doi:10.1016/j.jmb.2011.03.011 J. Mol. Biol. (2011) 408, 420–431

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Modulation of K11-Linkage Formation by Variable Loop Residues within UbcH5A

Ivan Bosanac[1], Lilian Phu[2], Borlan Pan[1], Inna Zilberleyb[1], Brigitte Maurer[1], Vishva M. Dixit[3], Sarah G. Hymowitz[1] and Donald S. Kirkpatrick[2]*

[1]*Department of Structural Biology, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA*
[2]*Department of Protein Chemistry, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA*
[3]*Department of Physiological Chemistry, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA*

Received 4 December 2010; received in revised form 2 March 2011; accepted 3 March 2011
Available online 10 March 2011

Edited by R. Huber

Keywords:
E2 enzyme;
polyubiquitin;
UbcH5;
linkage;
lysine-11

Ubiquitination refers to the covalent addition of ubiquitin (Ub) to substrate proteins or other Ub molecules via the sequential action of three enzymes (E1, E2, and E3). Recent advances in mass spectrometry proteomics have made it possible to identify and quantify Ub linkages in biochemical and cellular systems. We used these tools to probe the mechanisms controlling linkage specificity for UbcH5A. UbcH5A is a promiscuous E2 enzyme with an innate preference for forming polyubiquitin chains through lysine 11 (K11), lysine 48 (K48), and lysine 63 (K63) of Ub. We present the crystal structure of a noncovalent complex between Ub and UbcH5A. This structure reveals an interaction between the Ub surface flanking K11 and residues adjacent to the E2 catalytic cysteine and suggests a possible role for this surface in formation of K11 linkages. Structure-guided mutagenesis, *in vitro* ubiquitination and quantitative mass spectrometry have been used to characterize the ability of residues in the vicinity of the E2 active site to direct synthesis of K11- and K63-linked polyubiquitin. Mutation of critical residues in the interface modulated the linkage specificity of UbcH5A, resulting in generation of more K63-linked chains at the expense of K11-linkage synthesis. This study provides direct evidence that the linkage specificity of E2 enzymes may be altered through active-site mutagenesis.

Introduction

The ubiquitin (Ub) system controls the nature, amplitude, and duration of cellular signals by modulating protein–protein interactions and targeting proteins for destruction.[1] At the heart of the system is conjugation of Ub to lysine residues or the free N-terminus of protein substrates by the stepwise actions of an E1-activating enzyme, E2-conjugating enzyme, and E3 ligase. Polyubiquitin (polyUb) chains are synthesized when the C-terminus of one Ub molecule is conjugated to a lysine residue or the N-terminus of another Ub molecule. PolyUb chains linked through lysine 48 (K48), lysine 63 (K63), lysine 11 (K11), and the N-terminus each regulate certain cellular processes, with intricate control and precise timing of processes thought to be modulated by the lengths and linkages of these chains.[2,3]

The mechanisms of linkage specificity in polyUb chain synthesis by E2s and E3s are not well understood and remain an area of active investigation. HECT E3 ligases ($E3^{HECT}$) possess an active-site cysteine that receives Ub from a charged E2 (E2~Ub) and subsequently transfers it onto a substrate lysine.

*Corresponding author. E-mail address: kirkpatrick.donald@gene.com.

Abbreviations used: Ub, ubiquitin; polyUb, polyubiquitin; APC, anaphase-promoting complex; WT, wild type.

RING ligases ($E3^{RING}$) lack a catalytic cysteine and function instead by bringing the substrate lysine and catalytic cysteine of E2~Ub together in a conformation suitable for Ub transfer. Since the enzyme carrying Ub as a reactive thioester during the final enzymatic step plays a critical role in determining which polyUb linkage(s) will be synthesized, the forms of polyUb generated during $E3^{RING}$-catalyzed reactions are commonly a function of the E2. *In vitro* experiments have indicated that many E2s and $E3^{HECT}$ ligases generate polyUb chains in a linkage-specific manner. Among the $E3^{HECT}$ ligases, E6AP generates K48 linkages,[4] while NEDD4, Hul5, and Rsp5 generate K63-linked chains.[4–6] Likewise, E2 enzymes from yeast and mammals including Ubc13/UbcH13 (K63), Ubc1/E2-25K (K48), Cdc34 (K48), Ubc7/UbcH7 (K48), and UbE2s (K11) have well-defined linkage preferences.[7] Many of these same E2s will extend polyUb chains of defined linkages in a ligase-independent manner.

Conceptually, the determinants of linkage specificity (i.e., Ub lysine selection) for E2s and $E3^{HECT}$s can include residues oriented in the vicinity of the catalytic cysteine, flanking sequences that allosterically modulate the active site, or accessory proteins that associate with E2 or $E3^{HECT}$. The combined effect of these intrinsic and extrinsic features is to orient the correct lysine residue of the acceptor Ub toward the catalytic cysteine of Ub~E2, and specific examples of each have been demonstrated. Recent studies on the yeast E2 Ubc1 identified two loops near the catalytic cysteine that are required for efficient synthesis of K48-linked chains,[8] while work on $E3^{HECT}$ Rsp5 indicates that beta sheets positioned immediately adjacent to the catalytic cysteine are necessary and sufficient for K63-linkage synthesis.[6] The K48-specific E2 Cdc34 contains an acidic loop within its C-terminal extension,[9] as well as residues near the active site,[10] that are important for assembling K48-linked chains. In the case of the Ubc13–MMS2 heterodimeric E2, the noncatalytic partner MMS2 positions K63 of the acceptor Ub for isopeptide bond formation.[11]

While linkage-specific enzymes play central roles in many Ub-dependent processes, not all E2 and E3 enzymes synthesize homogenous polyUb linkages. Most notable among these are the Ubc4/UbcH5 enzymes, a family of constitutively expressed E2s that function with numerous $E3^{RING}$ and $E3^{HECT}$ ligases.[12] The role of these enzymes appears to be in conjugating monoUb and short chains onto substrates, as shown for Ubc4/UbcH5 reactions catalyzed by the anaphase-promoting complex (APC).[13,14] These enzymes can also effectively synthesize polyUb chains, with backside binding interactions contributing to the extent of substrate ubiquitination.[15,16] Despite the apparent promiscuity of Ubc4/UbcH5 enzymes in the substrate lysines they target for modification, mass spectrometry has indicated that these E2s display an innate preference for generating three of the eight possible polyUb linkages: K48, K63, and K11.[4,13,17–19]

To better understand the intrinsic mechanisms underlying polyUb chain synthesis by Ubc4/UbcH5 E2s, we determined the X-ray crystal structure of a noncovalent complex between UbcH5A and Ub.

Table 1. Data collection and refinement statistics

	H5A-Ub
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	27.5, 102.9, 159.8
α, β, γ (°)	90, 90, 90
Resolution (Å)	50–2.7
R_{sym}	12.8 (45.8)
$\langle I\rangle/\langle\sigma I\rangle$	17.4 (3.2)
Completeness (%)	97.4 (98.1)
Redundancy	5.3 (5.5)
Refinement	
Resolution (Å)	50–2.7
No. of reflections	13,133
R_{work}/R_{free}	20.9/27.5
No. of atoms	
Protein	3,608
Water	40
Mean *B*-factors	
Protein	30.1
Water	21.3
RMSDs	
Bond lengths (Å)	0.009
Bond angles (°)	1.1

X-ray diffraction data were collected on one crystal for each structure. Values in parentheses are for the highest-resolution shell.

Fig. 1. Crystal structure of a noncovalently linked UbcH5A and Ub complex. (a) Ribbon representation of UbcH5A (green and pink) and Ub (yellow and brown) in the asymmetric unit. Two distinct protein–protein interfaces are observed between each UbcH5A and Ub molecule. (b) View rotated by 90° from (a). (c) UbcH5A and Ub interact via the I44 hydrophobic patch on Ub and the "backside" of UbcH5A. (d) Open-book view of the Ub and UbcH5A interface highlighting the backside interaction. Atoms within a distance of 3.5, 4.0, and 4.5 Å from the binding partner molecule are colored red, orange, and yellow, respectively. The contacting residues are hydrophobic as well as hydrophilic in nature. The interface is composed of 23 UbcH5A residues and 18 Ub residues accounting for 630 and 680 $Å^2$ buried solvent-accessible surface area, respectively. (e) Interactions between the K11 surface of Ub (yellow) and residues surrounding the catalytic cysteine of UbcH5A (green). Side chains of C85 and K11 are shown as spheres with sulfur colored yellow and nitrogen blue. (f) Open-book view of the Ub and UbcH5A interface adjacent to the UbcH5A catalytic site and Ub TEK-box region. Color scheme as in (d). The contact buries solvent-accessible surface area of 410 $Å^2$ on UbcH5A (14 residues) and 440 $Å^2$ on Ub (14 residues).