Global conformational rearrangements during the activation of the GDP/GTP exchange factor Vav3

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Activation of Rho/Rac GTPases during cell signaling requires the participation of GDP/GTP exchange factors of the Dbl family. Although the structure of the catalytic core of Dbl proteins has been established recently, the molecular changes that the full-length proteins experience during normal or oncogenic conditions of stimulation are still unknown. Here, we have used single-particle electron microscopy to solve the structures of the inactive (unphosphorylated), active (phosphorylated), and constitutively active (N-terminally deleted) versions of the exchange factor Vav3. Comparison of these forms has revealed the interdomain interactions maintaining the inactive Vav3 state and the dynamic changes that the overall Vav3 structure undergoes upon tyrosine phosphorylation. We have also found that the conformations of phosphorylated Vav3 and N-terminally deleted Vav3 are distinct, indicating that the acquisition of constitutive activity by exchange factors is structurally more complex than the mere elimination of inhibitory interactions between structural domains.

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Introduction

A key event during the activation of cells by extracellular stimuli is the transition of Rho/Rac proteins from the inactive (GDP-bound) to the active (GTP-bound) state, a process that leads to the activation of intracellular pathways linked to cytoskeletal change, mitogenesis, and transcriptomal changes (Van Aelst and D'Souza-Schorey, 1997). This activation step is facilitated by guanosine nucleotide exchange factors (GEFs) of the Dbl family, a group of enzymes that catalyze GDP/GTP exchange on Rho/Rac proteins. Dbl proteins are characterized by the presence of a common structural element composed of Dbl-homology (DH) and plekstrin-homol-

ogy (PH) domains (Zheng, 2001). The DH is an ≈ 200 -amino-acid-long domain that usually contains all the structural information required for substrate recognition, substrate selectivity, and the catalysis of nucleotide exchange (Zheng, 2001). The PH domain is shorter in length (≈ 100 amino-acid residues) and is always located at the DH C-terminus. PH domains play diverse roles in these GEFs, functioning as phospholipid-dependent regulators of GEF activity, as stabilizers of the DH/GTPase complex, or as localization signals to ensure the compartmentalized action of the GEFs in specific regions of the cell (Hoffman and Cerione, 2002). Interestingly, DH–PH cassettes usually display constitutive enzyme activities when tested independently of the rest of domains present in the GEFs (Zheng, 2001), indicating that the full regulatory complexity of Rho/Rac GEFs cannot be understood unless direct information on the architectural plan of those enzymes outside the DH–PH regions is obtained.

The need for such structural information is well illustrated in the case of Vav proteins, a Dbl family subgroup with roles in signaling pathways activated by tyrosine kinases (Bustelo, 2000; Turner and Billadeau, 2002). In addition to the prototypical DH–PH cassette, the three known mammalian Vav proteins (Vav, Vav2, Vav3) contain calponin-homology (CH), acidic (Ac), zinc-finger (ZF), SH2, and SH3 domains (Figure 1A) (Bustelo, 2000; Turner and Billadeau, 2002). The understanding of these GEFs has improved significantly at the genetic, biochemical, and signaling levels during the last decade. Thus, we now know that Vav proteins become stimulated by direct phosphorylation on a tyrosine residue located in the Ac region (Y¹⁷⁴ in Vav, Y¹⁷² in Vav2, Y¹⁷³ in Vav3) (Bustelo, 2002). Moreover, they can become activated oncogenically by mutations deleting their N-terminal regions (Bustelo, 2000). Unlike their wild-type counterparts, these truncated proteins display constitutive, phosphorylation-independent exchange activity (Bustelo, 2002). In contrast to these advances, very fragmentary information is available regarding the intramolecular mechanisms regulating their exchange activity and about their overall architectural structure. A significant step in this direction was the analysis by NMR of the Vav DH domain fused to the last amino acids of the Ac region (residues 170–189). These studies revealed that phosphorylation drives Vav enzyme activation by disrupting an inhibitory loop established by interactions between residue Y¹⁷⁴ and the catalytic site of the DH domain (Aghazadeh *et al*, 2000). However, current experimental evidence suggests that this inhibitory loop, although operational *in vivo*, cannot explain the complete mechanism of regulation of Vav proteins. Thus, it has been shown that the CH region cooperates in the modulation of the on/off states of Vav proteins, probably by interacting with the Vav ZF (Movilla and Bustelo, 1999; Zugaza *et al*, 2002). Consistent with this view, CH-deleted Vav proteins containing an intact Ac region show phosphorylation-independent exchange activity (Movilla and Bustelo, 1999; Zugaza *et al*, 2002). In addition, it has also been proposed that the activity of Vav proteins

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Figure 1 Biochemical purification of Vav3 proteins. (A) Schematic representation of the structure of Vav proteins. Each domain has a color assigned, which is maintained in all figures. Arrows indicate the start site of Vav3 ($\Delta 1-144$) and Vav ($\Delta 1-189$). Y^{173} is depicted as a yellow circle. Other phosphorylation sites present in the Ac are shown as white circles. PRR, proline-rich region. (B) Analysis by SDS-polyacrylamide gel electrophoresis of the samples of Vav3 proteins used in this work. WT, wild type. (C) Exchange activity of unphosphorylated and phosphorylated full-length Vav3 proteins. GDP/GTP exchange reactions with RhoA alone (open boxes) and in the presence of unphosphorylated (closed boxes) or phosphorylated (closed triangles) wild-type Vav3. Exchange values were determined in duplicate after the indicated periods of time using a filter immobilization assay. (D) Phosphorylation-independent exchange activity of Vav3 ($\Delta 1-144$). Wild-type Vav3 (WT) or Vav3 ($\Delta 1-144$) pretreated with (+) or without (−) GST-Lck were subjected to exchange reactions as described above. Exchange values were determined in duplicate after 45 min as in panel C.

may be regulated outside the inhibitory loop via the binding of phospholipids to the PH region (Han *et al*, 1998). Biochemical evidence suggests that this second regulatory level could be mediated by interactions between the PH and DH regions that are enhanced and eliminated by phosphatidylinositol-bisphosphate and -triphosphate, respectively (Das *et al*, 2000). Despite these advances, we lack adequate information on the Vav three-dimensional (3D) structure to understand all these regulatory/functional issues at the structural level. Likewise, other problems that are general to all Dbp family members remain to be addressed structurally in the case of Vav proteins. Those include the 3D spatial distribution of the domains within the full-length molecule, their rearrangement during activation, and whether the orientations of the two domains of the DH–PH cassette are fixed or subjected to dynamic changes during the regulatory GEF cycle. Here, we have used single-particle electron microscopy (EM) coupled to computing tools to peep into the structure of a mammalian Vav protein in the inactive, active, and constitutively active versions. This strategy has given us an unprecedented view of the mechanisms modulating the biological activity of Vav proteins and, in addition, of the rearrangements that the architecture of a Rho/Rac GEF undergoes during its transition to activated states.

Results

Protein purification, EM, and image analysis

To date, structural information on Vav proteins and other Rho/Rac GEFs is limited to isolated domains due to difficul-

ties in obtaining large amounts of proteins and crystals for the full-length proteins. Due to this, we have chosen a single-particle EM approach to obtain 3D information on the different functional states of Vav family proteins. This avenue has been facilitated by recent developments in EM techniques that allow to elucidate molecular structures of relatively small proteins that were traditionally out of its scope (Orlova and Saibil, 2004). To perform EM, we selected Vav3 instead of the other Vav family members because this protein can be obtained with higher yields and displays more stable biochemical and catalytic behaviors than the other family counterparts (unpublished observations). Wild-type Vav3 and the Vav3 ($\Delta 1-144$) mutant protein with an N-terminal hexahistidine tag were expressed in insect cells using a baculovirus transduction system. After viral infection, the two proteins were purified to homogeneity by affinity chromatography onto nickel beads (Figure 1B). The phosphorylated version of wild-type Vav3 (from now on, pY-Vav3) was subsequently generated using *in vitro* kinase reactions with glutathione S-transferase (GST)-Lck. After the reaction, the kinase was eliminated by chromatography with a glutathione-coated matrix. As expected (Movilla and Bustelo, 1999), full-length Vav3 was only active when phosphorylated on tyrosine residues (Figure 1C and D). In contrast, Vav3 ($\Delta 1-144$) showed a constitutive, phosphorylation-independent exchange activity (Figure 1D). Aliquots of each preparation were spotted onto EM grids, negatively stained with uranyl acetate, and subjected to EM analysis. This approach allowed us to obtain microscopy fields where single-molecule images could be clearly identified above the background (Figure 2A and data not shown). Single images were extracted without any *a priori* bias (Figure 2B). However, a minor fraction of them was discarded during image processing because they were not consistent with the rest of the data set. The discarded images were extremely large in size, suggesting that they derived from protein aggregates. Selected images for Vav3 (5431 images), pY-Vav3 (3867 images), and Vav3 ($\Delta 1-144$) (4604 images) were processed independently using the EMAN software package (Ludtke *et al*, 1999) (see Supplementary data for further technical details). These particles were classified during 3D refinement into homogeneous averages linked to certain projection angles (Figure 2C–E). There existed a good agreement between final averages and projections, which were then used to build the definitive 3D reconstructions of the three Vav3 states (see below). The data set used to generate each of these volumes was found to be consistent with a unique output solution, revealing the structural homogeneity within each Vav3 state. Many of these averages displayed elongated shapes (Figure 2C–E), suggesting that Vav3 is not organized into a compact globular structure. In agreement to this, the analysis of the Euler angle distribution showed some preference for the elongated side views (Figure 2F–H, upper panels) that distributed along rotations of their longitudinal axis ('rocking of the particle'). Consequently, accurate 3D reconstructions for Vav3 could be obtained from our EM data since no significant missing cone exists in Fourier space, as shown before in the reconstructions of the chaperonin GroEL from side-view images (Ludtke *et al*, 2001). The resolution of the three reconstructions was estimated to be ≈ 22 Å using Fourier shell correlation between two independent volumes and a correlation coefficient cutoff value of 0.5 (Figure 2F–H,

Figure 2 EM of Vav3, pY-Vav3, and Vav3 ($\Delta 1-144$). (A) Representative field from an electron micrograph of Vav3 ($\Delta 1-144$). Single images can be seen over the background. Some selected molecules have been labeled with white asterisks. (B) A few extracted raw images for Vav3 ($\Delta 1-144$) prior to image processing. (C–E) Collection of selected pairs of projections (top row) and class averages (bottom row) in the final step of the refinement process for Vav3 (C), pY-Vav3 (D), and Vav3 ($\Delta 1-144$) (E). (F–H) Top panels: Euler angle distribution for the class averages in Vav3 (F), pY-Vav3 (G), and Vav3 ($\Delta 1-144$) (H) as provided by EMAN. The triangle has been tilted slightly for better visualization. The brightness and height of each column represent the number of images in each class on a logarithmic scale (more height and brightness imply larger number of images). Lower panels: Plots of Fourier shell correlation (FSC) curves between two independent models for the processing for Vav3 (F), pY-Vav3 (G), and Vav3 ($\Delta 1-144$) (H).

lower panels). The reconstructed volumes for Vav3, pY-Vav3, and Vav3 ($\Delta 1-144$) are shown in Figures 3, 4, and 6, respectively.

Location of domains into EM maps by fitting atomic data

To understand in structural terms the changes occurring during the two types of Vav3 activation, we next used fitting tools to locate each of the eight Vav3 structural domains within the reconstructed volumes. This step, although not providing structures accurate at atomic resolution, is useful to link the changes observed in the EM volumes with movements of specific Vav3 domains. Since we had no atomic structures for Vav3, we decided to use available data from homologous domains of other Vav family members and signaling proteins for this fitting step. The domains selected for inclusion in our study were the calponin CH domain (67% similarity to Vav3), the Vav DH domain with (residues 170–375) or without (residues 189–375) the most distal portion of the Ac region (88% similarity), the Sos1 PH domain (62%

similarity), the protein kinase C (PKC) ZF (58% similarity), and full-length Grb2 (59% similarity). This approach is legitimate because there is a high level of structural conservation within each domain family independently of the proteins they derive from. Consequently, it is unlikely that structural differences between those structures and the corresponding domains of Vav3 will result in significant effects in the accuracy of their location at the resolution level used in our EM maps (22 Å). These structures were accommodated into the Vav3 volumes using SITUS, an unbiased biocomputing technique (Wriggers and Birmanns, 2001). In all cases, the preferred domain orientations obtained were those providing a better continuity of the polypeptide chain among nearby domains (see Supplementary data for further technical details). Selected images for Vav3, pY-Vav3, and Vav3 ($\Delta 1-144$) EM volumes with the fitted domains are shown in Figures 3, 4, and 6, respectively. The accuracy of our fitting strategy was further demonstrated by performing reverse volume reconstructions. Thus, when the atomic structures of the calponin, Vav, Sos1, PKC, and Grb2 domains were

Figure 3 Structure of inactive Vav3. (A–F) Views of the Vav3 reconstructed volume before (A–D) and after (E, F) fitting the atomic structures of the indicated domains into the EM density (shown as white transparency). (G, H) Close-up of the Vav3 CH–Ac–DH–ZF region without the EM density. The linkers between the CH–DH, DH–PH, and PH–ZF are not included in this representation since no NMR or crystal structures are available for them. CS, catalytic site; 1, CH α 5– α 6 loop; 2, CH α 3– α 4 loop; 3, CH α 6 helix; 4, inhibitory Ac α -helix.

Figure 4 Structure of pY-Vav3. (A, B, D, E) Views of the Vav3 volume before (A, D, green color) and after (B, E) fitting the atomic structures of the indicated domains into the EM density. We include in panels A and D the structure of unphosphorylated Vav3 (orange color) to facilitate comparisons of the structures. (C, F, G) Modeling of the putative Vav3/Rac1 complex based on the Vav3 EM data and in the previously described crystal structure of the Tiam1/Rac1 complex. The GTPase is shown in ice blue color. The GTPase α -helix containing the C-terminal CAAX box is indicated by an arrow.

filtered to the 22 Å resolution in the predicted orientations of our modeling, the volumes obtained were very similar to those derived from EM images (Supplementary Figure S1). Furthermore, when we fitted the atomic models as rigid

bodies into the EM densities using crosscorrelation methods provided by the SITUS CoLoRes command (Wriggers and Birmanns, 2001), we obtained correlations above 0.68, 0.57, and 0.58 for Vav3, pY-Vav3, and Vav3 (Δ 1–144), respectively.

This is a good indication of the resemblance of the atomic models and the EM data, especially if we consider that only 85% of the total sequence of Vav3 has been accounted for by the atomic models.

Structure of inactive, wild-type Vav3

The 3D reconstruction of wild-type Vav3-derived EM images indicated that nonphosphorylated Vav3 is a monomeric, elongated protein composed of a massive head and a triangular tail (Figure 3A–F, Supplementary Video S1). The structure of the Vav3 head is highly complex since it harbors the five most N-terminal domains of this molecule (Figure 1A). A significant part of the head volume could be matched unambiguously using the SITUS program with the NMR structure described for the inhibitory Vav Ac/DH region (Aghazadeh *et al*, 2000) (Figure 3E–H, domain in orange). This result suggests that the autoinhibitory loop described by NMR does occur in the context of full-length Vav proteins in the absence of phosphorylation. The fitting of the CH within the head using the SITUS program also located unambiguously this domain at a side position of the Vav3 volume (Figure 3E–H, domain in green; Supplementary Video S1). The CH is in close proximity to the inhibitory loop of the Ac (residues 170–189) (Aghazadeh *et al*, 2000), the catalytic site and, as previously proposed using biochemical experiments (Zugaza *et al*, 2002), with the position assigned to the Vav3 ZF region (Figure 3F–H, Supplementary Video S1). The mass occupied by the CH disappears in the Vav (Δ 1–144) EM volume (see below; Figure 6), further confirming that the computing prediction reflects the actual localization of the CH in this area. At the top of the molecule, the DH and CH regions are interconnected by a well-defined mass that probably corresponds to the proximal region of the Ac domain for which there are no atomic data available (residues 144–170). This segment of the Ac region does not seem to make stable contacts with the DH or the CH, as inferred by the empty space observed in this section of the Vav3 volume (Figure 3B–D, Supplementary Video S1). As a consequence, the Vav3 volume acquires a convex, donut-like shape in this area (Figure 3B–D, Supplementary Video S1).

The PH occupies a region of the Vav3 head with a rough two-fold symmetry at the 22 Å resolution obtained (Figure 3A–H, domain in yellow; Supplementary Video S1). The top SITUS score solution placed the PH in an orientation that allowed connecting its C-terminal end with the ZF and Vav3 tail (Figure 3E–H, Supplementary Video S1). Similar top-ranked solutions were also obtained when this domain was fitted into the EM-derived volumes of pY-Vav3 and Vav3 (Δ 1–144) (see below), suggesting that the solution provided represents the most likely orientation of the PH domain within Vav3. However, as a consequence of its intrinsic two-fold symmetry at this resolution, we cannot exclude formally the possible existence of other orientations given as low-ranked solutions by the SITUS program. Notwithstanding this uncertainty, it is clear that the Vav3 PH is placed far away from the DH catalytic site, lacking any stable contacts with the overall DH surface other than the expected connection through the linker region (Figure 3A–H and Supplementary Figure S2A and B). Accordingly, the orientation of the Vav3 PH region relative to the DH domain is different from the structures of previously described DH–PH cassettes, being located in an intermediate position be-

tween the closed conformation of the Sos1 DH–PH structure (in which the PH faces the catalytic domain of the DH) and the opened conformation of the Tiam1 DH–PH region (in which the PH is located at the back of the DH domain) (Supplementary Figure S2, compare panels A and B with C–H) (Soisson *et al*, 1998; Worthylake *et al*, 2000; Rossman *et al*, 2002). The rest of the Vav3 head volume is occupied by a thin but prominent region that is very well fitted by the PKC ZF (Figure 3A–F, domain in red). The ZF makes contacts with the CH α 5– α 6 loop (Figure 3E–H, Supplementary Video S1). In addition, it is connected to the PH and the tail region by linker regions that have been well resolved in our EM images (Figure 3A–F, Supplementary Video S1). As a result of the conformation of the Vav3 head, the catalytic binding site of the DH domain is buried under a bulky mass composed of the CH and the Ac regions, making it difficult for the protein to interact with the GTPase substrates.

The structure of Vav3 is completed by the tail region containing the most C-terminal SH3–SH2–SH3 domains. The volume of this region fits well with one of the 3D conformations described for Grb2, a protein with a Vav3-like arrangement of SH2/SH3 domains (Maignan *et al*, 1995) (Figure 3E and F, domains in purple and ice blue; Supplementary Video S1). This tail connects to the area occupied by the ZF domain through a short linker of $\approx$ 15 Å of length that probably corresponds to the highly hydrophilic area present between the ZF and the most N-terminal SH3 domain (Figures 1A and 3A–F). The well-defined structure found by EM for the Vav3 SH3–SH2–SH3 region and the associated linker region indicates that the Vav3 C-terminus acquires a rather rigid structure in solution.

Structure of tyrosine-phosphorylated Vav3

The examination of the EM structure of pY-Vav3 indicates that it conserves the segregation of structural domains between head and tail regions (Figure 4A and D, Supplementary Video S2). Despite this, pY-Vav3 undergoes significant displacements of structural domains that affect the internal conformation of both head and tail areas. Such changes are quite extensive in the Vav3 head where major reorientations are seen for the CH, Ac, DH, PH, and ZF domains (Figure 4A, B, D, and E). In the case of the CH, Vav3 phosphorylation leads to the disruption of the previously described interactions with the other regions of the Vav3 head. Due to this, the CH is pulled away from both the ZF and the catalytic site (Figure 4D and E, Supplementary Video S2). The orientation of the CH domain within the new position in the pY-Vav EM volume was not totally unambiguous in this case. We selected as the most likely orientation the top score offered by SITUS since it was fully consistent with the expected displacement that the CH would experience from the unphosphorylated position to the phosphorylated one (Figure 4B and E). The catalytic DH region also undergoes a major displacement upon Vav3 phosphorylation. Such movement is revealed because the DH domain can be fitted now into the EM density only if rotated along its longitudinal axis by $\approx$ 90° with respect to the original position found in inactive Vav3 (compare Figures 3E and F and 4B and E). Such rotation can be easily visualized because the atomic structure of the Vav DH forms an elongated helical bundle arranged in two flat surfaces (Aghazadeh *et al*, 2000). In the inactive Vav3 conformation, one of the flat surfaces containing the catalytic

center is occluded by the CH and the distal Ac region (residues 170–189). However, in pY-Vav, the same flat surface is found facing an empty cavity that is far from the CH domain (Figure 4B and E, Supplementary Video S2). Interestingly, the Vav DH domain cannot be fitted into the pY-Vav3 EM volume unless the inhibitory loop of the Ac region (residues 170–189) is excluded from the analysis (Figure 4D and E, Supplementary Video S2). The displaced Ac loop might remain connected to the CH under these conditions, as inferred from the protruding mass associated to the CH that is still observed by EM (Figure 4D and E, Supplementary Video S2). The rest of the Ac region (residues 144–170) remains well defined as a structure connecting the CH with the top of the Vav3 head (Figure 4C–F, Supplementary Video S2). Taken together, these results indicate that, upon phosphorylation, the catalytic domain of Vav3 undergoes a large rotation along its longitudinal axis that leads to the displacement of the GTPase binding interface away from both the CH and the Ac domain. Another large displacement is observed in the case of the PH region, which becomes now closer to the DH region due to an $\approx 30^\circ$ rotation (Figure 4B, C, E, and F; Supplementary Figures S2I, J, S3C, and D). As a consequence of the combined movements of the DH and PH domains, these two regions become placed in an orientation similar, although not identical, to the Tiam1 DH–PH structure (Worthylake *et al*, 2000) (Supplementary Figure S2, compare panels E–F and I–J). The PH domain in its new orientation cannot either interfere or collaborate with the binding of the GTPase. Finally, it is observed that the whole Vav3 ZF–SH3–SH2–SH3 module changes its position when compared to the 3D reconstruction of unphosphorylated Vav3 (Figure 4). A movement of this whole area is also observed when the Vav3 CH domain is cleaved off (see below, Figure 6), indicating that such displacement is probably due to the ‘relaxation’ of the molecule after breakage of the CH/ZF interaction rather than being induced by possible phosphorylation events in the Vav3 tail area.

Due to all the changes affecting the positions of the CH, Ac, DH, and ZF regions, the GTPase has now free access to the catalytic region (Supplementary Video S2). This is consistent with our previous observations indicating that pY-Vav3, unlike its unphosphorylated counterpart, can physically interact with nucleotide-free Rho/Rac proteins (Movilla and Bustelo, 1999). We modeled the putative structure of the pY-Vav3/GTPase complex by using the available information of the crystal structure of the Tiam1–Rac1 complex (see Materials and methods). According to this modeling, the Vav3 substrates would sit on a cup-like structure composed of the DH, Ac, and CH regions (Figure 4C, F, and G). In such position, most of the GTPase surface would be hidden away from effector molecules and negative regulators by the surrounding walls of the cup (Figure 4C, F, and G). In addition, the membrane-binding region of the GTPase (CAAX box) would be located perpendicular and away from the cup area (Figure 4F and G, arrow), an orientation that would favor the presentation of the GTPase to the plasma membrane upon GTP loading.

Residues involved in the regulation of Vav catalytic activity by tyrosine phosphorylation

The examination of the structures of Vav3 and pY-Vav3 indicates that the CH domain may establish extensive inter-

actions with the Ac region (via its $\alpha 3$ – $\alpha 4$ loop), the DH (via its C-terminal $\alpha 6$ helix) and the ZF (via its $\alpha 5$ – $\alpha 6$ loop) to stabilize the conformation of the inactive Vav3 state (see Figure 3G and H). Moreover, the displacement of the CH region upon phosphorylation suggests that the disruption of some of those interactions may contribute to the acquisition of the activated Vav3 state (Figure 4). However, the resolution obtained with EM does not allow for the identification of the CH residues involved in such intramolecular interactions. To tackle this problem, we performed scanning mutagenesis of the surface-exposed CH residues present in the predicted interfaces and tested the activity of the resulting mutant proteins in either ZF binding or cell transformation assays. Since Vav3 shows poor transforming activity (Movilla and Bustelo, 1999), we decided to utilize the highly related Vav protein in these experiments.

To identify the amino acids of the CH $\alpha 5$ – $\alpha 6$ loop involved in the interaction with the ZF, we incubated a purified maltose binding protein (MBP)-Vav ZF with bacterial lysates expressing a collection of Vav CH–Ac point mutants fused to GST. The MBP fusion protein was then immobilized onto amylose beads, washed, and the bound proteins visualized by anti-GST immunoblots. Similar pull-down experiments have been used before to demonstrate the CH–ZF interaction *in vitro* (Zugaza *et al*, 2002). These experiments indicated that the two acidic amino-acid residues present in the CH $\alpha 5$ – $\alpha 6$ loop (D101, D104) were important for the CH–ZF interaction (Figure 5A, upper panel). These two residues face the ZF region according to our structural modeling (Figure 5C). In contrast, no deleterious effect on the binding of the CH to the ZF was observed when other residues of $\alpha 5$ – $\alpha 6$ loop were mutated (Figure 5A, upper panel). Parallel immunoblots confirmed that the fusion proteins used in the experiments were utilized in equivalent amounts in all samples (Figure 5A, middle and lower panels). No significant effect on ZF binding was observed when CH mutants outside the $\alpha 5$ – $\alpha 6$ were tested (data not shown).

Next, we used focus formation assays to test the transforming activity of Vav proteins mutated in surface-exposed residues of the CH $\alpha 3$ – $\alpha 4$ loop, the CH $\alpha 6$ helix, the CH $\alpha 5$ – $\alpha 6$ loop, and the Ac region. To get information on the contribution of the mutated residues in the stabilization of the inhibitory structure, all mutations were generated in the context of full-length Vav. This version of Vav reflects the conformational status of the inhibitory state of Vav proteins, because it lacks transforming activity *in vivo* unless co-expressed with tyrosine kinases (Schuebel *et al*, 1998). As comparative control, we used either point (Y174F) or N-terminally deleted ($\Delta 1$ –144, $\Delta 1$ –189) Vav mutants previously shown to be oncogenic *in vivo* (Schuebel *et al*, 1998; Lopez-Lago *et al*, 2000; Zugaza *et al*, 2002). We found that mutations affecting residues of either the Ac/DH (I173A) (Aghazadeh *et al*, 2000) or Ac/CH interfaces (N49A, E181K) stimulate Vav transforming activity (Figure 4B and C). This activity is $\approx 50\%$ of the transforming activity of the Vav mutant in which the inhibitory Y¹⁷⁴ residue has been targeted (Figure 5B). The simultaneous mutation of the Ac/CH and Ac/DH interfaces (N49A + I173A) results in transforming activities similar to those of the Y174F mutant (Figure 5B and C), suggesting that the interaction of the inhibitory tyrosine with the DH domain is totally disrupted under those conditions. Instead, the CH/ZF or CH/DH contacts

Figure 5 Activity of Vav CH mutants. (A) Interaction between the CH and ZF regions. Bacterial lysates containing equal amounts of the indicated point mutants of the GST-Vav CH-Ac (lower panel) were incubated with the MBP-Vav ZF bound to amylose beads. After incubation and washes, bound proteins were visualized by anti-GST immunoblots (upper panel). The use of similar amounts of MBP proteins was demonstrated by reblotting of membranes with anti-MBP antibodies (middle panel). PD, pull-down; TCL, total cell lysates. (B) Top panels: Transforming activity of Vav proteins. Residues conserved in mammalian Vav proteins are underlined. Note that residue numbers in Vav shift a +1 position with respect to Vav3 from residues 142 to 181. Bottom panels: Expression of mutant proteins in NIH3T3 cells. Cells were transfected, selected in the presence of neomycin, and the resulting pools evaluated by immunoblot analysis using anti-Vav antibodies. (C) Close-up view of the localization of some of the mutated residues in the Vav3 CH and Ac regions, as predicted by our 3D modeling. For the sake of simplicity, we have not depicted the PH domain.

seem to be passively driven by the interactions described above, since mutations of surface-exposed residues of the CH $\alpha 5$ - $\alpha 6$ loop (D101A, D104A, F105A) or $\alpha 6$ helix (S116A, I120A, A121D, Q122A) do not trigger Vav transforming activity (Figure 5B). Immunoblot experiments confirmed that all these mutant proteins were expressed in cells (Figure 5B, bottom). These experiments indicate that the inhibitory structure of Vav3 is stabilized through interactions of the Ac α -helix with the DH domain and, to a lower extent, by contacts between the CH and Ac regions. In contrast, although participating in the steric occlusion of the GTPase binding site, the CH/ZF interaction does not contribute much to the stabilization of the inhibitory conformation. As expected (Zugaza *et al*, 2002), deletions of the CH region promote much higher transforming activities (≈ 6 - to 12-

fold) than the rest of mutants (Figure 5B, right top panel). This suggests that the integrity of the CH region may have additional structural functions in Vav proteins other than the mere stabilization of the inhibitory structure and the masking of the GTPase binding site.

Structure of Vav 3 ($\Delta 1$ -144)

Deletion of the CH yields a Vav3 protein with phosphorylation-independent exchange activity (Figure 1D) (Movilla and Bustelo, 1999). EM analyses indicate that such mutagenic event induces dramatic changes in the overall conformation of the Vav3 molecule. In this case, the Vav3 head acquires now a linear conformation totally different from the convoluted structure found in the full-length proteins (Supplementary Video S3; compare Figure 6A and D with Figures 3A, B, 4A, and D). As expected, the area corresponding to the CH region is lost from the Vav3 ($\Delta 1$ -144) volume (Figure 6A, B, D, and E). Interestingly, the position of the DH undergoes minor changes with respect to that observed in nonphosphorylated Vav3 (Figure 6B and E, Supplementary Video S3). However, this domain can be fitted into the volume only after removing the inhibitory Ac loop (residues 170-189). Thus, unlike in the case of the bacterially purified Vav Ac-DH fragment, the inhibitory Ac loop is not stable in the context of the full-length protein in the absence of the CH region. More importantly, the rest of the Ac region that acquired a cylindrical structure in full-length Vav3 proteins cannot be visualized in the case of Vav3 ($\Delta 1$ -144) (Figure 6D-F, Supplementary Video S3), suggesting that the entire Ac domain acquires a highly disorganized state upon removal of the CH region. This view is fully consistent with the constitutive catalytic activity of Vav3 ($\Delta 1$ -144) and with the identical transforming activities of Vav ($\Delta 1$ -144) and ($\Delta 1$ -189) mutants (Movilla and Bustelo, 1999; Zugaza *et al*, 2002). The PH undergoes another rotation that puts it in a parallel position with respect to the longitudinal axis of the molecule (Figure 6A-F and Supplementary Video S3, Supplementary Figure S3E and F). As a consequence of such rotation, the relative orientation of the PH with respect to the DH is different from the positions previously seen in Vav3 and pY-Vav3 (Supplementary Figure S3). Despite the fact that the PH becomes now closer to the C-terminal end of the catalytic region (Figure 6 and Supplementary Figure S3, Supplementary Video S3), its position is still far away from the GTPase binding site. Finally, it is observed that the ZF-SH3-SH2-SH3 region of Vav3 ($\Delta 1$ -144) undergoes a shift in its position when compared with inactive Vav3 (Supplementary Video S3, compare Figure 6B and E with Figure 3E and F).

Due to these conformational changes in Vav3 ($\Delta 1$ -144), its catalytic domain is fully accessible for the binding of the GTPase substrates (Figure 6C and F). However, the changes experienced by the Vav3 head will probably impose a different structure of the Vav3/GTPase complex. Thus, the cup-like structure embracing the bound GTPase in pY-Vav3 is not present in this case as a consequence of the destabilization of the Ac domain (Figure 6C and F). Moreover, the modeling of the Vav3/GTPase complex suggests that the orientation of the bound GTPase relative to the rest of the Vav3 molecule would also be distinct due to the different location of the catalytic site in Vav3 ($\Delta 1$ -144) and pY-Vav3 (compare Figures 4C and 6F). These results indicate that the overall

Figure 6 Vav3 ($\Delta 1-144$) structure. (A, B, D, E) Views of the Vav3 reconstructed before (A, D) and after (B, E) fitting the atomic structures of the indicated domains into the EM density. (C, F) Modeling of the Vav3 ($\Delta 1-144$)/Rac1 complex as described in Figure 4. The arrow in panel C signals the GTPase CAAX box.

conformation of Vav proteins and their way of interacting with the GTPase substrate are highly contingent on the presence or absence of the CH region.

Discussion

We have described here the tertiary structure of Vav3, pY-Vav3, and Vav3 ($\Delta 1-144$) using EM techniques at middle resolution (≈ 22 Å). These molecules have elongated shapes and show their structural domains segregated between a head and a tail area. These two areas harbor the five N-terminal and three C-terminal domains of Vav3, respectively. More importantly, we have shown that the spatial arrangement of these domains changes extensively depending on the functional status of the protein. Thus, it has been observed that the Vav3 head fluctuates between the ‘closed’ conformation of inactive Vav3, the ‘partially opened’ structure of pY-Vav3, and the fully ‘opened’ structure found of Vav3 ($\Delta 1-144$). These changes are not limited to the head area, since the Vav3 tail also changes its orientation relative to rest of the molecule depending on whether Vav3 is in inactive or active states.

Although EM techniques do not provide atomic resolution, our study has given us as a better insight into the nature of the inhibitory structure modulating the exchange activity of Vav proteins. The seminal NMR work with a Vav Ac/DH fragment indicated that such inhibition involved interactions between the catalytic site and an α -helix containing the inhibitory Y¹⁷⁴ residue (Y¹⁷³ in Vav3) (Aghazadeh *et al*, 2000). However, subsequent biochemical analysis suggested that the stability of this inhibitory loop requires the presence of the CH domain when working in the context of full-length proteins (Movilla and Bustelo, 1999; Zugaza *et al*, 2002). Consistent with these observations, we have observed that the CH domain stabilizes the entire Ac region both in the unphosphorylated and phosphorylated states. By contrast, the entire Ac region disappears in the 3D reconstructions of

the EM images derived from CH-deleted Vav3 proteins, indicating that the former region becomes totally disorganized in the constitutively active state of this GEF. The CH-dependent stabilization of the Ac region gives a structural explanation for the paradoxical identical constitutive activities of CH-Ac- and CH-truncated Vav proteins despite the fact that the latter ones keep an intact Ac region and the inhibitory tyrosine (Zugaza *et al*, 2002). Our EM data are also consistent with the biochemical observations postulating an intramolecular interaction between the CH and the ZF region in the inactive state of Vav proteins (Zugaza *et al*, 2002).

The analysis of the movements of the structural domains of the Vav3 head in the three activation states analyzed allows making inferences regarding the regulatory cycle of Vav proteins (Figure 7). In nonstimulated cells, the catalytic site of Vav3 is hidden underneath two overlapping layers of domains, an inner one containing the inhibitory Ac loop and an outer one formed by the CH, the ZF, and the rest of the Ac region (Figure 7). Under these conditions, the access of the GTPase to the DH is totally blocked by a bulky mass of interfering domains. However, upon cell stimulation, the phosphorylation of Vav3 will release the inhibitory Ac loop from the DH, leading to the exposure of the catalytic region to the GTPase (Figure 7). Three phenomena collaborate synergistically to such exposure: the displacement of the CH-Ac region away from the catalytic site, the breakage of the CH/ZF interaction, and a large rotation of the DH that moves the catalytic site away from the other three domains. It is apparent that this structure could be moved back to the ‘closed’ configuration via the dephosphorylation of Vav3 by tyrosine phosphatases at the end of the signaling cycle (Figure 7). However, this fine-tuned yin/yang equilibrium is tilted toward a totally unregulated status upon the deletion of the CH region and the subsequent destabilization of the Ac region (Figure 7). Alternatively, a deregulated activation can be obtained by point mutations that abolish some of the interdomain interactions that occur in the unphosphorylated state (Figure 7). In this cycle, the CH region seems to play important structural roles, contributing to the occlusion of the GTPase binding site when Vav proteins are in the inactive state, the stabilization of the Ac region in both phosphorylated and unphosphorylated states, the proper conformation of the pY-Vav3 head and, finally, the orientation of the PH, ZF, and tail region in all Vav3 states.

Mutagenesis experiments performed with the homologous Vav protein indicated that there is a hierarchy in the importance of the intramolecular contacts maintaining the ‘closed’ conformation of inactive Vav proteins. Thus, we have observed that mutations disrupting the contacts of the Ac inhibitory loop with either the DH region or the CH domain result in oncogenic activation. The combination of mutations in those two areas leads to a synergistic effect, resulting in transformation levels similar to those obtained when the inhibitory Y¹⁷⁴ residue is targeted. These results indicate that the CH and Ac domains work as a single unit to ensure the stabilization of the ‘closed’ conformation of Vav proteins. In contrast, we have observed that mutations disrupting the interaction of the CH with the ZF do not trigger Vav activation *in vivo*, suggesting that this contact contributes to the steric occlusion of the catalytic site and to the orientation of the tail area but not to the stabilization of the overall structure of the head. To date, we could not ever get Vav CH and Ac point

Figure 7 Model for the regulation of Vav3 catalytic activity by phosphorylation and N-terminal deletions. Asterisks mark the position of the catalytic site. The asterisk in pY-Vav is missing since it is oriented on the other side of the molecule. Lanes in green and red show activation and inactivation steps, respectively. The thickness of each arrow represents the relative level of activation under each condition. The question mark indicates that the structure of the Vav point mutants is hypothetical. PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; Pi, inorganic phosphate. See Discussion for further details.

mutants with transforming activities as high as the truncated proteins lacking the CH region. This is even so when the inhibitory tyrosine residue or the other potential phosphorylation sites of the Ac region are mutated individually or in combination (Lopez-Lago *et al*, 2000). One possible explanation for these results is that point mutants, unlike the CH deletions, do not have dramatic effects on the stability of the Ac region other than exposing the catalytic site (Figure 7). Alternatively, it is possible that different CH areas will have to be targeted simultaneously to reproduce the effects of the domain deletion. Finally, it is also feasible that the higher transforming activity of CH-deleted Vav proteins observed *in vivo* is not due to higher catalytic rates but, rather, to the different orientation of other domains within the molecule (i.e., the PH region) that may affect the level of translocation of Vav proteins to the plasma membrane and/or their binding to other proteins during signal transduction. Future studies in this area will have to address this pending regulatory issue.

Our results have also shed light on other questions regarding the mechanistics of activation of Vav proteins during cell signaling. Thus, we have observed that the Vav3 PH cannot interfere with the catalytic site in any of the Vav3 versions. This result is inconsistent with the proposed model of regulation of Vav proteins by phospholipids that relied on hypothetical inhibitory interactions between the PH and DH areas (Han *et al*, 1998; Das *et al*, 2000). This model was initially appealing from a signaling point of view, since it gave a mechanistically simple explanation for the activation of Rac1

by the Ras/phosphatidylinositol 3-kinase pathway during cell signaling (Rodriguez-Viciano *et al*, 1997). However, this model has been rather controversial, because accumulating data indicate that the activation of Vav proteins occurs through phospholipid-independent events (Inabe *et al*, 2002; Zugaza *et al*, 2002). In fact, recent observations made with *vav*- and *vav3*-deficient lymphocytes indicate that Vav proteins act upstream, not downstream, of phosphatidylinositol 3-kinase (Costello *et al*, 1999; Inabe *et al*, 2002). Given that the PH seems to establish contacts with other domains only through the expected linker regions at the 22 Å resolution obtained, we surmise that the Vav3 PH works as a hinge region whose position will be passively established by the forces induced by the movements of the other Vav3 domains. This is in agreement with our previous biochemical observations indicating that Vav proteins with mutations disrupting the overall structure of the PH region can bind to, and promote nucleotide exchange on, their GTPase substrates *in vitro* (Movilla and Bustelo, 1999; Zugaza *et al*, 2002). Therefore, it is likely that the important role of the PH regions of Vav proteins *in vivo* might be derived from an action *in trans* unrelated to the direct catalytic regulation. Whether such roles are phospholipid-dependent or not remains to be determined.

An important issue in the GEF field is whether the GEF DH-PH core is fixed in time and space. Until now, the analyses of the structures of isolated DH-PH cassettes from a number of Dbl proteins have indicated that they have a rather rigid conformation (Hoffman and Cerione, 2002).

However, a problem with this approach is that it assumes that the architecture of the DH–PH cassettes does not depend on the protein context in which they operate. This assumption may be correct when working with DH–PH cassettes insulated from the influence of other domains or, alternatively, with DH–PH regions located at the GEF C-terminus. However, the DH–PH structure may be totally dependent on the conformational biases imposed by other domains in other instances. Our EM studies indicate that this is the case for Vav3. Thus, we have observed that the orientation of the PH relative to the DH domain is totally different in inactive Vav3, pY-Vav3, and Vav3 ($\Delta 1$ –144). Moreover, it is observed that the distance between the DH and PH regions changes in the three Vav3 versions studied, indicating that the DH–PH linker region has some level of structural flexibility. Such movements are probably induced by the intramolecular forces created by the interactions of the CH and Ac regions with the rest of the molecule (in the case of Vav3 and pY-Vav3) or, alternatively, by the relaxation of the Vav3 molecule after the breakage of those interactions (in the case of the N-terminally deleted proteins). Taken together, these observations emphasize the importance of studying these GEFs at the full-length level instead of focusing exclusively on the isolated catalytic regions as it has been the traditional approach in most cases. In addition, they indicate that the biological and biochemical properties of the wild type and oncogenic versions of these proteins may be similar, but not identical.

Materials and methods

Sample preparation

Hexahistidine-tagged Vav3 proteins and GST-tagged Lck proteins were obtained from baculovirus-infected *Spodoptera frugiperda* (Sf9) cells using affinity chromatography with nickel- and glutathione-coated beads, respectively (Movilla and Bustelo, 1999). pY-Vav3 was generated by *in vitro* kinase reactions with GST-Lck and subsequent removal of the kinase using glutathione beads (Movilla and Bustelo, 1999). The activity of Vav3 proteins was tested using *in vitro* GDP/GTP exchange assays (Movilla and Bustelo, 1999).

Electron microscopy

Few microliters of each sample were applied to carbon-coated grids after glow discharge. The sample was then negatively stained with 1% uranyl acetate and observed in a JEOL 1230 electron microscope operated at 100 kV. Micrographs were recorded at a nominal magnification of 50 000 under low-dose conditions.

References

- Aghazadeh B, Lowry WE, Huang XY, Rosen MK (2000) Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. *Cell* **102**: 625–633
- Bustelo XR (2000) Regulatory and signaling properties of the Vav family. *Mol Cell Biol* **20**: 1461–1477
- Bustelo XR (2002) Regulation of Vav proteins by intramolecular events. *Front Biosci* **7**: d24–d30
- Costello PS, Walters AE, Mee PJ, Turner M, Reynolds LF, Prisco A, Sarner N, Zamoyska R, Tybulewicz VL (1999) The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. *Proc Natl Acad Sci USA* **96**: 3035–3040
- Das B, Shu X, Day GJ, Han J, Krishna UM, Falck JR, Broek D (2000) Control of intramolecular interactions between the pleckstrin

Image processing and 3D reconstructions

Micrographs were digitized using a Minolta Dimage Scan Multi Pro scanner and averaged to a final 4.1 Å/pixel at the specimen. Single particles were selected from several micrographs for Vav3, pY-Vav3, and Vav3 ($\Delta 1$ –144), using the 'boxer' command from EMAN (Ludtke *et al*, 1999). Particles were normalized, centered, and subjected to 3D refinement as implemented in EMAN (Ludtke *et al*, 1999). Initial volumes were generated by image classification of the whole data set into few average images to build a 3D model using the common lines approach (see Supplementary data for further details). The reconstructions were rendered to show 100% of the protein mass assuming an average density of 1.35 g/ml. Volumes were visualized using VMD (Humphrey *et al*, 1996).

Fitting of atomic structures into EM maps

Atomic structures of selected domains (calponin CH domain, PDB entry 1H67; Vav Ac/DH domain, PDB entry 1F5X; Sos1 PH domain, PDB entry 1AWE; PKC ZF, PDB entry 1TBO; Grb2, PDB entry 1GRI) were fitted into the EM map computationally using the SITUS software (Wriggers and Birmanns, 2001). To help visualize the putative binding site of the Rho/Rac GTPase in Vav3, we positioned the Tiam1 DH domain in an equivalent orientation to the one in Vav3 and then left Rac1 to model a chimeric Rac1/Vav3 structure. The Tiam1 DH/Rac1 complex utilized was derived from PDB entry 1FOE (Worthylake *et al*, 2000). All volumes were rendered and visualized using VMD (Humphrey *et al*, 1996).

Pull-down and transforming activity assays

Pull-down assays of GST-CH mutants and MBP-ZF fusion proteins were performed as indicated (Zugaza *et al*, 2002). Transforming activity of vav-encoding constructs was quantified using focus formation assays (Zugaza *et al*, 2002). Mutations were performed in the mouse vav proto-oncogene cDNA with appropriate combinations of primers using a commercial mutagenesis kit (Quickchange, Stratagene). Other Vav mutants used (Y174F, $\Delta 1$ –144, $\Delta 1$ –189) have been previously described (Zugaza *et al*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Accession numbers

The EM reconstructions have been deposited in the Macromolecular Structure Database (<http://www.ebi.ac.uk/msd/>) with accession codes EMD-1103 (Vav3), EMD-1104 (pY-Vav3), and EMD-1105 (Vav3($\Delta 1$ –144)).

- homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. *J Biol Chem* **275**: 15074–15081
- Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, Krishna UM, Falck JR, White MA, Broek D (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science* **279**: 558–560
- Hoffman GR, Cerione RA (2002) Signaling to the Rho GTPases: networking with the DH domain. *FEBS Lett* **513**: 85–91
- Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* **14**: 27–38
- Inabe K, Ishiai M, Scharenberg AM, Freshney N, Downward J, Kurosaki T (2002) Vav3 modulates B cell receptor responses by regulating phosphoinositide 3-kinase activation. *J Exp Med* **195**: 189–200

- Lopez-Lago M, Lee H, Cruz C, Movilla N, Bustelo XR (2000) Tyrosine phosphorylation mediates both activation and down-modulation of the biological activity of Vav. *Mol Cell Biol* **20**: 1678–1691
- Ludtke SJ, Baldwin PR, Chiu W (1999) EMAN: semiautomated software for high-resolution single-particle reconstructions. *J Struct Biol* **128**: 82–97
- Ludtke SJ, Jakana J, Song JL, Chuang DT, Chiu W (2001) A 11.5 Å single particle reconstruction of GroEL using EMAN. *J Mol Biol* **314**: 253–262
- Maignan S, Guilloteau JP, Fromage N, Arnoux B, Becquart J, Ducruix A (1995) Crystal structure of the mammalian Grb2 adaptor. *Science* **268**: 291–293
- Movilla N, Bustelo XR (1999) Biological and regulatory properties of Vav-3, a new member of the Vav family of oncoproteins. *Mol Cell Biol* **19**: 7870–7885
- Orlova EV, Saibil HR (2004) Structure determination of macromolecular assemblies by single-particle analysis of cryo-electron micrographs. *Curr Opin Struct Biol* **14**: 584–590
- Rodriguez-Viciano P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**: 457–467
- Rossman KL, Worthylake DK, Snyder JT, Siderovski DP, Campbell SL, Sondek J (2002) A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange. *EMBO J* **21**: 1315–1326
- Schuebel KE, Movilla N, Rosa JL, Bustelo XR (1998) Phosphorylation-dependent and constitutive activation of Rho proteins by wild-type and oncogenic Vav-2. *EMBO J* **17**: 6608–6621
- Soisson SM, Nimnual AS, Uy M, Bar-Sagi D, Kuriyan J (1998) Crystal structure of the Dbl and pleckstrin homology domains from the human Son of sevenless protein. *Cell* **95**: 259–268
- Turner M, Billadeau DD (2002) VAV proteins as signal integrators for multi-subunit immune-recognition receptors. *Nat Rev Immunol* **2**: 476–486
- Van Aelst L, D'Souza-Schorey C (1997) Rho GTPases and signaling networks. *Genes Dev* **11**: 2295–2322
- Worthylake DK, Rossman KL, Sondek J (2000) Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. *Nature* **408**: 682–688
- Wriggers W, Birmanns S (2001) Using situs for flexible and rigid-body fitting of multiresolution single-molecule data. *J Struct Biol* **133**: 193–202
- Zheng Y (2001) Dbl family guanine nucleotide exchange factors. *Trends Biochem Sci* **26**: 724–732
- Zugaza JL, Lopez-Lago MA, Caloca MJ, Dosil M, Movilla N, Bustelo XR (2002) Structural determinants for the biological activity of Vav proteins. *J Biol Chem* **277**: 45377–45392