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Structural basis for recognition of the third SH3 domain of full-length R85 (R85FL)/ponsin by ataxin-7 ☆

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ABSTRACT

Ataxin-7 (Atx7) is a component of the nuclear transcription co-activator complex; its polyglutamine (polyQ) expansion may cause nuclear accumulation and recruit numerous proteins to the intranuclear inclusion bodies. Full-length R85 (R85FL) is such a protein sequestered by polyQ-expanded Atx7. Here, we report that Atx7 specifically interacts with the third SH3 domain (SH3C) of R85FL through its second portion of proline-rich region (PRR). NMR structural analysis of the SH3C domain and its complex with PRR revealed that SH3C contains a large negatively charged surface for binding with the RRTR motif of Atx7. Microscopy imaging demonstrated that sequestration of R85FL by the polyQ-expanded Atx7 in cell is mediated by this specific SH3C–PRR interaction, which is implicated in the pathogenesis of spinocerebellar ataxia 7.

Structured summary of protein interactions:
Atx7 PP2 and **SH3C** bind by isothermal titration calorimetry (View Interaction: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12)
Atx7 binds to **SH3C** by pull down (View interaction)
Atx7$_{100Q}$ and **SH3C** colocalize by fluorescence microscopy (View interaction)
SH3C and **Atx7** bind by nuclear magnetic resonance (View interaction)

1. Introduction

Spinocerebellar ataxia 7 (SCA7) is an autosomal dominant neurodegenerative disorder characterized by cerebellar ataxia associated with progressive macular dystrophy [1,2]. SCA7 is generally considered to be caused by expansion of a CAG repeat encoding polyglutamine (polyQ) tract in the protein ataxin-7 (Atx7) [3]. Atx7 is a component of mammalian STAGA (SPT3-TAF9-ADA-GCN5 acetyltransferase) transcription co-activator complex, and its polyQ expansion inhibits STAGA histone acetyltransferase activity [4,5]. Moreover, its yeast form regulates ubiquitin levels of histone H2B and links histone deubiquitination with gene gating and mRNA export [6]. Thus, impairments of these normal functions by polyQ expansion of Atx7 may underlie the production of SCA7 phenotype.

There are nine inherited neurodegenerative disorders related to polyQ expansion, the hallmark of this kind of disease is the formation of nuclear inclusion bodies [7]. The intranuclear inclusions found in human SCA7 brain contain many other proteins, including transcriptional regulators, ubiquitin/proteasome pathway components, cell death associated proteins, and chaperones and their partners [1]. The function of all these proteins can possibly be influenced by polyQ expansion of Atx7. However, whether these proteins are recruited to inclusions by polyQ expanded Atx7 through specific or non-specific protein interactions remains largely unknown. One such protein sequestered by polyQ expanded Atx7 to intranuclear inclusions is full-length R85 (R85FL), a splice variant of ponsin or Cbl-associated protein (CAP). R85FL/ponsin belongs to the SoHo adaptor protein family that regulates cytoskeletal organization and signal transduction [8]. There are three members of this family; all contain three highly conserved Src homology 3 (SH3) domains in the C-termini. As known, SH3 domain specifically recognizes proteins with proline-rich region (PRR) [9–11]. The canonical binding sites for SH3 domains usually include the amino-acid sequences like R/KxxPxxP (class I) or PxxPxR/K (class II) [10,12]. Like huntingtin (Htt), Atx7 contains a PRR just downstream of the polyQ tract [13].

Abbreviations: Atx7, ataxin-7; polyQ, polyglutamine; PRR, proline-rich region; R85FL, full-length R85; SCA7, spinocerebellar ataxia 7; SH3, Src homology 3

☆ The atomic coordinates and structure factors of free and PP2-bound SHC3 have been deposited in the PDB under codes 2LJ0 and 2LJ1, respectively.

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We herein report identification and structural analysis for the interaction of Atx7 PRR with the third SH3 domain (SH3C) of R85FL. Moreover, R85FL can be sequestered to the nuclear inclusion bodies by polyQ expanded Atx7 through specific SH3C–PRR interactions.

2. Materials and methods

2.1. Cloning, expression, peptide synthesis, and protein purification

The R85FL gene was cloned from human brain cDNA library (Invitrogen), and inserted into pcDNA3.0 vector with a FLAG tag in N-terminus of the protein. The Atx7 gene with 10 or 100 glutamine repeats in the vector pEGFPN1 was a gift from Dr. A. Brice. The SH3 domains and their mutants were subcloned into pGBTNH [14] and pGEX-4T-3. The peptides were obtained from solid-phase chemical synthesis and analyzed by electrospray mass spectrometry. The plasmids were transformed into *Escherichia coli* BL21 (DE3) cells to overexpress proteins. The proteins were purified by Ni^{2+}–NTA or GST affinity column, followed by gel filtration chromatography. $^{15}N/^{13}C$-labeled proteins were prepared using the M9 minimal medium containing $^{15}NH_4Cl$ and/or $^{13}C_{6\text{-}D}$-glucose as the sole nitrogen and/or carbon resource, respectively.

2.2. GST pull-down experiments

The GST-peptide fusions were added to the glutathione Sepharose 4B beads (Amersham Biosciences) in a PBS buffer (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.3), and the suspensions were agitated at 4 °C for 30 min. The beads were washed three times with the same buffer to remove any unbound proteins. An equivalent amount of different SH3 domains were added and incubated at 4 °C for an additional 4 h. Finally, the beads were recovered by centrifugation, and then the samples were resuspended in the sample buffer and subjected to SDS–PAGE (15% gel), followed by Coomassie staining.

2.3. NMR spectroscopy and structure determination

The sample containing $^{15}N/^{13}C$-labeled SH3C protein (~1.0 mM) with or without Atx7_PP2 peptide (4 mM) in a phosphate buffer (20 mM NaH_2PO_4, 50 mM NaCl, pH6.5) was used for the structural determination by NMR. All data were acquired at 25 °C on a Bruker Avance 600-MHz spectrometer. The spectra including HNCO, HNCACB, CBCA(CO)NH, H(CCO)NH, C(CO)NH and HCCH-TOCSY were obtained for the backbone and side-chain assignments. NOE restraints were obtained from ^{15}N- and ^{13}C-edited NOESY spectra with a mixing time of 100 ms. The NMR data were processed using NMRPipe and analyzed with SPARKY. Backbone dihedral restraints were derived from TALOS program. The structures were calculated using ARIA2.0, assessed with PROCHECK, and displayed with MOLMOL.

2.4. MTSL labeling and paramagnetic relaxation enhancement (PRE)

A Cys residue was introduced to the N- or C-terminus of Atx7_PP2, and the peptide fused with GB1 was obtained with pGBTNH expression vector [14]. After purification, MTSL ((1-oxyl-2,2,5,5-tetramethyl pyrroline-3-methyl) methanethiosulfonate) (ALEXIS Biochemicals, San Diego) was incubated with Atx7_PP2 at 4 °C overnight. Ten-fold excess of MTSL was used for the reaction and the excess MTSL was removed by desalting [11]. In the PRE experiment, after collecting a HSQC spectrum, dithiothreitol (DTT, 10 mM) was added to the mixture in the NMR tube and stayed at room temperature for 5 h to remove MTSL from the Atx7_PP2 peptide, and then another HSQC spectrum was acquired for reference [15]. The peak intensities with and without MTSL in the HSQC spectra of SH3C were compared for identifying the residues that are close to the N- or C-terminus of the peptide.

2.5. Construction of the complex structure

The complex structure was generated by HADDOCK2.1 [16]. The structures of SH3C in the peptide-bound form solved by NMR and Atx7-PP2 generated by homology modeling from the known proline-rich peptide (PDB ID: 2DF6) were used as initials for docking. The residues that have chemical-shift changes larger than the average value were treated as significantly perturbed residues. The active residues included Y13, Y15, N19, D21, D39, W41, P56 and Y59 of SH3C, and all residues of Atx7-PP2. The PRE-derived intermolecular distances were introduced as unambiguous restraints. Only those residues with resonance peaks significantly broadened or almost disappeared by paramagnetic effect were used as inputs for structure calculation. Normally the distance between the amide nitrogen atoms of SH3C and the paramagnetic oxygen of MTSL was set as 10 ± 3 Å. The ambiguous constraints were further modified by the fact that Pro56 of SH3C has intermolecular NOEs with the prolines of Atx7_PP2. The docking protocol included three stages with a final explicit water refinement. At the last iteration, 200 structures were generated and clustered by an RMSD cut-off of 3 Å.

2.6. Isothermal titration calorimetry (ITC)

ITC was performed with an ITC200 MicroCalorimeter (MicroCal). Proteins were dissolved at 100 μM in a PBS buffer (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.3; or 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.5 or 7.0). Upon reaching the equilibrium temperature of 25 °C, different peptides were titrated from a syringe into a sample chamber holding by 20 injections. Data were obtained using χ^2 minimization on a model assuming a single set of sites to calculate the binding affinity.

2.7. Cell culture and immunofluorescence microscopy

HEK 293T cells were transfected by FuGENE HD transfection reagent (Roche) following the manufacturer's instructions. Anti-FLAG antibody (Sigma) was used for immuno-fluorescence. For confocal microscopy, HEK 293T cells were grown on glass coverslips for 48 h after transfection. Images were obtained on a Leica TCS SP2 confocal microscope (Leica Microsystems).

3. Results

3.1. Atx7 PRR specifically interacts with the third SH3 domain of R85FL

By using two-hybrid approach to screen human retina cDNA library, a protein named R85 was identified for binding with Atx7 [13]. In addition, the R85FL was also found to co-localize with Atx7 [13]. R85FL is a splice variant of the *SH3P12* gene product. All variants have three repeated SH3 domains located at the C-termini (Fig. 1A). As known, SH3 is a modular domain recognizing proline-rich motifs [9,11], while Atx7 contains a PRR motif located at the N-terminus just behind the polyQ tract (Fig. 1A). To get molecular details of the recognition between Atx7 and R85FL, we examined the interactions of Atx7 PRR with different SH3 domains of R85FL by GST pull-down analysis. The results show that Atx7 PRR can interact with the third SH3 domain (as referred to SH3C)