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

Cunjie Zhang1, Amanda Doherty-Kirby1, Michael Kahn 2, Kathy Emani3, and Gilles Lajoie4
1: The University of Western Ontario, London, Ontario; 2: University of Washington, Seattle, WA;

Human p300 has three phosphorylation sites: pS20, pS90, and pS112. The identification of the phosphorylation sites was confirmed by using several different approaches followed by nano LC-ESI MS/MS. Multiple phosphorylation sites of the p300 was revealed by LC-ESI-MS of the intact protein.

Overview

Results

Overview

Results

P1

• 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 [7], 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 p300.

Figure 1. Underline is the mass 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.

Methods

• Sample preparation

Ep300 was the human p300 expressed in E coli; 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 Masslab on a microplate 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

a) In-gel tryptic digest. Samples run 12% SDS page then perform in-gel tryptic digest.
b) Four types of in-solution digestion:

1. trypsin,
2. ASP-N then trypsin
3. trypsin then N-term labeling by Nc-Osu

Advantages of N-term labeling:

• Enhance b-ions intensity
• Disturb b-ions with internal fragments.
4. Cyanogen bromide and Glu-C.

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

The digests were analyzed on a Q-Tof 1 (Micromass) operating in positive mode. Peptides were separated using a c18 precolumn and analytical column. Experiments were run in DDA mode without or with specific precursor ion inclusion, and direct MS/MS mode. Data were analyzed using PEAKS or by manual for identification of phosphorylation sites.

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. 

• Conclusions

1. L. W. Yuan and J. E. Gambao (2000). Phosphorylation of p300 at Serine 89 by Protein Kinase C. JBC 275,40946-51

Acknowledgements

We thank the Ontario Research and Development Challenge Fund (CORDF) for $5 and Dr. Suya Liu for his technical assistance.

References

1. A Summary of the Analysis Procedure and Results

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

Figure 4. MS/MS spectra show the N-terminal Nic-Osu modified tryptic Hp300 peptides T9 (1298.5, 3+) (A) and phosphorylated T9 (1323.5, 3+) (B) (21 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: b4/224-195, 1+; b4-H2O/206-195, 1+; y14/1417.70, 1+). According the observation, (+ion + plus Pi starting from b4 and y-ion + Pi starting from y14), the two individual phosphorylation sites are mapped to pS90 and pS112.

Figure 5. Total ion Current (TIC) chromatograms for directly MS/MS of tryptic digest Hp300. The chromatograms of two experiments showing the parent ions 1290 (T9) and 1300 (T9+Pi) show two main peaks (P1 and P2).

A: acquired at low m/z (30 – 700); B: acquired at high m/z (1300 – 2300). This 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: m/z 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 (232.2) in both P1 and P2. No b3 +Pi. C: Expansion of A to show b4 ion (319.16) in P1 and b4+Pi in P2. According the observation: (+ion + plus Pi starting from b4 and (+ion + Pi starting from y14), the phosphorylation sites were confirmed: peak 1 “SGSNSPNLGMVGPGVQMASQQLQQGPGLAGAAXYTR” and peak 2 “SYGSNSPNLGMVGPGVQMASQQLQQGPGLAGAAXYTR”.