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Mass Spectrometry-Based Identification of a Potential Binding Partner of Glutamate Carboxypeptidase II

Hledání potenciálního vazebného partnera glutamát karboxypeptidasy II pomocí hmotnostní spektrometrie

Diploma Thesis

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I affirm this thesis was elaborated independently under the supervision of Dr. Jan Konvalinka and that all sources used were cited properly and that no part of the thesis has been submitted for other degree.

(Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.)

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English Abstract

The incoming paradigm of the network (or systems) biology calls for a new high throughput tool for a wide scale study of protein-protein interactions. Mass spectrometry-based proteomics have experienced a great progress in recent years and have become an indispensable technology of elementary as well as clinical research.

Glutamate carboxypeptidase II (GCPII; EC 3.5.17.21) is a transmembrane protein with two known enzymatic activities. Its expression is highly upregulated in some solid tumors and also in tumor-associated neovasculature in general. Nevertheless, none of the two enzymatic activities were shown to be physiologically relevant to these cells. Some facts point at a possible receptor function of GCPII, however, no specific binding partner has been found yet.

In the search for potential binding partners and/or ligands of GCPII, a series of methods have been employed, including pull-down experiment, immunoprecipitation and mass spectrometry. Sample preparation and mass spectrometry data processing methodology was specifically developed in order to identify potential binding partners. As one of the outcome of that methodology, the interaction of β-subunit of F1 ATP synthase was selected for further detailed analysis as a putative ligand of GCPII.

Key words: mass spectrometry, proteomics, GCPII, glutamate carboxypeptidase II, β-subunit of F1 ATP synthase, protein-protein interaction

Český abstrakt

Systémová biologie bude vyžadovat použití nových metod s vysokou propustností dat pro studium protein-proteinových interakcí. Proteomika založená na hmotnostní spektrometrii prošla v posledních letech rychlým vývojem a stala se nepostradatelným nástrojem studia makromolekul v základním i klinickém výzkumu.

Glutamátkarboxypeptidasa II (GCPII; EC 3.5.17.21) je transmembránový protein se dvěma známými enzymovými aktivitami. Tento enzym je exprimován ve větším množství v buňkách některých pevných nádorů a také obecně v neovaskulatuře pevných nádorů, nehraje zde však fyziologickou roli. Některé skutečnosti mohou poukazovat na potenciální receptorovou funkci GCPII, přirozený ligand tohoto proteinu však nebyl doposud identifikován.

Pro hledání možných vazebných partnerů a/nebo ligandů GCPII byly použity metody afinitní chromatografie, imunoprecipitace a hmotnostní spektrometrie. Byla optimalizována metodika přípravy vzorků pro hmotnostní spektrometrii a analýza získaných dat s cílem nalézt možné vazebné partnery. Jeden z identifikovaných proteinů- β-podjednotka F1 ATP-syntasy, byl vybrán pro důkladnější analýzu možné interakce s GCPII.

Klíčová slova: hmotnostní spektrometrie, proteomika, GCPII, glutamátkarboxypeptidasa II, β-podjednotka F1 ATP-syntasy, protein-proteinová interakce

Brief Contents

1. Introduction

1.1. Glutamate Carboxypeptidase II

Human glutamate carboxypeptidase II (GCPII) is a zinc transmembrane metallopeptidase that plays different role in various tissues. The enzyme has two known natural substrates: N-acetylated-L-aspartyl-L-glutamate (NAAG) and folylpoly-γ-glutamate that are linked to GCPII activity either in the central nervous system (CNS) or in the small intestine, respectively. However, GCPII is expressed in number of tissues with no clear physiological function.

In the central nervous system (CNS) GCPII exhibits an N-acetylated alpha-linked acidic dipeptidase (NAALADase) activity, i.e. a free glutamate is released from N-acetylated-L-aspartyl-L-glutamate (NAAG) [1-2]. NAAG as well as glutamate are one of the most abundant excitatory neurotransmitters in human CNS. However, excessive glutamate release can lead to neurone damage and some pathological states [3-4]. Thus the inhibition of NAALADase activity of GCPII in a synaptic cleft can have a neuroprotective effect and was even proven to function in rats [5].

In the small intestine GCPII liberates glutamates and folic acid from poly-γ-glutamylatedfolates. This folate hydrolase activity of GCPII facilitates the absorption of dietary folates that can further be transported into the body for use as a vitamin [6-7].

Due to markedly upregulated GCPII expression in both benign and malignant hyperplasia of the prostate in human [8-12], GCPII is known and clinically employed as a specific prostate cancer antigen [13-14]. Hence, its widely used nickname "prostate-specific membrane antigen" (PSMA).

1.1.1. Tumor-Associated Vasculature

GCPII is expressed (at least in minimal level) in number of human tissues [15]. However, no other clear physiological function of the protein has been experimentally confirmed except those two enzyme-related mentioned above. Interestingly, GCPII role was implicated in tumor-related angiogenesis [16]. GCPII was found in the endothelium of vasculature of solid tumors [17] and further it was confirmed *in vitro* that the expression of GCPII is related to the viability and tube-formation ability of the human umbilical vein

endothelial cells (HUVECs) [18]. This finding corresponds to previous experiments showing the invasiveness of HUVECs to be limited via the inhibition of GCPII proteolytic activity as well as using the treatment of GCPII antibody both in a dose-dependent manner [19]

An elegant attempt to utilize the distinct expression of GCPII in tumor neovasculature was done by *Liu et al.,2002*. A "selective infarctive therapy" using a soluble extracellular domain of tissue factor coupled to a GCPII catalytic site inhibitor was shown to reduce the tumor mass specifically and efficiently when administered intravenously in rats [20]. Since the expression pattern of GCPII differs markedly in human and rat [15], this experimental approach still remains in the field of basic research.

1.1.2. Structure of GCPII

GCPII is type II membrane glycoprotein consisting of 750 amino acids and of an apparent mobility of 100 kDa [21]. GCPII forms noncovalently-associated homodimer [22-23]. Each monomer consists of three regions: N-terminal cytoplasmic region (amino acids 1-18), transmembrane helix (amino acids 19- 43) and C-terminal extracellular domain (amino acids 44-750) [21]. The extracellular portion can be divided into three subdomains (Figure 1): protease, helical and apical (also protease-associated) [22-23]. Apical domain shares some structural characteristics conserved between many trafficking receptors and might be responsible, from the structural point of view, for a hypothetic receptor function [24].

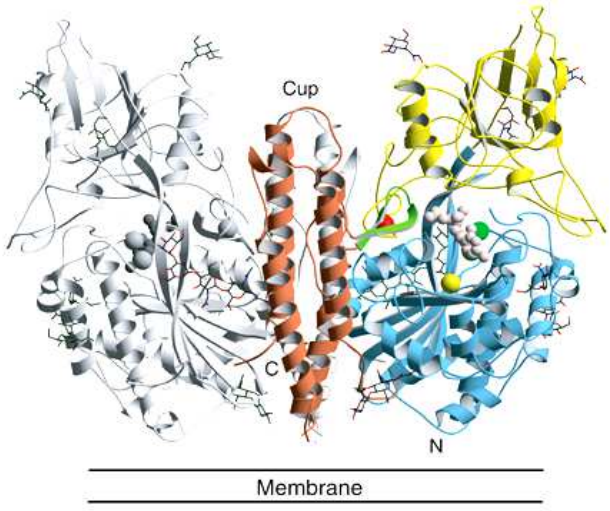

Figure 1.Structure of the Extracellular Portion of Glutamate Carboxypeptidase II:

One subunit of the dimer is shown in gray, while the other is colored according to the organization of domains. The protease domain is depicted in light blue; the apical one in yellow and the helical domain in brown. Dark green spheres represent the dinuclear zinc cluster at the active site, the Ca^{2+} ion is depicted by a red sphere, and the Cl^{-} ion by a yellow sphere. Picture adopted, text adopted and revised from [23,25].

The active site contains two zinc ions necessary for the enzymatic function and there is also one calcium ion far from the active site that holds together the protease and the apical domain. GCPII is also heavily glycosylated. N-glycosylation is vital for proper folding and also for subsequent GCPII secretion. These sites are critical for GCPII carboxypeptidase activity [26].

Despite the fact that GCPII shares only a 28% identity with transferrin receptor on amino acid level, the overall similarity of these proteins is evident [22-23,27] and was also suggested that the differences between them are predominantly those features directly responsible for the enzymatic activity of GCPII [23].

1.1.3. Internalization of GCPII

GCPII undergoes induced as well as spontaneous internalization *via* clathrin-coated pits and then accumulates in endosomes together with transferrin [28]. When associated with filamin A, GCPII is directed into recycling endosomal compartment after its internalization [29].

A general motif MXXXL in the cytoplasmic tail of GCPII was identified to be responsible for the internalization [30]. Internalization of GCPII in adaptor protein-2 (AP-2) negative mutants suggests an involvement of GCPII in a specific protein-protein interaction [30].

Kinetics of the internalization rather than dynamics was studied suggesting a putative receptor function of GCPII. Internalization rate and a proteolytic activity were shown to be decreased markedly by association with filamin A in prostate cancer-derived cell line (PC-3) [29]. This phenomenon together with a dose-dependent antibody-induced internalization [28] could be interpreted as a kind of an exposure of GCPII „waiting for" a ligand from the extracellular space. Hence, a flexible rate of the internalization can be generally seen as a regulation of trafficking of an unknown ligand or/and desensitization of the cell towards external stimuli [28].

2. Mass Spectrometry

Mass spectrometry (hereinafter only as MS) is an analytical technique for determination of components of a sample by their mass. A common mass-spectrometry instrument treats a sample in three stages: Produces a gas-phase ions from the sample, separates these ions according to their mass-to-charge ration (m/z), analyzes an abundance of each/chosen ion and finally processes the data for further *in silico* analysis but also to get a feedback in real time to be able to tune the machine "on-the-fly". Usually a device or procedure pre-separating and introducing sample is inserted prior to the mass-spectrum analysis. A liquid chromatography-coupled MS instruments are used for proteomic applications.

2.1. Ionization Methods

An important feature is the amount of energy transferred during ionization. Some analytes tend to undergo an extensive fragmentation and rearrangement under rough conditions which might be counter-productive for further analysis. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are two so-called „soft ionization" techniques most commonly used for analysis of biomacromolecules.

2.1.1. Matrix Assisted Laser Desorption Ionization

MALDI is based on dissolving of the analyte among small organic molecules of solvent having a strong absorption at the used laser wavelenght. The „matrix" can be described as a crystal of solvent with completely separated particles of the analyte. A short pulse of laser of a given wavelength induces an immense heating of the matrix (hundreds to thousands of Kelvins), consequently its sublimation and ionization of the sample. [31-34].

An initial approach to MALDI technique and its development was based on an empirical work lacking the knowledge of mechanistic principles in some respects. The mechanism of ionization of proteins and peptides is now believed (in the most common set-up) to be based on a proton transfer assisted by molecules of the matrix [35].

Due to the matrix which absorbs specifically the majority of laser energy, MALDI is able to produce a gas-phase monocharged ions (reviewed in [36]) of analytes of high molecular mass reaching typically tents to hundreds of kDa [37-38].

A phenomenon typically occurring during MALDI is a competitive ionization. In case of complex mixtures, ionization of some peptides is discriminated in favor of others and

thus an analysis can be less sensitive to them compared when analyzed individually. The suppression seems to depend on a sequence and also experimental conditions. For example pH of the matrix seems to influence ionization preferences unpredictably [39] while reversed-phase chromatography, where each fraction contains proteins of similar hydrophobicity, lowered variations of ionization [40]. On the other hand, MALDI showed to be tolerant towards usual physiological buffer composition (reviewed in [36]).

Another characteristic disadvantage is that MALDI, as a pulsed source of analyte, is compatible with only some types of detector such as time-of-flight (TOF), ion traps and Fourier transform ion cyclotron resonance cell, because these are able to capture the ions [41] after the period of ionization.

2.1.2. Electrospray Ionization

A major advantage of ESI [42] consists in a possibility to couple a continuous separation technique such as liquid chromatography (LC) with the subsequent mass analysis. In ESI a strong electric field is applied on a liquid leaving capillary tube by a slow flux (1-10 $\mu l.min^{-1}$). The electric potential of kV generates an electric field of about 10^6 $V.m^{-1}$ which induces an accumulation of charge at the surface of liquid, leading finally to the formation of charged droplets (reviewed in [43]). The threshold voltage is characterized by so called Taylor cone [44] that forms when a pressure of charged particles at the tip is equal to a surface tension. For a liquid under atmospheric pressure it was experimentally confirmed that the droplets breakdown prior to reaching a limit charge given by Rayleigh equation [45-48].

An evaporation of the solvent from droplets leads to an increase of coulomb forces and consecutive division and ruptures of droplets. An electric field causes desorption of charged particles from the surface of droplets [49]. This means that the less soluble the molecules are, the higher is the sensitivity of the analysis to them. For example molecules of liphophilic character (located closer to the surface of a droplet) can cover the signal of compounds more soluble in a polar solvent that are present further to the surface together with anions. It was shown, that so-called solvophobicity of certain proteins may help them escape from the droplet to a gas-phase and thus there is no doubt that the choice of solvent determines quantitative and consequently qualitative result of the detection [46-47](reviewed in [36]). Anions in general (which may concern impurities as well as buffers) can also easily enter the gas phase and therefore compete with analyte in mass detection [46-47]. This is another discussed phenomenon questioning the quantitative potential of MS analysis.

In general, there are several potential pathways of analyte after the evaporation of the solvent; it can precipitate from solution after its partial evaporation or coprecipitates with other nonvolatile agent such as inorganic ions. It was confirmed that sulfates and phosphates often suppress of ionization in biological samples [50].

It was also shown that not only ionized particles enter the gas phase, but also neutral molecules undergo an evaporation enveloped in a charged-solvent cluster. Such particles thus do not finally reach the detector; they may still cover signal of other molecules (reviewed in [50]).

In ESI we usually obtain multiply charged macromolecules: on average one charge per kDa (depending upon the sequence of a protein) [41]. The spectra of protein molecules normally fit a statistical distribution of group of peaks of molecular ions originating from multiple deprotonation $(M-zH)^{z-}$ or protonation $(M+zH)^{z+}$ with only a little abundance of ions coming from the fragmentation [51]. The average charge state of proteins linearly increases with their mass.

In case of a complex analysis of biological sample we exploit a fact that every single peak in the first mass spectrum at high resolution is in fact a group of several isotopic peaks (naturally differing by 1 Da in a mass), therefore their distance is 1/z [43] (reviewed in [36]). It is noteworthy that peaks of ions derived from proteins often respond to an attachment of proton to the molecular ion and that the overall net charge is therefore usually positive [43].

ESI as opposed to MALDI enables continuous detection of a sample and low flow rates may improve the resolution. An invention of nanoelectrospray (flow of tents of $nl.min^{-1}$) therefore allowed the instrument to perform several tandem analysis of the same fraction and increase the sensitivity at the same time [41].

In general, sensitivity of both MALDI and ESI depends on concentration, contamination and complexity of the sample [36], which plays a crucial role in determination of a composition of biological sample as we discussed above.

2.1.2.1. Fragmentation of Peptides in ESI

The techniques used for peptide/protein analysis are able to produce stable ions with no or low fragmentation that enables the analysis of complex mixtures. Such characteristic however, lack information concerning structure.

A method based on secondary fragmentation of selected ions is called tandem MS (or MS^n). Briefly, the ion scanned and selected by the first mass analyzer (on the basis of isotopic peaks), so called precursor ion, is sent to a collision cell where the kinetic energy is transferred to it by the intact atoms and is thus transformed to a vibration energy resulting in precursor ion fragmentation. Nevertheless, this method can be used only to clarify the assumptions based on molecular weight.

There are two types of fragments in the spectra : first arises from the fragmentation of one or more peptide bonds and the second represents the products of the cleavage of lateral amino acid residues. Conventionally, the nomenclature was determined as a_n, b_n and c_n for peaks of the fragments arising from decomposition of Cα-C, C-N and N-Cα keeping the positive charge at the N-terminus (n indicates a number of amino acid in a fragment) and x_n, y_n, z_n for the case where the positive charge is present at the C-terminus (Figure 2)[52-54]. During low-energy fragmentation, b_n and y_n are the most abundant peaks corresponding to those fragments losing small molecules (e.g. water, ammonia).

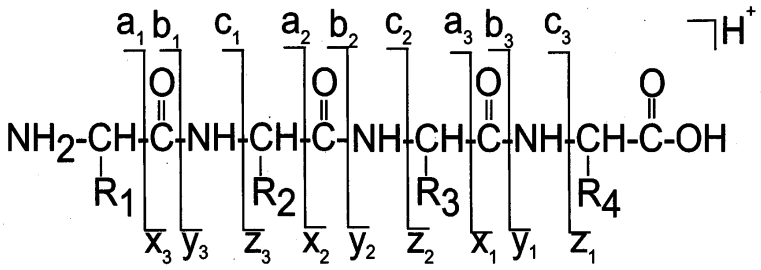

Figure 2.Common Fragments of Peptides in ESI:

Fragments a_n, b_n and c_n arise from decomposition of C_α-C, C-N and N-C_α keeping the positive charge at the N-terminus (n indicates a number of amino acids in the fragment), analogously x_n, y_n and z_n where the positive charge is present at the C-terminus. Figure adopted from [36].

MS-sequencing, the most efficient method for determining amino acid sequence [36,39,55], is based on the fact that the mass difference between two peaks successive within the series corresponds to a mass of the specific amino acid that can thus be determined (excluding isomers). This result, however, still depends on the availability of a database (based on previously measured data) or on the existence of the database of genomic translation (in all reading frames) *in silico* [36,56-57].

Also a digestion by an enzyme or a chemical agent at defined sites can be used to produce protein fragments. The fragments are then compared to a sequence in the database and a protein is determined with the expectancy according to the number of peptides found and the percentage of coverage of a protein by these fragments [58-61].

Some methods and their combination were employed to develop routine procedure determining amino acid sequence of any protein *de novo*. An incomplete proteolytic digest, utilization of a partial Edman degradation or computed shotgun sequencing helped to determine protein sequence but still, the detection depends on precedent hits or protein homology [36,62-63],(reviewed in [36]).

2.2. Mass Analyzers

There are four types of mass analyzers commonly used in MS-proteomics: ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron resonance analyzers [64]. These basic types can be also put together (combination of two or more same or different analyzers is called hybrid instrument).

2.2.1. Quadrupolar Mass Analyzers

A quadrupolar analyzer [65](Figure 3, page 11) hardware consists of four fully parallel rods where the two opposite are imposed on the identical voltage changing between the pairs at radio-frequency range. Ion travelling along the rods is exposed to a total electric field generated by the oscillatory and a constant potential applied upon the rods. Ions of a given m/z are enabled to pass through and not discharge on the rods. Scanning different ions of m/z ratio is made by changing of a magnitude of both potentials in time keeping their ratio constant (quadrupolar analyzers usually work with a stable angular frequency). The slope of the change in time determines then the resolution [66] as well as the number of cycles of the variable potential [36].

More types of regime of quadrupolar instruments are used. A tandem setup called "daughter scan" consists of three quadrupoles; the first one selects an ion of given m/z, the second serves as a collision cell, and the third analyzes products of the fragmentation (reviewed in [36]). Also the third quadrupole can be focused on the chosen ion while the m/z of entering ion is monitored by the first quadrupole. The method called "parent scan" presents information about the precursor ions – all ions that provide fragments of given m/z, (reviewed in [36,41]).

In a scan mode "neutral loss" both quadrupoles are used as scanners keeping constant mass difference between them. This means that if an ion of mass *m* enters the first quadrupole, for a given difference *d*, only ions providing fragments of (*m-d*) mass are detected [41].

2.2.2. Ion Trap

An ion trap [67](Figure 3, page 11) is based on circular electrode with two cups. A combination of constant and oscillatory potential functions as a three-dimensional quadrupole that allows expelling of particles of a given m/z. On the contrary to a quadrupole, all ions of distinct m/z are present inside the trap at the same time and their exit thus depends on their m/z. Charged particles influence their trajectories mutually and therefore it is suitable to reduce their movements. Molecules of low-pressured inert gas (fractions of pascals) are then used to dampen an extra kinetic energy from these ions by collisions [36]. The interaction of ions with such molecules thus produce fragmentation that can be multiplied also by irradiation and excitation at their specific frequency [41].

The movements of ions in ion trap are based on similar principle as was shown in case of quadrupole. However, maximal deviation must not reach any of the **two** limit values given by a physical shape of a trap. In case of a simple detection (just by ion trap), the m/z of fragments is detected either according to their secular frequency at which they are destabilized and ejected (applied on the cap of the instrument)[36]or by so called stability limit- a maximum oscillatory voltage at which the specific ions lose their stability in the direction of z-axis. The mass analysis is achieved by changing the amplitude (not the frequency) of the oscillatory voltage, as well as in case of quadrupolar detection. A zero to peak value of the oscillatory voltage then reaches thousands of volts, (reviewed in [41]).

An invention of less than 3-dimensional ion traps showed a potential of development of the method in markedly improved trapping efficiency (larger volume) and increased ion capacity (one order of magnitude) retaining similar mass range compared to original instruments [68-69].

2.2.3. Time of Flight Spectrometry

A nature of TOF analyzers (Figure 3, page 11) is suitable for a pulse character of ion generation such as MALDI. Mass to charge ratio is determined by the time in that a particle accelerated by a potential reaches detector in a constant distance from source-focusing lens.

An advantage of TOF is that all ions formed are analyzed regarding to a high transmission efficiency having no upper mass limit. On the other hand, a low mass resolution is typical for this type of MS. A distribution of flight times in the ions having the same m/z can be broad, (reviewed in [41]). Since the resolution is obviously proportional to flight time and lowering of voltage may reduce the sensitivity (lower transmission efficiency), the resolution could be only increased by elongation of the flight trajectory.

2.2.3.1. Enhancement of Resolution in TOF detection

So-called reflectron [70] consists of series of grids and electrodes that serve as an ion mirror which retards the particles in its field before sending them back to the detector. The velocity of a particle at the entrance of the reflectron is equal to the velocity at its exit, at a proper set-up, all particles of the same m/z should reach the detector at a same time. Such improvement however, introduces a mass limitation and enhances the resolution at the cost of sensitivity. Another way of improvement of resolution is to reduce a kinetic energy of the ions leaving the source by introducing a lag prior to a voltage pulse (start of the flight). The method is called delayed pulsed extraction and also aligns an energy dispersion of ions of the same m/z formed [36].

Two types of fragmentation can be observed in MALDI-TOF measurements. First is the metastable decomposition of ions in the source prior to the acceleration, second is called "post source decay" and occurred in the field free region. The m/z-separation of the fragments produced outside the accelerating field - having the same velocity but different kinetic energy, is realized by reflectron [36].

A strictly defined short pulse generation of ions can be easily coupled with TOF. Nevertheless, a continuous source such as ESI can be also combined [71]. To provide a pulse of ion beam, fragments from ESI are stored in a trapping device and extracted from it within short periods. Fragments then enter into a flight tube that is perpendicular to the axis of ESI eliminating the involvement of kinetic energy of incident particles [71].

2.2.4. Fourier Transform Mass Spectrometry

Fourier transform mass spectrometry (FTMS)[72]is similar in some regards to nuclear magnetic resonance (NMR)[73]. Gas-phased particles are injected and trapped in a space situated within a magnetic field (units of Tesla) (Figure 3, page 11). Ions trapped along an axis of the movement by a trapping voltage (Volts) therefore follow circular trajectories of perpendicular direction to a magnetic field. The angular velocity and frequency depend on

m/z and thus the direction of their circulation is determined by the charge [73]. According to Lawrence´s observation [74], only a modest electric field can be used for great acceleration of particles. In FTMS, all ions are excited at the same time by a fast scan of frequencies of wide range after entering the cyclotron. Trajectories affected in each case by a characteristic frequency produce a specific signal - "image current"(reviewed in [73]) whose superposition is than detected as a complex wave. The wave as a time-dependent function is finally transformed into frequency-dependent intensity function which allows elucidating the presence of particular ion from its specific frequency [73].

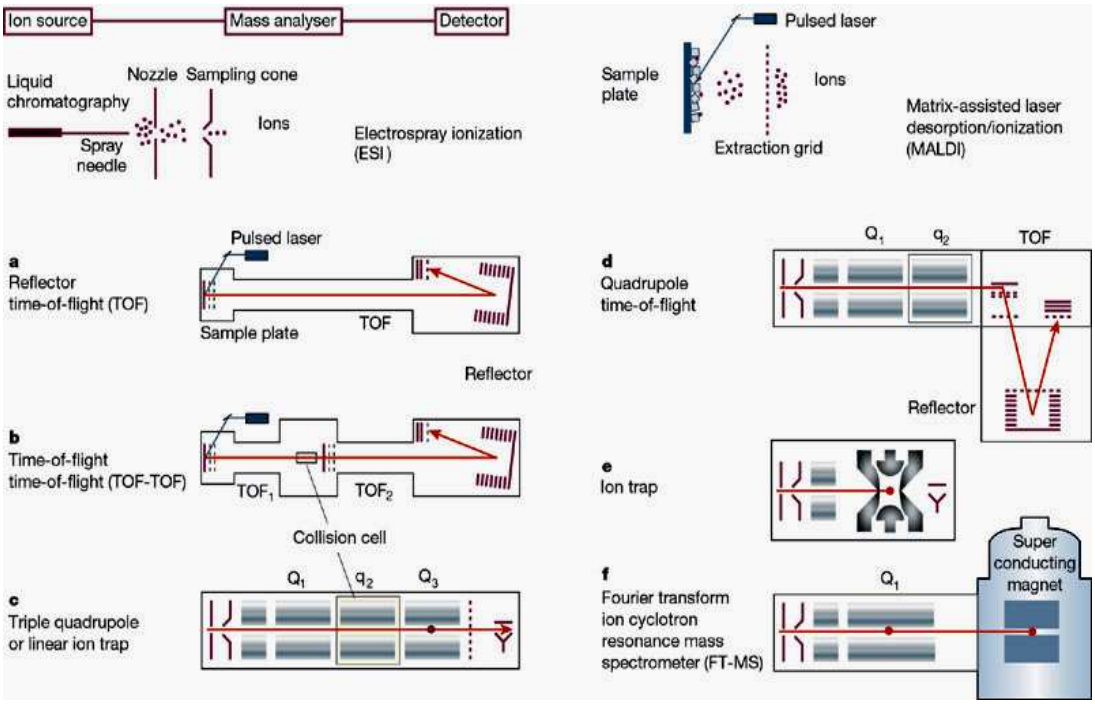

Figure 3.Mass Spectrometers In Proteomic Research:

Ion sources ESI and MALDI are depicted in upper part with commonly related detectors below: In TOF (a), the ions are separated as results of their different velocities; in TOF-TOF (b), additional fragmentation provides another mass spectrum describing these ions. Time-variable field permits stable trajectory only for selected ions in Quadrupolar instruments (c, d). Particles of desired m/z are fragmented in a collision cell, trapped in linear ion trap and scanned out with resonant field (c) or analyzed in TOF (d). Ions can be captured by Three-dimensional ion trap (e) where particles of chosen m/z are consequently scanned out similar to linear ion trap. FT-MS (f) traps ions employing strong magnetic field. Figure adopted and revised from [64].

FTMS poses a great advantage in its resolution and wide mass range since its early development [75]. The resolution depends on observation time due to a relaxation whose duration results mainly from a quality of vacuum (kinetic excitation is slowed down by collisions). Only a low number of particles should be present at the cell- higher density of ions will introduce repulsions and may produce some incoherency of the signal. Compared to Fourier transformed NMR, FTMS covers very broad range of frequencies which brings increased demands on appropriate computational unit. In addition, the process needs be rapid owing to the short lifetime of certain ions (reviewed in [41]).

3. Network Biology

So-called „molecular biology paradigm“ dominating for two generations was based on the „one gene- one protein- one function“ approach as well as on an assumption that one can identify a direct link between genotype and phenotype [76]. This paradigm assumes two premises: First, there is a straight relation between a gene and protein function and so that all biological processes could be explained by knowledge of the genome. Second, all pathways are linear and one-way therefore every function upstream is not affected by a downstream process *et vice versa* (reviewed in [77]). Such reductionism, however, is oversimplified and these assumptions are clearly not met in a number of complex biological setups.

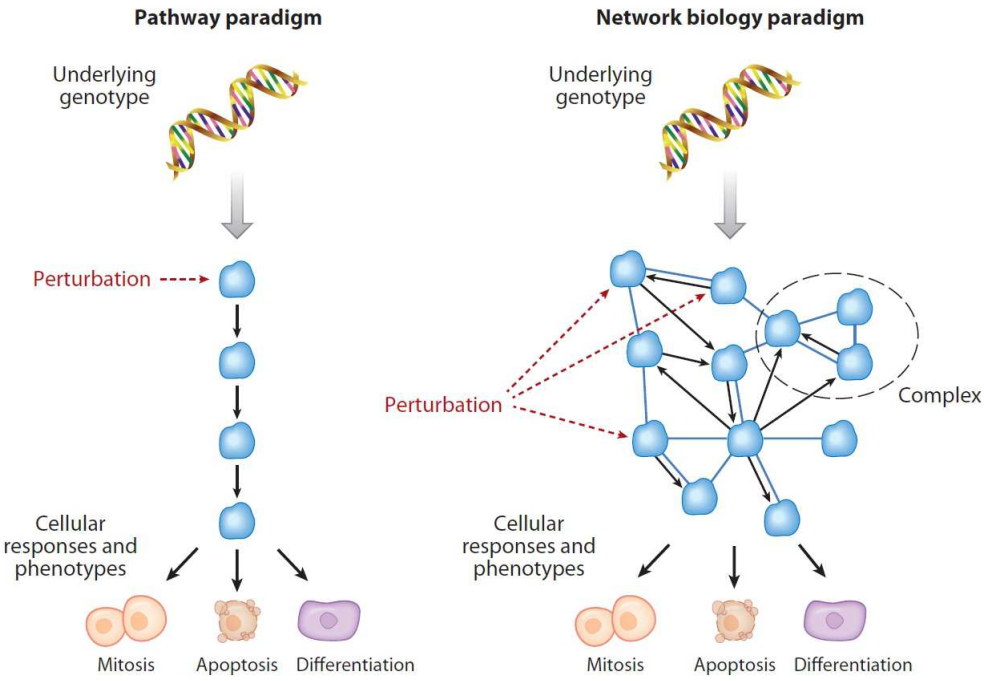

Figure 4.Outlining Example of Network Biology Paradigm (NBP):

In NBP, pathway are considered not to be only linear and one-way as opposed to previous insight. Each node of the network (blue point) can represent gene as well as protein, RNA or small metabolite molecule. Blue lines represent protein-protein interactions, black lines mean enzyme-substrate relationship. Figure adopted and revised from [77].

A recent model (Figure 4) of network biology (also systems biology or integrated biology) takes into account the possibility of influence of a perturbation on each part of the system (a node of the network) [77-78] including small molecules such as cofactors or

metabolites. When searching for a protein-protein interaction, we should always consider multi-protein complexes rather than a simple "partnership" [36,56].

4. Mass Spectrometry Based Proteomics

There are some non-destructive well established methods providing complex information concerning protein-protein interactions. NMR and crystallography, however, require milligram quantities of relatively pure material [79]. Since the „interactome" grows with the square of the number of proteins engaged[64], it is preferable to employ a sensitive high-throughput method when looking for an unknown interaction *in vivo* or *in situ* at least. In addition, a possibility of relatively weak interaction (nanomolar binding constants [36]) should be considered for proteins of low abundance in their natural environment. MS allows detection of specific complexes within a huge background of non-specifities of distinct origin. This feature enables a decrease of the number of purification steps, moderation of washing conditions and therefore an enhancement of the prospect of finding a weak or transient interaction [64] in near-physiological conditions [80].

Since the development of ionization method [42], mass spectrometry (MS) became a widely-spread method of choice for an analysis of complex macromolecular samples. The problem remains the same; protein identification stands on already available sequence databases. The typical primary output of an experiment (raw spectrometry data) therefore can be interpreted only *a posteriori* [64,81].

4.1. Bottom-Up Proteomics

So-called bottom-up proteomics (also shotgun proteomics) is a high-throughput analytical method based on an assumption that a complex mixture of proteins [55] (e.g. cell lysate) will produce reproducibly specific fragments after a digestion and fragmentation (Figure 6, page 16). When analyzed by MS instrument, these fragments provide "peptide sequence tag"- a partial random information about the whole peptide chain [55,62,82]. A peptide „hit list", as an output of usual experiment, is based on matches of these sequences and their combinations with database of sequences that are either hypothetical (Figure 5, page 15)or based upon already resolved spectra [62,64,83].

There are also other methods of identifying the peptides that should be mentioned but will not be discussed thoroughly in this thesis. It is for example *de novo* sequencing and

hybrid "error tolerant" reading. These techniques are not suitable for large scale studies and so represent only an alternative in particular situation (reviewed in [62,83]).

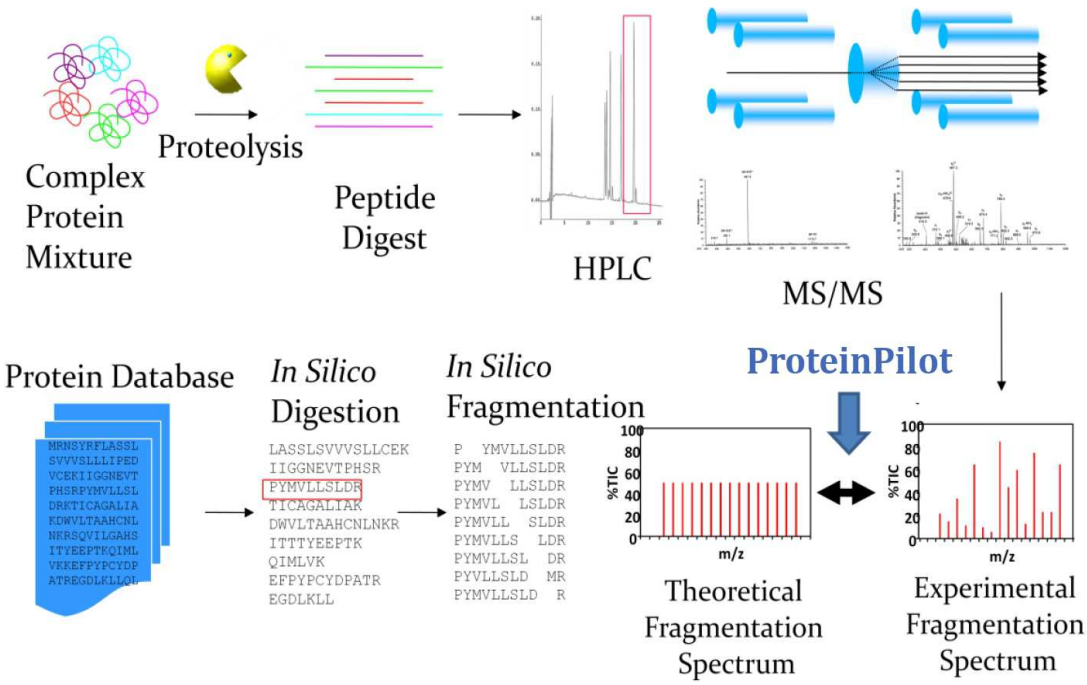

Figure 5. Review of Bottom-Up Analysis and Raw Data Processing using Protein Pilot:

In bottom-up proteomics, real spectra are matched with analytical software with theoretical pattern received *via in silico* digestion and fragmentation. Protein Pilot (AB Sciex 2010) employs so-called **Paragon** algorithm that performs two types of searches. More conventional **Fraglet** matches an experimental mass with a mass of hypothetical peptide within certain tolerance; in other words, MS^2 information is not used during the selection. **Taglet** as opposed to the preceding, designs sequences on the basis of "peptide sequence tag" obtained during MS^2 [84].

As the research progressed markedly at the conceptual level, the techniques still do not fulfill new demands on data collection that can be summarized as:

1) All sections of the „network" should be measurable, 2) the obtained results must be reproducible, 3) datasets have to have also a quantitative character and 4) the through-put of false positivity should be adjustable [77].

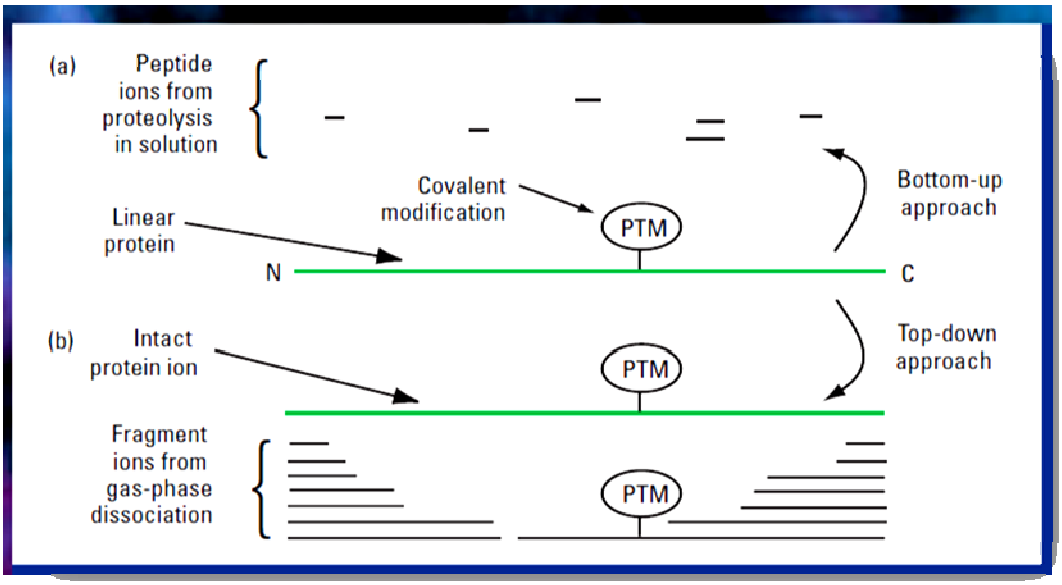

Figure 6. Difference Between Bottom-Up (a) and Top-Down (b) Approaches in MS-Based Proteomics:

In bottom-up proteomics, peptide mixture is digested prior to the analysis and present proteins are then deduced from so called "peptide- sequence tag". Such method poses great advantage in its high trough-put. In top-down, an intact protein is charged or charged and fragmented during MS-analysis. Absolute fragments enable sequence coverage reaching 100% and high mass accuracy allows precise determination of post-translation modification (PTM) according to its mass. Nevertheless, top-down proteomics is limited in case of biological sample facing a dilution of signal by numerous isotopic peaks and peaks corresponding to multiple-charged ions. Figure adopted and revised from [85].

4.2. Sample Preparation

Interaction experiments based on mass analysis consist of three inevitable steps that can be summarized as: presentation of bait protein, affinity separation of potential complex from "background proteins" (purification)[36,56]and consequent identification of interacting molecules [64].

If a complete collection of appropriate antibodies were available we would be able to use endogenous proteins as a bait in their natural environment and abundance. However, a more common approach is in an expression of tagged protein. A usage of commercially available promoters, however, results in an unnatural level of protein expression. Such methods thus tend to create artifacts and false positivities [64]. For this reason a careful system of negative controls is crucial for these experiments [64,79].

4.3. Data Processing

Mass spectrometry-based proteomics is an effective and quickly developing branch of basic as well as clinical research [86-88].Nevertheless, several inherent disadvantages should be taken into account when focusing on protein-protein interactions.

First, as protein identification is based on matches with already known sequence databases, the method depends on known and described proteins and their splice variants.

Second, a quantitative potential of MS data is restricted to a few methods and their further optimizations [80], therefore determining a cut-off between hits and contaminants is subjective matter of decision of a particular community [64]. False positivity is still a major obstacle in the method. A complete system of negative controls and optimized reproducibility may help resolve a particular problem of distinguishing between false positivity and positivity as well as between negativity and positivity.

MS experiments naturally result in a large collection of data. Therefore, a statistical method is currently considered as determinative limitation of MS-based proteomics [64].Individual criteria applied on secondary (processed) MS data impede their sharing and comparison (reviewed in [83]). A majority of published information consists of partially interpreted data. An ambition of the community is a creation of electronically accessible database [64,80,89].

4.3.1. Non-Specific Signals in MS-Based Proteomics

Some abundant proteins, such as keratins, are often identified in complex biological samples by MS. These proteins represent very common case of false positivity [36,80]. When looking for protein-protein interaction, keratins should not be considered as a group of non-interacting "scaffold-proteins". Keratins were shown to be involved in numerous signaling pathways [90]. Keratins also commonly undergo phosphorylation that regulates their solubility and interaction with 14-3-3 proteins that were shown to be possibly involved in subcellular organization of keratins, (reviewed in [91]). Some studies suggested that keratins might serve as adaptor proteins recruiting other molecules to 14-3-3 [92]. It was shown that a loss of a particular keratin can be only poorly accommodated by expression of another one [93]-[94]. LNCaP prostate cancer cell line was shown to express keratin 18 while these cells never express keratin 14[95-96].Expression of particular keratins thus seems to be a specific "fingerprint" of each cell and therefore defines a type as well as a stage of a cycle of such cell, (reviewed in [90,97]).

Since some specific motifs in keratins are conserved throughout species and also different cell types in human [91,97-100], a fragmentation of an epidermal keratin produces charged particles contributing to findings of a cytoskeletal one *et vice versa*.

5. Aims and Objectives

- Optimize preparation of sample for detection of potential binding partners of GCPII by mass spectrometry

- Perform binding assays of endogenous or recombinant GCPII in different cell lysates

- Systematically process gathered data in order to determine reproducibility, typical contaminants, sample suitability and finally potential protein partners of GCPII

- Test potential protein partners of GCPII *via* specific antibody

6. Chemicals

Fluka (Buchs; Switzerland)

Tetramethylethylenediamine; N;N'-Methylenebisacrylamide

Clontech (Mountain View; USA)

pTRE-Tight plasmid

GE Healthcare (Little Chalfont; UK)

Protein G Sepharose 4 Fast Flow

Gibco (Carlsbad; USA)

opti-MEM medium; L-glutamine; IMDM medium

Koh-i-noor Hardtmuth (ČeskéBudějovice; CZE)

96-well transparent microplate

Lach-Ner (Neratovice; CZE)

hydrochloric acid; sodium chloride; silver nitrate

Penta (Prague;CZE)

methanol; acetic acid; acetone; formaldehyde; isopropylalcohol; ethanol; acetic acid; glycerol

Pierce (Rockford; USA)

D-biotin; SuperSignal West Dura Chemoluminiscence substrate; goat anti-mouse-IgG1 antibody conjugated with horseradish peroxidase (0;8 mg/ml); sulpho-NHS-LC-biotin;

ROCHE s.r.o. (Prague;CZE)

Streptavidin Mutein Matrix; cOmplete; Mini EDTA-free Protease Inhibitor Cocktail

Serva (Heidelberg; Germany)

Coomassie Brilliant Blue G-250; bromphenol blue; bovine serum albumine

Sigma-Aldrich (St. Louis; USA)

2-mercaptoethanol; glycerol; EDTA; glycine; Tris; dithiothreitol (DTT); (tris(hydroxymethyl) aminomethane); acrylamide; sodium dodecylsulphate; sacharose; Tween-20;; Iodacetamide (IAA)

Thermo Scientific (Massachusetts; USA)

Casein blocker; n-Dodecyl-β-D-Maltoside, 96-well standard microplate

Santa Cruz Biotechnology (Santa Cruz; USA)

Mouse monoclonal IgG1 recognizing β subunit of F1 mitochondrial ATPase 0;2 mg/ml

Promega (Wisconsin; USA)

Gold; Mass Spectrometry Grade trypsin

Specific monoclonal antibody GCPII-04 recognizing denatured GCPII and antibody GCPII-08 recognizing native GCPII were prepared in laboratory of Prof. Václav Hořejší

N-terminally biotinylated recombinant extracellular portion of GCPII was prepared by Jan Tykvart in laboratory of Doc. Jan Konvalinka [101].

7. Instrumentation

pH-meter: 9450 pH meter; Unicam (USA)

spectrophotometer: NanoDrop 1000; Thermo Scientific (USA)

spectrophotometric reader: GENios; Tecan (Switzerland)

autoclave: MLS-3020U Sanyo Labo Autoclave; Sanyo (Japan)

balance: HL-400; A&D Engineering; Inc. (USA)
EK-400H; A&D Engineering; Inc. (USA)

bath: Thermomix BUB.Braun (Germany)

vertical polyacrylamide electrophoresis: Sigma (USA)

blotting machine: power supply: PowerPac HC; Bio-Rad (USA)
apparatus: Trans-BlotSD; Bio-Rad (USA)

incubator: MCO-17AI CO_2 Incubator; Sanyo (Japan)

centrifuges: Multifuge 3 S-R; Heraeus Instruments (Germany)
Megafuge 2;0R; Heraeus Instruments (Germany)
Centifuge 5415R; Eppendorf (Germany)
Optima MAX-XP; rotor TLA110 Beckman Coulter (USA)

microscopes: fluorescence microscope Olympus IX81; Tokio (Japan)
optical microscope Nikon TMS (Japonsko)

CCD Camera: UVP ChemiDoc-IT 600 (USA)

laminar box: BSB4A Laminar Flow Box; Gelaire (Australia)

sonicator: Soniprep 150; Sanyo (Japan)

concentrator: Refrigerated Centri Vap Vacuum Concentrator; Labconco (USA)

MS instrumentation: TripleTOF 5600; nanoSpray III; AB Sciex (USA)
ULTIMATE 3000 RSLCnano systems; Thermo Scientific (USA)
Acclaim PepMan 100 column; Thermo Scientific (USA)

labware: Protein Pilot 4.0; AB Sciex (USA)
Software Analyst TF 1.6; AB Sciex (USA)
Access 2003; 2007; Microsoft (USA)

Methods

7.1. Transient Transfection of Mammalian Cells

Lymphome Node Carcinoma of the Prostate (LNCaP) cells were used for transient transfection using polyethylenimine (PEI) as a transfection reagent. Cells were cultivated in 100 mm dish in IMDM medium (Gibco) until reaching 60% confluence on the transfection day. 7 μg of DNA was diluted into Opti-MEM (Gibco, reduced serum medium) to final volume 350 μl. 21 μl of transfection agent PEI was added to the mixture and this was incubated 20 min at 20 °C. Subsequently, the solution was added by drops to cells, gently agitated and cells were let incubated for 24 hours at 37°C in 5% CO_2. After 2 days, cells were harvested and washed two times by 1ml of PBS and immediately either disrupted or stored for further use at -20 °C.

7.2. Preparation of Cell Lysates

TBS:100 mM Tris-HCl (pH 7.2),150 mM NaCl
PBS:137 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4; pH 7,4

20 fully confluent 100 mm dishes of LNCaPs were washed with TBS and harvested. Cell suspension (0,5 ml) was mixed with 9072 μl of lyzis buffer consisting of TBS, Complete Mini Protease Inhibitor Cocktail EDTA-free (Roche) in recommended dilution, Na_3VO_4 to final concentration 1mM, NaF to final concentration 50 mM, sodium pyrophosphate to final concentration 2,5 mM, β-glycerolphosphate to final concentration 1 mM and DDM to final concentration 1 %. Suspension was sonicated 10 × 15 seconds in bath sonicator (Elmasonic S30, P-LAB) in ice. Lysates were centrifuged (Optima MAX-XP, Beckman Coulter; rotor TLA110, adaptor for 1,5 ml Beckman tubes) 45 000 × g for 1 hour in 4°C. Supernatant was stored in -80°C in 450 μl aliquots (each corresponding to 100mm dish). Total protein concentration was determined by standard Bradford Protein Assay as 5 mg/ml.

In case of transiently transfected cells only one dish and therefore 20 times lower amounts of all reagents were used.

For the preparation of lyzates from human umbilical vein endothelial cells (HUVECs were kindly provided by Jan Hraběta M.D., Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine)the same protocol was used excluding phosphatase inhibitors (Na_3VO_4, NaF, sodium pyrophosphate, β-glycerolphosphate) (see Results).

7.3. Bradford Protein Assay

1 μl of a cell lysate was mixed with 200 μl of dye reagent solution (Coomassie Brilliant Blue G-250). After following one-minute incubation at laboratory temperature, the absorbance was measured at 595 nm wavelength (spectrophotometric reader GENios, Tecan).The protein concentration was determined from calibration curve constructed using bovine serum albumin as a standard.

7.4. In-Gel Digestion

Destaining buffer :25 mM ammonium bicarbonate in 50 % acetonitrile (ACN)
Digestion buffer: 50 mM NH_4HCO_3 and 10 % ACN
Trypsin solution: trypsinsolublized in 250 μl of (3 μl acetic acid to 1 ml H_2O); stored at 70 °C.
Stock extract solution:1: 2 % TFA
Stock extract solution 2:60 % ACN

200 μl of destaining buffer was added to the gel pieces and this was left for 30 min at the 30 °C. The procedure was repeated two times. After the color was removed, gel was dried 200 μ ACN for approx. 5 min at the 30 °C until the gel pieces turned white. ACN was removed and gel was dried at the 30°C for approx. 5 min. Into the dry gel pieces 100 μl of 20mM DTT in 100 mM NH_4HCO_3 was added and left in 65°C for 30 min. Then 100 μl of 55 mM IAA in 100 mM NH_4HCO_3 was added and the reaction was stored in dark at the room temperature for 30 min. Excess liquid was removed and pieces washed with 200 μl of 100 mM NH_4HCO_3. Gel was dried as described previously. Gel pieces were soaked into 19 μl of digestion buffer and 1 μl of trypsin solution and let at 38 °C for 10 hours gently agitated. Gel pieces were taken from digestion solution and placed into the new eppendorf tube, 50 μl of the extraction solution 1 were added and this was left for 15 min in ice bath sonicated for 15

minutes. Than 50 µl of the extraction solution 2 was added and this was left for 15 min in bath in the sonicator. The solution was removed and then was added 50 µl of the extraction solution 3 and this was left for 15 min in bath in the sonicator. Peptides were dried at the speed vacuum and approx. 30 µl of 0.1 % formic acid was added.

7.5. In-Solution Digestion

Into the dry protein sample 20 µl of digestion buffer with 1 µl of 100mM DTT) was added. This was left it in 65°C for 30 min and then 1 µl of IAAwas added. Reaction was stored in dark at the room temperature for 30 min. 19 µl of digestion buffer and 1 µl of trypsin solution were added and left at 38 °C for 10 hours gently agitated. Digestion was stopped by addition of 2 µl of 5 % acetic acid. Solution was dried in the speed vacuum and to the dry sample was added approx. 30 µl of 0.1 % formic acid. The composition of buffers used were described previously (see In-Gel Digestion).

7.6. Nano-LC-MS/MS Analysis

Analysis of samples dissolved in 0.1 % formic acid was performed on UltiMate 3000 RSLCnano system (Dionex) coupled to a TripleTOF 5600 mass spectrometer with a NanoSpray III source (AB Sciex). The instrument was operated with Analyst TF 1.6 (AB Sciex). After injection the samples were trapped and desalted with 2 % acetonitrile in 0.1 % formic acid at flow rate of 5 µL/min on Acclaim PepMap100 column (5 µm, 2 cm × 100 µm ID, Thermo Scientific). Eluted peptides were separated using Acclaim PepMap100 analytical column (3 µm, 15 cm × 75 µm ID, Thermo Scientific). The 90 min elution gradient at constant flow of 300 nl/min was set to 5 % of phase B (0.1 % formic acid 99.9 % acetonitrile, phase A 0.1 % formic acid) for first 5 min, then stepped from 5 % to 50 % B over 55 min, from 50 % to 99 % B over 5 min, stayed at 99 % B for 10 min and descended to 5 % B and remained there for 15 min.

An information dependent acquisition method was utilized with total cycle time of 2.3 s. Maximum 25 MS/MS spectra per cycle were acquired, former target ions were excluded for 15 s after two occurences. TOF MS mass range was set to 350 – 1500 m/z, in MS/MS mode the instrument acquired fragmentation spectra with m/z ranging from 100 to 2000.

7.7. Primary MS Data Analysis

Protein Pilot 4.0 (AB Sciex) was used for protein identification from raw (*.wiff) spectra using HomoSapiens Database (UniProt, 30.7.2012). The search was set by choosing iodoacetamide as alkylation substance, trypsin as digestion agent and TripleTOF 5600 as instrument. All samples were evaluated by Paragon algorithm in the regime *Thorough* allowing MS precursor ion deviation up to ± 0,05 Da and ± 0,1 Da for MS^2. The charge of fragments was set between +2 and +5.

7.8. Secondary MS Data Evaluation

Information describing the MS spectra – so-called sequence coverage (95%), number of peptides (95%) and gene, among others, were provided by Protein Pilot 4.0 (AB Sciex) and were used for further deduction of information presented in this thesis. The sequence coverage (95%) was defined as "the percentage of matching amino acids from identified peptides having confidence greater than or equal to 95%, divided by the total number of amino acids in the sequence." [84]. The number of peptides (95%) is defined as "the number of distinct peptides having at least 95% confidence" while "multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides because they have different molecular formulas. Multiple spectra of the same peptide, due to replicate acquisition or different charge states, only count once." [84].The values of **sequence coverage (95%)** and **peptides (95%)** will be presented further only as **sequence coverage** and **number of peptides**.

An Access (Microsoft) database (Figure 7, page 26) was designed in cooperation with Pavel Šácha and was created by him. Twenty sets of experiments were imported in *.txt format from which 15 sets of pull-downs and immunoprecipitations from two cell lines were used for further evaluation presented in the Results. Accession numbers of the hits detected in each control were first grouped under the maximal coverage and maximum of peptides found. In each particular case of a tested hypothesis (e.g. – Is there more different protein detected in the gel sample or in the liquid?), a query was designed or criteria rewrote in a related one. After obtaining ultimate dataset, a sequence coverage limit was applied (see section Results). For identification of potential partners, either sequence coverage or number

of peptides of hits from negative control were numerically subtracted from those of positive control.

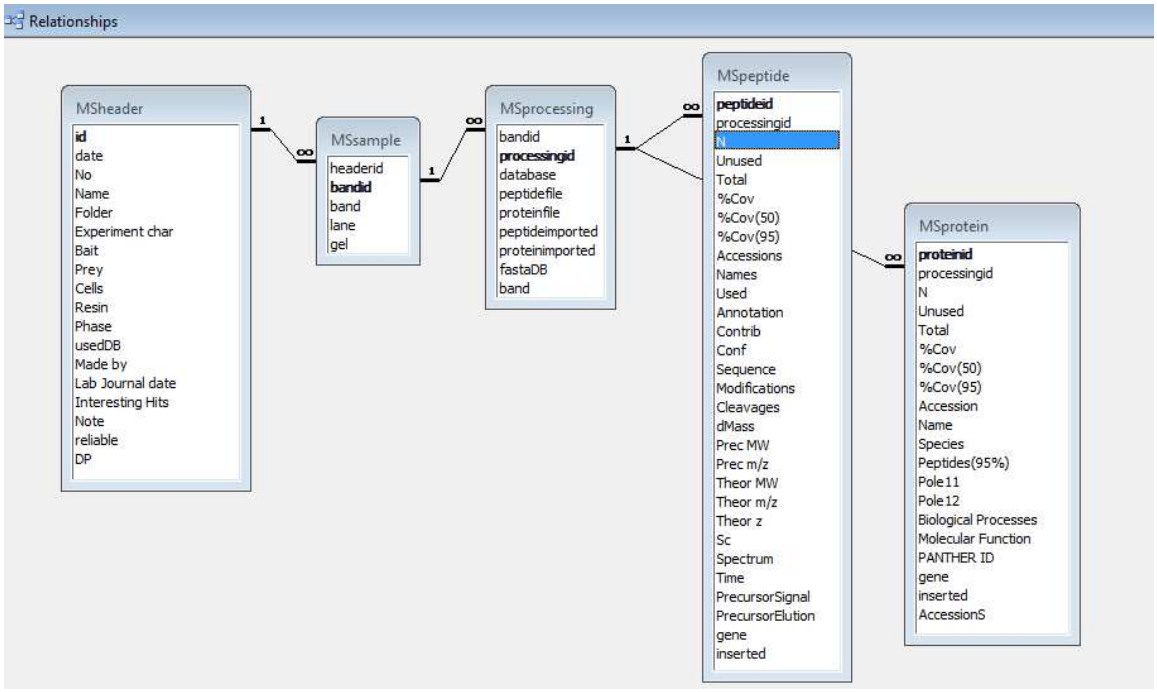

Figure 7. Relationships in the Database of Secondary MS Data :

Data was imported in *.txt format into the database and the characteristics of the experiments were described in table MSheader. Each experiment consisted of a number of samples (MSsample) defined by a type of a gel and its position in it (criteria not applied on liquid sample). Every sample was analyzed obtaining information about peptides (MSpeptide) and proteins (MSprotein) detected in MS analysis. Secondary data was processed by application of queries filtering data according to the criteria given for each table. Information about sequence coverage and number of peptides detected (table "MSprotein") were used in order to obtain the data shown in this thesis.

7.9. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Stacking gel (5% acrylamide): 250 mM Tris-HCl (pH 6,8); 5,5% acrylamide, 0,15% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,005% tetramethylethylendiamine; 0,1% ammonium persulfate

Separating gel (11% acrylamide): 313 mM Tris-HCl (pH 8,8); 9,25% acrylamide, 0,25% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,001% tetramethylethylendiamine; 0,1% ammonium persulfate

Separating gel (18% acrylamide): 313 mMTris-HCl (pH 8,8); 14,8% acrylamide, 0,7% N,N'-methylene-bisacrylamide; 0,1% sodium dodecylsulfate (SDS); 0,001% tetramethylethylendiamine; 0,1% ammonium persulfate

Running buffer (5x): 125 mMTris-HCl; 1,25 M glycine; 0,5% sodium dodecylsulfate (SDS); pH 8,8

Sample buffer (6x): 50 mM Tris-HCl (pH 6,8); 30% glycerol; 10% sodium dodecylsulfate (SDS); 6% 2-mercaptoethanol; 0,012% bromphenol blue

Samples were mixed with the sample buffer and boiled for 3-4 min before loading onto gel. Gels were let polymerize and then placed into a vertical electrophoretic apparatus. Electrophoresis ran by 145 V until the bromphenol blue dye migrated off. Proteins resolved in the gel were either visualized by silver staining, Coomassie Briliant Blue or subjected to Western blotting.

7.10. Silver Staining of SDS-PAG

Solution 1:12% acetic acid; 50% methanol; 0,02% formaldehyde
Solution 2:50% methanol
Solution 3:0,02% $Na_2S_2O_3 \cdot 5H_2O$
Solution 4: 0,2% $AgNO_3$; 0,02% formaldehyde
Solution 5: 566 mM Na_2CO_3, 16 μM $Na_2S_2O_3 \cdot 5H_2O$; 0,02% formaldehyde
Solution 6: 12% acetic acid, 50% methanol

The gel after SDS-PAGE was incubated for at least 20 min in Solution 1 and then washed three times in Solution 2 for 5 min. Consequently, the gel was incubated in Solution 3 for 1 min, washed with water three times and incubated in Solution 4 for 20 min. The gel was then rinsed with water three times for about one minute and the staining was developed by addition of Solution 5. When the protein bands in chosen lanes were clearly visible, the gel was rinsed with water three times and the process was stopped by addition of Solution 6. If the gel had to be stored, it was done so in Solution 2.

7.11. Western Blotting

Transfer buffer:92 mM glycine; 25 mM Tris-HCl; 10% methanol; 0,1% sodium dodecylsulfate (SDS); (pH not adjusted)
PBS: 137 mM NaCl; 2,7mMKCl; 10 mM Na_2HPO_4; 1,8mM KH_2PO_4; pH 7,4

The gel after SDS-PAGE and also a nitrocellulose membrane were equilibrated in the blotting buffer for about 10 minutes. Proteins were transferred by 12 V for 12 min (Power Pac HC, Bio-Rad) . The membrane was then incubated with 7 ml of Casein Blocker in standard dilution (Thermo Sc.) for at least 1 hour at 4°C. Consequently, an appropriate antibody was added (dilution depended on a type of an antibody; recommended dilution was used for commercial antibodies) and this was incubated overnight at 4°C. Membrane was washed three times with PBS + 0,05% Tween-20 for 5 minutes. If needed, a secondary goat anti-mouse antibody or neutravidin conjugated with horse-radish peroxidase was added and incubated for 30 minutes at 4°C (standard dilution was used for commercial antibody at the beginning however, it was finally used in 10 fold lower concentration to decrease image background). Membrane was washed again three times with PBS + 0,05% Tween-20 to remove free secondary antibody and 0,4 ml of the Luminol/Enhancer Solution (Super Signal West Dura Chemiluminiscence Substrate, Pierce) and the blot was incubated with the mixture for 5 min while gentle agitated. The membrane was then dried between two sheets of filter paper and placed into a transparent plastic foil. The chemiluminiscent signal was detected using CCD camera (UVP ChemiDoc-IT 600).

7.12. Biotinylation of Mouse Antibody recognizing β-subunit of F1 mitochondrial ATPase

Mouse monoclonal IgG1 (Santa Cruz Biotechnology, 0,2 mg/ml) was dialyzed to PBS ON, protein concentration was determined with standard Bradford Assay. The protein solution was mixed with 50 fold molar excess of 10 mM sulpho-NHS-LC-biotin (Pierce) in H_2O on ice for two hours. Reaction was stopped by addition of TBS. Antibody was dialyzed against this buffer ON. Final total protein concentration was set again to 0,2 mg/ml.

Buffer solutions were described previously.

7.13. GCPII Pull-down Using Streptavidin Mutein Matrix

Washing buffer:100 mM Tris-HCl (pH 7.2),150mMNaCl
Elution buffer :100 mM Tris-HCl (pH 7.2),150mMNaCl,2 mM D-biotin
Resin: Streptavidin Mutein Matrix (Roche)

Resin (Streptavidin Mutein Matrix, Roche, 20 μl *per* sample) was washed three times with 1 ml of cold washing buffer and incubated with 3 μg (i.e. 25 μl) of purified biotinylated extracellular portion of GCPII (prepared by Jan Tykvart in laboratory of Doc. Jan Konvalinka [101]) for 1 hour at 4°C. The untreated resin with 25 μl of washing buffer was used as negative control. Tubes were centrifuged at 6000 x g at 4°C and the supernatant was discarded. Resin was then washed three times as described previously. After that, resin with immobilized GCPII was mixed with 100 μl of cell lysate, standard set-up was optimized to contain total protein concentration 3 μg/μl approximately in each reaction. The same procedure was applied for the negative control. Both mixtures were incubated for 3 hours at 4°C. Samples were centrifuged (6000 x g; 4°C) and the supernatant was discarded. After that resin was washed three times (1000, 1000, 100 μl) with washing buffer. Last wash was stored for further analysis. Proteins were finally eluted by elution buffer (100 μl) at 4 °C for 2 hours or the resin was only washed and applied to sample buffer for SDS-PAGE analysis.

7.14. Immunoprecipitation with Protein G-Sepharose

Glycine buffer: 100 mM glycine, pH adjusted with HCl to pH=1
Elution buffer:5mMTris-HCl, 0,1 % DDM
Resin: Protein G-Sepharose 4 Fast Flow, GE Healthcare

Resin (Protein G-Sepharose 4 Fast Flow, GE Healthcare, 20 μl *per* sample) was washed three times with 1 ml of cold washing buffer (described previously) and incubated with about 5 μg of an antibody for 1 hour at 4°C. The untreated resin and resin incubated with different antibody (αl2- antibody recognizing NAALADase L and not recognizing any of the proteins studied) was used as negative control. Tubes were centrifuged at 6000 x g at 4°C and the supernatant was discarded. The resin was then washed three times as described previously. After that, resin with immobilized antibody was mixed with 100 μl of cell lysate

and the standard set-up was optimized to contain total protein concentration approx. 3 μg/μl in each reaction. The same procedure was performed with the negative control. Both mixtures were incubated for 3 hours at 4°C. The samples were centrifuged (6000 x g; 4°C) and the supernatant was discarded. After that the resin was washed three times (1000, 1000, 100 μl) with washing buffer and the last wash was stored for further analysis. Proteins were finally eluted by low pH (glycine buffer) at 4 °C for 1 hour, by boiling for 10 minutes in the elution buffer or the resin was only washed and applied to sample buffer for SDS-PAGE analysis. In the first case, the sample was dialyzed against TBS and protein concentration was adjusted using Amicon Ultra centrifugal filter with 3 kDa to at least hundreds of nanograms *per* microlitre.

8. Results

The first aim of the thesis was to develop a method of sample preparation for detection of the potential GCPII protein partners. We decided to use immunoprecipitation and pull down techniques.

8.1. Affinity Purifications (Immunoprecipitation and Pull-Down)

General approach for both the immunoprecipitation and the pull-down techniques was to immobilize GCPII as the bait protein on a resin which was further incubated with the cell lysate. The resin was washed and the hypothetical protein complex was eluted. Elution fractions were either dialyzed (eventually concentrated), trypsinized and applied directly to LC-coupled MS or resolved by SDS-PAGE, bands were excised, digested and then analyzed by MS.

8.1.1. Pull-Down

N-terminally biotinylated recombinant extracellular portion of GCPII (hereinafter denominated as Avi-tagged GCPII) was immobilized on Streptavidin Mutein Matrix and washed. The complex was then incubated with lysate of, LNCaP cells alone, LNCaP cells transfected with dermcidin or HUVECs. Proteins were released from the resin with an excess of biotin in concentration of approx. three hundreds of ng/µl.

8.1.2. Immunoprecipitation

Antibody recognizing native GCPII (GCPII-08) was immobilized on resin (Protein G Sepharose 4 Fast Flow, Healthcare) and incubated with LNCaP lyzate. The elution of potential complex of the antibody, endogenous GCPII and an unknown protein partner was performed either by low pH (100 mM glycine buffer pH=1) or by boiling in a sample buffer containing detergent (0,1 % DDM). The elution with low-pH provided a liquid sample of protein concentration of hundreds of ng/µl and showed to be more efficient than the latter. The elution by detergent provided sample of protein concentration close to the limit of

detection of the chosen method (Bradford Assay). When resolved by SDS-PAGE, the bands cannot be even visualized by Commassie Briliant Blue. However, the same liquid fraction was analyzed by LC-MS. We observed similar number of proteins in total detected with large sequence coverage compared to the analysis of hits from elution by low pH.

An antibody against homolog of GCPII NAALADase L (αL2) not recognizing GCPII was used as a negative control for all immunoprecipitations as well as untreated resin. The suitability of the negative control will be discussed below.

8.2. Analysis of Elution Fractions prior to MS Analysis

Part of a sample was directly digested in a liquid phase, part was mixed and boiled with sample buffer and resolved by SDS-PAGE. Standards of the cell lysate, resin, antibody, bait protein, wash fraction and elution fraction were resolved simultaneously for Western blotting and band excised prior to digestion with trypsin. All digested samples were consequently handed over for LC-MS analysis. Lists of hits detected both in the gel and "liquid sample" (without prior SDS-PAGE) were used for their comparison.

As consistent with the optimization (see below), in both pull-downs and immunoprecipitations, liquid elution samples were dialyzed against TBS that allowed direct digestion of the liquid sample.

Trypsinization and LC-MS analysis of all samples was performed by our colleagues Jana Horáková, Karel Rucker and Zuzana Demianová from the Department of Mass Spectrometry, IOCB, Prague. The procedures were described in chapter Methods and will not be discussed herein.

8.3. Primary MS Data Processing

Raw spectrometrical data (*.wiff) obtained from mass spectrometry analysis (TripleTOF 5600, ABSciex) were processed in ProteinPilot 4.0 (AB Sciex) and searched against HomoSapiens database (UniProt, 30.7.2012)[102] completed with a sequence of our synthetic gene of Avi-tagged GCPII. We stopped using the SwisssProt database (Uniprot), which was chosen at the very beginning of our experiments, because of a numerous apparently false discoveries (proteins from other species identified with high coverage).

Processing of primary data was also performed by our colleagues Jana Horáková and Zuzana Demianová from the Department of Mass Spectrometry, IOCB, Prague.

8.4. Secondary MS Data Processing

An output of ProteinPilot provided *inter alia* information about sequence coverage of distinct reliability, number of peptides detected, gene and species (see Methods). A database (Access, Microsoft) was established by Pavel Šácha. Data were imported, sorted as consistent with the experiments and then queries were applied for final data processing as was described previously.

MS, as a high-throughput method, provides us the large amount of information. A sensitivity of the instrument (TripleTOF 5600 with a NanoSpray III source (AB Sciex) operated by Analyst TF 1.6 (AB Sciex) allows detection of thousands of proteins with different accession numbers per run. Such large datasets cannot be easily interpreted by common sense on primary neither secondary level as opposed to other biochemical experiments. Hence, we assume such a basic "data mining" to be a crucial step of establishing MS-mediated proteomics in our laboratory. On the other hand, the criteria introduced for the data-processing might influence the outcome of analyses, as discussed in the Introduction.

8.5. Optimization of Experimental Procedure

8.5.1. Reproducibility

In order to test the reproducibility of the whole analytic procedure (SDS-PAGE and MS measurement), we resolved one elution from the same immunoprecipitation three times (LNCaPs, antibody GCPII-08) by SDS-PAGE on 11 % gel and analyzed each of them. We chose 118 hits in one triplicate having coverage greater than 50 %. Then we compared the sequence coverage of these hits in the two remaining samples of the triplicate. At a glance, hits with higher coverage (maximal coverage detected was 87 %) were highly reproducible in all three control measurements. Up to coverage 60 %, only two hits of 63 were not present in all control measurements while the coverages of the remaining varied by up to 10%. From 75 hits between coverage 60 and 50 %, 24 hits were not present in all three control

measurements while most of them varied by up to 10 % and 5 differed by 20-30%. Under the coverage of 30 %, a half of the hits was found only in one or two controls (Chart 1).

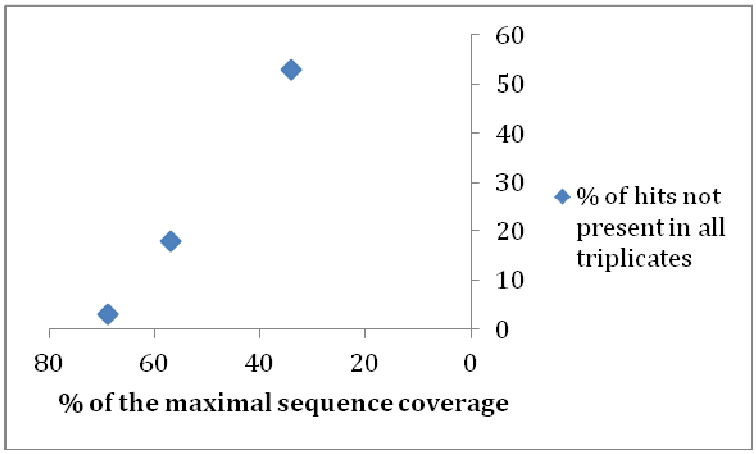

Chart 1. The Relationship between Sequence Coverage and Reproducibility of SDS-PAGE Preceding Mass Spetrometry Analysis:

One sample (10 µl from 100 µl elution) was resolved three times independently by SDS-PAGE and analyzed by MS. The percentage of the maximal sequence coverage measured in each run (x-axis) is ploted against percentage of different hits missing in one or two samples of a triplicate (y-axis).

The observation was reproduced with negative control of the same experiment (incubation with antibody not recognizing GCPII αL2) with similar results (data not shown) having lower number of proteins detected in total.

We conclude that SDS-PAGE analysis does not significantly influence the identification of hits with high sequence coverage. Therefore, we further focused only on the reproducibility of the MS analysis. One liquid elution (immunoprecipitation from LNCaPs with antibody recognizing β-subunit of F1 mitochondrial ATPase) was analyzed twice. Sample was frozen in -20 °C in between the two measurements. In this case, the bait protein was detected with 60 and 56 % coverage while all hits above 40 % coverage (50 % of the maximal coverage detected in these runs) were detected in both experiments with coverages differing by 10 % at maximum (% of sequence coverage/ % of sequence coverage). However, in the samples that were neither dialyzed nor concentrated we observed markedly lower coverages of all hits (the highest sequence coverage was 40 %). Both positive controls were detected (immunoglobulin as well as bait) and therefore these experiments can be considered as conclusive.

To prove once again if the reproducibility of the MS detection is satisfactory, we performed another experiment (IP with GCPII-08 in LNCaPs) in which we analyzed three times the same liquid elution (incubation of lysate with antibody GCPII-08 binding

endogenous GCPII). In the three runs, the bait protein was detected with sequence coverage of 32% (44 peptides detected); 33 % (42 peptides detected) and 33 % (40 peptides detected). The sequence coverage thus varied by 6 % at maximum. We thus conclude that the MS analysis provides reproducible results.

8.5.2. Sensitivity

We analyzed 2µl (400 ng of total protein) and 10 µl (2 µg of total protein) of a sample to estimate an influence of the amount of protein on the result of the detection. We used positive hits (present only in positive control) from immunoprecipitation with GCPII-08 in LNCaPs resolved by SDS-PAGE prior to the MS analysis. The bait protein was detected with coverage 58 and 53 % (10 µl and 2 µl control). The highest sequence coverage determined in the run was 88 % and 91 % (keratin 9 in both). A cut-off was set at 50 % of the maximal sequence coverage based on the observation that this coverage is sufficient for reliable and reproducible identification of a hit (see Chart 1, page 34).

Applying this cut-off on 928 and 627 hits detected in the 10 µl and 2 µl of sample, we found 70 and 73 hits, respectively. Seven hits were found exclusively in the 10 µl and 10 only in 2 µl sample (Chart 2).

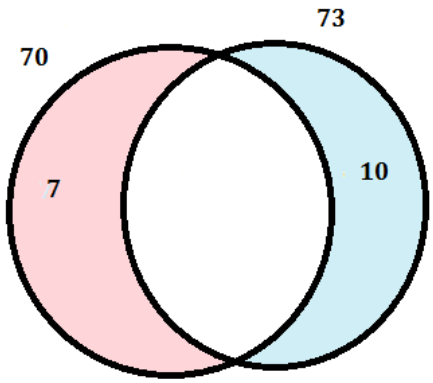

Chart 2. Illustration of the Number of Different Proteins Detected by Resolving 2 and 10 µl of a sample

10 µl and 2 µl of the elution of immunoprecipitation in LNCaPs (see Methods) were analyzed and numbers of potential binding partners of GCPII found were compared. From the larger amount, 7 hits were not found in the 2 µl sample, whereas, 10 hits were found exclusively in 2 µl sample and were not found in the 10 µl sample.

8.5.3. Gel density

Since it is known that small proteins can have higher mobility in SDS gel than it could be expected from their mass [103], we decided to determine the significance of the gel density by a comparison of 11% and 18% polyacrylamide gels. Both gels were run until the Bromphenol Blue migrated off. In order to retain a relevance for small peptide molecules, the samples were neither dialyzed nor concentrated prior to SDS-PAGE analysis.

Data from this set of experiments was processed fully- we first detected positive hits in both 18 % and 11 % polyacrylamide gels by subtraction of hits from negative control and then compared these sets of potential binding partners.

First, we used 11% as negative control for the 18% and then we did the same *per contra*. We observed 9 hits with coverages ranging from 50 and 30 % present exclusively in the 18% gel while there was only one hit present exclusively in 11% gel with the coverage above 35%. We assumed such reciprocal subtraction as a negative control for this data processing. It is worth mentioning that none of the proteins corresponding to hit genes "lost" by using 11% gel has molecular weight above 35 kDa.

8.5.4. Common contaminants

False positivity showed to be a major obstacle in bottom-up proteomics. We decided to map typical contaminants in our experimental setup.

Throughout our experiments, keratins showed to be present in every sample with a high coverage (up to 90%). Hits of cytoskeletal keratins of both type I and II were usually found with coverage higher than the one of positive control (bait protein).

We decided to find out whether the contaminant proteins are introduced during sample preparation or if these are inherent to the sample and their presence is therefore an inevitable disadvantage of our method.

First, we analyzed elution fractions from IP (LNCaPs) prepared in an "clean" way (we used flowbox, polyacrylonitrile gloves and sterile buffers). Data was compared with those from MS measurement of samples prepared by standard protocol while no significant difference was observed. Keratins were as usual the most prevalent false hits (keratins 1,2,9,10,14,16,17). Also other proteins were found regularly in negative control: anexin,

cystatin A, cytochrom c, fatty acid synthase, fatty acid synthase, galectin 7, glyceraldehyde-3-phosphate dehydrogenase malate dehydrogenase 2, tubulin α and β, peptidylprolyl isomerase A, 14-3-3 protein ε, S100 calcium binding protein A, profilin, prohibitin,ribosomal protein l23 and s16, ubiquitin.

In experiments with HUVECs (3 immunoprecipitations and 2 pull-downs), a large coverage of keratins was also observed in each run, but as opposed to LNCaPs, other proteins were detected repeatedly in negative control and are listed below: Actin, anexin, α–enolase, calreticulin, calumenin, fatty acid binding protein, galectin,glyceraldehyde-3-phosphate dehydrogenase, hemoglobin β, mitochondrial translocase, myosin (both chains), peptidylprolylisomerase, profilin, reticulocalbin, tubulin α, triosephosphate isomerase, vimentin.

Myosin in particular was detected in pull-downs in HUVECs in both experiment and negative control with coverage near or even higher than the one of the bait protein.

One of the abundant contaminants was also hemoglobin that was present in samples from HUVECs with high coverage (up to 80%) while there was none in duplicates or positive controls. Hemoglobin can be assumed as one of the "ghost" hits that are commonly discovered with no apparent relation to experimental design.

It is also worth mentioning that heterogeneous nuclear ribonucleoprotein and dermcidin which were intensively studied as candidates for binding partners of GCPII in LNCaPs, were assumed as non-specific binders in HUVECs. This finding supported our idea of how important is an application of diverse biological material.

8.5.5. The Choice of Negative Control

We used either an untretaed resin (Protein G Sepharose 4 Fast Flow, Healthcare) or immobilized αL2 (antibody that does not recognize GCPII) as negative control for immunoprecipitation experiments. Number of hits matching different proteins in the liquid samples were compared in order to determine an appropriate negative control for further experiments of IP character.

In both types of negative control, we compared proteins detected with coverage higher than 50 % of the maximal one detected. We found 18 different proteins in the elution of free resin and 22 proteins in the elution from the IP using αL2 (except for the

immunoglobulin itself). Two types of actin, tubulin and light chain of myosin differed between the two controls. Items with the highest coverage were keratin1 and dermcidin as usual. Data very similar concerning the complexity of negative control was observed in three separate experiments. Despite finding both controls equally efficient, we decided to perform both (with and without antibody) in all immunoprecipitations. Data from these controls were aggregated in each immunoprecipitation analysis and used together as a single negative control.

8.5.6. Comparison of Liquid and Gel Samples

We set to find out whether the SDS PAGE step is indeed necessary part in the sample preparation protocol. To this end the samples from reactions containing bait protein (positive control) were ether resolved by SDS-PAGE and analyzed by MS ("gel samples") or analyzed directly after the elution by MS ("liquid samples"). Protein hit lists from both experiments were then compared. From 55 hits present in the liquid sample with coverage greater than 40 % (bait protein found with coverage 67 and 64 %), 9 hits were found with coverage lower than 40 % in gel while 5 of them were not detected at all. Inversely, from 47 hits detected in gel sample with coverage greater than 40 %, 19 were found in liquid sample with coverage lower than 40 % and 11 were not detected in liquid sample at all. Based on these findings, we could have assumed there is no fact pointing at an advantage of any of the two preparation methods. Nevertheless, we explored individual protein hits in more detail and identified 16 of the 19 proteins present exclusively in gel sample as keratins and one of them as dermcidin. None of the hits discovered exclusively in liquid sample was keratin.

8.5.7. Dermcidin

LNCaPs were transiently transfected with plasmid encoding dermcidin and the cell lysate was used for pull-down experiment. Bait protein was found with coverage of 50%. In this experiment, dermcidin was detected in both negative control and incubation with bait protein (Avi-tagged GCPII) with similar coverage. (Avi-tagged GCPII). Dermcidin was also detected in different positions in the gel with average sequence coverage of 30% (see below).

8.5.8. Protein size distribution in SDS-PAGE

Previously mentioned experiment also showed that dermcidin was detected in different parts of analyzed gel (under and also above position expected in regard to molecular weight marker). In this case, the elution fraction was resolved by SDS-PAGE and the lane was cut into four pieces of the same size. Dermcidin was found in all four part of the lane in positive control (and in three parts of the negative one) which could have suggested that this protein is a post-experimental contaminant. Nevertheless, also other explanation was evident: proteins are not resolved properly by SDS-PAGE and they leave their traces throughout the lane. In the case, it would be more convenient to perform no SDS-PAGE prior to trypsinization in further experiments.

To prove the hypothesis, we mapped the presence of our bait-GCPII (positive control for a protein hit and experiment relevance) in immunoprecipitations in relation to its mobility in the gel. In order to eliminate "doubled" hits coming from potential scission in the middle of protein band, we compared always odd pieces of each gel.

In gels cut into four parts, GCPII was present in at least two of them with significant coverage (40 % of maximal coverage detected). An approximate position in the gel was the expected one (100 kDa) or lower. This was observed again in duplicates and regardless of the gel density. We therefore decided to cut lane into eight parts that were analyzed also separately (see Figure 8). Bait protein was detected in parts: 152, 158, 159, 160 and 161. In the last three samples was GCPII detected with the sequence coverage above 50 % (of the maximal). This data suggest that SDS-PAGE cannot be used for protein focusing without an exclusion of contamination of the lane.

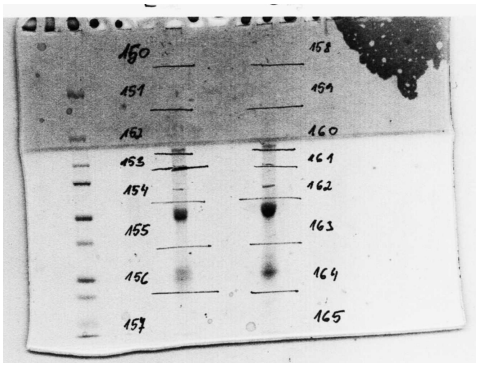

Figure 8. Example of a Scission of the gel prior to MS Analysis

Samples of the elution fractions of immunoprecipitation in LNCaPs (approx. 3 µg of total protein) was resolved by 18% SDS PAGE (see Methods for experimental details). The gel was

subsequently cut by clean scalpel (rinsed after each scission) as indicated on the figure and each part was digested and analyzed by MS separately. Parts 158-165 represent postive control elution-incubation with antibody GCPII-08)

8.5.9. Identification of Potential Protein Partners of GCPII

Even though we observed many hits present exclusively in positive control in certain experiments, we always detected at least some sequence coverage of these proteins in the negative control of other experiment. Also proteins possibly interacting with GCPII in one cell line showed to be a contaminant in the second one (myosin and keratin, see below). Even though we are not able to denote any of the hits as a binding partner of GCPII with certainty, we present herein a brief review of number of experiments (Table 1) and hits from experiments with HUVECs (Table 2, page 41) and also LNCaPs (Table 3, page 42).

Table 1. Review of the Experiments Performed:

We performed several experiments in two cell lines gathering more than 100 000 hits. Data was organized and processed as described in Methods. Samples of incubation containing bait protein or an antibody specific to bait protein are noted "Positive control", "Negative control" are noted samples of incubation with untreated resin or other antibody (as described previously).

Source	Procedure	Number of Samples		Number of Protein Hits	
		Positive Control	Negative Control	Positive Control	Negative Control
LNCaP	Immunoprecipitation	9	9	43864	37822
LNCaP	pull-down	13	12	12896	9977
HUVEC	pull-down	3	2	6912	4079

Table 2. First 16 Positive Hits from All Pull-Down Experiments in HUVECs:

Throughout all experiments of pull-down type in HUVECs, a best sequence coverage in each run for given accession number (coverage) in positive control (+) was chosen as well as the highest number of peptides found (peptides). Coverage or peptides of the negative controls were subtracted numerically obtaining "difference". The first raw represents our bait (positive control). It is worth mentioning that its sequence can be distinguished from endogenous GCPII by the protein database (see Methods) since the "bait" used in our pull downs lacks the N-terminal part of the protein and contains a biotinylation motif. Hits in the list are sorted according to "peptide difference".

Accession Number	Gene	Peptides (+)	Coverage (+)	Peptides Difference	Coverage Difference
sp|S01|AviEXST	AviEXST	205	71,5	205,0	71,5
sp|Q04609-6|FOLH1_HUMAN	FOLH1	113	62,1	113,0	62,1
sp|Q04609-7|FOLH1_HUMAN	FOLH1	113	58,5	113,0	58,5
sp|Q04609|FOLH1_HUMAN	FOLH1	113	57,3	113,0	57,3
sp|P05|GCPII	FOLH1	113	57,3	113,0	57,3
tr|A4UU13|A4UU13_HUMAN	FOLH1	112	60,1	112,0	60,1
tr|H6VRG2|H6VRG2_HUMAN	KRT1	116	69,6	65,0	6,4
sp|P13645|K1C10_HUMAN	KRT10	79	69,3	37,0	16,9
sp|P35908|K22E_HUMAN	KRT2	77	92,6	37,0	29,7
sp|P08779|K1C16_HUMAN	KRT16	51	72,7	32,0	26,6
tr|E7EQV7|E7EQV7_HUMAN	KRT6C	45	66,1	30,0	35,0
sp|P48668|K2C6C_HUMAN	KRT6C	45	65,8	30,0	35,5
tr|A1A4E9|A1A4E9_HUMAN	KRT13	39	70,7	29,0	54,2
tr|B2R853|B2R853_HUMAN	KRT6E	42	62,4	28,0	32,5
sp|P04259|K2C6B_HUMAN	KRT6B	44	65,8	26,0	28,7
sp|P12035|K2C3_HUMAN	KRT3	24	35,8	24,0	35,8

The data summarized in the Table 2. suggest that GCPII might specifically bind to some keratins in HUVECs. However, different proteins were identified as putative partners in LNCaPs (Table 3, page 42). Data from these experiments suggest that GCPII might specifically bind to a heavy chain of non-muscular myosin or to collagen I.

Table 3. First 21 Positive Hits from All Immunoprecipitations with GCPII-08 in LNCaPs:

Throughout all immunoprecipitations in LNCaP cells, a best sequence coverage in each run for given accession number (coverage) in positive control (+) was chosen as well as the highest number of peptides found (peptides). Coverage or peptides of the negative controls were subtracted numerically obtaining "difference". FOLH1 is the gene of our bait protein (positive control). Hits in the list are sorted according to "peptide difference". Data from these experiments suggest that GCPII might specifically bind to a heavy chain of non-muscular myosin or collagen type I.

Accession Number	Gene	Peptides (+)	Coverage (+)	Peptides Difference	Coverage Difference
sp|P02452|CO1A1_HUMAN	COL1A1	132	56,3	99,0	23,6
sp|Q04609|FOLH1_HUMAN	FOLH1	97	62,1	93,0	37,3
sp|P05|GCPII	FOLH1	97	62,1	93,0	37,3
tr|D3DTX7|D3DTX7_HUMAN	COL1A1	90	53,6	90,0	53,6
sp|P35579|MYH9_HUMAN	MYH9	123	54,6	69,0	13,5
tr|Q60FE2|Q60FE2_HUMAN	MYH9	123	54,6	69,0	13,5
sp|Q04609-6|FOLH1_HUMAN	FOLH1	67	60,3	63,0	33,5
sp|Q04609-7|FOLH1_HUMAN	FOLH1	67	58,5	63,0	33,2
tr|A4UU13|A4UU13_HUMAN	FOLH1	67	56,9	63,0	31,6
sp|S01|AviEXST	AviEXST	67	58,5	63,0	31,0
sp|P35908|K22E_HUMAN	KRT2	121	92,6	53,0	5,8
sp|P35580|MYH10_HUMAN	MYH10	59	36,3	52,0	27,0
sp|P35580-3|MYH10_HUMAN	MYH10	59	35,9	52,0	26,7
tr|B2RWP9|B2RWP9_HUMAN	MYH10	59	35,6	52,0	26,4
sp|P35580-2|MYH10_HUMAN	MYH10	59	35,5	52,0	26,3
tr|F8W6L6|F8W6L6_HUMAN	MYH10	59	35,5	52,0	26,3
tr|F8VTL3|F8VTL3_HUMAN	MYH10	59	35,2	52,0	26,1
tr|Q4LE45|Q4LE45_HUMAN	MYH10	59	35,0	52,0	26,0
tr|Q8N1C8|Q8N1C8_HUMAN	HSPA9	53	62,8	51	44,7
tr|H6VRG3|H6VRG3_HUMAN	KRT1	140	72,0	47	0,6
sp|P13645|K1C10_HUMAN	KRT10	133	73,4	46	-5,3

Before the entire dataset was obtained, experiments in LNCaPs (both pull-downs and immunoprecipitations) suggested that some proteins might be specifically precipitated from the lysate by immobilized GCPII. A particular attention was paid to β-subunit of F1 Mitochondrial ATPase that was further investigated as potential binding partner also in HUVECs. Therefore, we decided to analyze possible interaction of this protein as a model positive hit identified in our proteomic approach with GCPII in more detail.

8.6. Expression of GCPII and β-subunit of F1 Mitochondrial ATPase in LNCaPs and HUVECs

HUVEC lysates were analyzed by Western Blotting, immunoprecipitation from HUVECs with antibody recognizing GCPII (GCPII-08) was analyzed also by Western blotting followed by MS. We observed no endogenous expression of GCPII in HUVECs using any of the methods (data not shown).

Data acquired in preceding set of experiments suggested a possible interaction of GCPII with β-subunit of F1 ATPase (further only as βATPase) in LNCaP cell lysates. Some part of F1 mitochondrial ATPase protein complex was a common hit of 16 experiments. βATPase was the most frequented hit of the whole complex of ATPase. First, βATPase was found with coverage 78% (32 peptides) in IP in LNCaPs. Consequent experiments with LNCaPs revealed some coverage of the protein also in negative controls (the highest one reaching 40 %).

First, we confirmed the presence of βATPase in both HUVECs and LNCaPs by IPs with antibody recognizing the protein (monoclonal mouse IgG_1 against βATPase, Santa Cruz Biotechnology, hereinafter just as anti-βATPase). βATPase was detected by MS (coverage comparable to the one of bait-GCPII in previously mentioned immunoprecipitation) and by Western blotting as well (lanes 6,7, Figure 9, panel B, page 44). Then we proceeded to affinity purification using anti-βATPase.

Presuming that protein specifically co-precipitated with GCPII should be able inversely to precipitate endogenous GCPII from LNCaPs, we carried out IP with antibody recognizing βATPase (anti-βATPase). Four reactions were performed in parallel: immunoprecipitation using GCP08, βATPase, αL2 and no antibody whatsoever (free resin), the two latter serving as negative controls.

As we found out early that βATPase (56,5 kDa) is expected to migrate at the same molecular weight as the heavy chain of used IgGs which are also recognized by anti-mouse antibody (lanes 2,3,6,7,8,9; Figure 9, both panels), we tried to solve the problem by biotinylation of the monoclonal anti-βATPase antibody. Secondary antibody (neutravidin conjugated with horseradish peroxidase) would not cross-react with any of the controls used. The approach enabled us to determine that anti-βATPase is able to recognize and immunoprecipitate βATPase present in LNCaPs lysate (lane 6, Figure 10, page 46).

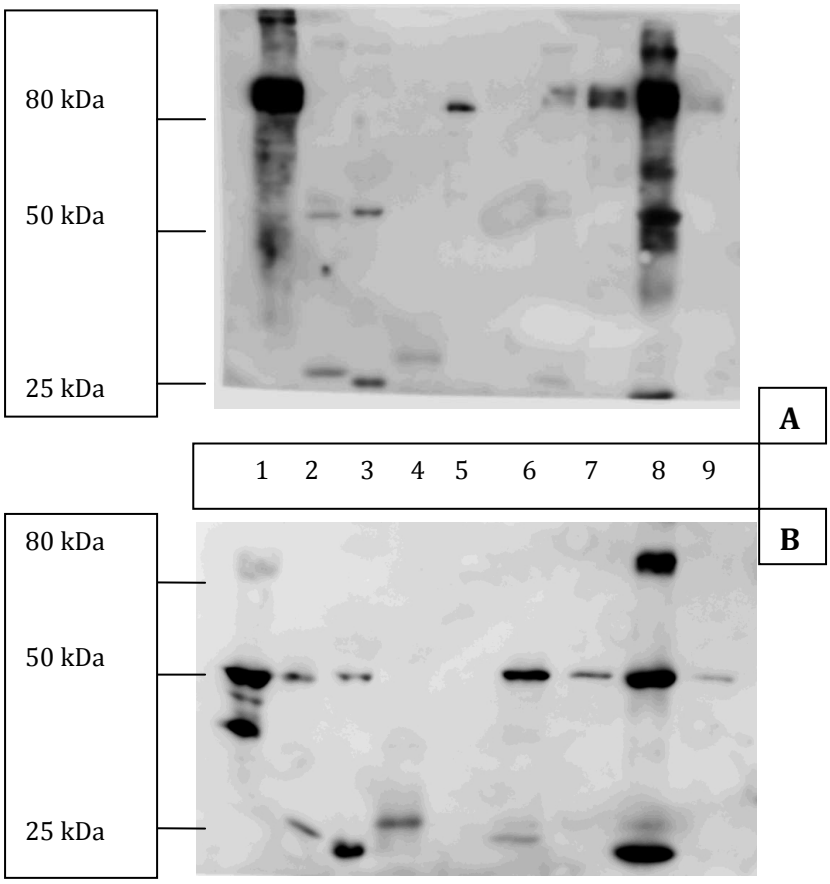

Figure 9. Western Blot of Samples of Immunoprecipitation (IP) from LNCaPs:

Lanes: 1- LNCaP lysate, 2- anti-βATPase IgG, 3- GCPII–O8 IgG, 4- Protein G Sepharose 4 Fast Flow (GE Healthcare Life Science), 5- Avi-tagged GCPII 5ng, 6-IP *via* anti-βATPase elution (10 µl from 100 µl), 7-IP *via* anti-βATPase last wash (10 µl from 100 µl), 8-IP *via*GCPII-08 elution (10 µl from 100 µl), 9-IP *via*GCPII-08 last wash (10 µl from 100 µl). Panel A: Western blot was first treated by mouse monoclonal antibody recognizing GCPII (GCPII-04). Panel B: Western blot was first treated with antibody anti-βATPase (Santa Cruz Biotechnology). Both membranes were developed by a secondary antibody anti-mouse (goat anti-mouse-IgG1conjugated with horseradish peroxidase, Pierce) and Super Signal West Dura Chemoluminiscence substrate (Pierce), 50 seconds, On-Chip integration regime.

The biotinylation resulted in highly elevated background of Western blots (Figure 10), in which we hardly identified bands of all controls. Thence we could not use this antibody for identification of light bands in the elution of IPs in LNCaPs.

The reliability of the performance of anti-βATPase was also confirmed by MS – bait protein was detected with 77 % coverage (39 peptides) which was about 80 % of the maximal coverage detected.

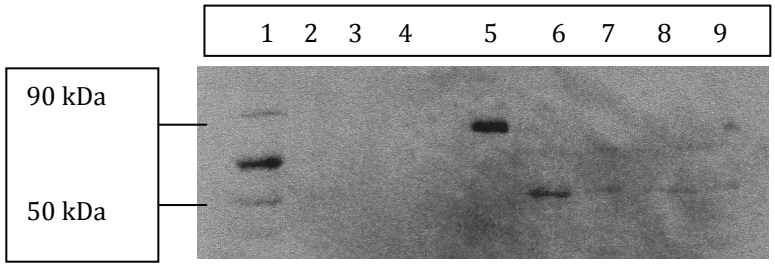

Figure 10. Western Blot of Samples of Immunoprecipitation (IP) from LNCaPs:

Lanes: 1- LNCaP lysate, 2- anti-βATPase IgG, 3- GCPII –08 IgG, 4- Avi-tagged GCPII 5ng, 5- IP *via* anti-βATPase elution (10 µl from 100 µl), 6-IP *via* anti-βATPase last wash (10 µl from 100 µl), 7- IP *via* GCPII-08 elution (10 µl from 100 µl), 9-IP *via* GCPII-08 last wash (10 µl from 100 µl). Western blot was developed with primary in-house biotinylated antibody anti-βATPase (Santa Cruz Biotechnology) and neutravidin (conjugated with horseradish peroxidase, Pierce), Super Signal West Dura Chemoluminiscence substrate (Pierce), 40 seconds, On-Chip integration regime.

We can see that βATPase can precipitate GCPII in this experimental set-up (lane 6, Figure 9, panel A, page 44). However, there is more of GCPII washed than eluted (lanes 6,7, Figure 9, panel A page 44.). Inversely, βATPase precipitated by GCPII is also washed out (lane 9, Figure 9, panel B, page 44). On the other hand, similar washout of βATPase could be seen in case of its specific antibody (lane 7, Figure 9, panel B, page 44), which is expected to bind this protein tightly.

The two bands in lanes 1 and 8 (Figure 9, panel B, page 44) were not explained. According to their position, we can expect them to be endogenous GCPII from LNCaPs (lane 1, Figure 9, panel A, page 44) and an elution enriched in this protein (lane 8, Figure 9, panel A, page 44). The bands could be visualized by a cross-reactivity with one of the antibodies used. Nevertheless, a similar signal would be expected a in the lane for bait-protein control (lane 5, Figure 9, panel B, page 44).

Mass-spectrometry analysis of the immunoprecipitation *via* GCPII-08 was discussed above. GCPII was not detected in the elution of immunoprecipitation *via* anti-βATPase in LNCaPs by this method.

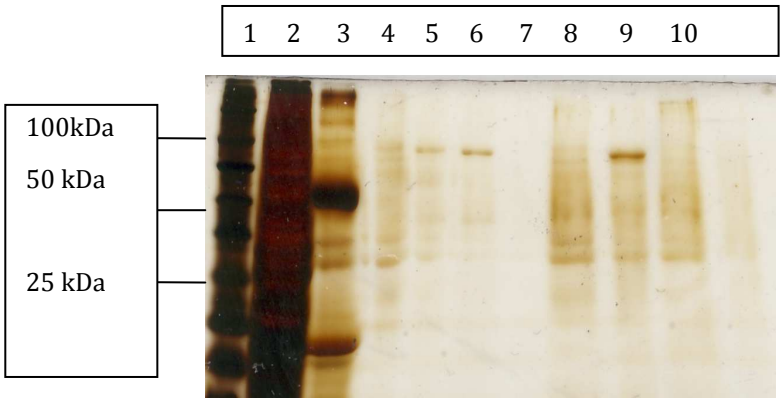

Figure 11. Silver-Stained SDS-PAGE of Samples of Pull-Down from HUVECs:

Lanes: 1- Marker, 2- HUVEC Lysate, 3- Streptavidin Mutein Matrix (Roche),4- Avi-tagged GCPII (1 ng), 5- Avi-tagged GCPII (5 ng), 6- Avi-tagged GCPII (10 ng), 7- Incubation with Avi-tagged GCPII last wash (10 µl from 100 µl),8- Incubation with Avi-tagged GCPII elution (10 µl from 100 µl), 9-Null control (reaction with free resin) last wash (10 µl from 100 µl), 10- Null control reaction elution (10 µl from 100 µl).

Since there is no endogenous GCPII expressed in HUVECs, we performed pull-down using with Avi-tagged GCPII to try to co-precipitate βATPase.

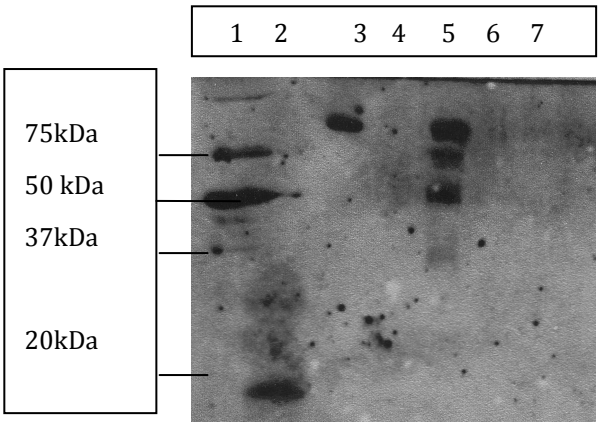

Figure 12. Western Blot of Samples of Pull-Down from HUVECs:

Lanes: 1- HUVEC Lysate, 2- Streptavidin Mutein Matrix (Roche),3- Avi-tagged GCPII (10 ng),4- Incubation with Avi-tagged GCPII last wash (10 µl from 100 µl), 5- Incubation with Avi-tagged GCPII elution (10 µl from 100 µl), 6-Null control (reaction with free resin) last wash (10 µl from 100 µl),7- Null control reaction elution (10 µl from 100 µl).Western blot was developed with primary in-house biotinylated antibody anti-βATPase (Santa Cruz Biotechnology) and neutravidin (conjugated with horseradish peroxidase, Pierce), Super Signal West Dura Chemoluminiscence substrate (Pierce), 3 minutes, On-Chip integration regime.

At a glance it can be assumed that the positive elution is richer in proteins, majority of which presumably "stick" to GCPII in a non-specific manner (compare lanes 8 and 10, Figure 11). However, no significant band could be seen in the position of 56 kDa in the elution lane 8 (Figure 11) suggesting that βATPase is not bound to GCPII in this experiment.

By Western blotting using biotinylated anti-βATPase, we identified 5 proteins in HUVECS (lane 1, Figure 10, page 45). We assume that four of them are endogenously biotinylated (and therefore interact with the neutravidine), the one slightly above 50 kDa is probably βATPase. In the positive elution (lane 5, Figure 12, page 46) we can see bait protein according to its marker (lane 3, Figure 12, page 46) and 3 other bands present exclusively in this positive control compared to lanes 6 and 7 (negative control elution and wash)(Figure 12, page 46). One of the proteins migrates at the position of βATPase so we would expect an interaction. Though, this assumption was not confirmed by MS analysis during which we detected no βATPase in the positive elution (elution from bait-containing resin) of the same pull-down.

9. Discussion

We attempted to introduce, optimize and finally apply a proteomic approach using affinity chromatography with MS detection of the complexes for the identification of binding partners of GCPII. In important part of the project was also the establishment of the database for the analysis of large datasets produced during MS proteomic analyses.

In our laboratory, some potential binding partners were previously identified by MS and were further studied. In consequent experiments, these proteins were shown to be false hits. This fact motivated us to explore deeply the method employed in the searching process and increase its overall reproducibility.

9.1. Optimization of the Experimental Procedure

When looking for a part of signaling pathway, it is of particular importance to include also membrane proteins in the experiment. We succeeded in preparation of cell lysates containing both cytoplasmic and membrane proteins which was shown on Western blots. This was also confirmed by detection of endogenous GCPII (a membrane protein) in LNCaPs.

Nonetheless, we reproducibly found two membrane-related proteins in one cell lysate (LNCaPs) (shown on Western Blots, Figure 9, page 44) which might be considered a sufficient proof-of-principle for the use of the protocol in further experiments.

We proved the employability of our affinity purification by a reproducible detection of all three bait proteins (GCPII, Avi-tagged GCPII, β-subunit of F1 ATPase) in the corresponding eluates by MS proteomics. It is apparent that also antibodies used (anti-β-subunit of F1 mitochondrial ATPase, GCPII-08) can be used as internal positive control. This, however, would require sequencing of the given antibody for introducing it into the sequence database.

In pull-down experiments, it was shown that we are able to introduce a sequence of a recombinant protein (Avi-tagged GCPII) into the sequence database and that it is possible to distinguish the tagged recombinant protein from its endogenous counterpart (GCPII) during MS analysis of a biological sample (pull-down from LNCaPs, Table2, page 41). The recombinant protein used as a bait lacks first 44 amino acid of the natural full

length GCPII and includes also biotin acceptor peptide in its sequence. these features enable to distinguish it from the fulllength GCPII.

After some adjustments, we reached reproducibility in our experiments. The differences in the sequence coverage of the bait protein (internal positive control) between individual runs were significant; on the other hand, the abundance of hits with the highest sequence coverage was very similar. Therefore, we decided to set our reliability limit above 50 % of the highest reached sequence coverage in each run Two features of LC-MS detection were observed in connection to the method of elution: First, dodecylmaltoside at given concentration (0,1%) does not interfere with the analysis. Second, total protein concentration of the sample does not affect significantly detection preferences in tested range (μg/μl–ng/μl), despite a competitive ionization [46,50] was shown to be related with the protein concentration.

Almost the only expected advantage of gel-based analysis (i.e. samples resolved by SDS-PAGE prior MS analysis) was the presumed ability to identify proteins of specific mass. We disproved this assumption. Surprisingly, our bair protein- GCPII was detected in different positions in the SDS gels. This can be explained by its potential degradation, still, it suggests at low efficiency of using SDS-PAGE prior to the trypsin digestion. After several experiments with gel samples of different densities (11 and 18 % polyacrylamide), we observed that gel density is involved in the quality of protein retention and detection and that it is preferable to use higher density gels. We also identified keratins to be a majority of hits present exclusively in gel-based sample. This can be taken as *exempli gratia* of importance of both statistical and individual data interpretation. Such data might question the suitability of SDS-PAGE for sample preparation. Because of the high reproducibility of liquid elution analysis and the facts mentioned above, we decided to eliminate the procedure of SDS-PAGE altogether from protocol for the sample preparation. For more, storing of liquid samples (freezing/thawing) showed to decrease a reproducibility only a little (4% of sequence coverage of the internal standard) which poses an important advantage compared to gel-based sample.

In 23 affinity experiments we mapped "sticky" proteins that can be found as common contaminants in further assays (e.g. heat shock proteins, ubiquitin, light chain of myosin and dermcidin- see Results). It is also known that keratins in general are one of the most common false discoveries in MS characterization of protein-protein interaction (reviewed in [36,64]). Naturally, some contaminant proteins can be caused by almost

ubiquitous dead epidermal cells. Others are caused by a non-specific interaction of cytoskeletal keratins present in a cell lysate. In the latter, we can never rule out a specific interaction, therefore, all such hits should be considered as any other. Nevertheless, keratins are highly conserved proteins [97] and we should expect a false detection of one by virtue of fragmentation of another. We proved some of the keratins to be inherent to the sample, since they were detected predominantly in one cell line. This led to the assumption that not all keratins are post-experimental contamination.

Also hemoglobin was detected in numerous experiments with both cell lines. In HUVECs, the sequence coverage in a positive elution (incubation with bait protein) reached 80 % while there was no hemoglobin detected in the negative control of several experiments. We found no literature reporting hemoglobin expression in HUVEC. The hemoglobin may come from a fetal calf serium medium in which cells were grown. Nevertheless, its presence in positive control still suggests a possible interaction with GCPII.

An appropriate negative control is a crucial step in all experiments of high throughput. For this reason we compared two types of negative control- free resin without any antibody and resin with a specific antibody against other protein. We decided to use both approaches albeit finding their efficiency comparable. We reduced thousands of hits to hundreds in single experiment by a subtraction of negative control. The chosen negative controls (resin and antibody used) served exclusively for a particular experiment for that it was designed. We have never used negative controls between unrelated experiments.

9.2. Potential Binding Partner of GCPII

Despite that GCPII already has a function in organism, many facts highlighted in the introduction point at its putative receptor function and naturally also at its involvement in a signal transduction. There were some high-throughput clinical studies of metastatic prostate cancer focusing on the proteome from the "network" point of view [104] as well as related MS-based expression profiling [88], none the less; the function of GCPII in some tissues is still unknown, albeit its specific expression related to cancer growth.

A putative ligand of GCPII remains to be identified. We should also take into account the possibility that GCPII may have more different ligands coming from the extracellular

space as well as from the cytoplasm (apart from AP-2 and filamin A [29-30]). Therefore, it is important to investigate the possible complex by more experimental setups.

The methods of choice in protein-protein interaction analyses is affinity pull-down and immunoprecipitation. These method are prone to false positives and their results need to be interpreted very carefully. Number of variables, including the immobilization method, plays important role in the experiment output. (Figure 14).

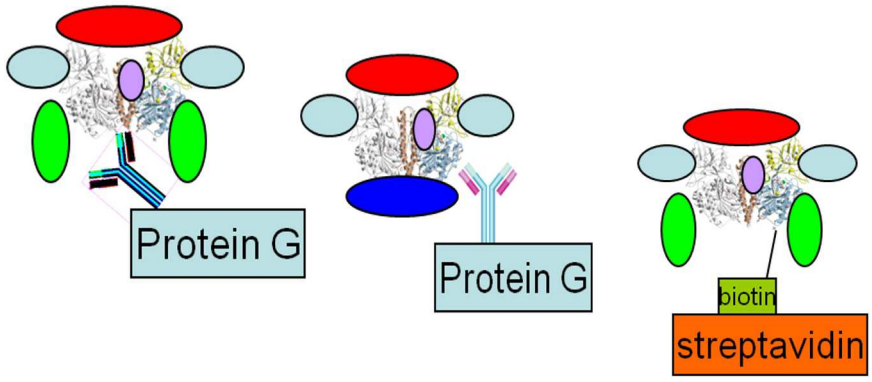

Figure 14. Example of Three distinct Ways of Immobilization of a Bait Protein:

Colored ellipsoids represent different proteins sticking to the bait protein (GCPII in this case) in experiments with cell lysate. Immobilization *via* first antibody (light chains in black) allows GCPII to "trap" prey proteins specifically interacting with protease domain laterally (proteins in green). Another antibody (light chains in pink) immobilizes GCPII by its protease domain and so permits interaction of proteins sticking to protease domain towards N-terminus (proteins in blue). In last case, N-terminally biotinylated GCPII provides interaction similar to the first antibody with particular experimental set-up (different carrier and elution conditions).

We tried to analyze some candidates for binding partners of GCPII. Previous experiments performed in our laboratory (data not shown) suggested that dermcidin [105-111], could be interacting specifically with GCPII. We disproved the interaction in LNCaPs transfected by a plasmid encoding full-length dermcidin. Also immunoprecipitation showed it to be almost ubiquitous in the experiments with LNCaPs.

Also other proteins have been indicated to be potentially interacting with GCPII.In Figure 11 on page 46; it is obvious that Avi-tagged GCPII precipitates some proteins from the solution. To check whether the elution from incubtion with bait is really richer in different proteins, we decided to **invert** controls *in silico* in order to elucidate if the "positive" control is really more positive than the negative. We subtracted the data of "positive" elution (containing bait protein) from the data of negative control. After this,

we observed in average three-fold lower abundance of different hits in data processed in this way compared to standard set-up (negative control subtracted from the positive).

One of the proteins possibly interacting with GCPII, β-subunit of F1 mitochondrial ATPase, was chosen as a model for procedure analyzing possible interaction inmore detail.

9.2.1. Potential Interaction of GCPII and β-subunit of F1 Mitochondrial ATPase in Studied Cell Lines

It was shown that in certain conditions, HUVECs express GCPII on their membrane [18] and that GCPII might be related generally to angiogenesis. Under normal conditions, little to no expression of GCPII was shown in these cells [18]. Other studies showed ATP synthase to be present on surface of several tumor-derived cell lines (reviewed in [112]) and serving as a receptor on cytoplasmic membrane of HUVECs involved in cell proliferation [112]. For these reasons we selected HUVECs for testing interaction between β-subunit of F1 mitochondrial ATPase and GCPII.

According to our expectations, we observed no expression of GCPII in HUVECs grown in standard condition (provided by Jan Hraběta M.D., Department of Paediatric Haematology and Oncology,2nd Faculty of Medicine) employing any of the techniques presented in Results. However, we confirmed presence of β-subunit of F1 mitochondrial ATPase (hereinafter βATPase) in HUVECs by a specific antibody. The reason for using of HUVECs to find a binding partner of GCPII is that we can expect no endogenous GCPII to compete with tagged bait (GCPII) during the pull-down.

Based upon facts mentioned above, we decided to perform and reproduce pull-down experiment in HUVECs. An immobilized recombinant GCPII was used to precipitate βATPase from the HUVEC lysate. Despite finding βATPase pulled-down by GCPII on Western Blots (Figure 12, page 46), we did not detect βATPase in the elution of the very same experiment by MS.

In LNCaPs, we showed GCPII to be immunoprecipitated by anti-βATPase antibody in the elution lane and also wash (Figure 9, page 44).This suggests a weak or non-specific interaction of βATPase and GCPII. On the other hand, assuming that βATPase is a subunit of a protein complex, it is expected to bind non-specifically to other proteins.

Figure 10 (page 44) shows a borderline signal of βATPase in the elution of IP from LNCaPs. In the case of Western Blots developed with a biotinylated antibody, however, we cannot rule out cross-reactivity with another biotinylated protein of a similar mass that can be endogenously expressed in LNCaPs (Figure 10, page 45) or HUVECs (Figure 12, page 46).

To conclude, the data from our experiments with βATPase does not conclusively proof the hypothesis that GCPII is a protein partner of GCPII.

Conclusions

- We optimized experimental procedure providing suitable samples of affinity purification for mass spectrometry

- We created a Microsoft Access database of data extracted by primary processing of mass spectra

- We processed the data acquired in MS analysis and identified number of potential binding partners of GCPII

- We analyzed one of these potential partners, β-subunit of F1-ATPase. Our data do not conclusively support the hypothesis that this protein is a protein partner of GCPII in studied cell lines.

List of Abbreviations

ACN	Acetonitrile
AP-2	Adaptor Protein-2
β-ATPase	β Subunit of F1 Mitochondrial ATPase
Avi –tagged GCPII	Biotinylated extracellular part of GCPII
derm	Dermcidin (Protein)
DDM	Dodecylmaltoside
EDTA	Ethylenediaminetetraacetic Acid
ESI	Electrospray Ionization
GCPII-04	Mouse Anti-GCPII antibody (recognizes denatured protein)
GCPII-08	Mouse Anti-GCPII antibody (recognizes native protein)
GCPII	Glutamate carboxypeptidase II
FTMS	Fourier Transform Mass Spectrometry
HUVEC	Human Umbilical Vein Endothelial Cells
IAA	Iodoacetamide
IgG	Immunglobuloin G
LNCaP	Lymph Node Carcinoma of the Prostate Cells
MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
NAAG	N-Acetyl-L-Aspartyl-L-Glutamate
NAALADase	N-Acetylated Alpha-Linked Acidic Dipeptidase

NMR	Nuclear Magnetic Resonance
ON	Overnight
PEI	Polyethyleneimine
PSMA	Prostate-Specific Membrane Antigen
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBS	Tris-Buffered Saline
TEMED	Tetramethylethylenediamine
TFA	trifluoric acid
TOF	Time-of-Flight
Tris	Trishydroxymethylaminomethane

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Svoluji k zapůjčenítétopráce pro studijníúčely a prosím, abybylařádněvedena evidence vypůjčovatelů.

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