Top-down proteomics goes beyond bottom-up proteomics; the case study of the Stem Loop Binding Protein (SLBP)

J. Paul Speir[1], Michael Easterling[1], Evgeniy Petrotchenko[2], William Marzluff[2], Christoph Borchers[2]

[1] Bruker Daltonics, Billerica, MA, USA, 01821, [2] Dept. of Biochemistry & Biophysics, UNC-CH, Chapel Hill, NC, USA, 27599

INTRODUCTION

The stem loop binding protein (SLBP) is involved in multiple aspects of histone mRNA metabolism. We have previously identified several phosphorylation sites in mammalian and *Drosophila* (d) SLBP by "bottom-up" proteomics, and determined the role of these sites in SLBP function[1,2].

However, from previous MW determinations of intact and truncated dSLBP proteins using a QqTOF instrument, it is clear that there are additional phosphorylation sites at the C-terminus. Moreover, the MW determined by QqTOF of these SLBP proteins was *ca.*10 Da lower than the calculated MW (Fig. 1)[3]. To explain this discrepancy and to determine all of the phosphorylation sites (including the C-terminal sites), we performed "top-down" experiments using FTICR-MS

METHODS

- The deletion mutants of dSLBP were expressed in Sf9 insect cells using the baculovirus expression system (Gibco-BRL), and were purified by affinity chromatography on Ni-NTA agarose (Qiagen).
- Prior to MS analysis, proteins were purified on micro-columns packed with POROS R1 (C4) resin (ABI), and the proteins were eluted with 75:24.8:0.2 acetonitrile-water-formic acid (v:v:v).
- Nano-ESI-MS analysis was performed on a QSTAR (ABI) instrument using electrospray needles from Protana as described before[3].
- All highly accurate mass spectrometric experiments were performed on Bruker Daltonics 9.4 T Qq-FTICR (Apex IV) equipped with an Apollo ESI source. After 1:10 dilution with 50:50 methanol:2% acetic acid (v:v:v), samples were analyzed by FIA at a flow rate at approximately 1 µL/min.
- Top-down *de-novo* sequencing of the 15+ charge state (m/z 927) of phosphorylated RBD-C were performed by CID outside the FT-ICR cell.
- ECD was accomplished with a hollow dispenser cathode.
- MS^3 experiment of RBD-C SLBP. The first MS stage was performed by skimmer induced fragmentation and the mass corresponding to the doubly charged b_{13} ion was selected for CID.

RESULTS AND DISCUSSION

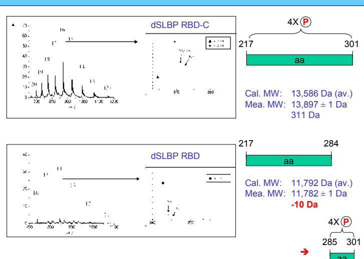
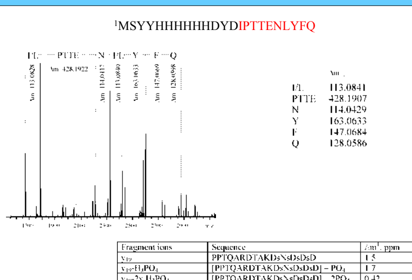


Figure 1: Nano-ESI-MS of truncated dSLBP proteins. Protein dSLBP RBD-C contains the RNA binding site and the C-terminus. Protein dSLBP RBD possesses only the RNA binding site

➔ 4 phosphoryl groups at the last 16 C-terminal aa residues.
➔ Mass discrepancy of ca. 10 Da in protein covering aa 217-284.



Fragment ions	Sequence	Δm[1], ppm
y_{19}	PPTQARDTAKDsNsDsDsD	1.5
y_{19}-H_3PO_4	[PPTQARDTAKDsNsDsDsD] – PO_4	1.7
y_{19}-2x H_3PO_4	[PPTQARDTAKDsNsDsDsD] 2PO_4	0.42

Figure 3: ESI-FTICR-MS/MS of dSLBP-RBD-C by CID.

➔ Y ions elucidated 4 phosphoryl groups at the C terminus.
➔ *De novo* sequencing revealed fragment ions corresponding to the N-terminus, however, these ions do not match b ions.

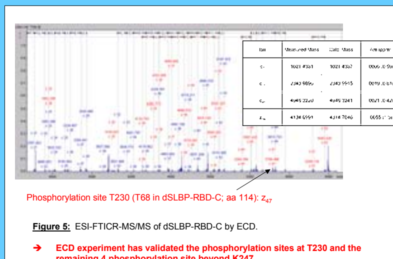
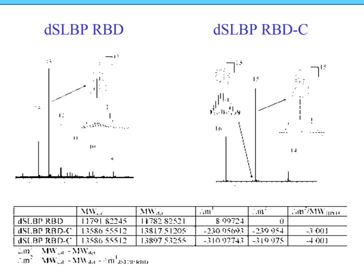


Figure 5: ESI-FTICR-MS/MS of dSLBP-RBD-C by ECD.

➔ ECD experiment has validated the phosphorylation sites at T230 and the remaining 4 phosphorylation site beyond K247.



	MW_{cal}	MW_{obs}	Δm^1	Δm^2	$\Delta m^2/MW_{HPO3}$
dSLBP RBD	11791.82245	11782.82521	8.99724	0	
dSLBP RBD-C	13586.55512	13817.51205	-230.95693	-239.954	-3.001
dSLBP RBD-C	13586.55512	13897.53255	-310.97743	-319.975	-4.001

Δm^1 = MW_{cal} - MW_{obs}
Δm^2 = MW_{cal} - MW_{obs} - $\Delta m^1_{dSLBP RBD}$

Figure 2: ESI-FTICR-MS of truncated dSLBP proteins.

➔ Highly accurate MW determination revealed a mass discrepancy of 8.997 Da between the cal. and obs. MW considering an incorporation of 3 (minor) and 4 (major) phosphoryl-groups.

Figure 4: ESI-FTICR-MS/MS/MS of dSLBP-RBD-C by skimmer-induced fragmentation combined with CID.

➔ Biotools™ software assignments show that the N-terminal methionine has been removed (MSYYHHHHHHHDYDI…), and the new N-terminus is acetylated (Ac-YYHHHHHHHDYDI).

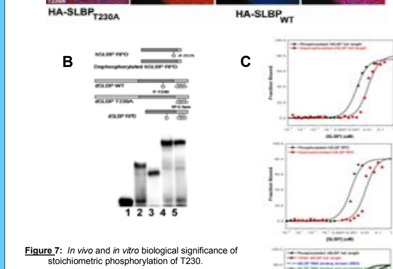


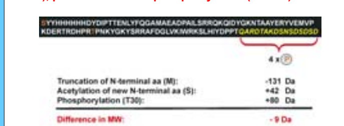
Figure 7: *In vivo* and *in vitro* biological significance of stoichiometric phosphorylation of T230.

➔ A) Wild-type protein is concentrated in the nucleus and the mutant protein is present throughout the cell[4].

➔ B,C) Mutant protein exhibits decreased binding to RNA.

CONCLUSION

- **The discrepancy of 9 Da corresponds to the sum of the loss of the N-terminal methionine (-131) and the acetylation of the new N-terminal amino acid, serine, (+42 Da), plus an additional phosphorylation (+80 Da).**



- **This phosphorylation site (T^{230}) had been previously identified by "bottom-up" proteomics, but the techniques utilized in those experiments did not allow us to evaluate the extent of phosphorylation at each site.**
- **Using the FT-ICR and top-down proteomics, it now appears that T^{230} is stoichiometrically phosphorylated, because no other ion series were detected. This finding is in total agreement with the lethality of dSLBP after mutating the phosphorylation site.**
- **Stoichiometric phosphorylation of SLBP has shown great biological significance to it's cellular localization *in vivo* and binding activity *in vitro*.**

Acknowledgements

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References

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