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Review

Analysis of posttranslational modifications exemplified using protein kinase A

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Abstract

With the completion of the major genome projects, one focus in biomedical research has shifted from the analysis of the rather static genome to the highly dynamic proteome. The sequencing of whole genomes did not lead to much anticipated insights into disease mechanisms; however, it paved the way for proteomics by providing the databases for protein identification by peptide mass fingerprints. The relative protein distribution within a cell or tissue is subject to change upon external and internal stimuli. Signal transduction events extend beyond a simple change in protein levels; rather they are governed by posttranslational modifications (PTMs), which provide a quick and efficient way to modulate cellular signals. Because most PTMs change the mass of a protein, they are amenable to analysis by mass spectrometry. Their investigation adds a level of functionality to proteomics, which can be expected to greatly aid in the understanding of the complex cellular machinery involved in signal transduction, metabolism, differentiation or in disease. This review provides an overview on posttranslational modifications exemplified on the model system cAMP-dependent protein kinase. Strategies for detection of selected PTMs are described and discussed in the context of protein kinase function.

Keywords: cAMP-dependent protein kinase; Phosphorylation; Myristoylation; Deamidation; Mass spectrometry

1. Introduction

More than a decade has passed since the term “proteome” was first introduced into the scientific community by Marc Wilkins as the “total protein complement of a genome” [1,2]. A major hallmark – and a major challenge – of proteome analysis, as opposed to genome projects, is the dynamics underlying the protein composition of a cell, a tissue or an organism at a given time point under given external and internal conditions. By comparing two defined states of a sample (i.e. before and after treatment or healthy and diseased condition) one should be able to identify those proteins which differ between these states. This differential proteomics approach can provide an insight into the mechanism underlying the observed phenotypes, adding a level of functionality to proteomic analyses that is often missing from the merely descriptive genome projects. This analysis should not be limited to the up- or downregulation of gene products, but should also include the analysis of post-translational modifications (PTMs) in a proteome, since these covalent protein modifications can alter protein function and interactions significantly, as outlined below.

The basic methodology of proteome analysis has remained largely unchanged in the last decade: due to the immense complexity of the proteome of even a single cell, the first step of proteome analysis is usually a separation step. In most cases this is achieved via two-dimensional gel electrophoresis [3,4], which still provides the highest resolution for separation of complex protein samples. The method has been continually refined, thus overcoming limitations of the earlier pioneering techniques and – most importantly – improving reproducibility [5,6]. Additionally, the use of fluorescent dyes can ameliorate the lack in dynamic range and linearity of common gel stains [7]. The use of two different fluorophores (e.g. Cy3 and Cy5) for the labelling of two samples allows for the comparison of these samples in a single gel, a technique known as difference gel electrophoresis (DIGE) [8]. Differences in protein levels are then reflected in the corresponding

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spot colour and intensity, depending on which protein is more prominent.

Identification of the proteins in question is then performed by mass spectrometry, usually by a tryptic digest and subsequent analysis of the resulting peptides via the peptide mass fingerprint (PMF). Based on MS/MS spectra of the peptides, sequence information can be obtained and – possibly – post-translational modifications can be identified.

As their name implies, they are covalent modifications introduced after – in some cases also during – protein biosynthesis. PTMs can range from the attachment of a methyl or phosphate group up to the addition of fatty acids, sugar moieties or – in the case of ubiquitination – even whole proteins. More than 200 different post-translational modifications of proteins have been described to date (for review see [9]). A common feature of these very different modifications is that they have an impact on protein function, for example on protein activity, localisation, protein interactions or on protein turnover. A proteomic study that aims to go beyond the quantitative description of the protein content of a sample has to include the analysis of post-translational modifications in order to obtain an idea on the investigated protein's function. This kind of analysis must not be restricted to a qualitative analysis of PTMs but should include some method for quantification, since some PTMs occur quantitatively, as stable modifications, while others affect only a subpopulation of an enzyme, or are only transient, still generating a massive cellular response. This is certainly true for the most important PTM in signal transduction, protein phosphorylation, which is mediated by the action of protein kinases. In cellular signal transduction [10,11] a small change in the phosphorylation status within a kinase cascade has a huge impact on the resulting cellular response. This can be attributed to conformational control exerted by phosphorylation that can function as an on–off switch (see Section 2.1 and Fig. 2) or change the binding behaviour of the modified protein.

1.1. The model system cAMP-dependent protein kinase (PKA)

Several articles in this issue will focus on the most prominent PTMs. In the present article we would like to illustrate some of the strategies that can be applied for PTM analysis on a single protein that is considered paradigmatic for a whole class of enzymes: the cyclic AMP dependent protein kinase (PKA). PKA is a key enzyme in the modulation of intracellular processes in eukaryotes and is also implicated in several human diseases [12–14]. In the absence of the second messenger cAMP, this kinase is kept in an inactive state by a regulatory (R) subunit dimer, which binds and inhibits two catalytic (C) subunit monomers. This heterotetrameric holoenzyme is activated by cAMP binding to the R-subunits, which then release the active catalytic subunits [15,16]. Thus, the C-subunit can phosphorylate target proteins in the cytosol or the nucleus, thereby also influencing gene transcription. PKA- C-subunit has been extensively characterized since it was discovered in 1968 [17]. The original work on the protein had to be performed using preparations from mammalian tissues, due to the lack of recombinant techniques at that time, identifying two major isoforms termed type I and type II, according to their elution behaviour in ion exchange chromatography [18–20]. With the introduction of modern genetic methods, it became possible to overexpress this enzyme in several heterologous systems. However, when doing so, it became apparent that the PTM pattern of the PKA C-subunit was altered [21]. On the other hand, with the aid of recombinant techniques previously unknown isoforms of the catalytic, as well as the regulatory subunit were discovered. To date, four isoforms of the regulatory [22–24] and at least a dozen isoforms of the catalytic subunit [25–31] have been described in mammals, increasing complexity even further. The majority of cellular responses to cAMP seem to be attributable to the ubiquitously expressed Cα subunit, but distinct signals may be promoted by the less abundant isoforms. Using mass spectrometric analyses, it was shown much later, that enzyme preparations from porcine heart consist of a mixture of at least the catalytic subunits Cα and Cβ. Those are 93% identical on the amino acid level, still unique peptides from each subunit were identified using tandem mass spectrometry [32]. As the different isoforms cannot be separated by conventional biochemical methods, homogeneous preparations of C-subunit isoforms can only be obtained with the use of recombinant techniques.

2. Posttranslational modifications of the PKA C-subunit

Several different post-translational modifications have been observed on this enzyme. Thus, PKA-C has been described to be multiply phosphorylated [33], N-terminally myristoylated (in fact it was the first protein shown to contain this PTM [34]) and, more recently, a deamidation has been described as well [32].

2.1. Phosphorylation

In eukaryotes, proteins can be phosphorylated on serine, threonine or tyrosine residues, allowing a distinction between Ser/Thr and Tyr kinases, according to their substrate specificity. Protein kinases do not only phosphorylate other proteins, thus altering their target's properties—they themselves are subject to phosphorylation, as a matter of fact most kinases need this PTM to be in an active state. In the case of the prototypic Cα subunit of PKA, belonging to the subfamily of Ser/Thr kinases, a threonine residue at position 197 in the so-called activation loop needs to be phosphorylated for the enzyme to assume an active conformation [21,35] (see Fig. 1). A second phosphorylation site at Ser 338 is obviously important for structural stability of the enzyme [35]. Both these phosphorylation sites are stable and remarkably resistant to phosphatase activity [36,37]. However, there is some evidence for a PP2A-like activity capable of dephosphorylating Thr 197 [38]. Mutant Cα-subunit lacking this phosphorylation site shows a dramatically decreased catalytic activity (500 fold [39,40]). While the Cα-subunit, like many protein kinases, is capable of autophosphorylation, there is also evidence for an upstream kinase performing this task in vivo [41,42]. Interestingly, even when expressed in *E. coli*, the major PKA C-subunits Cα and Cβ1 are capable of