Protein Nitration in Amyotrophic Lateral Sclerosis; Possible Multifunctional Role in the Pathogenesis

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Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease. A familial form is caused by mutations of the copper-zinc superoxide dismutase (SOD1) gene. Multiple mechanisms have been proposed to contribute to ALS pathogenesis, including oxidative stress. Evidence came from the identification of markers of oxidative stress, such as nitrotyrosine (NT), in the cortex and spinal cord of ALS patients. However, no comprehensive study on the protein targets of nitration in ALS has been reported. We found high levels of NT-immunoreactivity in spinal cord of a transgenic mouse model of ALS at a presymptomatic stage of the disease compared to age-matched controls. Using a proteome-based strategy, we identified the nitrated proteins under physiological or pathological conditions, and compared their level of specific nitration. Alpha and gamma enolase, ATP synthase beta chain, heat shock cognate 71 kDa protein and actin were over-nitrated in presymptomatic ALS mice. In addition, we identified by MALDI mass spectrometry 16 sites of nitration in proteins oxidized in vivo. Alpha enolase nitration at Tyr43, target also of phosphorylation, constitutes an additional indication of the possible interference of nitration with phosphorylation. In conclusion, we propose that protein nitration may have a role in ALS pathogenesis, acting directly by inhibiting the function of specific proteins and indirectly interfering with protein degradation pathways and phosphorylation cascades.

Mass Spectrometry-based Strategies for Identification of Reversibly Oxidized Endogenous Protein-tyrosine Phosphatases in Cancer Cells

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Specific cysteine residue in a number of biologically important proteins including protein-tyrosine phosphatases (PTP) is known to be susceptible to oxidation in response to stimuli which generate reactive oxygen species (ROS). Previous studies have shown that human cancer cells spontaneously produce high levels of ROS, which are essential for their activated tyrosine phosphorylation signal, rapid proliferative rate and transformed phenotype. In the best studied PTP1B, oxidation of its catalytic cysteine has been shown to lead to its inactivation but critical chemical evidence is still lacking to define the natural occurrence of the respective cysteine sulfenic, sulfinic, and/or sulfonic acid modified forms. Mass spectrometry-based analytical strategy coupled with judicious choice of chemical derivatization is shown herein to be highly effective in quantitatively determine the redox status of cysteine, affording, for the first time, visualization of both reversibly and irreversibly oxidized forms of native PTP1B in cancer cells with constitutively elevated ROS level. Unambiguous MALDI-MS/MS sequencing for identification of site-specific cysteine modification are demonstrated to be equally effective against in gel and in solution digested peptides derived respectively from gel-resolved and mixed redox forms of PTP1B. Similar strategy has now been extended to several other PTPs in conjunction with proteomic scale analysis initiated to identify alterations in protein tyrosine phosphorylation level in response to specific PTP inhibition by ROS following insulin stimulation. Particular focus is devoted to determine the additional interacting partners or substrates of PTP1B including those trapped by non-reactive mutant or oxidized forms, as well as those multimerized through putative disulfide bond formation induced by ROS.
Analysis of Farnesylation of Drosophila Visual Ggamma Subunit by Mass-Spectrometry

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Activation of phototransduction in the compound eye of Drosophila is mediated by a heterotrimeric G protein which couples to the effector enzyme phospholipase Cbeta. The gamma-subunit of this G protein (GgammaE), as well as gamma-subunits of vertebrate transducins contain a carboxy-terminal GAAX-motif with a consensus sequence for protein farnesylation. To study farnesylation of Drosophila GgammaE, we mutated the farnesilation site and overexpressed the mutated GgammaE, as well as non-mutated GgammaE in Drosophila photoreceptor cells. Mass spectrometry of overexpressed GgammaE-subunits revealed that non-mutated GgammaE is modified by farnesylation and acetylation, whereas mutated GgammaE is not farnesylated. Native GgammaE is also modified by acetylation and farnesylation. In the transgenic flies, mutated GgammaE forms a dimeric complex with GbetaE, and the fraction of membrane bound GbetaE is decreased. Thus, farnesylation of GgammaE facilitates the membrane attachment of the Gbetagamma-complex. Electroretinogram recordings revealed a significant loss of light-sensitivity in eyes of transgenic flies which express mutated GgammaE. This loss in light-sensitivity reveals that post-translational farnesylation is a critical step for the formation of membrane-associated G protein required for transmitting light-activation from rhodopsin to phospholipase Cbeta.

Histone Modifications; Complex Patterns of Development and Disease

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The addition of methyl groups to the amino-terminal tails of histones by the histone methyltransferases (HMTs) family of proteins results in changes in the chromatin structure and packaging. This modification that is commonly altered in cancer provide a stable epigenetic mark that serve to regulate gene expression. In this work we analyze the consequences at the chromatin, gene expression and protein level of the elimination of Suv-39h and G9, two HMTs that methylate the histone H3 at K9. Our results show that, apart from the logic alterations at their target (K9-H3), both knockouts present a particular chromatin modification profile that leads to a HMT-specific pattern of gene expression and protein content. Using high performance capillary electrophoresis (HPCE), mass spectrometry (MS), tandem mass spectrometry (MS/MS) and immunoblotting with antibodies against specific modifications of the histone tail we found that both KOs show a decrease of K9-H3 methylation and that, in the case of the G9/H11002, there is also a reduction of histone H4 acetylation. In addition, we have characterized a comprehensive panel of normal tissues, human cancer cell lines and primary tumors in terms of their posttranslational modifications in the tail of histone H4. Using three independent techniques, immunodetection, high-performance capillary electrophoresis and mass spectrometry, we found that cancer cells undergo a loss of mono-acylated and trimethylated forms of histone H4. These changes appear early and tend to accumulate during the tumorigenic process, as we show in our analysis of a mouse multistage skin carcinogenesis model. The use of specific antibodies against particular H4 modifications and tandem mass spectrometry reveals that these losses occurred predominantly at the acetyl lysine 16 (K16) and trimethyl lysine 20 (K20) of histone H4, respectively.
Polyubiquitin Conjugates as Molecular Lead Structures for Ubiquitin-Proteasome Proteomics; Synthesis, Structures, and Biochemical Properties

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Modification of proteins by the covalent attachment of ubiquitin plays a fundamental role in the control of many biological processes including cell cycle regulation, transcription, DNA repair, and apoptosis. Substrate proteins are often modified by the attachment of polyubiquitin chains consisting of several ubiquitin moieties linked to each other via isopeptide bond formation. Ubiquitin contains 7 lysine residues and each of these lysines can be used for polyubiquitin chain formation. Importantly, it appears that the actual lysine residue of ubiquitin used for ubiquitin-ubiquitin conjugation determines the biochemical/biological function of the respective polyubiquitin chain. For example, K48-linked polyubiquitin chains serve as a signal to target modified proteins for degradation by the proteasome, while K63-linked polyubiquitin chains have been linked to non-proteolytic processes. An attractive possibility is that the different function(s) of different polyubiquitin chains are mediated by proteins that selectively interact with the respective chain. However, with the exception of proteins interacting with K48-linked polyubiquitin chains, little is known about the identity of such proteins. In this study, we have performed the synthesis and structural characterisation of a series of chemically defined polyubiquitin chains that differ by the lysine residue used for ubiquitin-ubiquitin attachment. In order to synthesise such conjugates, new specific chemical strategies for the built-up of polyubiquitin chains, such as the use of thioether ligation of C-terminal ubiquitin-thiol residues with N-(chloroacetamido)-side chain protected Lysine residues have been applied. High resolution FT-ICR mass spectrometry (FTICR-MS) was predominantly used for the structural determination of the chemically synthesised conjugates. Furthermore, FTICR-MS has been a key tool for high resolution intracellular proteomics of both ubiquitinated moieties and specific substrate protein identification. The present results indicate substantial functional differentiation caused by different iso-ubiquitine conjugate structures.

Protein Thiol Modifications Visualized in Vivo

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Post-translational thiol modifications play a crucial role in the chemistry and stability of many proteins. Disulfide bonds stabilize protein-structure and thiol-disulfide exchange reactions are central in systems that are responsible for maintaining the cellular redox-potential. In addition, thiol-modifications serve as molecular switches in a growing number of redox-regulated proteins. We have developed an innovative technique that allows us to globally monitor the in vivo thiol status of cellular proteins upon variations in the redox homeostasis of the cell. This technique involves the differential trapping of protein thiols in whole cell extracts, leading to the selective radioactive carbamidomethylation of oxidatively modified cysteines while reduced cysteines are simultaneously labeled with non-radioactive iodoacetamide. This results in the chemical identity of the modified proteins, regardless of their previous oxidation state in vivo. The thiol-oxidation state of a protein can then be quantified as the ratio of radioactivity per protein in any given spot on a 2D gel derived from this differentially labeled protein extract. This method should be applicable to visualize and monitor the thiol-disulfide state of proteins under different stress conditions in a variety of cells and organisms. We used this technique to define substrate proteins of the major thiol-disulfide oxidoreductases thioredoxin and DsbA in Escherichia coli. Exposure of cells to hydrogen peroxide and nitric oxide was used to identify a large set of redox-sensitive proteins in both eukaryotes and prokaryotes. In summary, our differential thiol trapping technique can be used to discover redox-regulated proteins and to determine the redox-potential of proteins in different cellular compartments.
28.7

Disulphide Bond Mapping of Transmembrane Activator and CAML Interactor (TACI) CRD1

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Objective: TACI is a member of the tumor necrosis factor receptor superfamily and is expressed on B cells and activated T cells. TACI contains two cysteine-rich domains, CRD1 and CRD2, and binds to two different ligands, APRIL and BAFF. Previous studies have shown that only CRD2 is necessary for high-affinity binding to both ligands, making contacts through its “DxL” motif and “h1h2-loop” region. CRD1 is homologous to CRD2, having a conserved DxL motif, yet binds only weakly to both ligands. Thus, we postulated that sequence differences in the h1h2-loop might account for the >100-fold difference in affinity between the two domains. Bacterial expression constructs for native human TACI CRD1 and a chimeric variant in which the seven h1h2-loop residues of CRD1 were replaced by the corresponding six residues of CRD2 each produced two isomers, with identical molecular weights. The objective of this study was to assign disulphide maps for both isomers of native and chimeric CRD1, to identify which isomer had the expected disulphide-pairing of the correctly-folded protein, and determine whether the h1h2-loop residues of CRD2 would confer additional affinity in the context of CDR1.

Methods: CRD1 proteins were produced and purified from E. Coli. HPLC-purified isomers were digested with CNBr, trypsin and/or pepsin to generate peptides that were analyzed by N-terminal sequencing, peptide mass mapping and tandem mass spectrometric analysis.

Results & Conclusion: Using a combination of proteolytic digests, N-terminal sequencing, peptide mass mapping and tandem mass spectrometry the disulphide-pairing of each TACI isomer was identified, therefore allowing assignment of the correctly-folded isomers. Binding studies comparing the wild type and chimeric proteins showed that indeed, the h1h2-loop region is an important determinant for high-affinity binding to APRIL and BAFF.

28.8

ASC_OX_BIOT, a New Reagent for the Specific Isolation of Oxidized Proteins

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Plasma contains a broad spectrum of molecular information whose presence may indicate metabolic states, cellular responses to variety of stresses, hormonal stimulation and diseases. For these reasons, blood represents possibly the most insightful proteomic sample for disease diagnosis. In diseases involving oxidative stress, the antioxidant capacity of plasma is decreased, many proteins are oxidized and oxidative damage can lead to several events including loss in specific protein function. This is why reagents are needed in order to isolate a subclass of proteins. We present here the synthesis of a molecule ASC_OX_BIOT for the selective isolation of oxidized proteins.

This new reagent ASC_OX_BIOT contains three moieties: (i) one hydrazine function which reacts specifically with carbonyl moiety, resulting from oxidation of the proteins, to form hydrazone residues; (ii) a biotinylated tag whose high affinity with avidin allows the specific isolation; (iii) and finally a linker group holding these two moieties together.

We tested our reagent of on human plasma; the proteins tagged by means of our reagent were isolated on avidin column and then subjected to proteolytic digestion. The analysis of ASC_OX_BIOT-labelled peptides was achieved using nanoLC-nanoESI-QqTOF and allowed us to detect and identify efficiently oxidized proteins by a simple comparison between native and oxidized plasma. Similar reagents for selective isolation of glycoproteins and phosphoproteins are currently synthesized in the laboratory: ASC_GLY and ASC_PHOS, the introduction of these probes into proteomics analyses will serve to further experiments on protein mixtures.

28.9

Analysis of Protein Arginine Methylation by Mass Spectrometry; Kinetics and Sequence Preferences of PRMT1 and PRMT3

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Protein arginine methylation, catalyzed by protein arginine methyltransferases (PRMT), is a post-translational modification that plays a role in cellular processes like subcellular localization of proteins, signal transduction, transcriptional regulation, and interaction of proteins with nucleic acids. Arginine methylation and the methylation activity of an enzyme towards a substrate or cell extracts are often measured by the incorporation of labeled methyl groups using S-Adenosyl-L-[methyl-3H]Methionine, but quantification or detection of specific methylation sites is difficult with this method. MALDI-TOF MS is an alternative to overcome these problems. By using a synthetic peptide containing four potential arginine methylation sites we found that the ionization efficiency of unmethylated and methylated peptide during MALDI-TOF MS is the same. Thus, unmethylated and methylated peptide can be quantified in a spectrum by the ratio of their monoisotopic peak intensities. To calculate the total amount of incorporated methyl groups at a certain time point during in vitro methylation, the sum of all peak intensities in a spectrum was set equal to the total amount of incubated peptide. Addition of the concentrations of each methylation intermediate multiplied by the number of its methylation groups revealed the total amount of incorporated methyl groups. Investigating the methylation by PRMT1 and PRMT3 of the synthetic peptide and of recombinant human EWS protein, an RNA-binding protein which is highly methylated in vivo, we could elaborate not only differences in the kinetics of these enzymes but also in their sequence- and substrate specificity.

Molecular & Cellular Proteomics 4.8 S271
Peptoscope is a simple statistical algorithm meant to detect common peptide precursor mass differences. These mass differences often correspond to post-translational modifications. Peptoscope requires high-accuracy mass spectrometry data as input, such as that generated by LTQ-FTs. For the datasets used in this study, Peptoscope was able to detect post-translational modifications at electron-mass accuracy better. The method is used for automating a search engine with respect to variable modifications settings. Peptoscope is publicly available at www.peptoscope.ms.

HMGA proteins constitute a group of architectural nuclear factors involved in chromatin dynamics (Sgarra et al., FEBS Letters 2004, 574, 1–8). These proteins, highly expressed in cancer cells, have been shown to take part both in transcriptional regulation processes as well as in general chromatin organization. The peculiarities of these proteins are their ability of modifying the DNA bending and their capability to establish multiple protein-protein interactions. HMGA activities are modulated by a wide series of post-translational modifications (PTMs) (phosphorylation, acetylation, methylation). The expression of HMGA proteins in tumor cells has a causal role in tumoral transformation but this seems not to be the only requirement. Indeed recent experimental evidences suggest that the combination of the various PTMs that impinge on HMGA proteins could play a role in modulating their oncogenic properties. Since their discovery, HMGA proteins have been suggested as possible molecular marker of the transformed phenotype, in this light, their PTMs could represent additional and extremely fine markers. In order to identify quantitative and qualitative changes of HMGA post-translational modifications in tumor cells having different origin and degree of aggressiveness we started an LC/MS-based screening and found that HMGA’s methylation and phosphorylation are differentially modulated in the various cell lines analysed. By proteolytic digestions and MS/MS sequencing we found that arginine 25 is differentially methylated. In addition, comparing HMGA proteins among them (HMGA1a, HMGA1b, and HMGA2) it resulted that they not only have different expression patterns but are also differentially modified, suggesting different functions in the transformation process.

The majority of proteins are chemically modified by a variety of post-translational modifications. Additionally, several chemical modifications may occur during protein isolation and separation processes due to reactions with, e.g., buffer components. The analysis of the precise nature and structure of protein isoforms is crucial for the understanding of their specific activity. However, resolving these isoforms can be difficult because such modifications often have local effects without imparting global physical/chemical changes to the overall protein structure. Sometimes such separations become critical to projects, beyond the obvious technical hurdles. For example, requirements for protein-based pharmaceuticals may include characterization of every protein isoform and documentation of their specific activities. Therefore isolation of protein isoforms prior to analysis is essential to maintain unambiguous correlation of specific modifications to single isoform activities.

In this study we have analyzed protein isoforms of different origins like ovalbumin, human IgGs, and dipeptidyl peptidase IV by Free Flow Electrophoresis (FFE) as proof of principle. The high resolution of FFE, separating proteins based on charge, is ideally suited for difficult challenges like separation of protein isoforms. FFE separation media may contain a sophisticated combination of buffering substances without any reducing or denaturing agents, allowing native separation conditions. Resulting fractions are well suited both for separation of closely related species such as protein isoforms, and for direct use in further studies such as enzyme activity and/or immunoassays, including the potential for improving existing diagnostic assays. We present analysis of FFE fractions analyzed by two-dimensional gel electrophoresis and activity assays, demonstrating the ability to separate various isoforms into individual fractions.