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Quantitative Mass Spectrometry: Elucidation of the Transduction Cascade Triggered by B cell Receptors

Quantitative Mass Spectrometry: Over the past 20 years, bioanalytical mass spectrometry has developed into a key technology in the life sciences. This has led to the establishment of proteomics as a new field of research. Proteomics is defined as the investigation of the proteome i.e. the totality of all the proteins in a certain state of a cell or an organism.

Mass Spectrometry and Proteomics
The above definition presents a challenge to modern mass spectrometry, as the proteome of a cell is not constant, but rather is regulated dynamically. For example, different proteins with different numbers of copies are present during the different stages of cell development, differentiation and proliferation, as well as in the individual subcellular processes. Bioanalytical mass spectrometry allows the fast and reliable identification and quantification of proteins as well as the analysis of protein modifications. The development of so-called soft ionisation methods, such as Matrix-Assisted Laser/Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI), has made possible the ionisation of entire proteins and protein fragments (termed peptides) in the gas phase. The increasing number of sequenced genomes provides in silico information about the molecular weight and the amino acid sequence of proteins and in this way allows comparison with the masses obtained by mass-spectrometry measurements.

In a typical proteomic procedure, the identification of proteins is performed by determining molecular weights - importantly, not of intact proteins, but rather of their peptides. Often, the detection of an individual peptide is sufficient to identify a protein reliably. To break down the protein, endoproteinases such as trypsin are used; these cleave proteins at specific amino acids (in this case arginine and lysine), yielding peptides. These peptides are initially separated by means of high-performance liquid chromatography (HPLC) and are then ionised in the mass spectrometer. The mass spectrometer first determines the precise masses of the peptides. Subsequently, one or more peptides are isolated and fragmented along the peptide chain in the gas phase.

The mass differences of the measured fragments correspond to the masses of single amino acids so that sequence information of the peptide is obtained. Finally, the corresponding protein is identified by

searching the experimental accurate mass of the sequenced peptides and the experimentally determined sequence information against a protein database.

Bioanalytical Mass Spectrometry

The two "soft" ionisation methods mentioned above, MALDI and ESI, are used in bioanalytical mass spectrometry. In MALDI, laser irradiation is used to desorb the peptides from a solid matrix into the gas phase and simultaneously to ionise them. In contrast, with ESI, the ionisation of the peptide is performed continuously from the liquid phase, which for proteomics has the practical advantage that the mass spectrometer can be coupled directly to HPLC. Accordingly, over the past three to five years, ESI mass spectrometry has gained importance, especially in high-throughput proteomics. Proteomics includes not only the detection of proteins, but also their quantification (i.e. the determination of the numbers of copies of the proteins in the cell‘s various states, alluded to above), and it further includes the detection of modified proteins. Protein modifications are the driving force for protein-protein interactions in the cell; they determine the activity of a protein and also its lifespan.

Up to now, high-resolution two-dimensional (2D) gel electrophoresis, combined with various staining techniques, was regarded as a tried and tested method for the visualisation and quantification both of proteins in various cellular states and also of changes in proteins, such as post-translational modifications. For quantitative analyses, various states of the cell can be compared by comparing the staining intensities of corresponding protein spots in 2D gels. For identification, differentially stained protein spots are cut out, digested with endoproteinases and analysed with a e.g. MALDI-TOF mass spectrometer.

However, over the past five years, ESI has gained ground over MALDI, both for quantitative proteome analyses and for the detection of post-translationally modified proteins. This has been due to three factors. First of all, direct coupling of the liquid-chromatographic separation apparatus to the mass spectrometer used to measure and identify the electrospray-ionised peptides has drastically reduced the analysis time. Secondly, the development of labelling techniques for proteins and peptides from cells in different states allows absolute and relative quantification of the proteins to be performed directly in the mass spectrometer. Finally, the continual technical development of ESI mass spectrometers, with corresponding computer capacities, today allows a remarkably high throughput of highly complex protein and peptide mixtures (fig. 1, for full figure legend).

Quantification of Proteins

The following workflow for the analysis of proteins and their modifications in various cell states has been established. It is based on state-of-the-art quantitative ESI mass spectrometry. First, hydrolysis of different samples - entire tissue, cells, cellular organs or other cellular compartments - is performed with an endoproteinase such as trypsin. Prior or after hydrolysis, various stable isotopes are introduced - either while the cell is still in culture, by means of labelled amino acids (SILAC) [1], or after hydrolysis of the proteins, by chemical labelling methods that employ amino-reactive reagents (iTRAQ [2], TMT [3], ICPL [4] or dimethyl labelling [5]). Subsequently, the various isotope-labelled cells, proteins or peptides, respectively, are mixed together. The labelled peptides can then not only be identified and related to the corresponding sample, but can also be quantified relative to one another in the mass spectrometer by comparing the intensity of the signal from two identical peptides which have been differently labelled (fig. 2).

In this way it is possible to quantify not only proteins and peptides, but also their modified (e.g. phosphorylated) versions, since such peptide modifications can be recognised by a shift in molecular weight; the extent of the shift allows the modification to be identified.

However, the identification and quantification of these protein modifications is made more difficult by the fact that the modifications may be unstable, and also that they are usually found in substoichiometric amounts. Therefore, modified peptides must be enriched before quantitative analysis, so that a sufficient number of molecules is present for detection in the mass spectrometer. In the specific case of the analysis of phosphoproteins and phosphopeptides, various enrichment methods have been established that allow

several thousand of these to be identified and quantified in a single experiment. Phosphopeptides from a complex peptide mixture, labelled isotopically by the cell culture-based method (above), are enriched selectively on the basis of their altered chemical properties - e.g., by "strong cation exchange chromatography" (SCX), titanium dioxide particles (TiO_2) or "immobilised metal ion affinity chromatography" (IMAC) [6, 7, 8, 9]. This makes possible the large-scale identification of modified peptides in a mass spectrometer.

Special Applications:
Elucidation of the B cell Receptor Transduction Cascade
Our research group uses this mass spectrometry-based procedure for the identification and quantification of the phosphoproteome (totality of all phosphoproteins) in B cells of the immune system. We compare these cells in their unstimulated state with similar cells whose B cell receptor (BCR) has been activated with antigens [10] (fig. 3).

With our routine preparation we can identify approximately 10000 phosphorylation sites in various proteins after B cell receptor stimulation. From these sites, approximately 800 reveal a significant quantitative change upon BCR stimulation. Analysis of the results showed that BCR stimulation led to stronger phosphorylation of proteins already known to play key parts in the BCR signal cascade. In addition, further
phosphoproteins not previously thought to be associated with BCR signal transfer were identified. This example shows the enormous potential of mass spectroscopy-based protein analysis for solving problems in cell biology - for example, the question of which molecules are decisive for the receptor-promoted signal transduction cascade. The knowledge gained from this will provide an important basis for understanding the molecular biology of B cells, because both the development of B cells and their activity in the humoral immune response mechanism depend largely on BCR-promoted signal transduction. Furthermore, incorrect regulation of these BCR signal pathways can result in serious disorders. These include neoplasias (such as B cell lymphoma) and also auto-immune disorders and immune deficiency syndromes. It may therefore be anticipated that mass-spectrometry-based decoding of physiologically and pathologically dysregulated BCR signal pathways will not only lead to a better understanding of B cell biology, but will also make an important contribution to research into the molecular pathogenesis of a number of serious diseases.

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