B.1

Elucidating Methyltransferase Target Amino Acid Residues and Downstream Modification Events by Quantitative Mass Spectrometry

S. M. Eliuk 1, F. Chu 3, B. Panning 2, and A. L. Burlingame 1

1 Mass Spectrometry Facility, Department of Pharmaceutical Chemistry and 2 Department of Biochemistry & Biophysics, University of California, San Francisco, CA; 3 College of Life Sciences and Agriculture, University of New Hampshire, Durham, NH

Introduction: Post-translational modifications of histones are used to regulate DNA-chromatin interactions and ultimately gene expression. The various individual histone PTMs do not regulate in isolation. Instead, combinations of modifications are believed to act together to create a ‘histone code’. Knocking out a specific methyltransferase permits the investigation of its target amino acid residues and how these modifications affect further downstream modification events. Combined with stable isotope labeling of amino acids in cell culture (SILAC), changes in stoichiometries of modification site occupancies are revealed by quantitative mass spectrometry. Through these techniques, we show that knock out of a specific methyltransferase leads to a variety of histone modification changes.

Method: Wild type mouse embryonic stem cells (ESCs) were grown under normal conditions and ESCs mutant for eed, a core component of the histone H3 lysine 27 (H3K27) methyltransferase complex, were grown in SILAC ESC medium. Cells were combined, histones were acid extracted and subsequently fractionated by reverse-phase chromatography. Both pre-fractionated and unfractonated proteins were subjected to digestion with both trypsin and AspN and analyzed by LC ESI MS/MS on a LTQ Orbitrap with ETD fragmentation (fragment ions detected in the Orbitrap). Peptide data were analyzed using two in-house softwares, Protein Prospector and FAVA, allowing assignment of modification sites from fragmentation data and changing stoichiometries between wild-type and mutant cells through quantitative analysis based on the SILAC labeling.

Preliminary Data: As expected, H3K27 methylation was detected in the wild-type sample and absent in the eed mutant sample. In addition, although some modifications were unaffected, we identified other modifications whose abundances were altered in the eed mutant sample. ETD fragmentation with fragment ion detection in the Orbitrap of AspN digested histones was sufficiently sensitive to enable site-specific assignment of modifications on a chromatographic time scale. The online LC separation of the unfractonated histone AspN digests resolved H4 N-terminal tails with varying numbers of acetylations, but not methylations and enabled relative quantitation of differentially modified species. An increase in the level of acetylated species with K16 acetylation in the mutant cell lines. Support for this research was provided by the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF (A. L. Burlingame, Director) through the Biomedical Research Technology Program of the NIH National Center for Research Resources, NIH Grants NCRR P41RR001614 and S10 RR019934.

This is an open access article under the CC BY license.

B.2

Ecotoxicoproteomics to Study Microcystin-LR Effects in Medaka Fish

M. Edery, M. Malécot, K. Mezhoud, A. Marie, D. Praseuth, and S. Puiseux-Dao

Muséum national d’Histoire naturelle, Paris, France

Microcystins (MCs) are hepatotoxins with a potent inhibitor activity of protein phosphatases PP1 and PP2A. These nonribosomal peptides are getting more and more attention due to their acute toxicity and potent tumorpromoting activity. These toxins are produced by freshwater cyanobacteria. To date, the detailed mechanisms underlying the toxicity of microcystins are unknown. MC-leucine-arginine (MC-LR) is the most toxic and the most commonly encountered variant of MCs in aquatic environment. It has been used for toxicological investigations on the liver of an aquatic animal model; the medaka fish. We performed differential proteome analyses of MC-LR-treated (by 2 exposure routes; water or food contamination) and untreated medaka fish in order to investigate the mechanisms of establishment of early responses to the toxin. Cytosolic, membrane and organelle proteins from livers of exposed or non-exposed medaka were resolved by 2D electrophoresis and detected using stains specific for phosphoproteins and for whole protein content. Overall, more than 100 spots were found to vary significantly on the proteomic 2D maps or on the phosphoproteomic 2D maps. Of these, 32 proteins could be identified by mass spectrometry. Among them, phenylalanine hydroxylase, keratin 18 (type I) and grp78 showed variations in phosphoryl content in agreement with inhibition of PP2A activity after exposure of the fish to MC-LR. The other identified proteins exhibited variations in their expression level. The identified proteins appear to be involved in cytoskeleton assembly, cell signalling, oxidative stress and apoptosis. The methodology described in this report should be widely used to a number of tissues and organisms, thus helping in the search for biomarkers of MC-LR contamination.
Apoptosis is a genetically controlled process in which cells undergo self-destruction. Under normal conditions apoptosis eliminates damaged cells. However, many cancer cells escape apoptosis, allowing tumors to survive and grow. Apoptosis is triggered by two major pathways: the extrinsic pathway in response to cell stress such as DNA damage, and the intrinsic pathway in response to the activation of death receptors (DR4 & DR5) by death ligands such as Apo2L/TRAIL.

Upon stimulation with Apo2L, DR4 & DR5 trigger apoptosis through the formation of the DISC which recruits the initiator caspase-8 (casp-8). The DISC mediates self-processing of casp-8 into its activated form which is then released to the cytoplasm and activates the effector casp-3 & 7, leading to cell destruction. While DISC formation initiates casp8 stimulation, full activation of this protease requires further molecular aggregation events which are not fully understood. Here we describe the utilization of mass spectrometry (MS) in the analysis of components of the DISC leading to a model of casp-8 ubiquitination and activation in Apo2L-induced apoptosis.

Method: For the identification of the DISC components, H460 cells were treated with Apo2L at 4 °C for 2 h, the DISC was isolated by size exclusion chromatography (SEC) and was purified with monoclonal antibodies (MAbs) against DR4 & DR5. The integrity of the DISC was confirmed by casp8 Western blot. Proteins were eluted by boiling in 1X SDS sample buffer containing 10 mM DTT at 95 °C for 510 min, followed by alkylation with iodoacetamide at RT for 20 min. Proteins were separated on precast 420% Trisglycine gel. Protein bands were visualized by Coomasie Blue R250 and were excised for in gel tryptic digestion at 37 °C overnight. For ubiquitination mapping of casp-8, T7 tagged version of casp-8 was expressed in 293S cells cotransfected with Cullin-3 (CUL3) and ubiquitin (Ub). Lysate was immunoprecipitated (IP) with T7 Ab. Protein was eluted using 50:50 of acetonitrile: 0.1% TFA at RT for 5 min followed by insolubilization tryptic digestion at 37 °C overnight. Peptide mixtures were separated on the nanoAquity UPLC and detected on the LTQ-Orbitrap (Fisher Thermo, San Jose) using a top 10 data-dependent mode analysis.

Result: Stimulation of H460 cells with Apo2L resulted in the DISC which eluted at around 800 KDa in SEC. Isolation of the complex using SEC prior to IP eliminated the high abundant background proteins which allowed us to better identify the components of the DISC. MS analysis revealed several known DISC components such as Apo2L, DR4, DR5, FADD, casp-8 & 10, FLIP and E3-ubiquitin ligase subunit cullin 3. When 293S cells were transfected with different plasmids express casp-8, CUL3 and Ub, it was noticed that CUL3 enhanced casp-8 ubiquitination. Co-overexpression of CUL3 with wild type Ub further increased casp-8 ubiquitination. However, mutant Ub where all Lys residues were substituted with Arg did not give the same effect, suggesting that CUL3 promoted polyubiquitination of casp-8. Ubiquitination mapping identified K461 of casp-8 was ubiquitinated.

Based on this result it was proposed that upon Apo2L stimulation, the DISC was formed and CUL3 was recruited to the complex. CUL3-mediated ubiquitination of casp-8 might be responsible for the clustering and aggregation of the complex and full activation of casp-8. Activated casp-8 then activated effector casp-3 & 7 leading to apoptosis.

References

B.3 Analysis of the Death Inducing Signaling Complex (DISC) Using Mass Spectrometry

V. Pham, Z. Jin, R. Pitti, Y. Li, D. Lawrence, A. Ashkenazi, and J. Lill

Protein Chemistry Department, Genentech Inc., South San Francisco, CA

B.4 Proteome-scale Identification of Mitochondrial Transit Peptide Cleavage Sites

E. Crawford1, and J. A. Wells1,2

Departments of 1Pharmaceutical Chemistry and 2Cellular and Molecular Pharmacology, University of California, San Francisco, CA

We have used an N-terminus-specific labeling method to identify over 100 sites of mitochondrial transit peptide removal in human proteins. Until recently, proteome-scale study of proteolysis events was confounded by the lack of a robust labeling technology that could enrich for cleavage products from the profusion of proteins in the cell. Our lab developed a technology to overcome this problem. The engineered enzyme subtiligase ligates biotinylated peptide esters onto protein N-termini liberated by proteolysis. Once labeled, the cleavage products are readily extracted and identified by mass spectrometry. Studies of four human cell lines have revealed over 2,000 native N-termini. Over 100 of these (65% of which were previously unidentified) represent cleavages that occur at unique sites between positions 5 and 120 in mitochondrial proteins, making them likely mitochondrial transit peptide cleavage sites. The prediction program TargetP correctly predicts only 30% of these sites. This work represents a significant increase in the number of mitochondrial transit peptide cleavage sites known, and will lead to an improved description of cleavage site recognition and to improved prediction methods.

References
Under salt stress condition, plants respond by turning on phosphorylating cascade. Membrane is the site where key phosphorylation events occur. Nevertheless, so far limited phosphorylation sites in response to salt stress in plants have been reported. In the present work, the salt stress-stimulated protein phosphorylation of membrane proteins in Arabidopsis was investigated. Membrane fractions from Control, 200 mM or 400 mM salt-treated Arabidopsis suspension plants were isolated, followed by protease shaving with the assistance of 60% methanol and enrichment by zirconium ion-charged magnetic beads. Through the organic solvent promotion, the membrane proteins were prone to be dissolved in this aqueous-organic solvent system and facilitated to the downstream trypsin digestion. The phosphopeptides from different samples were enriched by Zr4+/H11001-IMAC (immobilized metal ion affinity chromatography) magnetic beads in parallel within five minutes. Through this simple procedure, we identified 17 phosphopeptides from 15 membrane proteins in Arabidopsis. These proteins include a sugar transporter, AHA1, and aquaporins. A phosphorylation site of AHA1 was identified in 200 mM salt-treated plants. A phosphorylation site of a sugar transporter was identified both in 200 and 400 mM salt-treated plants. In this study, we successfully identified phosphorylation sites from membranes of specific abiotic-stressed plants by organic solvent-assisted trypsin digestion followed by Zr4+/IMAC enrichment. In addition, we also identified distinct phosphorylation sites between different salt stress conditions. Further study such as more comprehensive mapping of phosphorylation sites and quantitative analysis of protein phosphorylation with and without salt stress are in progress.
B.7

Quantitative 2D-Gel Analysis of Isotope Encoded Proteins Using SERRS Detection

G. Knudsen¹, B. M. Davis², S. K. Deb², Y. Loethen², R. Gudihal³, P. Perera², D. BenAmotz², and V. J. Davisson²

¹Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California San Francisco, CA; ²Purdue University, West Lafayette, IN; ³Agilent Technologies India Pvt. Ltd., New Delhi, India

A new strategy for quantification of proteins in biological mixtures using comparative 2D gel electrophoresis and in gel detection by surface-enhanced resonance Raman (SERRS) is presented. Proteomic samples from HCT 116 human cancer cellular lysates were isotope-encoded by treating with Rhodamine-6G (R6G) lysine-labeling reagents. Isoform mixtures of human GMP synthetase were spiked into samples at controlled levels to assess the accuracy of optical imaging methods to quantify small differences in the isoform populations. Post-separation, dye labeled protein concentrations were estimated by water-referenced fluorescence spectral imaging and protein compositions were estimated from averaged comparisons between gels. The gel embedded proteins were subsequently stained by silver nanoparticle deposition and then imaged by Raman spectroscopy. Quantification of the isotope-edited Raman spectra for the R6G labeled proteins in single gels is used to determine the relative concentrations of protein isoforms. The results represent a prototype for future application of surface enhanced isotope-edited optical spectroscopy to comparative proteomics, as an alternative to mass spectrometric methods for quantifying changes in the distribution of low abundance protein isoforms.

This work was supported by NCI F32-CA123662 (GMK), GM067195-04 and GM053155-10 (VJD), NSF CHE 0455968 (DBA, PP), and GAANN Fellowship U.S. Dept. of Education (BMD).

B.8

Global Analysis of Caspase Proteolysis During Inflammation

N. Agard¹ and J. A. Wells¹,²

Departments of ¹Pharmaceutical Chemistry and ²Cellular and Molecular Pharmacology, University of California, San Francisco, CA

Proteolysis is a prevalent post-translational modification, regulating cell-signaling pathways including apoptosis and inflammation. Misregulation of these pathways has been linked to diverse human diseases including cancer, infection, and inflammatory diseases. However, a molecular connection between proteolytic processing and disease progression is limited by our inability to link proteases to their substrates.

The caspases (cysteine aspartyl proteases) are conserved dimeric proteases known to play essential roles in apoptosis and inflammation. In their best-known functions, initiator and executioner caspases cleave hundreds of identified substrates to induce the complex physiological responses associated with apoptotic cell death. By contrast the inflammatory caspases (Caspases-1, -4, and -5) have only a few known substrates including very important regulatory cytokines (ILb and IL18). Both biochemical and cellbiological data suggest that many additional substrates should exist. To identify these substrates we have pursued a proteomic strategy based on enzymatic tagging of free N-termini.

Identification of inflammatory caspase targets can be performed in three steps: 1) selective labeling of proteolyzed proteins; 2) isolation of tagged proteins from uncleaved proteins; 3) MS identification. Towards this end, we have developed an engineered enzyme, termed subtiligase, that selectively biotinylates free N-termini of proteins. The biotinylated proteins can then be isolated and identified. Although all endogenous proteins have an N-terminal amino acid, ~80% of eukaryotic proteins are N-terminally acetylated reducing the background of unproteolyzed proteins, and enhancing our ability to detect new proteolytic events.

The application of subtiligase to lysates pretreated with caspase-1 has identified 70 caspase substrates more than half of which have not been identified previously. The sites of cleavage are consistent with previously reported primary sequence specificities of caspase-1, and we identify ~40% of previously validated caspase-1 substrates. A small number of these substrates have been identified after cellular activation of caspase-1 mixed with apparent apoptotic caspase substrates, consistent with previous reports of caspase-1 activating caspase-7.
Two Mass Spectrometry Based Approaches for the Investigation of the Heparin Interactome

A. Ori, M. C. Wilkinson, and D. G. Fernig
University of Liverpool, United Kingdom

Heparan sulfate proteoglycans (HSPGs) are ubiquitous component of cell surface and extracellular space in metazoans. By interacting with a vast number of protein partners, more than two hundred in humans, HSPGs participate in molecular networks regulating complex biological phenomena such as development, immune response and disease.

Here, we present two approaches for the identification of new HS/heparinbinding proteins and for the localisation of heparinbinding sites (HBSs) on protein surfaces using MS-based techniques. First, the plasma membrane proteome isolated from rat liver was fractionated by heparin-affinity chromatography. Low-, medium-, and high-affinity sub-proteomes, as defined by the ionic-strength required for their elution, were characterised by filterassisted sample preparation (FASP) and LCQ-TOF mass spectrometry. Second, a strategy for the selective labelling of residues involved in heparin binding was devised. We demonstrated that the presence of heparin can protect against chemical modification amino acids involved in the interaction and that HBS residues can be selectively labelled after dissociation of the protein from the sugar chain. The identification of labelled peptides from well-characterized heparin-binding proteins by tandem mass spectrometry revealed the ability of this new technique to map low- and high-affinity HBSs. These two complementary approaches can shed light on new functional roles of HSPGs and ease the structural investigation of glycosaminoglycans-protein interactions.

References


Electron Transfer Dissociation Analysis of the Urinary Peptidome in Pregnancy

S. Hart¹, R. Blankley¹, and S. Guan²

¹University of Manchester, Manchester Interdisciplinary Biocentre, Manchester, United Kingdom; ²Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA

Introduction: The urinary peptidome has strong promise in clinical studies as an abundant, accessible biological sample. Many published studies rely upon MALDI-ToF profiling to compare normal samples with those taken during disease, without characterisation of the sequences of the large, non-specifically-proteolysed peptides. Electron transfer dissociation offers an effective mechanism to determine the sequence of large, highly-charged peptides [1], and where high-resolution, high-efficiency ion/ion reaction is possible, extended sequence tags can be generated for long polypeptides [2]. We propose the use of ETD to complement CID in urinary peptidomics. This work forms part of an ongoing study to investigate the causes of preeclampsia and establish a viable panel of biomarkers predicting development of this lifethreatening condition.

Methods: Urine samples were obtained with consent from pregnant women at 15 and 20 weeks gestation. Proteins (>10kDa) were segregated from low molecular weight components, including peptides and bile salts, using a 10kDa nominal molecular weight cut-off centrifugal filter. Peptides were purified from other low molecular weight components of the raw urine filtrate using solid-phase extraction. Briefly, this involved three stages: an initial reversed-phase (RP) step to pre-concentrate urinary peptides from bulk volume, a strong cation exchange step at low pH to separate peptides and bile salts, and a final RP polishing step [3]. Peptides were then subjected to RP HPLC separation, coupled online to a hybrid linear ion trap-orbitrap with electron transfer dissociation capability. Product ion spectra were collected for each selected precursor using both ETD and CAD.

Preliminary data: ETD measurements were found to yield sufficient product ion spectral information to generate confident identifications for highly-charged precursor ions, bearing up to eight protons, on a chromatographic timescale. CID measurements yielded identification information for shorter polypeptides bearing lower charge, Indiana, USA agreement with our previous studies on tryptic and Lys-C peptides [4]. High-resolution ETD measurements, whilst having reduced sensitivity in comparison to similar measurements made using the linear ion trap, provide additional certainty as to both the charge state of products and their odd- vs. even-electron nature, which varies with both fragment length and sequence composition. For larger precursors, high-resolution ETD yields the most confident identification data, and therefore represents the most effective method currently available to characterise such peptidomes without performing proteolytic digestion. Database searching in the absence of predicted cleavage sites is significantly more effective where high mass accuracy data are available. Datasets such as this will also provide valuable information as to the dissociation behaviour of non-tryptic peptides using different fragmentation methods, which can provide insight as to their underlying mechanisms.

Support for this research was provided by the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF (A. L. Burlingame, Director) through the Biomedical Research Technology Program of the NIH National Center for Research Resources, NIH grants SC10 RR018906, SC10 RR019934 and by a grant from the Engineering and Physical Sciences Research Council of the United Kingdom, EPSRC grant EP/E043143/1.

References

Non-Targeted Characterization of Glycopeptides in Lysosomal Storage Disease Samples

C. Dorschel¹, R. R. Sprenger², J. M. F. G. Aerts³, J. P. C. Vissers¹, and S. J. Geromanos¹

¹Waters Corporation, Milford, MA; ²University of Southern Denmark, Odense, Denmark; ³University of Amsterdam, The Netherlands

Introduction: The post-translational glycosylation of proteins can have a profound effect on their biological function and structure. Hence the ability to identify and characterize glycosylated peptides in systems samples (protein digests) with minimal manipulation of the starting material is advantageous in systems biology. Here we apply a strategy to target glycopeptides in a discovery experiment by observing characteristic fragment ions in the elevated energy channel time-aligned to eluting glycopeptides derived from plasma samples for controls (n = 8) and Gaucher disease patients pre and post-treatment (n = 12).

Methods: Samples were reduced, alkylated and digested with trypsin following standard procedures. Data were acquired on a nanoACQUITY system using gradient elution of peptides on a C18 column coupled to the nano electrospray source of a Synapt HDMS hybrid quadrupole/time of flight mass spectrometer operated in data independent, alternate scanning mode (MS2). Data were processed in ProteinLynx Global Server version 2.4 with glycopeptide detection enabled. Glycopeptide identification is based on the observation of target oxonium ions in the elevated energy channel, the mass difference between the observed precursor and the candidate peptide corresponding to a reasonable combination of carbohydrate ions, the observation of the glycopeptide Y1 ion (peptide + HexNAc) in elevated energy, and, for N-glycans, the presence of the Nxs/T sequence motif.

Results: Prior efforts to identify glycopeptides in complex mixtures have relied on data dependent schemes. The present work uses the same data independent scheme used to identify ordinary tryptic peptides in digest mixtures to identify glycopeptides as well, without any additional sample preparation or data acquisition.

The identified glycopeptides in the samples were tabulated according to chromatographic retention time and intensity, and subjected to hierarchical clustering. The result of the clustering indicated that the identified glycopeptides provided sufficient differentiation to separate the data according to membership in the control, pre-treatment, Oregon, USA, post-treatment groups, but with data from severely affected patients grouping as outliers. This suggests that glycosylation patterns may offer a means to track severity of disease. The data also show the presence of multiple glycoforms for many of the glycopeptides which show that the extent of glycosylation on these peptides is affected by the presence of disease and whether or not the disease is treated.

Further analysis of these results and consideration of clinical implications will be incorporated in the final presentation.
**B.13**

**Peak Extraction and Deisotoping of Low-Level MSMS Spectra from Quadrupole/TOF Instruments**

**J. Chen**, J. Trinidad, S. Guan, and A. L. Burlingame

Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA

Acquisition of LCMSMS spectra from quadrupole/time-of-flight (QTOF) instruments allows for protein identification, quantification, and PTM site assignment in a proteome scale. Although thousands and perhaps tens of thousands of peptides can be identified from a multi-dimension LCMSMS experiment, by use of database search, precise site assignment for many posttranslational modifications (PTM), especially for labile modification such as phosphorylation remains challenging. Modified peptides in general are of low stoichiometry and their MSMS peaks have low intensity. MSMS spectra of multiply charged peptide precursor ions contain singly and multiply charged product ions. Current and past data processing algorithms of high throughput mass spectrometry data have largely focused on the interpretation of mass spectra with high intensity. The effective analysis of lower signal tandem mass spectrometry data, however, is crucial to accurately determining accurate sites of PTMs such as phosphorylation. In this work, we extract spectral characteristics by performing statistical analysis of the identified ions in those low-level MSMS spectra. New algorithms are developed to differentiate low-level signal peaks from noise using “rules” learned from the statistical analysis and to determine the charge state, monoisotopic m/z of signal peaks and to remove other isotope peaks from the spectra. The resulting, simplified data is then used to accurately assign sites of PTMs.

Support for this research was provided by the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF (A. L. Burlingame, Director) through the Biomedical Research Technology Program of the NIH National Center for Research Resources, NIH grants NCRR P41RR001614.

*B.14*

**Network Analysis of Gcn5 HAT Complexes Refines the Organizational Makeup of the SAGA, SliK (SALSA) and ADA Complexes**

**M. Sardiu**, K. Lee, S. Swanson, J. Workman, L. Florens, and M. P. Washburn

Stowers Institute for Medical Research, Kansas City, Missouri, USA

A significant number of proteins perform their functions in conjunction with other proteins by forming distinct complexes which are responsible for specific processes in a cell. Understanding how protein associate into stable protein complexes is a pivotal part of understanding cellular activity. Here, we employed a combination of computational approaches and a systematic collection of quantitative proteomics data from wild-type and deletion strain purifications in order to decipher the relationships between individual proteins and to perform a comparative structure-function analysis of a complex. We applied this approach to a data set generated from components of the Saccharomyces cerevisiae SAGA (Spt-Ada-Gcn5) histone acetyltransferase (HAT) complex, which consists of distinct domains and functional modules. To group the proteins of the complex based on its abundance patterns, we performed hierarchical cluster analysis on the wild-type and deletion strain purifications. Perturbation of the complex by genetic deletion of several subunits resulted in the dissociation of the Gcn5 HAT complexes into five modules: (1) CORE; (2) DeUB; (3) ADA; and two potential novel modules. Furthermore, we could show that Sgf29, a protein of unknown function, formed a subcomplex with ADA. Strikingly, Sgf29 exhibits similar abundance levels and a comparable phenotype after deletion as other members of the ADA subcomplex, indicating that our approach is able to predict the function of novel proteins within a complex. We also explored the functional role of several interactions within the DeUB module.

In summary, we could show by our integrative study that quantitative proteomics analysis of genetic deletion strains provides valuable insights in both structure and function of a multiprotein complex.
B.15

Quantitative Label-free Analysis of Complex Protein Mixtures through the MFPaQ Software

E. Mouton Barbosa, D. Bouyssié, A. Gonzalez de Peredo, F. Roux-Dalvai, O. Burlet-Schiltz, and B. Monsarrat

Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, Toulouse, France

While nanoLC-MS/MS has become state of the art methodology for systematic identification of large numbers of proteins in complex samples, current methods for quantitative analysis using this approach still largely rely on isotopic labeling and have several shortcomings for clinical studies on biological fluids. Label-free approaches, based on direct comparison of the MS signal intensity of tryptic peptides across different runs, appear to be better suited to the analysis of a large set of clinical samples. However, the comparison of multiple MS runs of highly complex biological samples requires highly reproducible and resolutive systems, as well as dedicated bioinformatic tools. Here, we evaluated a quantitative proteomic workflow based on nanoLC-MS/MS analysis of complex mixtures on an Ultimate3000 Dionex system coupled to an LTQ-Orbitrap-XL mass spectrometer, and bioinformatic processing of data with the new labelfree quantitative module of the MFPaQ v4 software. This module uses an identity-based method, Indiana, USA which the precursor ion m/z and retention time (RT) of each identified peptide are extracted from MS/MS database search result files, and used as a starting coordinate to extract the peptide elution peak in the MS survey scans. We present the performances of this quantitative approach in terms of reproducibility, linearity, and number of quantified proteins for complex protein samples.

Conversely to pattern-based strategies in which LC-MS features have to be defined from the analysis of peptide elution and isotopic profiles in LC-MS maps, the approach used in MFPaQ, based on extracted ion chromatograms (XIC) of identified peptides, is driven by experimentally measured RT and by monoisotopic m/z values validated from MS/MS sequencing. Thus, it allows to perform peak detection in a robust an accurate way. A drawback of this method, however, is that only identified peptides can be quantified. For analysis of highly complex peptide mixtures, Mississippi, USA/MS undersampling thus limits the number of identified and quantified proteins. To circumvent this problem, we implemented an approach based on the extraction of MS signals in individual runs using a previously generated MS/MS identification database containing m/z and RT values associated with peptide sequences. In that way, a large number of additional, nonsequenced peptides MS signals can be extracted by the software. Using this strategy, more than 1300 proteins could be efficiently quantified with good accuracy in replicate injections of a highly complex cellular digest.

B.16

Pigment Epithelium-derived Factor: A Biomarker of Preeclampsia with a Role in the Pathogenesis of the Syndrome

K. Williams1, Y. Zhou1, and S. Fisher2

Departments 1 of Obstetrics, Gynecology and Reproductive Sciences and 2 of Cell and Tissue Biology, University of California, San Francisco, CA

During human pregnancy, the placenta, a transient organ that ultimately grows to the size of the liver, is inserted into the maternal circulation. The requisite vascular connections to the uterine vessels are formed by a unique population of placental cells, derived from the embryo, termed cytotrophoblasts. They have tumor-like invasive properties, evade maternal immune surveillance, and line the arterioles that deliver uterine blood to placental chorionic villi. The villi are covered by fused cytotrophoblasts, syncytiotrophoblasts, that line the intervillus space where maternal blood flows and transport substances to and from the embryo/fetus. Preeclampsia (PE), a pregnancy complication that is characterized by the new onset of maternal high blood pressure, proteinuria, and edema, is associated with a failure in the vascular component of cytotrophoblast invasion and placental hypoxia, which is thought to lead to the maternal endothelial dysfunction that is the hallmark of this syndrome. The molecular mechanisms that link underperfusion of the placenta to the maternal signs of preeclampsia include improper differentiation of invasive cytotrophoblasts, which leads to their dysregulated secretion of pro- and antiangiogenic factors.

We conducted a pilot study to determine if we could use maternal serum and an isobaric mass tagging strategy to identify biomarkers that are beacons of sPE. In this study, we analyzed depleted serum from women with sPE as defined ACOG and women with uneventful pregnancies and normal deliveries at term. LC MS/MS analyses of these samples revealed 10 proteins with different relative abundances in the two sample sets. APOA-4 concentrations dropped along with anti-thrombin-III, which circulates at lower levels as the clinical signs of this pregnancy complication become more severe. Pigment epithelium derived factor (PEDF), a hypoxia-inducible powerful VEGF inhibitor, was found in higher relative abundance in serum in 5/6 PE patients as compared to controls. These data were validated by antibody-based analyses of placental expression of PEDF. ELISA assays showed that smaller differences in protein abundances (cases vs. controls) were evident at 20 weeks of gestation, and significant differences at time of disease were found at early-onset PE vs. control. Immunolocalization showed that PEDF expression was higher in the chorionic villi of placentas obtained from PE patients. In vivo, PEDF inhibited cytotrophoblast differentiation / invasion, phenocopying PE effects on the placenta.

The complexity of the blood proteome in pregnancy, a portion of which is made up of placental proteins, can be exploited to identify circulating biomarkers that are sentinels of relevant cells such as placental trophoblasts and components of the maternal vasculature. We used information obtained from unbiased approaches to discover biomarkers such as PEDF that have a high degree of relevance to the pathophysiology of PE, expanding the current set of placental-derived molecules with vasculo-angiogenic functions that are also candidate PE biomarkers.
Kidneys in hibernators demonstrate remarkable functional plasticity in a seasonal fashion that is not found in non-hibernators. During the summer, animals capable of hibernation are homeothermic with body temperature (Tb) of ~37°C and feeding cycles like other mammals, but in winter they fast and transition into a heterothermic state in which Tb is maintained at as low as ~2.9°C for five to 20 days at a time. These bouts of torpor are concomitant with extreme reductions in heart, metabolic and respiratory rates, and are only interrupted by relatively brief (~11 hr) sojourns at euthermic Tb throughout winter. Each of these interbout arousals is characterized by a natural state of hypoxia during torpor when demand is also reduced, and oxidative reperfusion during rewarming, but no measurable tissue damage is accrued as is typically seen in non-hibernators. Such oxidative damage is prevalent in human kidney transplant recipients and results in severely reduced organ function. In addition, ground squirrels like other hibernators establish a closed system in which nitrogen is conserved and the accumulation of urea is greatly reduced or abolished. The biochemical mechanisms for this conservation and reuse of nitrogen remain to be elucidated. Knowledge of these mechanisms that provide protection during hibernation and by which the animals successfully maintain a closed system will inform approaches to organ cold storage and transplant and are likely to improve outcomes.

A quantitative DIGE approach was used to compare kidney proteins taken at several timepoints of the circannual cycle: early summer, later in summer, entrance into torpor, late in torpor, arousing from torpor and interbout aroused, with a sample size of five or six animals for each stage. Protein samples separated by 2D gel were compared and quantified using DeCyder 7.0 and spot data were exported for statistical analysis in R. Significant differences were determined using ANOVA and BH multiple test correction. Spots with q<0.05, Tukey pairwise p < 0.05, and fold change greater than 1.8 were picked for identification by LC-MS/MS. Functional implications of steady-state hibernator kidney protein level alterations will be discussed.