

Posters

Abstracts of posters are listed alphabetically by first author's name

Outer membrane proteome of *Myxococcus xanthus*: In search of the C-signal receptor

Aguiluz K, Hedderich R

Max Planck Institute for Terrestrial Microbiology

The Gram-negative soil bacterium *Myxococcus xanthus* is a model organism for studying social behaviour. In the presence of nutrients, it feeds cooperatively using extracellular digestive enzymes. When the nutrients become depleted, *M. xanthus* undergoes a developmental program in which the cells aggregate in order to form fruiting bodies. Within these fruiting bodies, cells differentiate into spores. The exchange of two extracellular signals (A and C-signal) is required to execute normal development. The C-signal is expressed 6 hours upon starvation and has been described as a developmental timer because different thresholds allow aggregation, fruiting bodies formation and sporulation. Mutants defective in C-signalling were localized in the *csgA* gene. The *csgA* gene product is a 25-kDa protein (p25). This protein is processed by a protease to a 17-kDa protein (p17). It has been demonstrated that the p17 protein, which is an outer membrane-associated protein, is the C-signal. C-signal transmission in *M. xanthus* involves a cell to cell contact dependent mechanism. According to our working model this requires the interaction of p17 with a cell surface exposed receptor. This receptor is thought to be part of a signalling cascade that transmits the signal from the cell surface through the cell envelope to the cytoplasm of the responding cell. The aim of this project is the identification of the C-signal receptor using a proteomic approach. We are developing two methods: biotinylation of cell surface proteins and enrichment of outer membrane proteins by biochemical pre fractionation. We compare the outer membrane proteome of vegetative and developmental cells in order to find the differentially expressed proteins using Difference Gel Electrophoresis (DIGE) and nano-LC plus MS/MS.

Discovery by advanced proteomic and bioinformatic techniques of serum proteins and protein clusters that are related to age-related skeletal diseases. A novel approach in bio-gerontology.

Alves R¹, Luider TM², van der Spek P³, Hofman A⁴, Pols HA¹, van Leeuwen JP¹

¹Department of Internal Medicine, Erasmus MC, Rotterdam

²Department of Neurology and Center for Biomics, Erasmus MC, Rotterdam

³Department of Bioinformatics, Erasmus MC, Rotterdam

⁴Department of Epidemiology Biostatistics, Erasmus MC, Rotterdam

The increase of life span is accompanied by an increase of age-related diseases that greatly reduce the quality of life and constitutes a serious economic and healthcare burden. Thus, it is essential to develop diagnostic methodologies to detect and follow aging phenomena. Serum proteome, besides the „classical proteins“, contains tissue proteins that normally function within the cells but can be released into plasma as a result of cell death or damage. This group of proteins hold the promise of disease diagnosis and therapeutic monitoring. However, the analysis of serum is analytically challenging due to the high dynamic concentration range, more than 10 orders of magnitude, that separate albumin and the rarest proteins now measured clinically. To address this problem, serum complexity will be reduced by depletion of six high-abundant proteins (albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin) using the antibody-based Multiple Affinity Removal column. For the age-related disease under study, osteoporosis, three phenotypic characteristics will be used to set up the study groups: 1) bone mineral density (high vs. low), and absence/presence of 2) vertebral fractures, and 3) hip fractures. We will start with about 50 serum samples, per group that after depletion will be digested with trypsin and measured either by mass spectrometry (MS) or by MS/MS. MS data will be analyzed by two different methods. Firstly, a „real unbiased“ protein clustering and pathway profiling and, secondly, a „guided-unbiased“ approach based by data on known serum markers of bone turnover. Next, clusters of proteins and/or pathways identified will be related and compared between the bone and calcium phenotypes under study, using standard statistical tools. Discriminative proteins and/or protein profiles will be selected for confirmation using Western blot analyses and developing multiplex assays. With this project we hope to identify a novel therapeutic target and develop new assays to patient care and also to get solid basis for further functional studies in which our human osteoblast bone formation model will be an important tool.

Development of innovative sensor and protein-protein interaction technology for high-throughput proteome analysis

Baumann M, Li X, Palme K

University of Freiburg, Institute for Biology II

While there is no doubt that DNA microarrays provide very valuable information on gene expression patterns on a global scale, their cellular resolution and accuracy are limited to the level of tissue dissection. This project concentrates on the development of innovative protein/antibody array technology for the detection of cell proteins at the highest sensitivity currently possible. Arrays of antibodies that bind to specific proteins will allow visualization of the subset of proteins that are actually produced in various tissues or cells, thus overcoming limitations inherent to current electrophoretic separation technology. A proof-of-concept for a high-affinity sensor antibody chip in combination with ultra-sensitive detection technology will be established. In the frame of this project a large number of proteins has been isolated following by poly- and monoclonal antibodies generation. The current step is antibody characterization and purification. The quality of the antibodies has been assessed by using Western blotting and immunolocalization technology. More than 200 monoclonal antibodies (from 89 different antigens), and 172 polyclonal antibodies (86 antigens) were tested and for the positives, immunodetection was performed. These antibodies will be useful tools for high-resolution microscopy and sensor chip technology. In addition screening tools were developed for largely automated screening of the cells proteome.

Proteomics interdisciplinary: comparison of complex protein mixtures of fungal organisms

Bernauer T¹, Langer E¹, Herberg F²

¹Universität Kassel, Fachbereich 18 Naturwissenschaften, Abteilung Ökologie

²Universität Kassel, Fachbereich 18 Naturwissenschaften, Abteilung Biochemie

The aim of the study is the description and analysis of protein mixtures of the xylobiontic fungus *Phanerochaete chrysosporium* for the first time. The decoding of the genome of this Ligno-Cellulose decaying fungus by Martinez et al. (2004) enabled the utilization of the proteomic research to solve ecological and evolutive questions. Total protein extracts from pure cultures will be examined analysing the protein expression during the fermentation in liquid cultures over their natural substrate (wood pellets) with the 2D polyacrylamide gel electrophoresis under different parameters, e.g. temperature, pH or nutrient offering in several kinds of wood. Furthermore the extracellular protein complex, responsible for the hydrolysis of the cellulose and gained from the supernatant of the fermentation, would be exemplary examined for the functional clarification of the existing enzymes of *Phanerochaete chrysosporium*. Alternatively it will be tested if the protoplasts can be generated under above culture conditions because they are reliable and more suitable for the extraction of the intracellular protein expression. The enzyme expression of *Phanerochaete chrysosporium* which has been specialized to different substrates (e.g. *Picea* and *Betula*) or has broad geographical distribution (North America, Europe) will also be compared with already available data of ITS-rDNA-sequences for testing the usage of protein expression patterns in evolutive questions. Finally a computer-assisted comparison of the obtained 2D-Gelstructures with public available proteomic tools will be performed.

Phosphorylation pattern analysis on PKA catalytic subunit

Bertinetti O, Hutschenreiter S, Herberg FW

Kassel University, Department of Biochemistry

Protein phosphorylation is one of the most important posttranslational modifications (PTMs), especially for the regulation of eukaryotic signal transduction pathways. One goal of current proteomic efforts is to qualitatively and quantitatively describe all phosphorylation sites of a protein. Recent developments have focused on technologies for enriching and quantifying phosphopeptides. We used side by side two approaches for phosphopeptide mapping to interrogate a protein sequence directly with a protease that recognizes phosphorylation sites (Knight et al. 2003; Arrigoni et al. 2005) based on beta-elimination/Michael addition for the derivatization of phosphoserine and phosphothreonine residues into lysine analogs. The modified proteins can be cleaved with a lysine-specific protease at the isosterically substituted amino acids. The inserted cleavage site yielded specific fragments reflecting the phosphorylation sites in the native protein. To localize the putative phosphorylation sites in peptides with more than one possible phosphorylation site we used a hybrid quadruple linear ion trap instrument (Q TRAP 4000™). We analyzed fragmentation in the Q2 LINAC™ collision cell and the Q3 linear ion trap separately. Fragments generated by collision with nitrogen in Q2 produced unclear findings in contrast to excitation frequency fragmentation in Q3. In Q3 peptide fragmentation occurs subsequent to the phospho-amino acid and helps to resolve the position of the phosphorylation. Recombinant catalytic subunit of cAMP-dependent protein kinase (PKA-C) was used as a model protein in all experiments. This enzyme serves as a prototype for the entire protein kinase family and is best characterized structurally and biochemically. To investigate the phosphorylation status of the recombinant PKA-C (mouse C), the phosphorylation pattern of the intact protein was determined. Up to six phosphorylation sites on the enzyme overexpressed in *E. coli* were identified using nanoLC-ESI/MS.

Molecular Studies on Pre- and Postsynaptic Plasticity: Characterisation of the Munc13-Calmodulin interactions by photoaffinity labelling & Proteomic analysis of mice lacking the ubiquitin ligase Nedd4-1

Dimova K, Brose N, Jahn O

Max-Planck-Institute for Experimental Medicine, Proteomics Group & Dept. Molecular Neurobiology

Sensing of and response to differential Ca^{2+} currents at the active zones of presynaptic terminals is an important adaptive mechanism defining the short-term presynaptic plasticity of neuronal cells. Even though many of the proteins regulating synaptic transmitter release have been identified, the exact mechanisms by which the Ca^{2+} signals are integrated into a cellular response remain unclear. The identification of Ca^{2+} -dependent calmodulin/Munc13 interactions suggested a Ca^{2+} sensor role for calmodulin in the modulation of Munc13-regulated synaptic vesicle priming. Based on photoaffinity labelling studies of the complex formation between CaM and Munc13-derived peptides, the aim of the on-going structural analysis of the CaM photoadducts is to identify whether the Munc13-derived peptides interact via the known 1-5-10 or 1-8-14 CaM binding motifs as proposed or use other binding sites. For the differential analysis of CaM and photoadduct in-gel digests, we have established a LC-MALDI technique which allows for mass spectrometric peptide sequencing from complex mixtures without signal suppression effects observed in peptide mass fingerprints and without the time limitations of online LC-MS. Mapping the CaM binding sites for the different Munc13 isoforms may give us further structural insights underlying the differential roles that these isoforms have in controlling short-term presynaptic plasticity. Adequately timed and spaced protein turnover is essential for sustaining the cellular dynamics of nerve terminals in the processing and storage of information. Many recent studies are recognizing the important role of the ubiquitin proteasome system (UPS) in the regulation of synaptic function and efficacy but details on the substrate specificity of the various UPS enzymes are still unknown. We use a knockout mouse model to identify synaptic targets of Nedd4-1, a HECT-type E3 ubiquitin ligase and apply a combination of MS-based comparative quantitative proteomic techniques (enrichment of ubiquitinated proteins, HysTag-labelling, as well as 2D-DIGE and 16BAC/SDS-PAGE analysis of KO and WT samples) in order to detect differential protein level regulation.

Genomic and Proteomic Analysis of *Pichia pastoris* cell cultures

Dragosits M, Gasser B, Maurer M, Mattanovich D

University of Natural Resources and Applied Life Sciences, Vienna

Pichia pastoris is a widely used host organisms for heterologous protein production. Although protein overexpression has been established since more than 20 years, production of specific protein still remains much of a trial and error effort. Recent studies on genome wide gene regulation in micro-organisms have demonstrated that many physiological reactions, including environmental stress and protein folding/aggregation are highly interrelated. Among environmental factors that affect protein expression and secretion, especially temperature, pH, osmolarity and oxidative stress may play an important role. Initial experiments analyzing the transcription of a subset of genes proved a strong impact of temperature on protein production. *Pichia pastoris* will be used as a model organisms for the expression of a complex protein. In our case a mAB Fab fragment will be used. Protein expression yield under various culture conditions, such as different temperatures and low and high osmolarity, will be determined accompanied by DNA microarray analysis and 2D-DIGE to identify physiological bottlenecks hampering protein secretion in *Pichia pastoris* cultures.

The Photomorphogenesis Regulator DET1 binds the promoters of Light-regulated genes

Dubin MJ, Ron M, Benvenuto G, Bowler C

Laboratory of Cell Signaling, Stazione Zoologica „Anton Dohrn“, Naples

Light provides a major source of information from the environment during plant growth and development. Light-regulated gene expression is partly controlled by the phytochrome photoreceptors, which once activated, are imported into the nucleus where they bind and activate transcription factors such as PIF3. Coupled with this, the degradation of positively acting intermediates such as the transcription factor HY5 by COP1 and other ubiquitin ligases acts to repress photomorphogenesis in the darkness. Another negative regulator of photomorphogenesis is DET1 (De-Etiolated 1), which forms part of a nuclear-localised complex with the plant homolog of UV-Damaged DNA Binding protein 1 (DDB1) and the E2 ubiquitin-ligase activating protein COP10 (Constitutive Photomorphogenic 10). DET1 binds chromatin via a direct interaction with the core histone H2B, suggesting that DET1 may repress light activated genes by interacting directly with their promoters. Here it is shown that DET1 forms part of a CUL4 based ubiquitin ligase complex and is localised to discrete foci throughout the euchromatin containing regions of nucleus and at the periphery of the nucleolus and nuclear envelope. Chromatin Immunoprecipitation experiments show that in the dark DET1 binds to the promoters of the light induced CAB2 and HEMA1 genes, and that this binding is abolished in the light, coincidental with the activation of these genes. Based on these data it is proposed that the DET1 complex binds the promoters of light-regulated genes in the dark and directly represses their transcription, either by chromatin remodeling and/or ubiquitin-mediated regulation of the transcriptional apparatus. Light causes the dissociation of the DET1 complex from these promoters, allowing activation of these genes.

Identification of differentially expressed proteins by 2D-DIGE/Mass spectrometry in keratin 5 promoter COX-2 transgenic urinary bladder

Dülsner E¹, Fürstenberger G¹, Schnölzer M², Müller-Decker K¹

¹Eicosanoids and Tumor Development Section

²Protein Analysis Facility, German Cancer Research Center, Heidelberg, Germany

Cyclooxygenase-(COX)-2 represents, beside COX-1, a key enzyme of prostaglandin biosynthesis. While the expression of COX-1 is constitutive in most tissues, COX-2 is transiently induced by many stimuli including cytokines, growth factors, and hormones. Constitutive over-expression and activation of COX-2 occurs in the course of regenerative hyperplasia, chronic inflammation, and epithelial carcinogenesis. Keratin 5 promoter-driven expression of COX-2 in basal epithelia of various organs, such as epidermis, urinary bladder, mammary gland, and pancreas causes the spontaneous development of hyperplasia, dysplasia, and in addition transitional cell carcinomas in the urinary bladder. The overall aim of the project is to unravel the molecular mechanisms contributing to these phenotypic changes. We choose a proteomic approach to detect differentially expressed proteins in wild-type (wt) and COX-2 transgenic urinary bladder. Two-dimensional (2D) gel electrophoresis based on isoelectric focussing (IEF) by means of precasted immobilized pH gradient strips (Immobiline Dry Strips pH 3-10, linear, 18 cm, loaded by reswelling technique, run by the Ettan IPGphor) and 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) using the ETTAN-Dalt 6 in the conventional mode and the recently established fluorescence difference gel electrophoresis (2D-DIGE) mode are applied to establish protein expression maps of normal and hyperplastic/dysplastic urinary bladder. After tryptic digestion of protein spots of interest, proteins were identified by matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry to receive peptide mass fingerprints of the individual proteins or by electrospray ionisation (ESI) MS/MS to obtain sequence information of tryptic peptides. Database searches with the measured monoisotopic peptide masses or with fragment ions from MS/MS experiments are performed against the NCBI non-redundant database using the peptide search routines provided by the Protein Prospector, ProFound, and MASCOT softwares. From the catalogue of proteins candidate proteins with pronounced differences in expression levels between dysplastic and normal bladder are currently validated using an independent cohort of dysplasias and matched controls.

Comparison of Large-Scale Human Protein Interaction Maps

Futschik ME¹, Chaurasia G², Wanker E², Herzel H¹

¹Institute for Theoretical Biology, Charité-Medical Division, Humboldt-University

²Max-Delbrück-Centrum, Berlin, Germany

Large-scale maps of protein interactions aim to constitute a scaffold for such comprehensive models. Recently, there have been a growing number of both experimental and computational efforts to gain systematical maps of human protein interactome. A major task will be the integration of these distinct protein networks. However, caution is required as studies of interaction maps in lower eukaryotes revealed a surprising divergence between different maps.

We present here a first critical and comparative assessment of eight different large scale human protein-protein interaction networks derived either from Y2H-assays, literature reviews or prediction based on interactions between orthologous proteins in other organisms. The analysis showed that the current maps have only a small, but nevertheless significant, overlap. We detected strong sampling and detection biases linked to the method of generating the maps. We also observed that some previous conclusions for network structures in lower eukaryotes cannot be simply extrapolated to humans. For example, the conjecture that the number of interaction is correlated with essentiality of proteins cannot be supported. This and other results suggest that the structure of interactomes of higher eukaryotes might differ substantially from those for lower organisms and, thus, general re-evaluation of concepts regarding network structure and evolution may be warranted. Furthermore, we found that network hubs can be divided into different evolutionary categories: Ancient hubs include proteins of core machineries as the proteasome and the polymerase whereas evolutionary novel hubs are mainly involved in signal transduction and regulation. This classification suggests that the current theory of simple preferential attachment may be not sufficient, but that network hubs have arisen to meet the particular requirements of an organism.

Dissecting of phosphorylcholine-modified proteins in different developmental stages of *Caenorhabditis elegans*

Grabitzki J¹, Ahrend M², Geyer R¹, Lochnit G¹

¹Justus-Liebig University Giessen, Medical Faculty, Institute of Biochemistry, Giessen, Germany

²University of Bonn, Institute of Animal Sciences, Bonn, Germany

The free-living nematode *Caenorhabditis elegans* has been found to be an excellent model system for developmental studies, investigating parasitic nematodes and drug screening. Structural analyses of glycoconjugates derived from this organism revealed the presence of nematode specific glycosphingolipids of the arthro-series, carrying, in part, phosphorylcholine (PC) substituents. PC, a small haptenic molecule, is found in a wide variety of prokaryotic organisms, i. e. bacteria, and in eukaryotic parasites such as nematodes. There is evidence that PC-substituted proteins and implicated glycolipids are responsible for a variety of immunological effects invasion mechanisms and long-term persistence of parasites within the host. In contrast to PC-modified glycosphingolipids, only a limited number of PC-carrying (glyco)proteins were identified so far [1-3]. We have analysed the expression of PC-modified proteins of *C. elegans* during developmental stages using 2D-SDS-PAGE separation, 2D-western-blotting and MALDI-TOF MS. The pattern of PC-modified proteins was found to be stage specific. The PC-modification on proteins was most abundant in the egg and dauer larvae-stages followed by the adult-stage and L4. Only small amounts of the PC-substitution were found in L3 and L2. In L1 we could not detect any PC-modification. The prediction of the cellular localisation of the identified proteins revealed a predominantly cytosolic and mitochondrial occurrence of the PC- modification. Most of the identified proteins are involved in metabolism.

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The phosphoproteome of *Bacillus subtilis*

Gronau K, Becher D, Hecker M

EMAU Greifswald

Proteinkinases and proteinphosphatasen spielen eine wichtige Rolle in Signaltransduktionsereignissen in allen Organismen. Die Mehrheit der Bakterien hat zahlreiche Zwei-Komponenten-Systeme, die Histidin- und Aspartatphosphorylierung, zum Beispiel wie das Phosphotransferasesystem des Zuckers, für den Transport. Die hochenergetische Phosphorylierung ist chemisch instabil und daher schwierig zu analysieren. Durch die vollständige Sequenzierung von Bakterien-Genomen wurde es offensichtlich, dass es viele unbekannte Kinasen und Phosphatasen gibt. Am wahrscheinlichsten können sie Tyrosin-, Serin- oder Threoninreste phosphorylieren und dephosphorylieren. Es ist sehr wahrscheinlich, dass dies ein neues und innovatives Feld der Bakterienphysiologie eröffnen wird. Das Ziel der Doktorarbeit ist es, die Funktion von bisher unbekannten Proteinkinasen/-Phosphatasen in *Bacillus subtilis*, dem Modellorganismus der funktionellen Genomik gram-positiver Bakterien, zu klären. Die wichtigsten Schritte sind die Beschreibung des Phosphoproteoms, die Anreicherung von Proteinkinasen/-Phosphatasen sowie die Untersuchung von Mutanten unbekannter Proteinkinase-/Phosphatase-Gene im Detail. Das Ergebnis wird eine detaillierte Analyse der Funktion von ausgewählten Proteinkinasen/-Phosphatasen sein, die das Netzwerk der Signaltransduktionsprozesse in *B. subtilis* beschreibt. Mit Hilfe von 2D-Gelelektrophorese, spezifischen Färbemethoden sowie Massenspektrometrie wurde eine Phosphoproteomkarte erstellt. Es ist möglich, das Phosphoproteinmuster von wachsenden Zellen und Zellen unter verschiedenen Stress- und Hungerbedingungen im Detail zu analysieren. Außerdem werden die Proteinphosphorylierungs- und -synthesemuster von Mutanten mit dem Wildtypmuster verglichen, um mögliche „Ziel“-Proteine von Proteinkinasen/-Phosphatasen zu finden. Ein weiterer Punkt von Interesse ist die Entwicklung analytischer Methoden zur Anreicherung von Phosphoproteinen sowie Proteinkinasen/-Phosphatasen.

Role of Tyk2 in systemic responses to endotoxin: a serum proteomics study

Grunert T¹⁻⁴, Marchetti M¹, Miller I², Strobl B^{3,4}, Karaghiosoff M³, Gemeiner M², Allmaier G¹, Müller M^{3,4}

¹Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria

²Institute of Medical Chemistry, University of Veterinary Medicine, Vienna, Austria

³Institute of Animal Breeding and Genetics, University of Veterinary Medicine, Vienna, Austria

⁴Research Center for Biomodels Austria, University of Veterinary Medicine, Vienna, Austria

Tyrosine kinase 2 (Tyk2) belongs to the Janus kinase (Jak) family and is involved in the signal transduction of a number of cytokines and growth hormones. Tyk2-null mice are viable, fertile and do not show any gross abnormalities in the development of the hematopoietic system. Although Tyk2 deficiency leads to only partial impairment of cytokine signaling, *in vivo* pathogen responses are strongly affected. Tyk2-null mice are, in contrast to wildtype mice, resistant to high-dose lipopolysaccharide (LPS) induced endotoxin shock.

Within the present project, the role of Tyk2 in systemic responses to LPS challenge is investigated with a proteomic approach. This includes two-dimensional electrophoresis of differently collected serum samples from wildtype and Tyk2-deficient mice before and after LPS challenge. Protein spots significantly different in abundance, newly present or totally absent in the absence of Tyk2 will be identified by in-gel tryptic digestion, vMALDI peptide mass fingerprinting (PMF) and tandem mass spectrometric-generated sequence tags for protein identification. In a first approach unfractionated serum is analysed by the outlined strategy. This allows to monitor changes of high and medium abundance proteins, including those known to be involved in acute phase reaction. Using MS-compatible silver staining for gel electrophoretic detection, we have already identified several spots changing over time upon high-dose LPS treatment as proteins or protein subunits described to be upregulated during inflammation. Furthermore we are screening groups of untreated mice of the two different genotypes in a 2D DIGE approach using minimal labelling with CyDyes. This will provide us with additional information about experimental as well as biological variations within and between the two genotypes under standard conditions, a prerequisite for further studies.

Proteomics as analytical tool for gene expression strategies, downstream process development and characterisation of product quality

Grzeskowiak JK¹, Machold C¹, Hahn R¹, Jungbauer A¹, Marchetti M²

¹Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria

²Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria

Detection, visualization and characterization of proteins in complex biological systems is a challenge for many academic and industry centres. Especially a novel 2-D electrophoresis method, 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE), has been applied for high resolution protein analysis. A comparison of heat and IPTG induced expression systems of Interleukin 2 in *Escherichia coli* is presented. The protein pattern in heat induced expression system shows an increase of chaperones and other heat shock proteins in bacteria cells. 2-D DIGE can be also used as analytical tool in downstream process development. This approach was applied for a life time study of protein A affinity chromatography media. MabSelect Xtra, MabSelect SuRe, ProSep-vA Ultra were packed in 1 ml columns and 50 consecutive purification cycles of hum mAB IGN 311 were performed. Impurity profiling of the crude sample and elutes at the beginning and the end of the 50 cycles was performed by DIGE. The analyses showed efficient removal of host cell proteins and a constant product purity over the 50 purification cycles. 2-D DIGE in addition to other proteomics methods is a helpful tool for the investigation of product stability in industrial processes. A novel beer stabilizing system developed by Amersham Biosciences was investigated. The process named „Combined Stabilizing System“ (CSS) is based on chromatographic separation principles to remove compounds responsible for haze formation. Samples before and after the chromatographic separation and two regenerates from the column, eluted with NaCl and NaOH were analyzed using SDS-PAGE, 2-D DIGE, Anion Ion Exchanger (IAX). Additionally Size Exclusion Chromatography (SEC) with Light Scattering (LS) and Refraction Index (RI) detection was performed. It could be shown that, during the process, an aggregate of protein and other substances with a molecular weight of approximate 62 kDa was removed from the beer sample. This protein tends to aggregate and thus it can impact the quality of beer. Using Peptide Mapping two barley proteins, named z-type serpine and non-specific Lipid Transfer Protein, were identified.

Proteomic and genomic characterization of the effect of nitrosative stress in *Candida albicans*

Hernandez R^{1,2}, Nombela C¹, Rupp S², Diez-Orejas R¹, Gil C¹

¹Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain

²Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik IGB, Stuttgart (Germany)

The encounter between *C. albicans* and phagocytes is generally considered to be the initial step through which cell-mediated host immune defences begin. Some authors have correlated the candidacidal activity of activated macrophages with peroxynitrite, resulting from the interaction between nitric oxide (NO) and superoxide anion. Peroxynitrite can produce synergistic cytotoxic effects by irreversibly oxidizing and nitrating a number of molecular targets. Thus, this compound is able to modify proteins and some transcription factors involving posttranslational modifications of proteins and modifications of gene expression. To analyse the in vitro effect of peroxynitrite on *C. albicans* we employed 3-morpholinopyridone (SIN-1) as a suitable agent for the generation of peroxynitrite in situ. We studied the cytotoxic effect of this nitrogen derived compound, selecting the conditions which lead to a 70% of yeast death, and used the proteomic technology to understand the effects produced at a molecular level. To highlight and identify proteins differentially expressed under this nitrosative stress comparative 2D-PAGE and mass spectrometric analyses were performed. SIN-1 treatment revealed differential expression of proteins implicated in metabolism, stress response, energy production, mitochondrial proteins and proteins of unknown function. Preliminary results of genomic studies using DNA arrays technology confirms those proteomic results helping us to understand the response of *C. albicans* to this nitrosative stress. In order to obtain a more deep knowledge about the damage caused by this compound on *C. albicans* and trying to understand what leads to the death of this fungus, we decided to analyse the main molecular modifications induced by SIN-1. The detection of oxidated and nitrated proteins were performed by 2D-PAGE and western blotting analysis. The characterization of these proteins will help us to know the main targets of nitrosative damage in this yeast.

Proteomics of processing relevant organelles in antigen presenting cells

Herrmann T, Zaidi N, Brandenburg J, Kalbacher H

Medical and Natural Sciences Institute, University of Tübingen, Ob dem Himmelreich 7,
D-72074 Tübingen

The processing of protein antigens to peptides and their loading onto MHC class II molecules is believed to take place in specialized endosomal/lysosomal loading compartments. Thus understanding the pathway involved in peptide processing and loading is an extremely important issue from an immunological perspective. Recently we described a fast and efficient subcellular fractionation procedure that permits lysosomes to be separated from endosomes. Differential centrifugation is used to isolate a subcellular fraction containing both endosomes and lysosomes. We demonstrated that hypotonic lysis of an endosome/lysosome-pool releases 85% of the lysosomal marker NAG into the supernatant. The endosomal fraction was thoroughly characterized using a variety of subcellular markers (e.g. labeled transferrin, proteins of the small GTPase family rab5 and rab 7) and immunological markers (antibodies to MHC class II or the invariant chain Ii). Cytosol was characterized by lactate dehydrogenase activity. Our aim is to analyse immunological relevant subcellular fractions by proteomics methods (like free-flow electrophoresis, 2D-SDS-PAGE and Tryptic fingerprint) both to define the abundance of immunological relevant molecules (i.e. MHC class II, Ii, HLA-DM, processing relevant proteases like the cathepsins) and also to follow the fate of a given protein antigen in pulse chase experiments. Moreover, it should be possible to analyse which proteins are up- or downregulated during stimulation by cytokines i.e. interferon gamma. The project will contribute to a better understanding of the processing of protein antigens, the peptide transport into the lysosomes and the efficient presentation of exogenous and endogenous (like autoantigens in MS and rheumatoid arthritis) proteins to T-helper cells. This project is supported by the DFG (SFB 685)

Mass-accumulating Hidden Markov Models

Hildebrandt I, Rahmann S

University of Bielefeld

To come up with a statistical model for protein fragmentation yielding a significance measure for mass spectra comparison in protein identification techniques, random weighted strings accompanied by the computational framework of mass-accumulating Hidden Markov Models have been proposed. Our current work concerns the extension to biologically more reasonable sequence models for proteins and the incorporation of post-translational modifications. Furtheron, we plan to investigate the usefulness of the probabilistic model and the associated computational framework in different fields in proteomics, e.g. the detection of differential expression of proteins in unaffected versus diseased samples.

Analysis of pathogen-induced, integrin-rich membrane microdomains

Hoffmann C¹, Agerer F², Citieuh ², Neske F², Fässler R³, Ohlsen K², Hauck CD^{1,2}

¹Lehrstuhl Zellbiologie, Universität Konstanz, Maildrop X908, 78457 Konstanz, Germany

²Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, 97070 Würzburg, Germany

³Max-Planck-Institut für Biochemie, Abtl. Molekulare Medizin, Am Klopferspitz 18, 82152 Martinsried, Germany

A key feature of pathogenic *Staphylococcus aureus*, a leading cause of hospital-acquired infections, is the expression of extracellular matrix binding proteins. In particular, the fibronectin-binding proteins (FnBPs) afford the pathogen with the ability to connect to and to trigger invasion into host cells. Previously, we have demonstrated that integrin engagement by fibronectin-coated bacteria will lead to the assembly of focal adhesion-like complexes (Agerer *et al.*, 2005 *J. Cell Sci.* 118:2189; Agerer *et al.*, 2003 *J. Biol. Chem.* 278:42524). Along this line, we demonstrate now that the bacteria recruit cholesterol- and sphingolipid-rich membrane microdomains to the invasion site. This process depends on the presence of FnBPs and can be mimicked by FnBP-coated inert microspheres. Clearly, depletion of cholesterol from the host cell membrane by Nystatin, Filipin, or methyl- β -cyclodextrin interferes with bacterial invasion demonstrating the functional relevance of membrane microdomains for pathogen entry. In contrast to wild-type cells, recruitment of membrane microdomains to the staphylococcal-host cell-contact site is absent in integrin 1-deficient mouse embryo fibroblasts suggesting that integrin 1 engagement is the initial event leading to the organization of membrane microdomains. The molecular mechanisms connecting integrins with the distribution of membrane lipids is currently unknown. Therefore, we will use our FnBP-coated latex-bead system as a local and easy to re-isolate trigger to analyse the molecular composition of FnBP-initiated, integrin-rich protein complexes by mass-spectrometry to identify all the components of these subcellular structures as a pre-requisite for further functional studies in the context of bacterial infection and in light of the physiological role of integrins.

Characterization of Biomolecules in Microparticles with Emphasis on Nucleic Acids and Modified Proteins

Jensen SS, Larsen MR

University of Southern Denmark

The project aims for the detection and characterization of biomarkers in microparticles. The focus will be on the development of methods for the isolation and characterization of modified nucleic acids, phosphoproteins and glycoproteins. In addition, those methods will be used to detect biomarkers for type 1 diabetes and other diseases. Recent years have shown an increased interest in the detection, characterization and biomedical usage of different forms of biomarkers in body fluids for the identification and distinction of normal versus disease states in humans. However, the exploitation for biomarkers in plasma is complicated due to large differences in protein concentration (in the range of 5-6 orders of magnitude) of high versus low abundant proteins. This results in difficulties with regard to isolation and identification of potential and, most likely, low abundant biomarkers. Upon inflammation, various cell types release vesicles from the plasma membrane called microparticles (MPs). Microparticles are vesicles shed from the plasma membrane of stimulated and apoptotic cells in the body which are afterwards transported to the vascular compartment. The presence of cellular microparticles in the circulatory system thus offers a novel method for purification of disease specific biomarkers. It is presently not known if cell activation or apoptosis leads to similar microparticles in terms of biomolecular composition (lipid, protein and nucleic acid fragments). However, it is known that the mechanism behind MP formation in the two situations differs and that the biomolecular content of the cells changes in the two situations. We therefore expect that the content of microparticles change according to the cellular stage and this provides us with the opportunity of isolating microparticles released into the bloodstream from disease cells/tissues and characterize them for the detection of possible biomarkers. No reports have so far been made on either the fate of nucleic acids in microparticles or on specific protein modifications such as phosphorylations and glycosylations. We will therefore in this study be focusing on the characterization of two important post-translational modifications: protein glycosylations and protein phosphorylations.

Synaptic cell-adhesion molecules Neurexins: Molecular Structures and Interaction-Studies

Klose M, Reissner C, Missler M

Center for Physiology and Pathophysiology Georg-August-University Göttingen

Neurexins are cell-adhesion molecules at the pre-synapse that form heterophilic connections with postsynaptic neuroligins and the extracellular matrix molecules dystroglycan and neurexophilins. They play a crucial role in neurotransmitter release at the pre-synapse and are involved in synapse formation and maturation, respectively. Neurexins may appear in more than thousand isoforms. This high variability is derived from three genes (Nrnx1-3) each under control of two promoters for expression of alpha(a)- and beta(b)-neurexins and by subsequent alternative mRNA splicing at five sites. The structure of a-neurexins consists of 3 extracellular cassettes with an EGF-domain flanked by two LNS-domains. The conserved c-terminus contains an O-linked sugar attachment sequence, a single transmembrane region and a short cytoplasmatic tail, the later may anchor at the pre-synapse via PDZ-containing adaptor molecules to the cytoskeleton. B-neurexins share with a-neurexins only the last LNS-domain (LNS6) and the consecutive C-terminus. Both neurexin1a and -b bind via LNS6 in a calcium-dependent manner to the postsynaptic transmembrane protein neuroligin. We performed site-directed mutagenesis studies on LNS6, b-neurexin1 and neuroligin1. This allowed us to identify residues of LNS6 and, for the first time, of neuroligin1 forming a hydrophobic contact required for the neurexin/neuroligin complex in addition to the binding of calcium to the LNS6. Neurexophilins, extracellular secreted proteins, and the black spider venom alpha-latrotoxin bind to LNS2-domains, whereas dystroglycan is able to bind in the presence of calcium to LNS2 as well as to LNS6. In this project binding assays using single domains and cassettes will reveal intramolecular arrangements, which is complemented by crystallographic structure determination of ligand-free and -bound LNS-domains and cassettes. To identify new ligands for neurexin1, pull down experiments from mouse brain homogenates using recombinant neurexin1-a cassettes or single LNS-domains have been done. Samples are subjected to 2d-gel electrophoresis and subsequent mass spectrometry. First results will be presented.

Characterization of SUMO-specific isopeptidases.

Kozackiewicz L, Melchior F

Georg-August-University of Göttingen, Biochemistry I

Posttranslational modifications of proteins with ubiquitin and ubiquitin-like proteins modulate their function by influencing either protein stability, localization, activity or interaction partners. SUMO (Small-ubiquitin like modifier) is a family of proteins. In humans 4 different SUMO genes exist, 3 of those are known to be conjugated to proteins. This modification is reversible and highly dynamic *in vivo*. It plays an important role in the number of processes, including cell cycle, intracellular trafficking, immune response signaling, transcription and DNA repair. Only one small family of enzymes having desumoylating activity has been identified and is referred to as the Ulp/SENPs (SUMO/Sentrin specific proteases) family. These enzymes are cysteine proteases and have been characterized to some extent. Considering the large number of ubiquitin specific isopeptidases, which fall into 5 different families (predominantly cysteine proteases but also metalloproteases), it is possible that there are yet unidentified SUMO-specific isopeptidases. In fact, both literature and our unpublished results support this hypothesis. The major goal of this project is therefore to identify, purify and characterize this novel activity. Using a FRET based assay we are able to follow the kinetics of desumoylating reactions and perform quantitative measurements. In order to identify desumoylating enzymes we are using different approaches including biochemical purification of desumoylating activity from HeLa cell extracts. This will hopefully result in the purification of protein amounts sufficient for mass spectrometry identification. In parallel we aim to biochemically characterize SENP family members in respect to substrate specificity.

The role of *Drosophila* claudins during septate junction protein complex formation

Küstner K

Max-Planck-Institut für biophysikalische Chemie

Epithelial barriers are essential structures to establish and maintain specific fluid compartments in higher metazoa. In vertebrates membrane-associated protein complexes, the tight junctions (TJs), form these barriers. Integral TJ components are members of the transmembrane claudin family. We identified the first member of *Drosophila* claudin family, termed Megatrachea (Mega). Mega is localised in septate junctions (SJs), the tight junction analogous structure of invertebrates that mediates epithelial barrier function. mega mutant embryos show both lack of the normal SJ architecture and defects in the transepithelial barrier. Our findings indicate not only a functional but also a structural similarity between the vertebrate TJs and the invertebrate SJs. Protein sequence analysis revealed additional *Drosophila* claudin-like proteins. Based on structural and expression pattern similarities we defined a subgroup of four claudin-like genes as the mega-related claudin genes. They comprise Mega and Sinuous, which were initially described to be involved in tracheal tube morphogenesis, and two novel transmembrane proteins, which we named Sesam and Kasim, respectively. We produced antibodies against the four *Drosophila* claudins and found that they all are integral components of the SJs. We are in the process to analyse septate junction formation, epithelial barrier function, tracheal morphogenesis and genetic interactions of the *Drosophila* claudins by lack-of-function and gain-of-function experiments. Initial genetic experiments indicate that Mega participate in protein complex formation with the SJ components Coracle and Neurexin. We plan to analyse the possible complex formation of Coracle, Neurexin and Mega by in vitro protein interaction studies. Such studies may also include the additional three claudin proteins. In order to study the structural components of the SJ protein complex we also plan to purify the membrane associated SJ proteins and to identify them by mass spectroscopy. By molecular and genetic tools we will analyse the corresponding genes in order to understand their participation to SJ formation. With such experiments we hope to contribute to a better understanding of the molecular mechanisms and morphological requirements that underlie transepithelial barrier function mediated by SJ proteins.

Global Analysis of Protein Complexes in *Mycoplasma pneumoniae*

Maier T, Wilm M, Herrmann R, Bork P, Serrano L, Gavin AC

EMBL Heidelberg, Meyerhofstr. 1, 69117 Heidelberg

Most cellular processes are carried out by multi protein complexes. The identification and analysis of their components provides insight into how the ensemble of expressed proteins (proteome) is organized into functional units. In analogy to a similar study in yeast (Gavin AC *et al.*, Nature 2002, Nature 2006), we will use tandem-affinity purification (TAP) and mass spectrometry in a large-scale approach to identify and characterize multi protein complexes in the bacterium *Mycoplasma pneumoniae*, a parasite of the human respiratory tract. Its relatively small and well annotated genome of about 690 ORFs makes *Mycoplasma pneumoniae* an ideal model organism for this study. Analysis of isolated protein complexes by mass spectrometry will include both MALDI-MS with samples from individual 1D SDS-PAGE gel bands and LC-MS with more complex liquid samples. This approach allows the direct comparison of two techniques in mass spectrometry with respect to sensitivity, duration and cost in a large-scale proteomics research project. Findings will be compared to published protein complexes in *E. coli* the eucaryotic organism *S. cerevisiae*. The analysis is expected to reveal features of procaryotic proteome organization, especially on the background of a small genome.

Alzheimer's Disease - Differential Proteome Analysis of a UBB⁺¹ transgenic mouse model

May C, Schulenburg T, Meyer HE, Marcus K

Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum, Germany

In eukaryotic organisms 80-90% of all proteins in the cytoplasm and nucleus are degraded by the ubiquitin-proteasome system (UPS). These are proteins which control cellular mechanisms like survival, differentiation, division and cell death or are mostly damaged or misfolded. The majority of the proteins which should be degraded by the UPS is marked with ubiquitin (UBB), a highly conserve protein with 76 amino acids. A faulty UPS can disturb the cell homeostasis and can cause protein aggregates which could result in cell death. In neurodegenerative diseases like for example Alzheimer's disease dysfunctions of the UPS seem to play an important role. Therefore these proteins are an interesting topic of research. Ubiquitin B⁺¹ (UBB⁺¹) is a mutant of UBB with a frameshifted extended C-terminus. In the presented work differential proteome studies of anatomical subfractions of a UBB⁺¹ transgenic mouse model at different age stages were performed using the DIGETM system (Differential In Gel Electrophoresis, GE Healthcare Bio-Sciences). After decapitation of the animals cortex and hippocampus were dissected from the brain and homogenized. Proteins were extracted, labelled with the fluorescence dyes and separated by either 1D- or 2D-PAGE. The labelled proteins were detected using a fluorescence scanner (TyphoonTM, GE Healthcare Bio-Sciences) and differentially expressed proteins were identified by mass spectrometry.

Identification of new interaction partners for posttranslationally modified HIV-1 Nef protein by differential screening in eucaryotic cells

Moetter J, Jonas E, Hoffmann S, Willbold D

Forschungszentrum Jülich, IBI-2/NMR, 52425 Jülich and Heinrich-Heine-University, 40225 Düsseldorf, Germany

Human immunodeficiency virus type 1 (HIV-1) Nef is a small protein of about 25 kDa, that is essential for high titer viral replication and pathogenesis. Nef is posttranslationally modified by phosphorylation and by myristoylation. Nef interacts with a multitude of host cellular proteins. Most of them were identified by using conventional yeast two-hybrid screens. It is well documented that the multiple functions of Nef involve cytoplasmic as well as membrane-bound stages. Conventional yeast two-hybrid screens rely on transcriptional activation of reporter genes in the nucleus to detect interactions. Thus, detection of interactions including integral membrane or membrane-associated proteins and of soluble bait-prey complexes that do not translocate into the nucleus is hampered. According to these restrictions it is very likely that yet other interaction partners Nef await to be uncovered. The aim of our work is to identify such novel Nef interactors. Thereby, we focus on posttranslationally modified Nef protein as well as on specific Nef mutants or Nef-fusion proteins, which are expected to determine more differentiated Nef proteins. We are using three modern yeast two-hybrid systems to screen for novel ligands. In the „Cytotrap System“ (Stratagene) interactions in the cytoplasm are detected by the recruitment of human Sos to the membrane („Ras-rescue“). A nuclear translocation of the complex is not necessary. For the identification of soluble or membrane inserted/associated interactors of membrane localized Nef two „split-ubiquitin“ based systems are applied. In the „Dualmembrane“ system (Dualsystems) membrane localization for the Nef bait is provided by the myristoyl moiety of Nef. By using the „Dualhunter“ system (Dualsystems) the myristoyl-anchor is replaced by the small membrane anchor Ost4p. The suitability of both systems is currently under evaluation. Furthermore a FRET-based screening approach, which is able to detect protein-protein interactions in a very direct fashion, is under development. Here, Nef is N-terminally fused to CFP allowing a screen against cDNA libraries fused to YFP in mammalian cell lines (HEK-293, HeLa).

Physiological proteomics of the membrane fraction of *Bacillus subtilis*

Otto A, Becher D, Hecker M

Universität Greifswald

Bacillus subtilis is regarded as a model organism of gram positive bacteria and provides excellent insight to the understanding of basic concepts of cell differentiation and physiological adaptation to stress. So far, the membrane proteome of growing and non growing cells of *Bacillus subtilis* was analyzed in order to provide the basis for its application in microbial physiology. Therefore we use optimized protocols for membrane purification to separate cytosolic proteins from membrane proteins and membrane associated proteins. Whereas the work is aimed to come to a complete gelfree workflow we use a combination of 1D SDS PAGE and the subsequent analysis by LC-MS/ MS. To bring physiology to the work, metabolic labeling with completely $^{15}\text{N}/^{14}\text{N}$ labelled cells was established. First data for glucose starved cells are available. The studies will give new insights into the physiological adaptations in a glucose starved cells of *Bacillus subtilis* with information assured by transcriptomics and gel- based proteomic data already acquired.

The influence of Tyk2 deficiency on the murine macrophage proteome

Radwan M¹⁻³, Miller I³, Strobl B^{1,2}, O'Donoghue N⁴, Dunn MJ⁴, Vogl C², Gemeiner M³, Müller M^{1,2}

¹Research Center for Biomodels Austria; University of Veterinary Medicine Vienna, Austria

²Institute of Animal Breeding and Genetics; University of Veterinary Medicine Vienna, Austria

³Institute of Medical Chemistry; University of Veterinary Medicine Vienna, Austria

⁴UCD Conway Institute of Biomolecular and Biomedical Research; University College Dublin, Ireland

Tyrosine kinase 2 (Tyk2) belongs to the Jak family of non-receptor tyrosine kinases. It has been recently shown in our lab that Tyk2-deficiency results in impaired production of nitric oxide (NO) and interferon beta (IFN beta) in lipopolysaccharide (LPS) treated macrophages. Macrophages play a major role in host defence, immunity and inflammatory responses. Within the present project the role of Tyk2 for macrophage function is investigated in more detail applying a proteomics approach. Using 2D-DIGE technology we compared protein patterns in two different macrophage populations from Tyk2-deficient and wildtype mice before and after LPS treatment. Whole cell lysates from either bone marrow-derived (BMM) or thioglycollate-elicited peritoneal macrophages (PM) were minimally labelled with CyDyes and subjected to 2D DIGE. Proteins were separated in two different gel formats (using either 11 cm or 24 cm IPG gel strips in the first dimension), and also in different pH gradients. Gel images were analysed with DeCyder in combination with univariate statistical analyses. The datasets show high reproducibility with lower variance in BMM than in PM cell extracts. Global protein patterns were quite similar in both populations of wildtype versus Tyk2-deficient macrophages, with only some proteins showing significant genotype-specific differences (typically 60 out of 1000 spots). Although the differences were quite small (usually less than 2-fold) they were highly reproducible and in most cases independent of LPS treatment. The proteins of interest were analysed by mass spectrometry. Several of the identified proteins are involved in immune responses. In order to extend our analysis to lower abundant proteins and to subcellular compartments, we have started with cellular fractionation techniques. We hope that this will enrich new potentially interesting candidates, e.g. regulatory proteins that may play an important role in the immune response.

Analysis of Phosphorylated Proteins in drug-induced Human Primary Hepatocytes

Redlich G¹, Langenfeld E¹, Schulenburg T¹, Vöcker E¹, Vacun G², Nuessler A³, Meyer HE¹, Marcus K¹

¹Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum, Germany

²Institut for Biochemical Engineering, University of Stuttgart, Stuttgart, Germany

³Charité, Campus Virchow-Klinikum, Klinik für Allgemein-, Visceral- und Transplantationschirurgie, Berlin, Germany

In our systems biology project we are interested in detoxification and dedifferentiation processes in human hepatocytes where our group is performing the proteomics part. Proteome analysis of cell-cultured primary hepatocytes is done at different time-points of drug induction by Rifampicin or Phenobarbital respectively. To comment on dedifferentiation processes non-induced control cells are analyzed as well. Additionally, the investigation of phosphorylation processes as a result of drug-induction is of special importance. The soluble protein fraction was obtained by differential centrifugation after cell lysis followed by differential two-dimensional gel electrophoresis (2D-DIGETM, GE Healthcare). Detection and quantification of phosphorylated proteins was done using the phosphospecific Pro-Q®Diamond stain (Invitrogen) in combination with the DIGETM technology followed by spot picking and in-gel digestion. Proteins were identified by MALDI-TOF-MS/MS analysis. To obtain quantitative data, the image analysis software DeCyderTM (GE Healthcare) was used. A new workflow will be presented allowing more reliable quantification of phosphoproteins and their phosphorylation state by standardizing Pro-Q®Diamond stained gels. In addition, several phosphorylated, liver-specific proteins could be identified and quantified. To get statistical relevant results several biological replicates have to be analyzed. Investigation of phosphorylation sites including their localization will be done by mass spectrometry.

Identification of new and improved biocatalysts using bacterial colony based techniques

Reisinger C¹, Schürmann M², van Assema F², Remler P¹, Schwab H¹

¹Applied Biocatalysis Research Centre, Petersgasse 14, 8010 Graz, Austria

²DSM Research B.V. Pharma Chemicals, ASC&D, P.O. Box 18, 6160 MD Geleen, The Netherlands

The discovery of new enzymes, which catalyze chemical reactions of synthetic value, is a cornerstone of „White Biotechnology“. Efficient screening methods are required to identify an appropriate clone within thousands to millions of less suited variants. We have developed screening protocols for several types of enzymes, which allow the detection of the monitored activity already on the level of a bacterial colony. The big advantage of this strategy that uses the inherent compartmentalization by the colonies lies in the parallel design of the experiment, yielding activity information for several thousand samples in one step. It therefore exiles time consuming handling and procedures of serial analytics from the primary discovery event. We developed techniques for the screening of enzymes such as nitrile hydratases, amidases, threonine aldolases or esterases. The colorimetric reaction can thereby be catalyzed by the investigated enzyme itself, as for example in the case of amidases. When no such direct reaction is known, the staining can be realized by an (enzymatic) follow-up step. Enzymatic catalysis of the detection step often gives a solution in the case of unreactive analytes or those difficult to discriminate from the complex biological matrix of a microorganism. For instance, we used the selective properties of dehydrogenases to detect the release of aldehydes or alcohols from aldolase or esterase reactions via the simultaneously released fluorescent NADH cofactor [1]. Also the analytical problem of the nitrile hydratase reaction could be addressed by the use of a „sensing enzyme“. In this case an amidase recognizes the otherwise hard to capture amide and transforms it into a colour signal. These presented methods are successfully applied for the isolation of active clones from genomic libraries or the identification of improved variants from mutant libraries.

[1] Reisinger *et al.* (2006) *J. Mol. Cat. B*, 39, 149-155

Characterization of late endosomes in *D. discoideum*

Rethmeier R

Department of Cell Biology, University of Kassel

My current project is the characterization of late endosomes in *Dictyostelium discoideum*. These are unusual organelles in that they have a neutral luminal pH value and the capability to efficiently fuse with the plasma membrane. I try to identify unknown proteins on them and elucidate their function. To this end I want to isolate the late endosomes together with the resident proteins. After conventional biochemical fractionation of the organelles, I try to establish immuno-isolation procedures with antibodies against the sole known marker for late endosomes, vacuolin. The proteins that are co-purified with vacuolin will be identified by peptide-fingerprinting and comparison with the completely sequenced genome of *Dictyostelium*. A side project during my thesis will be the identification of the antigens recognized by as yet uncharacterized antibodies that are available in our research group and show promising localizations in immunofluorescence analysis. Also in this case mass spectrometry is the main method to determine the sequence of these proteins in order to get their genes for further research involving molecular biology techniques like fusions to the green fluorescent protein and targeted gene disruption. Further biochemical characterization may include the analysis of protein modifications on the gene products selected for in-depth analysis, at later states of my work.

On-line enrichment strategy for the MS-based identification of protein-RNA cross-links and phosphopeptides using TiO₂ columns

Richter F, Kühn-Hölsken E, Raabe M, Plessmann U, Gronborg M, Urlaub H

Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry

A novel strategy for the mass-spectrometric identification of protein-RNA interaction sites was recently developed by Kühn-Hölsken et al. using small ribonuclear particles (RNA 11, 1915-1930, 2005). Briefly, this comprises: (i) Generation of protein-RNA cross-links by UV irradiation at 254 nm, (ii) hydrolysis of the cross-linked sample with endopeptidases under denaturing conditions and separation from the non-cross-linked peptide by size-exclusion chromatography, (iii) hydrolysis of the RNA by endonucleases and additional treatment with endoproteinases, (iv) purification of peptide-oligoRNA cross-links by reversed-phase HPLC and (v) analysis of the cross-links by MALDI-ToF MS and MS/MS. One bottleneck of this strategy is currently the low yield of protein-RNA cross-linking and thus the large starting amounts needed. To reduce this we use nano-scale liquid chromatography with self-packed Vydac C18 columns (75 μm i.d.) coupled online to a MALDI target spotter. Fractions of 50-150 nl (mixed with DHB as matrix) are spotted and analysed by MALDI-ToF MS and MS/MS. We also implemented a novel liquid chromatography (LC) online enrichment strategy; this uses titanium dioxide (TiO₂), previously shown effective in enriching phosphopeptides (Larsen et al., Mol Cell Proteomics 7, 873-886, 2005). Since peptide-RNA cross-links also carry phosphate groups they are efficiently bound to the TiO₂ matrix, while non-cross-linked peptides do not bind. The online LC-system (Dual Gradient, LC packings) comprises a trapping column to remove non-cross-linked RNA oligonucleotides, a TiO₂ column for the enrichment of cross-links over residual non-cross-linked peptides, a pre-column column for desalting the eluted cross-links, and a self-packed analytical column for subsequent separation of the cross-links. Trapping and TiO₂ columns are coupled to the loading and capillary pump of the system, respectively, and the desalting and analytical columns to the nano-pump system. In the subsequent MALDI-ToF MS analysis of spotted fractions, the cross-links are the predominant species in the spectrum. Importantly, the system can be used for on-line enrichment of phosphopeptides from a crude mixture.

Desensitization of human GC-A by dephosphorylation

Schröter J^{1,2}, Kuhn M¹, Sickmann A²

¹Institut of Physiology, Julius-Maximilians Universität Würzburg, Germany

²Rudolf-Virchow-Center, DFG Research Center for Biomedicine, Würzburg, Germany

In several cardiovascular diseases, especially in primary hypertension and cardiac hypertrophy/cardiac insufficiency, the plasma concentrations of the cardiac natriuretic peptides ANP and BNP are highly increased. But the endocrine and cardiac effects of these peptides are clearly decreased. This is a sign of dysfunction of the guanylyl cyclases-A receptor (GC-A). In mice the deletion of the GC-A gene leads to drastical arterial hypertension and cardiac hypertrophy. Consequently it is supposable that a failure in receptor or postreceptor in patients could contribute to clinical complications especially to cardiac changes. Which molecular mechanisms, leading to this putative GC-A failure, are unknown. Observations of GC-A expressing cell lines show that ANP itself (homologous), but also vasoconstrictory and growth stimulating hormones like angiotensin II and endothelin (heterologous), leads to dephosphorylation and desensitization of GC-A. The (patho)physiological relevance of these processes in vivo is not known so far. In this project the mechanisms should be characterized leading to a decreased stimulation of GC-A receptors in cardiovascular diseases. At first a quantification method based on mass spectrometry for single phosphorylated sites of GC-A under wild-type basal conditions and after receptor desensitization should be applied. Based on this results specific antibodies for phosphorylation can be developed. Afterwards the ratio between phosphorylated/dephosphorylated GC-A in hypertrophic, insufficient hearts can be analysed and correlation with the cGMP synthesizing activity of the receptor can be calculated. With the help of these antibodies and specific MS analyses the involved protein kinases and protein phosphatases should be characterized.

Alzheimer's Disease - Analysis of modified proteins in an UBB⁺¹ transgenic mouse with endogenous proteasome inhibition

Schulenburg T¹, van Leeuwen FW², Meyer HE¹, Marcus K¹

¹Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum, Germany

²Netherlands Institute for Neuroscience, Meibergdreef 47, 1105BA Amsterdam, The Netherlands

Ubiquitin-B⁺¹ (UBB⁺¹) - a mutant form of ubiquitin lacking glycine-76 - was found to accumulate in the neuropathological hallmarks of Alzheimer's Disease (AD) and aggregates of other neurodegenerative diseases. UBB+1 is crucial for ubiquitination of aberrant proteins and is ubiquitinated itself on Lys29 and Lys48. In neurodegenerative disorders it additionally presents an ubiquitin fusion degradation substrate blocking proteasomal degradation. A reduction of proteasomal activity allows the accumulation of ubiquitinated UBB⁺¹ (UBB⁺¹-ub) and other ubiquitinated proteins, higher levels of UBB⁺¹-ub promotes proteasome inhibition and contributes to aggregate formation and cell death. In order to assess the function of UBB⁺¹ and its downstream effects a transgenic mouse line was established expressing UBB⁺¹ protein in the brain. In our project we analyse effects of high UBB⁺¹ expression levels on the proteome in the cortices of these mice. We are especially interested in changes of protein modification such as phosphorylation because protein phosphorylation is known to play an important role in AD (e.g. hyperphosphorylated tau and presenilin). Additionally oxidised proteins are in the focus of our analyses as one of the prevalent effects in AD is the increase of oxidative stress resulting in higher levels of oxidised protein species. Sophisticated proteomic technologies like 2D-DIGE combined with staining procedures specific for phosphorylated and oxidised proteins are used for differential proteome analysis. Protein identification and the localisation of modified amino acids is performed by mass spectrometry. So far several phosphorylated proteins were identified to be regulated in the cortices of the UBB+1 mice. The validation of these results by immunoblotting is still under work.

Assembly, Stability and Dynamics of Photosynthetic Membrane Complexes

Schwenkert S, Lezhneva I, Umate P, Stoppel R, Meurer J

Ludwig-Maximilians-Universität, Department Biologie I, Botanik, Menzingerstr. 67, 80638 München, Germany

Thylakoids are specialised biomembranes capable of solar energy transformation. Our aim is to investigate the biogenesis as well as the structure/function relationship of the thylakoid membrane system consisting of four major multi subunit membrane complexes, which are of dual genetic origin. Therefore, a large collection of knockout nuclear mutants in *Arabidopsis* and plastome mutants in tobacco, mostly for low-molecular-weight subunits (LMWs) of the photosystems II and I, the cytochrome b6f complex as well as the ATP synthase are under study with regard to the kinetics of the assembly processes and to functional aspects since their roles are at present almost unknown. Interestingly, many of these mutants are still viable but distinct steps during the assembly of these complexes are affected in the individual lines. Future attempts mainly focus on proteomics approaches in combination with *in vivo* labelling of thylakoid membrane proteins to probe into the stepwise assembly of the complexes and the functional role of individual subunits. Recent work has uncovered that the generation, maintenance and repair of the thylakoid system are highly sophisticated processes. Therefore, dynamic posttranslational processes, such as movement of proteins within the thylakoid membrane (state transition), the reversible dissociation of chlorophyll antennae from the core complexes, the regulation of phosphorylation and the turn over of individual subunits will be investigated. For instance, (i) the nuclear gene B3, encoding a yet unknown protein in *Arabidopsis thaliana*, is required for the stability of the cytochrome b6f complex. (ii) Another nuclear-encoded factor, HCF101, is essential for the assembly of [4Fe-4S]-containing membrane and soluble complexes in all organisms. (iii) The assembly of photosystem I will be studied in mutants, which are lacking the cofactor phylloquinone (vitamin K1). (iv) We identified those LMWs, which are important for the dimerisation of photosystem II and the cytochrome b6f complexes. Other LMWs are crucial for the association of the outer antenna to the photosystem II reaction center and for distinct functions during the photosynthetic electron transport process.

Towards Analysis of Arabidopsis ROP protein complexes

Singh MJ, Molendijk A, Palme K

Institut für Biologie II, Universität Freiburg, Germany

Cycling between GTP bound „on“ and GDP bound „off“ forms, monomeric GTPases play very important role in wide range of signaling pathways in eukaryotic organisms. ROPs(Rho of plants) are the only type of RHO family monomeric GTPases present in plants. Rops are involved in cell polarity, pathogen defense, cell death and hormonal responses. Although involved in many different signaling pathways, little is known about protein interactors that are upstream or downstream in the different ROP signaling cascades. To isolate the ROP containing protein complexes, we have generated transgenic Arabidopsis lines expressing N terminally TAP (Tandem affinity tag) tagged wild type, Constitutively active and dominant negative mutant versions of ROP4. We have also generated various transgenic lines with N terminal GFP tag, which are being used to monitor the effects of phytohormone application on intra cellular localization of ROP using microscopy and for protein complex isolation by using GFP as an affinity tag. Using the anti GFP monoclonal antibody we have successfully co-immunoprecipitated an yet unidentified protein of about 150 kD.

The glyco-proteomic characterization of recombinantly expressed IgG molecules

Stadlmann JMR¹, Strasser R¹, Stöger E², Altmann F¹

¹University of Natural Resources and Applied Life Sciences, Vienna

²RWTH Aachen

The Asn-linked glycans of IgG molecules have a pronounced impact on the in vivo efficacy of antibodies. The appendant structural analysis of the Asn-linked glycans is usually achieved by HPLC or mass spectrometry of the released oligo-saccharides. However, incomplete N-glycosylation, due to under-glycosylation or arising from de-glycosylation, is not detected by this approach. Employing a glyco-proteomic approach, based on cap-LC ESI-MS, we analysed the recombinant anti-HIV antibody 2G12 expressed in different plant expression systems, and compared it to its counter-part derived from CHO cell culture. While rAb 2G12 expressed via the plant secretory-pathway yielded typical plant N-glycans, containing core-a-1,3-fucose and xylose, the expression of rAb 2G12 fused to the ER-retention signal peptide KDEL yielded predominantly oligo-mannosidic N-glycan structures. However, employing the glyco-proteomic approach an additional glycoform (i.e. a single GlcNAc residue), providing evidence for the presence of endo-N-acetylglucosaminidase activity in the (maize) seed secretory pathway, as well as substantial amounts of non-glycosylated rAb 2G12 molecules were concomitantly identified. Furthermore, this analytical method enabled us to confirm the correct translation of the expression constructs and the correct processing of the (N-terminal signal) peptide in plants. LC-ESI-MS of tryptic glycopeptides proved to be a reliable, high-throughput analytical tool in the characterization of therapeutic IgG, additionally delivering deeper insights into the glycosylation status of IgG than analysis of liberated N-glycans.

(Post-translational) proteome changes during the resistance response of tomato plants to the pathogen *Cladosporium fulvum*

Stulemeijer IJE¹⁻³, America AH², Jensen ON³, Joosten MHAJ¹

¹WUR, Department of Phytopathology, Wageningen, The Netherlands

²WUR, Plant Research International, Wageningen, The Netherlands

³Syddansk Universitet, Biochemistry and Molecular Biology, Odense, Denmark

Resistance of plants to pathogens is essential to secure our food resources. The interaction between the plant-pathogenic fungus *Cladosporium fulvum* and its only host tomato (*Lycopersicon* spp.) serves as a model to study the features of plant resistance and is associated with an apoptotic-like event, the hypersensitive response (HR). Recognition of an avirulence protein (Avr4) of *C. fulvum* by the resistance protein (Cf-4) of tomato initiates the HR in the cells around the site of penetration, thereby preventing further growth of the attacking fungus. To study proteome changes during the HR in intact plants, transgenic tomato seedlings expressing both Avr4 and Cf-4 are employed, in which a synchronous systemic HR is induced. Time-series of HR-induced Cf-4/Avr4 seedlings are analysed by 2DE gel analysis. In addition, multidimensional LCMS is performed to obtain additional information on the proteome changes. We found that MAP-kinase activation via phosphorylation rapidly occurs upon the initiation of the Cf-4/Avr4-dependent HR (Stulemeijer *et al.*, in preparation), indicating that downstream phosphorylation events play an important role during the execution of HR. Recent developments in the analysis of the phosphoproteome by mass spectrometry provide possibilities to study phosphorylation events in HR-induced Cf-4/Avr4 seedlings. To explore the importance of protein phosphorylation during the Cf-4/Avr4-mediated HR and to characterise the phosphorylated proteins, we are currently performing a full range phosphoproteome analysis. Therefore, proteins are extracted from leaf material of the Cf4/Avr4 seedlings in urea buffer which avoids extraction of enormous amounts of RuBisCo (the most abundant protein in plants), after which proteins are digested by trypsin. Subsequently, phosphopeptides are isolated on TiO₂-columns that have the potential of binding complex mixtures. The peptides will be separated by capillary LC interfaced to an ESI Q-TOF MS/MS instrument to obtain high mass accuracy in both MS and MS/MS modes and good ion statistics for de novo peptide sequencing and quantitation.

Quantitative proteomics approach to study peroxisomes in *Saccharomyces cerevisiae* following induction by oleate vs. ethanol

Sühling T¹, Brocard C², Hartig A², Meyer HE¹, Warscheid B¹

¹Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum, Germany

²Campus Vienna Biocenter, University of Vienna, Austria

Peroxisomes are single membrane bound organelles ubiquitously present in almost all eukaryotic cells. They contain a large set of enzymes that catalyze various metabolic reactions such as alpha- and beta-oxidation of fatty acids and detoxification of reactive oxygen species. In *S. cerevisiae*, biogenesis and proliferation of peroxisomes can be induced by oleic acid or ethanol containing medium, which is proposed to result in altered expression profiles of enzymes present in the peroxisomal matrix. In order to determine changes in the expression of peroxisomal matrix proteins in dependency on the induction medium used, a differential proteomic approach will be followed. Thus, the aim of my master thesis is to carry out a quantitative differential proteome analysis of peroxisomes in *S. cerevisiae* following the induction of peroxisomes by oleate or ethanol. After growing yeast in media containing either oleate or ethanol for 16 h, peroxisomes are isolated by density gradient centrifugation followed by affinity purification via an HA-tag to minimize the amount of copurifying proteins from mitochondria or the endoplasmic reticulum (ER). Purity of peroxisomes will be checked by Western blotting using antibodies against peroxisomal, mitochondrial and ER marker proteins. In further analyses, peroxisomal samples will be tryptically digested in-solution and then analyzed by nano HPLC/ESI-tandem MS. For protein identification, MS/MS datasets will be searched against the IPI database by MASCOT applying the decoy database concept. To obtain quantitative information on proteins present in yeast peroxisomes induced by oleate or ethanol, a relative quantitative proteomic strategy will be developed (e.g. stable isotope labeling by iTRAQ combined with relative quantitative MS/MS analyses).

Modulation of the cellular proteome of endothelial cells by K13 of the human herpesvirus type-8

Thurau M, Sturzl M

Division of Molecular and Experimental Surgery, University of Erlangen-Nuremberg

Kaposi's sarcoma (KS) is a tumor of endothelial cell origin. The human herpesvirus type-8 (HHV-8) is necessary but not sufficient for the onset of this disease. Additional co-factors involved in KS pathogenesis are inflammatory cytokines and co-infection with the human immunodeficiency virus type-1 (HIV-1). Most of the tumor cells are latently infected by HHV-8. During latent infection only few of the more than 80 known HHV-8 genes are expressed. Due to their expression in the tumor cells it is likely that latent genes of HHV-8 are the key to the tumorigenic activity of HHV-8. Among the latent genes is the viral FLICE inhibitory protein K13. By interacting with FLICE (also called Caspase-8) via its death effector domains (DEDs) K13 is a known inhibitor of programmed cell death. Recent findings show that K13 does not only block apoptosis by direct interference with the apoptotic machinery but also activates NF-kappaB signalling pathways and has transforming and oncogenic properties in cell culture and in vivo. In this project the modulation of the cellular proteome by K13 is analysed in order to identify cellular proteins that may be involved in the pathogenesis of KS. To this goal primary human endothelial cells will be generated that express K13 constitutively. The analysis of the cellular proteome will be done by the two dimensional fluorescence difference gel electrophoresis (2D-DIGE) method. In order to increase the sensitivity of the 2D-DIGE technology biochemical fractionations of different cellular compartments will be isolated and analysed separately. Identified cellular proteins will be tested for their participation in apoptotic and proliferative regulation processes. This should provide new indications about the role of cellular proteins in the formation of KS and help to learn about the molecular mechanisms that lead to this frequent tumor.

Peak intensity prediction for PMF mass spectra using support vector regression

Timm W¹⁻³, Böcker S², Twellmann T³, Nattkemper T³

¹Intl. NRW Graduate School in Bioinformatics and Genome Research

²Bioinformatics Group, Jena University

³Applied Neuroinformatics Group, Faculty of Technology, Bielefeld University

With the increasing amount of data nowadays produced in the field of proteomics, automated approaches for reliable protein identification are highly desirable. One widely-used approach are protein mass fingerprints (PMFs) that allow database searching for the unknown protein, based on a MALDI-TOF mass spectrum of its tryptic digest. Current approaches and software packages for interpreting PMFs do rarely make use of peak intensities in the measured spectrum, mostly due to the difficulty of predicting peak intensities in the simulated mass spectra. In this work, we address the problem of predicting peak intensities in MALDI-TOF mass spectra, and we use regression support vector machines (ν -SVR) for this purpose. We compare the impact of different preprocessing and normalization modes such as binning and balancing data sets on prediction accuracy. Our preliminary results indicate that we can predict peak intensities using ν -SVR even from very small data sets. It is reasonable to assume that peak intensity prediction can greatly improve automated peptide identification.

Characterization of aromatic compounds degradation pathways by 2-D DIGE analysis

Tomás-Gallardo L¹, Santero E¹, Camafeita LE², Calvo E², Lopez JA², Floriano B¹

¹CABD, Universidad Pablo de Olavide, Sevilla, Spain

²CNIC, Proteomic Unit, Madrid, Spain

Rhodococcus sp. strain TFB is a metabolically versatile Gram-positive bacterium able to grow using a wide variety of aromatic compounds, such as phthalate, naphthalene and tetralin, as carbon and energy source. In our hands, this strain has been refractory to genetic manipulation. That is why a proteomic approach has been used to characterize the metabolic pathway involved in the degradation of the aromatic compounds. Proteome profile of the soluble fraction of cell extracts obtained from different carbon sources cultures were compared using the 2-D DIGE technology (GE, Healthcare). Specifically induced spots were detected when comparing the proteome profile of phthalate, tetralin and naphthalene to the glucose grown cells. Those spots were picked and subjected to identification by MALDI MS/MS or ESI MS. Key enzymes of aromatic metabolism were identified by comparing with the previously described degradation pathways in other bacteria. The results obtained allow us to propose a metabolic pathway for the degradation of the monoaromatic compound (phthalate) that does not share any enzymatic steps with the metabolic pathway deduced for tetralin and naphthalene. Protein identification data have been useful to design a molecular approach to find the genes that encode those proteins to study their expression pattern.

Analysis of proteins in human hepatocytes: Absolute quantification and a targeted proteomics approach

Unglaube A, Langenfeld E, Redlich G, Schulenburg T, Meyer HE, Marcus K

Medizinisches Proteom-Center, Ruhr-Universität Bochum

The central interest of our cooperative network is a holistic, systems-oriented analysis of human hepatocytes especially aiming at the understanding of detoxification and dedifferentiation processes in these cells. We want to establish a liquid chromatography/mass spectrometry (LC/MS) based method for the absolute quantification of selected proteins, e.g. cytochromes P450 (CYP) and transporter proteins, which are involved in phase I and phase II metabolism. We use a relatively new method called Protein-AQUA™, which allows absolute quantification of proteins via stable isotope labelled peptides as an internal standard [1]. Multiple reaction monitoring (MRM) is applied to support our method. Development and optimization of the experimental setup will be described exemplarily for two proteins (CYP2D6 and CYP3A4). Further, we use MRM for a targeted proteomics approach: We perform a targeted investigation of defined proteins predicted to be involved in signal transduction as well as metabolic pathways of primary human hepatocytes. Method establishment and optimization for distinct proteins will be presented.

[1] Gerber et al. 2003, PNAS, 100, 12, 6940-6945.

Identification of Serine16 as Phosphorylation Site in the HIV-GAG-derived Capsid Protein - Comparison of Different Approaches based on Electrospray Mass Spectrometry

Winter D¹, Assmus A², Kräusslich HG², Lehmann WD¹

¹Central Spectroscopy, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

²Department of Virology, Universitätsklinikum Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany

Covalent modification of the structural precursor polyprotein GAG of HIV-1 is functionally essential for budding and maturation of the viral particles, as demonstrated for myristylation, ubiquitinylation and phosphorylation. An important part of the 55 kD GAG polyprotein is the the 24 kD capsid protein fragment from which the viral coat is composed. We attempted to pinpoint the so far unknown phosphorylation sites in the 24 kD HIV capsid protein by electrospray tandem mass spectrometry. For this purpose, we employed in-gel digestion of the 24 kD capsid protein by trypsin followed by different analytical strategies; (i) automated nanoESI-MS/MS; (ii) capillaryLC-ESI-MS/MS; (iii) phosphopeptide enrichment by Ga(III) IMAC (immobilized metal affinity chromatography) and nanoESI-MS/MS; (iv) phosphopeptide enrichment using TiO₂ microcolumn enrichment and nanoESI-MS/MS. Using nanoESI-MS/MS without enrichment no phosphorylation site could be identified. In the LC-ESI-MS/MS run a single phosphorylation site at Ser16 could be identified. The combination of Ga(III)-IMAC and nanoESI-MS/MS provided the same positive result. In contrast, the combination of TiO₂ enrichment and nanoESI-MS/MS was unsuccessful. Comparison of the signal intensities between the phosphorylated and the unmodified form of the tryptic T1 fragment containing the Ser16 residue resulted in an estimation of the phosphorylation degree of about 16 %. The study underlines the benefits of a multi-method approach for the practice of protein phosphorylation analysis.

Gel-free approaches in qualitative and quantitative analyses of bacterial proteomes

Wolff S, Hecker M, Becher D

Ernst-Moritz-Arndt University Greifswald

A combination of strong cation exchange and reversed phase chromatography coupled to mass spectrometric analyses was used to study the cytosolic proteome of exponentially growing cells of the soil bacterium *Bacillus subtilis*. The separation by two-dimensional liquid chromatography (2D-LC) revealed many additional protein identifications to the so far exclusively gel-based datasets. Furthermore, isobaric tagging for relative and absolute protein quantitation (iTRAQ™) was employed to carry out quantitative studies on the heat shock response of *B. subtilis*. Gel-free results were compared to a parallel quantitation on two-dimensional gels disclosing both the advantages as well as the drawbacks of gel-based and non-gel-based methods. Moreover, known facts on the heat shock response in *B. subtilis* could be validated in the iTRAQ™ experiment, but also new physiological insights of the stress response have been gained. Protein extracts of the human pathogen *Staphylococcus aureus* were likewise separated by 2D-LC and analyzed using an LTQ FT mass spectrometer (Thermo Finnigan). In addition to a mere protein identification of cytosolic proteins, quantitative alterations in the proteome of *S. aureus* under anaerobic conditions are just being investigated by the implementation of SILAC (stable isotope labeling by amino acids in cell culture).

Investigation of cholesterol - drug metabolism crosstalk in primary human hepatocytes using two dimensional gelelectrophoresis

Wörner M

Saarland University

The liver represents one of the most complex organs of the human body. Maintaining many homeostases and drug metabolism are only two of its functions. Equal to drug metabolism, cholesterol homeostasis is regulated on transcriptional level via nuclear receptors. This regulation seems to be the base of a crosstalk between drug metabolism and cholesterol homeostasis. Because of the particular role of cholesterol and drug metabolism for health, the crosstalk is of prime pharmaceutical importance. To investigate this phenomenon, the proteome of primary human hepatocytes will be analysed after treatment with different xenobiotica. For this purpose, two dimensional gelelectrophoresis is chosen as method. To visualize low abundant proteins, the samples are prefractionated by differential centrifugation. The cytosolic, nuclear and microsomal fractions represent the objects of investigation. Separation of fractionated proteins occurs with IEF in first and SDS-PAGE in second dimension. The resulting gels are bioinformatically analysed with PDQuest. To be able to investigate members of the Cytochrome P450 family, a protocol for immunodetection of CYP 3A has been included. The CYP 3A subfamily is of interest, because some members of it belong to the main drug metabolising P450 enzymes. The experimental setup of the experiments has already been finished. The final protocol allows to separate about 1.300 proteins of cytosolic fraction, 1.300 proteins of nuclear fraction and 700 proteins of microsomal fraction. Each of the three fractions results in different protein patterns on the gel. Detection of CYP 3A was successful but has to be reproduced. In summary, the protocol delivers good results with proteins of human hepatocytes. It is now ready to be used to investigate the crosstalk between cholesterol homeostasis and drug metabolism in xenobiotica treated hepatocytes.