Mass spectrometry based proteomic strategies applied in the study of central nervous system derived cells

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ABSTRACT

This thesis focuses on evaluation, improvement and development of mass spectrometry based proteomic strategies for identification and quantitation of proteins. Development of such strategies is important to achieve a better understanding of regulatory mechanisms of individual cell types, both during normal development and during the onset and progression of diseases. Cells derived from the central nervous system served as model systems. Proteins secreted by these cells can be predicted to be involved in a variety of biological processes, including protective/survival effects, promotion of brain plasticity or intercellular communication. Secreted proteins might also be potential biomarkers of clinical importance, a tool for early detection and diagnosis of diseases. A challenge in the analysis of conditioned media is that the secreted proteins are much less abundant than the media proteins. A strategy involving preparative two-dimensional gel electrophoresis based on liquid phase separation identified several secreted proteins in well-defined conditioned medium from neural stem/progenitor cells. Their identification demonstrates the potential of this approach in identifying lower abundance proteins in the medium. In a serum-containing astrocyte conditioned medium, secreted proteins were identified using metabolic labeling. The proteins released were distinguished from the components of the medium in the mass spectrometric analysis by the labeled amino acid that was incorporated into the cellular proteins during culturing. Both strategies employed on the conditioned media can be used as initial screening tools to identify released proteins. Furthermore, they can be extended to most cells lines for studying secreted proteins. Mass spectrometric methods based on metabolic labeling have also shown great promise for identification and quantitation of proteins in complex mixtures. A prefractionation step involving micro-scale iso-electric focusing in liquid phase of whole cell extracts was found to be useful in the analysis. The number of identified proteins was drastically increased and the quantitation of lower abundance proteins was improved as compared with direct analysis of the same sample.

Key words: proteomics, mass spectrometry, protein identification, protein quantitation, fractionation, SILAC

POPULÄRVETENSKAPLIG SAMMANFATTNING

Proteiner är de aktiva beståndsdelarna i allt levande och har en mängd viktiga funktioner. Proteinsammansättningen ändras från tid till annan och i vissa situationer, exempelvis vid sjukdom. Inom proteomik studeras proteiner och kunskap kan erhållas om vilka proteiner som uttrycks vid olika tillfällen samt om ledtrådar till de molekylära mekanismer som ligger bakom normal utveckling eller som orsakar sjukdom. Proteomik kan även beskrivas som en kombination av olika tekniker där separationsmetoder, masspektrometri och databassökning är viktiga delar. I regel separeras först proteinerna följt av enzymatisk spjälkning, där proteinerna klyvs till mindre bitar - så kallade peptider. Peptiderna massbestäms därefter med masspektrometri. Peptidmönstret för ett specifikt protein är som ett fingeravtryck och genom att jämföra peptidmönstret med teoretiska mönster av proteiner i databaser kan proteinet identifieras. Peptiderna kan också sönderdelas i masspektrometern till ännu mindre fragment och sedan massbestämmas. Vilka fragment som bildas beror på aminosyrasekvensen hos peptiden. Fragmentmönstret ihop med peptidmassan kan användas i en mer specifik databassökning för proteinidentifiering. Syftet med avhandlingen är att utveckla och förbättra masspektrometriska metoder inom proteomiken med vilka proteiner identifieras och kvantifieras. Som modellsystem användes celler isolerade från centrala nervsystemet. Proteiner utsöndrade av dessa celler kan antas vara involverade i en rad olika processer, så som kommunikation mellan celler, cellers överlevnad samt plasticiteten hos hjärnan. Identifiering av utsöndrade proteiner i odlingsmedium är en analytisk utmaning, då dessa proteiner finns i mycket lägre koncentrationer än mediumproteinerna. Preparativ tvådimensionell gelelektrofores visade sig vara användbar vid identifiering av utsöndrade proteiner i ett väldefinierat medium från neuronala stam-/progenitorceller. Med metabolisk märkning kunde utsöndrade proteiner identifieras och särskiljas från mediumproteinerna i serum innehållande media från astrocyter. Den märkta aminosyran inkorporerades i cellernas proteiner under odling. Masskillnaden mellan utsöndrade proteiner innehållande aminosyran och mediumproteinerna detekterades av masspektrometern. Resultaten visar att utsöndrade proteiner kan identifieras med båda dessa strategier och kan användas som initiala screeningverktyg och även appliceras på andra cellsystem där utsöndrade proteiner till mediet skall studeras. Metabolisk märkning kan också användas vid identifiering och kvantifiering av proteiner från två olika cellkulturer. Ett fraktioneringssteg ökade antalet identifierbara och kvantifierbara proteiner jämfört med direktanalys av samma cellysat. Proteinerna separerades med avseende på laddning i vätskefas till olika fraktioner, vilket resulterade i en anrikning av proteinerna och en minskad provkomplexitet.

PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III):

Paper I. **Proteome analysis of conditioned medium from cultured adult hippocampal progenitors**
A. Dahl, P. Eriksson, A. Persson, G. Karlsson, P. Davidsson, R. Ekman, A. Westman-Brinkmalm
Rapid Commun Mass Spectrom, (2003) 17:2195-2202

Paper II. **Proteome analysis of serum-containing conditioned medium from primary astrocyte cultures**
A. Thorsell, J. Faijerson, F. Blomstrand, M. Nilsson, K. Blennow, PS. Eriksson and A. Westman-Brinkmalm
Submitted for publication

Paper III. **Evaluation of sample fractionation using micro-scale liquid phase iso-electric focusing on mass spectrometric identification and quantitation of proteins in a SILAC experiment**
A. Thorsell, E. Portelius, K. Blennow and A. Westman-Brinkmalm
Rapid Commun Mass Spectrom, (2007) 21:771-778

Please note the change in surname from Dahl to Thorsell.

The author has contributed to the papers set out above as follows:

Responsible for the major part of experiments and writing of the manuscripts (Papers I-III). Cell culturing and immunocytochemical experiments (Papers I-II) have been performed by A. Persson and J. Faijerson, but I have actively taken part in the planning. Cell culturing (Paper III) has been performed by E. Portelius.

Related publications not included in thesis:

Reactive astrogliosis induces astrocytic differentiation of adult neural stem/progenitor cells in vitro
J. Faijerson, R.B. Tinsley, K. Apricó, **A. Thorsell**, C. Nodin, M. Nilsson, F. Blomstrand, P.S. Eriksson
J Neurosci Res, (2006) 84:1415-24

Demonstration of multiple novel glycoforms of the stem cell survival factor CCg
A. Dahl, P. Eriksson, P. Davidsson, R. Ekman, A. Westman-Brinkmalm
J Neurosc. Res, (2004) 77:9-14.

Clinical mass spectrometry in neuroscience. Proteomics and peptidomics
P. Davidsson, A. Brinkmalm, G. Karlsson, R. Persson, M. Lindbjer, M. Puchades, S. Folkesson, L. Paulson, **A. Dahl**, L. Rymo, J. Silberring, R. Ekman, K. Blennow
Cell Mol Biol, (2003) 49:681-8

TABLE OF CONTENTS

ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
AHPs	adult hippocampal derived stem/progenitor cells
CID	collision induced dissociation
CNS	central nervous system
ESI	electrospray ionisation
FKBP12	immunophilin FK506 binding protein 12
FT-ICR	Fourier transport ion cyclotron resonance
IEF	iso-electric focusing
LC	liquid chromatography
LTQ	linear ion trap
MALDI	matrix-assisted laser desorption/ionisation
m/z	mass-to-charge
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NSPCs	neural stem/progenitor cells
ppm	parts per million
PEBP	phosphatidylethanolamine binding protein
p*I*	iso-electric point
PMF	peptide mass fingerprinting
RP	reverse phase
SILAC	stable isotope labeling by amino acids in cell culture
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOF	time-of-flight
Q-TOF	quadrupole time-of-flight

INTRODUCTION

The term “proteome” was used for the first time in 1995, to describe the protein complement of a genome (Wasinger et al. 1995). While the genome of an organism can be considered static over a short timescale, the proteome is highly dynamic and will vary over time as a reaction to changes in the environment or during development. Proteomics is the study of proteins on a large scale in order to obtain a global, integrated view of disease processes, cellular processes and networks at the protein level. As opposed to traditional approaches that examine one or a few proteins at a time, proteomic strategies attempt to examine large numbers of proteins concurrently. Proteomics might also be described as multidisciplinary research in which separation techniques, mass spectrometry (MS) and bioinformatics play crucial roles. The proteins and their proteolytic peptides are separated in various steps prior to fragmentation and/or mass measurement with the mass spectrometer. The mass spectra generated are then searched against databases for protein identification or interpreted for quantitative analysis of the proteins.

Application of proteomic technology to neuroscience has been of increasing interest, with the field of neuroproteomics established in 2004 (Choudhary et al. 2004). The physiological and pathophysiological changes, which are associated with certain neurological diseases or conditions, are to some extent reflected by affected production and metabolism of proteins. Consequently, neuroscience can benefit tremendously from proteomics, with its ability to track changes in protein expression function that underlie the growth and differentiation of individual cell types, both during normal development and during the onset and progression of disease. Proteomic analysis can provide the possibility of identifying novel biomarkers associated with specific nervous system diseases. Unique protein biomarkers could be used for clinical diagnosis of diseases and monitoring their progression as well as following different biological processes in the central nervous system (CNS). Candidate proteins for detailed functional studies, which might not have been considered as interesting in a traditional approach can also be identified. Improvements in our knowledge of pathogenesis pathways and mechanisms obtained by proteomic studies are important for the understanding of how to control the endogenous cells and in the future may provide novel therapeutical strategies and targets.

AIMS OF THE THESIS

The focus of this thesis was on evaluation, improvement and development of mass spectrometry based methods applied to the study of central nervous system derived cells for protein identification and their quantitation.

Specific aims

- to evaluate whether preparative two-dimensional gel electrophoresis was suitable for identification of secreted proteins in a well-defined conditioned medium from neural stem/progenitor cells;

- to evaluate whether secreted proteins could be identified in serum-containing astrocyte conditioned medium by the use of metabolic labeling in combination with mass spectrometry, and whether quantitative analysis of proteins could be performed in conditioned medium from non-lesioned and lesioned astrocytes in a scratch injury model for astrogliosis; and

- to evaluate the effects of a prefractionation step using micro-scale iso-electric focusing in liquid phase on mass spectrometric identification and quantitation of proteins in a SILAC experiment.

PROTEOMIC STRATEGIES

Proteome analysis has traditionally been accomplished using a combination of two-dimensional (2D) gel electrophoresis to separate proteins and MS for protein identification. The proteins are separated according to their iso-electric point (p*I*) in the first dimension and molecular weight (MW) in the second dimension (Gorg et al. 1988; Gorg et al. 2000). The 2D gel is subsequently stained for visualisation, and image analysis of the protein patterns used to reveal differences between two states of cell populations or tissues. Selected protein spots are proteolytically in-gel digested and the resulting peptides analysed by MS. The introduction of 2D differential in-gel electrophoresis (DIGE), which allows for separation of two sets of protein mixtures from different sources in the same gel, has minimised some of the reproducibility problems associated with 2D gel electrophoresis (Unlu et al. 1997). By labeling the different pools of proteins with fluorescent dyes (Cy2, Cy3 or Cy5), the proteins are separated on the same 2D gel under identical electrophoretic conditions, and the protein profile can be imaged by fluorescent excitation of the different dyes. A major drawback of 2D gel electrophoresis is that quantitation is limited by co-migrating proteins into the same spot on the gel.

Complementary methods to 2D gel electrophoresis, such as multidimensional protein identification technology (MudPIT), also known as "shotgun proteomics" (Washburn et al. 2001; Romijn et al. 2003; Swanson et al. 2005), and stable isotope labeling (Gygi et al. 1999; Oda et al. 1999; Ong et al. 2002) have been developed. Separation strategies using on-line coupling of multi-dimensional liquid chromatography (LC) to MS instruments (*e.g.* MudPIT) have attracted a great deal of interest in recent years. The system is most frequently described as a combination of ion exchange chromatography and reversed phase (RP) LC, where the peptides are separated according to their charge and hydrophobicity. Quantitative MS methods rely on the incorporation of stable isotopes into proteins or peptides, where relative protein abundance can be calculated from the intensities of normal versus isotope labeled forms of the tryptic peptides with identical sequences in the same mass spectrum.

Mass spectrometric analysis provides accurate mass measurements of small quantities of peptides as well as sequence information essential for protein identification using databases. Peptide mass fingerprinting is based on the insight that proteins after proteolysis with a certain protease, will produce a mass fingerprint of digestion products dependent on the amino acid sequences

of the specific protein (Aebersold et al. 2001). Peptide mass fingerprinting data are commonly acquired by matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis of the digested sample. Tandem mass spectrometers (*e.g.* ion trap and quadrupole-TOF (Q-TOF) instruments) have the ability to isolate and fragment peptide ions and to record the resulting fragment ion spectra used for protein identification. Peptide mass fingerprinting is usually the choice of method for non-complex samples, such as 2D gel separated proteins. Identification with peptide fragmentation does not require extensive separation of the proteins prior to proteolysis, since fragmentation ion spectra of only a few peptides can contain a sufficient amount of information for virtually unambiguous protein identification (Aebersold et al. 2001). Mass spectrometers able to accomplish fragmentation are typically used in conjunction with electrospray ionisation (ESI), which allows direct coupling with high performance separation techniques, such as nano-scale LC and capillary electrophoresis. On-line LC approaches have proven to lead to a significant increase in protein identification compared with traditional 2D gel approaches (Washburn et al. 2001; Romijn et al. 2003). A flow chart for the combination of the different proteomic strategies used in this thesis is given in figure 1.

SEPARATION STRATEGIES

Owing to the complexity of the proteome, it is desirable to utilise separation strategies that offer a wide dynamic range to be able to detect as many proteins as possible in the sample. A common issue in proteomic analyses is that highly abundance proteins suppress the detection and identification of relatively low abundance proteins. However, any reduction in the complexity of the protein sample or their enzymatic digestion products tends to improve the proteome coverage. A number of separation techniques are available in proteome analyses and the principles of the ones used in this thesis are given below.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates the proteins according to their electrophoretic mobility. The protein sample is mixed with SDS, an anionic detergent that denaturates the proteins and applies a negative charge to each protein in proportion to its mass. The SDS prevents different proteins with similar MWs from migrating differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. Consequently,

the proteins are separated strictly by their length, and the distance of migration through the gel can be assumed to be directly related to the size of the protein. Following electrophoresis, the gel-separated proteins may be visualised by staining or processed further for *e.g.* Western blotting.

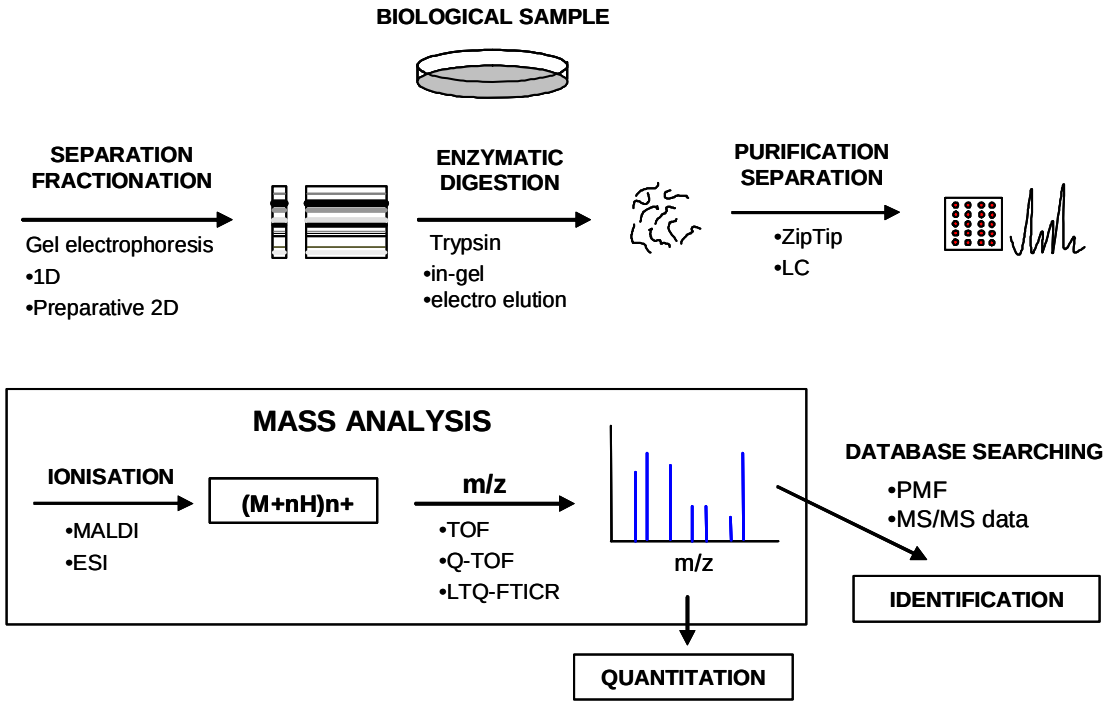

Figure 1. A flow chart for the different strategies used in the present thesis. The proteins in the biological sample are resolved using either 1D or preparative 2D gel electrophoresis. The proteins are enzymatically digested with trypsin and the peptide mixtures produced are desalted or further separated and enriched by on-line RP-LC prior to MS analysis. The peptides can be ionised with either matrix assisted laser desorption/ionisation or electrospray ionisation. Mass measurement and fragmentation analysis take place in the mass analysers. The resulting mass spectra are database searched for protein identification or interpreted for quantitative analysis of the proteins.

Preparative two-dimensional gel electrophoresis

Preparative 2D gel electrophoresis is based on the same principles as analytical 2D gel electrophoresis except that in the first dimension, where the proteins are separated according to their p*I*, occurs in liquid phase (Nilsson et al. 1999; Davidsson et al. 2001). Ampholytes are added to the sample and the pH-gradient generated in the applied electrical field fractionates the proteins in the sample. In the second separation step the iso-electric focused fractions are further resolved on a preparative one-dimensional (1D) gel combined with electro-elution of the protein bands into liquid phase or using 1D gel electrophoresis followed by in-gel digestion of excised gel bands. Advantages

of preparative 2D gel electrophoresis include the retention of highly hydrophobic, highly basic and lower MW proteins as well as low abundance proteins, which tends to be discriminated by traditional 2D gel electrophoresis. Recently, a miniaturised iso-electric focusing (IEF) instrumentation was developed, suitable for analysis of samples with limited availability owing to the reduced sample consumption (Harbers et al. 2005). This type of prefractionation allows for higher protein load than direct analysis, and facilitates the detection of lower abundance proteins, thanks to both protein enrichment and reduction in sample complexity.

Reverse phase liquid chromatography

In RP-LC, the packing is non-polar and the solvent is polar with respect to the sample. The retention of the analyte molecules is the result of interaction between the analyte and the stationary phase, and the separation is based on the hydrophobic character of the analyte. The most commonly used RP-LC columns in peptide separations are packed with silica particles with attached C18-alkyl chains. The complexity of the sample is drastically reduced in the LC-separation, and fewer analyte molecules are eluted within the same time window. In addition to fractionation of complex mixtures, purification of the samples occurs since buffer salts are eluted with the void. Furthermore, the LC-separation also increases the concentration of every analyte in the eluent. This concentration effect is increased with decreasing inner diameter of the column, owing to reduced peak volume as a result of the lower flow rate (Shen et al. 2002). The combination of ionisation at atmospheric pressure and the continuous flow of solvent used in ESI allows direct coupling of nano-scale LC with column i.d. of 75 μm or less to mass spectrometers, and has dramatically improved the sensitivity of analysis of complex biological samples (Shen et al. 2002). By contrast, LC-separations in conjunction with MALDI-MS are primarily performed off-line, by continuous or discrete deposition of LC-effluent onto the MALDI sample support (Mirgorodskaya et al. 2005).

BIOLOGICAL MASS SPECTROMETRY

Mass Spectrometers

Mass spectrometry is an essential tool in proteomic research, providing accurate molecular mass measurements of low quantities of peptides as well as amino acid sequence information. In a mass spectrometer the sample

molecules are separated in space or time after volatilisation and ionisation in terms of their mass-to-charge (*m/z*) ratios, and their molecular masses can be measured. One advantage of molecular mass measurement over other physico-chemical characteristics is that the molecular mass is not dependent on external conditions. Mass spectrometers consist of three basic components: the ion source, the mass analyser and the detector. The ion source converts sample molecules into ions in gas phase and one or more mass analysers separate the various ionised species according to their *m/z* ratio. The detector counts ions emerging from the final analyser and measures their abundance, a plot of which against the *m/z* yields the mass spectrum. Currently, the two most common ionisation methods used in the analysis of peptides and proteins are MALDI and ESI. There are a number of different types of mass analysers employed in proteomic research. The mass spectrometers utilised in this thesis were MALDI-TOF MS, ESI Q-TOF MS and ESI Linear Ion-trap coupled to a Fourier transform ion cyclotron resonance (LTQ FT-ICR) MS.

Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry

The MALDI-TOF MS instrument combines a MALDI ion source and a TOF analyser (Reflex II, Bruker). MALDI is a soft ionisation technique resulting in intact peptides/proteins in gas phase with little fragmentation (Karas et al. 1988; Tanaka et al. 1988). The MALDI ion source usually operates under high vacuum conditions. The technique involves embedding of the analyte in a solid organic matrix, which strongly absorbs energy at the wavelength of the laser. The mixture of matrix and analyte is irradiated by a laser pulse and the absorbed energy causes translational motion and ionisation of both the matrix and the analyte molecules. Consequently, a volume of the matrix and the trapped analyte molecules is ejected into gas phase. MALDI results predominately in formation of singly charged peptide/protein ions, although ions of two or three charges can also be formed. The signal intensities vary greatly, and frequently several peptides present in the sample are not observed in the mass spectrum. The reasons behind the selectivity of the MALDI process have not yet been fully explained, but are probably a combination of varying ionisation efficiencies and matrix/analyte co-crystallisation efficiencies (Cohen et al. 1996).

The TOF mass analyser is well suited for pulsed ion sources and is the method of choice for most MALDI instruments. The mass analysis in a TOF instrument is based on the principle that after acceleration in the ion source, analyte ions of different *m/z* have the same energy but different velocities. For ions with the same charge, the ones with lower mass acquire higher velocity and reach the detector faster. During the MALDI desorption/ionisation event ions of the same *m/z* often acquire different initial kinetic energies, and hence

hit the detector at slightly different times, causing peak broadening and lowering the resolution. Use of an ion mirror and a time-lag-focusing ion source can improve the resolving power of the TOF mass analyser. The mirror, an electrostatic ion reflector, not only lengthens the flight path but also compensates for the difference in initial kinetic energies so that ions with the same *m/z* hit the detector at almost the same time (Mamyrin et al. 1973). By making the somewhat slower (less energetic) ions take a shorter path in the reflector than the faster (more energetic) ones, ions can be focused in time and the peaks in the mass spectra become narrower (figure 2).

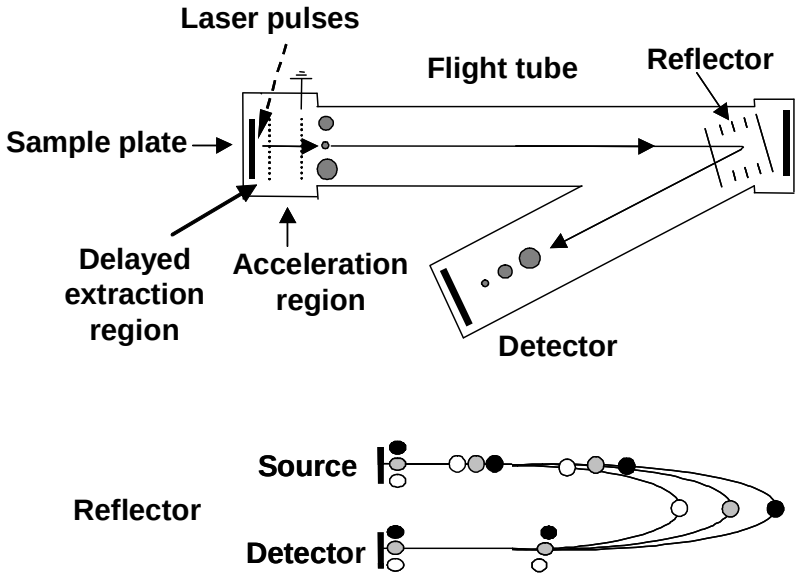

Figure 2. Schematic drawing of a MALDI-TOF mass spectrometer equipped with a reflector. The reflector compensates for the different initial velocities and improves the resolution of the TOF analyser, since the ions with lower kinetic energy do not penetrate as deeply into the reflector as the more energetic ions, which therefore travel a greater distance. Thus, the ions with different energies will reach the detector at almost the same time. A MALDI-TOF MS instrument (Reflex II, Bruker) was used in Paper I.

Time-lag focusing, or delayed extraction, was introduced by Wiley and McLaren in 1955 (Wiley et al. 1955) and has become a standard feature of MALDI–TOF mass spectrometers during the last decade (Brown et al. 1995). After the desorption/ionisation event, ions are first allowed to travel from the source into a field free region. After a short (variable) time the electric field is switched on and the ions are accelerated towards ground potential. Ions with lower initial velocity will have travelled a shorter distance from the sample plate and will suddenly be at higher potential than the initially faster ions. Consequently, the originally slower ions will instead be the faster ones when they exit the acceleration region, and with properly set delay time and source

voltages they will "catch up" with the initially more energetic ions at the detector. More recently, MALDI ion sources have also been coupled to Q-TOF and to two TOF (TOF-TOF instruments) analysers to allow for fragmentation of MALDI-generated precursor ions (Loboda et al. 2000; Medzihradszky et al. 2000).

Electrospray ionisation quadruple time-of-flight mass spectrometry

The Q-TOF is a hybrid instrument that combines a quadrupole mass filter orthogonally with a TOF analyser (Qtof2, Waters Micromass). The instrument is equipped with a nano ESI source (Morris et al. 1996; Wilm et al. 1996), and has revolutionised fragmentation analysis of peptides, thanks to the high sensitivity. The gas-phase analyte ions are created by ESI at atmospheric pressure (Aleksandrov et al. 1984; Yamashita et al. 1984; Fenn et al. 1989). The sample is dissolved in a polar, volatile solvent and transported through a thin capillary placed at high potential. An electric field is applied between the needle and the nozzle (the entrance to the vacuum system), which results in the formation of a very fine spray of solvent droplets containing preformed ions of the type $(M+nH)^{n+}$ or $(M-nH)^{n-}$, where M is the analyte molecule and n is the charge state. The droplets shrink, through evaporation, assisted by a flow of gas, and ultimately yield gas-phase analyte ions, which can be passed through the vacuum chamber of the mass analyser. This ionisation method can result in both singly and multiply charged ions. The precise mechanism of the ion formation is a matter of debate (Mora et al. 2000). The combination of ionisation at atmospheric pressure and the continuous flow of solvent used in ESI allows for direct coupling with separation techniques, such as nano-scale LC and capillary electrophoresis.

The Q-TOF combines the ability to obtain efficient precursor ion selection by the use of the quadrupole mass filter and dissociation in a hexapole collision cell with the high sensitivity of the TOF analyser (Morris et al. 1996). The quadrupole mass filter in the Q-TOF separates ions according to their *m/z* ratio by utilising the stability of their trajectories in an oscillating electrical field. Ions that do not have a stable trajectory through the quadrupole will collide with the rods and never reach the detector. Advantages of the Q-TOF hybrid as compared with earlier MS instruments include better sensitivity, improved resolving power and mass measurement accuracy. The relatively high resolving power and mass accuracy are attributed mainly to the narrow beam packet, pushed down into the TOF analyser which is equipped with a reflectron orthogonally to the transfer ion optics. Another advantage of the Q-TOF is the easy switching between MS and tandem MS (MS/MS) modes and that fragmentation of a specific ion can be carefully controlled. In MS mode, the ions drift through the quadropole filter, which acts as a focusing

device transmitting all ions to the TOF analyser, where they are separated according to their *m/z* ratio. In the MS/MS mode the quadropole filter is set to allow only ions within a very narrow *m/z* range to pass through to the collision cell for subsequent fragmentation. The precursor ion dissociates into product ions, whose ion trajectories are stabilised in the hexapole, and the fragment ions are then measured in the TOF analyser.

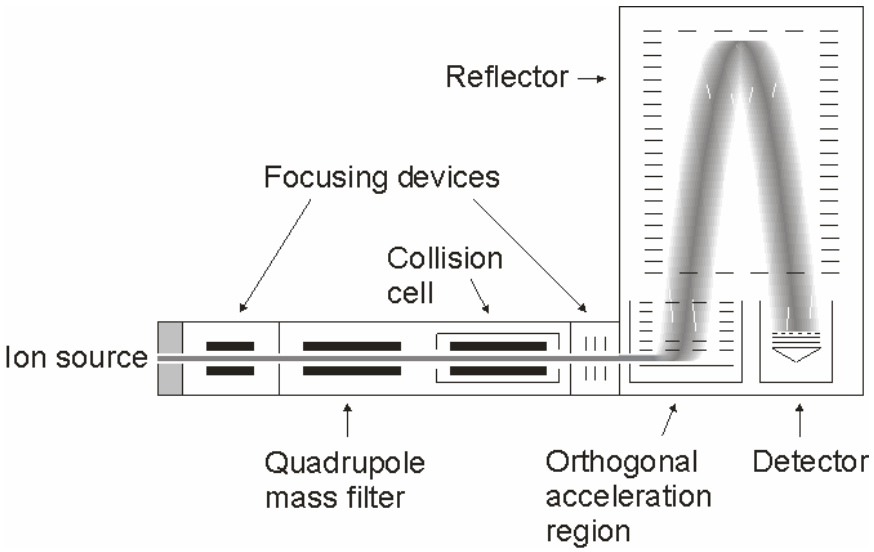

Figure 3. Schematic drawing of the Q-TOF MS (Q-TOF2, Waters Micromass). This instrument was used in Paper I, where the samples were sprayed from capillaries using the nano-flow ESI source.

Electrospray ionisation linear ion trap Fourier transform ion cyclotron resonance mass spectrometry

The LTQ-FT-ICR MS (LTQ-FT, Thermo Electron) is also a hybrid instrument, which consists of a linear ion trap capable of very fast, sensitive peptide sequencing combined with an ion cyclotron resonance trap (ICR). The instrument is well-suited for coupling to an ESI nano-scale LC system. Ion trap analysers use the same principle as the quadrupole in that specific combinations of DC current and radio frequencies are used to select particular *m/z* ratios. In contrast to the quadrupole mass filter, the geometry of the quadrupole ion trap is cylindrical. By changing current and radio frequency combinations, ions within particular *m/z* ratios are ejected from the ion trap to the detector. In the ICR-trap, ions circle in a 7 Tesla magnetic field and their image current is detected. The frequency spectrum obtained is then converted to a mass spectrum by Fourier transformation. The principle of ICR technique for mass spectrometers was introduced in the early 1950s (Hipple et al. 1949; Sommer et al. 1951) and the combination with the Fourier transform

technique was demonstrated by Comisarow and Marshall in 1974 (Comisarow et al. 1974). The FT-ICR possesses extraordinary resolving power and mass accuracy (Marshall et al. 1998). The mass accuracy is typically in the low ppm or sub-ppm level. The high performance is attributable to the fact that ions are detected in terms of their cyclotron resonance frequencies, which can be measured extremely accurately.

The LTQ is used for example, as a collecting device for the ICR cell, where the actual, high mass accuracy measurement takes place. The numbers of ions that ultimately go into the ICR cell is very important. Varying the numbers of ions can dramatically influence the resolution and sensitivity of the overall measurement. The number of ions transferred is regulated by the LTQ with the Automatic Gain Control. The ICR cell is capable of allowing for very high mass accuracy measurements of protein and peptide ions, as well as fragment ions produced either inside the LTQ collision cell prior to transfer to the ICR cell or inside the ICR cell itself. High resolution and high mass accuracy measurements of the precursor ions can also be performed in the ICR cell, at the same time as the LTQ part of the mass spectrometer isolates, fragments and obtains the MS/MS spectra of the selected peptides. The high mass accuracy of the precursor ion, usually 1-2 ppm, results in a more specific database search for protein identification. The LTQ can also be used on its own, resulting in better sensitivity. The loss in sensitivity when using the combination of the LTQ and the ICR is due to loss of ions in the transfer to the ICR cell. Consequently, the increased sensitivity is at the expense of losses in resolution and mass accuracy.

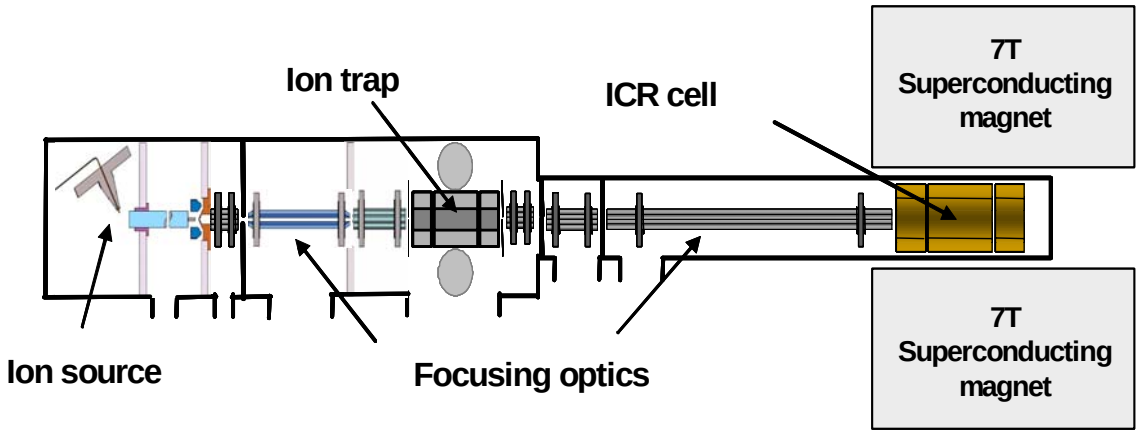

Figure 4. Schematic drawing of the hybrid LTQ-FT instrument (Thermo Electron) equipped with a 7T magnet. This instrument coupled to a nano-scale LC system was used in papers II and III.

Protein identification

Trypsin is preferentially used to digest proteins prior to mass measurement of the peptides generated by MS. Trypsin cleaves proteins at the basic amino acids arginine (R) and lysine (K). Digestion with trypsin results in a relatively large number of reasonable sized peptides, offering the possibility of unambiguous protein identification. Furthermore, trypsin yields basic residues at the C-terminal, resulting in more predictable fragmentation in the MS/MS analysis, which is an important factor in the interpretation of the spectrum of an unknown digestion product (Tang et al. 1993; van Dongen et al. 1999). Other enzymes that produce alternative peptide patterns are Lys C (cleaves K), Arg-C (cleaves R) and chymotrypsin (cleaves FYWLIVM).

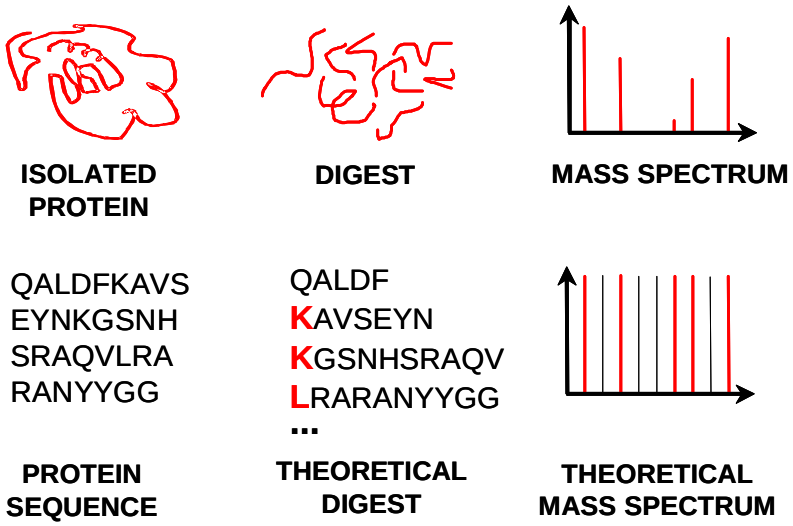

Figure 5. Schematic picture of a protein identification by PMF. The isolated protein sample is digested with trypsin and the peak list of the detected digestion products from the MALDI spectrum is searched against databases, where the peak list is compared with peak lists of digestion products of theoretical digested proteins.

Peptide mass fingerprinting

In many cases sequence-specific digestion of the protein, in addition to accurate measurement of the *m/z* ratio of the produced peptides, gives enough information to identify the protein in the sample (Mann et al. 1993). The experimentally obtained *m/z* values can be matched against theoretical peptide masses calculated from an in silico protein database resulting in a ranked listing of potential proteins in the sample (figure 5, (Aebersold et al. 2001). This method of identifying proteins is called peptide mass fingerprint (PMF). Data for PMF is usually obtained via MALDI-TOF analysis of a digest, resulting in a mass fingerprint for the specific protein (figure 6). In particular, PMF for identification of proteins from non-complex samples has become one

of the most important biological applications, and forms the core of protein identification in proteomics research. The limit of PMF is normally reached when the sample contains a complex protein mixture, or if the protein yields too few digestion products. Therefore, PMF is well suited for proteins separated by 2D gel electrophoresis or other multi-dimensional separation strategies, usually yielding non-complex tryptic digests (Aebersold et al. 2001; Aebersold et al. 2003). However, the addition of sequencing capability of the MALDI instruments allows for the analysis of more complex samples, and should make protein identification by both PMF and MS/MS more specific than those obtained by simple PMF (Aebersold et al. 2003).

Amino acid sequence analysis by tandem mass spectrometry

For protein identification using MS/MS, a single peptide precursor ion is isolated and fragmentations, mainly along the peptide bonds, are induced, resulting in a spectrum containing a pattern more or less unique for the individual peptide (Aebersold et al. 2001). Both the mass spectrum and the molecular mass of the fragmented peptide are used to search theoretical fragmentation patterns of peptides with the same predicted molecular mass from an in silico database. The specificity of the database search is high and the amino acid sequence of even a relatively small peptide can identify a protein to a high degree (Mann et al. 1994). Consequently, and in contrast to PMF, protein identifications can be obtained from complex mixtures of proteins. Mass spectrometers able to accomplish MS/MS analysis, including. ion trap, TOF-TOF and Q-TOF instruments, are commonly used in conjunction with ESI, but have also lately been coupled to MALDI ion sources (Aebersold et al. 2001; Aebersold et al. 2003).

The most common method of fragmentation is CID, which has been used in this thesis. Other examples of common fragmentation methods are electron capture dissociation (ECD, (Zubarev et al. 2000)) and infra-red multi-photon dissociation (IRMPD, (Little et al. 1994)). The process of CID includes multiple low-energy collisions of the peptide precursor ion with an inert gas, which converts translational energy into vibrationally excited states with increasing stretching of bond amplitudes, which finally leads to dissociation of the precursor ion. The fragments produced are the result of N-terminal and C-terminal fragmentations across the peptide bond to give sequence ions with charge retention on either the acylium (‘b’) or ammonium (‘y’) ion fragments (figure 6, (Roepstorff et al. 1984)). Subfragments associated with loss of water or ammonia from these ions are commonly observed as well. The b-ions can lose carbon monoxide and become a-ions. Immonium ions (H_2N=CHR, where R is the amino acid side chain are also seen in the spectra. The easiest spectra to interpret are those obtained from doubly charged tryptic

peptide precursor ions, since the resulting fragment ions are mostly singly charged, with only a few doubly charged fragments.

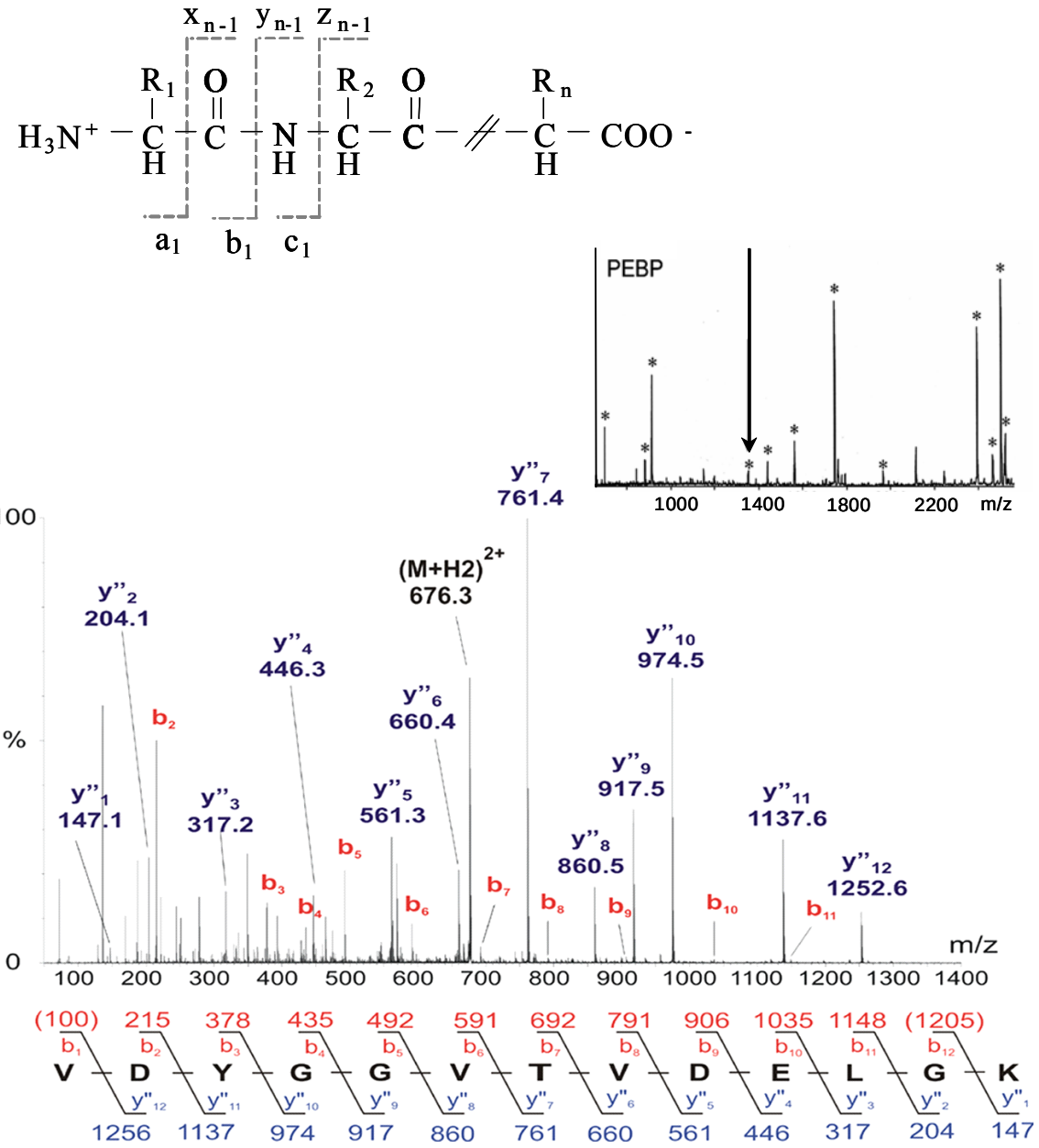

Figure 6. Nomenclature of peptide fragmentation generated by CID. For protonated peptides the cleavage of the amide bound in the peptide backbone will generate different series of fragmentation ions (Roepstorff et al. 1984). In the formation of a-, b-, c-ions the charge is retained at the N-terminus, and the major N-terminus containing ion series is the b-ion series. For x-, y- and z-ions the charge is retained on the C-terminus, and the major C-terminus containing ion series is the y-ion series. MALDI spectrum for a protein identified with PMF. The peptide indicated with an arrow in the MALDI spectrum was selected for fragmentation analysis. Mass spectrum from MS/MS analysis of the selected doubly charged peptide at *m/z* 676.3. The y-ions series (C-terminal fragments), and the b-ion series (N-terminal fragments), are shown. The amino acid sequence matching to this MS/MS data was VDYGGVTVDELGK.

Quantitation by mass spectrometry

Quantitative proteomics has traditionally been performed by 2D gel electrophoresis followed by protein identification of selected spots using MS. It is challenging to perform quantitative analysis using MS, for reasons of variations in detector response, the condition of the ion source as well as ionisation efficiency for peptides of different sequences or even identical peptides from different MS analysis. However, in recent years mass spectrometric methods based on stable isotope labeling have shown great promise for the identification and quantitation of proteins in complex mixtures. Stable isotopes can be incorporated via chemical reactions with isotope-containing reagents at different functional groups in peptides or proteins (*e.g.* Isotope Coded Affinity Tags (ICAT) (Gygi et al. 1999), iTRAQ (Ross et al. 2004)) or enzymatic derivatization (*e.g.* proteolytic ^{18}O labeling (Mirgorodskaya et al. 2000; Yao et al. 2001)). Metabolic incorporation of stable isotope ($^{15}N/^{13}C$) labeled nutrients (Oda et al. 1999) or stable isotopically labeled amino acids (Stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al. 2002; Ong et al. 2003)) is simple and efficient, but is restricted to living cells. The samples containing proteins with either a "light" or "heavy" form of the label are combined and quantitation of the proteins is based on the relative intensities of their tryptic peptides in the pairs (figure 7). One advantage of metabolic labeling over chemical and enzymatic labeling is that the label is introduced into the sample at an early stage of the experiment. The sample to be analysed can be combined even before protein extraction and thereby minimising errors due to sample handling. On the other hand, an attractive feature of chemical labeling is that the selectivity of the labeling reaction can be used to direct the isotopes and attached affinity tags to specific functional groups or protein classes, thus enabling their selective isolation and analysis. However, several recent studies have also indicated that quantitative proteomic studies can be performed using MS strategies even without incorporating isotopic labels (Skold et al. 2002; Wang et al. 2003; Johansson et al. 2006).

Stable isotope labeling with amino acids in cell culture

Metabolic incorporation of stable isotopes is a straightforward and accurate procedure for quantitation of proteins from cell lines. Specific amino acids containing substituted stable isotopic nuclei (*e.g.* ^{2}H, ^{13}C, ^{15}N) can be incorporated into proteins of mammalian cells using SILAC (Ong et al. 2002; Ong et al. 2003). A variety of amino acids are suitable in SILAC, although the use of an essential amino acid that does not metabolise to a different amino acid is desirable to avoid a mixture of labeled amino acids. Arginine and lysine are the preferred choices, because trypsin cleaves after these two

residues. Consequently, every peptide ending with arginine or lysine can be quantified in the comparison to the light state. For complete coverage of proteins, both arginine and lysine can be used as labels. However, for routine quantitative analysis, only one of these isotopes needs to be used (Amanchy et al. 2005).

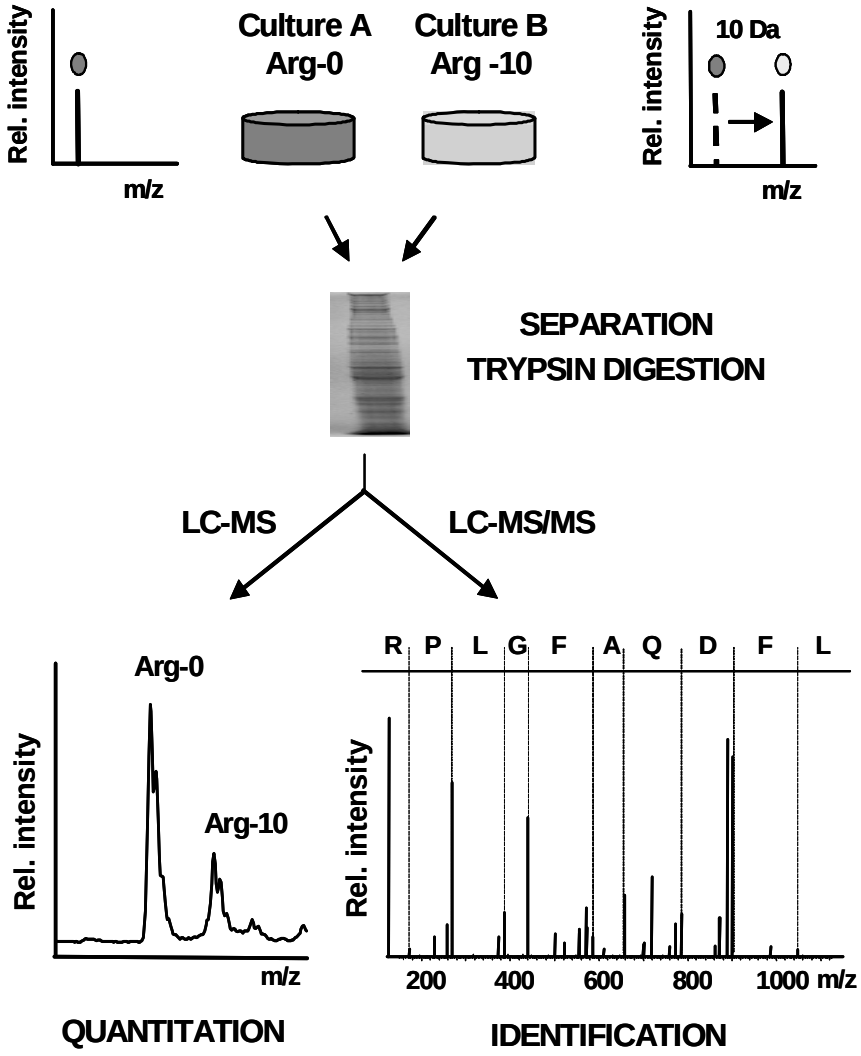

Figure 7. Schematic procedure of a SILAC experiment using isotopically labeled arginine with a mass difference of 10 Da as the label. The two cell cultures are mixed, the proteins resolved using 1D gel electrophoresis and excised bands in-gel digested with trypsin. The unlabeled and labeled peptides are co-eluting in the LC-separation allowing for quantitation and identification of the proteins corresponding to the peptides in the SILAC pair.

In SILAC experiments, cells are cultured in culture media containing the naturally abundance amino acid or its isotopically labeled analogue, giving rise to cells containing either "light" or "heavy" proteins (figure 7). The two cell populations are combined, allowing for the use of any method of protein

purification without introducing errors into the final quantitative analysis. No differences in elution time in the LC-separation are observable for unlabeled tryptic peptides or peptides containing amino acids substituted with ^{13}C and/or ^{15}N (Ong et al. 2003; Molina et al. 2005). The protein identities are obtained in MS/MS mode, while protein quantitation is performed in MS mode in the same LC-separation.

IMMUNOBASED STRATEGIES

Western blot

The method of western blotting can detect, in a mixture of protein or fragments of proteins, those that react with a specific antibody (figure 9). The proteins are separated using SDS-PAGE and transferred to a polyvinyldifluotide (PVDF) membrane. The immobilised proteins are irreversibly bound to the membrane and have the ability to bind antibodies for immunological detection. To avoid unspecific binding of the antibodies, the membrane can be preincubated in a buffer containing milk powder. Visualisation of the protein-antibody complex involves binding of a second antibody linked to a marker enzyme that drives a colorimetric or photometric signal to the complex.

Immunocytochemistry

Immunocytochemistry is a good and a widely used method for demonstrating the presence and cellular distribution of different antigens (figure 9). The cell culture is immersed in a solution of the antibody against the protein of interest. After the excess is washed off, the cell culture is incubated with a secondary antibody. The secondary antibody is conjugated with a fluorescent dye, *e.g.* fluorescein, and the localisation of the protein-antibody complex can be visualised using fluorescent microscopy. The cells are usually also co–immunostained with a marker expressed in a specific cell type. Subsequently, localisation and distribution within cells and differences in the protein expression by various type of cells can be monitored with immunocytochemistry.

CENTRAL NERVOUS SYSTEM DERIVED CELLS

Cell cultures derived from the CNS are important experimental tools in the search for regulatory mechanisms, which are of potential significance for the understanding of the functional biology of the cells *in vivo*. The ability to control the endogenous cells is a key factor in developing novel therapeutical strategies and in being able to alleviate neurological disorders, such as Alzheimer and Parkinson diseases, and impairments that accompany stroke and trauma in the future.

NEURAL STEM/PROGENITOR CELLS AND NEUROGENESIS

Neurogenesis, the generation of new neurons, occurs in the adult mammalian CNS following the migration and differentiation of neural stem/progenitor cells (NSPCs). Neurogenesis in the adult brain has mainly been described in two regions, referred to as neurogenic regions: the dentate gyrus of the hippocampus (Altman et al. 1965; Eriksson et al. 1998; Gould et al. 1999) and the olfactory bulb (Lois et al. 1993; Alvarez-Buylla et al. 2002). The new neurons in the olfactory bulb are generated from NSPCs of the subventricular zone, which have migrated through the rostral migratory steam to the olfactory bulb (Alvarez-Buylla et al. 2002; Curtis et al. 2007). Multipotent precursors are concentrated to these two regions, but they can also be found in lower densities in several other regions of the adult brain (Palmer et al. 1995; Palmer et al. 1999). Furthermore, the induction of neurogenesis has also been demonstrated in non-neurogenic regions in response to selective neuronal death or degeneration (Magavi et al. 2000; Arvidsson et al. 2002). The prevailing understanding today is that the adult CNS has very poor regenerative capacity and it is unknown whether or not neurogenesis is an integral part of the normal brain functions (Palmer et al. 1995; Taupin et al. 2002). The mechanisms controlling neurogenesis involve a variety of factors, including aging, environmental enrichment, exercise, stress, growth and neurotrophic factors, CNS injury and neurodegeneration (Gage et al. 1998; Emsley et al. 2005). In addition to their neurogenic capacity, NSPCs also have the potential to divide and differentiate into glial cells (Palmer et al. 1997). The potential and the fate of multipotent precursor are influenced by the cellular and molecular local microenvironment in the CNS (Takahashi et al. 1998; Young et al. 2000). The presence of multipotent stem/progenitor

cells in the adult brain raises many fundamental questions, such as what role these cells play in CNS maintenance, whether there is a capacity for limited self-repair after brain injury or degeneration, if non-neurogenic regions can be molecularly modified to promote neurogenesis and whether it is possible to manipulate endogenous precursor cells for brain repair.

Neural stem cells can be described as cells that (i) can generate neural tissue or are derived from the nervous system, (ii) have some capacity for self-renewal, and (iii) are multipotent with a capacity to generate differentiated progeny of the neuronal, astroglial and oligodendroglial lineages. Neural stem cells undergo symmetric or asymmetric cell divisions. Both progeny will be stem cells in a symmetric division. In an asymmetric division one new stem cell, which is identical to the mother cell and one cell that is more determined for a certain lineage of differentiation are produced. These cells have reduced stem cell properties and are referred to as progenitor cells.

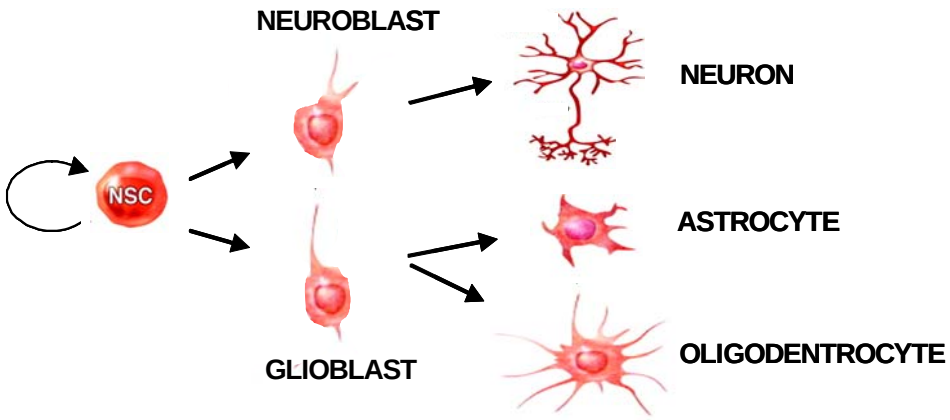

Figure 8. Schematic picture of NSPCs that have both multipotency and self-renewing capability. The stem/progenitor cells can differentiate into neurons and glial cells, besides giving rise to new stem/progenitor cells.

Multipotent NSPCs can be isolated and expanded in the presence of growth factors (Reynolds et al. 1992; Reynolds et al. 1992; Palmer et al. 1997; Zhu et al. 1999). *In vitro* culturing of NSPCs allows for detailed characterisation of the cells, essential to the understanding of the mechanisms controlling the endogenous NSPCs. It is known that proliferation, survival and differentiation of NSPCs depend on exogenous factors added to the medium as well as cell density (Palmer et al. 1997; Whittemore et al. 1999; Zhu et al. 1999; Taupin et al. 2000). Furthermore, several studies have also demonstrated that co-

culturing with other cells as well as culturing in conditioned medium from various cells influence the dynamics of NSPCs (Taupin et al. 2000; Kaneko et al. 2003; Faijerson et al. 2006). A number of secreted factors are believed to be involved in the differentiation of NSPCs into neurons and glial cells (Whittemore et al. 1999; Zhu et al. 1999; Brunet et al. 2004; Faijerson et al. 2006; Hasegawa et al. 2006).

REACTIVE ASTROCYTES

Astrocytes belong to the glial cell group and are the most abundant cell type in the brain. In contrast to neurons and oligodendrocytes, which are highly differentiated and have limited proliferative capacity, astrocytes are highly plastic and posses the potential to proliferate and migrate (Fawcett et al. 1999). They are important for the maintenance of CNS homeostasis and recycling of neurotransmitters. Astrocytes also play a role in the plasticity of CNS, *e.g.* by controlling the number and function of synapses (Haydon et al. 2006). In addition, astrocytes modulate the activity of neighbouring cells and affect cell-to-cell interactions by secreting numerous growth factors and extracellular matrix proteins (Yoshida et al. 1991; Schaar et al. 1993; Schwartz et al. 1994; Aubert et al. 1995; Ridet et al. 1997). Moreover, they can generate neurons not only during development, but also throughout life, as well as promoting neurogenesis (Lim et al. 1999; Song et al. 2002).

Activation of astrocytes occurs prominently in response to all forms of CNS insults such as injury, ischemia, infection, and degenerative disease. As a result of the activation many of the astrocytic properties are abnormally up-regulated. This glial activation, called reactive gliosis, is important for wound healing, but can also pose an obstacle to regeneration (Ridet et al. 1997). The functions of reactive astrocytes are not well understood, and both harmful and beneficial activities have been attributed to these cells (Ridet et al. 1997; Chen et al. 2003; Pekny et al. 2005). Furthermore, after a lesion, NSPCs can be activated and migrate towards lesioned areas and differentiate into neurons and glia cells (Nait-Oumesmar et al. 1999; Arvidsson et al. 2002; Nakatomi et al. 2002). In these areas reactive astrocytes are present, cells known to secrete a wide range of molecules and growth factors, indicating the potential for communication between these cells and the NSPCs (Ridet et al. 1997; Krum et al. 1998; Liberto et al. 2004).

Astrocytic cultures are important experimental systems for investigating the reactive state of astrocytes. Cultured astrocytes can display many of the characteristic changes seen in reactive gliosis and have been employed to

mimic the astrocytic reactions following a stab wound or an ischemic injury *in vivo*. Many cell culture systems have been established for this purpose, including chemical treatment of astrocytes (Egnaczyk et al. 2003; Falsig et al. 2006) and mechanical lesion of astrocytes (Kornyei et al. 2000; Faijerson et al. 2006). Recently, reactive astrocytes in a mechanical scratch injury model for reactive gliosis were demonstrated to induce astrocytic differentiation of NSPCs through secreted factors (Faijerson et al. 2006). Additionally, the same study revealed that the NSPC-derived astrocytes participated in glial scar formation.

RESULTS AND DISCUSSION

Proteomics has significant potential, in the search for proteins involved in regulatory processes, and can provide greater insight into the functional biology of the endogenous cells. The proteomic studies in the present thesis have been applied to cells derived form the CNS. A brief summary of the results obtained in the different studies performed during the PhD-work, as well as the findings in the papers included are given below.

The peptide patterns in conditioned medium from cultured NSPC were studied with MS, revealing changes in the pattern evolving during culturing, owing both to secretion and degradation of the medium components (Dahl et al. 2001; Dahl et al. 2001). A cleavage product from insulin (one of the major medium components) was later identified in the conditioned medium by culturing the cells in medium containing either bovine or human insulin. The mass shift produced between the intact protein and its cleavage product was contingent on the origin of the insulin, and the consequent differences in amino acid sequences. Interestingly, preliminary data indicates that this finding is of biological importance. Moreover, a number of proteins secreted by NSPCs were identified in the well-defined conditioned medium using preparative 2D gel electrophoresis (Paper I). Three of the proteins were selected for complementary immunobased studies that confirmed their secretion. Additionally, novel glycoforms of the autocrine/paracrine cystatin C survival factor was found in the conditioned medium (Dahl et al. 2004). Further structural characterisation of the glycosylations using MS was not possible, owing to the relatively low content of the protein isoforms.

Secreted proteins were also identified in serum-containing astrocyte conditioned medium, by the use of metabolic labeling and MS (Paper II). To our knowledge, this study is the first to successfully identify secreted proteins in serum-containing conditioned medium using a proteomic approach involving MS. A combination of SILAC, 2D gel electrophoresis and MS was employed to examine the protein turnover in gliablastoma cells (Dahl et al. 2005; Dahl et al. 2006; Dahl et al. 2006). The incorporation of isotopically labeled arginine into proteins was monitored over time, revealing different turnover rates for protein isoforms. Furthermore, prefractionation using micro-scale liquid phase IEF was demonstrated to increase the number of proteins identified and improved their quantitation in a SILAC experiment (Paper III).

PAPER I

Proteome analysis of conditioned medium from cultured adult hippocampal progenitors

At the time this study was performed a few proteins had been identified in conditioned medium from cultured NSPCs using proteomic methods involving MS. Previously, NSPCs had been suggested to secrete factors to enhance their own survival *in vitro* (Taupin et al. 2000) several other studies later demonstrated that conditioned medium from various types of cells contain released factors that influence the dynamics of NSPCs (Chang et al. 2003; Chang et al. 2003; Kaneko et al. 2003; Faijerson et al. 2006). However, only a few studies have as yet employed proteomic strategies involving MS to identify factors in conditioned medium form cell cultures (Lim et al. 2002; Andersen et al. 2003; Lafon-Cazal et al. 2003; Delcourt et al. 2005; Gronborg et al. 2006). This approach allows for identification of proteins, which might not have been considered as interesting in a bioactivity context with a traditional hypothesis-driven approach.

Preparative 2D gel electrophoresis was utilised to isolate the medium proteins prior to the MS analysis. Identification of proteins secreted into the well-defined conditioned medium posed a substantial analytical challenge, owing to the relatively low protein content and the fact that the proteins of interest were much less abundant than the medium components. Preparative 2D gel electrophoresis has previously been shown to be suitable for analysis of relative low abundance proteins and to improve their sequence coverage in human cerebrospinal fluid (Westman-Brinkmalm et al. 2002). The culture medium was dialysed prior to IEF, since the high salt content interferes with the focusing of the proteins. Dialysed conditioned medium from cultured NSPCs derived from the adult rat hippocampus (adult hippocampal derived stem/progenitor cells, AHPs) and control medium were separated in liquid phase into twenty fractions, with pH ranging from 3 to 10. Proteins in the resulting IEF fractions were resolved using gel electrophoresis. Distinct protein bands were visible in the conditioned medium but not in the control medium, suggesting a greater abundance of proteins present in the conditioned medium (see figure 1, Paper I). Three sets of sample containing two IEF fractions each were selected for evaluation, whether this preparative approach allowed for identification of proteins originating from the cells in the conditioned medium. The analysis resulted in identification of 12 unique proteins (Table 1).

Table 1. Proteins identified in gel eluter fractions obtained from IEF sets 1-3. IEF sets 1-3 contain the combination of IEF fractions 7 and 8, 11 and 12, and 17 and 18. Transferrin is a component of the culture medium.

IEF set	Gel Eluter Fr. no.	Protein ID	Accession no. (NCBI)	MW (kDa)	pI	MS[a]	MS/MS[b]	Function
1	1-6	Transferrin				x		
	8	Rho-GDI	12597249	23.4	5.1		3	Regulation of Rho/Rac family members
	9	Phosphatidylethanolamine binding protein (PEBP)	8393910	20.8	5.5	15	5	Regulation of MARK pathway, membrane biogenesis, HCNP precursor
	10	Thioredoxin peroxidase 1	8394432	21.8	5.2	9	2	Apoptosis, proliferation
	13	Brain lipid binding protein	13540630	14.9	5.5		2	Fatty acid uptake, transport and targeting Involved in developing CNS
		Prothymosin alpha	11120684	12.4	3.8		1	Proliferation
		Thioredoxin	16758644	11.7	4.8		1	Apoptosis, proliferation
	14	Transferrin				x		
2	1-7	Transferrin				x		
	8	Glutathione S-transferase P	121749	23.4	6.9	7		Apoptosis, proliferation
	9	Phosphatidylethanolamine binding protein	8393910	20.8	5.5	9		See above
	10	Epidermal lipid binding protein	1706754	15.0	6.7	6	2	Fatty acid uptake, transport and targeting, involved in developing CNS
	11	Brain lipid binding protein	13540630	14.9	5.5	7		See above
	13	Polyubiquitin	1050930	11.2	5.4	12	3	Involved in protein degradation, cellular growth and development
3	7	Cystatin C	226712	14.0	9.7	9		NSPC survival factor, involved in neuronal degeneration and CNS repair
	8	Cystatin C	226712	14.0	9.7	13		See above
	9	Cystatin C	226712	14.0	9.7	16		See above
	10	Cystatin C	226712	14.0	9.7	9		See above
	11	FK506 binding protein (FKBP12)	17985953	11.9	7.9	8		Stabilizing intracellular calcium channels, involved in neuronal activities
	12	Diazepam binding inhibitor	13937379	10.0	8.8		3	Functional relationship with lipid binding proteins
	13	Diazepam binding inhibitor	13937379	10.0	8.8		2	See above
	13	Polyubiquitin	1050930	11.2	5.4		1	See above

[a] Number of peptides matched in the MALDI spectra of the identified proteins with a Z score of 1.65 or higher given by Profound. [b] Number of fragmented peptides with peptide fragment ion spectra indicating identity or extensive homology ($p<0.05$).

Mammals were specified in the search, since the conditioned medium contains both secreted proteins and medium components including human transferrin and bovine insulin. Transferrin was identified in several fractions in the analysis of IEF sets 1 and 2. All other proteins were identified as rodent proteins, indicating that these proteins were extracellular.

The MALDI mass spectra indicated both highly concentrated protein fractions and high purity of the identified proteins (see figure 3, Paper I). Advantages of using preparative 2D gel electrophoresis to separate and identify proteins in complex mixtures include the high load capacity in the IEF separation and the resulting enrichment of proteins into separate fractions, yielding sufficient quantities of proteins for identification of relatively low abundance ones. Some of the proteins were identified in multiple fractions, which may imply post-translational modifications.

Three of the identified proteins known to be secreted were selected for immunobased studies. FKBP12 (Paper I), PEBP (Paper I) and cystatin C (Dahl et al. 2004) were detected in the conditioned medium using Western blotting, the increase in intensity of their protein bands during culturing being caused by accumulation of proteins (figure 9). FKBP12 and PEBP appeared as single bands, while Western blot of cystatin C revealed several bands. The cellular distribution of the proteins was also investigated. All nestin-positive AHPs showed immunoreactivity for all three antibodies against the proteins, which all seemed to have a cytoplasmic localisation (figure 9). Nestin is expressed by immature NSPCs and is a marker for these cells (Taupin et al. 2002). These results strengthen the assumption that the presence of FKBP12, PEBP and cystatin C in conditioned medium is attributable to secretion.

Secreted cystatin C has previously been identified both as a survival factor for AHPs in conditioned medium and stimulating neurogenesis in adult rat brain (Taupin et al. 2000). Other proteins secreted by these cells in culture might also be expected to affect the endogenous NSPCs, as cystatin C. In addition to its neurogenic capacity, cystatin C has also been suggested to promote astrogenesis in the developing CNS in an autocrine/paracrine manner (Hasegawa et al. 2006). Interestingly, both FKBP12 and PEPB have hippocampal localisation in the rodent brain (Steiner et al. 1992; Ojika et al. 2000). Furthermore, PEPB has been described as the precursor of hippocampal cholinergic neurostimulation peptide (HCNP), involved in the differentiation of neurons in developing hippocampus (Ojika et al. 2000). PEBP, also known as Raf-1-interacting protein, controls the Raf/MEK/ERK mitogenic-activated protein kinase (MAPK) pathway, shown to be involved in proliferation and differentiation of stem/progenitor cells (Rajan et al. 1998; Yeung et al. 1999; Learish et al. 2000). The FKBP12 interacts with the

inositol 1,4,5-triphosphate receptor (IP3R), which is also highly expressed in hippocampus and plays important roles in development events (Snyder et al. 1998; Rosemblit et al. 1999; Sharp et al. 1999). Furthermore, FKBP12 has been suggested to be involved in neuronal survival and regeneration (Lyons et al. 1995; Kato et al. 2000). Thus some proteins secreted from AHPs can be predicted to have autocrine/paracrine protective or survival effects. Other likely roles include promotion of brain plasticity and/or communication with the normal environment of NSPCs *in vivo*. Further studies are needed to investigate the biological relevance of the proteins identified, and might provide valuable clues to NSPC-research in the future.

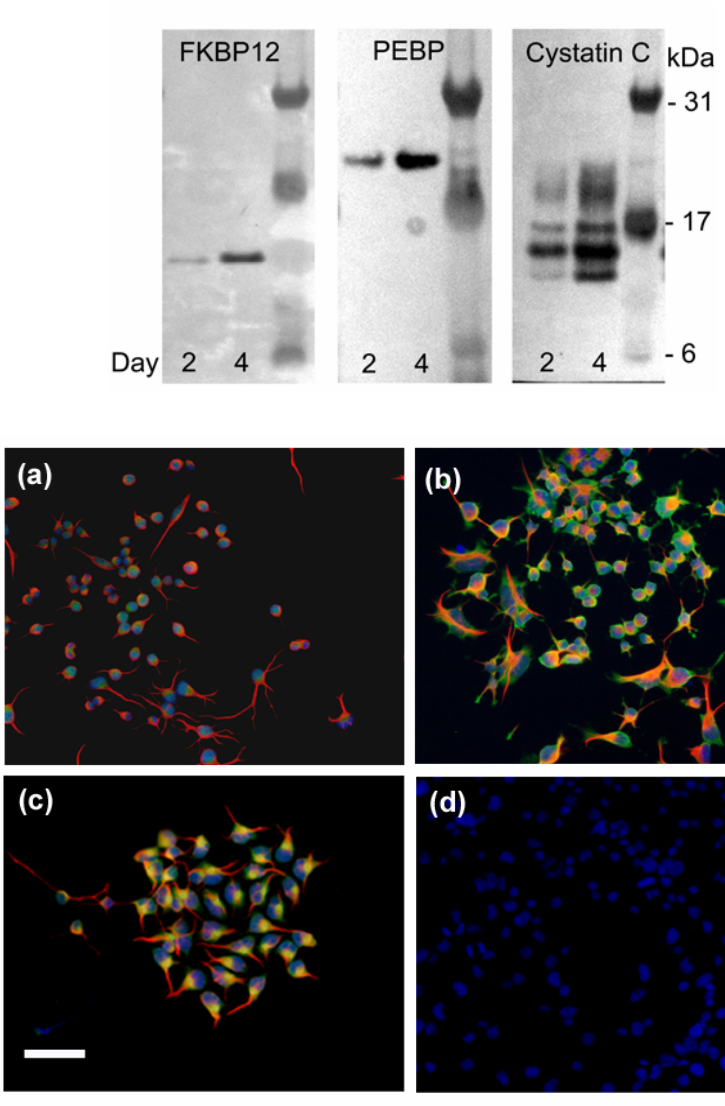

Figure 9. Immunoblotting of FKBP12, PEBP and cystatin C in conditioned media from two and four days of culturing. Demonstration of immunostainings for (a) FKBP12; (b) PEBP and (c) cystatin C (green). The AHPs are also immunostained with antiserum against nestin (red) and stained by Hoechst 33258 staining (blue). No immunoreactivity was found when primary antisera were omitted (d). Scale bar: 50 μm

In summary, preparative 2D gel electrophoresis followed by MS analysis allows for identification of secreted proteins in well-defined conditioned medium from cultured AHP. The identification of lower abundance proteins is facilitated, since these proteins are enriched into different fractions during the IEF separation. This approach is a suitable tool in the search for potential secreted and bioactive proteins in well-defined conditioned media.

Comments

The proteins identified in the conditioned medium can be either secreted proteins, or attributed to apoptosis, as a result of the relatively long culturing time. Shorter culturing time might have been preferable, to avoid spontaneous autolysis. Obviously, shorter culturing time would result in lower levels of the proteins in the conditioned medium. However, the Western blotting of FKBP12, PEBP and cystatin C revealed distinct proteins bands after only 48 hours of culturing. The levels of LDH (an intracellular enzyme) were low in conditioned medium from both 48 h and four days of culturing and no morphological signs of cell death or toxicity were detected, indicating low quantities of cellular proteins, due to toxicity or cell death in the cell cultures. Furthermore, the MALDI mass spectra of the IEF fractions indicated high levels of the identified proteins. These findings suggest that shorter culturing time would probably also result in sufficient quantities of proteins for identification. The cells were cultured in a serum-free well-defined medium with relatively low levels of the proteins added to the culture medium. Despite the serum-free conditions, the medium components will be more pronounced in the conditioned medium with shorter culturing time. Consequently, identification of the relatively low abundant secreted proteins using MALDI PMF might be difficult. However, fragmentation analysis allows for identification of proteins in a more complex mixture of proteins and can be the method of choice for protein identification (Aebersold et al. 2001). Furthermore, separation of the tryptic peptides using a nano-scale LC system prior to the MS/MS analysis, would improve the sensitivity of the analysis significantly as compared with spraying the digest directly into the mass spectrometer without separation as in this study (Shen et al. 2002).

Western blot analysis of PEBP and FKBP12 resulted in one distinct band on the blots at the expected MWs, and no unspecific protein bands were observed. However, Western blotting of cystatin C resulted in several protein bands on the blot. Previously, a glycoform of the protein was demonstrated to be a survival factor for AHPs (Taupin et al. 2000). In order to evaluate whether the proteins band were attributable to other novel glycoforms, the different bands were separated and isolated, and then incubated with several

deglycosylation enzymes. Western blotting of the isolated and deglycosylated protein fractions revealed mass shifts and verified that the different protein bands were glycoforms of the protein (Dahl et al. 2004).

PAPER II

Proteome analysis of serum-containing astrocyte conditioned medium from primary astrocyte cultures

It is very valuable to develop strategies that allow for identification of secreted proteins in serum-containing culture media. As is well known, mammalian cells require serum for optimal growth, and serum deprivation elicits many cellular responses (Higuchi et al. 2006; Lebon et al. 2006; Wei et al. 2006). Moreover, most data on cellular properties in cultures comes from culturing in serum-containing media. However, identification of secreted proteins in serum-containing conditioned media poses a substantial analytical challenge, owing to the fact that the proteins of interest are much less abundant than the medium components. Furthermore, differentiating the identified proteins as secreted proteins or proteins originating from the serum is complicated. For this reason, we evaluated whether SILAC combined with peptide fragmentation allows for identification of released proteins in serum-containing culture media. The second objective of this study was to investigate whether we could find any qualitative and quantitative differences in the secretion profiles between primary astrocytes and astrocytes in a scratch injury model of astrogliosis.

Most of the proteins identified in the conditioned medium were bovine proteins derived from the serum as expected. However, the database search also resulted in 21 proteins that were identified as proteins originating from rattus norvegicus in the serum-containing conditioned medium (see table 1, Paper II). For the majority of these proteins, a labeled arginine-containing peptide was found, confirming that they were derived from the cells rather than from the conditioned medium. The labeled amino acid was incorporated into the proteins during the culturing, and the released proteins containing the label could subsequently be distinguished from the medium proteins (figure 10). Several proteins previously identified as secreted by astrocytes under serum-free conditions with proteomic strategies involving MS were found (Lafon-Cazal et al. 2003; Delcourt et al. 2005), as well as a number of proteins that have not earlier been identified in astrocyte-conditioned medium. Comparison of the released proteins and the proteins identified in the whole astrocyte cell extract revealed a number of proteins identified in both the

samples (see table 1, Paper II). This finding strengthens the result that the proteins found in the conditioned medium are derived from the astrocytes. However, several of the proteins were only found in the conditioned medium, suggesting that these proteins are enriched in the medium. There were possibly too low levels of these proteins in the whole cell lysate compared to the other relatively highly abundant proteins to allow for their identification.

	unlabeled	labeled	sequence
			V
y_{13}	**1457.7**	**1467.8**	**P**
y_{12}	**1360.7**	**1370.7**	**T**
y_{11}	**1259.6**	**1269.7**	**P**
y_{10}	**1162.6**	**1172.6**	**N**
y_9	**1048.6**	**1058.6**	**V**
y_8	**949.5**	**959.5**	**S**
y_7	**862.5**	**872.5**	**V**
y_6	**763.4**	**773.4**	**V**
y_5	*664.3*	**674.3**	**D**
y_4	**549.3**	**559.3**	**L**
y_3	**436.2**	**446.2**	**T**
y_2	**335.2**	**345.2**	**C**
y_1	*175.1*	*185.1*	**R**

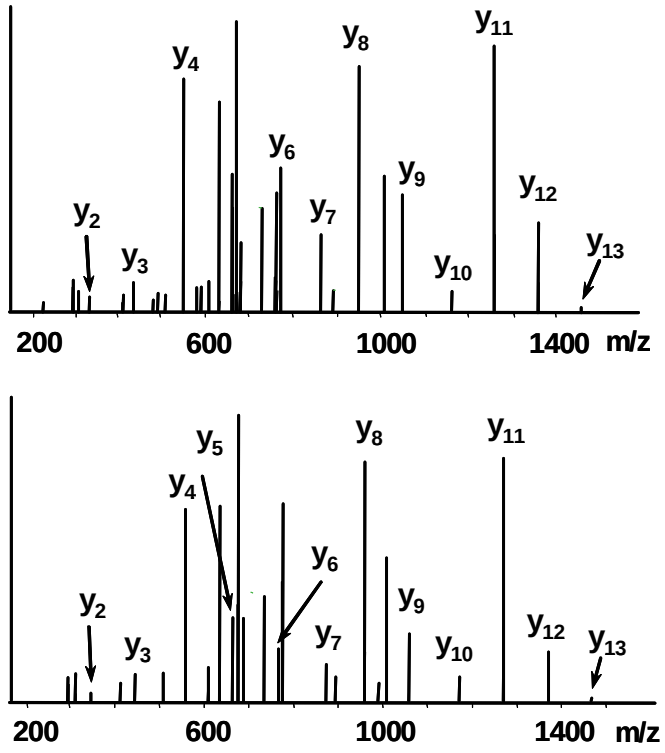

Figure 10. Annotated fragmentation ion (CID) spectra for the doubly charged peptides of a SILAC pair identified as glyceraldehydes-3-phospahte dehydrogenase in the conditioned medium from lesioned astrocytes. The peptides from the secreted protein were distinguished from the peptides from the corresponding proteins in the medium itself by the incorporated arginine label. The molecular masses of the precursor ions were 1555.8 and 1565.8 Da for the unlabeled (top spectrum) and labeled (bottom spectrum) peptides, respectively. The two fragmentation spectra are close too identical, except for the 10 Da mass difference attributed to the labeled arginine. The amino acid sequence of the peptides was RCTLDVVSVNPTPV, obtained by interpretation of the produced fragmentation ions. The y-series of ions (C-terminal fragments) are shown.

No significant differences in the secretion profiles were found in the conditioned medium from lesioned and non-lesioned astrocytes. Neither could any quantitative differences between the two cell cultures be detected. Our results also revealed inherent limitations in the quantitative analysis of lesioned and non-lesioned astrocyte conditioned media. Some of the secreted

proteins generated tryptic peptides with the same amino acid sequences as the corresponding bovine proteins in the culture medium itself (figure 10). Other limitations in the quantitative analysis were co-elution of different peptides with similar molecular mass during the LC-run, and the fact that the number of detected SILAC peptide pairs from each protein was generally too low. Co-elution of different peptides with close to the same *m/z* resulted in peptide overlap that prevented the quantitative analysis, and suppressed relatively low abundance proteins by more abundant ones (figure 11).

One explanation for the similarities in the qualitative and quantitative secretion profiles could be that the proteins identified are the main proteins released by astrocytes, and do not differ significantly between the two conditions. Earlier studies, where most of the proteins were identified in serum-free astrocyte conditioned medium, support this theory (Lafon-Cazal et al. 2003; Delcourt et al. 2005). However, this finding is confirmation that the proteins in the lesioned astrocyte conditioned medium were indeed secreted, rather than being the result of intracellular proteins leaking from ruptured cells. It is also likely that some differences in protein expression were masked by the fact that only a fraction of the cells in the astrogliosis model is lesioned and therefore directly affected. Moreover, it should be noted that non-lesioned primary astrocytes have been suggested to display a reactive phenotype (Wu et al. 1998).

Secreted proteins have been proposed to be involved in a various processes including intercellular communication. They might also be potential biomarkers of clinical importance, a source for early detection, diagnosis and monitoring the progression of diseases or conditions. One of the proteins identified as secreted in the present study was clusterin. This protein was found in conditioned media from both lesioned and non-lesioned astrocytes and has not previously been identified in astrocyte-conditioned media in MS based proteomic studies (Lafon-Cazal et al. 2003; Delcourt et al. 2005). Clusterin is highly expressed in response to damage, displays neuroprotective properties, and plays a central role in remodelling the CNS after ischemic damage (Wiggins et al. 2003; Imhof et al. 2006). Other astrocyte-released proteins have also been associated with promotion of the brain plasticity (Ridet et al. 1997; Hasegawa et al. 2006). Morover, astrocytes secrete numerous growth factors and extracellular matrix proteins that modulate the activity of neighboring cells and affect cell-cell interactions (Yoshida et al. 1991; Schaar et al. 1993; Schwartz et al. 1994; Aubert et al. 1995; Ridet et al. 1997). Consequently, identification of factors in conditioned media might lead to new advances in the understanding of the molecular mechanisms regulating the endogenous cells.

In summary, SILAC in combination with MS analysis facilitates identification of secreted proteins in complex mixtures such as the serum-containing conditioned medium. The released proteins were distinguished from the medium components by the labeled amino acid incorporated into the cellular proteins during culturing. To our knowledge, we are the first to identify secreted proteins in serum-containing medium using this proteomic strategy. The approach can be used as an initial screening tool to identify proteins released in serum-containing conditioned medium. It can be extended to most cell lines, in which the secretome will be the object to study.

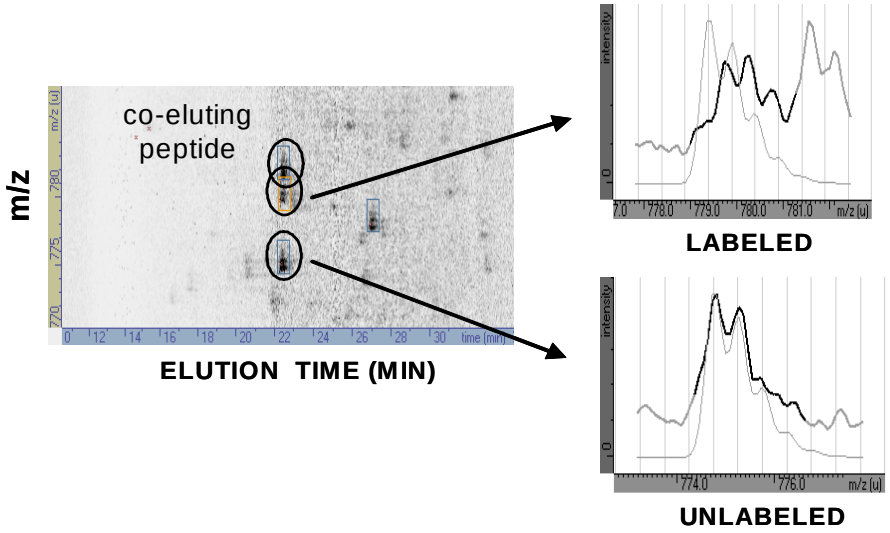

Figure 11. The LC-MS signal intensity map of a SILAC peptide pair. The intensity spots from the doubly charged SILAC peptide pair of *m/z* 774.4 and 779.4 are indicated on the map. Only the unlabeled peptide in the SILAC pair was detected and identified using the software. The labeled peptide had an interfering peptide with close to the same *m/z* which eluted at almost the same time. As a result of the peptide overlap, quantitation was not possible. The ion chromatogram of the labeled SILAC peptide shows two superimposed elution curves, illustrating the overlap of the two peptides in the LC-MS separation.

Comments

The excised and trypsin-digested gel bands resulted in a mixture of peptides from both secreted and serum proteins. The risk for co-elution of different peptides and consequent peptide suppression in the MS analysis increases with sample complexity. The presence of co-eluting SILAC peptide pairs also has to be considered. Subsequent, analysis of the complex digests is very demanding on the performance of the LC-MS system. However, the LTQ-FT mass spectrometer combined with the high throughput nano-scale LC-system utilised in this study facilitates identification of proteins in complex mixtures

and can differentiate peptides with in incorporated arginine label from the unlabeled peptide with the same amino acid sequence. On-line nano-scale LC separation drastically improves the sensitivity of MS analysis, as a result of both a concentration effect of the peptides and a reduction in the sample complexity (Shen et al. 2002). Fewer peptides elute within the same time window, limiting the suppression in the MS analysis. Furthermore, the linear ion trap is a fast scanning spectrometer (in the millisecond range) and handles isolation, fragmentation and detection of up to six precursor ions in 1 second. In general, only two or at most a very few peptides with probability scores for correct protein identification of $p<0.05$ from the same protein are required for protein identification.

The approach used in the present paper is different from previous studies of astrocyte secreted proteins, where the cells were cultured under serum-free conditions and 2D gel electrophoresis followed by PMF for protein identification was the proteomic strategy employed (Lafon-Cazal et al. 2003; Delcourt et al. 2005). The reason for this is that the high abundance serum proteins would suppress the detection of the lower abundance secreted proteins on the 2D gel and therefore prevent their identification. Furthermore, the level of secreted proteins loaded onto the 2D gel would drastically be reduced in the analysis of serum-containing conditioned media, since the capacity of the 2D gel is restricted to a certain total protein load. The presence of serum proteins in the conditioned media also increases the risk of proteins co-migrating into the same spot on the 2D gel. The purity of the isolated proteins is crucial to PMF, determining whether or not the database searches will result in unambiguous protein identification (Aebersold et al. 2001).

To improve the strategy used in the present study and its ability to identify secreted proteins in the serum-containing media, the number of detected peptides containing the labeled amino acid has to be increased. A reduction in the sample complexity prior to LC-MS analysis was demonstrated to have this desired effect (Paper III). The reduction resulted in fewer co-eluting peptides, which caused the higher abundance proteins to suppress the lower abundance proteins in the MS analysis. Another approach could be to culture the cells in medium containing both labeled arginine and lysine to maximise the number of labeled peptides (Amanchy et al. 2005). Reduction in the sample complexity and maximising the number of labeled peptides also facilitates the quantitation of proteins. The quantitative limitation due to medium proteins with identical tryptic peptides as the released proteins might be solved by labeling both the cultures with different isotopically labeled amino acids that will result in different mass shifts (*e.g.* ^{13}C6-Arg and ^{13}C6^{15}N4-Arg). This would make it possible to distinguish the extracellular proteins from both the cell cultures from the proteins of the culture media.

PAPER III

Evaluation of sample fractionation using micro-scale liquid phase iso-electric focusing on mass spectrometric identification and quantitation of proteins in a SILAC experiment

Mass spectrometric methods based on stable isotopes have shown great promise for the simultaneous and automated identification and quantitation of complex protein mixtures (Gygi et al. 1999; Oda et al. 1999; Ong et al. 2002). In a SILAC experiment the unlabeled and labeled samples are combined and treated as a single sample, which allows for the use of various methods of protein purification without introducing errors into the final quantitative analysis. Owing to the complexity of the proteome, it is desirable to develop methods that offer a wide dynamic range to improve the proteome coverage. The increased number of peptides in a quantitative experiment, arising from SILAC peptide pairs, implies that prefractionation might be crucial prior to the LC MS analysis in order to prevent suppression and improve the quantitative analysis (Ong et al. 2003; Amanchy et al. 2005). Therefore, the effects of a prefractionation step using micro-scale IEF in liquid phase on identification and quantitation of proteins in a SILAC experiment were evaluated.

The IEF separation of the combined cell lysates generated ten fractions with a shift in pH between the IEF fractions ranging from 2 to 10. The resulting IEF fractions, as well as the unfractionated sample of the pooled lysates, were resolved using gel electrophoresis. Distinct protein bands that were indistinguishable in the unfractionated sample were observed in the IEF fractions. Differences in the protein band patterns in the lanes of the different IEF fractions, as well as in the lane of the unfractionated sample were also seen (figure 12). The unfractionated sample, fractions 4 and 5 from the IEF, were selected for further analyses, since all three contained distinct protein bands in the whole molecular weight range with approximately the same amount of loaded protein. Fractions 4 and 5 also had almost the same pH, allowing for examination whether the proteins were focused into well-defined fractions. The majority of the proteins identified in fractions 4 and 5 differed and were identified as present in one fraction but not in both. This finding, as well as the differences in the protein band patterns between the IEF fractions observed on the gels, demonstrate that the focusing of the sample was satisfactory and that the sample complexity was reduced.

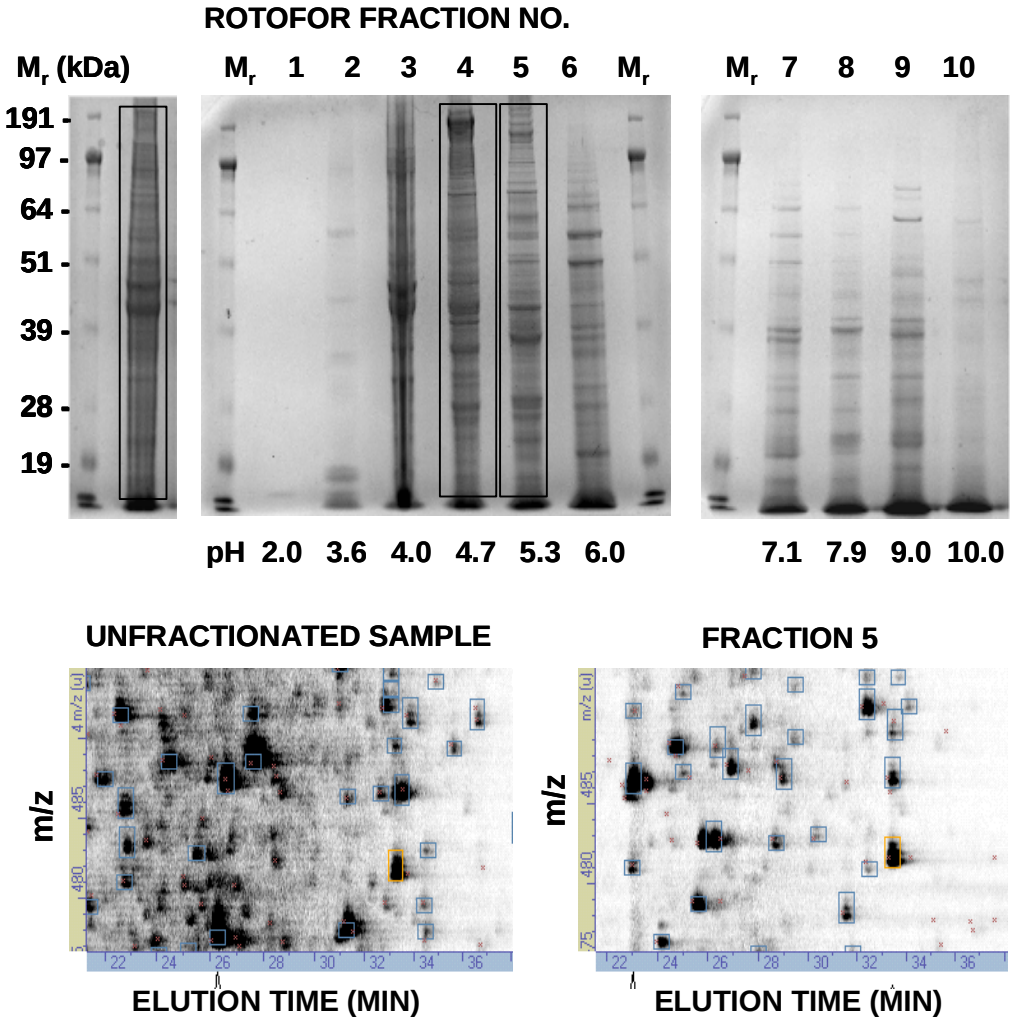

Figure 12. Unfractionated sample and IEF fractions resolved by gel electrophoresis. The shift in pH between the IEF fractions and the distribution of the proteins into different IEF fractions indicate that the focusing was satisfactory. The proteins were enriched into IEF fractions 3, 4, 5 and 6. Distinct protein bands, indistinguishable in the unfractionated sample, were present in the IEF fractions. The LC-MS intensity maps corresponding to the same excised band from the unfractionated sample and fraction 5 are shown. The intensity map of fraction 5 contains fewer peptides, illustrating the reduced sample complexity and risk for co-eluting peptides.

The distribution of the peptides identified as Moesin or Lamin also suggests that the proteins were focused into well-defined fractions (see table 1, Paper III). Although many peptides of Lamin were found in the unfractionated sample and in fraction 5, only a few peptides were identified in fraction 4 and most of the Moesin peptides were identified in fraction 4. The fractionation increased the number of proteins identified drastically and numerous proteins were identified in only the two IEF fractions. The number of peptides of a specific protein found and their probability score for correct protein

identification were also enhanced. Consequently, the fractionation increased the number of found SILAC pairs of the identified proteins as compared with the unfractionated sample (see tables I and II, Paper III). It is essential to obtain multiple SILAC pairs from each protein in order to obtain statistically significant intensity ratios. Several of the SILAC pairs that could not be quantified in the unfractionated sample had interfering peptides with similar physicochemical properties that eluted in the LC-separation closely enough to overlap. This reduced sample complexity due to the prefractionation limited the co-elution in the fractionated samples. The DeCyder MS 2D signal intensity maps of the LC-MS analysis were more sparsely covered, illustrating a reduction in the risk of overlap of different peptides (figure 12). The use of high resolution MS might also limit this problems, thanks to the high mass accuracy measurement of the peptides.

In summary, we demonstrate that micro-scale liquid phase IEF was satisfactory and the proteins were separated into well-defined fractions. Furthermore, the fractionation increased the number of proteins identified drastically, and numerous proteins were identified only in the IEF fractions. The combination of increased protein load, enrichment of proteins into separated fractions relative the unfractionated sample and the reduced sample complexity, limited errors in measurements of the intensity ratios as well as the suppression of relatively low abundance proteins by more abundant ones.

Comments

In a SILAC experiment it is important that the number of cell divisions and/or culturing time is sufficient to allow close to complete incorporation of the labeled amino acid into the cellular proteins. The measured intensity ratios will otherwise have to be corrected with a correction factor that takes the incomplete incorporation into account. However, our results also revealed the importance of using amino acids where ^{12}C and/or ^{14}N are completely substituted with ^{13}C and/or ^{15}N. The amino acid used in the present study was not fully substituted, which resulted in a small peak with 1 Da lower than the most abundant peak in the isotopic envelope of the labeled peptide for the singly charged peptide ions (figure 13). For peptide ions with a higher charge state this peak was not resolved from the labeled peptides in the mass spectra, and was impossible to detect. Accordingly, the peak area of the labeled peptides was overestimated and incorrect relative intensity ratios for these ions are calculated. Subsequently, in measurements of protein expression using SILAC, the purity of the isotopically labeled amino acid has to be examined before the start of the experiment. Experiments performed without considering the substitution efficiency of the labeled amino acid might not

become aware of this possible limitation during the analysis. The use of a high resolution mass spectrometer such as FT-ICR MS might be a solution, since this mass spectrometer is capable of completely resolving the isotopic envelope, also for doubly and triply charged ions.

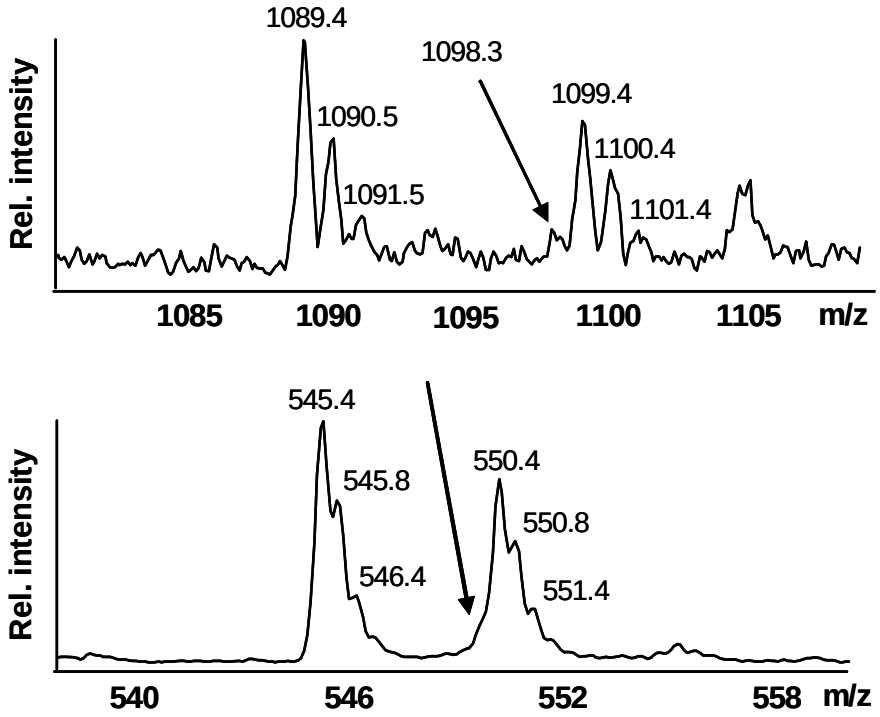

Figure 13. Mass spectra of singly and doubly charged ions of a SILAC pair. The ^{12}C or ^{14}N of the arginine in the labeled peptide is not fully substituted with ^{13}C or ^{15}N, respectively. For the singly charged peptide ions a small peak with 1 Da lower than the most abundant peak in the isotopic envelope of the heavy peptide is detected. For the doubly charged peptide ions, the peak from the not fully substituted arginine-containing peptides is not resolved from the dominant peak and is included in the integrated area.

Disadvantages of extensive fractionation are increased measurement times and increased sample consumption. However, the main benefit is the improvements in both sequence coverage and the number of quantifiable proteins. This paper demonstrates that direct analysis allows for efficient high throughput identification and quantitation, while lower abundant proteins require the more time consuming prefractionation approach. One advantage of SILAC experiments is that any fractionation approach can be applied to the combined sample without affecting the final quantitative MS analysis. The intensity ratios are preserved during isolation, separation, and ionisation, since the peptides in the SILAC pairs have the same physiochemical properties and thus behave identically (Ong et al. 2003; Amanchy et al. 2005; Molina et al. 2005).

CONCLUDING REMARKS

Our results show the high potential of mass spectrometry based proteomic strategies for identification and quantitation of proteins in biological samples. Consequently, development and improvement of proteomic approaches involving mass spectrometry for examination of proteins is of considerable significance for the understanding of the complex interactions within the CNS, and can be expected to have impact on restorative strategies for neuronal repair in the damage brain the future.

Responses to stated aims:

- Preparative 2D gel electrophoresis is suitable for identification of secreted proteins in the well-defined conditioned medium from cultured NSPCs. The high load capacity in the IEF step and the resulting reduction in sample complexity and enrichment of lower abundance proteins are important factors for the identification of these proteins;

- The lower abundance secreted proteins were possible to differentiate from the higher abundance medium proteins in the complex sample using metabolic labeling combined with mass spectrometric identification. Identification of the proteins containing the labeled amino acid in the serum-containing conditioned medium demonstrates the capacity of the approach. On the other hand, the quantitative analysis was found to have some inherited limitations; and

- Prefractionation using micro-scale IEF increased the number of proteins identified drastically and improved their quantitation as compared with direct analysis of the same SILAC sample. This is a result of the combination of increased protein load, enrichment of proteins into separated fractions as well as the reduced sample complexity relative the unfractionated sample. Co-elution of different peptides in the LC-separation and suppression of lower abundance proteins by more abundance ones were limited in the fractionated samples.

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