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¹H, ¹³C, and ¹⁵N resonance assignment of the ubiquitin-like domain from Dsk2p

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Abstract

The ubiquitin-like domain (UBL) of yeast protein Dsk2p is widely believed to recognize and bind to ubiquitin receptors on the proteasome and, as part of Dsk2p, to bridge polyubiquitinated substrates and proteasomal degradation machinery. Here we report NMR resonance assignment for ¹H, ¹⁵N, and ¹³C nuclei in the backbone and side chains of the UBL domain of Dsk2p. This assignment will aid in NMR studies focused on understanding of Dsk2's interactions with proteasomal receptors and its role as a polyubiquitin shuttle in the ubiquitin-dependent proteasomal degradation as well as other cellular pathways.

Keywords

Dsk2p; ubiquitin-like domain; UBL; proteasome

Biological context

Dsk2p, a yeast protein comprising a N-terminal ubiquitin-like (UBL) domain, two stress-induced phosphoprotein 1 (STI1) domains, and a C-terminal ubiquitin-associated (UBA) domain, was first isolated from *Saccharomyces cerevisiae* as a suppressor of kar1 allele defective for spindle pole body duplication (Biggins et al. 1996; Funakoshi et al. 2002). Dsk2p belongs to a class of UBL-UBA proteins proposed to act as polyubiquitin shuttles in ubiquitin-mediated protein degradation, the principal mechanism for the turnover of short-lived proteins in eukaryotes. Characteristic for the modular composition of these proteins, which include Rad23 and Dsk2 families, is the presence of a UBL domain at or near the N-terminus and a UBA domain at the C-terminus. The bi-functional nature of these proteins is based on the ability of the UBL domain to bind to the proteasome (Funakoshi et al. 2002) while the UBA domain can bind monomeric ubiquitin (monoUb) and polyubiquitin (polyUb) chains (Wilkinson et al. 2001).

Dsk2p appears to play a similar role to Rad23, another UBL-UBA protein in yeast. Both proteins have been found to mediate the interaction between polyubiquitinated substrates and the proteasome (Funakoshi et al. 2002; Elsasser et al. 2004; Fujiwara et al. 2004; Verma et al. 2004; Ghaboosi and Deshaies 2007). However, Dsk2p is distinct from Rad23 that contains two UBA domains. In hHR23a, the human homologue of Rad23, the C-terminal UBA-2 has a rather low affinity for monoUb, but binds strongly and selectively to Lys48-linked polyUb chains

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(Varadan et al. 2004; Raasi et al. 2005; Varadan et al. 2005). Dsk2p's UBA, on the other hand, binds strongly already to monoUb and appears to bind polyUb chains nonselectively (Ohno et al. 2005; Raasi et al. 2005; Zhang et al. 2008). The UBL domains of both Dsk2p and Rad23 bind proteasomal subunit Rpn1, but only Dsk2p's UBL has been shown to interact with the Rpn10 subunit of the proteasome (Ishii et al. 2006). These structural and binding differences between Dsk2p and Rad23 suggest that these two proteins may differ in the specificity of their recognition by and association with the proteasome. A crystal structure of the UBL domain of Dsk2p (Fig. 1) has been solved by X-ray diffraction method (Lowe et al. 2006). However, very little is known with regard to Dsk2p's interactions with various proteasomal components and possibly other binding factors in the cell. Identification of the proteasomal receptors for the UBL domain and understanding of the interplay and possibly competition between Dsk2, other UBL-containing proteins, and (poly)ubiquitin in their binding to these receptors are essential to our understanding of how proteasomal recognition and processing of the polyubiquitin signal is achieved and regulated. Given that many of these interactions are relatively weak, NMR appears to be the method of choice to address these issues. The resonance assignment of the UBL domain from Dsk2p opens the possibility for a close examination by NMR of Dsk2's role in the Ub-dependent proteasomal degradation and perhaps other pathways in the cell.

Methods and experiments

The cDNA encoding the UBL domain of yeast Dsk2p was cloned into pQE30 vector (Qiagen) under the phase T5 promoter, and the plasmid was then transformed into *E. Coli* M15[pREP4] cells. The construct (further referred to as Dsk2-UBL) used in our study contains 97 residues in total, including a N-terminal His₆-tag. The actual UBL domain of Dsk2p spans residues S2 to P77. Both ¹⁵N and ¹⁵N/¹³C uniformly enriched Dsk2-UBL samples were expressed in M9 minimal media. Cells were grown at 37°C, induced with 0.5 mM IPTG at A₆₀₀ ~ 0.6–0.8, and further incubated overnight at 25°C. Purification of Dsk2-UBL was carried out using a 5 mL HiTrap chelating column followed by size-exclusion chromatography.

NMR samples (2 mM) of purified Dsk2-UBL were prepared in 20 mM phosphate buffer at pH 6.8, containing 7% D₂O and 0.02% (w/v) NaN₃. NMR data were acquired at 23 °C on a Bruker Avance 600 spectrometer. NMR spectra were processed with XWINNMR software and analyzed using XEASY/CARA (Bartels et al. 1995; Keller 2004). Backbone assignments were obtained using the following 3D experiments: HNCO, HNCACO, HNCA, HN(CO)CA, HNCACB, and CBCA(CO)NH. Side chain chemical shifts were obtained from 3D H₂CCCONH-TOCSY, CCCONH-TOCSY, and ¹⁵N-separated TOCSY spectra. The overall secondary structure assignment was verified using the ¹³C chemical shift index method (Wishart et al. 1992; Wishart and Sykes 1994), while ³J_{HnHa} couplings (from HMQC-J experiment (Kay and Bax 1990)) and characteristic NOESY contacts were used to verify the assignment of residues to the helical regions.

In addition to the abovementioned experiments, for the assignment/structure verification purposes we conducted ¹⁵N-¹H residual dipolar coupling (RDC) and ¹⁵N relaxation measurements. RDC experiments were carried out as described elsewhere (Ruckert and Otting 2000). Measurements of the ¹⁵N auto-relaxation and the ¹⁵N-¹H cross-relaxation (measured via steady-state ¹⁵N{¹H} NOE) rates were performed as described in (Hall and Fushman 2003).

Assignments and data deposition

All assignments for ¹H, ¹⁵N, and ¹³C backbone and side chain resonances of the Dsk2-UBL construct (residues 0-86, not including the His₆-tag) were deposited in the BioMagResBank database; the BMRB entry number is 15769. In total, chemical shift assignments were made

for 98% of all the possible protein backbone resonances including H_N , H_α , N , C_α , and C' (Fig. 1); 90% of aliphatic protons, and 91% of aliphatic carbons.

Chemical shift indexing based on $^1H_\alpha$, $^{13}C_\alpha$, $^{13}C_\beta$, and $^{13}C'$ secondary shifts (Wishart and Sykes 1994) fully agrees with the secondary structure of the protein inferred from the crystal structure (Fig. 1). Also heteronuclear NOE data indicate that the backbone amide assignment agrees with the crystal structure (Fig. 1). Amides residing in the elements of secondary structure (α -helix and β -strands) exhibit higher NOEs, an indication of a well-ordered and relatively rigid structure, while the terminal and loop residues showed lower NOE values consistent with various degrees of backbone flexibility. As an additional independent validation of the backbone amides assignment, residual 1H - ^{15}N dipolar couplings (RDCs) were measured and fit to the crystal structure of Dsk2p UBL (PDB code 2BWF). The results (not shown) indicate a good agreement between the measured and back-calculated RDCs, characterized by the Pearson's correlation coefficient of 0.95 and the quality R-factor (Clare and Garrett 1999) of $R = 0.15$ for the secondary structure residues.

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Figure 1.

(A) Signal assignment of the ^1H - ^{15}N HSQC spectrum of the Dsk2-UBL construct studied here. Crosses mark positions of the signals belonging to G10, Q11, and S67 which cannot be seen at the contour levels shown here. (B) Validation of the assignment using steady-state $^{15}\text{N}\{^1\text{H}\}$ NOE (black solid circles) and chemical shift index (Wishart and Sykes 1994). The symbols on the top represent elements of secondary structure (circles for helices, squares for β -strands) derived from the crystal structure ('CRYST') and based on chemical shift index ('CSI'). Note that CSI does not report secondary structure elements that are shorter than 4 (helix) or 3 (β -strand) residues. The NOE data for overlapping signals are not shown here. Note that all residues C-terminal to P77 show negative heteronuclear NOEs indicative of high backbone disorder/flexibility in this part of the construct. (C) The crystal structure of the UBL domain of Dsk2 (PDB code 2BWF).