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REVIEW

Current technologies to identify protein kinase substrates in high throughput

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Abstract **Since the discovery of protein phosphorylation as an important modulator of many cellular processes, the involvement of protein kinases in diseases, such as cancer, diabetes, cardiovascular diseases, and central nervous system pathologies, has been extensively documented. Our understanding of many disease pathologies at the molecular level, therefore, requires the comprehensive identification of substrates targeted by protein kinases. In this review, we focus on recent techniques for kinase substrate identification in high throughput, in particular on genetic and proteomic approaches. Each method with its inherent advantages and limitations is discussed.**

Keywords phosphorylation, kinase substrate, *in vitro* kinase assay, high throughput screening, mass spectrometry, phosphoproteomics

Introduction

Protein kinases are by far the largest group of enzymes that catalyze the protein post-translational modifications in eukaryotes, contributing to around 2% of the human genome (Rubin et al., 2000; Lander et al., 2001; Manning et al., 2002b). These enzymes catalyze phosphorylation by transferring a phosphate group from adenosine-triphosphate (ATP) mainly to the hydroxyl group of serine, threonine, or tyrosine residues in substrate proteins (Kim et al., 2010). Protein phosphorylation is an essential post-translational modification that regulates almost every aspect of biological functions, including protein–protein interactions, signal transduction, subcellular localization, and apoptosis (Hunter, 2000; Pawson, 2004). Deregulation of phosphorylation dynamics within the cell often leads to the development of numerous diseases such as cancer, diabetes, central nervous system pathologies, and immune disorders (Blume-Jensen and Hunter, 2001). Therefore, understanding how signaling networks function and how they are disturbed in disease states requires phosphorylation analysis, which includes the identification of phosphorylated proteins and corresponding sites of phosphorylation and quantitative measurement of phosphorylation changes (Cohen, 2001; Manning and Cantley, 2002).

Generally, typical detection and quantification for phosphorylation is challenged by the low stoichiometry of phosphorylation on proteins (Jin et al., 2010; Wu et al., 2011). Although current liquid chromatography-mass spectrometry (LC-MS)-based phosphoproteomics has enabled the identification and quantification of thousands of phosphorylation events, the precise connections between the majority of protein kinases and identified phosphorylation sites have been missing. Thus, mapping kinase-substrate relationships is a critical step for understanding essential signaling networks and identifying pharmaceutical targets for drug discovery (Cohen, 2002). Toward this goal, various *in vitro* and *in vivo* techniques (Fig. 1) have been described for the screening of kinase substrates in high throughput.

Genetic screening

Genetic screening has been historically applied to discover candidate substrates of protein kinases. In this approach, the phenotype for a kinase mutant needs to be established first.

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Then the high throughput genetic manipulation including more recently siRNA library technology is performed on a genome-wide scale to screen the genes that can either suppress the phenotype in mutants or mimic the kinase mutant phenotype in wild-types. Those genes will be further tested as substrates of the kinase by biochemical approaches. Because of the relative ease of genetic manipulation in model organisms such as yeast, worms, and flies, this approach combined with subsequent epistasis analysis has been successful in identifying substrates in the downstream pathways of specific kinases in those organisms (Leberer et al., 1997; Paradis and Ruvkun, 1998; Clark et al., 2006; Sha et al., 2010).

Although this method offers a probability of identifying physiologically relevant proteins directly, the actual relationship between kinase and identified genes is not clarified. Other than phosphorylation, effects of transcription, translation, and other post-translational modifications all can contribute to a large portion of false positives. Plus, this approach is more difficult to utilize in higher organisms, such as mammalian cells, due to the bottleneck of high throughput mutagenesis. In this case, protein homology approach may provide valuable information for predictions of kinase substrates. However, not all the kinases in mammalian cells have their corresponding homology in lower organisms.

In vitro kinase assay

The most commonly used biochemical method to determine kinase activity toward substrates is the *in vitro* kinase assay in which the purified kinase is incubated with a putative substrate in the presence of ATP. The phosphorylated substrates can then be assessed by various measurements including colorimetric, radioactive, chemiluminescence, and fluorometric detection (Johnson and Hunter, 2005). This approach requires no specialized reagents and is applicable to any kinase regardless of its substrates.

However, *in vitro* kinase assays have limitations in that the phosphorylation *in vitro* may differ from what takes place physiologically. First, the use of concentrated purified kinase *in vitro* is partially responsible for a lower specificity. Second, the use of exogenous kinases outside cellular contexts often leads to a loss of physiological regulatory mechanisms. Third, purification of an active kinase is sometimes challenging due to the variation of protein activity using prokaryotic expression systems and the co-purified contaminating kinases using eukaryotic systems. Thus, a rigorous proof that the phosphorylations found *in vitro* are physiologically relevant must be performed by *in vivo* studies (Delom and Chevet, 2006).

Although the *in vitro* kinase assay combined with mutagenesis is well received as the golden standard in defining the kinase-substrate relationship, this low throughput method is laborious since each kinase is assayed with one putative substrate at a time. To fulfill the necessity for phosphorylation network mapping, there have been a number of approaches attempted to screen potential kinase substrates in a high throughput manner.

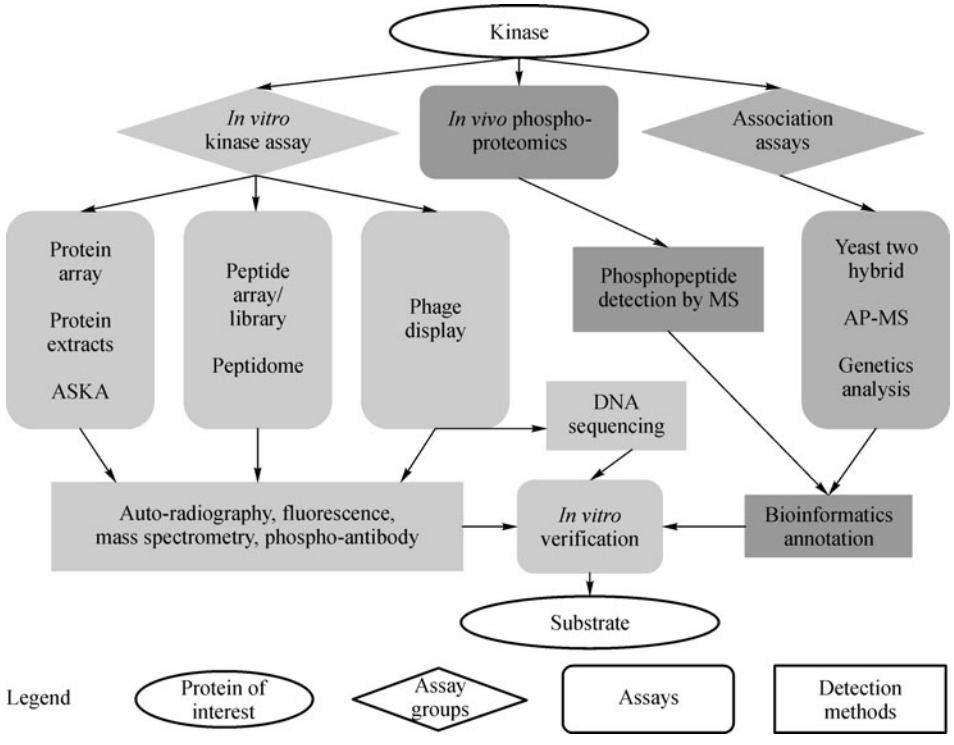

Figure 1 High throughput strategies to identify kinase substrates. ASKA, analog sensitive kinase allele; MS, mass spectrometry; AP-MS, affinity purification-mass spectrometry.

Protein and peptide microarrays

Surface-based assays like high-density peptide and protein arrays are prevailing tools used to systematically analyze biochemical activities of thousands of genes or proteins (Fig. 2). A comprehensive yeast proteome microarray was generated within the last decade (Zhu et al., 2001). Recently, chips containing larger proteomes like the human proteome have been created as well (Jeong et al., 2012). Taking advantage of prefixing the proteome or sub-proteome on solid-phase chips, substrate screening for a target kinase has become relatively straightforward by incubating the purified kinase with each putative substrate in a high throughput fashion. Moreover, an array technique usually requires only small amounts of reagents and is considered to be a sensitive and rapid assay. Using this technique, Snyder and coworkers conducted a pioneer global substrate analysis in yeast. They tested 82 unique kinases representing about two thirds of yeast kinome with yeast proteome chips and identified approximately 4200 phosphorylation events involving 1325 different proteins, demonstrating the power of such a method in phosphorylation analysis (Ptacek et al., 2005). It is worth pointing out that due to the use of multiple chips, not only can an overall picture of kinase-substrate preferences be built up, but a higher accuracy of measurement can be achieved when many internal standards are obtained by multiplexing.

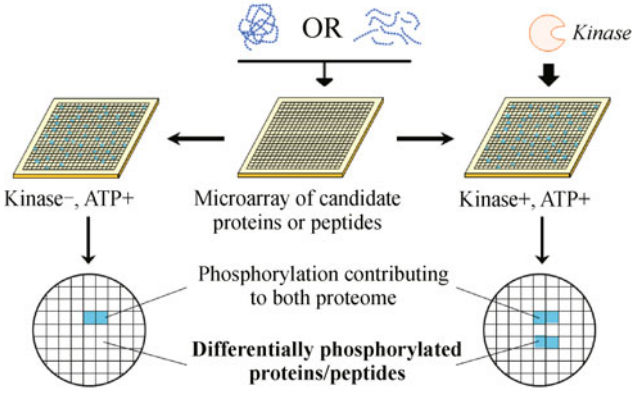

Figure 2 Kinase assay based on protein array or peptide array. Protein/peptide collections are spotted on the microarray, followed by the incubation with a purified active kinase under the reaction condition. Phosphorylation is detected by various methods.

Besides the interrogation of protein chips to identify substrates *in vitro*, peptide arrays are also commonly used in phosphorylation analysis. Currently, actual phosphorylation sites can rarely be predicted, even if the structure of the protein kinase and substrates are both known (Kreegipuu et al., 1998). Therefore, substrate screening using a peptide array is ideal for rapid characterization of the kinase specificity purely based on primary sequences. A typical peptide microarray consists of hundreds of peptides that are derived from specific organisms using bioinformatics design. A kinase of interest can be incubated with the peptides arrayed on the chip under reaction conditions, and the phosphorylated peptides can be scanned by several detection methods including autoradiography (Buss et al., 2004), fluorescence, and immunoblotting (Lesaicherre et al., 2002). Although they do not reveal actual kinase-substrate relationship, peptide microarrays provide invaluable information for assessing kinase sequence specificity. Combining this technology with a database-searching technique, it might be possible to identify novel substrates containing consensus sequences of the specific kinase.

General concerns with these surface-based techniques include nonspecific absorption of the liquid-phase proteins to the chip, incorrect protein conformation and inefficient kinase substrates interaction due to steric hindrance during protein binding (Huang et al., 2010). Occasionally, in cases where a third adaptor protein is necessary to assist the interaction between a kinase and its substrate, binary interaction-based microarray screening may fail.

Phage display

The phage display approach has been suggested as an alternative method to microarrays used for studying protein-peptide interaction and protein-ligand interaction (Fukunaga and Hunter, 1997; Jiang et al., 1998; Khati and Pillay, 2004). In this approach, the cDNA library was cloned into phage expression vectors. Phage plaque can be formed on lawns of *Escherichia coli*, and the proteins coded by individual cDNA clone are massively expressed both in bacteria and phage. After those proteins are subsequently immobilized on the solid-phase, they are subjected to phosphorylation by the purified kinase of interest in the presence of [γ-^{32}P]ATP (adenosine triphosphate), and the substrate can be identified by sequencing the phage plaques containing positive phosphorylation signals (Fig. 3).

Alternatively, this method can be employed for the determination of substrate specificity for kinase as well (Dente et al., 1997). In this strategy, a library of peptides with random sequences around a central residue (i.e. Ser, Thr or Tyr) is used instead of the cDNA library. To improve the sensitivity, multiple selection rounds can further enrich the positive phages with phosphorylation signals after kinase assay. After several cycles of selection, distinct substrate sequences could be defined by phage cloning. Hermann and coworkers used this technique to study kinase specificities of several tyrosine kinases including Blk, Lyn, c-Src and Syk. The preferences were mainly recapitulated in known or presumed protein substrates, demonstrating the value of this technique in substrate screening (Schmitz et al., 1996).

Phage display approach conveniently identifies the candidate substrate by isolating the clone from the phosphorylation-positive plaque. However, when the bacteria expression system is used, the improper folding of cDNA-encoded proteins becomes a potential problem (Pillay, 2004).

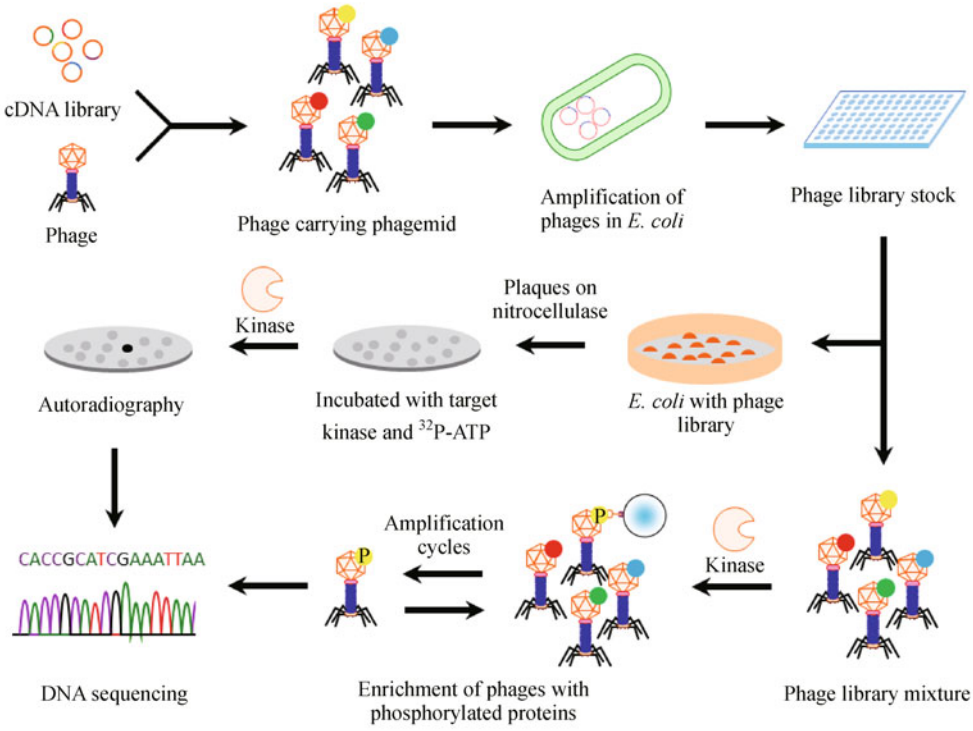

Figure 3 Workflow for phage display-based substrate screening. From a large population of phages, phosphorylated phage can be either identified by autoradiography or phosphorylation-specific antibodies.

Protein interaction-based substrate screening

One common practice to screen potential kinase substrates is to identify kinase-interacting proteins. This method is based on the hypothesis that a kinase-substrate pair can be co-identified when they are interacting. A number of approaches to study protein–protein interactions have been used to identify potential kinase substrates (Staudinger et al., 1995; Tien et al., 2004; Amano et al., 2010).

However, phosphorylation is commonly considered to be a transient protein–protein interaction. Once the substrate is phosphorylated by the kinase, the association is disrupted, allowing one single kinase to catalyze multiple phosphorylation events. Thus, such interactions are difficult to be trapped or identified. The transient interaction does not trigger the reporter gene transcription in the yeast-two-hybrid system, while kinase-interacting substrates are hardly captured during affinity purification. Nevertheless, there are some cases demonstrating that kinase substrates were successfully identified, either through two-hybrid system (Fig. 4) (Yang et al., 1992; Vadlamudi et al., 2002) or the affinity purification method (Daub et al., 2008; Belozerov et al., 2012).

High false positive rate is the most severe problem associated with the interaction screening method. Compared to real substrates, there are overwhelming numbers of other proteins interacting with target kinase physiologically, including components of protein complexes and adaptor proteins. The reality that no convincing strategy exists to differentiate *bona fidein vivo* substrates from kinase binding proteins certainly limits the usefulness of this method. As with all other approaches discussed here, confirmation of candidate substrates by a secondary assay is critical and in

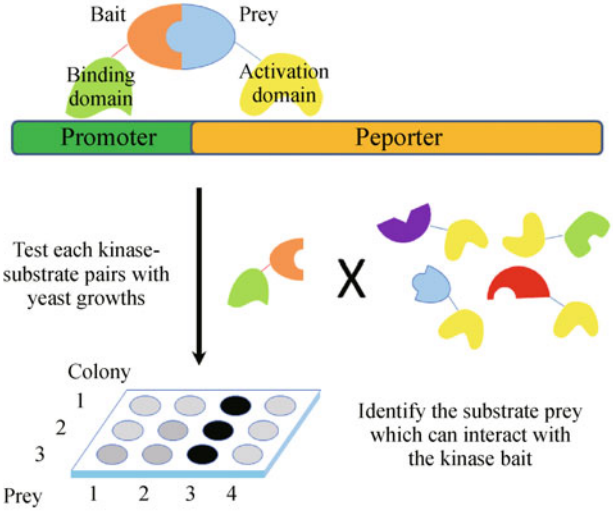

Figure 4 Application of the classical yeast-two-hybrid approach for the kinase substrate identification. Each candidate is paired with the kinase of interest. Putative substrates showing positive interaction must be examined by alternative experimental strategies.

this case another high throughput experiment is probably inevitable to further narrow down the candidate list.

Mass spectrometry-based high throughput screening

In the past decade, the advancement of high speed and high resolution mass spectrometry has significantly improved the efficiency and accuracy of protein post-translational modification (PTM) identification and has become the dominant technology for phosphorylation identification in proteomics study. Compared with microarray based screening and phage display system, the mass spectrometry not only identifies phosphoproteins, but also pinpoints their phosphosites. There have been increasing attempts to develop mass spectrometry-based proteomic strategies for the kinase substrates exploration (Huang et al., 2007; Amanchy et al., 2008; Coba et al., 2009).

Phosphoproteome profiling

Shotgun proteomics enables the identification of thousands of proteins in a single analysis. LC-MS/MS analysis of a peptide mixture can detect and characterize diverse types of PTMs, such as phosphorylation (Witze et al., 2007). Together with the improvement in phosphopeptide enrichment methods (Neville et al., 1997; Pinkse et al., 2004; Iliuk et al., 2010), mass spectrometry based large scale phosphorylation profiling has largely replaced metabolic labeling with radioisotopes and Edman sequencing to identify endogenous phosphoproteins and sites of phosphorylation (Cañas et al., 2006). Furthermore, a variety of quantitative methods for LC-MS/MS analysis have been successfully applied to quantify phosphorylation change on specific sites. All these technological progresses have led to numerous application of mass spectrometry in biological fields such as the kinase-substrate analysis we are discussing here (Kosako and Nagano, 2011).

The generic phosphoproteomics procedure for identifying kinase substrates contains three major steps. First, samples are treated to inactivate the target kinase, such as pharmacological inhibition, antibody injection, knockout technology or RNA interference. Second, the phosphorylation change due to the kinase perturbation is quantified by mass spectrometry. Third, important phosphorylation events are tracked and used for kinase substrate prediction using bioinformatics tools (Kettenbach et al., 2011).

There are several limitations of this strategy for substrate screening. First of all, it requires an effective and highly specific way to inactivate the target kinase, which is difficult to achieve and evaluate with any of available methods. Moreover, unlike *in vitro* methods, it is difficult to determine if a phosphorylation change is the direct effect of either the target kinase activity or coherence through downstream phosphorylation cascade. Therefore, phosphoproteome profiling does not typically reveal a precise relationship between protein kinases and their direct substrates (Manning and Cantley, 2002).

Synthetic peptide library screening

Almost all of the previously mentioned array based experiments can be transformed into the MS platform. Among them, solution-phase peptide library screening is the most popular method to study kinase specificity. Generally, the peptide library usually requires one fixed position of Ser, Thr or Tyr surrounded by random amino acids in degenerate positions. To get a better representative preference of substrate sequences, a higher coverage of all potential sequences is strongly recommended. However, the library complexity is exponentially growing which will soon saturate the experimental detection capability. In the past years, the progress of mass spectrometry's accuracy and scanning speed drastically improved the efficacy of this methodology (Songyang et al., 1995; Mah et al., 2005), and the computational prediction has improved the determination of the relevant phosphorylation events (Fujii et al., 2004). In the meantime, some frameworks, like Scansite for probabilistic predictions of phosphorylation sites based on library screening approaches, are generated and extensively utilized in data mining for high throughput experiments (Yaffe et al., 2001; Obenauer et al., 2003).

Besides the surface-based and solution-phase based peptide library techniques, more recently, polymer-bound peptide library method has been used in substrate screening as well. In this approach, a peptide library is pre-constructed on aresin bead, connected by a photolabile linker (PLL). After the kinase reaction is performed on the peptide library, a colorimetric assay using an alkaline phosphatase conjugated anti-phospho-Tyr antibody conveniently detects and picks out the beads with phosphorylated substrates by color change. At last, the phosphopeptides are eluted from the beads by UV irradiation and sequenced by mass spectrometry. Since an optimized peptide library synthesis route termedone-bead-one compound (OBOC) was built up (Lam et al., 1995; Lou et al., 1996), several tyrosine kinase like c-Src, ZAP-70 and Brk specificities have beenexamined (Kim et al., 2007; Shin et al., 2008).

In vitro kinase assay with peptide and protein extracts

Taking advantage of massive sequence diversity in natural cell lysate, the *in vitro* kinase assay with enzymatic digested peptidome from cell extracts can serve the same purpose for kinase specificity interrogation as a peptide microarray. Combined with the high throughput identification capability of modern mass spectrometry, the cost demand of such experiments is significantly lower than array-based assay. Theoretically, the peptidome represents the full spectrum of physiologically possible sequences, whereas synthetic pep-

tide library contains some extra artificial sequences and misses some real sequences due to the degenerative amino acid design.

In recent years, a number of attempts to identify phosphoproteins have directly used a cell lysate (or fractions of cell lysate) as a protein pool for an *in vitro* kinase reaction that is followed by mass spectrometric analysis. Compared to bacterial expression systems using cDNA library, the direct utilization of protein extracts from cells is a more convenient way to extract the proteome with natural folding and post-translational modifications. This strategy is attractive in the substrate screening because of its minimized labor cost. Due to the complexity of natural cell extracts, high speed and high resolution mass spectrometry is essential to make this method applicable.

One of the main challenges for these approaches is how to distinguish the direct kinase substrate phosphorylation from a background resulting from other kinase activities. To address this issue, some optimized procedures, like high concentration of purified kinase (Knebel et al., 2001; Cohen and Knebel, 2006), pulse heating to inactivate endogenous kinase activities (Troiani et al., 2005), and quantification (Kettenbach et al., 2011) can be employed. Furthermore, an elegant approach named Analogue-Sensitive Kinase Allele (ASKA) technology was developed by Shokat's group (Shah et al., 1997). In this approach, only the mutant kinase of interest can accept bulky-ATP analogs exclusively so that the direct phosphorylation caused by target kinase can be distinguished from indirect effect due to other endogenous kinases during *in vitro* kinase assay (Fig. 5A). Although *in vitro* specificity of

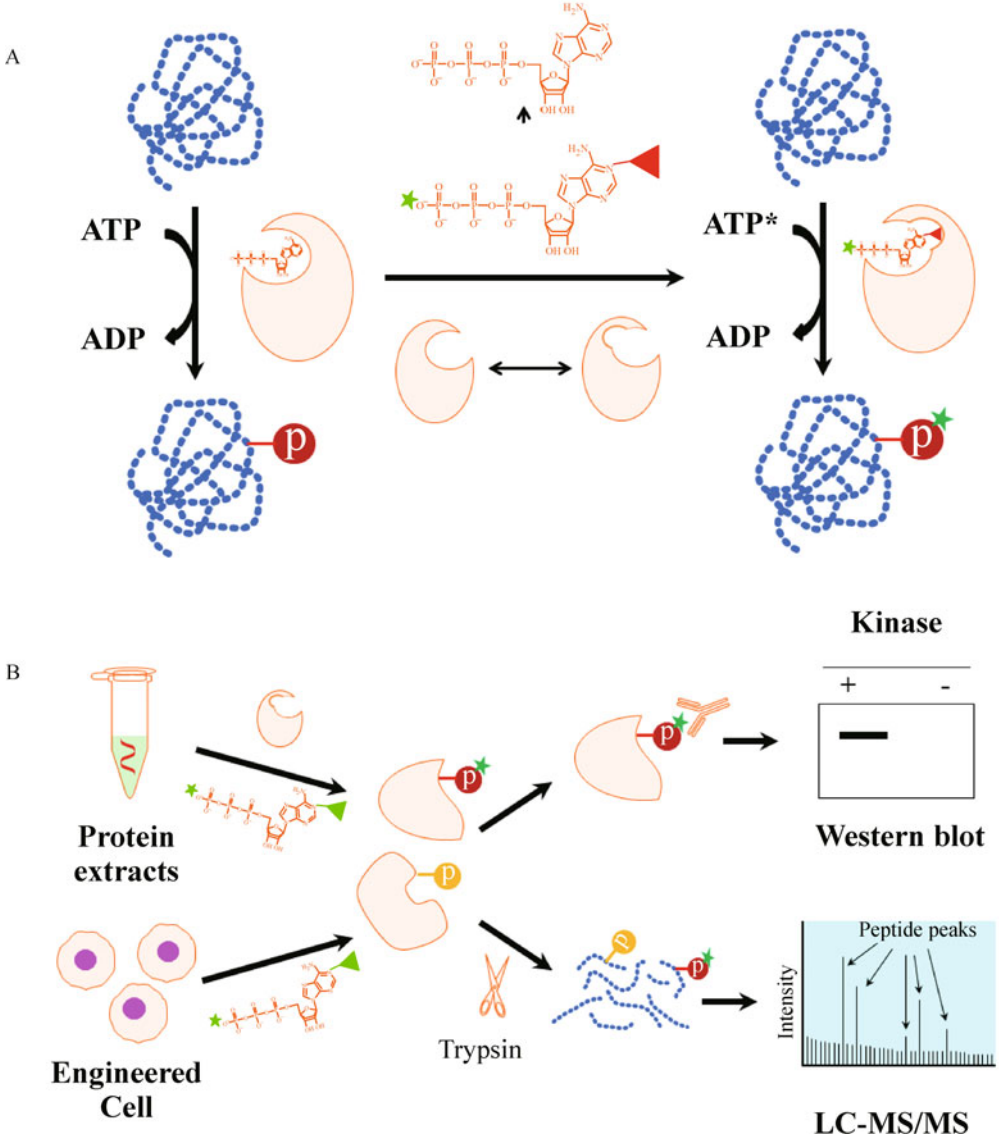

Figure 5 Brief illustration of analog sensitive kinase allele strategy (ASKA). (A)The bulky-ATP analog can be only up taken by engineered kinase. (B) Proposed application of ASKA to identify direct kinase substrates both *in vitro* and *in vivo*.

kinases is still a concern, the use of a bulky-ATP and an engineered kinase pair successfully eliminates the background phosphorylation problems, assuring all the phosphorylated proteins in the cell lysate are the direct substrates of the target kinase. This approach has been widely used and has successfully identified novel substrates of various kinases (Habelhah et al., 2001). Recently ASKA has been coupled with quantitative proteomics, termed Quantitative Identification of Kinase Substrates (QIKS) to identify substrate proteins of Mek1 (Morandell et al., 2010), as well as microarray platform to identify substrates of yeast Pho85-Pcl1 cyclin-dependent kinase (Dephoure et al., 2005).

All those applications of ASKA, however, have been limited to the identification of *in vitro* kinase substrates. In principle, this approach should be applicable to *in vivo* labeling of substrates as well as the *in vitro* biochemical method discussed above, when the mutant kinase is expressed (Fig. 5B). However, how to deliver sufficient quantities of radiolabeled bulky-ATP analog into the cell for visualization and how to purify substrate proteins are yet to be clarified (Manning and Cantley, 2002). Another challenge for this chemical genetic approach is the prerequisite of engineer active analog-sensitive kinases, which requires preliminary examination of specific kinases so that the overall throughput of this method is lowered (Blethrow et al., 2004).

Kinase Assay Linked with Phosphoproteomics (KALIP)

All above methods can only identify kinase's substrates under *in vitro* conditions or detect endogenous phosphorylation events modulated by the kinase (including direct and indirect substrates). To address the issue, our group has recently devised an integrated strategy termed Kinase Assay Linked with Phosphoproteomics (KALIP) for determining the substrate specificity and identifying direct substrates of protein kinase (Xue et al., 2012). In this approach, a pool of formerly phosphorylated peptides is derived directly from a cellular lysate for sensitive *in vitro* kinase reaction. The resulting newly phosphorylated peptides are then enriched and identified by mass spectrometry (Fig. 6). Those phosphopeptides reveal the substrate specificity of the kinase and putative phosphorylation sites.

Taking the *in vitro* kinase specificity issue into consideration, the *in vitro* kinase assay is further linked to endogenous phosphoproteomics from cells in which the kinase is active or inhibited. Overlapping the two results from both the direct phosphorylation sites observed from the *in vitro* reaction and the kinase dependent physiological phosphorylation sites revealed from *in vivo* phosphoproteomics should yield the physiological direct substrates of the target kinase with highest confidence. Using this method, numbers of known

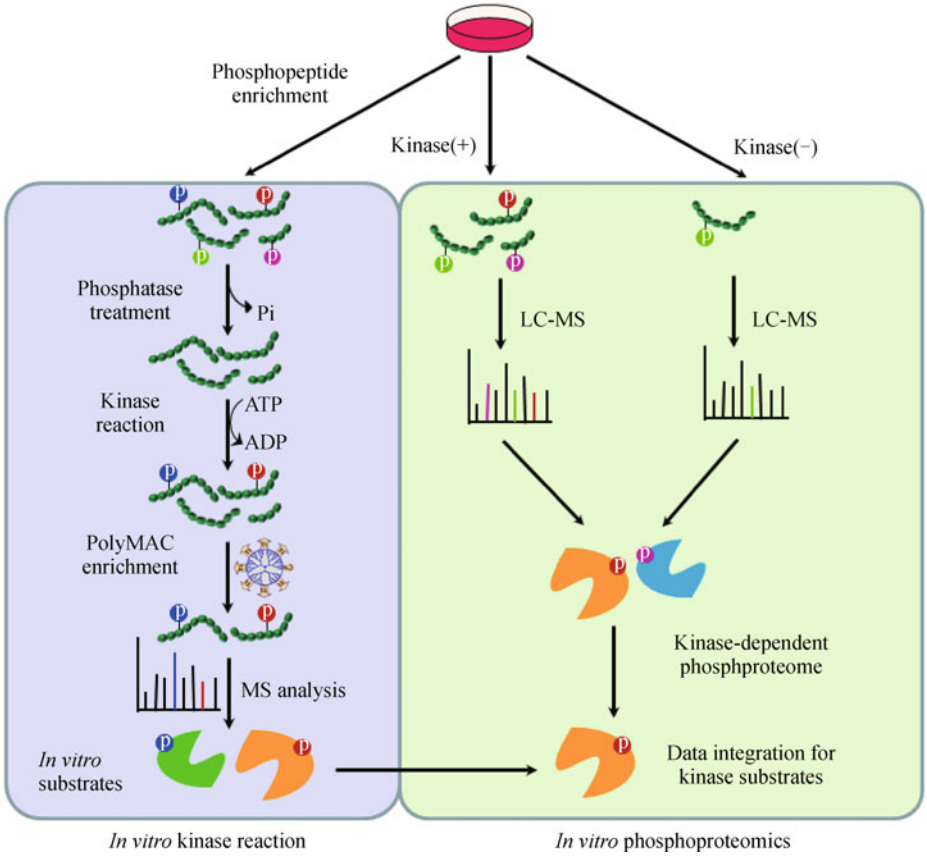

Figure 6 Flow chart for the Kinase Assay Linked with Phosphoproteomics (KALIP) approach to identify direct kinase substrates.

substrates and presumed primary sequence preference of the spleen tyrosine kinase (Syk) are revealed. Moreover, out of a small candidate list, several novel substrates of Syk were confirmed by *in vitro* phosphorylation assays and receptor-mediated phosphorylation assessment, indicating a high true positive rate of KALIP approach. Interestingly, by comparing the varied putative substrates in two cell lines in which the kinase plays different roles, the author proposed that substrates identified by this strategy should be both kinase and cell type specific.

Unlike traditional peptidome screening, KALIP used the pre-enriched total phosphotyrosine peptides as the substrate pool to eliminate physiologically unphosphorylatable peptides and to increase the dephosphorylation efficiency. However, compared to tyrosine phosphorylation, pre-enrichment of serine/threonine phosphorylation has not been demonstrated. Therefore, it is difficult to achieve a complete subsequent dephosphorylation of serine/threonine phosphopeptides. To address this issue, quantitative proteomics will be extremely helpful to dissect the true phosphorylation events from the residual phosphorylation background. In addition, any enzymatic digestion that generates a peptide pool will abolish certain motifs containing digested residue that are required by the kinase of interest, though this bias can be partially resolved by using a variety of proteases.

Bioinformatics of kinase-substrate prediction

In parallel with those ever-evolving experimental approaches, computational analyses on kinase substrates take advantage of the massive biological information to generate their predictions. The central hypothesis of this prediction is that the sequence or structure preference of a kinase determines its corresponding substrates.

Phosphorylation sequence preferences have been examined for many kinases through the use of peptide library screenings (Songyang et al., 1996). The favor or disfavor of a kinase for each amino acid flanking the phosphorylation site can be calculated by a position-specific scoring matrix (PSSM). The quantitative kinase preference is then used to exam any known protein to predict which of the characterized kinases may account for a particular phosphorylation event. Scansite is one of pioneering tools for doing such analyses (Yaffe et al., 2001). The approach accesses the enrichment of certain motifs on specific proteins relative to all occurrences in a protein database, which has achieved more reliable prediction than simple pattern matching. One application of the approach is the identification of tuberin as the AKT substrate. The prediction result was further confirmed by Western blotting using the antibody against phospho-motif of AKT upon platelet-derived growth factor (PDGF) stimulation (Manning et al., 2002a). Many other programs with amended algorithms were also demonstrated to succeed. NetworKIN, an integrative computational approach combines consensus sequence motifs and protein–protein interaction networks, identified 53BP1 and Rad50 as CDK1 and ATM substrates, respectively (Linding et al., 2007). On the other hand, iGPS which uses the supervised machine learning algorithm predicted a condensed human liver protein phosphorylation network containing 12819 potential kinase-substrate relations among 350 kinases, 962 substrates and 2633 phosphosites (Song et al., 2012).

Although bioinformatics prediction of substrates or specificity of a kinase alone is not completely reliable, it offers a quick screening that can be further tested with other techniques, and it can be integrated to any experimental strategy described above to ameliorate experimental burdens and to improve the overall sensitivity.

Table 1 Comparisons of kinase substrate screening techniques (See text for more details)

Assay	Working condition	Advantages	Limitation
Protein array	*In vitro*	Sensitive Direct substrate identification	Artifact prone No phosphosite information
Phage display	*In vitro*	Direct substrate identification	Protein misfolding by bacteria
Peptide array/ Synthetic peptide library	*In vitro*	Kinase specificity identification Pseudo-unbiased motif screening	Require bioinformatics for substrate prediction
Protein extract	*In vitro*	Fast, cheap, convenient	Endogenous kinase contamination
Phosphoproteomics	*In vivo*	Native phosphorylation monitoring, including phosphosites, quantity, etc.	Do not indicate direct relation of kinase and substrate
Kinase assay linked phosphoproteomics (KALIP)	*in vitro, in vivo*	Fast, cheap, convenient Direct substrate identification	Loss of motifs due to enzymatic digestion Loss of protein structure for kinase reaction
Analog sensitive kinase assay (ASKA)	*In vitro, in vivo*	Direct substrate identification Native reaction condition	Method optimization for individual kinase Not yet demonstrated *in vivo*
Yeast two hybrid AP-MS	*In vivo*	No knowledge required for kinases Fast to make decision	Require other techniques to provide phosphorylation information

Conclusion

Phosphorylation by protein kinase is considered as a vital regulatory mechanism in living systems. Recently, protein kinases have drawn much attention as targets for new drug development. Various approaches to screening kinase substrates are essential for the study of protein kinases and contribute much to identifying the sequence specificity of phosphorylated sites.

To illuminatethe relationship between kinases and substrates, many targeted and high throughput approaches (Table 1) have been attempted. Those methods can be grouped into three major categories. First is *in vitr*o phosphorylation assay, including solid-phase array, phage display and *in vitro* phosphorylation reaction using analogue-sensitive kinase alleles, peptide library or protein extracts. Second is the indirect protein–protein, protein-gene interaction assay like affinity purification for discovering kinase-associated proteins (Gavin et al., 2002; Gavin et al., 2006; Krogan et al., 2006; Breitkreutz et al., 2010). Third is the systematic genetic screening for identifying genes that functionally interact with kinases (Fiedler et al., 2009; Sopko et al., 2006; Costanzo et al., 2010). Among the detection methods, mass spectrometry has emerged as the method-of-choice to detect unknown kinase substrates and reveal phosphorylation sites. While bioinformatics and biochemical and molecular biology experiments are crucial to evaluate and validate high throughput data, proteomics will continue playing a major role for kinase substrate identification with new instrumentation and novel strategies.

Compliance with ethics guidelines

Conflict of interest

Liang Xue and W. Andy Tao declare that they have no conflict of interest.

Human and animal rights, and informed consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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