EMSL Research and Capability Development Proposals

An Integrated Workflow for Identification and Quantitation of Intact Phosphoproteins

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Alterations in protein posttranslational modifications (PTMs), especially phosphorylation, are often associated with specific phenotypes and biological responses to stimuli. To simultaneously study multiple PTMs occurring throughout a protein, we have developed a comprehensive liquid chromatography-mass spectrometry (LC-MS) platform for highly sensitive identification and quantitation of intact proteins.

In fiscal year (FY) 201010, we focused on optimizing the top-down LC-MS platform using histones isolated from human fibroblasts. Due to their relatively small mass (<25 kDa) and high abundance, histones serve as ideal targets for intact protein analyses. Core and linker histones serve as spools to package and order DNA and regulate chromatin structure and functions. Core histones are heavily modified through lysine acetylation, lysine ubiquitination, lysine or arginine methylation, serine or threonine phosphorylation, etc. These covalent PTMs form a "histone code" that plays important roles in the storage, retrieval, repair, and duplication of genetic information. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been one of the most powerful analytical tools for characterizing these heavily modified proteins. Because all PTMs along the entire protein sequence function in concert, top down mass spectrometry has proven a superior approach for analyzing histones in comparison to traditional antibody-based or even more recent bottom-up and middle-down MS approaches.

Comparison of multiple purification methods was used to determine the optimal sample preparation methods to provide both adequate sample quantities and preserve PTMs. Using a histone affinity purification kit from Assay Designs provided better preservation of phosphorylated histone variants over traditional histone preps. Chromatography conditions were optimized to improve the resolution of intact protein separations, and these developments contributed to increased proteome coverage due to better use of the MS dynamic range and reduced discrimination during ionization.

High-resolution separation prior to MS minimizes ion suppression and under-sampling challenges associated with the analysis of highly complex histone mixtures, which often span several orders of dynamic range in protein abundance. For this reason, we have developed a new online two-dimensional (2-D) weak cation exchange (WCX)-hydrophilic interaction liquid chromatography (HILIC)/reversed phase liquid chromatography (RPLC) platform and employed it to characterize histones purified from human fibroblasts in a proof-of-principle experiment. From ~20 μg of core histones, 41 H4 isoforms were detected, and the type and locations of PTMs were unambiguously mapped for 20 of these variants. Compared to corresponding offline studies reported previously, the online WCX-HILIC/RPLC platform

offered significant improvement in sensitivity, with several orders of magnitude reduction in sample requirements and decrease in overall analysis time. To the best of our knowledge, this study represents the first online 2-D LC-MS/MS characterization of histones at the intact protein level.[1]

Compared to one-dimensional RPLC, the 2-D WCX-HILIC/RPLC yielded approximately a twofold increase in the number of identified H4 isoforms. However, even higher gains are expected if the salt-gradient WCX-HILIC is replaced with a pH-gradient WCX-HILIC, which recently was successful in separating human histone H3.2 and H4 peptides.[2] Encouraged by these results, we have modified our 2-D platform for reversed operation (i.e., RPLC-WCX/HILIC) to first separate major histone families, then differentially modified forms within each family. In a proof-of-principle experiment, 950 HeLa core histones were confidently identify in a single 24-hour LC-MS analysis. These findings compare favorably to previous offline studies using much larger sample sizes and, in fact, represent a major breakthrough for applying mass spectrometry in epigenetic research. For instance, in comparison to the offline top-down approach for the analysis of H4[3], this online study identified 100 additional isoforms from 100-fold less sample (Figure 1).

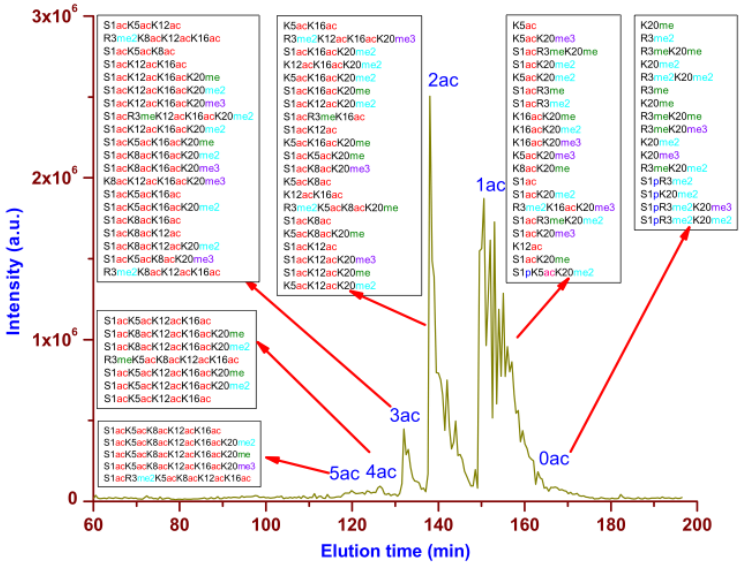

Figure 1. MS-only base-peak chromatogram reconstructed from the mass spectra acquired during the second dimension (WCX-HILIC) separation of the H4 fraction from online 2-D LC-MS analysis of HeLa core histones. Insets list histone isoforms unambiguously identified within each chromatographic peak.

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[1] Tian Z., R. Zhao, N. Tolić, R.J. Moore, D.L. Stenoien, E.W. Robinson, R.D. Smith, L Paša-Tolić. "Two-Dimensional Liquid Chromatography System for Online Top-Down Mass Spectrometry." Submitted to *PROTEOMICS*.

[2] Young NL, PA DiMaggio, MD Plazas-Mayorca, RC Baliban, CA Floudas, and BA Garcia. 2009. "High Throughput Characterization of Combinatorial Histone Codes." *Molecular & Cellular Proteomics* 8:2266-2284. DOI:10.1074/mcp.M900238-MCP200.

[3] Pesavento JJ, CR Bullock, RD LeDuc, CA Mizzen, and NL Kelleher. 2008. "Combinatorial Modification of Human Histone H4 Quantitated by Two-dimensional Liquid Chromatography Coupled with Top Down Mass Spectrometry." *The Journal of Biological Chemistry* 283:14927-14937. DOI: 10.1074/jbc.M709796200.

These technological developments have also been applied for comparative (i.e., quantitative) analysis of purified histones isolated from control and mitotic (e.g., heavily phosphorylated) normal human dermal fibroblasts. To date, we have identified 452 histone isoforms in control cells and 421 histone isoforms in mitotic cells related to H2A, H2B, and H4. Many of these isoforms show quantitative differences in control versus mitotic cells. Analysis of the H3 isoforms is in progress, and we expect to increase the total identified isoforms from each of these samples. (These initial results also indicate the urgent need for better informatics tools for top-down quantitative proteomics.) LC-MS analysis of a second biological replicate of these samples is currently in progress, and we expect this data to result in a high-quality publication. In addition, effective LC-MS platform for characterization of histones will notably enhance EMSL MS capability and help to increase the prestige of EMSL as a unique user facility, boost the User Community interest, and create a whole new realm of research opportunities.

Products and Output

New Capability for EMSL Users

Intact protein analysis capabilities developed as part of this EMSL Research and Capability Development project are now available for EMSL users.

Proposals for the Intact Protein Analysis Capability

New Proposals:

DTRA/Joint Science and Technology Office (JSTO) proposal to CBS.MEDRAD.01.10.PN.PP.017: "Biosignatures of acute radiation exposure"— David Stenoien, James Campbell, and Ljiljana Paša-Tolić

This proposal will test the hypothesis that alterations in protein levels, PTMs, and metabolic cofactors and products can serve as molecular fingerprints of radiation exposures that can accurately assess radiation doses received and highlight signaling pathways associated with acute radiation syndrome. A key component of this proposal will be to determine if combinatorial PTMs on histones and other proteins can be used to predict radiation exposure and doses received.
Status: Funded for FY 2010 and FY 2011 at $450,000 per year.

Existing Proposals:

The integrated platform will benefit the existing DOE Low Dose Radiation Scientific Focus Area (SFA), "Linear and Nonlinear Tissue Signaling Mechanisms in Response to Low Dose/Low Dose-Rate Ionizing Radiation" led by Project Manager William F. Morgan. More specifically, it will benefit the subproject, "Characterization of Signaling Events Induced by Low Dose Ionizing Radiation," led by David Stenoien, PI. A main focus of this work will be to identify radiation-dependent multisite phosphorylations and map chromatin level changes resulting in altered gene transcription, epigenetic regulation, DNA damage repair, and chromosomal instability caused by ionizing radiation.
Status: Funded through at least FY 2010.

EMSL User Proposals:

The following represents user submissions to employ EMSL's intact protein analysis capabilities:

- **Proposal 30404:** "DNA methylation-dependent patterns of histone post-translational modification"—Craig S. Pikaard (PI)
- **Proposal 30441:** "Integrated top-down and bottom-up proteomics strategy to identify multisite and multistep phosphorylation"—David L. Stenoien (PI)
- **Proposal 30509:** "Analysis of trout sperm nuclear proteins"—Irvin R. Schultz
- **Proposal 30455:** "The proteome of prostate cancer-derived exosomes: Exosomes as biomarker treasure chests"—Guido Jenster (PI)

- **Proposal 34696:** "Exploring the Boundaries of Metazoan Thermotolerance at Hydrothermal Vents: Respiration and Protein Expression of Paralvinellid Worms"—Peter R. Girguis (PI)
- **Proposal 34940:** "Targeted identification of epigenetic determinants of T cell memory"—Heather S. Smallwood (PI).
- **Proposal 39923:** Towards Complete Proteome Coverage and Identification of the Entire Protein Coding Potential of the Model Pathogen *Bartonella henselae*"—Christian H.-B. Ahrens (PI)
- **Proposal 40022:** "Understanding Biomass Conversion within Thermophilic Communities and Isolates"—Steven W. Singer (PI)
- **Proposal 40090:** "Role of Histone Modifications in Gene Expression and DNA Replication"—Scott D. Michaels (PI)
- **Proposal 40132:** Looking for Partners in Crime: Identification of Protein Complexes and Post-Translational Modifications Associated with Drug-Resistance in Breast Cancer" "—Arzu Umar (PI)

Publications

Tian Z., R. Zhao, N. Tolić, R.J. Moore, D.L. Stenoien, E.W. Robinson, R.D. Smith, L Paša-Tolić. Accepted. "Two-Dimensional Liquid Chromatography System for Online Top-Down Mass Spectrometry." Invited submission to *PROTEOMICS.*

Presentations

Tian Z., R. Zhao, N. Tolić, R.J. Moore, D.L. Stenoien, E.W. Robinson, R.D. Smith, and L. Paša-Tolić. 2010. "Top-down Characterization of Core Histones using an Online Two-Dimensional Liquid Chromatography System." presented at the *58th ASMS Conference on Mass Spectrometry*, May 23-27, 2010, Salt Lake City, Utah.

Tian Z., R. Zhao, N. Tolić, R.J. Moore, D.L. Stenoien, E.W. Robinson, R.D. Smith, and L. Paša-Tolić. 2010. "Online Two-Dimensional Liquid Chromatography Characterization of Histones using Top-Down Mass Spectrometry." Presented at the *34th International Symposium on High Performance Liquid Separations and Related Separations (HPLC 2010)*, June 19-24, 2010, Boston, Massachusetts.

L. Paša-Tolić. 2010. "Decoding the Histone Code Using Top-Down Mass Spectrometry." presented at the *4th Mass Spectrometry in Biotechnology and Medicine (MSBM) Summer School*, July 4-10, 2010, Dubrovnik, Croatia.