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An integrated workflow for identification and quantitation of intact phosphoproteins

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Characterizing multisite phosphorylation

Overview

Objective: Comprehensive high-throughput characterization of protein phosphorylation states, coordination and dynamics of posttranslational modifications (PTMs)

Methodology

- Intact protein RPLC-FTICR
  - "Metal-free" high pressure (10,000 psi) HPLC system
  - C5 fused-silica capillary column (200 µm i.d. × 80 cm) with in-line Kasil frit coupled with Triversa Nanomate for concurrent MS analysis and fraction collection
  - 12T FTICR equipped with a novel ICR cell for high resolution and mass measurement accuracy
- Bottom-up of collected fractions for tentative identification of proteins and PTMs
- Confirmation of identification and localization of PTMs by targeted intact protein MS/MS

Applications

- Identification and quantitation of protein isoforms, e.g., multisite phosphorylation
- Profiling intact phosphoproteins

Introduction

- While bottom-up proteomics approaches have lead to identification of thousands of phosphorylations sites in a proteome, information on the coordination of phosphorylated sites is ultimately unobtainable since only a portion of the entire protein is generally detected.
- The phosphorylation of any site on a given protein can affect its activity, degradation rate, ability to dock with other proteins or bind divalent cations, and its localization. These effects can operate within the same protein due to multisite phosphorylation, which is a key mechanism for achieving signal integration in cells.
- Hence, knowing the overall phosphorylation signature of a protein is essential for understanding the "state" of a cell. We report on a new capability for high-performance LC-MS comprehensive analysis of intact phosphoproteins.

Methods

Integrated top-down and bottom-up strategy

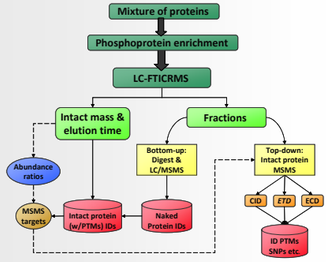

Figure 1. Integrated top-down and bottom-up strategy. Flow from the RPLC separation is directly split so that we can simultaneously collect fractions and MS data on intact proteins. The relatively small number of proteins present in each fraction allows us to infer proteins from peptide level (bottom-up) characterization of the same fraction. Accurate intact protein mass and time measurements facilitate protein abundance profiling at a level that incorporates protein modification complement and high-throughput selection of biologically relevant targets for subsequent off-line gas phase fragmentation (top-down; CID and ECD) using only an aliquot of the original fraction used for bottom-up analysis.

Instrument design for concurrent LC-MS and fractionation

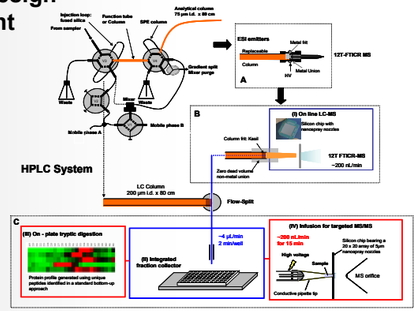

Figure 2. Metal-free HPLC system coupled with 12T FTICR-MS. Two ISCO syringe pumps deliver mobile phases. Valve V1 selects the mobile phase; V2 is for sample transfer to the SPE column when working with the 75 µm i.d. column; V3 is for sample loading (sample loop); and V4 is for sample trapping and separation (analytical column).

A) Standard setup, which uses metal screen as a column frit and a metal union to apply ESI voltage. **B)** Metal-free design uses the Kasil frit, fused silica sample loop, and PicoClear union for coupling to Triversa NanoMate ("metal free" LC-ESI interface). **C)** Fractions collected using Triversa Nanomate were analyzed using a conventional bottom up pipeline, and/or reconstituted for CID (in external accumulation hexapole) and/or ECD (in an ICR trap) tandem MS analysis.

Experimental

Sample preparation

- Mixture of standard proteins: ubiquitin, β -casein, α-casein, β-lactoglobulin A, β-lactoglobulin B, carbonic anhydrase, myoglobin, and BSA
- Yeast phosphoprotein: yeast cell lysate were prepared using buffer A of PMAC kit (Clontech), and phosphoprotein purified according to manufacture's manual

Capillary RPLC

- 10,000 psi
- 80 cm × 200 (or 75) µm column packed in-house with Phenomenex Jupiter particles (C5 stationary phase; 5 µm particle diameter; 300 Å pore size)
- Mobile phase A: 0.01% trifluoroacetic acid, 0.585% acetic acid, 25% acetonitrile, 5% isopropanol, and 69.414% water
- Mobile phase B: 0.01% trifluoroacetic acid, 0.585% acetic acid, 9.414% water, 45% acetonitrile, and 45% isopropanol)
- SPE column (150 µm i.d. x 4 cm) with "back flash" arrangement for sample trapping when column flow rates <300 nl/min

FTICR MS(/MS)

- Intact protein MS analyses on modified Bruker 12T APEX-Q FTICR with ion funnel interface, various ion transfer, selection and storage multipoles
- Proteins of interest characterized using off-line intact protein MS/MS to confirm identity and characterize PTMs
- CID-MS/MS analyses accomplished by reducing DC offset from 0 V to -25 V
- ECD-MS/MS analyses performed using heated hollow cathode located outside ICR cell

Bottom-up analysis

- 50 mM NH_4HCO_3 in 30% (v/v) aqueous acetonitrile digestion buffer (pH=8.2)
- Digestion at 37°C and overnight
- Samples evaporated to remove extra organic solvent
- Final volume adjusted to 15 µl by adding mobile phase A.
- Thermo Fisher Scientific LTQ ion trap used for LC-MS/MS analysis

Results

"Metal-free" vs. standard HPLC and ESI-interface

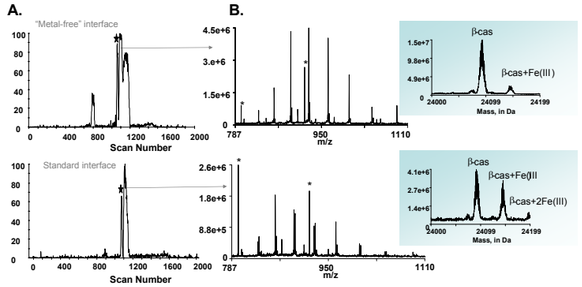

Figure 3. Improved sensitivity using "metal-free" interface. A: The total ion chromatogram reconstructed from the FTICR spectra acquired during the RPLC separation of 1 µg of β-casein with "reduced-metal" (top) and standard (bottom) interfaces. **B:** FTICR spectra and neutral mass spectra (insets) of β-casein variant A2 with "reduced-metal" (top) and standard (bottom) interfaces. The injector (needle, loop and valve) and the stainless steel unions employed in the conventional RPLC system are capable of mimicking the Fe(III)-IMAC behavior and hence trap phosphorylated proteins/peptides.

Phosphoprotein enrichment

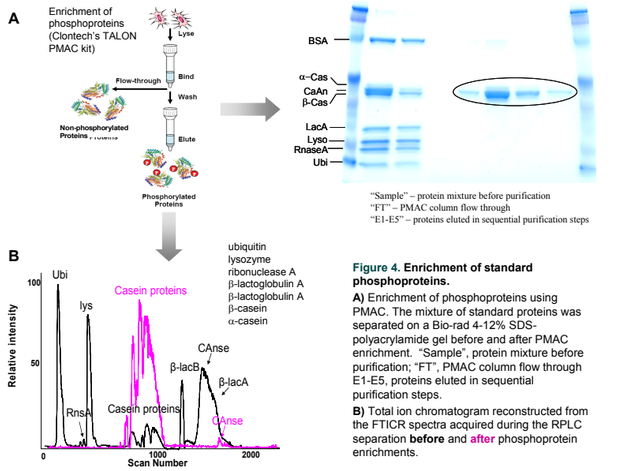

Figure 4. Enrichment of standard phosphoproteins.

A) Enrichment of phosphoproteins using PMAC. The mixture of standard proteins was separated on a Bio-rad 4-12% SDS-polyacrylamide gel before and after PMAC enrichment. "Sample", protein mixture before purification; "FT", PMAC column flow through E1-E5, proteins eluted in sequential purification steps.

B) Total ion chromatogram reconstructed from the FTICR spectra acquired during the RPLC separation **before** and **after** phosphoprotein enrichments.

Proof-of-principle demonstration

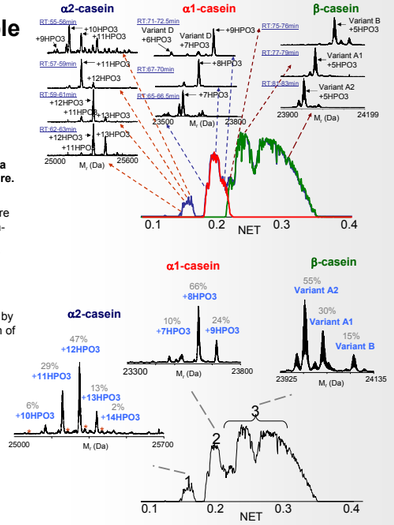

Figure 5. Integrated analysis of a standard phosphoprotein mixture.

Top: Tentative identifications of proteins and modified proteins were accomplished by matching bottom-up data with the measured intact protein masses. Offline MS/MS of targeted isoforms was used to confirmed identity and localize phosphorylation sites.

Bottom: "Bird's eye view" offered by LC-MS data facilitates quantitation of various phosphoprotein forms.

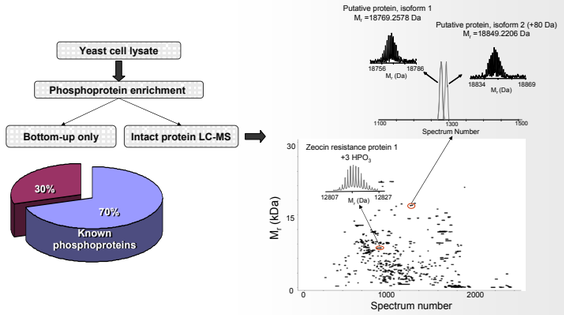

Figure 6. Integrated analysis of yeast phosphoproteome. Bottom-up analysis of phosphoprotein enriched yeast lysate identified more than 500 proteins; >70% of identified proteins have been previously reported to be phosphorylated. (left) 2D display obtained for LC-MS analysis of phosphoprotein enriched yeast lysate. (right) Tentative identifications of proteins and modified proteins were derived by matching bottom-up data with the measured intact protein masses. ~16% of the detected intact proteins carry >2 phosphorylations, while such information can never be revealed using traditional bottom-up approach.

Conclusions/ Future Directions

- A "metal-free" high-pressure (up to 10,000 psi) nanoRPLC system was developed and optimized for intact phosphoprotein separations. Preliminary results demonstrated a significant sensitivity enhancement compared to a conventional system.
- In a proof-of-principle experiment, the "metal-free" LC-MS and integrated workflow were applied to analyze casein proteins enriched from a standard protein mixture. High enrichment efficiency was confirmed by both SDS-gel and intact LC-MS data.
- The integrated strategy confirmed the presence of ~20 casein isoforms, arising from genetic variants (SNPs) and varying numbers of phosphorylation sites.
- The integrated platform has been successfully applied to characterize a yeast phosphoproteome.

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