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## Functional phosphoproteomics tools for current immunological disorders research

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### ABSTRACT

Signaling networks are key elements in all major aspects of cellular life, playing a major role in inter- and intracellular communications. They are involved in diverse processes such as cell-cycle progression, cellular metabolism, cell-cell communication and appropriate response to the cellular environment. The latter comprises a whole range of networks that are involved in regulation of cell development, differentiation, proliferation, apoptosis, and immunologic responses. The key mechanism involves the transduction of extracellular signals across the cell surface to different effectors in the cytosol and the nucleus. Dysregulation of these pathways is often associated with immunology disorders and malignant diseases such as cancer. One of the most common mechanisms of activation and/or inactivation of signaling transduction pathways is phosphorylation and de-phosphorylation at serine, threonine and tyrosine residues. Phosphoproteomics is playing an important role in our understanding of how phosphorylation participates in translating distinct signals into the normal and or abnormal physiological responses, and has shifted research towards screening for potential therapies for diseases and in-depth analysis of phosphoproteomes. Given the importance of phosphoproteomics in translational research we aim at outlining phosphoproteomic approaches based on mass spectrometry (MS). This review focuses on (1b) the role of phospho signaling in immunology, (2a) current phosphopeptide enrichment methods based on IMAC and titanium dioxide, (2b) phosphopeptide analysis by MS, and (2c) issues concerned with interpretation of phospho spectra by database dependent search. Finally, quantitative methods used in phosphoproteomics such as Stable Isotope labeling with Amino acid in cell Culture (SILAC), isobaric Tag for Relative and Absolute Quantitation (iTRAQ) and Absolute Quantification (AQUA) is discussed in section 3.

**Keywords:** Phosphorylation; Proteomics; Mass Spectrometry; Immunology disorders.

### Abbreviations

**AQUA** Absolute Quantitation; **CID** Collision-Induced Dissociation; **Da** Dalton (molecular mass); **ECD** Electron Capture Dissociation; **ESI** Electron Spray Ionization; **ETD** Electron Transfer Dissociation; **FT-ICR** Fourier transform-Ion Cyclotron Resonance; **H<sub>3</sub>PO<sub>4</sub>** Phosphoric acid; **ICR** Ion Cyclotron Resonance; **IMAC** Immobilized Metal Affinity Capture; **IT** Ion Trap; **iTRAQ** isobaric Tag for Relative and Absolute Quantitation; **kDa** kilodalton (molecular mass); **LC** Liquid Chromatography; **MALDI** Matrix-Assisted Laser Desorption/Ionization ; **MOAC** Metal Oxide Affinity Chromatography; **Mr** Relative molecular mass (dimensionless); **MS** Mass Spectrometry; **MS/MS** tandem mass spectrometry; **m/z** Mass to charge ratio; **PID** Primary Immuno deficiencies; **PTM** Post-Translational Modification; **SILAC** Stable Isotope Labelling with Amino acid in cell Culture; **SIMAC** Sequential Elution from IMAC; **TiO<sub>2</sub>** Titanium dioxide; **TOF** Time Of Flight.

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## 1. Introduction

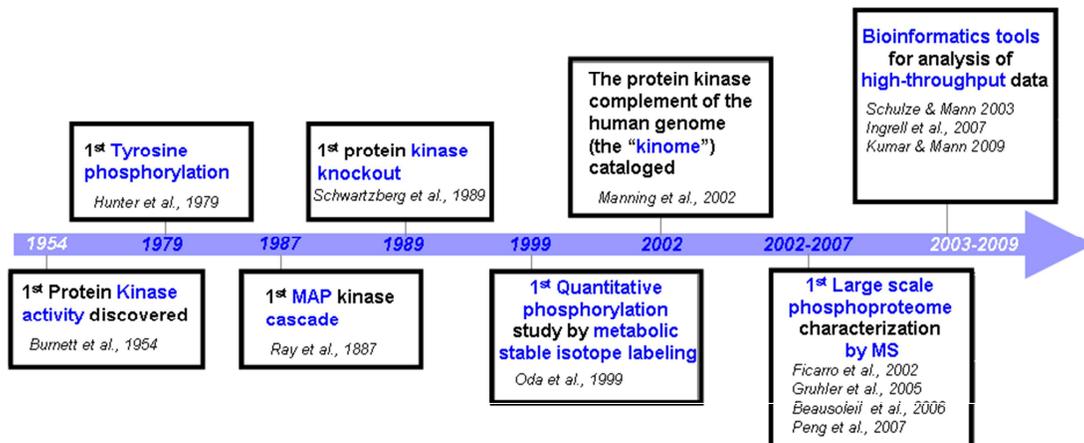
### 1.1 A short history of phosphorylation studies.

In 1954 the first protein kinase activity was observed by using  $^{32}\text{P}$  labeling [1]. The next major finding occurred in 1979, when Tony Hunter and co-workers discovered the first tyrosine phosphorylated by a signaling cascade [2]. This was shortly followed by the elucidation of the amino acid sequence of the first kinase (PKA) by Edman degradation [3]. The spotlight in the late eighties and nineties was focused on the characterization of signaling cascades, mainly the MAP

kinases [4]. Indeed, this period witnessed the purification and identification of the first protein tyrosine phosphatase [5]. One of the major landmarks of the late 1980s was the creation of the first protein kinase knockout in mouse [6]. In 1997, the use of sophisticated genetic engineering allowed the manipulation of the amino acid residues in tyrosine kinases [7]. Recently, Ficarro *et al.* [8] and Gruhler *et al.* [9] reported the identification of thousands of phosphopeptides including the phosphorylation sites from yeast by using a combination of phosphoproteomic tools such as phosphopeptide enrichment and tandem MS. In 2002 the proportion of the human genome encoding protein kinases was analyzed and for the first time a complete set of human kinase sequences were assembled and classified [10]. This study defined the human kinome, which contains around 500 protein kinases, many of which are considered likely therapeutic targets. In recent years large scale phosphoproteome analysis quantitatively profiled thousands of phosphopeptides [11, 12, 13, 14, 15, 16, 17, 18]. Finally, bioinformatics has become an important team player when the phosphopeptide results from latest generation of mass spectrometers needs to be tested for association with existing molecular information (Figure 1).

### 1.2. Relevance of phosphorylation in immunology disorders

During an infection caused by agents like fungi, bacteria, or viruses, pathogen components bind to receptors on immune cells, and trigger the activation of signaling pathways [19, 20, 21]. This activation stimulates the production of inflammatory mediators, including pro-inflammatory cytokines like tumor necrosis factor, interleukin-1, chemokines, prostaglandins and interferons. Interestingly, the precise production of inflammation mediators depends on body site and type of inflammation. All these mediator components can act locally and are also secreted into the blood where they mount responses to fight the invading pathogen. However, the uncontrolled production of these compounds can cause tissue



**Figure 1.** Evolution of protein phosphorylation studies in phosphoproteomics. Different studies on protein-phosphorylation and recent progress on MS based proteomics are shown for the last fifty years. In the beginning analysis of specific kinases by classical molecular biology techniques initiated the interest in phosphoproteomics. Nowadays, large-scale studies can be carried out thanks to advances on MS-based proteomics tools and computational methods.

damage. Increased levels of pro-inflammatory cytokines are also characteristic of chronic inflammatory and autoimmune diseases, like for example: Polymyalgia Rheumatica, Addison's disease, Systemic lupus erythematosus, Type 1 Diabetes Mellitus, inflammatory bowel disease, asthma, rheumatoid arthritis and psoriasis [22, 23, 24].

The most commonly used anti-inflammatory drugs are glucocorticoids but there are side effects associated with the long-term use of steroids. There is a need for anti-inflammatory drugs with fewer side effects, orally active, more effective and less expensive. The potential targets of such drugs could be components of the signaling pathways that regulate the production of inflammatory mediators.

While autoimmune diseases are complex and involve deregulation of both the innate and adaptive immune system. The study of the response of innate immune cells to the components of the pathogens which bind the immune receptors proved to be a productive way of dissecting the signaling pathways which control the production of pro-inflammatory cytokines.

Recently, protein-kinases have become the pharmaceutical industry's most studied class of drug targets and a number of drugs that inhibit these enzymes have been approved for the treatment of cancers. Additionally, recent evidence suggests that several protein kinases of the innate immune system are potentially attractive targets for the development of drugs to treat chronic inflammatory diseases [25].

Cancer, at a molecular level, is considered a state of altered signaling. One of the most common mechanisms of activation and/or inactivation signaling pathways is phosphorylation and de-phosphorylation at serine, threonine and tyrosine residues. This type of modification controls a variety of cellular processes including cellular growth, proliferation, cell cycle control, cytoskeletal mobility and receptor regulation [26]. Phosphorylation leads to allosteric modifications that may result in sufficient conformational changes that cause activation or inactivation of various proteins and associated altered functioning. We hypothesize that identification of phosphoproteins associated with the various stages of cancer may provide information on the mechanism of tumorigenesis and insights into the development of diagnostic and therapeutic procedures. The mitogen activated protein kinase (MAPK) pathways are known to be deregulated in many human malignancies [27, 28, 29, 30]. The best studied MAPKs, with regards to malignancy, are the extracellular signal regulated protein kinases (ERK). ERKs phosphorylate cytoplasmic targets or migrate to the nucleus where they can activate transcription factors involved in cellular proliferation. Aberrant signaling in the MAPK/ERK pathways has been described in prostate, breast and colon cancers *in vitro* as well as *in vivo* models [31, 32, 33, 34]. In addition, in cervical cancer, one study has described decreased activation of ERK1/2 in invasive cervical carcinoma [35]. A second relevant example is annexin A1, which is a calcium dependent phospholipid binding protein that has been linked to membrane trafficking through exocytosis and endocytosis [36].

Other studies have evaluated the role of annexin A1 in the modulation of the MAPK/ERK [37]. Indeed, many members of the Annexin family are known to undergo alternative splicing yielding a number of isoforms. The resultant variant forms may have different functions and binding capacity compared to the native forms [38]. Another relevant example is related to the DNA-Protein Kinase catalytic subunit (DNA-PKcs), a macromolecule found to be involved in the repair of double stranded DNA breaks through activation of p53 which was found to be expressed in cancer specimens in its tyrosine phosphorylated and cleaved form [39]. In contrast, in normal specimens DNA-PKcs existed in its intact, full length and non-phosphorylated form. The aim of this study was to identify differential expression and modification of proteins that could suggest aberrant pathways which could serve as novel targets for developing new therapies in the treatment of cervical cancer and aide in monitoring disease recurrence or progression.

In addition, we will provide examples of the application of phosphoproteomics in immunological studies. The CDC25 family of proteins is comprised of dual specificity phosphatases that regulate cell cycle transitions, and are key targets of the checkpoint machinery to maintain genome stability during DNA damage. Three isoforms of CDC25 have been identified in mammalian cells: CDC25A, CDC25B, and CDC25C. CDC25A and CDC25B overexpression has been reported in many types of human cancers, but is insufficient to cause cancer, and the mechanism responsible for CDC25 overexpression is unclear [40, 41].

Studying dose-response effects of the anti-cancer drug rapamycin on the phosphoproteomics level has identified hundreds of novel rapamycin-targeted cellular proteins and their phosphorylation sites. This information enabled us to identify CDC25B as the key enzyme in mediating rapamycin induced oncogenic AKT activation. Importantly, we show that phosphoproteomic profiling of a certain therapeutic agent cannot only identify potential drug target(s) to improve the efficacy of that therapeutic approach in disease treatment, but can also provide cellular information on possible beneficial and adverse side effects of a certain disease therapy when treating patients [42].

Primary Immunodeficiencies (PID) are "nature's experiments" which have allowed not only the elucidation of many signaling pathways but also their function and clinical relevance.

An interesting example is related to Bruton's tyrosine kinase (Btk; member of the Tec family of kinases) [43, 44], important in B-lymphocyte development, differentiation, and signaling. Btk is predominantly expressed in B lymphocytes and monocytes but not in plasma cells [45, 46]. Btk expression in the B-cell lineage is also developmentally regulated, with bone marrow-derived hematopoietic stem cells, common lymphoid progenitor cells, developing B and myeloid lineages showing the highest levels, whereas resting mature cells prior to activation have reduced cellular Btk. As B lymphocytes are the only cells known to be affected in X-linked

agammaglobulinemia (XLA), the physiological significance of Btk expression in other cell types remains to be established.

Mutations in the Btk gene lead to XLA in humans and X-linked immunodeficiency (Xid) in mice. Activation of Btk triggers a cascade of signaling-events that culminates in the generation of calcium mobilization and fluxes, cytoskeletal rearrangements, and transcriptional regulation involving nuclear factor- $\kappa$ B (NF- $\kappa$ B) and nuclear factor of activated T cells (NFAT). In B cells, NF- $\kappa$ B was shown to bind to the Btk promoter and induce transcription, whereas the B-cell receptor dependent NF- $\kappa$ B signaling pathway requires functional Btk. Moreover, Btk activation is tightly regulated by a plethora of other signaling proteins including protein kinase C (PKC), Sab/SH3BP5, and caveolin-1. Additionally, the prolyl isomerase Pin1 negatively regulates Btk by decreasing tyrosine phosphorylation and steady state levels of Btk [47]. It is intriguing that PKC and Pin1, both of which are negative regulators of Btk, bind to the pleckstrin homology domain of Btk. To this end, novel mutations in the pleckstrin homology are being investigated, in order to design selective and novel drugs [48].

Another relevant example is related to IgA deficiency [49, 50, 51]. IgA plays a role in systemic immunity which is still not understood, IgA antibodies in secretions can neutralize viruses, bind toxins, agglutinate bacteria, prevent bacteria from binding to mucosal epithelial cells, and bind to various food antigens preventing entry into the general circulation (Table 1).

Because IgA is known to protect mucous surfaces, it is a mystery why most IgA-deficient subjects remain healthy. This lack of disease in IgA deficiency is often attributed to a compensatory increase in IgM in the secretions [52]; the colostrum of IgA-deficient subjects has been shown to contain abundant amounts of IgM. The main immunological difference between the IgA-deficient and the normal intestinal tract is the substitution of IgM secreting plasma cells for IgA-secreting cells [53]. This difference is evident in both healthy and ill IgA-deficient subjects.

When nodular lymphoid hyperplasia develops, the nodules contain a proliferation of IgM plasma cells.

Despite the fact that most IgA-deficient subjects are not ill, IgA deficiency has been associated with an astonishing number of specific disorders (Table 2) [51, 54, 55, 56].

A fundamental defect in IgA deficiency is the failure of IgA-bearing B lymphocytes to mature into IgA secreting plasma cells. This appears to be a defect of stem cells, since IgA deficiency can be transferred by bone marrow engraftment. A cardinal feature is that there is a paucity of IgA-bearing gastrointestinal plasma cells. There are decreased (but not absent) numbers of IgA-bearing B cells in the peripheral circulation in these patients, which bear an immature phenotype; that is, IgA-bearing B cells that also are positive for IgM and IgD. The reason that IgA deficient subjects have B cells that fail to switch to the production of IgA is unknown. There is much to learn about the role of IgA in human immunity. IgA is the most abundant immunoglobulin

**Table 1.** Examples of specific human IgA antibody reactivity

(a) Bacteria	Bacteroides fragillis
	Bordetella Pertussis
	Campylobacter
	Clostridiumdiphtheriae, C. tetani
	Escherichia coli O, K antigens, enterotoxin
	Salmonella
	Shingella
	Streptococcus
	Streptococcus mutans
	Neisseria gonorrhoeae
Vibrio cholerae	
(b) Fungi	Candida albicans
(c) Protozoa	Giardia
(d) Viruses	Arboviruses - Semliki forest
	Coxsackie virus
	Cytomegalovirus
	Dengue
	Echovirus
	HIV
	Influenza A
	Parainfluenza
	Poliovirus 1, 2, 3
	Respiratory syncytial virus
	Rhinovirus
Rotavirus	
Ross river	
Japanese B	
(e) Others	Milk proteins
	Peanut lectin
	Soy lectin
	Wheat gluten, gliadin

made and also the most ignored. IgA is the second-most prevalent immunoglobulin in serum, but its role in systemic immunity is unknown. IgA has a known biological activity against a number of pathogens and can be shown to serve as a barrier preventing the permeation of foreign antigens and pathogens; on the other hand, lack of IgA does not usually result in a perceptible immune defect [57].

Because no genetic defect is known in IgA deficiency and its clinic diversity, many genetic studies have been focused on the relationship of IgA deficiency with HLA system [58, 59, 60, 61, 62, 63, 64], complement components [65, 66], transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) [67, 68, 69] and other genes [70, 71, 72, 73, 74]. Recently, a genome-wide association study identified association with Interferon-induced helicase C domain containing protein 1 (IFIH1) and C-type lectin domain family 16 (CLEC16A) [75].

The phosphoproteomics of IgA deficiency could explain not only why IgA deficient people are asymptomatic but also why some patients present infections and/or autoimmunity

**Table 2.** Examples of conditions associated with selective IgA deficiency

(a) Allergy
Asthma, atopy, eczema
(b) Autoimmunity
Rheumatoid arthritis ITP, hemolytic anemia, pernicious anemia, systemic lupus erythematosus, Still's disease, transfusion reactions due to anti IgA antibody, dermatomyositis, vitiligo, Sjogren's syndrome, Henoch-Schonlein syndrome, primary biliary cirrhosis, autoimmune hepatitis
(c) Endocrinopathy Thyroiditis
Graves disease, idiopathic Addison's disease, diabetes mellitus, 21-hydroxylase deficiency
(d) Gastrointestinal diseases
Giardiasis, Crohn's disease, ulcerative colitis, nodular lymphoid hyperplasia, celiac disease, lactose intolerance, malabsorption, villous atrophy, achlorhydria, cholelithiasis
(e) Malignancy
Gastric carcinoma and lymphoma
(f) Neurological
Seizures, migraine, sensory neuropathy, myasthenia gravis, cerebral vasculitis
(g) Respiratory tract
Recurrent sinopulmonary infections, sarcoidosis, pulmonary hemosiderosis

and/or other pathological situations.

XLA and IgA deficiency are two relevant examples of PID because in XLA, the genetic disorder was observed for the first time in a PID and in IgA deficiency, the most frequent PID, the genetic disorder is unknown yet.

Many other PID are interesting for analysis with phosphoproteomics tools because their genetic defects involve many signaling pathways depending on phosphorylation/dephosphorylation phenomena, such as IL-1 receptor associated kinase 4 (IRAK 4) deficiency with functional defects of TIR-IRAK signaling pathway and bacterial infection (pyogenes) in the patients [76]. Another example is the Hyper IgE syndrome, a clinical situation where three different genetic disorders including protein phosphorylation can cause the disease, it has mutations in either signal transducer and activator of transcription protein 3 (STAT 3) [77, 78] or tyrosin kinase 2 (TYK 2) [79] or in the dedicator of cytokinesis 8 (DOCK 8) [80].

For up to date information on PID with defects in signaling pathways involving phosphorylation, see the reference of the International Union of Immunology Societies Expert Committee on Primary Immunodeficiencies [81].

Current phosphoproteomics strategies allow the study of

different immunological diseases. The advantage over other methodologies, is that they allow the assignment of the specific amino acids which are phosphorylated and or dephosphorylated when comparing healthy and malignant tissues/cells. In addition, phosphoproteomics gives spatial and temporal biological information. Therefore, it will help to establish the right and more specific prognosis and diagnosis, and therefore to treat different immunological disorders with more accurate therapies. Indeed, these findings suggest that it plays a critical role in studies related to the discovery of therapeutic targets [26, 82].

Nowadays, many hospitals have included and are including MS technologies in order to collaborate with/and for clinical research, covering many kinds of pathologies. Thus, improving the therapy - treatments of the patients.

### 1.3. *The role of phosphorylation in protein structure and function*

Reversible phosphorylation of proteins modulates a great variety of cellular processes. Biological regulation and signaling is in this manner controlled by kinases, phosphatases and phosphopeptide recognition domains of effector proteins [83]. Peptides phosphorylated mainly on serine, threonine or tyrosine residues are specifically recognized by a variety of protein domains. Phosphotyrosine (pTyr)-containing peptides interact with SH2 (Src Homology 2) and PTB (Phosphotyrosine Binding) domains. Phosphoserine and phosphothreonine (pSer/pThr) are recognized by an increasing variety of modular protein domains, including 14-3-3, WW, FHA (Forkhead Associated), Polo-box, and BRCT (BRCA1 C-terminal) among others [83, 84]. In plants, only 14-3-3 and FHA domains are found to mediate phosphopeptide binding processes [85].

According to the classical theory, all these domains discriminate between the phosphorylated vs. the non-phosphorylated state of proteins, based on the conformational changes induced by the presence of a negatively-charged phosphate group in the basal state of the phosphopeptide. Structure/function experiments performed using NMR relaxation techniques on the phosphorylated state of the response regulator "Nitrogen regulatory protein C" from *Salmonella typhimurium* [86] indicated that both states (active and inactive) are present before phosphorylation, suggesting that this process only acts through the modification of the preexisting equilibrium. Changes induced by phosphorylation could be of different magnitude. In some cases, adding of phosphate groups leads to partially unfolding of previously structured domains, making them more accessible to phosphopeptide recognition domains, as it has been demonstrated by NMR studies in the case of the KH-domain 1 of the RNA binding K-homology splicing regulator protein and its interaction to 14-3-3 protein [87].

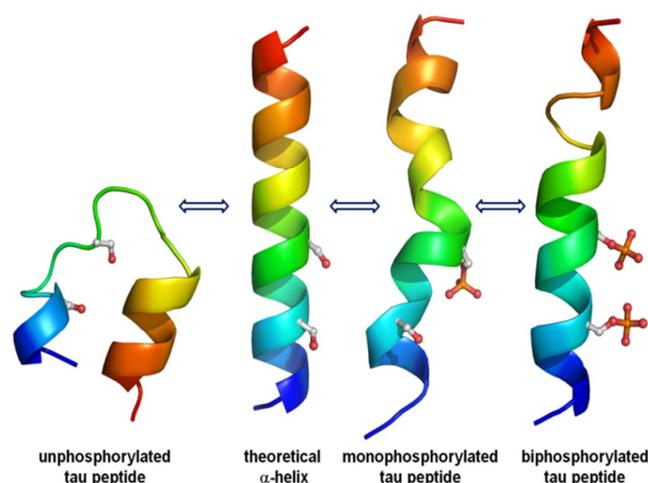
Since no high resolution 3D coordinates obtained by NMR or crystallization procedures are available, the increasing capacity and accuracy of computational simulations allow us to generate models of the different phosphorylation states of

a protein. As an example, these methods have been used to explore low-energy conformations of the phosphorylation-regulated structure of the R domain of the cystic fibrosis transmembrane conductance regulator [88]. The results suggested that the increment of the radius of gyration of the phosphorylated states of the domain was correlated with the activation of the chloride channel through reorganization of its helix packing.

Phosphorylation can not only be correlated with changes in tertiary structure but also with changes in secondary structure. A recent study [89], monitoring the secondary structure changes of a coiled-coil model peptide through Circular Dichroism, found a sequential cascade starting from a stable helical state continued by a phosphorylation-induced unfolded structure and finishing, in this particular case, in a magnesium stabilized alpha-helix structure. Computational simulation could also be very useful to model this type of changes. A good example of this could be the comparative study, using both Molecular Dynamics and Circular Dichroism techniques, of the displacement in the equilibrium between the unphosphorylated peptide of the third tau microtubule-binding repeat and its mono- and diphosphorylated states [90]. Molecular Dynamics models predicted a stabilization of the peptide in a short alpha helical structure mediated by phosphorylation. This arrangement, contrasted using Circular Dichroism approaches, will lead the peptide to a homo-polymerized complex, connecting the structural changes due to phosphorylation with modifications in the functional behavior of the peptide (Figure 2).

In recent years, simulation techniques based on Quantum Mechanics are increasing their capacity and are being implemented at a scale that allows the simultaneous calculation of the behavior of several dozens of atoms. Particularly, these methods permit the analysis of the formation and breaking of covalent bonds in the active center of proteins (Quantum Mechanics) within the framework of the atomic models of complete biological macromolecules, calculated using classical Molecular Dynamics. These methodologies, usually termed QM/MM (Quantum Mechanics / Molecular Mechanics) interface [91], enable the analysis of some biological relevant events as the hydrolysis of peptide bond by proteases [92], hydrolysis of nucleoside triphosphates molecules in the active center of nucleosidase enzymes [93] or phosphorylation and dephosphorylation [94, 95, 96] processes, taking in account not only detailed aspects of formation and rupture of covalent bonds but also the movements of the entire protein associated to them [97].

Some of the effects of protein phosphorylation in protein structure must be studied taking into account that these effects are not stable but transient, allowing the presence of both phospho- and dephosphorylated states of the protein in a dynamic equilibrium [95]. In the near future, QM/MM interface techniques will not only be able to simulate the behavior of the two extreme states of the protein, but also the intermediate situations of phosphate ions being added or removed from the surface of the macromolecules. Therefore,



**Figure 2.** *Phosphorylation in protein structure and function.* Simulation, using Molecular Dynamics techniques, of the different equilibrium states of a peptide from the third tau microtubule-binding repeat. Phosphorylation displaces the pre-existing equilibrium towards a stabilized short alpha helical structure (modified from [84]).

QM/MM interface will be able to analyze how these continuous processes can affect transient interactions with other proteins in signaling cascades [97] associated to immune response or diseases such as cancer.

## 2. Current proteomic strategies to study phosphorylated proteins and peptides

Phosphorylation plays important roles in regulating protein structure and function. Therefore, it also modulates protein catalytic activity, subcellular localization, and interaction processes [98, 99]. Protein reverse phosphorylation [100], concert actions of kinases and phosphatases, and plays a central role in all processes, especially in cell signaling.

Phosphoproteomics involves the identification and quantification of phosphoproteins, and also the linkage of phosphosites to specific activation of the identified proteins. The analysis of the spatial and temporal aspects of protein phosphorylation is of great interest to the discovery of functions of specific biological processes.

### 2.1 Phosphopeptides enrichment methods used previous to MS analysis

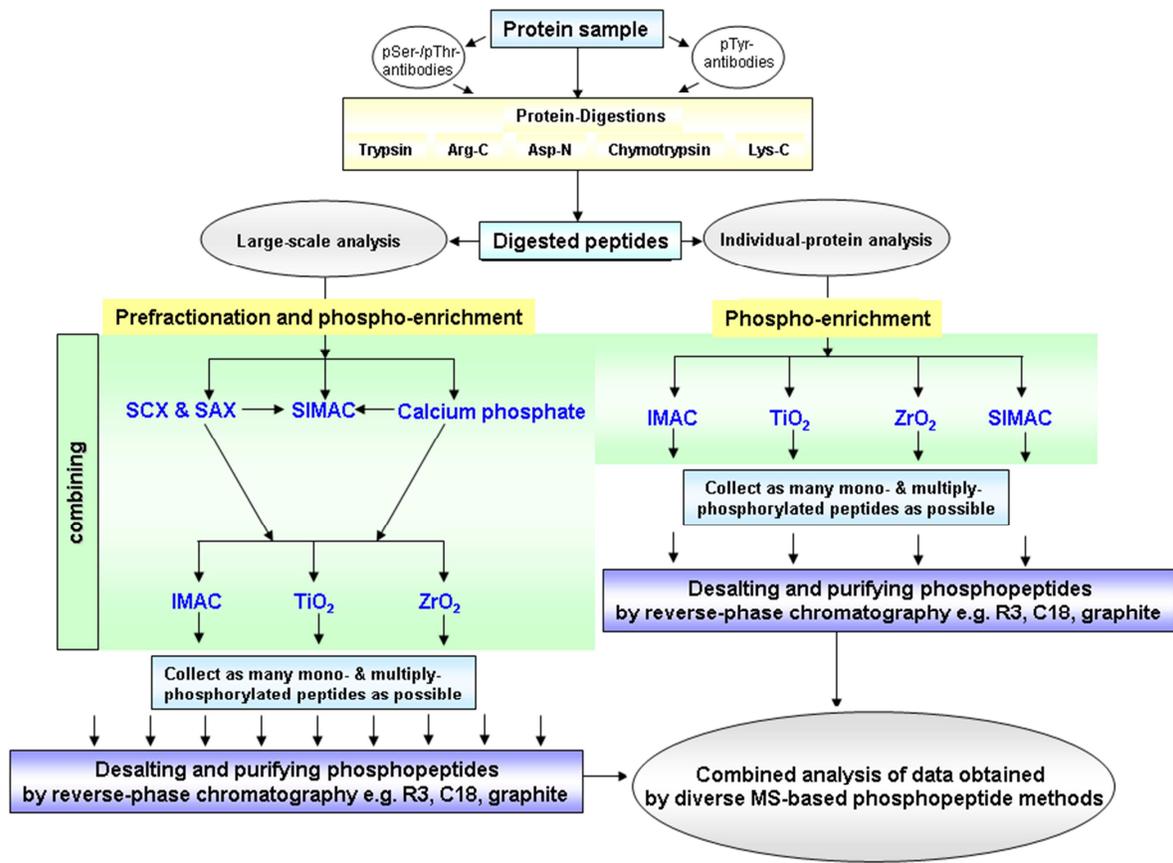
For many approaches, including the study of immune disorders, the goal is to generate a global view of serine, threonine, and tyrosine phosphorylation within the sample, focusing specifically on the selected subset of phosphopeptides. Since the detection of phosphopeptides by MS is often hindered by suppression effects, many different strategies for removing unphosphorylated peptides have been established: immunoprecipitation by antibodies, pre-fraction systems such as ionic chromatographic exchange (SCX/SAX) and calcium phosphate precipitation, metal affinity chromatography e.g. IMAC,  $\text{TiO}_2$ ,  $\text{ZrO}_2$ , and reverse phase chromatography (Figure 3).

Immunoprecipitation of phosphotyrosine is still much more frequent [101] than immunoprecipitation using phospho-serine or threonine antibodies. The reason is that affinity chromatography such as IMAC or titanium dioxide has higher capacity for phosphoserine and phosphothreonine peptide binding [102].

The most common techniques to enrich for individual and/or global phosphorylation are IMAC and Titanium Dioxide (TiO<sub>2</sub>) [103], which are based on the high affinity of phosphate groups for metal ions such as Fe<sup>3+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup> and Ga<sup>3+</sup>. The phosphopeptides that are negatively charged will bind the positively charged metal ions by electrostatic interactions. One of the main limitations associated with both phosphopeptide enrichments is the nonspecific retention of non-phosphorylated acidic peptides, due to the weak affinity between negatively charged carboxylate and positively charged metal ions. However, conversion of carboxylate groups to esters effectively eliminates nonspecific retention of non-phosphorylated peptides, although this has the drawback of increased complexity in the subsequent MS analysis. During the last five years, titanium dioxide (TiO<sub>2</sub>) has emerged as the most common of the metal oxide affinity chromatog-

raphy (MOAC) based phosphopeptide enrichment methods. This technique offers increased capacity compared to IMAC resins in order to bind and elute mono-phosphorylated peptides. TiO<sub>2</sub> exploits the same principle as IMAC, and is similarly prone to nonspecific retention of acidic non-phosphorylated peptides. However, when loading peptides in 2, 5-dihydroxybenzoic acid (DHB) [104], glycolic and phthalic acids, nonspecific binding to TiO<sub>2</sub> is reduced, thereby improving phosphopeptide enrichment without chemical modification of the sample. In addition, TiO<sub>2</sub> is often considered to be interchangeable with IMAC. It works on similar levels of sample amounts (e.g., micrograms of protein) for the identification of phospho-sites by MS analysis. Recently, SIMAC [105, 106] appeared as a phosphopeptide enrichment tool which is exploiting the properties of IMAC coupled to TiO<sub>2</sub>, making it possible to carry out more refined studies.

Another phosphopeptide enrichment prior to mass spectrometric analysis is ZrO<sub>2</sub> [107] and its principle is based on metal affinity chromatography like IMAC and TiO<sub>2</sub>. ZrO<sub>2</sub> allows isolation of single phosphorylated peptides more selectively than TiO<sub>2</sub> when using  $\alpha$  and  $\beta$  casein as protein models. Indeed, it has been successfully used in the large-scale charac-



**Figure 3.** Schematic workflow of current phosphoproteomic strategies. Phosphorylated proteins obtained by immunoprecipitation from tissue or cell extract must be digested using one or a combination of different enzymes prior to bottom up MS. For a unique phosphoprotein analysis, IMAC, TiO<sub>2</sub>, ZrO<sub>2</sub> or SIMAC are directly applied to the resulting peptides. For large scale phosphopeptide analysis, the peptides must be loaded onto pre-fraction columns (SCX/SAX, Calcium phosphate or SIMAC is used). In order to capture as many phosphopeptides as possible, it is necessary to use different phosphopeptide enrichments methods (IMAC, TiO<sub>2</sub>, ZrO<sub>2</sub>) when applying pre-fraction methods. Finally, the phosphopeptides which have been previously captured combining different phospho-strategies, must be purified, desalted and concentrated by reversed phase chromatography (POROs R3, C18 Disks and/or graphite) in order to be analyzed by MS.

terization of phosphoproteins.

Furthermore, a strategy which consists of fractionating and subsequently enriching phosphopeptides on a proteome wide scale is based on strong cation/anion exchange (SCX and SAX) chromatography. The principle of SCX/SAX phosphopeptide enrichment is based on the negative charged phosphate group ( $\text{PO}_4^-$ ) of the phosphopeptides. In cation exchange chromatography, a positively charged analyte is attracted to a negatively charged solid-support, while in anion exchange chromatography negatively charged molecules are attracted to a positively charged solid-support. SAX has previously been successfully combined with IMAC [108] and resulted in greater recovery and identifications by MS of mono-phosphorylated peptides originating from membrane proteins. SCX has in a similar way been combined with IMAC ( $\text{Fe}^{3+}$ ) and MS analysis, allowing the identification of thousands of phosphorylated residues from complex biological samples [9].

Calcium phosphate precipitation is a strategy that provides a useful pre-fractionation step to simplify and enrich phosphopeptides from complex samples. Zhang and co workers [109] have demonstrated that phosphopeptide precipitation by calcium phosphate combined with a two step IMAC ( $\text{Fe}^{3+}$ ) procedure resulted in the observation of an increased number of phosphopeptides. This method consists of precipitating phosphopeptides by adding 0.5M  $\text{NaHPO}_4$  and 2M  $\text{NH}_3\text{OH}$  to the peptide-mixture followed by 2M  $\text{CaCl}_2$ . The sample is vortexed and centrifuged, and, subsequently, the supernatant is removed before washing the pellet with 80 mM  $\text{CaCl}_2$ . The washed pellet is dissolved in 5% of formic acid and the resulting peptide mixture is desalted by reversed phase chromatography before isolating the phosphopeptides by IMAC ( $\text{Fe}^{3+}$ ).

Finally, since most phosphopeptide analysis is nowadays performed by MS, and this technique is sensitive to contaminants such as salts, it is necessary to clean up the samples prior to analysis, generally by reversed phase chromatography combining POROs R3 with C18 Disks and also graphite powder [110, 111, 112]. Poros R3, C18 Disks and graphite powder are materials containing long hydrocarbon chains, proven to be effective for the desalting and cleaning of very hydrophilic peptides, including phosphopeptides [104, 113]. In 1999, Gobom and co-workers [110] introduced a micro column purification method in which a chromatographic resin was packed in the tip of a small constricted GELoader tip, creating a micro-column. Using a chromatographic approach, GELoader tips packed with R3, C18 or graphite material, contaminants like salts can be separated from the phosphopeptides. Indeed, using RP chromatography, molecules such as proteins, peptides and nucleic acids are separated according to their hydrophobicity. In addition to the removal of salts, these techniques also facilitate a concentration of the sample by the use of a low elution volume. This further improves the sensitivity and quality of the subsequent mass spectrometric analysis.

The phosphopeptide enrichments may be coupled with stable isotope labeling steps (such as SILAC, ICAT, iTRAQ

and  $^{18}\text{O}$ ) for relative quantification and/or with AQUA analysis for absolute quantitative analysis [114, 115]. Since different metal ions appear to enrich for slightly different subsets of phosphorylated peptides, maximal coverage of the phosphoproteome may be obtained by multiple analyses with different metals, or by mixing multiple metal ions in a single enrichment step [26].

## 2.2 MS analysis of phosphopeptides

There have been important improvements in the MS technology concerning sampling throughput, sensitivity and mass accuracy over the past decade, but technical limitations in instrumentation still exist that make it impossible to fully sample the entire phosphoproteome. In addition, reproducibly and accurately measuring protein phosphorylation via MS presents many challenges [116]. Among these challenges is the fact that, many phosphorylated proteins are present in low overall abundance yet can have pronounced and important biological effects through their involvement in signaling cascades and other forms of downstream amplification such as transcriptional control. These properties make phosphoprotein or peptide enrichment a prerequisite for accurate analysis and increase sensitivity in MS detection. Robust sample lysis conditions to ensure stability of phosphoproteins that truly depict the phosphorylation state of proteins at the time of cell lysis are crucial. Furthermore, sample cleanup and pre-fractionation steps to crudely enrich for phosphopeptide pools and ensure buffer compatibility for phosphoenrichment protocols are also necessary [103, 117, 118].

Phosphorylation on serine and threonine residues are labile and conventional fragmentation CID (Collision Induced Dissociation) typically results in the partial neutral loss of phosphoric acid ( $\text{H}_3\text{PO}_4$ , 98/z) in  $\text{MS}^2$  mode, due to the gas phase  $\beta$ -elimination of the phosphor-ester bond. Therefore, dehydroalanine and dehydroaminobutyric acid are generated. When peptide ions are fragmented by CID, series of  $\gamma$ - and  $b$ -ions are formed [119, 120]. The peptide sequence is obtained by correlating mass difference between peaks in the  $\gamma$ -ion series or between peaks in the  $b$ -ion series with amino acid residue masses. The CID fragmentation is occurring on the peptide backbone, and only limited sequence information is obtained. This event can also compromise the identification of phosphorylation sites. Related to phosphotyrosine residues, partial neutral loss is also observed ( $\text{HPO}_3$ , 80/z) in  $\text{MS}^2$  mode, but the phosphate group on tyrosine residues is more stable than on serine and threonine residues. In addition, the phospho-finger-print characteristic of phosphotyrosine, is the phosphotyrosine immonium ion (~216 Da), which is a positive indicator for the presence of a peptide phosphorylated on tyrosine [121, 122].

The ion originating from neutral loss of phosphoric acid ( $\text{H}_3\text{PO}_4$ ) can be selected for further fragmentation by  $\text{MS}^3$  mode. The selected ion, after neutral loss fragmentation, is automatically selected for further fragmentation. This gives the possibility to add extra energy for the fragmentation of peptide backbone. Although, the  $\text{MS}^3$  mode requires that the

selected ion is abundant in order to observe the fragmented ions. A pseudo-MS<sup>3</sup> development is MultiStage Activation (MSA) [123], which was implemented on quadrupole-IT and linear IT-orbitrap. In MSA, the fragmentation of the precursor ion occurs simultaneously with the fragmentation of the ion originating from the neutral loss. Then, the MS<sup>2</sup> and MS<sup>3</sup> mass-data are combined in a hybrid spectrum, resulting in improved sequence information and also in an improvement of the confidence for the phosphorylation site assignment.

Alternative fragmentations to CID are ECD (electron capture Dissociation) and ETD (Electron transfer dissociation). By ECD, radical peptide ions are obtained when multiply-charged peptide ions are reacting with low-energy thermal-electrons. In addition, this fragmentation occurs in the peptide between the backbone amide and the alpha carbon, generating c and z-ions [124]. An advantage of ECD is that it occurs only on the peptide backbone, and labile phosphate groups are left intact on the resulting c- and z- fragment ions, thus, enabling the identification of the specific phosphorylation sites. Therefore, it is extremely useful for the analysis of multiply-phosphorylated peptides. One disadvantage of ECD is that it has selectivity for disulfide bonds, due to the high radical affinity of the bond [125, 126]. The main drawback of ECD is only that it is used in the FT-ICR instruments due to the requirement of a static magnetic field for the thermal electrons, meaning high costs and high specialization.

By ETD, c- and z- ions are also generated. Actually, this fragmentation was developed in order to carry out ECD-like dissociation experiments, in a Quadrupole Linear Ion Trap [123, 127]. ETD is a chemical process in which reaction with fluoranthene radical anions disrupts the peptide backbone at regular intervals. ETD preserves the intact information about labile modifications, which are not observed directly when using CID. For instance, phosphate groups are good leaving groups, which mean that they are easily lost in the excitation process. However, by using ETD one can directly observe fragments that contain the intact phosphopeptides. The drawback of ETD is less sensitive compared to CID, because of lower ionization efficiency. As a result, we recommend using CID to start with, and would recommend switching to ETD in case you weren't able to determine the phosphorylation site.

### 2.3 Database dependent search for the identification of phosphopeptides

The new generation of mass spectrometers renders identification of thousands of proteins. However, proteins are normally identified based on several peptides, whereas phosphorylation sites are only identified by few peptides. Since different phosphorylation sites on the same protein usually behave differently [17], each phosphorylation site has to be evaluated individually. In addition, the identification of multi phosphorylated peptides has to be carefully evaluated because of the distinct behavior of individual phosphorylation sites. Identification of phosphopeptide sites involves

database dependent search, application of filter parameters, estimation of false discovery rate and validation.

Care must be taken when defining the search parameters for a data set with spectra of phosphopeptides. The definition of phosphoserine and phosphothreonine modifications must include neutral loss of HPO<sub>3</sub> (79.966/z) and H<sub>3</sub>PO<sub>4</sub> (97.977/z) whereas phosphotyrosine displays no characteristic neutral loss but instead a marker ion at 216/z. Furthermore, an apparent neutral loss of approximately 80/z and 98/z might have other causes than loss of HPO<sub>3</sub> or H<sub>3</sub>PO<sub>4</sub> (Neutral Loss-Based Phosphopeptide Recognition: A Collection of Caveats, Journal of Proteome research, 2007, 6, 2866-2873). Moreover, the negative charge of a phospho group can shield some tryptic sites if located close to the cleavage site. The search parameters must therefore also allow for 2-3 missed tryptic cleavage sites.

The general available search engines are not optimized specifically for phosphopeptide identification and spectra of phosphopeptides are often of lower quality because of the labile phospho group. Therefore quality filtering using information from complementary spectra becomes essential. For example, complementary spectra of a specific phosphopeptide can be obtained by CID, HCD, ETD and MS<sup>3</sup>. Furthermore, digesting the protein solution with alternative enzymes can provide alternative peptides that may ionize or fragment better. Ideally the software used is able to combine information from all the above mention spectra sources and provide a filtered list with high quality results.

The decoy approach can be used to estimate false discovery rate. The decoy approach consists of searching two sequence databases which contains the protein sequences in both forward and reversed (decoy) orientations, respectively. In addition, the number of false-positive hits that are found in the decoy database, is then used to predict the number of false positives found in the forward database. The decoy approach has been described as a useful method for analyzing LC-MS/MS data from yeast [128, 129], and later, it has also been used for phosphoproteome analysis [130, 131]. Search engines like Mascot [132] and VEMS [133] have built in false discovery rate on the peptide level which can be used for filtering. However, no search engine is able to provide a false discovery rate based on combined data from the different spectra sources mentioned above.

Finally, the phosphopeptide assignments must be validated. Validation can for example be done by obtaining spectra on a synthetic phosphopeptide standard which has the same phosphorylation sites as a phosphopeptides of interest from the biological sample.

It can, in some cases, be difficult to assign the correct phosphorylation sites in a peptide containing many serines, threonines and tyrosines. To this end, VEMS has a spectrum viewer that can generate all possible phosphorylation sites for a peptide and overlay the theoretical fragment ions from these with the observed fragment ions [134]. The possible phosphorylation sites are restricted by the observed parent ion mass and the identified peptide sequence. This tool is

useful for manually validating that the correct phosphorylation sites in the peptides are annotated. It also allows detection of spectra originating from two phosphopeptides with the same amino acid sequence but different phosphorylation sites that could not be separated on the reverse phase column. VEMS considers all fragment ion types characteristic for phosphopeptides including multiple combinations of losses of  $-79.966/z$  and  $-97.977/z$  for the scoring.

The software MSQuant (<http://msquant.sourceforge.net>) [17, 18] is compatible with MS<sup>2</sup> and MS<sup>3</sup> searches performed using Mascot. The MSQuant scoring is probability based and takes into account the number of matched b- and y-ions for all possible combinations of phosphorylation sites in a given peptide sequence. Another program, Ascore (<http://ascor.med.harvard.edu/ascor.php>), uses a probability-based scoring algorithm to validate phosphopeptides assigned by SEQUEST [130, 131].

A recent comparison of the database dependent search engines Mascot [132], SEQUEST [135], OMSSA [136], and X!Tandem [137] suggested that it is useful to combine results from several search engines [138]. The comparison indicated that, when searching high mass accuracy data sets, Mascot and SEQUEST obtained higher sensitivity for phosphopeptide identifications [138]. This study also demonstrated that replicate-runs of the same sample significantly increased the total number of phosphopeptide identifications. Although, a particular search algorithm may perform better under certain conditions, researchers should not limit their searches to using just one algorithm since a combined use of search engines offer better coverage [139]. Recent alternative strategies for database dependent search are based on peptide sequence tags e.g. GutenTag [140] and InsPecT [141]. However, the sequence tag based methods have so far not been applied to large scale phosphopeptide analysis. The sequence tag based methods can become important in the future when the quality of phosphopeptide spectra improves.

### 3. MS-based quantitative strategies for phosphopeptides

Proteins containing amino acids with one or more of the stable isotopes of <sup>2</sup>H, <sup>13</sup>C, <sup>15</sup>N or <sup>18</sup>O can be used as internal standards by addition, at an early stage of the analysis, of a complex protein sample. There are two approaches for introducing a stable isotope into a proteins or peptides: metabolic labeling using whole cells grown in culture (e.g. SILAC) or chemical labeling (e.g. iTRAQ, ICAT).

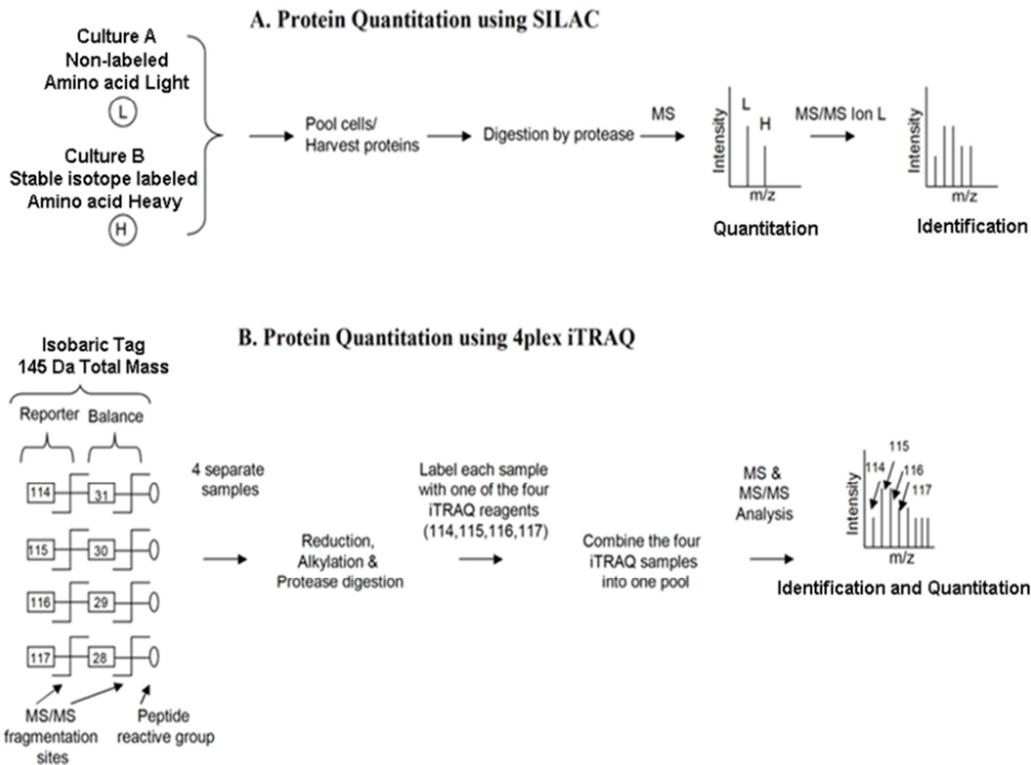
Measuring the changes in phosphorylation is critical for understanding the biology of a phosphorylation event, since protein phosphorylation is very dynamic and constantly changing throughout the life of a cell. We restrict the discussion here to three MS-based quantitation strategies which have direct utility towards measuring changes in protein phosphorylation: SILAC, iTRAQ, and AQUA. Other chemical labeling techniques that rely on stable isotope incorporation using e.g. <sup>18</sup>O labeled water during trypsin digestions and stable isotope incorporation ICAT can also be considered with relevant information, but will not be described here.

#### 3.1 SILAC

Stable isotope labeling by amino acids in cell culture (SILAC) is a quantitative method based on *in vivo* labeling of proteins in cell cultures with amino acids that contain stable isotopes (non radioactive, e.g. <sup>2</sup>H, <sup>13</sup>C and <sup>15</sup>N) [115, 142]. In its simplest form, two separated cell cultures are grown in a pair-wise fashion; for example, culture A might be yeast cells grown under “normal” conditions (light conditions) while culture B might be yeast cells grown in the presence of a stress condition. The growth conditions of the cells are identical (except for the presence of the stress-stimuli), but the growth media of culture B has an essential amino acid (one not synthesized by the cell) replaced with an isotopically “heavy” form of that amino acid (e.g. <sup>13</sup>C<sub>6</sub>-arginine). A number of cell lines have been used for SILAC experiments, and the growth and morphology of the cells have not been affected by the isotopically labeled amino acid [9, 142, 143]. After approximately five rounds of doubling, cellular proteins are essentially 100% labeled with the selected amino acid. After culturing, the light and heavy cell populations are combined (1:1) into one pool and the proteins are isolated. The protein pool is then digested with a protease, typically trypsin, to form a peptide pool that is analyzed by MS. Each peptide analyzed will be present in two forms, the light and the heavy form. The two forms have the same chemical properties, so they have approximately the same chromatographic retentions, ionization efficiencies, and fragmentation characteristics, but they are distinguishable based on the mass difference due to the heavy isotope incorporation in the selected amino acid. The peak signals produced by the light and heavy forms of a peptide are measured by the mass spectrometer, and a relative quantification of that peptide from the two cultures is calculated. Tandem MS is also performed in the same experiment on either the light or the heavy form, therefore, the identity of the peptide and the protein is determined. Indeed, all peptides, both phosphorylated and non-phosphorylated, that contain the isotopically labeled amino acid, are available for relative quantification by SILAC. The SILAC method is compatible with the above mentioned enrichment of phosphoproteins/phosphopeptides including the immunoprecipitation of a target protein [144]. To assist with the enrichment of phosphopeptides in the SILAC method, the combination of SCX chromatography and IMAC, have been employed after proteolytic digestion [9]. This approach enriches the phosphopeptides and helps removing non-phosphorylated peptides that can act as noise in the quantification experiment (Figure 4A).

#### 3.2 iTRAQ

The second method for the global quantification of proteins and protein modifications is an *in vitro* chemical labeling procedure called iTRAQ. The iTRAQ reagent consists of two to eight isobaric (same nominal mass) tags that can be used to label two to eight separate protein samples; for example, one sample might be “normal” yeast cells while the three remaining samples might be yeast cells grown at



**Figure 4.** Scheme of two quantitative proteomic methods based on isotopic labeling. (A) *Protein quantification by SILAC.* Stable isotope labeling with amino acids in cell culture is an approach for the in vivo incorporation of a tag into proteins for relative quantitation by MS. The method relies on the incorporation of amino acids with substituted stable isotopic nuclei (e.g.  $2\text{H}$ ,  $13\text{C}$ ,  $15\text{N}$ ). Two cell populations are grown in culture media that are identical except that one of them contains a “light, L” and the other a “heavy, H” form of a particular amino acid (e.g.  $12\text{C}$  and  $13\text{C}$  labeled L-lysine, respectively). After a number of cell divisions, each instance of this particular amino acid will be replaced by its isotope labeled analog, and the quantitation information is provided in the MS mode by the intensity peaks observed in the spectra. (B) *Protein quantification scheme of the iTRAQ method.* This method is relying on the derivatization of primary amino groups in intact proteins using isobaric tag for relative and absolute quantitation. Due to the isobaric mass design of the iTRAQ reagents, differentially labeled proteins do not differ in mass; accordingly, their corresponding proteolytic peptides appear as single peaks in MS scans. Because quantitative information is provided by isotope-encoded reporter ions that can only be observed in MS/MS spectra. In addition phosphorylation analysis by MS and MS/MS can be carried out on iTRAQ labeled peptides to obtain quantitative phosphopeptide information.

three different concentrations of a stress treatment. The iTRAQ tags contain three regions: a peptide reactive region, a reporter region, and a balance region [145]. The peptide reactive region of the tag consists of an NHS ester and is designed to react with the N-termini and lysines of peptides after protease digestions. In the case of 4-plex iTRAQ, the four reporter groups appear in the tandem mass spectrum at  $m/z$  114, 115, 116, and 117. The attached balance groups are designed to make the total mass of the balance and reporter group 145 Da for each tag, resulting in balance groups of 31 Da, 30 Da, 29 Da, and 28 Da, respectively. Protein samples for quantification are separately isolated and digested proteolytically, and each sample is chemically labeled with one of the iTRAQ reagents. After labeling, the samples are combined and subsequently analyzed by MS. As the iTRAQ reagents are isobaric, identical peptides from each sample will have identical masses, therefore there is no division of the precursor signals in the first stage of mass analysis that could lead to increased spectral complexity by the combination of multiple samples. Additionally, the isobaric nature of the reagent increases the ion population for a given peptide by summarizing the amount of a peptide from each sample. This

makes the detection of the peptides more sensitive. During tandem MS, fragmentation takes place along the peptide backbone and also between the reporter and balance region of the tag which facilitates the quantitation based on the intensity of the reporter ions. The reporter ions in the tandem mass spectrum are in general more intense than the fragment ions. The relative amounts of these reporter ions correspond to the relative amounts of the peptides present in the four samples. In contrast to SILAC and AQUA (described below), it is during tandem MS experiments, and not during the first stage of mass analysis, that relative quantification of peptides takes place. Phosphoproteins can be analyzed in an identical manner as well as non-phosphorylated proteins with the iTRAQ methodology. The iTRAQ reagent labels phosphopeptides to the same degree as non-phosphorylated peptides and it does not affect the stability of phosphopeptides. Enrichment strategies, such as IMAC [146, 147] or immunoprecipitation with anti-phosphotyrosine antibodies [147], have been utilized to remove non-phosphorylated peptides to focus the analysis on site-specific phosphorylation. Also, since iTRAQ is an in vitro labeling procedure it can be applied to clinical samples such as tumor tissues and fluids (e.g.

serum, urine, blood). iTRAQ has been described as a very powerful method for the quantification of phosphorylation on a proteomic scale. In addition, White and co-workers [148] applied iTRAQ combined with MRM for phospho quantitative analysis of signaling networks, identifying and quantifying 222 tyrosine phosphorylated peptides, getting an extremely high percentage of signaling nodes covered (Figure 4B).

### 3.3 AQUA

The AQUA strategy provides an absolute quantification of a protein of interest [149]. In the AQUA method, a peptide from the protein of interest is constructed synthetically containing stable isotopes, and the isotopically labeled synthetic peptide is called AQUA peptide. The synthetic peptides can be synthesized with modifications such as phosphorylation to allow for the direct, quantitative analysis of post-translationally modified proteins. The stable isotopes are incorporated into the AQUA peptide by using isotopically “heavy” amino acids during the synthesis process of the interesting peptide (native peptide). Therefore, the synthetic peptide has a mass increase of e.g. 10 Daltons, due to the incorporation of a  $^{13}\text{C}_6$  and  $^{15}\text{N}_4$ -arginine into the synthetic peptide, compared to the native peptide. Although the mass difference between the native and the synthetic peptide allows the mass spectrometer to differentiate between the two forms, both forms have the same chemical properties, resulting in the same chromatographic retention, ionization efficiency, and fragmentation distribution. In AQUA experiments, a

known amount of the isotopically labeled peptide is added to a protein mixture, which is proteolytically digested, and later analyzed by MS. Since the native peptide and its synthetic counterpart have the same chemical properties, the MS signal from the quantified synthetic peptide can be compared to the signal of the native peptide. This finally allows for the absolute quantification of the peptide to be determined [150]. Multiple AQUA peptides can be used to quantify multiple proteins in a single experiment (Figure 5).

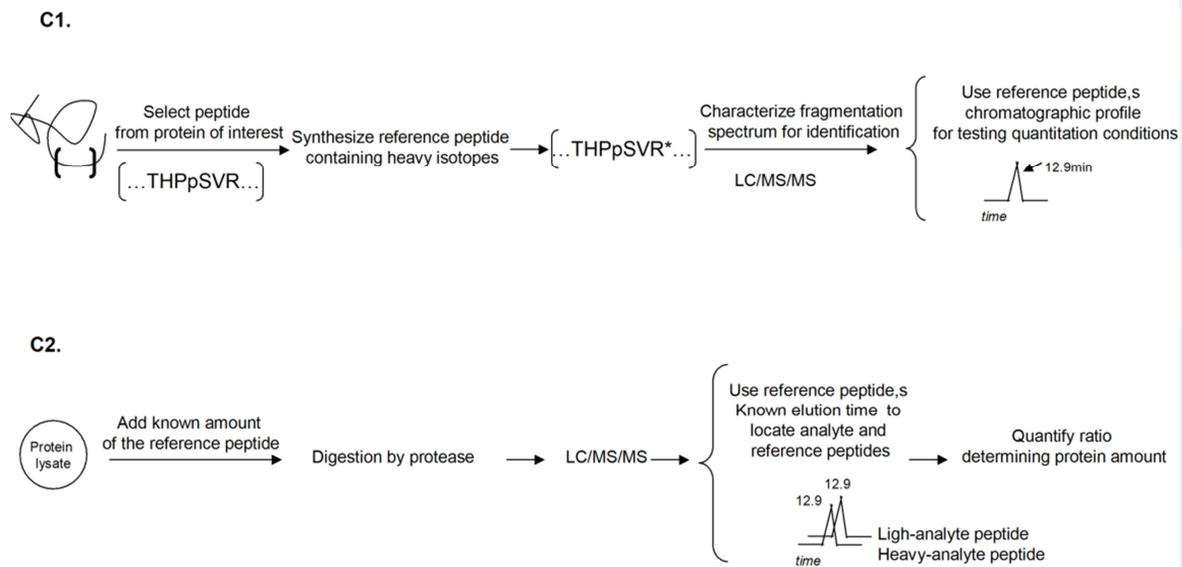
### 4. Concluding remarks and future perspectives

Cancer and immune disorders, remain among the leading causes of death worldwide. Therefore, the identification of useful biomarkers and the improvement of the understanding of the development of these diseases is still a need.

The immune system is easily influenced by the existence of cancer in the body, even when it is in a preclinical stage, and these studies should be expanded and extended in the future to answer the numerous questions concerning (a) the roles of immune cells in cancer surveillance (b) the characteristics of inflammation associated with cancer development, (c) the effects of environment/lifestyle factors on the immune system, and (d) the interaction between aging and immunity in the occurrence of cancer and other diseases.

The importance of protein kinase-regulated signal transduction pathways in immunology disorders and cancer has led to the development of drugs that inhibit protein kinases at the apex or intermediary levels of these pathways. Protein phosphorylation assignment studies of these signaling path-

### C. Protein Quantitation scheme using AQUA reference peptides



**Figure 5.** Scheme of absolute quantitative proteomic method based on the synthesis of reference peptides with stable isotopes. (C1) The first steps of the AQUA method consist of synthesizing peptides of interest with incorporated stable isotopes as ideal internal standards to mimic native peptides formed by proteolysis. These synthetic peptides can also be prepared with covalent modifications, like phosphorylation, that are chemically identical to naturally occurring posttranslational modifications. (C2) AQUA internal standard peptides are added to the protein sample, after proteolysis, and used to precisely and quantitatively measure the absolute levels of proteins and their post-translational modifications by MS.

ways will provide important insights into the operation and connectivity of these pathways that will facilitate the identification of the best targets for cancer therapies (e.g. the identification of a phosphate group on a specific serine, threonine or tyrosine by phospho enrichments combined with MS). Moreover, phosphoproteomic analysis of individual tumors will help match targeted cancer drugs to the appropriate patients.

The current phosphoproteomic goals imply the identification of phosphoproteins, mapping of phosphorylation sites, quantitation of phosphorylation under different conditions, and the determination of the stoichiometry of the phosphorylation. In addition, knowing when a protein is phosphorylated, which kinase/s is-are involved, and how each phosphorylation fits into the signaling network, are also important challenges for researchers in order to understand the significance of different biological events.

The new MS technologies are fundamental for cataloging all this information, and it is heading towards collecting accurate data on phosphopeptides on a global scale. Finally, it must be pointed out the possible difficulties to get sufficient amount of specific phosphorylated proteins of specific low abundant protein-kinases *in vivo* which might limit the usability of the phosphoproteome analysis.

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