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Top-down characterization of genetically encoded Bcl-xL phosphorylation and phosphomimetics using electron capture dissociation

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Introduction

Genetic Code Expansion (GCE) creates authentic proteoform standards for studying phosphorylation-dependent processes.

Genetic code expansion (GCE) facilitates the incorporation of non-canonical amino acids into proteins during translation at programmed stop codons.[1] Authentic and non-hydrolyzable phosphoserine can be encoded site-specifically into one or more locations in the protein sequence.[2] Non-hydrolyzable mimics of phospho-amino acids containing phosphonate groups are crucial for studying phosphorylation-dependent processes in a cellular environment (Figure 1).

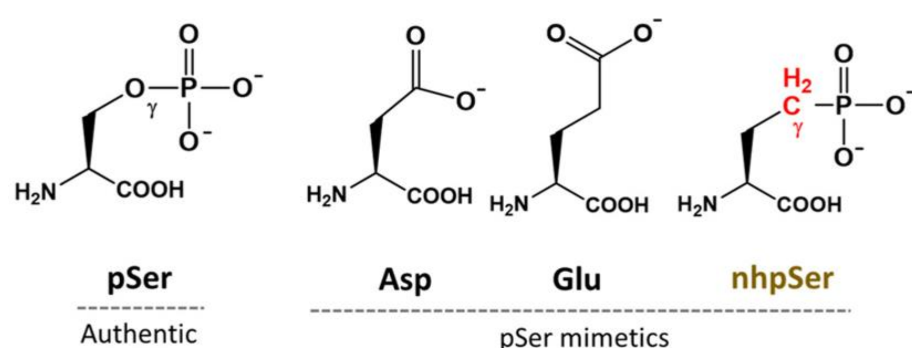

Figure 1- The structures of authentic phosphoserine (left) and phosphoserine mimetics (right). This study characterizes genetically encoded pSer, Glu, and nhpSer into full-length Bcl-xL. Figure adapted from reference 2. Licensed under CC BY.

Combining GCE with top-down ECD MS enables the in-depth characterization of phospho-proteoforms.

Top-down electron capture dissociation (ECD) MS is a powerful approach for studying phosphorylation due to its ability to retain labile modifications and characterize proteoform-specific phosphorylation patterns.

Here, we employ top-down ECD MS to characterize Bcl-xL, a protein central to cellular apoptosis. GCE was used to encode phosphorylation at Ser62, a key site that initiates apoptosis when phosphorylated.[3] However, the mechanisms underlying this process have not been resolved.

Our results provide near complete sequence coverage with site-specific Bcl-xL phosphorylation and phospho-mimics localization. Additionally, Bcl-xL and peptide binding partners were analyzed using native-like top-down analysis. Impurity analysis of peptide binding partners revealed prevalent deamidation and truncation in one sample which has implications for downstream binding studies.

Experimental

Sample Preparation

Bcl-xL variants were expressed and purified by the GCE4All Center using amber codon reassignment to create pure, site-specifically modified Bcl-xL.

Before analysis, samples were desalted and diluted to ~1µM in 15% acetonitrile with 0.1% formic acid.

Top-down Analysis with the 6545XT Q-TOF ExD cell

All top-down experiments were performed using an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer equipped with an Agilent ExD cell (G1997AA) for electron fragmentation. (Figure 2)

Figure 2- An instrument schematic showing the placement of the ExD cell into the Agilent 6545XT AdvanceBio LC/Q-TOF.

Software

A pre-release version of MassHunter BioConfirm v.12.1 was used for intact mass deconvolution.

ExDViewer v.4.5.24 was used for targeted top-down sequence analysis and localization of modifications. (Figure 3)

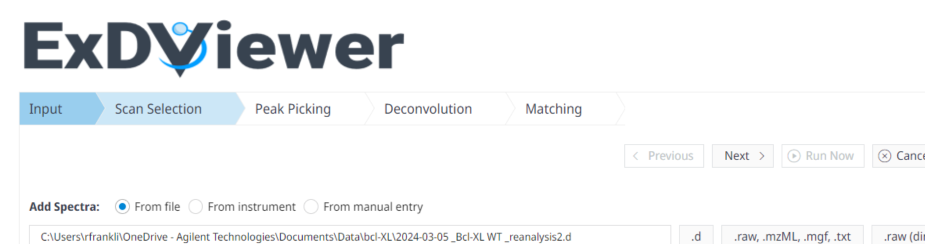

Figure 3- ExDViewer deconvolution input page. Raw .d files can be loaded directly for fragment analysis.

Top-down analysis of Bcl-xL proteoforms

Top-down sequence analysis was performed on Wild-type Bcl-xL, S62A and S62E mutants, and Bcl-xL modified with genetically encoded phosphoserine and non-hydrolyzable phosphoserine. The fragmentation resulted in 87-90% sequence coverage for all Bcl-xL variants tested. Complimentary ECD and CID-type ions were detected in the spectrum in addition to side chain fragments which appear as w-ions (Figure 4). The rich fragmentation spectrum enabled confidence sequence confirmation of Bcl-xL including the site of modification.

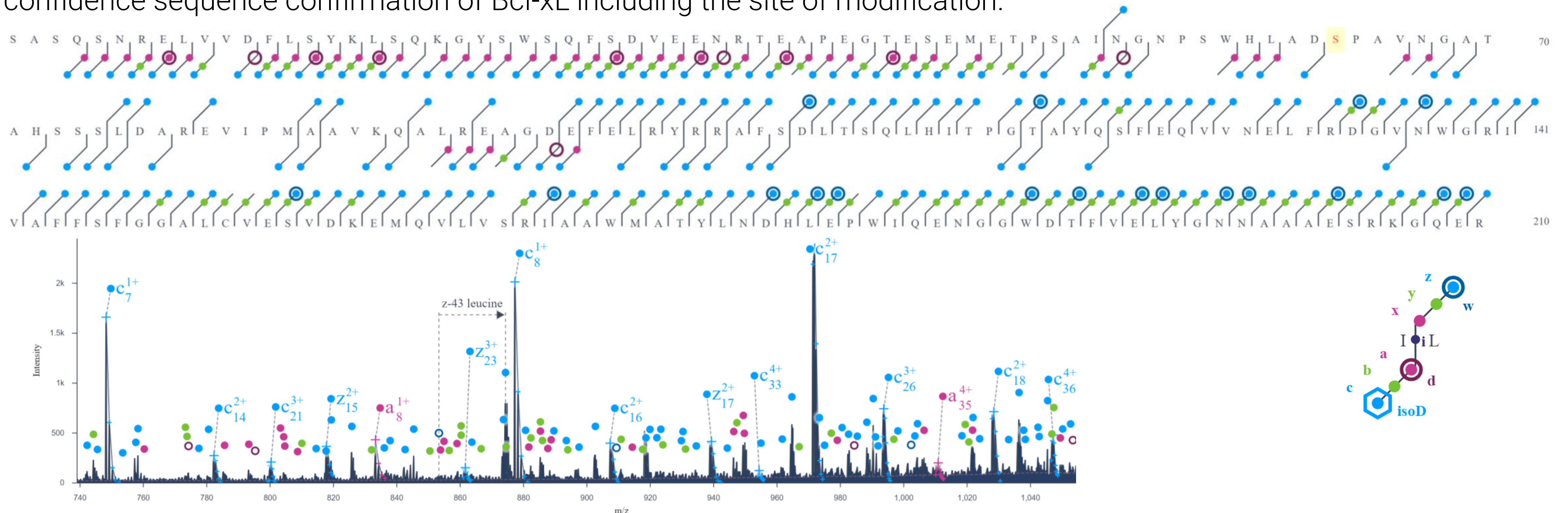

Figure 4- (Top) The sequence coverage map of Bcl-xL with the phosphorylation site in amino acid position 62 (highlighted in yellow). (Bottom) A section of the fragment spectrum from phosphorylated Bcl-XL showing efficient ECD fragmentation.

Characterization of Bcl-XL peptide binding partners

Bcl-xL binding interactions were investigated using the BH 3 domain Bcl-2 agonist of cell death (BAD) (3.1 kDa) and BH3 interacting-domain death agonist (tBID) (2.8 kDa) whose binding properties may change when Bcl-xl is phosphorylated.

The intact mass spectrum indicated that the BAD BH3 domain was pure, with only the expected peptide observed as major peaks (Results not shown). The sequence of BAD was confirmed with 100% bond coverage.

In contrast, tBID was heterogeneous with prevalent deamidation (-17 Da) and truncation observed (Figure 5). Using ECD fragmentation, the truncation was determined to be on the N-terminus and does not undergo the same deamidation event as the intact tBID precursor.

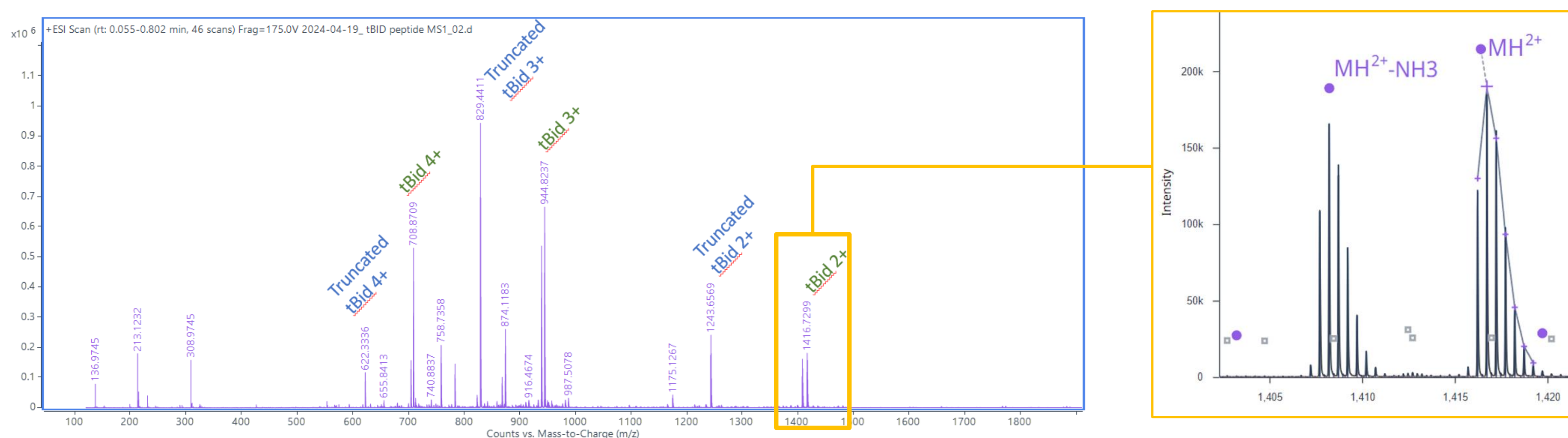

Figure 5- The MS1 spectrum of tBID showing a heterogeneous mix of ions. Zooming in on the 2+ tBID precursor reveals that the double peak is due to the loss of NH_3 (right).

Results and Discussion

Top-down analysis of Bcl-xL + peptide complex

Bcl-xL and BAD peptide were prepared in a 1:2 ratio in a solution of 100 mM ammonium acetate. The 11+ precursor of the Bcl-xL/BAD peptide complex was isolated. The noncovalent complex was subjected to 20V of collisional activation before electron capture dissociation. The resulting fragmentation spectrum contained sequence informative fragments for both Bcl-xL and the peptide (Figure 6).

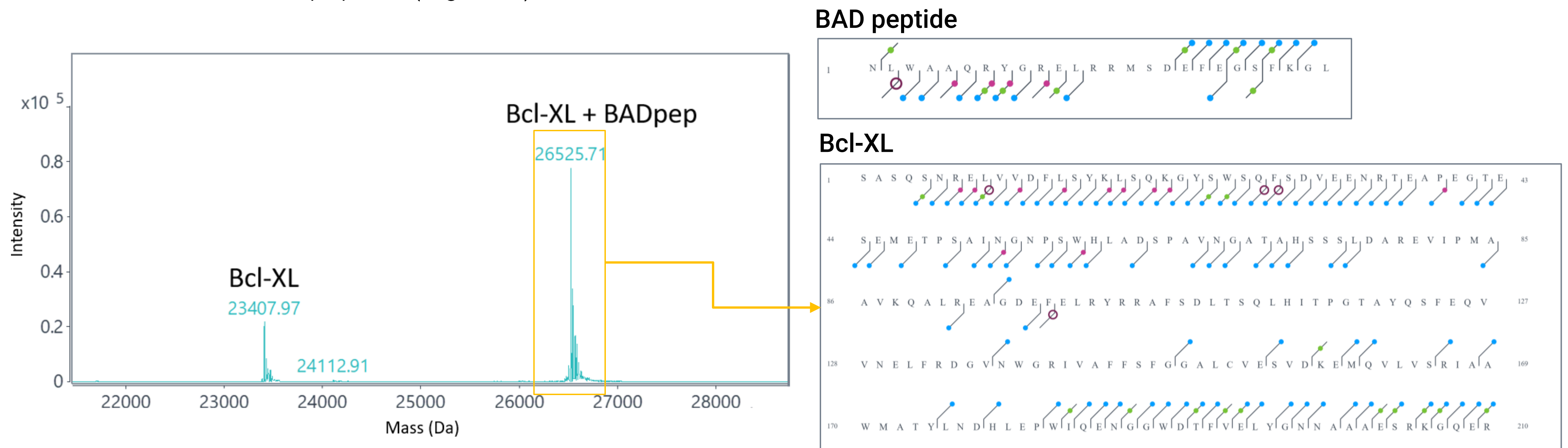

Figure 6- (Left) The deconvoluted spectrum of the 1:2 Bcl-xL/BAD peptide mixture. (Right) Bond coverage maps resulting from ECD fragmentation of the non-covalent complex.

Genetically encoded Bcl-xL modifications

The presence of genetically encoded phosphoserine and non-hydrolyzable phosphoserine was confirmed by intact mass (Figure 7).

Using the top-down results, the precise modification site was determined to be in amino acid position 62. Off-target proteoforms that could have arose from translational mis-encoding of other amino acids at the intended site of phosphorylation were not observed.

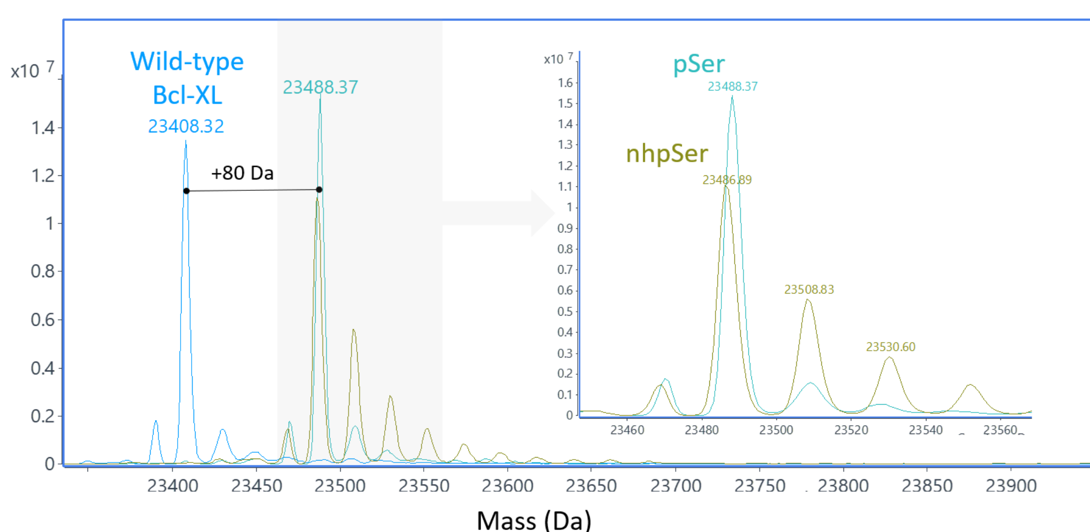

Figure 7- Overlaid deconvoluted spectra of wild-type, phosphoserine (pSer), and non-hydrolyzable (nhpSer) containing Bcl-xL. The inset shows the 2Da difference in mass between authentic pSer and non-hydrolyzable pSer.

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Conclusions

- Genetic code expansion creates high-quality proteoform standards for studying phosphorylation-dependent reactions.
- Top-down ECD MS enables the thorough analysis of engineered proteoform standards, confirming sequence and proteoform-specific modification sites.
- This combination of technologies provides a rigorous characterization of engineered protein authenticity before downstream phospho-dependent binding studies with other biophysical techniques.

References

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3- Upreti, M., Galitovskaya, E. N., Chu, R., Tackett, A. J., Terrano, D. T., Granell, S., & Chambers, T. C. *Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance*. J. Bio. Chem. 2008. 283(51), 35517–35525.

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