Tyrosine Residues at the Carboxyl Terminus of Vav1 Play an Important Role in Regulation of Its Biological Activity*\(\textsuperscript{**}\)

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The guanine nucleotide exchange factor (GEF) Vav1 is an essential signal transducer protein in the hematopoietic system, where it is expressed physiologically. It is also involved in several human malignancies. Tyrosine phosphorylation at the Vav1 amino terminus plays a central role in regulating its activity; however, the role of carboxyl terminal tyrosine residues is unknown. We found that mutation of either Tyr-826 (Y826F) or Tyr-841 (Y841F) to phenylalanine led to loss of Vav1 GEF activity. When these Vav1 mutants were ectopically expressed in pancreatic cancer cells lacking Vav1, they failed to induce growth in agar, indicating loss of transforming potential. Furthermore, although Y841F had no effect on Vav1-stimulated nucleotide factor of activated T cells (NFAT) activity, Y826F doubled NFAT activity when compared with Vav1, suggesting that Tyr-826 mediates an autoinhibitory effect on NFAT activity. SH2 profiling revealed that Shc, Csk, Abl, and Sap associate with Tyr-826, whereas SH2-B, Src, Brk, GTPase-activating protein, and phospholipase C-γ associate with Tyr-841. Although the mutations in the Tyr-826 and Tyr-841 did not affect the binding of the carboxyl SH3 of Vav1 to other proteins, binding to several of the proteins identified by the SH2 profiling was lost. Of interest is Csk, which associates with wild-type Vav1 and Y841F, yet it fails to associate with Y826F, suggesting that loss of binding between Y826F and Csk might relieve an autoinhibitory effect, leading to increased NFAT. Our data indicate that GEF activity is critical for the function of Vav1 as a transforming protein but not for NFAT stimulation. The association of Vav1 with other proteins, detected by SH2 profiling, might affect other Vav1-dependent activities, such as NFAT stimulation.

Tyrosine phosphorylation, a posttranslational modification tightly regulated by the combined action of tyrosine kinases and phosphatases, plays a central role in controlling many important biological processes including proliferation, differentiation, apoptosis, cell adhesion, and motility (1–5). In addition to its importance in normal signal transduction, aberrant tyrosine phosphorylation has been observed in various pathological conditions, particularly in development and progression of cancer (6–8). One of the most important consequences of protein tyrosine phosphorylation is to regulate protein-protein interactions (9). Many tyrosine-phosphorylated proteins serve as high affinity binding sites for proteins containing modular (Tyr(P))\textsuperscript{2}-specific binding domains. These modular domains serve to couple tyrosine phosphorylation to the assembly of signaling complexes and the relocation of signaling proteins and thus play a central role in downstream signaling. By far the most abundant such module in humans is the Src homology 2 (SH2) domain (10). There are 120 SH2 domains encoded by the human genome (11), and each SH2 domain has binding specificity for a unique spectrum of tyrosine-phosphorylated sites (12). Uncovering the role of tyrosine residues in various proteins and deciphering their interactions with SH2-containing proteins enhances our knowledge of the regulation and function of cells in the human body.

Wild-type Vav1 (herein Vav1) is an important signal transducer with a pivotal role in the hematopoietic system, where it is exclusively expressed (13–15). Vav1 encodes a unique protein with several motifs known to play a role in tyrosine-mediated signal transduction, including a diffuse B-cell lymphoma (DBL) homology (DH)\textsuperscript{2} domain, a pleckstrin homology (PH) domain, an SH2 domain, and two Src homology 3 (SH3) domains (13–15). The best known function of Vav1 is as a GDP/GTP exchange factor (GEF) for Rho/Rac, a function strictly controlled by tyrosine phosphorylation (16). Rho/Rac activation leads to cytoskeletal rearrangement during activation of T cells (17–19). Vav1 also functions independently of its exchange activities, acting as a scaffold protein that leads to modulation of the JNK, ERK, Ras, NF-κB, and NFAT pathways (14, 15). These effects are likely mediated by the modular domains of Vav1 such as SH2 and SH3, which interact with other proteins, including Shc, NCK, SLP-76, ZAP-70, GRB2, and Crk (14, 15).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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\(\textsuperscript{1}\) The abbreviations used are: DH, DBL homology; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor; NFAT, nuclear factor of activated T cells; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; EGF, epidermal growth factor; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PTB, polyprolyrimidine tract-binding protein; TCR, T cell receptor; CSK, C-terminal Src kinase; luc, luciferase; Abs, antibodies; mAb, monoclonal antibody.

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The regulation of the activity of Vav1 is attributed in part to its phosphorylation on tyrosine residues at the amino terminus (Tyr-140, -160, -174). Substitution of the three tyrosines at the amino terminus to phenylalanine generated the Vav1/H110033YF mutant, which increased stimulation of NFAT transcription, enhanced GEF activity toward Rac, and promoted increased focus formation in agar when ectopically expressed in NIH3T3 cells and leading to its increased GEF activity (20). A solution structure of the Vav1 DH domain, including residues 170–189 of the Ac region, shows that Tyr-174 lies within an helix that binds to part of the GTPase interaction site, occluding access to the GTPase. Phosphorylation of Tyr-174 causes dissociation of this helix from the DH domain, relieving the autoinhibition (21). Internal dynamics are required for and control both basal activity and full activation of the autoinhibited DH domain (22).

Recent high resolution x-ray structure of the DH-PH-C1 domains of Vav1 in complex with Rac1 revealed a novel mechanism of Vav1 regulation; the PH and C1 domains contribute to efficient GEF activity by stabilizing the DH domain structure rather than by direct contact with GTPase (23).

Although the contribution and importance of Tyr-142, -160, -174 have been studied in depth, the significance of other tyrosine residues in Vav1 has not been analyzed. Recently, several of the carboxyl Vav1 tyrosine residues were shown to be phosphorylated in cancer cells and in Jurkat T cells (24–26), raising the possibility that these tyrosine residues also play an important role in Vav1 function. Also, Tyr-745 was recently identified as a tyrosine residue crucial for maturation of acute promyelocytic leukemia-derived cells (27). We undertook the experiments described here to explore the contribution of tyrosine residues at the carboxyl terminus of Vav1 to its biological activities and investigate their possible interactions with other proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Jurkat leukemia T cells, J.Vav1 cells (Jurkat T cells lacking Vav1 expression) (28), and pancreatic cancer cells, Panc1 (29), were grown in RPMI and Dulbecco’s modified Eagle’s medium (respectively) containing 10% fetal calf serum. Human embryonic kidney 293 cells (HEK293T) were grown in

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**FIGURE 1. Molecular structure of Vav1.** A, the location of the following Vav1 domains is indicated: calponin-homology (CH) domain; acidic (Ac) motif that contains three tyrosine residues as indicated; a DH domain; a PH domain; a C1 domain; a proline-rich region (–PPP–); two nuclear localization signals (NLS); and two SH3 domains and an SH2 domain. Tyrosine residues mutated in the current study are underlined, and the number of the residue is indicated underneath. B, sequence alignment of Vav1 among different species. The tyrosine residues mutated in this study are highlighted in human Vav1 in red as well as by a red asterisk. C, sequence alignment of the carboxyl terminus of human Vav1, Vav2, and Vav3.
Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

**Activation of Cells**—Jurkat T cells and J.Vav1 cells (1 × 10⁶ cells/ml) were activated with anti-CD3 OKT3 and anti-CD28 mAbs for 30 min on ice and then further incubated with Goat anti-mouse IgG for 3 min at 37 °C (30). Panc1 cells were grown to subconfluence and then starved in Dulbecco’s modified Eagle’s medium without serum for 24 h. Cells were then treated with RPMI containing 100 ng/ml human EGF for 5 min at 37 °C.

**Transfection**—Panc1 cells were transfected with the appropriate plasmids using the jetPEI transfection reagent according to the manufacturer’s instructions (Autogen Bioclear). Jurkat and J.Vav1 cells were electroporated with the indicated DNAs as described (30). The cells were then resuspended in RPMI supplemented with 10% fetal calf serum and grown for an additional 48 h before use in the various experiments. Transfection of HEK293T was performed using calcium phosphate reagents.

**Expression Vectors**—To facilitate comparison of Vav1 mutants, we cloned all cDNAs in the same plasmid backbone. We utilized the pMEX vector, a noncommercial mammalian expression plasmid that regulates gene expression through the murine mammary tumor virus long terminal repeat promoter for studies in Jurkat T cells, J.Vav1, and Panc1. GST fusion proteins that we used were described previously (12). Also, we used a pSam68-Myc-tagged vector (31).

**Immunobilization of Bacterial Fusion Proteins on Glutathione-Sepharose Beads**—Fusion proteins were purified from Escherichia coli bacteria and bound to glutathione-Sepharose beads (Amersham Biosciences) as described previously (32, 33).

**Antibodies**—Polyclonal anti-Vav1 antibodies were raised in rabbits against a specific peptide of Vav1, residues 528–541 (34). Other antibodies used are anti-Tyr(P) (4G10) monoclonal anti-Vav1 antibodies (Upstate Biotechnology, Lake Placid, NY); anti-Myc (clone 9E10), anti-phosphotyrosine (4G10; Upstate Biotechnology); anti-FLAG antibodies (Sigma); anti-CD28 (R&D Systems); anti-CD3 (OKT3, IgG2a; American Tissue Culture Collection, Manassas, VA); rabbit Anti-CSK polyclonal antibody (Upstate Biotechnology); and monoclonal anti-rabbit IgG and native peroxidase antibody produced in mouse (clone RabT-50, Sigma) that do not detect the heavy chain of the antibodies.

**Immunoprecipitation and Immunoblotting**—Cell lysis, immunoprecipitation, and immunoblotting procedures were performed as described previously (32).

**NFAT Assay**—J.Vav1 T cells were transfected as described (35) with the following plasmids (8 µg unless otherwise indicated): pSK115 encoding wild-type Vav1, empty vector, NFAT-luc, and Renilla. Eighteen hours later, cells were activated with anti-CD3 for 6 h and then lysed. Luciferase activity was measured with a Dual-Luciferase assay kit (Promega, Madison, WI) using a luminometer (Lumat LB 9506; Berthold Technology). Relative luciferase activity was defined as the ratio of firefly luc activity to Renilla luc activity.

**GEF Activity of Vav1 in Panc1 Cells**—Panc1 cells (2.5 × 10⁶) were transfected with 4 µg of empty vector, Vav1, or Vav1 mutants together with 1 µg of FLAG epitope-tagged Rac plasmid as indicated. The extent of GTP-bound Rac to P²³-activated kinase was determined as described previously (36).

**Soft Agar Assay**—The soft agar assay was carried out in 6-well culture dishes. The bottom of each well was coated with 3 ml of medium containing 0.8% soft agar. Triplicates of 1 × 10⁵ cells were plated on top in 3 ml of medium containing 0.3% soft agar. Experiments were repeated three times. Cells were fed every 3rd day with 0.3 ml of medium. After 14 days, colonies were counted.

**Mutagenesis**—Mutagenesis was performed according to the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenesis was performed on a plasmid encoding wild-type Vav1 in pMEX, a vector capable of driving high levels of protein expression via its mouse mammary tumor virus long terminal repeat. The primers used were as follows (the substituted nucleotide is bold and underlined): Y826F, 5'-GGGGGAATTCATTTTGGCCGGGTGGTGCTG-3' and 3'-CCACCATCCCTTTTAAGAACCAGGC-5'; Y836F, 5'-GCCAACACTCTCTTGTGAGGAAGATTATTCGG-3' and 3'-CCACGCGAGGGTGGTGAGGAAGATTATTCGG-5'; Y841F, 5'-GATTTTCTGAGAATACCTGG-3' and 3'-GCACTCCTCCTTCAAAGAAGCTTATGACGAC-5'; Y844F, 5'-GAATCTCTGAGCCCTT-3'.
Peptide Binding Assay Using SH2/PTB Domains (Rosette Assay)—N-terminal biotinylated peptides corresponding to human Vav1 Tyr-826 (RGEIYGRVGWFP) and Tyr-841 (ANYVEEDYSEYC), both phosphorylated and unphosphorylated forms, were synthesized (GL Biochem Ltd. and CASLO Laboratory, respectively). A nitrocellulose membrane with pore size 0.1 μm (BA79, Schleicher & Schuell) was coated with 0.5% gelatin (G-2500, Sigma) for 30 min at 37 °C. Peptides were dissolved in N,N-dimethylformamide, diluted in spotting solution (180 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 15% β-mercaptoethanol, 0.03% bromphenol blue) at 15 mM, and manually spotted onto the dried membrane in multiple duplicate arrays (0.1 μl/spot). After drying, the arrayed membrane was fixed in 4% paraformaldehyde phosphate-buffered saline solution at room temperature for 5 min, rinsed with phosphate-buffered saline, incubated in equilibration buffer (10 mM CAPS, pH 11.0, 20% methanol) for 30 min, and then blocked for 1 h in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20) containing 10% nonfat milk, 1 mM EDTA, and 1 mM Na3VO4. N-terminal GST-tagged human SH2/PTB domains were prepared and labeled as described (12, 37). Each SH2/PTB probe was incubated at a concentration of 4 μg/ml with a set of peptide spots in separate wells of a 96-well chamber plate. After ECL-based detection and densitometric quantitation, resulting values were used to compare different peptides and SH2/PTB probes.

RESULTS

Effect of C-terminal Tyrosine Amino Acid Substitutions on NFAT Activation in T Cells—Vav1 encodes four tyrosine residues at its most carboxyl terminus: Tyr-826, Tyr-836, Tyr-841, and Tyr-844 (Fig. 1A). Tyr-826 and Tyr-836 are embedded within the carboxyl SH3 region of Vav1, whereas Tyr-841 and Tyr-844 are located outside of the SH3 region (Fig. 1A). All four of these tyrosine residues are conserved among
mammalian Vav1 proteins (Fig. 1B), suggesting their importance. However, only Tyr-836 is conserved between human Vav1, Vav2, and Vav3 proteins (Fig. 1C). To explore the role of these C-terminal tyrosines in the physiological functions of Vav1, we substituted phenylalanine for tyrosine at each of these residues individually, creating the mutants Y836F, Y844F, Y826F, and Y841F. Because Vav1 participates in multiple signaling pathways with very different outcomes, we used two different experimental systems to assess the effects of these tyrosine mutants. T cells, where Vav1 is normally expressed, serve as a model for the physiological actions of Vav1 (13–16). Epithelial cells, in which Vav1 is usually absent but might be ectopically expressed in human malignancies, are a model for its pathological actions (29, 38, 39).

We first analyzed whether the mutants that we created are phosphorylated following activation of Vav1 in T cells. For that purpose, J.Vav1 cells, derivatives of Jurkat T cells in which Vav1 expression was knocked down (28), were transfected with an empty vector, Vav1, Y826F, Y836F, Y841F, or Y844F (Fig. 2A) and activated using anti-CD3 and CD28 mAbs to stimulate the T cell receptor (TCR). Western blotting revealed no difference in overall tyrosine phosphorylation between Vav1 and any of the mutant proteins. This is probably because phosphorylation at other tyrosine residues in Vav1 masks any change in phosphorylation at the single residues that we mutated (Fig. 2A).

Vav1 appears to be necessary for full activation of the transcription factor NFAT following TCR stimulation (35). To analyze the ability of our Vav1 tyrosine mutants to stimulate NFAT, we co-transfected J.Vav1 cells with a luciferase reporter driven by a promoter containing NFAT binding sites and plasmids encoding either Vav1 or the Vav1 mutants (Fig. 2B). Following activation of the TCR by anti-CD3 and CD28 mAbs, we assessed luciferase activity in cell extracts. As expected, in cells transfected with Vav1, luciferase activity increased significantly following TCR activation. In cells transfected with the Vav1 mutants Y836F, Y841F, and Y844F, TCR activation induced luciferase activity to levels similar to those obtained with wild-type Vav1. Surprisingly, transfection with Y826F doubled the luciferase activity when compared with Vav1, suggesting that this tyrosine residue contributes to an autoinhibitory effect on Vav1-stimulated NFAT activity (Fig. 2B).

**Effect of C-terminal Tyrosine Amino Acid Substitutions on Vav1 Transforming Activity in Pancreatic Cancer Cells**—We and others have detected ectopic expression of Vav1 in neuroblastoma (38), pancreatic ductal adenocarcinomas (29), and lung cancer (39). Vav1 plays a role as a signal transducer protein in these malignancies, and amino acid substitutions of tyrosines at the amino terminus of Vav1 have been shown to enhance its ability to transform fibroblasts (20). It was therefore of interest to analyze whether the C-terminal Tyr mutants maintain transforming ability. Panc1 cells, pancreatic cancer cells that do not express Vav1, were previously shown to be highly useful in analyzing the transforming ability of Vav1 and several of its mutants (29). Panc1 cells were transfected with an empty vector, Vav1, Y826F, Y836F, Y841F, or Y844F and stimulated with EGF (Fig. 3A). Vav1 and all of the mutants were highly phosphorylated in Panc1 cells following EGF treatment. The increase in tyrosine phosphorylation of Vav1 and the mutants was more dramatic following stimulation of Panc1 cells with EGF than following TCR stimulation in J.Vav1 cells (Fig. 3A versus Fig. 2A). This may stem from the fact that there are several kinases in J.Vav1 cells (Lck, Fyn, hematopoietic cell kinase, and more) that phosphorylate Vav1 in the absence of stimulation, creating higher basal levels of phosphorylation. These kinases are not expressed in Panc1 cells.

To test whether substitution of any of the C-terminal Tyr residues affects the transforming potential of Vav1, we assessed the effect of our mutants on Panc1 growth in soft agar. Although Panc1 cells transfected with Vav1 exhibit enhanced growth in agar when compared with cells transfected with vector only as reported previously (29), cells transfected with the Vav1 C-terminal tyrosine mutants did not; the number of foci formed in cells transfected with any of the mutants was not significantly different from cells transfected with empty vector (Fig. 3B). Furthermore, the size of the foci formed by Panc1 cells transfected with vector only or with any of the Vav1 mutants was considerably smaller than those formed by cells transfected with wild-type Vav1 (Fig. 3C). Thus, all four of the tyrosine residues at the carboxyl terminus of Vav1 are required for its transforming activity in Panc1 cells.

**Vav1 C-terminal Tyrosine Mutants Lose Their Capacity to Activate Rac**—We chose to investigate two of these carboxyl-terminal tyrosine residues, Tyr-826 and Tyr-841, more completely for the following reasons. Bioinformatics analysis predicts that Tyr-826 and Tyr-841 are the likeliest to be phosphorylated (0.95 and 0.969 score respectively; NetPhos 2.0 Server). In addition, these tyrosine residues are conserved in Vav1 but not in Vav2 and Vav3 (Fig. 1C), suggesting that they are important for a Vav1-specific function. Tyr-826 is within the SH3 region, whereas Tyr-841 is outside it (Fig. 1B).

One of the characteristic functions of Vav1 is its ability to regulate Rac1 activity (16). Amino acid substitutions for Tyr(P)-174 at the amino terminus were shown to enhance Vav1 activity toward Rac (20). Therefore, it was of great interest to determine whether the C-terminal Tyr mutations also participate in or modulate Vav1 GEF activity for Rac. Panc1 cells were...
transfected with Vav1, Y826F, or Y841F along with a FLAG-tagged Rac (Fig. 4). The level of Rac activation was analyzed using a Pak1-GST pulldown assay. Our results clearly indicate that substitution of either Tyr-826 (lane 4) or Tyr-841 (lane 6) with phenylalanine decreases the level of Rac1-GTP significantly, attesting to the importance of these tyrosines for Vav1 exchange activity.

Identification of Proteins That Associate with Vav1 at Tyr-826 or Tyr-841—To identify possible binding partners at Tyr-826 and Tyr-841, we performed an in vitro binding assay using a large set of GST-tagged SH2 and PTB domains (Fig. 5). N-terminal biotinylated peptides corresponding to tyrosines 826 and 841 of Vav1 (both phosphorylated and unphosphorylated forms) were synthesized, immobilized, and screened with 116 different SH2/PTB domains using a high throughput dot blotting assay (Fig. 5A) (12). Multiple SH2 domains interacted with each of the peptides in a phosphorylation-dependent manner, and the SH2 binding patterns for the Tyr-826 and Tyr-841 peptides were distinct (Figs. 5, B and C, and 6; supplemental Table 1 and supplemental Fig. S1, A–D). For example, SH2 domains of the Shc, C-terminal Src kinase (Csk), Abl, and Sap families bound strongly to tyrosine 826. Intriguingly, most of these are predicted to down-regulate signaling, consistent with our finding suggesting that Tyr-826 participates in suppressing NFAT activity. A different set of SH2 domains, including those from the SH2-B and Src families, bound tyrosine 841 (Figs. 5, B and C, and 6; supplemental Table 1 and supplemental Fig. S1, A–D). As shown in Figs. 5 and 6, SH2 domain probes differed in their ability to bind the two phosphorylated peptides, but none of them bound to unphosphorylated peptides.

To validate our results, we analyzed the binding of several of the identified proteins to wild-type Vav1, Y826F, and Y841F. Binding was analyzed in both J.Vav1 cells (Fig. 7, A and B) and Panc1 cells (Fig. 7, C and D). Cells were transfected with Vav1, Y826F, Y841F, or vector alone. Following stimulation either with anti-CD3 and CD28 mAbs (Fig. 7, A and B) or with EGF (Fig. 7, C and D), cell lysates were bound either to control GST carrying mutated Vav (GST-G830V) or to control GST-SH2-B and GST-Src. Our results clearly demonstrate that although Vav1 and Y826F from activated J.Vav1 or activated Panc1 cells bind to SH2-B and Src, Y841F does not associate with either fusion protein, although it is equally well expressed (Fig. 7, A and C). These binding results extend the data obtained by the SH2 profiling experiments, demonstrating that full-length Vav1 associates with SH2-B and Src at Tyr-841 in a phosphorylation-dependent manner in living cells.
We next wished to verify that the substitutions we made for C-terminal tyrosines had no general effects on protein folding or normal function of the carboxyl SH3 region (SH3C). For that, we chose two Vav1 mutants embedded within the SH3C (Y826F and Y836F) and a Vav1 mutant at the carboxyl-terminal region, but outside the SH3C, Y841F and analyzed their binding to Sam68, which we previously identified as a Vav1 SH3C-as-sociating protein (31). This association is mediated through proline-rich motifs in Sam68 and is not dependent on Vav1 tyrosine phosphorylation (31). We transfected HEK293T cells with Myc-tagged Sam68 along with empty vector, Vav1, Y826F, Y836F, or Y841F. Cell extracts were immunoprecipitated with anti-Vav1 antibodies, and the level of Sam68 associated with Vav1 was determined by immunoblotting with anti-Myc mAbs (Fig. 8). Our data indicate that all three mutants associate with Sam68 to a similar extent as wild-type Vav1, suggesting that normal folding of Vav1 and other functions of the SH3C region are intact in the mutant proteins.

Our SH2 profiling indicated that Tyr-826 associates with several proteins that are predicted to down-regulate signaling such as CSK (40). Because acute elimination of CSK in Jurkat T cells and primary T cells triggers an increase in TCR-dependent NFAT stimulation (40), it was conceivable that loss of binding between Vav1 and CSK might lead to an increase in NFAT stimulation driven by Y826F. Indeed, our results demonstrate that Vav1 associates with CSK in Jurkat T cells in vivo (Fig. 9A). Moreover, CSK associates in vivo with wild-type Vav1 and Y841F but loses its binding to Y826F (Fig. 9B). Similar results validated the specific and phosphorylation-dependent interaction of CSK with Vav1 Tyr-826 in activated J.Vav1 and Panc1 cells in a GST binding assay (data not shown).

**DISCUSSION**

Our investigation into the roles of four C-terminal tyrosine residues in Vav1 revealed that tyrosine phosphorylation at these sites regulates functions important for the physiological and pathological actions of Vav1. Although substitution of phenylalanine for tyrosine at Tyr-836, Tyr-841, or Tyr-844 had no effect on NFAT stimulation by Vav1, substitution at Tyr-826 resulted in 2-fold higher NFAT stimulation than seen with wild-type Vav1 (Fig. 2B). We also found that replacing any of these four tyrosines with phenylalanine dramatically reduced the transforming ability of Vav1 in Panc1 cells (Fig. 3, B and C). In addition, at least two of the

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**FIGURE 6.** Quantified SH2/PTB screening data. The signal intensities of multiple independent experiments with duplicate spots were quantified using densitometry, and the net binding intensity was obtained by subtracting the value of binding to unphosphorylated peptide from the value of binding to phosphorylated peptide. The bar graph indicates the mean value and S.E. for each SH2/PTB domain probe.
mutants, Y826F and Y841F, had significantly reduced GEF activity toward Rac (Fig. 4), indicating that these residues play a role in GEF activity. Importantly, Y826F and Y841F associate with Sam68 to a similar extent as wild-type Vav1, suggesting that the ability of the carboxyl SH3 region to bind proteins with proline-rich domains is intact in these mutant proteins (Fig. 8).

Thus, the loss of GEF activity toward Rac is likely a direct result of loss of tyrosine phosphorylation at Tyr-841 and Tyr-826 rather than changes in protein folding or SH3C function. Although studies clearly show that the amino terminus of Vav1 regulates its GEF activity through autoinhibition of GTPase activation (21, 22, 41) and stabilization of the DH domain structure by the PH and C1 domains (21, 23), it is not clear how the Y826F and Y841F mutations at the C-terminal disrupt Vav1 GEF activity.

Our results unequivocally indicate that full GEF activity is not required for activation of NFAT-mediated transcription because eliminating tyrosine phosphorylation at Tyr-826 dramatically reduces GEF activity while actually boosting NFAT-mediated transcription. This is in contrast to earlier reports indicating that Vav1 GEF activity is required for TCR-induced NFAT- and NFκB-mediated transcriptional activation (42–44) but in agreement with recent studies (45, 46). For instance, truncated Vav1 with constitutive GEF activity did not enhance NFAT-dependent transcription (35, 42), whereas a GEF-deficient mutant in the Dbl region did, suggesting that GEF activity is not required for Vav1 to stimulate NFAT-dependent transcription (45). Additionally, using a mouse carrying a Vav1 GEF-defective gene, Saveliev et al. (46) demonstrated that the GEF activity is important for the selection of thymocytes and
for optimal activation of T cells but not for TCR-induced calcium flux, activation of ERK, or NFAT stimulation. Our results strongly support the notion that Vav1-mediated NFAT activity is independent of its GEF activity (Figs. 2 and 4).

Although GEF activity is not critical for NFAT stimulation, it is essential for various aspects of transformation, such as proliferation, migration, and metastasis (47). Consistent with this, the mutants we generated are not efficient in propagating growth in agar (Fig. 3). Our results are compatible with a previous study showing that Vav1 protein lacking GEF function failed to enhance Panc1 cell proliferation (29). Also, we demonstrated previously that Vav1 is expressed in many lung cancer cell lines, where it functions as a GEF for Rac, lending further support to a link between Vav1 GEF activity and proliferation (39). Loss of GEF activity in the Y826F and Y841F mutants is likely to be primarily responsible for the loss of transforming activity in Panc1 cells.

We identified SH2-containing proteins that interact with Tyr-826 and Tyr-841 using SH2 profiling, a recently developed proteomic method that uses the cellular Tyr(P) binding apparatus in in vitro binding assays (12, 37). The sensitivity, specificity, and high throughput capacity of SH2 profiling are highly advantageous for rapid and functional screening of SH2 domain-containing proteins, and the assay is quantitative, providing a rank order of binding of high affinity SH2 domains at each site. SH2 profiling revealed that Tyr-826 and Tyr-841 associate in a phosphorylation-dependent manner with remarkably distinct groups of proteins (Figs. 5 and 6). Although some of these associations were suggested by previous findings, our results are the first to uncover the nature of these direct protein-protein interactions. It is conceivable that loss of interaction with one or more of the SH2-containing proteins we identified here also contributes to loss of transforming activity. Furthermore, loss of interaction with SH2-containing proteins might be responsible for increased NFAT activity in the Y826F mutant.

We found that Tyr-826 associates with high affinity with Shc, Csk, Abl, Sap, Shp-2, and Crk. Most of these proteins are associated with negative regulation of signaling. This is consistent with our finding that the Y826F doubled NFAT activity (Fig. 2), strongly suggesting that Tyr-826 participates in an autoinhibitory function. Of particular interest, previous studies have shown that acute elimination of Csk in Jurkat T cells and primary T cells triggers an increase in TCR-stimulated responses, including NFAT stimulation (40). Loss of binding between Y826F and Csk (Fig. 9 and data not shown) might mimic the effects of Csk knockdown, resulting in the increase in NFAT activity we observed with the Y826F mutant (Fig. 2A).

Our finding that Tyr-826 associates with Shc is consistent with previous studies suggesting that interaction between Vav1 and Shc affects T cell functions including chemotaxis (48). Another protein we found to interact with Tyr-826, Abl, was shown previously to be involved in integrin-Vav1 signaling in neutrophils through its ability to partially phosphorylate Vav1 (48). Although an earlier study reported constitutive association between the c-Abl kinase SH3 domain and Vav1 SH2 domain, it was performed under experimental conditions in which Vav1 is not phosphorylated (49). Our data clearly show a phosphorylation-dependent association between Tyr-826 and c-Abl (Figs. 5 and 6); thus, the association between Abl and Vav1 that is important in integrin signaling may occur at Tyr-826 within the SH3C region of Vav1, rather than at SH2. Additional studies reported an association between bcr-abl, Vav1 (50, 51), and the adaptor Crk (52). Binding of inhibitory natural killer cell receptors to human leukocyte antigen class I on target cells (53) induces tyrosine phosphorylation of Crk and dephosphorylation of Vav1. However, these studies did not determine whether these events involved direct association between Vav1 and Crk, as is now revealed by our profiling assay.

Phosphorylated Tyr-841 associated with a completely different group of SH2 domain proteins in our profiling assay. Prominent among these are Src and other Src family kinases (Fig. 6). Src family members such as Lck, Yes, and Fyn play a major role in controlling Vav phosphorylation upon T cell activation, regulating its activity in hematopoietic cells (54–58). Most studies reveal that tyrosines Tyr-142, Tyr-160, and Tyr-174 are phosphorylated when Vav1 associates with these tyrosines.
phorylated by Lck and that this phosphorylation triggers Vav1 GEF activity (56, 57). Based on the results presented here and on bioinformatic predictions, other tyrosine residues, including Tyr-841, may also be phosphorylated by members of the Src kinase family. We also demonstrated an association between Tyr-841 and Src homology 2-B adaptor protein (SH2-B). In addition to its classical adaptor functions, SH2-B demonstrates a unique ability to enhance the activity of the cytokine receptor-associated tyrosine kinase JAK2 and several receptor tyrosine kinases (59). Recently, SH2-B family members were found to participate in the regulation of actin cytoskeleton, potentially by binding to Vav proteins, such as Vav3 (60). APS, another member of the family of adaptor proteins that includes SH2-B, also binds to Vav1 in our assay. APS was shown previously to bind both Vav1 and Vav3 and to enhance Vav1- or 3-induced focus formation, with a greater effect on Vav3-induced transformation (60). Our results also demonstrate association between Tyr-841 and Sap, shedding new light on their function in natural killer cells in which 3BP2 acts downstream of Sap. Increases CD244 phosphorylation and links downstream of Sap, increases CD244 phosphorylation and links to Vav proteins, such as Vav3 (60). APS, another member of the family of adaptor proteins that includes SH2-B, also binds to Vav1 in our assay. APS was shown previously to bind both Vav1 and Vav3 and to enhance Vav1- or 3-induced focus formation, with a greater effect on Vav3-induced transformation (60). Our results also demonstrate association between Tyr-841 and Sap, shedding new light on their function in natural killer cells in which 3BP2 acts downstream of Sap, increases CD244 phosphorylation and links the receptor with phosphatidylinositol 3-kinase, Vav, phospholipase C-γ, and protein kinase C to achieve maximum natural killer killing function (61–63).

Our SH2 profiling along with the biochemical and functional assays provide us with new insight into how Vav1 C-terminal phosphorylation functions in normal physiology and transformation. These studies also define a new set of downstream signaling pathways critical for the physiological and transforming functions of Vav1 that remain to be studied.

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