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PRINCIPAL INVESTIGATOR: Behzad Aghazadeh, Ph.D.
Michael K. Rosen, Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
New York, New York 10021

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6. AUTHOR(S)
Behzad Aghazadeh, Ph.D.
Michael K. Rosen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sloan-Kettering Institute for Cancer Research
New York, New York 10021

E-MAIL:
behzad@mrnmr1.ski.org

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Rho-family of small GTPases transduce signals from receptors to modulate various physiological responses including cytoskeletal reorganization leading to changes in cell shape and motility, mitogenesis and development. Proteins containing the Dbl homology (DH) domain are responsible for activating Rho GTPases by catalyzing the exchange of GDP for GTP. The mechanism of DH domain stimulation by receptors remains largely unknown however. For the Dbl protein Vav, this mechanism involves tyrosine phosphorylation, resulting in increased nucleotide exchange activity *in vivo*. We show through structure determination that the mVav1 DH domain is autoinhibited by an N-terminal extension, which lies in the GTPase interaction site. This extension contains the Tyr174 Src-family kinase recognition site, and phosphorylation of this residue, as well as truncation of the peptide, results in stimulation of GEF activity. NMR spectroscopy data show that the N-terminal peptide is released from the DH domain and becomes unstructured upon phosphorylation. Thus, tyrosine phosphorylation relieves autoinhibition by exposing the GTPase interaction surface of the DH domain, which is obligatory for Vav activation.

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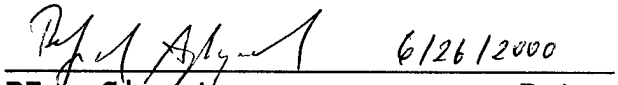
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4. Table of Contents

5. Introduction

The Rho family GTPases Rho, Rac and Cdc42 regulate a diverse array of cellular processes such as regulation of the actin cytoskeleton, gene expression and cell cycle progression. Not surprisingly, aberrant Rho signaling can contribute to many disease processes, including tumorigenesis, tumor metastasis and developmental disorders. Activation of Rho proteins, through release of bound GDP and subsequent binding of GTP, is catalyzed by guanine nucleotide exchange factors (GEFs) in the Dbl family. All members of this group of over 30 molecules, most of which were initially identified based on their oncogenic potential, share a sequence of approximately 200 residues, termed the Dbl-homology (DH) domain. We have previously reported the solution structure of the DH domain of human βPIX. We were able to identify through mutagenesis studies together with sequence alignments, the probable GTPase interaction site. In order to address the issues surrounding DH domain regulation *in vivo*, we have determined the solution structure of the autoinhibited mouse Vav1 DH domain. We show that N-terminal the Src tyrosine kinase phosphorylation motif containing Y174 covers regions of the active site inhibiting access to the GTPases. Furthermore, we were able to phosphorylate the molecule using purified src and show by solution NMR that phosphorylation results in the release of the autoinhibitory peptide, thereby activating nucleotide exchange function.

6. Body

The Rho-family GTPases, Rho, Rac and Cdc42, are primarily known as regulators of the actin cytoskeleton. These GTPases also control cell growth and apoptosis through activation of the extracellular-signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) cascades. Deregulation of these signaling pathways can lead to disease processes such as tumorigenesis and developmental disorders.

Rho-GTPases are activated by GEFs that contain a conserved region termed the Dbl homology (DH) domain which is responsible for catalyzing the nucleotide exchange. The mechanisms whereby upstream signals are relayed to the Dbl GEFs are poorly understood. The significance of DH domain regulation is evidenced by involvement in diseases. Indeed, many members of the Dbl family GEFs were initially identified based on their oncogenic potential, or have been directly linked to developmental disorders and tumor metastasis.

One of the best characterized Rho-GEF proteins is the proto-oncogene product, Vav. Stimulation of Vav results in Rac-mediated formation of lamellipodia and activation of JNK *in vivo*, and Vav GEF activity is specific for Rac *in vitro*. Vav becomes rapidly phosphorylated in response to antigen stimulation of the B- and T-cell receptors through the action of Syk and Src-family tyrosine kinases. This phosphorylation event results in the stimulation of nucleotide exchange activity. One of the putative phosphorylation sites is Tyr174. Phosphorylation of this residue enhances GEF activity *in vitro* and truncation of the N-terminal 186 residues (Δ1-

186) leads to phosphorylation independent activation of Vav2 GEF function.

In this study, we have determined the solution structure of the autoinhibited Vav DH domain (residues 170-375) and characterized its structural changes on phosphorylation at Y174. The conserved portion of the domain (residues 186-375) is architecturally similar to other members of the Dbl family. The N-terminal autoinhibitory extension forms an α-helix that binds in the DH domain active site through contacts to conserved residues, blocking access to GTPases. Tyr 174 forms an integral part of this autoinhibitory interface, and is largely buried in the intramolecular complex. Phosphorylation of Tyr 174, or removal of the N-terminal peptide greatly enhances the DH domain exchange activity. NMR spectroscopic data show that phosphorylation of Tyr 174 causes the N-terminal peptide to become unstructured and release from the DH domain. Thus, the Vav DH domain is activated through phosphorylation-mediated exposure of the active site.

The structure of Vav170-375 was calculated from a total of 3432 NMR distance restraints. In addition, 326 chemical shift-based backbone torsion angle restraints obtained from the program TALOS were included as were 79 side-chain torsion restraints. Residues 178-198 are disordered due to a lack of NOEs observed for this region. The root mean square deviation for the structured regions (residues 170-177, 199-375) is 0.63Å for backbone and 1.12Å for all heavy atoms.

The Vav DH domain is structurally similar to previously reported DH domains. It is composed of 11 α-helices that form a flattened, elongated bundle giving rise to two surfaces formed by

helices D, E, F, G and A, I, J, K. The latter has been identified as the GTPase interaction site and many of the residues required for GEF activity cluster on this surface. The N-terminal eight residues (170-177) containing the IY(174)EDL phosphorylation motif form an additional α-helix attached by a flexible linker (residues 178-198) to the N-terminus of the DH domain fold. This element packs orthogonally across the center of the active site. Side-chains of residues on the N-terminal peptide make contacts to a hydrophobic patch created on the DH domain. In Trio, most DH domain residues indicated in the interaction with Rac map to regions immediately adjacent to the autoinhibitory contacts in our structure. We therefore conclude that Vav GEF function is inhibited through the occlusion of residues important for GTPase binding and nucleotide exchange catalysis. The residues involved in autoinhibition are conserved throughout the Vav proteins, indicating that this mechanism of autoinhibition is conserved among this family of GEFs.

We next investigated the structural changes that occur upon phosphorylation resulting in the activation of the Vav DH domain. The majority of signals in the phosphorylated and unphosphorylated NMR spectra are similar, indicating no gross structural changes in the body of the domain. However, changes were observed for the methyl groups comprising the inhibitory binding pocket. In addition, a number of new resonances appeared in the $^{1}H/^{15}N$ HSQC spectrum. These have much higher intensities compared to the rest of the spectrum while showing poor chemical shift dispersion in the proton dimension, indicative of an unstructured and mobile region. Sequential assignment has mapped these signals to the N-terminal 14 residues of Vav170-375. Chemical shift index (CSI) analysis

verified that these residues lack secondary structure in solution in the phosphorylated state. We therefore conclude that phosphorylation of Tyr174 causes release of the N-terminal inhibitory arm from the body of the DH domain, exposing the GEF active site.

7. Appendix

Research accomplishments

- The solution structure of the autoinhibited mouse Vav1 DH domain has been determined by multi-dimensional NMR spectroscopy.
- The structure is similar to the previously reported DH domain structures of βPIX, Trio and mSos.
- In addition, our construct of Vav DH domain contains the Tyr174 phosphorylation motif, which contacts the active site of the DH domain, thereby prohibiting access of GTPases.
- We show by solution NMR that Tyr174 is phosphorylated by Src tyrosine kinase, and that phosphorylation results in the release of the inhibitory peptide form the active site.
- The phosphorylated peptide is unstructured in solution, indicating that tyrosine-phosphorylation destabilizes helix formation.

Publications

Aghazadeh, B., Lowry, W.E., Huang, X.Y. & Rosen, M.K. Structural Basis for Relief of Autoinhibition of the Dbl Homology Domain of Proto-oncogene Vav by Tyrosine Phosphorylation. *Submitted.*