Journal of Structural Biology 180 (2012) 84–95

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Identification and structural basis for a novel interaction between Vav2 and Arap3

Bo Wu, Fengsong Wang, Jiahai Zhang, Zhiyong Zhang, Liying Qin, Junhui Peng, Fudong Li, Jianping Liu, Guowei Lu, Qingguo Gong, Xuebiao Yao, Jihui Wu *, Yunyu Shi *

Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, PR China

ARTICLE INFO

Article history:
Received 11 February 2012
Received in revised form 13 June 2012
Accepted 14 June 2012
Available online 28 June 2012

Keywords:
Vav2
Arap3
SH2 domain
NMR
Protein-peptide interaction
Complex structure

ABSTRACT

Vav2 is a ubiquitous guanine nucleotide exchange factor (GEF) for the small GTPase Rac1. It regulates processes including cell migration, neuronal development and phagocytosis through interactions with different proteins. In this study, Arap3, a dual GTPase-activating protein (GAP) for RhoA and Arf6, was first identified to be a novel interaction partner for Vav2 both in vitro and in vivo. ITC and NMR chemical shift perturbation experiments demonstrated that Vav2 SH2 domain was able to interact directly with phosphorylated Y1403 and Y1408 within the C-terminal region of Arap3 with high affinities, with the dissociation constants (Kd) of ~0.27 and ~1.40 μM, respectively. In addition, using different phosphotyrosine peptides, the pY +3 specificity of Vav2 SH2 domain was discovered. The solution structures of Vav2 SH2 domain in free and in complex with the phosphotyrosine peptide pY1408 were therefore determined to understand the structural basis of this recognition specificity. Structural analysis revealed that the presence of a Phe residue in the BG loop (BG6) leads to the formation of a shallow hydrophobic pY +3 pocket on the surface of Vav2 SH2 domain, which determines the pY +3 specificity of Vav2 SH2 domain.

1. Introduction

Small GTPases of the Rho family (Rac1, Cdc42, RhoA) and the Arf family (Arf1–Arf6) control cellular cytoskeleton remodeling and membrane trafficking (D'Souza-Schorey and Chavrier, 2006; Jaffe and Hall, 2005; Myers and Casanova, 2008). They act as molecular switches cycling between a GTP-bound active form and a GDP-bound inactive form. Guanine nucleotide exchange factors (GEFs) activate them by catalyzing GDP/GTP exchange, whereas GTPase-activating proteins (GAPs) inactivate them by enhancing their intrinsic GTPase activity (Bos et al., 2007). When loaded with GTP, each member of these two family of small GTPases binds to specific effectors to mediate downstream signaling pathways, leading to changes in cytoskeleton and/or membrane.

Vav2, a member of Vav family (Vav1, Vav2 and Vav3), serves as a GEF for Rac1 (Marcoux and Vuori, 2003; Tamas et al., 2003). It regulates diverse processes including lamellipodia formation, spreading and cell migration, phagocytosis, and neuronal development in numerous cell types (Arora et al., 2008; Cowan et al., 2005; Hunter et al., 2006; Marignani and Carpenter, 2001). Vav2 contains eight domains: a calponin homology (CH) domain, an acidic (Ac) region, a Dbl homology (DH) domain, a pleckstrin homology (PH) domain, a zinc finger (ZF) domain, a Src homology 2 (SH2) domain and two Src homology 3 (SH3) domains. The DH domain exhibits GTP/GDP exchange activity and this activity is triggered by phosphorylation of several tyrosine residues within the Ac region (Lopez-Lago et al., 2000). The presence of an SH2 domain is a unique structural feature of VAV family different from any other GEFs for Rho GTPases observed (Bustelo, 2000; Rossman et al., 2005). SH2 domain is a common protein interaction module that specifically recognizes phosphotyrosine (pY) containing motif (Pawson et al., 2001). Vav2 SH2 domain belongs to the IIA subgroup according to the most recent SH2 domain classification (Huang et al., 2008). The specificities of SH2 domains in this subgroup are determined mainly by the pY +3 residue (the third residue C-terminal to phosphotyrosine) of ligand. Vav2 has been reported to interact, via its SH2 domain, with several phosphorylated receptors or co-receptors, including epidermal growth factor receptor (EGFR) (Tamas et al., 2003), platelet-derived growth factor (PDGFR) (Pandey et al., 2000), Eph family receptors (Cowan et al., 2005; Hunter et al., 2006) and CD19 (Doody et al., 2000), and membrane-associated cytoplasmic adaptor proteins, including SLP76 (Tartare-Deckert et al., 2001). Through these interactions, Vav2 is recruited to plasma membrane from cytoplasm in response to diverse extracellular signals. Membrane translocation of Vav2 is cru-

Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; SH2, Src homology 2; pY, phosphotyrosine; PV, pervanadate; MD, molecular dynamics.

* Corresponding authors. Present addresses: Room 424, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, PR China (J. Wu); Room 422, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, PR China (Y. Shi). Fax: +86 551 3600374, fax: +86 551 3600374.

E-mail addresses: wujihui@ustc.edu.cn (J. Wu), yyshi@ustc.edu.cn (Y. Shi).

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http://dx.doi.org/10.1016/j.jsb.2012.06.011

cial to its phosphorylation and therefore activation by receptors and Src family kinases (Tamas et al., 2001). Activated Vav2 then mediates the transductions of diverse extracellular signals to intracellular responses, through activating Rac1. Despite many interaction partners have been found for Vav2, we still want to know whether we can find novel partners of Vav2.

In this paper, we report the identification of Arap3 as a novel interaction partner for Vav2. Arap3 is an important signaling protein controlling cell spreading and lamellipodia formation (Krugmann et al., 2006; Stacey et al., 2004). It serves as a PIP3- and Rap1-activated dual GAP for Arf6 and RhoA (Krugmann et al., 2004, 2002; Stacey et al., 2004). Like Vav2, Arap3 is also a substrate of Src family kinases. Its two C-terminal tyrosine residues, Y1403 and Y1408, undergo rapid phosphorylation in cells upon stimulation with growth factors or during adhesion to extracellular matrix (Stacey et al., 2004). Unlike Vav2, which is a proto-oncoprotein, Arap3 has been reported recently as a suppressor of peritoneal dissemination of scirrhous gastric carcinoma cells and the suppressive activity of Arap3 requires the phosphorylation of these two phosphorylation sites (Y1403 and Y1408) (Yagi et al., 2011). However, the interaction partners of these two tyrosine phosphorylation sites of Arap3 have not been identified yet.

Our research indicated that Vav2 can interact with Arap3 through its SH2 domain which binds directly to the two phosphorylated tyrosine residues (Y1403 and Y1408) within the C-terminal of Arap3. Both the phosphotyrosine peptides derived from residues Y1403 and Y1408 in Arap3 bound to Vav2 SH2 domain with comparable affinities ($Kd = 0.27 \pm 0.01$ μM for pY1403 and $Kd = 1.4 \pm 0.05$ μM for pY1408). Solution structures of Vav2 SH2 domain in free and in complex with the phosphotyrosine peptide derived from Y1408 of Arap3 were solved by NMR spectroscopy. The pY +3 specificity of Vav2 was investigated and the structural determinants governing its pY +3 specificity was uncovered. The interaction of Vav2 and Arap3 in a full-length manner was further confirmed by co-immunoprecipitation, immunofluorescence staining and GST pull-down experiments. The biological implication of this novel interaction was discussed.

2. Materials and methods

2.1. Antibodies and reagents

Goat polyclonal anti-Arap3 antibody was kindly provided by Dr. Sonja Vermeren (The Babraham Institute, Cambridge, United Kingdom). The following antibodies and reagents were purchased commercially: monoclonal mouse anti-phosphotyrosine antibody (Cell Signaling, Danvers, MA), monoclonal mouse M2 anti-FLAG agarose (Sigma–Aldrich), monoclonal mouse anti-GFP antibody (Santa Cruz), polyclonal rabbit anti-GFP antibody (Cell Signaling, Danvers, MA), polyclonal rabbit anti-GFP antibody (BD) and monoclonal rabbit anti-Vav2 antibody (Cell Signaling, Danvers, MA), polyclonal rabbit anti-FLAG antibody (Cell Signaling, Danvers, MA), Protein A/G agarose (Pierce/Thermo Scientific).

2.2. Plasmid construction

The full-length myc-tagged human Vav2 plasmid (pCMV5-Vav2) and the full-length GFP-tagged human Arap3 plasmid were kindly provided by Der (University of North Carolina, Chapel Hill, NC, USA) and Dr. Sonja Vermeren (The Babraham Institute, Cambridge, United Kingdom), respectively. For NMR and ITC experiments, the DNA fragment encoding the SH2 domain of Vav2 (residues 659–771) was amplified by PCR from pCMV5-Vav2 plasmid and cloned into a modified pET28a (+) (Novagen) plasmid, generating a fusion protein with an N-terminus 6XHis tag. For GST pull-down experiments, the Vav2 SH2 was subcloned into pGEX4T-1 vector. For transient expression in mammalian cell, the full-length Vav2 coding sequence was amplified by PCR from pCMV5-Vav2 plasmid and inserted into p3XFlag-myc-CMV-24 using the EcoRI and SalI cloning sites. Mutants were generated by PCR mediated site-directed mutagenesis. All the constructs were verified by DNA sequencing.

2.3. Recombinant protein expression and purification and peptide synthesis

The recombinant plasmids harboring His-tagged or GST-tagged Vav2 SH2 were transformed into *Escherichia coli BL21* (DE3) (Novagen) strain. Cells were grown at 37 °C up to an $A_{600\ nm}$ of 0.8, and then were induced with 0.5 mM IPTG at 25 °C for 8 h. For the production of uniformly ^{15}N or ^{13}C, ^{15}N-labeled samples, cells were grown in minimal medium using $^{15}NH_4CL$ (0.5 g/L) and $^{13}C_6$-glucose (2.5 g/L) as the sole nitrogen and carbon source. The His-tagged proteins were purified by a chelated-nickel column followed by a size-exclusion column. The GST and GST fusion proteins were purified using immobilized glutathione and size-exclusion columns. The purity of protein was confirmed by SDS–PAGE. Protein concentrations were measured using BCA reagent (Pierce). Peptides used in the experiments were chemically synthesized and purified by Sangon (Shanghai).

2.4. NMR sample preparation and NMR experiments

The NMR sample of the apo Vav2 SH2 contained ~1 mM of protein was dissolved in NMR buffer: 25 mM phosphate buffer with 75 mM NaCl, 5 mM EDTA, 5 mM DTT in 90% H_2O, 10% D_2O (pH 6.2). For the SH2/peptide complex sample, the ^{15}N, ^{13}C-labeled Vav2 SH2 (~1 mM) mixed with peptide pY1408 (~3 mM) was dissolved in the same buffer as described above.

NMR experiments were carried out at 293 K for SH2 in the free and 298 K for the SH2/peptide complex on Bruker DMX500 and DMX600 spectrometers equipped with cryoprobes. The following spectra were recorded to obtain Vav2 SH2 domain backbone and aliphatic side-chain resonance assignments: 2D ^1H–^{15}N HSQC, 3D-HNCO, HN(CA)CO, CBCANH, CBCA(CO)NH, HBHA(CBCACO)NH, (H)C(CO)NH-TOCSY, H(C)(CO)NH-TOCSY, HCCH-TOCSY, HCCH-COSY, ^{15}N- and ^{13}C-separated NOESY. 2D homonuclear NOESY and TOCSY spectra were used to obtain Vav2 SH2 domain aromatic side-chain resonance assignments. Peptide resonance assignments were obtained from $^{13}C/^{15}N$-filtered ^1H NOESY and heteronuclear X-Filter ^1H PFG double-quantum experiments (Dalvit et al., 1998) in D_2O. 2D ^{13}C-filtered (F1), ^{13}C-edited (F2) NOESY spectrum was used to detect the intermolecular interactions. NMR data were processed with NMRPipe (Delaglio et al., 1995) and analyzed using Sparky3 (Goddard and Kneller, University of California, San Francisco).

2.5. Structure calculations and validation

The 9 residues (MGHHHHHHM) at the N-terminus as an artifact from the vector were not included in structure calculation. The structures were calculated and refined using the program CNS1.2 (Brunger et al., 1998). The NOE crosspeaks from the 3D ^{15}N- and ^{13}C-separated NOESY spectra were assigned and converted into distance restraints. Phi and psi dihedral angle restraints were obtained based on TALOS (Cornilescu et al., 1999) predictions. Hydrogen-bond restrains were obtained by identifying the slow-exchanging amide protons in the H/D exchange experiments. For the final stage, 200 structures were calculated and 20 conformers were selected on the basis of energetic criteria (low total energy, using the accept.inp routine) to form a representative ensemble.