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Dissecting kinase signaling pathways

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Aberrant protein kinase signaling is a hallmark of many human diseases including cancer, diabetes, and neurological disorders. Kinase inhibitors have shown to be successful at treating some of these diseases, implying that understanding kinase signaling pathways may lead to additional, non-kinase drug targets. However, identifying substrates of protein kinases is difficult due to the universality of the chemical mechanism kinases utilize and the ability of multiple kinases to phosphorylate the same protein substrates. In this review, we explore the advantages and disadvantages of several techniques for identifying kinase substrates. Once putative substrates are identified, their validation as physiological substrates remains a major challenge. We propose three criteria for confirming the physiological relevance of a putative substrate's interaction with a kinase.

Kinases as effective drug targets

Kinases are important regulatory proteins, relaying signals from outside the cell to cause downstream events, such as changes in cell metabolism, migration, or proliferation. Unregulated activity of specific individual kinases is the cause for many cancers and neurological disorders. Recently, the importance of kinases in the etiology of cancer has been proven by the successful use of kinase inhibitors for the treatment of various cancers. Three of those, imatinib (Gleevec®), trastuzumab (Herceptin®) and lapatinib (Tykerb®), are discussed below.

Imatinib

Phenylamino-pyrimidine scaffolds were originally developed as inhibitors of protein kinase C [1]. Imatinib, a derivatized form of this scaffold, was found to selectively inhibit the platelet-derived growth factor (PDGF) receptor, c-kit, [2] BCR-Abl, c-Abl, and Abl-related-gene (Arg) at sub-μM concentrations [3]. Imatinib has been used to target cancers with upregulated signaling of the aforementioned kinases by inhibiting the tyrosine kinase activity. Here we briefly describe the efficacy of imatinib at treating chronic myelogenous leukemia and gastrointestinal stromal tumors.

The causative agent for >95% of chronic myelogenous leukemia (CML) is a fusion of the *bcr* and *abl* genes, which results in a constitutively active kinase called BCR-Abl [4]. By targeting BCR-Abl, imatinib is effective in delaying disease progression, with 95% of chronic phase patients having a hematologic response [5] and minor side effects. The use of imatinib to treat CML has been heralded as a successful model for directed cancer treatment.

Because of the safety and success of imatinib in treating CML, it has been tested in combination therapies and proven effective for treatment of other types of cancer. For example, mutations in c-kit are found in many gastrointestinal stromal tumors (GIST). Though imatinib treatment for GIST was not as efficacious as for CML, >50% of the tumors responded to the treatment and shrank [6]. This finding was encouraging, as most advanced GISTs do not respond to chemotherapy [6].

Trastuzumab

Elevated levels of human epidermal growth factor receptor 2 (HER2) are found in approximately 30% of breast cancers, correlating with poor prognosis [7,8]. The humanized anti-HER2 antibody, trastu-

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zumab, is effective against HER2-positive breast cancer. Cell lines expressing HER2 showed reduced growth and colony formation on soft-agar when treated with the antibody [9]. The exact mechanism of action of trastuzumab is unknown; it is conceivable that antibody binding may block the receptor from binding an agonist and thereby delay cell division. However, it is also possible that the antibody marks cells for destruction by the immune system [10]. Regardless of the mode of action, trastuzumab is particularly effective in treating HER2-positive breast cancers when it is used in combination with other chemotherapies [11].

Lapatinib

Lapatinib is a small molecule inhibitor of the kinase domain of EGFR and HER2. Lapatinib blocks downstream signaling through the Akt and mitogen-activated protein kinase pathways *in vitro* and *in vivo* [12,13] and has shown activity as a monotherapy for breast cancer in preclinical trials. As lapatinib targets the same kinase as trastuzumab but has a different mode of action, lapatinib has recently shown to be effective against trastuzumab-resistant, HER2-positive breast cancers in some patients. Additional trials including several that investigate the use of lapatinib as an adjuvant to other therapies are ongoing [14].

These examples demonstrate that targeting kinases is an effective way of treating cancers and confirm that understanding the molecular basis of a disease allows for the development of tailored treatments to specifically target this disease. In fact, kinase inhibitors represent an important new category of drugs with more than 600 clinical trials currently underway (http://www.clinicaltrials.gov/). It is important to note that hyperactive kinases mediate their oncogenic actions via their substrates; therefore, identification of downstream kinase substrates may uncover additional drug targets. These substrates may be better drug targets than kinases because the downstream substrates might have a more limited signaling role in the cell, might be a terminal point in a pathway, or have fewer functional redundancies than kinases. It is therefore conceivable drugs that target substrates would have fewer pharmacological side effects. Phosphorylation events usually promote allosteric changes in substrate conformation or mediate new protein–protein interactions between the substrate and other cellular binding partners. Of these, it is likely that allosteric changes would be more readily targeted for inhibitory drug development. The identification of suitable targets and generation of specific therapies are essential for successful implementation of this strategy.

Techniques for identifying substrates of protein kinases

Kinases comprise 1.8% of the human proteome [15]. The vast majority of kinases utilize ATP and Mg^{2+} to catalyze the transfer of the γ-phosphate of ATP to Ser, Thr, or Tyr residues, depending on the type of kinase. The universality of this enzymatic reaction complicates the identification of kinase substrates. This is further exacerbated by functional similarities among the members of a kinase family and the overlapping recognition of individual substrates by multiple kinases [16]. Thus, any study identifying a kinase–substrate relationship requires careful validation.

Many different techniques have been utilized to identify kinase substrates, but only in very few cases have the identified substrates been rigorously validated. Strategies to identify substrates have employed genetic, biochemical, and proteomic analysis. Here we describe low- and high-throughput approaches used to identify kinase substrates and discuss the advantages and disadvantages of each technique.

Low-throughput approaches

Several low-throughput approaches have been used to identify kinase substrates. Because they involve the analysis of phosphoproteins from cells with altered kinase activity, the substrates identified using these approaches are likely to be physiologically relevant. A major disadvantage is that these approaches are not readily adapted to high throughput analysis.

Purifying phosphoproteins from cells expressing activated kinases

A classic gain of function technique capitalizes on the availability of constitutively active kinases and phospho-specific antibodies [17]. Following expression of a constitutively active kinase, phospho-proteins are purified using phospho-specific resins or antibodies (Figure 1a). Depending on their abundance, the phosphoproteins can be identified by sequencing or mass spectrometry. Alternatively, after the phosphoproteins are enriched, the purified phosphoprotein is used to generate monoclonal antibodies, which then could be used for subsequent analysis and large-scale purification and identification of the putative substrates [18].

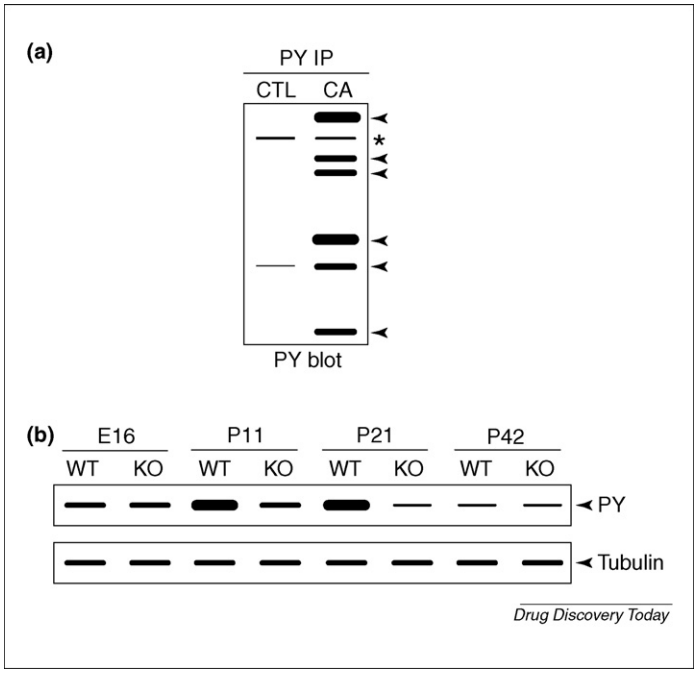

FIGURE 1

(a) Scheme for identifying substrates with constitutively activate kinases and phosphotyrosine enrichment. Cells expressing a constitutively active (CA) kinase are compared with control (CTL) cells. After enrichment for phosphorylated proteins (anti-phosphotyrosine, PY, immunoprecipitation), the blot is probed for phosphorylation content. The putative substrates are indicated by arrowheads; the background phosphorylation is labeled with an asterisk. **(b)** Comparative western blotting using wild type (WT) and kinase deficient tissues (KO). Monitoring phosphorylation at various developmental times, embryonic day 16 (E16), post-natal day 11 (p11), p21, and p42. The phosphotyrosine content is reduced in KO extracts relative to WT at p11 and p21. A loading control protein (e.g. tubulin in this case) is necessary for quantitation.

Although this technique was fruitful in identifying 6–10 putative v-Src substrates [19,20], the major shortcoming of this technique is that it does not distinguish between substrates that are directly phosphorylated by the activated kinase or substrates that are targeted by an additional downstream kinase. For example, cortactin was originally identified as a target of v-Src in transformed fibroblasts [18,21]. However, we recently demonstrated that Abl family tyrosine kinases act downstream of the Src family of tyrosine kinases to phosphorylate cortactin in response to PDGF [22].

Comparing phosphoprotein composition of wild type and kinase-deficient organisms

Another way of identifying kinase substrates is by comparing the spectrum of phosphorylated proteins in WT versus kinase-deficient (mutant) extracts: putative substrates will be hypo-phosphorylated in the mutant extracts relative to WT extracts [23]. This is often done by analyzing a cell line or tissue and monitoring extracts throughout development (Figure 1b). These extracts can be fractionated by column chromatography, solubility, or another physical attribute to purify or enrich for the phosphoprotein of interest. The phosphorylation status of proteins within the extracts is examined by western blot analysis with phospho-specific antibodies or dyes [24] or gel mobility shift. Identification of the substrate's identity may be based on literature searches, mass spectrometry, or protein sequencing techniques. Using this technique, our lab identified p190RhoGAP as a substrate of the Arg kinase in the developing mouse brain [25]. An advantage of this technique is that the analysis can be simplified by focusing on a particular tissue; however, this technique is only useful for identifying highly abundant proteins.

A chemical genetic technique to identify direct kinase substrates

The bump-hole technique, originally developed by Shokat and colleagues, allows for direct labeling and detection of kinase substrates [26]. This technique requires the generation of a mutant kinase that, unlike WT kinases, can utilize a structurally altered nucleotide. Chemical addition of a 'bump' on ATP generates an unnatural nucleotide (here denoted A*TP). Removal of a bulky amino-acid side chain in the ATP binding site of the kinase creates a 'hole' in which the 'bump' can fit. Mutant kinases that can accept A*TP are called *analog-specific*- (*as*-) kinases (Figure 2). By combining an *as*-kinase, cell extract, and A*TP-γ-^{32}P, one can identify the *direct* substrates of the *as*-kinase by monitoring the incorporation of radiolabeled phosphate. The extracts are separated by gel electrophoresis and the radiolabeled band is excised for protein identification. This technique is the only approach that identifies direct kinase–substrate interactions from complex mixtures and, because it relies on radioactivity, is more sensitive than many of the techniques described above. However, as with other approaches, the putative substrate must be purified and identified, which requires sufficient material for mass spectrometry. Immunoprecipitation with phospho-specific antibodies or purification of the radiolabeled proteins from large amounts of starting material may enrich for phospho-proteins to generate sufficient material for protein identification.

Two recent variations of this technique allow for the purification of direct substrates of an *as*-kinase by (1) using a library of strains expressing a single epitope-tagged protein or (2) employing nucleotide analogs containing sulfur (A*TP-γ-S) rather than A*TP-γ-^{32}P. First, kinase assays were performed with *as*-Pho85 and lysates of 4250 yeast strains containing a single epitope-tagged protein. All tagged proteins were purified and incorporation of radiolabeled phosphate was monitored. This systematic application of the bump-hole technique identified 24 putative substrates and overcame the difficulty of substrate identification [27]. Second, the incorporation of a thiol through thiophosphorylation provides a novel reaction site for the addition of an immunoreactive

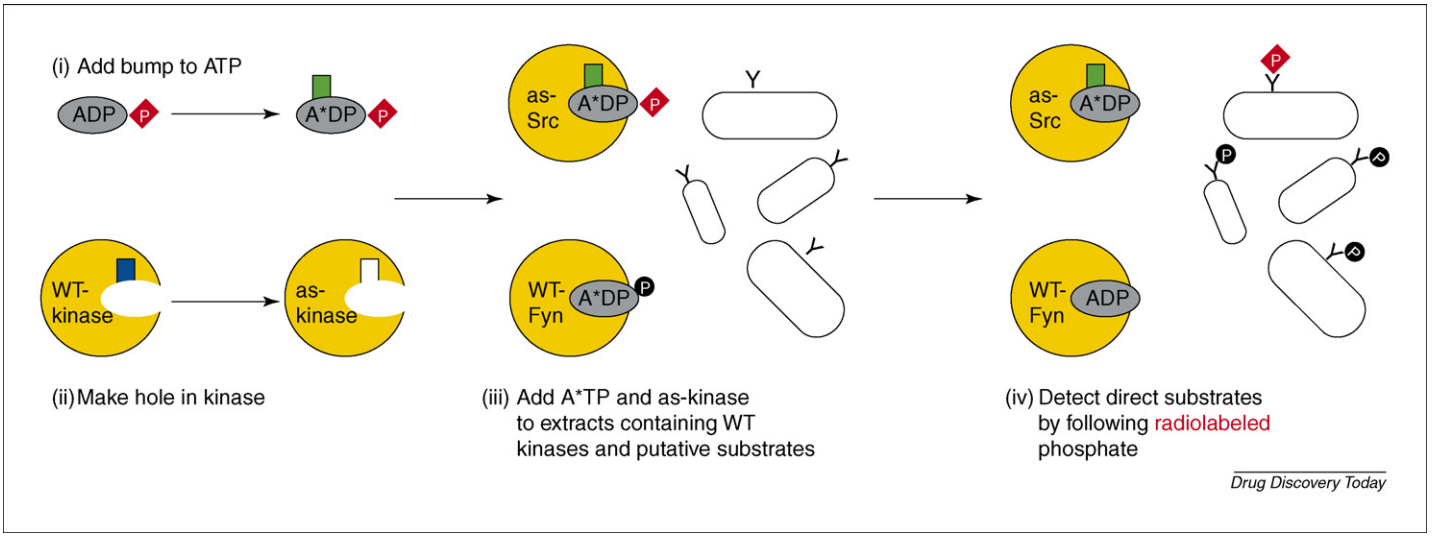

FIGURE 2

The bump-hole technique directly labels substrates. First, a bump (green rectangle) is added to ATP, causing a chemically altered nucleotide (A*TP) to sterically clash with WT kinases and prevent usage. Second, a hole is engineered into the ATP binding site of the kinase to permit A*TP binding and usage by mutating a bulky residue (blue) to alanine or glycine. A kinase that can utilize A*TP has altered nucleotide specificity and is called *analog specific* (*as*-kinase). Third, A*TP is combined with the *as*-kinase to create a unique nucleotide-kinase pair. The bump-hole technique is sensitive and labels substrates directly. When one *as*-kinase (Src in this case) is added to cell extract containing a mixture of putative substrates and other WT kinases but only A*TP is radiolabeled (radiolabeled phosphate is red diamond, unlabeled phosphate is black circle), only the direct substrates of the *as*-kinase will be radiolabeled. Fourth, the radiolabeled bands must be isolated and identified by mass spectrometry.

tag [28,29]. Immunoprecipitation with antibodies raised against the thiophosphorylation site tag enrich and purify the direct *as*-kinase substrates. Proof-of-principle work confirmed a previously reported Erk2 substrate was thiophosphorylated, purified, and identified from cell extracts [29].

Despite the detection of inabundant phospho-proteins, only medium- to high-abundance substrates may be identified. Also, this technique may yield false positives because putative substrates are identified from cell or tissue lysate where the possible physiological boundaries between the kinase and substrates have been eliminated. Further, not all kinases can be engineered to utilize structurally altered nucleotides [30]. Despite these limitations, Shokat and co-workers have used this technique to identify substrates of v-Src, JNK, and other kinases [31,32].

Another variation of this technique may help to identify the role of the kinase *in vivo*. Instead of adding a 'bump' to ATP, this technique adds a 'bump' to a general kinase inhibitor, creating a unique, highly specific inhibitor for the *as*-kinase [33]. This unique inhibitor and the *as*-kinase can be used as a conditional allele *in vivo* to identify what roles the *as*-kinase plays in cellular processes. For example, Shah and colleagues used this approach with *src*−/−*yes*−/−*fyn*−/− (SYF) cells reconstituted with *as*-Src to confirm a role for Src in response to PDGF treated cells [34]. This approach could be used to follow up on substrates identified using the direct labeling approach.

High-throughput approaches

In addition to the previously discussed approaches, several techniques for identifying kinase substrates involve scaleable approaches well suited for high-throughput analysis.

Database searching for kinase substrates

Peptide libraries have been used to identify consensus phosphorylation site motifs for several kinases (e.g. Cdc2, Cdk2, PKA, SLK1, casein kinases I and II, CamKII, Cdk5, and Erk1, and several PKC isoforms-see refs 12–15 in [35] for more information). These motifs can help predict phosphorylation sites within a potential substrate. For example, the consensus phosphorylation site motif for protein kinase A (PKA) is R-R-X-S/T-*hydrophobic* [36]. A peptide containing this sequence has a K_M (Michaelis constant: the substrate concentration at which an enzyme catalyzed reaction proceeds at one-half its maximum velocity) nearly equivalent to a full-length protein containing the same sequence [37,38]. Database searches can be performed using the consensus phosphorylation site motif to identify novel substrates or to provide supporting evidence to validate putative substrates from other techniques. For example, Kang *et al.* identified putative substrates from cultures overexpressing the kinases PknA and PknB. They found that the putative substrates contained sequences that matched consensus phosphorylation site motifs for PknA and PknB [39]. Further, Rychlewski *et al.* found that some of the residues from their consensus phosphorylation site motif for Abl were found in eleven of fourteen published Abl substrates [40]. Despite these examples, this technique considers only the sequence surrounding the phosphoacceptor site, but not possible secondary and tertiary structural elements, such as domains or surfaces on the protein substrates that may mediate interactions with the kinase. Also, the assay to establish the consensus phosphorylation site motif is often performed with peptide substrates at supraphysiological concentrations: tens to hundreds of μM, two to three orders of magnitude higher than for folded protein substrates. These facts likely explain why several validated kinase substrates fail to contain the consensus phosphorylation site motif.

Identification of candidate substrates from kinase-binding partners

Identification of proteins that physically interact with a kinase-of-interest has also led to the identification of novel substrates. Techniques such as yeast-two-hybrid or GST-pull-down assays employing protein–protein interaction domains of the kinase-of-interest have identified putative substrates. The first kinase used to identifying new substrates from binding partners is the SNF1 protein kinase. A yeast-two-hybrid screen for SNF1 binding partners identified four SNF1-interacting proteins (SIP1-4) [41]. Of these, SIP1 interacted genetically with SNF1 and was phosphorylated *in vitro* by SNF1.

A variation of this technique is to identify binding partners by performing pull-down assays with tagged protein–protein interaction domains. In these assays, cell or tissue extracts are combined with the tagged protein. After incubation, the tagged protein is purified and any binding partners are identified. For example, the Src substrate Sin was identified initially as a binding partner for the Src SH3 domain and then shown to be a substrate of the kinase [42,43]. It is possible that not all binding partners will be kinase substrates, but this can be easily tested by monitoring the phosphorylation state of binding partners.

Screening entire cDNA libraries using in vitro transcription, translation, and kinase assays

Putative substrates of protein kinases can also be identified by systematically screening a cDNA library in pools of 20–30 genes for proteins that are phosphorylated by a particular kinase. According to this strategy, a cDNA library is split into pools of about thirty genes. These pooled genes are transcribed and translated *in vitro* to generate pools of potential substrates. Finally, the pool proteins are incubated with the kinase-of-interest (Figure 3). Using autoradiography, phospho-specific antibodies, or mobility shifts, one can identify which protein(s) in the pool of thirty are phosphorylated by the kinase, usually without extensive purification. By repeating this approach, an entire library can be systematically screened for kinase substrates. This technique was utilized by Kirschner and colleagues to identify substrates of cyclin-dependent kinases (CDKs) [44,45]. Although it requires substantial effort to generate and systematically work through a whole cDNA library, the major advantage of this technique is that even inabundant, putative substrates can be identified.

Identifying candidate substrates from kinase assays on protein microarrays

Many of the methods described above are not suited to identify substrates that are relatively inabundant. Protein microarrays have been developed that contain the entire yeast proteome, portions of the *Arabidopsis* proteome, and portions of the human proteome [22,46–48]. Processes such as ligand binding, protein–protein and protein–DNA interaction, metabolic pathways, kinase activity, and antibody cross-reactivity have been studied using protein

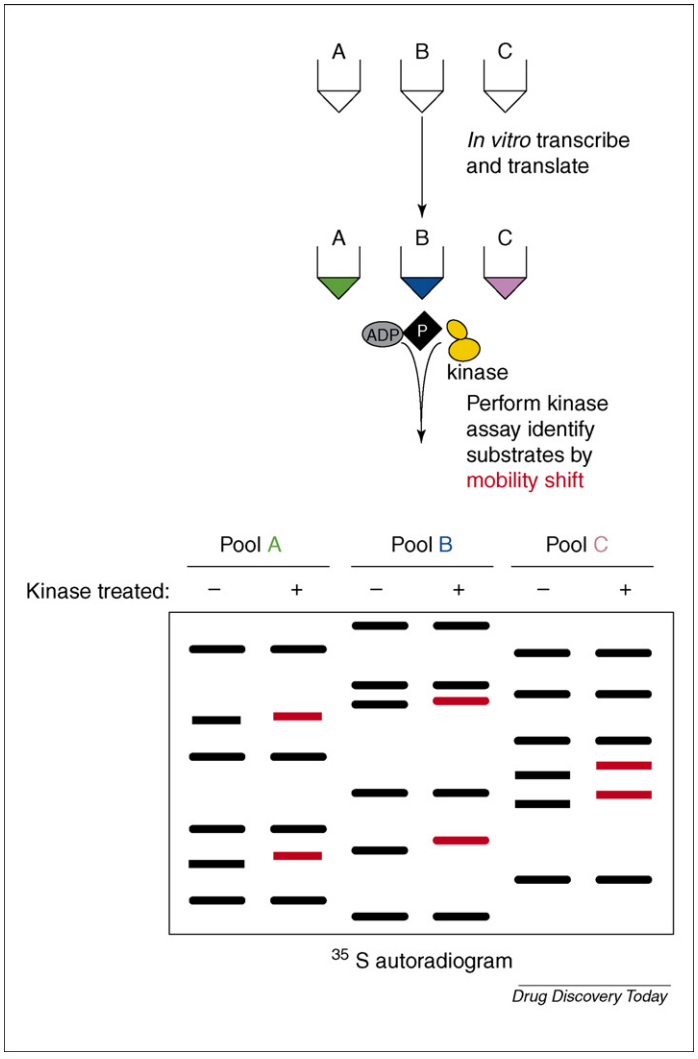

FIGURE 3

Screening entire cDNA libraries using *in vitro* transcription, translation, and kinase assays systematically identifies putative substrates. In this technique small pools of cDNAs are separated into different wells and transcribed and translated *in vitro* producing a known, unique set of ^{35}S-labeled putative substrates. The kinase-of-interest is added to an aliquot of the protein mixture, and the kinase-treated and untreated mixtures are separated by SDS PAGE electrophoresis. Labeled phosphoproteins are identified by mobility shift (red bands). Judging from the molecular weight of the putative substrate, one can identify the putative substrate from the known contents of the small pool.

microarrays [46,48–57]. The substrates of a particular kinase can be profiled by incubating these microarrays with ATP-γ-^{33}P and the kinase-of-interest (Figure 4) [22,46,48]. Because the proteins are purified and spotted on the array according to a known pattern, identification of all putative substrates, even those of low-abundance, is easy. The major disadvantage of this technique is that proteins must be purified and that immobilization to the chip may alter the folded conformation, leading to false positives. However, several protein arrays are commercially available, and the rapid screening and identification of substrates should be balanced against the risk of false positives. This technique is also potentially amenable to automation. A variation of this technique is to use the protein array to identify binding partners of the kinase [22,46]. Identification of the same protein as a binding partner and a substrate reduces the likelihood of it being a false positive.

Phosphoproteomics

Owing to technical strides in mass spectrometry, analysis of global phosphorylation events has become possible [58]. In this technique, cells are perturbed by an activating signal, treatment with a kinase inhibitor, or genetic ablation of a kinase, and then analyzed for alterations in phosphorylation status. Generally, the phosphoproteins are enriched by anion exchange chromatography, separated by 2-dimensional electrophoresis, and stained. Spots with altered mobility between the control and perturbed cells are identified by mass spectrometry. Using an inhibitor to CDK5, Gillardson *et al.* identified alterations in phosphoprotein content in proteins consistent with the known role of CDK5 *in vivo* [59,60]. The major advantages of this technique are the potential of examining global changes in phosphorylation status due to a particular kinase and the generation of refined phosphorylation consensus motifs. However, owing to the complex nature of proteomic studies involving extracts, inabundant or sub-stochiometrically phosphorylated substrates may escape detection.

Criteria for evaluating putative kinase substrates

Each of the techniques described above can be used to identify putative kinase substrates. However, subsequently it has to be proven that these newly identified substrates are indeed physiological kinase substrates. We propose the following criteria to confirm if new, putative substrates are bona fide *in vivo* substrates of the kinase-of-interest. According to these criteria, the substrate must:

- be phosphorylated *in vitro* by the kinase-of-interest with favorable kinetics;
- be phosphorylated in response to a physiological signal involving the kinase-of-interest;
- alter a cellular process in a phosphorylation-dependent manner on sites phosphorylated by the kinase-of-interest.

Substrates are efficiently phosphorylated *in vitro*

Relevant substrates should interact favorably with their respective kinase *in vitro*. Our work has demonstrated a correlation between the efficiency of a kinase phosphorylating a substrate *in vitro* and the likelihood that it is serving as a substrate *in vivo* [22,25]. These catalytic interactions are easily characterized by performing steady-state kinetic analysis. In this assay, increasing concentrations of the putative substrate are incubated with a constant concentration of the kinase and excess ATP-γ-^{32}P, and the incorporation of radiolabeled phosphate is monitored. The data are fit to the Michaelis–Menten equation, which provides the Michaelis constant (K_M) and the k_{cat}. These values provide a metric for comparing putative substrates. The best substrates would have a low K_M and a reasonable turnover rate. Relevant, validated *in vivo* substrates have K_Ms in the 100–300 nM range and k_{cat}s of ~20–100 min^{-1} for tyrosine kinases [22,25,61] or >600 min^{-1} for serine/threonine kinases [62–64]. This step is useful for separating the putative substrates that can interact from those that interact well. However, it is possible that physiologically important substrates are not phosphorylated efficiently *in vitro*. For example, elevated local concentration of a substrate may overcome the requirement to interact optimally with the kinase. Substrates whose interactions with the kinase require a bridging protein

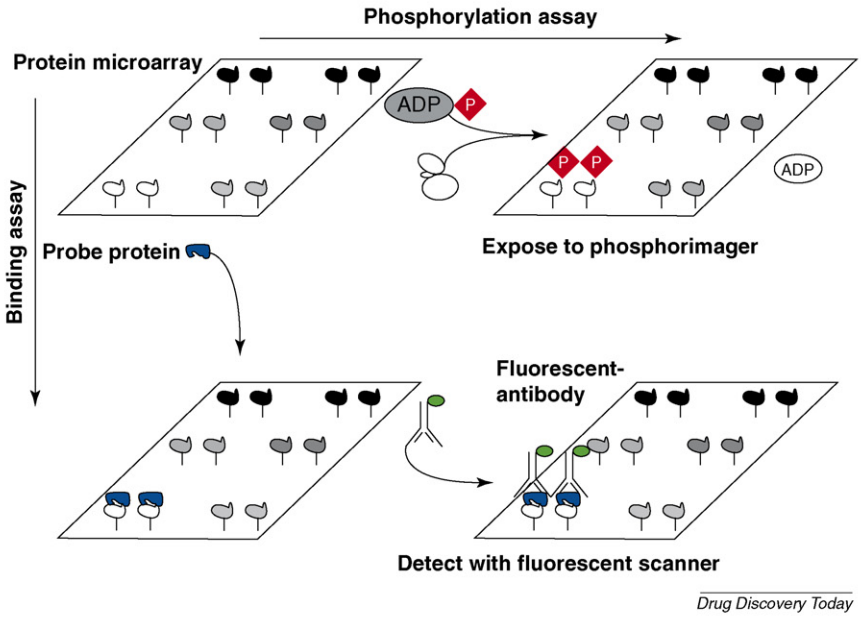

FIGURE 4

Identification of kinase substrates and binding partners using protein microarrays. Scheme to identify kinase substrates and binding partners. Entire proteomes are expressed, purified, and arrayed in duplicate on glass slides according to a known pattern. (Top) Addition of kinase and ATP-γ-^{33}P radiolabels (red) substrates; these are detected by exposing microarray to phosphorimager and using known pattern to identity substrates. (Bottom) Addition of kinase (probe protein) and fluorescently labeled antibodies (green) identifies binding partners upon detection with fluorescent scanner. The identification of the same protein as a binding partner and a substrate reduces the likelihood of the putative substrate being a false positive.

may be phosphorylated efficiently *in vivo* whereas the *in vitro* K_M is poor.

Substrates must be phosphorylated by the kinase in response to a physiological stimulus

Identification of signals that increase phosphorylation of the substrate in a kinase-dependent manner within a physiological setting is essential. These efforts can be guided by knowledge of what stimuli lead to increased kinase activity or increased phosphorylation of the substrate. The gold standard involves stimulating WT and kinase-mutant (knockout or knockdown) cells with various signals and monitoring the phosphorylation status of the putative substrate. The substrate of interest is immunoprecipitated or purified from the cell extracts and then its phosphorylation status is tested by autoradiography, western blot analysis with phospho-specific antibodies or dyes, or gel mobility shift. In order to be validated, a substrate must exhibit increased phosphorylation in WT but not mutant cells, and reexpression of the kinase in mutant cells must rescue the phosphorylation. This criterion would not hold for most oncogenic kinases because they are constitutively active.

Alter a cellular process in a phosphorylation dependent manner

The final and most difficult criterion for confirming a kinase substrate is to identify a cellular phenomenon regulated by phosphorylation by the kinase-of-interest. Phosphorylation is a convenient, reversible switch for activating or deactivating a process, such as changing the localization of a protein (JNK moves to nucleus when phosphorylated) [65], altering its catalytic activity (phosphorylation activates phosphorylase to hydrolyze glycogen) [66], stimulating the formation of a new complex (GRB2 binds to phosphotyrosines on growth factor receptors) [67], or releasing inhibitory interactions (phosphorylation, and subsequent ubiquitination, of IκBα releases its inhibitory hold on NFκB) [68]. As above, identifying a process that is affected in mutant but not WT cells is required.

Importantly, identification of the *in vitro* and *in vivo* phosphorylation sites further confirms the importance of substrate phosphorylation in a cellular process. For example, if phosphorylation is required for a protein to translocate to the cell periphery, expression of a non-phosphorylatable protein should block its ability to translocate. This would confirm that the process requires phosphorylation. Thus, careful correlation of *in vitro* and *in vivo* phosphorylation sites is essential for substrate evaluation.

Conclusion

Elucidating kinase signaling networks may lead to a greater understanding of the causes of human disease and provide additional targets for drug intervention. The unraveling of kinase networks can be accomplished using the techniques described here. Each technique has been successful in uncovering information about kinases and their substrates. Instead of identifying ever increasing numbers of putative substrates, more emphasis should be placed on confirming the *in vivo* relevance of the substrates. To this end, we have proposed three criteria for evaluating putative kinase substrates: first, the kinase can phosphorylate the putative substrate efficiently *in vitro*; second, the putative substrate should be

phosphorylated in response to a physiological signal that is dependent on the kinase-of-interest; third, phosphorylation of the substrate by the kinase-of-interest alters a cellular process.

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