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Functional and Physical Interactions of Syk Family Kinases with the Vav Proto-Oncogene Product

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Summary

Syk family kinases are essential for lymphocyte development and activation. Therefore the identification of their direct effectors is of critical importance. Here, we report that Syk interacts in the yeast two-hybrid system with Vav, a proto-oncogene product exclusively expressed in hematopoietic cells. This interaction was direct, required the catalytic activity of Syk, the SH2 domain of Vav, and tyrosine residues in the linker domain of Syk. Vav also associated with Syk and Zap in antigen receptor–stimulated B or T cells, respectively. Functionally, Vav was phosphorylated by Syk family kinases both in vivo and in vitro. Furthermore, Syk and Vav cooperated to activate NF-AT synergistically. These results indicate that the interaction between Syk family kinases and Vav plays an important role in coupling immune recognition receptors to signaling pathways involved in lymphokine production.

Introduction

Clonotypic T and B cell antigen receptors (TCR and BCR, respectively) and receptors for the Fc fragment of immunoglobulins are associated noncovalently with conserved transmembrane proteins that transduce activation signals. The cytoplasmic tails of these signaling subunits contain at least one immunoreceptor tyrosine-based activation motif (ITAM) critical for signal transduction (Cambier, 1995; Reth, 1989). Based on current genetic and biochemical studies, it is believed that immunoreceptor-initiated hematopoietic cell activation results from the sequential activation of protein tyrosine kinases (PTKs) of the Src and Syk families and their interaction with phosphorylated ITAMs. Src family kinases (Lck, Fyn, or both in T cells) are thought to induce the early tyrosine phosphorylation of the ITAMs, which leads to the recruitment of Syk family PTKs via their tandem Src homology 2 (SH2) domains. This process induces the activation of Syk family PTKs and leads to increased tyrosine phosphorylation and activation of a number of cellular proteins and downstream effectors (DeFranco, 1995; Mustelin, 1994; Weiss and Littman, 1994).

The central role of Syk family PTKs in both developmental and activation events in hematopoietic cells was recently demonstrated by genetic studies (Arpaia et al., 1994; Chan et al., 1994; Cheng et al., 1995; Elder et al., 1994; Negishi et al., 1995; Turner et al., 1995). The $p72^{syk}$ (Syk) PTK is widely expressed in hematopoietic cells and was found to associate either constitutively or inducibly with the BCR, TCR, and Fc receptor. Syk-deficient mice exhibit a block in B cell development, most likely due to a defect in signaling through the pre-BCR complex (Cheng et al., 1995; Turner et al., 1995). A related PTK, $p70^{zap}$ (Zap) is expressed in T and natural killer cells, and its absence in humans causes 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 (Arpaia et al., 1994).

Antigen receptor triggering leads to activation and tyrosine phosphorylation of Zap and Syk, leading to additional SH2-mediated interactions with Src family PTKs (Couture et al., 1994; Thome et al., 1995), and cotransfection experiments have demonstrated a functional cooperation between these two families of PTKs. However, Syk, but not Zap, was shown to be active in the absence of Src family kinases in COS cells, human cytotoxic T lymphocytes, and mast cells (Couture et al., 1994; Kolanus et al., 1993; Rivera and Brugge, 1995). This suggests that Syk can directly associate with or phosphorylate (or both) critical effector proteins. Presently, however, little is known about the physiological substrates of Syk and Zap. Syk family PTKs are required for the activation of phospholipase C-γ (PLC-γ), and the SH2 domain of PLC-γ can interact with phosphotyrosyl (PTyr) residues located in the linker domain of Syk, between the tandem SH2 and catalytic domains (Law et al., 1996; Sillman and Monroe, 1995). Tyrosine-containing synthetic peptides based on the sequence of two hematopoietic-specific signaling proteins, $p95^{vav}$ (Vav) and HS1, were recently shown to serve as good substrates for purified Syk in vitro (Brunati et al., 1995).

Vav contains several functional domains, including a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, a cysteine-rich domain, one SH2 domain, and two SH3 domains. Triggering of structurally diverse hematopoietic receptors, including antigen receptors, Fc, cytokine, and PTK receptors, induces rapid phosphorylation of Vav on tyrosine (reviewed by Bonnefoy-Bérard et al., 1996). The SH2 domain of Vav was found to associate with ligand-activated epidermal growth factor receptors in Vav-transfected fibroblasts (Bustelo et al., 1992; Margolis et al., 1992), with Zap in TCR-activated Jurkat cells (Katzav et al., 1994), and with the recently cloned (Jackman et al., 1995) protein SLP-76 (Motto et al., 1996; Wu et al., 1996). Although the exact function of Vav in hematopoietic cells is unclear, it is likely to play an important role in immune cell signaling pathways. Thus, $vav^{-/-}$ mice display a severe reduction in the number

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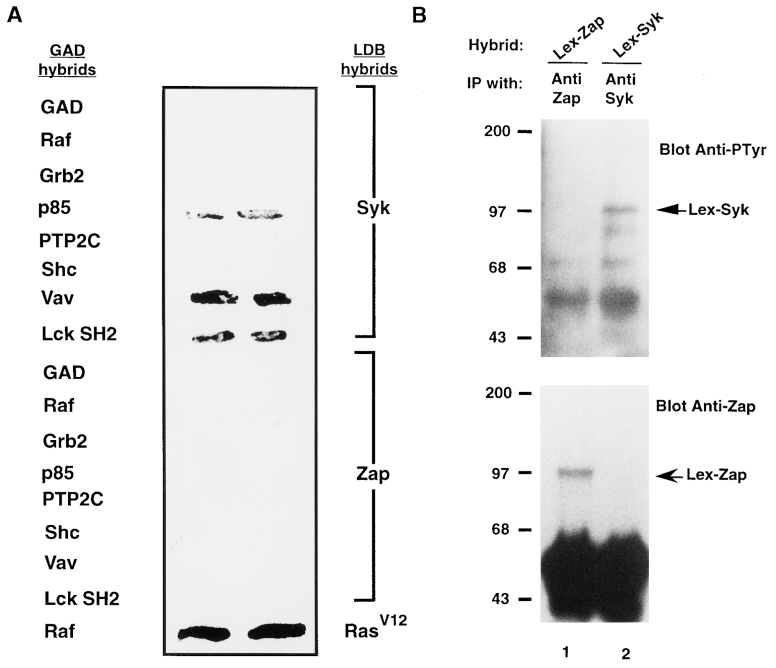

Figure 1. Interaction between Syk or Zap and Various Signaling Proteins in Yeast

(A) Yeast (L40) was cotransformed with the plasmids encoding LexA–Syk or LexA–Zap in combination with plasmids encoding the indicated GAD hybrids. After isolation on selection medium, transformants were assayed for β-galactosidase activity using a filter assay as described in Experimental Procedures. As a negative control, the Syk and Zap hybrids were coexpressed with GAD alone. The interaction between GAD–Raf and LexA–Ras^{V12} is also shown as a positive control (Votjek et al., 1993). A positive interaction of Syk with p85, Vav, and Lck SH2 is shown. Similar results were obtained by analyzing yeast growth on selective plates lacking histidine.

(B) LexA–Zap and LexA–Syk expression and phosphorylation in yeast. We lysed 2×10^8 yeast expressing LexA–Zap or LexA–Syk hybrids. The lysates were immunoprecipitated using anti-Zap or anti-Syk antibodies and analyzed by anti-PTyr (top) or anti-Zap (bottom) immunoblotting.

and antigen receptor–mediated activation of both mature T and B cells (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). In addition, Vav overexpression in T cells enhances activation of transcriptional elements in the interleukin-2 (IL-2) promoter (Holsinger et al., 1995; Wu et al., 1995), and Vav cooperates with SLP-76 to induce IL-2 gene activation (Motto et al., 1996; Wu et al., 1996).

In this study, we addressed the potential interaction of Syk and Zap with downstream effectors involved in immune cell activation using the yeast two-hybrid system and biochemical analysis of T and B cells. We found the following: first, Syk interacts with Vav in the yeast two-hybrid system; second, the interaction between Syk and Vav is direct, depends on the catalytic activity of Syk, and involves the SH2 domain of Vav and tyrosine residue(s) located in a region of Syk between its C-terminal SH2 (SH2[C]) and kinase domains; third, Vav binds Syk and Zap in BCR- or TCR-stimulated B and T cells, respectively, demonstrating that this interaction also occurs under physiological conditions; and, fourth, Vav is phosphorylated by Syk and Zap in vivo and in vitro, and coexpression of Vav and Syk results in increased activation of NF-AT in T cells. These results identify Vav as a direct physical and functional target for Syk family kinases, suggesting that this association is critical for both BCR- and TCR-mediated immune cell activation.

Results

Syk Binds Vav, Lck, and p85 in Yeast

We have examined the interaction of the two PTKs, Zap and Syk, with potential downstream effectors using the yeast two-hybrid system originally described by Fields and Song (1989). One partner was a hybrid between the LexA DNA-binding domain (LDB) and the full-length sequence of Zap or Syk (LexA–Zap or LexA–Syk, respectively). The second partner was a Gal4 activation domain (GAD) fusion with the full-length sequences of Raf (GAD–Raf; Votjek et al., 1993), Grb2, the regulatory subunit (p85) of phosphatidylinositol 3-kinase, Shc, Vav, the tandem SH2 domains of PTP2C (Tartare-Deckert et al., 1995, 1996), or the SH2 domain of Lck. The yeast strain L40 was cotransformed with combinations of these hybrids, and their interaction was monitored by the expression of two reporter genes, *HIS3* and *LacZ*, which contain upstream LexA-binding sites (Votjek et al., 1993). The LexA–Syk and LexA–Zap hybrids were incapable by themselves, or in combination with an unrelated GAD fusion protein (i.e., GAD–lamin), of activating the expression of the two reporter genes (data not shown). Cotransformation of LexA–Zap with any of the GAD hybrids failed to induce β-galactosidase expression (Figure 1A), even though the LexA–Zap fusion protein was readily expressed in yeast (Figure 1B, bottom). By contrast, the coexpression of LexA–Syk with GAD–p85, GAD–Vav, or GAD–Lck SH2 induced β-galactosidase expression (Figure 1A) and enabled yeast growth in the absence of histidine (data not shown). The discrepancy between Syk and Zap correlated with the ability of the two kinases to become autophosphorylated. Thus, Syk, but not Zap, was phosphorylated on tyrosine (Figure 1B, top). We conclude that Syk interacts in yeast with Vav, p85, and Lck.

Characterization of the Interaction between Syk and Vav

To study the Syk–Vav interaction in more detail, we first determined which domain(s) of Vav interacts with Syk. Four different GAD–Vav deletion constructs were generated (Figure 2A): Vav 386–846 contains the PH domain, the two SH3 and the SH2 domain of Vav; Vav 610–846

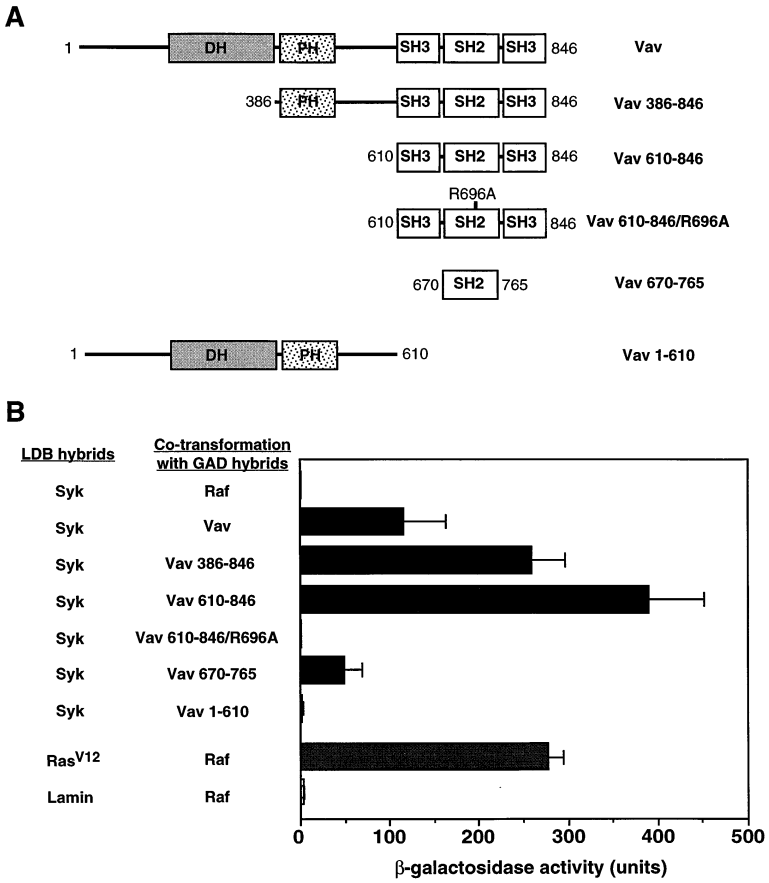

Figure 2. Mapping of the Vav Domains Mediating the Interaction with Syk

(A) Schematic representation of the human full-length Vav protein and truncated Vav constructs used in this study. DH, Dbl homology domain; PH, Pleckstrin homology domain.

(B) Yeast (L40) was cotransformed with LexA–Syk plus the indicated GAD–Vav constructs. Coexpression of GAD–Raf with LexA–Ras^{V12} or LexA–lamin B are shown as positive and negative controls, respectively. β-Galactosidase activity in cell lysates was measured by a liquid assay using the substrate o-nitrophenyl-β-D-galactopyranoside and was calculated as described by Miller (1972). Results are expressed as the mean (± SD) of three independent transformants and are representative of three similar experiments. Similar results were obtained by analyzing growth on selective plates lacking histidine.

consists of the two SH3 and the SH2 domain of Vav; Vav 670–765 corresponds to the SH2 domain of Vav; and Vav 1–610 comprises the N-terminal region, DH, PH, and cysteine-rich domains of Vav. The interaction of these hybrids with LexA–Syk was analyzed by a quantitative β-galactosidase assay. The results (Figure 2B) indicate that, by comparison with full-length Vav, deletion of the N-terminal plus DH domains (Vav 386–846) and, to a larger extent, additional deletion of the PH and cysteine-rich domains of Vav (Vav 610–846) increased the interaction with Syk. Thus, these N-terminal domains of Vav are not required for the interaction with Syk and, moreover, appear to contain sequences that interfere with this interaction. Conversely, a Vav construct containing residues 1–610 but lacking the tandem SH3-SH2-SH3 domains of Vav failed to interact with Syk, indicating that one or more of these C-terminal domains is involved in the interaction. A mutation in a conserved arginine residue within the SH2 domain of Vav (R696L), which is known to inactivate this domain (Katzav, 1993), abolished the interaction of Vav 610–846 with Syk; conversely, GAD–Vav 670–765, which contains only the SH2 domain of Vav, still interacted with LexA–Syk, albeit to a lower extent than the other interacting constructs. The discrepancy between the strong interaction detected between LexA–Syk and GAD–Vav 610–846 and the relatively modest interaction between LexA–Syk and GAD–Vav 670–765 likely results from a difference in the conformational stability of the two hybrids. Our results indicate that the interaction between Syk and Vav in yeast requires the SH2 domain of Vav and is most likely stabilized by one or both flanking SH3 domain(s).

Next, we examined whether the interaction between Syk and Vav was dependent upon the tyrosine kinase activity of Syk, its SH2 domains, or both. A kinase-inactive hybrid (LexA–Syk K395R) was generated by mutating a conserved lysine residue (Lys-395) in the ATP-binding site of Syk. We also generated three deletion mutants: Syk 1–298, Syk 1–212, and Syk 213–628 (Figure 3A). The ability of these mutants to interact with GAD–Vav 610–846 and GAD–Vav 610–846/R696A was tested. Unlike wild-type Syk, LexA–Syk K395R was unable to interact with Vav (611 ± 75 U versus 35 ± 3 U, respectively) (Figure 3B). As a negative control, it was shown that both Syk hybrids failed to interact with the SH2-mutated Vav hybrid (7 ± 2 U and 5 ± 0.9 U). Consistent with these results, the two Syk hybrids lacking the kinase domain but containing all or part of the tandem SH2 domains (Syk 1–298 or Syk 1–212) did not interact with GAD–Vav 610–846. By contrast, deletion of the two SH2 domains of Syk (Syk 213–628) increased the interaction between LexA–Syk and GAD–Vav 610–846 (1127 ± 86 U) (Figure 3B). These results demonstrate that the catalytic activity of Syk, but not its SH2 domains, is necessary for its interaction with the Vav SH2 domain.

Finally, we wished to identify the potential binding site for the Vav SH2 domain on Syk. The linker region of Syk located between its SH2(C) and kinase domains

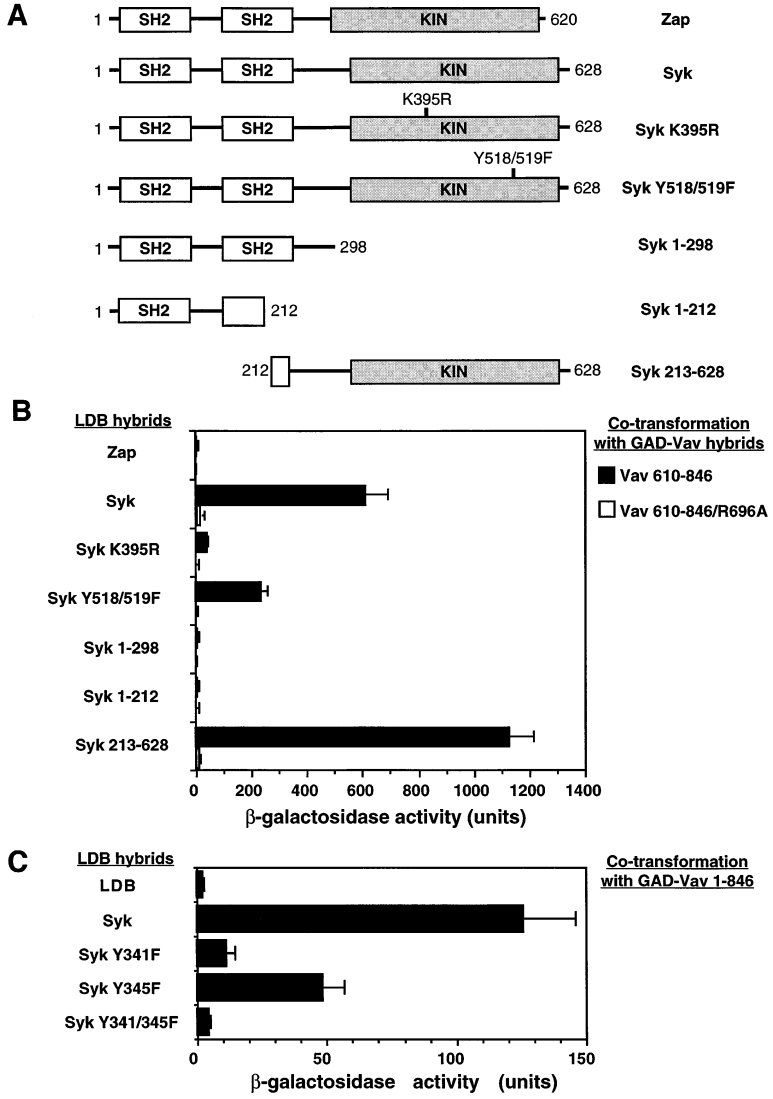

Figure 3. Mapping of Syk Domains Interacting with Vav

(A) Schematic representation of the human Zap or porcine Syk proteins and the different truncated forms of Syk used in this study. These mutated or deleted Syk constructs are described in detail in the text. KIN, kinase (catalytic) domain.

(B) The indicated Zap or Syk LDB hybrids were assayed for their interaction with GAD–Vav 610–846 or SH2-mutated GAD–Vav 610–846/R696A hybrids in yeast. β-Galactosidase activity in cell lysates was measured as described previously. Results are expressed as the mean (± SD) of three independent transformants. Similar results were obtained by analyzing growth on selective plates lacking histidine.

(C) LDB Syk hybrids mutated at the indicated tyrosine residues within the Syk linker region were assayed for their interaction with GAD–Vav 1–846 in yeast. β-Galactosidase activity in cell lysates was measured as described above, and results are expressed as the mean (± SD) of three independent transformants.

contains a sequence, Tyr-Glu-Ser-Pro, which has been suggested to represent a binding site for the Vav SH2 domain (Songyang et al., 1994). We mutated the corresponding tyrosine residue (Tyr-341), an adjacent tyrosine residue (Tyr-345), or both and assessed the effect of these mutations on the interaction with Vav (GAD–Vav 1–846) by a quantitative β-galactosidase assay. The Y341F mutation nearly abrogated the interaction of Syk with Vav (90% inhibition), whereas the Y345F mutation decreased this interaction by only 60% (Figure 3C). The double mutation Y341/345F caused a 96% reduction of this interaction. The relative specificity of the interaction between Vav and Tyr-341 of Syk is indicated by the finding that Syk Y341F was still capable of interacting with a GAD hybrid consisting of the Lck SH2 domain (Table 1). These results indicate that Tyr-341 and, to a lesser degree, Tyr-345 within the linker region of Syk are required for binding to the SH2 domain of Vav.

As an additional control, we tested the effect of another mutation in two tandem tyrosine residues in the catalytic domain of Syk (Tyr-518 and Tyr-519) on the interaction with Vav. These residues represent the major autophosphorylation site of Syk and mediate its binding to the SH2 domain of Lck (Couture et al., 1996). The corresponding fusion protein, LexA–Syk Y518/519F, was still capable of interacting with GAD–Vav 610–846, albeit to a lower degree than wild-type Syk (Figure 3B). The weaker interaction of this mutant is consistent with its reduced enzymatic activity (Couture et al., 1996).

Table 1. Comparison of the Interaction of Vav or Lck with Syk in the Yeast Two-Hybrid System

GAD Hybrids	Cotransformation with LDB Hybrids		
	Syk	Syk K395R	Syk Y341F
Vav	+++	−	−
Vav 670-765	++	−	−
Lck 121-224	+++	−	+
Lck 121-224/Y192E	−	−	−

The indicated combinations of plasmids encoding GAD–Vav or GAD–Lck and LDB–Syk hybrids were used to cotransform yeast, and interactions were detected by a filter β-galactosidase assay. The results are shown in a semiquantitative manner. An SH2-mutated Lck construct (Y192E) is included as a negative control. This mutation abolishes the binding of Lck SH2 to autophosphorylated Syk (Couture et al., 1996).

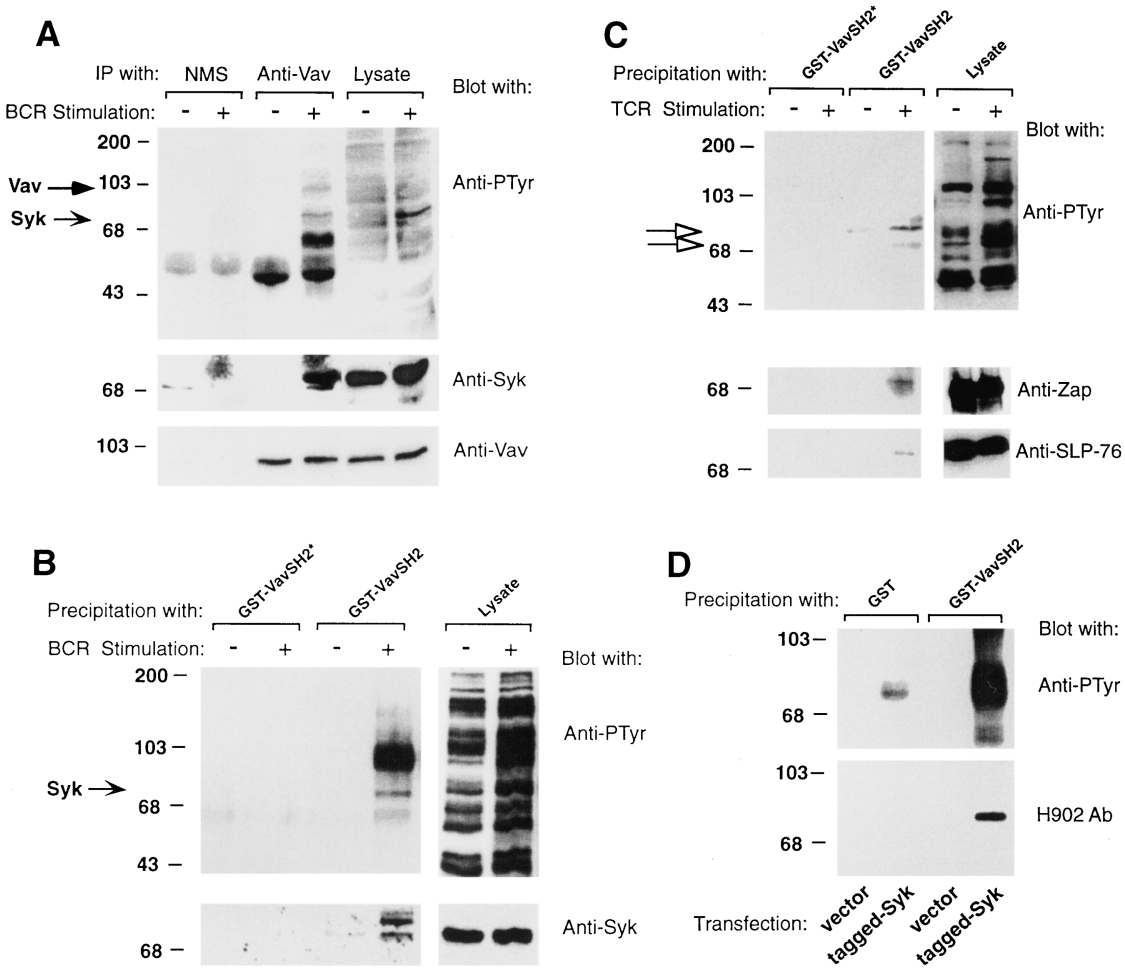

Figure 4. Interaction of Vav with Syk or Zap in Lymphocytes

(A) Coimmunoprecipitation of Syk with Vav. H2 B cells (5×10^7) were either left unstimulated or were stimulated for 4 min with a $F(ab')_2$ fragment of a goat anti–human immunoglobulin antibody (10 μg/ml) and lysed as described in Experimental Procedures. Cell lysates were subjected to immunoprecipitation using anti-Vav antibodies. The immunoprecipitates and whole lysate (2×10^6 cell equivalents) were analyzed by immunoblotting with the indicated antibodies.

(B) In vitro association of the Vav SH2 domain with B cell proteins. Lysates were prepared from H2 cells (2.5×10^7), which were either left unstimulated or stimulated as described above. Lysates were then precipitated with the indicated GST–Vav fusion proteins, and bound proteins or whole lysates (1×10^6 cell equivalents) were probed with anti-PTyr (top) or anti-Syk (bottom) antibodies.

(C) Association of the Vav SH2 domain with Zap and SLP-76 in Jurkat T cells. Jurkat cells (2.5×10^7) were either left unstimulated or stimulated for 4 min with anti-CD3 (OKT3; 5 μg/ml) and lysed. The lysates were precipitated with GST–Vav fusion proteins, and the bound proteins were detected by immunoblotting with anti-PTyr (top), anti-Zap (middle), or anti-SLP-76 (bottom) antibodies. PTyr-containing proteins in whole-cell lysates (1×10^6 cell equivalents) are shown for comparison. The positions of Zap and SLP-76 are indicated by the open arrows.

(D) Lysates from H902-tagged Syk-transfected COS cells were precipitated with control GST or GST–Vav SH2 proteins, and Syk binding was detected by immunoblotting with anti-PTyr (top) or anti-tag (H902; bottom) MAbs.

Together, these results underscore the critical role of Tyr-341 in the interaction with the Vav SH2 domain.

Interaction of Syk Kinases with Vav in Lymphocytes

We extended our analysis to determine whether the interaction between Syk and Vav detected in the yeast two-hybrid system also occurs in lymphocytes. First, the presence of associated proteins was examined in Vav immunoprecipitates from lysates of resting or BCR-stimulated human B lymphoid (H2) cells. Anti-PTyr immunoblotting revealed that BCR stimulation induced the phosphorylation of a 95 kDa protein, which comigrated with authentic Vav (Figure 4A). Three additional PTyr-containing proteins of ~72, ~62, and ~50 kDa were also detected in Vav immunoprecipitates. Similar phosphoproteins were not detected in control (NMS) immunoprecipitates. The tyrosine phosphorylation of the 72 and 62 kDa proteins was increased following BCR stimulation, whereas the PTyr content of the 50 kDa protein was similar in resting and activated cells (Figure 4A, top). When the same immunoprecipitates were immunoblotted with an anti-Syk antibody (Figure 4A, middle), the 72 kDa phosphoprotein comigrated with Syk, indicating that tyrosine-phosphorylated Syk is associated with Vav following B cell activation. The stoichiometry of Syk association with Vav appeared low (≤1%), but

was maintained under harsh lysis conditions using RIPA buffer (data not shown).

To confirm this association more directly, we probed lysates of BCR-stimulated H2 cells with glutathione S-transferase (GST) fusion proteins containing the wild-type SH2 domain of Vav or a mutated counterpart (SH2*) with the inactivating R696L point mutation. Bound cellular proteins were detected by immunoblotting with anti-PTyr (4G10) or anti-Syk antibodies (Figure 4B). Three major phosphoproteins of 90, 72, and 62 kDa present in lysates of activated B cells associated with the active Vav SH2 domain. An additional ~160 kDa protein was also observed after a longer exposure of the membrane (data not shown). Anti-Syk blotting revealed the association of Syk with the GST–Vav SH2 protein, but not (or very weakly) with GST–Vav SH2* (Figure 4B). Similar binding of Syk to GST–Vav SH2 was also observed in lysates of BCR-stimulated Ramos B cells (data not shown).

Since Vav SH2 can bind the Syk-related Zap kinase in activated T cells (Katzav et al., 1994), we extended this analysis to T cells by probing lysates of resting or anti-CD3-stimulated Jurkat T cells with the GST–Vav SH2 fusion protein and detecting bound cellular proteins by anti-PTyr immunoblotting (Figure 4C). Activated T cell lysates contained two major Vav SH2–bound 76 and 70 kDa phosphoproteins. An additional 36 kDa PTyr-containing protein was also observed in longer exposures. Reprobing of the same membrane with anti-Zap or anti-SLP-76 antibodies demonstrated that the 76 and 70 kDa phosphoproteins comigrated with SLP-76 and Zap, respectively. SLP-76 is known to associate with the SH2 domain of Vav in activated T cells (Motto et al., 1996; Wu et al., 1996). Immunoblot analysis of H2 or Ramos B cell lysates showed no detectable expression of SLP-76. Taken together, these results indicate that association with the Vav SH2 domain is a common property of Syk family kinases in activated T and B lymphocytes.

Finally, we used the GST–Vav SH2 fusion protein to probe lysates of COS cells transiently transfected with an epitope-tagged Syk expression vector. Immunoblotting with anti-PTyr (4G10) or anti-tag (H902) monoclonal antibodies (MAbs) revealed that tyrosine-phosphorylated Syk was bound to GST–Vav SH2, but not (or very weakly) to GST alone (Figure 4D).

To determine whether the SH2 domain of Vav binds directly to Syk, we assessed the binding of GST–Vav SH2 to Syk by Far Western blotting. Wild-type or mutant Syk immunoprecipitates from COS cells were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. The membranes were incubated with a GST–Vav SH2 fusion protein, and binding was detected with an anti-GST MAb. This analysis revealed binding of GST–Vav SH2 to a protein comigrating with authentic Syk in immunoprecipitates containing wild-type and, to a lesser degree, Y341F-mutated Syk, but not to kinase-inactive Syk K395R (Figure 5A). The identity of the faster-migrating protein is unknown. When probed with the anti-tag MAb (H902) all three Syk proteins were found to be expressed at a similar level in the transfected COS cells (Figure 5C). The differential binding of Vav SH2 to the different

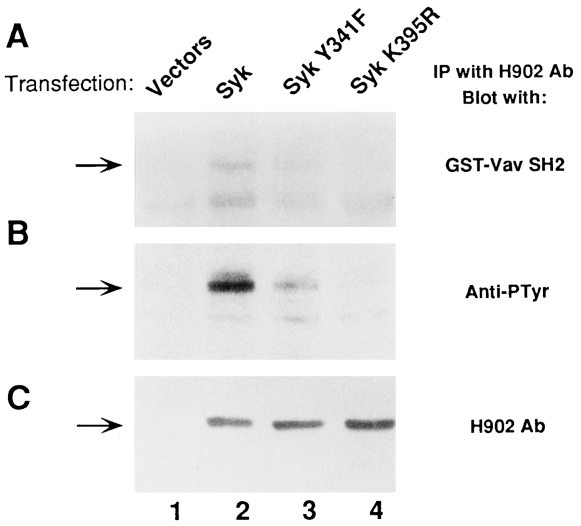

Figure 5. Direct Binding of the Vav SH2 Domain to Syk

Syk immunoprecipitates were prepared from lysates of COS cells transfected with vector controls (lane 1), wild-type Syk (lane 2), Syk Y341F (lane 3), or kinase-deficient Syk K395R (lane 4) using the H902 MAb. Following SDS–PAGE and transfer to nitrocellulose, the samples were overlayed with a GST–Vav SH2 fusion protein, and binding was detected with an anti-GST MAb (A) or with anti-phosphotyrosine MAb (4G10) (B). A control GST protein tested in parallel did not bind to Syk (data not shown). In (C) the same membrane was stripped and reprobed with MAb H902 to assess the expression level of the transfected Syk proteins.

Syk proteins correlated with their PTyr content as revealed by anti-PTyr immunoblotting (Figure 5B). The decreased tyrosine phosphorylation of Y341F Syk suggests that Tyr-341 is a major autophosphorylation site or that this residue regulates the enzymatic activity of Syk (or both). Taken together, these results are consistent with the data obtained in the yeast two-hybrid experiments and demonstrate a similar association between Vav and Syk in lymphocytes.

Syk and Zap Phosphorylate Vav

Next, we examined whether the interaction between Syk and Vav results in phosphorylation of Vav. COS cells were transiently transfected with Vav alone or with Vav plus wild-type or mutated Syk expression vectors. Anti-PTyr immunoblotting of Vav immunoprecipitates from these cells demonstrated that the PTyr content of Vav was markedly elevated when it was coexpressed with wild-type, but not with kinase-deficient Syk (Figure 6A, lane 3 versus lanes 2 and 5). Of note, Vav phosphorylation was dramatically decreased when it was coexpressed with the catalytically active Y341F Syk mutant (lane 4). These results demonstrate, first, that Vav is phosphorylated on tyrosine when coexpressed with Syk and, second, that Syk residue Y341 is important for this functional interaction.

To address more directly the possibility that Vav can be a physiological substrate for Syk family kinases, we produced a GST fusion protein corresponding to residues 161–191 of Vav, which encompass a potential single substrate site for Syk, i.e., Tyr-174 within an Ile-Tyr-Glu-Asp-Leu motif (Brunati et al., 1995). As a negative

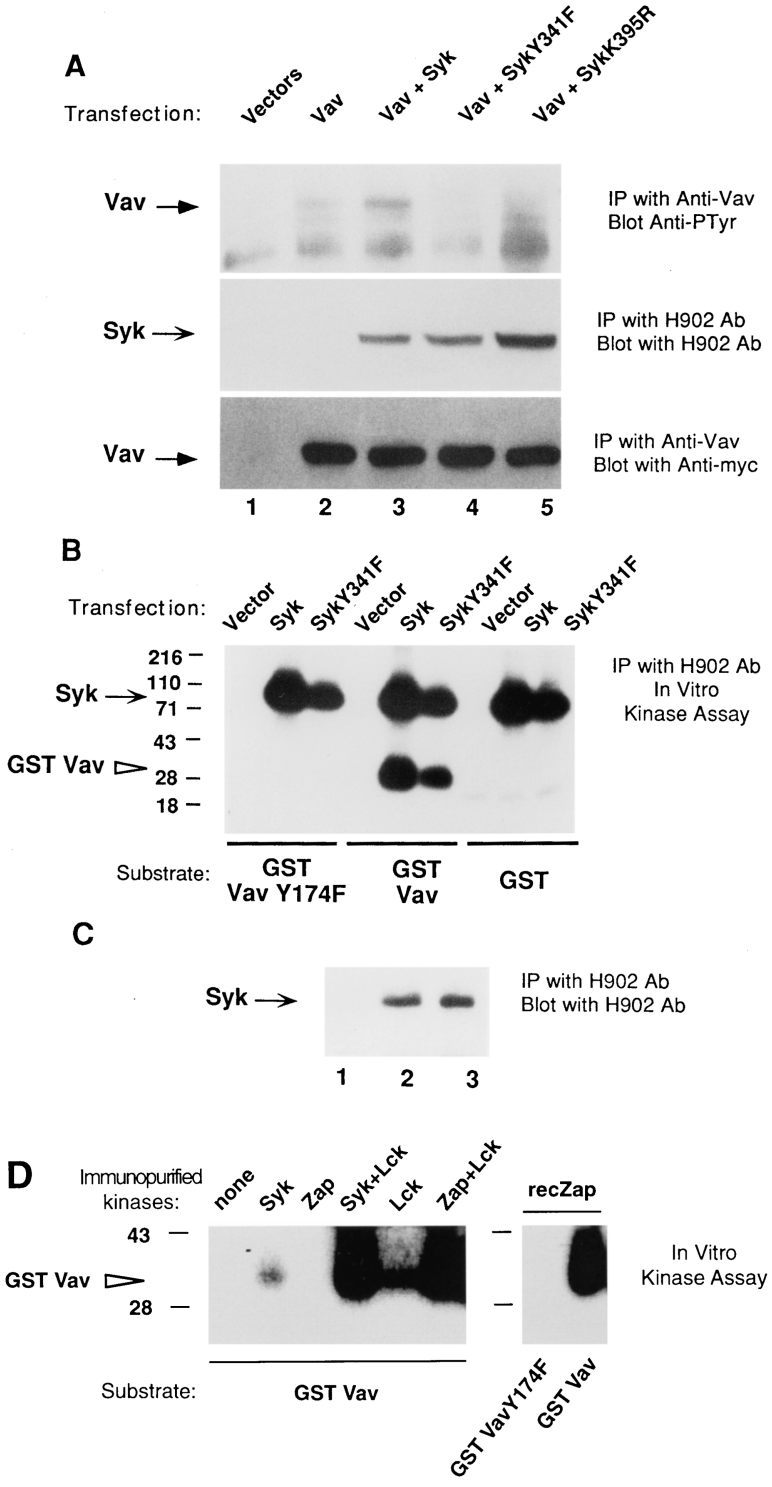

Figure 6. Syk Phosphorylates Vav

(A) Phosphorylation in intact cells. COS cells were transiently transfected with vector controls (lane 1), Myc-tagged Vav alone (lane 2), or Vav plus the indicated H902-tagged Syk expression vectors (lanes 3–5). Cell lysates were immunoprecipitated with H902 or anti-Vav antibodies, and the membranes were immunoblotted with anti-PTyr (4G10), H902, or 9E10 MAbs as indicated to reveal the PTyr content of Vav (upper panel) and the expression levels of Syk (middle panel) or Vav (bottom panel).

(B) In vitro phosphorylation. Wild-type or Y341F-mutated Syk were immunoprecipitated from COS cells lysates using MAb H902. We added 5 μg of recombinant GST, GST–Vav, or GST–Vav Y174F to the washed Syk immunoprecipitates and performed an in vitro kinase reaction as described in Experimental Procedures. The samples were subjected to SDS–PAGE analysis and autoradiography. The bands corresponding to autophosphorylated Syk or the phosphorylated Vav fusion peptide are indicated.

(C) Syk expression. Lysates of vector control (lane 1), Syk- (lane 2), or Syk Y341F–transfected cells (lane 3) were immunoprecipitated with MAb H902 and immunoblotted with the same antibody.

(D) Phosphorylation of Vav by Syk and Zap. Syk, Zap, and Lck were immunoprecipitated from singly transfected COS cells lysates, and an in vitro kinase reaction was performed on the individual immunoprecipitates or the indicated mixtures using GST–Vav as a substrate (left panel). An in vitro kinase reaction was performed using 1 μg of recombinant Zap (recZap) and an unmutated or Y174F-mutated GST–Vav fusion peptide substrates (right panel). The samples were subjected to SDS–PAGE analysis and autoradiography. The band corresponding to the phosphorylated Vav fusion peptide is indicated.

control, we used a similar fusion protein in which Tyr-174 had been mutated to phenylalanine (Vav Y174F). These proteins were subjected to an in vitro kinase assay using immunopurified Syk from COS cells. As shown in Figure 6B, Syk phosphorylated the wild-type Vav fusion protein. In contrast, GST and GST–Vav Y174F were not phosphorylated detectably by Syk. The mutated Syk Y341F, which was expressed at a similar level (Figure 6C), phosphorylated the recombinant Vav protein to a lesser degree (55% by densitometry) than wild-type Syk, consistent with a potential regulatory role of Tyr-341. This lower activity is unlikely in itself to explain the much greater reduction (≥90%) in the association between Syk Y341F and Vav (Figure 6A).

Finally, we compared the ability of Syk and Zap to phosphorylate the recombinant Vav protein by performing in vitro kinase assays with immunopurified Syk, Zap-70, or Lck from transiently transfected COS cells.

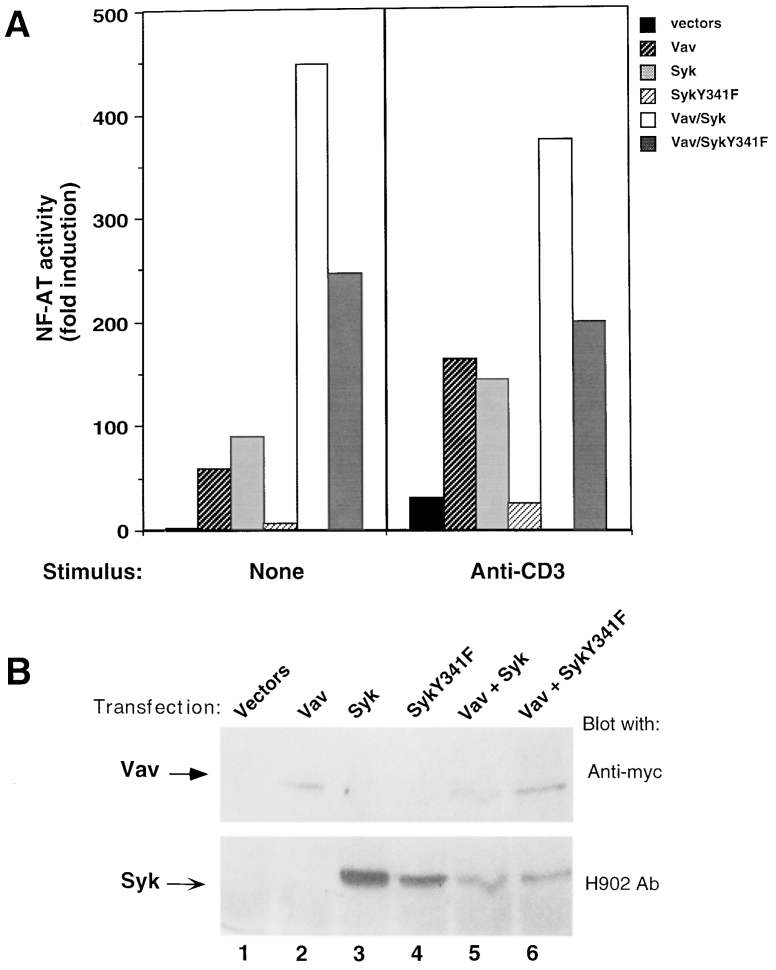

Figure 7. NF-AT Activation in Syk- or Vav-Transfected Jurkat Cells (or Both)

(A) Jurkat-TAg cells (10×10^6) were electroporated with 5 μg of an NF-AT-Luciferase reporter construct in combination with 5 μg of Myc-tagged pEF-Vav, 5 μg of H902-tagged wild-type or Y341-mutated Syk, or combinations of Vav plus Syk, as indicated. After 40 hr, 5×10^5 cells were either left unstimulated or were stimulated for 6 hr with an anti-CD3 MAb (OKT3; 10 μg/ml). Luciferase activity in cell lysates was determined as described in Experimental Procedures. Results are represented as the fold induction of luciferase activity as compared with the activity in unstimulated cells transfected with empty vectors. Results represent the mean of triplicate determinations, and standard deviation of the mean of each condition was less than 15%. Results are representative of two independent experiments. The basal activity and the maximum NF-AT responses were appproximately 20 arbitrary units (AU) and 2×10^4 to 3×10^4 AU, respectively.

(B) Aliquots of cell lysates from each transfection group were subjected to SDS–PAGE and immunoblotting with anti-Myc (9E10; upper panel) or H902 (lower panel) MAbs to assess the expression levels of the transfected Vav and Syk proteins, respectively.

We found that Syk or Lck alone, but not Zap, phosphorylated Vav. Lck synergized with either Zap or Syk to increase dramatically the phosphorylation of the Vav fusion peptide (Figure 6D, left panel). To confirm that Zap can directly phosphorylate Vav, we used a baculovirus-derived recombinant Zap protein that is known to be constitutively active (Isakov et al., 1996). The recombinant enzyme phosphorylated the Vav fusion protein in vitro (Figure 6D, right panel). These results indicate that Vav is a substrate for Syk family kinases and that Tyr-174 represents one likely phosphorylation site.

Syk and Vav Synergize to Activate NF-AT in T Cells

TCR stimulation leads to activation of distinct transcription factors involved in IL-2 production, including NF-AT (Schreiber and Crabtree, 1992), and transient Vav overexpression was recently found to result in activation of this transcription factor (Holsinger et al., 1995; Wu et al., 1995). Since Vav is phosphorylated on tyrosine in activated T cells (Margolis et al., 1992) and our results (Figures 1–6) demonstrated a physical and functional Syk–Vav interaction leading to phosphorylation of the latter, we assessed the effects of transient transfection with Vav alone, or with Vav plus Syk, on the activity of an NF-AT transcriptional element cloned upstream of a luciferase reporter gene (NF-AT-Luc). In unstimulated cells, transient overexpression of Vav or Syk alone led to a 60- and 90-fold increase in the basal transcriptional activity of the NF-AT reporter construct, respectively (Figure 7A). Coexpression of Syk plus Vav resulted in a synergystic effect, i.e., a 450-fold activation of NF-AT. This was clearly not due to increased expression of Syk or Vav (or both) in doubly transfected cells by comparison with cells transfected with Vav or Syk separately (Figure 7B). The Syk Y341F mutant was considerably less active, either alone or in the presence of coexpressed Vav. In anti-CD3-stimulated cells, Vav or Syk overexpression stimulated NF-AT to a level higher than in unstimulated cells. Syk and Vav coexpression resulted in an additive effect, which was no longer observed with the Y341F Syk mutant. As a control, phorbol myristate acetate plus ionomycin stimulation caused strong NF-AT stimulation, which was not augmented by transient Syk or Vav overexpression (data not shown). We also observed that SH2-mutated Vav (R696L) was incapable of cooperating with wild-type Syk and neither was kinase-deficient Syk capable of synergizing with wild-type Vav to activate NF-AT (data not shown). These findings indicate that Syk and Vav cooperate in stimulating a signaling pathway leading to NF-AT activation. Thus, the interaction between Syk and Vav revealed by our

earlier experiments has a distinct functional and physiologically relevant outcome.

Discussion

Identification of the substrates and downstream effectors of immunoreceptor-associated PTKs of the Src and Syk families is essential for elucidating the mechanisms of immune cell activation. Although several substrates of Lck and Fyn have been identified, the physiological substrates of Zap or Syk are still unknown. Nevertheless, genetic studies have recently demonstrated a critical role of Syk family PTKs in T or B cell development and function (Arpaia et al., 1994; Chan et al., 1994; Cheng et al., 1995; Elder et al., 1994; Negishi et al., 1995; Turner et al., 1995). We have combined two independent approaches, i.e., the use of the yeast two-hybrid system and a biochemical–functional analysis of protein–protein interactions in mammalian cells, including T and B cells, to address the interactions between Syk family kinases and potential immediate targets. Evidence is presented that the Vav proto-oncogene product, as well as Lck and the p85 subunit of the phosphatidylinositol 3-kinase, interact with phosphorylated Syk in yeast. Zap expressed in yeast was not detectably phosphorylated, thus explaining the absence of interactions with the LexA–Zap hybrid. The discrepancy between Syk and Zap most likely reflects their differential mode of activation. While Zap is incapable of autoactivating, and requires phosphorylation by Src family kinases in order to become enzymatically active, Syk can become activated by autophosphorylation and can mediate downstream signaling events in the absence of Src family kinases (Couture et al., 1994; Kolanus et al., 1993; Rivera and Brugge, 1995). Consistent with these findings, human Syk was recently shown to exhibit a greater intrinsic catalytic activity than Zap (Latour et al., 1996).

In the present study, we focused on the interaction between Vav and Syk. We determined that the SH2 domain of Vav is necessary and sufficient for the interaction with Syk, although one or both SH3 domains most likely stabilize it. Other domains of Vav appeared to exert a negative influence on this interaction. The interaction between Syk and Vav was dependent on the catalytic activity of Syk, but did not require its SH2 domains. Unlike mutations in three other Syk tyrosine residues (345, 518, and 519), mutation of Tyr-341, located in the linker domain between its SH2(C) and catalytic domains, almost completly abolished the interaction with Vav. Although a formal proof is lacking, this suggests that Syk autophosphorylates on Tyr-341, thereby creating a binding site for the Vav SH2 domain. Tyr-341 lies within a motif (Tyr-Glu-Ser-Pro) found to be optimal for interaction with Vav SH2 (Songyang et al., 1994). It has been suggested that the same motif, which is repeated twice in SLP-76, mediates the activation-dependent association of SLP-76 with the Vav SH2 domain (Wu et al., 1996). Since this sequence is conserved between human and porcine Syk and Zap (Tyr-315), it is likely that the activation-induced phosphorylation of this tyrosine residue on Zap is also important for the binding of Vav to Zap. In strong support of our finding, the Vav SH2 domain was shown to be necessary for efficient phosphorylation of Vav induced by TCR stimulation (Wu et al., 1996), presumably resulting from the binding of Vav to activated Zap. A second tyrosine residue (Tyr-345) lies within a Tyr-Ala-Asp-Pro motif, and mutation of this residue to phenylalanine caused only partial inhibition of the binding to the SH2 domain of Vav, indicating that this residue may be less critical for that interaction.

Although the association between Syk and the SH2 domain of Vav was readily demonstrated in yeast, it was important to ascertain whether it also occurs in lymphocytes and, if so, what are its functional consequences. By analyzing the physical and functional interactions between Syk and Vav in human B or T cells cells, we were able to confirm and extend the findings obtained in the yeast two-hybrid system. First, Vav interacted with Syk or Zap in vivo following BCR or TCR stimulation, respectively. Second, a Vav SH2 fusion protein was capable of binding activated (i.e., tyrosine-phosphorylated) Syk (and Zap) in vitro, and this binding was direct as demonstrated by Far Western blotting. The undetectable or weak binding of the GST–Vav SH2 fusion protein to catalytically inactive or Y341F-mutated Syk, respectively, is consistent with our findings in yeast that both intact catalytic activity of Syk and Tyr-341 are essential.

A point mutation in the conserved PTyr-binding pocket of Vav SH2 abolished its binding to Syk or Zap, as well as to other PTyr-containing proteins present in activated B or T cell lysates. In this regard, Vav SH2 interacted with distinct tyrosine-phosphorylated proteins, including Zap and SLP-76, in lysates of TCR-stimulated Jurkat cells, consistent with recent studies (Katzav et al., 1994; Motto et al., 1996; Wu et al., 1996). SLP-76 was recently found to mediate activation of IL-2 gene transcription in T cells in association with Vav (Motto et al., 1996; Wu et al., 1996). One notable difference between B and T cells was the absence in the former of detectable SLP-76. This probably reflects the finding that B cell lines express very low levels of SLP-76 mRNA compared with T cell lines (Jackman et al., 1995). Instead, we observed association of Vav SH2 with a major 85–90 kDa unidentified B cell phosphoprotein. One possibility is that a family of SLP-76-related proteins is differentially expressed in distinct hematopoietic cell types.

Although only a small fraction of cellular Syk associated with Vav under our lysis conditions, the association was stable as evidenced by its resistance to extensive washes in RIPA buffer. This may be due either to a low stoichiometry of phosphorylation at Tyr-341 by Syk or to a low affinity of the interaction between phospho-Tyr-341 and Vav SH2 in vivo. In addition, another SH2-containing effector(s) may compete for binding Syk at this phosphorylated residue. This notion is supported by a recent report that the equivalent tyrosine residue in human Syk, Tyr-348 (as well as Tyr-352), was required for the interaction between active Syk and PLC-γ1 and for subsequent PLC-γ1 activation (Law et al., 1996). Another likely explanation for this low stoichiometry is that Vav and Syk dissociate rapidly following the dephosphorylation of Syk family kinases by associated SH2-

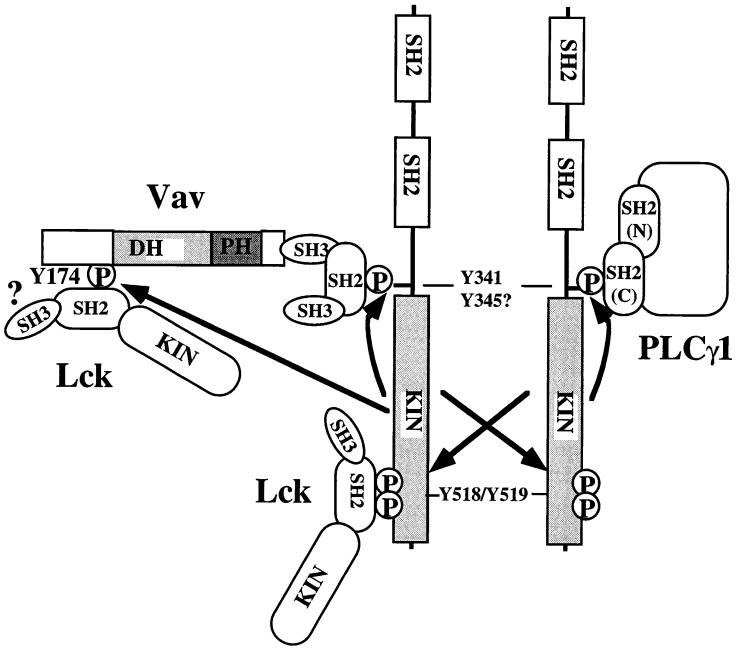

Figure 8. Putative Model of Sequential Activation and Recruitment of, and Interactions among, Syk, Vav, and Lck

As a consequence of immunoreceptor crosslinking, the phospho-ITAM-bound Syk is activated and becomes autophosphorylated on Tyr-518, Tyr-519, and Tyr-341 (and potentially other residues). Phospho-Tyr-341 recruits Vav (and PLC-γ1) by direct binding to its SH2 domain (Figures 2–5; Table 1), thereby facilitating phosphorylation of Vav on Tyr-174 by activated Syk (Figure 6). This event then creates a docking site for the SH2 domain of Lck (or another Src family kinase). The associated Lck may then phosphorylate additional tyrosine residues in the Vav–Syk complex. Independently of this mechanism, Lck can also be recruited via direct association of its SH2 domain with the phosphorylated Tyr-518/519 residues of Syk (Couture et al., 1996). See text for more details.

containing protein tyrosine phosphatases, which represents an important regulatory event during lymphocyte activation. Indeed, PTyr phosphatase inhibitors can increase the association between Syk family kinases and Vav SH2 (Wu et al., 1996; M. D., unpublished data), and an association between Zap and the SHP1 PTyr phosphatase was recently described (Plas et al., 1996).

What are the functional consequences of the interaction between Syk family kinases and Vav? The present study addresses two aspects of this important question. First, we demonstrated increased tyrosine phosphorylation of Vav in COS cells in the presence of coexpressed wild-type Syk. Not surprisingly, this effect was not observed with catalytically inactive Syk. Of more importance and interest, this increased phosphorylation was practically abolished by mutating Tyr-341 in Syk, a mutation that reduced its catalytic activity by only ~45%. This result suggests that recruitment of Vav to phospho-Tyr-341 is physiologically relevant in that it facilitates the phosphorylation of Vav by Syk (Figure 8). Although the results obtained with COS cells do not prove that Vav is a direct substrate for Syk, this is strongly supported by our finding that a Vav fusion peptide containing a single tyrosine residue (Tyr-174) was phosphorylated in vitro by immunopurified Syk. This is consistent with a recent finding that a synthetic peptide containing Tyr-174 of Vav was an efficient substrate for purified Syk in vitro (Brunati et al., 1995). Furthermore, we also observed that baculovirus-derived recombinant Zap or Lck-activated Zap (Figure 6D) was similarly capable of phosphorylating Tyr-174 in Vav, suggesting that Vav is a direct physiological target for both Syk and Zap.

One potential consequence of the phosphorylation of Vav on Tyr-174 by Syk or Zap may be the creation of a binding site for the SH2 domain of Src family kinases (Figure 8). The corresponding motif, Tyr-Glu-Asp-Leu, was found to be optimal for this binding (Songyang et al., 1993). Although such binding has not been formally demonstrated, we observed a direct association between the SH2 domains of Lck or Fyn and tyrosine-phosphorylated Vav from activated T cells (T. Collins and N. Bonnefoy-Bérard, unpublished data), and Lck was found to coimmunoprecipitate with Vav in an activation-dependent manner (Gupta et al., 1994). Studies in a Syk-deficient chicken B cell lymphoma have indicated that Syk plays a critical role in coupling the BCR to PLC-γ-dependent events, i.e., inositol phosphate production and calcium mobilization (Takata et al., 1994). Interestingly, deficient Vav phosphorylation is observed in mast cells from *syk*$^{-/-}$ mice triggered via their FcεRI (V. Tybulewicz, personal communication). However, these studies also demonstrated that a cross-talk between Syk and Lyn, a Src family PTK, was required for optimal BCR-dependent activation and maximal tyrosine phosphorylation (Takata et al., 1994). Thus, our findings suggest a potential mechanism for the sequential recruitment of Syk and Src family PTKs by Vav, an event that may facilitate cross-talk between these two PTK families (Figure 8).

Another clue regarding the biological significance of the interaction between Syk and Vav comes from the effects of transient overexpression of Syk or Vav (or both) in Jurkat cells on the transcriptional activity of NF-AT, an important element in cytokine gene promoters such as IL-2, IL-3, IL-4, and tumor necrosis factor α (Northrop et al., 1993; Schreiber and Crabtree, 1992; Rao, 1994). In a similar system, it was recently shown that Vav activates the IL-2 gene and potentiates activation of the IL-2 promoter induced by TCR/CD3 ligation (Holsinger et al., 1995; Wu et al., 1995). Our results confirm these findings and extend them by demonstrating that coexpression of Syk and Vav causes synergistic NF-AT activation in the absence of TCR stimulation and produces an additive effect in anti-CD3-stimulated cells. This effect was not observed (or was less pronounced) when Y341-mutated, but otherwise catalytically active,

Syk was coexpressed with Vav. This finding strongly suggests that Tyr-341 is important, in the context of intact lymphocytes, for an optimal functional interaction betwen these two signaling proteins. Furthermore, the linker domain of Zap exhibit a tyrosine residue (Tyr-315) lying within the same conserved Tyr-Glu-Ser-Pro motif. Therefore, it is likely that Syk and Zap act directly or indirectly upstream of Vav in a pathway leading to immune receptor–induced transcription of cytokine genes.

On the other hand, the recruitment of PLC-γ1 by activated Syk (Law et al., 1996) may facilitate the tyrosine phosphorylation and enzymatic activation of the former, leading to production of second messengers that subsequently activate protein kinase C and increase the intracellular calcium concentration. It is interesting that NF-AT stimulation by Vav overexpression, which does not have a direct effect on TCR-induced calcium mobilization, is nevertheless dependent on calcineurin (Wu et al., 1995). This suggests an interaction between PLC-γ1- (or calcium) and Vav-mediated signals. Therefore, activated Syk family kinases seem to control at least two independent signaling pathways that interact cooperatively to induce activation of the IL-2 gene. The presence of activated and aggregated Syk family kinases within a membrane-localized signaling complex may allow simultaneous binding to Vav, PLC-γ1, and possibly other effectors (e.g., SLP-76) and facilitate their tyrosine phosphorylation and functional interactions. In this regard, SLP-76, a Vav SH2–interacting protein that cooperates with it to activate the IL-2 gene, was recently shown to be phosphorylated by activated Zap (Bubeck Wardenburg et al., 1996).

The role of Vav remains controversial (Bonnefoy-Bérard et al., 1996). Our findings suggest that one potential function of Vav is that of an adaptor protein, acting via its phosphorylated tyrosine residues and SH2 and SH3 domains (as well as other functional domains) to recruit other signaling molecules. This action may be important for the formation of active, multisubunit signaling complexes. The two SH3 domains or PH domain (or both) of Vav may connect immune receptor–associated PTKs and other signaling proteins to cytoskeletal elements. Actin cytoskeleton rearrangement is one of the earliest events observed during activation of immune cells, including mast, B, or cytotoxic T cells. This response requires small GTPases of the Rho family, which appear to be functionally linked to Vav, and are also likely involved in regulating pathways leading to IL-2 production in T cells. In this regard, it is tempting to speculate that Vav may bridge immune receptor–associated PTKs of the Syk and Src families to downstream signaling cascades involving both Ras- and Rho-related small GTPases.

Experimental Procedures

Plasmids, DNA Constructs, and Mutagenesis

The yeast expression plasmids pBTM116, pGAD–Raf, pGAD–Lamin, and pLexA–Ras^{V12} (Votjek et al., 1993) were provided by A. Vojtek (Seattle, WA). The plasmid pACTII was from Clontech (Palo Alto, CA). The plasmid encoding a hybrid between GAD and Grb2 was provided by J. Camonis (Paris, France). The plasmids encoding GAD hybrids with p85, Shc, or the SH2 domains of the protein tyrosine phosphatase PTP2C have been described previously (Tartare-Deckert et al., 1995, 1996).

Plasmid construction, cloning, and DNA sequencing were carried out according to standard protocols (Ausubel et al., 1992). In-frame insertion of the desired cDNAs into yeast expression plasmids was performed using thermostable PWO polymerase (Boeringher Mannheim, Federal Republic of Germany). Porcine Syk (residues 1–628) was PCR amplified from the pTag/SRα-Syk vector (Couture et al., 1994) and fused to LDB in pBTM116. Truncated Syk plasmids Syk 1–298, Syk 1–212, and Syk 213–628 were obtained by restriction endonuclease digestion of the pBTM116-Syk plasmid, followed by ligation. Human Vav cDNA (residues 1–846) was PCR amplified from a pEFneo-Vav vector and fused to the GAD-encoding sequence in pACTII. Vav 386–846, Vav 610–846, Vav 670–765, and Vav 1–610, were PCR amplified from pACTII-Vav and fused to pACTII. cDNAs encoding the wild-type or a Y192E-mutated version of the Lck SH2 domain (residues 121–224) were PCR amplified from pEF-based constructs (Couture et al., 1994) and fused to pACTII. The Myc epitope-tagged Vav expression vector (pEF-Vav-myc) was constructed as described (A. Munshi et al., personal communication). The NF-AT luciferase (NF-AT-Luc) reporter plasmid was a gift from G. Crabtree.

Bacterial expression plasmids of GST fusion proteins containing the SH2 domains of Lck (GST–Lck SH2; Couture et al., 1996) or Vav (GST–Vav SH2; Katzav et al., 1994) have been described. cDNAs encoding residues 161–191 of Vav (GST–Vav), or a similar peptide in which Tyr-174 has been mutated to phenylalanine (GST–Vav Y174F), were PCR amplified from pEFneo-Vav construct and inserted in-frame into the pGEX3T-1 expression vector (Pharmacia) to generate the corresponding GST fusion proteins. All mutants were generated by site-directed mutagenesis of double-stranded DNA using the Transformer kit (Clontech). Mutations were verified by DNA sequence analysis.

Antibodies and Chemicals

The MAb used for CD3/TCR stimulation was OKT3 (ATCC, Rockville, MD), and the antibody used for BCR stimulation was a purified F(ab′)$_2$ fragment of goat anti–human immunoglobulin (Cappel, West Chester, PA). The anti-Vav MAb was from Upstate Biotechnology (Lake Placid, NY), the polyclonal anti-Vav and anti-Zap antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Syk antiserum was generated as previously described (Couture et al., 1996). The anti-SLP-76 MAb was provided by R. Lahesmaa and P. Findell (Roche Biosciences, Palo Alto, CA). The anti-PTyr MAb 4G10 was from Upstate Biotechnology. The anti-tag MAb H902 has been described before (Couture et al., 1994). Chemicals were from Sigma, and enzymes were from GIBCO. Purified, baculovirus-derived recombinant Zap was a gift of Dr. P. Burn (Hoffman–La Roche, Nutley, NJ).

Yeast Transformation and Reporter Gene Expression

The yeast strain L40 (*MATa, trp1, leu2, his3, LYS::lexA-HIS3, URA::lexA-lacZ*) was provided by A. Vojtek (Seattle, WA) and was cultured on synthetic defined drop-out yeast media lacking the appropriate amino acids obtained from BIO 101 (La Jolla, CA). Growth conditions and maintenance of the yeast strain L40 were as previously described (Guthrie and Fink, 1991). L40 was cotransformed with pairs of the indicated plasmids by the lithium acetate method (Gietz et al., 1992). Cotransformants were selected on Trp$^-$, Leu$^-$ plates to select for the pBTM116 and pACTII derivatives, respectively. After 3 days at 30°C, three colonies of each transformation were plated on Trp$^-$, Leu$^-$ master plates and incubated at 30°C for 2 days. The transformants were then tested for histidine prototrophy by replica plating following a 3 day incubation at 30°C on Trp$^-$, Leu$^-$, and His$^-$ medium and for β-galactosidase activity using a filter assay (Tartare-Deckert et al., 1995). Transformants were also tested for β-galactosidase activity by a quantitative assay, performed essentially as described previously (Bartel and Fields, 1995). In brief, a solution containing 8 mM of chlorophenol red-β-D-galactopyranoside as substrate was incubated with 1 ml of yeast cell extract, and the increase in A_{574} was monitored after 30 min. Results were expressed as units, as defined by Miller (1972).

Analysis of Hybrid Protein Expression in Yeast

Yeast cells (2×10^8) expressing LexA–Zap or LexA–Syk were lysed as described previously (Couture et al., 1996). The lysates were diluted 1:20 in 150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 5 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF); immunoprecipitated using anti-Zap or anti-Syk antibodies; and analyzed by anti-PTyr immunoblotting as described above.

Cell Lines and Stimulation

Jurkat cells (E6-1; ATCC) or simian virus 40 T antigen (TAg)-transfected Jurkat (Jurkat-TAg) cells (obtained from G. Crabtree) were cultured in RPMI 1640 medium, 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin, and streptomycin. COS cells and the non-Hodgkin human B lymphoma H2 cell line (W. Erba, National Institutes of Health, Bethesda, MD) ($CD19^+$, IgG^+, IgD^-, and IgM^-) were cultured in the same medium. In the case of Jurkat-TAg cells, the medium also contained 2 mg/ml geneticin (GIBCO).

For stimulation, cells were washed twice in RPMI medium and incubated for 4 min at 37°C in RPMI medium, 1 mM sodium orthovanadate, without or with 5 μg/ml OKT3 (T cells) or with 10 μg/ml goat anti-human IgG (B cells). Reactions were stopped by addition of ice-cold buffer A (150 mM NaCl, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES, pH 7.4], 5 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF).

GST Precipitation, Immunoprecipitation, and Kinase Assay

Cells were centrifuged to remove the supernatant and lysed in 1 ml lysis buffer (1% Nonidet P-40 [NP-40] in buffer A) for 20 min on ice. Nuclei were pelleted by centrifugation for 15 min at 16,000 × g at 4°C. For GST precipitations, lysates were incubated with 5 μg of the indicated GST fusion protein for 4 hr at 4°C, followed by incubation with glutathione–Sepharose 4B beads (Pharmacia) for 1 hr. Samples were washed four times in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in buffer A).

For immunoprecipitations, lysates were incubated with optimal concentrations of the indicated antibodies for 4 hr at 4°C, followed by incubation with anti–mouse IgG-conjugated agarose beads for 1 hr. Samples were washed three times in RIPA buffer. Precipitates were dissolved in SDS sample buffer, resolved by 10% SDS–PAGE, and analyzed by immunoblotting as described below.

For in vitro kinase assay, immunoprecipitates or recombinant proteins were incubated in 40 μl of 50 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1% NP40, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear, Boston, MA) plus 5 μg of the indicated GST fusion proteins for 15 min at 30°C with gentle agitation. Reactions were stopped by the addition of sample buffer, boiled at 95°C for 5 min, and analyzed by SDS–PAGE and autoradiography.

Immunoblotting and Far Western Blotting

SDS–PAGE-resolved samples were transferred to nitrocellulose membranes (0.4 μm pore size; Bio-Rad). The membranes were blocked with 5% dry milk dissolved in TBST (25 mM Tris–HCl [pH 7.5], 125 mM NaCl, 0.1% Tween 20) for 16 hr at 4°C. The membranes were then incubated for 2 hr in blocking buffer containing 1 μg/ml of the indicated antibodies. The membranes were washed three times for 10 min in TBST, incubated with horseradish peroxidase (HRP)-conjugated donkey anti–rabbit or sheep anti–mouse IgG antibody (1:2000; Amersham) for 30 min at room temperature, washed three times in TBST, and developed using an enhanced chemiluminescence kit (Amersham) following the instructions of the manufacturer.

For Far Western blotting, the blocked membranes were incubated overnight with 10 μg/ml of the indicated GST fusion proteins in 5% milk in TBST, washed three times with TBST, incubated with an anti-GST MAb (0.1 μg/ml; Santa Cruz) for 1 hr at room temperature, and washed three times for 5 min in TBST. Binding was detected by development with HRP-conjugated sheep anti–mouse IgG antibody and enhanced chemiluminescence.

Cell Transfection and Luciferase Assay

COS cells were transfected by lipofection with 10 μg of the indicated plasmid DNAs as previously described (Couture et al., 1994). Jurkat-TAg cells (10×10^6) were electroporated with 5 μg of the reporter plasmid NF-AT-Luc together with 5 μg of pEF-Vav-myc without or with 5 μg of pME18S-Syk. Similar amounts of the corresponding empty vectors were used as controls. After 40 hr, 5×10^5 cells were left unstimulated or were stimulated with 10 μg/ml of an anti-CD3 MAb (OKT3) or with 50 ng/ml of phorbol myristate acetate plus 1 μg/ml of ionomycin. After 6 hr at 37°C, cells were lysed in 100 μl of 100 mM KPO_4 (pH 7.8), 1 mM dithiothreitol, 0.5% Triton X-100. Aliquots of the lysates were 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 was determined in triplicate and expressed as arbitrary units (AU).

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