Characterizing Sequential Phosphorylation in the Notorious Nucleocapsid SR-rich Domain using Top-down Electron Capture Dissociation

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Introduction

- The SARS-CoV-2 nucleocapsid (N) becomes highly phosphorylated by host enzyme, GSK-3β, within a central serine/arginine rich (SR-rich) domain during infection.
- GSK-3β requires an initiating site-specific 'primer' phosphorylation which has limited the precise measurement of GSK-3β substrates in the SR-rich domain
- Traditional bottom-up MS techniques fail to accurately characterize sites of multi-phosphorylation in SR-rich proteins

Here, we establish GSK-3β -substrate pairs in the SR-rich domain using genetically encoded phosphoserine 'primers' and top-down electron capture dissociation (ECD-MS)

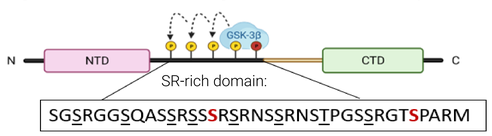


Figure 1- Schematic of GSK-3β phosphorylation of the R-rich domain. The initiating 'primer' phosphorylation is indicated in red and theoretical GSK-3β substrates are underlined

Methods

- The site-specific 'primer' phosphorylations were genetically encoded into positions S188 and S206 using amber codon reassignment.
- The homogenous 'primed' SR-rich domain was reacted with GSK-3β and allowed to fully phosphorylate before MS analysis

Figure 2- Top-down MS workflow for characterizing the phosphorylated SR-rich domain. Data was analyzed with the ExD Viewer software

- A targeted acquisition was performed using an Agilent 6545XT AdvanceBio LC/Q-TOF equipped with the ExD AQ-250 option (e-MSion)
- Data analysis was accomplished using the Agilent BioConfirm software for intact mass deconvolution and the e-MSion ExD Viewer for fragment matching.

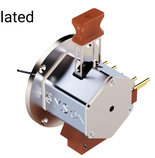


Figure 2- The ExD cell for Agilent instruments. The cell is attached to the entrance of a slightly shortened collision chamber.

Results

Primer-dependent phosphorylation by GSK-3β

- A primer phosphorylation on **Ser188** resulted in the addition of 3-6 phosphorylations by GSK-3β, with a 4X phosphorylated state being the most abundant.
- A primer on **Ser206** resulted in 3-5 added phosphorylations by GSK-3β, with a 5X phosphorylated state being most abundant
- Primers on both **Ser188 + Ser206** result in a 9X phosphorylated SR-rich domain.

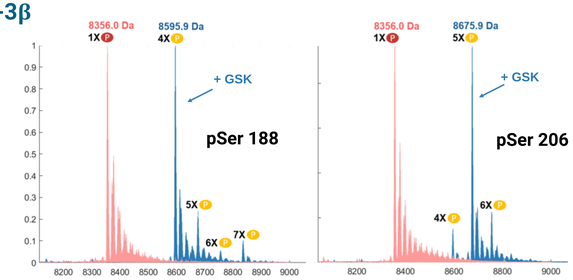
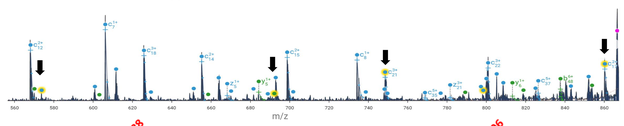


Figure 3- Intact mass measurements (Da) of the 'primed' SR-rich domain before (pink) and after GSK-3β phosphorylation (blue)

Figure 4- Mass of the dual primed SR domain before (pink) vs. after GSK-3β phosphorylation (blue)

Top-down ECD enables phospho-site localization

- Using ECD we confidently localized each phosphorylation site of the most abundant species from each sample.
- Multiply phosphorylated proteins required an additional 5-10 V of collision energy to reduce the effects of ECnoD.



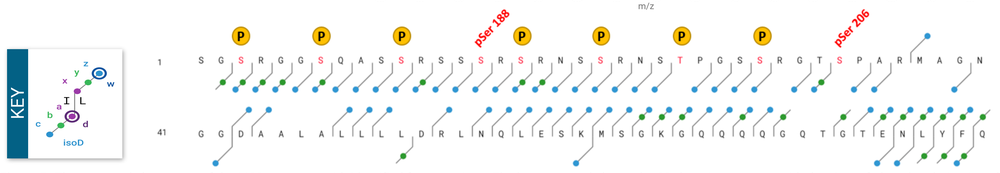


Figure 5- The top panel shows part of the MS2 spectrum with identified fragment ions. The bottom panel shows the SR domain sequence with the sites of phosphorylation in red.

Testing GSK-3β activation using pSer mimics

A **nonhydrolyzable pSer mimic** creates permanently phosphorylated proteins that can be used to study signaling pathways inside cells

Using top-down ECD-MS we compared GSK-3β activation using the nonhydrolyzable mimic and an aspartate mimic. We show that the nonhydrolyzable mimic yields very similar results to the genuine pSer primer, while the Asp primer is not recognized by GSK-3β.

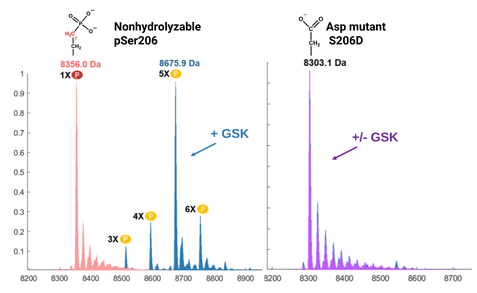
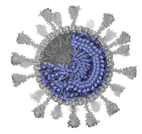


Figure 6- GSK-3β phosphorylation of the SR domain using pSer mimics

Conclusions

- Identified GSK-3β –substrates within the SARS-CoV-2 nucleocapsid SR-rich domain resulting from a single or dual priming event. A 'fully' phosphorylated protein with 9 phosphorylations was identified.
- Top-down ECD-MS enables confident phosphosite localization in SR-rich proteins. The combination of ECD + CID enables deeper sequence coverage of multiply phosphorylated SR-rich proteins.
- Combining genetically encoded phosphorylation with top-down ECD-MS is a powerful tool for establishing kinase-substrate pairs in sequential sequencing pathways.
- Nonhydrolyzable phosphoserine is a much more effective pSer mimic than the commonly used aspartate mimic for GSK-3β activation.



References

1. Wu, C., Yeh, S., Tsay, Y., et al. (2009) Glycogen Synthase Kinase-3 Regulates the Phosphorylation of Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Protein and Viral Replication. Mech. Sig. Trans.
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