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The involvement of the proto-oncogene Vav in antigen receptor-mediated signal transduction

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Dedication

This dissertation is dedicated to my wife, Liz, for your love, support, patience, and encouragement. I love you!

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Much of the materials described in this dissertation is reproduced or modified from the following published or submitted papers, and I appreciate the permissions granted by the publishers to use these materials. The text in all chapters were written by me, with comments from the other coauthors. All of the experiments in Chapter II and III were conducted by me. All of the experiments in Chapter IV (except Figure 3) were conducted by me. All of the experiments in Chapter V (except Figure 1 and Figure 4A) were conducted by me. I am very grateful to Dr. Qihong Zhao, for initiating and collaborating the work described in Chapter V. The sequence alignment was kindly provided by Dr. Chris Botka. Dr. Arthur Weiss was my thesis advisor. Drs. S. Katzav, D.G. Motto, G.A. Koretzky, and T. Kurosaki provided cDNAs, cell lines and antibodies that made this thesis possible.

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ABSTRACT

The involvement of the proto-oncogene Vav in antigen receptor-mediated signal transduction.

by Jun Wu.

Stimulation of the antigen receptors in T and B cells induces activation of multiple tyrosine kinases including the Src and the Syk family members, resulting in phosphorylation of numerous intracellular substrates. One such substrate is the 95 kD proto-oncogene Vav, which is expressed exclusively in hematopoietic and trophoblast cells. Vav contains a number of structural motifs including a leucine-rich N-terminus, a putative guanine nucleotide exchange domain for Rho/Rac/CDC42 family of small GTPases, a pleckstrin homology domain, a cysteine-rich region, and one SH2 domain flanked by two SH3 domains. Although its expression pattern, structure and homology to other proteins suggests a role in lymphocyte signal transduction, the involvement of Vav in antigen receptor-mediated signaling processes is poorly understood. In these studies, we show that overexpression of Vav alone in Jurkat T cells leads to activation of nuclear factors, including NFAT, involved in interleukin 2 (IL-2) expression, which is further enhanced with TCR stimulation. The Vav-mediated NFAT activation in Jurkat cells is specific to antigen receptor-mediated gene activation and depends upon an intact TCR signaling pathways. In addition, a truncated Vav containing only the C-terminus functions as an inhibitor in TCR signaling cascade. These studies strongly suggest that Vav plays an important role in antigen receptor signal transduction. To explore the molecular mechanisms by which Vav functions in lymphocytes, we have identified another hematopoietic-specific tyrosine phosphoprotein, SLP-76, which associates with Vav via the Vav SH2 domain following TCR stimulation. More importantly, Vav

and SLP-76 functionally cooperate in TCR-mediated downstream gene activation, suggesting that a complex containing Vav and SLP-76 participates in the events triggered by antigen receptor stimulation. Finally, our genetic analysis using mutant cell lines and mice demonstrate that both Src and Syk/ZAP-70 PTKs contribute to Vav tyrosine phosphorylation in T and B cells. The Syk/ZAP-70 PTKs, in particular, appear to directly recruit Vav following antigen receptor stimulation, resulting in subsequent Vav tyrosine phosphorylation. We further show that a point mutation of the Vav binding site in ZAP-70 impairs ZAP-70's ability to interact with Vav, to phosphorylate Vav, and to function in antigen receptor-mediated signal transduction. Taken together, these data provide strong evidence that Vav is a critical component in lymphocyte activation.

Table of Contents

CHAPTER I

Introduction

T lymphocytes play a critical role in normal immune responses against pathogens, in transplantation rejection, and in autoimmune diseases. The specificity of the T cell-mediated immune response is determined by the T cell antigen receptor (TCR), which recognizes peptide complexed to the proper major histocompatibility complex (MHC) molecule and transduces this extracellular binding event to intracellular signal transduction machinery. This signal transduction process induces a series of biochemical events, including tyrosine phosphorylation of intracellular proteins, cytoskeletal rearrangement, Ca^{2+} influx, and Ras activation (1). These signaling events, through a poorly understood mechanism, ultimately leads to effector functions such as lymphokine production, cell proliferation and differentiation. Both biochemical and genetic evidence indicates that this signal transduction pathway is crucial for lymphocyte development and activation (1). This introduction will briefly review the current understanding of the signal transduction events initiated by engagement of the TCR and provide the rationale for undertaking the course of experimentation described in this dissertation to determine the role of the proto-oncogene Vav in TCR-mediated signal transduction pathways.

The TCR complex

The TCR is a multi-subunit complex consisting of Ti α and β chains, the CD3 γ, δ and ε chains, and a ζ-containing homodimer or heterodimer (2-4). Assembly of all subunits is required for efficient surface expression. The disulfide-linked α and β heterodimer is responsible for ligand recognition and is noncovalently associated with the invariant CD3 and ζ chains (5). The α and β chains have very short cytoplasmic tails which are incapable of transducing extracellular binding events directly to intracellular signaling machinery. In contrast, the cytoplasmic

domains of CD3 subunits and TCR ζ chains contain the immunoreceptor tyrosine based activation motifs (ITAM) consisting of two YxxL paired sequences separated by six to eight amino acids (4, 6, 7). This ITAM is present in three copies in ζ and is found as a single copy in each of the CD3 subunits. Recent studies have shown that the ITAM sequences are responsible for transducing TCR-mediated downstream signaling events. A chimeric receptor containing a single isolated ITAM motif is sufficient for the activation of the PTK pathway and subsequent TCR-mediated proximal and distal signaling events (8, 9). The importance of the ITAM motifs is further underscored by mutational analysis in which mutations of either tyrosine residue within the ITAM motif results in an impairment of receptor function (10). In addition, removal of the C-terminal leucine residue within the ITAM sequence also leads to loss of receptor function (8). Therefore, an intact ITAM is necessary and sufficient to transduce extracellular ligand binding events to intracellular signaling events.

In B cells, the Igα and Igβ subunits of the membrane Ig receptor also serve as the signal transducing components for the B cell antigen receptor (BCR), and each contains one copy of the ITAM (11, 12). Similar to what has been found in T cells, chimeric receptors containing either the cytoplasmic domains of Igα or Igβ subunits are capable of inducing signaling events such as tyrosine phosphorylation and Ca^{2+} mobilization (12). Thus, the ITAM motifs found in the CD3 subunits, the TCR ζ chains, the BCR Igα and Igβ subunits, and other immunoreceptor-associated molecules provide the molecular basis for initiating signaling cascades following receptor engagement.

Protein tyrosine kinases (PTKs) in TCR signaling.

Unlike many growth factor receptors, the TCR complex does not possess intrinsic tyrosine kinase activity. However, one of the earliest biochemical events following TCR ligation is the activation of multiple cytoplasmic PTKs, resulting in tyrosine phosphorylation of numerous cellular proteins (4, 13). The importance of PTK activation is underscored by studies in which treatment of T cells with a PTK inhibitor, herbimycin A, markedly impairs TCR-mediated function (14). Recent biochemical and genetic evidence has demonstrated that two families of PTKs, Src and Syk, play obligatory and proximal roles in TCR-mediated signal transduction. Studies of T cell lines have implicated the Src family members Lck and Fyn as the initiating PTKs for TCR signaling (4). They function by phosphorylating the tyrosine residues within the ITAM motifs within the CD3 and ζ subunits following TCR stimulation. Phosphorylation of the ITAMs results in recruitment of Syk family PTKs via their two SH2 domains, where they can become tyrosine phosphorylated and activated through the actions of the Src family of PTKs (4, 15-17).

The critical role of the Src PTKs in T cell development and activation has been demonstrated in cell lines or mice deficient in Lck, Fyn, or both kinases. Mutant mice deficient in Lck have a severe reduction in thymocyte numbers and a profound block in thymocyte development at the transition to the $CD4^+CD8^+$ stage (18). The residual progression of thymocytes from $CD4^-CD8^-$ to the double positive stage in *lck*-/- mice depends upon the redundant function of Fyn, since mice lacking both Lck and Fyn show a complete arrest of thymocyte development at the $CD4^-CD8^-$ stage (19, 20). These results suggest that the pre-TCR complex-mediated signaling events depend upon the Src PTKs Lck and Fyn. In addition, TCR stimulation in T cells from Lck-deficient mice or in T cell lines

lacking a functional Lck molecule leads to a marked reduction in tyrosine phosphorylation, calcium mobilization and proliferation (21). Another Src family member Fyn also plays an important role in TCR signaling. Overexpression of Fyn in transgenic mice or expression of a constitutive active form of Fyn in T cell hybridomas significantly enhances TCR-mediated function (22, 23). Although no significant alteration of T cell development is found in *fyn-/-* mice, the $CD4^+$ or $CD8^+$ single positive thymocytes from these mice show a severe impairment in TCR-mediated signaling events including tyrosine phosphorylation, calcium mobilization, interleukin-2 (IL-2) production and proliferation (24, 25).

A Syk family PTK ZAP-70 is also implicated in proximal TCR signaling. ZAP-70 is expressed exclusively in T cells and natural killer cells (15). The central role of ZAP-70 in T cell development and activation has been demonstrated in mice and humans. ZAP-70 deficient mice show a profound block in T cell development at the transition from the $CD4^+CD8^+$ stage to the single positive stage (26). ZAP-70 mutations in humans cause a severe combined immunodeficiency characterized by the failure of $CD4^+$ T cells to respond to antigen stimulation and by an absence of peripheral $CD8^+$ T cells (27-29). Taken together, these results indicate that Lck, Fyn, and ZAP-70 PTKs play critical roles at the proximal steps in TCR-mediated signal transduction.

Signal transduction pathways leading to gene activation.

Activation of the Src and Syk PTKs by engagement of the TCR leads to tyrosine phosphorylation of multiple intracellular proteins including phospholipase C γ1 (PLCγ1), Vav, Cbl and SLP-76 (1, 30). Tyrosine phosphorylation of PLCγ1 induces its enzymatic activity (31), leading to the generation of the two second messengers, inositol 1,4,5-trisphosphate (IP3) and

diacylglycerol (DAG), which are responsible for the rapid and sustained intracellular calcium increase and activation of protein kinase C (PKC), respectively (1). However, at the time of the initiation of these studies, the functional significance of the tyrosine phosphorylation of other cellular substrates was unclear. Nonetheless, these early biochemical events ultimately lead to a variety of T cell functions, including transcriptional activation of the IL-2 gene.

TCR activation leads to cell cycle 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 calcineurin, a calcium/calmodulin dependent protein phosphatase and Ras, a small molecular weight membrane associated GTPase (32-35). It is believed that calcineurin is activated by the increase of intracellular calcium caused by activation of PLCγ1 (1). Calcium ionophores which elevate cytoplasmic free calcium can activate calcineurin independently of TCR stimulation (1). In addition, two pharmacological agents, FK506 and Cyclosporin A (CsA), can block TCR-mediated IL-2 gene activation by inhibiting calcineurin function (36, 37). Ras activation is also critical for TCR-mediated IL-2 gene activation. Expression of an activated form of Ras, in conjunction with a calcium ionophore or an activated form of calcineurin, leads to IL-2 gene activation, whereas a dominant negative form of Ras inhibits TCR signaling (35, 38). Ras can be activated by a PKC-dependent pathway in T cells (39). It is believed that DAG, a second messenger generated by PLCγ1 activation, is responsible for the activation of PKC and subsequent Ras activation. However,

TCR-induced Ras activation can not be completely blocked by a PKC inhibitor, suggesting the presence of a PKC-independent pathway leading to Ras activation in T cells (39). It has been shown that following TCR stimulation a 36 kD protein (pp36) becomes tyrosine phosphorylated and associated with Grb2, an adaptor protein for a Ras exchange factor SOS (40-42). The pp36/Grb2/SOS complex has been proposed to serve as the link between the proximal TCR signaling machinery and downstream Ras activation. However, the identity of pp36 and the exact molecular mechanism by which TCR ligation induces Ras activation remain to be determined.

While much progress has been made in defining the nature of the biochemical events involving the activation of Ras and calcineurin pathways, these are not the only pathways important for TCR-mediated functions. For example, T cells exhibit a massive and rapid cytoskeletal rearrangement following TCR engagement. Interestingly, inhibition of Rho by C3 exoenzyme or expression of a dominant negative form of CDC42 inhibited the TCR-induced cytoskeletal rearrangement (43, 44). In Jurkat T cells, actin polymerization also appears to be important for TCR activation (45). Moreover, T cells from immunodeficient patients with Wiskott-Aldrich Syndrome (WAS) have profound defects in cytoskeletal organization and in their responses to antigens (46-48). Interestingly, the protein that is defective in WAS, WASP, appears to be a novel effector for the Rho/Rac/CDC42 family of small GTPases (49-53). These results strongly suggest that activation of Rho/Rac/CDC42 pathways is another critical event in T cell activation. However, the mechanism by which TCR ligation triggers the activation of Rho/Rac/CDC42-mediated pathways remains undefined.

The proto-oncogene Vav

Since tyrosine phosphorylation of cellular proteins has been shown to be an early and obligatory step in TCR signal transduction, identification and understanding the function of these molecules may provide clues regarding potential mechanisms for TCR-mediated signal transduction. One of these TCR-induced tyrosine phosphorylated proteins is Vav, a 95 kD proto-oncogene expressed exclusively in hematopoietic and trophoblast cells (54-57). Vav was first identified as a result of an artifactual activation during the course of a gene transfer assay (56). Removal of the first 67 amino acids (a.a.) in the amino-terminal leucine-rich region activates its transforming potential when expressed in NIH-3T3 cells (56, 58, 59). Although the function of Vav was unknown at the time, its structure strongly suggested a role in signal transduction. It contains a putative guanine nucleotide exchange factor (GEF) domain for Rho/Rac/CDC42 family of small GTPases (60, 61). A homologous domain is also present in CDC24 and Dbl, two known GEFs for Rho. Moreover, Vav also contains a number of structural motifs shared by other signaling molecules, including one Src homology 2 (SH2) domain, two Src homology 3 (SH3) domains, a pleckstrin homology (PH) domain, and a cysteine-rich domain (61).

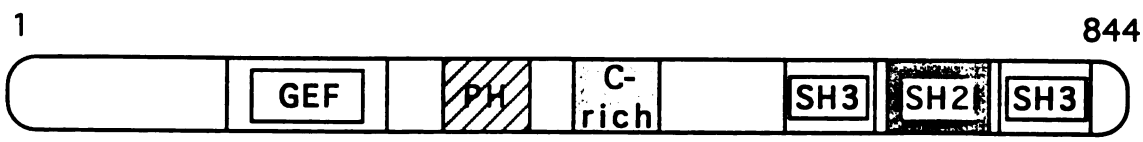

Figure 1. Schematic representation of the Vav proto-oncogene product. N-terminus (a.a. 1-198); GEF domain (199-385); PH domain (403-504); Cysteine-rich region (516-564); N-terminal SH3 domain (617-665); SH2 domain (671-765); C-terminal SH3 domain (789-837).

Recent studies show that Vav becomes tyrosine phosphorylated following a variety of stimuli, including TCR, B cell antigen receptor (BCR) and IL-2 receptor (IL-2R) stimulation (54, 55, 62, 63). When Vav was expressed ectopically in fibroblasts, epidermal (EGF) or platelet-derived growth factors (PDGF) stimulated its rapid tyrosine phosphorylation (54, 55). Activation of EGF or PDGF receptors also resulted in association of these receptors with Vav via its SH2 domain. Although Vav has not been found to associate with any receptors upon ligand stimulation in hematopoietic cells, its SH2 domain was shown to interact with a number of signaling molecules including ZAP-70 and an unknown tyrosine phosphorylated protein Vap-1 (62, 64). Taken together, these findings suggested that Vav may play an important role in the receptor-mediated signaling processes in hematopoietic cells.

Although Vav shares homology with the GEFs for Rho/Rac but not Ras family members, it was initially reported to account for most of the receptor-stimulated Ras GDP/GTP exchange activity in both T and B cells (65, 66). In addition, tyrosine phosphorylation of Vav or binding of DAG to its cysteine-rich domain were shown to be required for this exchange activity (65-68). However, these findings could not be confirmed by other investigators, who demonstrated that neither Vav nor oncogenic Vav had any detectable exchange activity for Ras and the morphology of Vav-transformed NIH-3T3 cells was distinct from Ras-transformed cells (69, 70). The DAG/phorbol ester binding activity of the cysteine-rich region in Vav also was controversial, since *in vitro* assays failed to show any binding of Vav to DAG or phorbol ester (71). Therefore, the significance of the findings implicating Vav as a Ras exchange factor in lymphocytes is uncertain.

The expression pattern, structure and tyrosine phosphorylation of Vav strongly suggested its involvement in TCR-mediated signal transduction. I decided to use Jurkat T cells as a model system to explore the potential role of Vav in TCR-mediated signaling leading to IL-2 gene activation. To do so, I transiently overexpressed Vav or oncogenic Vav in Jurkat T cells and examined their effects on IL-2 promoter elements. Overexpression of wildtype Vav alone in Jurkat cells markedly enhanced basal and TCR-mediated NFAT activation and this Vav-mediated effect required a functional TCR signaling machinery. This functional assay provided an easy tool to dissect the function of Vav in TCR signaling. Since Vav contains a number of structural domains which are likely to be involved in protein-protein interactions, I then took a biochemical approach to identify molecules interacting with Vav following TCR stimulation. The identification and function of the interacting molecules may provide clues for Vav regulation and function in T cells.

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CHAPTER II

Involvement of the proto-oncogene Vav in

T cell antigen receptor-mediated signal transduction.

Introduction:

Stimulation of the T cell antigen receptor (TCR) induces activation of multiple tyrosine kinases, resulting in phosphorylation of numerous intracellular substrates. One substrate is Vav, which is expressed exclusively in hematopoietic and trophoblast cells. It contains a number of structural motifs including SH2, SH3 and PH domains and a putative guanine nucleotide exchange (GEF) domain. However, the function of Vav in lymphocyte signal transduction is unclear. The initial goal of this dissertation research is to establish a functional assay in Jurkat T cells in order to study the function of Vav in TCR-mediated signaling pathways. In this study, we show that overexpression of Vav alone in Jurkat T cells leads to activation of nuclear factors, including NFAT, involved in interleukin 2 (IL-2) expression. In addition, Vav synergizes with TCR stimulation in inducing NFAT- and IL-2- dependent transcription. We further define the requirements of Vav-induced NFAT activation and propose that Vav plays an important role at an yet unidentified proximal position in the TCR signaling cascade. The following paper, reproduced from the journal in which it was published, details the biochemical and functional analysis of the involvement of Vav in TCR-mediated signal transduction.

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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, though 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-Rat 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 Tiβ 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-Rat, 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 transients 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

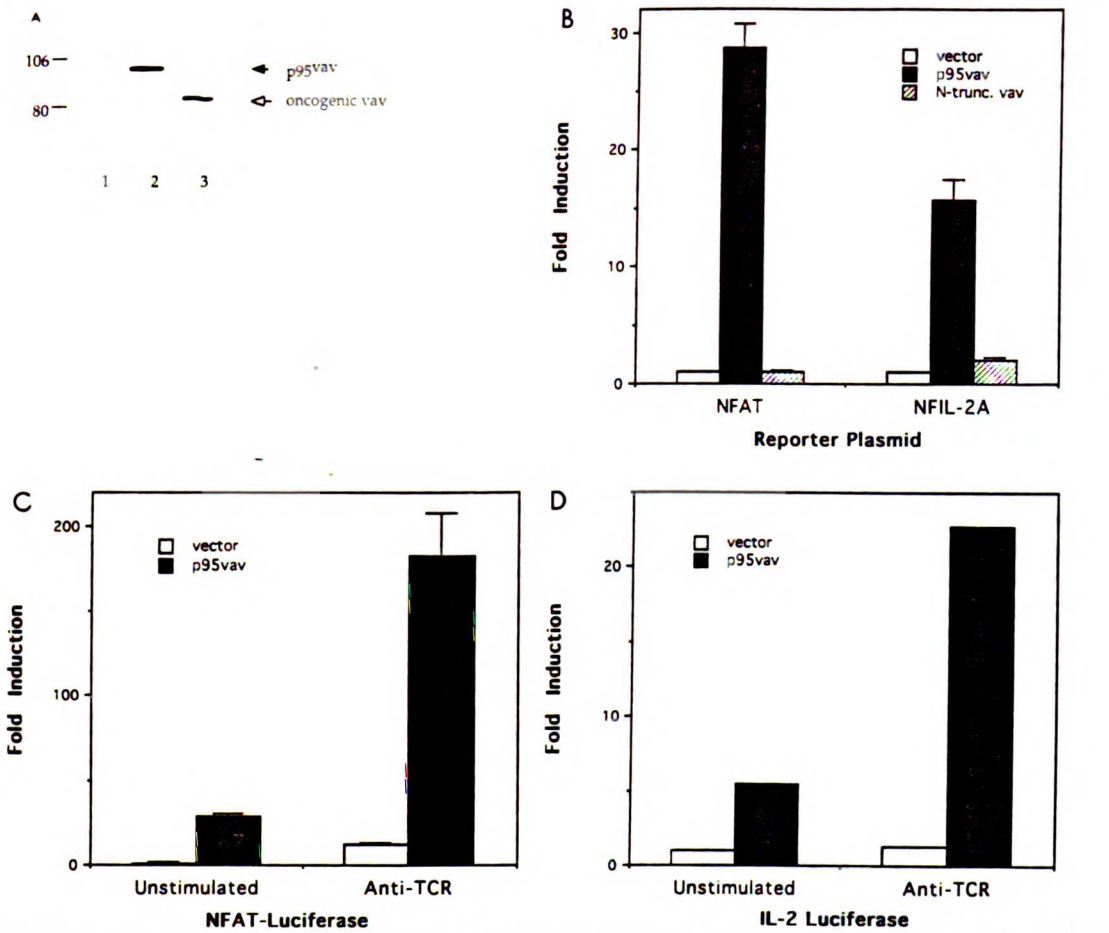

FIG. 1. Overexpression of p95^{vav} in TAg Jurkat cells augments basal and TCR-stimulated IL-2 transcriptional activity. (A) TAg Jurkat cells were cotransfected with plasmids encoding pCD8/T together with either an empty vector (lane 1), wild-type p95^{vav} (lane 2), or N-terminally truncated vav (lane 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.

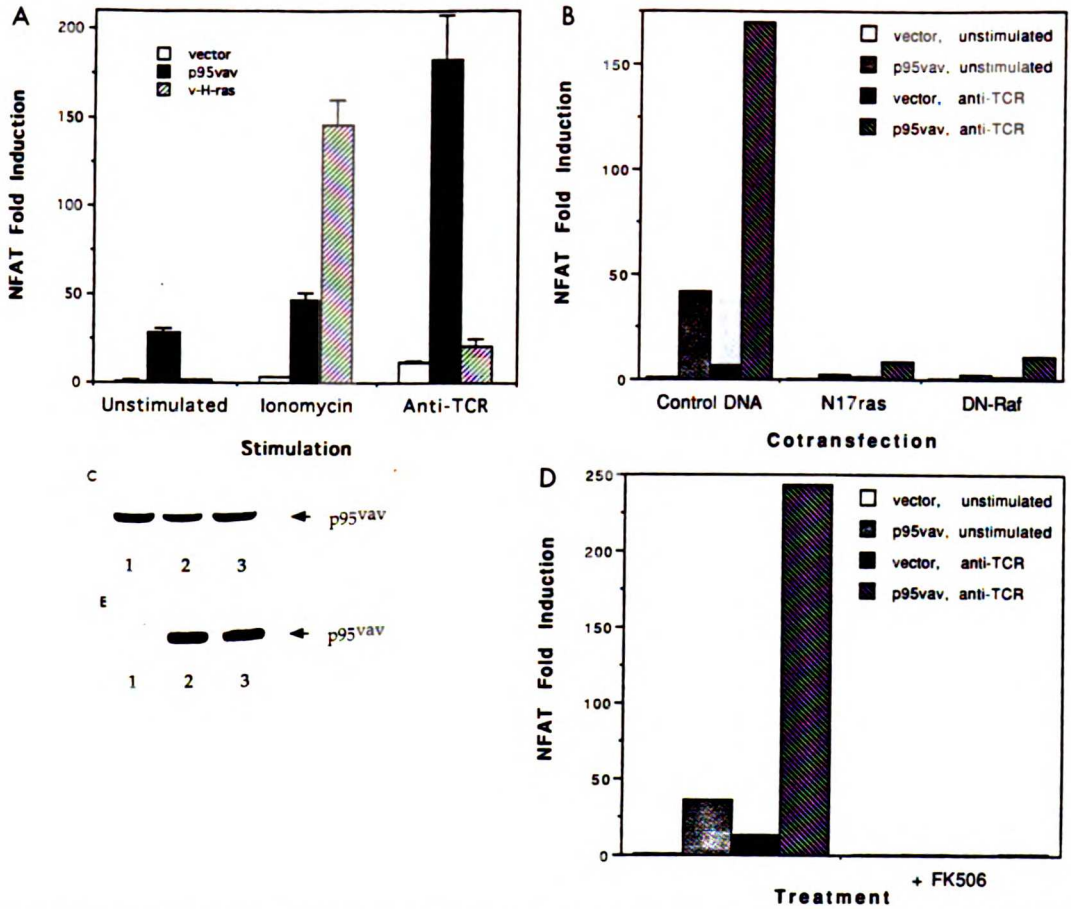

FIG. 2. $p95^{vav}$-mediated NFAT activation is not mimicked by Ras activation but is dependent on Ras and calcineurin function. (A) TAg Jurkat cells were transfected with NFAT-Luc along with either an empty vector alone, $p95^{vav}$, or v-H-ras. Cells were either unstimulated or stimulated with ionomycin 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 p95vav overexpression (Fig. 2B) but did not suppress the level of transfected p95vav (Fig. 2C). These data suggest that p95vav does not simply function to directly activate Ras; rather, it appears to influence signaling events that depend upon Ras function.

To determine whether the p95vav-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 p95vav-overexpressing cells (Fig. 2D and data not shown). The drug treatment was not toxic to the cells and did not inhibit p95vav overexpression, as shown in Fig. 2E. These results indicate that the NFAT activation in p95vav-overexpressing cells depends on both Ras and calcineurin pathways and suggest that p95vav may function upstream of or parallel to them in TCR signaling pathways.

The p95vav 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 p95vav 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 p95vav failed to augment the HM1R stimulation, in contrast to its effect on TCR-mediated induction (Fig. 3). In addition, overexpression of p95vav 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 p95vav effect is specific to the TCR-mediated signaling pathways and further implicate p95vav 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 p95vav 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 p95vav (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 p95vav participates in the TCR-mediated signaling pathway, proximal TCR signaling events may influence the effect of p95vav on NFAT activation. In fact, incubation of p95vav-transfected cells with herbimycin A blocked the basal activation of NFAT (Fig. 4A), suggesting that PTKs are required for p95vav 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 p95vav was

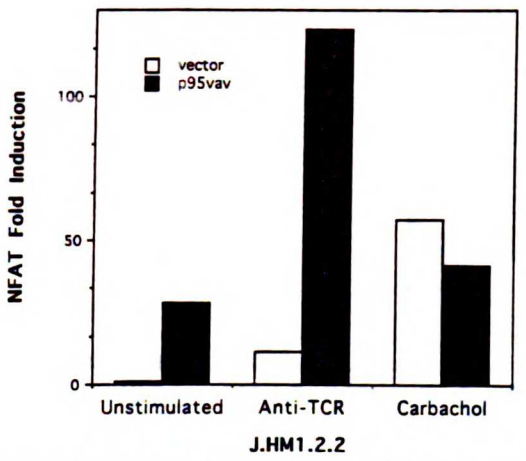

FIG. 3. Overexpression of p95vav in J.HM1.2.2 cells specifically augments the TCR-mediated NFAT activation. J.HM1.2.2 cells were cotransfected with NFAT-Luc and either the control vector (pEF-BOS) or p95vav. Transfected cells were either unstimulated or stimulated with an anti-TCR MAb (C305) or carbachol 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 p95vav 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 p95vav, 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 p95vav 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 p95vav 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 p95vav 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 p95vav in J.RT3-T3.5 cells had no effect on basal NFAT activity (Fig. 5A). Another

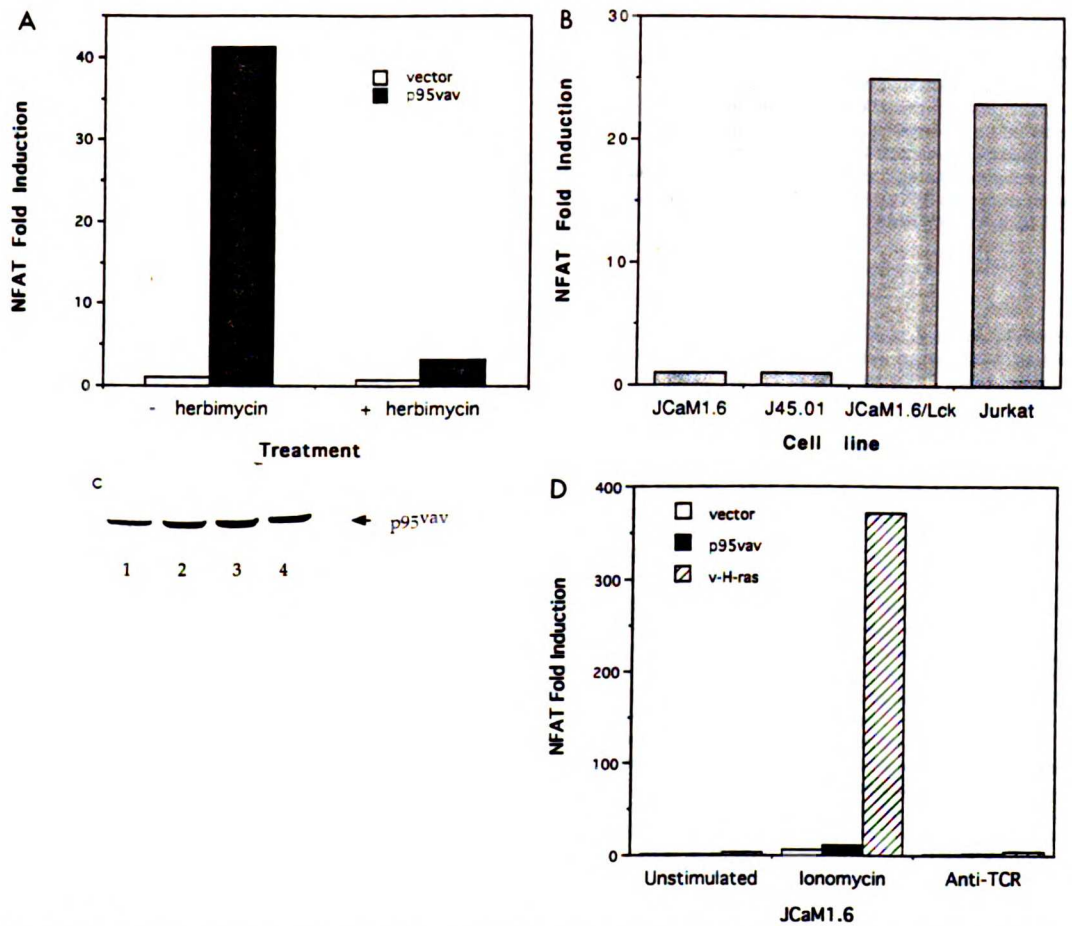

FIG. 4. The effect of $p95^{vav}$ overexpression on NFAT activation requires the proximal tyrosine kinase activity in Jurkat T cells. (A) NFAT induction by overexpressing $p95^{vav}$ is sensitive to herbimycin A treatment. TAg Jurkat cells were cotransfected with NFAT-Luc and either an empty vector (pSV7d) 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-

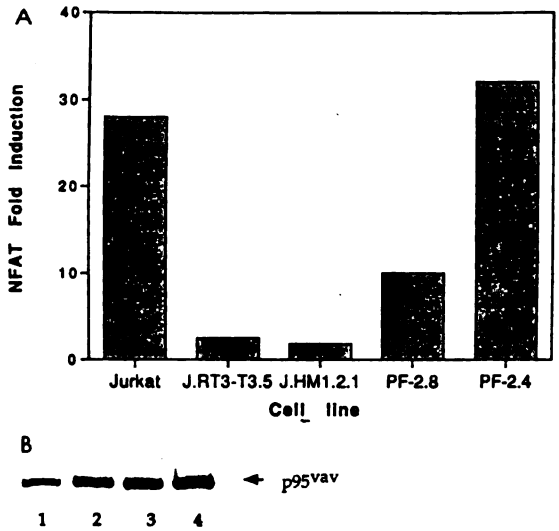

FIG. 5. Expression of a TCR is required for p95^{vav} function. (A) Wild-type Jurkat, J.RT3-T3.5, J.HM1.2.1, PF-2.8, and PF-2.4 cells were cotransfected with NFAT-Luc and either an empty vector (pEF-BOS) or myc-p95^{vav}. Transfected cells were assayed for basal luciferase activity. The results are shown 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

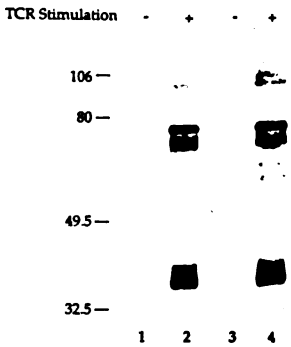

FIG. 6. Basal and TCR-stimulated cellular tyrosine phosphorylation in p95^{vav}-overexpressing Jurkat cells. TAg Jurkat cells were transfected with truncated CD8 along with either pSV7d vector (lanes 1 and 2) or p95^{vav} (lanes 3 and 4). After 24 h, CD8-expressing cells were purified as described in 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.

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.

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

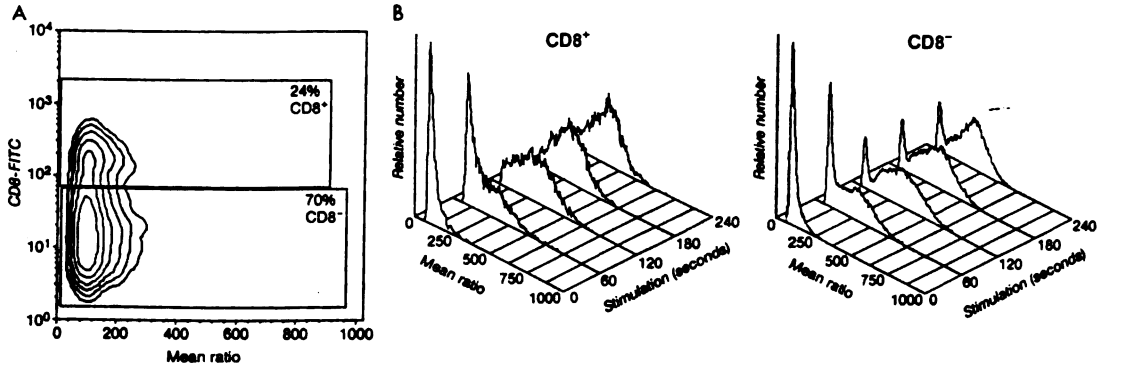

FIG. 7. Intracellular calcium level in p95^{vav}-overexpressing Jurkat cells. TAg Jurkat cells were transfected with truncated CD8 and p95^{vav}. After 24 h, cells were stained with FITC-CD8 Ab and loaded with Indo-1 as described in the text. (A) The top 25% of the transfected cells (the CD8-positive 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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Addendum: The GEF, PH and SH2 domains are important for Vav function in T cells.

Introduction:

Vav contains an interesting array of structural motifs including a putative GEF domain for the Rho/Rac/CDC42 family of small GTPases, a PH domain which may be involved in membrane targeting, and two SH3 and one SH2 domains presumably mediating protein-protein interactions. Recent studies in mice and in cell lines have indicated that Vav is a critical component in antigen receptor-mediated signal transduction. It is therefore of great interest to determine the functional domains within Vav to further define the role of Vav in lymphocyte signal transduction. A panel of Vav mutants including a GEF deletion mutant, a PH domain deletion mutant, an SH2 mutant and an SH3 mutant were generated and analyzed by their ability to activate basal or TCR-mediated NFAT activation in Jurkat T cells. We found that the GEF, PH, SH2 domains, but not the SH3 domain, are required for the full ability of Vav to induce NFAT activation in Jurkat T cells.

Results and Discussion:

The GEF domain is the only potential catalytic domain in Vav and has been highly controversial regarding its function. Altman and his colleagues have shown that Vav is an activator for Ras in lymphocytes (1, 2), however, these results have not been supported by other investigators. We have shown that overexpression of Vav in Jurkat T cells fails to mimic the effect induced by expression of an activated form of Ras (3). In addition, preliminary experiments failed to show MAPK activation in cells overexpressing Vav (unpublished

results). These data suggest that Vav is not simply an activator for Ras in T cells. The GEF domain of Vav shares sequence homology with other known Rho/Rac/CDC42 exchange factors such as Dbl and CDC24 (4, 5). Microinjection of fibroblasts with cDNAs containing the GEF and PH domains of Vav also leads to morphological changes similar to those of activation of Rho/Rac/CDC42 (6). These results suggest that Vav may be an activator for Rho/Rac/CDC42 but not for Ras. To further examine the role of the Vav GEF domain in T cells, we generated a Vav deletion mutant in which the third conserved region of the GEF domain was deleted. Since a small deletion or point mutations within this conserved region in Dbl abolished its exchange activity on Rho, Rac and CDC42 (7), this deletion in Vav is likely to result in a loss of its GEF activity. Interestingly, in contrast to wildtype Vav, overexpression of this GEF deletion mutant failed to induce basal NFAT activation (Fig. 1). Furthermore, its ability to enhance TCR-stimulated NFAT activation was markedly reduced when compared with wildtype Vav. These data suggest that the GEF domain is required for Vav function in T cells. These results further imply that the Rho/Rac/CDC42-mediated events such as cytoskeletal organization, may play an important role in antigen receptor-induced downstream gene activation.

However, our preliminary experiments showed that overexpressionn of Vav in Jurkat T cells did not appear to simply activate either Rac-1 or CDC42, since expression of an activated form of Rac-1 or CDC42 failed to mimic the effect induced by overexpression of Vav (data not shown). In addition, coexpression of the Vav GEF mutant with an activated form of Rac-1 failed to induced NFAT activation (data not shown). It is possible that an activated form of either Rac-1 or CDC42 may not fully reproduce the physiological function of the GTPase in T cells. Nonetheless, physiological effector(s) of Vav remains to be determined.

It is intriguing to note that all known or putative Rho/Rac/CDC42 GEFs contain a PH domain adjacent to the GEF domain. Although the exact function of PH domains is still poorly defined, recent findings on Dbl suggest that they may be involved in membrane localization processes (8). Interestingly, similar to the GEF domain deletion mutant of Vav, a PH domain deletion mutant also showed a marked reduction in its ability to induce basal or to synergize with TCR-mediated NFAT activation when compared with wildtype Vav (Fig. 1). These data suggest that the PH domain, and perhaps membrane localization of Vav, is also important for Vav function in T cells.

In addition to the GEF and PH domain, Vav also contains two SH3 and one SH2 domains in the C-terminus. The SH2 domain of Vav has been shown to interact with a number of phosphorylated proteins including ZAP-70 (9), whereas the C-terminal SH3 domain associates with the heterogeneous ribonucleoprotein K (hnRNP-K) via a proline-rich sequence within hnRNP-K (10, 11). To examine the functional significance of the interactions mediated by the Vav SH2 and SH3 domains, we generated an SH2 domain point mutant in which an arginine residue within the predicted phosphate-binding site of the Vav SH2 domain was substituted with a lysine, and an SH3 domain point mutant in which a proline residue within the SH3 domain shown to be required for its interaction with hnRNP-K was mutated to a leucine (10). As shown in Figure 1, mutation within the SH2 domain reduced the Vav activity to induce NFAT activation, suggesting that interactions mediated by the Vav SH2 domain may be important for Vav function in T cells.

In contrast, the C-terminal SH3 domain point mutant appeared to be more potent in enhancing both basal and TCR-induced NFAT activation when compared with wildtype Vav, while it completely failed to interact with hnRNP-

K (10). These data suggest that the C-terminal SH3 domain may play a negative regulatory role in Vav function. Recent studies on the tyrosine kinase Itk have revealed that the SH3 domain of Itk is capable of intramolecularly interacting with a proline-rich sequence within Itk, an interaction resulting in negative regulation of Itk (12). Interestingly, a short proline-rich sequence (PxPP), which resembles the SH3 domain-binding motif found in hnRNP-K (10), is also present in Vav. It is therefore, important to determine whether the C-terminal SH3 domain of Vav is able to interact with Vav itself via the proline-rich sequence, which may potentially lead to inactivation of Vav.

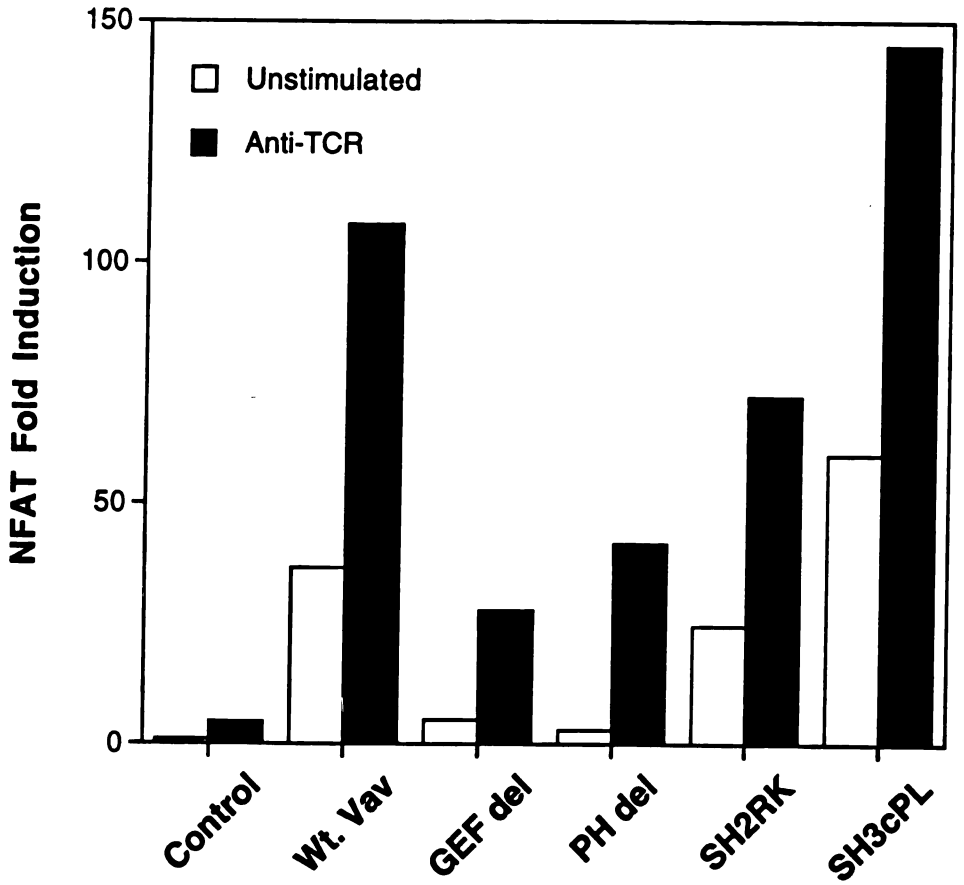

Figure 1 The GEF, PH, SH2 domains, but not the SH3 domain of Vav is required for the full activity of Vav to enhance both basal and TCR-mediated NFAT activation. TAg Jurkat cells were cotransfected with NFAT-Luc with either empty vector, wild-type Vav, the GEF del. mutant, the PH del. mutant, the SH2RK, or the SH3cPL mutant. Transfected cells were then assayed for luciferase activity. Western blot analysis showed comparable levels of expression among wildtype and the mutants (data not shown).

Materials and Methods:

Mutagenesis. The BamHI/KpnI fragment of Vav from pEF115myc (3) was subcloned into pUC19 and the resulting plasmid was digested with XcmI to remove fragments containing n.t. 1240-1537. The digested plasmid was then ligated with a linker (top strand: 5'-CAC CTG TTC CAG TTC CAG CTG TCC ATT GAG-3', bottom strand: 5'-TC AAT GGA CAG CTG GAA CAG GTG T-3'). The resulting plamid was then digested with BamHI and KpnI and the insert was subcloned back to pEF115myc to yield the GEF deletion mutant (lacking a.a. 296-385). To generate the PH domain deletion mutant, pUC19Vav(BamHI/KpnI) was digested with FseI and BsmI and the resulting plasmid was ligated with a double-strand linker (top strand: 5'-CCC AAC ATC TAT CCG GAG AAT GCC-3'; bottom strand: 5'-C ATT CTC CGG ATA GAT GTT GGG CCG G-3'). The resulting plasmid was then digested with BamHI and KpnI and the insert was subcloned back into pEF115myc to yield the PH domain deletion mutant (lacking a.a. 404-504). The SH2 domain point mutant (pEF115RK) was described elsewhere. The SH3 domain point mutant was generated by first subcloning the KpnI/XbaI fragment from pEF115myc into pUC19. The resulting plasmid was digested with BglII and BstXI and was then ligated with a double-strand oligo to create a single substitution of n.t. 2500 (C to T), resulting in a point mutation at a.a. 834 (P changed to L). The KpnI/XbaI fragment was then cloned back into pEF115myc to yield pEF115SH3cPL.

Cell line, transfection, and luciferase assay. TAg Jurkat cells were maintained and transfected as described (3). Luciferase assays were performed as described (3).

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CHAPTER III

Vav and SLP-76 physically interact and functionally cooperate in TCR-mediated IL-2 gene activation.

Introduction

In addition to the Vav overexpression study in Jurkat cells, recent studies in Vav-deficient mice have provided compelling evidence that Vav plays a critical role in both lymphocyte development and activation. However, the molecular mechanism by which Vav functions in T and B cells remains unknown. Since Vav becomes tyrosine phosphorylated following antigen receptor stimulation, it is reasonable to speculate that Vav may interact with other tyrosine phosphoproteins following TCR stimulation. Thus, identification of Vav-interacting protein may help to define the biochemical function of Vav in lymphocytes.

A number of proteins have been reported to interact with Vav, demonstrated by either using GST fusion proteins or coimmunoprecipitation. An unknown tyrosine phosphoprotein of 75 kD has been consistently detected to interact with Vav in thymocytes, T and B cells. In this study, I identified this protein as the newly cloned hematopoietic-specific molecule SLP-76. I further showed that SLP-76 interacts with Vav via the Vav SH2 domain and functions in cooperation with Vav in TCR-mediated IL-2 gene activation. These results strongly suggest that a signaling complex containing at least Vav and SLP-76 play an important role in antigen receptor-mediated signal transduction. The following paper, reproduced from the journal in which it was published, details the biochemical and functional analysis of the interaction between Vav and SLP-76.

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Vav and SLP-76 Interact and Functionally Cooperate in IL-2 Gene Activation

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Summary

T cell antigen receptor (TCR) stimulation induces tyrosine phosphorylation of many intracellular proteins, including the proto-oncogene Vav, which is expressed exclusively in hematopoietic and trophoblast cells. Vav is critical for lymphocyte development and activation. Overexpression of Vav in Jurkat T cells leads to potentiation of TCR-mediated IL-2 gene activation. However, the biochemical function of Vav is unknown. Here, we demonstrate that the major induced tyrosine phosphoprotein associated with Vav is the hematopoietic cell-specific SLP-76. The Vav SH2 domain is required for this interaction and for TCR-mediated Vav tyrosine phosphorylation. Similar to Vav, overexpression of SLP-76 markedly potentiates TCR-mediated NF-AT and IL-2 gene activation. Furthermore, overexpression of both Vav and SLP-76 synergistically induces basal and TCR-stimulated NF-AT activation. These results suggest that a signaling complex containing Vav and SLP-76 plays an important role in lymphocyte activation.

Introduction

The T lymphocyte antigen receptor (TCR) recognizes pathogens or foreign antigens and initiates a series of intracellular biochemical events that result in biological responses. These signal transduction events are crucial to both T cell development and T cell–mediated immune responses. One of the earliest biochemical events following ligation of the TCR is the activation of Src family protein tyrosine kinases (PTKs) such as Lck and Fyn, which leads to phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR ζ and CD3 chains (Weiss and Littman, 1994). The phosphorylation of ITAMs results in the subsequent recruitment and activation of Syk/ZAP-70 family PTKs (Chan et al., 1991, 1992, 1994; Iwashima et al., 1994; Wange et al., 1992). The activation of Src family and Syk/ZAP-70 PTKs leads to the phosphorylation of many downstream substrates including phospholipase-C γ1 (PLC-γ1), the proto-oncogene Vav, and the recently cloned SLP-76 (Jackman et al., 1995; Weiss and Littman, 1994). Although tyrosine phosphorylation of PLC-γ1 induces its enzymatic activity, leading to the activation of the inositol phospholipid pathway, the significance of the tyrosine phosphorylation of Vav and SLP-76 remains unclear.

The proto-oncogene Vav was first identified as a result of its transforming activity during the course of an oncogene search of esophageal carcinoma DNA (Katzav et al., 1989). However, its transforming potential in fibroblasts was shown to be due to the removal of its N-terminal 67 aa (Coppola et al., 1991; Katzav et al., 1989, 1991). Vav contains a number of interesting structural motifs, including a putative guanine nucleotide exchange factor (GEF) domain for the Rho/Rac/CDC42 families of small GTPases, a pleckstrin homology (PH) domain, which may participate in membrane localization, a cysteine-rich (C-rich) domain, which may form zinc fingers, as well as two Src homology 3 (SH3) domains and a Src homology 2 (SH2) domain at its C terminus, which are presumably involved in protein–protein interactions (Adams et al., 1992; Boguski and McCormick, 1993; Boguski et al., 1992; Galland et al., 1992; Koch et al., 1991). Vav is expressed exclusively in hematopoietic and trophoblast cells (Katzav et al., 1989; Zmuidzinas et al., 1995), and is readily tyrosine phosphorylated in response to a variety of stimuli, including stimulation of TCR, B cell antigen receptor (BCR), and various cytokine receptors (Bustelo and Barbacid, 1992; Bustelo et al., 1992; Evens et al., 1993; Margolis et al., 1992). Its structure, expression, and tyrosine phosphorylation pattern suggests an important role in signal transduction pathways in lymphocytes.

The importance of Vav in lymphocyte development and activation was demonstrated recently in both mice and cell lines. Germline inactivation of the *vav* gene results in early embryonic lethality, suggesting its critical role in trophoblast development (Zmuidzinas et al., 1995). Chimeric mice derived from blastocysts lacking the recombinase-activating gene (*RAG*) and embryonic stem (ES) cells deficient in Vav expression displayed a dramatic reduction in thymocyte number as well as peripheral T and B cells (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). Moreover, the Vav-deficient mature T and B cells showed a profound defect in proliferation and cytokine production in response to TCR and BCR stimulation. The defect in Vav $^{-/-}$ chimeras appeared to be specific to TCR and BCR signaling pathways, since the Vav $^{-/-}$ T cells could proliferate when exogenous interleukin-2 (IL-2) was added. Similarly, lipopolysaccharide or CD40 ligand stimulation could also induce Vav $^{-/-}$ B cell proliferation. The block in Vav $^{-/-}$ T cells appears to be in a proximal component of the TCR signaling pathway, since the defect could be rescued by treatment with phorbol ester and ionomycin, pharmacological agents that mimic the downstream events induced by TCR stimulation and consequently induce IL-2 production.

The involvement of Vav in TCR signal transduction

was also demonstrated in the human leukemic Jurkat T cell line (Wu et al., 1995). Overexpression of Vav led to a marked increase in basal nuclear factor of activated cells (NF-AT) or IL-2-driven transcriptional activity, which was further enhanced by TCR stimulation. This Vav-induced NF-AT activation appeared to be specific to the TCR, since it had no effect on NF-AT induction induced by the G protein–coupled human muscarinic receptor. Overexpression of Vav had no effect on phorbol ester and ionomycin-induced NF-AT activation but required calcineurin and Ras to induce NF-AT, further supporting the involvement of Vav in the proximal events of TCR signaling. Finally, a functional TCR complex was required for this Vav activity. These results are consistent with the findings in Vav$^{-/-}$ chimeric mice and provide further evidence that Vav plays an important role in proximal events involved in TCR signaling.

Recent approaches towards studying the function of Vav have focused on identifying proteins that interact with Vav in lymphocytes. Although Vav has not been found to associate directly with antigen receptors following ligand stimulation in T and B cells, a number of proteins have been reported to interact with Vav, including an RNA-binding protein (hnRNP K), Ku-70, Grb-2, JAK family members, Lck, ZAP-70, and tubulin (Bustelo et al., 1995; Hobert et al., 1994; Gupta et al., 1994; Huby et al., 1995; Katzav et al., 1994; Matsuguchi et al., 1995; Romero et al., 1996; Ye and Baltimore, 1994). Unfortunately, the functional significance of these interactions remains unclear, since many of these associations were characterized by using GST fusion proteins or the yeast two-hybrid system. However, an unknown 70–75 kDa tyrosine-phosphorylated protein has been reproducibly shown to associate with Vav in cells following antigen receptor stimulation in thymocytes, T cells, and B cells (Bustelo et al., 1992; Gouy et al., 1995; Katzav et al., 1994). Therefore, we attempted to identify this phosphoprotein in an effort to investigate the function of Vav in T cells.

Here, we identify this tyrosine-phosphorylated protein as the newly cloned 76 kDa SLP-76. SLP-76 was identified first through its association with GST–Grb2 fusion proteins in vitro (Jackman et al., 1995). It contains a number of potential tyrosine phosphorylation sites in its N terminus, an SH2 domain in its C terminus, and a central proline-rich region that may interact with SH3 domain-containing proteins such as Grb2 (Motto et al., 1996). Interestingly, SLP-76 is also expressed specifically in hematopoietic cells and becomes readily tyrosine phosphorylated upon antigen receptor stimulation. In this report, we show that the SH2 domain of Vav interacts with tyrosine-phosphorylated SLP-76 in Jurkat T cells. Moreover, an SH2 domain mutant of Vav fails to become tyrosine phosphorylated or to interact with SLP-76 following TCR or BCR stimulation. Overexpression of a truncated Vav protein containing its C-terminal SH2 and two SH3 domains blocks TCR-mediated NF-AT activation. This inhibitory effect is dependent upon the SH2 domain phosphotyrosine-binding function. These results suggest that the interactions mediated by the Vav SH2 domain, such as the Vav–SLP-76 interaction, are important for TCR signaling. Interestingly, similar to Vav, overexpression of SLP-76 markedly potentiates the TCR-mediated IL-2 gene activation (Motto et al., 1996). In this report, we show that SLP-76 and Vav act synergistically to induce NF-AT activation, suggesting that a complex containing Vav and SLP-76 plays an important role in TCR-mediated IL-2 gene activation.

Results

Vav and SLP-76 Interact in Jurkat T Cells

To identify potential regulators or effectors of Vav, we immunoprecipitated Vav from Jurkat T cell lysates, resolved the precipitates by SDS–PAGE, and immunoblotted the gels with anti-phosphotyrosine antibodies. Following TCR stimulation, the tyrosine phosphorylation of Vav was markedly increased when compared with Vav precipitates from unstimulated cells. In addition, a second phosphoprotein of 76 kDa coprecipitated with Vav following receptor engagement (Figure 1A). A 76 kDa phosphoprotein was also found to be associated with Vav upon antigen receptor stimulation in thymocytes (data not shown; Gouy et al., 1995). By blotting the Vav immunoprecipitates with antisera against known tyrosine-phosphorylated proteins with similar molecular mass, we determined that this 76 kDa Vav-associated phosphoprotein was not Syk, ZAP-70, Itk, Tec, paxillin, or CD5 (data not shown). Recently, a 76 kDa protein, SLP-76, was cloned from Jurkat T cells. Interestingly, similar to Vav, SLP-76 is expressed specifically in hematopoietic cells and also becomes tyrosine phosphorylated upon TCR or BCR stimulation. Therefore, we attempted to determine whether SLP-76 was the 76 kDa phosphoprotein associated with Vav. As shown in Figure 1B, SLP-76 could be detected in Vav immunoprecipitates from Jurkat lysates by Western blotting using an anti-SLP-76–specific antiserum. Conversely, Vav could also be coimmunoprecipitated with this anti-SLP-76 antiserum (Figure 1C). There does appear to be a varying degree of basal association between Vav and SLP-76 in Jurkat cells (Figures 1B and 1C; data not shown). However, this association could be further induced by TCR stimulation or by treatment with pervanadate, a tyrosine phosphatase inhibitor that induces high levels of cellular tyrosine phosphorylation.

To analyze further the association between Vav and SLP-76, Jurkat cells containing SV40 large T antigen (TAg) were transiently transfected with a myc epitope-tagged form of Vav. These transfected cells were then lysed and Vav was immunoprecipitated with an anti-myc epitope monoclonal antibody (MAb). As shown in Figure 1D, SLP-76 could be readily detected in the Vav immunoprecipitates from myc–Vav-transfected cells following TCR stimulation. In addition, anti-phosphotyrosine blots revealed that the SLP-76 associated with Vav was tyrosine phosphorylated (data not shown). It is noteworthy that only a weak basal association between Vav and SLP-76 was detected in TAg-transfected Jurkat cells, in contrast with the results obtained with parental Jurkat cells (Figures 1B and 1C). One potential explanation for these differences is that the interaction between Vav and SLP-76 may depend upon tyrosine phosphorylation of Vav or SLP-76, or both, since we consistently observed that the basal tyrosine phosphorylation level of TAg Jurkat cells was much lower than that of parental Jurkat cells (data not shown).

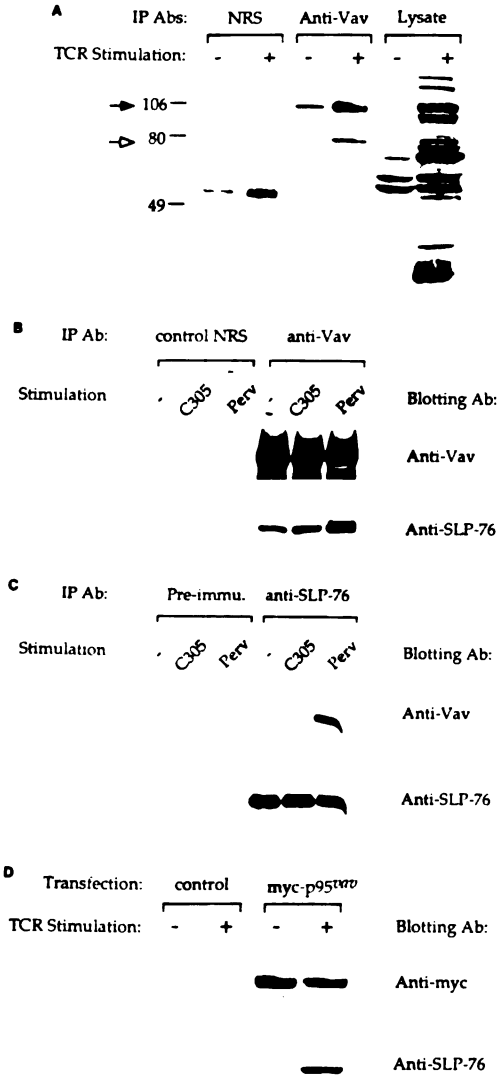

Figure 1. Vav and SLP-76 Interact in Jurkat T Cells

(A) Anti-phosphotyrosine (4G10) blot of normal rabbit serum (NRS) and anti-Vav immunoprecipitates from unstimulated and TCR-stimulated Jurkat T cells. Jurkat cells (5×10^7) were either left unstimulated or stimulated with anti-TCR MAb (C305, 1:500 dilution) for 2 min, and subsequently lysed in NP-40 lysis buffer and immunoprecipitated with the indicated antibodies as described in Experimental Procedures. The immunoprecipitates and 2×10^6 cell equivalent lysates were then blotted with anti-phosphotyrosine antibody (4G10). The migrations of Vav and the 76 kDa Vav-associated tyrosine phosphoprotein are indicated by closed and open arrowheads, respectively.

(B) SLP-76 coimmunoprecipitates with Vav. Jurkat cells (5×10^7) were either left unstimulated, stimulated with C305, or treated with pervanadate. The lysates were immunoprecipitated with either NRS or anti-Vav antiserum. The upper part of the blot was blotted with anti-Vav antiserum, whereas the lower part was blotted with anti-SLP-76 antiserum.

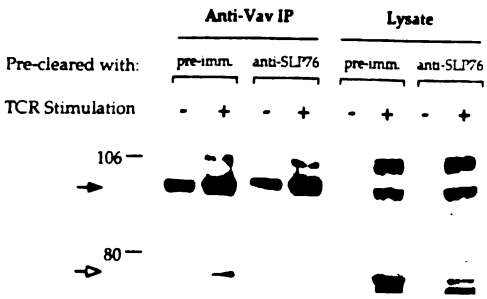

Figure 2. SLP-76 Is the 76 kDa Vav-Associated Phosphoprotein

Jurkat cells were either left unstimulated or stimulated with C305 for 2 min. The lysates were then subject to two rounds of precleaning with either preimmune or anti-SLP-76 antisera and subsequently with protein G–coupled sepharose beads. The precleared lysates were then immunoprecipitated with anti-Vav antiserum and the immunoprecipitates (left) or lysates (right) were blotted with anti-phosphotyrosine MAb (4G10). Each IP lane represents approximately 5×10^7 cell equivalents. Each lysate lane represents approximately 2×10^6 cell equivalents. The closed and open arrowheads represent the migrations of Vav and SLP-76, respectively.

To demonstrate further that SLP-76 is the only tyrosine-phosphorylated protein contained in the 76 kDa band that coimmunoprecipitates with Vav, we precleared SLP-76 and then immunoprecipitated Vav from the SLP-76-depleted Jurkat lysates. After two rounds of depletion, over 90% of SLP-76 could be removed from the lysates as assayed by Western blotting (data not shown). Depletion of SLP-76 eliminated most of the Vav-associated 76 kDa tyrosine phosphoprotein (Figure 2), indicating that the 76 kDa phosphoprotein is predominantly SLP-76. Note that the band migrating at 105 kDa is likely a modified form of Vav, which has been previously reported (Coppola et al., 1991) and could be detected by both the anti-Vav antiserum or the anti-myc epitope MAb (see Figure 4A; data not shown). Taken together, these results demonstrate that in T cells, Vav and SLP-76 interact with each other in vivo.

The SH2 Domain of Vav Interacts with SLP-76

To define the region of Vav that mediates its interaction with SLP-76, we transiently transfected TAg Jurkat cells with a myc epitope-tagged truncated Vav (Vav–C), containing the C-terminal SH2 and two SH3 domains (amino acids 538–845). Vav–C was immunoprecipitated and im-

(C) Vav coimmunoprecipitates with SLP-76. Cells were processed as in (B) and the lysates were immunoprecipitated with either preimmune serum or anti-SLP-76 serum. The upper and lower part of the blot were blotted with anti-Vav and anti-SLP-76 antisera, respectively.

(D) The myc epitope-tagged Vav associates with SLP-76. TAg Jurkat cells were transiently transfected with either control DNA (pEFBOS) or myc epitope-tagged Vav (pEF115myc). After transfection (20–40 hr), $1–2 \times 10^7$ live cells were either left unstimulated or stimulated with anti-TCR MAb (C305, 1:500) for 2 min and then lysed. The lysates were immunoprecipitated with anti-myc MAb (9E10), and the upper and lower parts of the blot were blotted with anti-myc MAb and anti-SLP-76 antiserum, respectively.

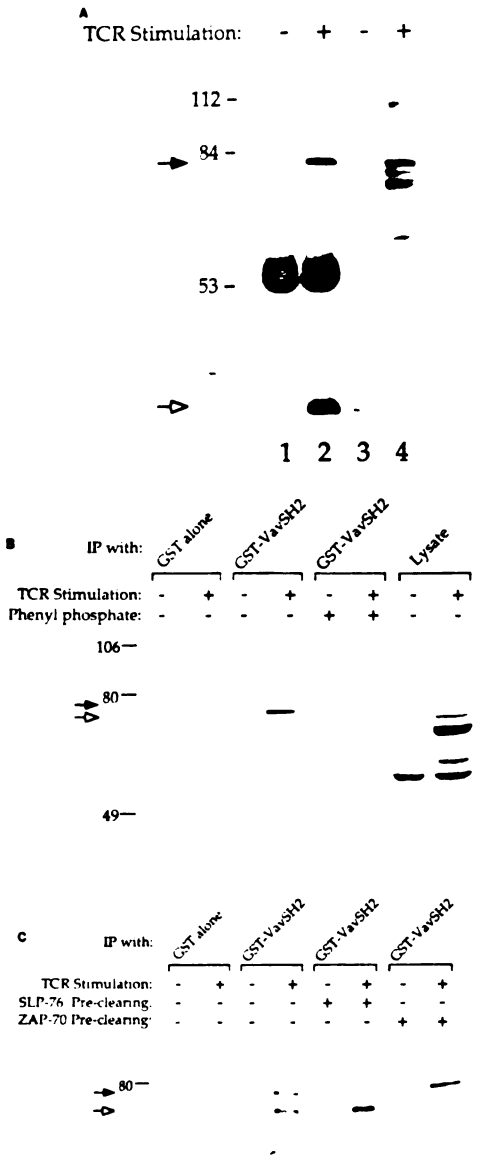

Figure 3. The Vav SH2 Domain Can Interact with SLP-76

(A) The C-terminal portion of Vav is sufficient for interacting with SLP-76. TAg Jurkat cells were transiently transfected with Vav–C (pEFVav–Cmyc) and processed as described in Figure 1D. The lysates were immunoprecipitated with anti-myc MAb (9E10) and the immunoprecipitates were blotted with anti-phosphotyrosine MAb (4G10) (lanes 1 and 2). The whole cell lysates are shown in lanes 3 and 4. The migrations of SLP-76 and Vav–C are indicated by closed and open arrowheads, respectively.

(B) GST–VavSH2 fusion protein precipitations of Jurkat lysates. Jurkat cells were either left unstimulated or stimulated with C305 for 2 min. The lysates were first precleared with GST alone and then mixed with either GST alone or GST fusion protein containing the Vav SH2 domain (GST–VavSH2) in the absence or presence of 50 mM phenyl phosphate. The protein complexes were then blotted with anti-phosphotyrosine MAb (4G10). Each protein precipitation lane represents approximately 5×10^7 cell equivalents and each lysate lane represents 2×10^6 cell equivalents. The closed and open arrowheads indicate the 76 kDa and 70 kDa phosphoproteins associated with GST–VavSH2 fusion protein.

(C) GST–VavSH2 fusion protein interacts with SLP-76 and ZAP-70. Jurkat cells were either left unstimulated or stimulated with C205 for 2 min. The lysates were first precleared twice with either preimmune serum, anti-SLP-76 antiserum, or anti-ZAP-70 antiserum and then precleared with GST alone. The precleared lysates were then precipitated with either GST alone or GST–VavSH2 and the protein precipitates were blotted with anti-phosphotyrosine MAb (4G10). Each protein precipitation lane represents approximately 5×10^7 cell equivalents. The migrations of SLP-76 and ZAP-70 are indicated by the closed and open arrowheads, respectively.

mune complexes were analyzed by anti-phosphotyrosine Western blot. Following receptor stimulation, a prominent 76 kDa tyrosine phosphoprotein was coimmunoprecipitated with Vav–C (Figure 3A). Immunoblot analysis confirmed that this phosphoprotein was SLP-76 (data not shown), indicating that the C-terminal portion of Vav is sufficient to mediate the interaction between Vav and SLP-76. Interestingly, Vav–C also became tyrosine phosphorylated following TCR ligation, demonstrating that the C-terminal region contains Vav tyrosine phosphorylation sites.

The inducible association between Vav and SLP-76 suggests a possible SH2 domain–phosphotyrosine interaction. Previous in vitro studies indicated that the SH2 domain of Vav could interact with a number of tyrosine phosphoproteins in lymphocytes (Bustelo et al., 1992; Katzav et al., 1994). To examine whether the association between Vav and SLP-76 is mediated by the SH2 domain of Vav, a bacterial GST fusion protein containing the Vav SH2 domain was used to bind tyrosine phosphoproteins in Jurkat lysates. As shown in Figure 3B, GST–VavSH2 selectively bound to two major tyrosine-phosphorylated proteins of 76 kDa and 70 kDa following TCR stimulation. These interactions were likely to be mediated by the SH2 domain and phosphotyrosine interactions, since the addition of phenyl phosphate could completely disrupt both these associations (Figure 3B). Both preclearing and direct Western blotting experiments using the appropriate antisera revealed that the 76 kDa and 70 kDa phosphoproteins were SLP-76 and ZAP-70, respectively (Figure 3C; data not shown). Note that SLP-76 depletion did not reduce the amount of ZAP-70 associated with GST–VavSH2 and vice versa (Figure 3C), suggesting that these interactions likely involve separate complexes. Unlike the Vav–SLP-76 association, the interaction between Vav and ZAP-70 appeared to occur at a much lower stoichiometry or may be more transient, since it was difficult to detect in vivo (see Figure 1A; data not shown), although it has been possible to demonstrate in other studies (Katzav et al., 1994; Gouy et al., 1995). Taken together, these data indicate that the Vav SH2 domain is sufficient to interact with SLP-76 following TCR stimulation.

The Vav SH2 Domain Is Necessary for Both the Vav–SLP-76 Interaction and Optimal TCR-Stimulated Vav Tyrosine Phosphorylation

When Vav is introduced into fibroblasts, it is inducibly tyrosine phosphorylated upon epidermal growth factor

or platelet-derived growth factor receptor stimulation (Bustelo et al., 1992; Margolis et al., 1992). Activation of these receptors also leads to binding of Vav to these receptors via its SH2 domain, suggesting that the Vav SH2 domain may mediate the recruitment of Vav to these receptors where it can be tyrosine phosphorylated. The TCR-mediated Vav–C tyrosine phosphorylation described above is also consistent with this hypothesis. Therefore, we generated an SH2 domain point mutant of Vav and examined its tyrosine phosphorylation status as well as its ability to associate with SLP-76 in Jurkat T cells. Substitution of an arginine residue with lysine (VavSH2RK) within the predicted phosphate-binding site of the Vav SH2 domain markedly reduced its TCR-induced tyrosine phosphorylation, as compared with the wild type (Figure 4A). Similarly, we found that the SH2 domain of Vav was also required for its BCR-induced tyrosine phosphorylation in chicken DT40 B cells (data not shown). These data demonstrate that the Vav SH2 domain is important for TCR- or BCR-induced tyrosine phosphorylation of Vav.

The SH2 mutant of Vav (VavSH2RK) also failed to associate with SLP-76 following TCR stimulation (Figure 4B). Since SLP-76 also contains an SH2 domain that may interact with tyrosine-phosphorylated Vav, one possible explanation for the loss of the association between VavSH2RK and SLP-76 is that the SH2 domain of SLP-76 could not bind to the weakly phosphorylated VavSH2RK. This appeared to be unlikely, since a GST fusion protein containing the SLP-76 SH2 domain failed to bind to tyrosine-phosphorylated Vav (data not shown; Motto et al., 1996). Taken together, these data demonstrate that the Vav SH2 domain is both necessary and sufficient for mediating the interaction between Vav and SLP-76 following TCR stimulation.

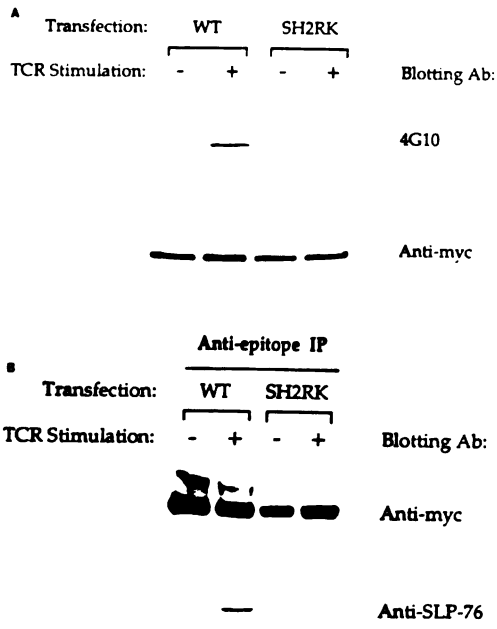

Figure 4. The Vav SH2 Domain Is Necessary for TCR-Induced Vav Tyrosine Phosphorylation and Vav–SLP-76 Interaction

(A) TCR-induced tyrosine phosphorylations of wild-type and an SH2 domain point mutant of Vav. TAg Jurkat cells were transiently transfected with either myc epitope-tagged wild-type Vav (pEF115-myc) or an SH2 domain mutant of Vav (pEF115mycRK). After transfection (20–40 hr), 1–2 × 10^7 live cells were either left unstimulated or stimulated with anti-TCR MAb (C305, 1:1000) for 2 min and then lysed. The lysates were immunoprecipitated with anti-myc MAb (9E10) and blotted with anti-phosphotyrosine MAb (4G10) (top). The blot was then stripped and reblotted with anti-myc MAb (9E10) (bottom).

(B) VavSH2RK fails to associate with SLP-76. TAg Jurkat cells were transfected and stimulated as described in (A). The lysates were immunoprecipitated with anti-myc MAb (9E10). The upper part of the blot was blotted with anti-myc MAb (9E10) (top), whereas the lower part was blotted with anti-SLP-76 antiserum (bottom).

The Interaction(s) Mediated by Vav SH2 Domain Is Important for TCR Signaling

The early biochemical events induced by TCR stimulation, through poorly understood mechanisms, lead to gene induction and other T cell effector functions. Reporter constructs containing multiple copies of the NF-AT binding site or the entire IL-2 promoter and regulatory elements have been useful tools to monitor TCR-mediated signaling events. We previously showed that overexpression of Vav in Jurkat T cells leads to a marked increase of basal and TCR-stimulated NF-AT and IL-2 promoter activities (Wu et al., 1995). Since the C terminus of Vav (Vav–C) is sufficient to interact with SLP-76 and to become tyrosine phosphorylated following TCR stimulation, we attempted to determine whether overexpression of Vav–C was also sufficient to induce NF-AT activation in Jurkat cells. In contrast with the full-length Vav, overexpression of Vav–C not only completely failed to induce basal NF-AT activity, but also inhibited TCR-stimulated NF-AT induction (Figure 5), suggesting that the effector domain of Vav may reside in its N-terminal region. To map the region responsible for this inhibitory effect, we examined the mutant of Vav–C (Vav–CSH2RK) in which the arginine residue within the phosphotyrosine binding pocket of the SH2 domain was substituted with lysine. The levels of Vav–C and Vav–CSH2RK were comparable when assayed by Western blot (data not shown). This point mutation completely abolished the inhibitory effect induced by overexpression of Vav–C, suggesting that the NF-AT inhibition was dependent upon the phosphotyrosine binding function of the Vav SH2 domain. Overexpression of Vav-C may compete with the endogenous Vav to form complexes with SLP-76 or to interact with PTKs responsible for Vav tyrosine phosphorylation, thereby acting as a dominant negative to inhibit TCR signaling. These data suggest that the interaction(s) mediated by Vav SH2 domain, such as the Vav–SLP-76 interaction, is important for TCR signaling events leading to NF-AT activation.

SLP-76 and Vav Functionally Cooperate in TCR-Mediated IL-2 Gene Activation

To address further the functional significance of the interaction between Vav and SLP-76, we overexpressed SLP-76 in Jurkat cells and examined its effect on the

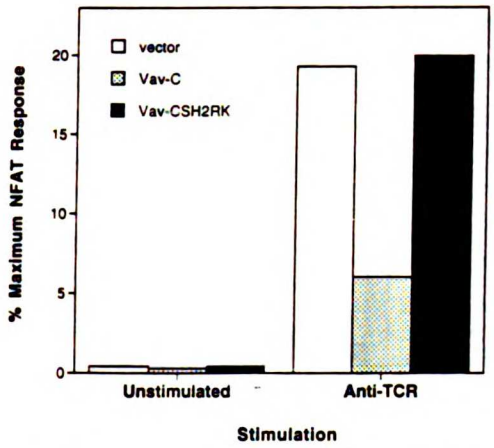

Figure 5. Overexpression of the C-Terminal Vav Inhibits TCR-Mediated NF-AT Activation

Jurkat T cells were cotransfected with 20 μg of NF-AT–Luc together with 20 μg of either an empty vector (vector), the C-terminal Vav (Vav–C), or an SH2 domain point mutant of Vav–C (Vav–CSH2RK). Cells were either unstimulated or stimulated with anti-TCR MAb (C305, 1:1000) and subsequently assayed for luciferase activity. The results are shown as the percent of the maximal stimulation for each transfection condition induced by treatment with PMA (50 ng/ml) plus ionomycin (1 μM). The maximum NF-AT responses for this experiment were approximately 1.5×10^5 arbitrary light units (AU) and did not differ significantly between different transfection conditions. Luciferase activity was determined in triplicate in each experimental condition. The data are representative of two independent experiments.

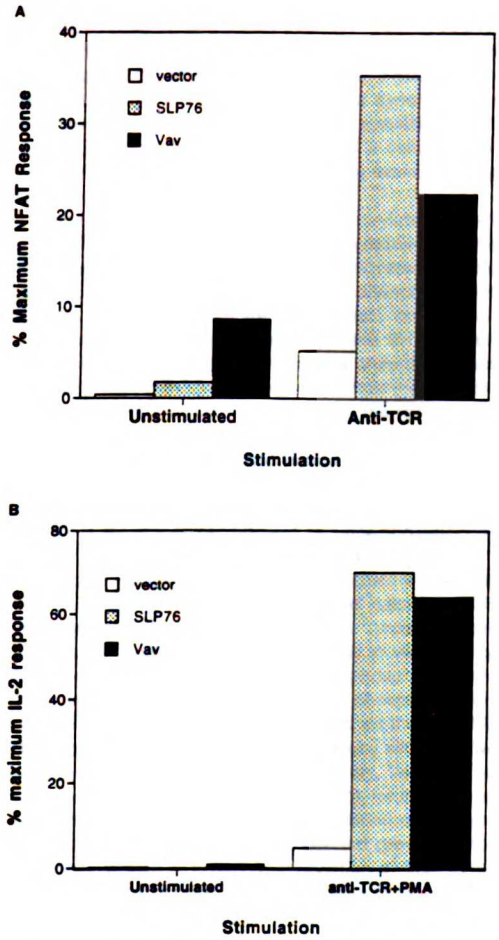

Figure 6. Overexpression of Either Vav or SLP-76 Potentiates TCR-Induced NF-AT and IL-2 Responses

Jurkat T cells were cotransfected with 20 μg of NF-AT–Luc (A) or IL-2–Luc (B) together with 40 μg of either an empty vector (vector), SLP-76, or Vav, as described in Experimental Procedures. Cells were either unstimulated or stimulated with either anti-TCR MAb (C305, 1:1000) (A) or C305 plus PMA (50 ng/ml) (B), and subsequently assayed for luciferase activity. The results are shown as the percent of the maximal stimulation for each transfection condition induced by treatment with PMA (50 ng/ml) plus ionomycin (1 μM) (maximum responses). The maximum NF-AT or IL-2 responses were approximately 2–4 $\times 10^5$ AU. Luciferase activity was determined in duplicate or triplicate in each experimental condition. The data are representative of at least two independent experiments.

NF-AT or IL-2-driven promoters. Unlike Vav, and as we have recently reported (Motto et al., 1996), overexpression of SLP-76 only led to minimal basal induction of NF-AT (Figure 6A). Similar to Vav, however, overexpression of SLP-76 dramatically synergized with TCR stimulation to activate NF-AT and IL-2 transcriptional activity (Figures 6A and 6B; Motto et al., 1996), suggesting its involvement in events leading to IL-2 gene activation. Since Vav and SLP-76 interact in T cells, we cotransfected various amounts of SLP-76 or Vav cDNAs, or both, into Jurkat cells to address whether they could cooperate to influence the TCR-mediated NF-AT responses. Coexpression of SLP-76 and Vav led to a dose-dependent synergy in basal NF-AT induction (Figure 7A). The synergy between SLP-76 and Vav could also be observed at lower doses of DNA when the TCR was engaged (Figure 7B). However, at higher doses of DNA the synergy decreased, suggesting that some components of the signaling pathway may be near saturation following receptor stimulation. Taken together, these data demonstrate that SLP-76 and Vav not only physically interact in T cells but functionally cooperate in the TCR signaling pathway leading to IL-2 gene activation.

Discussion

One immediate and obligatory consequence of triggering the TCR is the tyrosine phosphorylation of multiple intracellular substrates, including the proto-oncogene Vav. Recent studies showed that Vav is critical for T and B cell development and antigen receptor–mediated activation (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995; Wu et al., 1995). In this report, we demonstrate that another hematopoietic cell-specific protein, SLP-76, interacts with Vav in T cells and that the association is induced by TCR stimulation. Our study demonstrates further that the interaction between Vav and SLP-76 is mediated by the SH2 domain of Vav.

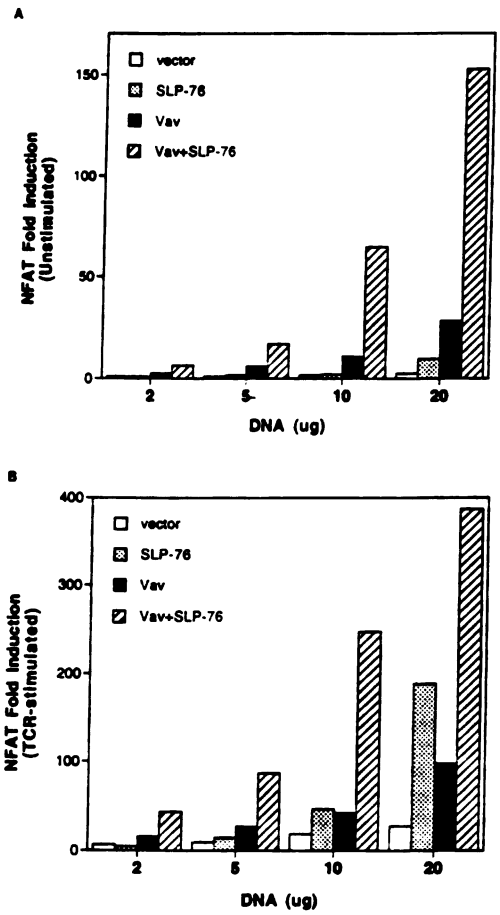

Figure 7. Overexpression of Both Vav and SLP-76 Synergistically Induces Basal and TCR-Stimulated NF-AT Activation

Jurkat cells were cotransfected with 20 μg of NF-AT–Luc together with the indicated amount of either empty vector, Vav, SLP-76, or Vav plus SLP-76. Cells were either unstimulated (A) or stimulated with anti-TCR MAb (C305, 1:1000) (B), and subsequently assayed for luciferase activity. The results are shown as the fold induction of luciferase activity as compared with the activity in unstimulated cells transfected with 2 μg of empty vector, which is approximately 200 AU. Luciferase activity was determined in triplicate in each experimental condition. The data are representative of two independent experiments.

Interestingly, a point mutation in the phosphotyrosine binding pocket of the SH2 domain of Vav not only abolished the TCR-induced association of Vav with SLP-76, but also markedly reduced TCR-stimulated Vav tyrosine phosphorylation, suggesting that the SH2 domain of Vav may also serve a critical role in TCR-induced Vav tyrosine phosphorylation. Furthermore, overexpression of a truncated form of Vav, containing the C-terminal SH2 and two SH3 domains, blocked TCR-mediated NF-AT activation. This inhibitory effect was abrogated completely by a point mutation in the Vav SH2 domain, suggesting that interactions mediated by this region, such as the Vav–SLP-76 association, are important in events leading to downstream NF-AT induction. Similar to Vav, overexpression of SLP-76 in Jurkat T cells markedly potentiated TCR-mediated NF-AT and IL-2 gene activation. Moreover, SLP-76 and Vav could synergistically activate basal and TCR-induced NF-AT activation. Thus, our data implicate the involvement of a complex between Vav and SLP-76 in TCR signaling events leading to IL-2 gene activation.

Since Vav becomes tyrosine phosphorylated following TCR stimulation and PTK activity is required for the Vav-mediated NF-AT activation in Vav-overexpressing Jurkat cells (Wu et al., 1995), its tyrosine phosphorylation is likely to be important for its function. We demonstrated that a point mutation within the SH2 domain of Vav markedly reduces Vav tyrosine phosphorylation following TCR stimulation, suggesting that the SH2 domain of Vav is important in mediating the recruitment and tyrosine phosphorylation of Vav in T cells. Vav–C contains the SH2 domain and becomes tyrosine phosphorylated following TCR stimulation. Thus, its overexpression could potentially compete with endogenous Vav for TCR-induced recruitment, tyrosine phosphorylation, or both, thereby interfering with TCR signaling.

Our data provide two potential mechanisms by which Vav becomes tyrosine phosphorylated following TCR stimulation. Since SLP-76 is the major tyrosine-phosphorylated protein associated with Vav in vivo, it may function as an adaptor for Vav tyrosine phosphorylation. TCR stimulation could lead to tyrosine phosphorylation of SLP-76, which could then recruit Vav via the Vav SH2 domain to the proximity of a PTK, thereby allowing Vav to become phosphorylated. Alternatively, Vav may be directly recruited by a PTK, e.g., ZAP-70, for its tyrosine phosphorylation. In fibroblasts, stimulation of the epidermal growth factor or platelet-derived growth factor receptors results in autophosphorylation of their cytoplasmic tails, providing the binding sites for the downstream molecules such as PLC-γ1 via their SH2 domains, which then lead to tyrosine phosphorylation and activation of these molecules (Koch et al., 1991). Although the TCR and BCR do not possess intrinsic tyrosine kinase activity, the Syk/ZAP-70 kinase family members have been shown to interact with the receptor ITAM motifs following receptor stimulation (Weiss and Littman, 1994). This recruitment results in tyrosine phosphorylation and activation of these kinases. Phosphorylated tyrosine residues within the PTKs may provide further binding sites for other downstream SH2 domain-containing proteins such as Vav. In this study and elsewhere (Katzav et al., 1994), we showed that the GST–VavSH2 fusion protein could bind to phosphorylated ZAP-70 following TCR stimulation. The predepletion experiments revealed that this in vitro binding was not dependent upon the presence of SLP-76 (Figure 4A), suggesting that Vav may interact directly with ZAP-70 via the Vav SH2 domain. It is conceivable that the tyrosine phosphorylation of Vav then induces a conformational change, which allows it to dissociate from ZAP-70 and to interact with SLP-76 for other cellular functions. In fact, the low stoichiometric association in vivo between Vav and ZAP-70 suggests that the interaction may be very transient. SH2 domains can provide specificity in

signaling processes by binding to specific phosphotyrosine-containing sequences. The degenerate phosphopeptide library approach has predicted multiple potential Vav SH2 domain-binding sequences, including the sequence YESP (Songyang et al., 1994). SLP-76 contains two motifs of YESP, which likely serve as the binding sites for the Vav SH2 domain. Interestingly, the same motif is also present in ZAP-70 (Y315) and in Syk (Y348). Experiments are underway to determine whether these tyrosine residues are important in mediating Vav phosphorylation and other downstream TCR signaling events.

It is interesting that, although structurally unrelated to Vav, overexpression of SLP-76 in T cells also dramatically potentiates TCR-mediated NF-AT activation. This suggests that SLP-76 plays a role in the TCR signaling cascade and is likely to be a critical component in the pathway. SLP-76 was initially cloned through its in vitro association with GST fusion protein containing Grb-2, a known adaptor critical for Ras activation (Jackman et al., 1995). This in vitro interaction between Grb-2 and SLP-76 is mediated by the SH3 domain of Grb-2 and the proline-rich sequence in SLP-76 (Motto et al., 1996). It has been shown that, following TCR stimulation, both Grb-2 and PLC-γ1 become associated with an unknown 36 kDa tyrosine-phosphorylated protein (pp36), which may serve as a link between the receptor and the downstream Ras or PLC-γ1 activation pathways, or both (Buday et al., 1994; Sieh et al., 1994). Interestingly, pp36 could also be easily detected in TCR-stimulated anti-SLP-76 immunoprecipitates (Motto et al., 1996). Although demonstration of an in vivo association between SLP-76 and Grb-2 and the functional significance of this complex are yet to be determined, these observations suggest that SLP-76 may play a role in the proximal TCR signaling events leading to activation of Ras or PLC-γ1 pathways, or both.

The function of Vav has been an enigma. Although Vav contains sequence homology to exchange factors for the Rac/Rho/CDC42 family of small GTPases but not for Ras, it was reported to be the major antigen receptor–stimulated Ras exchange factor in lymphocytes (Gulbins et al., 1993). However, this conclusion has not been reproduced or supported by other studies. For example, the transformation phenotype of the oncogenic Vav-transfected fibroblasts is distinct from that of fibroblasts transformed by oncogenic Ras, but is similar to the phenotype of cells transformed by oncogenic Dbl (Bustelo et al., 1994), a known Rac/Rho/CDC42 exchange factor sharing homologous GEF domain with Vav (Boguski and McCormick, 1993). Moreover, oncogenic Vav- or Dbl-transformed cells did not exhibit elevated levels of Ras–GTP (Bustelo et al., 1994; Khosravi-Far et al., 1994). In Jurkat T cells, we and others also showed that the effect of Vav overexpression is distinct from that of simply activating Ras pathway alone (Wu et al., 1995; Holsinger et al., 1995). These data suggest that Vav is not simply a GEF for Ras. Here, we showed that in contrast with the full-length Vav, overexpression of the C-terminal Vav (Vav–C) not only failed to potentiate NF-AT activity, but also appeared to function as a dominant negative mutant for TCR signaling. This suggests that an effector domain of Vav is N-terminal to its SH3 and SH2 domains and may involve the GEF domain. A detailed mutagenesis study of Vav should help to identify its functional domains.

The results of this study provide direct evidence for an activation-dependent interaction between two hematopoietic-specific molecules, Vav and SLP-76. The association appears to play an important role in TCR signal transduction. Since both Vav and SLP-76 contain a number of structural motifs that may potentially interact with other signaling molecules, the association between Vav and SLP-76 may further induce formation of a multiprotein signaling complex, which in turn functions cooperatively to influence the downstream events leading to IL-2 gene induction. In fact, it has been shown that SLP-76 also becomes associated with pp36 and tyrosine-phosphorylated proteins of 62 kDa and 130 kDa (Motto et al., 1996). Hence, overexpression of the C terminus of Vav may compete with the endogenous Vav to form nonfunctional complexes with SLP-76 and other proteins, and thereby block TCR signaling. Future biochemical and genetic analyses may help to elucidate the mechanisms by which Vav and SLP-76 function in lymphocytes.

Experimental Procedures

DNA Constructs and Fusion Proteins

The NF-AT and IL-2 luciferase reporter constructs were gifts from Dr. G. Crabtree. The myc epitope-tagged Vav expression plasmid (pEF115myc) was described previously (Wu et al., 1995). The C-terminal Vav (Vav–C) was constructed by replacing the BamH1–Kpn1 fragment of Vav cDNA from pEF115myc with an oligonucleotide fragment (BamHI–KpnI) comprising both a Kozak translational initiation site and the myc epitope tag sequences, resulting in the plasmid pEFVav–Cmyc. Vav cDNA possessing the SH2 RK mutation (pEF115mycRK) was generated by overlapping extension polymerase chain reaction (PCR), using pEF115myc cDNA as a template. The resulting PCR product was subcloned into the pEF115myc after removal of the corresponding wild-type sequence, resulting in the plasmid pEF115mycRK. The Vav–C containing the SH2 RK mutation was constructed by replacing the KpnI–XbaI fragment of pEFVav–Cmyc with the corresponding fragment from pEF115mycRK, resulting in the plasmid pEFVav–CmycRK. The pEF/flag/SLP-76 and GST/SLP-76/SH2 cDNAs were described elsewhere (Motto et al., 1996). The GSTVavSH2 was provided by Dr. S. Katzav (Katzav et al., 1994). GST fusion proteins were induced and affinity purified as described (Smith and Johnson, 1988).

Antibodies

The MAb used for the stimulation of the TCR was C305, which recognizes the Jurkat Ti β chain (Weiss and Stobo, 1984). Anti-Vav polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Sheep anti-SLP-76 antiserum was generated against amino acids 136–235 of human SLP-76 expressed as a GST fusion protein (Motto et al., 1996). Anti-phosphotyrosine MAb, 4G10, was purchased from Upstate Biotechnology, Incorporated (Lake Placid, New York). A MAb, 9E10, for the detection of the myc epitope was provided by Dr. J. M. Bishop.

Cell Lines and Transfections

Jurkat cells (E6-1) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (complete medium). TAg Jurkat cells (a gift from Dr. G. Crabtree) (Clipstone and Crabtree, 1992) were maintained in the complete medium with 2.0 mg/ml geneticin (GIBCO), and transferred to geneticin-free medium 48 hr before experiments to prevent aminoglycoside-mediated inhibition of phosphoinositide hydrolysis. Jurkat cells (10^7) were transiently transfected by electroporation, as previously described (Wu et al., 1995), with 20 μg of the NF-AT or

IL-2 reporter plasmids and the indicated amount of vectors containing either no insert (pEFBOS), Vav (pEF115myc), SLP-76 (pEF/flag/SLP76), Vav–C (pEFVav–Cmyc), or Vav–CSH2RK (pEFVav–CmycRK) cDNA inserts. TAg Jurkat cells were transiently transfected by electroporation with 20 μg of the indicated plasmids (either pEFBOS, pEF115myc, pEF115mycRK, or pEFVav–Cmyc) in serum-free RPMI at a density of 2–3 × 10^7 cells/400 μl/cuvette with a Bio-Rad gene pulser set at 250 V and 960 μF. After electroporation, the cells were transferred to complete RPMI and incubated at 37°C for 24–40 hr.

Luciferase Assays

Transfected Jurkat cells (2 × 10^5) were aliquoted into a 96-well plate (Corning) 20–40 hr after transfection and cultured in a final volume of 100 μl RPMI growth medium. Cells were unstimulated or stimulated at 37°C in the growth medium containing either 1:1000 dilution of C305 ascites or 50 ng/ml phorbol myristate acetate (PMA) and 1.0 μM ionomycin. After an 6–8 hr stimulation period, cells were lysed in harvest buffer (100 mM KPO_4 [pH 7.8], 1.0 mM DTT, 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 $MgCl_2$). Luciferase activity was quantitated with a monolight luminometer (Analytical Luminescence Laboratory, Ann Arbor, Michigan) immediately after the addition of 100 μl of 1.0 mM luciferin (Sigma Chemical Company, St. Louis, Missouri). Luciferase activity was determined either in duplicate or in triplicate for each experimental condition.

Immunoprecipitations, Protein Precipitations

Cells were harvested and washed in phosphate-buffered saline, and were left either unstimulated or stimulated with C305 ascites (1:500 dilution) at 37°C for 2 min or with pervanadate at room temperature for 10 min, and then lysed at 10^6/ml in the lysis buffer containing 1% NP40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, protease and phosphatase inhibitors, as previously described (Straus and Weiss, 1992). After 20 min at 4°C, lysates were centrifuged for 15 min at 14,000 rpm. Lysates were then immunoprecipitated with the indicated antibodies. When precipitated with GST fusion proteins, lysates were first precleared with GST alone before they were precipitated with the indicated GST fusion proteins. Resulting immunoprecipitates or protein complexes were then washed extensively in lysis buffer, resolved by (SDS–PAGE), and transferred to polyvinylidene difluoride membranes.

Immunoblots

Blots were blocked with 3% albumin in TBST buffer (10 mM Tris, [pH 7.9], 150 mM NaCl, with 0.05% Tween detergent). Blots were incubated with the indicated antisera or MAb followed by a secondary antibody conjugated with horseradish peroxidase and then assayed by enhanced chemiluminescence assay (ECL kit; Amersham Life Sciences, Arlington Heights, Illinois). In the case of reblotting with a different primary antibody, blots were incubated at 70°C in stripping buffer (2% SDS, 0.1 M 2-ME, 62.5 mM Tris [pH 6.8]) for 20 min, washed extensively in TBST buffer, and then blocked and probed as described above.

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CHAPTER IV

Involvement of Src and Syk PTKs in Vav tyrosine phosphorylation following antigen receptor stimulation.

Summary:

One of the earliest biochemical events following antigen receptor stimulation in T and B cells is tyrosine phosphorylation of numerous intracellular substrates, including the 95 kD proto-oncogene product Vav. Vav is expressed primarily in hematopoietic cells and has been shown to be essential for both T and B cell development and antigen receptor-mediated signal transduction. Recent experiments have suggested that tyrosine phosphorylation of Vav is critical for regulating its exchange activity for the Rho/Rac/CDC42 family of GTPases. To identify the protein tyrosine kinase (PTK) responsible for Vav phosphorylation and activation, we examined antigen receptor-mediated Vav tyrosine phosphorylation in chicken B cells lacking the Src- and/or the Syk/ZAP-70-family PTKs as well as in thymocytes from either Lck-, Fyn- or ZAP-70-deficient mice. We demonstrate here that both Src and Syk/ZAP-70 families of PTKs contribute to Vav tyrosine phosphorylation following antigen receptor stimulation. Moreover, the SH2 domain of Vav is required for both Src and Syk PTK-mediated Vav tyrosine phosphorylation. These data raise the possibility of differential regulation of Vav by Src and Syk families of PTKs in antigen receptor signaling.

Introduction

Stimulation of the T and B cell antigen receptors (TCR and BCR) initiates a cascade of signal transduction events including the activation of cytoplasmic protein tyrosine kinases (PTKs) (1). The Src family members Lck and Fyn in T cells and Lyn, Fyn, and Blk in B cells are believed to be responsible for phosphorylation of the tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR or BCR signaling subunits following antigen receptor stimulation (1-3). Phosphorylation of the ITAMs results in recruitment of Syk/ZAP-70 PTKs where they can become phosphorylated and activated.

The critical roles of Lck, Fyn and ZAP-70 in T cell development and activation has been demonstrated in immunodeficient patients, mice, and cell lines deficient in one of these PTKs. Loss of either Lck or ZAP-70 leads to a severe defect in both T cell development and TCR signaling function (4-9), whereas T cells from Fyn-deficient mice develop normally but are impaired in TCR signaling (10, 11). The importance of Lyn and Syk in BCR signaling has also been revealed by inactivating these genes in either mice or the chicken B cell line DT-40 by homologous recombination. Loss of Lyn expression results in abnormal B cell maturation and reduced BCR function, whereas inactivation of the *syk* gene leads to an early arrest of B cell development and a severe block of BCR signaling (12-16).

Activation of the Src and Syk/ZAP-70 PTKs in T and B cells leads to tyrosine phosphorylation of numerous cellular proteins including the proto-oncogene Vav, phospholipase C γ isoforms, Cbl, Shc and SLP-76 (1, 17). Tyrosine phosphorylation and/or activation of these substrates ultimately results in downstream cytokine gene induction and other effector functions.

The proto-oncogene Vav is expressed exclusively in hematopoietic and trophoblast cells and contains an array of structural motifs, including a leucine-rich N-terminal domain, a guanine nucleotide exchange (GEF) domain for the Rho/Rac/CDC42 family of small GTPases, a pleckstrin homology (PH) domain, a cysteine-rich region, and two Src homology 3 (SH3) domains that flank one src homology 2 (SH2) domain (18, 19). Vav plays a critical role in lymphocyte development and activation. Chimeric mice derived from blastocysts lacking the recombinase-activating gene (RAG) and embryonic stem cells deficient in Vav expression exhibit a marked reduction in thymocyte number and peripheral T and B cells, as well as a specific defect in TCR- and BCR-mediated signal transduction leading to cytokine production and proliferation (20-22). In addition, overexpression of Vav in Jurkat T cells results in a marked increase in basal NFAT or IL-2 promoter-driven transcriptional activity, that is further enhanced by TCR stimulation (23). These results provide strong evidence that Vav plays a critical role in antigen receptor signaling pathways.

The exact molecular mechanism by which Vav functions in lymphocytes still remains elusive. However, its structure as well as recent data suggest that Vav is likely to be involved in the activation of the Rho/Rac/CDC42 family of small GTPases. The GEF domain in Vav shares close homology with those found in Dbl and CDC24, two known GEFs for Rho, Rac or CDC42 (24). Moreover, recent *in vitro* experiments and in fibroblasts have demonstrated that Vav, upon tyrosine phosphorylation, can activate Rho/Rac/CDC42 family of GTPases (25, 26). These results strongly suggest that Vav may be an important regulator of the Rho/Rac/CDC42-mediated signaling events such as cytoskeletal rearrangement in lymphocytes.

Since Vav becomes rapidly tyrosine phosphorylated following TCR or BCR stimulation and its activity on Rho/Rac/CDC42 depends upon its tyrosine phosphorylation (25-28)., identification of the kinase(s) responsible for the phosphorylation of Vav in lymphocytes is of great interest. The Src family member Lck appears to be sufficient to activate the exchange activity of Vav on Rho/Rac/CDC42 *in vitro* and in fibroblasts (25, 26)., whereas the Syk/ZAP-70 family members have been shown to interact with Vav via the Vav SH2 domain following antigen receptor stimulation (29-31). To identify upstream PTK(s) responsible for phosphorylating Vav and to explore the potential role of Vav tyrosine phosphorylation in antigen receptor-mediated signaling pathways, we utilized various mutants of the chicken B cell DT-40, in which either the PTK Lyn, the Syk kinase or both kinases have been inactivated by homologous recombination (15, 32). In this study, we show that both the Src- and the Syk-families of PTKs contribute to Vav tyrosine phosphorylation following antigen receptor stimulation. Moreover, these kinases appear to utilize a similar mechanism to recruit Vav, since the Vav SH2 domain is required for its phosphorylation mediated by either Src or Syk PTKs. We further demonstrate the involvement of these PTKs in antigen receptor-mediated Vav tyrosine phosphorylation in thymocytes by analyzing either Lck-, Fyn-, or ZAP-70-deficient mice.

Results and Discussion:

To understand the molecular mechanism of Vav regulation in lymphocytes, we have taken a genetic approach to determine the requirements for Vav tyrosine phosphorylation following antigen receptor stimulation. We utilized three mutants of the chicken cell line DT-40, generated by inactivation of either *lyn*, *syk* or both genes by homologous recombination (15, 32). Loss of Lyn expression, the only Src family member expressed in DT-40 cells, led to reduced tyrosine phosphorylation of cellular proteins and delayed intracellular calcium increase following BCR stimulation (Figure 1 and reference 15). Inactivation of *syk* not only resulted in reduced tyrosine phosphorylation but also completely abolished both the calcium increase and downstream gene induction (Figure 1 and reference 15 and 33). The mutant cells deficient in both Lyn and Syk expression exhibited the most severe phenotype, in which minimal or no tyrosine phosphorylation or other signaling events could be observed following BCR stimulation (Figure 1, data not shown and reference 32). Note that both Lyn-deficient and Syk-deficient cells exhibited partial phosphotyrosine induction in response to BCR stimulation (Figure 1). In addition, the patterns of tyrosine phosphorylation in Lyn- or Syk-deficient cells were distinct (Figure 1), suggesting that they may mediate tyrosine phosphorylation of different proteins. To determine whether Lyn, Syk or both PTKs are required for BCR-induced Vav tyrosine phosphorylation, we transiently transfected human Vav cDNA into wildtype, Lyn-deficient, Syk-deficient, and Lyn/Syk double-deficient DT-40 cells. These transfected cells were then lysed prior to and following BCR ligation, and human Vav was immunoprecipitated with an anti-Vav antiserum, that does not react with avian Vav (Figure 2A, and data not shown). As shown in Figure 2A, Vav was inducibly tyrosine phosphorylated following BCR stimulation in wild-

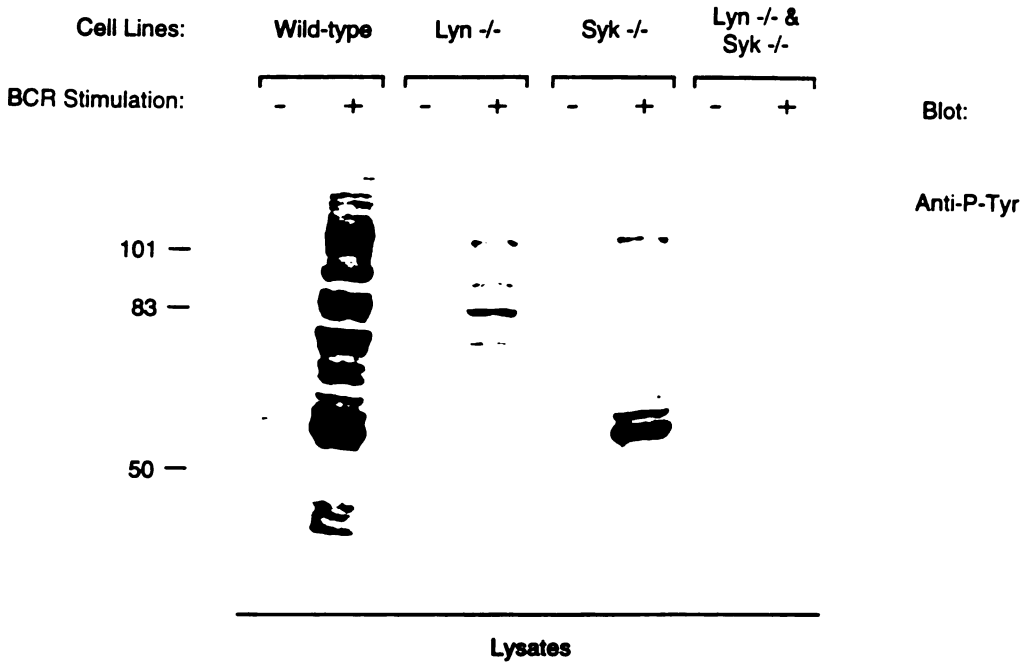
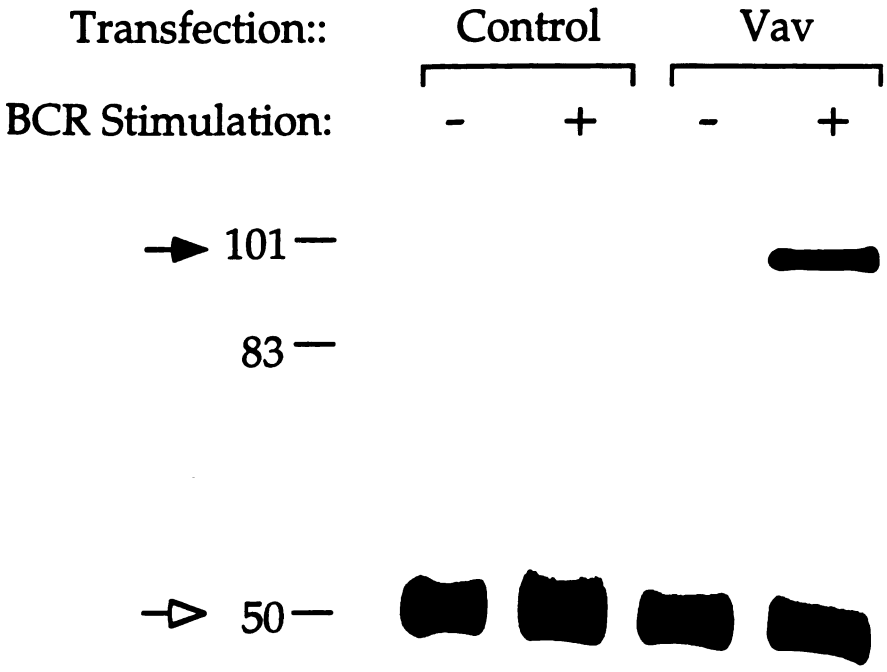


Figure 1. Induction of tyrosine phosphoproteins in wild-type and various mutants of DT-40 B cell lines. Wild-type, Lyn-deficient, Syk-deficient and Lyn/Syk double-deficient cells were either left unstimulated or stimulated with anti-BCR monoclonal antibody (mAb) (M4, 2 μg/ml) for 2 min., and subsequently lysed in NP-40 lysis buffer. $2x10^6$ cell equivalent lysates were then blotted with anti-phosphotyrosine antibody (4G10). The mobilities of molecular weight markers are indicated on the left.

type DT-40 cells. Loss of either Lyn or Syk did not substantially reduce the magnitude of BCR-induced Vav tyrosine phosphorylation (Figure 2B). In contrast, Lyn/Syk doubly-deficient DT-40 cells completely failed to exhibit any Vav tyrosine phosphorylation following BCR ligation (Figure 2B). Cotransfection of Vav with either a Src or a Syk kinase in Lyn/Syk doubly deficient cells reconstituted Vav tyrosine phosphorylation (data not shown). These data suggest that either Lyn or Syk PTK is capable of mediating Vav tyrosine phosphorylation in B cells.

In T cells, Lck and Fyn, two Src family members, as well as ZAP-70 have been shown to be critical in initiating TCR-mediated signal transduction events including tyrosine phosphorylation of intracellular substrates (1). To determine the physiological role of these PTKs in TCR-mediated Vav tyrosine phosphorylation, we examined the phosphorylation status of Vav in thymocytes from mice lacking either Lck, Fyn, or ZAP-70. We found that the TCR-stimulated Vav tyrosine phosphorylation was reduced in the Lck-, Fyn-, or ZAP-70-deficient thymocytes (Figure 3A, 3B), demonstrating the involvement of these kinases in mediating Vav phosphorylation in T cells. The residual phosphorylation of Vav in these thymocytes may suggest potential redundancy or different sites of phosphorylation by each of these kinases. These data suggest that both Src and Syk PTKs are important in TCR-mediated Vav tyrosine phosphorylation in thymocytes. While these results are consistent with the findings in DT-40 cells, one caveat is that the thymocyte population in either Lck- or ZAP-70-deficient mice is different from the control animals, which may complicate our interpretation. Detailed biochemical analyses on the thymocyte subpopulations are underway to provide more accurate comparisons.

Figure 2 (A)


Transfection::
Control
Vav
BCR Stimulation:
- + - +
101
83
50

(B)

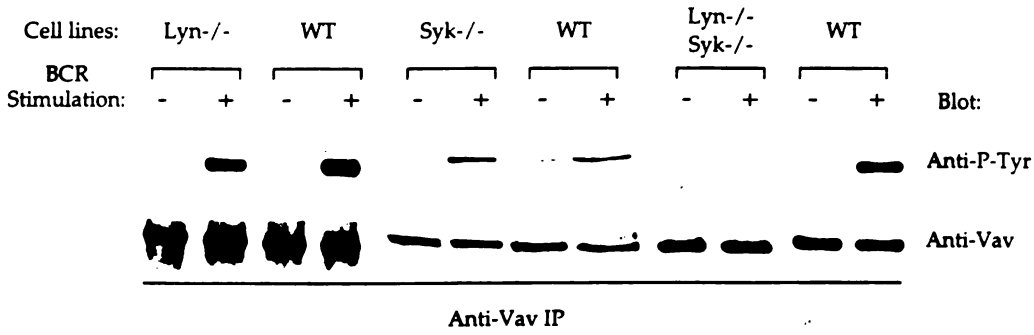
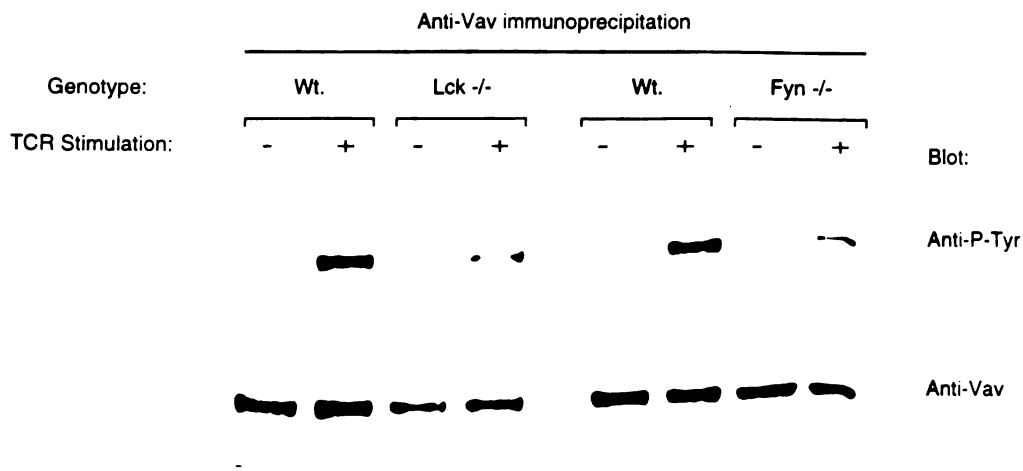

Cell lines:
Lyn-/-
WT
Syk-/-
WT
Lyn-/- Syk-/-
WT
BCR Stimulation:
- + - + - + - + - + - +
Blot:
Anti-P-Tyr
Anti-Vav
Anti-Vav IP

Figure 2. (A) Tyrosine phosphorylation of Vav in wild-type DT-40 cells. Wild-type DT-40 B cells were transiently transfected with either an empty vector or human Vav (pCI115). After transfection (24-40 hrs), 1-2x10^7 cells were either left unstimulated or stimulated with anti-BCR mAb (M4, 2 μg/ml) for 2 min. and then lysed. The lysates were immunoprecipitated with anti-Vav polyclonal antibody (Ab) and then the immunoprecipitates were blotted with anti-phosphotyrosine mAb (4G10). The mobilities of molecular weight markers are indicated on the left. The migration of Vav and heavy chain are indicated by closed and open arrowheads, respectively.

(B) Tyrosine phosphorylation of Vav in mutant DT-40 cells. Wild-type, Lyn-deficient, Syk-deficient and Lyn/Syk double-deficient DT-40 cells were transiently transfected with human Vav (pCI115). After transfection (24-40 hrs), 1-2x10^7 cells were either left unstimulated or stimulated with anti-BCR mAb (M4, 2 μg/ml) for 2 min. and then lysed. The lysates were immunoprecipitated with anti-Vav polyclonal Ab and then the immunoprecipitates were blotted with anti-phosphotyrosine mAb (4G10) (top). The blot was then stripped and reblotted with anti-Vav polycolonal Ab (bottom). Each mutant cell line was examined in separate experiments in which wild-type cells were used as controls.

(A)



(B)

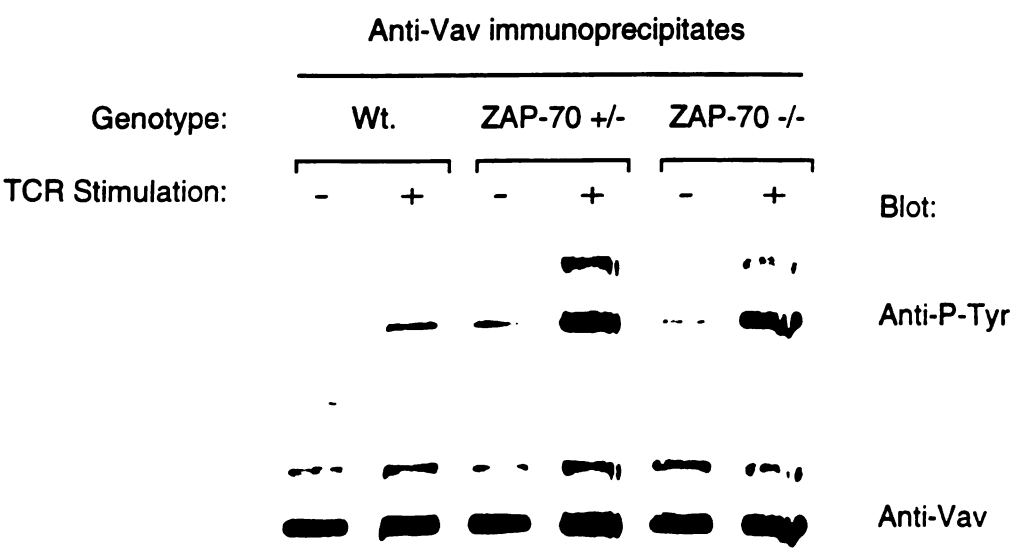


Figure 3. Lck, Fyn and ZAP-70 regulate TCR-stimulation-induced tyrosine phosphorylation of Vav. Thymocytes isolated from wildtype from wild type (Wt.), lck-/-, fyn-/- (A), or zap-70-/- (B) mice were either left unstimulated or stimulated with the anti-CD3 mAb 145-2C11 for 3 min. Vav immunoprecipitates were first analyzed with anti-phosphotyrosine Ab 4G10. The blots were stripped and then blotted with anti-Vav. The lower band in (B) corresponds to the migration of Vav.

One means of phosphorylating Vav following antigen receptor stimulation is by its recruitment by an upstream adaptor or a PTK, possibly via the Vav SH2 domain. To determine whether the SH2 domain of Vav is required for both Src or Syk PTK-mediated Vav phosphorylation, we examined the tyrosine phosphorylation status of an SH2 domain point mutant of Vav expressed in wild-type DT-40 cells in response to BCR stimulation. Point mutation of the arginine residue within the predicted phosphotyrosine-binding site of the Vav SH2 domain abrogated BCR-induced tyrosine phosphorylation of Vav in wild-type DT-40 cells (Figure 4). As both Lyn and Syk kinases can independently contribute to Vav phosphorylation (Figure 2B), these results suggest that both the Src and the Syk PTK-mediated Vav phosphorylation require the function of the Vav SH2 domain.

Recent reports have shown that the Src kinase-mediated Vav tyrosine phosphorylation is sufficient to induce the Vav activity on Rho/Rac/CDC42 *in vitro* and in fibroblasts (25, 26). Here, we demonstrate that in DT-40 B cells and in thymocytes both Src and Syk PTKs contribute to Vav tyrosine phosphorylation. Interestingly, in contrast to Syk-deficient DT-40 B cells, BCR stimulation in Lyn-deficient cells led to a comparable level of IP3 generation and NFAT induction when compared with that in wildtype cells (data not shown an reference 15). These data suggest that Lyn-mediated Vav phosphorylation is not absolutely required for some of the BCR-mediated signaling events. These results further imply that Syk family PTKs may be able to phosphorylate and activate Vav, at least in DT-40 B cells. In addition, our data raised a possibility that these two distinct families of PTKs, Src and Syk, may phosphorylate Vav at different sites under physiological conditions, resulting in different functional consequences. In fact, Vav contains more than 30 potential tyrosine phosphorylation sites and

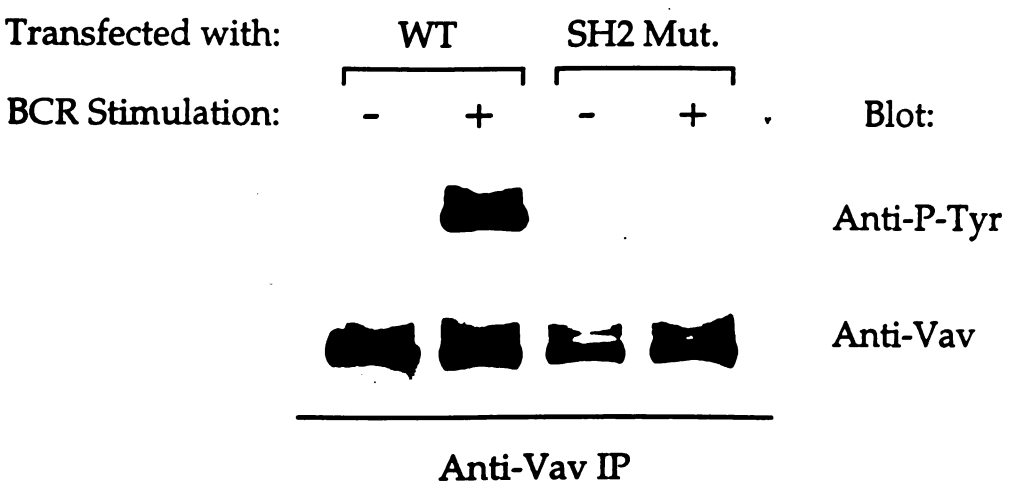


Figure 4. The SH2 domain is necessary for BCR-induced Vav tyrosine phosphorylation

Wild-type DT-40 cells were transiently transfected with either wild-type human Vav (pCI115) or an SH2 domain point mutant of Vav (pCI154). After transfection (24-40 hrs), 1-2x10^7 live cells were either left unstimulated or stimulated with anti-BCR (M4, 2 μg/ml) for 2 min. and then lysed. The lysates were immunoprecipitated with anti-Vav polyclonal Ab and blotted with anti-phosphotyrosine mAb (4G10) (top). The blot was then stripped and reblotted with anti-Vav polyclonal Ab (bottom).

previous studies have suggested that multiple tyrosine phosphorylated residues may exist following antigen receptor stimulation. For instance, the C-terminus of Vav (Vav-C), containing the two SH3 and one SH2 domains, became tyrosine phosphorylated following antigen receptor ligation when transfected into T and B cells (data not shown and reference 34), demonstrating that this C-terminal region contains tyrosine phosphorylated site(s). Meanwhile, a peptide or a GST fusion protein encompassing Y174 in the amino-terminus of Vav has been reported to be an efficient substrate for Syk (30, 35). Future phospho-peptide mapping of Vav from either wild-type , Lyn- or Syk-deficient DT-40 cells will help to examine this hypothesis.

Although both Src and Syk PTKs are capable of mediating Vav phosphorylation, they appear to utilize a common mechanism involving the Vav SH2 domain. A number of tyrosine phosphorylated proteins have been shown to interact with the Vav SH2 domain following antigen receptor stimulation, including Syk, ZAP-70, SLP-76 and CD19 (29-31, 36). Both Syk and ZAP-70 contain a consensus Vav SH2 domain binding motif, YESP, allowing them to directly recruit Vav. Recent studies have shown that the tyrosine residues within the YESP motifs in Syk and ZAP-70 are important for their interaction with the Vav SH2 domain and the subsequent Vav tyrosine phosphorylation, as well as for antigen receptor-mediated signal transduction (30, 31). However, ZAP-70/Syk-mediated Vav tyrosine phosphorylation cannot account for all the Vav tyrosine phosphorylation events in lymphocytes; Vav still becomes tyrosine phosphorylated following BCR stimulation in Syk-deficient DT-40 cells. CD19 and SLP-76 also contain one or more Vav SH2 domain binding sites and have been shown to interact with the Vav SH2 domain following antigen receptor stimulation. Thus, they may function as adaptors for the recruitment and

tyrosine phosphorylation of Vav in the absence of ZAP-70 or Syk. Future mutagenesis studies may help to resolve these alternatives.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

Mice. Wildtype mice (C57BL/6 strain) were purchased from The Jackson Laboratory (Bar Harbor, ME). *lck-/-* mice (7) and *fyn-/-* mice (10) were obtained from Drs. T. Mak (Amgen and the Ontario Cancer Institute, Toronto, Ontario, Canada) and R. Perlmutter (University of Washington, Seattle, WA), respectively. *zap-70-/-* mice were generated by homologous recombination-based gene targeting method (T. Kadlecek and A. Weiss, unpublished results).

DNA constructs. The Vav expression plasmid (pCI115) was constructed by subcloning the EcoRI fragment containing the full length human vav from pSK115 into pCIneo, a mammalian expression vector (InVitrogen) (37). The SH2 domain point mutant (R695L) of Vav (pCI154) was constructed by subcloning the EcoRI fragment from pSK154 into pCIneo (37).

Antibodies The mAb used for the stimulation of the BCR was M4 (kindly provided by M Cooper and CL Chen). Anti-CD3 mAb 145-2C11 was obtained from the American Type Culture Collection (Rockville, MD). Anti-Vav polyclonal Ab and mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology Inc (Lake Placid, NY), respectively. Anti-phosphotyrosine mAb, 4G10, was purchased from Upstate Biotechnology Inc.

Cell lines and transfections. Wild-type, Syk-deficient, Lyn-deficient and Lyn/Syk double-deficient cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% chicken serum, penicillin, streptomycin and glutamine, as previously described (15). Wild-type and all three mutants of DT-40 cells were resuspended in serum-free medium (RPMI 1640) and then transferred into a 0.4 cm cuvette (BioRad) at a density of $2x10^7$ cells/0.45 ml/cuvette. 30 µg of wildtype Vav cDNA (pCI115) were added to the cuvette. Cells were then electroporated with a Bio-Rad gene pulser set at 350V

and 500μF and then left on ice or RT for 10 min. After electroporation, the cells were transferred to the medium used to maintain DT-40 cells and incubated at 37°C for 24-40 hrs.

Stimulation, immunoprecipitations, protein precipitations, and immunoblotting

Cells were harvested and washed in phosphate-buffered saline, and were left either unstimulated or stimulated with M4 (2 μg/ml) at 37°C for 2 minutes. Preparation of thymocytes was described previously. Stimulation of mouse thymocytes with 10 ug/ml purified anti-CD3 mAb 2C11 was carried out as described (38). Cells were then lysed in the lysis buffer containing 1% NP40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, protease and phosphatase inhibitors, as previously described (31). After 20 min at 4°C, lysates were centrifuged for 15 min at 14,000 rpm. Lysates were then immunoprecipitated with the indicated antibodies. Resulting immunoprecipitates were then washed extensively in lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and blotted as previously described (23).

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CHAPTER V

The Vav binding site (Y315) in ZAP-70 is critical for antigen receptor-mediated signal transduction.

Summary

Stimulation of antigen receptors in T and B cells leads to the activation of the Src- and Syk/ZAP-70- families of protein tyrosine kinases (PTK). These PTKs subsequently phosphorylate numerous intracellular substrates, including the proto-oncogene product Vav. Vav is essential for both T and B cell development and T and B cell antigen receptor-mediated signal transduction. Following receptor ligation, Vav associates with phosphorylated Syk/ZAP-70 PTKs, an interaction that depends upon its SH2 domain. In this study we demonstrate that a point mutation of tyrosine 315 (Y315F) in ZAP-70, a putative Vav SH2 domain binding site, eliminated the Vav-ZAP-70 interaction. Moreover, the Y315 mutation impaired the function of ZAP-70 in antigen receptor signaling. Surprisingly, this mutation also resulted in marked reduction in the tyrosine phosphorylation of ZAP-70, Vav, SLP-76 and Shc. These data demonstrate that the Vav binding site in ZAP-70 plays a critical role in antigen receptor-mediated signal transduction.

Introduction

Stimulation of the T and B cell antigen receptors (TCR and BCR) initiates a cascade of signal transduction events involving the activation of two families of protein tyrosine kinases (PTKs), Src- and Syk/ZAP-70 (1). The Src family members initiate these events by phosphorylating the tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) following TCR/BCR stimulation (1). The Syk/ZAP-70 PTKs are subsequently recruited to the phosphorylated ITAMs, where they become phosphorylated and activated (1). Activation of these kinases further leads to tyrosine phosphorylation of numerous cellular proteins including Vav, phospholipase C γ isoforms, Shc, and SLP-76 (1, 2, 3, 4). Tyrosine phosphorylation and/or activation of these substrates ultimately results in downstream cytokine gene induction and other effector functions.

Vav is expressed exclusively in hematopoietic cells and contains an array of structural motifs, including a guanine nucleotide exchange (GEF) domain for the Rho/Rac/CDC42 family of small GTPases, a pleckstrin homology (PH) domain, and two Src homology 3 (SH3) domains that flank one src homology 2 (SH2) domain (5, 6). Vav plays a critical role in lymphocyte development and activation (9-12). Recent functional data *in vitro* and in fibroblasts suggests that Vav functions as a GEF for the Rho/Rac/CDC42 family of small GTPases (5-8). However, the exact molecular mechanism by which Vav functions in lymphocytes remains to be determined.

We have previously shown the Vav SH2 domain is required for its TCR/BCR-induced tyrosine phosphorylation (13). In addition, we and others have previously reported that tyrosine phosphorylated ZAP-70 can associate with the Vav SH2 domain following TCR stimulation (13-15). Interestingly, both

ZAP-70 (Y315) and Syk (Y348) contain a consensus Vav SH2 domain binding sequence, YESP (16). By using the chicken B cell DT-40 in transient transfection experiments, we show here that Y315 in ZAP-70 is critical for antigen receptor-mediated signaling. We find that mutation of Y315 in ZAP-70 prevents its interaction with the Vav SH2 domain. The point mutation in ZAP-70 also results in global defects in antigen receptor-mediated signaling events, as measured by the marked reduction in inducible tyrosine phosphorylation of ZAP-70, Vav, SLP-76 and Shc. These data strongly suggest that Y315 of ZAP-70 plays a critical role in regulating ZAP-70 function.

Results and Discussion:

Mutation of Y315 impairs ZAP-70 function. To examine functional requirements of Y315 in ZAP-70 in antigen receptor mediated signal transduction, we transfected the Syk-deficient DT-40 B cells with either wild-type or the mutated ZAP-70 [ZAP-70(Y315F)] along with a NFAT reporter construct (19). Consistent with the previous reports (19, 20), loss of *syk* in DT-40 resulted in a complete block in BCR-stimulated NFAT activation, a defect that could be rescued by expression of wild-type ZAP-70 (Fig. 1). In contrast, mutation of Y315 in ZAP-70 markedly impaired its ability to reconstitute BCR-induced NFAT activation (Fig. 1).

Y315 of ZAP-70 is required for interaction with the SH2 domain of Vav. To determining whether tyrosine 315 within the YESP motif of ZAP-70 functions as the Vav binding site, we transiently transfected Syk-deficient DT-40 cells with either wild-type ZAP-70 or ZAP-70(Y315F) and examined their abilities to interact with a GST fusion protein containing the Vav SH2 domain (GST-VavSH2). As shown in Fig. 2A, GST-VavSH2 fusion protein selectively bound to wild-type ZAP-70 following BCR stimulation or by treatment with the protein tyrosine phosphatase inhibitor pervanadate (Fig. 2A). In contrast, mutation of Y315 in ZAP-70 markedly impaired its ability to bind to the Vav SH2 domain.

Lck also associates with ZAP-70 via its SH2 domain following TCR stimulation (21, 22). Interestingly, both wild-type ZAP-70 and ZAP-70(Y315F) from either BCR-stimulated or pervanadate-treated lysates could bind efficiently to GST-LckSH2 domain (Fig. 2B), indicating that Y315 in ZAP-70 is specifically required for its interaction with the Vav SH2 domain but not Lck SH2 domain.

Although initial phospho-peptide mapping studies failed to identify Y315 as one of the major tyrosine phosphorylated residues in ZAP-70, it is important to

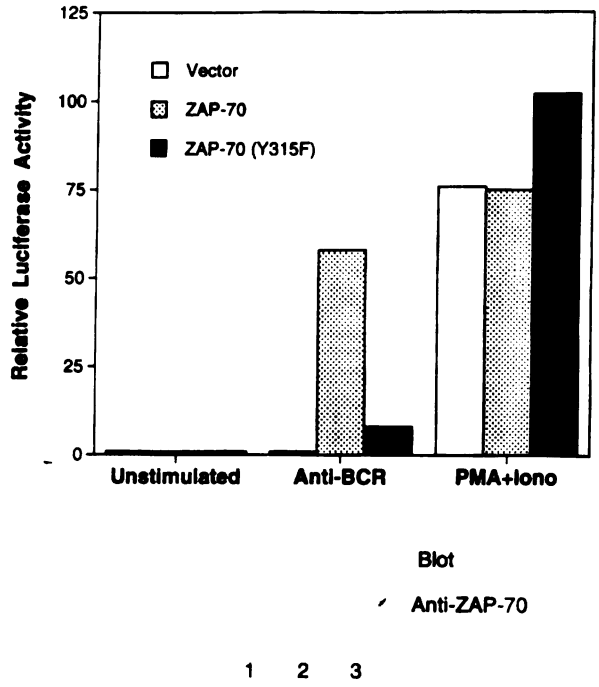


Fig. 1. Mutation of Y315 in ZAP-70 impairs its function in BCR-mediated signal transduction. Syk-deficient DT-40 cells were transiently cotransfected with 20 μg of NFAT-Luc along with 30 μg of either an empty vector, wild-type ZAP-70 or ZAP-70 (Y315F). After transfection (24-40 hr), cells were either left unstimulated or stimulated with either anti-BCR (M4, 2 μg/ml) or PMA (50 ng/ml) plus ionomycin (1 μM) for 6-8 hrs, and subsequently assayed for luciferase activity. The results are shown as the fold induction of luciferase activity as compared with the activity in unstimulated cells transfected with the empty vector, which is approximately 200 AU. Luciferase activity was determined in triplicate in each experimental condition. The data are representative of at least three independent experiments. The lower panel represents anti-ZAP-70 blot (MAb 2F3.2) of equivalent amount of lysates from different transfectants in the Luciferase assay described above.

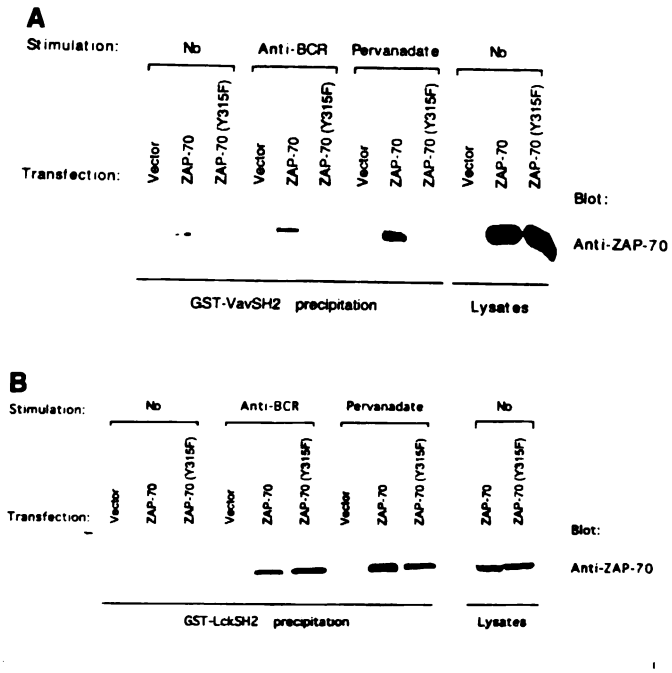


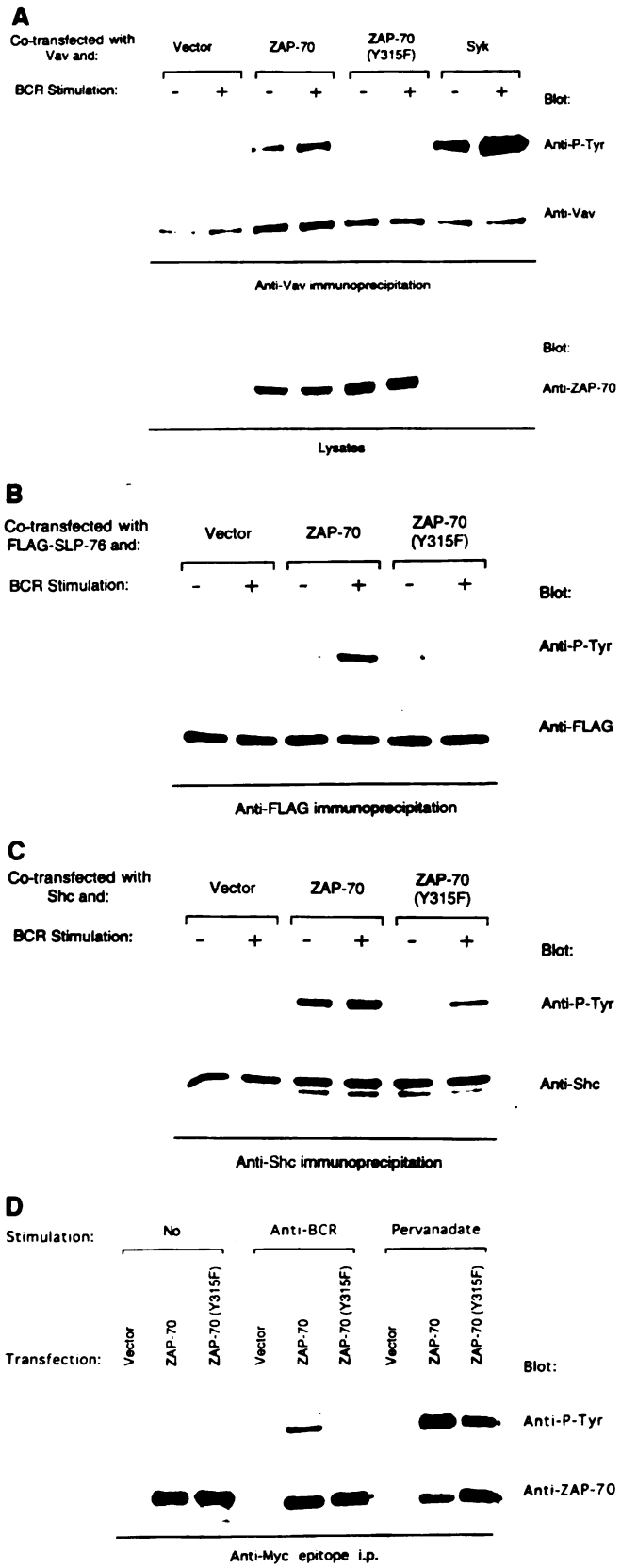
Fig. 2. Y315 in ZAP-70 is required for its binding to the Vav SH2 domain. **(A)** Syk-deficient DT-40 cells were transiently transfected with either an empty vector, wild-type ZAP-70 or ZAP-70 (Y315F). Cells were then either left unstimulated or stimulated with either anti-BCR or pervanadate and then lysed. The lysates were first pre-cleared with GST alone and then mixed with a GST fusion protein containing the Vav SH2 domain. The protein complexes were blotted with anti-ZAP-70 mAb (2F3.2). The lysate lanes represent approximately one tenth volume of the total cell lysates used for GST precipitations. **(B)** Mutation of Y315 in ZAP-70 did not affect its ability to interact with the Lck SH2 domain. Syk-deficient cells were transfected as described in (A) and were either left unstimulated or stimulated with anti-BCR or pervanadate. The lysates were first precleared with GST alone and then precipitated with GST fusion protein containing the Lck SH2 domain. The protein complexes were then blotted with anti-ZAP-70 mAb (2F3.2).

note that not all of the phosphorylation sites observed by two dimensional peptide mapping were identified (23, 24). In fact, the corresponding residue (Y348) in Syk has been shown to be a major *in vitro* autophosphorylation site and it serves as the binding site for the Vav SH2 domain (15, 25). Moreover, not only did mutation of Y315 in ZAP-70 abolish the ZAP-70-Vav interaction, this interaction could also be completely disrupted by the presence of a ZAP-70 peptide encompassing phosphorylated Y315 (14). These observations strongly argue that Y315 in ZAP-70 does represent an *in vivo* phosphorylation site following antigen receptor stimulation.

Mutation of Y315 in ZAP-70 markedly reduces tyrosine phosphorylation of Vav, SLP-76, Shc and ZAP-70 itself. To assess whether the Y315 mutation affects ZAP-70-mediated Vav tyrosine phosphorylation, we transiently coexpressed human Vav with empty vector, wild-type ZAP-70, ZAP-70(Y315F) or wild-type Syk into Lyn/Syk double-deficient DT-40 cells, in which BCR-induced Vav phosphorylation was completely absent (Fig. 3A, data not shown and reference 26). Coexpression of Vav with either wild-type ZAP-70 or Syk, but not ZAP-70(Y315F), led to Vav tyrosine phosphorylation, which was further induced by BCR stimulation (Fig. 3A).

To further examine the impact of Y315 mutation on ZAP-70-mediated tyrosine phosphorylation of other downstream substrates, we analyzed the tyrosine phosphorylation status of SLP-76 and Shc. Coexpression of wild-type ZAP-70 with either SLP-76 or Shc in Syk-deficient or Lyn/Syk double deficient DT-40 cells resulted in BCR-stimulated SLP-76 or Shc phosphorylation (Figure 3B, 3C and data not shown). Surprisingly, mutation of Y315 in ZAP-70 substantially impaired its ability to mediate phosphorylation of SLP-76 and Shc (Figure 3B and 3C and data not shown). In addition, mutation of Y315 also

Fig. 3


A
Co-transfected with Vav and:
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ZAP-70
ZAP-70 (Y315F)
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BCR Stimulation:
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Anti-Vav immunoprecipitation
Blot:
Anti-ZAP-70
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B
Co-transfected with FLAG-SLP-76 and:
Vector
ZAP-70
ZAP-70 (Y315F)
BCR Stimulation:
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Co-transfected with Shc and:
Vector
ZAP-70
ZAP-70 (Y315F)
BCR Stimulation:
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Anti-ZAP-70
Anti-Myc epitope i.p.

Fig. 3. (A) Vav is not phosphorylated in cells transfected with ZAP-70(Y315F) Lyn/Syk double-deficient DT-40 cells were transiently cotransfected with human Vav (pCI115) along with either an empty vector, wild-type ZAP-70, ZAP-70(Y315F) or Syk. After transfection (20-40 hrs), cells were either left unstimulated or stimulated with anti-BCR (M4, 2 μg/ml) for 2 min. and then lysed. The lysates were immunoprecipitated with anti-Vav polyclonal Ab and the immune complexes were blotted with anti-phosphotyrosine Ab (4G10) (upper panel). The blot was then stripped and reblotted with anti-Vav polyclonal Ab (middle panel). Equivalent amount of lysates were taken from each experimental condition and resolved on a SDS-PAGE and then blotted with anti-ZAP-70 mAb (2F3.2) (lower panel). **(B)** Mutation of Y315 in ZAP-70 reduces ZAP-70-mediated SLP-76 tyrosine phosphorylation. Syk-deficient DT-40 cells were transiently transfected with FLAG-epitope tagged human SLP-76 (FLAG-SLP-76) along with either an empty vector, wildtype ZAP-70 or ZAP-70(Y315F). Cells were stimulated and lysed as described in (A). The lysates were immunoprecipitated with anti-FLAG epitope antibody (M2) and the immune complexes were blotted with 4G10 (upper panel). The blot was then stripped and reblotted with anti-FLAG antibody (lower panel). Anti-ZAP-70 Western blot revealed equivalent expression between wildtype ZAP-70 and ZAP-70(Y315F) (data not shown). **(C)** Mutation of Y315 in ZAP-70 also reduces ZAP-70-mediated Shc tyrosine phosphorylation. Lyn/Syk double-deficient DT-40 cells were transiently transfected with human Shc cDNA along with either an empty vector, wildtype ZAP-70 or ZAP-70(Y315F). Cells were stimulated and lysed as described in (A). The lysates were immunoprecipitated with anti-Shc monoclonal antibody and the immune complexes were blotted with 4G10 (upper panel). The blot was then stripped and reblotted with polyclonal anti-Shc

antibody (lower panel). Anti-ZAP-70 Western blot showed equivalent expression between wildtype ZAP-70 and ZAP-70(Y315F) (data not shown). **(D)** Mutation of Y315 reduces BCR-mediated ZAP-70 tyrosine phosphorylation. Syk-deficient DT-40 cells were transiently transfected with either a vector, a myc-epitope tagged wildtype or ZAP-70(Y315F). Cells were either left unstimulated or stimulated with anti-BCR or pervanadate for 2 min and then lysed. The lysates were then immunoprecipitated with anti-myc antibody (9E10) and the immune complexes were blotted with 4G10 (upper panel). The blot was then stripped and reblotted with anti-ZAP-70 mAb (2F3.2) (lower panel).

markedly reduced ZAP-70 tyrosine phosphorylation following antigen receptor stimulation in Syk-deficient cells and in Jurkat T cells (Fig. 3D and data not shown). Taken together, Y315 of ZAP-70 is not only required for Vav tyrosine phosphorylation, but also for tyrosine phosphorylation of other downstream substrates such as SLP-76, Shc, and even for ZAP-70 itself.

Mutation of Y315 in ZAP-70 does not affect ZAP-70 kinase activity or binding of ZAP-70 to receptor ITAMs. One explanation for the global defects of ZAP-70(Y315F) could be that the Y315F mutation reduced ZAP-70 kinase activity. Myc epitope-tagged ZAP-70 or ZAP-70(Y315F) was expressed in Lyn/Syk double-deficient cells and the kinase activity of anti-myc epitope tag immunoprecipitates was measured as both autophosphorylation and phosphorylation of an exogenous substrate, band III. The *in vitro* kinase assay failed to reveal a substantial difference between wild-type ZAP-70 and ZAP-70(Y315F) in their abilities to phosphorylate Band III, although there may be a modest reduction in autophosphorylation of ZAP-70(Y315F) (Fig. 4A).

Another critical step for ZAP-70 phosphorylation and activation is binding of ZAP-70 to the ITAMs following receptor stimulation. We used a biotinylated doubly phosphorylated ITAM peptide to precipitate ZAP-70 from lysates of Syk-deficient DT-40 cells transfected with either wild-type ZAP-70 or ZAP-70(Y315F). Similar amounts of wild-type ZAP-70 and ZAP-70(Y315F) bound to the phosphorylated peptide (Fig. 4B). In addition, similar amounts of tyrosine phosphorylated TCR ζ chain were found to coimmunoprecipitate with either form of ZAP-70 when analyzed in Jurkat T cells (data not shown). Taken together, these data demonstrate that mutation of Y315 of ZAP-70 did not dramatically affect its kinase activity or its binding to receptor ITAM.

Fig. 4.

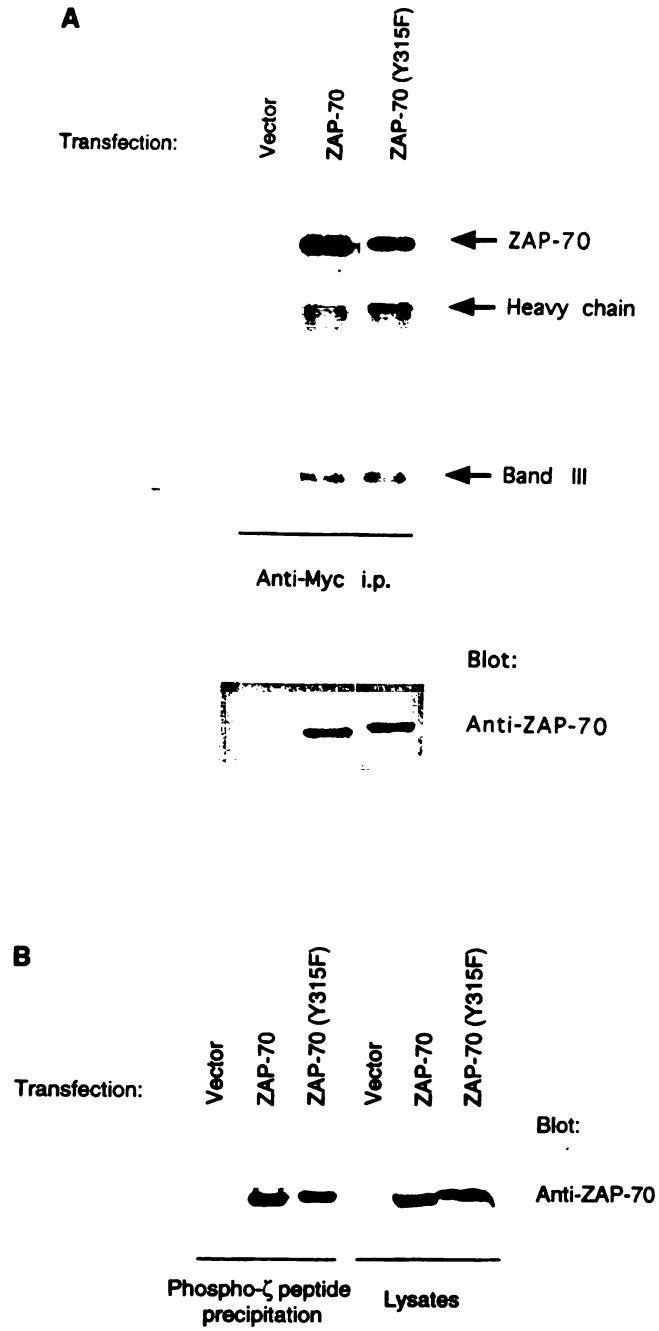
A
Transfection:
Vector
ZAP-70
ZAP-70 (Y315F)
ZAP-70
Heavy chain
Band III
Anti-Myc i.p.
Blot:
Anti-ZAP-70
B
Transfection:
Vector
ZAP-70
ZAP-70 (Y315F)
Vector
ZAP-70
ZAP-70 (Y315F)
Blot:
Anti-ZAP-70
Phospho-ζ peptide precipitation
Lysates

Fig. 4. (A) Mutation of Y315 in ZAP-70 does not affect its intrinsic tyrosine kinase activity. Lyn/Syk double deficient DT-40 cells were transient transfected with either an empty vector, a myc epitope tagged wild-type ZAP-70 or ZAP-70(Y315F). After transfection (24-40hrs), the lysates were immunoprecipitated with anti-myc epitope (9E10) and then subjected to an *in vitro* kinase assay. The products were separated on a SDS-PAGE gel and transferred to PVDF membrane. The membrane was subjected to KOH treatment and then *in vitro* phosphorylated proteins were detected by autoradiography (upper panel). Expression of ZAP-70 was detected by immunoblotting the same membrane with anti-ZAP-70 mAb (2F3.2) (lower panel). **(B)** Mutation of Y315 in ZAP-70 does not affect its binding to receptor ITAMs. Syk-deficient cells were transfected with either an empty vector, ZAP-70 or ZAP-70(Y315F). After transfection (24-40 hrs), the lysates were mixed with 1 μg of doubly phosphorylated peptide encompassing the second ITAM of TCR ζ chain, followed by the addition of avidin beads to collect complexes. The complexes were then blotted with anti-ZAP-70 mAb (2F3.2).

In summary, we demonstrate here that Y315 in ZAP-70 is required to interact with the Vav SH2 domain, and is critical for ZAP-70-mediated gene activation. Notably, the Y315-homologous residue in Syk is also required for its interaction with the Vav SH2 domain and for Vav phosphorylation(15). We provide evidence here that the Y315 mutation results in a global defect in ZAP-70-mediated signaling pathways, suggesting an important role of Y315 in regulating ZAP-70 function.

Our results also underscore some differences between TCR- and BCR-mediated signaling pathways. A mutant Jurkat T cell line lacking the Src family member Lck failed to exhibit TCR-induced tyrosine phosphorylation, calcium mobilization and downstream gene activation (27). In addition, no ZAP-70 activation and Vav tyrosine phosphorylation could be observed in this mutant cell line (28, 29), suggesting that ZAP-70 activity depends upon functional Lck in T cells. In contrast, Lyn-deficient B cells showed similar BCR-induced Vav tyrosine phosphorylation, IP_3 generation and NFAT induction when compared to wild-type cells (data not shown, and reference 19). Moreover, expression of ZAP-70 alone in Lyn/Syk double-negative DT-40 cells was capable of inducing Vav tyrosine phosphorylation, although to levels less than that by expression of Syk (Fig. 3A and data not shown). These results suggest that the function of Syk/ZAP-70 PTKs in B cells may be less dependent upon Src family kinases.

Antigen receptor stimulation results in the assembly of multiprotein complexes, a process likely to facilitate efficient tyrosine phosphorylation and/or activation of appropriate signaling molecules (2). The Vav-ZAP-70 binding via Y315 may be important in initiating the proper formation of such signaling complexes, as both proteins are able to interact with many other signaling molecules (2). Since Vav possesses an GEF domain for Rho/Rac/CDC42 (7, 8),

the interaction between Vav and ZAP-70 may provide a mechanism by which ZAP-70 activates downstream Rho/Rac/CDC42-mediated signaling events such as cytoskeletal rearrangement. Mutation of Y315 in ZAP-70 may result in an impaired recruitment, phosphorylation and/or activation of many proteins including ZAP-70, Vav, SLP-76 and Shc.

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Materials and Methods:

DNA constructs and fusion proteins. The NFAT luciferase reporter construct was a gift from Dr. G. Crabtree. The Vav plasmid (pCI115) was constructed by subcloning human vav into pCIneo (InVitrogen). The parental plasmid for the ZAP-70 mutant was pCDNA3-ZAP-70. The Y315F mutant of ZAP-70 [ZAP-70(Y315F)] was created by M13-based, oligonucleotide-directed, site-specific mutagenesis procedures [(17)]. The myc epitope-tagged wild-type ZAP-70 (pSXSRa-ZAP-myc) was provided by Dr. L. Samelson. DNA encoding wild-type rat Syk was subcloned into the mammalian expression vector pEFBOS. GSTVavSH2 was provided by Dr. S. Katzav. The human Shc plasmid and the FLAG epitope tagged human SLP-76 cDNA were provided by Dr. M. Gishizky and Dr. G. Koretzky, respectively.

Antibodies and peptide. The mAb used for the stimulation of the BCR was M4 (provided by M Cooper and CL Chen). Anti-Vav polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine mAb, 4G10, was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Anti-ZAP-70 mAb (2F3.2) was described previously (18). The anti-myc epitope mAb (9E10) was provided by Dr. J. M. Bishop. The peptide used in this paper represents a biotinylated doubly phosphorylated version of the second ITAM of the TCR ζ chain (18).

Cell lines, transfections and luciferase assays. Wild-type and various mutants of DT-40 cells were maintained and transfected transiently as previously described (17, 19). Briefly, 30 μg of either an empty vector, wild-type ZAP-70 or ZAP-70(Y315F) and 20 μg of NFAT-Luc construct was used. Cells were then electroporated, processed, and assayed as described (17).

Immunoprecipitations, protein precipitations, peptide binding, and immunoblotting. Cells were harvested, washed, and were left either unstimulated or stimulated with M4 (2 μg/ml) and then lysed as previously described (13). Lysates were then immunoprecipitated with the indicated antibodies. When precipitated with GST fusion proteins, lysates were first pre-cleared with GST alone (10 μg) before they were precipitated with the indicated GST fusion proteins (2-5 μg). Resulting immunoprecipitates or protein complexes were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Peptide binding and immunoblotting were carried out as previously described (18).

In vitro kinase assay. After transient transfection, wild-type and mutant ZAP-70 were immunoprecipitated and *in vitro* kinase assays were performed as previously described (17). Samples were then analyzed by SDS-PAGE, transferred to PVDF membrane, treated with 1 M KOH for 1 hour, and then subjected to autoradiography and immunoblotting.

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Chapter VI

Concluding remarks

The antigen receptors in T and B cells recognize pathogens and foreign antigens and initiate a series of signal transduction events resulting in morphologic change, proliferation, and the acquisition of immunologic effector functions. Since these events are crucial for lymphocyte development and activation, understanding the mechanisms by which signaling proteins participate in antigen receptor-mediated pathway can provide insights into the regulation of the immune response. The structure and expression pattern of the proto-oncogene Vav, as well as its tyrosine phosphorylation and homology to other signaling molecules prompted me to explore its role in antigen receptor-mediated signal transduction. In this dissertation research, I developed a functional assay in Jurkat T cells to show that Vav plays an important role in TCR-mediated gene activation. The assay further represents a useful tool to perform structural and functional analysis of Vav. In addition, both biochemical and genetic approaches were taken to demonstrate the importance of the interactions between Vav and other signaling molecules including SLP-76 and ZAP-70. The present research suggests that Vav may be an critical regulator in a novel antigen receptor-mediated signaling pathway leading to events such as cytoskeletal rearrangement and morphological changes. While considerable progress has been made by us and others in analyzing the function of Vav in T and B cells, many questions still remain unanswered. The following discussion will address some of these major unsolved issues and discuss future experimental directions to define the molecular mechanisms by which Vav and other Vav-related proteins function.

Overexpression of Vav in Jurkat T cells.

The finding that overexpression of Vav alone in Jurkat T cells enhances both basal and TCR-stimulated NFAT activation suggests an important role for Vav in TCR signaling pathways, it also allows us to start dissecting the function of Vav in a T cell line. However, one concern regarding such an overexpression approach is whether it reflects the physiological function of Vav in T cells. In particular, since Vav shares a number of structural motifs found in other signaling molecules, it is possible to generate an artifactual effect when Vav is overexpressed at a five to ten-fold level over the endogenous protein. However, the following observations provide strong support for the validity of the overexpression approach.

First, overexpression of either truncated forms of Vav, one lacking the first 67 amino acid or another containing only the first 198 amino acid in the N-terminus, did not enhance either basal or TCR-stimulated NFAT activation (1). Moreover, overexpression of the GEF or PH domain deletion mutant of Vav also failed to induce basal NFAT activation (Fig. 1, addendum for chapter II). These data argue that the effect observed with full-length Vav does not simply reflect the overexpression of domains (such as SH2 and SH3 domains) shared by other signaling molecules. Secondly, Vav-induced NFAT activation was specific to TCR-mediated signaling pathway and depended upon an intact TCR signaling complex (1). These data demonstrate the specificity of the Vav-mediated effect in T cells and further suggest its role in TCR-mediated signal transduction. The most compelling evidence which supports the physiological relevance of the Vav-induced IL-2 gene activation came from the analysis of the Vav-deficient T cells. Chimeric mice derived from blastocysts lacking the recombinase-activating gene and embryonic stem cells deficient in Vav expression displayed a dramatic

reduction in thymocyte number as well as peripheral T and B cells (2-4). Consistent with our findings that overexpression of Vav in Jurkat T cells led to enhancement of IL-2 gene activation, the Vav-deficient mature T cells from these mice showed a profound defect in IL-2 production and proliferation in response to TCR stimulation (2-4). Moreover, similar to the effect induced by Vav overexpression, the defect in the Vav-deficient T cells also appeared to be specific to TCR-mediated signaling, since they could proliferate normally when exogenous IL-2 was added. Finally, the defect in the Vav-deficient T cells also appears to be in a proximal event of the TCR signaling pathway, since treatment of these cells with PMA and ionomycin could induce IL-2 production and proliferation. Taken together, these results strongly argue that Vav-induced NFAT activation in Jurkat cells does reflect, at least in part, the physiological role of Vav in T cells.

Nonetheless, it is important to note that an outcome induced by overexpression of Vav may be a sum of multiple effects. For instance, in contrast to the GEF, PH or SH2 domain, the C-terminal SH3 domain appears to play an inhibitory role in TCR-mediated NFAT activation, since a C-terminal SH3 domain point mutant of Vav showed higher activity in inducing basal and TCR-stimulated NFAT activation when compared with wildtype Vav (Fig. 1, addendum for chapter II). Furthermore, the presence of endogenous Vav may also complicate the interpretation when overexpressing a mutant form of Vav in Jurkat cells. Therefore, generation of a Vav-deficient cell line may be an ideal and economical approach to define the functional domains within Vav. The chicken DT-40 cells may be a suitable candidate, since they show an unusually high level of homologous recombination (5), and many mutants of this cell line have been successfully generated using gene targeting (6-8).

The N-terminal domain of Vav and oncogenesis.

Vav was originally identified as an oncogene as a result of the removal of its first 67 amino acids (9). Despite its potent transforming activity in fibroblasts, overexpression of the N-terminal truncated Vav in Jurkat T cells not only failed to induce any basal NFAT activity, but also inhibited TCR-stimulated NFAT activation (1). The N-terminal region of Vav alone is not sufficient nor does it function *in trans* with the rest of the molecule to activate NFAT, since overexpression of a truncated form of Vav containing the first 198 amino acids alone or together with the oncogenic Vav failed to induce any NFAT activation (unpublished results). These results imply that the N-terminal region plays an important role in regulating Vav function in T cells. Furthermore, they raise questions regarding whether the oncogenic phenotype of the N-terminal truncated Vav in fibroblasts reflects the true function of wildtype Vav in lymphocytes.

It has been shown that oncogenic Vav can activate Rac or CDC42 small GTPases when transfected into fibroblasts (10, 11), a likely mechanism to cause cell transformation. In fact, transformation induced by oncogenic Vav can be blocked by cotransfection of a dominant negative form of Rac-1 (12). Interestingly, similar to oncogenic Vav, expression of an activated form of CDC42 in Jurkat T cells did not enhance basal NFAT activity, and instead, it inhibited TCR-mediated gene activation (unpublished results). Therefore, it is possible that oncogenic Vav does activate Rho/Rac/CDC42-mediated pathways in both fibroblasts or T cells, but the N-terminal region may allow wildtype Vav to reside in an appropriate cellular compartment to interact with appropriate signaling partners and to exert its physiological function. In support of this hypothesis, recent studies have revealed different subcellular localization patterns between

wildtype Dbl and an oncogenic form of Dbl (Cerione, R.A., personal comm.). Thus, it is important to determine the subcellular localization of both wildtype and oncogenic Vav in T cells, and in particular, whether antigen receptor stimulation alters their cellular localization.

The N-terminal region of Vav is perhaps the most mysterious and exciting part of Vav. This leucine-rich region was initially reported to contain a helix-loop-helix domain followed by a leucine zipper (13). Although none of these similarities have later been shown to be significant (14, 15), the N-terminal region does share homology with calponin, a muscle structural protein which interacts with actin and other cytoskeletal components (14). Similar calponin homology (CH) domain have been identified in two recently-cloned putative GTPase-activating proteins (IQGAPs) for Rac1 and CDC42 (Fig 1) (16). Therefore, it is likely that the CH domain of Vav may bind to some cytoskeletal components, allowing it to interact with appropriate signaling molecules, whereas deletion of this region may release Vav to interact with other signaling processes and cause transformation. Identification of molecules that interact with this region will not only be informative to understand Vav function in lymphocytes, but also may shed lights on the mechanisms of oncogenesis.

Vav: from lymphocytes to *C. elegans*.

When Vav was ectopically expressed in fibroblasts, it became tyrosine phosphorylated following PDGF or EGF stimulation (17, 18). Moreover, activation of the PDGF and EGF receptors resulted in their association with Vav via the Vav SH2 domain, leading to subsequent Vav tyrosine phosphorylation. A recent degenerate phosphopeptide library approach has predicted a potential Vav SH2 domain binding sequence within the cytoplasmic tail of PDGF or EGF

receptor (19). So why do activated PDGF or EGF receptors interact specifically with and phosphorylate a heterologous protein like Vav? Furthermore, since tyrosine phosphorylated Vav has been shown to activate Rho/Rac/CDC42 family of GTPases (10, 11), what is the functional significance of Vav tyrosine phosphorylation by these receptors?

Recent isolation of a ubiquitous Vav homologue, Vav2, may provide partial answer for these questions (20, 21). Vav2 shares 63% and 55% identity at the nucleic acid and amino acid levels, respectively, and contains all the structural motifs found in Vav. Therefore, the inducible association between the growth factor receptors and Vav and subsequent Vav tyrosine phosphorylation suggests that Vav2 may function downstream of PDGF or EGF receptors, serving as a link between these receptors and Rho/Rac/CDC42-mediated signaling events. In addition, since TCR stimulation in the Vav-deficient T cells is still capable of inducing certain downstream signaling events such as tyrosine phosphorylation of cellular proteins and IL-2 receptor expression (2, 3), it is important to determine whether Vav2 can compensate the defects seen in the Vav deficiency.

More interestingly, a Vav homologue also appears to be present in *C. elegans* (VavC) (Genbank accession number U39470 and Figure. 2). Although preliminary database search failed to identify any known genetic defect to be associated with the VavC locus (unpublished results), inactivation experiments such as antisense approach may help to elucidate the function of VavC in *C. elegans*. Since both the EGF receptor-homologue let-23 have been shown to be essential for normal development of *C. elegans* (22), VavC may be involved in the signaling events initiated by let-23 to activate downstream Rho/Rac/CDC42 family of GTPases.

Sequence alignment reveals some interesting features among these related molecules (Figure 2). All three members share homology in the N-terminus, GEF, PH, SH2 domains, however, the *C. elegans* Vav does not appear to contain any SH3 domain found in Vav and Vav2. It has been proposed in the addendum of chapter II that the Vav SH3 domain may interact with a proline-rich region (a.a. 607-610) between the cysteine-rich and the N-terminal SH3 domains (Figure 2), an interaction which may result in negative regulation of the molecule. Interestingly, in contrast to Vav and Vav2, VavC does not contain the proline rich sequence, suggesting that VavC may be regulated differently. Note that all three members contain CH domains in the N-terminus, again suggesting that this region may interact with some cytoskeletal components and such interaction may be important for these family members to function properly. It is also striking that a series of potential tyrosine phosphorylation sites are conserved among the N-termini of these family members (Fig. 2). In fact, Y174 in Vav has been suggested to be phosphorylated by Syk family kinases (23). Since these potential phosphorylation sites are adjacent to the GEF domain, it is possible that tyrosine phosphorylation may lead to a conformational change within the region, allowing the GEF domain to be functional. It is also noteworthy that these conserved tyrosine residues and their surrounding sequences resemble the immunoreceptor tyrosine based inhibitory motif (ITIM) (24). The ITIM motifs are found in many inhibitory molecules and have been shown to dampen growth factor- or antigen receptor-mediated signaling by recruiting the protein tyrosine phosphatases SHP-1 and SHP-2 (25, 26). Does Vav or its related molecule recruit these phosphatases via this motif? If so, does this interaction result in down regulation of Vav or its partner? Future mutagenesis studies may provide clues for the mechanisms by which Vav and its related molecules are regulated in

different cell types or organisms.

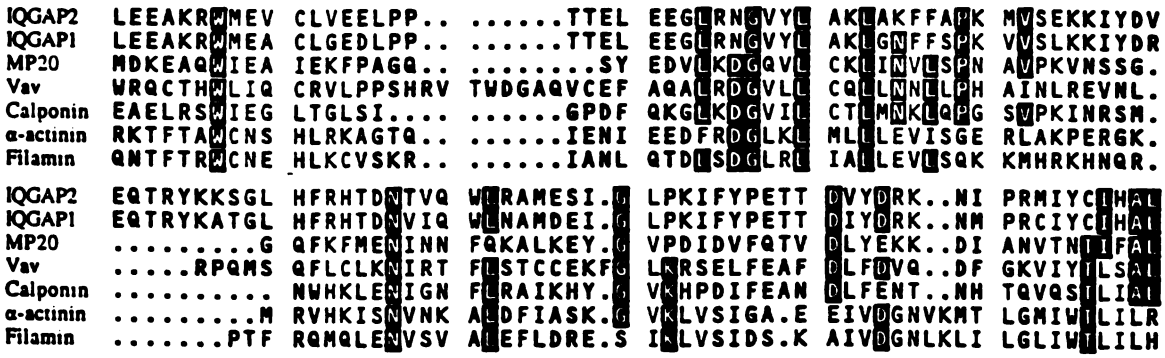
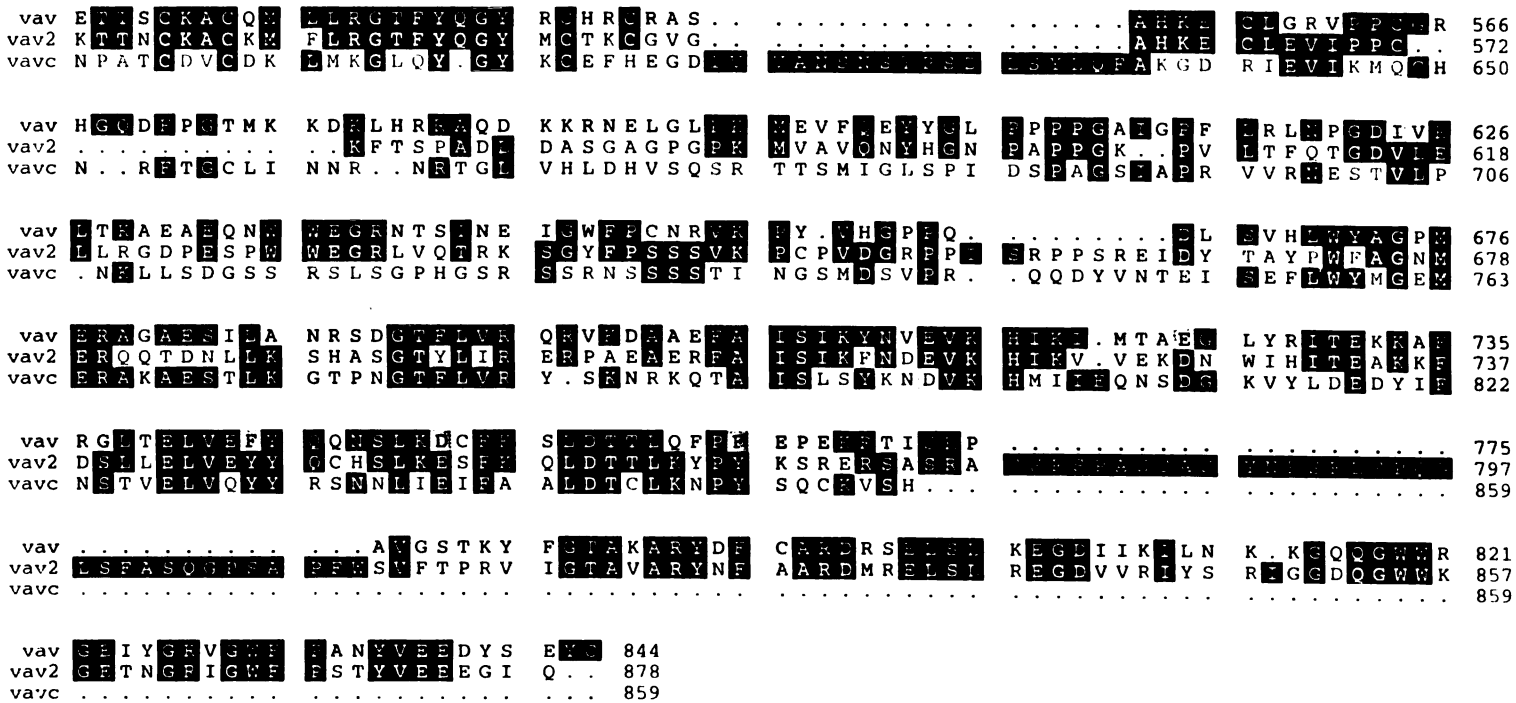


Figure 1. Alignment of calponin homology (CH) domains in IQGAP2, IQGAP1 , Drosophila asynchronous muscle protein MP20, human proto-oncogene Vav, human calponin, human a-actin, and human filamin.

Figure 2. Alignment of Vav, Vav2 and C. elegans Vav (VavC)


vav
vav2
vavc

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