Vav1 Is a Component of Transcriptionally Active Complexes

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Abstract
The importance of the hematopoietic protooncogene Vav1 in immune cell function is widely recognized, although its regulatory mechanisms are not completely understood. Here, we examined whether Vav1 has a nuclear function, as past studies have reported its nuclear localization. Our findings provide a definitive demonstration of Vav1 nuclear localization in a receptor stimulation–dependent manner and reveal a critical role for the COOH-terminal Src homology 3 (SH3) domain and a nuclear localization sequence within the pleckstrin homology domain. Analysis of DNA-bound transcription factor complexes revealed nuclear Vav1 as an integral component of transcriptionally active nuclear factor of activated T cells (NFAT)– and nuclear factor (NF)κB-like complexes, and the COOH-terminal SH3 domain as being critical in their formation. Thus, we describe a novel nuclear role for Vav1 as a component and facilitator of NFAT and NFκB-like transcriptional activity.

Key words: Vav • nuclear translocation • nuclear factor of activated T cells • calcium influx • protein subdomains

Introduction
Engagement of immune receptors initiates a wide variety of biochemical responses that lead to the activation of T, B, and mast cells. Activation of tyrosine kinases is the event most proximal to receptor engagement and results in the subsequent assembly of signaling modules comprised of multiple tyrosine phosphorylated proteins including kinases, phosphatases, adaptors, and other effector proteins like the 95-kD protooncogene Vav1 (1–7). Vav 1 is expressed exclusively in hematopoietic cells whereas Vav2 and Vav3 are ubiquitous homologues that are coupled to several growth factor receptors (8, 9). Vav 1 is a structurally complex protein containing a Calponin homology domain (CH), an acidic region, a Dbl homology domain (DH), a pleckstrin homology domain (PH), a cysteine-rich domain, and three Src homology domains (SH3-SH2-SH3) (10). Via its DH domain Vav 1 has been shown to catalyze the GDP-GTP exchange of Rho family GTPases with some preference for the Rac GTPase (11). Interestingly, Vav1 also contains two putative nuclear targeting sequences (NLS) whose presence is suggestive of Vav1 nuclear localization but whose function has not formally been demonstrated.

In recent years gene targeting deletion experiments revealed the importance of Vav1 in T lymphocyte development and function. A profound defect in positive and negative selection of T cells has been described (12–14) that results in low numbers of mature T cells in the periphery. The cells that manage to escape the thymus are impaired in

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*Abbreviations used in this paper: BMMC, bone marrow–derived mast cell; CH, calponin homology; DH, Dbl homology; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; JNK, c-jun NH2 terminal kinase; NFAT, nuclear factor of activated T cells; NF, nuclear factor; NLS, nuclear localization sequence; PH, pleckstrin homology; PLC, phospholipase C; RBL, rat basophilic leukemia; SH, Src homology; wt, wild-type.
antigen-induced calcium signals and proliferation. These cells also show decreased activation of nuclear factor of activated T cells (NFAT)- and NFκB-transcription factors and reduced expression of activation markers in response to TCR stimulation. Moreover, the cells failed to form actin-dependent patches and caps that are the hallmark of the immune synapse (15–18). Many of these defects may be explained by the role of Vav1 in the activation of Rho family GTPases, which function to reorganize the actin cytoskeleton, and by its regulation of phospholipase C (PLC)γ-dependent calcium responses as seen in Vav1−/− mast cells (19).

Whereas it is clear that Vav1 localizes to the plasma membrane in activated T (4), B (7), and mast cells (20), early studies suggested a possible nuclear localization for Vav both in rat basophilic leukemia (RBL) cells and in T lymphoid cells upon prolonged FceRI engagement or pro-lactin stimulation, respectively (21, 22). Subcellular fractionation, immunofluorescence, and electron microscopic studies also indicated the partial nuclear localization of Vav in T cell lymphomas (Jurkat), granulocytes (HL60), and megakaryoblastic cells (UT7) even in the absence of any stimulus (23–25). Several studies have also shown an interaction of Vav with nuclear proteins. These include Ku-70, a component of the DNA-dependent protein kinase complex (23), the ribonucleoprotein hNRNP, involved in RNA maturation and nucleocytoplasmic transport (25), and ENX-1, the human homologue of a member of the polycomb group of proteins involved in transcriptional regulation of Drosophila homeobox genes (26, 27). While the SH2 domain of Vav was demonstrated to be essential in receptor proximal events by mediating its plasma membrane localization and interaction with a linker for activation of T cells (LAT)-organized signaling module both a leucine stretch within the NH2-terminal CH domain (responsible for the interaction with Enxl) and the COOH-terminal SH3 (hNRNP and Ku70 interacting region) have been demonstrated to be domains that interact with nuclear proteins. Altogether, these results suggested a molecular function for Vav1 in the nucleus.

In this study we undertake the challenge of elucidating a nuclear role for Vav1. First, we demonstrate unequivocally that Vav1 translocates to the nucleus upon prolonged stimulation of the FceRI and that its movement to the nucleus is dependent on one of the two previously identified putative NLS. Furthermore, we find that Vav1 nuclear targeting is also under the control of the COOH-terminal SH3 (C-SH3) domain, which serves to sequester its presence in the cytoplasm. Most importantly, however, is the finding that Vav1 is a nuclear partner of the transcription factor NFAT (28) and a nuclear factor (NF)κB-like factor (29). For NFAT, Vav1 serves to facilitate its movement to the nucleus and forms part of a transcriptionally active complex that binds the NFAT binding site of the IL-2 promoter.

Materials and Methods

Antibodies and Reagents. Supernatants from the hybridoma Hi-DNP-e-26.82 were used as a source of anti-DNP specific IgE for sensitization experiments (1/200). Antibodies to NFATp and Vav1 were purchased from Upstate Biotechnology. Antibody to Vav 1 was also provided by Dr. X. Bustello (University of Salamanca, Salamanca, Spain). The monoclonal anti-CD3 (UCHT1), anti-Myc tag (9E10), and anti-CD28 were provided by Dr. G. Bismuth (ICGM, Paris, France), Dr. S. Fischer (ICGM), and Dr. D. Olive (U119 Inserm, Marseille, France), respectively. Monoclonal antibodies to NFATc and poly (ADP-ribose) polymerase (PARP) and polyclonal antibody to c-jun NH2 terminal kinase (JNK) were purchased from Santa Cruz Biotechnology, Inc. Antibodies to glutathione S-transferase (GST) and c-Jun (c-Jun was a gift of V. Tybulewicz, National Institute of Medical Research, Mill Hill, UK) were provided by Dr. P. Mayeux (ICGM).

Cell Culture and Stimulation. Bone marrow-derived mast cells (BMMCs) from Vav-null and from wild-type (wt) litter mates were generated from femur bone marrow by incubation in IL3 for 4 wk as described previously (30). Mast cell differentiation and phenotype were confirmed by toluidine blue staining. Purity was usually more than 95%. Based on our previous experiments, monitoring exocytosis at the single cell level by annexin V binding, more than 80% of the cells degranulated after IgE cross-linking (31). RBL-2H3 cells were maintained as reported (29). For stimulation, cells were sensitized for 1 h in culture medium (2 × 106 cells/ml) with DNP-specific IgE. After 1 h, the cells were washed and challenged with DNP-human serum albumin (HSA) (100 ng/2 × 106 cells/ml) for the indicated period of time at 37°C. Jurkat cells were maintained as described previously (32). Stimulation was performed with a combination of anti-CD3 mAb (10 μg/ml) and anti-CD28 (10 μg/ml).

DNA Constructs. pEF-Myc-tagged Vav1 was provided by Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA). pEF-Myc–tagged Vav1 deleted of the CSH3 domain (delCSH3), of the NH3 domain (delNH3), of the first consensus NLS sequence (delNLS1), or the second NLS sequence (delNLS2) were obtained by recombinant DNA technology by replacing the wt fragments with an appropriate mutated fragment generated by PCR, as follows: for delCSH3: the wt fragment between Bsu36I at 1,988 bp and BstXI at 2,508 bp in the ORF was replaced by a fragment that deleted amino acids 787 to 846. For delNH3: the fragment between AflIII at 1,406 bp and Bsu36I at 1,988 bp of the ORF was replaced by the corresponding fragment deleted of amino acids 605–662. For delNLS1 and delNLS2: the fragment between AflIII–Bsu36I was replaced by the corresponding fragment deleted of amino acids 487–494 and 576–589, respectively. pEFdelNLS1CSH3 and pEFdelNLS2CSH3 were constructed by the additional replacement of the Bsu36I–BstXI fragment in delNLS1and delNLS2 by the corresponding fragment isolated from delCSH3.

Immunofluorescence Staining and Confocal Analysis. RBL cells and BMMC-derived mast cells were processed for confocal imaging as described previously (32). Antibodies to Vav and myc were used at a 1/500 dilution followed by incubation with donkey anti-mouse F(ab)2 coupled to FITC (Jackson ImmunoResearch Laboratories; 1/100 dilution). The coverslips were mounted in Mowiol with Dabco antifading and the nucleus was labeled with DAPI. Immunofluorescence was analyzed by confocal laser-scanning microscopy with high numerical aperture lens (63 × 1, 3NA; Bio–Rad Laboratories) at 522/535 wavelength. Images were single optical sections obtained as TIFF files.

Subcellular Fractionation and Nuclear Extracts. All procedures were performed at 4°C. For nucleus and cytoplasmic fractionation procedures, cells were lysed (5 × 106 cells/ml) in buffer A
(10 mM Heps, pH 7.6, 15 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, 0.05% NP40, and protease inhibitors). Nuclei were pelleted by low speed centrifugation (1,000 g for 10 min at 4°C) and resuspended in buffer C (50 mM Heps, pH 7.8, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol and protease inhibitors). The nuclei were lysed (5 × 107 nuclei/ml) by addition of 10% (vol/vol) of 3 M ammonium sulfate, pH 7.9 followed by rotation at 4°C for 30 min. The nuclear debris was pelleted at 100,000 g for 15 min. Soluble proteins in the nuclear supernatant were precipitated by addition of an equal volume of ammonium sulfate 3 M, pH 7.9, pelleted by centrifugation at 50,000 g for 10 min and resuspended in 100 μl of buffer C.

The cytoplasmic fraction was recovered after pelleting of the nuclei. The latter was stabilized by addition of 10% (vol/vol) glycerol and 10% (vol/vol) of buffer B (0.3 M Heps, pH 7.8, 1.4 M KCl, 30 mM MgCl2). Soluble proteins were recovered by centrifugation at 200,000 g for 15 min. The recovered proteins were precipitated by adding an equal volume of 3 M ammonium sulfate, pH 7.9, and pelleted at 100,000 g for 10 min. The precipitated cytoplasmic proteins were resuspended in 100 μl of buffer C. Quantitation of protein concentrations in the extracts was determined by the BCA protein assay (Pierce Chemical Co.). To extract both soluble and DNA embedded nuclear proteins for the electrophoretic mobility shift assays, nuclear extracts were prepared according to the method of Dignam et al. (salt extraction with 370 mM NaCl) as described previously (29, 33).

**Immunoblotting, Gel Retardation Assays, and Cross-linking Experiments.** Immunoprecipitations and immunoblotting were performed by using previously described procedures (32) and immunoblotted proteins were revealed by enhanced chemiluminescence (Amersham Pharmacia Biotech). For electrophoretic mobility gel shift assays the following oligonucleotides (5′-end labeled) were used as probes: distal NFAT site of human IL-2 promoter: GGAGGAAAAAAC; distal NFAT site (28, 34). Transfections and determination of luciferase activity were performed as described previously (31).

**JNK Assays.** For JNK assay, JNK1 immunoprecipitated with goat polyclonal anti-serum to JNK1 (Santa Cruz Biotechnology, Inc.) was used to phosphorylate the GST-c-Jun 5–89 fusion protein (c-jun GST, UBD) for 10 min at room temperature. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylation was detected by autoradiography. To control for JNK1 levels the membrane was subsequently immunoblotted with anti-JNK1.

**Reverse Transcription PCR.** RBL cells were transfected with expression vectors encoding Vav1 or deleted Vav1 constructs. After IgE cross-linking, RNA was extracted and reverse transcribed. PCR was performed using specific primers for rat IL-2 and a house keeping gene GAPDH as described previously (19). As a positive control rat IL-2 was transcribed in vitro and 1 μl of a 1:100 dilution of the resulting mRNA was subjected to amplification by PCR.

**Gene Transfer and Calcium Measurements.** For virus-mediated gene transfer experiments into BMMC, the pSFV1 expression system, Vav1-green fluorescent protein (GFP) constructs, and infection procedure were as described previously (35). All procedures were performed 4 h after the initial infection. Intracellular calcium levels were measured as described (30). Briefly, cells were loaded with 16 μM Fura Red (Molecular Probes) in RPMI/2% FCS media for 45 min at 37°C. The cells were then incubated with IgE (1 μg/106 cells) on ice for 1 h and brought to room temperature for 20 min. The cells were resuspended in Tyrodes/BSA, and changes in dye fluorescence with time determined by flow cytometry after stimulation with 30 ng/ml of Ag, with subsequent stimulation by 1 μM thapsigargin. The changes in fluorescence of Fura Red with time, after Ag stimulation, was measured only for transfected cells (based on a histogram gate compared with noninfected cells) expressing GFP. The percentage of GFP+ cells varied from 9 to 24%.

**Results**

**Prolonged Stimulation of FcεRI Results in Nuclear Translocation of Vav1.** We wished to confirm and extend the prior observations of the nuclear localization of Vav1. For these studies we used the RBL-2H3 mast cell line, which contains a larger cytoplasmic compartment than T cells. Using confocal imaging, we found that Vav1 exhibited a cytoplasmic localization with a distinct exclusion from the nucleus (Fig. 1 A, NS) consistent with our prior observations (20). After challenge of IgE-sensitized cells with antigen for a brief period of time (5–15 min, data not shown), no nuclear localization could be visualized whereas longer stimulation periods (30 min or more) led to the movement of Vav1 to the nucleus (Fig. 1 A, S30 and S60). Quantitation of the relative amount of fluorescence in the nucleus of RBL cells (determined by DAPI staining) during stimulation showed that nuclear localization started to increase significantly after 30 min of continuous stimulation. The relative amount of the fluorescent signal present in the nucleus increased by over sixfold in 1 h (stimulated versus unstimulated cells). A similar translocation of Vav1, after prolonged FcεRI stimulation, was also observed in primary bone marrow derived mast cells (BMMC, Fig. 1 B) indicating that this response was
also seen in normal mast cells. No staining was detected when anti-Vav antibody was either depleted with an excess of Vav before incubation with the cells or incubated with vav−/− BMMCs (Fig. 1 B; control and data not shown). Additionally, we also observed some nuclear translocation of Vav1 in Jurkat T cells although a low level of nuclear fluorescence was also detectable in unstimulated cells (data not shown). Thus, these results dem-

Figure 1. Vav1 is translocated to the nucleus after prolonged FcεRI stimulation. DNP-specific IgE-sensitized RBL cells (A) or BMMCs purified as described in Materials and Methods (B) were maintained in complete medium and were then either left unstimulated (NS) or stimulated for 30 (S30) or 60 min (S60) with DNP-HSA. The localization of Vav1 was determined by single section confocal imaging using an antibody to Vav1 (UBI; 1/500 dilution) and a FITC-labeled secondary antibody (α Vav). Nuclei were stained in parallel with DAPI (DAPI) and merged images are on the right panel. Insets show higher magnification of one stimulated cell with anti-Vav Ab (green) and the corresponding merged image with DAPI, respectively. On the control confocal image anti-Vav antibody was depleted on GST-Vav before incubation with the cells (B, control).
onstrate that Vav1 is capable of translocating to the nucleus upon prolonged cell stimulation.

The COOH-terminal SH3 Domain of Vav1 Mediates Its Cytoplasmic Retention. Upon stimulation of antigen receptors, Vav1 localizes to lipid rafts where it interacts with other tyrosine phosphorylated partners preferentially via its SH2 domain (6, 20). In contrast, most of the putative nuclear partners of Vav1 interact with its COOH-terminal SH3 domain, an exception is Enx1 which interacts with the CH domain. Our initial experiments using a Vav1 construct in which the adaptor region (SH3-SH2-SH3) was deleted, showed a constitutive nuclear localization for Vav1 (data not shown), thus providing a clue for regulatory control in the COOH terminus of Vav1. This was not due to the SH2 domain as Vav1 with mutation or deletion of this domain localized to the cytoplasm (20). Thus, to further delineate the importance of each SH3 domain to the nuclear localization of Vav1 we constructed Vav1 genetic mutants where either the NH2- or COOH-terminal SH3 domain was deleted (delNSH3 and delCSH3, respectively, Fig. 2 A). The mutant constructs were transfected into RBL-2H3 cells and their subcellular distribution was visualized by confocal imaging (Fig. 2 B). The delNSH3 mutant showed cytoplasmic localization in resting cells and a partial nuclear translocation upon FceRI stimulation similar to the actions of transfected wt or endogenous Vav1 (Fig. 2 C, Wt-Vav, and Fig. 1 A). Some cytoplasmic accumulation was also seen in cells expressing the highest levels of exogenous proteins (Figs. 2 B, delNSH3 [S], and 2 C, Wt-Vav [S]). In contrast, the delCSH3 mutant exhibited a strong nuclear localization both in unstimulated and stimulated cells (Fig. 2 B). Immunochemical analysis revealed almost equivalent expression levels of the mutant and wt proteins in total extracts and confirmed the increase of delCSH3 Vav1 in nuclear fractions of unstimulated cells compared with untransfected or wt transfected RBL cells (data not shown). Because delCSH3 was constitutively localized to the nucleus, this enabled us to explore the function of the putative nuclear localization signal sequences (delNLS1 and delNLS2, respectively) in causing the nuclear localization of this protein. The first NLS is located within the PH domain of Vav1 and is closely related to the SV40 consensus sequence (Fig. 2 A, NLS1: KXRXXKKK; reference 36). The second NLS is located between the cysteine rich and the N-SH3 domains and is composed of a bipartite sequence (NLS2: KXXXXXRRRXXXKKK, resembling those found in transcription factors [37, 38]). Fig. 2 C shows that deletion of NLS1 was sufficient to restore a constitutive cytoplasmic localization to the double mutant protein (delNLS1delCSH3) whereas deletion of the second NLS (delNLS2delCSH3) caused this protein to remain in the nucleus. Similarly, a NLS1 deleted mutant of wt-Vav was restricted to the cytoplasm upon prolonged FceRI stimulation (Fig. 2 C, delNLS1). In contrast, a NLS2 deleted mutant behaved like wt-Vav and was able to translocate to the nucleus after FceRI stimulation (data not shown). These results were confirmed by immunochemical analysis of the nuclear and cytoplasmic fractions of stably transfected delNLS1 and delCSH3 RBL cells. Indeed, while delCSH3 was predominantly nuclear, delNLS1 was exclusively cytoplasmic, regardless of whether cells were stimulated or not (Fig. 2 D).

Functional Properties of Vav1 Mutants with Nuclear and Cytoplasmic Localization. We previously demonstrated that the absence of Vav1 in mast cells had significant consequences in the activation of c-Jun NH2-terminal kinase (JNK1), PLCγ, and subsequent calcium responses upon stimulation of the FceRI (19). The calcium regulating effects of Vav1 were demonstrated to be dependent on the guanine nucleotide exchange activity of its DH domain. Therefore, the calcium response of either a cytoplasmic (delNLS1) or nuclear (delCSH3) localized mutant form of Vav1 was tested in order to determine their ability to potentiate the calcium influx. To test the effect of these mutants on early activation signals we used BMMC derived from vav1-null mice. Calcium levels were determined in Fura-Red loaded cells that were reconstituted with wt or mutant Vav1, gated for transfected GFP expression, and activated by FceRI stimulation. As shown in Fig. 3 A, we confirmed our previous results showing a substantial decrease in the Ag-dependent calcium response of Vav1-null BMMCs compared with cells derived from vav1+/+ mice (Fig. 3 A, Wt and KO; reference 19). Transfection of vav1−/− BMMCs with wt Vav–GFP resulted in a calcium response that was enhanced, albeit slightly delayed, when compared with endogenous Vav (KO [Vav-GFP], Wt [GFP]). In contrast, the delNLS1–Vav mutant (delNLS1) did not restore any Ag-dependent calcium response, showing levels similar to that observed for vav−/− BMMCs whereas the delCSH3 mutant partially restored the FceRI-induced calcium response up to the level of vav+/+ BMMCs, albeit with a significantly delayed response to Ag (delCSH3). Consistent with our prior results (19), the thapsigargin-induced calcium response, which is independent of FceRI engagement, was confirmed to be dependent on Vav1 competence. Both the delNLS1 and delCSH3 mutants showed reconstitution of the thapsigargin-induced calcium response to the extent of vav−/− derived cells but significantly diminished compared with vav+/+ and wt-Vav transfected vav−/− cells. This result is consistent with an important role for a fully functional Vav1 in the regulation of calcium stores.

Vav1 mutants were also tested for their capacity to activate JNK; another well-documented pathway regulated by Vav1. For these experiments we used RBL cells, as the efficiency for transfection of vav1−/− BMMCs did not permit biochemical analysis of JNK activation. The RBL cells were stably transfected with wt-Vav or mutated Vav and JNK activity was measured in an in-gel kinase assay by phosphorylation of GST-c-jun. In agreement with previous observations (11, 39) we observed a substantial induction of JNK activity in wt Vav1 transfected compared with mock-transfected cells, after antigenic stimulation (Fig. 3 B, WtVav versus control GFP). Similarly, although expression levels of the delNLS1 Vav1 mutant were lower compared with wt Vav1, it also enhanced to a similar extent JNK activity.
Conversely, the delCSH3 Vav1 mutant only mildly enhanced JNK activation above the control GFP transfectant. However, this enhancement was significantly less (around twofold less) than the JNK activation induced by delNLS1 mutant Vav1, although the levels of mutant protein were higher and JNK1 protein levels were equivalent.

As Vav1 was demonstrated to be important for the activation of the NFAT transcription factor and IL-2 gene transcription, we also examined the effect of Vav1 localization on NFAT activity. For this purpose, wt Vav, delCSH3Vav, and delNLS1Vav, were transfected into Jurkat T cells together with a luciferase reporter construct un-
PCR amplification of in vitro transcribed IL-2 mRNA as a control for IL-2 primers and PCR conditions. One representative of four experiments is shown.

IL-2 control represents the were untransfected (RBL) or transfected with the indicated constructs. Cells were left unstimulated (NS) or were antigen stimulated for 1 h (S). Cytokine to myc (bottom panel). One single representative experiment is shown. (D) Effect of Vav1 deletion mutants on IL-2 steady-state mRNA level. RBL cells were transfected with the indicated constructs. Cells were left unstimulated (−) or were antigen stimulated (+). JNK1 kinase activity was measured by an in vitro kinase assay. The top panel shows an autoradiography of (γ32P) incorporated into c-Jun-GST after its phosphorylation by JNK1 immunoprecipitates from cell lysates. Bottom panels show the amount of JNK1, exogenous, and endogenous Vav1 found in the transfectants. The fold induction of JNK activity is relative to control GFP (-Ag) arbitrarily set to 1.0 and is the mean of three individual experiments with standard deviations ranging from 4.3 to 9.9%. (C) Transcriptional activation of NFAT by Vav1 deletion mutants. Jurkat T cells were cotransfected with the IL-2 promoter-luciferase plasmid and expression plasmids encoding either wt Vav (wtVav), delCSH3, or delNLS1 Vav mutants. After 24 h, cells were either left unstimulated (NS) or were stimulated for 60 min with CD3 and CD28 (S). Luciferase activity was determined in cell extracts (top panel). Results are representative of three individual experiments and error bars represent standard deviation. Samples of the same lysates were also analyzed for the expression of myc-tagged Vav1 constructs by immunoblotting with mAb to myc (bottom panel). One single representative experiment is shown. (D) Effect of Vav1 deletion mutants on IL-2 steady-state mRNA level. RBL cells were untransfected (RBL) or transfected with the indicated constructs. Cells were left unstimulated (NS) or were antigen stimulated for 1 h (S). Cytokine (IL-2) or housekeeping gene (GAPDH) mRNA levels were determined by RT-PCR as described in Materials and Methods. IL-2 control represents the PCR amplification of in vitro transcribed IL-2 mRNA as a control for IL-2 primers and PCR conditions. One representative of four experiments is shown.

**Figure 3.** The differential localization of Vav1 (delCSH3 versus delNLS1) promotes or inhibits functional responses. (A) Intracellular calcium measurements. Vav +/+ (Wt) and −/− (KO) BMBCMs were transfected with GFP alone (GFP), wt Vav-GFP (Vav-GFP), delCSH3 Vav-GFP (delCSH3), and delNLS1 Vav-GFP (delNLS1). Intracellular calcium levels of IgE and Fura Red AM loaded cells were measured by flow cytometry before and after stimulation at the indicated time (arrows) with 30 ng DNP-BSA (Ag) followed by 1 μM thapsigargin (Thpsg). Data were collected after gating of transfected GFP-positive cells. One representative calcium experiment is shown. (B) Activation of NFAT activities. RBL cells were transfected with the indicated constructs. Cells were left unstimulated (−) or were antigen stimulated (+). JNK1 kinase activity was measured by an in vitro kinase assay. The top panel shows an autoradiography of (γ32P) incorporated into c-Jun-GST after its phosphorylation by JNK1 immunoprecipitates from cell lysates. Bottom panels show the amount of JNK1, exogenous, and endogenous Vav1 found in the transfectants. The fold induction of JNK activity is relative to control GFP (-Ag) arbitrarily set to 1.0 and is the mean of three individual experiments with standard deviations ranging from 4.3 to 9.9%. (C) Transcriptional activation of NFAT by Vav1 deletion mutants. Jurkat T cells were cotransfected with the IL-2 promoter-luciferase plasmid and expression plasmids encoding either wt Vav (wtVav), delCSH3, or delNLS1 Vav mutants. After 24 h, cells were either left unstimulated (NS) or were stimulated for 60 min with CD3 and CD28 (S). Luciferase activity was determined in cell extracts (top panel). Results are representative of three individual experiments and error bars represent standard deviation. Samples of the same lysates were also analyzed for the expression of myc-tagged Vav1 constructs by immunoblotting with mAb to myc (bottom panel). One single representative experiment is shown. (D) Effect of Vav1 deletion mutants on IL-2 steady-state mRNA level. RBL cells were untransfected (RBL) or transfected with the indicated constructs. Cells were left unstimulated (NS) or were antigen stimulated for 1 h (S). Cytokine (IL-2) or housekeeping gene (GAPDH) mRNA levels were determined by RT-PCR as described in Materials and Methods. IL-2 control represents the PCR amplification of in vitro transcribed IL-2 mRNA as a control for IL-2 primers and PCR conditions. One representative of four experiments is shown.

**Vav Is Part of Transcriptionally Active Complexes.** Because of our earlier observations of Vav1 localization to the nucleus and the ability of the cytosolic delNLS1 Vav1 to drive NFAT/AP1 dependent reporter activity we further explored whether Vav1 was directly involved in NFAT-dependent transcriptional activation. We first measured the induction of nuclear NFAT/AP1 DNA binding activity upon FceRI stimulation of RBL cells. The initial kinetic experiments measured the appearance of an NFAT complex in nuclear extracts that bound the distal site of the human IL-2 promoter. While we observed a slight induction of NFAT binding activity at 5 min after stimulation, the activity fur-
ther increased reaching a maximum at 1 h (Fig. 4A). Complex formation was blocked both by NFAT or AP1 unlabeled competitor oligonucleotides. Interestingly, the kinetics of NFAT/AP1 DNA binding activity was found to be strikingly similar to the movement of Vav1 to the nucleus. Hence, we further analyzed the composition of the protein–DNA complex by electrophoretic mobility supershift assays. Surprisingly, in addition to the supershift of the NFAT/AP1 binding complex by incubation with anti-NFATp or anti-c-Jun antiserum, we found that the complex with lower mobility could be supershifted, or was partly disrupted, by incubation with two different antibodies to Vav1. An irrelevant antibody to GST did not cause this shift (Fig. 4B, top left). The presence of Vav in this complex was subsequently confirmed by UV cross-linking of the lower mobility complex, which was then excised resolved by SDS-PAGE, and probed by immunoblot with an antibody to Vav1. A specific band corresponding to p95 Vav was isolated from an excised region containing the complex induced by FceRI stimulation (Fig. 4B, *lane 2), but not from an excised region corresponding to the same molecular mass but derived from unstimulated cells (Fig. 4B, *lane 1, bottom left). Furthermore, in RBL cells stably transfected with wt Vav1 we found enhanced formation of the lower mobility NFAT/AP1 complex (Fig. 4B, top right). The protein–DNA complex was again supershifted upon incubation with antibodies to NFATp or Vav but not with antibodies to NFATc or the irrelevant protein GST. Analysis by Western blotting of the band of lower mobility also revealed higher quantities of p95 Vav (Fig. 4B, bottom right, *lane 4).

The NFAT distal site present in the IL-2 promoter has been shown to cooperatively bind NFAT and AP1 transcription factors (34; Fig. 4A). The possibility that Vav1 could also participate independently in AP1-DNA binding was explored by testing an NFAT-independent AP1 site derived from the human metallothionein IIA promoter. As shown in Fig. 4C, nuclear extracts from unstimulated cells showed a faint but specific protein–DNA complex in a gel shift assay. The amount of the low mobility complex was enhanced after FceRI stimulation and was supershifted upon incubation with antibody to c-Jun. However, incubation with antibodies to Vav1 (Fig. 4C) or NFAT (data not shown) did not cause a supershift. We further analyzed the protein–DNA complex (Fig. 4C, *lanes 1 and 2) for Vav1.

Figure 4. Vav1 is a specific component of NFAT and NFκB-like DNA binding complexes. (A) Kinetics of NFAT nuclear binding activity present in nuclear extracts after stimulation of RBL for the indicated times as revealed by EMSA. The presence of the NFAT-DNA complex is indicated by an asterisk. In competition experiments the unlabeled oligonucleotides (IL-2 NFAT or AP1 sites, excess of 20-fold) were incubated 15 min with the nuclear extract before addition of the probe. (B–E) Supershift analysis of various nuclear transcriptional complexes in unstimulated (NS) and in stimulated (S) RBL cells or transfectants (wt-Vav) is shown. The following different oligonucleotides were used as probes: human NFAT (B), AP1 (C), NFκB-like (D), and Oct 1 (E). The specific DNA–protein complexes generated are indicated by an asterisk. For supershift analysis extracts were incubated with the following antibodies: anti-NFATp (UBI) or NFATc (Santa Cruz Biotechnology, Inc.), anti-Vav (a and b: mAb UBI and pAb provided by Dr. X. Bustello, respectively), anti c-Jun and irrelevant anti-GST or anti-myc for 15 min before addition of the labeled oligonucleotides. Nuclear extracts were also preincubated with an excess (20X) of unlabeled competitor oligonucleotide as indicated. In preparative experiments specific complexes (*) were cut out from wet gels using samples corresponding to the indicated lanes after UV irradiation. Samples were resolved by SDS-PAGE and analyzed by immunoblotting with mAb to Vav1 (bottom of B, C, and D). Total extracts of RBL were loaded in parallel as controls for immunoblotting (Ext).
content by UV cross-linking and Western blotting; no band corresponding to the 95 kD Vav1 was detected (Fig. 4 C, bottom). The results demonstrate that Vav1 cooperatively participates in a NFAT/AP1 transcriptionally active complex that specifically binds to the distal region of IL-2 promoter, but not to an NFAT-independent AP1 complex. The participation of Vav1 in a transcriptionally active complex raised the possibility that it may also participate in other complexes, like NFkB, where Vav1 activity has been demonstrated to play a role (18). We showed previously that an NFkB-like factor binding to a NFkB site present in the 3′ region of the TNF-α gene is important for transcriptional induction of cytokines in mast cells (29). Furthermore, we also demonstrated that Vav1-deficiency has a partial effect on the FcεRI-induced TNF-α mRNA levels (19). Thus, it seemed possible that Vav1 might also form part of a NFkB-like complex in mast cells. As shown in Fig. 4 D, a NFkB-like DNA binding activity, which was weakly detected in unstimulated cells, was strongly induced upon FcεRI stimulation (Fig. 4 D, *). The resulting protein-DNA complex was supershifted by incubation with two different antibodies to Vav but not with an anti-Myc Ab (Fig. 4 D, top). The presence of Vav1 in the bands with lower mobility was also analyzed. The UV cross-linked proteins in the excised gel bands were resolved by SDS-PAGE and subsequently immunoblotted with an antibody to Vav1 revealing the presence of p95 Vav in these complexes with a detectable increase during stimulation (Fig. 4 D, lanes 1 and 2, bottom). Finally, we also examined the possible contribution of Vav1 to another protein–DNA complex corresponding to the Oct1 site of the IL-2 promoter (40). In unstimulated cells a rather strong DNA binding protein complex was observed. Increased DNA binding was induced by FcεRI stimulation. However, incubation with an antibody to Vav1 failed to supershift this complex (Fig. 4 E). Thus, while Vav1 can participate cooperatively or independently in two transcriptionally active complexes (NFAT/AP1 and NFkB-like), its participation is complex specific, as it does not participate in other transcriptional complexes (AP1 and Oct1) that also bind the IL-2 promoter.

**Nuclear delCSH3 Vav1 Hinders the Formation of Both NFAT and AP1-DNA Binding Complexes.** To understand the failure of delCSH3 Vav1 to potentiate NFAT/AP1 reporter activity and increase IL-2 mRNA levels, we analyzed its effect on NFAT/AP1 and AP1 DNA binding activities in stable delCSH3 transfectants. These results were compared with cells that had been stably transfected with either wtVav or delNLS1 Vav1. As shown in Fig. 5 A, upon FcεRI stimulation, untransfected cells exhibited a NFAT/AP1-DNA binding activity detected in a gel shift assay using an NFAT/AP1 oligonucleotide probe. This complex was enhanced in the cells overexpressing wt-Vav1 (compare wt-Vav and RBL). Similarly, untransfected and stimulated RBL cells exhibited an enhanced NFAT-independent AP1 DNA binding activity (Fig. 5 A, RBL, AP1). Again, overexpression of wt-Vav1 resulted in a strong enhancement of the AP1 binding complex that was readily seen in unstimulated cells and further increased with FcεRI stimulation (AP1, wt-Vav). Immunoblot analysis with an antibody to NFATp, confirmed the substantial increase of NFAT in the nucleus of wt-Vav1 cells compared with untransfected cells after stimulation (Fig. 5 A, bottom). In striking contrast, in the cells that had been stably transfected with the constitutively nuclear-localized delCSH3 Vav1 mutant we did not detect a functional NFAT/AP1 DNA binding activity in three separate stably transfected clones even after prolonged exposures (Fig. 5 A, delCSH3 and data not shown). Furthermore, while a weak AP1 DNA binding activity was detected in unstimulated cells, the binding was not increased upon stimulation. Thus, delCSH3 Vav1 was ineffective in potentiating both NFAT/AP1 and AP1 DNA binding activities and in increasing IL-2 mRNA (Fig. 3 D).

In contrast, in nuclear extracts of cells transfected with delNLS1 we could identify an AP1 DNA binding activity, comparable to wt-Vav transfected cells, that was enhanced
upon stimulation. However, the NFAT/AP1 DNA binding activity observed, although slightly enhanced upon stimulation, remained very weak and became apparent only after prolonged exposure (Fig. 5 A, delNLS1). This is consistent with the inability of delNLS1 Vav1 to effectively translocate to the nucleus and to promote IL-2 mRNA accumulation to the level of wt Vav1 (see Fig. 5 A, RBL, and Fig. 3 D). Immunoblot analysis of the nuclear extracts of delCSH3 or delNLS1 cells revealed a slight increase in the quantity of NFAT present in the nucleus upon stimulation to the same extent as untransfected RBL cells (Fig. 5 A, bottom). Thus, this increase in all three nuclear extracts is likely attributed to the presence and activation of endogenous Vav1. Finally, the appearance of an Oct1 nuclear complex was not affected by any of the Vav1 constructs as its DNA-binding activity was present in unstimulated cells and was similarly further enhanced by FcγRI stimulation (Fig. 5 B).

As the delCSH3 Vav1 mutant does not seem to affect the nuclear translocation of NFAT mediated by endogenous Vav1, we analyzed whether its inhibitory effects on NFAT and AP1 DNA binding activities were related to the quantity of the mutant protein present in the nucleus. We used two additional delCSH3 stable clones expressing lower levels of the nuclear myc-tagged delCSH3 as monitored by immunoblotting of nuclear extracts with anti-Myc antibody (Fig. 6 B, clones 1 and 2). Nuclear extracts from these transfectants demonstrated an inhibitory effect on both NFAT/AP1 and AP1 DNA binding activities compared with endogenous Vav1 in untransfected RBL cells. The inhibitory effect was proportional to the amount of delCSH3 Vav present in the nucleus, leading to complete inhibition of complex formation in the highest expressing clone (clone 3; Fig. 6 A).

Together with the data from the previous section these results indicate that delCSH3 Vav1 is unable to promote the formation of an NFAT/AP1-dependent transcriptionally active complex. They also demonstrate that this nuclear mutant hinders the DNA binding mediated by endogenous Vav1 and has a more general inhibitory effect as exemplified by inhibition of AP1 DNA binding.

**Discussion**

Vav1 contains multiple protein structural interaction motifs that have allowed the isolation of several binding partners in vitro and in vivo, indicating a role for Vav1 as an adaptor in a wide variety of signaling pathways. Among the structural domains found on Vav1, the COOH-terminal SH3 domain has been most exclusively associated with nuclear proteins. However, the functional relevance of these interactions is unknown (23–25).

In this report we demonstrate that the COOH-terminal, but not the NH2-terminal, SH3 domain of Vav1 is directly responsible for the cytoplasmic retention of the proto-oncogene. Although the mechanism remains unknown, one possibility may be that the COOH-terminal SH3 mediates specific interaction with cytoskeletal proteins that sequester it in the cytoplasm (27, 41). Furthermore, SH3-dependent interaction with other cytoplasmic proteins could also prevent its movement to the nucleus as described for NFκB/IKB complexes interacting with RasGAP SH3-binding proteins (42). Alternatively, the C-SH3 domain or the COOH-terminal portion of Vav may also be responsible for intramolecular bridging that could mask the NLS within the PH domain. This mechanism of intramolecular masking of an NLS was previously described in the nuclear transport of B-Myb (43). Our data support the unmasking of the NLS upon FcγRI stimulation, as the deletion of the functional NLS1 sequence restored retention of the delCSH3 mutant and Vav1 in the cytoplasm. Although deficient in nuclear transport, the delNLS1 Vav1 mutant was still capable to activate JNK, an event that requires both the tyrosine phosphorylation of Vav1 and the conformational activation of the DH domain (44–46). Genetic studies have indicated a major role for Vav1 both in early signaling events such as in calcium mobilization and cytoskeletal rearrangement but also in more delayed responses, such as the production of...
cytokines, including IL-2, IL-3, IFN-\(\gamma\), and TNF-\(\alpha\). Experimental evidences have focused on the role of Vav1 as a guanine nucleotide exchange factor (GEF) for Rac and the consequences of Rac activation on cytokine production. More recently, however, several reports described a guanine nucleotide exchange factor-independent function of Vav1, most notably in potentiating NFAT transcriptional activity and facilitating PLC\(\gamma\) phosphorylation (19, 47). Our findings provide strong evidence for GEF-independent regulation of NFAT as Vav1 is demonstrated to become an active participant in the NFAT/AP1 transcriptional complex that binds to the distal promoter of IL-2. Indeed, supershift analysis of the activated complex, using two different antibodies, established the presence of Vav1. Interestingly, a third antibody directed to the C-SH3 domain of Vav1 was unable to supershift the complex indicating that this domain may be inaccessible because of the conditions used in the gel shift assays or because it may be bound to another protein (unpublished data). The specific presence of Vav1 within the complex was also confirmed by direct immunochemical analysis. The importance of Vav1 in the formation of NFAT/AP1 complex is also reinforced by our data that shows a substantial enhancement of both the DNA-bound complex and all identified components, including Vav1, in cells overexpressing wt Vav (see Figs. 4 B and 5 A). On the other hand, we did not observe its presence in a protein–DNA complex containing only the AP1 transcription factor-binding site, despite a strong induction of AP1 binding activity upon Fc\(\epsilon\)RI stimulation. Vav1 was also absent in the NFAT-independent Oct1 transcriptional complex activated upon Fc\(\epsilon\)RI stimulation but was present in another inducible NFkB-like transcriptional complex that has been shown to play a role in the transcription of TNF\(\alpha\) in RBL mast cells (29). These results clearly favor the specific interaction of Vav1 with NFAT and NFkB complexes and are in agreement with the impaired NFAT and NFkB activation previously observed in \(vav1^{-/-}\) animals (18). Thus, beyond the ability of Vav to lead to NFAT and NFkB activation we found that Vav1 becomes a potentiating partner of the transcriptionally active complex.

To further explore the mechanisms involved we exploited the findings that deletion of specific motifs or domains of Vav1 would result in opposite cellular compartmentation under nonactivation conditions. The cytoplasmic delNLS1 Vav1 mutant that was still fully effective in activating JNK showed a strong NFAT-independent AP1–DNA binding activity and a weak AP1–binding to the NFAT/AP1 probe. However, this mutant was ineffective at potentiating calcium influx, and, accordingly, NFAT nuclear translocation and DNA binding were not enhanced over endogenous levels. This suggests that increased JNK activation alone was not sufficient to enhance NFAT binding. Despite the absence of significant NFAT/AP1 mobilization the delNLS1 Vav1 strongly induced a NFAT/AP1 reporter activity after transient transfection into Jurkat cells and led to some increase of the levels of IL2 mRNA in RBL cells. This result is most easily explained by the binding of AP1 to the three NFAT/AP1 tandem repeats present in the reporter plasmid. This is in agreement with a recent study that has revealed an AP1-dependent induction of this IL-2 reporter construct (48).

Our competition experiments using the AP1 recognition motif also confirmed the ability of AP1 to bind to the IL-2 distal promoter in the absence of NFAT and to promote some transcriptional activation of the IL-2 gene (Figs. 4 A and 3 D). Collectively, the results with the delNLS1 Vav1 mutant provide further evidence for a model whereby Vav1 has GEF-dependent and -independent activities that must function synergistically to promote maximal NFAT/AP1 binding activity at the IL-2 distal promoter and drive full gene transcription (47). The inability of the delNLS1 mutant to potentiating calcium influx might further reflect some alteration within the PH domain that inhibits Vav1-mediated activation of PLC\(\gamma\) and PI3K, the latter activity being necessary for thapsigargin-induced depletion of ER stores (19, 49, 50). This hypothesis is further reinforced by a subcellular localization of the delNLS1 mutant that is more diffuse in the cytoplasm and does not rapidly localize to the plasma membrane at early time of stimulation as compared with wt-Vav (Fig. 2 C, and data not shown).

The constitutive and primarily nuclear localization of the delCSH3 Vav1 mutant allowed us to assess the effects of its presence in the nucleus. Clearly, this mutant, in contrast to wt-Vav1, was unable to induce luciferase activity with an IL-2 promoter construct and to promote the transcription of this cytokine gene. However, the novel finding was that this mutant, in a dose dependent manner, was ineffective in inducing detectable levels of both NFAT/AP1 and AP1–DNA complexes by endogenous Vav (see Figs. 5 A and 6 A). As delCSH3 also inhibited induction of AP1 complexes, where Vav is not a participant, this suggests a more general inhibitory effect for this mutant. This hypothesis is in agreement with our finding that the inducible NFkB-like DNA complex, in which Vav1 is a component, is also abrogated in delCSH3 transfected cells (unpublished data). Because del-CSH3 Vav1 does not induce further NFAT translocation but does not abrogate NFAT translocation due to endogenous Vav, it does not appear that the delCSH3 mutation affects transport of transcription factors into the nucleus. However, the absence of the C-SH3 domain is the characteristic in common to the inhibitory effect on NFAT, NFkB, and AP-1 DNA binding activity. One possibility, in agreement with our results of a delCSH3-mediated dose-dependent inhibitory effect is that increasing concentrations of delCSH3 largely accumulated in the nucleus, can compete for the binding of endogenous Vav1 to these transcriptional complexes thereby sequestering them as a complex unable to bind to its DNA. Our current hypothesis is that Vav plays the role of an adaptor protein in the nucleus as it does in the cytoplasm and that this role is important for the DNA binding activity of transcriptional complexes. It is already known that the C-SH3 domain associates with Ku70, a component of the DNA-dependent protein kinase complex, and Ku70 interactions with homeobox transcription factors (known as important effectors in the hematopoietic system) have been described (23, 51),
Thus, it is possible that Vav1 plays a more general role in the nucleus by facilitating the phosphorylation and/or assembly of transcription factor–containing DNA binding complexes. Interestingly, the full transcriptional activity of NFAT seems to require phosphorylation of the NH2-terminal transactivation domain by an inducible kinase (52). It is also of interest to note that Vav1 associates with ENX-1 (26), a putative regulator of homeobox gene expression.

In conclusion, this study provides direct evidence for the presence of Vav1 as a component of an active transcriptional complex. It demonstrates the participation of Vav1 in an NFAT and NFκB–like transcriptional complex but not as a member of AP-1 or Oct DNA binding complexes. Furthermore, we found that the presence of Vav1 in the nucleus is promoted by the prolonged stimulation of receptors and depends on a functional NLS found within the PH domain of Vav1 as well as on derepression of the C-SH3 domain–mediated cytoplasmic localization. This provides the first evidence of a nuclear role for Vav1 and further demonstrates that its role in regulation of IL-2 gene transcription transcends the regulation of calcium responses and JNK stimulation that activate NFAT.

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