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REGULATION OF Vav PROTEINS BY INTRAMOLECULAR EVENTS

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1. ABSTRACT

The Vav family is a group of signal transduction molecules with oncogenic potential that play important roles in development and cell signaling. The function of Vav proteins co-evolved with tyrosine kinase pathways, probably to assure the optimal conversion of extracellular signals into intracellular responses coupled to the cytoskeleton and the transcriptome. To date, the best-known function of Vav proteins is their role as GDP/GTP exchange factors for Rho/Rac molecules. This activity is highly regulated during signal transduction by processes involving intramolecular interactions among several domains of Vav proteins. On one hand, the phosphorylation of Vav proteins on a specific tyrosine residue leads to a conformational change that allows the activation of the catalytic activity of Vav proteins. This mechanism of activation has been recently explained in structural terms and shown to involve the acidic and Dbl-homology domains of Vav. On the other hand, the activity of Vav proteins is affected by a second type of intramolecular interaction occurring between the plekstrin-homology and the catalytic regions of Vav that is regulated by phospholipids. In this review, we will give a brief overview of the recent advances in this field.

2. INTRODUCTION

Members of the Rho/Rac family participate in the generation of coordinated cellular responses to extracellular stimuli. These GTPases play roles in the formation of cytoskeletal structures that contribute to changes in cell shape and motility, in the activation of lipid and protein kinase cascades, and in regulating transcriptome dynamics (for a review, 1,2). Recently, *loss-of-function* mutations in some signaling elements of Rho/Rac pathways were found associated with a number of human hereditary diseases, including immunodeficiencies (Wiscott-Aldrich syndrome, neutrophil immunodeficiency), some types of mental retardation, skeletal/segmentation abnormalities (Aarskog-Scott syndrome), and in cancer (1). Rho/Rac proteins are subjected to a tight regulation during signal transduction. These GTPases are in an inactive state maintained by the presence of bound GDP molecules in non-stimulated cells. After the reception of extracellular signals, Rho/Rac proteins undergo an exchange of GDP by GTP molecules, an event inducing conformational changes in their switch I and II regions that makes them competent for the binding to their effectors. Eventually, the GTPases become inactive again due to the hydrolysis of GTP to yield GDP. This cycle is regulated by guanosine nucleotide exchange factors (GEF), Rho GDP dissociation inhibitors (GDI), and GTPase activating proteins (GAPs). GEFs allow the rapid activation of the GTPases during cell stimulation by catalyzing the release of the bound GDP. By contrast, Rho GDIs block the dissociation of the bound GDP from the GTPases as well as the interaction of these proteins with the plasma membrane. GAP proteins accelerate the hydrolysis of GTP into GDP, therefore allowing the rapid inactivation of the GTPases at the end of the stimulation cycle (1-3).

The importance of these signal transduction pathways for human disease has triggered the interest in the study of the cellular and structural mechanisms that mediate the activation/deactivation cycle of the GTPases, their regulators, and effectors. This interest has fructified in a wealth of structural information about many of these signaling molecules, especially those involving GTPase/effector interactions. However, one of the areas

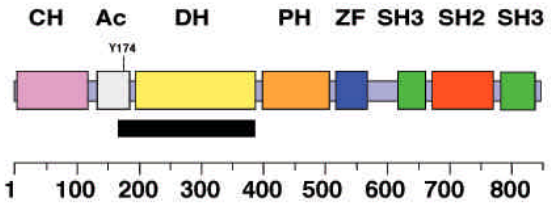

Figure 1. Structure of mammalian Vav proteins. The structural domains have been described in the text. The inhibitory tyrosine residue involved in Vav inactivation (Y174) is shown. A closed box indicates the fragment of Vav used for NMR studies. Amino acid numbers are indicated at the bottom of the figure.

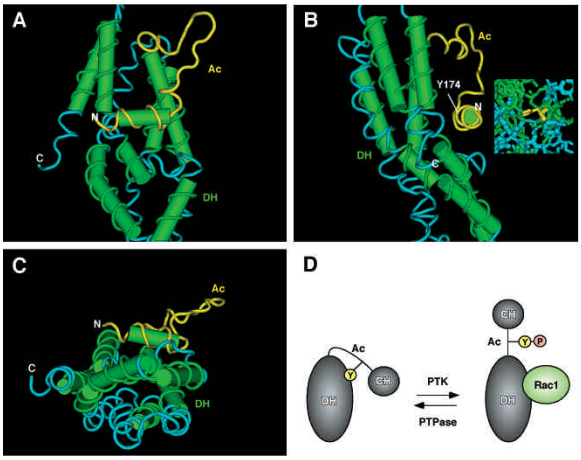

Figure 2. The structure of non-phosphorylated Vav. Panels A-C show the same structure viewed from three different angles. The inset of panel B depicts a detail of the Ac/DH binding interface, with the non-phosphorylated Y174 residue highlighted on yellow. Panel D shows a schematic representation of the mechanism of activation of Vav by tyrosine phosphorylation. The structures of Vav have been modeled using the Cn3D 3.0 software based on the data provided by Aghazedeh *et al.* (29). N: N-terminus; C: C-terminus; P, phosphate group.

less understood in this field is the mechanism mediating the activation of Rho/Rac GEFs. Recent reports have shown that the activation of many Rho/Rac GEFs is mediated by their translocation to the plasma membrane in a lipid-dependent manner (Tiam1), migration from the nucleus to the plasma membrane (Cdc24), interaction with either β/γ subunits of heterotrimeric G-proteins (Ras GRF, PDZ-RhoGEF, p115^{RhoGEF}) or lipids (Sos, Dbl), and by phosphorylation (Tiam1, Ras GRF, Vav). Most of these regulatory steps have not been elucidated as yet in structural terms. In this context, the regulation of the activity of one particular subgroup of these GEFs, the Vav family, is perhaps the best understood both in cellular and structural terms. In this review, we will center our discussion in the structural aspects of Vav family regulation. Other complementary information regarding the implication of Vav proteins in cell signaling, their binding proteins, and the modulation of their activity by heteromolecular interactions can be found in two recent review articles (4,5).

3. STRUCTURE AND FUNCTION OF Vav PROTEINS

The Vav family has currently five known members, three in mammalian cells (Vav, Vav2, and Vav3) and two in invertebrates (*C. elegans* Vav and *D. melanogaster* Vav). In addition, EST clones encoding fragments of Vav-like proteins are found in frogs (*X. laevis*), fish (*D. rerio*, *I. punctatus*), and worms (*S. stercoralis*). Vav proteins are not present in *S. pombe* and *S. cerevisiae*. All Vav proteins have similar structures (figure 1). Mammalian Vav proteins contain a calponin-homology (CH) domain, an acidic (Ac) region, the Dbl-(DH) and plekstrin-homology (PH) domains typical of members of Rho/Rac GEFs, a zinc finger (ZF) domain, and two SH3 domains flanking a single SH2 region (figure 1). The nematode Vav protein differs mostly at the C-terminus, where it lacks the two SH3 domains present in mammalian Vav proteins. The structure of *D. melanogaster* Vav is better conserved, lacking only the most N-terminal SH3 domain when compared with its mammalian counterparts. The Vav family is the only group of signal transduction proteins known to date combining a DH-PH cassette with ZF and SH2 regions in the same molecule.

The best-characterized function of Vav proteins is their activity as guanosine nucleotide exchange factors for Rho/Rac proteins (6-9). Such activity is strictly dependent on the direct phosphorylation of Vav on tyrosine residues by protein tyrosine kinases (PTKs). Activation of Vav proteins leads to changes in the actin cytoskeleton, activation of serine/threonine kinases such as JNK and p38, and gene expression. When deregulated by genetic alterations, the constitutive activation of these pathways leads to the oncogenic transformation of several cell types. These biological effects have been reviewed recently (4). More recently, it has been suggested that some functions of Vav in hematopoietic cells are independent of its GEF activity. Those include the stimulation of the transcription of the IL-2 gene proximal promoter via the activation of the transcription factor NF-AT (an acronym for Nuclear Factor of Activated T-cells) and the induction of robust calcium fluxes during T- and B-cell signaling. The discovery of these GEF independent functions was based on two independent lines of evidence. First, oncogenic mutants of Vav with deletions in the CH region that constitutively activate the Rac1 pathway cannot trigger NF-AT or Ca2+ responses (10,11). Secondly, Weiss' group has demonstrated that a catalytically inactive version of Vav is still capable of inducing NF-AT activation despite any detectable levels of GTP-bound Rac1 in these cells (12). The signal transduction elements that mediate these two responses are currently unknown. It is possible, however, that these GEF-independent functions are still activated by tyrosine phosphorylation because Vav cannot trigger NF-AT activation in mutant cell lines defective in PTK function.

4. REGULATORY MECHANISMS OF Vav PROTEINS

4.1. Regulation by phosphorylation

The most distinctive regulatory feature of all members of the Vav family is the tight regulation of their exchange activity by direct tyrosine phosphorylation. This posttranslational modification is mediated by PTKs that become activated during cell signaling. The first link between Vav proteins and tyrosine phosphorylation was obtained a decade ago, when it was shown that Vav was phosphorylated upon treatment of a fibroblast cell line (NIH 3T3) with either EGF or PDGF (13,14). This experimental treatment led to the rapid phosphorylation of Vav on tyrosine residues and to the physical interaction with the autophosphorylated receptors. The same type of experiments demonstrated the phosphorylation of Vav upon the engagement of the T-cell and B-cell receptors (14,15). Since these early observations, the phosphorylation of Vav has been observed upon the stimulation of more than thirty different cellular receptors (for a review, 4). More recently, it has been shown that the other known members of the family are also closely linked to tyrosine phosphorylation events (8,16-20). Although the mechanism that leads to Vav phosphorylation is probably different depending on the type of receptor involved, we can recognize two main mechanisms of Vav/receptors interaction. On one hand, we have the interaction between Vav and membrane receptors with intrinsic tyrosine kinase activity, such as the case of the EGF and PDGF receptors. In this case, Vav proteins interact physically with the autophosphorylated receptor via their SH2 domains and, in the process, become tyrosine phosphorylated by the receptor. However, it is presently unclear whether this phosphorylation is mediated by the activated receptor itself or by an associated PTK such as Src. Point mutations or deletions of the SH2 domain of Vav proteins lead to the generation of proteins incapable of binding to, and becoming phosphorylated by, the activated membrane receptors, demonstrating that the SH2 domains of these proteins are the essential structural domains for this heteromolecular interaction (8,13,14). In agreement with this, the SH2 domain alone of Vav proteins can interact efficiently with the activated receptors (8,13,14,16). Vav proteins can bind to other intracellular SH2/SH3-containing proteins during this process (Grb2, Shc, p85) (8,19,21,22). However, there is no evidence indicating that these adaptor proteins play ancillary roles in the Vav/receptor interaction.

The second general mechanism for Vav phosphorylation is observed in receptors lacking intrinsic tyrosine kinase activity, such as the case of antigen and cytokine receptors. In this case, the phosphorylation of Vav proteins involves a rather complex mechanism combining the utilization of cytoplasmic PTKs and adaptor proteins. Although there is still confusion about the specific kinases that mediate the phosphorylation of Vav proteins, both signaling and genetic data suggest that Syk family PTKs (Syk and Zap70) are the prominent ones in lymphoid and myeloid lineages (23-25). Roles for Src family and Jak family have also been described (26-28). As in the case of membrane receptors, the Vav protein interacts physically with the activated kinases, a process that involves the SH2 domains of Vav proteins and specific phosphorylated residues of Syk and Zap70. However, unlike membrane receptors, the affinity of Vav proteins towards these proteins is not strong enough to allow the effective translocation of Vav proteins to the plasma membrane. To solve this problem, the translocation of Vav to the plasma membrane is facilitated by its binding to several adaptor molecules, including Grb2 (in T- and B-cells), Lat (in T-cells), CD28 (in T-cells), and CD19 (in B-cells) (4,5).

Regardless of the type of interaction mediating the phosphorylation of Vav proteins during cell signaling, the functional endpoint of this process is the activation of the catalytic activity of these GEFs (6-9). Early biochemical and cell biology experiments hinted at the possibility that the role of phosphorylation was the release of an inhibitory structure mediated by the N-terminal domains of Vav proteins. Thus, Schuebel *et al*. demonstrated that the deletion of the N-terminal regions of Vav and Vav2 (residues 1-186) leads to the oncogenic activation of Vav proteins (7). This effect was coupled to the acquisition by these truncated proteins of a constitutive, phosphorylation-independent exchange activity (7). This mechanism of activation has been demonstrated now for the three mammalian members of the Vav family as well as for *Drosophila* Vav (7,8; J. Couceiro and X. R. Bustelo, manuscript in preparation). Later on, it was shown that the point mutation of specific tyrosine residues in the acidic region of Vav induced the hyperactivation of Vav proteins both in fibroblasts and lymphoid cells (11). Despite this evidence, the specific intramolecular mechanism that mediated the activation of Vav by tyrosine phosphorylation could not be elucidated until very recently. Using NMR techniques, Michael Rosen and collaborators solved the atomic structure of the Vav DH region and part of the adjacent Ac domain (figures 1,2). The protein used in these experiments was not phosphorylated on tyrosine residues, thus giving the perfect picture of the inactive conformation of Vav. The DH domain showed the typical structure of previously studied DH proteins, with 11 alpha helices forming a flattened, elongated bundle (figure 2A-C) (29). While this result was expected, the surprise arose when the folding of the adjacent fragment of the Ac domain was examined. It was found that the region encompassing residues 170-177 of Vav formed an additional alpha helix that was connected to the DH domain by a highly flexible linker. More importantly, this alpha helix packed orthogonally to the Rac1 binding domain of the Vav DH domain (figure 2A-C). The stabilization of this structure was due to the hydrophobic interaction of three residues of the N-terminal alpha helix (I173, Y174, L177) with residues present in the Vav DH region (Y209, T212, P320, L325, V328). As a consequence of this folding, the N-terminal alpha helix made the DH domain inaccessible to the GTPase substrate. This structure immediately demonstrated the mechanism of Vav inhibition and, as a bonus, suggested that perhaps the tyrosine residue involved in those interactions was the key one in the activation of Vav by tyrosine phosphorylation. To test this, the same group analyzed by NMR the same protein after tyrosine-

phosphorylation by Src (29). The structure of the Vav DH domain was unchanged when the folding of the phosphorylated and non-phosphorylated forms of Vav were compared. However, the phosphorylation of Y174 induced the release of the N-terminal inhibitory alpha helix from the GTPase binding site. This conformational change appears to be mediated by the steric clash of the newly incorporated phosphate group with the side chains of residues present in the DH region of Vav (R332, Y209). Due to this new conformation, the Vav protein acquired an "open" configuration capable of making stable interactions with Rac1 (figure 2D). The residue located at position 174 of Vav and its surrounding amino acid sequences are conserved in the three mammalian Vav members and in *D. melanogaster* Vav, suggesting that this regulatory mechanism is taking place in most Vav family members. Whether this is the exclusive mechanism for the phosphorylation-dependent activation of Vav proteins is presently unknown. In this respect, it is possible that other intramolecular interactions mediated by the most N-terminal CH region could contribute to this autoinhibitory loop. In agreement with this possibility, deletions in this region can trigger the activation of Vav even when the acidic domain and the inhibitory residue Y174 are kept intact (8, X.R. Bustelo, unpublished observations). Likewise, whether phosphorylation plays alternative functional roles in the regulation of the Vav pathway remains to be fully established. In this context, it has been suggested that three tyrosine residues of the Vav acidic domain could also induce the downmodulation of active Vav, probably by attracting some inhibitory phosphotyrosine binding proteins (11,12,30).

4.2. Regulation by lipids

The level of activity of Vav proteins seems also dependent on the type of lipids bound to their PH domains. This regulatory mechanism was demonstrated initially by Broek and coworkers (31). Using *in vitro* exchange reactions, this group showed that the enzyme activity of phosphorylated Vav could be increased approximately two-fold when exchange reactions were conducted in the presence of PI-3,4-P_2 or PIP_3, two products of phosphatidylinositol-3 kinase (PI-3K). Conversely, the GDP/GTP exchange activity of Vav was totally inhibited when the PI-3K substrate PIP_2 was included in the reactions. It was subsequently shown that these effects were due to the specific binding of phospholipids to the PH domain of Vav. Based on these results, it was proposed that the PIP_2-bound Vav PH domain was inhibiting the catalytic activity of Vav due to the establishment of intramolecular interactions with other regions of Vav. Such intramolecular interaction resulted in the inability of Vav to undergo optimal tyrosine phosphorylation and, as a consequence, precluded the optimal stimulation of Vav exchange activity. Conversely, the substitution of PIP_2 by the catalytic products of PI-3K after receptor activation released such inhibitory structure, favoring the phosphorylation and activation of Vav (31). In agreement with these *in vitro* observations, Broek and coworkers showed that the treatment of cells with wortmannin, a PI-3K inhibitor, led to the inhibition of Vav phosphorylation and to the block of Rac1 activation *in vivo* using an overexpression system (32). These results were corroborated by others, showing that PI-3-K appeared to be upstream of Vav in COS cells and some hematopoietic cell lines (33-35). It should be noted, however, that other groups could not find any effect of wortmannin on Vav activity *in vivo* (36,37).

The mechanism by which phosphatidylinositol derivatives impinge on Vav function has not been elucidated as yet using Structural Biology methods. However, biochemical experiments using purified Vav domains have recently shown that the Vav PH domain can bind to the Vav DH domain in a PIP_2-dependent manner (32). As a consequence, the Vav DH domain cannot bind to Rac1 under these conditions. By contrast, the PH/DH interaction is abolished when PIP_2 is substituted by PIP_3 (32). These results suggest that the PH and the Y174 residue inhibit Vav by making contacts with the catalytic region of Vav. It will be important to elucidate the structure of the PIP_2-bound Vav at the atomic level to see whether these contacts are established in overlapping or distinct regions to those used by the Ac domain. Future experiments in this direction will help to fully understand this multifaceted regulation of Vav family proteins.

5. PERSPECTIVE

In this review, we have paid attention to the regulatory pathways of Vav proteins involving exclusively intramolecular events. Although different in structural terms, the two regulatory mechanisms reviewed here have common features. First, both mechanisms work by limiting the access of the GTPases to the catalytic domains of Vav proteins in non-stimulated cells. Secondly, the structural stratagem to achieve such regulation is the same: the utilization of mobile structural domains capable of interacting with the DH region and of fluctuating between an "open" and "locked" configuration. This regulatory mechanism appears to have been set in place very early in evolution, since the acidic region, the regulatory phosphotyrosine residue, and the PH domain are already present in the oldest member of the family known so far, the *C. elegans* Vav protein. The need for such a complex regulatory mechanism is easily understood when we look at the biological consequences derived from the abnormal function of Vav proteins. Cells with deregulated Vav proteins undergo oncogenic transformation, acquire increased motility and metastatic properties and, in some cases (Vav2), show profound alterations in the cell cycle that lead to the formation of giant, polynucleated cells (4). It is therefore possible that these two regulatory mechanisms arose very early during evolution as a double safety mechanism assuring the proper amount of Vav signal during cellular responses to mitogens, cytokines, and antigens. The regulation of Vav by intramolecular interactions can be even more complex, since we know that the deletion of the CH region of Vav can lead to the generation of proteins with constitutive, phosphorylation-independent exchange activity (8; X.R. Bustelo, unpublished). As expected, these forms of Vav are also highly transforming *in vivo* (X.R. Bustelo, unpublished), giving further support to the important regulatory role of

this N-terminal domain of Vav. Further structural studies with Vav proteins will reveal whether this regulatory mechanism is independent or overlapping with those already known for Vav proteins.

Given the large number of Rho/Rac GEF that exist in cells, an obvious question that remains to be answered is the extent to which the regulatory mechanisms operating on Vav can be generalized to other members of this prolific family of enzymes. Current evidence indicates that both lipid binding and phosphorylation events do play regulatory roles in the catalytic activity of other Rho/Rac GEFs. Thus, the binding of phospholipids has been found to affect the exchange activity of Dbl (38), Tiam1 (39), Trio (40), and Sos1 (41). As in the case of Vav, these effects are mediated by the PH regions present in these GEFs. Structural studies with Sos1 suggest that the binding of lipids to the PH domain may induce a structural change in this region that could allow the stable interaction of the GTPase with the Sos1 DH-PH cassette (42). However, biochemical experiments have shown that the Dbl PH domain can bind to another region of Dbl that is located outside the catalytic area (43). Thus, it seems that the regulation of GEF activity by PH domains and lipids will follow different colors and flavors in function of the protein involved. As in the case of Vav, phosphorylation has been shown to affect the activity of other exchange factors, including Ras GRF (44), Tiam1 (45), and Dbl (46). At this moment, however, there are no structural studies available to elucidate the mechanism by which this posttranslational modification affects the activity of these GEFs.

Despite the complexity of the regulation of Vav activity by intramolecular interactions, we now know that it represents only the tip of the iceberg. Indeed, current evidence indicates that the modulation of Vav activity during signal transduction is still more complex, involving heteromolecular interactions that either enhance or inhibit the signaling output of these GEFs. For instance, the interaction of Vav with Slp76, Blnk, and Nef leads to enhancement of Vav-mediated responses in lymphocytes (for a review, 4,5). Conversely, Vav proteins interact physically with proteins that inhibit their signaling outputs (Cbl-b) (for a review, 4). The specific points of functional interaction of Vav proteins with these regulatory molecules are still poorly defined. Given the rapid pace observed in both signaling and structural studies nowadays, it is predictable that all these regulatory steps will be quickly elucidated in the near future.

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Abbreviations: Ac: acidic; CH: calponin-homology; DH: Dbl-homology; GAP: GTPase activating protein; GDI: GDP dissociation inhibitor; GEF: GDP/GTP exchange factor; IL: interleukin; NF-AT: nuclear factor of activated T-cells; PH: plekstrin-homology; PI: phosphatidylinositol; PTK: protein tyrosine kinase; SH: Src-homology.

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