NOVEL METHODS OF PHOSPHOPROTEOME ANALYSIS THE FARM ANIMALS SEMEN

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Introduction

Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, regulate enzymatic activity, subcellular localization, complex formation, degradation of proteins, the cell cycle, cytoskeletal regulation and apoptosis. Phosphorylation on serine, threonine and tyrosine residues is an extremely important modulator of protein function and it is estimated that 30- 50% of the proteins are phosphorylated at some time point (Jia et al, 2012, McLachlin and Chait, 2001), Protein phosphorylation is a fast and reversible process. It is catalysed by kinases by attaching phosphate groups onto specific amino acids. Opposed to phosphorylation, dephosphorylation removes the phosphate groups from proteins by phosphatases. Dephosphorylation plays important role in balancing the protein phosphorylation status in signalling proteins.

Exact knowledge of when and where phosphorylation occurs and the consequences of this modification for the protein of interest can lead to an understanding of the detailed mechanism of the sperm cell maturation, capacitation and acrosome reaction. which are required for the spermatozoon to reach, bind, penetrate and fuse with the oocyte. Therefore, there is a great need for methods capable of accurately elucidating sites of phosphorylation.

Proteins phosphorylation in mammals semen

Freshly, ejaculated sperm cells are not capable to fertilize the oocyte To acquire fertilizing ability, the spermatozoa have to undergo an activation process called capacitation (Flesch et al., 1999). During this process bicarbonate activates a sperm specific adenylate cyclase and thereby induces increased cAMP levels in the sperm cell. The subsequent activation of protein kinase A (PKA) induces, by signalling mechanism, tyrosine phosphorylation of several proteins (Flesch et al., 1999) i.e. tyrosine phosphorylated of flagellar proteins which induce hypermotility. Tyrosine phosphorylation can induce conformational changes in proteins thus leading either to their activation or inactivation (Kimura et al., 1996), Fig. 1. Studies on capacitation and

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tyrosine phosphorylation have focused on the whole sperm cell in relation to the binding of sperm cells to the zona pellucida and the acrosome reaction. Historically by the first time spermatozoa phosphoproteins were detected in 1989 by Leyton and Saling. That was the first evidence for the presence of tyrosine phosphorylation in mammalian spermatozoa namely the mouse sperm (Leyton and Saling, 1989). Using anti-phosphotyrosine antibody they identified in mouse sperm three phosphoproteins with molecular weight of 52, 75, and 95 kDa. The 95 kDa protein showed enhancement in immunoreactivity with the antibody after sperm capacitation and interaction with oocyte ZP proteins. Next in 1991, study carried out by Naz et all determined tyrosine phosphorylation in sperm of several mammalian species like; human, rat, rabbit, and mouse.

Figure 1 – Protein phosphorylation cascade during sperm capacitation (Naz and Rajesh 2004)

Phosphoprotein enrichment

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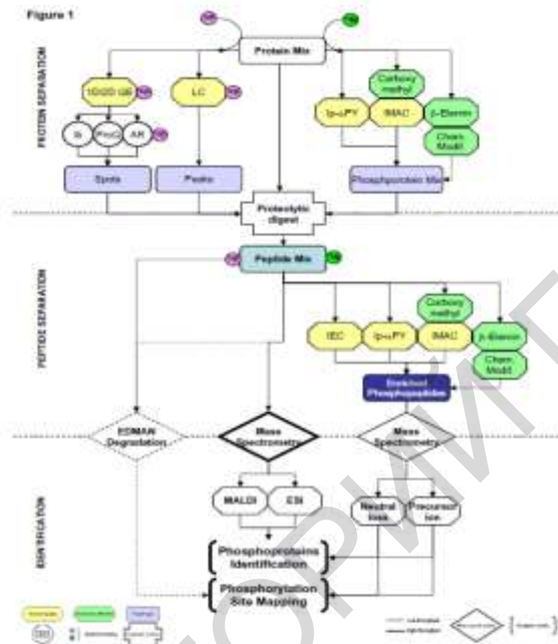

Figure 2 – The schematic workflow of phosphoprotein enrichment strategies (Delom and Chevet 2004)

They described four phosphorylated proteins in the molecular weight range of 95 kDa/94 ± 3 kDa (FA-2 antigen), 46 ± 3 kDa, 25 ± 7 kDa and 12 ± 2 kDa in human sperm (Naz et al., 1991) and also detected a protein of molecular mass with 94 ± 3 kDa in mouse sperm, that was reported before by Leyton and Saling (1989). But, this protein was not identified in rat and rabbit sperm. Using 32P metabolic labeling and *in vitro* kinase assays, human sperm was found to have at least seven proteins (200, 112, 104, 48, 42, 31 and 25 kDa) that are phosphorylated and fourteen proteins (122, 105, 95, 89, 73, 62, 48, 46, 40, 33, 30, 28, 25 and 22 kDa) (Naz, 1996). Further studies showed that the 94 ± 3 kDa and 46 ± 3 kDa proteins are also phosphorylated at ser/thr residues besides phosphorylation at tyrosine residues (Naz, 1999). The 46 ± 3 kDa protein was found out to be the FA-1 antigen, which has been known to play an important role in sperm-ZP binding (Naz, Ahmad, 1994).

Detection of phosphoproteins

Radioactive labelling of proteins with ^{32}P isotope

Many of radioactive isotopes are very useful for the biochemical processes. Cells are typically labeled with 32P or 33P prior to protein isolation or these radioisotopes are incorporated into subcellular fractions using protein kinases after their isolation (Steinberg et al., 2003). The phosphoproteins are then usually detected by autoradiography using film or storage phosphor imaging screens. The labeling methods generally use radioactive inorganic phosphate, ATP or GTP. Since *in vitro* radiolabeling only provides a measure of the phosphate groups attached during the actual labeling period, no information is provided with radioisotopes are the simple and very sensitive detection of the labels as well as the fact that labeled groups are chemically identical to their naturally occurring analogues. But even at low doses radioactive isotopes are dangerous, especially when they are incorporated into biologically active molecules. Protection and disposal are the main disadvantages; therefore, non-radioactive labeling, such as biotinylation or fluorescent labeling, should be preferred whenever it is possible (Holtzhauer, 2006). The phosphorylated proteins are detected by autoradiography method. Radiolabeling detects all types of phosphotylation (Springer, 1991).

Immunoprecipitation

In general, antibodies bind various epitopes. Immunoprecipitation used for phosphoprotein or phosphopeptide enrichment employs antibodies raised against phosphorylated amino acids. Phosphoamino acid-selective antibodies have been particularly well suited to identifying tyrosine phosphorylated proteins have not given satisfactory results during phosphoprotein enrichment. Immunoprecipitation is unsuitable for large-scale studies covering the whole phosphoproteome. Antibodies are phosphoamino acid-specific, so it is necessary to perform several parallel immunoprecipitation reactions (one with anti-pY antibodies, and at least one with anti-pS/pT antibodies). Due to this, they are mostly used when one particular phosphorylated amino acid is being searched for. This advantage becomes especially apparent in the case of tyrosine phosphorylation, since phosphotyrosine is notably less common than phosphoserine and phosphothreonine (Fila, Honys, 2012).

Immunoblot

Immuno- or Western blot is a technique [15] which requires the availability of specific antibodies to detected proteins transferred from a one-dimensional gel electrophoresis (1-DGE) or 2-DGE [16] to a solid membrane support. The development of antibodies against common protein epitopes allows the identification of proteins sharing the same characteristics such as phosphorylated proteins. For phosphoproteome analysis phosphoserine, phosphothreonine and phosphotyrosine represent the common epitopes which are recognized by specific antibodies that are routinely employed.

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Although Western blot allows the detection of very low abundance phosphoproteins, this method is not very suitable for quantitative analysis due to the variability of the amount of proteins transferred to the membrane. In addition, the selectivity and affinity characteristics of the antibodies are of major importance since a large number of "false positive" interactions may be detected, thus reducing the applicability of this approach.

Immobilized metal affinity chromatography (IMAC)

The most common method for selectively enriching phosphopeptides from biological samples is immobilized metal affinity chromatography (IMAC). In this technique, metal ions, specially Fe3+ or Ga3+, are bound to a chelating gel beds. Phosphopeptides are selectively bound because of the affinity of the metal ions for the phosphate moiety. The phosphopeptides can be released using high pH or phosphate buffer, the latter usually requiring a further desalting step before MS analysis. Limitations of this approach include possible loss of phosphopeptides because of their inability to bind to the IMAC column, difficulty in the elution of some multiply phosphorylated peptides, and background from unphosphorylated peptides (typically acidic in nature) that have affinity for immobilized metal ions (Lachin, Chait, 2001) Two types of chelating resin are commercially available, one using iminodiacetic acid and the other using nitrilotriacetic acid, but there are some suggestions that iminodiacetic acid resin is less specific than nitrilotriacetic acid (Zhou et al., 2000).

Metal oxide affinity chromatography (MOAC)

The most commonly used MOAC phosphopeptides enrichment strategy employed titanium dioxide (TiO_2) whereas phosphoprotein enrichment relied mostly on aluminium hydroxide ($Al(OH)_3$). The alternative metal oxides are zirconium dioxide (ZrO_2), gallium oxide (Ga_2O_3), ferric oxide (Fe_3O_4), niobium oxide (Nb_2O_3), stannic oxide (SnO_2), hafnium dioxide (HfO_2) and tantalum oxide (Ta_2O_5)—were used only rarely. Titanium dioxide (TiO_2) has very high affinity for phosphopeptides and in recent years it has become one of the most popular methods for phosphopeptide enrichment from complex biological samples. Peptide loading onto TiO_2 resin in a highly acidic environment in the presence of 2,5-dihydroxybenzoic acid (DHB), phthalic acid, lactic acid, or glycolic acid has been shown to improve selectivity significantly by reducing unspecific binding of non-phosphorylated peptides. The phosphopeptides bound to the TiO_2 are subsequently eluted from the chromatographic material using an alkaline buffer. TiO_2 chromatography is extremely tolerant towards most buffers used in biological experiments, highly robust and as such it has become the method of choice in large-scale phosphoproteomics.

Identificaion of phosphorylates proteins by 2D-PAGE electrophoresis

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Commonly, for separation by 2D-PAGE, proteins are subjected to isoelectric focusing and separated by size. The 2D-PAGE is the only method capable of resolving several thousands of proteins at the same time including protein variants produced by the co- or post-translational processing such as phosphorylation, glycosylation, and sulfation (Delom and Chevet, 2006). The phosphorylation of a protein leads to a decrease in its pI and consequently its coordinates in a 2-D gel. To map phosphoproteins on 2D-PAGE, it has been exploited this fact to discriminate phosphoproteins from nonphosphoproteins. Although this method can provide valuable information, it suffers from many limitations, including poor protein representation and an inability to identify low-copy proteins . Another limitation to 2D-PAGE resides in the fact that only some proteins with molecular weight between 10 and 100 kDa are visualized. Furthermore, 2D-PAGE is poorly suitable to resolve integral membrane proteins due to proteins aggregation during the first isoelectric-focusing (IEF) migration. Thus, the limitations of 2D-PAGE have inspired the development of several methods (Graves, Haystead, 2002).

Specific stainings of phosphoproteins

SDS-PAGE and 2D-PAGE are a widely used technique for the separation, identification, and characterization of phosphoproteins. After SDS-PAGE electrophoreses we can visualize separated phosphorylated proteins with appropriate stainings. Few chromogenic dyes have been introduced to visualize mentioned polypeptides in polyacrylamide gels. For example carbocyanine dye “Stains-All” is capable of detecting phosphoprotein bands in blue, while non-phosphoprotein bands in red. However, in this method there are some drawbacks i.e. light-instability, low sensitivity, poor linearity with the protein concentration and heat treat requirement. Another dye is Gel-Code which is often used to analyze gel-separated proteins that are phosphorylated at serine and/or threonine residues. Nonetheless it suffers from a lack of sensitivity with a detection limit of 80–100 ng of protein. The most commonly used commercial dye is Pro-Q Diamond (Invitrogen™), which may simply and directly stain phosphoproteins phosphorylated on tyrosine, serine or threonine residues. However the major disadvantage is high cost of this reagent. Similar sensitivity and simplicity of detection was ascertained in case of staining with use of quercetin (3,5,7,4′,5′-pentahydroxyflavone). About 16–32 ng of phosphoproteins could be selectively detected by mentioned fluorescent dye.(Wang et al., 2014).

Mass spectrometry

In recent years, mass spectrometry (MS) has become an increasingly viable alternative to more traditional methods of phosphorylation analysis. Provided that the MS resolution is sufficiently high, it is also feasible to

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determine the distribution of the number of attached phosphates [14]. For more detailed analysis of the sites of phosphate attachment and stoichiometry, it is necessary to examine peptide fragments of the phosphoprotein of interest. Such fragments are usually generated by digestion of the phosphoprotein with site-specific proteases such as trypsin. Most of the work described here relates to MS measurements of phosphopeptides. every phosphorylated component of the protein should be detected. Unfortunately, MS analysis of proteolytic digests of proteins rarely provides 100% coverage of the protein sequence, and regions of interest are easily missed. In addition, negatively charged modifications can hinder proteolytic digestion by trypsin, the protease of choice in many applications. Phosphorylation is often sub-stoichiometric, such that the phosphopeptide is present in lower abundance than other peptides from the protein of interest. Finally, the mass spectrometric response of a phosphopeptide may be suppressed relative to its unphosphorylated counterpart, and this suppression tends to be enhanced in the presence of other peptides. Analysis of phosphopeptides is therefore easier when the number of non-phosphorylated peptides has been reduced to a minimum (i.e. the phosphopeptides have been enriched). Several strategies have been developed to enrich the sample for phosphorylated peptides or phosphoproteins before analysis.

Conclusions

Protein phosphorylation is one of the most challenging posttranslational modifications to study, mainly due to the low abundance and stoichiometry of this event. However, protein phosphorylation is critical for many cellular processes, which therefore rely on the efficient addition or removal of phosphate groups on specific amino acid residues (serine, threonine and tyrosine) of certain proteins. The phosphoproteome consists of the entire complement of phosphorylated proteins in cells, which is mapped or analysed not only for the identification of phosphorylation sites, but also for the quantitation of phosphorylation events in signal transduction pathways in a time dependent manner. The analysis of the phosphoproteome relies on techniques such as: radioactive labeling, immunoprecipitation, immunodetection, immobilized metal affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), specific stainings of phosphoproteins (ProQ-Diamond, quercetin) 1D and 2D-PAGE electrophoresis, mass spectrometry are used to increase the amount of phosphorylated species in the monitoring step.

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EXTENDERS IN BOAR SEMEN PRESERVATION

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Introduction. Cryopreservation induces wide-range changes in the structural membrane integrity of spermatozoa, resulting in a reduction in their fertilizing ability (Yeste, 2016; Yeste et al., 2017; Fraser et al., 2018). Moreover, the specific susceptibility of spermatozoa to cold shock-induced damage has been shown to be a major factor responsible for compromised post-thaw (PT) semen quality (Pettitt & Buhr, 1998; Fraser et al., 2010;

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