4.1 Proteomic Mapping of Dynamic Protein-Protein Interactions in Mammalian Cells

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Protein-protein interactions in vivo occur dynamically and depend on extracellular cues. Present large-scale data sets include mostly static protein networks. To perform functional proteomics in mammalian cells, we developed an automated system we call LUMIER (LUMinescence-based Mammalian IntERactome) to analyze dynamic protein-protein interactions. In LUMIER, a Luciferase (LUC) tagged fusion protein is co-expressed with a Flag-tagged protein in HEK-293T cells. The association of these proteins is determined by co-immunoprecipitation using an anti-Flag antibody and the presence of the LUC-tagged interactor is detected via its luciferase activity. LUMIER easily identifies transmembrane receptor partners, signal-dependent interactions and those that occur only in the presence of post-translational modifications. Using LUMIER we systematically tested the interactions of 518 3Flag-tagged proteins with members of the TGFβ pathway and identified about 900 interactions from 11,914 assays. Analysis of the TGFβ LUMIER data by k-means clustering and self-organizing maps revealed a subnetwork in which TGFβ pathway components are connected to the p21 activated kinase (PAK) network, the polarity complex and Occludin, a tight junction constituent. We showed that Occludin is important during TGFβ-kinase (PAK) network, the polarity complex and Occludin, a tight junction constituent. We showed that Occludin is important during TGFβ-kinase (PAK) network, the polarity complex and Occludin, a tight junction constituent.

4.2 Characterization of Protein Complexes by in Vivo Cross-linking and Mass Spectrometric Analysis

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The assembly of individual proteins into large complexes is a key feature of cellular organization. Co-immunoprecipitation and subsequent mass spectrometric protein identification is commonly employed for their study, but stringent purification conditions are required to reduce non-specific binding. Inadvertently, this causes loss of more transient protein interactions hence information of protein complex composition. We have shown previously that formaldehyde cross-linking in vivo preserves protein-protein interactions. This method enabled us to identify known and potentially novel interaction partners of a constitutively active mutant of the small GTPase M-Ras. Commonly observed proteins, tentatively classified as contaminants, partially obscured our assignments, however. Comparative analysis of cross-linked protein mixtures with and without cross-link reversal was developed to highlight contaminants as proteins with identical molecular weights in both samples. Several GTPases were co-purified repeatedly after cross-linking, but could not be co-immunoprecipitated, suggesting a transient interaction or an indirect interaction via a common binding partner. The scaffold protein IQGAP - known to be present in a number of membrane protein complexes - was identified and confirmed as novel binding partner of M-Ras. Testing each known binding partner of IQGAP for indirect interactions by cross-linking and Western blots enabled us to explore the next layer of interactions, thus extending the interaction distance from the bait protein. In vivo cross-linking is ideally suited for such studies because it tolerates the harsh extraction conditions that would typically cause complex dissociation. We are applying this method to other GTPases to test our hypothesis that these switch molecules act as messengers between individual protein complexes in cell signaling networks.

4.3 Systematic Analysis of Uncharacterized Protein Complexes from Saccharomyces cerevisiae

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Although the yeast Saccharomyces cerevisiae is perhaps the best characterized model organism at the molecular genetic level, approximately one third of its ~6000 proteins remain uncharacterized or poorly characterized with respect to cellular functions and/or biochemical properties. A substantial fraction of these uncharacterized proteins are essential for cellular viability under normal laboratory conditions or have orthologs to higher eukaryotes, including humans. In a systematic proteomic approach to assign functions to these unknowns, we chose an initial set of ~700 yeast bait proteins that, at the start of this study, had no significant defined molecular attributes. Of this set of unknowns, ~500 have homology to human proteins (E < 10^-11) and ~200 possess a lethal phenotype when the respective gene is deleted. Proteins that interacted with the unknown baits were captured by FLAG-epitope immunofluoropurification and identified by liquid-chromatography tandem mass spectrometry. A comprehensive list of protein-protein interactions was generated from >1400 individual purifications, which yielded >9000 excised gel bands and >75 000 protein identifications. Interaction data were filtered using a new combined statistic that integrates the scores for spectra database matches with the frequency of occurrence for each protein in our data set. The resulting receiver-operator characteristic curve was used to rank each interaction pair into one of several confidence groups. Over 2000 high confidence interactions were uncovered, which were further verified by microarray co-expression and immunoblot analysis. Network analysis of interactions in this study, together with a novel database of all previously published yeast interactions, allowed us to assign protein functions for several hundred proteins. Most notably, a large percentage of the previously uncharacterized proteins were involved in translation initiation and ribosomal biogenesis. This study provides significant insight into previously uncharacterized yeast proteins and elaborates protein networks relevant to human disease.
A GPCR Interactome; A Comprehensive Membrane Protein Interaction Map of Human G-protein-coupled Receptors Using the Membrane-based Yeast Two-hybrid Approach

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As biology enters the so-called “post-genomics” era, researchers have begun to embrace the exciting opportunity of investigating proteins in high-throughput experiments. G-protein-coupled receptors (GPCRs) constitute one of the most important families of membrane receptors. Their medical importance is reflected by the fact that they are targeted by ~30% of all medicines, making them extremely valuable targets for the pharmaceutical industry. In humans, this family of receptors has been proposed to contain approximately 1500 members, of which only approximately 700 GPCRs have been identified so far, and their communication with other cellular components is poorly understood. Previously, we have begun to embrace the exciting opportunity of investigating proteins in high-throughput experiments. G-protein-coupled receptors (GPCRs) constitute one of the most important families of membrane receptors. Their medical importance is reflected by the fact that they are targeted by ~30% of all medicines, making them extremely valuable targets for the pharmaceutical industry. In humans, this family of receptors has been proposed to contain approximately 1500 members, of which only approximately 700 GPCRs have been identified so far, and their communication with other cellular components is poorly understood. Previously, we have developed a genetic method for the in vivo detection of membrane protein interactions in Saccharomyces cerevisiae, the so-called “membrane-based yeast two-hybrid” (MbYTH) system (Stagljar et al., 1998 Proc. Natl. Acad. Sci. USA 95, 5187–5192), and have recently adapted it for prey library screening (Thaminy et al., 2003 Genome Res. 13, 1744–1753). Our current effort is directed to generating a comprehensive protein interaction network of about 50 human GPCRs. We present data which demonstrate the correct expression of GPCRs in yeast, and we show results of MbYTH screens with several pharmaceutical important GPCRs as bait proteins. We have found several interesting GPCR-interacting proteins with biological significance. Our results demonstrate, that such a proteomic approach will reveal many unknown regulatory pathways and will provide the framework upon which a systems biology understanding of the GPCRs function will be developed.

Study on Expression and Activity Identification of Recombinant LG4-5 Domain of Laminin-4 Chain

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The Laminin α4 chain a component of laminin-8/9 is expressed in basement membranes, such as those beneath endothelia, the perinerium of peripheral nerves, and around developing muscle fibers. The C-terminal G domains of Laminin α4 chain are important in the interactions with their receptors and ligands which is heparin, sulfatides, and the microfibrillar fibulin-1 and fibulin-2. Our construct the yeast expression vector pGAP ZUA-LG4-5 with His-tag. The recombinant LG4-5 fusion protein with His-Tag is expressed in pichia SMD1168, and identified by Western-blot with anti-His-Tag. The result showed that the the recombinant LG4-5 fusion protein had expression in pichia SMD1168. Finally, the function of the purified the recombinant LG4-5 fusion protein by Ni-NTA affinity chromatography was detected, the result indicated that the recombinant LG4-5 fusion protein can enhance extension and adhesiveness of human A549 cell of lung. Our study suggests that we can use the yeast expression system to acquire the recombinant LG4-5 protein with biologic activity. This will offer a new technique in study on laminin.
Use of Marimastat Conjugates for the Analysis of Proteome and Interactome of Metalloproteinases

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Despite the fact that many technological advances are currently involved in proteome analysis, there is still a great need for the development of novel engineered chemical probes for proteomics and interactomics. Here, we describe our approach concerning the study of proteome and interactome of membrane proteins, that relies on the use of a small synthetic inhibitor chemically modified to allow for its immobilisation to magnetic beads or affinity chromatography materials. Proteins will be detected together with their native interaction partners because of non-denaturing conditions. This general procedure has been optimised for the enrichment of metalloproteinases, especially matrix metalloproteinases, which are potential target in tumour therapy.

Hydroxamic acids are known to be potent inhibitors of metalloproteinases. Marimastat is a reversible inhibitor with a good potency and shows activity towards a wide range of metalloproteinases. The synthesis of new marimastat derivatives will be reported here. The parent compound is modified with a linker to allow immobilisation on a solid surface (affinity column chromatography or surface plasmon resonance sensor chip). This approach is appropriate for the generation of metalloproteinase proteome subsets. These techniques also allow enrichment and isolation of interactions partners of the target proteins.

Proteomics of Soluble Fragment and Full-length E-cadherin for Deciphering Invasion Mechanisms in Breast Cancer Cells

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Among the hallmarks of cancer is defective cell-cell adhesion. E-cadherin, the prototypic member of the classical cadherin family, is crucial for cell-cell adhesion, counteracting primary invasion and hence metastasis formation. Its activity should not be solely considered as mechanistic since it extensively participates at signal transduction through its linkage with cytoplasmic proteins. Moreover, E-cadherin may undergo ectodomain shedding and its soluble fragments (sE-CAD) may regulate invasion. Therefore we have performed a proteomic approach to provide new elements in the understanding of E-cadherin and sE-CAD role in breast cancer invasion. We have previously shown that in MCF-7/AZ breast cancer cells the complex E-cadherin/b-catenin is fully functional and the sE-CAD in the medium is constitutively observed. After cellular lysis in digitonin buffer, E-cadherin and its partners have been immunoprecipitated. We have harvested the conditioned medium (CM) after 24 hours of starvation in serum-free medium and performed the sE-CAD immunoprecipitation. We have separated immunoprecipitated-proteins on high resolution 2D-gels and identified blue colloidal-stained proteins after MALDI-TOF analysis of tryptic fragments. Concerning the immunoprecipitation of the E-cadherin around fifty proteins have been identified. Some of them are already known to associate directly or indirectly with E-cadherin and other identified proteins may represent new partners E-cadherin providing new insights on intracellular networks involved in cancer invasion. From the CM 2D-gels we have characterized few secreted factors which could be involved in the sE-CAD pro-invasive effect. Our results show new protein actors in the E-cadherin invasive and adhesive properties opening promising perspectives in cancer treatment.
4.9

Fractionomics: Rapid Functional Profiling of Global Protein Interactomes by Gel-free Shotgun Tandem Mass Spectrometry

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Gel-free shotgun sequencing based on high-throughput tandem mass-spectrometry has become a powerful method for analyzing global changes in protein expression and abundance in cells and tissues as a function of experimental perturbations and developmental, physiological and disease processes. Despite recent progress, researchers are keen to gain additional insight into changes in protein function and the regulation and activity of entire biochemical pathways. Yet current methods for investigating protein-protein interactions and protein complexes on a genome-scale (“functional proteomics”) remain tedious, and are not generally applicable to the routine examination of dynamic biological processes. Hence, there is an urgent need for the development of more rapid, efficient and effective functional proteomic methods that can be readily and broadly applied to diverse biological problems. To this end, our group is developing a robust analytical framework for examination of entire soluble cellular interactomes using well-established procedures of biochemical fractionation combined with basic LC-MS procedures. Here, I will report on our latest progress, demonstrating the practical utility of such a platform for generalized systematic functional profiling of complex biochemical systems.

4.10

Microfluidic Analysis of Antibodies in a Compact Disc Format

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A new and flexible high throughput microfluidic method for the analysis of antibody specificity is presented. The method is based on compact discs (CDs) in which the centrifugal force moves the fluid through the microstructures containing the columns. IMAC matrix is loaded in the CD structures, allowing for antigen to be captured through the attached His<sub>6</sub>-tag. The CDs contain microstructures enabling analysis of antibodies against 104 different proteins using a single CD. The bound primary antibody is recognized by a fluorescently labeled secondary antibody and detection is carried out by a laser induced fluorescence detector. Importantly, it is possible to separate high affinity binding from unspecific or low affinity binding by examining the binding pattern in the different columns. The method shown here is proven to be reproducible, sensitive and flexible.

4.11

Detection of Protein-Protein Interactions by ProteinChip Technology

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For a functional characterisation of the proteome interaction studies are of particular interest, because it is known that most of the proteins usually work interconnected. In this work we used the E2F1-transcription factor which plays an essential role in the regulation of DNA replication, the nucleotide biosynthesis or cell cycle as a model system to investigate whether the ProteinChip technology SELDI (surface enhanced laser desorption/ionization) can be used for protein-protein-interaction studies. The E2F1-transcription factor seemed to be an ideal model system because of the number of its already known interacting partners. The cell lines U-2 OS and MCF-7 were used to detect endogenous E2F and possible binding partners using ProteinChip technology SELDI. For a sufficient precipitation we tested several affinity surfaces for their compatibility with the ProteinChip technology, like protein A/agarose, IDM-affinity beads, the PS10/PS20 ProteinChips and the RS100 ProteinChips. Only the IDM-affinity beads led to conclusive findings. To analyse interactions, an immunoassay was done by using E2F1 antibodies coupled on IDM beads. This complex was incubated with U-2 OS and MCF-7 lysate, respectively. Afterwards an H50 ProteinChip was loaded with the eluate. The protein pattern generated with a specific E2F1 antibody using SELDI-TOF-MS showed differential signals compared to assays using non-specific antibodies.

To identify some of these different signals, immunoblots against already known interaction partners, correlating to the SELDI data, where done with the same eluate used for SELDI-analysis. With this procedure we identified pRB as an E2F1-interactor in both cell lines and another E2F1-interaction partner in the U-2 OS cell line. For further identification of the other differential signals, the eluates have to be subjected to other processes to get more insight into the regulation of cell cycle or DNA synthesis. This work is supported by the IZKF Jena and the BMBF.

4.12

Functional Knockouts of Specific Protein Interactions

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A wealth of data has been generated concerning global interaction networks. However, to use this wealth of data to validate drug targets and develop novel therapeutics, it will be important not only to identify all protein interactions, but also to determine the critical interactions of each protein in both normal and disease processes. For example, it is known that members of the AP-1 family interact to form multiple hetero- and homo-dimers, and that different complexes activate alternative sets of genes. However, because these proteins interact through a leucine zipper domain, and multiple complexes exist within a cell, it has been difficult to elucidate the function of each individual interaction. To this end, we have developed a novel peptide library, designated the Phylomer library. This library contains both random peptides and natural peptides derived from the ORFs of diverse bacterial genomes. Due to redundancy and the limited number of protein folds found in nature, the natural ORFs from this diverse library provide a rich source of peptides that interact specifically and with high affinity to human proteins. This library was screened to identify peptides that specifically disrupt Jun homodimerisation, and specific peptide disruptors of c-jun homodimerisation were found. These specific inhibitors can now be used to further analyse the effects of specific leucine zipper complexes within the cell, and are being developed as putative neuroprotective peptides. The use of specific peptides allows for specific knockouts of an individual function of a protein, as opposed to other techniques that eliminate all the members of a particular family. Functional proteinomics will be important in fully understanding the implications of each interaction identified within developing “interactomes,” and in the development of specific drugs targeted to particular protein functions.
4.13 Mapping Protein-Protein and Protein-DNA Interactions

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The dynamic range of current expression proteomic technologies is often insufficient for exhaustive study of the proteome or even to provide a depth of analysis sufficient to study key signalling proteins. Thus, it is important to develop different approaches that will allow us to bypass the dynamic range issue and to zoom on subgroup of the proteome. In particular, we have developed methods to study protein interactions in human cells based on immunopurification coupled to mass spectrometry. This approach allowed the mapping of thousands of novel protein interactions in human cells in key signalling pathways. We will present some of the results derived from this study. As well, we are developing methodologies to study changes in protein ubiquitination and DNA binding proteins by mass spectrometry. These novel approaches will allow us to study cell signalling by looking at the changes in protein that are directed to the degradation pathway as well as looking at changes in proteins that bind to DNA. We will present preliminary results derived from these approaches.

4.14 Proteomic Analysis of Hepatitis C Virus-interacting Proteins Using Tandem Affinity Purification and Mass Spectrometry

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HCV (hepatitis C virus) is of great medical importance with an estimated 170 million people infected worldwide. HCV frequently establishes a persistent infection associated with chronic hepatitis and liver fibrosis which could progress to cirrhosis and hepatocellular carcinoma. Patients with chronic hepatitis C are currently treated with interferon alpha in combination with ribavirin. Sustained response rates, however, are limited to 40% of the patients. Considerable work is needed to develop new anti-HCV therapies and understanding the interactions of the HCV proteins with the host cell may aid the development of novel antivirals. HCV is an enveloped virus with a single-stranded RNA genome of positive polarity that directs the translation of a single open reading frame. Subsequent co- and post-translational cleavage of the viral polyprotein results in the production of structural and nonstructural proteins. Core and envelope proteins E1 and E2 compose the structural elements of the virion particle. Nonstructural protein 2 (NS2), NS3, and NS4A are involved in the proteolytic processing of the HCV polyprotein. RNA-dependent RNA polymerase and RNA helicase activities are assigned to NS5B and NS3, respectively. NS4B is a membrane associated protein involved in targeting the replication complex to the Endoplasmic Reticulum. The NS5A protein has multiple and diverse properties, such as alteration of the antiviral response via PKR signalling by looking at the changes in protein that are directed to the degradation pathway as well as looking at changes in proteins that bind to DNA. We will present preliminary results derived from these approaches.

4.15 Development of a Biochip-based Approach for the Genome-wide Mapping of Interactions between Protein Domains and Peptide Ligands

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Functional genome and proteome research aims at a complete description of the network of protein interactions within a cell or organ(s) that is diagnostic for a specific cellular state such as fetal or adult, brain or liver, healthy or oncogenic/pathogenic etc. Many proteins, prominently those of regulatory function, are built from smaller domains which are stably folded structural modules still displaying their specific functional property. The catalogue of such domains that recognize linear epitopes is rapidly growing (SH2, SH3, PH, EVH1, PDZ, WW, etc.). Linear epitopes can be effectively represented by small peptide fragments that are readily available through simultaneous and parallel chemical synthesis. We have developed a process for the genome-wide mapping of interactions between protein domains and peptide ligands with the aim to identify new domains and their target binding sites. Our approach is entirely based on high throughput biochip technologies. This work was carried out in the frame of the German Human Brain Proteome Project (HBPP). A T7-phage library displaying protein domains from a randomly fragmented and cloned cDNA library was “panned” on an array of synthetic peptide ligands derived from brain specific proteins. Peptide arrays are synthesized in situ by SPOT-synthesis on a planar substrate. After multiplexed affinity enrichment, peptide specific phage populations were eluted, propagated, labelled and individual binders identified by differential hybridization to DNA micro-arrays. Special novel structured plastic surfaces were developed to allow parallel synthesis, multiplexed panning and phage elution without physical separation of the peptide sites.

4.16 Isolation and Molecular Analysis of a Transcription Factor for Placenta-specific Expression of the Bovine Cyp19 Gene

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Placental estrogen plays a central role in bovine reproduction. Biosynthesis is catalyzed by the estrogen synthase, product of the Cyp19 gene. Estrogen production is controlled at the level of Cyp19 transcription. Reporter gene analyses of the placenta-specific promoter and mapping of DNaseI hypersensitive sites revealed an E-box motif with a major effect on transcription (Kalbe et al., J. Mol. Endocrinol. 2000, 25, 265–273; Fürbass et al., Eur. J. Biochem. 2001, 268, 1222–1227). Aim of the current work is the isolation and characterization of this E-box binding transcription factor (E-BP). Two strategies are followed: 1) A biochemical approach to purify the E-BP and characterize it by means of mass spectrometry. 2) A genetic approach to isolate the E-BP encoding cDNA by yeast one hybrid screening. Band shift analyses with nuclear extracts from various tissues suggested E-BP to be restricted to the placenta. DNA affinity chromatography with placenta nuclear extracts and magnetic beads coated with either a wild-type or a mutated E-box target DNA revealed a band of 43 kDa, which was present only in eluates from the wild-type target. Thus, the 43 kDa band likely represented the E-BP. Currently, the procedure is being optimized to increase the yield of E-BP for subsequent MALDI-TOF mass spectrometry. In a first attempt to isolate the cDNA encoding E-BP, an expression cDNA library was screened in a yeast one hybrid system. Four clones were found to express proteins, which could specifically interact with a wild-type E-box target. Data bank search did not reveal matches to known genes. This work will help to elucidate the complex regulatory network underlying the Cyp19 expression in the placenta.
4.17 Development of a New Tandem Affinity Purification (TAP) Strategy for Isolation of Protein Complexes Involved in Cell Signalling Out of Mammalian Cell Culture

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Although the MAPK pathway is one of the best studied signalling pathways it is still not fully understood how a linear pathway can convert inputs from different receptors into a specific cell response like proliferation or differentiation. The specificity can be archived by cross talks between the signalling pathways, feedback regulation and the organization of the cascades elements in different protein assemblies, f. e. mediated by scaffolding proteins. Therefore, the analysis of protein-complexes is one important step towards the understanding of the MAPK-pathway. For the systematic analysis of protein complexes the TAP (tandem affinity purification) technique had become a widely used standard (Gavin et al., 2002). In order to ensure a moderate size of the tag and minimize possible specific interactions with host proteins, we have refined the tap-tag principle developed by Rigaut and coworkers (Rigaut et al., 1999). The classical TAP tag combines a ProteinA tag with a Calmodulin binding peptide tag (CBP-tag), separated by a TEV (tobacco etch virus-protease) site. With a high-triple A-epitope separated by a TEV cleavage site. We tested the new tag combination with Raf and B-Raf isoforms as upstream signalling elements of the MAPK pathway (MAPKKK) as bait proteins. Using one and two step purification protocols we have obtained protein complexes suitable to be analysed by direct analysis on by LC-MS/MS. As a result we have obtained information on protein composition and activity of Raf-associated protein complexes. Literature: Gavin, A. C., et al., (2002) Nature, 415, p.141–147; Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B., Nature Biotechnology, 17, p.1030–32.

4.18 Microscale Peptide Synthesis for Protein Interaction Experiments

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As the biological function of proteins is determined by their interactions, the elucidation of interaction networks is a major focus of proteome research. Many of these interactions can be modeled with synthetic peptides corresponding to the interaction motif. Thus, specific protein interaction partners of a certain motif can be identified using respective synthetic peptides as baits in affinity pull-down experiments. If desired, phosphorylated amino acids, isotope labels or a handle such as biotin can be incorporated into these peptides. Only microgram amounts of peptide are required if the analysis of binding partners is done by sensitive mass spectrometry. Without prior knowledge of binding motifs, many peptides may be required to map all interaction sites. We here describe the results of facile parallel preparation of peptides using a small scale synthesis-sequence. Synthesis and work-up is straightforward and a sufficient quantity is obtained in the first attempt in most cases. Purification is achieved by coupling of peptides biotinylated at the N-terminus to immobilized streptavidin in combination with a synthesis procedure containing a capping step in each cycle. Streptavidin affinity beads loaded with biotinylated peptides are used in pull-down experiments. Using pairs of peptides with either normal tyrosine or phosphotyrosine, modification-specific interaction partners were identified. Specific binding could be distinguished from background binding by applying Stable Isotope Labeling by Amino acids in Cell culture (SILAC). The procedure is rapid and can be applied to many sequences in parallel. At the small scale employed even isotopically labeled peptides or sequences with other expensive building blocks can be made at a reasonable cost.

4.19 Biomolecular Interaction Analysis as a General Tool for Functional Proteomics

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Several methods for the in vitro and in cell characterization of protein-protein and protein-ligand interactions were validated using cAMP dependent protein kinase (PKA) as a model system. This kinase is a key player in cellular signalling cascades controlling energy metabolism and mediates the important posttranslational modification (PTM) of protein phosphorylation. The modular structure of the enzyme permits investigation of numerous interactions: Protein-protein interactions were determined between the catalytic (C, 40 kDa) and regulatory (R, 45 kDa) subunits of the kinase and the heat-stable protein kinase inhibitor (PKI, 8 kDa). Protein-ligand interactions were measured with cAMP, a low molecular weight (LMW) ligand of PKA. It was demonstrated that the kinetics of protein interaction are influenced by protein phosphorylation. These crucial PTMs were analysed using mass spectrometry. Several BIA methods were evaluated for their applicability in interaction monitoring: surface plasmon resonance (SPR) as a solid phase assay using Biacore instruments, AlphaScreen and fluorescence polarisation (FP) as homogeneous assays, and Bioluminescence resonance energy transfer (BRET), an in cell interaction assay. SPR proved to be the method of choice for the determination of separate association and dissociation constants in real time, with the inherent drawback of limitation in sample throughput. While AlphaScreen is capable of measuring any kind of interaction in a high-throughput format, it is not suitable for determination of kinetic constants. FP provides a fast and simple assay setup for screening of LMW ligand binding, but is limited by the ligand size. BRET provides a medium-throughput assay platform for reproducible quantification of protein interactions in intact cells, it is, however, more cost and labour intensive than the other methods.

4.20 Targeted Proteomic and Structural Analysis of 14-3-3SIGMA, a P53 Effector Commonly Silenced in Cancer

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In order to comprehensively identify proteins interacting with 14-3-3sigma in vivo, tandem affinity purification (TAP) and the multidimensional protein identification technology (MudPIT) were combined to characterize 117 proteins presumably associated with 14-3-3sigma in human cells. The majority of identified proteins contained one or several phosphorylatable sites. The binding sites contributing to the potential direct interaction with 14-3-3sigma. 25 proteins were not previously assigned to any function and were named SIPS2–26 (for 14-3-3sigma-interacting-protein). Among the 92 interactors with known function were a number of proteins previously implicated in oncogenic signaling (APC, A-RAF, B-RAF, c-RAF) and cell cycle regulation (AJUBA, c-TAK, PTOV-1, WEE1). The largest functional classes comprised proteins involved in the regulation of cytoskeletal dynamics, polarity, adhesion, mitogenic signaling and motility. Accordingly, ectopic 14-3-3sigma expression prevented cellular migration in a wound- ing assay and enhanced MAP kinase signaling. The functional diversity of the identified proteins indicates that induction of 14-3-3sigma could allow p53 to affect numerous processes in addition to the previously characterized inhibitory effect on G2/M progression. The data suggest that the cancer-specific loss of 14-3-3sigma expression by epigenetic silencing or p53 mutations contributes to cancer formation by multiple routes. Furthermore, we determined the crystal structure of 14-3-3sigma and compared it to the known structures of 14-3-3tau and zeta. Thereby, we identified determinants of 14-3-3 isoform specific ligand interaction and dimerization.
4.21 Cloning and Expression of Apoptin Fusion Gene and the Effects on Apoptosis of Tumor Cells
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Apoptin is a protein that can specifically induce apoptosis of tumor cells but does not affect normal cells or diploid cells. It has been confirmed that there are many receptors of epidemic growth factor (EGF) existing on the surface of tumor cells. Bacillus pyocyaneus exotoxin II domain (P II) encodes a transmembrane protein. Therefore if the genes encoding EGF and P II could be integrated into the gene encoding apoptin, the ability of apoptin to promote the apoptosis of tumor cells would be enhanced greatly. In order to study the mechanism by which apoptin induces tumor cell apoptosis, the apoptin fusion gene was amplified by PCR, the apoptin fusion gene was cloned into an expression vector pcDNA3.1, and then positive recombinant plasmids were transfected into host cell of CHO to expression the apoptin fusion protein. The specific protein expressed (about 30KDa) was detected by SDS-PAGE. We incubated the fusion protein with cells such as HeLa cells, prostate cancer cells(PC-3M), gastric cancer cells(JGC4901), colonic cancer cells(SW111), hepatocarcinoma cells(Bel7402). The results showed that apoptin fusion protein had been successfully enterthese tumor cells as confirmed with ELISA and Western blot. The results showed that remarkable apoptotic characteristics such as nuclear shrinkage appeared in tumor cells, the apoptosis percent were higher than control, the cell cycle were exchanged also detected by FACS. It was suggested that the apoptin fusion protein could induce apoptosis in different tumor cells effectively.

4.22 IntAct: An Open Source Framework for Molecular Interactions
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IntAct is an extensible open source framework for molecular interactions. The project implements the PSI-MI standard for the representation and annotation of interaction data and provides a public repository populated with experimental data from project partners and curated literature data. Additionally, a set of applications is available to manage the IntAct repository and analyse the data. The framework relies on a relational database system which can be either Oracle or PostgreSQL. It integrates a user-friendly web interface that allows the user to browse data using a search engine which features intelligent lookup by gene name, various cross references such as UniProt, InterPro, GO, PubMed, FlyBase. IntAct displays interactions graphically using an interactive 2D visualisation system showing interactions networks in the context of GO annotations. IntAct also provides additional analysis modules, such as the prediction of targets for pull-down experiments. The web-based curation interface allows multi-user access and a well-controlled curation process. The system, including all source code and data in both HTML and PSI-MI XML representation, has been publicly available since August 2003 and contains more than 60000 binary interactions and complexes accumulated over the first 2 years. The data are released on a monthly basis. If you want to create a local IntAct instance, download the public data, submit your experimental data prior to publication or simply use the central and freely available instance of IntAct, it is accessible at http://www.ebi.ac.uk/intact.

4.23 Comparative Analysis of PDZ Domains Binding Modalities
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Small modular binding domains mediate protein-protein interactions by conferring specificity in multiprotein complex formation. To study the mechanisms that determine the recognition of specific target motifs, we have performed a comparative study of the binding properties of distinct PDZ domains (PSD-95/Dlg/ZO-1). The variability of PDZ primary sequences and the structural adaptability of their fold results in different binding modalities that enlarge the type and the number of interactions that each domain can engage. PDZs generally bind to the target carboxyl terminus, but they can also bind to internal sequences of other PDZ or other interaction modules. We have collected a set of PDZ domains deriving from proteins with diverse functions, such as scaffold adapters (hINADL, Mupp1, Cypher, Enigma, Let . . .), enzymes (PTP-BL), putative signal transducers (VSP). We have exploited an integrated proteomic approach in order to identify and characterize the specific targets of PDZ-mediated multi-protein-complexes. The experimental approach includes the screening of combinatorial libraries of phage displayed peptides and/or cDNA products, coupled with pull-down from brain extracts and mass spectrometry analysis. We have compared the relative efficiency and reliability of the different methods. Using phage display methodologies we have identified the residues and the structures involved in each domain/ligand interaction, making distinction among PDZs selecting “canonical” ligands (classifiable according to their C-terminal preference) and other PDZs that select unusual peptides and/or show preferences for more than one target. For some of the PDZ domains we have identified novel interacting proteins by mass spectrometry analysis that allow to infer a putative role of the corresponding complex.

4.24 Mammalian Proliferating Cell Nuclear Antigen May Function as a Double Homotrimer Complex
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The diverse function of proliferating cell nuclear antigen (PCNA) may be regulated by interactions with different protein partners. Interestingly, the binding sites for all known PCNA-associating proteins are on the outer surface of the PCNA trimer (hINADL, Mupp1, Cypher, Enigma, Let...) , enzymes (PTP-BL), putative signal transducers (VSP). We have exploited an integrated proteomic approach in order to identify and characterize the specific targets of PDZ-mediated multi-protein-complexes. The experimental approach includes the screening of combinatorial libraries of phage displayed peptides and/or cDNA products, coupled with pull-down from brain extracts and mass spectrometry analysis. We have compared the relative efficiency and reliability of the different methods. Using phage display methodologies we have identified the residues and the structures involved in each domain/ligand interaction, making distinction among PDZs selecting “canonical” ligands (classifiable according to their C-terminal preference) and other PDZs that select unusual peptides and/or show preferences for more than one target. For some of the PDZ domains we have identified novel interacting proteins by mass spectrometry analysis that allow to infer a putative role of the corresponding complex.
Deciphering Stem Cell Differentiation Program Using Proteomics and Bioinformatics Tools

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The embryonal carcinoma (EC) P19 cells provide an ideal model to study the molecular combination along differentiation. The cells can be induced into the three germ layer lineages. Upon induction (in the presence of retinoic acid) the cells become post-mitotic and undergo a program of differentiation to develop into functioning neurons. Within 18 hours, a set of genes are induced that leads to dramatic morphological and physiological changes. About 24 hrs later, the cells establish their neuronal polarity and exhibit a distinguished axon and dendrites properties. The cells were manipulated to acquire a defined neurotransmitter phenotype of the cholinergic or glutamatergic neurons. We applied a comparative 2D electrophoresis analysis to P19 neurons, specifically for the membranous enriched fractions throughout the neuronal maturation process. Differential analysis of the 2D gels using Z3 software revealed 3–4% change in the level of protein expression between the two cultures as well as about 400 genes that were differentially expressed (at a fold change of >2). Examples of significantly different protein expression are the alternative spliced version of drebrinA and E, actin binding proteins that shape the dendrites, agrin, tau and PDZ scaffolding proteins. We applied PANDORA (www.pandora.cs.huji.ac.il) for analyzing collectively the genes and proteins that were changed throughout this study. We realized that most changes are associated with only few pathways. The most prevalent ones are the WNT pathway, the Ubiquitin dependent folding pathway and the NGF-dependent signaling pathways. Furthermore, by comparing the gene expression results with the proteomics based results we were able to indicate statistically significant changes in localization and trafficking of proteins following their expression. The role of growth factors and neurotrophins in affecting the neuronal differentiation and survival program of the EC cells will be discussed. This study is supported by the DIAMONDS and the BioSapiens EU consortium and by the Sudarsky Center for Computational Biology.

Reverse Engineering of Cell Cycle-specific Protein Networks from Two-dimensional PAGE Data

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Despite the large amounts of research in genomics, more effort is needed for development of methodologies to identify and analyze complex biological networks. High-throughput gene expression assays are yielding vast amounts of data on gene expression networks, but new techniques such as proteomics will further add to this glut of information. Advances in proteomics technologies are enabling to investigate systematically the complex molecular processes utilizing both protein expression and post-translational modifications, which reflect on protein molecular weight and isoelectric point. These modifications can be referred to important cell system processes such as signal transduction, programmed cell death, differentiation, proliferation, disease progression etc.

Two-dimensional gel electrophoresis is the major protein mapping technique in proteomics. The goal of the study is to establish link between protein 2D-PAGE profiles and cell system biology data. Analysis the events of the cell-cycle-specific protein networks were performed by 2D electrophoresis. For this purpose, the skin fibroblast culture was synchronized by hydroxyurea and culture samples through each 30 min after synchronization were taken for 2D-PAGE analysis. 22 gels for synchronized cells and same number of control gels were further submitted to reverse engineering algorithm. Protein spot coordinates X, Y and Z, which correspond to protein mass, pI and expression, respectively, were used for distance calculations between each spot and their natural neighbors. The cross-covariations of obtained distances were used as weights for calculation of spot interconnection strengths. The resulting matrix was submitted to multidimensional scaling (MDS), which allows reconstructing a configuration of protein spots in two- or three-dimensional space using their interconnection values. Obtained results showed that more than 75% of variations established in 2D-PAGE gels can be covered by produced 3D plot. Revealed protein networks were verified using previously published data, which describes cycle-specific cell events. Thus, a system-level approach provided allows discovering key molecular connections in a cell processes through reverse engineering of proteins network from 2D-PAGE profiles.
**4.27**

**Analysis of Human Plasma Proteins under Non-denaturing Conditions**

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Two-dimensional gel electrophoresis (2DE) under denaturing conditions is widely employed for the analysis of complex protein systems, mainly because of the necessity to separate each polypeptide before their identification. However, the advances of protein identification techniques, especially mass spectrometry, enabled the analysis of proteins separated under non-denaturing conditions. Even native protein mixtures without the separation steps can be analyzed by mass spectrometry to obtain information on each component protein.

We have been working on the separation and analysis of human plasma or cellular proteins using non-denaturing 2DE and have tried to correlate the non-denaturing 2DE patterns with denaturing 2DE patterns. This process was facilitated by mass spectrometric techniques, MALDI-TOF MS followed by peptide mass fingerprinting and ESI-MS/MS followed by mass-tag sequencing (Mukai and Manabe, *J. Electrophoresis* 2004, 48, 59–66, Jin and Manabe, *Electrophoresis* 2005, 26, 1019–1028). Further, recently we found that direct targeting of human plasma for MALDI-TOF MS provided detailed information on low-molecular-mass (up to 30 kDa) proteins (Jin and Manabe, *Electrophoresis* 2005, in press) when the mass spectra are compared with the accumulated data on human plasma proteins. The results on the analysis of protein-protein interactions in human plasma using non-denaturing 2DE and mass spectrometry will also be summarized.

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**4.28**

**Molecular and Functional Characterization of Multiprotein Complexes Interacting with Serotonin 5-HT2 and 5-HT4 Receptors**

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Many G-protein coupled receptor (GPCR) functions are mediated by protein networks that interact with their intracellular domains, especially the C-terminal domain. These networks are organized around modular proteins containing protein-protein interaction domains. To date, many proteins identified as binding partners of GPCRs are PSD-95/Disc-large/Zonula occludens-1 (PDZ) domain containing proteins, which recognize a PDZ recognition motif (PDZ ligand) located at the carboxy-terminal extremity of the receptors. We have recently developed a proteomic approach combining peptide-affinity chromatography, two-dimensional electrophoresis and mass spectrometry to provide a global picture of multiprotein networks that interact with the C-terminal domain of several GPCRs. We found that two GPCR subfamilies activated by serotonin (the 5-HT2 receptor subtypes and the 5-HT4 receptor splice variants) interact with specific sets of intracellular proteins. These include scaffolding proteins containing one or several PDZ domains and signaling proteins. Co-immunoprecipitation, immunofluorescence and electron microscopy experiments confirmed that these specific interactions take place in living cells. Functional studies indicated that the interaction of the 5-HT2C receptor with its PDZ binding partners modulates its internalization and the desensitization of receptor-mediated Ca2+ response in heterologous cells and neurons. We also found that sorting nexin 27, a PDZ protein recruited by the 5-HT4a receptor, redirects part of these receptors to early endosomes. Hence, the interaction of the C-terminal domain of 5-HT2 receptors and 5-HT4 receptor splice variants with specific protein networks may contribute to their trafficking, targeting to specific cellular compartments and their different signal transduction properties.
4.29

The Regulation Mechanism of the Eukaryotic Translation Initiation Factor 4E in Drosophila melanogaster

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Objective: Translational control is critical for the proper regulation of cell cycle, tissue induction and growth, normal embryogenesis and germ-line development. The translation requires the recruitment of a wide number of proteins that fulfill various functional roles. One of the main proteins involved in this process is the eukaryotic translation initiation factor 4E (eIF4E). This protein directly binds the 7-methyl-guanosine cap structure at the 5’ end of mRNAs and recruits additional factors to form a functional complex that, after mRNA circularization, initiates translation. The eIF4E function can be regulated at different levels by a variety of molecular events, such as phosphorylation, growth factors stimuli, interaction with activators or repressors (i.e. 4E-BPs). These regulation mechanisms are still unclear and several proteins involved in these processes remain unknown. This work is aimed to the identification of the protein partners involved in eIF4E regulation in Drosophila melanogaster by a functional proteomic approach.

Methods: The eIF4E complexes have been purified from Drosophila melanogaster ovaries by immunoprecipitation using anti-eIF4E antibodies immobilised on protein G coated beads. Complex components were eluted with Laemmli buffer, separated by SDS-PAGE, digested chemically with a 13C/12C reagent specific to primary amino groups in proteins. In an advanced approach, we also generated more significant chemically with 13C/12C modified amino acids or their extracts specificity of protein interaction still needs to be confirmed. We therefore identified new interaction partners of Pex3p; however, in some cases the specificity of protein interaction still needs to be confirmed. We therefore successfully identified new interaction partners of Pex3p; however, in some cases the specificity of protein interaction still needs to be confirmed. We therefore successfully identified new interaction partners of Pex3p; however, in some cases the specificity of protein interaction still needs to be confirmed.

Conclusion: The immunoprecipitation approach coupled to nanoLC-MS/MS analyses provided to be an effective strategy in the investigation of eIF4E interactors.

4.30

Proteomic Approach to Identify Pex3p-interacting Proteins in Saccharomyces cerevisiae

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Pex3p is an integral membrane protein of peroxisomes that plays an essential role in biogenesis and integrity of these organelles. Yeast mutants deficient in Pex3p lack detectable peroxisomal membrane structures and mislocalize peroxisomal matrix and membrane proteins to the cytosol. Therefore, it has been suggested that Pex3p is involved in early stages of peroxisomal membrane assembly. It also represents an important component of the peroxisomal matrix protein import machinery. Generally, highly complex functions such as organelle biogenesis and protein translocation are mediated by larger protein complexes. To identify new binding partners of Pex3p in Saccharomyces cerevisiae, we used epitope tagging and affinity purification combined with MS analysis. Peroxisomal extracts of yeast cells expressing Pex3p fused to a TEV-protein A tag (Pex3p-TEV-ProT) were subjected to affinity purification via an IgG matrix. Proteins bound to the matrix were eluted, separated by SDS-PAGE and analyzed by MS. As a control, peroxisomal extracts of cells expressing wild-type Pex3p were processed in parallel. With this approach, we successfully identified new interaction partners of Pex3p; however, in some cases the specificity of protein interaction still needs to be confirmed. We therefore successfully identified new interaction partners of Pex3p; however, in some cases the specificity of protein interaction still needs to be confirmed.

4.31

The Calcium-sensing Receptor (CASR) Dimerizes in the Endoplasmic Reticulum; Biochemical and Biophysical Characterization of CASR Mutants Retained Intracellularly

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The calcium-sensing receptor (CASR) belongs to group C of the G protein-coupled receptor superfamily. The CASR exists at the plasma membrane as a homodimer. Although other members of group C have been shown to be constitutive homodimers it has not been precisely documented at which point in the biosynthetic pathway the CASR dimerizes. To address this issue we have made a biochemical and biophysical analysis of wild-type and mutant CASRs (harboring R66H, R66C or N538X inactivating mutations identified in familial hypocalciuric hypercalcaemia/neonatal severe hyperparathyroidism patients) that were transiently expressed in human embryonic kidney (HEK) 293 cells. By a trans-reporting assay (measuring MAP kinase activity) and an aequorin assay (measuring intracellular calcium release), all mutants were shown to be markedly deficient in cell signaling in response to increases in the extracellular CASR ligands, calcium and gadolinium, relative to wild-type. By immunoblot analysis of cell extracts, all mutants, although as well-expressed as wild-type, lacked mature glycosylation, indicating impaired trafficking from the endoplasmic reticulum (ER). In addition, whereas dimerized forms of wild-type, R66H and R66C mutants were present, none was observed with the truncated N538X mutant. By immunofluorescent confocal microscopy of nonpermeabilized cells, while strong staining at the plasma membrane was observed for the wild-type, little or no staining was seen for the mutants. In permeabilized cells strong perinuclear staining was observed for both wild-type and mutants. By fluorescent confocal microscopy using two different fluorophors, one for the CASR and the other for markers of the ER or Golgi apparatus, the mutant CASRs were localized within the ER but not the Golgi apparatus. By the use of the photobleaching fluorescence resonance energy transfer (pBFRET) method, it was demonstrated that the wild-type, R66H and R66C mutants were dimerized in the ER whereas the N538X mutant was not. Hence, CASR dimerization in the ER occurs as an early event and is likely to be necessary (but is not sufficient) for exit of the receptor from the ER and trafficking to the cell surface.
4.32

Uncovering the Structure of Protein-Protein Interaction Networks

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Understanding the inner workings of the cell constitutes the foremost fundamental problem of modern biology. The information contained in large protein-protein interaction (PPI) networks is being exploited for understanding the cell and developing new drugs. Currently available PPI networks of model organisms, containing thousands of nodes (proteins) and tens of thousands of edges (interactions), are noisy and largely incomplete. PPI networks of higher organisms will be much larger. As these data sets grow, it is important that our models keep representing the data well, since the models can be used for data cleaning and experimental planning. The currently accepted scale-free model of PPI networks is based on global statistical properties of PPI networks. These measures are very weak, since qualitatively different graphs can have equal values in these measures. In addition, due to the nature of wet lab experiments, PPI networks are currently probed well only locally. Therefore, we analyze the local structural properties of existing PPI networks using a novel tool that exhaustively counts frequencies of small connected subgraphs over an entire network. We demonstrate that existing models of PPI networks do not fit the data under this measure, and propose a new, geometric random graph model of PPI networks that fits the data significantly better than existing models. In a geometric random graph, nodes correspond to uniformly randomly distributed points in a metric space; two nodes are adjacent in the graph if they are close enough in the metric space. Since counting subgraphs of large graphs is computationally intensive, we propose two heuristics for uncovering local structure of PPI networks and demonstrate that the heuristics work well on real data.

4.33

Identification and Characterisation of Cellular Proteins Associated to the Epstein-Barr Virus-encoded Nuclear Antigen 5 (EBNA 5)

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The Epstein-Barr Virus (EBV) is a lymphotropic herpesvirus infecting more than 90 percent of the population worldwide. The virus establishes a life-long latency upon acute infection in resting memory B cells. It is the causative agent of infectious mononucleosis and is associated with several malignancies, such as Burkitt’s lymphoma and lymphoproliferative disorder in post transplant- and AIDS-patients. EBV-encoded nuclear antigen 5 (EBNA 5) is one of the first viral proteins detected after primary EBV infection and has been shown to be required for efficient transformation of B lymphocytes. The primary known function of EBNA5 is cooperation with EBNA2 in the transcriptional activation of the LMP1 promoter. However, we have recently shown that EBNA5 inhibits pre-mRNA cleavage and poly-adenylation. EBNA5 has been shown to interact with a number of cellular proteins such as the Hsp/Hsc70 complex, but the functional implications have not yet been investigated. To further elucidate its functions, we have isolated EBNA5-protein complexes and identified several of the protein components. We identified proteins not previously known to interact with EBNA5. An improved tandem affinity purification method was developed for isolation of protein complexes in mammalian cells. A Protein A/StrepTag II affinity purification tag was fused to the C-terminus of EBNA5. Protein complexes were purified from HEK 293 cells transiently transfected with a plasmid encoding the EBNA 5 fusion protein. The eluate from the affinity purification was separated on SDS-PAGE gels. In coomassie stained gels, protein bands present in the EBNA 5 eluate were selected for identification by in-gel tryptic digestion, followed by MALDI-TOF MS or LC-MS/MS. The MASCOT search engine was used for database searches. Among the identified interaction partners were several proteins belonging to the HSP-family, regulators of HSP-function and one protein involved in RNA-processing.
4.34 Self-assembling Protein Microarrays for Mapping Protein-Protein Interactions among 1300 Breast Cancer-related Proteins

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Protein microarrays are an emerging tool for systematic High Throughput studies of protein-protein interactions. Our lab recently developed a new Protein microarray system, the “Nucleic Acid Programmable Protein Array” (NAPPA) (Ramachandran et al., 2004). In this method, cDNA plasmids encoding the target proteins are spotted on slides. Subsequently, the target proteins are expressed in situ using rabbit reticulocyte lysate. The epitope-tagged query protein is co-expressed in the in vitro translation mix and interacts with the corresponding target proteins. Bound query proteins are detected by using anti-tag antibodies. This novel approach will enable us to exploit a collection of genes generated in our laboratory, the “Breast Cancer 1000 Gene Collection” (BC1000). This unique, sequence-verified set of more than 1300 cDNAs comprises genes that have been demonstrated in the biomedical literature to be relevant to breast cancer. My aim is to use the NAPPA technology to generate a protein array of the BC1000 gene collection to map protein-protein interactions among these 1300 proteins. The final goal of this project is to construct an interaction map which will be made publicly available to the scientific community. This level of throughput requires automation and refinement of current NAPPA techniques to obtain an efficient, reproducible, and cost-effective methodology. As a first step I designed 3 new expression vectors which have N-terminal tags (e.g., 3xFlag, 3xmyc). These tags are essential for capture and detection of the expressed proteins. In a pilot test I transferred a set of more than 30 different breast cancer genes (e.g., EGFR, ErbB2, Fos, Jun) into all 3 vectors and generated a protein microarray chip using the NAPPA system. As a result, NAPPA detected most known, biochemically-verified binary protein-protein interactions. Furthermore, NAPPA is currently being optimized for large-scale analysis, and protein arrays with 100 and finally 1300 breast cancer proteins will be generated in the near future. We anticipate that this study will contribute significantly to the progress of breast cancer research and will facilitate efforts to identify potentially novel therapeutic targets.

4.35 A Novel Tag System for the Analysis of Protein-Protein Interactions by in Vivo Cross-linking

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Molecular mechanisms in signal transduction pathways are characterized by transient interactions between several proteins, some of which are part of assembled complexes. Analysis of such protein-protein interactions requires the isolation and purification of these transient complexes. Affinity chromatography is commonly used to selectively purify complexes from a bait protein fused to an immuno-specific tag. After extensive washing to remove unspecific proteins and contaminants, the complex is eluted and individual components are identified by mass spectrometry. Weak or transient interaction partners are often lost during stringent washing using high salt concentrations or detergents. To overcome this problem we have recently established a novel approach for the analysis of protein-protein interactions that is based on treatment of live cells with formaldehyde, which coheres interacting proteins by chemical cross-linking mediated mainly by lysine residues. Here we present a novel tag system fusing a stretch of five lysine residues as docking sites for formaldehyde cross-linking to the myc epitope tag. We are applying this novel tag to study protein interactions of the glycogen synthase kinase (GSK3β), which plays a role in a large number of cellular processes including the insulin and wnt signaling pathways. We are able to show that the addition of the lysine stretch is increasing the number of cross-linked proteins. Furthermore, different numbers of Ser(Gly)k repeats were inserted as linker between the novel tag and the bait. For GSK3β, we observed that the increase in cross-linked proteins is dependent on the linker length. This tag embodies a novel tool to elucidate protein-protein interactions such as those between kinases and kinase substrates in cell signaling pathways.

4.36 Identification of Protein-Protein and Protein-DNA Interactions Using Functional Microarrays

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Besides the understanding which proteins are expressed in a certain tissue to a defined time point, the characterization of protein-DNA interaction is essential for a detailed description of complex biological systems. Cells respond along defined pathways to external stimulus. This can result in cell division, in the formation of tissues but also in the development of diseases. Signals are transmitted by specific protein-protein interactions and results in a changed expression of different proteins. These changes are normally analysed using RNA expression techniques. A key role in the responses is the regulation of those genes by transcription factors. To gain a better insight into the underlying regulation mechanisms a variety of protein microarray based techniques were developed and applied to specific biological questions. We have used the well-established functional genomic technique protein microarrays and the recently established ds DNA microarray technique to identify protein-DNA and protein-protein interactions. Using the developed techniques, we were able to identify cation dependent interactions between members of the S100 protein family and putative interaction partners. Beside already known interactions we were able to identify several new once and verify those with an independent method. In addition we were able to use functional protein microarrays for the analysis of protein-DNA interactions. In a close cooperation with the group headed by Dr. C. Hultschig we established a technique for a site-specific immobilisation of ds DNA onto microarrays. After incubation either with a purified protein or with a complex mixture e.g., cell extract, we were able to detected proteins that has bound to their immobilized target DNA with specific antibodies.
Progression through the cell cycle is central to cell proliferation and fundamental to the growth and development of all multicellular organisms, including higher plants. The periodic activation of complexes containing cyclins and cyclin-dependent kinases (CDKs) is central in cell cycle control. Elucidating the molecular interface between the CDK/cyclin complexes, their substrates and other interacting proteins is essential to fully understand the cell cycle and its link with plant development. In this study we report on the results of a targeted proteomic study using the tandem affinity purification (TAP) technique combined with mass spectrometry to characterize TAP-tagged protein complexes from stably transformed Arabidopsis suspension cultures. These are an unlimited source of a- or asynchronously dividing cells, allowing us to isolate the low abundant cell cycle protein complexes. The cultures were transformed via Agrobacterium co-cultivation. The Multi-Site Gateway technology (Invitrogen) was used to efficiently clone C-terminal TAP-tagged bait proteins, under control of their endogenous or a strong constitutive promoter. Besides the original described TAP-tag (Rigaut et al., 1999), we have developed a novel SFHA-tag, which consists of the Strep-tagII (IBA GmbH), two tobacco etch virus (TEV) protease cleavage sites, three copies of the FLAG epitope and three copies of the hemagglutinin (HA) epitope. Next, we optimized protocols to cryo-preserve the transformed and wild-type Arabidopsis cell suspension culture. This opens the possibility to handle and store large sets of transgenic cell cultures, necessary for setting up a high-throughput TAP-platform. We have performed tandem affinity purifications on total protein extracts from a first series of transgenic cultures expressing TAP-tagged bait proteins. Purified proteins were identified by Maldi-TOFTOF in collaboration with CEPROMA (Antwerp University). Proteins co-purified with CDKA;1 included known associated proteins such as D-type cyclins, CKS, and KRP s, but also several other proteins. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Séraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nature Biotechnology, Vol. 17, 1030–1032.

Systematic Identification of Human Protein-Protein Interactions Using Protein Arrays

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Protein arrays and microarrays have become a valuable tool for high-throughput biology (LaBaer and N. Ramachandran, Curr. Opin. Chem. Biol. 2005, 9, 14–19; Feilner et al., Curr. Proteomics 2004, 1, 283–295). For the systematic identification of protein-protein interactions (PPIs) His-tagged recombinant human proteins were expressed in a 384-well format, spotted onto filter membranes in high-density and screened for their ability to interact with GST-tagged recombinant proteins. PPIs were detected using a filter-based enzyme-linked immunosorbent assay. Interaction partners for the proteins CHIP, amphiphysin II and VCP were identified, and subsequently confirmed by pull-down, co-immunoprecipitation and two-hybrid assays. Using our array-based technology we were able to identify novel PPIs potentially involved in neurodegenerative disease processes, protein folding and endocytosis.
TET-KRAB-mediated Gene Expression Enables the Identification of Proteins Functionally Associated with TIF-1BETA Protein Complexes

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The transcriptional intermediary factor 1 beta (TIF-1beta) is considered to be essential for transcriptional silencing (e.g., Margolin et al., PNAS 1994, 91, 4509–4513) promoted by the sequence-specific binding of KRAB-containing C2H2 zinc finger proteins to target gene loci. An integrative functional genomic approach has been undertaken to study repressor functions of Krüppel-associated box (KRAB) proteins in close conjunction with TIF-1beta protein complexes in the TIS-10 cell system (Deuschle et al., MCB 1995, 15, 1907–1914). Hereto, transcriptome, proteome and toponome methods were combined to functionally analyze protein complexes within the stably transfected cell line TIS-10. HDAC inhibitors demonstrated that histone deacetylation is not required for mediating transcriptional repression of doxycycline-regulated Tet-KRAB protein constructs (P. Lorenz, Biol. Chem. 2001, 382, 637–644). In contrast, the knockdown of Tet-KRAB mRNA as well as of TIF-1beta RNA led to a 180-fold increase of luciferase reporter activities exceeding the doxycycline-mediated gene activation stating a 40-fold activation. Furthermore, transcripts of proteins found to be differentially regulated in knock-down experiments of TIF-1beta as well as proteins found to be physically associated with TIF-1beta are selectively studied by means of their repressor activities. Thus, the TIS-10 cell system harbours a great screening potential for stratifying cellular components that have a strong impact on TIF1-beta repressor functions. This analysis should finally lead to the elucidation of KRAB zinc finger specific protein functions.

A Proteomic Approach to Understand the Anti-invasive and Pro-apoptotic Effect of Xanthohumol in Human Breast Cancer Cells

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Nowadays, there is growing interest in chemopreventive phytochemicals like flavonoids. We demonstrated earlier that the hop-flavonoid xanthohumol (X) has an anti-invasive effect on human breast cancer cell lines. Here we demonstrate that X induces cell death of various human breast cancer cell lines but not of a panel of human primary normal breast epithelial cells. Treatment with X initiated formation of autophagic vacuoles, as visualized by monodansyl-cadaverin staining and by transmission electron microscopy. In addition, X stimulated the expression of the autophagy gene product beclin1. Finally, apoptotic events like stimulated cleavage of PARP, and the appearance of nuclear condensation were observed. In parallel, X induced an overexpression of an 80-kDa protein in the cancer cells. We have identified it as the endoplasmic reticulum (ER) chaperone BiP (GRP78) after 2-DE separation, in-gel tryptic digestion and analyzing the peptitic fragments by MALDI-TOF/LC-MS-MS. This upregulation coincided with an increased phosphorylation of the extracellular signal-regulated kinase (Erk). Furthermore, inhibition of m-calpain activity blocked both the stimulated BiP expression and Erk phosphorylation, which points to the involvement of this Ca2+/H11001-dependent protease during ER stress.

In conclusion, we have demonstrated by a proteomic approach that X induced ER stress and calpain activity specifically in human breast cancer cells but not in normal epithelial mammary cells. This stress is associated with apoptotic and autophagic events which eventually result in cell death.
4.42 Proteins of the Epidermal Differentiation Complex (EDC) Are Differentially Expressed in CD4+CD25+ Regulatory T Cells

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Objective: CD4+CD25+ T regulatory cells (Tregs) play a central role in the maintenance of peripheral tolerance. These minor populations of CD4+ T cells which co-expresses the IL-2 receptor a-chain (CD25) is critical for the control of autoreactive T cells in vivo. CD25+ Tregs are both hyporesponsive and suppressive. Activated CD25+ Tregs strongly suppress proliferation and cytokine release of conventional CD4+CD25- T cells. However, the molecules mediating this suppression are still elusive.

Methods: To identify proteins, preferentially expressed by CD25+ Tregs and involved in their suppressive properties, we isolated T cells from buffy coats and leukapheresis of healthy volunteers and performed differential proteome and bioinformatical analyses of resting and activated CD25+ and CD25- Tregs compared to resting and activated conventional CD4+CD25- T cells.

Results: Approximately 1,600 protein spots were matched and more than 450 Treg-specific proteins were identified by MALDI mass spectrometry. Bioinformatical analysis of 25 differentially expressed proteins shows that some proteins map to chromosomal locations previously associated to common autoimmune diseases. Interestingly, gene products on the genomic cluster EDC are statistically over-represented. We performed meta-analysis of six additional large-scale studies (Psoriasis, Atopic Dermatitis etc.) where the EDC is highly over-represented and extracted the EDC- and the Treg-interactome by manual literature mining.

Conclusion: We suggest that EDC-genes are highly co-regulated and that EDC proteins form a tightly linked sub-network, supporting the hypothesis that one main regulatory event may affect the expression of many genes within the EDC, proposing that this mechanism may accomplish or participate in Treg-development.

4.43 The Studies of P15ink4b Tumor Suppressor in Chicken

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The INK4 locus on human 9p21, and the corresponding locus on mouse chromosome 4, encode two members, p15ink4b and p16ink4a, of the INK4 cyclin-dependent kinase inhibitor family. INK4 proteins block the phosphorylation of the retinoblastoma gene product and play a key role in tumors suppression and control of cell proliferation. However, it is confirmed by the some investigations that the chicken locus lacks the capacity to encode p16ink4a. So it is to say p15ink4b should be much more important in control of cell proliferation in chicken. We used MD tumor as research model to investigate the association between p15ink4b and telomere, telomerase and some other intracell proteins. We cloned p15ink4b gene from apoptosis chicken cell which be produced by physical and chemical methods. We expressed the p15ink4b protein in BAC-TO-BAC Baculovirus Expression Systems and some tumor cells. It is significant that cells could be apoptosis when p15ink4b expressed in DT40, the MDV-derived lymphoblastoid cell line, MDCC-MSB1 (MSB-1), in which aberrant CpG island methylation of p15ink4b gene occurred. We purified the p15ink4b protein by using the tandem affinity purification method (Danier Forler et al., EMBL, Heidelberg). The association between p15ink4b protein and other proteins in cell has also been researched.

4.44 Identification of Ubiquitin-interacting Proteins in A549 Cells with the Multidimensional Protein Identification Technique following Ubiquitin Affinity Chromatography

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Ubiquitin regulates many cellular functions by the conjugation (ubiquitination) onto the target proteins. The discovery of new ubiquitinated proteins as well as their functions seems to be everlasting, which has led ubiquitin to the nicknamed “Darwin’s phosphate.” In order to understand the functional diversity of ubiquitin, we have tried to identify the ubiquitin-interacting proteins (UIPs) from A549 lung adenocarcinoma cells. We first enriched UIPs using ubiquitin-affinity chromatography in which mutant ubiquitin of I44A was used as control because 44th isoleucine of ubiquitin is known to be the most important residue for its hydrophobic interactions. Following the digestion of enriched UIPs with trypsin, they were identified through the tandem mass spectrometry directly linked with SCX/RP tandem chromatography and database searching using Sequest program. Reliability of this approach was confirmed by the identification of the expected proteins such as ubiquitin activating enzyme (E1) and many deubiquitinating enzymes including ubiquitin carboxyl hydrolase L1. In addition, we identified various proteins containing UBA (ubiquitin associated) domain, UIM (ubiquitin-interacting motif), CUE (coupling of ubiquitin to ER degradation) domain, and VHS (vPS27P/Hrs/Stam) domain. We believe the list of UIPs identified in this study provides the important initial step to find and elucidate the new functions of ubiquitin.
Protein Function in the Evolution of Yeast Protein Interaction Network

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The integrity of the yeast protein interaction network is maintained by a few highly connected proteins (hubs), which hold other less-connected proteins (non-hubs) together. Here these hubs’ functional tendencies are analyzed by a structured and controlled vocabulary – Gene Ontology. Compared with non-hubs, hubs tend to be involved in more functional roles, especially the “date” hubs. Hubs and non-hubs are enriched in different categories. Interestingly, most of them are reciprocal, i.e., if in one category hubs are enriched, non-hubs are often depleted. The phylogeny of functional category is consistent with that of proteins’ connectivity. All these observations strongly suggest protein function might be an important factor of the emergence of hubs throughout the evolutionary time.

Entropic Trapping of Globular Proteins

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The existence of entropic traps for globular proteins is proved both theoretically and experimentally. The phenomenon of entropic traps is caused by the high degree of degeneracy within a multi-charged state of a polyvalent ion. The greater the number of available states $Z(q)$ corresponding to the fixed value of the polyvalent ion charge $q$, the greater is the internal entropy of this charged state. The charge state of any protein is changed as the pH of the surrounding solution changes: $q = q(pH)$. An entropic trap appears in any gel-based system if the pH value is not constant inside the system. A protein is trapped at the point where the number of its states $Z(pH)$ is maximal. The observation of these traps can be seen on a standard immobilized pH gradient strip (IPG) and on a chip having discrete fixed pH values at defined points without any voltage source.