Solution structure of a membrane-anchored ubiquitin-fold (MUB) protein from *Homo sapiens*

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Abstract

The protein Bc059385, whose solution structure is reported here, is the human representative of a recently identified family of membrane-anchored ubiquitin-fold (MUB) proteins. Analysis of their similarity to ubiquitin indicates that homologous amino acid residues in MUBs form a hydrophobic surface very similar to the recognition patch surrounding Ile-44 in ubiquitin. This suggests that MUBs may interact with proteins containing an α -helical motif similar to those of some ubiquitin binding domains. A disordered loop common to MUBs may also provide a second protein interaction site. From the available data, it is probable that this protein is prenylated and associated with the membrane. With <20% identity to ubiquitin, the MUB family further expands the sequence space that maps to the β -grasp fold, and adds membrane localization to its list of functional roles.

Keywords: structural genomics; NMR; β -grasp; CAAX box; prenylation; ubiquitin binding domain

Structural genomics projects often reveal new examples of how nature adapts common protein structural motifs to perform diverse biological functions. Ubiquitin (Ub) and the Ub-like proteins (SUMO, Nedd8, ISG15, and others) comprise an important family of proteins that typifies the adaptive and modular character of molecular biology (Schwartz and Hochstrasser 2003). This family of covalent protein modifiers, which alter targets involved in various biochemical pathways and cascades, belongs to the β -grasp superfamily, whose members exhibit a single structural motif while retaining high sequence diversity. Consequently, the analysis of Ub-fold proteins provides opportunities to learn about both structure–function and structure–sequence relationships.

The membrane-anchored Ub-fold (MUB) proteins are a newly described family with structural homology with ubiquitin (Downes et al. 2006). Conserved structural features unique to the MUB family include an extended loop and unstructured domain containing a C-terminal CAAX-box, the canonical motif for protein prenylation. Consequently, MUB proteins lack the C-terminal typical Gly–Gly motif required for ubiquitin conjugation to the target proteins. Recent studies in *Arabidopsis* indicate that the MUBs are prenylated and localized to the membrane (Downes et al. 2006). Here we report the structure of Bc059385, the human MUB ortholog, solved by NMR spectroscopy at the Center for Eukaryotic Structural Genomics (CESG; <http://www.uwstructuralgenomics.org>). Although specific roles for the MUBs remain unknown, structural comparisons reveal conserved sequence elements that coincide with one of the hydrophobic surfaces used by ubiquitin to bind other proteins (Hurley et al. 2006). Structural and functional characterization of the MUBs will expand our understanding of the versatility of the ubiquitin fold in protein modification and localization.

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Results and Discussion

Structure of Bc059385

We completed the Bc059385 ^1H , ^{15}N , and ^{13}C chemical shift assignments by standard NMR methods (Fig. 1A; Markley et al. 2003) and determined its three-dimensional structure (Fig. 1B) using an automated procedure for iterative NOE assignment (Herrmann et al. 2002). Molecular dynamics calculations in explicit solvent were used in the final refinement (Linge et al. 2003). The final conformers were generated from 115 dihedral angle constraints and 1144 nonredundant NOE-derived distance restraints. Statistics describing the agreement with experimental constraints, coordinate precision, and stereochemical quality are summarized in Table 1. Like all proteins in the β -grasp domain superfamily, Bc059385 consists of five β -strands forming a mixed β -sheet in the order 2-1-5-3-4, one major α -helix (residues 31–41) that sits in the concave groove of the β -sheet, and a short helical turn at the end of the β 4– β 5 loop. Heteronuclear ^{15}N - ^1H NOE values shown in Figure 1C reflect the relative rigidity of secondary structure elements and disorder at the N and C termini. Residues 42–56 form an extended loop of undefined structure owing to a lack of chemical shift assignments and structural constraints (Fig. 1D),

and although ^{15}N - ^1H NOE values could not be tabulated, it is evident from NOESY spectra that residues at the loop boundaries experience chemical exchange broadening and participate in no stable structural interactions. This flexible loop, along with a C-terminal CAAX box, is a feature Bc059385 shares with a subset of Ub-fold proteins called MUBs.

MUB (membrane anchored Ub-fold) family proteins

Upon solving the β -grasp domain structure of At3g01050.1 from *Arabidopsis*, we suggested that prenylation of the CAAX box would direct it to the membrane compartment (Vinarov et al. 2004). Vierstra and coworkers subsequently used bioinformatic queries to identify sequence homologs to At3g01050.1 (now also known as AtMUB1) throughout multicellular species (Downes et al. 2006). The MUB family adopts the conserved ubiquitin fold but has no obvious sequence homology with ubiquitin (<20% sequence conservation) or other Ub-fold protein (Downes et al. 2006). They further demonstrated experimentally that MUBs in *Arabidopsis* are indeed prenylated and localize to the membrane as a consequence of lipid modification at the conserved CAAX box cysteine.

Figure 1. (A) ^{15}N - ^1H HSQC spectrum of Bc059385. Resonance assignments are indicated by residue number and one-letter amino acid code. The cross-peak for gly⁷³ (boxed) is aliased from its true ^{15}N chemical shift of 102 ppm. (B) Stereoview of the ensemble of 20 conformers of Bc059385. Unstructured residues of the C terminus (94–117) are omitted for clarity. Secondary structure elements are colored red (helix) or cyan (sheet), and residues of the disordered loop (42–57) are shown in dark gray. (C) ^{15}N - ^1H heteronuclear NOE values plotted as a function of residue number for Bc059385. (D) Backbone RMSD values for the Bc059385 ensemble plotted as a function of amino acid sequence. Disordered C-terminal residues with RMS deviations exceeding 5 Å are omitted for clarity (dashed line).

Table 1. Structural statistics for 20 NMR structures

Constraint summary		
Dihedral angles from TALOS	ϕ	56
	ψ	59
NOE distance constraints	Long	493
	Medium	216
	Short	141
	Intraresidue	294
	Total distance	1144
Ramachandran from PROCHECK	Most favored	73.8
	Additionally allowed	19.0
	Generously allowed	4.7
	Disallowed ^a	2.5
Deviation from idealized geometry		
Bond lengths	RMSD (Å)	0.0015
Torsion angle violations	RMSD (°)	1.4
Constraint violations		
NOE distance	Number >0.5 Å	0 ± 0
	RMSD (Å)	0.023 ± 0.0012
	Max violation (Å)	0.304
	Number >5°	0 ± 0
	RMSD (°)	0.7556 ± 0.0899
	Max violation (°)	4.5
Atomic RMSD to mean structure (residues 5–44, 56–91) (Å)	Backbone	0.5 ± 0.07
	Heavy atom	1.03 ± 0.11
WHATCHECK quality indicators	RMS Z-scores	
	Bond lengths	0.77 ± 0.02
	Bond angles	0.84 ± 0.02
	Bumps	0 ± 0.00
		–2356
Lennard-Jones energy (kJ/mol)		

^aWith the exception of gln⁶³, all residues in the disallowed region are located in the disordered loop or unstructured N and C termini.

Bc059385, the human MUB ortholog whose structure is reported here (HsMUB), was cloned in 1999 and originally named ubiquitin-like protein 3 (UBL3) (Chadwick et al. 1999). HsMUB is widely expressed in different tissues (Chadwick et al. 1999) and is probably subject to prenylation and membrane localization like the *Arabidopsis* MUBs, but its function is unknown. In humans, disease susceptibility studies place the gene in chromosomal loci associated with atopic dermatitis (Beyer et al. 2000; Bu et al. 2006) and familial cholesterolemia (Knoblauch et al. 2000). Studies in *Drosophila* suggest that the gene is not essential (Chadwick et al. 1999). The biological importance of its functional interactions could be difficult to assess, because organisms (especially those, like the fly and humans, that express just one MUB) may have evolved redundant mechanisms to maintain viability. In this regard, it is notable that there are six MUB paralogs in *Arabidopsis* and four in *Oryza* (Downes et al. 2006). The multiplicity of MUBs in plants suggests that new functions evolved there. It is also intriguing that the clustering steps of a ClustalW (Chenna et al. 2003) analysis led to the gravitation of the ubiquitin sequences (including the plant Ub paralogs) toward those of the MUBs from higher animals. This led us to perform a more detailed sequence-structure comparison of the MUBs and ubiquitin-like proteins.

MUB interaction sites

In multiple sequence alignments between the MUBs and ubiquitin and the other well-characterized Ub-like proteins (NEDD8, SUMO, FAT10, and ISG15) (Schwartz and Hochstrasser 2003), conserved amino acid residues within the MUB family (Fig. 2A) matched the ubiquitin sequence at 14 positions, and the other Ubl sequences at fewer than 10 positions. Many are buried in the hydrophobic core, presumably to maintain the β -grasp fold. However, a subset of conserved residues on the HsMUB surface resembles the well-studied ubiquitin Ile⁴⁴ hydrophobic patch (Hurley et al. 2006). This patch, centered on Ub residues Leu⁸, Ile⁴⁴, and Val⁷⁰ and flanked by basic residues Arg⁴², His⁶⁸, and Arg⁷², is a major interaction site for α -helical ubiquitin binding domains (UBDs) of various types. Most of the corresponding residues are highly conserved throughout the MUB family (Fig. 2B). Among the other Ub-like proteins, only NEDD8 displays a similar hydrophobic patch. Our analysis also indicated that other sites for ubiquitin binding domains are absent in the MUBs.

The subset of α -helical UBDs that bind to the Ub-Ile⁴⁴ patch includes UBA (ubiquitin associated), UIM (ubiquitin-interacting motif), DUIM (double-sided UIM), MIU

Figure 2. (A) Sequence conservation within the MUB family is plotted against the HsMUB sequence. Colored bars correspond to residues in ubiquitin that form key contacts with α -helical ubiquitin binding domains. Green letters denote HsMUB sequence positions identical to ubiquitin. (B) Fractional representation of specific amino acids in the MUB family is shown for six conserved sequence positions that correspond to the UIM binding site in ubiquitin. HsMUB residues are indicated, with the corresponding ubiquitin residue in parentheses. (C) Residues highlighted in B are displayed on the HsMUB structure in ribbon (*left*) and surface (*center*) representations and on the surface of human ubiquitin (*right*). Green (hydrophobic) and blue (basic) residues contribute to a putative UIM binding site. Nonconserved positions in the MUB family (magenta) correspond to ubiquitin residues that contribute to CUE domain recognition. (D) MUBs contain an extended loop not present in ubiquitin. Loop residues are highlighted in an overlay of MUB structures from human (cyan), mouse (orange), and *Arabidopsis* (green) with ubiquitin (magenta).

(motif interacting with ubiquitin), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation), and GAT (GGA [Golgi-localized, gamma-ear-containing, ADP-ribosylation-factor-binding protein], and TOM [target of Myb]) (Hurley et al. 2006). One or more of these α -helical motifs may interact with the MUBs in an analogous manner. From differences in the mode of interaction between the various α -helical UBDs and the Ub-Ile⁴⁴ patch and the pattern of MUB sequence conservation, we speculate that a motif similar to the UIM is the most likely binding partner.

The UIM consists of a single α -helix that binds the Ub β -sheet at a hydrophobic surface formed by Ile⁴⁴ and Val⁷⁰ and contacts the positively charged side chains of His⁶⁸, Arg⁷², and Arg⁴² as well as the backbone NH of Gly⁴⁷ (Swanson et al. 2003). The same combination of residues is preserved in the HsMUB structure (Fig. 2C). Alternatively, UBA domains may also recognize the MUBs, because residues important for this interaction are a subset of the UIM-Ub interface. However, Ub residues His⁶⁸, Val⁷⁰, and Arg⁷², which are present in the MUBs, are less critical for UBA binding (Ohno et al. 2005). CUE domain binding is less likely because two ubiquitin residues important for CUE binding, Lys⁶ and Ala⁴⁶ (Prag et al. 2003), are substituted with incompatible amino acids in the MUBs (Fig. 2B,C). Finally, it is

notable that Leu⁸ has a diminished role in the UIM and UBA interaction. This is significant because the surface side chain equivalent to Ub-Leu⁸ in the HsMUB, Val¹⁷, is not well conserved (Fig. 2A). This also precludes a GAT-like interacting domain because it would require a consistent Leu⁸ counterpart (Prag et al. 2005).

Because flexible structures are known to provide binding sites (Ishima and Torchia 2000), the disordered loop common to all MUBs (Fig. 2D) may support additional interactions. This combination of a flexible loop and hydrophobic binding surface likely enables the MUBs to interact with multiple binding partners or display greater specificity toward a single target. As an example of multiple ubiquitin binding sites, the CUE domain of Rabex-5 forms two complexes with distinct ubiquitin surfaces (Penengo et al. 2006).

The β -grasp protein superfamily, despite the small size of the domain, encompasses a remarkable diversity of sequence and biological function. The MUBs add additional sequence-fold-function relationships to this expanding superfamily. Along with Ub-like protein modifiers, PB1 and CAD heterodimerization domains (Moscat et al. 2006), and enzymatic domains like the MOad/ThiS family (Rudolph et al. 2003), the lipid-modified MUBs expand the functional repertoire of the β -grasp superfamily to include roles in membrane localization. In

contrast to the lipid-modified MUBs, which associate directly with the plasma membrane, previous examples of ubiquitin membrane localization requires the participation of other membrane-associated proteins (Guarino et al. 1995; Slagsvold et al. 2005). MUBs and their targets are anticipated to provide a novel system through which protein trafficking and membrane localization can be controlled by interactions with a prenylated β -grasp domain (Sinensky 2000).

Materials and Methods

Target selection

The protein Bc059385 was selected as part of the CESG effort to determine the structures of proteins with potentially unique folds possessing <30% sequence homology with known structures. After screening for soluble expression and purification, the protein was placed in the pipeline for production, purification, and structure determination via NMR. Management of all data associated with production was performed using the SESAME LIMS (Zolnai et al. 2003).

Protein expression and purification

The protein product of *Homo sapiens* gene *bc059385* was prepared in [U - ^{13}C , ^{15}N]-labeled form on a cell-free expression system as previously described (Vinarov et al. 2004). Briefly, the protein was expressed with an N-terminal His₆ fusion tag in wheat germ extract supplemented with [U - ^{13}C , ^{15}N] amino acids (Cambridge Isotope Labs) and purified by Ni-NTA affinity chromatography, followed by size-exclusion chromatography (Tyler et al. 2005).

NMR spectroscopy

All NMR data were acquired at 25°C on a Bruker 600 MHz spectrometer equipped with a CryoProbe and processed via the NMRPipe software (Delaglio et al. 1995). Total acquisition time was 207 h. Comparisons of 1D ^1H and 2D ^1H - ^{15}N HSQC spectra acquired at the start and end of 3D data acquisition indicated no protein degradation or unfolding during data collection. About 65% of the ^1H , ^{15}N , and ^{13}C resonance assignments were obtained in an automated manner using the program Garant (Bartels et al. 1996), with peak lists from 3D HNCO, HNCACO, HNCA, HNCOC, HNCACB, and CCONH spectra that were generated automatically with SPSCAN. The assignments were manually inspected, edited, and completed via XEASY (Bartels et al. 1995). Side-chain assignments were also analyzed and completed from HCCONH, HBHACONH, and HCCN-TOCSY spectra using XEASY.

Structure determination

Distance constraints were obtained from ^{15}N -edited NOESY-HSQC, ^{13}C -edited NOESY-HSQC, and ^{13}C -aromatic-edited NOESY-HSQC spectra. Backbone ϕ and ψ dihedral angle constraints were generated from secondary shifts of the $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, $^{13}\text{C}^\gamma$, and ^{15}N nuclei using the program TALOS

(Cornilescu et al. 1999). Structures were generated in an automated manner using the NOEAssign module of the torsion angle dynamics program CYANA (Herrmann et al. 2002), which produced an ensemble that was subsequently refined manually. The resulting 20 CYANA conformers with the lowest target function were subjected to a molecular dynamics protocol in explicit solvent using XPLOR-NIH (Schwieters et al. 2003). Atomic coordinates, geometric constraints and chemical shift assignments were deposited in the PDB (2GOW) and the BioMagResBank (7095).

Data deposition

Coordinates and constraints have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>) under PDB code 2GOW. All time-domain NMR data and chemical shift assignments have been deposited in BioMagResBank (<http://www.bmrb.wisc.edu/>) under BMRB Accession 7095.

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