J|A|C|S COMMUNICATIONS

Published on Web 06/14/2010

Nonhydrolyzable Ubiquitin−Isopeptide Isosteres as Deubiquitinating Enzyme Probes.

Anitha Shanmugham,[†] Alexander Fish,[‡] Mark P. A. Luna-Vargas,[‡] Alex C. Faesen,[‡] Farid El Oualid,[†] Titia K. Sixma,[‡] and Huib Ovaa*,[†]

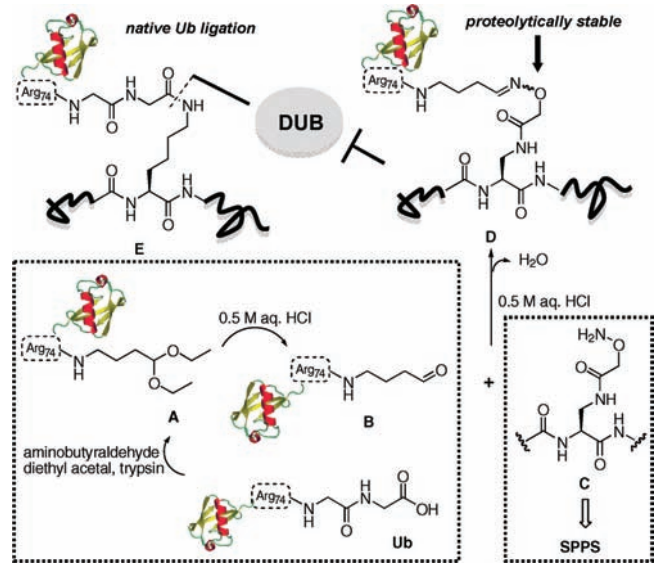
The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Received March 3, 2010; E-mail: h.ovaa@nki.nl

Ubiquitin (Ub) is a conserved 76 amino acid protein that is post-translationally conjugated onto target proteins[1] by the concerted action of ubiquitinating enzymes termed E1, E2, and E3. The Ub C-terminal carboxylate residue is conjugated to a target protein, primarily to the ε-amino group of lysine residues or their N-termini. Since Ub itself contains seven lysines (K6, K11, K27, K29, K33, K48, and K63), Ub polymers having various linkages with different sizes and shapes can be generated,[2] and all of the possible linkages have been observed in vivo[3] that target distinct substrates for different cellular fates, regulating many cellular processes including proteasomal degradation, cell-cycle progression, and signal transduction.[4,5] Ubiquitination can be reversed by the action of deubiquitinating enzymes (DUBs).[6] Nearly 100 DUBs are encoded by the human genome, but understanding of their function and mechanism of action is still limited. The chain topology, sequence, and structure of the ubiquitinated target protein are believed to determine DUB specificity. Here we describe a ubiquitin-based tool that enables the systematic investigation of linkage selectivity dictated by the local peptide sequence flanking Ub-branched lysine residues in target proteins, a determinant previously described for ubiquitin E3 ligases.[7] Several DUBs are known to hydrolyze specific Ub−Ub linkages, such as the K48-linked topology,[8] a linkage that targets proteins for proteasomal recognition and ensuing degradation. The K63 linkage has been associated mainly with the regulation of nonproteolytic processes.[2] Since distinct linkages target proteins for specific fates, it is important to know the determinants that allow DUBs to discriminate between them.

As native isopeptide-linked substrates are cleavable, they cannot be used for detailed affinity measurements. We therefore linked Ub in a nonhydrolyzable and isosteric manner with native Ub conjugates through an oxime linkage (**D** in Scheme 1). Oxime formation is a chemoselective condensation reaction between aminoxy and aldehyde moieties[9] to form a linkage that is stable under physiological conditions.[10,11] In our approach, Ub is functionalized at the C-terminus with an aldehyde (**B**) that can be generated in situ from an acetal (**A**) and subsequently ligated with an aminoxy-modified peptide (**C**) to form **D**. The spacer between the peptide chain and Ub in **D** lacks the scissile isopeptide bond and is designed to isosterically mimic the native isopeptide linkage in **E**. Structural studies of native poly-Ub chains have revealed that the isopeptide linkage region is disordered,[12,13] and it is therefore unlikely that the introduction of an oxime bond would change the spatial demands. Using solid-phase peptide synthesis (SPPS), we synthesized peptides **C** (Scheme 1) in which lysine residues targeted for ubiquitination were replaced by an aminoxyacetyl-L-diaminopropionic acid (hereafter termed **X**). Next, we generated **B**, the required aldehyde derivative of Ub, masked as the diethyl acetal

Scheme 1. Oxime-Linked Ubiquitinated Peptides (**D**) as Nonhydrolyzable Ub Branched Target Sequences



A. This acetal was converted into the aldehyde in situ by treatment with aqueous HCl. The Ub diethyl acetal **A** was conveniently prepared by reversed trypsinolysis of Ub and aminobutyraldehyde diethyl acetal (Scheme 1).[14] Thus, when Ub was incubated at 37 °C (pH 7.5) with trypsin and 25% aqueous aminobutyraldehyde diethyl acetal, over 50% conversion of Ub into **A** was observed within 3 h. Following workup and purification by cation-exchange chromatography, **A** was isolated in >95% purity and 30% overall yield under optimized conditions.

Nonhydrolyzable K48- and K63-linked Ub−isopeptide isosteres were generated from aminoxy-functionalized peptides QRLIFAG**X**-QLEDGR (Ub_{41-54}) and LSDYNIQ**X**ESTLHL (Ub_{56-69}), respectively, representing K48 and K63 Ub linkages. Biotin was attached at the N-terminal position for immobilization onto streptavidin, which was required for the affinity measurements by surface plasmon resonance (SPR). Incubation of each of the peptides (1.5 equiv) with diethyl acetal **A** (1 mg/mL) in 0.5 M aqueous HCl for 30 min at 37 °C resulted in in situ acetal deprotection and ensuing complete ligation as judged by LC−MS analysis (Figure 1). Finally, the ligation products were purified by preparative reversed-phase HPLC. SPR was used to analyze the binding of various DUBs to the two different isosteres mimicking the most predominant (i.e., K48- and K63-linked) Ub−Ub linkages[8] side-by-side. To determine whether a Ub−isopeptide isostere can specifically bind to specific DUBs, we compared the affinities of a small panel of DUBs for each of the isosteres and unconjugated Ub (Table 1). All of the tested DUBs were shown to be active prior to the SPR measurements. At the concentrations tested, the catalytic domain (CD) of

[†] Division of Cell Biology and The Netherlands Proteomics Centre.
[‡] Division of Biochemistry and Center for Biomedical Genetics.

10.1021/ja101803s

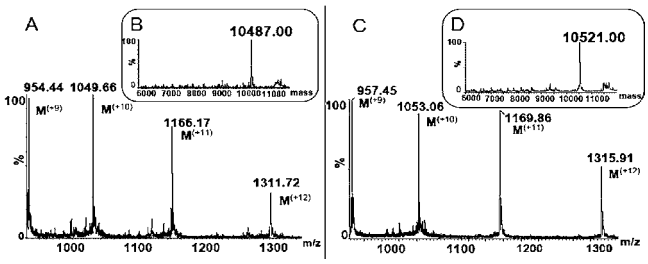


Figure 1. (A, C) ES$^+$-MS and (B, D) deconvoluted MS spectra of biotinylated UbK48 and UbK63 isopeptide isosteres, respectively.

Table 1. Affinities of DUBs toward Ub−Isopeptide Isosteres

	affinity, K_d (μM)		
DUB	Ub	UbK48 isostere	UbK63 isostere
USP7 CD	no binding	5.61 ± 0.20	no binding
USP2a CD	1.00 ± 0.40	3.69 ± 1.50	13.3 ± 2.0
USP4 CD	2.28 ± 0.43	1.71 ± 0.43	2.34 ± 0.50
USP21 CD	0.14 ± 0.02	0.06 ± 0.01	0.08 ± 0.01
USP25	no binding	no binding	no binding

Table 2. Oxime-Linked Ub Conjugates (**X** = Conjugation Site)

protein (human)	targeted lysine	sequence
PCNA	K164	GDAVVISCA**X**DGVKFSASGE
H2B	K120	EGTKAVT**X**YTSSK
H2A	K119	QAVLLP**X**KTESHKPG
FANCD2	K561	QDDMHLMR**X**QLSSTVFKY

USP7 (HAUSP) was found to bind the UbK48 isopeptide isostere with great selectivity over the UbK63 isostere or free Ub. We then tested the effect of the two isosteres and Ub on USP7 catalytic activity and found that the UbK48 isostere was able to inhibit USP7 while Ub or UbK63 did not. This implies that USP7 selects the peptide sequence flanking the UbK48 linkage over the K63 linkage or free Ub. Albeit with varying affinities, USP2a CD bound both the UbK48 and UbK63 isosteres, in agreement with earlier work that showed that both linkages are accepted by the DUB USP2a.[8] USP21 CD showed very high affinity toward Ub (in the nM range) and even greater affinity toward both of the tested isoesteres. USP4 CD bound Ub and Ub isoesters with similar affinities. Overall, our results indicate that interaction with the peptide sequence flanking the conjugation site can form the basis for DUB selectivity. We have shown that the linkage specificity intrinsic to DUBs[15] is reflected by the observed specific affinities toward Ub−isopeptide isosteres.

To demonstrate the general applicability of the oxime ligation strategy, we synthesized a panel of oxime-linked Ub conjugates based on peptide sequences derived from FANCD2,[16] Histone2A, Histone2B, and PCNA (Table 2), all of which are proteins that have potential as diagnostic markers. Fast (<30 min.) and complete conversions were observed in all cases, without the need of a large excess of any of the reactants. As USP7 is known to act on ubiquitinated H2B,[17] when this isostere along with the UbK48 and UbK63 isosteres was used to measure the interaction with USP7, the affinity of the H2B isostere for USP7 was observed to be comparable to that of the UbK48 isostere.

In summary, we have developed a practical oxime-based Ub−peptide ligation methodology and used the obtained Ub−isopeptide isosteres to determine DUB affinities for K48 and K63 topoisomer mimics. This allowed us to determine that the sequences flanking the Ub conjugation site have a direct effect on the affinity for DUBs.

We have shown that the sequence surrounding the isopeptide linkage is an important determinant for substrate recognition by DUBs. The generated proteolytically stable Ub−isopeptide isosteres also hold potential for other purposes. They can act as specific DUB inhibitors,[18] which may help us to understand DUB-specific biochemical events. As stable nonhydrolyzable ligands, they can freeze DUBs in molecular transition states for crystallographic studies that aim to understand mechanistic aspects of DUB action. Furthermore, proteolytically stable Ub−isopeptide conjugates may find use as affinity supports for proteomics approaches.

Acknowledgment. We thank Henk Hilkmann for help with SPPS and Frauke Melchior for the Usp25 construct. This work was supported by a VIDI Grant from The Netherlands Organization for Scientific Research (NWO) to H.O. and an EU Spine2complexes Grant to H.O. and T.K.S.; an EU Marie Curie Reintegration Grant to F.E.O.; and EU Rubicon, KWF, and CBG Grants to T.K.S.

Supporting Information Available: Detailed protocols and characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pickart, C. M. *Annu. Rev. Biochem.* **2001**, *70*, 503.
(2) Ikeda, F.; Dikic, I. *EMBO Rep.* **2008**, *9*, 536.
(3) (a) Xu, P.; Duong, D. M.; Seyfried, N. T.; Cheng, D.; Xie, Y.; Robert, J.; Rush, J.; Hochstrasser, M.; Finley, D.; Peng, J. *Cell* **2009**, *137*, 133. (b) Peng, J. M.; Schwartz, D.; Elias, J. E.; Thoreen, C. C.; Cheng, D. M.; Marsischky, G.; Roelofs, J.; Finley, D.; Gygi, S. P. *Nat. Biotechnol.* **2003**, *21*, 921.
(4) Ciechanover, A. *EMBO J.* **1998**, *17*, 7151.
(5) Pickart, C. M. *Cell* **2004**, *116*, 181.
(6) Nijman, S. M. B.; Luna-Vargas, M. P. A.; Velds, A.; Brummelkamp, T. R.; Dirac, A. M.; Sixma, T. K.; Bernards, R. *Cell* **2005**, *123*, 773.
(7) Catic, A.; Collins, C.; Church, G. M.; Ploegh, H. L. *Bioinformatics* **2004**, *20*, 3302.
(8) Komander, D.; Reyes-Turcu, F.; Licchesi, J. D. F.; Odenwaelder, P.; Wilkinson, K. D.; Barford, D. *EMBO Rep.* **2009**, *10*, 466.
(9) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030.
(10) Forget, D.; Boturyn, D.; Defrancq, E.; Lhomme, J.; Dumy, P. *Chem.—Eur. J.* **2001**, *7*, 3976.
(11) Zhang, L.; Torgerson, T. R.; Liu, X. Y.; Timmons, S.; Colosia, A. D.; Hawiger, J.; Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9184.
(12) Phillips, C. L.; Thrower, J.; Pickart, C. M.; Hill, C. P. *Acta Crystallogr., Sect. D* **2001**, *57*, 341.
(13) Cook, W. J.; Jeffrey, L. C.; Kasperek, E.; Pickart, C. M. *J. Mol. Biol.* **1994**, *236*, 601.
(14) Wilkinson, K. D.; Cox, M. J.; Mayer, A. N.; Frey, T. *Biochemistry* **1986**, *25*, 6644.
(15) Komander, D.; Lord, C. J.; Scheel, H.; Swift, S.; Hofmann, K.; Ashworth, A.; Barford, D. *Mol. Cell* **2008**, *29*, 451.
(16) Alpi, A. F.; Patel, K. J. *DNA Repair* **2009**, *8*, 430.
(17) (a) Sarkari, F.; Sanchez-Alcaraz, T.; Wang, S.; Holowaty, M. N.; Sheng, Y.; Frappier, L. *PLoS Pathog.* **2009**, *5*, e1000624. (b) van der Knaap, A. J.; Kumar, B. R. P.; Moshkin, Y. M.; Langenberg, K.; Krijgsveld, J.; Heck, A. J. R.; Karch, F.; Verrijzer, C. P. *Mol. Cell* **2005**, *17*, 695.
(18) Yin, L. M.; Krantz, B.; Russell, N. S.; Deshpande, S.; Wilkinson, K. D. *Biochemistry* **2000**, *39*, 10001.

JA101803S