Structural Determinants for the Biological Activity of Vav Proteins*

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We have used an extensive mutagenesis approach to study the specific role of the eight structural domains of Vav during both the activation and signaling steps of this Rac1 exchange factor. Our results indicate that several Vav domains (Dbl homology, pleckstrin homology, and zinc finger) are essential for all the biological activities tested, whereas others are required for discrete, cell type-specific biological effects. Interestingly, we have found that Vav domains have no unique functions. Thus, the calponin homology domain mediates the inhibition of Vav both in vitro and in vivo but, at the same time, exerts effector functions in lymphocytes upon receptor activation. The Vav SH2 and SH3 regions play regulatory roles in the activation of Vav in fibroblasts, mediating both its phosphorylation and translocation to the plasma membrane. In contrast, the Vav SH2 and SH3 regions act as scaffolding platforms in T-cells, ensuring the proper phosphorylation of Vav and the subsequent engagement of downstream effectors. We also provide evidence indicating that the zinc finger region exerts at least three different functional roles in Vav, aiding in the down-regulation of its basal activity, the engagement of substrates, and the induction of ancillary pathways required for cell transformation. Finally, the results obtained are consistent with a new regulatory model for Vav, in which the calponin homology region inhibits the basal activity of Vav through interactions with the zinc finger region.

Vav proteins are phosphorylation-dependent exchange factors that catalyze the release of GDP from Rho/Rac family members, thereby facilitating their transition from the inactive (GDP-bound) to the active (GTP-bound) state (1). This activity is crucial for the coordination of developmental and mitogenic processes. Thus, the elimination of the vav gene results in impaired lymphoid development, lymphopenia, and defective immune responses in mice (2–5). Similarly, deletion of either vav2 or vav3 genes results in impaired signaling responses in activated B-cells (6–8). It has also been demonstrated that the subversion on the normal activation/deactivation cycle of some members of the Vav family results in severe alterations of cell behavior, including tumorigenesis, changes in F-actin organization, and the acquisition of metastatic properties by transformed cells (1). Finally, the activation of Vav or Vav2 proteins by the Nef protein of the human immunodeficiency virus plays an essential role in the pathogenic cycle of this virus (9, 10).

One important feature of this GEF family is the structural complexity of its members (1, 10) (see Fig. 1A). Mammalian and avian Vav proteins contain eight structural domains, including a calponin homology (CH) region, an acidic (Ac) domain, the catalytic Dbl homology (DH) region, a pleckstrin homology (PH) domain, a zinc finger (ZF) region similar to those present in c-Raf and protein kinase C family members, and a SH2 domain flanked by two SH3 domains. Caenorhabditis elegans and Drosophila melanogaster Vav differ mostly at the C terminus, where they lack the P-most N-terminal SH3 domain. Recent evidence suggests that these domains are specialized in the coordination of catalytic, regulatory, signaling, and/or targeting functions. In the case of the CH region, this domain negatively affects the function of Vav, because deletions of its first 66 amino acids lead to the oncogenic activation of the vav proto-oncogene (11). However, it is not known whether this mutation affects the activity of Vav in cis or in trans, because this oncoprotein is still dependent on tyrosine phosphorylation for the activation of its exchange activity (12). In this respect, this region has been shown to interact with both Rho/Rac GDP dissociation inhibitors (GDI) and with the transcriptional factor Enxl (13, 14), indicating that at least part of the function of this domain could be associated with the formation of heteromolecular interactions. Interestingly, it has been proposed that the Vav CH region has effector functions in T-cells, because the deletion of this domain abrogates the activation of NF-AT by Vav in lymphocytes (10, 15). The Vav acidic domain plays a negative regulatory role on the activity of Vav through one of its residues, Tyr174 (16, 17). NMR studies have shown that this residue interacts with amino acids located in the DH domain, leading to a “closed” conformation of the protein incapable of interacting with its GTPase substrates. This intramolecular interaction is disrupted by the phosphorylation of Tyr174, leading to the generation of an “open,” catalytically competent Vav conformation. Due to this regulatory mechanism, Vav proteins are totally dependent on phosphorylation for the activation of their catalytic and biological activities (16, 17). On the other hand, the DH region is in charge of establishing the interaction with Rho/Rac proteins, leading to the subsequent catalysis of GDP/GTP exchange on these GTPases. The function of the Vav PH region has not yet been fully

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1 The abbreviations used are: CH, calponin homology; DH, Dbl homology; PH, pleckstrin homology; ZF, zinc finger; Ac, acidic; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; MBP, myelin basic protein; GTPγS, guanosine 5′-3-O-(thio)triphosphate; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; GEF, guanosine nucleotide exchange factor; GDI, GDP dissociation inhibitor; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; EGFR, epidermal growth factor receptor.
elucidated. Broek and co-workers (18, 19) have proposed that this domain regulates the catalytic activity of Vav by an on/off mechanism based on the binding of phosphatidylinositol bisphosphate (PIP2) or phosphatidylinositol triphosphate (PIP3). Although this model is supported by results obtained in different experimental settings, its physiological significance remains unclear (17). The ZF region seems to play an important, although not yet fully elucidated, regulatory function on Vav. Studies with Vav3 have shown that the integrity of the ZF region is important for the optimal binding of this GEF to its substrates, probably by offering an additional area of contact with the GTPase (20). However, it remains to be determined whether this is the exclusive function of the Vav ZF domain. Finally, the C-terminal SH2 and SH3 domains participate in protein-protein interactions with regulatory and/or effector functions. The SH2 domain is important for the interaction

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**Fig. 1.** A, Schematic representation of the Vav mutants used in these experiments. The distribution of Vav structural domains is depicted at the top. Solid bars represent the length of the proteins expressed. Point mutations are represented by asterisks. To facilitate the analysis of this figure, the numbers shown on the left of each protein will be used in the rest of the panels. The same style will be followed in the rest of the figures. B, NIH3T3 cells were transfected with non-linearized vectors encoding wild type Vav (1 µg, bar 1), Vav Y3xF (0.5 µg, bar 2), Vav-(Δ1–66) (0.5 µg, bar 3), Vav-(Δ1–144) (0.05 µg, bar 4), Vav-(Δ1–186) (0.05 µg, bar 5), Vav-(Δ1–186 + Δ608–845) (0.5 µg, bar 6), or Vav-(Δ1–186 + Δ608–845 + C-CH) (0.5 µg, bar 7). Values (normalized as foci per µg of transfected plasmid) represent the mean ± S.D. of three independent experiments, each performed in duplicate. C, Expression of wild type Vav (lanes 1), Vav-(Δ1–186 + Δ608–845) (lanes 6), or Vav-(Δ1–186 + Δ608–845 + C-CH) (lanes 7) in the soluble (S) and insoluble (P) fraction of COS1 cells. The mobility of these proteins is indicated by an arrow, an arrowhead, and an asterisk, respectively. D, Left panel, NF-AT activity in non-stimulated (open bars) or CD3-stimulated (closed bars) Jurkat cells expressing wild type Vav (bar 1), Vav Y3xF (bar 2), and Vav-(Δ1–186) (bar 5). Right panel, JNK activity in Jurkat cells expressing wild type Vav (bar 1), Vav Y3xF (bar 2), Vav-(Δ1–144) (bar 4), Vav-(Δ1–186) (bar 5), and Vav-(Δ1–186 + Δ608–845) (bar 6). Values represent the mean ± S.D. of at least three independent experiments, each performed in triplicate.
with autophosphorylated tyrosine kinases and with adapter proteins such as Slp76 or Bink (1, 10). The N-terminal SH3 domain binds to the adapter protein Grb2 (21, 22). This interaction appears to be important in lymphoid cells, because the binding of Vav to Grb2 makes possible its association with LAT and CD28, two membrane-anchored proteins that allow the translocation of Vav to the plasma membrane and its interaction with the upstream tyrosine kinases (1, 10). In agreement with this model, mutations in CD28 and LAT impair the phosphorylation of Vav during T-cell receptor signaling (1, 10).

Finally, the C-terminal SH3 domain is characterized by its interactive promiscuity, because it can form complexes with a wide variety of proteins including cytoskeletal regulators (zyxin), RNA-binding proteins (hnRNPK, hnRNPC, and p44), transcriptional modulators (Ku-70), and ubiquitination factors such as Chl-b (1, 23). The actual participation of those proteins in the biological functions of Vav remains undefined.

Despite these advances, we still lack considerable information about the mechanism by which all these separate functions are assembled during signal transduction to facilitate the activation of the catalytic activity of Vav and the induction of downstream effectors. To shed light onto this process, we studied the effect of mutations in all the aforementioned structural domains on the biological activity of wild type Vav and its four known oncogenic versions (Δ1–66, Δ1–186, Δ1–186+Δ608–845, and Y33F). All these mutant proteins were tested in terms of exchange activity in vitro and in a number of biological read-outs (transforming activity, JNK activation, morphological change, NF-AT activation, and phosphorylation) using fibroblast, kidney, and lymphoid cell lines.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antibodies to the Vav DH, ZF, and SH3-H2/SH3 domains were raised in rabbits using either GST or MBP fusion proteins purified from *Escherichia coli*. Other antibodies used include AT5 and HA (Babco), hexahistidine, MBP, and GST (Sigma), phosphotyrosine residues (Upstate Biotechnology, Inc., and Santa Cruz Biotechnology), CD3 (Dako), and EGFP (Clontech). Immunoblots were developed using chemiluminescent methods (ECL, Amersham Biosciences).

**Expression Vectors**—Vav point mutants were obtained using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Truncated proteins were generated by using either appropriate internal restriction sites (i.e. BamHI for deletions of the C-terminal SH3-SH2-SH3 region) or PCR according to standard procedures. The inclusion of the K-Ras tail was done by ligating two annealed phosphorylated oligonucleotides (mVav-CAAX-R, 5′-GATCCCATG- TAATGTCAATGTTGACTCCTCGGCTC-3′, and mVav-CAAX-F, 5′-AATTGTACAGGACAGGACTACTATAGACTAGTG-3′) to linearized, Vav-encoding plasmid. All mutant cDNAs were subjected to automatic sequence analysis to avoid the possibility of extra mutations. Details regarding the generation of specific constructs and mutants are available upon request. All vav cDNAs were used in the mammalian expression vectors pcDNA3 (Invitrogen) and pMEX (see "Results"). For the generation of mammalian expression vectors containing EGFP/Vav domains, the DH, PH, and ZF regions were amplified by PCR from the vav proto-oncogene cDNA and cloned into pEGFP-C (Clontech). pcDNA-HA-JNK1 and pcDNA-AT7/c-Jun plasmids were gifts from Dr. R. Cerione (Cornell University, Ithaca, NY). All GST proteins were purified by chromatography onto glutathione beads following standard procedures (20). To generate MBP fusion proteins, the indicated domains of Vav were amplified by PCR using the vav proto-oncogene cDNA as template and cloned into the pMAL-c expression vector (New England Biolabs). The MBP-β-galactosidase fusion protein was obtained by inducing the unmodified commercial vector pMAL-c. MBP fusion proteins were induced in *E. coli* (strain DH5α) and purified using amyllose beads (New England Biolabs), as indicated by the commercial supplier.

**Biochemical Experiments**—Exchange reactions were performed in duplicate at room temperature for 40 min using 15 pmol of GDP-bound Rac1 and 3.5 pmol of Vav proteins in the presence of [32P]GTPγS, as described previously (20). In vitro binding experiments with Vav purified from S9 cells were performed as indicated (20). In the case of binding assays with Rac1, the GTPasease (75 pmol) was stripped of nucleotides by incubation for 10 min at room temperature in binding buffer (25 mM HEPES [pH 7.5], 100 mM NaCl, 2.5 mM bovine serum albumin per ml, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10% (v/v) glycerol) supplemented with a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals). After these incubations, the buffer was supplemented with 50 nmol MgCl2 plus either GDP (450 μM) or no nucleotides and then incubated an additional 30 min. Then 30 μl of glutathione beads and Vav proteins (55 pmol) were added to the GTPasease solution. After an incubation of 3 h at 4 °C, the beads were washed three times with binding buffer plus 50 mM MgCl2, boiled in SDS-PAGE sample buffer, and analyzed by immunoblots. In the case of binding with GST-Vav CH proteins, the stripping protocol was bypassed. Binding experiments with *E. coli*-derived proteins were done as follows: affinity-purified GST fusion proteins were incubated with GST-Vav CH-ac (100 pmol) in binding buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl2, 20 mM KCl, 1 mM diithiothreitol, 1% Triton X-100, 5 mM imidazole, and 500 μM [γ-32P]ATP using GST-ATP2 as phosphate acceptor (25). Rac1 stimulation in *vivo* was determined by GST-Pak pull-down experiments, as described (26). Expression of proteins in hematopoietic cells was done by electroporation, exactly as described (25). In the case of NF-AT assays, Jurkat cells were electroporated with 5 μg of pNF-AT/luc together with 20 μg of either empty or CDNA-containing expression vectors. For JNK assays, Jurkat cells were transfected with a mixture of 5 μg of pFPR-Luc and 2 μg of pF2A-c-Jun together with 20 μg of either empty or CDNA-containing plasmids. After 48 h, cells were either left unstimulated or stimulated for 8 h with anti-CD3 antibodies (10 μg/ml), and measured with phosphate-borate-binding assay, a luciferase solution, and evaluated for luciferase activity, exactly as described before (25).

**Generation and Purification of Proteins**—Proteins were purified from baculovirus-infected S9 cells using affinity chromatography as described before (20). The Vav CH domain was generated by PCR and cloned into pGEX-2T. The pGEX vector encoding the Rac1 binding domain of PK (pGSP-Pak-RBD) was provided by Dr. R. Cerione (Cornell University, Ithaca, NY). All GST proteins were purified by chromatography onto glutathione beads following standard procedures (20). To generate MBP fusion proteins, the indicated domains of Vav were amplified by PCR using the vav proto-oncogene cDNA as template and cloned into the pMAL-c expression vector (New England Biolabs). The MBP-β-galactosidase fusion protein was obtained by inducing the unmodified commercial vector pMAL-c. MBP fusion proteins were induced in *E. coli* (strain DH5α) and purified using amyllose beads (New England Biolabs), as indicated by the commercial supplier.

**Generation of Expression Vectors for These Studies**—In order to study the functional relevance of the structural domains of Vav in all aspects of the biology of this GEF, we inactivated each of them by generating either truncations (CH, Ac, SH3, SH2, and SH3 domains) or appropriate missense mutations (Ac, DH, PH, ZF, SH3, SH2, and SH3) in wild type Vav and its...
four known oncogenic versions (Vav-(Δ1–66), Vav-(Δ1–186), Vav-(Δ1–186+Δ608–845), and Vav Y3xF) (Fig. 1A). Wild type and Δ1–66 are forms of Vav whose exchange activity is phosphorylation-dependent (Fig. 1A, proteins 1 and 3). Vav-(Δ1–186) and Vav-(Δ1–186+Δ608–845) have acquired phosphorylation-independent exchange activities because of the deletion of the CH and Ac domains (Fig. 1A, proteins 5 and 6) (29). Vav-(Δ1–186+Δ608–845) lacks, in addition to the CH-Ac domains, the C-terminal SH3-SH2-SH3 region (Fig. 1A, protein 6) (29). Vav Y3xF has three tyrosine to phenylalanine mutations affecting the main negative regulatory site of Vav (Tyr<sup>174</sup>) and two adjacent tyrosine residues (Tyr<sup>142</sup> and Tyr<sup>160</sup>) (Fig. 1A, protein 2). These two sites cooperate with Tyr<sup>174</sup> in the activation/inactivation of Vav during signal transduction (16, 25). By using all these proteins, we expected to be able to estimate the relative contribution of each domain to effector, regulatory, and/or phosphorylation-dependent functions. In order to facilitate the comparison of all Vav mutants, we cloned all cDNAs in the same plasmid backbones. We utilized the pMEX vector for studies in NIH3T3 and Jurkat T-cells, a non-commercial mammalian expression plasmid that regulates gene expression through the murine mammary tumor virus-long terminal repeat promoter. For some studies in COS cells (NF-AT, phosphorylation, and Rac1 activation), the same cDNAs were subcloned in pCDNA3, a mammalian expression vector containing a cytomegalovirus promoter and the SV40 replication origin that allows the episomal amplification of plasmids in the transfected cell. The biological activity of each of these proteins was measured in NIH3T3 cells (for transforming activity and morphological change), Jurkat T-cells (for NF-AT and JNK activation measurements), and/or COS1 cells (for morphological change, phosphorylation levels, Rac1 activation, and JNK stimulation).

The CH Domain Inhibits Vav in Cis and Is Essential for the Regulation of Vav Activity in Vivo—According to the current model for Vav activation, the main regulatory step mediating the activation of Vav during signal transduction is the phosphorylation of Tyr<sup>174</sup> and adjacent tyrosine residues (Tyr<sup>142</sup> and Tyr<sup>160</sup>) (16, 17). Thus, we expected that mutations in these three residues would lead to the generation of a highly transforming protein. To test this hypothesis, we compared the transforming activity of Vav Y3xF with those induced by wild type Vav, Vav-(Δ1–66), and two different versions of Vav-(Δ1–186) (Δ1–186 and Δ1–186+Δ608–845). As expected from previous results (29), we found that wild type Vav has very low transforming activity, whereas Vav-(Δ1–66) and Vav-(Δ1–186) display moderate and very high transforming potential, respectively (Fig. 1B, compare bars 1, 3, and 5, respectively). The deletion of the SH3-SH2-SH3 region of Vav-(Δ1–186) reduces, but does not abolish, the transforming activity of this protein (Fig. 1B, bar 6). These results are in agreement with previous observations indicating that the activities of Vav-(Δ1–186) and Vav<sub>2</sub>-(Δ1–187) are independent of their phosphorylation status (29). Interestingly, Vav Y3xF shows in these assays a transforming activity similar to the phosphorylation-dependent Vav oncoprotein (Vav-(Δ1–66)) and significantly lower than that shown by Vav-(Δ1–186) (Fig. 1B, compare bar 2 with bars 3 and 5). This observation indicated to us that, at least in an in vivo context, the removal of the inhibitory tyrosine residues is not sufficient for the full activation of Vav and that the CH region also contributes to the inhibition of Vav. To corroborate this possibility, we generated one additional Vav mutant protein (Δ1–144) lacking the entire CH domain (Fig. 1A, protein 4). As shown in Fig. 1B (bar 4), this new version of Vav displays a transforming activity similar to the Vav-(Δ1–186) oncoprotein described previously (Fig. 1A, compare bars 4 and 5) and significantly higher than the Y3xF mutant (Fig. 1A, compare bars 4 and 2). Similar results were obtained when all these Vav mutants were tested for their ability to induce morphological change and JNK activation in NIH3T3 cells and COS1 cells (Table I).

To investigate whether the inhibitory effect of the CH region found in vivo was mediated by mechanisms in cis or in trans, we evaluated the transforming activity of a truncated Vav protein in which its CH region was transferred from the N to the C terminus of the molecule (Fig. 1A, protein 7). This domain swapping should not affect the ability of the CH to interact in trans with other proteins, because the CH-binding proteins RhoGDI and Eex-1 were cloned using two-hybrid experiments in which the Vav CH region was placed downstream of the Gal4 DNA binding domain (13, 14). This chimeric protein was properly expressed in vivo (Fig. 1C, lanes labeled 7). This version of Vav was as transforming as Vav-(Δ1–186+Δ608–845) (Fig. 1B, compare bars 6 and 7), suggesting that the inhibitory effect of this domain is intramolecular and position-dependent. Similar results were obtained when all these Vav mutants were tested in cytoskeletal and JNK activation assays in NIH3T3 cells and COS1 cells (Table I). These results indicate that the exchange activity of Vav is controlled by two intramolecular events, one mediated by the inhibitory residues of the Ac domain and another one regulated by the CH region of Vav.

We next investigated the behavior of all these mutant proteins in lymphoid cells in relation to their ability to stimulate NF-AT and JNK activities. In the case of NF-AT transactivation assays, we found that full-length Vav proteins (wild type Vav and Vav Y3xF), but not the oncogenic versions, are functional in this biological response, indicating that the structural integrity of Vav protein is required for this specific biological effect (Fig. 1D, left panel). This is in agreement with previous publications indicating that Vav-(Δ1–66) and Vav-(Δ1–186) are inactive in this response (15, 25). Different results were found when these proteins were tested in JNK assays. Vav Y3xF, Vav-(Δ1–144), and Vav-(Δ1–186) stimulate JNK in non-stimulated T-cells (Fig. 1D, right panel, bars 2, 4, and 5). Wild type Vav is inactive in this assay (Fig. 1D, right panel, bar 1). However, and in contrast to results found in NIH3T3 and COS1 cells (Table I), Vav mutants lacking the SH3-SH2-SH3 region were incapable of inducing detectable levels of JNK activation in lymphoid cells (Fig. 1D, right panel, bar 6). This should not be the consequence of the lack of phosphorylation, because the activity of Vav-(Δ1–144) and Vav-(Δ1–186) proteins is phosphorylation-independent (see below). This protein is normally expressed in Jurkat cells (see below, Fig. 6, E and F). The importance of these results is 2-fold. On the one hand, they show that the CH and SH3-SH2-SH3 domains, although contributing in both cell types to the inhibition (CH) and activation (SH3-SH2-SH3) of Vav proteins, have different functional roles in lymphoid and non-lymphoid cells. In addition, they also suggest that the mechanism for the activation of the JNK pathway in non-lymphoid and lymphoid cells is different. In the former case, the activation of JNK requires only the activation of Rac1-mediated pathways. In the latter case, activation of this serine/threonine kinase requires an additional signal provided by one of the domains located in the Vav C terminus. This result has been corroborated by experiments with missense point mutations in the SH2 and SH3 domains of Vav (see below, Fig. 5D).

The CH Region Inhibits GDP/GTP Exchange Activity through Intramolecular Interactions with the ZF Region—To corroborate our observations that the CH region regulates Vav activity in cis, we tested the dependence of the GDP/GTP exchange activity of Vav-(Δ1–144) on tyrosine phosphorylation events. If our model were correct, we would expect that the
### Table I

| Type of Vav protein | NIH3T3 | Actin | COS1 | JUN | JURKAT |
|---------------------|--------|-------|------|-----|--------|
|                     | Foci   | Actin | JNK  | NFA-T | JNK |
| WT                  |        |       |      |       |       |
| +L213Q              |        |       |      |       |       |
| +W495L              |        |       |      |       |       |
| +C529S              |        |       |      |       |       |
| WT (linearized)     | +      | NA    | NA   | NA   | NA    |
| +L213Q (linearized) |        |       |      |       |       |
| +W495L (linearized) |        |       |      |       |       |
| +C529S (linearized) |        |       |      |       |       |
| +G519A              |        |       |      |       |       |
| +M520A              |        |       |      |       |       |
| +E525A              |        |       |      |       |       |
| +K559A              |        |       |      |       |       |
| +Q533A              |        |       |      |       |       |
| +E542A              |        |       |      |       |       |
| +Y544A              |        |       |      |       |       |
| +R548A              |        |       |      |       |       |
| +K555A              |        |       |      |       |       |
| +E556A              |        |       |      |       |       |
| +P651A              |        |       |      |       |       |
| +P656A              |        |       |      |       |       |
| +P651L              |        |       |      |       |       |
| +G690V              |        |       |      |       |       |
| +P833L              |        |       |      |       |       |
| +Δ608–845           |        |       |      |       |       |
| +Δ608–845 + K-Ras tail |       |       |      |       |       |
| Δ1–66               | +++    | +++   | +++  | +++  | +     |
| +L213Q              |        |       |      |       |       |
| +W495L              |        |       |      |       |       |
| +C529S              |        |       |      |       |       |
| +Q533A              |        |       |      |       |       |
| +E542A              |        |       |      |       |       |
| +Y544A              |        |       |      |       |       |
| +R548A              |        |       |      |       |       |
| +K555A              |        |       |      |       |       |
| +E556A              |        |       |      |       |       |
| +P651A              |        |       |      |       |       |
| +P656A              |        |       |      |       |       |
| +P651L              |        |       |      |       |       |
| +G690V              |        |       |      |       |       |
| +P833L              |        |       |      |       |       |
| +Δ608–845           |        |       |      |       |       |
| Δ1–186              | +++++  | ++++  | ++++ | ++++ | +++++ |
| +L213Q              |        |       |      |       |       |
| +W495L              |        |       |      |       |       |
| +C529S              |        |       |      |       |       |
| +E525A              |        |       |      |       |       |
| +K559A              |        |       |      |       |       |
| +Q533A              |        |       |      |       |       |
| +E542A              |        |       |      |       |       |
| +Y544A              |        |       |      |       |       |
| +R548A              |        |       |      |       |       |
| +K555A              |        |       |      |       |       |
| +E556A              |        |       |      |       |       |
| +P651A              |        |       |      |       |       |
| +G690V              |        |       |      |       |       |
| +P833L              |        |       |      |       |       |
| +Δ608–845           |        |       |      |       |       |
| +Δ608–845 + K-Ras tail |       |       |      |       |       |
| +Δ608–845 + C-CH    |       |       |      |       |       |
| +Δ608–845 + C-CH    |       |       |      |       |       |
| Δ1–144              | ++++   | ++++  | ++++ | ++++ | ++++  |
| Y2×F                |        |       |      |       |       |
| +L213Q              |        |       |      |       |       |
| +W495L              |        |       |      |       |       |
| +C529S              |        |       |      |       |       |
| +P651L              |        |       |      |       |       |
| +G690V              |        |       |      |       |       |
| +P833L              |        |       |      |       |       |
| +Δ608–845           |        |       |      |       |       |

*Values are given relative to each specific biological read-out and should not be compared among different columns. See “Materials and Methods” for details of each experimental procedure.

*ND, not determined; NA, not appropriate.
removal of the CH region would originate a phosphorylation-
independent, constitutively active protein. To test this possi-
bility, we purified the Vav-(Δ1–144) protein from baculovirus-
infected Sf9 cells and compared its exchange activity with
those of wild type Vav, Vav-(Δ1–186/Δ608–845), and Vav
Y174F. The latter protein was used instead of the Y3xF mutant
utilized in vivo because this last protein is insoluble when
expressed in Sf9 cells (data not shown). To test their depend-
ence on phosphorylation, the exchange activity of these pro-
tein toward Rac1 was determined before and after phospho-
rylation by the tyrosine kinase Lck, as described previously
(12). In agreement with published results (12), the GDP/GTP
exchange activity of wild type Vav toward Rac1 is dependent on
the prior phosphorylation by Lck (Fig. 2A). By contrast, the
exchange activity of Vav-(Δ1–186/Δ608–845) is phosphoryla-
tion-independent (Fig. 2A). Vav Y174F was still phosphoryla-
tion-dependent, although the non-phosphorylated protein
displays a higher basal activity than the wild type protein (Fig.
2A). Under the same conditions, Vav-(Δ1–144) shows a cata-
lytic behavior identical to Vav-(Δ1–186/Δ608–845) (Fig. 2A).
Thus, this protein is fully active despite containing the three
regulatory tyrosine residues and the rest of the Ac domain intact.

The results shown above are consistent with the idea that
the Vav CH region regulates Vav activity through an intramo-
lecular event. To corroborate this hypothesis further, we inves-
tigated whether the Vav CH region could establish stable
interactions with the rest of the protein. To this end, we first
tested whether a GST fusion protein containing the Vav
CH/Δ608 Ac domains could form complexes with Vav-(Δ1–144)
using in vitro pull-down experiments. As shown in Fig. 2B (lanes
4–7), the GST/Vav fusion protein did associate with Vav-(Δ1–144)
in a concentration-dependent manner. As a control, no
binding was observed when parallel experiments were carried
out with beads either uncoated or coated with the GST protein
alone (Fig. 2B, lanes 3 and 8, respectively). The strength of
the CH/Vav interaction is very weak when compared with the
affinity of Vav for nucleotide-free Rac1 under identical experi-
mental conditions (Fig. 2B, compare lanes 2 and 4–7). We do
not know at present whether this low affinity is an actual
measure of the strength of the CH/Vav interaction or, alterna-
tively, the consequence of measuring an intramolecular inter-

Fig. 2. A, exchange activity of Vav proteins. GDP-loaded GST-Rac1 (4 pmol) was incubated for 45 min at room temperature with [35S]GTP-γS in
the presence of nonphosphorylated (open bars) or phosphorylated (closed bars) Vav proteins (1.8 pmol each). After incubation, aliquots from each
sample were taken in duplicate, and the exchange obtained under each experimental condition was determined by a filter immobilization assay.
B, interaction of purified His6-tagged Vav-(Δ1–144) with the indicated amounts of GST, GST-Rac1, or GST-Vav-CH+Ac. GST-Rac1 was used in the
GDP-bound (lane 1) or nucleotide-free (lane 2) form. After incubation, glutathione beads were washed and analyzed by immunoblotting using
anti-hexahistidine antibodies. WB, Western blot. C, interaction of purified GST-Vav CH+Ac with MBP fused to either β-galactosidase (βGal), the
Vav CH, the Vav DH, or the Vav ZF. After the incubation, MBP proteins were immobilized with amylase beads, washed, and analyzed by
immunoblotting using anti-GST antibodies. D, left panel, interaction of purified GST-Vav CH+Ac with EGFP proteins fused to either the Vav DH,
the Vav PH, or the Vav ZF domain ectopically expressed in COS1 cells. Right panel, immunoblot analysis of total cellular lysates (TCL) from COS1
cells using anti-EGFP antibodies to visualize the levels of expression of the fusion proteins.
action in an assay designed for detecting heteromolecular associations. We next performed pull-down experiments with bacterially expressed Vav domains to identify the regions involved in the CH/Vav interaction. To this end, we purified from E. coli the Vav CH+Ac region as a GST fusion protein and the CH, DH, and ZF regions as maltose-binding protein (MBP)-tagged proteins. As negative control, we used MBP fused to β-galactosidase. After incubation of the GST with each MBP protein in vitro, the MBP proteins were immobilized on amylose beads and washed, and the binding of the GST-Vav CH+Ac domain to them was evaluated by immunoblotting with anti-GST antibodies. As shown in Fig. 2C, the GST-Vav CH+Ac domain could bind to the ZF region and, with lower affinity, to the DH region. This last interaction is probably due to association of the DH with the tyrosine residues of the Ac region, as reported previously (16). In contrast, no binding was detected with the Vav CH region or β-galactosidase (Fig. 2C).

The interaction between the Vav CH and ZF regions was also detected when pull-down experiments were performed using the GST-Vav CH+Ac fusion protein and COS cell lysates expressing the DH, PH, and ZF domains as EGFP fusion proteins (Fig. 2D, left panel). Taken together, these results indicate that the DH down-modulates Vav activity in cis by establishing interactions with the Vav ZF region.

The Central Domains of Vav Are Essential for Its Biological Activity—To evaluate the role of the DH, PH, and ZF regions of Vav, we generated 15 Vav proteins containing missense mutations in each of these domains (Fig. 3A). The residues targeted by these mutations were Leu213 (located in the first α-helix of the DH domain), Trp495 (located in the last α-helix of the PH domain), and Cys529 (located in the second loop of the Vav ZF). These mutations were first tested in the context of the full-length protein in focus formation assays. Because wild type Vav shows very low transforming activity, in these experiments we used linearized plasmids, because this procedure leads to a 300-fold increase in the number of foci scored due to a more efficient incorporation of the transfected plasmids into the host cell genome. Using these conditions, wild type Vav shows low transforming activity (~300 foci/μg−1) (Fig. 3B, upper panel, bar 1). In contrast, the linearized plasmids encoding DH, PH, and ZF mutants of full-length Vav do not transform fibroblasts (Fig. 3B, upper panel, bars 2–4, respectively). This is not due to problems with the expression or phosphorylation of these proteins, because all Vav proteins show identical behavior in these two parameters (Fig. 3C). Mutations in the DH, PH, and ZF regions also abolish the transforming activity of all oncoproteins tested (Vav Y3xF, Vav-(Δ1–66), Vav-(Δ1–186), and Vav-(Δ1–186 + Δ608–845)) (Fig. 3B, lower panel, and data not shown). Similar results were found when these proteins were tested for actin reorganization (Table I) and Rac1 activation (data not shown). Moreover, these mutants are inactive when tested for the stimulation of NF-AT and JNK activities in lymphoid cells (Fig. 3D and Table I; see also Fig. 6D below).

Taken together, these results indicate that, unlike the SH3 and SH2 regions, the three central domains of Vav are required for the effector functions of Vav in all biological responses and cell types.

To evaluate the contribution of these domains to the catalytic activity of Vav, we tested the effect of mutations in the DH, PH, and ZF regions on the GDP/GTP exchange activity of Vav proteins. To facilitate these assays, the Δ1–144 version of Vav was chosen because it does not require phosphorylation for the stimulation of its GDP/GTP exchange activity (see Fig. 2A). As shown in Fig. 3E, the DH and ZF mutants were catalytically inactive under conditions in which Vav-(Δ1–144) displayed high levels of GDP/GTP exchange. Unlike these two mutants, the Vav-(Δ1–144) protein with an inactive PH region showed levels of exchange activity similar to Vav-(Δ1–144). A similar result was obtained when the activity of this mutant was tested in wild type Vav (data not shown). These results indicate that the ZF and PH region have different regulatory roles, with the ZF region acting in cis and the PH region regulating the function of Vav in trans.

Multiple Regulatory Roles of the Vav ZF Region Unveiled by Scanning Mutagenesis—Given the complex role of the ZF region on Vav regulation, we undertook a scanning mutagenesis approach to identify all the residues of the ZF region essential for its function. To this end, we mutated several residues of the Vav ZF with putative solvent-exposed radicals into alanine. Because the tertiary structure of the Vav ZF is still unknown, we selected our targets for mutagenesis using the known structures of the Raf-1 and protein kinase C-δ ZF regions. Based on those comparisons, we mutated in wild type Vav and Vav-(Δ1–66) a total of 13 residues of 24 possible surface-exposed amino acids (Fig. 4A). Residues not mutated belonged to three groups: amino acids not conserved among Vav family members, cysteine and histidine residues that coordinate the two Zn2+ atoms of the Vav ZF, and glycine residues (due to their low tendency to participate in binding interfaces). When these mutations were tested in focus formation assays, we found that four of them severely affected the transforming activity of Vav-(Δ1–66) (mutations M520A, Q542A, Y544A, and K555A), four reduced the transforming activity of Vav-(Δ1–66) by at least 2-fold (mutations Q519A, E525A, M534A, and P563A), and five had no significant effect (Fig. 4B). All mutant proteins are expressed at similar levels that Vav-(Δ1–66), as assessed by immunoblotting of COS1 cell lysates (Fig. 4C, lower panel). Notably, we could not identify mutations capable of activating or enhancing the transforming activity of wild type Vav or Vav-(Δ1–66) (Fig. 4B, Table I). This result indicates that the mutations generated on Vav have not targeted the ZF interface responsible for the interaction with the CH region or, alternatively, that this region may overlap with the region involved in the binding to Rac proteins.

To evaluate the activity of these ZF mutants on Rac1 activation, we measured their ability to promote the incorporation of GTP molecules onto Rac1 in vivo using GST-PAK pull-down experiments. Upon cotransfection in COS1 cells, Vav-(Δ1–66) can induce an increase in the levels of GTP-bound Rac1 (Fig. 4C, upper panel). In contrast, Vav proteins with the ZF mutations Q542A, Y544A, and K555A were totally inactive in this functional read-out. This result suggests that this region is probably involved in the interaction with the GTPase substrates. Interestingly, we found several Vav ZF mutants (M520A and M534A) that, despite not being transforming, are capable of activating Rac1 in vivo. The case of the mutation M520A is the most notable, because it totally abolishes the transforming activity of Vav-(Δ1–66) while keeping exchange activity toward Rac1 (Fig. 4C, upper panel). All Vav proteins used in these transfections were expressed at similar levels, as shown in Fig. 4C (lower panel). Likewise, Rac1 shows similar levels of expression in the transfected cells (Fig. 4C, middle panel). Taken together, all these results indicate that the ZF region has at least three different functional roles on Vav. First, it promotes the inhibition of wild type protein by establishing interactions with other regions of the molecule. Second, it cooperates with the DH region to facilitate GDP/GTP exchange on Rho/Rac substrates. Finally, it is involved in a third function, unrelated to GDP/GTP exchange, which is essential for the transforming activity of Vav. This last result has biological relevance, because it suggests that the transformation induced by Vav proteins requires the engagement of parallel
routes to those mediated by Rho/Rac proteins.

Role of the C-terminal Domains on the Biological Activity of Vav Proteins—Because our previous results have indicated that the deletion of the C terminus of Vav had a negative impact on the biological activity of this protein in lymphoid and non-lymphoid cells, we performed mutagenesis experiments to...
FIG. 4. A, amino acid sequence of the mouse Vav ZF region. Residues involved in Zn\(^{2+}\)/H\(^{11001}\) coordination are shown in boldface type. The Zn\(^{2+}\) loops are indicated at the top. Hydrophobic residues probably contributing to the structural core and that are buried in the interior of the ZF are outlined. All the possible target sites for the scanning mutagenesis are underlined. The residues actually targeted for mutagenesis are indicated by gray circles. The predicted secondary structure is shown at the bottom. B, NIH3T3 cells were transfected with non-linearized plasmids encoding Vav-(Δ1–66) or Vav-(Δ1–66) proteins with the indicated mutations in the zinc finger region (0.6 μg each). Values (normalized as foci per μg of transfected plasmid) represent the mean of two independent experiments, each performed in duplicate. C, activation of Rac1 by Vav ZF mutants. COS1 cells were transfected with a plasmid encoding wild type Rac1 (AU5 tagged, 5 μg) either alone or in the presence of plasmids (5 μg each) encoding the indicated Vav proteins. After culture, cells were lysed and total cell lysates subjected to either GST-PAK pull-down experiments (upper panel), anti-AU5 immunoblot (middle panel), or anti-Vav immunoblots (lower panel). WB, Western blot.

A

Zn\(^{2+}\)-I

516 HDQMS ETTSCK CQMLRGTFTQYRCRCP HEKEL GRPPS 564

β1 β2 β3 β4 β5 α1

B

C

Vav PROTEIN

None Δ1-66 Q519A Q520A E525A K530A M531A M534A Y544A K554A E562A P563A

GTP-Rac1 Rac1 Vav Δ1-66

anti-AU5 anti-AU5 anti-Vav
analyze the contribution of each of the Vav C-terminal domains to the biological properties of this protein (Fig. 5A). The sites targeted in these experiments were proline residues 651 and 833 (located in the N- and C-SH3 domains, respectively) and glycine residue 690 (located in the SH2 domain). These sites have been commonly mutated before in other signaling molecules to assess the role of their SH3 and/or SH2 domains in vitro and in vivo. By using focus formation assays, we found that these mutants display different behavior depending on the type of Vav protein used. Thus, the presence of the three C-terminal domains is mandatory for the transforming activity of wild type Vav (Fig. 5B, upper panel). Instead, the most N-terminal SH3 domain is dispensable for all the oncogenic versions of Vav proteins (Fig. 5B, lower panel, compare bars 6 and 7, 11 and 12, and 16 and 17). The SH2 domain is essential for the transforming activity of Vav-(Δ1–66) and, to a lower extent, Vav Y3xF (Fig. 5B, lower panel, compare bars 11 and 13 and 6 and 8). The function of this domain is less important for Vav-(Δ1–186), because this mutant protein has still moderate levels of transforming activity (Fig. 5B, lower panel, compare bars 16 and 18). The C-SH3 domain is important for Vav-(Δ1–66) proteins (Fig. 5B, lower panel, compare bars 11 and 14). However, Vav Y3xF and Vav-(Δ1–186) are less sensitive to the effects of this mutation (Fig. 5B, lower panel, compare bars 6 and 9 and 16 and 19). Indeed, these mutant proteins keep a biological activity similar to or higher than that displayed by Vav-(Δ1–66) (Fig. 5B, lower panel, compare bar 11 with bars 9 and 19). Interestingly, we found that the SH2 and C-SH3 mutations do not have an additive effect in the biological activity of Vav-(Δ1–186), because the transforming activity of Vav proteins with single mutations in the SH2 and C-SH3 domains is identical to that displayed by the Vav-(Δ1–186) protein lacking the entire C-terminal region (Fig. 5B, lower panel, compare bars 18–20). This result suggests that the SH2 and C-SH3 domains work together during signal transduction to generate fully functional Vav proteins. These results indicate that the SH2 and C-SH3 domains are essential for all the phosphotyrosine-dependent forms of Vav, whereas the N-SH3 is only important for wild type Vav.

We expressed these mutants in the context of full-length Vav to see the phosphorylation status of these proteins on COS1 cells (Fig. 5C). No changes in the phosphorylation levels were found in Vav proteins with null mutations in either the N- or C-SH3 domain. However, a lower interaction with the auto-phosphorylated EGF-R was appreciated in the case of the Vav C-SH3 mutant protein. As expected, the Vav SH2 mutant does not become phosphorylated or associated with the EGF-R under these conditions (Fig. 5C, upper panel). Anti-Vav immunoblots confirmed that the mutant Vav proteins were expressed in cells, although the SH2 mutant protein always displayed lower levels of expression than wild type and the other mutant proteins utilized in these experiments (Fig. 5C, lower panel).

Finally, we evaluated the activity of these mutant Vav proteins in T-cells. In the case of NF-AT responses, we tested only mutants generated in the context of full-length Vav and Vav Y3xF, because the N-terminally truncated proteins are inactive in this biological response (see Fig. 1D). As shown in Fig. 5D, the SH2 and C-SH3 domains are important for the induction of NF-AT by full-length Vav (compare bars 1, 3, and 4). The N-SH3 mutation shows a milder inhibitory effect, reducing the activity of Vav by 60% in non-stimulated and CD3-stimulated Jurkat cells (Fig. 5D, bars 1 and 2). Similar results were found when these mutants were generated in Vav Y3xF (Table I). In the case of JNK, we utilized the mutants constructed in the oncogenic versions of Vav, because wild type Vav shows no activity in this assay (see above, Fig. 1D). By using these mutants, we found that the Vav SH2 and C-SH3 domains are important for the activation of JNK in T-cells induced by oncogenic Vav-(Δ1–186) (Table I). As in the case of NF-AT responses, the N-SH3 showed a milder inhibitory action. Similar results were obtained with Vav-(Δ1–66) and Y3xF (Table I). These results, together with those reported in Fig. 1, confirm that the C-terminal regions of Vav play important effector roles in the pathway leading to the activation of JNK.

Membrane Localization of Vav Leads to Enhanced Responses in Fibroblasts but Not T-cells and Rescues in a Cell-specific Manner the Absence of the SH3-SH2-SH3 Region—Although the C-terminal domains are not essential for the transforming activity of the phosphorylation-independent versions of Vav (see Fig. 1), their deletion or mutation lowers the overall level of cell transformation induced by these proteins (see Figs. 1, 3, and 5), suggesting that these domains must be implicated in regulatory and/or effector functions besides its role on promoting the phosphorylation of full-length Vav. To look for additional clues for the function of these proteins, we decided to investigate the possible role of these sequences in membrane localization. We reasoned that if the C-terminal domains were involved in membrane translocation and/or phosphorylation of Vav, the incorporation of membrane-tethering signals in these proteins would rescue the functionality of Vav proteins lacking SH3-SH2-SH3 regions. Instead, if these domains were involved in effector functions, no rescue would be expected. To differentiate these possibilities, we investigated the effect of the incorporation of the C-terminal tail of K-Ras to Vav proteins in vivo. This tail contains the polybasic region and the CAAX box of K-Ras, an amino acid sequence that allows the translocation of proteins to the plasma membrane. These sequences were incorporated at the C terminus of Vav-(Δ608–845) and Vav-(Δ1–186 + Δ608–845) proteins (Fig. 6A, proteins 3 and 6). When tested in focus formation assays, we found that Vav proteins containing the K-Ras tail have higher transforming activities than their unmodified counterparts (Fig. 6B, compare bars 2 and 3 and 5 and 6). In fact, the activity of these proteins is even higher than those displayed by Vav proteins containing intact SH3-SH2-SH3 regions (Fig. 6B, compare bars 1–3 and 4–6). Similar results were found when these proteins were tested in JNK (in COS1 cells) and morphological assays (both in COS1 and NIH3T3 cells) (Table I). These observations suggest that the C-terminal domains of Vav are mainly implicated in the efficient translocation of Vav to the plasma membrane during cell signaling in non-lymphoid cells.

To test the effect of the incorporation of the K-Ras tail in the phosphorylation of Vav on tyrosine residues, we compared the phosphorylation levels of wild type Vav and Vav-(Δ608–845) with or without the K-Ras tail. As expected, the version of Vav lacking the C-terminal domains cannot associate with the EGF-R and, as a result, is not phosphorylated in vivo (Fig. 6C, upper panel, lane 2) (29). This observation is fully consistent with the data described in Fig. 5. In contrast, when this version of Vav is expressed with the K-Ras tail, the phosphorylation of this protein is rescued (Fig. 6C, upper panel, lane 3). However, this protein cannot associate physically with the activated EGF-R despite its normal levels of tyrosine phosphorylation (Fig. 6C, upper panel, lane 3). As positive control, wild type Vav shows normal levels of phosphorylation and association with the receptor (Fig. 6C, upper panel, lane 1).

To evaluate the effect of the incorporation of the K-Ras tail in the biological activity of Vav proteins in lymphoid cells, we tested the activity of Vav-(Δ608–845 + K-Ras tail) in Jurkat cells using both NF-AT and JNK assays. This form was selected for the study because it contains the CH domain, a region important for the NF-AT response (see Fig. 1D). It was found
A schematic representation of the Vav mutants used in these experiments. B, upper panel, transforming activity of mutants constructed in full-length Vav. NIH3T3 cells were transfected using with 1 µg of ScaI-linearized vectors encoding wild type Vav (bar 1), Vav P651L (bar 2), Vav G690V (bar 3), Vav P833L (bar 4) or Vav(Δ608–845) (bar 5). Lower panel, NIH3T3 cells were transfected using non-linearized plasmids encoding Vav Y3xF (0.5 µg, bar 6), Vav Y3xF+P651L (0.5 µg, bar 7), Vav Y3xF+G690V (0.5 µg, bar 8), Vav Y3xF+P833L (0.5 µg, bar 9), Vav Y3xF+Δ608–845 (0.5 µg, bar 10), Vav(Δ1–66) (0.5 µg, bar 11), Vav(Δ1–66+P651L) (0.5 µg, bar 12), Vav(Δ1–66+G690V) (0.5 µg, bar 13), Vav(Δ1–66+P833L) (0.5 µg, bar 14), Vav(Δ1–66+Δ608–845) (0.5 µg, bar 15), Vav(Δ1–186) (0.05 µg, bar 16), Vav(Δ1–186+P651L) (0.05 µg, bar 17), Vav(Δ1–186+G690V) (0.05 µg, bar 18), Vav(Δ1–186+P833L) (0.05 µg, bar 19), Vav(Δ1–186+Δ608–845) (0.05 µg, bar 20). Values (normalized as foci per µg of transfected plasmid) represent the mean ± S.D. of three independent experiments, each performed in duplicate.

C, expression of wild type Vav (lane 1), Vav P651L (lane 2), Vav G690V (lane 3), and Vav P833L (lane 4). Proteins were immunoprecipitated from COS1 cells and immunoblotted using either anti-phosphotyrosine (upper panel) or anti-Vav (lower panel) antibodies. D, NF-AT activity in non-stimulated (open bars) or CD3-stimulated (closed bars) Jurkat cells expressing wild type Vav (bar 1), Vav P651L (bar 2), Vav G690V (bar 3), Vav P833L (bar 4), and Vav(Δ608–845) (bar 5). Values represent the mean ± S.D. of three independent experiments, each performed in triplicate.
FIG. 6. A, schematic representation of the Vav mutants used in these experiments. The K-Ras tail is indicated by a C-terminal wavy line. B, NIH3T3 cells were transfected with non-linearized plasmids encoding wild type Vav (1 µg, bar 1), Vav-(Δ608–845) (1 µg, bar 2), Vav-(Δ608–845 + K-Ras tail) (1 µg, bar 3), Vav-(Δ1–186) (0.05 µg, bar 4), Vav-(Δ1–186 + Δ608–845) (0.05 µg, bar 5), Vav-(Δ1–186 + Δ608–845 + K-Ras tail) (0.05 µg, bar 6), Vav-(Δ1–186 + W495L + Δ608–845) (0.5 µg, bar 7), Vav-(Δ1–186 + C529S + Δ608–845) (0.5 µg, bar 8), Vav-(Δ1–186 + W495L + Δ608–845 + K-Ras tail) (0.5 µg, bar 9), and Vav-(Δ1–186 + C529S + Δ608–845 + K-Ras tail) (0.5 µg, bar 10). Values (normalized as foci per µg of transfected plasmid) represent the mean ± S.D. of four independent experiments, each performed in duplicate. C, expression of wild type Vav (lane 1), Vav-(Δ608–845) (lane 2), and Vav-(Δ608–845 + K-Ras tail) (lane 3). Proteins were immunoprecipitated from COS1 cells and immunoblotted using either anti-phosphotyrosine (upper panel) or anti-Vav (lower panel) antibodies. D, left panel, NF-AT activity in non-stimulated (open bars) or CD3-stimulated (closed bars) Jurkat cells expressing wild type Vav (bar 1), Vav-(Δ608–845) (bar 2), Vav-(Δ1–186 + Δ608–845) (bar 5), Vav-(Δ1–186 + Δ608–845 + K-Ras tail) (bar 6), Vav-(Δ1–186 + W495L + Δ608–845) (bar 7), Vav-(Δ1–186 + C529S + Δ608–845) (bar 8), Vav-(Δ1–186 + W495L + Δ608–845 + K-Ras tail) (bar 9), or Vav-(Δ1–186 + C529S + Δ608–845 + K-Ras tail) (bar 10). Values represent the mean ± S.D. variations of at least three independent experiments, each performed in triplicate. E, expression of wild type Vav (lane 1), Vav-(Δ608–845) (lane
that the incorporation of K-Ras tail to this version of Vav does not rescue the functional defect derived from the removal of SH3-SH2-SH3 sequences in NF-AT activation (Fig. 6D, left panel, bars 2 and 3). As positive control, the overexpression of Vav does lead to high levels of NF-AT activation in non-stimulated and stimulated cells (Fig. 6D, left panel, bar 1). To analyze in more detail the cause of this defective rescue, we measured the phosphorylation levels of these proteins in the transfected cells. As shown in Fig. 6E (upper panel, lanes labeled as 1), the overexpression of wild type Vav leads to the constitutive phosphorylation of this protein in Jurkat cells. The phosphorylation of wild type Vav is not increased upon CD3 stimulation, suggesting that it is fully phosphorylated in this experimental setting. In contrast to the results observed in COS1 cells, the Vav protein modified with the K-Ras tail is not phosphorylated even upon stimulation with CD3 antibodies (Fig. 6E, upper panel, lanes labeled as 3). This is not due to expression problems, because this protein is expressed at higher levels than wild type Vav in these cells (Fig. 6E, lower panel). When the subcellular localization of these proteins was investigated, the Vav-(Δ608–845) protein with the K-Ras tail showed a constitutive presence in the plasma membrane that was independent on the activation status of T-cells (Fig. 6F, lanes labeled as 3). This result indicates that the phosphorylation process of Vav proteins differs significantly in lymphoid and non-lymphoid cells, because the integrity of the SH2 and SH3 regions is required for the activation of Vav in T-cells. When tested in JNK assays, the incorporation of the K-Ras tail to Vav-(Δ1–186+Δ608–845) does not rescue the inability of this mutant to stimulate JNK activity under conditions in which Vav-(Δ1–186) elicits a robust response (Fig. 6D, right panel, bars 4–6). This lack of rescue cannot be attributed to lack of proper phosphorylation of the C-terminally deleted Vav proteins, because the activity of Vav proteins with Δ1–186 deletions is phosphorylation-independent (see Fig. 2A). This result is an additional proof for the model implicating the entire C terminus in the signaling pathway that mediates JNK activation in T-cells.

Membrane Localization of Vav Does Not Rescue the Inactivity of PH and ZF Mutants—Because the PH and ZF regions of Vav may also have roles in membrane tethering activities, we used a similar experimental strategy to check whether the Vav proteins that were inactive due to mutations in these two domains could be rescued by the incorporation of the K-Ras tail (Fig. 6A, proteins 9 and 10). In contrast to what was found with the C-terminal domains, the incorporation of the K-Ras C terminus to Vav PH and ZF mutants did not rescue the biological activity of these proteins in fibroblast cells (Fig. 6B, bars 7–10). The same results were found in JNK transactivation assays in Jurkat cells (Fig. 6D, right panel, bars 7–10). Taken together, these results indicate that the PH and ZF domains contribute to the biological activity of Vav independently of its phosphorylation or membrane tethering activities.

DISCUSSION

In this report, we have used a total of 66 mutants to address the specific role of the eight structural domains of Vav in the activation and signaling properties of this Rac GEF. In order to maximize the information obtained in this study, these mutations were generated in the wild type protein and its four known oncogenic derivatives. Moreover, these mutants were studied in three different cell lineages and in a number of biological responses. This strategy was important in order to unveil new functions for these structural domains. To our knowledge, this is the first time in which a comprehensive study of all the structural domains of Vav was undertaken. In general terms, the conclusion derived from these experiments is that the biology of Vav proteins is highly complex, with several structural domains in charge of a number of regulatory and/or effector functions that, in most cases, are cell type-specific.

One of the main observations of this work pertains to the mechanism of activation of Vav by tyrosine phosphorylation. Recent structural NMR studies with a truncated version of Vav DH have shown that the regulation of the catalytic activity of Vav is mediated by an intramolecular Ac/DH interaction (16, 17). Our results indicate that the mechanism of Vav regulation in the context of the full-length protein is more complex, requiring the additional cooperation of the CH and ZF regions. In agreement with this new model, we have demonstrated that the transforming activity of Vav proteins lacking the inhibitory tyrosine residues is significantly lower than that displayed by Vav-(Δ1–186), a phosphorylation-independent version of Vav. We have also shown that forms of Vav lacking the entire CH region (Δ1–144) can be highly transforming despite containing an intact Ac domain. The effect of the CH region on the activity of Vav is in cis, because the deletion of this domain leads to the generation of constitutive, phosphorylation-independent versions of Vav. The negative regulatory action of the CH region is mediated by its interaction with the Vav ZF region. This effect is position-dependent, because the transfer of the CH to the Vav C terminus does not result in the inhibition of its activity. Taken together, these observations are consistent with an alternative model for Vav activation involving the simultaneous release of CH/ZF and Ac/DH interactions during signal transduction. In this new model, the main regulatory point appears to be the CH/ZF interaction, whereas the Ac/CH interaction has only a secondary role, probably by facilitating the formation of a stable interaction between the CH and ZF regions. This is in agreement with our data showing that the disruption of the Ac/DH association by site-directed mutagenesis only results in the generation of phosphorylation-dependent Vav proteins with moderate transforming activity. In addition, we have observed that the affinity of the N-terminal domains toward the ZF domain is higher than toward the DH region. The results of the CH/ZF interaction are probably 3-fold. First, it may cooperate with the Vav Ac domain to limit the physical access of Rac1 to the DH domain. Second, it will impinge on the binding of Rac1, because the ZF region also cooperates with the DH domain in the catalytic process. Because the CH domain exerts effector functions in some T-cell responses (NF-AT activation), this model of regulation offers also a good molecular stratagem for blocking simultaneously the CH- and Rac1-mediated pathways in non-stimulated T-cells.

These studies have also revealed the critical role of the DH, PH, and ZF regions for the biological activity of Vav. Mutations in the DH domain abolish all the biological activities of wild
type and oncogenic Vav proteins. Because this region is in charge of the catalysis of GDP/GTP exchange on the Vav substrates, these results suggest that the Rac1 pathway plays an essential role in the biological responses triggered by Vav. We have obtained similar results with Vav2 and Vav3, indicating that this catalytic role is crucial for Vav family members (20). The PH region is also essential for the biological activity of Vav (wild type and oncogenic) in all cell types tested. However, we have recently found that this is not a property conserved among Vav family members. For instance, although mutations in the Vav2 PH domain abolish all the biological activities tested, they are inconsequential for the actin remodeling induced by Vav3 in fibroblasts (20). It has been proposed that the Vav PH domain contributes to the activation/deactivation of this protein by binding to phospholipids. According to this model, the binding of PIP2 to the Vav PH domain induces an inhibitory interaction between the Vav PH and DH domains. As a consequence, Vav cannot become phosphorylated when bound to PIP2 molecules. The exchange of PIP2 by PIP3 during signal transduction makes possible the release of this inhibitory conformation, allowing the subsequent activation of Vav (17–19). Our observations are not consistent with such a regulatory role. Thus, we have shown that PH mutations do not enhance the catalytic activity of wild type or oncogenic Vav proteins despite the fact that they eliminate PIP2 binding. We have also demonstrated that Vav PH mutants do not show any alteration on its ability to undergo tyrosine phosphorylation in vivo. Moreover, it has been demonstrated that the effect of the Vav PH domain is independent on phosphorylation events, because its mutation affects the biological activity of phosphorylation-independent Vav proteins. Finally, we have been unable to detect any effect of phospholipids over the catalytic activity of Vav in vitro or any synergistic interaction between wild type Vav and constitutively active forms of PI-3K in vitro. Results from other laboratories have also indicated that Vav proteins are independent on phosphatidylinositol 3-kinase activity, at least from the upstream side (8, 30–32). These negative results are not entirely surprising, because the Vav PH domain does not conform to the recently defined consensus motif for PIP2 binding by PH domains (33, 34). Another possibility is that the Vav PH domain to allow full exchange activity, as described previously for other Rho/Rac GEFs (35). However, this is unlikely for Vav because the mutation of its PH domain does not have any detectable effect on its catalytic activity in vitro. Finally, it has been shown that some PH domains help in the translocation of proteins to the plasma membrane (36). This possibility does not fit Vav biology either, because the negative effects of PH mutations cannot be rescued by the inclusion of membrane-tethering signals such as the K-Ras C-terminal tail. Thus, although the Vav PH domain is essential for the activity of Vav proteins, its precise functional role remains to be elucidated. In any case, its function seems to be linked to the Rac1 pathway because Vav proteins with inactive PH domains cannot activate Rac1 in vivo.

Another domain critical for the biological activity of Vav (wild type and oncogenic) is the ZF region. The function of this domain is essential for Vav, Vav2, and Vav3 (this work, data not shown, and Ref. 20). Our results indicate that this domain plays at least three roles in Vav. On one hand, it appears to be part of a complex intramolecular network of interactions that control the maintenance of the inactive state of Vav proteins. Next, it contributes to the enzyme reaction of Vav toward Rac1. Mutagenesis experiments suggest that this activity is mediated by residues located at the ZF C terminus (Gln542, Tyr544, and Lys555). Finally, our scanning mutagenesis experiments indicate that the N-terminal region of the Vav ZF is involved in a new pathway, previously unknown, that contributes to the transforming activity of Vav in a Rac1-independent manner (Fig. 7). Interestingly, a similar scanning mutagenesis approach was done before with the Raf1 ZF region with different results. For example, several mutants of the Raf1 ZF increased the activation of Raf by Ras, others the basal activity of the kinase, and others were inhibitory (37). In our case, we have obtained only Vav mutants that are either biologically active or inactive. The Ras binding domain of the Raf1 ZF seems also to be located in a different location than the putative Rac1 binding domain of the Vav ZF. These results indicate that, despite a similar folding, the regulatory and/or effector functions of the Vav and Raf ZF are not conserved.

Although the integrity of the DH-PH-ZF core is essential for the biological activity of Vav, its effector properties depend on the cell type analyzed. In fibroblasts, the DH-PH-ZF cassette is sufficient to induce all biological activities tested (Fig. 7). However, this is not the case in lymphoid cells. For instance, the activation of JNK by Vav requires the integrity of the SH3-SH2-SH3 region in T-cells, indicating that some adaptor/effector proteins should collaborate with the Rac1 pathway to achieve the activation of this downstream serine/threonine kinase (Fig. 7). This result resembles the need for the co-engagement of T-cell and CD28 receptors to obtain JNK activation in lymphocytes (38). The induction of NF-AT responses by Vav is even more complex, requiring the concurrent participation of the SH3-SH2-SH3 region and the CH domain (Fig. 7). Taken together, these results suggest that, unlike fibroblasts, the activation of JNK and NF-AT responses by Vav in T-cells requires the engagement of the Rac1 pathway plus one (mediated by the SH3-SH2-SH3 regions) or two additional pathways (mediated by the SH3-SH2-SH3 and CH regions), respectively (Fig. 7). The need for these differential signaling requirements between fibroblast and lymphoid cells remains unknown. However, it has been reported recently (39) that JNK activation inhibits cell proliferation at the early times of T-cell stimulation. The engagement of two different pathways could be a molecular trick to make sure that the optimal activation of JNK activation is only accomplished at times more permissive with cell proliferation.

The functional role of the Vav SH3-SH2-SH3 region is also complex and cell type-dependent. In the case of fibroblasts, this region participates in two coordinated functions, the facilitation of the association of Vav with receptors (with the subsequent Vav phosphorylation) and, in addition, the translocation of Vav to the plasma membrane. As expected, the first function is essential for the biological activity of the phosphorylation-dependent versions of Vav. The function of the SH2 region in this process can be substituted by the incorporation of membrane-tethering signals at the Vav C terminus, suggesting that the translocation of Vav to the plasma membrane is enough to ensure its phosphorylation in fibroblasts. In addition to phosphorylation, we have demonstrated that mutations in the SH2 and C-SH3 domains reduce, but do not abolish, the biological activity of Vav Δ1–186. Because the activity of this protein is independent of phosphorylation (29), these results indicate that membrane translocation is required for ensuring not only the activation of Vav by tyrosine phosphorylation but also for the efficient engagement of downstream elements. The function of the Vav SH2-SH3 domains in this capacity can be substituted, even with more efficiency, by the K-Ras tail, sug-

2 J. L. Zugaza, M. A. López-Lago, M. J. Caloca, M. Dosil, N. Movilla, and X. R. Bustelo, unpublished observations.
gesting that the function of those domains is to ensure the physical proximity of Vav to its downstream effectors in the membrane rather than mediating the direct physical association with them. These results also provide evidence indicating that several putative partners of Vav that bind to the C-SH3 region are not important for the biological activities of Vav tested in fibroblasts (i.e. hnRNP-K, hnRNP-C, p44). Whether they play roles in other biological routes unrelated to the responses studied in this work remains to be determined. Interestingly, and unlike tyrosine phosphorylation, the function of the Vav C terminus in membrane translocation needs the coordinated effort of the SH2 and C-SH3 domain, because the inactivation of the two domains simultaneously gives the same reduction in the biological activity of Vav proteins than the separate mutation of each domain. In contrast to this simplistic model found in fibroblast, the role of the Vav SH3-SH2-SH3 region is more complex in lymphocytes. In addition to the effector functions on JNK and NF-AT responses already discussed, we have seen that the SH3-SH2-SH3 region plays very important functions in the phosphorylation of Vav protein in lymphoid cells. However, unlike fibroblasts, we have observed that membrane translocation does not ensure Vav phosphorylation. Thus, it seems that the activation of Vav in lymphoid cells requires the physical interaction with the stimulated kinases with Vav via the SH2 and C-SH3 domains. In support of this observation, previous results have shown (40) that mutation of the Vav-binding site of Zap70 cancels the ability of this kinase to phosphorylate Vav. Collectively, these results indicate that the pathways of Vav phosphorylation are mechanistically different in non-lymphoid and lymphoid cells.

The study of the N-SH3 domain also had some special interest of its own, because it is the only domain not conserved through the phylogeny of the Vav family (1). The structure of this domain is also totally different from the usual scaffold found in all the other SH3 domains, including the Vav C-SH3 domain (22). Our studies have shown that this domain is essential for the transforming activity of wild type Vav and, to a lower extent, for the induction of JNK and NF-AT responses in T-cells. In contrast, all the oncogenic versions of Vav (both phosphorylation-dependent or -independent) are immune to the effects of the N-SH3 mutation. The behavior of Vav N-SH3 mutants is therefore totally different from the other mutants used in this study. The specific requirements of the wild type and the oncogenic versions of Vav for the N-SH3 may indicate that the disruption of this domain may be hypomorphic in nature. In agreement with this possibility, we have observed that the N-SH3 Vav mutation decreases, but does not abolish, the induction of NF-AT or JNK activities by Vav in T-cells. In addition, we have observed that the transforming activity of N-SH3 Vav mutants can be rescued by the co-transfection of either synergistic oncogenes (i.e. H-Ras) or activated cytoplasmic tyrosine kinases (i.e. Lck and Syk). Such rescue is not observed when Vav proteins containing mutations in the DH, PH, ZF, SH2, or C-SH3 regions are used.2

In summary, the use of all these mutant Vav proteins has allowed us to obtain a comprehensive glimpse of the regulation of Vav during cell signaling and the complexity of its signaling pathways in lymphoid and non-lymphoid cells. Future studies will allow us to correlate these observations with the engagement of specific effectors and signaling molecules. In addition,
the extensive collection of Vav mutants now available will ensure the precise dissection of any new cellular responses that are under the regulation of Vav proteins.

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