IP assisted LC/MS/MS making study protein complexes easy

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Objective

To identify Protein A interacting proteins and the change of post-translation modifications in a key gene knock-out mouse. This study was performed by analysis of anti-Protein A antibody immunoprecipitations using Liquid Chromatography tandem-Mass Spectrometry (LC-MS/MS).

Methods: Protein A and associated proteins were precipitated from tissue protein lysates of wild-type (WT) mouse or a gene knock-out (KO) mouse by anti-Protein A antibody. The immunoprecipitation (IP) samples were digested by trypsin and analyzed by nano-LC/MS/MS using a Dionex Ultimate 3000 RSLCnano system coupled with a Thermo Q-Exactive mass spectrometer.

Results: The analysis of negative IP sample (without antibody) and IP samples from WT and KO (with antibody) by trypsin digestion and LC/MS/MS enabled the identification of Protein A interacting proteins. The analysis revealed that mouse Protein A is the dominant protein in both sample WT and KO and identified more than a dozen of proteins which are specifically precipitated either in WT sample or KO sample. Furthermore, several acetylation modification on lysine residuals were detected in both samples, but two acetylation modification sites (K277 and K333) were only detected on Protein A from KO sample.

Introduction

Protein complexes are the main molecular machines that support all major cellular pathways. The determination of their composition, structural organization and dynamics are important challenges of modern biology. Affinity purification coupled to mass spectrometry (AP-MS) such as immunoprecipitation assisted LC-MS/MS (IP-MS) is a well-suited tool to assess the protein composition of cellular protein complexes [1–3]. Many cellular protein complexes have now been well characterized using this approach. Several large scale studies based on AP-MS have also been performed and have revealed that at least 500 protein complexes are present in Saccharomyces cerevisiae [4] or Drosophila melanogaster [5] cells. Chemical cross-linking methods have also been successfully used in association with AP-MS strategies [6].

In this report, we evaluated the power of IP-MS and identified the Protein A interacting proteins and the change of PTMs in KO mouse using the IP-MS approach.

Materials and Methods

Samples

1. CT: IP sample without adding antibody from WT mouse lysate as negative control
2. WT: IP sample with adding antibody from WT mouse lysate
3. KO: IP sample with adding antibody from a gene knock-out (KO) mouse lysate

Reagents:

1. Pierce Trypsin Protease, MS grade (Pro# 90057)
2. Formic Acid Optima LC/MS (A11-50) from Fisher Scientific
3. DTT (Pro#1000748546) and Iodoacetamide (Pro#122270250) from sigma
4. LC-MS/MS grade water, Methanol and Acetonitrile from sigma

Equipment

1. Bench-top centrifuge.
2. Speed Vacuum Concentrator (Thermo Savant ISS110).
3. C18-ziptip (ZTC18S960, Millipore).
4. Mass spectrometry system: Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific), Dionex Ultimate 3000 RSLCnano system (Thermo Scientific), Proteome Discoverer 1.4 software (Thermo Scientific). PicoFrit Column, 360 um OD/75 um ID, 15um tip ID, packed with 5um ProteoPep II C18 300A, 20 cm (New Objective).

Preparation of IP Precipitation Protein Digestion

Immunoprecipitation was performed by Client at JHU. IP samples (1-5 µg of protein) were digested by trypsin according to Poochon's SOP PS6001. The tryptic peptides mixtures were cleaned using C18 Zip-Tip and reconstituted in 25 µl of 0.1% formic acid according to Poochon's SOP PS6002. 15 µl of tryptic peptides was analyzed by LC/MS/MS.

Nanospray LC/MS/MS analysis and database search

The LC/MS/MS analysis of samples were carried out using a Thermo Scientific Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Peptide mixture sample was loaded onto a peptide trap cartridge at a flow rate of 5 µL/min. The trapped peptides were eluted onto a reversed-phase PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (3-36%) in 0.1% formic acid. The elution duration was 110 min at a flow rate of 0.3 µl/min. Eluted peptides from the PicoFrit column were ionized and sprayed into the mass spectrometer, using a Nanospray Flex Ion Source ES071 (Thermo) under the following settings: spray voltage, 1.6 kV, Capillary temperature, 250°C. For protein identification MS files were analyzed using the Thermo Proteome Discoverer 1.4.1 platform (Thermo Scientific, Bremen, Germany) for peptide identification and protein assembly. Database search against public mouse protein database obtained from NCBI website is performed based on the SEQUEST and Decoy algorithms through the Proteome Discoverer 1.4.1 platform. Carbamidomethylation of cysteines was set as a fixed modification, and Oxidation, Deamidation Q/N-deamidated (+0.98402 Da), S/T/K phosphorylation (+79.966 Da), K acetylation

(+42.011 Da), and R/K methylation (+14.016 Da) were set as dynamic modifications. The minimum peptide length was specified to be five amino acids. The precursor mass tolerance was set to 15 ppm, whereas fragment mass tolerance was set to 0.05 Da. The maximum false peptide discovery rate was specified as 0.01. The resulting Proteome Discoverer Report contains all assembled proteins with peptides sequences, possible PTMs and matched spectrum counts. The estimation of relative abundance of protein is based on peptide spectrum match counts (PSM #).

Results

Identification of potential Protein A interacting proteins

The protein precipitations, CT (IP without antibody from WT mouse tissue lysate as a negative control), WT (IP from WT mouse tissue lysate), and KO (IP from a gene knock-out mouse tissue lysate), were analyzed using the proposed protocol. The amount of Protein A precipitated from either WT or KO by the antibody is almost the same, 370 PSM# verses 379 PSM# (Table 1), suggesting the IP performed successfully. The Western blot result also confirmed that the Protein A precipitated from both samples is similar (Figure 1). The co-precipitated proteins in the IP were identified by LC/MS/MS. A selected protein list is summarized in the Table 1. The relative abundance of these proteins co-precipitated with Protein A from WT and KO was compared based on the PSM#. Based on the ratio of PSM# (WT/KO) the interacting specificity/affinity to Protein A is evaluated (Table 1).

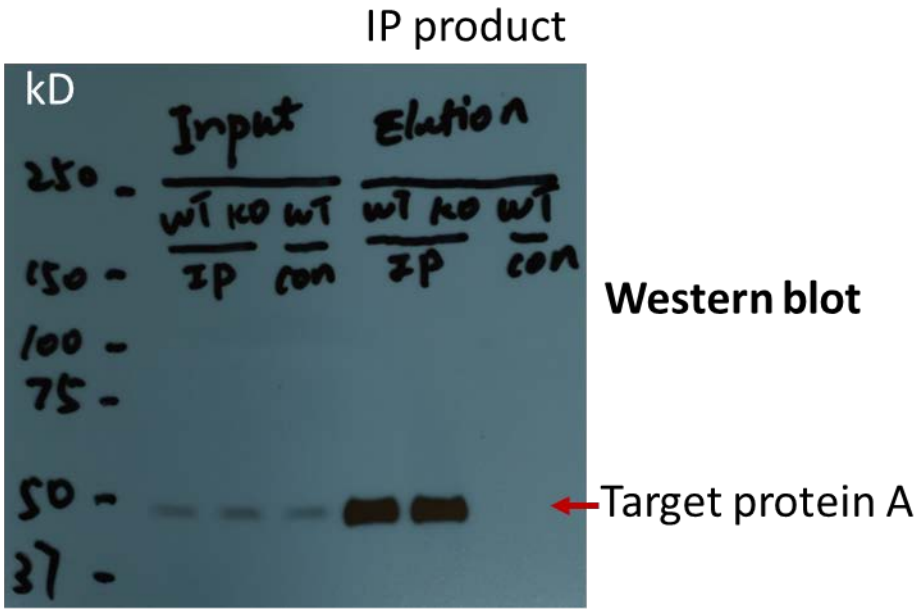

Figure 1: Equal amount of IP samples were analyzed by SDS-PAGE followed by western blot against anti-Protein A antibody.

Table 1: Comparison of Protein A interacting proteins between WT mouse and a gene KO mouse

Description	# AAs	# PSM CT	# PSM WT	# PSM KO	Ratio(WT/KO)	Comment
Protein A	403		**370**	**379**	0.98	IP Target
Protein 1	531		**49**	35	1.40	WT Strong
Protein 2	1023		**28**	20	1.40	WT Strong
Protein 3	780		**34**	26	1.31	WT Strong
Protein 4	348		**48**	37	1.30	WT Strong
Protein 5	744		**19**	15	1.27	WT Strong
Protein 6	1013		**36**	29	1.24	WT Strong
Protein 7	1061		20	18	1.11	non specific
Protein 8	298	1	29	30	0.97	non specific
Protein 9	529		84	89	0.94	non specific
Protein 10	677		17	20	0.85	non specific
Protein 11	350		27	32	0.84	non specific
Protein 12	553		59	**78**	0.76	KO strong
Protein 13	453		17	**23**	0.74	KO strong
Protein 14	373		8	**11**	0.73	KO strong
Protein 15	354		16	**22**	0.73	KO strong
Protein 16	357		9	**14**	0.64	KO strong
Protein 17	493		6	**10**	0.60	KO strong
Protein 18	213		7	**14**	0.50	KO strong
Protein 19	314		5	**12**	0.42	KO strong
Protein 20	340		7	**18**	0.39	KO strong
Protein 21	298		4	**11**	0.36	KO strong
Protein 22	215		4	**11**	0.36	KO strong

Identification of the differential PTMs between WT and KO

Several acetylation modification sites on Protein A were identified in both WT and KO. Two acetylation modification sites (K277 and K333) were only detected on Protein A from KO sample (Figure 2). This may explain why the binding affinity of Protein A of WT to proteins listed in table 1 is different from that of KO Protein A.

Figure 2: Comparison of modifications on Protein A between WT and KO IP samples. Green colored background peptide sequence areas were detected by LC/MS/MS. Sequence (WT) in black and KO in red. Two additional acetylation modification sites at K277 and K333 highlighted in yellow were only detected on Protein A from KO sample.

References

1. Dunham WH, Mullin M, Gingras AC. Affinity-purification coupled to mass spectrometry: basic principles and strategies. Proteomics 2012; 12:1576–90.
2. Gingras AC, Gstaiger M, Raught B, Aebersold R. Analysis of protein complexes using mass spectrometry. Nat. Rev. Mol. Cell Biol. 2007; 8:645–54.
3. Kocher T, Superti-Furga G. Mass spectrometry-based functional proteomics: from molecular machines to protein networks. Nat. Methods 2007; 4:807–15.
4. Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, et al. Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature2006; 440:637–43.
5. Guruharsha KG, Rual JF, Zhai B, Mintseris J, Vaidya P, Vaidya N, et al. A protein complex network of Drosophila melanogaster. Cell 2011; 147:690–703.
6. Herzog F, Kahraman A, Boehringer D, Mak R, Bracher A, Walzthoeni T, et al. Structural probing of a protein phosphatase 2 A network by chemical cross-linking and mass spectrometry. Science 2012; 337:1348–52.