29.1

Analysis of Protein Phosphorylation in Halobacterium salinarum

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Protein phosphorylation plays an important role in the two-component system based signal transduction pathways in bacteria and in archaea. These cascades have been identified also in the extremely halophilic archaeae Halobacterium salinarum and are well understood. Beside these protein phosphorylation events in which phosphorylation of histidine and aspartate residues are crucial, only little is known about further protein phosphorylation in Halobacterium salinarum. From genome and proteome analysis on H. salinarum several putative protein kinases and phosphatases have been annotated and identified. Nevertheless, the extent of regulatory protein phosphorylation is not yet well elucidated. In order to identify phosphorylated proteins and to determine their phosphorylation sites different specific experimental approaches have been applied to the whole cell extract. A gel based approach utilizing 1- and 2-dimensional gel electrophoresis after radioactive labeling of the cells with 32P resulted in the identification of a number of phosphate carrying proteins by MALDI-TOF MS. In addition, anti-phosphoserine/threonine antibodies, were used for the determination of the phosphorylated proteins in both procedures (1- and 2-DE). Discrete signals were observed indicating Ser/Thr phosphorylation but the magnitude of protein phosphorylation appeared to be rather low. Alternatively, a gel-free method involves strong cation exchange (SCX) chromatography was applied to enrich phosphopeptides resulted after trypsin digestion of the whole cell extract, under low salt elution conditions. LC-MS/MS analysis of the additional IMAC-enriched SCX fractions yielded the identification of threonine and serine phosphorylation sites of different halobacterial proteins, the first archaeal Ser/Thr phosphorylation sites ever described. The question if the identified phosphorylation sites are of regulatory importance or just present metabolic intermediates has to be answered.

29.2

Analysis of Ser/Thr Phosphorylation by MALDI Mass Spectrometry

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Protein phosphorylation is one of the most important cellular events and, probably, the most flexible and potent way the cell uses to regulate its functions. Approximately 30% of all proteins in mammalian organisms are phosphorylated and protein kinases represent the largest known protein family in eukaryotic genomes. It has been calculated that serine phosphorylation accounts for about 90% of the total phosphorylation events, while threonine and tyrosine phosphorylations account respectively for about 10% and 0.1% of the total phosphorylation.

From the methods recently developed for phosphopeptide analysis by mass spectrometry, two areas command considerable interest. First, introduction of a suitable derivatisation procedure that would increase the ionisation, and hence the detection of phosphorylated peptides. Second, use of a higher order fragmentation (MS3) to obtain a confident assignment of the phosphorylation site within the peptide.

The strategy we employed for the analysis of Ser/Thr phosphorylation by MALDI mass spectrometry involves a beta-elimination/Michael addition reaction using 4-mercaptoethylpyridine as a nucleophile. The substitution of the negatively charged phosphate group with a new positively charged pyridyl group strongly increases the ionisation. With use of HCCA matrix, the ability to detect phosphopeptides is greatly improved. When fragmented, the derivatised peptides produce a strong neutral loss of 105 in their MS/MS spectrum. This neutral loss is automatically detected by the system, and triggers the MS3 experiment on the linear ion trap. The method also appears very suitable for multiply phosphorylated peptides, as shown for peptides with up to 5 phosphorylated residues per molecule.

29.3

A Novel Combined Genetic and Proteomic Approach to Identify PI3Kgamma-dependent Phosphorylated Proteins

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PI3Kgamma is a phosphatidylinositol kinase mainly expressed in leukocytes and activated by GPCRs. We previously demonstrated that neutrophils and macrophages lacking PI3Kgamma expression display an impaired response to chemokines and a consequently reduced recruitment to the inflammation site in vivo.

Nonetheless, the signaling events causing a PI3Kgamma-dependent chemotactic response are still mostly obscure.

To clarify the role of PI3Kgamma in leukocyte movement, we compared the patterns of tyrosine and serine/threonine phosphorylated proteins following chemokine stimulation.

By using bone marrow derived macrophages (BMDMs), obtained from wild-type and PI3Kgamma-null mice and cultivated in vitro, we developed a proteomic approach by combining an anion exchange chromatography and a phosphoprotein-immunoprecipitation. This allowed the isolation of proteic candidates involved in PI3Kgamma pathway.

We consistently identified 3 bands that displayed modified serine/threonine phosphorylation levels and 7 bands that displayed modified tyrosine phospho-levels between wild-type and PI3Kgamma-null BMDMs. Three of these bands were randomly chosen for sequence analysis using MS/MS approach and one of those was identified as the class III intermediate filament vimentin.

To confirm this finding, vimentin was immunoprecipitated from wild-type and mutant BMDMs and analysed for its tyrosine phosphorylation levels. Consistently with the results of the proteomic screening, tyrosine phosphorylation of vimentin was reduced in the PI3Kgamma-null BMDMs extracts.

Moreover vimentin tyrosine phosphorylation was reduced in wild-type BMDMs treated with the PI3Kgamma inhibitor LY294002.

The finding that, upon chemokine stimulation, vimentin is tyrosine phosphorylated in a PI3Kgamma dependent manner is intriguing: being vimentin involved in the cytoskeletal dynamics, it is possible that the lack of vimentin phosphorylation contribute to the migration defect of PI3Kgamma-null macrophages.
Phosphoprotein Modulated by SEL1L Induced Expression in MCF-7 Human Mammary Carcinoma Cell Line

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Most studies on SEL1L, the human ortholog of the C. elegans sel-1 gene, have focused on its role in cancer development and have provided significant in vitro and in vivo evidences to link its increased expression to a decrease in tumor aggressiveness. Recent observations indicate that in oncogenesis, SEL1L modulates the transcription of mediators involved in the remodelling of the extra-cellular matrix such as the metalloproteinase 1 and 7, the tumor suppressor gene PTEN and members of TGF-β pathway. Moreover, using proteomic and microarray analyses we found that SEL1L induced expression changes the levels of proteins and transcripts involved in different signalling pathways, in cytoskeletal reorganization, metabolic processes, protein folding, cell stress response and tumor associated proteins. Protein phosphorylation is very important in the control of signal transduction network in regulating a number of biological processes including response to extracellular stimuli, DNA damage, cell growth and division. In general oncogenes encode for overactive mutant forms of protein tyrosine kinases. We aim to study the phosphoproteome in the human breast cancer cell line MCF7 in which the gene SEL1L was stably transfected. Preliminary data indicate the modulation of some protein spots upon SEL1L ectopic expression as compared to control cells. *L. Bianchi and C. Canton contributed equally to this work.

Mass Spectrometry Mapping and Quantitation of Stimulation-dependent Phosphorylation of EGF Receptor by Stable Isotope Labeling

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Protein phosphorylation is a relevant and dynamic protein modification that regulates signalling events. EGF receptor is a 180 kDa protein tyrosine kinase which is phosphorylated and mediates many cellular responses in normal and pathological biological processes. A mass spectrometry (MS) based experimental strategy was used for the identification and quantitation of phosphorylation sites on EGF receptor to better evaluate the response of this tyrosine kinase receptor to different cellular conditions. In order to map the phosphorylation sites we developed an analytical strategy that combines miniaturized sample preparation techniques and MALDI tandem mass spectrometry; LC-MS/MS was also employed to identify additional phosphorylation sites. A total of 9 phosphorylation sites were detected in EGF receptor. Some of these were observed only under distinct experimental conditions: the constitutive phosphorylation of T669, S967, S1002, Y1045 and stimulation-dependent differential phosphorylation of Y1068, Y1086, S1142, Y1148, Y1173 were revealed. The SILAC (Stable Isotope Labeling) was used for quantitation of phosphorylation sites. Hela cells were split in two groups and grown in media containing “light” or “heavy” form of Lys and Arg. The cells were subjected to different conditions: e.g. pervanadate treatment, adhesion or suspension both in presence of EGF stimulation. After immunoprecipitation, the EGF receptor was digested and the peptide mixture was subjected to phosphopeptide purification by immobilized Fe(III) chromatography (IMAC) or TiO₂.”VEMS,” an in house developed software program, was employed for relative quantitation of phosphopeptides.
29.6

SELDI-MS and SELDI-MS/MS Analysis of Phosphopeptides Specifically Enriched on GA(III)-IMAC Arrays

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A widely used strategy for the identification of phosphorylation sites is the enrichment of phosphopeptides after tryptic digestion of the protein by immobilized metal affinity chromatography (IMAC) prior to mass spectrometrical analysis. Enrichment on IMAC ProteinChip Arrays and direct analysis by SELDI-MS (Surface Enhanced Laser Desorption/Ionization-Mass Spectrometry) and SELDI-MS/MS should have advantages over other IMAC procedures due the reduced number of processing steps.

Ga(III) coated IMAC30 arrays were used for enrichment of a mixture of five synthetic phosphopeptides in the presence of an excess of unphosphorylated peptides. Twenty-five fmol phosphopeptides could be detected even if spiked into 1 pmol of a tryptic digest of bovine serum albumin. The phosphopeptides were captured on the ProteinChip Arrays with high affinity (about 90% binding) and specificity since non-phosphorylated peptides exhibited only weak binding activity. Similar results were obtained for a beta-casein digest where two singly phosphorylated peptides were specifically enriched and easily detected from 25 fmol total protein.

Furthermore, a phosvitin-digest was analysed and several singly and double phosphorylated peptides, not been reported before, were detected. SELDI-MS/MS analysis enabled a clear assignment of the phosphorylation sites. One phosphopeptide of phosvitin was identified despite the non-tryptic cleavage site at its C-terminus.

In comparison to other IMAC procedures the enrichment of phosphopeptides on Ga(III) coated IMAC30 arrays for SELDI-MS exhibits higher specificity and excellent sensitivity and is therefore highly complementary to other methods like HPLC-ESI MS.

29.7

Quantitation of Protein Phosphorylation Using Stable Isotope Dimethyl Labeling Coupled with Immobilized Metal Affinity Chromatography (IMAC) and Mass Spectrometry

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In this study, we coupled stable isotope dimethyl labeling with IMAC enrichment to quantify protein phosphorylation at determined phosphorylation sites. The proposed method was first characterized using α- and β-casein as two model phosphoproteins and applying both MALDI-MS and ESI-MS/MS instruments. Our results indicate that the labeling has no adverse effect on the IMAC enrichment for tryptic peptides having single and multiple phosphorylation sites. Moreover, the MS/MS spectra derived from the labeled phosphopeptides were simple and straightforward to interpret, and the phosphorylation sites can be determined by either database search or manual assignment. We applied this method further to the analysis of pregnant rat uteri with and without treatment with 8-bromo-cGMP. In this analysis, most of the identified phosphorylation sites and their corresponding phosphoproteins were found to be related to the biological pathway investigated. It is interesting to note that the decrease of phosphorylation level of nucleolar protein B23 upon the treatment suggests a possible inhibitory action of cGMP on cell proliferation through dephosphorylation of protein B23. The ratio of the phosphorylation levels at Ser 15 of HSP27 obtained from the treated and untreated samples was further confirmed by the consistent results obtained from Western blot analyses. The advantages associated with stable isotope dimethyl labeling-global labeling, complete reaction (near 100%), and no interference to MSMS sequencing-suggest that our proposed coupling strategy is a stable and accurate method for detecting small and large variations in protein phosphorylation levels and provides a critical tool for performing phosphoproteomics.
Identification of New in Vivo Phosphorylation Sites of EBV-LMP1 by Mass Spectrometry

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Epstein-Barr virus (EBV) encoded latent membrane protein 1 (LMP1) is the key oncoprotein in EBV-associated carcinoma. The LMP1 protein mimics cellular receptor to modulate plenty cellular signal transductions through self-aggregation and phosphorylation. The phosphorylation of LMP1 can be detected in EBV-infected or LMP1-gene transfected cell lines. Two phosphorylation sites, S313 and T324, have been predicted by site-directed mutagenesis. However, there are additional phosphorylation sites that may involve in regulating the turnover rate of LMP1 protein. By in vitro kinase assay, we have demonstrated that the protein casein kinase 2 (CK2) is a potent LMP1 kinase. For further understanding whether this phosphorylation can be detected in vivo, the LMP1 protein was immunoprecipitated from flag-LMP1 gene transfected HEK293T cell lysate and characterized by using MALDI-TOF/TOF and LC-ESI MS/MS spectrometry. We found that there are one to two phosphorylation residues located in the carboxyl-terminus activation domain 1 (CTAR1) of LMP1. S211 and S215 are the two candidate amino acids. Since S211 is located in consensus CK2 recognition sites, whether LMP1 can be phosphorylated by CK2 in vivo will be further studied.

A Strategy for Phosphoproteomics That Integrates Various Gel- and MS-based Techniques

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Objective: Protein phosphorylations have an outstanding role in signal transduction pathways and are thus of significant importance in disease-related studies. Therefore, reliable methods are needed for the detection and characterisation of phosphorylation sites in large numbers of proteins. Because of its well characterised genome and the relatively high abundance of phosphorylations, Arabidopsis thaliana is a convenient model organism. So far, many phosphorylation studies deal with individual proteins that are purified in order to submit them to extensive analysis approaches. Lately, a commercial staining kit became available that allows to selectively stain phosphoproteins in a 2D gel. This allows to parallelise phosphoprotein analysis and thus identify a high number of phosphorylated proteins.

Methods: After homogenisation, leaves of A. thaliana were submitted to 2D-gel-electrophoresis. For phosphoprotein analysis, the gel was stained using the Pro-Q-Diamond-stain (MolecularProbes). After a comparison with a total-protein stain, spots of various intensities were picked and digested. After PMF identification (MALDI-TOF-MS), aliquots of the potentially phosphorylated spots were submitted to an in-depth LC-MS/MS analysis.

Results and Conclusion: When a gel of Arabidopsis leaf material is stained using the phosphoprotein-specific dye, surprisingly many spots appear. Part of them, by comparison to the total-protein image of a Sypro-Ruby-stained gel and also MS identification results, seem to be false positives. The application of a phospho-sensitive stain allows a stepwise approach: Candidates that do not seem phosphorylated are eliminated in the staining and/or the MS identification step. Thus, the workload for LC-MS/MS analytics is reduced. This strategy can be applied not only to plants but to various kinds of (sub-)proteomes.
29.10 IMAC for Phosphoproteins
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Phosphoproteome studies are hampered by the lack of methods which allow a comprehensive and fast analysis of intact phosphoproteins. We established an immobilized metal-affinity chromatography-based technique for the enrichment of phosphorylated proteins, which allows recovery of up to 90% phosphoproteins. This technique is compatible with two-dimensional gel electrophoresis, MS-analysis, and can be applied to cultured cells and to tissues. Combined with simplicity of this procedure, it may provide a tool for the efficient and comprehensive analysis of phosphoproteins.

29.11 Neural Network and Mass Spectrometry for Identification of Protein Kinase A and B Substrates Involved in Apoptosis
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Protein phosphorylation catalyzed by protein kinase A (PKA) and protein kinase B (PKB) is important for regulation of many biological functions. In this study we identified PKA and PKB phosphorylation sites in proteins involved in apoptosis using bioinformatics and mass spectrometry. All human protein entries were extracted from Swiss Prot and sorted into functional categories using Gene Ontology. Eight proteins involved in apoptosis were selected and potential PKA and PKB phosphorylation sites were predicted by the artificial neural network algorithm: NetPhosK available on-line at http://www.cbs.dtu.dk/services/NetPhosK/. HA-tagged proteins were expressed in Cos-7 cells, precipitated and phosphorylated in vitro by PKA with radioactive ATP and in vivo after stimulation of cells with forskolin, insulin, or serum. Three new PKA substrates were identified: Death-associated protein kinase 1 (DAPK1), Nucleotide-binding oligomerization domain 1 (NOD1), and High temperature requirement protein A2 (HTRA2). The phosphorylation sites in the three proteins were identified using mass spectrometry including MALDI-TOF and LC-MS/MS. Finally, the functional role of the phosphorylation sites was analyzed by mutation of serine to alanine, expression of proteins, and measurement of apoptosis.

29.12 High Sensitivity Chemiluminescent Detection and Quantitation of Phosphoproteins after Western Blotting from 1D and 2D-Gels; Analysis of 2D-Spot Patterns of Proteins from Bacillus subtilis
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The detection and quantitation of specific subsets of post-translationally modified proteins has become a major area of activity in the proteomic analysis of biological systems. Phosphoproteins are a focus for much of this research owing to their singular importance in cell signalling and metabolic control. Here we present data to show the high sensitivity chemiluminescent immuno-detection, on Western Blots, of phosphoprotein markers separated by 1D-PAGE, using a set of three specific anti-phosphoamino acid antibodies. The blots were also stained with a fluorescent, total protein stain prior to the immuno-detection. Both the fluorescent patterns and the chemiluminescent patterns were visualised using a high resolution, CCD based, imaging system which includes modes optimized for the detection of both fluorescence and chemiluminescence. The use of the same imaging device to obtain both types of image facilitated accurate correlation of the fluorescent, total protein patterns with the corresponding chemiluminescent, immuno-detection pattern. The application of this technology to the specific chemiluminescent immuno-detection of phosphoproteins present in complex samples of proteins, extracted from Bacillus subtilis and separated by high resolution 2D-PAGE, is demonstrated. We show the correlation of the 2D-spot patterns between the phosphoproteins and the total protein patterns after detection of the latter using the fluorescent, total protein stain. 2D-spot pattern matching enabled comparison of data with that obtained previously on 2D-gels using a specific fluorescent phosphoprotein gel stain. Most investigations of phosphoproteins have been concentrated on eukaryotic organisms. This work illustrates the potential of this technology for continuing to advance our knowledge of the phosphoproteins of bacteria.
29.13

Phosphorylation as a Regulatory Mechanism in the Spindle Assembly Checkpoint

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Objective: Accurate segregation of chromosomes during cell division is essential for the conservation of genomic integrity. The spindle assembly checkpoint is a surveillance mechanism that ensures proper sister chromatid segregation. Loss of the checkpoint protein Mad3 compromises the checkpoint and leads to aneuploidy.

To investigate the mechanism of Mad3 function in the spindle checkpoint, we analyzed its modification status and protein interaction partners using a quantitative mass spectrometry-based approach.

Methods: HeLa cells were metabolically labeled using stable isotope of amino acids in cell culture (SILAC). Cells were grown either in medium containing "light" amino acids (U-12C-arginine and lysine) and arrested in mitosis with taxol, or in medium containing "heavy" amino acids (U-13C-arginine and lysine) and arrested at G1/S with thymidine. Cells from both states were mixed, lysed and Mad3 was immunoprecipitated. Bound proteins were separated by SDS-PAGE, differentially digested, analyzed by microcapillary LC-FTICR-MS/MS (LTQ FT), data-searched with SEQUEST and quantified by an in-house developed software (Vista).

Results: LC-FTICR-MS/MS analysis of Mad3 resulted in the identification and quantification of 13 serine/threonine phosphorylation sites. Ser/Thr- to Ala-mutants for these sites were constructed and analyzed with regards to Mad3 protein mobility and sub-cellular localization.

Analysis of Mad3-interacting proteins identified known components of the checkpoint complex, thus validating our experimental approach. In addition, novel Mad3-interacting proteins were identified and quantified using SILAC. Cloning, expression and characterization of these proteins revealed insights on Mad3’s role in the spindle checkpoint, as well as novel functions of Mad3 in anaphase.

Conclusion: Using a quantitative proteomics approach, we were able to demonstrate phosphorylation as an important molecular mechanism in regulating Mad3 function. Furthermore, we found a novel protein network that ensures fidelity of sister chromatid segregation after metaphase and initiation of cytokinesis.

29.14

The Human Cerebrospinal Fluid Phosphoproteome

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Protein phosphorylation is an important mechanism for the reversible regulation of protein function, a fact that is reflected by the high abundance of phosphoproteins in eukaryotic cells. Protein kinases, protein phosphatases and their phosphoprotein substrates are also critical components of the central nervous system. Consequently it is very likely that these protein subclasses are also represented in cerebrospinal fluid (CSF) that surrounds the brain and reflects its metabolic state. We have used CSF from patients suffering from normal pressure hydrocephalus to carry out a thorough phosphoproteome analysis using two dimensional gel electrophoresis and phosphoprotein-specific stains as well as shotgun mass spectrometry. In order to deal with the great complexity of peptides resulting from a proteolytic digest of CSF proteins we have used several protein prefractionation methods. Tandem mass spectrometry data of CSF-derived proteolytic peptides were searched against the human IPI database with phosphorylation at serine, threonine and tyrosine residues as variable modifications. The determination of the sites of phosphorylation in CSF proteins provides important information about specific kinase-substrate relationships and is thereby able to reveal insights into neurological disease mechanisms.
Protein and Proteome Phosphorylation Stoichiometry Determined by μLC-ICP-MS and Imaging Laser Ablation ICP-MS

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Protein phosphorylation is the functionally most versatile reversible post-translational modification. As a result, an additional heteroelement is attached to proteins, which is not present in proteinogenic amino acids. Therefore, element mass spectrometry (inductively coupled plasma-MS, ICP-MS) with phosphorus detection can be used to probe this important covalent protein modification. Sulphur can be used as internal standard, since it occurs in the proteinogenic amino acids cysteine and methionine.

Two different strategies were applied to evaluate the use of element mass spectrometry for quantitation of the phosphorylation stoichiometry, both starting from 1D-SDS-PAGE as the first purification step. One strategy resembles the established analytical proteomics protocol, consisting of in-gel digestion, peptide extraction and LC-MS analysis using ICP-MS as element-selective detector. Phosphorus and sulphur were monitored, and the P/S ratio was used as measure for the protein phosphorylation stoichiometry. Whereas this method is based on the analysis of proteolytic peptides, intact proteins are directly assessed in the second approach. For this purpose, proteins are blotted onto a PVDF membrane and analyzed by imaging laser ablation (LA) ICP-MS. Both strategies were evaluated using reference proteins (casein, ovalbumin) and provided very similar results.

To compare the global phosphorylation stoichiometry of different cellular proteomes, both methods were applied to cytosolic protein precipitates of pro- and eukaryotes. For the bacterium Corynebacterium glutamicum values of 0.005–0.02 (mol P/mol protein) were observed. Investigations of eukaryotic cells (Mus musculus) consistently showed higher values (0.25–0.8 mol P/mol protein). Proteomes of cell cultures (3T3 cells) showed somewhat higher values than tissue samples (epidermis, papillomas). In contrast to established methods, the presented methodology requires no isotopic labelling step and may be useful in cancer research, since the phosphorylation status of many proteins is higher in the mitotic phase compared to the interphase.

Revival of Negative Ion Electrospray Tandem Mass Spectrometry in Protein Phosphorylation Analysis

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Positive ion electrospray MS/MS is a key analytical technology for recognition of protein phosphorylation at serine (S), threonine (T) and tyrosine (Y). One important feature in this context is the loss of H₃PO₄ from pS- and pT-peptides. However, this neutral loss is strongly sequence- and charge state-dependent, limiting the recognition and counting of phosphate groups in pS- and pT-peptides. In addition, neutral loss analysis is not useful for pY peptides. Special features of negative ion MS/MS of phosphopeptides comprise the formation of [PO₃]⁻ and [H₂PO₄]⁻ ions from pS, pT, and pY peptides, as well as the abundant loss of H₃PO₄ from pS- and pT-peptides. In our studies for additional phosphopeptide-specific fragment ions in the negative ion mode, abundant but currently unnoticed high-mass fragments were observed from multiply charged precursors. These fragments are highly useful specific marker ions for phosphopeptide analysis, since (i) they are completely absent in the spectra of their nonphosphorylated analogs, (ii) they consistently occur in an m/z region with very low background, (iii) they are generated with equal efficiency by pS-, pT-, and pY-peptides, (iv) they are generated at low to moderate collision offsets, (v) they allow the counting of the numbers of phosphorylated residues present, (vi) they often allow the differentiation between pS-, pT-, and pY-residues, and (vii) QTOF parent scans or LC-MS/MS data may be automatically searched for these ions using a “variable neutral gain” function. In view of these observations and in view of first analyses using these fragment ions as phosphopeptide-specific probes, negative ion MS/MS may experience a revival in protein phosphorylation analysis in the near future.
29.17

Investigation of Tyrosine Phosphorylation Triggered by LPS Stimulation in Drosophila Immune Cells

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Protein tyrosine phosphorylation has a prominent role in the coordination of signal transduction in eukaryotes after extracellular stimuli. Interaction of bacterial lipopolysaccharides (LPS), a component of the outer cell wall of Gram-negative bacteria with Drosophila immunocompetent cells represent a model system for studying tyrosine phosphorylation and activation of innate immune reactions.

To identify and characterize proteins that were phosphorylated/dephosphorylated in Drosophila cell line in response to immune challenge we used the following strategy. An immobilized Phospho-Tyrosine monoclonal Antibody (CST, USA) was used for enrichment of Tyr-phosphorylated proteins. Phosphorylated proteins eluted from this affinity sorbent were separated by two-dimensional gel electrophoresis (2-DE) and transferred to nitrocellulose membrane. The membranes were then probed with Phospho-Tyrosine antibody. The Tyr-phosphorylation patterns were matched with the total proteome map. After assignment of phosphoproteins to their corresponding spot on the preparative gels the proteins were identified by MALDI-TOF MS and ESI-MS/MS and database search.

The identified targets include several receptor signaling proteins with tyrosine kinase activity associated with the JAK/STAT cascade, proteins involved in cytoskeleton regulation, defense and stress responses. LPS treatment of mbn-2 cells induced phosphorylation of several chaperonins, the subunits of Drosophila CCT (TCP) complex. Eukaryotic CCT proteins are absolutely required for folding of ~10% of newly synthesized proteins. Among CCT substrates are tubulin, actin, actin-binding proteins, cyclins, cell division control proteins, histone deacetylases and others. Our present work on the differential phosphorylation in Drosophila mbn-2 cells is shedding new light on the signaling processes connected with innate immune reactions.

29.18

Insulin-dependent Tyrosine-phosphorylated IRS-1 Characterized with CAPLC-Q-TOF Mass Spectrometry

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Phosphorylation of insulin receptor substrate 1 (IRS-1) by insulin receptor kinase occurs at TyrXXMet (YXXM) and TyrMetXMet (YMXM) motifs. Tyrosine-phosphorylated IRS-1 binds specifically to cellular proteins containing Src homology 2 (SH2) domains. Thus, IRS-1 is located in the middle of upstream link to the insulin receptor and downstream links to various regulatory processes. In insulin-sensitive tissues, such as solues muscle, insulin significantly boosts PI 3-kinase activity in IRS-1. A new tandem mass spectrometric approach coupling with capillary liquid chromatography allows one to pin-point the substoichiometrically phosphorylated tyrosine (p-Y) residues in control and insulin stimulated rats. IRS-1 is immunoprecipitated with mAb (3 mg, 05–699, Upstate) from solues muscle homogenates (50 mg of total proteins). A total of 14 spots, with the same MW but different pI crossing in the range from 6.5 to 8.5, reveal high heterogeneous phosphorylation patterns in 2D gel electrophoreses. These multistate phosphorylations which cause apparent pI shifting towards to the anode (calculated pI = 9.0 without phosphorylation) are mainly attributed to a number of different phosphorylated Ser and Thr residues. However, limited p-Tyr residues are observed in twist patterns: p-YICM (Y460) and p-YY (46, 47) in control (N = 4 from 40 rats); on the other hand, p-YMPM (Y628) and p-YADM (Y1010) in the C-terminal region with high intensified MS counts are observed in the insulin stimulated solues muscle (N = 2 from 20 rats). These insulin dependent changes in the p-Tyr patterns may provide the potential sites of drug treatment for type 2 diabetes therapy.

29.19

Quantitative Phosphoproteomics Applied to the Epidermal Growth Factor Receptor Pathway

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Virtually all signal transduction pathways in eukaryotic cells are regulated by post-translational modifications (PTMs) such as reversible protein phosphorylation. Protein phosphorylation is also known to be both spatial and temporal in its distribution; it is therefore advantageous to develop methods for identification and quantitation of individual phosphorylation sites in different cell states.

Here we present an LC-MS based quantitative phosphopeptide-specific approach that combines stable isotope labeling by amino acids in cell culture (SILAC) for quantitation with strong-cation exchange (SCX) and titanium-chromatography (TIO2, Titansphere) for phosphopeptide enrichment and state-of-the-art FTICR mass spectrometry for identification. We utilize the high mass accuracy and high-dynamic range of the Finnigan LTQ-FT in FTICR-mode by using selected ion monitoring (SIM) scans while simultaneously acquiring MS/MS and neutral loss dependent MS3spectra in the linear ion trap.

Based on the accurate parent mass and tandem MS as well as MS3 fragments phosphopeptides are identified via protein sequence database matching with very high confidence. This enables sequencing and thereby identification and quantitation of thousands of peptides in single analysis when combined with online nanoscale liquid chromatography (nanoLC-MS).

We have applied this methodology to EGF-stimulated HeLa cells using different activation time-points and identified several known as well as unknown differentially regulated phosphorylation sites in the pathway including the EGF receptor itself and downstream signaling molecules like Shc, STAT5, Ymer, ERK1 and ERK2, etc.
**29.20**

**Differentiation of Phosphorylated and Sulfated Peptides Using a Novel API-QTOF Technology**

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Phosphorylation and sulfation influence the functionality of proteins and peptides. Sulfation is a permanent modification critical to the function of the protein. The neuropeptide cholecystokinin-8-sulfate, for example, has been reported to cause schizophrenia. To determine the number and sites of PTMs of proteins and peptides mass spectrometry based methods have been used over the last period. The mass difference between phosphorylation and sulfation is only 9.5 mDa. Here we show the differentiation of phosphorylation and sulfation based on mass accuracy, isotopic pattern information, and separation.

A new hybrid QTOF with exact measurement of the isotopic ratio is used for the experiments. Samples are introduced via LC or capillary zone electrophoresis (CZE). Tyr[SO3H]Cholecystokinin fragment 26–33 Amide (Sigma Aldrich), Tyr[PO3H2]Cholecystokinin fragment (Bachem) and the nonsulfated Cholecystokinin fragment 26–33 Amide (Sigma Aldrich) are used as model substances.

We present a method based on combined information of accurate mass and isotopic pattern for unambiguous differentiation between phosphorylation and sulfation on the intact peptide level as well as on fragments for determination of the modification site. The new TOF detector technology is able to reproduce the correct isotopic pattern over a wide dynamic range which is used in a new algorithm for determination of the elemental composition from a given mass spectrum. To reduce the number of possible candidates for a given m/z the accurate mass of all isotopes, their distance and ratio are taken into account and compared to the measured pattern. In case of isobaric phosphorylated and sulfated peptides the described method is used for differentiation between both modifications on the level of the intact peptide mass as well as on MS/MS information for localisation of the modification site.

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**29.21**

**Detection of Phosphopeptides from Complex Mixtures Using Titanium Oxide Precolumns and FT-ICR Mass Spectrometry**

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The reversible phosphorylation of proteins ranks amongst the most important post-translational modifications that occur in the cell and the characterization of phosphorylation events is one of the many tasks to be accomplished in the post-genomic era. Selective detection of phosphopeptides from complex mixtures is a challenging and highly relevant task in many proteomics applications. Often phosphopeptides are present in sub-stoichiometric amounts and need selective isolation/enrichment before identification. We introduced a novel method for the selective isolation of phosphopeptides from complex mixtures using a titanium oxide pre-column, which is able to absorb organic phosphates in acidic conditions and desorb them in alkaline conditions (Pinkse et al., *Anal. Chem.* 2004, 76, 3935–43). This approach allows for the selective enrichment of phosphorylated peptides at the femtomole level. Even with this powerful enrichment strategy the successful identification of phosphorylation sites of “unknown” phosphoproteins is still hampered by the lack of sufficient backbone fragmentations of phosphopeptides. In particular phosphoserine and phosphothreonine containing peptides reveal only the predominant loss of phosphoric acid upon collision-induced dissociation. In the present study we have combined the phosphopeptide enrichment method with the MS n capabilities of the linear ion trap, together with the high mass accuracy of FTICR mass spectrometry using a LTQ-FT. Thereby, neutral loss driven MS3 and accurate mass measurement provide an extra contribution to specificity. The selectivity and practicality of using this combined approach is demonstrated via the analysis of several model peptide and protein systems. In addition, selective enrichment and detection of phosphorylated peptides from more complex peptide mixtures will be presented.
Investigation of Differential Phosphoproteome Expression in Rat Primary Neurons following Amyloid-beta Peptide Treatment

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Alzheimer’s disease (AD) is a progressive and incurable neurodegenerative disorder. Although early cellular events leading to degeneration of neurons are still largely unknown, it is generally accepted that protofibrils/plaques comprised of amyloid-
\(\beta\) (A\(\beta\)) peptides are causing the disease. It was also demonstrated that alteration of the protein phosphorylation machinery occurs in brains of patients as shown by dysregulation of enzymatic activity of several kinases such as GSK3\(\beta\), cdk5, Fyn, ERK and MEK1 culminating in hyper-phosphorylation of the microtubule associated protein tau, a classical AD hallmark. In this study a proteomics approach using DIGE\(\text{®}\) technology was employed to investigate variations occurring at the phosphorylation level in primary rat neurons as a consequence of short (5 minutes) and medium time (4 hours) exposure to the neurotoxic A\(\beta\) peptide (25–35). Prior to 2D-DIGE analysis protein samples were enriched in phosphorylated proteins using immobilized metal affinity chromatography (IMAC) which should facilitate identification of less abundant proteins. Three independent preparations of rat cortical neurons were analyzed in a single DIGE experiment designed to take into account both, technical and biological variation as well as dye swapping. Analysis of gel images using the BVA module of DeCyder software, detected a total of 40 spots varying in abundance of at least 1.3 fold at 5 minutes and/or at 4 hours with a false discovery rate (FDR) \(\leq 0.12\). Selected spots were automatically excised from a preparative gel and identified by ESI-MS/MS. Amongst the identified proteins were several structural proteins, enzymes and receptors which will be assembled into a “pathopathway” of AD. This should further the understanding of the mechanisms of amyloid-
\(\beta\) mediated neurotoxicity.

Analysis of Phosphorylated Membrane Proteins in Human Hepatocytes

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In the present differential proteome study phosphorylated membrane proteins from hepatocytes (control and drug-induced) are analysed for the enlightening of drug degradation in the liver. For this purpose several new methods have to be established for the separation, detection and identification of phosphorylated membrane proteins. To date different preparation methods were applied and checked for applicability, specificity and protein recovery like extraction using Triton X-114 by phase separation or differential centrifugation. Several detergents have been tested to solubilize the membrane pellets won by differential centrifugation. Furthermore several newly developed gel-based two-dimensional separation techniques for protein separation were used and compared – for example 2D-CTAB/SDS-PAGE and double-SDS-PAGE. Analysis of phosphorylated membrane proteins was done using the phosphospecific ProQ Diamond stain (Invitrogen) followed by spotpicking and in-gel digestion with several enzymes. Localization of phosphorylation sites was realized by mass spectrometry using a 4000 Q Trap.

First results have shown that the extraction of membrane proteins with Triton X-114 resulted in worse protein recoveries in contrast to differential centrifugation methods. CHAPS, SDS and CTAB appeared as the best detergents to solubilize the membrane pellet after centrifugation. The compatibility of the extraction methods with the newly developed two-dimensional separation techniques could be demonstrated. A problem is situated in digesting the membrane proteins for further characterization. Because of their physical and chemical character standard protocols are hardly applicable. Our experiments confirmed this. The digestion with Trypsin as a stand-alone enzyme partially resulted in bad sequence coverages in MS and MS/MS experiments. Indeed, several different membrane and membrane-associated proteins as well as some phosphorylation sites could be identified confirming the power of the methods applied.
A new high-throughput methodology to analyse protein phosphorylation patterns in the moss Physcomitrella patens after stimulation with the phytohormone cytokinin has been developed. Phosphorylation rates of the proteins are investigated at the peptide level using a combination of HPLC and capillary electrophoresis (CZE) (Ficarro et al., Nature Biotechnology, 2002, 301–305). Total protein extracts from the samples of interest are digested by a specific protease and phosphorylated peptides are isolated by IMAC (Fe3+ technology, 2002, 301–305). Total protein extracts from the samples of interest are identified by partial sequencing of the corresponding phosphorylated peptides using MS/MS and search in a specific Physcomitrella EST-derived database.

Modulation of TCTP Oligomerization through PLK-dependent Phosphorylation


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Translationally controlled tumor protein (TCTP) is a highly conserved and abundantly expressed protein. Although the cDNA sequences for both the murine and human protein were published in the late eighties, the precise function of the family remains elusive. TCTP is reported to possess a precise function of the family remains elusive. TCTP is reported to possess abundant expression and is overexpressed in cells on stimulation with a combination of mass spectrometry techniques (Heintz et al., Electrophoresis 2004, 25, 1149–1159). The separation techniques allow the rapid comparison of different samples with high reproducibility and sensitivity. Phosphorylation patterns are investigated at different time points after hormone stimulation in order to establish a precise time course and relative quantification of the cytokinin-induced phosphorylation events in Physcomitrella. Comparative analysis of these patterns leads to the characterisation of peptides whose phosphorylation state is related to the hormone signal. Subsequently, proteins of interest are identified by partial sequencing of the corresponding phosphopeptides using MS/MS and search in a specific Physcomitrella EST-derived database.

Detection of Novel Tyrosine-phosphorylated Proteins in Macrophages by Proteomic Analysis

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Disregulated production of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) has been implicated in the pathogenesis of rheumatoid arthritis. In the rheumatoid joint, TNF is mainly produced by macrophages stimulated by physical contact with activated T cells. Inhibitor studies and confocal microscopy suggest that these contact-dependent effector functions are mediated by tyrosine phosphorylation, through which cell surface proteins are phosphorylated is unknown. We have developed a novel two-stage purification strategy, in which cell-surface biotinylated and streptavidin affinity capture is used in combination with anti-phosphotyrosine antibodies to recover tyrosine phosphorylated proteins. Phosphotyrosine-containing proteins were immunoprecipitated from plasma membrane, nuclear and cytosolic fractions of pervanadate-stimulated RAW264.7 cells. Bound proteins were eluted with phenyl phosphate and separated by solution-phase isoelectric focussing and 1D gradient gel electrophoresis. Silver-stained bands were excised digested in gel using an Investigator ProGest system, and were characterized by tandem mass spectrometry. More than a hundred phosphoproteins or their interaction partners were identified, including three with no assigned function, which have not hitherto been detected as expressed proteins. We have expressed FLAG- and GFP-tagged versions of two of these in HeLa and RAW 264.7 cells. Both proteins were inducibly tyrosine phosphorylated upon challenging the cells with perva expandate, validating the results of the proteomic study. One of these candidate proteins was inducibly tyrosine phosphorylated in HeLa cells following stimulation with TNF-α and MAPK inhibitors. RAW 264.7 cells. Both proteins were inducibly tyrosine phosphorylated upon challenging the cells with pervanadate, validating the results of the proteomic study. One of these candidate proteins was inducibly tyrosine phosphorylated upon challenging the cells with pervanadate, validating the results of the proteomic study. One of these candidate proteins was inducibly tyrosine phosphorylated in HeLa cells following stimulation with TNF-α and MAPK inhibitors.
Comparing the Tandem Mass Spectra of Phosphorylated Peptides Acquired by Using a Different Type of Mass Spectrometer

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Mass spectrometry has become the commonly used technique for phosphopeptides analyzing and phosphorylated sites defining. Quadrupole time-of-flight, quadrupole ion trap and MALDI-TOF-TOF are three in common used tandem MS for phosphorylated peptides sequencing in phosphoproteomics. Here we report the data of comparing the tandem mass spectra acquired from phosphopeptides by using three different mass spectrometers. The phosphopeptides from trypsin digest of standard proteins were analyzed by using LC-ESI-q-tof Micro, LC-ESI-LCQ deca XP and 4700 MALDI-TOF-TOF, respectively. Some phosphopeptides from human fetal liver and human chang’s cell were enriched by using immobilized metal affinity chromatography (IMAC) and analyzed by using one or two type of tandem MS. The preliminary data showed that all three types of phosphorylated peptides (phospho-serine, phospho-threonine and phospho-tyrosine) could generate predominant peaks of neutral loss of phosphoric acid in MALDI-TOF-TOF spectra. And most phospho-serine/threonine peptides could produce strong neutral loss peaks in ion trap spectra. While only a small part of phospho-serine/threonine peptides could generate the visible neutral loss peaks in quadrupole-tof spectra and these phospho-peptides normally produce poorer fragment ion peaks. Many phospho-serine/threonine peptides analyzed by using q-tof MS could generate good fragment ion peaks but their neutral loss peaks was invisible. Some of these peptides were analyzed by using MALDI-TOF-TOF and all of them could generate clearly visible neutral loss peaks. This result indicates that the commonly used strategy in phosphoproteomics for selecting phosphopeptide candidates from ion trap data by picking out data containing neutral loss peaks maybe not suit for data acquired by using q-tof MS.