Journal of Structural Biology 187 (2014) 194–205

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Structural investigation of the interaction between the tandem SH3 domains of c-Cbl-associated protein and vinculin

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ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form 16 May 2014
Accepted 19 May 2014
Available online 28 May 2014

Keywords:
CAP
Focal adhesion
Proline-rich peptide
Tandem SH3 domains
Vinculin

ABSTRACT

c-Cbl-associated protein (CAP) is an important cytoskeletal adaptor protein involved in the regulation of adhesion turnover. The interaction between CAP and vinculin is critical for the recruitment of CAP to focal adhesions. The tandem SH3 domains (herein termed SH3a and SH3b) of CAP are responsible for its interaction with vinculin. However, the structural mechanism underlying the interaction between CAP and vinculin is poorly understood. In this manuscript, we report the solution structure of the tandem SH3 domains of CAP. Our NMR and ITC data indicate that the SH3a and SH3b domains of CAP simultaneously bind to a long proline-rich region of vinculin with different binding specificities. Furthermore, the crystal structures of the individual SH3a and SH3b domains complexed with their substrate peptides indicate that $Q807^{SH3a}$ and $D881^{SH3b}$ are the critical residues determining the different binding specificities of the SH3 domains. Based on the obtained structural information, a model of the SH3ab-vinculin complex was generated using MD simulation and SAXS data.

1. Introduction

Focal adhesions are complex multi-molecular assemblies that link the extracellular matrix to the actin cytoskeleton via integrins (Balaban et al., 2001; Geiger et al., 1995; Yamada and Geiger, 1997). These adhesions play an important role in the signal transmission between the extracellular matrix and the cell's cytoskeleton, which is crucial for many biological processes, including cell migration, embryonic morphogenesis, angiogenesis, inflammation, and wound healing (Ridley et al., 2003; Rossiter et al., 1997; Sheetz et al., 1998; Yang et al., 1995). In response to extracellular signals, including both chemical and physical signals, focal adhesions change dynamically and initiate signaling events (Choquet et al., 1997; Geiger et al., 2009; Lauffenburger and Horwitz, 1996). In the cytoplasm, focal adhesions associate with the actin cytoskeleton through various plaque proteins (Liu et al., 2000). More than 50 proteins have been found in focal adhesions, and these include scaffolding molecules, adapter proteins, small GTPases, kinases, phosphatases, and proteases (Zamir and Geiger, 2001). The adaptor protein vinculin, which undergoes a conformational changes when localizing to FA (Chen et al., 2005), is a pivotal regulator of focal adhesions through its interaction with other adhesion proteins (Humphries et al., 2007).

Vinculin is comprised of three seven-helical bundle domains (D1, D2, and D3) and a four-helical bundle (D4) domain that is connected to a five-helical bundle (D5 or vinculin tail) domain via a proline-rich hinge region, and the D1–D4 domains form the vinculin head (Vh) domain (Bakolitsa et al., 2004; Borgon et al., 2004). To date, 19 binding partners have been identified for vinculin, and these include talin and α-actinin, which bind to the D1 domain, and F-actin, paxillin, and PIP2, which associate with the vinculin tail (Vt) domain (Carisey and Ballestrem, 2011). In addition to the D1 and Vt domains, the proline-rich linker also harbors considerable binding sites for proteins, including Arp2/3, CAP, vinexin,

Abbreviations: CAP, c-Cbl-associated protein; DLS, dynamic light scattering; ECM, extracellular matrix; FA, focal adhesion; HSQC, heteronuclear single-quantum coherence; ITC, isothermal titration calorimetry; NOE, nuclear overhauser effect; prr, proline-rich region; RDCs, residual dipolar couplings; SAXS, small angle X-ray scattering; SH3 domain, Src homology 3 domain; Vh, vinculin head; Vt, vinculin tail.

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http://dx.doi.org/10.1016/j.jsb.2014.05.009
1047-8477/

and VASP (Brindle et al., 1996; DeMali et al., 2002; Kioka et al., 1999; Mandai et al., 1999).

As a vinculin-binding protein, CAP belongs to the vinexin family and has a unique structure consisting of a sorbin homology (SoHo) domain in the N terminus and three consecutive SH3 domains in the C terminus (Kioka et al., 2002). The first and second SH3 domains of CAP that bind to the proline-rich region of vinculin have been reported to be responsible for the localization of CAP in cell-ECM adhesions (Mandai et al., 1999). CAP impairs focal adhesion turnover and negatively regulates cell migration. Zhang et al. demonstrated that the designated mutants of CAP that disrupt its interaction with vinculin do not exhibit these abilities. In addition, a more recent study indicated that the CAP-vinculin protein complex is important for stretch-sensitive organ assembly and function and that CAP may serve as a scaffolding protein at membrane-cytoskeleton interfaces (Bertolucci et al., 2005).

As the exclusive component contributing to the interaction between CAP and vinculin, the SH3 domain is one of the most popular interaction modules in the protein–protein interaction. The combination of multiple SH3 domains offers increased affinity and high specificity to a given interaction (Mayer, 2001). Although the structures of complexes consisting of a single SH3 domain bound with proline-rich motifs have been previously reported (Feng et al., 1994; Lim et al., 1994; Wu et al., 1995), there is very limited knowledge about the structural information regarding multiple SH3 domains complexed with their binding partners. Additionally, although Zhang et al. has reported that the designated mutations of conserved residues suggested by the typical SH3 domain binding motif effectively disrupt the CAP-vinculin interaction *in vivo* (Zhang et al., 2006), it remains interesting to learn the mechanism through which tandem SH3 domains bind with the long proline-rich linker of vinculin and the roles that two individual SH3 domains play in this interaction.

In this study, we determined the solution structure of the tandem SH3 domains of CAP using nuclear magnetic resonance (NMR) spectroscopy. Our structure and NMR relaxation data show that there is no fixed relative orientation between the SH3a and SH3b domains. NMR chemical shift perturbation and X-ray crystallography were further used to investigate the interaction interface of the tandem SH3 domains of CAP with the proline-rich region of vinculin. Moreover, small angle X-ray scattering (SAXS) and negative staining EM were employed to analyze the complex structure of the tandem SH3 domains of CAP and vinculin. Through molecular dynamics (MD) simulations, we generated a structural model of the tandem SH3 domains of CAP complexed with vinculin (herein designated CAP^{SH3}-vinculin).

2. Methods

2.1. Protein expression and purification and peptide synthesis

The SH3a domain (aa 791–849), SH3b domain (aa 870–930), and tandem SH3 domains (aa 791–930) of wild-type CAP and CAP mutants were constructed into a modified pET28a vector with an upstream sequence encoding a His tag followed by a TEV protease site. The full-length vinculin was cloned into the pET28a vector. The vin_{837} and vin_{857} were constructed into a modified pET32b vector with an upstream sequence encoding a thioredoxin protein followed by a TEV protease site. All of the expression vectors were transformed in BL21 DE3 bacterial cells for overexpression. The bacteria were grown in LB to an OD_{600} of 0.8 and induced with 1 mM IPTG for 24 h at 16 °C. ^{15}N- or $^{15}N/^{13}C$-labeled samples were produced in M9 medium supplemented with $^{15}NH_4Cl$ or both $^{15}NH_4Cl$ and ^{13}C glucose, respectively. These proteins (except full-length vinculin) were successively purified through a Ni-chelating column (Qiagen), TEV cleavage, and Superdex 75 column gel filtration. Full-length vinculin was purified by Superdex 200 column gel filtration and further purified using an anion exchange column (Q Sepharose). The prr1 and prr2 peptides used in this study were synthesized by GL Biochem (Shanghai).

2.2. NMR spectroscopy and structure calculation

All of the spectra were recorded at 303 K on a Bruker DMX500 or DMX600 spectrometer. Both the purified ^{15}N-labeled and $^{13}C/^{15}N$-labeled proteins were concentrated to 0.5 mM in 500 μL of buffer A (50 mM NaH_2PO_4, pH 6.4, 1 mM EDTA) with 10% (v/v) D_2O. 2D and 3D classical experiments were used to obtain the backbone and side chain resonance assignments (see Supplemental Experimental Procedures).

The structure calculation for the tandem SH3 domains was performed based on the proton-proton NOE restraints and dihedral angle restraints (φ and ψ) obtained using the Talos+ program (Shen et al., 2009) with the CNS version 1.2 program (Brunger, 2007; Brunger et al., 1998). Further structure refinement was performed using Xplor-NIH (version 2.34) (Schwieters et al., 2003), with restraints including NOEs, dihedral angles, hydrogen bonds, and ^{1}H-^{15}N RDCs (collected in 4.5% C12E5/hexanol alignment medium), and two SH3 domains were refined individually against the experimental RDCs. The quality of the final structures was assessed using the PROCHECK-NMR program (Laskowski et al., 1996). The calculated structures were visualized using MOLMOL (Koradi et al., 1996).

2.3. Backbone relaxation

The ^{15}N relaxation experiments were conducted at 303 K on a Bruker DMX600 NMR spectrometer. The hetero-nuclear ^{1}H-^{15}N NOEs, as well as longitudinal (R1) and transverse (R2) ^{15}N relaxation rates, were measured using standard two-dimensional methods. The relaxation delays were set to 11, 61, 142, 242 (run twice), 362, 523, and 753 ms for the T1 measurements and to 17.6, 35.2, 52.8 (run twice), 70.4, 105.6, and 140.8 ms for the T2 measurements. A recycle delay of 1 s was used for the measurement of the R1 and R2 relaxation rates. The hetero-nuclear NOE experiments were run twice in an interleaved mode with and without (reference experiment) proton saturation during the recovery delay.

2.4. RDC measurements

The RDCs of the 0.3 mM ^{15}N-labeled tandem SH3 domains in the free and in vin_{857}-bound states were obtained from spectra obtained in the anisotropic phase (see Supplemental Experimental Procedures) using the in-phase/anti-phase scheme (Ottiger et al., 1998) at 303 K. Each RDC experiment was successively recorded twice. PALES (Zweckstetter, 2008) was used for the data analysis.

2.5. Crystallization and structure determination

The SH3a and SH3b domains were purified using the aforementioned procedure and dialyzed into 20 mM Tris–HCl (pH 8.0) buffer supplemented with 200 mM NaCl. The SH3a-prr2 or SH3b-prr1 complexes were prepared by mixing the SH3 domains with the prr2 and prr1 peptides, respectively, at a molar ratio of 1:1.5. Crystals of the SH3a-prr2 complex were obtained in a solution containing 0.1 M Bis-Tris (pH 6.5), 0.2 M lithium sulfate monohydrate, and 25% (w/v) PEG3350, and crystals of the SH3b-prr1 complex were obtained in a solution containing 0.1 M HEPES (pH 7.5), 2.0 M ammonium sulfate, and 2% (v/v) PEG400.

The crystallographic data were collected by Shanghai Synchrotron Radiation Facilities (SSRF) 17U beam line and processed using