Mammalian Histidine Kinase and Phosphohistidine Detection

P. Besant, X. Zu, and P. Attwood
University of Western Australia, Perth, Australia

An increase in the incidence of histidine phosphorylation of histone H4 in mammalian cells appears to be causally associated with an increase in cellular proliferation (1). Although this and many other reports of histidine phosphorylation in mammalian cells are documented in the literature, little is understood about their biological roles by comparison to hydroxyamino-acid kinases. Many of the reasons for this lack of understanding stem from difficulties associated with the study of the acid-labile phosphoramidate (P-N) bond of phosphohistidine. The acid-labile nature of phosphohistidine has meant that traditional kinase purification and phosphoamino acid detection methods are not applicable to this class of kinase.

With recent developments and adaptations of traditional methods, the detection and assay of histidine kinases is now slowly revealing some of the biological processes histidine kinases and protein histidine phosphorylation are involved in. These methods now include one and two-dimensional in-gel kinase assays of histone H4 histidine kinases and mass spectrometric methods for the detection of phosphohistidines in proteins. In order to examine the relationship between histidine kinase activity and cellular proliferation in liver tissue we have adopted many of these methods. By examining the levels of histidine kinase activity both before and after the liver has undergone various physical and biological perturbations, we hypothesize that histidine kinase activity may function as an oncodevelopmental marker (2). The next step in the process is to utilize these techniques that are geared toward the study of acid-labile phosphorylation to identify and characterize the kinases associated with increased activity in proliferating liver tissue.


Separation of Histone H3 for the Characterization of the Intact Protein and Its Modifications

C. Brinkworth, R. Talroze, S. Guan, and A. Burlingame
Mass Spectrometry Facility, University of California, San Francisco, CA

Histone 3 is an important member of the octamer core known to play significant roles in defining and stabilizing multiple levels of chromatin structure. The goal of the research reported is to develop the method for separation of H3 histone and its modifications. Bovine core histones were used as a model system. By variation of the HPLC reverse-phase column characteristics, the composition of the solvent and the flow gradient and rate we found conditions at which at least four partially overlapping peaks related to H3 were presented in the chromatogram. These peak fractions were collected and analyzed by Tris-HCl gel followed by the complete proteolysis of Comassie stained gel bands with trypsin. LC MS–MS measurements showed the presence of H3 histone in all 4 fractions. The purification of the fractions of H3 will provide sufficient amount of the intact protein and its modifications for infusion studies using ESI-FTMS.

This work was supported by the NIH (Grant: RR 01614).

Improved Sample Preparation Methods for Mass Spectrometric Characterizations of Glycosylated Proteins

W. Chen, Y. Yu, P. Lee, and J. Gebler
Waters Corporation, Milford, MA

Structural characterization of glycoproteins has been one of major objectives in biomedical research. The main obstacles in mass spectrometric (MS) analysis of glycoproteins lie in the low ionization efficiency of glycopeptides and the complicated nature of heterogeneous oligosaccharide moieties. Sample preparation procedures are crucial to the success of MS-based analysis of glycosylation. The focus of this study covers two aspects of sample preparations in mass spectrometric analysis of glycoproteins: affinity enrichment of glycopeptides and sample clean-up of glycans (oligosaccharides) after enzymatic cleavage. Published methods recommending various affinity media for glycopeptides enrichments were systematically evaluated. When applied to the model glycoproteins ribonuclease B and folate binding protein (FBP), the evaluation revealed detailed insights into the selectivity of each enrichment method. An optimized method for efficient glycopeptide enrichment was therefore developed. In the second portion of poster, a unique approach for the sample preparation of glycan analysis is presented. This approach utilized an enzyme-friendly surfactant to first denature glycoproteins, followed by enzymatic cleavage of the glycans by peptide-N-Glycosidase F. The released glycans were further processed using a micro-scale HILIC (hydrophilic interaction) SPE device to yield a sample ready for MS analysis. This method is optimized and has been demonstrated to be able to process both neutral and acidic (containing sialic acid) glycans effectively. The results of the sample processing as well as the procedure will be discussed in the poster.
Characterization of Posttranslational Modifications on Histone H2A Variants

F. Chu1,2,3, R. Chalkley1,2, D. Nusinow2, T. Fazzio3, B. Panning3, and A. Burlingame1,2

1Mass Spectrometry Facility; 2Department of Pharmaceutical Chemistry; 3Department of Biochemistry and Biophysics, University of California, San Francisco, CA

Multicellular organisms consist of many cell types all encoded by identical genetic information but displaying a high degree of differentiation, characterized by a unique pattern of gene expression for each cell type. Establishment and maintenance of these diverse expression patterns is fundamentally important for cell identity and organism survival, and aberrant gene expression in a single cell can lead to developmental abnormalities or cancer. Epigenetic regulatory mechanisms employ dynamic modifications of chromatin structure through histone methylation, acetylation, phosphorylation and ubiquitination, to regulate gene expression. These posttranslational modifications are integrated in a combinatorial fashion to provide cells with transcriptional memory to stably maintain gene expression patterns throughout many divisions, and developmental flexibility to facilitate programmed alterations in gene expression during embryogenesis and development. Though posttranslational modifications on the core histones have been under intense investigation for many years, the modifications on variant histones have not been catalogued. We developed a technique for rapid, one-step purification of large quantities of histones and histone-associated proteins.

Using tandem mass spectrometry, we have conclusively identified multiple posttranslational modifications on tagged histone H2A variants, including methylation, phosphorylation and ubiquitination, to regulate gene expression during embryogenesis and development. Though posttranslational modifications on the core histones have been under intense investigation for many years, the modifications on variant histones have not been catalogued. We developed a technique for rapid, one-step purification of large quantities of histones and histone-associated proteins.

Using tandem mass spectrometry, we have conclusively identified multiple posttranslational modifications on tagged histone H2A variants, including methylation, phosphorylation and ubiquitination, to regulate gene expression during embryogenesis and development. Though posttranslational modifications on the core histones have been under intense investigation for many years, the modifications on variant histones have not been catalogued. We developed a technique for rapid, one-step purification of large quantities of histones and histone-associated proteins.

Identification and Quantitation of Cell Wall-associated Proteins from Different Vancomycin-resistant Strains of Staphylococcus aureus

C. Gatlin, R. Pieper, S. Kuntamalla, S. Huang, H. Alami, D. Clark, E. Gebregeorgis, P. Parmar, E. Mongodin, S. Gill, and S. Peterson

The Institute for Genomic Research, Rockville, MD

The emergence of highly virulent community acquired Staphylococcus aureus and continued progression of resistance to multiple antimicrobials, marks the re-emergence of S. aureus as a serious health care threat. Investigation of proteins localized to the cell surface could help to elucidate mechanisms of virulence and antibiotic resistance in S. aureus. In this study, proteomic methods were developed to identify and quantitate proteins present in the supernatants of the lysostaphin-digested cell envelope from cultured vancomycin-intermediate S. aureus (VISA) cells. Combining approaches of two-dimensional gel electrophoresis (2-DE) or chromatographic separation of proteins with mass spectrometry (MS) analyses resulted in the identification of 142 proteins of particular interest. Of these proteins, 48 contained predicted cell wall localization or secretion signal motifs, including 13 with distinct covalent peptidoglycan-anchor sites, 3 of which are uncharacterized to date. One of the two most abundant cell envelope proteins, which showed remarkably high variations in Mr and pI in the 2-DE gel display, was the S. aureus surface protein G (SasG). The display of numerous secreted proteins that are not covalently cell wall-anchored, suggests that, in the exponential growth phase, secreted proteins can be retained physiologically in the cell envelope and may interact with cell wall-anchored proteins and carbohydrate structures in a manner yet to be determined. The remaining 94 proteins, devoid of recognizable motifs, were repeatedly profiled in the VISA cell envelope fractions. Repeated profiling suggests that some of these proteins may be naturally present in the cell compartment rather than experimentally caused by cell lysis.

Cell wall proteins from three different VISA strains of S. aureus with different susceptibilities to vancomycin (P100, MIC = 2 μg/ml; HIP827, 3 μg/ml; VP32, 32 μg/ml) were iTRAQ labeled followed by LC/LC-MALDI-TOF/TOF analysis. Many proteins were found to be up- or down-regulated in the most resistant VP32 strain compared to the other two strains. The iTRAQ results will be compared with our 2-DE and transcriptome analyses. Possible cell wall-localized functions of these differentially-expressed proteins will also be discussed.
**B.6**

**FTMS Data Analysis Tools for Characterization of Posttranslational Modifications of Intact Proteins**

S. Guan, X. Zhang, and A. Burlingame

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

Electron capture dissociation (ECD) and infrared multi-photon dissociation (IRMPD) implemented on a high field FTMS allow for direct characterization of mid-sized proteins. The methods are especially powerful for analysis of proteins containing complex posttranslational modifications (PTMs), such as histones, the major players in chromatin biology. A typical ECD/IRMPD spectrum of an intact protein may contain hundreds of multiple charged isotope clusters. The de facto method for analysis of such complex data sets is THRASH (1), which extracts monoisotopic masses and charge states from them. While THRASH is quite effective in general; it sometimes identifies incorrect monoisotopic m/z peaks (or inaccurate charge states) and reports erroneous deconvoluted masses (most frequently one Da more or less than correct masses). Large amount of effort has been spent on verification of reports from THRASH and on searching for missing fragments in our current investigation of intact histone proteins by FTMS. These samples contain only limited number of protein sequences and proteins with a single PTM content are dissociated by use of a combination of quadrupole and SWIFT isolation. Thus, there is still a need to explore and develop better tools for the data analysis. The tool developed in our laboratory generates isotope patterns from given sequences and modifications and then matches the isotope profile with possible charge states to experimental spectrum. Assignment can be carried out either manually through graphic interface or automatically. In an automated mode, a discrimination system using intensity, fitness of isotope patterns, and mass deviation from theoretical values can significantly reduce both false-positive and false-negative assignment. The data analysis tool can reduce and eventually relieve researchers from tedious and time-consuming tasks for analyzing raw spectral data.

Financial support was provided by NIH NCRR Grants RR 01614.


**B.7**

**Enhanced Sequence Coverage of the Peptide Mass Fingerprint for the Nicotinic Acetylcholine Receptor**

M. Hajivandi¹, M. Blanton², J. Leite¹, X. Liang¹, and M. Pope¹

¹Invitrogen, Carlsbad, CA; ²Texas Tech University Health Sciences Center, Lubbock, TX

Introduction: The nicotinic acetylcholine receptor (nAChR) is a paradigmatic ligand-gated ion channel that regulates signaling across the neuronal synaptic junction. Electrophysiology, in combination with site-directed mutagenesis, can be used to screen a number of potential ion-channel modulators. However, this relies on measurable functional changes whereas “silent” mutations or binding-events cannot be recorded. Mass spectrometry (MS), in combination with agonist/antagonist crosslinking, is a powerful analytical tool in mapping the protein contact points with pharmacological agents. However, the majority of ion channel modulators bind at sites that reside within hydrophobic domains which are difficult to analyze by MS. This study describes a protocol that yields a) enhanced sequence coverage, and b) detection of pharmacologically significant regions in an, otherwise intractable, integral membrane receptor.

Methods: Affinity-purified nAChR from *Torpedo pacifica* was prepared for MS analysis using several protocols. The efficacy of these protocols was determined by their ability to yield a) maximal sequence coverage and b) detection of pharmacologically relevant sites. nAChR samples were trypsinized in the presence and absence of various surfactant blends or separated by SDS-PAGE followed by trypsin digestion in the presence or absence of surfactants. Proteolysis products were separated by C18 reverse-phase nanoflow liquid chromatography (Dionex) followed by MALDI-TOF-TOF (Applied Biosystems) or LC-ESI-QTOF analysis (Waters-Micromass). MS data was analyzed by GPS Explorer (Applied Biosystems) and Mascot Distiller (Matrix Science) sequence database search software.

Preliminary Results: Identifying the precise site where pharmacologically active reagents bind to receptors is often complicated by the hydrophobic nature of these protein regions. MS analysis of integral membrane proteins is often plagued by low sequence coverage and sample recovery. The present study utilizes a parallel approach to processing nAChR samples for MS analysis. This approach relies on parallel workflows that include and omit a surfactant blend during proteolysis with trypsin. Although both workflows generate a subpopulation of peptides with overlapping sequences, each treatment generates unique peptides. The combined proteolysis product pool yields a peptide mass fingerprint with sufficiently enhanced sequence coverage that makes mapping drug binding sites amenable. On average, our combined sample preparation protocol yielded 1.5 times greater sequence coverage for nAChR than conventional “in-gel” or solution proteolysis protocols. More importantly, pharmacologically significant nAChR sequence regions were detectable by our method. Specifically, we were able to identify the region between the delta subunit amino-acids 278–297, which encompasses the pore forming structure, M2. Further, we were able to identify the region between the delta subunit amino-acids 299–311, which contains the M2-M3 loop (a region links the conformational change associated with agonist binding with channel opening). We have also been able to identify several regions of the N-terminal domain core that form the agonist binding sites in the alpha and delta subunits.
**B.8**

An Integrated Proteomics Approach to Decipher the 26 S Proteasome Interacting Networks

L. Huang, C. Guerero, and P. Kaiser

Department of Physiology and Biophysics, University of California, Irvine, CA

One of the major challenges in proteomics is the characterization and mapping of protein complex interacting networks to obtain a complete understanding of their functions. Mass spectrometry-based interaction proteomics has become the most promising technology for accomplishing such tasks. The 26 S proteasome complex is responsible for the ubiquitin-mediated degradation of many important regulatory proteins, including those involved in cell division, transcription, cell signaling and development. Ubiquitinated proteins are believed to be transferred to the 26 S proteasome by various receptor proteins prior to their degradation. Although some of the known receptors have been purified in vivo, the identification of all receptor proteins and other interacting partners has not yet been possible. Towards this goal, we have developed an integrated proteomics approach to globally identify 26 S proteasome interacting proteins. Using a quantitative strategy, specific proteasome interacting proteins can be identified and distinguished from nonspecific interactions. The integrated approach can be used to elucidate dynamic protein interaction networks under physiological conditions, which is a general approach that can be applied to the study of other macromolecular machine protein interaction networks.

---

**B.9**

Targeted Proteomic Techniques for Discovery and Post-translational Modifications Using a Hybrid-Triple Quadrupole Linear Ion Trap Mass Spectrometer

C. Hunter¹, D. Cox², S. Webb¹, and N. Morrice³

¹Applied Biosystems, Foster City, CA; ²MDS Sciex, Toronto, Ontario, Canada; ³MRC Phosphorylation Unit, Dundee, Scotland

Often, when approaching a proteomics experiment, significant information is available about the sample to enable specific biological questions to be asked. The direct combination of triple quadrupole and ion trapping capabilities, in a hybrid triple quadrupole - linear ion trap mass spectrometer, presents new opportunities for hypothesis-driven discovery and quantitation of proteins and post-translational modifications. Utilizing the specific triple quadrupole scan modes such as precursor ion scanning or multiple reaction monitoring (MRM), targeted experiments can be designed to obtain more relevant information from a sample. In addition, various chemical tags utilizing stable isotope coding have been employed to enable different types of quantitative experiments.

To measure the relative changes in protein phosphorylation between the active and inactive forms of MAP kinase-activated protein kinase 1, an N-terminal peptide labeling chemistry (iTRAQ reagents) and a precursor ion (-79) detection strategy were combined. 12 phosphorylation sites were identified and changes in the key sites of phosphorylation involved in kinase activation were characterized.

The activity of the muscle specific transcription factor family: Myocyte Enhancer Factor 2 (MEF2), is tightly controlled by phosphorylation. This modification is responsible for either an increase in transcriptional activity or a decrease, depending on the specific site of phosphorylation. Although mass spectrometry based methods such as precursor ion and neutral loss scans are extremely useful for identifying unknown phosphopeptides from a complex mixture, they do not take advantage of any prior knowledge about the protein being investigated. In this case, a significant amount of information was available for designing a targeted experiment. This included: the primary sequence, type of phosphorylation (Serine/Threonine), and predicted phosphorylation consensus sites. This information was used to predict precursor and fragment ion m/z values for an MRM based discovery experiment. By using these highly selective MRM experiments to trigger dependant product ion scans, we were able to identify low levels of phosphorylation of MEF2, and other proteins.

In summary, a combination of triple quadrupole and linear ion trap scans, hypothesis driven experiments, and iTRAQ chemistry was employed to identify and quantitate several phosphorylation sites, from proteins involved in mammalian cell signalling.
B.10 Location of Cofilin Binding Site on Actin Monomer

A. Kamal¹, J. Guan², S. Almo², and M. Chance²

¹Albert Einstein College of Education, Bronx, NY; ²Albert Einstein College of Medicine, Bronx, NY

The actin cytoskeleton plays important roles in controlling many critical biological processes including cell motility, cytokinesis, and endocytosis in eukaryotic cells. These diverse events are directed by the assembly of highly ordered macromolecular complexes of actin and actin regulatory proteins. Among the actin regulatory proteins, the cofilin/ADF family of proteins binds to both actin monomers (G-actin) and filaments (F-actin) in a pH-dependent manner. Cofilin is a member of cofilin/ADF protein family that severs actin filaments to generate free barbed ends leading to filament extension. The molecular interactions between cofilin and G- or F-actin relevant to function have been extensively studied. Various literature reports of cofilin binding sites on G-actin consistently identify the cleft between subdomains 1 and 3, where gelsolin segment 1 and profilin bind.

We use the technique of synchrotron footprinting, here to probe the binding site of cofilin in G-actin. In this approach, hydroxyl radicals are generated from millisecond exposure of aqueous solutions to unattenuated "white" synchrotron radiation resulting in stable oxidative modifications of solvent-accessible and reactive amino acid sidechains. The specific extents and sites of oxidation are quantified after proteolytic digestion using liquid chromatography (LC)-mass spectrometry (MS) and LC-MS/MS methods.

The modification rates are calculated for various peptides of G-actin in absence and presence of cofilin. The values obtained are in accordance with the number, type (specific reactivity), and solvent exposure of the reactive amino acids in the respective peptides. A number of actin peptides located in the sudomain 1 showed decreased modification rates upon cofilin binding to actin. Of particular interest are the peptides 85–95 and 96–113, which comprise a helix in the cleft between subdomains 1 and 2, and an associated loop respectively, signifying this region to be the center of cofilin binding surface. The binding surface is further extended to the front face of a lower helix (118–125), N-terminus (1–18) and C-terminus (360–372). Few peptides whose reactive probes located near the nucleotide binding cleft also showed decreased modification rates. Further, the two slantly crossed helices located in the cleft between subdomains 1 and 3, which form the two-stranded hinge centering at residues L140 and S338 are found to show increased modification rates. This hinge causes the propeller motion between the large and small domains producing an opening and closing of the nucleotide cleft. Thus, cofilin binding may induce closure of the nucleotide-binding cleft originating from a rotation around this hinge. As a result the residues near the nucleotide-binding cleft are more exposed, whereas those near the hinge are slightly solvent exposed.

Steady-state fluorescence experiments of acrylodan labeled (at Cys-374) actin revealed a ~12 nm blue shift of emission maximum in presence of cofilin suggesting that cofilin binding decreases the polarity of the fluorophore environment and is consistent with its binding surface involving the C-termini that contains Cys-374 as revealed from the footprinting data. However no change in the emission maximum is observed when actin is complexed with DNaseI, gelsolin segment 1, and profilin and is consistent with their locations not blocking the solvent access channel of Cys-374, as revealed from their crystal structures.

B.11 Conformational Changes in Akt/PKB Activation Probed by Mass Spectrometry

H. Kim and B. Huang

National Institutes of Health, Bethesda, MD

Upon growth factor stimulation, cytosolic Akt is recruited to the plasma membrane through the interaction of its PH domain with PtdIns(3,4,5)P3 generated by PI3-K. The membrane interaction results in conformational changes of Akt, allowing its activation through the phosphorylation at Thr 308 and Ser 473 in the kinase and regulatory domains, respectively. Although the partial crystal structures of Akt have been reported, neither the three-dimensional (3D) structure of the whole molecule containing PH, kinase (KD) and regulatory domains (RD) nor its conformational changes accompanying activation have been demonstrated. In this report, we probed 3D structures in solution for both inactive and active Akt by chemical cross-linking and mass spectrometry. Tandem mass spectrometry identified seven cross-linked lysine pairs, spaced either between 20 and 24Å or less than in inactive Akt. Among them, two inter-domain cross-linked pairs, K30(PH)-K389(KD) and K284(KD)-K426(RD) were no longer observed within 24Å distance constraint when Akt was fully activated by phosphorylation, although each lysine residue in these pairs was individually modified by the cross-linkers. Our results strongly suggest that inactive Akt exists as a folded structure with the PH and regulatory domains extending over parts of the kinase domain. When phosphorylated, these domains move away from the kinase domain, most probably exposing the substrate recognition sites in the kinase domain. This open conformation may be critical for the substrate access and may serve as an important determinant for its activation states in cytosol. Our results demonstrate for the first time the conformational changes of the full-length Akt structure in solution due to activation. In addition, our results provide valuable spatial distance information between domains in both inactive and active Akt molecules, which is unavailable through the existing X-ray crystallography data.
Members of the growth factor and cytokine receptor superfamily function as protein kinases during the first step for transduction of cell signaling. Over the past two decades, the epidermal growth factor (EGF) receptor has been the most studied, and appears to be closely linked to not only cell growth, but also various cancer developments. Recently, antibodies and inhibitors which target against and antagonize receptor tyrosine kinases have been available as anticancer drugs. However, difficulties remain when using these drugs due to serious side-effects. To help alleviate this situation, and develop safer and more effective drugs, it is necessary to understand the signaling networks of growth factor receptors more precisely. Here, we have conducted a large-scale proteomic analysis of functional molecules acting downstream of the EGF receptor. In brief, total proteins immunoprecipitated with a mixture of anti-phosphotyrosine antibodies from the lysates of EGF-stimulated A431 cells, which express large amounts of the EGF receptor, are separated by SDS-PAGE. Next, the whole lane was in-gel digested and peptides were analyzed by LC/MS/MS. As a result, over 150 proteins including not only well-studied ones such as the effectors of EGF signaling, but also several previously unknown proteins have been identified. In this presentation, we focus on the function of these newly identified proteins on EGF signaling. To address the issue, the following studies were carried out: i) Identification of tyrosine phosphorylation sites upon EGF-stimulation by mass spectrometry and mutational studies. ii) Production of site-specific anti-phospho antibodies. iii) Screening of interacting proteins with immuno-affinity methods. One of these proteins is a novel small adaptor protein consisting of 273 amino acid residues with a proline-arginine (PR) motif which recognizes a new class SH3 binding protein. The wild-type mRNA and a spliced variant lacking exon 8 of ASH3BP were found to be ubiquitously expressed among various culture cells and human tissues. The PR motif within its proline-rich region specifically bound to the SH3 domain of the CD2AP/CIN85 family proteins, which are known as association factors for Cbl. Although deletion and mutation studies have made it clear that ASH3BP was phosphorylated at tyrosine 204 upon EGF-stimulation, this phosphorylation had no effect on the binding of ASH3BP to CD2AP/CIN85. Since several recent studies have shown that the Cbl and CD2AP/CIN85 complex plays an important role in the down-regulation signaling of growth factor receptors, our results suggest that ASH3BP is involved in a negative receptor signaling pathway cooperating with CD2AP/CIN85.

The Bcr-Abl fusion kinase drives oncogenesis in patients with chronic myelogenous leukemia (CML) and leads to constitutive tyrosine phosphorylation of many downstream signal transduction proteins. Currently CML patients are treated with the Abl tyrosine kinase inhibitor imatinib. Although imatinib effectively treats patients in the early stages of CML, it loses its effectiveness in later stages of the disease because of amplification or mutation of the Bcr-Abl gene.

We have generated phosphotyrosine profiles for six cell lines that represent three Bcr-Abl fusion types in four distinct cell lineages using immunoaffinity purification of tyrosine phosphopeptides followed by tandem mass spectrometry. We identified 60 to 140 unique tyrosine phosphopeptides from each of these cell lines. By comparing the profiles, we found phosphotyrosine sites that are common to all six Bcr-Abl transformed cell lines. We also found consistent differences in the phosphorylation patterns of different Bcr-Abl fusions. To confirm differential phosphorylation of the three Bcr-Abl fusion kinase forms, phosphotyrosine peptides from each cell line were subjected to a targeted tandem mass spectrometry analysis of all observed Bcr-Abl phosphopeptides. This targeted strategy expands the effective dynamic range of the mass spectrometer by focusing analysis time exclusively on the phosphopeptides of interest.

To identify imatinib-responsive signaling pathways, we merged the immunoaffinity purification strategy with quantitative analysis. Untreated and imatinib-treated K562 cells were compared using Stable Isotope Labeling of Amino acids in Cell culture (SILAC). Several signature peptides are responsive to imatinib treatment, indicating that phosphorylation at these sites is in fact mediated through Bcr-Abl. This comprehensive phosphoproteomic study of the Bcr-Abl fusion protein highlights novel disease markers and suggests potential therapeutic targets for imatinib-resistant patients.
Effect of shRNA Knockdown of Protein Complex Subunits on Complex Formation and Quantitation Using SILAC Technique

J. Leite, M. Hajivandi, X. Liang, and M. Pope
Invitrogen, Carlsbad, CA

Introduction: In combination with mass spectrometry, recent advances in protein tagging and purification have made it possible to isolate and characterize native complexes in high throughput. Stable isotopic labeling in cell cultures (SILAC) has been used successfully to study the dynamics of cell signal-dependent protein-protein interactions. Our goal was to develop shRNA knockdown as an investigative means to study the role of the individual subunits on the activity and stoichiometry of complexes. Because many of these knockdown effects are anticipated to be subtle, we reasoned that conventional methods may not have sufficient dynamic range to discern small changes in protein expression. Instead we have employed metabolic labeling, SILAC, to precisely quantify stoichiometric changes in complex formation caused by shRNA knockdown perturbations.

Methods: A tagged Arp 2/3 complex subunit was expressed in mammalian cells and the tag was used to purify the protein complexes from lysates. The cell lines described above were transfected with shRNAs U6 Entry clones specifically targeting a second subunit of the same complex in medium containing 13C-Arg. Another culture was treated with lacZ shRNA U6 Entry clones in non-labeled control medium. The cultures were combined and protein complexes purified using Streptavidin agarose. The complex subunits were digested with trypsin and digested peptides were analyzed by Q-TOF.

Preliminary Results: We present a new method for the analysis of protein expression knockdown and how it may be applied to determine protein complex assembly and stoichiometry. Specifically, the marriage of two powerful technologies, shRNA and SILAC, combine to produce a method capable of detecting significant stoichiometric effects under even relatively subtle knockdown conditions. In this study, we focus on the Arp 2/3 complex assembly. Using differential expression analysis by SILAC, while the introduction of targeted shRNA serves as an experimental stimulus, we have quantified the degree of knockdown of specific complex subunits to as little as 20%. Also, we have quantified the effects of this knockdown on the stoichiometry of the affinity-purified complex. Using sequential knockdowns of select subunits coupled to SILAC analysis we have constructed a model for Arp 2/3 complex assembly.

Mesoporous TiO₂ Membranes As MALDI-Targets for Specific Detection of Phosphopeptides in Mixtures

M. Linscheid¹, A. Melikyan¹, T. Moritz², S. Kurzawaski³, and H. Schlüter³
¹Department of Chemistry, Humboldt-Universitaet zu Berlin,
²Department of Applied Analytical Development, Atotech;
³Endocrinology and Nephrology, Charité Universitätsmedizin Berlin-Campus Benjamin Franklin, Berlin, Germany

Mesoporous titanium oxide films [1] can be used for immobilization and preconcentration [2] of phosphorylated peptides in peptide mixtures. When prepared on conductive supports they may be used as MALDI targets with high selectivity for phosphorylated compounds. In contrast to other approaches we can use the targets for “on target” sample preparation. The targets have the potential for high throughput screening for phosphorylation in phosphoproteomics.

We will demonstrate here the potential of that approach using alpha-Casein, beta-Casein and bovine Fetuin. After preconcentration of the phosphopeptides from tryptic digests on target the peptides can be analyzed using MALDI TOF and AP MALDI Ion Trap MS.

The first experiments were made using a sol-gel protocol for the membranes and conductive ITO glass support. For a robust method compatible with routine inlet systems of mass spectrometers, more conductive supports made from aluminium and silicon have been manufactured and tested. Spot sizes of 1 or 0.5 mm in diameter were etched in the targets and the mesoporous films were made inside the etched spots with a thickness of typically 4 μm.

We have used this methodology to analyze the phosphorylation in fibrinogen, specifically in Fibrinopeptide A as the interactive peptide to thrombin [3, 4]. We could demonstrate that this approach is superior to the standard protocol in terms of detection limits for phosphorylation.

Thus we have shown to have developed a means to rapidly and selectively analyze phosphorylated peptides in mixtures using MALDI MS and AP MALDI MS/MS. The approach can be paralleled for high throughput.

References
**B.16**

**Methodology for Mapping and Monitoring of Phosphorylation Sites Using a Hybrid Quadrupole Linear Ion Trap Mass Spectrometer**

S. Mollah\(^1\), M. Torres\(^2\), M. Requard\(^2\), and C. Borchers\(^2\)

\(^1\)Applied Biosystems, Foster City, CA; \(^2\)Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC

Protein phosphorylation is one of the most widely studied modifications that is crucial for various biological processes such as cell signaling and cell cycle control. However, this modification is usually present at low levels which makes its identification difficult. Also, because of its dynamic nature the levels of phosphorylation recovered at a given time can vary from one sample preparation to another. For example, the sample preparation step or given time at which phosphatase inhibitors are added is crucial to the level of phosphorylation present in the final step. Presented here is a targeted LC/MS/MS approach to monitor and compare levels of phosphorylation of peptides during various cell stages and sample preparation stages. Phosphopeptides are first selectively identified from a complex mixture using precursor ion scanning. After initial identification of phosphopeptides using precursor ion scan, standard Multiple Reaction Monitoring (MRM) methods can be built to monitor or rapidly screen levels of these phosphopeptides in different cell stages. In addition, the MIDAStm workflow (MRM-Initiated Detection And Sequencing) is used for simultaneous detection, quantitation and identification of peptides. This methodology is not limited to only phosphorylation analysis but can also be applied to other post-translational modification studies.

In this example, phosphorylation on Anaphase-promoting complex 1 (Apc1) of Saccharomyces cerevisiae was studied utilizing this methodology. Apc1 is the largest subunit in the anaphase-promoting complex (APC), an E3 ubiquitin ligase that regulates the metaphase to anaphase transition and exit from mitosis in eukaryotic cells. Phosphorylation of specific APC subunits, including Apc1, is an important regulatory mechanism involved in both activation and deactivation of the APC. However, the sites of phosphorylation, their degree of modification during different cell cycle stages, and their individual contributions to APC activity remain unknown. Phosphorylation of Apc1 is particularly important because it is the only subunit known to exhibit both complex-activating and deactivating phosphorylation, though the sites responsible for these functions are also unknown. Therefore, information describing the sites of phosphorylation in Apc1 and their modification status at different cell cycle stages will improve our understanding of APC regulation through phosphorylation.

**B.17**

**Phosphorylation of Bcr-Abl Kinase and Its Downstream Substrates in Response to STI571 Treatment in Human Chronic Myelogenous Leukemia Cells**

M. Pope\(^1\), X. Liang\(^1\), M. Hajivandi\(^1\), D. Wisniewski\(^2\), B. Clarkson\(^2\), and M. Resh\(^2\)

\(^1\)Invitrogen, Carlsbad, CA; \(^2\)Memorial Sloan-Kettering Cancer Center, New York, NY

Introduction: Phosphorylation regulates many aspects of cellular behavior. For the past two decades, most of the work in studying phosphorylation has relied heavily on thin-layer chromatography and procedures based upon phospho-antibody assays. Quantification of phosphorylation level changes in response to stimulation of growth factors or drug treatment remains a major obstacle. Several quantitative proteomics approaches, such as ICAT, iTRAQ, 2D-DIGE, and SILAC (Stable Isotopic Labeling by Amino Acids in Culture), have been developed to quantify differential expression of proteins in normal and disease states. Here we use the SILAC approach in human chronic myelogenous leukemia cells to quantify the change in phosphorylation of Bcr-Abl kinase and its downstream substrates in response to treatment of STI571, a specific inhibitor of Bcr-Abl kinase.

Methods: SILAC is an emerging technology for quantitative proteomics, which is based on labeling proteins with “light” and “heavy” amino acids. Here we labeled the cells with light and heavy tyrosine for a duration of six cell doublings. An equal number of cells labeled with either light or heavy tyrosine were combined before and after the heavy labeled cells were subjected to the treatment of STI571 for various times. Cell lysates were immunoprecipitated with anti-pTyr antibody or incubated with the GST-fusion protein of the SH2 domain of SHP1 to enrich phosphorylated proteins. Protein bands were in-gel digested with trypsin and the peptide extract was separated on nano-LC, followed by the analysis with MALDI-TOF-TOF. Quantitation was achieved by measuring the ratio of each isotopic phosphopeptide pair.

Preliminary Results: Based on metabolic labeling of proteins with light and heavy Tyrosine, we are able to determine the change in phosphorylation of Bcr-Abl kinase, as well as its downstream substrates SHP2 and Dok2, in response to STI571 treatment in human chronic myelogenous leukemia (CML) cells. In this study, we have identified two phosphopeptides from Bcr-Abl kinase, one from SHP2, one from Dok2. Dok2 is a substrate of Bcr-Abl kinase, which displays increased tyrosine phosphorylation levels in primary CML progenitor cells in human patients. We have shown that the phosphorylation level of this site is decreased 75% upon 1 h treatment with STI571 and was inhibited more than 90% upon treatment with STI571 for 2 h. This approach is applicable to the study of regulation of phosphorylation in response to stimuli or drug treatment in cell culture in general.
B.18

**Phosphoproteome of Rested and Active Lymphocytes**

P. Ruperez, J. Trinidad, J. Abian, and A. Burlingame

1University of California, San Francisco, CA; 2Instituto de Investigaciones Biomedicas de Barcelona, Consejo Superior de investigaciones científicas, Barcelona, Spain

The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a post-translational modification of high biological impact. While lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified and the analysis of acetylation sites in proteins is increasingly performed using mass spectrometry techniques which have some limitations and pitfalls. Especially the non-enzymatic cysteine acetylation which takes generally place in any cysteine containing peptide and protein independent from their amino acid composition and sequence can result in false-positives. The acetylation of proteins at specifi...
**B.21**

**Protein Isoform Recognition and Quantitation**

W. Tang, S. Seymour, A. Loboda, C. Hunter, and D. Schaeffer

Applied Biosystems, Foster City, CA

In a typical “bottom up” proteomics experiment, the proteins in a sample are enzymatically or chemically cleaved into smaller pieces prior to analysis by mass spectrometry. The protein assembly/grouping problem for the data gathered by this type of experiment can be stated as follows: given a list of putative identified peptides (obtained, for example, by performing a database search on each MS/MS spectrum) together with the confidences that each peptide is correct, we would like to determine which proteins are actually being detected. We present here software which intelligently performs protein assembly/grouping and, furthermore, leverages the grouping algorithm to obtain more accurate protein quantitation. The software determines the minimal number of proteins truly required to explain the identified peptides. Multiple protein isoforms are reported only if there is sufficient distinct peptide evidence. This approach makes use of peptide confidences to detect true isoforms while preventing the false detection of isoforms based on weak evidence. The quantitation portion of the software calculates a relative expression ratio for each protein based on measured relative peptide expression ratios. Only the spectra unique to a single protein are used to calculate protein ratios. Spectra which correspond to multiple proteins almost certainly do not provide an accurate estimate of a single protein’s expression ratio. To test the effectiveness of the software, we analyzed data from samples containing highly homologous proteins. For instance, one data set comes from mouse liver microsome samples (phenobarbital-treated vs. untreated control), which are rich in cytochrome P450 proteins. These cytochrome P450 proteins pose a special challenge due to the high degree of sequence similarity between different isoforms. For this data set, our software recognizes and calculates relative expression ratios for 20 distinct cytochrome P450 isoforms. Certain isoforms are found to be significantly up-regulated ($p$-value $< 0.05$) in the phenobarbital-treated samples compared to untreated samples, while other isoforms show no significant change in expression. The cytochrome P450 2c subfamily exhibits particularly interesting behavior. Phenobarbital dosing leads to significantly increased expression of cytochrome P450 2c29 but has little or no effect on the expression level of cytochrome P450 2c40 despite the high sequence similarity of the two isoforms (68% sequence identity). To confirm the accuracy of the expression ratios calculated, expression changes were also measured using highly sensitive targeted Multiple Reaction Monitoring (MRM) absolute quantitation experiments using isotope-labeled custom-synthesized peptides. All of the ratios obtained through identification and grouping are consistent with the results obtained via MRM.

**B.22**

**Enrichment and Characterization of Glycopeptides from Gel-separated Glycoproteins**

M. Thaysen-Andersen¹, N. Brünger², and P. Højrup¹

¹Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark; ²Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Frederiksberg, DK, Denmark

Glycosylation is one of the most common post-translational protein modifications to occur in nature. The biological role of glycosylations varies from conformational stability and protection against degradation, to molecular and cellular recognition in development, growth and cellular communication. Characterization of glycoproteins by mass spectrometry (MS) is typically performed on glycopeptides or free glycans isolated by liquid chromatography. Each isolated fraction is then treated sequentially with specific endo/exo-glycosidases, followed by monitoring the mass changes by MS. However, this approach has only in a few cases been applied to the low amounts available from gel-separated glycoproteins. Consequently, it is of great interest to develop sensitive techniques for glycostructure analysis of low abundant glycoproteins.

Here we present a sensitive and fast technique to investigate glycostructures from gel-separated glycoproteins. The method will be illustrated by the characterization of more than 40 different glycan structures from recombinant human TIMP-1 expressed in two different organisms, the technique shows the potential to differentiate and characterize different glycosylation patterns. The enrichment of glycopeptides was based on hydrophilic interaction chromatography (HILIC) packed into custom-made micro-columns. The characterization of the glycan structures was performed using matrix-assisted laser desorption ionization time-of-flight MS (MALDI TOF MS) supported by fragmentation data obtained by MALDI Q-TOF MSMS.
Mass spectrometric-based protein identification from Coomassie-stained SDS-PAGE gels has become a routine analysis in many labs. These methods are typically less reliable for the analysis of post-translational modifications such as phosphorylation-state mapping due to the unpredictability and variability of sequence coverage. While much recent work has focused on sample preparation chemistries that enrich phosphopeptides and selective mass analysis such as precursor ion- and neutral loss-scanning to facilitate the detection of phosphopeptides, we find that our typical work flows serendipitously reveal phosphorylated peptides from in-gel digests without any special considerations with respect to enrichment techniques or mass spectrometric bias. Methods for in-gel digestion and nano-LC/MS using a QqTOF instrument have proven robust for acquiring automated tandem mass spectra of phosphopeptides in a completely blind fashion. Analysis of the data using the Mascot database search program can flag possible phosphorylated peptides in addition to identifying the protein. The tandem mass spectra of the potentially phosphorylated peptides are then subjected to manual de novo sequencing. This is necessary because the MS/MS of a phosphoserine-containing peptide is usually a mixture of at least two species: the phosphoserine-containing peptide and the dehydroalanine-containing peptide resulting from the neutral loss of H3PO4. Considering that the frequency and extent of the occurrence of the neutral loss appears to be sequence dependent, one typically obtains partial b- and y-ion series of one species (phosphorylated) in the presence of an overlapping partial b- or y-ion series of the other species (neutral loss). The convoluted tandem spectrum may result in Mascot scores and expectation values which underestimate the likelihood that the peptide is phosphorylated. Additionally, the tandem mass spectra do not always contain the sequence ions necessary to definitively pin down the precise position of the phosphorylated residue. Examples will be presented that illustrate instances where multiple possible sites of phosphorylation are easily ruled out, and instances where only one possible phosphorylation site exists and yet the data is simply not forthcoming which enables definitive assignment.

Reversible phosphorylation of proteins is a critical post-translational modification, which affects a range of biological processes such as protein-protein interactions, protein enzymatic activity and protein structure. Obtaining a comprehensive understanding of which proteins are modified and their sites of modification is a central focus of current proteomic studies. The understanding of this modification is hampered by dynamic range issues. Proteins that become phosphorylated are often of low abundance to begin with and their phosphorylation is often sub-stoichiometric.

A range of biochemical, chromatographic and mass spectrophotometer based techniques have been developed to examine low abundance phosphopeptides in the background of highly abundant non-phosphorylated peptides. In this present study, we have combined Strong Cation Exchange chromatography with Immobilized Metal Ion Chromatography to achieve a high degree of phosphopeptide enrichment. Combining these two techniques lead to a level of enrichment was significantly better than either technique in isolation.

We examined the phosphorylation state of proteins involved in synaptic signaling from a biochemical preparation known as the postsynaptic density. We identified approximately 1000 proteins in this mixture, and identified over 800 phosphorylated peptides from more than 350 different proteins. Phosphorylation sites occurred most commonly on serine and to a lesser extent on threonine and tyrosine.

Support Contributed By: NIH NCRR grants RR12961, RR14606, and RR01614 to ALB and grants from the Wellcome Trust (UK) to RS.
Optimization of a Quantitative Method for Phosphorylation Site Occupancy Analysis

R. Wei, M. Burgess, M. Fitzgibbon, D. Friedman, and M. Botfield

Vertex Pharmaceuticals, Cambridge, MA

To assess the in vivo efficacy and specificity of therapeutic agents directed against selected members of the protein kinase family, an accurate assessment of phosphorylation site occupancy is required. To this end, a nano-LC/MS/MS based method, Isotope-Tagged Internal Standards (ITIS), was developed and optimized to quantify site-specific protein phosphorylation without the need for phospho-specific antibodies. The target proteins undergo in-gel digestion to generate tryptic peptide fragments, which are spiked with heavy, mass tagged standards (13C- and 15N-tagged synthetic peptides) corresponding to the tryptic fragments containing the phosphorylation sites of interest. These internal standards co-purify through all chromatographic steps and permit quantification of corresponding light, peptides by MS/MS, thereby enabling quantitative measurement of the phosphorylation status of endogenous target proteins.

Due to the relatively low abundance of kinases and their substrates in the biological samples of interest, all targets were enriched and isolated by immunoprecipitation, resolved by SDS-PAGE prior to in-gel digestion with trypsin. Lack of enzymatic fidelity with trypsin is commonly observed, but a detailed study of possible causes has been under-reported. For the ITIS nano-LC/MS/MS method, it is crucial that all proteolytic products be accounted for, as missed-cleavage peptides will not be measured unless they are specifically monitored. We observed that location of a phosphate group proximal to the proteolytic site could interfere with digestion, resulting in a mixture of cleavage products containing the phosphorylation site of interest. For some proteins, either due to missed-cleavage events or lack of an amenable sequence, trypsin would not generate proteolytic fragments suitable for LC/MS/MS quantification. To circumvent these problems we explored the use of additional enzymes. Trypsin, Asp-N and Lys-C were assessed alone or in multi-enzyme combinations. We found that all were capable of in-gel digestion and for some problematic sequences the dual-enzyme method was able to generate more favorable peptides. Therefore, the sequence of the target protein requires development of a tailored enzymatic strategy to accommodate the particular site(s) of interest.

Using this optimized technique, we can routinely quantify up to 10 sites simultaneously (40 MRM channels) from 5 fmol to 500 fmol with an LOQ of 5–20 fmol on a conventional triple quadrupole mass spectrometer. This enables the simultaneous interrogation of multiple kinase signaling pathways to assess the in vivo specificity of therapeutics directed against protein kinases.

Liquid Chromatography with Electrospray-Ionization Mass Spectrometry and Fraction Collection (LC-MS+) Reveals Fatty Acylation of Human Apolipoproteins A-I and A-II

J. Whitelegge, P. Ghasri, L. Yam, S. Bassilian, K. Faull, V. Schumaker, and D. Puppione

Department of Chemistry, University of California, Los Angeles, CA

High density lipoproteins (HDL) are one of the two major carriers of cholesterol in mammalian plasma, being regarded as beneficial because of their role in the reverse-cholesterol transport pathway. Various apolipoproteins, located on the outer surface of HDL, have been identified, including ApoA-I (28078.62 Da in humans) which plays an essential role in the formation and metabolism of HDL, and ApoA-II (8690.91 Da in humans) which forms dimers in species with cysteine at residue 6, including humans. Despite many years of study, there are major deficits in our understanding of the function of different apolipoproteins within HDL. A thorough understanding of the different isoforms of the apoproteins of HDL is of significance to proteomics because of the central role of blood in human biomarker screening.

HDL preparations from humans and other mammals have been analyzed by liquid chromatography with electrospray-ionization mass spectrometry and fraction collection (LC-MS+). The resolution afforded yields profiles of the apolipoproteins that define the covalent state of the cognate gene product and reveal post-translational modifications with molecular detail. These analyses provide the clearest evidence to date of sub-populations of human ApoA-I and A-II that are fatty acylated, as defined by positive shifts in mass and chromatographic retention. The observed delta masses (264–266 Da) suggest predominant modification by stearate or oleate, while absence of available cysteine residues for thioesterification suggests ester or amide linkage of the fatty acid residues. Fractions collected during LC-MS+ provide liquid protein samples for fatty-acid analysis as well as characterization of post-translational modification sites. The usefulness of LC-MS+ as a core technology for detail-orientated study of integral membrane proteins is extended to soluble proteins whose post-translational modification could modulate their interaction with lipids and biological membranes.
Advances in LC MS/MS on a Novel Tandem Time-of-flight Mass Spectrometer

M. Willetts1, B. Williamson2, D. Gostick1, K. Parker2, and N. Araki3

1Applied Biosystems, Framingham, MA; 2MDS Sciex, Toronto, Canada; 3Kumamoto University, Kumamoto, Japan

MALDI tandem time-of-flight mass spectrometry is now well accepted for sensitive protein identification and quantification. When used in conjunction with iTRAQ reagents LC-MALDI enables multiplexed quantitation of up to four individual samples in one experiment. Samples derived from biological fluids and tissues often contain many thousands of proteins over a very wide dynamic range and usually require fractionation of the enzymatically digested sample using two-dimensional chromatography in order to simplify the sample sufficiently for analysis by mass spectrometry. While such separation strategies significantly reduce the complexity of each fraction there is still a need for increased sensitivity and dynamic range in order to identify low level peptides in the presence of much more intense species. Further more such precursors need to be specifically isolated in the presence of other potentially more intense peptides, closely related in mass to avoid complicating both the fragmentation pattern and the quantitation of the iTRAQ reporter ions.

Here we will present an investigation of a novel tandem time-of-flight mass spectrometer for the analysis of putative biomarkers in complex biological samples using two-dimensional LC^MALDI MS/MS. The presentation will include an investigation of the dynamic range and sensitivity of the system and highlight advances in precursor ion selection that allow more reliable identification and quantitation.

Chemical Tagging and Profiling of Lymphocyte Plasma Membrane Glycoproteins by Mass Spectrometry

B. Wollscheid, J. Watts, and R. Aebersold

Institute for Systems Biology, Seattle, WA

The molecular composition and dynamic organization of the plasma membrane (PM) determines how a cell can interact with its environment at any given moment in time. Cell surface-exposed proteins embedded in the membrane are crucial for cell-cell communication, interaction with pathogens, binding of chemical messengers, and responding to environmental perturbations. Of particular interest are membrane microdomains, known as lipid rafts, which can confer specificity and efficiency in signaling due to enrichment for certain signaling proteins.

Alterations in both the lipid rafts and total PM protein composition, as a result of a disease state may well define phenotypic and functional differences between normal, and for example tumour cells. The identification of the PM subproteome is hampered by inherent problems in isolating PMs, the limited relative abundance of surface membrane proteins and the difficulty in resolving and identifying hydrophobic polypeptides. A three step protocol that conferred specificity for the PM subproteome, circumvented these problems to a large extent. This involved a tandem affinity labeling strategy employing gentle, covalent chemical labeling of carbohydrate-containing proteins on living cells, combined with specific enzymatic peptide release step that allowed for systematic and selective identification of glycosylated peptides derived from cell surface proteins. Subsequent peptide and protein identification was achieved via reversed phase capillary liquid chromatography coupled to electrospray ionization ion trap tandem mass spectrometry (LC-MS/MS). By the inclusion of stable isotope labeling methodologies, the quantification of PM proteins can be accomplished to reveal changes occurring on the cell surface in response to a specific perturbation, or alteration of its environment.

Funding from NIH RO1-AI-41109-01 (to J. W.) and RO1-AI-51344-01 (to R. A.), NHLBI Proteomics Center at ISB (N01-HV-28179).
Site Determination of Disulfide Bonds with Matrix-assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

H. Yu¹, K. Murata², J. Hedrick², F. Xiang³, and A. Franz¹
¹University of the Pacific, Stockton, CA; ²University of California, Davis, CA; ³Shimadzu Biotechnology Corporation, Pleasanton, CA

Disulfide bond formation is one of several post-translational events in proteins. Disulfide bonds are critical for maintaining the three-dimensional structure of the protein and its biological function. Site-determination of multiple disulfide bonds in high-molecular mass proteins is complicated by the number of possible Cys-Cys combinations. The approach originally used was elegant diagonal electrophoresis [1]. In the early 90s, the commonly used strategy was based on enzymatic digestion, reverse phase HPLC separation, and sequencing by Edman degradation and mass spectrometry. However, this approach is cumbersome for large proteins with multiple disulfide bonds, especially when appropriate enzymes are not available to generate peptides with only one disulfide bond. The advent of Matrix-Assisted Laser Desorption/Ionization (MALDI) mass spectrometry (MS) and Electrospray Ionization MS (ESI) has greatly enhanced the efficiency for disulfide bond screening with high sensitivity [2–3]. Inter-chain disulfide bonds in tryptic peptide ions can be fragmented resulting in characteristic mass spectral patterns [4]. Fragmentation techniques include In-Source Dissociation (ISD), Post-Source Decay (PSD), and Collision-Induced Dissociation (CID). Dissociation of disulfide linkages in positive ion MALDI-MS can occur across both the S-S and the two C-S bonds. These fragmentation paths yield three mass spectral peaks characteristically separated by 32 Da [5]. As a consequence, chemical modification of Cys-Cys linkages prior to analysis is less necessary. MALDI-MS-ISD has been successfully applied to screening for the presence of inter-chain disulfide bonds [6]. In this report, several peptides (one or multiple disulfide bonds) from the tryptic digestion of Salmon Egg Lectin SEL 24 K [7] were investigated. Mass spectra were recorded on MALDI Time-Of-Flight (TOF) and MALDI Quadrupole Ion Trap (QIT) mass spectrometers. Disulfide linkages in the peptides were identified by ISD, PSD, and CID techniques.

References
Investigation of the Posttranslational Modifications of Yeast Histone H3 and H2B by Combined Intact Protein Electron Capture Dissociation and Digest LC/MS/MS Analysis

X. Zhang1, R. Chalkley1, S. Guan1, J. Recht2, R. Diaz2, C. Allis3, A. Marshall3, and A. Burlingame1

1University of California, San Francisco, CA; 2The Rockefeller University, New York, NY; 3Florida State University, Tallahassee, FL

Posttranslational modifications (PTMs) of histones H3 and H2B from Saccharomyces cerevisiae were investigated by use of electron capture dissociation (ECD) mass spectrometry of intact protein and by LC/MS/MS analysis of enzymatic digestions of the protein with collisional induced dissociation (CID).

Histones are known to be heavily modified by a wide variety of post-translational modifications, but it is difficult to locate the sites of modifications and to determine the co-occurrence of different modifications on the same protein species. From analysis of histones prepared with site-directed lysine mutations or specific gene deletions, the protein PTM isoform population can be greatly simplified and the modifications at other sites can be more readily deduced from ECD-MS experiments of the intact protein.

Protein components were selected from intact mixtures in the gas phase for sequence analysis using ECD-MS. In parallel, NanoLC-CID-MS analysis of protein digests yielded complementary details of the nature and location of specific posttranslational modifications.

This study demonstrates that integration of the information from these two mass spectrometry approaches can provide global insight into the differing occupancies and relative stoichiometries of different modifications on these proteins expressed by different cell states or by exposure to other stimuli or inhibitors.

Financial support was provided by NCRR 01614 (to A. L. B.), NIH R01 GM63959 (to C. D. A.) and NSF CHE-99-09502 (to A. G. M.).

Modification of ATP Synthase α Is Associated with Stimulated Acid Secretion by Gastric Glands

L. Zhu1, S. Karvar1, J. Trinidad2, V. Lao1, J. Crothers1, A. Burlingame2, and J. Forte1

1University of California, Berkeley, CA; 2University of California, San Francisco, CA

Drastic morphologic and functional changes are readily achievable with gastric gland parietal cells, the cell pumping HCl into stomach by H+, K-ATPase. When the cells are stimulated with histamine, the apical membrane is expanded while most of the intracellular tubulovesicles are depleted. This membrane traffic brings most of the H+, K-ATPase to the apical membrane where active secretion of HCl occurs. Besides the dramatic changes, another advantage of this material for studying proteomic changes is that the differences between resting and stimulated states of the parietal cells can be maintained for extended periods and analyzed by biochemical methods after homogenization.

Beside these morphological and functional changes of parietal cells, it is obvious that other departments within the cell have to undergo adjustments to meet the demand. Cell signaling system, trafficking machinery, cytoskeleton proteins and their organizing proteins are some known examples; massive energy production by mitochondria is another.

To get a global view of these changes and to identify new factors involved in stimulated acid secretion, we prepared resting and stimulated gastric glands from rabbit. The quality of the material is ensured by morphological and functional assays of H,K-ATPase trafficking and acid secretion.

Whole lysate from resting and stimulated gastric glands were resolved by 2D electrophoresis. From Coomassie blue stained gels, the most pronounced difference between resting and stimulated samples was the pl shifting of a 55kd protein. Although the percentage of the shift is different with different preparations, the 55kd protein repeatedly showed an acidic pl shift upon stimulation. In resting sample, much of the protein has an apparent pl of 8.3; in stimulated sample, the dominant mass was pl 7.7, together with spots at pl 8.0 and pl 8.3. We cut out the spots at pl 7.7, pl 8.0 and pl 8.3 for in-gel digestion and nano-LC-MSMS analysis.

Results clearly identified these spots as ATP synthase alpha subunit. Western blot analysis with a monoclonal antibody confirmed the above results. We propose that there is a previously unrecognized mechanism of active regulation of ATP synthase, involving modification of the alpha subunit in a way that adds one or more negative charges. Active regulation of ATP synthase may be necessary for parietal cells to meet the energy demand of stimulated acid secretion ~ 1 ATP per H+ pumped.
B.33

Developing Peptide MRM-based Assays for Cardiovascular Biomarker Proteins in Plasma Using a Hybrid Triple Quadrupole Linear Ion Trap Mass Spectrometer

C. Hunter¹, G. Becker², A. Breite², and L. Anderson³

¹Applied Biosystems, San Mateo, CA; ²Roche Applied Science, Indianapolis, IN; ³The Plasma Proteome Institute, Washington, DC

As the study of protein biomarkers increases in importance, technical limitations in the detection of low abundance proteins and high throughput, high precision quantitation remain to be overcome. Practical approaches to these problems are critical for the validation of biomarkers in plasma, where extensive lists of candidate markers are being developed based on pathways, microarray data, and experimental proteomics. Validation of these markers for clinical use will require analysis of extensive candidate marker panels in thousands of clinical serum and plasma specimens. The combination of triple quadrupole and ion trapping capabilities of the hybrid triple quadrupole - linear ion trap mass spectrometer presents new opportunities in hypothesis-driven discovery and validation of protein biomarkers, particularly where optimized multiplex immunoassays are not available.

The multiple reaction monitoring scan (MRM) of a triple quadrupole MS system is well known to be the most sensitive and specific way to detect components in a complex matrix such as plasma. In this study, we explored the use of MRMs to detect selected tryptic peptides in a human plasma tryptic digest, each such peptide representing a specific candidate protein biomarker. Both in silico and experimental peptide and MRM selection approaches have been applied. A suite of over 100 MRMs representing ~50 plasma protein markers have been monitored using the MRM-based technique. Stable isotope labeled peptides to some of the proteins were added as internal standards to provide absolute quantitation of the endogenous peptides in undepleted and depleted plasma. Current set of MRM transitions designed based on MS analytical data has provided MRM’s capable of detecting proteins down to the level of fibronectin (~1 ug/ml).

B.34

Proteomic Analysis of Cellular Response to Brassinosteroid in Arabidopsis

W. Tang¹, Z. Deng¹, X. Zhang², N. Suzuki², R. Chalkley², A. Burlingame², and Z. Wang¹

¹Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA; ²Department of Pharmaceutical Chemistry, University of California, San Francisco

Brassinosteroid (BR) is a plant hormone that structurally resembles animal steroid hormones but functions through a distinct signaling mechanism. BR plays important roles in multiple plant developmental processes, and mutants with defect in BR synthesis or signal transduction show dwarfism, reduced fertility, and delayed senescence. Genetic studies have shown that BR is perceived by the cell surface receptor-like kinase BR1, and that downstream signal transduction involves a receptor-like kinase BAK1, a GSK3-like kinase BIN2, and nuclear transcription factors of the BZR family. In order to identify new molecular factors potentially associated with brassinosteroid signal transduction pathway or brassinosteroid regulated physiological response, differential in-gel electrophoresis (DIGE) was used to identify BR-regulated proteins. Total protein and plasma membrane proteins were prepared from seedlings treated with BR for various periods from 15 min to 24 h. Whereas significant changes were detected in total protein samples only after at least 3 hr of BR treatment, obvious responses were observed in PM proteins after 15 min BR treatment. Using MS/MS, we identified 61 brassinolide regulated proteins from purified plasma membrane fractions and 65 proteins from total protein fractions. While none of the known BR signaling proteins were identified in total protein sample, the BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) is one of the most consistently BR down-regulated proteins in the PM fractions. Since BAK1 has been shown to physically interacts with BR receptor BR1 and to play essential role in BR signal transduction, our results demonstrate that it is feasible to use 2-D DIGE proteomic methods with subcellular fractionation to identify important signal transduction components. Additional BR-regulated proteins suggest BR regulates plant growth through modifying cytoskeleton and other cellular processes. Further studies will reveal the roles of these proteins in BR signaling and growth regulation.
Conformational Flexibility of IKBα

S. Truhlar, D. Ferreiro, S. Bergqvist, and E. Komives

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA

IKBα inhibits the transcription factor NF-KB thus turning off transcription of a plethora of genes. IKBα has six ankyrin repeats in its ankyrin repeat domain. Amide hydrogen/deuterium exchange followed by pepsin digestion and MALDI-TOF mass spectrometry has been used to probe the foldedness of regions of the protein. Because ankyrin repeat domains are non-globular and the sequences in each repeat are similar but not identical, it was possible to obtain pepsin digest products that revealed the solvent accessibility of similar regions of secondary structure in each repeat. In all cases, the beta hairpin was the most solvent inaccessible, the helices were also solvent inaccessible and the variable loops were more solvent accessible. Comparison between repeats showed that the third repeat was the most compact, the second and fourth were the next most compact and the first, fifth and sixth were solvent exposed. Based on consensus sequences for stable ankyrin repeat domains, we have introduced two proline mutations into the third and fourth ankyrin repeats. The double mutant protein is 1.5 kcal/mol more stable to urea denaturation and is less prone to aggregation. Amide hydrogen/deuterium exchange experiments reveal that the stabilization correlates with decreased solvent accessibility of the first, fifth and sixth repeats. This suggests that inter-repeat interactions are a substantial part of the stabilizing force of ankyrin repeat domains.