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The NMR solution structure of the ubiquitin homology domain of Bcl-2-associated athanogene 1 (BAG-1-UBH) from *Mus musculus*

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ABSTRACT

BAG-1 (Bcl-2-associated athanogene 1), a multifunctional anti-apoptotic protein known to interact with various cellular proteins, was isolated using its interaction with the anti-apoptotic protein, Bcl-2. A 97-amino acid segment that includes the ubiquitin homology (UBH) domain of mouse BAG-1 (mBAG-1) interacts with a peptide corresponding to the cytoplasmic tail (CT) domain of proHB-EGF. This protein–peptide interaction is likely to have functional significance, as the two species exhibit a synergistic cytoprotective effect. In this study, we determined the solution structure of mBAG-1-UBH and investigated its interaction with the proHB-EGF-CT peptide using isothermal titration calorimetry and NMR spectroscopy. The solution structure of mBAG-1-UBH was shown to be similar to the previously reported structure of hBAG-1-UBH (PDB code 1WXV). However, their electrostatic potential maps demonstrated some differences in the UBH motifs that may be important for protein–peptide interaction. An NMR titration experiment demonstrated that residues 23–26 and residues 89–94 of mBAG-1-UBH are important for its molecular interaction with the peptide proHB-EGF-CT. BAG-1-UBH shares some biological functions with ubiquitin including the formation of polyubiquitin chain and the proteasomal protein degradation. The unique cytoprotective activity suggests mBAG-1-UBH to be an interesting ubiquitin-like protein with distinct biological functions. Here, we first reported the solution structure of mBAG-1-UBH and the growth factor precursor-interacting motif on the protein. For detail understanding about the binding interface and the mechanism of interaction, the study on mBAG-1-UBH/proHB-EGF-CT complex structure is necessary.

1. Introduction

The BAG (Bcl-2-associated athanogene) proteins (BAG-1, BAG-2, BAG-3, BAG-4 and BAG-5) share conserved C-terminal (the BAG domain) and central regions that bind to Hsc70/Hsp70 and Bcl-2, respectively, but they differ widely in their N-terminal domains. These proteins act as anti-apoptotic factors by inhibiting the chaperone activity of Hsc70/Hsp70 and by increasing the anti-cell death function of Bcl-2 [1]. The BAG-family proteins have been implicated in different cell processes linked to cell survival and have been found to be expressed at high levels in several types of tumor cell lines [2,3]. Thus, the over-expression of BAG-family proteins may serve as a novel molecular biomarker in diagnosing certain carcinomas.

BAG-1 (Bcl-2-associated athanogene 1) contains a unique ubiquitin homology (UBH) domain at its N-terminus that enables its interaction with the proteasome. The ability of BAG-1 to govern proteasomal degradation of certain proteins has been reported [4]. Furthermore, the binding between mouse BAG-1 and the membrane form of the heparin-binding EGF-like growth factor (HB-EGF) has been demonstrated [5]. HB-EGF, a potent mitogen and chemotactic factor for various cell types, localizes to the plasma membrane as a 20–30 kD precursor protein (proHB-EGF) containing an extracellular EGF-like domain, a transmembrane segment, and a short cytoplasmic tail (CT) [6]. Pull-down assays using domain constructs performed by Lin et al. identified the domain that interacts with proHB-EGF-CT as residues 1–97 of mBAG-1, an N-terminal fragment that includes its UBH domain. The UBH domain of mouse BAG-1 (residues 1–97) interacts with the 24-amino acid cytoplasmic tail of proHB-EGF and increases HB-EGF secretion, leading to cell survival [5]. The protein and peptide exhibit synergistic cytoprotective effects that are abolished in the presence of mBAG-1 or pro-HB-EGF alone. ProHB-EGF, Hsp70 and Bcl-2 interact with mBAG-1 at distinct binding domains, and thus, it is possible that mBAG-1 represents a link between the growth factor precursor and anti-apoptotic mechanisms.

The coordinates of the solution structure of human BAG-1-UBH have been deposited in Protein Data Bank (PDB) with accession

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code 1WXV. The mouse and human proteins share 70% sequence identity (Fig. 3D), and thus, their structures might be similar. However, a previous study showed that there are significant differences between the ^{15}N-^{1}H HSQC spectra of mBAG-1-UBH and hBAG-1-UBH [7], suggesting some differences in the chemical environments between the two structures.

The complete backbone and side chain resonances of mBAG-1-UBH were assigned previously by NMR spectroscopy [7]. In this study, we first determined the protein structure using solution NMR spectroscopy. To study its interaction with the proHB-EGF-CT peptide using HSQC titration and isothermal titration calorimetry (ITC) experiment, proHB-EGF-CT was cloned and overexpressed in *Escherichia coli* cell. The sequence information was included in Fig. 3D and the molecular weight of purified proHB-EGF-CT was confirmed by TOF-ESI mass (Supplementary Fig. 1). Circular dichroism (CD) study showed that the purified peptide is a random coil (Supplementary Fig. 2). The mBAG-1-UBH/proHB-EGF-CT interaction was represented as a one binding site model using ITC experiment. The stoichiometry of binding equals to one proHB-EGF-CT molecule per monomer of mBAG-1-UBH. We further identified the putative proHB-EGF-CT-binding site on mBAG-1-UBH by HSQC titrations. The binding motif comprises the C-terminus and the turn between β1 and β2 of mBAG-1-UBH which is different from the normal interaction surfaces on ubiquitin [8]. This finding suggests mBAG-1-UBH to be an interesting ubiquitin-like protein with distinct functional motif which is probably related to its cytoprotective activity.

2. Materials and methods

2.1. Protein/peptide expression and purification

The mBAG-1-UBH protein was expressed and purified as described previously [7]. NMR samples consisted of 0.5–1.0 mM ^{15}N- or ^{15}N/^{13}C-labeled mBAG-1-UBH in 20 mM PBS buffer (pH 6.0) containing 100 mM NaCl, 5 mM DTT, and 10% (v/v) D_2O (i.e., NMR buffer). The cDNA fragment encoding the peptide proHB-EGF-CT (residues 185–208) was subcloned into the vector pGEX-4T1. The plasmid was then transformed into *E. coli* BL21 (DE3) cells (Novagen) to express the recombinant fusion protein GST-proHB-EGF-CT. The unlabeled fusion protein was expressed in Luria Broth (LB) medium. After overnight growth, cultures were diluted 50-fold and incubated at 37 °C, 200 rpm until reaching an OD_{600} of 0.6. Protein expression was then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 37 °C, 200 rpm. The cells were harvested, and the bacterial pellet was resuspended in 20 mM PBS buffer (pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride. After sonication and ultracentrifugation, the soluble GST-proHB-EGF-CT fusion protein, present in the supernatant of the cell lysate, was purified on a Glutathione Sepharose 4B column (GE). The GST fusion protein was eluted, and the tag was cleaved by addition of 1 unit thrombin per mg fusion protein. The GST tag was separated from the protein on a dC18 HPLC column (Atlantis). Fractions containing purified proHB-EGF-CT were collected, lyophilized and dissolved in 20 mM PBS buffer (pH 6.0) containing 100 mM NaCl and 5 mM DTT. The molecular weight of the purified peptide was confirmed by electrospray ionization mass spectrometry (Supplementary Fig. 1).

2.2. ^{1}H-^{15}N HSQC chemical shift perturbation experiments

The ^{1}H-^{15}N HSQC spectrum of a protein provides information about the chemical environment of each residue and allows fingerprinting of the conformation of the protein's backbone. ^{15}N-labeled mBAG-1-UBH and the unlabeled proHB-EGF-CT peptide were prepared in NMR buffer, and their ^{15}N-^{1}H HSQC spectra were recorded at 25 °C on a VARIAN-700 MHz NMR spectrometer equipped with a cold probe. Aliquots of a 1.5 mM solution of unlabeled proHB-EGF-CT were titrated into a 0.5 mM ^{15}N-labeled mBAG-1-UBH solution to obtain mixtures with 1:0, 1:0.5–1:3 ratios of mBAG-1-UBH:proHB-EGF-CT. An HSQC spectrum was recorded at each titration point.

2.3. Isothermal titration calorimetry

To study the interaction between mBAG-1-UBH and proHB-EGF-CT, an isothermal titration calorimetry (ITC) experiment was performed using a VP-ITC calorimeter (MicroCal). Both the protein and peptide samples were dissolved in 20 mM PBS buffer (pH 6.0) containing 100 mM NaCl. All experiments were performed at 25 °C. The calorimetry cell contained 1.4 ml of 0.015 mM mBAG-1-UBH, to which was titrated 240 μL of 0.134 mM proHB-EGF-CT. The titration curve was analyzed using ORIGIN 7.0 (OriginLab).

2.4. NMR spectroscopy

All NMR spectra were acquired at 25 °C on a VARIAN-700 MHz NMR spectrometer equipped with a cold probe. The ^{15}N-labeled and ^{15}N/^{13}C-labeled mBAG-1-UBH protein samples were prepared in NMR buffer to a final sample volume of approximately 500 μL and a final concentration of 1 mM. The 2 and 3D NMR experiments included ^{1}H-^{15}N HSQC, HNCO [9], HNCA [10], HN(CO)CA [11], HNCACB [12], HBHA(CO)NH [13], CC(CO)NH [14], HC(CO)NH [14], HCCH-TOCSY [15] and HCCH-COSY [16] were utilized for assignment of the backbone and side chain resonances as conducted previously [7]. NOE distance constraints were obtained from ^{15}N-edited NOESY-HSQC and ^{13}C-edited NOESY-HSQC spectra [17]. All NMR data were processed using the software VNMRJ and analyzed using the software SPARKY [18].

2.5. Structure calculation of mBAG-1-UBH

The input file for the mBAG-1-UBH structure calculation contained the distance restraints from the ^{15}N- and ^{13}C-edit NOESY spectra, the TALOS + -generated dihedral angle restraints based on the resonance assignments of ^{1}H, ^{15}N, $^{13}C_{\alpha}$, $^{13}C_{\beta}$ and ^{13}CO [19] and the distance restraints imposed by hydrogen bonds derived from CSI predictions [20]. The NOE cross peaks were automatically assigned, and peak intensities were iteratively converted to inter-proton distance restraints using the program ARIA [21]. The NOE assignments given in the first ARIA round were checked manually, with the unambiguous and ambiguous NOE restraints derived from ARIA outputs further analyzed and employed as inputs for the next

Table 1
Structural statistics for the final 20 simulated annealing structures of mBAG-1-UBH.

NMR Restraints for ARIA/CNS calculations Protein distance restraints	
Total	1237
Intraresidue	402
Sequential	261
Medium range	113
Long range	311
H-bond restraints	22
Dihedral angle restraints	128
Structural statistics for 20 structures	
Average rmsd (all residues)	
Backbone rmsd to mean (Å)	0.51 ± 0.07
Heavy atom rmsd to mean (Å)	0.96 ± 0.08
Average rmsd (structured region)	
Backbone rmsd to mean (Å)	0.21 ± 0.02
Heavy atom rmsd to mean (Å)	0.69 ± 0.07
Residues in the allowed region of the Ramachandran plot	97.4%