_2$) followed by 100 μl of 1.0 mM luciferin. Luciferase activity, expressed in arbitrary units (AU), was determined either in duplicate or in triplicate for each experimental condition. In the cotransfection experiments, 30 μg of a vector containing either N17ras, DN-Raf, or pCD8T was cotransfected with pSV115. For wild-type Jurkat T cells and mutant derivatives, plasmid pEF115 or pEF115myc (40 μg) was transiently transfected into 10^7 cells. Forty hours after transfection, 5×10^5 cells were aliquoted into a 24-well plates in 1.5 ml of medium. Cells were then stimulated and assayed as described above. For the inhibition assays, cells were maintained in medium containing either 100 ng of FK506 per ml or 3.0 μM herbimycin A for 24 h after transfection. They were stimulated and assayed as described above.

Cell purification and immunoblots. TAg Jurkat cells were transiently transfected with pCD8T, along with an empty vector, pSV115 or pSV67. Twenty-four hours later, cells were harvested and CD8-positive cells were enriched by adding FITC-CD8 Ab and sheep anti-mouse immunoglobulin-coated magnetic beads (Dynal Inc., Great Neck, N.Y.). The bound CD8-positive cells were then lysed in lysis buffer containing 1% Nonidet P-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors as previously described (59). In the case of TCR stimulation, CD8-positive cells were stimulated with C305 (1:250) for 2 min and then lysed immediately. Lysates equivalent to 10^6 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes. The blots were blocked with 5% bovine serum albumin (or 3% ovalbumin) in 10 mM Tris (pH 7.9)–150 mM NaCl containing 0.05% Tween detergent. Blots were incubated with primary Ab followed by [^{125}I]protein A or secondary Ab conjugated with horseradish peroxidase and then assayed by autoradiography or enhanced chemiluminescence assay, (ECL kit; Amersham), respectively.

Measurement of calcium by flow cytometry. Calcium measurements were performed as previously described (21). The indicated transiently transfected cells were incubated in media at 10^6/ml with a 1.0 μM the calcium-sensitive fluorescence dye Indo-1 (Molecular Probe, Menlo Park, Calif.) at 37°C and then stained with FITC-CD8 Ab at 4°C. Fluorescence-activated cell sorter analysis was performed as previously described (4).

RESULTS

Overexpression of p95^{vav} in Jurkat T cells increases the basal activity of IL-2 promoter elements and further potentiates TCR-mediated signal transduction. TCR activation contributes to the production of IL-2. *cis*-acting elements in the IL-2 promoter bind nuclear factors, including NFAT and NFIL-2A (14). Reporter constructs containing multimers of either element are responsive to TCR-mediated signaling and require activation of both calcineurin and Ras pathways (14, 15, 63). To determine the role of p95^{vav} in T-cell signaling, we transiently overexpressed p95^{vav} in SV40 TAg-transfected Jurkat T cells and examined its effect on the NFAT-Luc or NFIL2A-Luc reporter construct. The expression vector used contains an SV40 origin of replication to allow its high-level gene expression in TAg Jurkat cells (10). By cotransfecting truncated CD8 as a surface marker for transfected cells and then enriching the CD8-positive transfectants, we estimated that the level of p95^{vav} overexpression is about 5 to 10 times that of the endogenous p95^{vav} by Western blotting (immunoblotting) and autoradiography (Fig. 1A). Overexpression of p95^{vav} alone resulted in 30- and 15-fold increases in basal activity of the NFAT and NFIL-2A reporter constructs, respectively (Fig. 1B). In contrast, reporter constructs driven by a constitutively active Rous sarcoma virus promoter or CD4 enhancer promoter produced only a twofold increase of activity in the p95^{vav}-overexpressing cells (data not shown).

Overexpression of a truncated form of p95^{vav}, lacking the first 67 amino acids in the N terminus, did not lead to increases

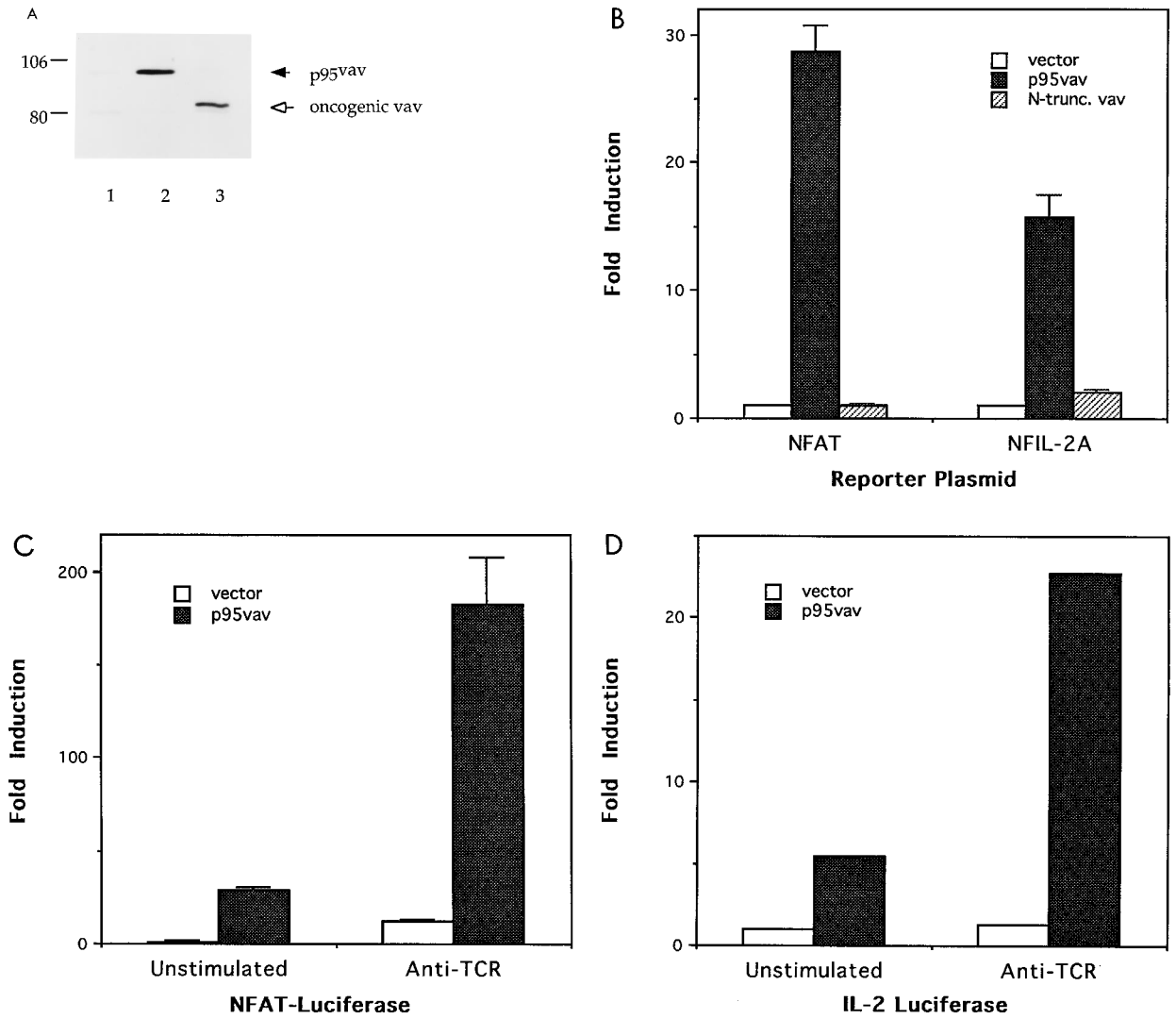

FIG. 1. Overexpression of p95^{vav} in TAg Jurkat cells augments basal and TCR-stimulated IL-2 transcriptional activity. (A) TAg Jurkat cells were cotransfected with plasmids encoding pCD8/T together with either an empty vector (lane 1), wild-type p95^{vav} (lane 2), or N-terminally truncated vav (lane 3). CD8-positive cells were purified, normalized, and blotted for vav expression. Sizes are indicated in kilodaltons. (B) TAg Jurkat cells were cotransfected with either NFAT-Luc or NFIL2A-Luc together with either an empty vector, wild-type p95^{vav}, or N-terminally truncated (N-trunc.) vav. Transfected cells were then assayed for luciferase activity. (C and D) TAg Jurkat cells were transfected with either NFAT-Luc (C) or IL2-Luc (D), along with either an empty vector or wild-type p95^{vav}. Cells were either unstimulated or stimulated with an anti-TCR MAb (C305) and then assayed for luciferase activity. The results are shown as the fold induction of luciferase activity compared with the activity in unstimulated cells cotransfected with pSV7d empty vector and the reporter construct. Luciferase activities of the unstimulated cells transfected with the empty vector were approximately 500 AU for NFAT-Luc and 200 AU for either NFIL2A-Luc or IL2-Luc. Maximal stimulated activities (by treatment with 50 ng of phorbol myristate acetate per ml and 1.0 μM ionomycin) were typically about 10^5 AU for NFAT-Luc and 5×10^4 AU for either NFIL2A-Luc or IL2-Luc. The results are expressed as the mean of values determined from at least three independent experiments. Error bars represent standard errors of the means.

in the basal activity of either NFAT or NFIL-2A (Fig. 1B). The overexpression level of the truncated vav is similar to that of wild type p95^{vav} in TAg Jurkat cells (Fig. 1A). This result was surprising, since the truncated p95^{vav} is transforming in fibroblasts, but indicates that the effect observed with the full-length p95^{vav} does not simply reflect the overexpression of domains (such as SH2 and SH3 domains) shared by other signaling molecules. Moreover, it implies that the N-terminal region may be required for p95^{vav} function in T cells.

Not only did overexpression of p95^{vav} lead to NFAT activation without TCR stimulation, it also markedly augmented TCR-stimulated NFAT activity compared with the level in the control vector-transfected cells (Fig. 1C). A similar 10-fold augmentation was observed on TCR-mediated induction of a reporter construct containing the entire upstream 275 bp of the IL-2 regulatory region (Fig. 1D). Note that TCR stimulation alone failed to activate the IL-2 promoter in this experiment because costimulation with phorbol ester or CD28 stimulation is required for activation of this promoter construct. Overexpression of the N-terminal truncated vav failed to enhance TCR-mediated signal transduction (data not shown). The p95^{vav}-induced NFAT activation is not due to the expression of TAg in Jurkat cells, since we also obtained similar results when we overexpressed p95^{vav} in unmodified (lacking TAg expression) Jurkat cells by using a different mammalian expression vector, pEF-BOS (see Fig. 3 and 4). These data demonstrate that overexpression of p95^{vav} has a profound effect on TCR-mediated activation of factors involved in IL-2 gene regulation and allowed us to study further the signaling role of p95^{vav} in T cells.

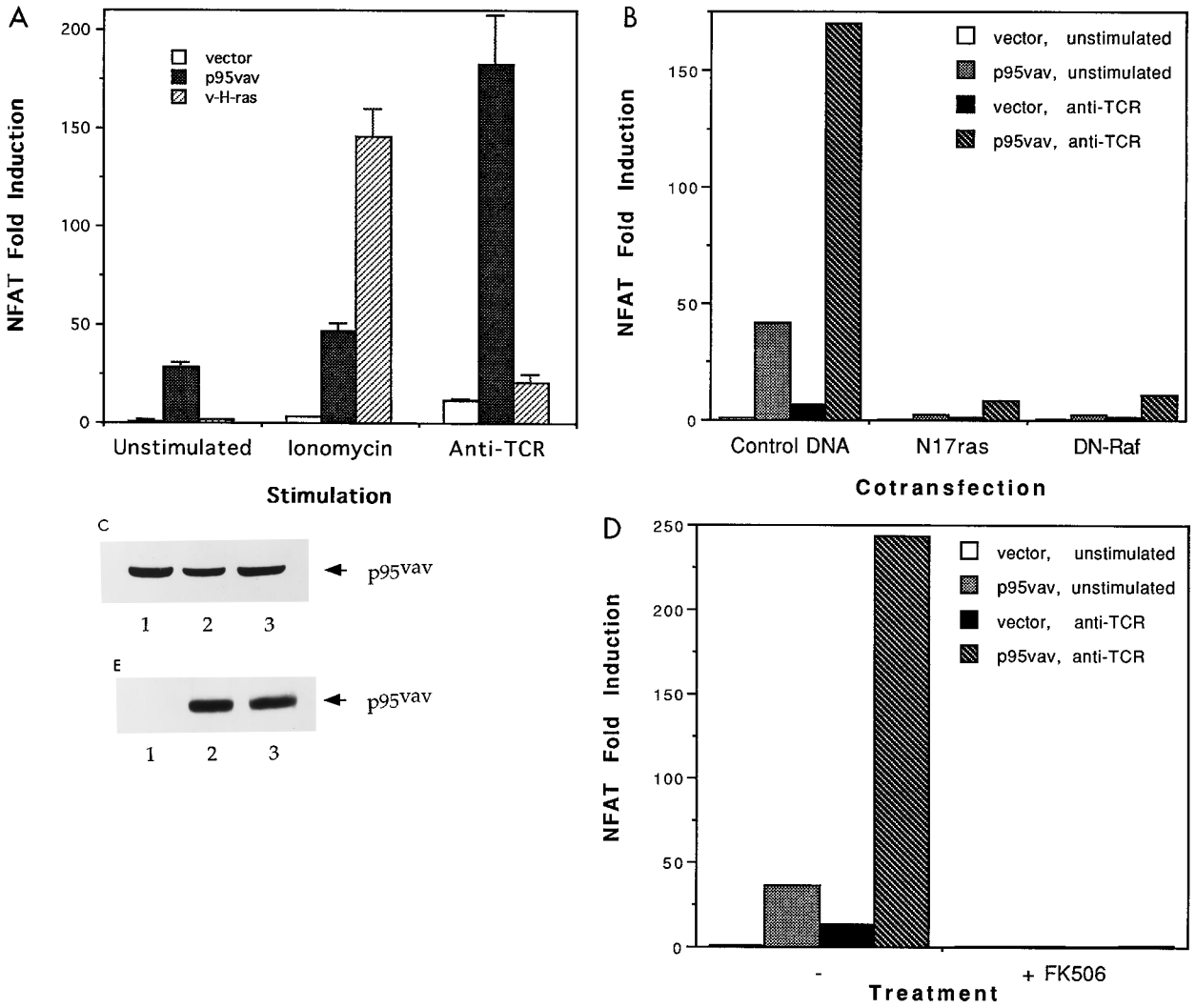

FIG. 2. p95^{vav}-mediated NFAT activation is not mimicked by Ras activation but is dependent on Ras and calcineurin function. (A) TAg Jurkat cells were transfected with NFAT-Luc along with either an empty vector alone, p95^{vav}, or v-H-ras. Cells were either unstimulated or stimulated with ionomycin or an anti-TCR MAb (C305) and then assayed for luciferase activity. The results are expressed as the means of values determined from at least three independent experiments. Error bars represent standard errors of the means. (B) Cotransfection of N17ras or DN-Raf impairs the basal and TCR-mediated NFAT activation induced by p95^{vav} overexpression in TAg Jurkat cells. A control vector, N17ras, or DN-Raf was cotransfected into TAg Jurkat cells with either a control vector or myc-p95^{vav} along with NFAT-Luc. Transfected cells were stimulated and assayed for luciferase activity. (C) An aliquot of cells from each transfection in panel B was lysed and blotted for myc epitope. Lanes 1 through 3 represent myc-p95^{vav} plus vector, myc-p95^{vav} plus N17ras, and myc-p95^{vav} plus DN-Raf, respectively. (D) TAg Jurkat cells were transfected with NFAT-Luc along with either a control vector or myc-p95^{vav}, stimulated in the presence or absence of FK506, and assayed for luciferase activity. (E) An aliquot of transfected cells in panel D was lysed and blotted for the myc epitope. Lane 1 through 3 represent control vector, myc-p95^{vav}, and myc-p95^{vav} in the presence of FK506, respectively. All luciferase assay results are shown as the fold induction of luciferase activity compared with the activity in unstimulated cells cotransfected with pSV7d vector and NFAT-Luc. The data are representative of at least two independent experiments.

Rather than simply activating Ras alone, p95^{vav} appears to function upstream of or parallel to Ras and calcineurin. It has been shown that the minimum requirement for NFAT induction is activation of both Ras and calcineurin (63). Expression of a constitutively activated Ras protein, v-H-ras, generates a signal which can synergize with a calcium signal to induce NFAT (51, 63). Since a region of p95^{vav} shares homology with GEF domains for the Rho/Rac family of small GTPases and has been reported to be a Ras GEF in lymphocytes, one possibility is that p95^{vav} overexpression in T cells directly activates Ras. It might then be expected to further synergize with a basal calcineurin activity leading to NFAT activation. Unlike p95^{vav}, transfection of v-H-ras into TAg Jurkat cells did not lead to a significant basal stimulation of NFAT activity (Fig. 2A). Moreover, ionomycin, a calcium ionophore which activates calcineurin, strongly synergized with v-H-ras but not with p95^{vav} in NFAT induction (Fig. 2A). These results are consistent with the recent report showing that p95^{vav} does not directly activate Ras (7).

However, Ras could still play a role in the effects of p95^{vav} overexpression. Since expression of either a dominant negative Ras (N17ras) or a dominant negative Raf (DN-Raf) prevents TCR-mediated activation of NFAT and the IL-2 gene (48, 51), we coexpressed N17ras or DN-Raf along with p95^{vav} in TAg Jurkat cells to examine whether p95^{vav} may depend on the Ras pathway to exert its activity on NFAT. To monitor the level of p95^{vav} overexpression, we generated a myc epitope-tagged version of p95^{vav}. Coexpression of either N17ras or DN-Raf abol-

ished the elevated basal and TCR-mediated NFAT activity induced by p95^{vav} overexpression (Fig. 2B) but did not suppress the level of transfected p95^{vav} (Fig. 2C). These data suggest that p95^{vav} does not simply function to directly activate Ras; rather, it appears to influence signaling events that depend upon Ras function.

To determine whether the p95^{vav}-induced activation of NFAT also requires calcineurin function, we used two immunosuppressive agents, FK506 and cyclosporin A, which are known to inhibit calcineurin function and thus impair TCR-mediated NFAT activation (15, 17). Treatment of Jurkat cells with either FK506 or cyclosporin A completely blocked both the elevation of basal levels and TCR-mediated NFAT activation in p95^{vav}-overexpressing cells (Fig. 2D and data not shown). The drug treatment was not toxic to the cells and did not inhibit p95^{vav} overexpression, as shown in Fig. 2E. These results indicate that the NFAT activation in p95^{vav}-overexpressing cells depends on both Ras and calcineurin pathways and suggest that p95^{vav} may function upstream of or parallel to them in TCR signaling pathways.

The p95^{vav} activity appears to be specific to TCR-mediated signaling. TCR engagement induces PLC activity, which leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of inositol 1,4,5-trisphosphate and DAG. These second messengers are responsible for the rapid and sustained intracellular calcium increase and activation of PKC upon TCR stimulation, respectively (61). The calcium increase is believed to be responsible for activation of calcineurin, whereas PKC activation can lead to Ras activation (61). In J.HM1.2.2, a Jurkat T-cell line expressing the seven-transmembrane domain heterologous human muscarinic receptor (HM1R), PLC-β is believed to be activated by the HM1R via a heterotrimeric G-protein-mediated mechanism (12). Stimulation of the HM1R in Jurkat cells induces increases in intracellular Ca^{2+} concentration and Ras activation (reference 20 and data not shown). We examined the effect of p95^{vav} overexpression in J.HM1.2.2 cells, in which stimulation through either TCR or HM1R can cause IL-2 gene activation. Consistent with previous studies, carbachol stimulation of the HM1R led to a potent induction of NFAT (Fig. 3) (12). Strikingly, overexpression of p95^{vav} failed to augment the HM1R stimulation, in contrast to its effect on TCR-mediated induction (Fig. 3). In addition, overexpression of p95^{vav} in Jurkat cells had only minimal effects on the NFAT induction following treatment with phorbol ester and calcium ionophore (data not shown). These data argue that the p95^{vav} effect is specific to the TCR-mediated signaling pathways and further implicate p95^{vav} function in the proximal signaling events upstream of or parallel to Ras and calcineurin in T cells.

An intact TCR signaling pathway is required for p95^{vav} to function. The earliest events occurring after TCR stimulation involve the activation of multiple cytoplasmic PTKs, manifested by the tyrosine phosphorylation of a variety of cellular proteins, including p95^{vav} (61). The role of PTK activation is underscored by the effects of PTK inhibitors such as herbimycin A, which markedly impairs TCR signaling (22, 33). If p95^{vav} participates in the TCR-mediated signaling pathway, proximal TCR signaling events may influence the effect of p95^{vav} on NFAT activation. In fact, incubation of p95^{vav}-transfected cells with herbimycin A blocked the basal activation of NFAT (Fig. 4A), suggesting that PTKs are required for p95^{vav} function.

One of the cytoplasmic PTKs required for proximal TCR signaling is Lck. A mutant Jurkat cell line, JCaM1.6, lacking functional Lck fails to mobilize intracellular calcium, to induce tyrosine phosphorylation, or to express activation antigens following TCR stimulation (59). Interestingly, when p95^{vav} was

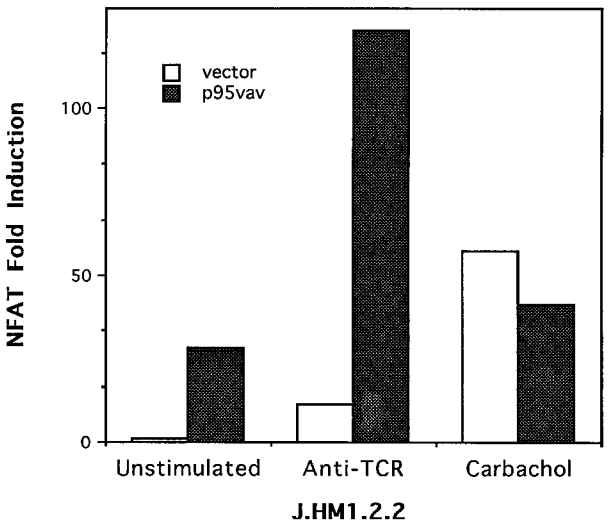

FIG. 3. Overexpression of p95^{vav} in J.HM1.2.2 cells specifically augments the TCR-mediated NFAT activation. J.HM1.2.2 cells were cotransfected with NFAT-Luc and either the control vector (pEF-BOS) or p95^{vav}. Transfected cells were either unstimulated or stimulated with an anti-TCR MAb (C305) or carbachol and assayed for induced luciferase activity as described in Materials and Methods. Luciferase activity was determined in either duplicate or triplicate for each experimental condition. The results are shown as the fold induction of luciferase activity compared with the activity in unstimulated cells cotransfected with the empty pEF-BOS and NFAT-Luc reporter construct. Luciferase activities of the unstimulated cells transfected with the empty pEF-BOS vector were typically 500 to 1,000 AU. Maximal stimulated activities (by treatment with 50 ng of phorbol myristate acetate per ml and 1.0 μM ionomycin) were typically about 1×10^5 to 3×10^5 AU. The data are representative of two independent experiments.

overexpressed in JCaM1.6, no basal induction of NFAT was observed (Fig. 4B and C). Reconstitution of this mutant cell with wild-type murine Lck restored all TCR-mediated function as well as the p95^{vav} effect on NFAT (Fig. 4B and C) (59). In contrast, v-H-ras was still able to activate NFAT in synergy with ionomycin in JCaM1.6 (Fig. 4D), suggesting that p95^{vav}, unlike activated Ras, requires proximal signals which depend on Lck to exert its activity. Another Jurkat mutant cell line, J45.01, which lacks CD45 membrane protein tyrosine phosphatase, is defective in TCR-mediated signaling at a very proximal step (41). The block in J45.01 is thought to result from negative regulation of Lck (58). As with JCaM1.6 cells, no NFAT induction was detected when p95^{vav} was overexpressed in J45.01 cells (Fig. 4B and C). These data strongly suggest that a PTK (Lck) and a phosphatase (CD45), which are required for proximal events in TCR signaling, are crucial for p95^{vav} function.

Recent studies showed that the signaling function of the TCR complex reside within a common motif (ITAM) in CD3 and TCR ζ chains (30, 55). Following TCR cross-linking, ITAMs in CD3 and TCR ζ chains become tyrosine phosphorylated, possibly by Lck, and serve as the binding sites for another cytoplasmic PTK, ZAP-70 (9, 61). Thus, the TCR ITAMs function as membrane anchors to facilitate the interaction of Lck and ZAP-70 (31). These initial biochemical events then lead to tyrosine phosphorylation of downstream intracellular substrates. The potential involvement of p95^{vav} in the initial events in TCR-mediated signaling pathways was further demonstrated by using a mutant Jurkat cell line, J.RT3-T3.5, which fails to express a TCR because of TCR β-chain deficiency (46). Overexpression of p95^{vav} in J.RT3-T3.5 cells had no effect on basal NFAT activity (Fig. 5A). Another

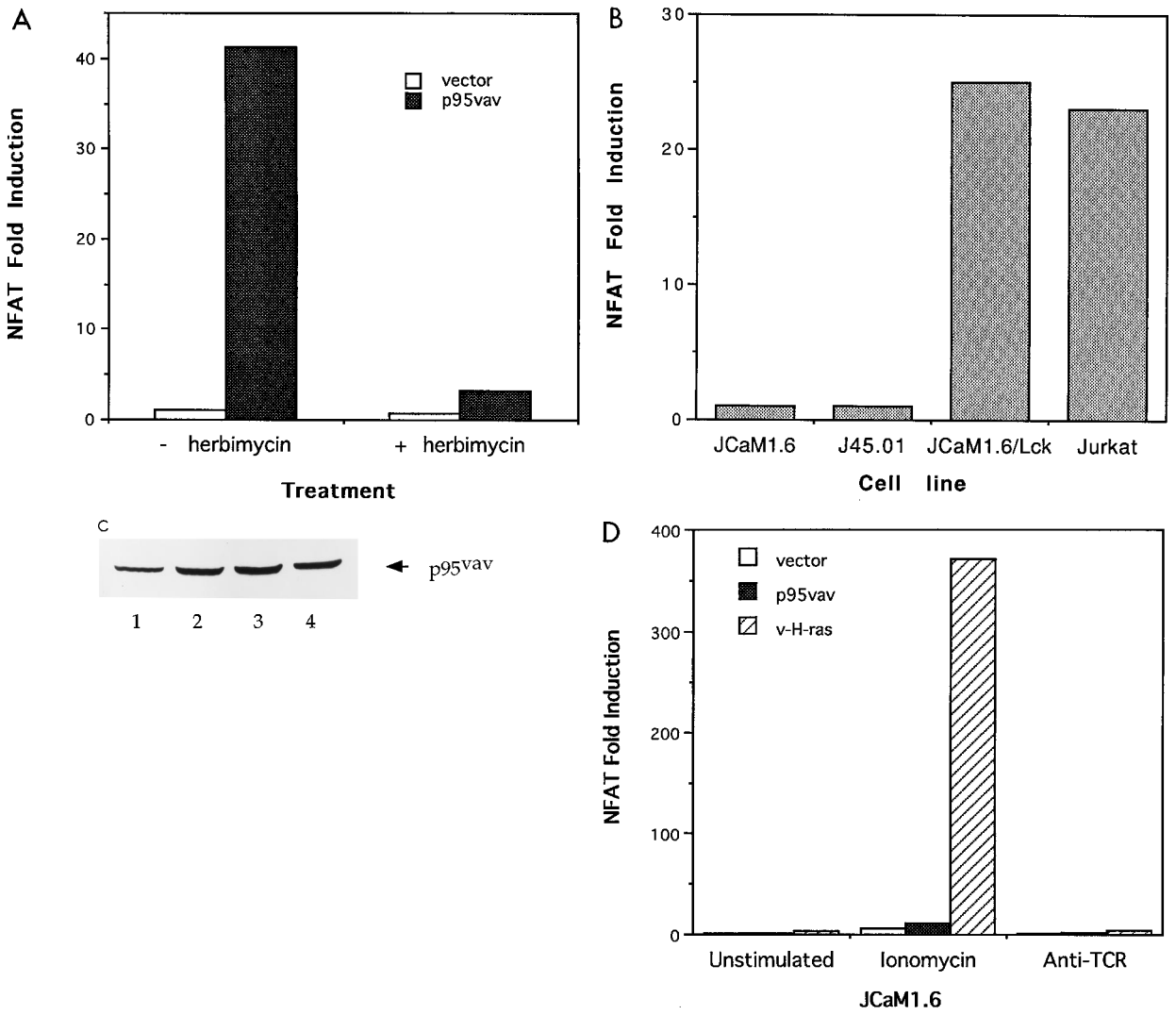

FIG. 4. The effect of p95^{vav} overexpression on NFAT activation requires the proximal tyrosine kinase activity in Jurkat T cells. (A) NFAT induction by overexpressing p95^{vav} is sensitive to herbimycin A treatment. TAg Jurkat cells were cotransfected with NFAT-Luc and either an empty vector (pSV7d) or p95^{vav}. Transfected cells were either untreated or treated with herbimycin A and assayed for luciferase activity. Western blot analysis showed that herbimycin A treatment does not inhibit p95^{vav} overexpression in Jurkat cells (data not shown). (B) Overexpression of p95^{vav} in Jurkat mutants deficient in TCR signaling does not lead to basal NFAT activation. JCaM1.6, J45.01, Lck-reconstituted JCaM1.6 (JCaM1.6/Lck), and wild-type Jurkat cells were transfected with NFAT-Luc and either an empty vector or myc-p95^{vav}. Unstimulated cells were assayed for luciferase activity. (C) Anti-myc epitope blot of equivalent amount of lysates from different transfectants in panel B. Lanes 1 through 4 represent lysates from transfected JCaM1.6, J45.01, JCaM1.6/Lck, and Jurkat cells, respectively. (D) v-H-ras, but not p95^{vav}, functions in a Lck-deficient Jurkat mutant. JCaM1.6 cells were transfected with NFAT-Luc along with either an empty vector, p95^{vav}, or v-H-ras. Transfected cells were either unstimulated or stimulated with ionomycin or an anti-TCR MAb (C305) and subsequently assayed for luciferase activity. The results are shown as the fold induction over that in untreated (A) or unstimulated (B and D) vector-transfected cells. The data are representative of two independent experiments.

HM1R-expressing TCR-negative Jurkat cell line, J.HM1.2.1, also failed to induce NFAT when p95^{vav} was overexpressed (Fig. 5A). The p95^{vav}-induced NFAT activation was restored in two of the TCR-β reconstituted Jurkat cells lines, PF-2.8 and PF-2.4 (Fig. 5A) (46). Note that the intermediate effect on NFAT activity in PF2.8 cells may reflect the fact that this cell expresses only 15% of the level of TCR expressed by Jurkat cells (22). Figure 5B shows that p95^{vav} was comparably overexpressed in these cells. In addition, J.HM1.2.1 and Jurkat cells overexpressed similar levels of p95^{vav} (data not shown). Thus, a functional TCR is required for p95^{vav} to function.

The proximal TCR signaling events do not appear to be altered in the p95^{vav}-overexpressing cells. The data above suggest that p95^{vav} may participate in the proximal events in TCR-mediated signal transduction. One hypothesis is that p95^{vav} normally acts as a positive regulator or interacts with negative regulators for a proximal PTK, Lck or ZAP-70, involved in TCR signaling. Therefore one might expect that overexpression of p95^{vav} may activate these PTKs, resulting in hyperphosphorylation of the downstream substrates, and lead to NFAT activation. To study whether p95^{vav} overexpression increases the levels of cellular tyrosine phosphorylation, we cotransfected truncated CD8, using it as a surface marker to purify the p95^{vav}-transfected cells by using magnetic beads, and analyzed the unstimulated and TCR-stimulated cell lysates. We have found that when two plasmid constructs are used in such cotransfections, the plasmids are taken up and expressed by the same population of cells. Similar approaches have also been used by others (4). As shown in Fig. 6, we found no significant alteration of tyrosine phosphorylation in unstimulated or stim-

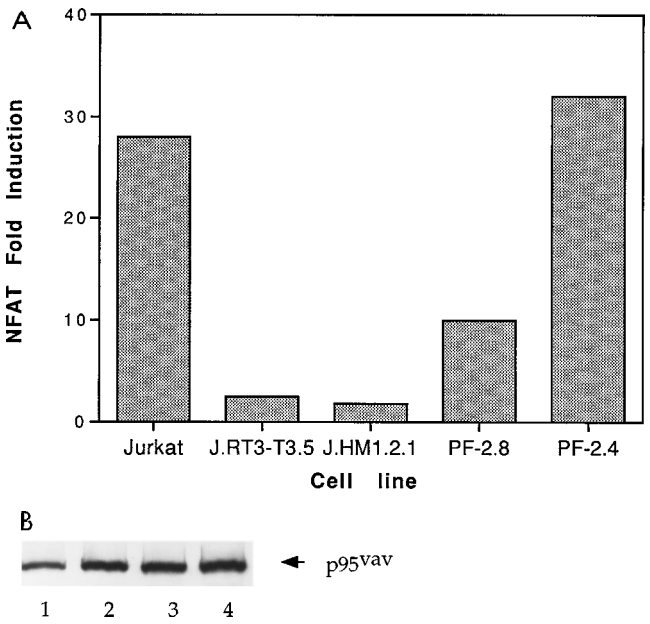

FIG. 5. Expression of a TCR is required for p95^{vav} function. (A) Wild-type Jurkat, J.RT3-T3.5, J.HM1.2.1, PF-2.8, and PF-2.4 cells were cotransfected with NFAT-Luc and either an empty vector (pEF-BOS) or myc-p95^{vav}. Transfected cells were assayed for basal luciferase activity. The results are shown as the fold induction over that in unstimulated vector-transfected cells. The data are representative of two independent experiments. (B) Anti-myc blot of lysates from p95^{vav}-transfected Jurkat, J.RT3-T3.5, PF-2.8, and PF-2.4 cells (lanes 1 through 4, respectively). p95^{vav} was also overexpressed in J.HM1.2.1 cells to a level similar to that in Jurkat cells (data not shown).

ulated p95^{vav}-overexpressing cells compared with control cells, suggesting that the balance between the proximal PTKs and protein tyrosine phosphatases is largely unaltered by p95^{vav} overexpression.

TCR stimulation activates PLC-γ1, resulting in the sustained rise of intracellular calcium. If p95^{vav} potentiates the proximal TCR signaling events, this calcium response may be modulated in the p95^{vav}-overexpressing cells. TAg Jurkat cells were cotransfected with truncated CD8 and p95^{vav}. We analyzed the intracellular calcium levels in CD8$^+$ and CD8$^-$ cells by flow cytometry. The gated CD8-positive transfectants overexpressed p95^{vav} as determined by Western blotting (data not shown). As shown in Fig. 7A and B, there is no significant difference in either the basal or TCR-stimulated level of intracellular calcium in p95^{vav}-overexpressing cells compared with nontransfected cells. These data suggest that overexpression of p95^{vav} in Jurkat T cells does not activate the proximal PTKs involved in TCR signaling.

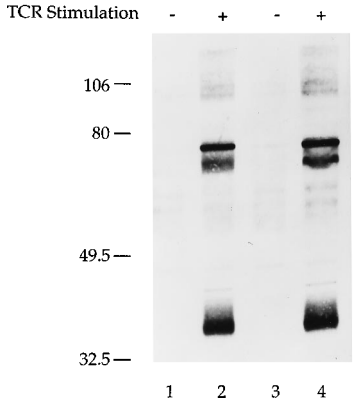

FIG. 6. Basal and TCR-stimulated cellular tyrosine phosphorylation in p95^{vav}-overexpressing Jurkat cells. TAg Jurkat cells were transfected with truncated CD8 along with either pSV7d vector (lanes 1 and 2) or p95^{vav} (lanes 3 and 4). After 24 h, CD8-expressing cells were purified as described in Materials and Methods and either left unstimulated (lanes 1 and 3) or stimulated with an anti-TCR MAb (C305) for 2 min (lanes 2 and 4). Whole cell lysates were prepared and blotted with antiphosphotyrosine MAb 4G10.

DISCUSSION

Signaling through the TCR results in a rapid increase in tyrosine phosphorylation of numerous intracellular proteins, including the proto-oncogene product p95^{vav}, and ultimately leads to transcriptional activation of the IL-2 gene (61). The detailed molecular mechanism by which the biochemical events initiated by the TCR on the plasma membrane are transmitted to the nucleus is still poorly understood. However, the induced tyrosine phosphoproteins are likely to play an important role. In this report, we show that overexpression of p95^{vav}, one of these substrates, in Jurkat T cells leads to a marked induction of the basal and TCR-mediated IL-2 promoter activity. Furthermore, this p95^{vav} activity requires the functions of both Ras and calcineurin, as well as a functional TCR complex. Our data strongly implicate the involvement of p95^{vav} in the TCR-mediated signaling pathway. This finding is consistent with the impaired function of antigen receptors on T and B lymphocytes which fail to express p95^{vav} (16a, 60, 64).

It is striking that an intact N-terminal region is required for p95^{vav} to exert its activity on NFAT. Although overexpression of the full-length vav may nonspecifically titrate out a negative inhibitor of TCR signaling pathways, this is unlikely since overexpression of the N-terminal region alone or a truncated form of p95^{vav} lacking its C-terminal Src homology domains fails to mimic the effects of the full-length p95^{vav} in Jurkat cells (data not shown). The leucine-rich amino-terminal of p95^{vav} was initially reported to contain a helix-loop-helix domain followed by a leucine zipper, similar to the carboxy-terminal region of myc proteins and the steroid binding domains of nuclear receptors (11, 34, 35). However, when more objective and quantitative criteria were applied in the sequence comparison, none of these similarities was shown to be significant (1, 3). Nonetheless, removal of this region can activate p95^{vav} transforming potential in NIH 3T3 cells, suggesting a regulatory function of the N-terminal region (11, 34). It is interesting that this region has some similarity with molecules which interact with actin or other cytoskeletal proteins (1). One possibility is that the N-terminal region allows p95^{vav} to bind to certain cytoskeletal components, and deletion of this region may release p95^{vav} to interact with other signaling processes and cause transformation. However, in T cells, this region appears to be critical for p95^{vav} function, perhaps by anchoring it to an appropriate compartment to exert its physiological function. The identification of molecules that interact with this region will be informative to understand its function.

p95^{vav} shares homology with proven or putative Rho/Rac GEF domains found in yeast CDC24, rodent CDC25^{Mm}, and human Dbl (2). Unfortunately, little is known about the functions of these GEFs, largely because of the difficulties in demonstrating their catalytic activities against Rho or Rac. In fact, only two members of this group, CDC24 and Dbl, have recently been demonstrated to activate Rho or Rac GTPase activity in vitro (2). On the other hand, p95^{vav} has been reported to be responsible for antigen receptor-stimulated Ras activation in lymphocytes (23, 26). Here, we show that the

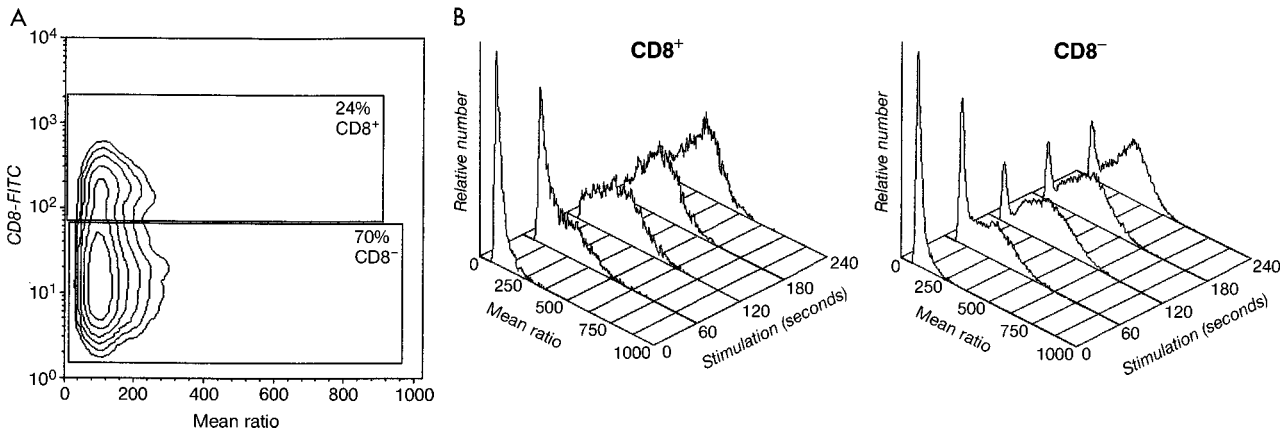

FIG. 7. Intracellular calcium level in p95^{vav}-overexpressing Jurkat cells. TAg Jurkat cells were transfected with truncated CD8 and p95^{vav}. After 24 h, cells were stained with FITC-CD8 Ab and loaded with Indo-1 as described in the text. (A) The top 25% of the transfected cells (the CD8-positive cells) contain the p95^{vav}-overexpressing cells (Fig. 1A and data not shown). The horizontal axis shows the Indo-1 fluorescence ratio representing the intracellular calcium level. (B) Transfected cells were gated as in panel A and were either unstimulated or stimulated with an anti-TCR MAb (C305) for 60, 120, 180, 240 s. The left and right panels shows the calcium levels in gated CD8-positive and CD8-negative populations, respectively.

effect of p95^{vav} overexpression is distinct from that of simply activating Ras pathway alone. Moreover, overexpression of another known Ras GEF, SCD25, failed to induce NFAT activation in Jurkat cells (data not shown). These data are consistent with the reports arguing against p95^{vav} as a GEF for Ras (7, 38). The exchange activity of p95^{vav} is further complicated by the presence in T cells of SOS, a highly conserved Ras GEF in other signaling processes (56). It has been shown that upon TCR ligation, a 36-kDa protein (pp36) and Shc become tyrosine phosphorylated and associate with Grb2, an adapter protein for SOS (56, 57). In T cells, the pp36-Grb2-SOS or a Shc-Grb2-SOS complex, rather than p95^{vav}, may serve as the link between the receptor and the downstream Ras pathway (50, 57).

How does overexpression of p95^{vav} lead to basal activation of NFAT and further synergy with TCR stimulation? It is believed that the basal state of the TCR signaling represents a dynamic equilibrium between activating and inhibitory signals and that stimulation through the TCR leads to an alteration of this balance. One explanation is that p95^{vav} normally interacts with a negative regulatory protein involved in the proximal TCR signaling pathway. Overexpression of p95^{vav} would titrate out this inhibitory molecule and shift the equilibrium to favor activating signals. It has been shown that in T and B cells, p95^{vav} is able to interact with a number of proteins, including some of the proximal PTKs (8, 27, 29, 36). However, p95^{vav} does not appear to activate the proximal PTKs, since we found no induction of basal or TCR-stimulated tyrosine phosphorylation when analyzing the lysates from p95^{vav}-overexpressing cells. In addition, our data on the intracellular calcium levels in those cells also indicate that some of the known proximal events may not be altered by p95^{vav} overexpression. However, it is possible that the phosphotyrosine assay is not sensitive enough, especially if p95^{vav} modulates only a subset of phosphoproteins. Also, since we only examined the cells 1 day after transfection, a shift of equilibrium by p95^{vav} overexpression may already be compensated for. More detailed kinetic and biochemical analyses will be important in assessing p95^{vav} function.

An alternative explanation is that p95^{vav} may be a key limiting mediator in the TCR-mediated signaling cascade. Overexpression of p95^{vav} may amplify the basal TCR signaling and allow it to proceed. For instance, the requirement for Lck or TCR expression could reflect tyrosine phosphorylation of p95^{vav} in order to exhibit its functional activity. In fact, in p95^{vav}-overexpressing Jurkat cells, p95^{vav} was weakly tyrosine phosphorylated in the basal state, and its tyrosine phosphorylation was further induced upon stimulation (data not shown). It is interesting that not only do p95^{vav} and Dbl contain similar GEF regions for Rho, but their oncogenic forms also share the same transforming phenotypes in NIH 3T3 cells (1, 7, 38). Since Dbl has been recently shown to activate Rho and Rac but not Ras (28), p95^{vav} may act as a Rho/Rac GEF in hematopoietic cells. One of the earliest responses of cells to many extracellular factors is a rapid reorganization of their actin cytoskeleton. It has been shown that the formation of stress fibers and focal adhesions is dependent on Rho proteins (53), whereas membrane ruffling requires Rac proteins (54). Rho and Rac have been implicated in regulating degranulation in mast cells (49). In cytotoxic T cells, TCR stimulation triggers a massive and rapid cytoskeleton rearrangement. Moreover, expression of Rac2, a hematopoietic cell-specific Rac family member, is up-regulated upon T-cell activation (52). Therefore, p95^{vav}-mediated activation of Rho and/or Rac may be an important TCR-mediated signaling event, in combination with the Ras and calcineurin signals, leading to downstream effector functions. In fact, recent evidence suggest that vav and Ras may mediate distinct but interactive signaling pathways in fibroblasts (7, 35). Overexpression of p95^{vav} in T cells may lead to activation of these small GTPases and further potentiate the basal TCR signals resulting in IL-2 gene activation. Future mutagenesis study of p95^{vav} and identification of its interacting proteins may help us to resolve these alternatives.

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