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Crucial structural role for the PH and C1 domains of the Vav1 exchange factor

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The Vav family of proteins are guanine nucleotide exchange factors (GEFs) for the Rho family of GTPases, which regulate various cellular functions, including T-cell activation. They contain a catalytic Dbl homology (DH) domain that is invariably followed by a pleckstrin homology (PH) domain, which is often required for catalytic activity. Vav proteins are the first GEFs for which an additional C1 domain is required for full biological activity. Here, we present the structure of a Vav1 fragment comprising the DH–PH–C1 domains bound to Rac1. This structure shows that the PH and C1 domains form a single structural unit that packs against the carboxy-terminal helix of the DH domain to stabilize its conformation and to promote nucleotide exchange. In contrast to previous reports, this structure shows that there are no direct contacts between the GTPase and C1 domain but instead suggests new mechanisms for the regulation of Vav1 activity.

Keywords: C1 domain; exchange factor; GTPase; Vav1; X-ray crystallography

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INTRODUCTION

Vav1 is a guanine nucleotide exchange factor (GEF) for the Rho family of GTPases, and has been shown to be crucial in T-cell development and activation (Tybulewicz, 2005). Analysis of Vav1-deficient T cells has shown that Vav1 transduces T-cell antigen receptor signals to several downstream pathways and it has been suggested that some of these might depend on the GEF activity of Vav1, whereas others depend on the function of Vav1 as an adapter protein (Tybulewicz *et al*, 2003).

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The primary structure of Vav1 shows that the protein contains eight domains (Fig 1A; Tybulewicz, 2005). Vav1, in common with many GEFs for the Rho family of GTPases (RhoGEFs), contains a conserved Dbl homology (DH) domain, which is responsible for catalysing nucleotide exchange (Crespo *et al*, 1997; Han *et al*, 1997). As seen in most RhoGEFs, the DH domain is flanked by a pleckstrin homology (PH) domain (Rossman *et al*, 2005). In addition, there is a calponin homology (CH) domain, and an acidic (Ac) region to the amino-terminal and a C1 domain to the carboxy-terminal side of the DH/PH module. Finally, the C terminus of the protein contains one SH2 and two SH3 domains. It has been proposed that the CH–C1 part of Vav1 is involved in regulating exchange activity, whereas the SH3/SH2/SH3 domains might have adapter function.

The GEF activity of Vav1 is regulated by several mechanisms. Phosphorylation of Tyr 174 within the Ac region results in an increase in GEF activity (Crespo *et al*, 1997; Han *et al*, 1997; Lopez-Lago *et al*, 2000). A solution structure of the DH domain of Vav1, including residues 170–189 of the Ac region, shows that Tyr 174 lies within an α-helix that binds to part of the GTPase interaction site, thereby occluding access to the GTPase (Aghazadeh *et al*, 2000). Phosphorylation of Tyr 174 causes dissociation of this helix from the DH domain, thereby relieving the autoinhibition. The CH domain has also been implicated in an autoinhibitory function, as deletion of this domain results in increased exchange activity (Zugaza *et al*, 2002). It has been proposed that the CH domain might bind directly to the C1 domain and thereby hold the Vav1 protein in an inactive 'closed' conformation, in which the CH–C1 interaction helps to stabilize the inhibitory Tyr 174–DH interaction. Activation of Vav1 would then be achieved by a conformational change leading to an 'open' conformation in which the DH domain is no longer occluded. Support for such a model has come from single-particle electron microscopy of Vav3 (Llorca *et al*, 2005).

The PH domains of DH-containing RhoGEFs seem to have diverse functions (Rossman *et al*, 2005). In some cases, they participate directly in binding to the GTPase, and in other cases they might bind to phospholipids and either regulate membrane targeting or allosterically activate GEF activity. In the case of

Vav1, a direct effect of phosphoinositides on exchange activity in solution has been suggested, with activity enhanced in response to the binding of phosphatidylinositol (4,5)-bisphosphate ($PtdIns(3,4)P_2$) or $PtdIns(3,4,5)P_3$ and inhibited following the binding of $PtdIns(4,5)P_2$ (Han *et al*, 1998; Das *et al*, 2000). It has been proposed that $PtdIns(4,5)P_2$ might promote association between the PH and DH domains, resulting in occlusion of the GTPase-binding site, whereas binding of $PtdIns(3,4)P_2$ or $PtdIns(3,4,5)P_3$ would cause dissociation of the domains and hence increase GEF activity.

C1 domains are present in a wide range of signalling proteins and can be divided into typical C1 domains, which bind to lipids and regulate membrane association, and atypical C1 domains, which lack the features required for lipid binding and instead might be involved in protein–protein interactions (Colon-Gonzalez & Kazanietz, 2006). On the basis of its sequence, the C1 domain of Vav1 has been classified as atypical, which is supported by the observation that it does not interact with phorbol esters (Kazanietz *et al*, 1994). Instead, the C1 domain of the Vav family of GEFs has been proposed to be important for enzymatic activity. Mutations in the C1 domain reduce GEF activity, and this has been suggested to be due to direct interactions between the C1 domain and the GTPase (Booden *et al*, 2002; Zugaza *et al*, 2002; Heo *et al*, 2005). Finally, the inhibition of GEF activity by the CH domain might be dependent on its ability to interact with the C1 domain.

To understand the contribution of the PH and C1 domains to the GEF activity of Vav1, and to shed light on its substrate specificity, we determined a high-resolution X-ray structure of the DH–PH–C1 domains of Vav1 in complex with Rac1. We show that the PH and C1 domains contribute to efficient GEF activity by stabilizing the DH domain structure and not through direct contacts with the GTPase. We also report that Vav1 is a GEF for Rac1, RhoA and Cdc42, and interpret this promiscuity in the light of the three-dimensional structure.

RESULTS AND DISCUSSION

Overall structure of Vav1 bound to Rac1

Vav1 (amino acids (aa) 170–575) and Rac1 were coexpressed and the nucleotide-free complex was purified by affinity and gel filtration chromatography. The complex crystallized readily and the structure was solved at 1.85 Å resolution by molecular replacement. The overall architecture of the GTPase/DH–PH portion of this complex is similar to that of other GTPase–GEF complexes except for the orientation of the PH domain, which differs extensively from structure to structure (Fig 1B). This is in part due to significant differences in the length and orientation of the C-terminal helix α6 of the DH domain. Interestingly, the orientation of this helix is similar between all structurally characterized DH domains up to a position equivalent to residue Arg 375 in Vav1, which marks the predicted C-terminal end of the DH domain. However, in a subset of GEFs, including Vav1, this helix is extended and often contains a slight kink around this position, which, in turn, determines the orientation of the PH domain. In contrast to all other RhoGEF structures reported, the Vav1 structure presented here is the first complex to contain another regulatory domain, the C1 domain that makes direct contacts with the DH domain. Strikingly, the interface between the DH and the PH–C1 cassette is extensive and buries 1,726 $Å^2$ of solvent-accessible surface between the two domains (Fig 1B).

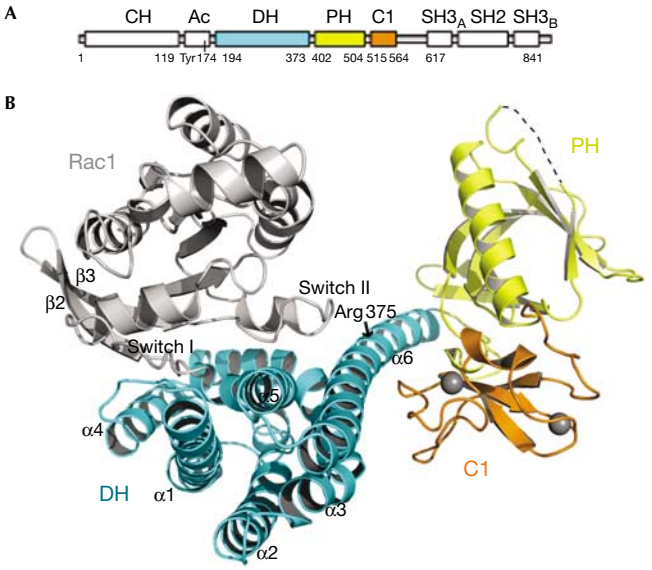

Fig 1 | Overall structure of the Rac1–Vav1 DH–PH–C1 complex. (**A**) Diagram showing the domain structure of Vav1. The DH–PH–C1 fragment is shown in the same colours as used throughout the manuscript. The domain boundaries as determined by Prosite (http://www.expasy.ch/prosite/) are indicated. (**B**) Ribbon diagram of the Rac1–Vav1 DH–PH–C1 structure, with Rac1 coloured in grey, the DH domain in cyan, the PH domain in yellow and the C1 domain in orange. The two Zn^{2+} ions bound to the C1 domain are shown as grey spheres. Ac, acidic; CH, calponin homology; DH, Dbl homology; PH, pleckstrin homology.

The Vav1–Rac1 interface

Complex formation between Rac1 and Vav1 buries about 2,600 $Å^2$ of solvent-accessible surface between them. The interface is similar to that of other GTPase/DH–PH structures and, not surprisingly, the conformations of the two switch regions superimpose well with those of other GTPases, indicating that the mechanism of nucleotide exchange is conserved in Vav1 (Erickson & Cerione, 2004; Rossman *et al*, 2005). The structure of switch I is supported by interactions of $Glu\,201^{Vav1}$, a residue highly conserved in RhoGEFs, with the hydroxyl group of $Tyr\,32^{Rac1}$ and the backbone amides of $Thr\,35^{Rac1}$ and $Val\,36^{Rac1}$. Similarly, the backbone carbonyl of $Ala\,59^{Rac1}$ forms a hydrogen bond with the side chain of highly conserved $Lys\,335^{Vav1}$ to remodel the conformation of switch II. This is further supported by a hydrogen bond from the side chain of $His\,337^{Vav1}$ to the backbone oxygen of $Gly\,60^{Rac1}$. As seen in other GTPase–GEF complexes, the effect of the conformational changes induced in switch I and II is to disrupt binding to the nucleotide and Mg^{2+} ion, and thereby promote nucleotide release. One of the main differences to other structures is the lack of significant contacts between the DH domain and strands β2 and β3 of the GTPase, which are often present and are believed to contribute to GTPase specificity. Residues $Asp\,65^{Rac1}$ and $Arg\,66^{Rac1}$ in the switch II region are the most intimately involved residues in the Rac1–Vav1 interface and form a total of seven hydrogen bonds with helix α6 of the DH domain, including the side chains of $Asn\,371^{Vav1}$, $Lys\,374^{Vav1}$, $Arg\,375^{Vav1}$ and $Glu\,378^{Vav1}$ (Fig 2A). Some of these interactions, such as those made by $Asn\,371^{Vav1}$ and $Glu\,378^{Vav1}$, are also

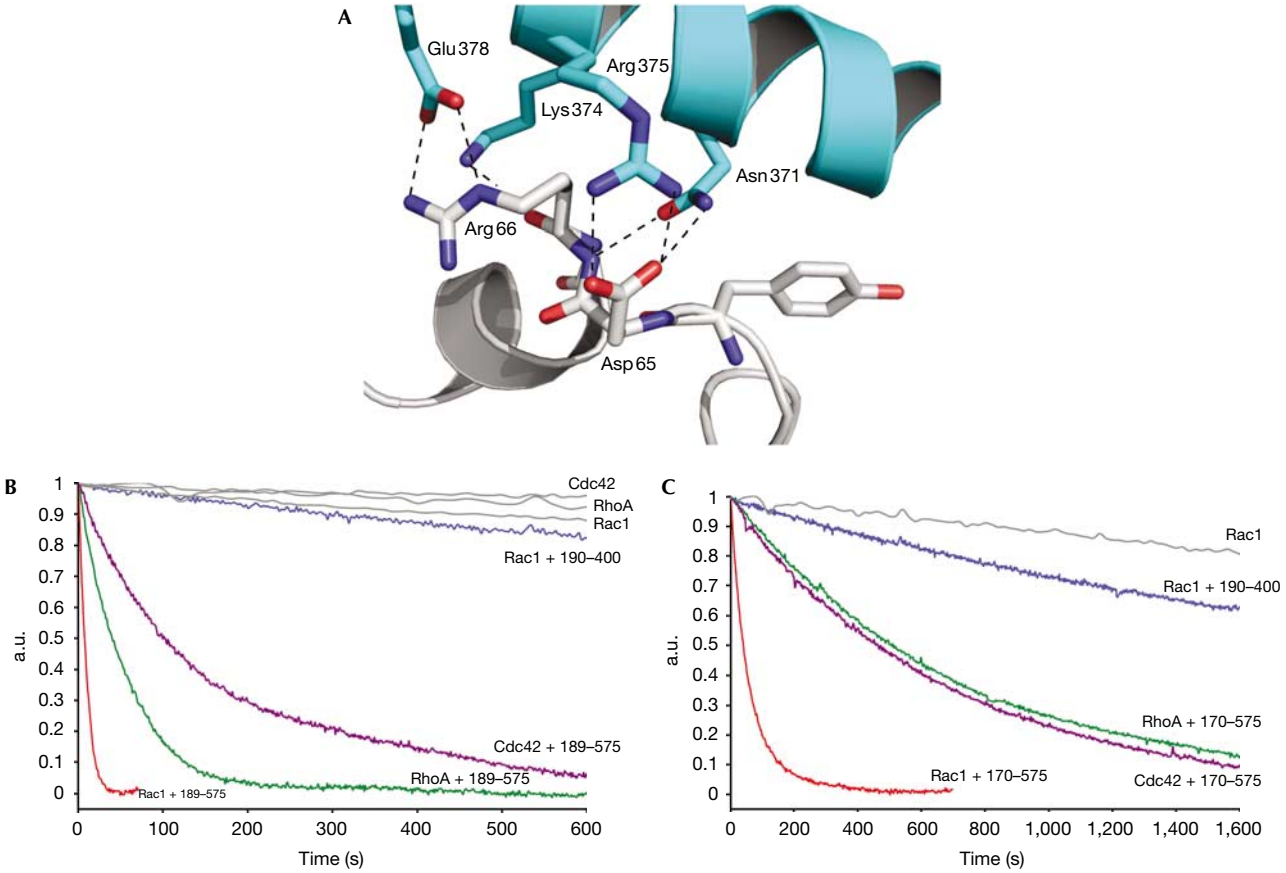

Fig 2 | The Rac1–Vav1 interaction and nucleotide exchange. (**A**) Detailed view of the interface between Asp 65 and Arg 66 in the switch II region of Rac1, and residues from helix α6 of the DH domain. Atoms are coloured by type except carbons, which are the same colour as in Fig 1B. The dashed lines indicate hydrogen bonds. Nucleotide exchange activity of (**B**) active (amino acids (aa) 189–575) and (**C**) autoinhibited (aa 170–575) Vav1 DH–PH–C1 and the isolated DH domain (aa 190–400) towards Rac1, Cdc42 and RhoA, as well as intrinsic exchange rates. DH, Dbl homology; PH, pleckstrin homology.

observed in many other GTPase–GEF structures, whereas others, especially those contributed by Lys 374^{Vav1}, are absent in most complexes. Interestingly, in those GEFs in which the PH domain is in direct contact with the GTPase such as Dbs, Trio or LARG, some of the contacts with Asp 65^{Rac1} and Arg 66^{Rac1} are made by residues from the PH domain, and loss of these interactions often impairs nucleotide exchange, indicating that they are important for the stabilization of a catalytically active conformation (Rossman *et al*, 2002; Kristelly *et al*, 2004; Chhatriwala *et al*, 2007).

There have been conflicting reports on the substrate specificity of the Vav family of proteins. In particular, the ability of Vav proteins to be active on Cdc42 has been questioned, and it has been suggested that different Vav isoforms have different GTPase specificities (Schuebel *et al*, 1998; Aghazadeh *et al*, 2000; Movilla *et al*, 2001). By using two different DH–PH–C1 fragments of Vav1 ('active' (aa 189–575) and 'autoinhibited' (aa 170–575)), we found that although Rac1 is the preferred substrate of Vav1, nucleotide exchange on Cdc42 and RhoA is also enhanced, but to a lesser extent (Fig 2B,C; Table 1). A similar selectivity has been previously established for Vav2 (Abe *et al*, 2000; Heo *et al*, 2005). The structure shows that the residues of Vav1 that make contacts with Rac1 are conserved between all human and mouse Vav isoforms,

Table 1 | Intrinsic and Vav1-stimulated nucleotide exchange rates

Vav1	GTPase	Exchange rate (s^{-1})	Enhancement over intrinsic rate
–	Rac1	0.00015	–
DH	Rac1	0.0003	2
DH–PH–C1 I	Rac1	0.0152	101
DH–PH–C1 A	Rac1	0.117	780
–	Cdc42	0.00013	–
DH–PH–C1 I	Cdc42	0.0014	11
DH–PH–C1 A	Cdc42	0.0072	55
–	RhoA	0.0003	–
DH–PH–C1 I	RhoA	0.0014	5
DH–PH–C1 A	RhoA	0.019	63

The table lists rates of nucleotide exchange on Rac1, Cdc42 and RhoA (at 0.4 μM) either alone or in the presence of the following Vav1 fragments (at 1.6 μM): DH domain alone (amino acids (aa) 190–400) or DH–PH–C1 fragments containing aa 170–575 indicated by I for autoinhibited or aa 189–575 indicated by A for active. DH, Dbl homology; PH, pleckstrin homology.

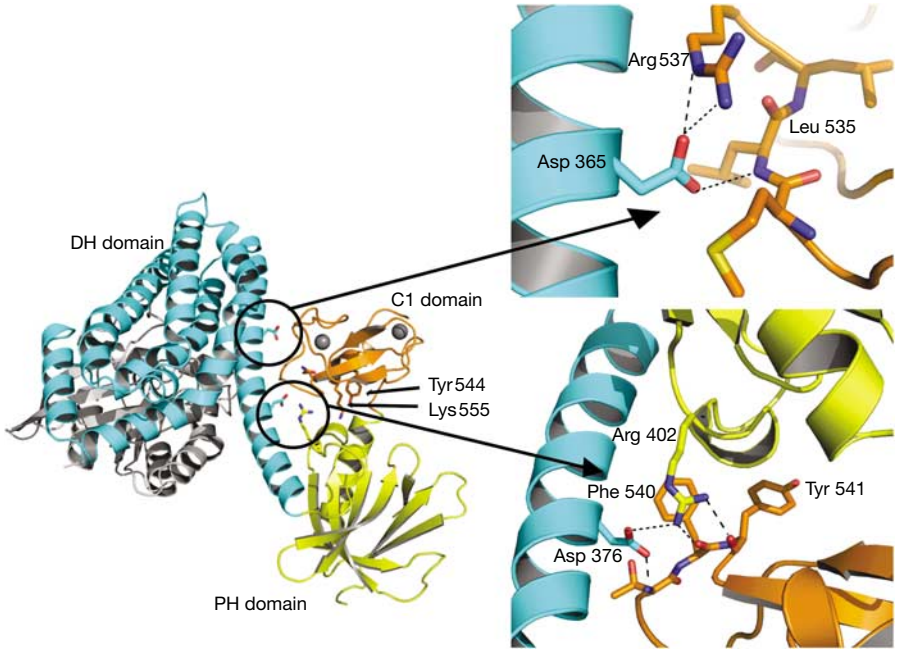

Fig 3 | The DH–PH–C1 interface. Detailed view of the interface between residues from helix α6 of the DH domain and the PH and C1 domains. The positions of residues Gln 542, Tyr 544 and Lys 555 within the C1 domain that have been discussed in the text are indicated. DH, Dbl homology; PH, pleckstrin homology.

raising the possibility that there are no major differences in their specificity, which is in contrast to previous suggestions (Movilla & Bustelo, 1999; Movilla *et al*, 2001). Inspection of the interface shows that there are no residues in Vav1 that would be expected to obviously discriminate against Cdc42 or RhoA through steric clashes. For some GEFs, discrimination between Rac1 and Cdc42 has been shown to depend on Trp 56^{Rac1} and the equivalent Phe 56^{Cdc42} (Karnoub *et al*, 2001; Snyder *et al*, 2002). In Rac1-specific GEFs such as Tiam1, conserved Ile 1187 allows binding of the bulky indole group of Trp 56^{Rac1}. Conversely, the equivalent Leu 1376 in the Cdc42-specific GEF Intersectin (Itsn) will accommodate the smaller Phe 56^{Cdc42} but not Trp 56^{Rac1}. In Vav1 the analogous residue is a methionine, Met 327, whereas in Vav2 and Vav3 it is a valine, and both the residues provide sufficient space to accommodate a bulky tryptophan.

Interactions between DH, PH and C1 domains

In principle, DH domains contain all the residues that are required for the remodelling of switch regions and nucleotide exchange. Nevertheless, there are many examples of GEFs that show enhanced exchange activity in the presence of a PH domain. In some GEFs, there are direct contacts between the PH domain and GTPase, and mutation of the residues involved can severely interfere with catalysis. In the case of Vav1, the C1 domain has been shown to be required for exchange activity and has been proposed to make direct contacts with the GTPase (Booden *et al*, 2002; Zugaza *et al*, 2002; Heo *et al*, 2005). To evaluate the importance of the PH and C1 domains in enzymatic activity, we compared the catalytic rates of DH–PH–C1 fragments with the isolated DH domain. Addition of the PH and C1 domains significantly increased the activity of the DH domain (Fig 2B,C; Table 1). However, our structure shows unequivocally that there are no direct contacts between the PH or C1 domain and the GTPase (Fig 1B), and thus these domains must contribute to activity by other means. We note that the PH and C1 domains make extensive contacts with the DH domain (Fig 3). Furthermore, the PH and C1 domains and the linker bridging the two domains form an extensive interface, explaining why it has not been possible to produce either an isolated domain or the DH–PH construct in a soluble form, as these would probably expose hydrophobic surfaces. The crucial residues in this interface are Asp 365 and Asp 376 located in helix α6 of the DH domain, Arg 402 at the beginning of the PH domain and Phe 540 in the C1 domain. Asp 365 contacts the side chain of Arg 537 and the backbone amide of Leu 535. Asp 376 and Arg 402 are involved in an extensive hydrogen-bonding network that connects the PH and C1 domains, whereas Phe 540 is at the centre of a hydrophobic pocket formed by all three domains (Fig 3). To examine the contribution of these interdomain interactions to catalytic activity, we introduced the following single amino-acid substitutions into both active (aa 189–575) and autoinhibited (aa 170–575) Vav1: D365A, D376A, R402A and F540A. However, introduction of these mutations severely decreased protein stability, making it impossible to produce sufficient quantities of pure and non-aggregated protein for exchange assays. This observation supports the idea that an intimate DH, PH and C1 domain interface is crucial for the structural integrity of the DH–PH–C1 cassette.

On the basis of the structure presented here, we propose that the PH and C1 domains contribute to GEF activity by forming a single structural unit that binds to the critical helix α6 of the DH domain and thereby restricts its conformational flexibility. This

helix, in turn, makes crucial contacts with the switch II region to remodel it and interfere with the binding of the Mg^{2+} ion. Similar stabilizing interactions in other GEFs are provided by direct PH–GTPase contacts, which might help to 'lock down' and stabilize helix α6. The observation that in some GEFs such as Itsn there is no obvious beneficial effect of the PH domain on exchange activity (Pruitt *et al*, 2003) suggests that in those GEFs helix α6 is either intrinsically much more rigid or that the PH domain requires other factors such as membrane-bound lipids or other proteins to adopt the catalytically fully active conformation. In this respect, it is interesting to note that the DH–PH unit in RhoGEFs is often flexible and that for some GEFs the orientation of the PH domain in the unbound structure is different from that in the GTPase-bound structure. By contrast, we believe that the orientation of the PH–C1 unit observed in our structure might constitute the conformation that will be found in both the active and inactive states, as the interface between the DH, PH and C1 domains is so extensive that it might be energetically unfavourable to disrupt. This model of a structural unit formed by the PH and C1 domains that stabilizes and fixes the DH domain is supported by mutagenesis of the C1 domain, which identified three mutations that abolished Vav1 GEF activity: Q542A, Y544A and K555A (Zugaza *et al*, 2002). Gln 542 probably has a structural role but Tyr 544 and Lys 555 form hydrogen bonds across the PH–C1 interface to stabilize the observed domain arrangement, and thus explain why mutation of these residues abolishes GEF activity (Fig 3). By contrast, the structure presented here is not compatible with previous reports suggesting a direct interaction between Rac1 and the C1 domain, which were based on nuclear magnetic resonance (NMR) chemical shift mapping and glutathione-*S*-transferase (GST) pull-down experiments (Movilla & Bustelo, 1999; Heo *et al*, 2005). Instead, our structure shows that the C1 domain is a long way from Rac1, and is engaged in extensive contacts with the DH and PH domains. Our results are supported by a recent study that also failed to detect a direct GTPase–C1 interaction (Brooun *et al*, 2007). The discrepancy between these studies might be due to the tendency of the isolated C1 domain to aggregate, and thus the reported GTPase–C1 contacts might be nonspecific interactions.

Ligand-binding properties of the PH and C1 domains

Binding of phosphoinositides to the PH domain of Vav1 has been proposed to allosterically regulate GEF activity by modulating interactions between the PH and DH domains (Han *et al*, 1998; Das *et al*, 2000). However, our structure does not provide support for such a model, as the phosphoinositide-binding site is located away from the interface with the DH domain (supplementary Figs S1,S2 online). Furthermore, given the extensive interactions made between the DH domain and the PH–C1 module, it seems unlikely that this module would undergo major conformational changes before GTPase binding. In agreement with this, Bustelo and co-workers (Zugaza *et al*, 2002) could not detect an effect of phospholipids on Vav1 activity *in vitro*. Nonetheless, our structure does not preclude the possibility that phospholipid binding to the PH domain might regulate membrane targeting of Vav1.

The C1 domain of Vav1 belongs to the group of atypical C1 domains that do not bind to diacylglycerol (DAG) or phorbol ester (Kazanietz *et al*, 1994; Colon-Gonzalez & Kazanietz, 2006). In spite of this, its structure overlaps well with that of the typical protein kinase Cδ (PKCδ) C1 domain, bound to phorbol-13-acetate (46 atoms overlap with an r.m.s.d. of 0.9 Å; Fig 4A; Zhang *et al*, 1995). In particular, the loops making up the ligand-binding site in PKCδ are conserved in Vav1, in contrast to the structures of the atypical C1 domains of, for example, Raf1 (Mott *et al*, 1996; Fig 4A). Furthermore, Vav1 contains a solvent-exposed cavity in the same position in which phorbol-13-acetate is bound to PKCδ (Fig 4B) and that is flanked on one side by hydrophobic residues from helix α6 of the DH domain, indicating that it could accommodate a small molecule (Fig 4C). Intriguingly, this potential ligand-binding site is located next to the region in the α6 helix that makes contacts with switch II of Rac1, suggesting that ligand binding to the C1 domain could modulate Vav1 activity. Further studies are now required to identify such a putative ligand and to test this model.

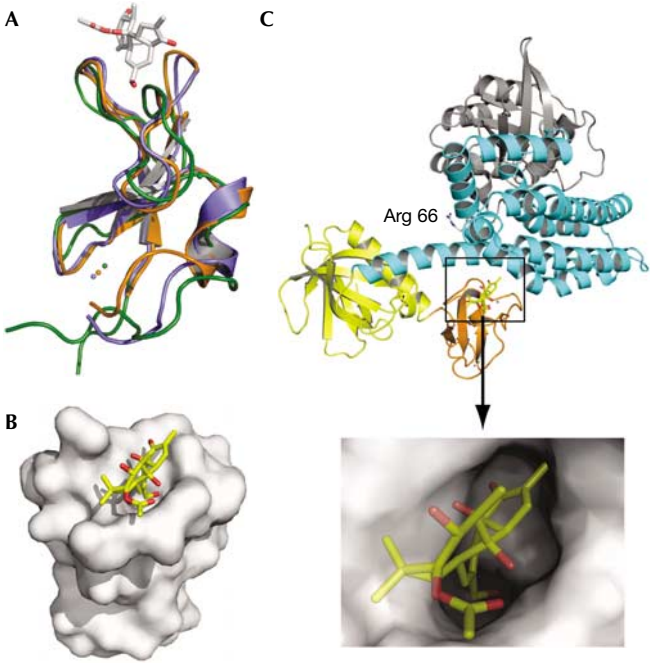

Fig 4 | The C1 domain of Vav1. (**A**) Overlap of the C1 domain of Vav1 (orange) with that of the typical C1 domain of PKCδ bound to phorbol-13-acetate (1PTR, blue) and the atypical C1 domain of Raf1 (1FAR, green). The ligand bound to PKCδ is shown in a ball-and-stick representation. (**B**) Surface representation of the C1 domain of PKCδ with bound phorbol acetate. (**C**) Surface representation of Vav1 DH–PH–C1 shows a pocket in the C1 domain in the same position in which phorbol acetate is bound to PKCδ (shown for comparison). DH, Dbl homology; PH, pleckstrin homology; PKCδ, protein kinase Cδ.

Vav1 activation

Nucleotide exchange activity of Vav1 is regulated through an intricate interplay between reversible phosphorylation, auto-inhibitory protein interactions and possibly lipid binding. The NMR structure of an autoinhibited DH fragment explained how the region containing Tyr 174 inhibits exchange activity by occluding part of the GTPase-binding site (Aghazadeh *et al*, 2000). However, the observation that we were able to purify a complex of Rac1 and

Vav1 containing aa 170–190, and that such a fragment is active in exchange assays, indicates that autoinhibitory interactions made by the Tyr 174-containing helix are not sufficient on their own to maintain the inactive state. Instead, open and closed conformations seem to be in a dynamic equilibrium that can easily be shifted towards the open conformation in the presence of Rac1. Hence, other *in vivo* mechanisms for successful repression are crucial. These are believed to involve intramolecular interactions between the CH and C1 domains. So far, structural information that could confirm such a mechanism is limited to the low-resolution electron microscopic structure of Vav3 (Llorca *et al*, 2005). This study compared the structures of full-length inactive, full-length phosphorylated and N-terminally truncated Vav3. The structure most relevant to the complex presented here is that of the truncated protein. A distinguishing characteristic of the reconstructed model is the position of the DH and C1 domains, which are suggested to occupy opposite sides of the PH domain and do not form any contacts. The structure presented here clearly shows that this is not correct, but now provides the opportunity to reinterpret the electron microscopic data by fitting the structure presented here into the overall protein envelope.

CONCLUSION

Here, we have made a first step towards an understanding of how regulatory domains in RhoGEFs outside the DH–PH module can contribute to the regulation of enzymatic activity. In addition, our structure has highlighted the existence of a putative ligand-binding pocket located at the C1–DH domain interface in a location where ligand binding could possibly modulate the exchange activity of Vav1 and its isoforms.

METHODS

The fragments of Vav1 used for nucleotide exchange assays were expressed in BL21-AI cells with an N-terminal His_6 tag, and then purified by affinity and gel filtration chromatography. The Rac1–Vav1 complex used for crystallization was produced by coexpression of Vav1 with GST-Rac1 and purification by affinity chromatography, followed by thrombin cleavage of the GST tag and gel filtration.

The Rac1–Vav1 complex was crystallized in hanging drops containing equal amounts of complex at 12 mg/ml and well solution (100 mM Bicine pH 9.0, 10% PEG6000). Crystals were cryoprotected with PEG400 and data were collected at SRS Daresbury. The structure was solved by molecular replacement using the Vav1-DH domain and Rac1 as search models. Refinement was carried out in REFMAC/ARP and model building in COOT. The final model of the complex has an *R*-factor of 20.6% (R_{free} 25.2%). The structural data have been deposited in the Protein Data Bank database, with the accession code 2vrw.pdb.

Guanine nucleotide exchange assays were carried out by fluorescence spectroscopy using *N*-methylanthraniloyl-GDP-labelled GTPases. Exchange was monitored by following fluorescence emission of the mant-labelled GTPase (400 nM) in the presence of 1.6 μM GEF together with 2 mM unlabelled GDP (using an ISS PC1 fluorimeter) at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 440$ nm.

For detailed descriptions, see the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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