Membrane protein challenge

PROTEOMICS Membrane proteins are of central interest for pharmaceutical drug targeting but their physiochemical properties and their association with partner molecules in complexes make them difficult to analyze: Protein complexes must be solubilized in intact form from appropriately prepared membranes, captured by specific antibodies, and cut enzymatically into smaller peptides which can be analyzed and quantified. Experts provide support; one of them is the Logopharm GmbH in Freiburg/Germany.

While the genome encodes the information for the biosynthesis of proteins and determines the functional potential of an organism (genotype), its actual state including adaptive or pathological changes (phenotype) is reflected by its actual protein repertoire, its proteome. The proteome of a cell typically encompasses about 100,000 proteins. It is subjected to constant reconstruction depending on internal factors (cell type, cellular differentiation and development stage) as well as on external factors (growth conditions, stress factors, etc.). In addition, mature proteins exist in multiple isoforms, together with their immature precursors and degradation products. Proteomic analysis aims at mapping the spatial and temporal distribution of these proteins in cells or cellular compartments under defined conditions. It serves to elucidate composition and organisation of extended protein networks, clarify links between geno- and phenotype, identify proteins that could be used as biomarkers for the diagnosis and therapy of diseases or to monitor therapy successes, and potentially reveal novel drug targets. But proteomics is time-consuming.

A lot of challenges

The experimental strategy must be carefully designed, source material and membranes properly prepared and separated, and target proteins need to be isolated and identified. Membrane proteins, although they have important functions as transport proteins, cell recognition- and adhesion molecules, receptors and enzymes, and participate in most biological key processes (photosynthesis, neuronal excitation, respiration, immune response, signal transduction), are under-represented in proteome analysis due to their low abundance. Despite this almost one third of all genes encodes for membrane proteins and nearly two thirds of all pharmaceutical drugs exert their effects through this protein class.

There are additional challenges concerning proteome analysis of membrane proteins. The hydrophobic character of these proteins and biochemical modifications (e.g. glycosylations) make them difficult to isolate. Even though membrane proteins are generally stable when embedded in the membrane phospholipid bilayer, they tend to aggregate and precipitate in aequeous environment, making it necessary to use detergents, salts disrupting electrostatic interactions or even organic solvents. Typically, membrane proteins form large functional complexes together with partner proteins. Examples are certain types of voltage-dependent calcium and potassium channels in the plasma membrane of neurons: each channel itself consists of several protein subunits, and together they form a "super-complex" allowing entering calcium ions to directly activate the potassium channel.

Start-up company

This has been demonstrated in collaboration with the research group of Bernd Fakler, professor at the Institute of Physiology II at the University of Freiburg, Germany, and researchers from the University of Innsbruck/Austria. From Fakler's team experienced in membrane protein research the start-up company Logopharm GmbH was founded in autumn 2005 with headquarters in March near Freiburg and laboratories in the Freiburg BioTechPark. The

company's 4 employees provide services in the field of differential proteomics with focus on the analysis of membrane proteins and their complexes, using small sample amounts and native source material, keyword "micro proteomics". Customers come from academic institutions as well as from biotech and pharmaceutical companies.

"Among our clients are researchers from industry who aim to target a disease-relevant membrane receptor protein with a drug molecule and therefore need to establish a screening test", cites Dr. Uwe Schulte, CEO and cofounder of Logopharm, as typical request. The customer chooses the tissue or cell material, from which the receptor protein should be characterized. In some cases he also provides an antibody against the target molecule and/or a cell line which is overexpressing the wanted membrane protein. Antibodies are very specific high-affinity recognition molecules. They are used by protein researchers as selective purification ligands. Such antibodies are generated by repeated immunization of rabbits or mice with a mixture of an immunostimulatory adjuvant and a synthetic peptide that corresponds to a specific sequence of the target protein. In some rare cases the protein encoding gene is isolated from a cDNA-library, cloned into a circular DNA vector from bacteria and this construct is injected into the muscle or the skin of a laboratory animal. Here, the extrinsic protein is heterologously expressed and induces the formation of specific antibodies (genetic immunisation).

Cooperation partner Hans-Günther Knaus generates suitable antibodies for the Freiburg group if no commercial alternatives are available. Knaus is professor at the Innsbruck's University Institute for Biochemical Pharmacology and as well as Schulte's mentor Fakler cofounder of Logopharm, After proteins have been gently solubilized from the prepared plasma membranes with Logopharm's proprietary buffers, the antibodies are used for affinity capture of the target proteins. In order to reduce protein losses, the number of purification steps is kept at a minimum. Control experiments measuring proteins bound by a non-specific antibody help estimating the fraction of non-specific proteins. An alternative control is performed with knockout material.

The target protein in such cells and tissues had been eliminated by genetic engineering or removed by preincubation with an excess of specific antibody. "Even though these procedures narrow down the number of proteins that can be considered for the target complex, we still have to evaluate hundreds of proteins by mass spectrometry", explains Schulte. Ultimately, the quantitative comparison with results from additional experiments (which could be based on different cell materials, alternative antibodies targeting other epitopes on the receptor protein or antibodies against partner proteins, functional tests in the presence of possible complex partners, etc.), complemented by databases and literature will reveal the specific proteins that are part of the target complex.

Dr. Uwe Schulte, CEO and cofounder of Logopharm.

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Precise analysis of protein mixtures

Mass spectrometry (MS) allows the fast, comprehensive and precise analysis of protein mixtures. At first, the isolated proteins must be cut into small pieces (peptides), e.g. by using the proteolytic enzyme Trypsin. Peptides are then separated by nano-scale high-performance liquid chromatography (nano-HPLC) and sprayed into a tandem mass spectrometer (LC-MS/MS) where they are ionized and separated according to their mass and electrical charge. In a subsequent compartment, automatically selected peptide ions are fragmented by collision with gas molecules. The obtained fragment ion MS/MS-spectra are characteristic for each peptide. Finally, extracted mass information is used for database searches and assignment of spectra to individual protein sequences.

Software developed

This approach is referred to as top-down- or shotgun proteomics. The membrane protein specialists from Freiburg don't use any isotopic- or other labelling methods when quantifying proteins. "The MS spectra are recorded in intervals. The intensity of the peptide peaks over time reflects the elution profile of the peptides from the HPLC column. With approximately one thousand affinity separations and resulting LC-MS/MS datasets, we have gained tremendous experience with these elution profiles and can deduce the quantitative ratio of the peptides", Schulte ensures. The Logopharm team has developed software that facilitates the quantitative evaluation of LC-MS data. It will be provided to selected users for testing in the 4th quarter 2008; a respective publication is currently in preparation.

Beate Peiseler-Sutter