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Backbone and side chain resonance assignments for a structured domain within Atg32

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Abstract

Autophagy is a catabolic cellular process that targets cytosolic material, including mitochondria, to the vacuole or lysosomes for degradation. The selective degradation of mitochondria by autophagy is termed mitophagy. Dysfunctional mitophagy, which leads to the accumulation of damaged mitochondria, has been implicated in Parkinson's disease, cancer, cardiac disease and metabolic disease. In *Saccharomyces cerevisiae*, mitophagy is initiated by the autophagy receptor Atg32, an outer mitochondrial membrane protein. A lack of structural information for Atg32 has hindered our understanding of the molecular mechanisms of mitophagy initiation. To gain new structural insight into Atg32, we have identified the location of a structured domain within the cytosolic region of Atg32 and completed the backbone and side chain resonance assignments for this domain.

Keywords

autophagy; mitophagy; Atg32; NMR

Biological Context

Macroautophagy (hereafter autophagy) is a cellular process in which cytosolic material is captured in double membrane vesicles, termed autophagosomes (Yin et al. 2016). Completed autophagosomes fuse with the vacuole or lysosomes resulting in the degradation of their cargo. The capture of cargo during autophagy can occur through either a non-selective or a selective mechanism. In selective autophagy, cargos to be degraded are identified by autophagy receptors. These receptors constitute a diverse set of proteins that may be localized in the membrane of the cargo, recognize the cargo directly or recognize ubiquitin moieties on the cargo (Johansen and Lamark 2011). Despite the diversity in these receptors, they all share a common 4 amino acid sequence, termed the Atg8 family interacting motif (AIM). The AIM is recognized by the ubiquitin like proteins Atg8 in *Saccharomyces cerevisiae* or the LC3 family of proteins in mammals (Noda et al. 2010). During autophagosomal biogenesis, Atg8 becomes conjugated to the expanding autophagosomal membrane and the interaction between Atg8 and the AIM creates a link between the cargo and the membrane.

The degradation of mitochondria by selective autophagy is termed mitophagy. In *S. cerevisiae*, the primary mitophagy receptor is Atg32, a 529 amino acid single pass outer mitochondrial membrane protein (Kanki et al. 2009). In response to nitrogen starvation,

Atg32 identifies mitochondria to be degraded and recruits downstream autophagy proteins, including Atg11, to initiate mitophagy. The only structural information which is currently available for Atg32 is a single crystal structure of Atg8 bound to the AIM of Atg32 (Kondo-Okamoto et al. 2012). As such, much of the molecular mechanisms of mitophagy initiation by Atg32 has remained a mystery. Defects in mitophagy have been observed in Parkinson's disease, cancer and various metabolic disorders. Therefore, gaining insight into the molecular mechanisms of mitophagy initiation will provide new insight into the interplay between mitophagy and disease.

No structured domains have been previously identified in Atg32. To determine if any structured domains are present in Atg32, we screened different constructs within the cytosolic region of Atg32 using *Escherichia coli* as an expression system. Constructs which expressed and were soluble were checked for the presence of secondary and tertiary structure using circular dichroism and 1D ^1H NMR spectroscopy. Constructs were further optimized using limited proteolysis and mass spectrometry. This screening approach led to the identification of a single structured domain in Atg32 comprising residues 200-341. We have completed the backbone and side chain assignments for this newly identified structured domain.

Methods and experiments

Protein Expression and Purification

A construct of Atg32 comprising residues 200-341 was codon optimized for expression in *E. coli* and subcloned into pET His6 TEV LIC cloning vector (1B) which was a gift from Scott Gradia (Addgene plasmid # 29653). Codon optimized Atg32₂₀₀₋₃₄₁ pET His6 TEV LIC cloning vector (1B) was transformed into BL21 (DE3) star cells (Invitrogen). Cells were grown in 250 ml of Terrific Broth (TB) in baffled ultrayield flasks at 37°C shaking (220 rpm) to an optical density of approximately 3.0 at 600 nm. Cells were pelleted and the supernatant was removed. Cell pellets were resuspended in M9 minimal media containing either 3 g/L ^{15}N ammonium chloride or 3 g/L ^{15}N ammonium chloride and 10 g/L ^{13}C glucose (Cambridge Isotope Laboratories) for uniform labeling with ^{15}N or ^{15}N and ^{13}C . Cells were subsequently grown at 37°C shaking (220 rpm) until the OD at 600 nm increased by 1.0, which took approximately 1.5 hours. 250 μl of 1 M isopropylthio- β -D-galactoside (IPTG) was added to each 250 ml culture and the cultures were grown for 3 hours at 37°C shaking (220 rpm). Cells were harvested by centrifugation and stored at -80°C.

Frozen cell pellets were resuspended in 50 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl_2 , 0.1% Triton X-100 buffer containing 1 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin and 1 $\mu\text{g/ml}$ pepstatin. Cells were lysed by passing the sample through a French Press (Thermo Electron) 3 times at 4°C. Lysates were cleared by centrifugation at $40,000 \times g$ for 45 minutes at 4°C. The supernatant was applied to 3 ml of TALON resin (Clontech) which was preequilibrated with 50 mM Tris pH 8.0, 500 mM NaCl. Protein was incubated on the resin at 4°C rocking for 30 minutes. The resin was washed with 50 mM Tris pH 8.0, 500 mM NaCl, 2.5 mM imidazole and the protein was eluted in 50 mM Tris pH 8.0, 200 mM NaCl, 200 mM imidazole. TEV protease was added to fractions containing protein and incubated overnight at 4°C to cleave off the hexahistidine tag. Cleaved protein was diluted to a final NaCl

concentration of 100 mM and then applied to a 5ml HiTrap SP column (GE Healthcare) preequilibrated in 50 mM Tris pH 8.0, 100 mM NaCl. The protein was eluted using a gradient from 100 mM to 1 M NaCl. Fractions containing protein were pooled and applied to a HiLoad Superdex 75 PG column equilibrated in 20 mM sodium phosphate pH 6.5, 100 mM NaCl, 0.2 mM TCEP. Fractions containing purified protein were pooled and concentrated. The uniformly labeled ^{15}N ^{13}C Atg32₂₀₀₋₃₄₁ was concentrated to 750 μM .

NMR spectroscopy

All NMR experiments were performed at 308 K on either a Bruker Avance 700 MHz or a Bruker Ascend 850 MHz spectrometer. The sequence-specific backbone assignment was determined using 2D [^1H ^{15}N] HSQC, 3D HNCA, 3D HN(CO)CA, 3D HNCO, 3D HN(CA)CO, 3D CBCA(CO)NH, 3D HNCACB and 3D HBHA(CBCACO)NH experiments. Aliphatic side chain assignments were determined using 3D (H)CCH-TOCSY, 3D HC(C)H-COSY, 3D HC(C)H-TOCSY, 3D H(CCCO)NH and 3D (H)CC(CO)NH experiments. Amide side chain assignments were determined using a 3D ^{15}N -resolved NOESY. Methionine epsilon assignments were determined using a 3D ^{13}C -resolved NOESY. For aromatic side chain assignments, a 2D ^1H - ^1H NOESY, 2D ^1H - ^1H TOCSY and 2D ^1H - ^1H DQF-COSY were recorded on a sample dialyzed into 20 mM Sodium Phosphate pH 6.5, 150 mM NaCl and 0.1 mM TCEP made in 100% D_2O (Cambridge Isotope Laboratories). ^1H chemical shifts were externally referenced to 0 ppm methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), whereas ^{13}C and ^{15}N chemical shifts were indirectly referenced according to the IUPAC recommendations (Markley et al. 1998). All NMR spectra were processed using Topspin 3.5 (Bruker). Processed spectra were analyzed using CARA (<http://cara.nmr.ch/>).

Assignment and data deposition

96% of backbone resonance assignments (H, N, CA HA) were completed. Of the 132 peaks (10 prolines) that were expected in the 2D [^1H ^{15}N] HSQC only H247, D248, S249 and G291 were absent (Figure 1). These residues are predicted to be in flexible loops and are presumably absent due to exchange. 92% of non-labile ^{13}CH groups were assigned. ^{15}NH side chain moieties of arginines and lysines were not assigned. Secondary structure probabilities were calculated using TALOS-N (Figure 2) (Shen and Bax 2013). Atg32₂₀₀₋₃₄₁ contains 6 β -strands and 5 α -helices. Chemical shift assignments were deposited in the Biological Magnetic Resonance Data Bank (BMRB) under accession number 27081.

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Figure 1.
Annotated 2D [^1H ^{15}N] HSQC spectrum recorded on a Bruker Avance 700 MHz magnet at 308 K. Residues are labeled with the single letter code and the number at which they appear in the Atg32 sequence. Atg32₂₀₀₋₃₄₁ contains no tryptophan residues.

Figure 2. Secondary structure probabilities as calculated using the TALOS-N webserver. Blue bars represent the β -strand probabilities and red bars represent the α -helix probabilities.