1.1

Unraveling Complexity in Cytokine-dependent Signaling: How Can Mass Spectrometry Help?

G. Stark

Cleveland Clinic Foundation and Case Western Reserve University, Cleveland, OH

Several current topics in the lab will be discussed. (1) Interferons stimulate the activation of STAT transcription factors through phosphorylation of a single tyrosine residue, followed by STAT dimerization. Although phosphoSTAT dimers carry the main signal, additional signals are needed to give different responses in different cell types. Activation of several kinases and non-STAT transcription factors contributes to this modulation. How are these various proteins modified in response to activation of the interferon receptors and how do they interact, in the cytosol and on the regulated promoters? (2) Cytokine-dependent changes in the levels of unphosphorylated STATs 1 and 3 activate transcription by mechanisms distinct from those used by phosphorylated STAT dimers. How do unphosphorylated STATs drive gene expression? What are the partner proteins and DNA elements? (3) Main line pathways for TGFβ and interleukin-1 activate SMAD and NFkB transcription factors, respectively. However, we find that these two cytokines also cross-activate (TGFβ activates NFkB and interleukin-1 activates SMADs). Our current model is that aggregation of one type of receptor, driven by its ligand, causes ligand-independent aggregation of the other receptor. How can mass spectrometry help to unravel the details of these biologically important interactions?

1.2

Bio-orthogonal Affinity Purification of Direct Kinase Substrates

J. Allen, S. Lazerwith, and K. Shokat

Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA

Protein phosphorylation is a major mechanism of post-translational protein modification used to control cellular signalling, protein localization, enzymatic activity, and protein half-life. A challenge in phosphoproteomics is to identify the direct substrates of each protein kinase. We have developed a chemical strategy for delivery of a bio-orthogonal affinity tag to the substrates of individual protein kinases. The kinase of interest is engineered to transfer a phosphorothioate moiety to phosphoacceptor hydroxyl groups on direct substrates. In a second non-enzymatic step, alkylation of the phosphorothioate with p-nitrobenzylmesylate (PNBM) forms a phosphorothioate ester labelled protein. Antibodies directed against the phosphorothioate ester epitope recognize these labelled substrates, but not alkylation products of other cellular nucleophiles. Direct kinase substrate labelling, combined with immunoaffinity purification potentially provides a general route for identification of kinase substrates, and is demonstrated with PKB, Cdk1, and Cdc5 substrates.

1.3

Cell Cycle Proteomics Using Quantitative Mass Spectrometry

D. Pappin[1], J. Jebanathiraja[2], H. Steen[2], M. Kirschner[2], P. Ross[1], and S. Hattan[1]

[1]Applied Biosystems, Framingham, MA; [2]Harvard Medical School, Cambridge, MA

A proteome-wide program of gene expression during the cell division cycle in a human cancer cell line (HeLa) was characterized using quantitative labeling reagents and MS. Here, we made use of a set of isobaric peptide tags (iTRAQ) to study the changes in protein abundance at four time-points over one full cell cycle in chemically synchronized cells. We have obtained protein expression level profiles for more than 1,700 proteins. In this data set we have directly observed expected changes in Cyclin and Cdc protein families, but more than 400 proteins show cell-cycle abundance changes that have not been previously described.

Methods and Results

HeLa, HeLa S3 and 293T cells were synchronized in prometaphase by treatment with nocodazole, in early and late G1 by release from nocodazole arrest, and in early S by double thymidine arrest. Cells were harvested, washed with PBS, and frozen. The cell pellet (approximately 100 mg) was suspended in 1ml of ice cold lysis buffer (0.1 M TEAB, 0.1% v/v Triton X-100, 6 M guanidine, 1 μM okadaic acid, 1 μm microcystin-LR) and centrifuged to remove insoluble debris. Total protein was reduced and alkylated with MMTS and digested with trypsin overnight. 100 μg of each cell sample was then labeled with one of the 4-plex iTRAQ reagents, combined, and the peptides analyzed by 2D SCX, capillary RP-HPLC and MALDI TOF-TOF MS.

The use of genetic and biochemical screens to identify proteins involved in the cell cycle has proven to be extremely useful. Not all proteins functionally involved in the cell cycle are identified in these screens, however, because of inherent biases associated with each screen. In this study we have used a 4-plex set of isobaric amine-reactive peptide labels to quantitatively profile protein expression levels at four time points over a complete mammalian cell cycle. Cells were chemically synchronized in prometaphase, early and late G1 and in early S-phase. We examined the data set for proteins that are functionally relevant in the cell cycle by looking for proteins that exhibit changes in post-translational modifications and solution concentration. Data has been obtained for more than 1,700 proteins whose expression levels have been monitored across the cell cycle. Hierarchical clustering of the quantitation patterns obtained from the data has revealed co-expressed groups of previously well-characterized protein families (*e.g.* Cyclins and Cdcs), but several hundred other proteins show cell-cycle expression level changes that have not been previously described.

1.4

Epithelial-Mesenchymal Transition Regulates NSCLC Cell Sensitivity to EGF Receptor Inhibition: Applications of Quantitative LC-MS/MS Profiling to the Dissection of EGFR Signaling Networks

J. Haley, S. Thomson, E. Buck, F. Petti, and N. Gibson

OSI Pharmaceuticals, Farmingdale, NY

Treatment of second and third line patients with non-small cell lung carcinoma (NSCLC) with the EGF receptor kinase inhibitor erlotinib significantly increased survival relative to placebo. The molecular determinants which confer cell sensitivity in wild type (wt) EGFR tumors remain unclear. Anti-phosphotyrosine affinity chromatography and membrane isolation methods were used to select specific protein populations for identification and quantitation. Isobaric peptide labeling and uLC-MSMS mass spectrometry methods were employed to measure signaling networks associated in NSCLC lines sensitive (H292, H358), moderately sensitive (H441, A549) and insensitive (H460, H1703, Calu6) to the downsteam effects of EGFR kinase inhibition. Phosphoprotein and protein complexes associated with the differential temporal sensitivity and with cell-specific responses to EGFR pharmacological inhibition were identified using a multiplex isobaric peptide tagging approach. This methodology allowed for quantitation of protein abundance under several conditions within a single LC-MS/MS experiment. In wt EGFR tumors, kinase blockade markedly disrupted complexes containing immediate signaling proteins, membrane adhesion complexes and internalization complexes in a manner correlating with sensitivity to erlotinib. The importance of an EGFR-dependent PI3 kinase pathway to erlotinib sensitivity was reinforced.

We demonstrate that wild-type EGF receptor containing NSCLC lines grown both in culture and as xenografts show a range of sensitivities to EGF receptor inhibition dependent on the degree to which they have undergone an epithelial to mesenchymal transition (EMT). NSCLC lines which express E-cadherin or to a lesser extent -catenin showed greater sensitivity to EGF receptor inhibition *in vitro* and in xenografts. This was demonstrated by immunoblot and fluorescence confocal microscopy. In contrast NSCLC lines having undergone EMT, expressing vimentin and/or fibronectin, were insensitive to the growth inhibitory effects of EGF receptor kinase inhibition in vitro and in xenografts, as shown by immunoblot, confocal microscopy and mass spectrometry. Analysis of pancreatic tumor lines sensitive or insensitive to EGF receptor inhibition also indicated E-cadherin (epithelial) expressing cells were erlotinib sensitive, while vimentin/fibronectin (mesenchymal) expressing cells were insensitive. The loss of E-cadherin associated with EMT has been shown to correlate with poor prognosis in multiple solid tumor types. These data suggest EMT also may be a general biological switch rendering non-small cell lung and pancreatic tumors sensitive or insensitive to EGF receptor inhibition.

1.5

Analysis and Quantification of Protein and Phosphorylation Expression at the Synapse

J. Trinidad[1], C. Specht[2], A. Thalhammer[2], R. Schoepfer[2], and A. Burlingame[1]

[1]University of California, San Francisco, CA;
[2]University College, London, UK

Excitatory synaptic connections in the mammalian central nervous system rely on glutamate as their primary neurotransmitter. Ligand-gated ion channels, that detect glutamate, do not occur in isolation on the post-synaptic membrane. Rather, they are an integral part of an extensive complex comprising over 500 unique proteins (known as the post-synaptic density). Dynamic modulation of synaptic signaling is a critical process underlying such phenomena as learning and memory. This modulation of synaptic strength likely occurs at the molecular level, in part via changes in protein expression and post-translational modification of components of the glutamate-sensing complex.

We have developed methods, which allow for comprehensive quantitation of relative protein and phosphorylation expression of synaptic components. Post-synaptic density enriched fractions are purified using density centrifugation. Samples as digested with trypsin, and isotopically labeled using the iTRAQ reagent. Strong cation exchange chromatography is used to fractionate the mixture, and this is followed by immobilized metal affinity chromatography to enrich for phosphorylated peptides. These chromatographic methods have been automated using an HPLC system, which allows for a rapid and robust workflow. Using this approach we plan to examine a range of experimental conditions known to affect synaptic function and determine their molecular underpinnings, with a long-term goal of understanding the integrated functioning of the post-synaptic density.

We have initially applied these methods to analyze post-synaptic density proteins from four distinct brain regions: cortex; hippocampus; midbrain, and cerebellum. While the fundamental process of synaptic transmission are similar in all these brain regions, it is known from electrophysiological studies, that modulation of synaptic strength occurs by different molecular mechanisms. We have obtained relative quantitation information on over 800 synaptic proteins and 700 unique sites of phosphorylation. Significant changes in protein expression could be found in each region. Approximately 6% of proteins showed greater than a 2-fold relative expression increase in at least one brain region. More striking was the variability in phosphorylation expression. Over 35% of the identified phosphorylation sites were highly up regulated in a single brain region. These observations support the notion that post-translational modifications play as large (or larger) a role as protein expression levels alone in regulating cellular processes.

Support Contributed By: NIH NCRR grants RR12961 and RR01614 to ALB and grants from the Wellcome Trust (UK) to RS.