



A Case Study: Identification of Three Phosphorylation Sites of Adenoviral E1A-binding Protein (p300) by LC-ESI-MS/MS

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Overview

- Human p300 has three phosphorylation sites: **pS₂₉**, **pS₉₀** and **pS₁₁₂**
- The identification of the phosphorylation sites were confirmed by using several different approaches followed by nano LC-ESI MS/MS.
- Multiply phosphorylation sites of the p300 was revealed by LC-ESI-MS of the intact protein.

Introduction

CREB-binding protein (CBP)/p300 plays an important role in the connection of many different signal transduction pathways and the promotion of certain differentiation and proliferation processes. CBP/p300 serves as coactivator for transcription factors that is regulated by phosphorylation.

One phosphorylation site of p300 at Ser₉₀ was reported by Yuan⁽¹⁾, but the evidence was not strong.

In our study, different enzymes digests combined with chemical cleavage of the protein as well as N-terminal modification of the digested peptides, followed by LC-ESI-MS/MS were used to identify three phosphorylation sites of Hp300.

Methods

Sample preparation

Ep300 was the human p300 expressed in Ecoli; Hp300 was the human p300 expressed in human HCT116 colon cancer cells
The protein were Purified by His-tag
Purified protein was stored in 500 mM NaCl around 0.2 ug/ul

Protein molecular mass analysis

LC-ESI-MS analysis of intact p300 was carried out using a LC-MS (Q-ToF, Micro, Micromass) on a microbore C18 column. Elution was performed in a gradient of acetonitrile and water. MS experiments were performed at a cone voltage of 40 V, capillary voltage of 3.2 kV, collision energy of 10 V. The MS data were processed by MaxEnt1.

Protein digestion

- In-gel trypsin digest: Samples run 12% SDS page then perform in-gel trypsin digest.
- Four types of in-solution digestion:
 - trypsin
 - ASP-N then trypsin
 - trypsin then N-terminal labeling by Nic-OSu⁽²⁾

Advantages of N-terminal modification:

- Enhance b-ions intensity
 - Distinguish b-ions with internal fragments.
- Cyanogen bromide then Glu-C.

Identification of phosphorylation sites using LC-ESI-MS/MS

The digests were analyzed on a Q-ToF Global(Micromass) operating in positive mode. Peptides were separated using a c18 precolumn and analytical column. Experiments were run in DDA mode with or without specific precursor ion inclusion, and direct MS/MS mode. Data were analyzed using PEAKS and by manual for identification of phosphorylation sites.

Acknowledgements

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Results

AISRELVDPNISQISARLQVDGTGSKLMAENVVPEGPPSAKRPKLSPS
PALASASDGTDFGSLFDLEHDLPELINSTELGTNGGDINQLQTS
LGMVQDAASKHKQLSELLRSGSPNLNMGVGGPGQVMASQAQQ
SPSPGLGLAAQLYTRASQPELAPEDPELEHHHHHHHH

Figure 1. Underline is the ms/ms mapped sequence. The red part is Hp300 sequence which was cloned into the Hind III-Not I sites of the pTriEx-3 vector with C-terminal fusion HSV-tag and His-tag.
The calculated MW(avg) = 18287.14 Da,
WM(with N-terminal acetylation) = 18329.24
WM(with N-terminal acetylation + One phosphorylation) = 18409.21
WM(with N-terminal acetylation + two phosphorylation) = 18489.18

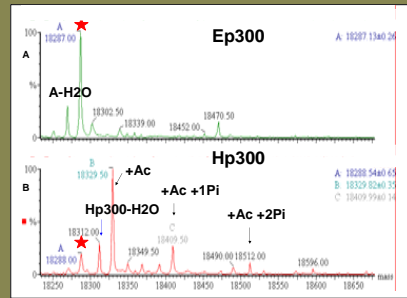


Figure 2. MaxEnt1 processed LC-ESI-MS data for Ep300 (A) and Hp300 (B). The PTM including one acetylation (Ac: plus 42Da) and one or two phosphorylation (Pi: plus 80Da) are showing in B. ★ Indicates the protein without PTM.

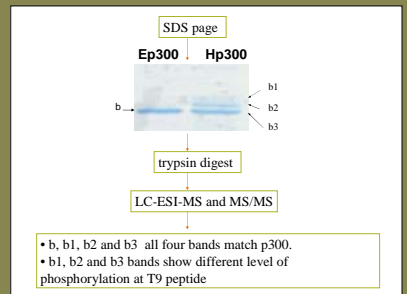


Figure 3. In-gel trypsin digest procedure and analysis results

Conclusions

Three approaches were used to localize the controversial phosphorylation site of Hp300 at pS₉₀ but not pS₉₉

- Trypsin digest with N-terminal modification.
- Separation of the two individual tryptic p phosphorylated peptides (T9) with same MW by capillary LC.
- Cyanogen bromide cleavage then Glu-C digest to get the short phosphopeptide with only one phosphorylation site.

Except the pS₉₀, two more phosphorylation sites with the same sequence pattern (...SSP...) were identified. The phosphorylation sequence pattern is similar as proline-directed phosphorylation³

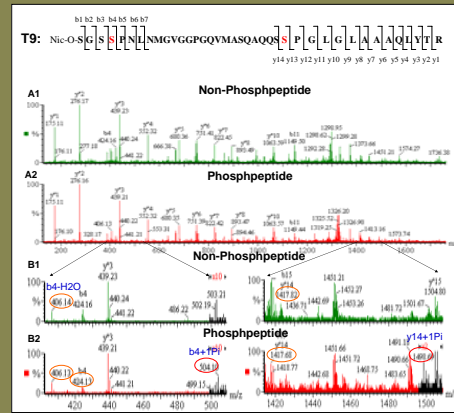
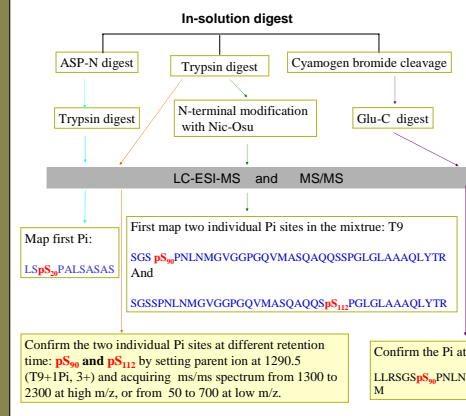


Figure 4. MS/MS spectra show the N-terminal Nic-OSu modified tryptic Hp300 peptides T9 (1298.9, 3+) (A1) and phosphorylated T9 (1325.5, 3+) (A2). B1 and B2 are the expansion of A to show the mixture ms/ms fragments of two individual Pi sites in the same peptide. Observed evidence: b4(424.15, 1+), b4-H2O(406.13¹⁺), y14 and b15 (1417.72¹⁺) in both B1 and B2. b4+1Pi (504.09, 1+) and y14+1Pi (1498.70, 1+) in B2 only.

A Summary of the Analysis Procedure and Results



Confirm the two individual Pi sites at different retention time: pS₉₀ and pS₁₁₂ by setting parent ion at 1290.5 (T9+1Pi, 3+) and acquiring ms/ms spectrum from 1300 to 2300 at high m/z, or from 50 to 700 at low m/z.

Confirm the Pi at LRLRSRSPSNLNM

References

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- K. P. Lu, Y. C. Liou and I. Vincent (2003). Proline-directed phosphorylation and isomerization in mitotic regulation and in Alzheimer's Disease. Bioessays. 25(2):174-81.

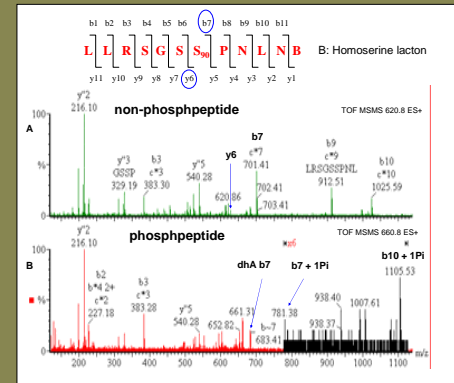


Figure 5. ms/ms spectra for cyanogen bromide cleavage, then Glu-C digest of Hp300 to compare the fragments of the non-phosphorylated (620.8 2+) (A) and phosphorylated (660.8 2+) (B) peptides. y6 (627.33 1+) and b7 (701.41 1+) in A, b7 + 1Pi (781.38 1+) and Dehydroalanine of b7 (dHAb7: 683.38 1+) in B. b6 (614.39, 1+) in both A and B. No b6 + 1Pi and y6 were observed in B. The evidence support the phosphorylation site at pS₉₀.

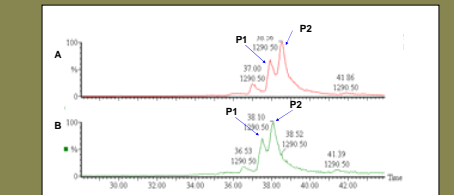


Figure 6. Total Ion Current (TIC) chromatograms for directly MS/MS of trypsin digest Hp300. The chromatograms of two experiments showing the parent ions 1290 (T9 + 1Pi, 3+) have two main peaks (P1 and P2). A: acquired at low m/z (50 – 700). B: acquired at high m/z (1300 - 2300). The purpose is to check b3, b4, b5 and b6 at low m/z as well as y13, y14 and y15 at high m/z to confirm the phosphorylation sites: pS₉₀ and pS₁₁₂

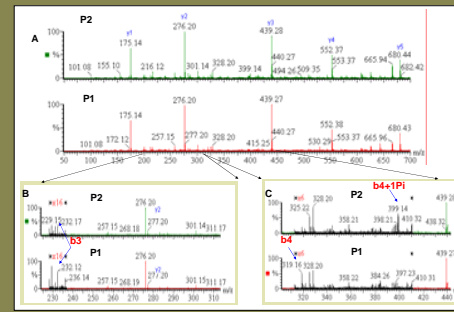


Figure 6. A: ms/ms spectra show the fragments of parent ion 1290 (3+) at low m/z for both peaks (P1 and P2). B: Expansion of A to show b3 ion (232.12) in both P1 and P2. No b3 + 1Pi. C: Expansion of A to show b4 (319.16) in P1 and b4-1Pi in P2. According the observation (plus Pi at Peak2 starting from b4 and at Peak1 starting from y14), the phosphorylation sites were confirmed: Peak 1 *SGSPNLNMGVGGPGQVMASQAQQSSPGLGLAAQLYTR* and Peak 2 *SGSPSPSNLNMVGGPGQVMASQAQQSSPGLGLAAQLYTR*