Molecular and cellular responses to renal injury : a (phospho)-proteomic approach

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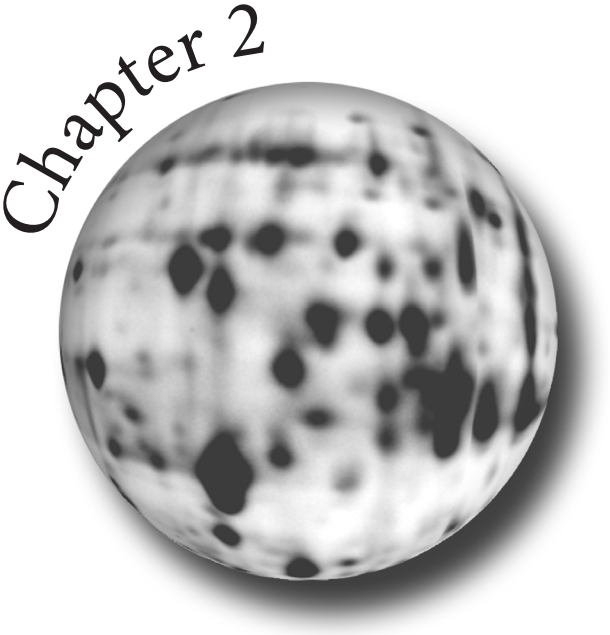
Phospho-proteomics and cellular signalling



Phospho-proteomic analysis of cellular signalling

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ABSTRACT

Reversible protein phosphorylation plays an important role in the regulation of many different processes, such as cell growth, differentiation, migration, metabolism and apoptosis. Identification of differentially phosphorylated proteins by means of phospho-proteomic analysis provides insight into signal transduction pathways that are activated in response to for example growth factor stimulation or toxicant-induced apoptosis. This review summarizes recent advances made in the field of phospho-proteomics and provides examples of how phospho-proteomic techniques can be combined to quantitatively investigate the dynamic changes in protein phosphorylation in time. By linking experimental data to clinical data (*e.g.* disease progression or response to therapy) new disease markers could be identified, which could then be validated for applications in disease diagnosis and progression or prediction of a response to drugs.

INTRODUCTION

The activity of most cellular proteins is regulated by post-translational modifications (PTMs) [1]. At least 200 different types of PTMs are known of which one of the most widespread PTM is reversible protein phosphorylation [2]. Today it is generally accepted that protein phosphorylation and dephosphorylation is regulated by a balanced activity of protein kinases and protein phosphatases. However, it is only a few decades ago that one would look surprised when mentioning "a MAP kinase-kinase-kinase-dependent phosphorylation of a MAP kinase-kinase". How was protein phosphorylation originally discovered? Protein kinase activity was first observed in 1954 when Burnett and Kennedy described a liver enzyme that catalyzed the phosphorylation of casein [3]. In 1955 Fischer and Krebs found that conversion of glycogen phosphorylase from an inactive to an active form, which is important for glycogenolysis, was dependent on the activity of a kinase [4]. In this way, they demonstrated for the first time the relation between protein phosphorylation and protein activation. In addition, Sutherland *et. al.* showed that glycogenolysis could be stimulated with adrenalin or glucagons, demonstrating that a hormone can affect the activity of kinases and phosphatases, thereby changing the phosphorylation status of a protein [5]. In 1968, with the discovery of protein kinase A (PKA) and the finding that it activated phosphorylase kinase, for the first time a signal transduction cascade was described in which a kinase activates another kinase. With this discovery, the general significance of protein phosphorylation in cell biological processes was recognized and more and more studies focused on protein phosphorylation. Proteins were found to be phosphorylated on two or more residues by one or more kinases (*e.g.* multisite phosphorylation), which nowadays is believed to be a common mechanism for regulating protein function rather than an exception. New kinases were discovered, like v-Src, the protein encoded by the transforming gene of Rous sarcoma virus, and the epidermal growth factor (EGF) receptor [6,7]. These kinases and many other growth factor receptors turned out to be tyrosine kinases. The search for their downstream targets and pathways was initiated, resulting in the recognition of an increased number of signal transduction cascades. These studies actually depict the early stages of what we nowadays call phospho-proteomics: the analysis of the whole complement of phosphorylated proteins in a cell.

PHOSPHORYLATION IN SIGNAL TRANSDUCTION

Today we know that phosphorylation and dephosphorylation of a protein by protein kinases and protein phosphatases can affect the function of a protein in many ways by: 1) increasing or decreasing its biological activity, 2) stabilizing it or marking it for breakdown, 3) facilitating or inhibiting movement between subcellular compartments or 4) initiating or disrupting protein-protein interactions. The importance and complexity of protein phosphorylation in cellular signalling is supported by the fact that over 500 protein kinases are encoded by the human genome and that one-third of all proteins in the cell are phosphorylated at any one time [8]. Early estimates suggest that there are approximately 100000 potential phosphorylation sites in the human proteome of which

fewer than 2000 are currently known, emphasizing the need for sensitive, high throughput methods to identify, characterize, and monitor new sites of protein phosphorylation [9]. In eukaryotic cells, phosphorylation of a protein takes mainly place at a Ser, Thr or a Tyr residue with a ratio of about 1000:100:1 respectively. Although tyrosine phosphorylation represents only a small percentage of total protein phosphorylation, it plays a significant role in cellular signalling mediated by for example receptor-mediated signalling.

Many cellular processes are tightly regulated by protein phosphorylation and abnormal phosphorylation due to for example mutations in kinases or phosphatases is recognized as a cause or a consequence of many human diseases like cancer. With respect to cancer, especially the deregulation of protein kinase function due to overexpression, constitutive activation or autocrine stimulation is frequently implicated in the onset of tumor formation. As a result of this, kinase signalling pathways are a major focus of biomedical research, which in the past years resulted in the development of several protein kinase inhibitors as targeted therapeutics. For example, the monoclonal antibody Herceptin (Genentech) inhibits the receptor tyrosine kinase Her2 and is used for the treatment of breast cancer. In addition, the small-molecule inhibitor Gleevec (Novartis) targets BCR-Abl, c-kit and the platelet derived growth factor (PDGF) receptor and is used for the treatment of chronic myelogenous leukaemia. As these examples show, knowledge on the activation state of phosphorylation-dependent signal transduction pathways in relation to the onset of diseases may result in identification of novel drug targets or identification of disease markers.

In the past, many gene expression profiling studies have been performed to screen for disease specific alteration in gene expression. Phospho-proteomics provides additional information compared to expression profiling studies. It offers a way to qualify and quantify the state of kinase-dependent pathways and provides detailed post-translational phosphorylation information, which is obviously not obtainable by gene arrays. There is increasing interest in phospho-proteomic studies as determined by the number of publications describing various strategies to identify phospho-proteins and their phosphorylation sites in a global fashion. Since the cellular proteome consists of many different proteins of which the phospho-proteins represent one-third, it is difficult to identify all phospho-proteins from such complex samples. Thus, the major challenge for the application of phospho-proteomics lies in successfully substracting phospho-proteins from the whole cell lysate with a focus on identification of low-abundant phospho-proteins also. In addition, phosphorylation of a protein is a reversible and highly dynamic process, which often takes place at multiple residues. Therefore, the phosphorylation profiles of a group of proteins may vary in time and period of protein kinase and phosphatase activation. To understand the order in which cellular signalling takes place, current phospho-proteomic techniques require the ability to measure protein phosphorylation at multiple time points within a cell. Below, we will further describe the latest phospho-proteomic techniques, which offer a way to quantify high numbers of phospho-proteins from a complex mixture of proteins. We will focus on studies that have combined different phospho-proteomic techniques to describe dynamic changes in cellular protein phosphorylation in a time-

dependent manner and discuss how, in future, phospho-proteomics can be used to understand the way in which signalling networks are perturbed in human diseases.

PHOSPHO-PROTEOMIC TECHNIQUES

The fundamental aims of phospho-proteomics are the detection, identification and quantitation of phospho-proteins, as well as mapping of their phosphorylation sites. The identification of phospho-proteins and phosphorylation sites is greatly improved by the introduction of mass spectrometry (MS), but for the detection of phosphorylated proteins classical methods, such as 2D-electophoresis are still being used and optimized. Recently, some good reviews on the analysis of phosphorylation by different phospho-proteomic methods have been published [10-12]. These should be consulted for detailed description of methodology and protocols. In this section, we will only shortly touch upon these different techniques and focus more on novel experimental procedures and insights within the context of these. A summary of the different methods is provided in Table 1.

^{32}P labeling

Classical ^{32}P labeling is still a valuable tool for the initial screening of changes in phosphorylation [13,14]. This technique is highly selective and sensitive and offers a way to easily quantify phosphorylation levels of a protein. ^{32}P labeling is a good method for the identification of novel phosphorylated proteins in signalling pathways. It can be used for the analysis of immunoprecipitated and gel separated signalling complexes or for quantification of differentially phosphorylated proteins using classical 2D-gels of total cell lysates. Because phosphorylation is such a dynamic process, these experiments are especially suitable for the determination of the time point at which the highest level of a novel phosphorylated protein is detected upon triggering a certain signalling cascade. When using ^{32}P labeling in combination with 2D-electrophoresis there is a chance of identifying false positives, due to possible co-migration of other proteins.

A novel approach for the screening of kinase substrates with radio-active labeling is differential phospho-protein labeling (DIPPL). This technique uses differential labeling of cells with ^{32}P and ^{33}P in a control (*e.g.* absence of kinase activity) and experimental group (*e.g.* presence of kinase activity), respectively [15]. After mixing, 2D gel-electrophoresis and drying, gels are exposed twice. During the first exposure, both ^{32}P and ^{33}P labeled proteins are detected while in a second round of exposure the ^{33}P activity is filtered out resulting in the detection of only ^{32}P labeled proteins. Comparison of these two exposures will reveal spots that were specifically phosphorylated in cells displaying the kinase activity.

Radioactive labeling is well suited for experiments using the recently developed synthetic peptide arrays, covering known phosphorylation sites of a large panel of different proteins [16]. An important advantage of these arrays is that apart from cells, they can be used with a small amount of tissue, allowing for example kinome profiling in biopsies [17]. However, despite the relatively large number of peptides present on available chips, the total number of kinases that is actually covered is at present still relatively small.

Antibodies and antibody-arrays

For the analysis of signalling pathways in which protein phosphorylation plays an essential role, a whole array of phospho-specific antibodies against many proteins are currently available. The parallel staining with antibodies against the total pool of a specific protein allows the quantification of the relative increase of phosphorylation, which is a very important parameter in studying, phosphorylation dependent, signalling pathways. In many cases, of course, this type of approach is not applicable because no antibodies are available. Moreover, no novel signalling partners will be identified.

One alternative for using antibodies against specific phospho-proteins is the use of antibodies against specific phosphorylated amino acids. Especially for phosphotyrosine there are good commercially available antibodies (*e.g.* 4G10, PT66, PY99) [18-20]. One of the approaches is using these antibodies for 2D electrophoresis in conjunction with western blot analysis and subsequent mass spectrometric analysis of the spots of interest on parallel protein stained gels [21]. However, similar to the situation with ^{32}P labeling, this also suffers from the potential identification of false positives.

Because several of the pTyr specific antibodies work very well for immunoprecipitation purposes this problem can be largely circumvented [19,20]. Immunoprecipitated proteins can be eluted using standard techniques, but for tyrosine phosphorylated proteins phenylphosphate can be used as an elegant alternative. In this way, the tyrosine phosphorylated proteins can be eluted without IgG contamination in the sample. We have used this approach in the immunoprecipitation of tyrosine phosphorylated proteins from primary human T-cells (Hensbergen *et. al.*, unpublished data). Immunoprecipitated proteins were gel separated, stained with silver, and subsequently identified by LC-Ion-trap-MS/MS. We found several proteins known to be tyrosine phosphorylated (*e.g.* p56lck, Fyn binding protein, general transcription factor II) and some proteins that most probably represent a-specifically immunoprecipitated proteins (*e.g.* actin, myosin). In the case of Fyn binding protein we also identified a tyrosine phosphorylated peptide (**Fig. 1**). Interestingly, the non-phosphorylated peptide was also identified showing that this site is not fully phosphorylated in the immunoprecipitated Fyn binding protein thus indicating that one of the other sites in Fyn binding protein is probably also phosphorylated. This example indicates that immunoprecipitation in conjunction with MS-MS analysis can well be used to study the stoichiometry of protein phosphorylation.

It has recently been shown that a phosphotyrosine specific antibody can also be used to immuno-purify tyrosine phosphorylated peptides [22]. Therefore, a second round of immunopurification could be used as a valuable tool for further enrichment of the tyrosine phosphorylated peptides. Although the use of phospho-serine and phospho-threonine specific antibodies for immunoprecipitation purposes has been described [23], they are at present not generally applicable due to the lack of acceptable specificity.

Phospho-specific gel staining

As an alternative to antibody staining on 2D-blots or radio-active ^{32}P protein labeling, a phospho specific gel staining has become available (Pro-Q Diamond) [24]. Besides the

avoidance of radioactivity and blotting techniques, the advantage of this staining is the ability to analyze specimens, such as body fluids and biopsy materials that are not amenable to radio-labeling in culture. Pro-Q diamond can easily be combined with a total protein stain (*e.g.* Sypro ruby) making quantification of differential phosphorylation easier. The staining binds noncovalently to phosphoproteins and is thus fully compatible with MS techniques, allowing protein identification after gel electrophoresis. Protein phosphorylation needs to be confirmed using MS/MS to create absolute certainty that the protein spot is indeed phosphorylated. As holds true for radio-active labeling, Pro-Q diamond staining does not discriminate between Ser, Thr or Tyr phosphorylation and additional techniques should be applied to do so.

Very recently, novel alkoxy bridged dinuclear metal (Zn^{2+} and Mn^{2+}) complexes were developed as phosphate binding tags [25]. These so-called Phos-tags are coupled to biotin and were found to be very sensitive in the analysis of electro blotted phospho-proteins. Moreover, polyacrylamide bound Phos-tags gave rise to a mobility shift of phosphorylated protein in standard SDS-PAGE gels.

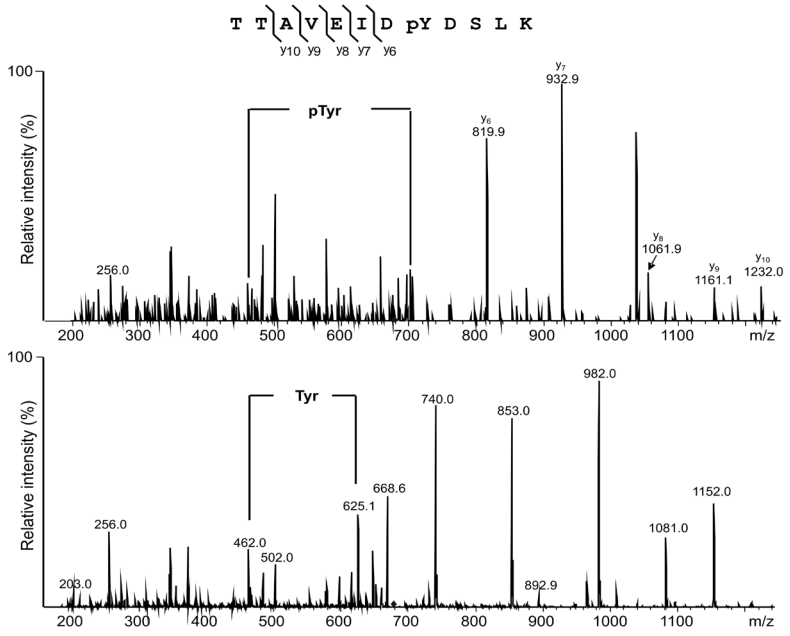

Figure 1. *Identification of a tyrosine phosphorylated peptide from Fyn binding protein*. Tyrosine phosphorylated proteins were immunoprecipitated from a total cell extract of human T-cells using the 4G10 antibody. Immunoprecipitated proteins were separated by SDS-PAGE and protein bands of interest were digested with trypsin and analyzed by LC-Ion-trap MS/MS. One of the proteins found was Fyn binding protein and within this digest a tyrosine phosphorylated peptide (TTAVEIDpY-DSLK) was found (upper part). Within the same sample the non-phosphorylated cognate peptide was also identified (lower part). pTyr= phosphorylated tyrosine.

Table 1. *A summary of the phospho-proteomic approaches described in this review*

Category	Technique	Enrichment	Quantification	Remarks
^{32}P	^{32}P-2D ([3,16])		x	[1]Sensitive technique, but [2]change for false positives when using 2D in combination with MS and [3]no discrimination between Tyr, Ser, Thr
	^{32}P-IP-1D ([17])	x		
	DIPPL ($^{32+33}$P) ([18])		x	
Immunoaffinity	pTyr-IP-1D ([4,19])	x		[1]Good anti-Tyr antibodies available, [2]poor specificity for anti-Ser or anti-Thr antibodies and [3]change for false positives when using 2D in combination with MS
	pTyr-2D ([20])		x	
	pSer-Thr-IP-1D ([21])	x		
	pTyr-IP peptides 1D ([22])	x		
Gel stain	Pro-Q-Diamond ([23])		x	See [2] and [3] of ^{32}P labeling and background staining of non-phospho proteins
Metal affinity	IMAC ([6,7,27])	x		[1]Only for peptides, [2]a-specific binding of non-phospho peptides and [3] high throughput possibilities by coupling IMAC to RP-HPLC
	pTyr-IP-IMAC ([4,22])	x		
	TiO_2 ([25])	x		
Chemical modification	β-elimination ([26])			[1]Possible side reactions and [2]not all phosphoproteins are modified
	Biotinylation ([27])	x		

IMAC

To reduce the complexity of the sample prior to mass spectrometric analysis, many efforts have been made to enrich for phospho-peptides. One of the most widely used procedures is Immobilized Metal Affinity Chromatography (IMAC). This is based on the fact that phospho-peptides have a tendency to bind to certain heavy metals (*e.g.* Fe^{3+}, Ga^{3+}) at low pH. Following binding and washing off non-phosphorylated peptides, phospho-peptides are eluted at high pH [26-28]. One drawback of the IMAC procedure is the non-specific bind-

ing of, especially, acidic peptides. To circumvent this problem, peptide methylation has been proposed, although there is still debate on the necessity of this procedure [29-31]. Furthermore, in a large-scale analysis using IMAC of phospho-peptides from plasmamembrane proteins, mainly multiple phosphorylated peptides were identified [31]. By applying a combination of strong anion exchange chromatography followed by IMAC, this bias for multiple phosphorylated peptides was largely circumvented. Recently also TiO_2 has been shown to be a valuable matrix for phosphopeptide enrichment [32].

Chemical modifications

Apart from the above-described methods, some specific chemical modifications have been developed for tagging of the phosphate group. For this purpose, the phosphate moiety is first removed by β-elimination. The resulting dehydroalanine (from pSer) or dehydroaminobutyric acid (from pThr) is then modified by a thiol containing reagent [33,34]. Especially bi-functional thiol reagents are of interest because the second thiol group can be used to introduce an affinity tag, thereby allowing subsequent affinity purification. Knight *et. al.* developed a chemistry in which phosphothreonine and phosphoserine were converted to lysine analogs thereby introducing a novel cleavage site for a lysine-specific protease [35]. Interestingly, the chemistry could also be adapted for solid phase modification and enrichment in one step. Van der Veken *et. al.* recently developed a novel chemical probe featuring an affinity label (biotin), an acid-labile linker, a site for incorporation of stable isotopes, and an electrophilic reactive group [36]. Because of the acid-labile linker, the tagged peptides are more efficiently recovered from the affinity resin and the mass spectra of peptides lacking the biotin could be more easily interpreted.

One of the drawbacks of the above described procedures is that not only phosphate groups are lost during the β-elimination reaction but also for example O-glycans, resulting in false identifications of phosphorylation. Furthermore, phophotyrosine is not sensitive to the β-elimination reaction. Zolodz *et. al.* used this aspect as a tool for reduction in the complexity of a mixture of phosphopeptides prior to IMAC when searching for phosphotyrosine containing peptides [37].

Mass spectrometry

In principle, mass spectrometric analysis of spots corresponding to for example ^{32}P or pY labeled spots will identify the phosphoprotein but, as mentioned above, these type of analyses potentially result in the identification of false positives. In addition, many proteins identified in analyses of tryptic digests of immunoprecipitated phospho-proteins by LC-MS are not necessarily phosphorylated proteins themselves. Therefore, in any analysis of protein phosphorylation, the exact localization of the phosphorylation site and its dynamics is ultimately very important. The most obvious way of determining protein phosphorylation is the detection of the 80 Da mass difference. Still, this type of analysis is not trivial for intact proteins and requires high mass accuracy instrumentation. Furthermore, the mass difference of 80 Da does not necessarily mean phosphorylation but could be due to other modifications or alterations as well. It is especially difficult to discriminate

between phosphorylation and sulfation, since their mass difference is very small.

In general, the identification of phosphorylation is performed after some type of digestion of the protein of interest. Ionization of phosphorylated peptides is considered poor, especially in comparison to non-phosphorylated peptides. Therefore, it is generally difficult to measure the phosphorylated peptide in mixtures of phosphorylated and non-phosphorylated peptides. This can partially be circumvented by peptide ionization in the negative ion mode, but because of the relatively poor performance of MS/MS under these conditions, this is not widely applied.

Some mass spectrometric techniques can be applied specifically for the detection of phosphorylated peptides in mixtures with non-phosphorylated peptides. One of these relies on the fact that in many cases of phosphorylation at serine or threonine residues, a neutral loss of phosphoric acid (-98) or phosphate (-80) is observed upon collision induced dissociation. This phenomenon can be used in neutral loss scans, particularly when using triple quadrupole mass spectrometers. Although these types of measurements are valuable for identification of a peptide as a potential phospho-peptide, the fragmentation spectra harboring a high level of neutral loss do often contain insufficient information for subsequent sequence analysis. In this case, additional fragmentation of the major ion in the MS/MS spectrum is necessary by for example using an Ion-trap mass spectrometer. Alternatively, samples can be analyzed before and after treatment with alkaline phosphatase although this hampers mapping of the exact localization of phosphorylation within the peptide.

The neutral loss of the phosphate moiety observed in collision induced dissociation does not occur with some other fragmentation techniques. Using FT-ICR-MS, for example, it is possible to use Electron Capture Dissociation (ECD) [38,39]. With this fragmentation technique only the peptide backbone is cleaved, resulting in typical c and z ions, while amino acid modifications, like phosphorylation, stay intact. Therefore, this analysis has a high potency for identification of phosphorylation sites but is hampered by the fact that ECD fragmentation is not very efficient. Furthermore, the cycle time is rather long so it has not yet been applied in LC-MS analysis of complex mixtures. Recently, a novel fragmentation technique, Electron Transfer Dissociation (ETD), has been described [40]. This gives fragmentation patterns similar to ECD but its application will benefit from the fact that it is compatible with Ion-trap mass spectrometers.

For tyrosine phosphorylation, precursor ion scans targeting the immonium ion of phosphotyrosine (m/z 216.043) have been used [41,42]. However, due to the long acquisition time necessary, these types of scans are not suitable for LC-MS/MS analysis but rather require off-line nanoelectropsray experiments.

Quantification of phosphorylation

One of the recent developments in mass spectrometry is its use for not only detection and identification but also for quantification. Most of the techniques rely on a certain stable isotope labeling. These strategies are also applicable for the quantification of phosphorylation. One popular labeling technique uses isotopically labeled amino acids that

are applied during cell culturing (SILAC, stable isotope labeling with amino acids) [43]. Amino acids containing isotopes of carbon (*e.g.* $^{13}C_6$Arg) are preferred above deuterated amino acids because the latter may give rise to a chromatographic isotope effect, thereby hampering proper quantification [44]. Moreover, the advantage of $^{13}C_6$Arg or $^{13}C_6$Lys is the fact that in combination with the standard enzyme for digestion, trypsin, there will be an almost uniform mass difference (6 Da) between labeled and unlabeled peptides, facilitating data analysis. SILAC has been successfully applied in the study of signalling pathways [45,46]. The obvious drawback of *in vivo* labeling techniques with amino acids is that it is not easily applicable to other systems then cell cultures. However, several groups have established completely labeled animal model species (rat, C. elegans, D. melanogaster) using ^{15}N enriched food [47,48].

For *in vitro* labeling techniques in the context of phosphorylation, also ^{18}O and DTT have been used [49,50]. Although the isotope coded affinity tag (ICAT) has proven to be a valuable approach for protein quantification and could therefore be used to quantify phospho-proteins, it is not generally applicable for analysis of phospho-peptides because it relies on the presence of a cysteine residue within a certain peptide. The recently introduced iTRAQ technique, where peptides are N-terminally labeled with isobaric labels that can be quantified in the MS/MS mode does not have this caveat. Combining iTRAQ with IMAC has recently been shown to be a valuable tool for the quantification of tyrosine phosphorylation of the EGFR signalling (see further below) [22]. Recently, a stable isotope-free method for quantification of phosphorylation stoichiometry was described [51].This method uses ion intensities as a measure of quantification. First, peptides are selected that show small variability in ion intensities between replicate MS runs. Second, assuming a correlation between the intensities of the phosphorylated peptide and its non-phosphorylated cognate and correction for the difference in ionization/detection efficiencies between these two peptides (the so-called flyability ratio), it was possible to quantify absolute phosphorylation stoichiometry.

PHOSPHO-PROTEOMICS IN CELLULAR SIGNALLING, A FEW EXAMPLES

Over the past two decades, it has become clear that tyrosine phosphorylation plays an important role in a variety of signal transduction pathways. Although tyrosine phosphorylation represents only a small percentage of total protein phosphorylation, a minor change in the tyrosine phosphorylation status of a protein is already sufficient to induce malignant transformation [52]. How can we identify downstream targets in protein tyrosine kinase and phosphatase signalling pathways? Because only a small percentage of all phosphorylations occurs on tyrosine residues, these important modifications are difficult to asses compared to the more numerous sites of Ser and Thr phosphorylation. In this section, we mainly focus on studies that use techniques to identify tyrosine phosphorylated proteins. However, minor adjustments make the described techniques applicable for identification of Ser or Thr phosphorylated proteins.

In order to understand the onset of certain cell biological events one needs to understand the cross-talk between kinases, phosphatases and phospho-proteins in different

signal transduction pathways. For this, it is important to follow kinetic changes in protein phosphorylation at various time points after for example stimulation of cells with growth factors. One of the first studies on time-dependent phospho-proteome based analysis of signal transduction pathways focused on identification of differentially phosphorylated proteins in mouse fibroblasts following PDGF stimulation [53,54]. Using classical 2D-electrophoresis in conjunction with anti-phosphotyrosine and anti-phosphoserine antibodies, at least 100 proteins were identified with a prominent change in phosphorylation as a function of time. Within a short time period (20 min) PDGF initiated a very complex series of kinetic changes in phosphorylation levels. Many proteins were (in)directly involved in the cytoskeletal changes induced by PDGF, such as vimentin, cortactin, rac and several G-proteins. Moreover, combining the Ser and Tyr phosphorylation data revealed information on the stoichiometry of specific protein phosphorylation. For example, the time-dependent decline in tyrosine phosphorylation of the proto-oncogene tyrosine kinase fgr shortly after PDGF stimulation corresponded with a strong increase in Ser phosphorylation. This indicates that PDGF stimulation caused a time-dependent phosphorylation switch within one single protein.

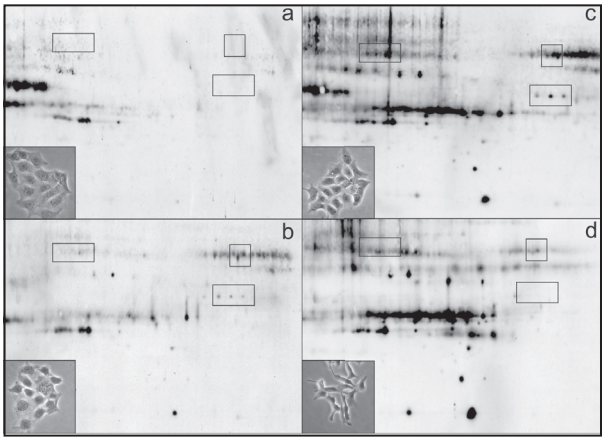

Figure 2. *2D phospho-tyrosine blots of v-Src MDCK cells.* Temperature sensitive v-Src MDCK cells were cultured at (a) the non-permissive temperature (40 °C) or at the permissive temperature (35 °C) for (b) 30, (c) 120 and (d) 240 min. 2D-blots were run and stained with a general anti-phosphotyrosine antibody (PY99). Over time, cells lose their cell-cell interactions. Inserts represent phase-contrast images of the cells at the indicated timepoints. Rectangles represent areas with differential tyrosine phosphorylation (*e.g.* up or downregulated).

Since phosphorylation of proteins in relation to activation of growth factor receptors or kinases is very dynamic, it will remain difficult to obtain a complete kinetic profile of protein phosphorylation in time. However, instead of having to identify all phosphoproteins that undergo changes in relation to a stimulus, it should be possible to link a certain phosphorylation pattern to a cell biological event. By comparing the changes in protein phosphorylation with changes in cellular behavior, one could define the role of

such a differential protein phosphorylation. For example, activation of Src kinase results in the onset of an epithelial to mesenchymal transition (EMT); a phenotypic switch in which epithelial cells undergo changes in their F-actin network, loose their cell-cell interactions and obtain a mesenchymal phenotype. We demonstrated that within 30-60 minutes, many proteins were tyrosine phosphorylated, while cells did not yet disrupt cell-cell junctions (de Graauw *et. al.*, submitted). After 2 hours of Src kinase activation, cells changed their F-actin network and after 4 hrs of Src kinase activation they lost their cell-cell junctions. These cellular changes corresponded to different overall tyrosine phosphorylation patterns (**Fig. 2**) in which most proteins showed an increase in pTyr, while others were dephosphorylated again after 4 hours of Src kinase activation. This suggests that phosphatases were also activated. Using the classical approach (2D-electrophoresis with phospho-state specific Ab) in combination with a specific biological effect we were able to determine the role of the identified proteins such as ezrin, radixin, annexin A1 and A2 to the onset and progression of EMT.

Since it is rather laborious and difficult to obtain a global kinetic profile of protein phosphorylation using 2D-electrophoresis, analysis of protein phosphorylation on a broad scale is nowadays mostly done using mass spectrometric techniques. Salomon *et. al.* were one of the first to describe an approach based on multidimensional liquid chromatography/mass spectrometry to rapidly and simultaneously identify differentially phosphorylated proteins in signal transduction pathways [19]. They followed changes in pTyr patterns that occur over time during either activation of T-cells or treatment of CML cells with Gleevec, a protein tyrosine kinase inhibitor. Phospho-tyrosine immunoprecipitation was used in combination with methyl esterification and IMAC of tryptic peptides prior to LC-MS-MS. This resulted in the identification of 64 unique sites on 32 different proteins, among which Cas, PYK2, SHC, SHIP-2 and cortactin. The described technique enabled the examination of changes in sites of phosphorylation over time. Since many proteins could be studied within one experiment, profiling of tyrosine kinase inhibitors, such as Gleevec becomes easier. This helps providing a better understanding of the downstream effects of these agents on a variety of signalling pathways.

Blagoev *et. al.* describe a different technique that allows the comparison of three protein samples so that protein phosphorylation profiles can be determined at multiple timepoints [55]. The proteomes of three cell populations were metabolically labeled with different stable isotope forms of arginine, allowing the simultaneous comparison of three different samples by their mass difference of 6 Da or 10 Da, respectively. This technique was used to analyze early phosphorylation events in EGFR signalling in HeLa cells. By running the analysis twice, one corresponding to 0, 1 and 10 min of EGFR stimulation and one corresponding to 0, 5 and 20 min of stimulation, a profile of five different timepoints could be compared by using the 0 min as a common reference [55]. After incubation of the cells with different arginine isotopes, cell lysates of the three experimental groups were mixed and used for immunoprecipitation with an anti-phospho tyrosine antibody. Upon EGF stimulation, 81 proteins showed a >1.5 fold change in phosphorylation of which most were involved in receptor down regulation and signal termination or cy-

toskeletal remodeling and cell adhesion. Although some of these proteins were already known to play a role in EGFR signalling many other proteins were newly implicated, such as a group of RNA-binding proteins. The unique contribution of this study is the generation of time courses of 'activation' of every single protein detected [55]. The most striking response upon EGF stimulation is the dynamics of Arp2/3 complex, which is responsible for the branching of actin microfilaments. Arp3 has a maximum phosphorylation at 5 min, which was followed by a decrease at 10 min and another increase in tyrosine phosphorylation at 20 min. Nine different Arp2/3 members were identified and all display the same quantitative behavior. Given that also gelsolin, which actively participates in actin dynamics, has the same profile as the Arp2/3 complex one might screen for proteins, which display similar phosphorylation kinetics, thereby identifying a novel EGFR target protein, which is associated with F-actin dynamics. Subsequently, the role of such a protein can be determined using for example phospho-defective or phospho-mimicking proteins. Using a classical 2D phospho-proteomics approach we identified, among others Arp2 as a differentially phosphorylated protein in nephrotoxicant-induced lamellipodia formation prior to the onset of apoptosis of primary cultures rat renal proximal tubular epithelial cells (de Graauw *et. al.*, submitted). To obtain detailed information on the kinetics of Arp2 tyrosine phosphorylation and that of related F-actin regulatory proteins during a dynamic process such as lamellipodia formation, a method like Blagoev *et. al.* described is necessary.

The use of three isotopes in combination with anti-phosphotyrosine immunoprecipitation can be extended to many other cellular systems that involve tyrosine phosphorylation. For instance, comparing phosphorylation profiles in cells stimulated with different growth factors. EGF and not PDGF stimulated differentiation of human mesenchymal stem cells into bone forming cells [56]. A comparison of EGF and PDGF stimulated cells with control cells showed that more than 90% of the tyrosine phosphorylated proteins were identical for both growth factors. However, the PI3K pathway was exclusively activated by PDGF. Inhibition of PI3K in the PDGF-stimulated cells indeed removed the differential effect of the two growth factors. This study not only demonstrates the importance of quantitative phospho-proteomics to elucidate differences in (closely related) phosphoproteomes, but also shows that it is crucial to link the identified phospho-proteins to cell biological events. The triple metabolic labeling can be used to study Ser or Thr phosphorylation patterns as well. Phospho-proteins can than be separated by for example IMAC technology combined with methylation of peptides [57].

Using immunoprecipitation techniques not only results in identification of phospho-proteins, but also many other non-phosphorylated proteins will be identified since this technique does not distinguish between isolating tyrosine-phosphorylated proteins and proteins associated with the tyrosine phosphorylated proteins. Information on protein-protein interactions is just as valuable as information on protein phosphorylation itself. However, the phosphorylation of all proteins identified in a certain study should be confirmed. Identification of such phosphorylation sites in complex protein mixtures remains a difficult task.

Instead of using immunoprecipitation techniques to enrich for phospho-proteins one can enrich for phosphopeptides, thereby circumventing the large amount of non-phosphorylated peptides in a mixture. By immunoprecipitating phosphopeptides, the time course of individual phosphorylation sites can be determined in detail. This was successfully performed by Zang *et. al.* who identified and quantified the tyrosine phosphorylation of specific residues downstream of EGF, in a time-dependent manner [22]. Tryptic peptides from four different EGFR stimulation time points were labeled with four isoforms of the iTRAQ reagent to enable quantification. Cell lysates were mixed and tyrosine phosphorylated peptides were immunoprecipitated and enriched by IMAC before LC/MS/MS analysis. This led to the identification of 78 tyrosine phosphorylation sites on 58 proteins.

Although mass spectrometry allows us to identify large amounts of differentially (tyrosine)-phosphorylated proteins, the fundamental question of which kinase is responsible for a given phosphorylation event remains elusive since many kinases are active in the same pathway and exhibit overlapping substrate specificities. Shag *et. al.* developed a method to identify direct substrates of the protein tyrosine kinase v-Src [58]. To differentiate between substrates of Src kinase and other kinases they mutated the ATP binding site of v-Src in such a way that the engineered v-Src uniquely accepted an ATP analog (A*TP = N6 (benzyl) ATP). This ATP analog was not accepted by the wild-type Src kinase so that the direct substrates of one kinase (in this case Src) can be traced in the presence of any number of cellular kinases [58]. A broad screen for v-Src targets in NIH 3T3 cells has led to the identification of several thus far unknown substrates of which some are known to be involved in cellular processes critical for cell transformation [59]. The method described by Shag *et. al.* could potentially be used for any kinase, but the exact site for an amino acid substitution in the kinase domain should then first be identified.

FUTURE PERSPECTIVES

Proteomic analysis of cellular signalling is a difficult task. Understanding the dynamics of signal transduction as a result of phosphorylation requires the ability to asses changes in the sites of phosphorylation across numerous proteins simultaneously as well as over time. Although 2D-electrophoresis combined with phospho-specific antibodies remains a common methodology to asses changes in protein phosphorylation, it provides a limited amount of phospho-proteins. In contrast, relative large numbers of phospho-proteins can be identified using mass spectrometry techniques. By combining techniques described in this review, it is not only possible to identify differentially phosphorylated proteins, but also to identify their phosphorylation sites and determine their time-dependent kinetic profile. The main challenge for the coming years will be to optimize and extend the described techniques to improve sensitivity for low abundant proteins and to increase throughput to facilitate a more detailed analysis of time-resolved phosphorylation events.

Identification of protein phosphorylation without knowing the function has little meaning. Therefore, a second challenge will be the determination of the function of spe-

cific protein phosphorylation events in relation to the cell biological effect. To allow selection of interesting proteins, it would be useful to generate signalling networks from the lists of identified phospho-proteins and their phosphorylation sites. With such a phosphorylation database, researchers can easily combine their data with others and determine missing links that might be important for the investigated cell biological event. In addition, the database could be used to generate phospho-state specific antibodies, thereby making it easier to study the role of protein phosphorylation in signalling pathways.

A step towards the understanding of the patho-physiology of diseases as well as drug target identification would be profiling of the phospho-proteome of diseased human tissue. However, often the material is scarce and not fresh, while also the cellular composition of the diseased tissue can be rather heterogeneous. Although proteomics technologies are not yet fully applicable for screening of patient tissue, some studies have used clinical specimens to demonstrate the feasibility to generate the information needed [60]. Another way to study differential protein phosphorylation in disease is to link experimental data obtained from cell lines or freshly isolated cells to clinical data such as disease outcome and/or drug response [61]. The combined data might be used for human tissue arrays stained with (phospho)-specific antibodies. In this way, one might identify disease specific kinases and/or downstream substrates, which can be novel drug targets. Cancer drug therapy is still often directed at a single molecular target, but often multiple proteins are deregulated depending on the type of cancer. In the future, one might combine drug therapies more often to target a set of (kinase-activated) proteins along a deranged signalling pathway. This combinatorial therapy can result in a decreased toxicity, but also diminish the likelihood of tumor cells to develop resistance to a cocktail of inhibitors.

In conclusion, the development of new phospho-proteomic techniques, such as the multiple isotope labeling discussed in this review will enable us to obtain large amounts of data on the temporal patterns of phosphorylation. However, phospho-proteomic data needs to be linked to cell biological events in order to understand the role of differential protein phosphorylation in different signal transduction pathways. This will provide deeper insight into the functioning of the cell, which can be put to use to modulate deregulation of phosphorylation in cancer and other human diseases.

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