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Kinomics: methods for deciphering the kinome

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Phosphorylation by protein kinases is the most widespread and well-studied signaling mechanism in eukaryotic cells. Phosphorylation can regulate almost every property of a protein and is involved in all fundamental cellular processes. Cataloging and understanding protein phosphorylation is no easy task: many kinases may be expressed in a cell, and one-third of all intracellular proteins may be phosphorylated, representing as many as 20,000 distinct phosphoprotein states. Defining the kinase complement of the human genome, the kinome, has provided an excellent starting point for understanding the scale of the problem. The kinome consists of 518 kinases, and every active protein kinase phosphorylates a distinct set of substrates in a regulated manner. Deciphering the complex network of phosphorylation-based signaling is necessary for a thorough and therapeutically applicable understanding of the functioning of a cell in physiological and pathological states. We review contemporary techniques for identifying physiological substrates of the protein kinases and studying phosphorylation in living cells.

Phosphorylation forms the basis of cell signaling networks[1]. There are two main questions that confront those trying to understand phosphorylation-based signaling: what substrates and sites does a particular kinase phosphorylate, and which kinase is responsible for phosphorylating an identified phosphorylation site? Answering these questions is difficult. Classically, protein phosphorylation has been studied by laborious biochemical methods such as *in vivo* labeling, peptide mapping, phosphoamino acid analysis and purification of kinase activities. Although these approaches laid invaluable groundwork and are still useful today, to tackle a problem of this scale effectively requires the use of a combination of different techniques (**Fig. 1**). In this review, we describe and evaluate modern techniques for studying the protein kinases, or, in other words, state-of-the-art kinomics (**Table 1**).

KINASE TO SUBSTRATE

We begin with a description of ways to identify *bona fide* substrates of protein kinases. Application of these methods to all 518 human kinases is the essence of kinomics.

Phosphorylation screening

A widely used core of techniques has been developed for identifying substrates of protein kinases by *in vitro* phosphorylation. These techniques are of two main types: library or lysate screens and candidate substrate screens. These screens normally require purified, active protein kinase, and this may not always be easy to produce: expressing and purifying a kinase from bacteria or insect cells is not always possible, and upstream activating kinases may not be known. Purifying kinases from stimulated mammalian cells may be the most productive method but has the potential drawback that contaminating kinases may be present. A major caveat

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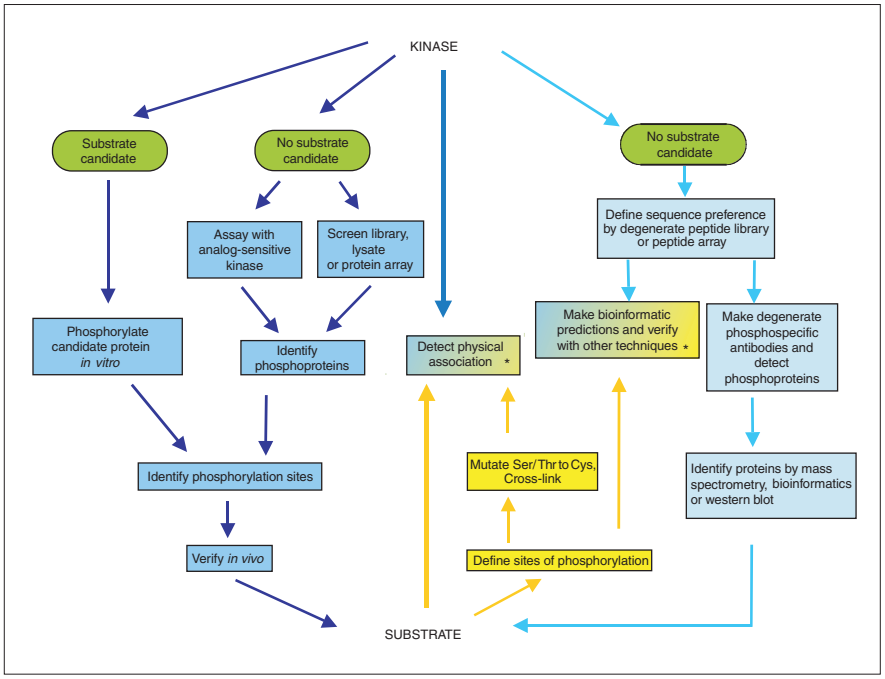

Figure 1 | Flow chart of strategies available for the identification of either substrate or kinase. Techniques labeled with * lead to kinase or substrate candidates that need to be further investigated and validated using alternative experimental strategies.

with phosphorylation screening is the promiscuity of kinases in the absence of *in vivo* regulatory constraints, producing false-positive substrate identification.

In vitro protein phosphorylation. Classical *in vitro* lysate phosphorylation has been coupled with mass spectrometry (MS) in the kinase substrate tracking and elucidation (KESTREL) method[2] (Kinasource). Cell lysates are incubated with a high level of purified, active kinase of interest, and the resultant phosphoproteins are separated by gel electrophoresis and then identified by tryptic mass fingerprinting. Care must be taken with this approach to ensure a protein is not identified as a substrate owing to comigration along the single electrophoretic dimension with a genuine phosphorylated protein. Typically, this technique is limited by high background phosphorylation, but important protocol modifications, such as the use of Mn^{2+}-[γ-^{32}P]ATP instead of Mg^{2+}-[γ-^{32}P]ATP or simple chromatographic separation of the crude cell extract, have improved the success of the technique in some instances. This exact technique will probably not work for all protein kinases, such as those unable to use Mn^{2+}-ATP efficiently, but it illustrates what is possible by combining classical techniques and MS (Box 1).

Phage-based assays. Phage expression libraries can be used to produce a library of potential kinase substrate proteins, which is incubated with a purified kinase, and the clones encoding substrates are then identified[3]. The library is plated out on a bacterial lawn, and the phage plaques are transferred to nitrocellulose membranes, which are blocked and then incubated with a purified kinase and [γ-^{32}P]ATP. Autoradiography is used to determine which plaques become strongly phosphorylated. The cDNAs from replicated plaques are rescued and sequenced, and the proteins they encode are verified as substrates. In an analogous manner, anti-phosphotyrosine antibodies have been used to identify SH3 multiple domains 1 (also known as FISH; five SH3 domains) as a substrate of SRC[4]. Phosphospecific consensus-site antibodies, many of which are now available (Box 2), could likewise be used to screen libraries after phosphorylation. Bacterial cDNA expression libraries coexpressing a constitutive or inducible kinase of interest could also be screened with such antibodies. mRNA display offers an alternative to phage display for phosphorylation screening[5]. The mRNA and the protein directly produced from it by *in vitro* translation are chemically linked by puromycin acting as a terminator tRNA. mRNAs that encode substrates of a kinase can be isolated by screens involving several rounds of selection and translation.

Peptide and protein array screens. The development of arrays of biological macromolecules in defined patterns on solid-phase 'chips' presents numerous opportunities for the study of phosphorylation. Whereas 'gene chips' are currently the more advanced technology, several 'protein chips' have been produced, offering a variety of possibilities for experiments. Generally, such array techniques offer a convenient, sensitive and rapid assay requiring small quantities of reagents.

The kinome of the yeast *Saccharomyces cerevisiae* has been assayed using protein chips[6]. Individual multiwell chips with a bound substrate were incubated with 119 glutathione-S-transferase (GST)-tagged fusion proteins representing nearly all of the 122 yeast kinases. Phosphorylation was detected using a phosphorimager, which allows a quantitative comparison of the extent of phosphorylation of a particular substrate by each kinase. Seventeen different chips were used to build up an overall picture of kinase-substrate preferences. Clearly, this is a very powerful technique, but the same caveats exist as with any *in vitro* phosphorylation screen. However, testing a large array of conditions provides many internal standards that can be used to produce a measure of confidence.

Peptide arrays allow rapid characterization of the preferred primary sequence for phosphorylation by a kinase[7]. A typical array might consist of hundreds of peptides derived from known phosphorylation sites or an array of peptides of random sequence covering a wide range of sequence space. A kinase of interest can be incubated with [γ-^{32}P]ATP and the peptide arrayed on a chip (for example Jerini), and the phosphorylated peptides can be identified by phosphorimaging[8]. The technique may be used to assess kinase sequence specificity, and by database scanning with the derived preferences, it may be possible to identify new substrates of a kinase. Proteome microarrays are increasing substantially in their complexity, and chips containing the majority of the yeast proteome have been produced[9] (Protometrix). Chips containing human proteome and subproteome arrays will doubtlessly be available soon.

General problems with these techniques are nonspecific absorption of the liquid-phase proteins to the chip, and binding to the chip or incorrect folding rendering the protein biologically nonfunctional.

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These problems are likely to be ameliorated with advances in chips and in the methods used to immobilize the proteins[10]. One particularly exciting advance is the ability to separate proteins by MS and 'soft-land' them in specific patterns on a chip[11]. A wide variety of sample types could be used as the protein source.

Genetic screens

Genetic screens in model organisms offer a way of identifying physiologically relevant protein kinase substrates. Such screens generally involve establishing a weak gain-of-function or loss-of-function mutant phenotype for the kinase of interest and then screening for second site mutations that suppress this phenotype. The products of the genes identified in the screen can then be tested as substrates of the kinase. Typically, homologous genes in higher organisms have conserved functions, but it is not possible to study all the kinases of mammalian species in lower organisms with smaller kinomes and genomes.

Functional knockouts to investigate kinases

Another broad class of techniques used to discover the function and substrates of a protein kinase involve obliteration of the kinase activity and investigation of the consequences. There are various ways to do this: the kinase can be genetically knocked out; it can be suppressed by RNA interference (RNAi); or its enzymatic activity can be reduced with specific inhibitors. This general approach only identifies involvement of a kinase in a particular pathway. Other techniques are required to demonstrate direct phosphorylation of a particular substrate.

Mice with genetic deletions or knock-in mutations of protein kinases and cell lines derived from them are valuable tools. Commercial efforts are underway to knock out every kinase in the kinome and analysis of the complete set of mice will be a valuable undertaking (for example, Lexicon Genetics).

RNAi technology offers a convenient means of depleting specific proteins[12]. Potential drawbacks include incomplete depletion and some nonspecific effects[13]. A set of custom short interfering RNA (siRNA) oligonucleotides can be designed for a kinase of interest, or, more conveniently, one can use pools of precharacterized and tested siRNAs for each kinase that are now available on a kinome-wide scale (for example, Dharmacon). Virally mediated stable expression of short hairpin RNAs (shRNAs) represents a potent alternative approach[14].

Increasing numbers of specific small-molecule inhibitors of protein kinases and phosphatases are becoming available and even more are likely to be produced as byproducts of the growing pharmaceutical interest in signaling molecules as potential therapeutic targets. The possibility of nonspecific effects of these inhibitors should always be considered, but the use of inhibitors provides a rapid preliminary test for the involvement of a kinase of interest in a particular process.

Table 1 Relative strengths, weaknesses, sensitivity, difficulty, speed etc. of the techniques. See text for explanation.

Technique	Advantages	Disadvantages
	Kinase ⇒ substrate	
Phosphorylation screening	Fast, easy and cheap	Artifact-prone
- *In vitro* protein phosphorylation	Detects direct phosphorylation	Requires further validation
- Phage-based assays		
- Peptide or protein array screens		
Genetic screens	Many pioneering successes	Time consuming
	Not limited by sensitivity	Not possible in all cases
	Aided by the availability of genome sequences	Does not show direct phosphorylation
Functional knockout	Rapid means of testing involvement of a kinase	Does not detect direct phosphorylation
- Specific inhibitors	*In vivo* technique	Nonspecific effects, cross-talk and feedback
- RNAi		
- Genetic knockout		
Analogue-sensitive kinase	Shows direct phosphorylation	Specificity
		Not yet functional in cells
Phosphospecific substrate antibody (**Box 2**)	Detects substrates phosphorylated *in vivo*	Limited by preference of the kinase and specificity of antibody
		Proteins need identification by other techniques
	Kinase ⇔ substrate	
Physical association	Requires no knowledge of sequence preference of the kinase	Needs other techniques to detect phosphorylation
- Yeast two-hybrid screen		
- Phage screen	High-throughput potential	
- Proteomic techniques		
Bioinformatics	Extremely quick method for making predictions	Relatively low reliability necessitates the use of other techniques
		Requires distinct kinase preferences
	Substrate ⇒ kinase	
Cross-linking	New, potentially powerful technique	Unproven specificity
		Requires phosphorylation site to be known
		In vitro technique
In-gel kinase assays	Convenient assay	Poor renaturation of many kinases
		In vitro technique

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BOX 1 MASS SPECTROMETRY

The ability to identify proteins and the post-translational modifications of a protein by MS is a considerable improvement on traditional biochemical techniques and presents a powerful array of tools to investigate protein phosphorylation.

Identifying phosphoproteins by MS

MS can be used with tremendous sensitivity to identify a protein unambiguously. This is useful in a variety of techniques that produce a band on an autoradiograph or a purified kinase and are often limited by the ability to identify the protein. Proteins can be identified by 'fingerprinting' where a protein is digested with trypsin and the characteristic masses of the peptides produced are accurately determined and used to search a database of predicted trypitic-peptide masses. Protein identification is also possible by tandem mass spectrometry (MS/MS)[41]. The first MS separation is used to select a particular mass species, which is then bombarded, causing fragmentation; the pattern of overlapping fragments produced allows the peptide sequence to be deduced, and this is used to search a database.

Identification of phosphorylation sites by MS

MS can be used to identify the individual phosphorylation sites on a protein[42]. A modification of the peptide fingerprinting technique can be used to identify phosphorylation of a protein. Covalent attachment of a phosphate group to a protein leads to an increase in mass of 80 Da, so the altered charge-to-mass ratio will be detectable in the peptide spectra. It may be possible to attribute the phosphorylation to a particular residue of the peptide; if not, MS/MS can pinpoint the site. Achieving peptide fragmentation in MS/MS by electron capture dissociation may lead to improved phosphopeptide sequencing[43]. When studying the phosphorylation of a protein with MS, it must be remembered that only the phosphorylation of analyzed peptides will be detected, and this should be considered before claiming to have described 'all' the phosphorylation sites of a particular protein.

Large-scale identification of phosphorylation sites

A key to understanding the role of protein phosphorylation is the identification of *in vivo* phosphorylation sites. The high-throughput capabilities of MS are ideally suited to this task. Phosphorylated proteins may represent a small fraction of the total protein so enrichment is required by techniques such as IMAC[44], anti-phosphotyrosine antibody enrichment[45], chemical replacement of the phosphate group with an affinity tag such as biotin[46] or strong cationic resin exchange chromatography[47]. The phosphorylated subset of proteins can be isolated, and then these proteins can be individually identified by MS. Alternatively, the protein mixture can be digested with trypsin and the phosphopeptides enriched and identified, resulting in the identification of large numbers of phosphorylation sites from a single sample[47]. The capacity of such techniques will surely improve, ideally to the point of describing the phosphoproteome (all the phosphorylated proteins in the cell, estimated to be at least one-third of all intracellular proteins, perhaps 20,000 phosphorylated proteins in a cell with serine, threonine or tyrosine phosphorylated in the ratio 1,800:200:1). This would be an invaluable advance in understanding phosphorylation-based signaling networks. It would be equivalent to *in vivo* mapping of every protein in the cell and would allow many standard techniques to be used more powerfully. Phosphosite (http://www.phosphosite.org) is a new database that aims to authoritatively catalog all *in vivo* phosphorylation.

Comparison of samples by MS

Comparative analysis of samples by MS is possible by isotopic encoding. In stable isotope–labeled amino acids in cell culture (SILAC), cells are grown for several generations in the presence of an essential amino acid labeled with a nonradioactive isotope, so that all proteins become uniformly labeled[48]. Analysis by MS can then identify both the protein and the sample from which it originated, presenting numerous possibilities. The use of isotopes of two elements simultaneously allows more samples to be encoded and more detailed analyses, such as a time courses[49]. Samples can also be encoded by affinity tags[50].

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Inhibitor-resistant mutants can be produced to assess the nonspecific effects of an inhibitor in a particular circumstance[15].

ATP analog–sensitive kinases

Substrates of a kinase can be identified using a mutant form of the kinase that is able to use an ATP analog to phosphorylate its substrates[16]. A bulky residue in the ATP binding pocket is mutated, typically to a glycine, to allow the kinase to use an N^6-modified ATP analog, such as N^6-(benzyl) ATP. Only the analog-sensitive mutant form of the kinase is able to use the ATP analog, but otherwise, the mutant kinases behave as wild-type kinases. To identify substrates, γ-^{32}P-labeled, N^6-modified ATP analog is added to a cell extract, and the proteins phosphorylated by the modified kinase can be detected, even in the presence of other active kinases. A considerable advantage offered by this technique is that it can be used to show that the phosphorylation is direct; no other technique unequivocally demonstrates this. In principle, this approach could also be attempted in intact cells stably expressing endogenous levels of a single analog-sensitive kinase. However, substantial problems exist in getting the highly charged ATP analog into cells in sufficient quantities to compete with the high endogenous ATP levels and with hydrolysis of the ATP analog and consequent exchange of the labeled phosphate with the cellular ATP pool.

The analog-sensitive kinase can also be specifically and reversibly inhibited by cell-permeant N^6-modified purine-based small molecules[17]. In general, these inhibitors have no effect on cells lacking the analog-sensitive kinase and appear to be much more selective than conventional inhibitors. This is a powerful technique, but it cannot be used to prove that a protein is a direct target for the analog-sensitive kinase *in vivo* and the cell or organism has to be engineered to replace the wild-type protein kinase allele with the ana-

log-sensitive protein kinase allele. Unfortunately, it has proved difficult to generate functional analog-sensitive alleles for some kinases.

KINASE TO OR FROM SUBSTRATE

Physical association

In principle, another way to identify protein kinase substrates or the kinase that phosphorylates a particular substrate is to make use of the fact that kinase and substrate must interact. The physical interaction between kinase and substrate is typically transient, of moderate affinity and reduced upon phosphorylation and may involve a third protein. Many kinases use 'docking sites' distinct from the sites of phosphorylation to enhance the specificity and efficiency of substrate phosphorylation[18]. Better understanding of these interactions will facilitate the identification of substrates. Interaction does not necessarily mean phosphorylation, as many nonsubstrate proteins are known to interact with kinases. The approach is not always successful but can be attempted in several ways.

The great sensitivity of MS can be used to identify the proteins associated with a particular kinase or substrate. Tandem affinity purification (TAP) tagging produces highly purified complexes by sequential purification against different protein tags and is ideal for such analysis. This approach can be used to investigate a particular kinase of interest, and it has been used as a high-throughput approach to assess global protein-protein interactions[19].

Protein kinase substrates can be identified through yeast two-hybrid screens[20]. This approach has also been used in large-scale efforts to define yeast protein-protein interactions[21]. These datasets contain an enormous amount of useful information but do not immediately provide a list of all the substrates of all the kinases.

Phage display fusion libraries have also proved to be a useful method to detect kinase-substrate interactions. A purified kinase is immobilized and incubated with a phage library of potential substrates, and the interacting proteins are identified[22]. This could also be used in the opposite orientation with a library of kinases expressed on the surface of phage to identify candidate kinases responsible for the phosphorylation of a particular substrate.

Bioinformatics

Bioinformatic prediction of substrates of a kinase or identification of the kinase responsible for phosphorylating a particular site are not completely reliable but offer quick predictions that may be tested with other techniques. The core approach is to define the sequence preference of kinases and use these preferences in a computational manner to make predictions.

Defining sequence preferences. Phosphorylation site sequence preferences have been defined for many kinases through the use of degenerate oriented peptide libraries[23]. A library of peptides is synthesized consisting of an invariant serine, threonine or tyrosine residue flanked by a fixed number of residue positions at which there are a mixture of amino acids. A population of peptides is then phosphorylated *in vitro* by a purified kinase of interest. Next, the small fraction of the peptides that become phosphorylated is separated from the nonphosphorylated peptides by immobilized metal affinity chromatography (IMAC) and sequenced by Edman degradation, which gives a quantitative description of the preference the kinase has for each amino acid at each position flanking the phosphorylation site, known as a position-specific scoring matrix (PSSM). Typically, no preference is seen at some positions, whereas at other sites certain amino acids are strongly favored or disfavored.

An extension of this method uses a set of biotinylated, oriented peptide libraries in which each position, in addition to the phosphorylation site, is fixed, in turn, with every amino acid[24]. The array of libraries in the 96-well format is phosphorylated in solution in the presence of [γ-^{32}P]ATP by a purified kinase and transferred to an avidin capture membrane, and phosphorylation is detected by autoradiography, which provides a readily visualizable picture of sequence specificity. Analysis of the array then allows particular favored or disfavored residues within the context of a mixed library to be identified. This approach permits residues normally omitted from the library, such as phosphorylatable residues and cysteine, to be included because they will not adversely affect the isolation of the peptide. Indeed, phosphorylated residues themselves can be included, allowing the investigation of priming phosphorylation.

Computational analysis. The PSSM can be used to predict phosphorylation sites of a known protein, to search sequence databases in an attempt to identify new substrates and to predict which of the characterized kinases may account for a particular phosphorylation event. Scansite (http://scansite.mit.edu) is an excellent and pioneering web-based tool for doing such analyses[25]. A protein sequence is 'scanned' at various stringencies for phosphorylation sites using the PSSM. Sites that are found are a given a score based on the PSSM, and the score is defined relative to all occurrences in a protein database. Results are typically much more reliable and manageable than for simple pattern matching, such as searching for the occurrence of the consensus phosphorylation site, particularly when only the highest-scoring sites are considered. The results can be improved further by restricting the proteins considered according to molecular weight or isoelectric point, when this information is known about a potential substrate. An elegant example of such usage is the recent identification of TSC2 (also known as tuberin) as a substrate for AKT (PKB)[26]. A phosphospecific antibody raised against a degenerate phosphopeptide mixture representing the consensus phosphorylation site of AKT (RxRxxpT) detected several proteins phosphorylated upon platelet-derived growth factor (PDGF) stimulation. The proteins were then sought using bioinformatics: Scansite was used to identify candidate substrates of the predicted size that contained consensus AKT phosphorylation sites. The predicted identities of the phosphorylated bands were confirmed by western blotting.

SUBSTRATE TO KINASE

An equally important question that often needs to be addressed experimentally is, 'Which kinase phosphorylates my substrate?'. This may be answered by methods that identify substrates of kinases, but techniques are available for moving specifically from substrate to kinase.

Kinase renaturation screening

In vitro phosphorylation can be used to test a kinase's ability to phosphorylate a particular substrate. In-gel kinase assays offer a means of doing so. Candidate kinases (or cell fractions) are resolved on an SDS gel containing a substrate of interest trapped within it. After gel electrophoresis, the proteins are renatured either in the gel or after transfer to a membrane, and the entire gel or membrane is incubated with [γ-^{32}]ATP, allowing kinase to phosphorylate the substrate[27].

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Table 2 Timeline of discovery of select AKT substrates. Numerous substrates have been identified for AKT and the means by which they have been identified has changed over the years with technology advances.

Substrate	Method of discovery	Date	Reference
GSK3α/β (glycogen synthase kinase 3α and 3β)	Chromatographic separation of kinase activity towards a GSK3-peptide, depletion of kinase activity by immunodepletion of AKT, specific inhibition of both AKT activation and GSK3 phosphorylation, *in vitro* phosphorylation and peptide mapping	1995	56
BAD (Bcl2 antagonist of cell death)	Similarity of known phosphorylation sites to consensus phosphorylation sequence of AKT, *in vitro* and *in vivo* phosphorylation, mutagenesis, specific inhibitors, peptide mapping, phosphospecific antibody	1997	57
mTOR (mammalian target of rapamycin)	Antibody reactivity and occurrence of consensus AKT phosphorylation site in antibody epitope, inducible ER-AKT fusion mimicking insulin stimulation of mTOR activity. AKT phosphorylation of MTOR immunoprecipitates, phosphospecific antibody, specific inhibitors	1998 1999	58 59
DAF-16 (forkhead)	Genetic screen in *C. elegans*	1998	60
RAF	Similarity to known regulatory phosphorylation site of AKT consensus, constitutively active and kinase-inactive forms of AKT, phosphospecific antibody, *in vivo* labeling, *in vitro* phosphorylation, coimmunoprecipitation	1999	61
BRCA1 (breast cancer 1)	*In vitro* phosphorylation, inhibitors and molecular analysis of heregulin-induced BRCA1 phosphorylation	1999	62
eNOS (endothelial nitric oxide synthase)	Constitutively active AKT enhanced eNOS activity, *in vitro* phosphorylation, phosphoamino acid analysis, mutation of putative AKT phosphorylation sites, phosphoserine antibody and specific inhibitors.	1999	63
IKKα (IκB kinase)	Specific inhibitors, kinase-inactive and constitutively active AKT, mutation of putative AKT site	1999	64
Mdm2 (mouse double minute 2)	Identification of putative AKT phosphorylation sites, evolutionary conservation of sites, mutation of sites, *in vitro* phosphorylation of protein and analysis of sites of phosphorylation by MS, coimmunoprecipitation of AKT and Mdm2 from serum-starved cells, MS analysis of Mdm2 phosphorylation in IGF1-stimulated cells, specific inhibitors.	2001	65
ACLY (ATP-citrate lyase)	Phosphospecific anti-substrate antibody and identification by MS	2002	66
TSC2, also known as Tuberin (tuberous sclerosis 2)	Phosphospecific substrate antibody, putative identification by bioinformatics, confirmation of identity by western blotting	2002	67
YAP (YES-associated protein 1)	Tandem affinity purification with 14-3-3 τ isoform after AKT activation, separation on a two-dimensional gel, identification of bands reactive to phosphospecific anti-substrate antibody by MS	2003	54
WNK1 (protein kinase, lysine deficient 1)	Bioinformatic identification of consensus phosphorylation site, *in vitro* phosphorylation and peptide mapping, mutagenesis, inhibitors, phosphospecific antibody and PDK1 knockout cells to provide evidence for phosphorylation	2004	68

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Phosphorylated bands can be detected by autoradiography, and the kinase can be identified (if an extract or column fraction is used) by candidate western blotting, on the basis of size or by MS. This technique is relatively low-throughput in testing substrates and many kinases are not easily renatured to produce full activity (for unknown reasons, it is very difficult to renature tyrosine kinases). Once again, the potential for false positives is a serious problem.

Kinase-substrate cross-linking

In principle, chemically cross-linking a substrate of interest to the kinase responsible for its phosphorylation should allow identification of the kinase. In one new method, the serine or threonine of a substrate that is normally phosphorylated is mutated to a cysteine, and, in the presence of a bifunctional cross-linker, the substrate is covalently linked to the catalytic lysine residue of the kinase. What is normally a transient interaction becomes permanent, and the kinase can be isolated and identified. Numerous challenges remain: the extent of crosslinking to endogenous cysteines needs to be low, the cross-linked product needs to be stable enough to be isolated, and, ideally, the system needs to work in cells[28]. Perhaps the technique could be made even more general with the development of an ATP-mimicking molecule that cross-links the kinase to the residue normally phosphorylated. Kinase-substrate pairs could then be identified from a complex mixture by immunoprecipitation of the kinase, trypsinization and identification of crosslinked peptides by MS.

Kinase-mediated dephosphorylation

Another potential approach for identifying the kinase responsible for phosphorylating a substrate at a particular site would be to use protein kinases, in the presence of high concentrations of ADP, as protein phosphatases[29]. A kinase is a catalyst, and like all catalysts, it increases the rate of reaction but does not affect the position of equilibrium. The equilibrium constant of a phosphorylation reaction is close to 1, although in cells the equilibrium generally lies far from this owing to the high ratio of ATP to ADP and the low concentration of phosphoprotein product. A substrate of interest could be incubated as a phosphoprotein with a variety of kinases, and the rate of dephosphorylation of a particular site could be measured. Kinases that cause the most rapid dephosphorylation are candidate physiologically relevant kinases. Apart from the original report, this technique has not been applied, but with the advent of arrays of active kinases, large numbers of kinases can be compared simultaneously, giving this technique real potential.

Studying phosphorylation *in vivo*

Identifying all the substrates of all the protein kinases is just the

start of kinomics. Understanding phosphorylation-based signaling requires studying protein kinases at appropriate resolution and in living cells.

Single-cell kinase assays

Analysis of the dynamic changes in phosphorylation of populations of cells may miss rapid kinetic and spatial changes in subsets of the population because of the asynchronous nature of the response. Therefore, it is essential to study the behavior of kinases in single cells. Single-cell assays for particular kinases have been developed using intracellular substrate peptides[30]. Fluorescently tagged peptides known to be efficient and specific substrates of a particular kinase are introduced into cells by means of transduction domains or by microinjection. The peptides are reversibly phosphorylated by the kinase inside the cell. The single cells are analyzed individually by extremely rapid lysis with a pulse laser and immediate loading of the lysate from a single cell into a capillary where the peptides are separated by electrophoresis. The amount of phosphorylated and unphosphorylated peptide is quantified by means of the fluorescent tag. This technique also offers the potential to assay multiple kinase activities simultaneously in a single cell by using multiple electrophoretically distinguishable fluorescently tagged substrate peptides.

Fluorescence-activated cell sorting (FACS) also provides analysis at single-cell resolution. Multi-parameter FACS sorting on the basis of phosphospecific anti–active kinase antibody fluorescence allows simultaneous assay of multiple kinase activities[31]. Immunocytochemistry with phosphospecific antibodies also provides single-cell analysis. However, an antibody (for example, anti-phosphotyrosine) will often have broad reactivity in a cell and may therefore provide relatively low-resolution information on a specific phosphorylation event.

Analysis of phosphorylation in living cells

Identification of the substrates of protein kinases is clearly of great importance in achieving a detailed understanding of the signaling networks that operate in a cell. Deeper understanding will come with studying the spatial complexity and temporal dynamics of phosphorylation. Techniques suitable for investigating kinases in living cells are needed to provide a further level of understanding of phosphorylation-based signaling. This information needs to be obtained at sufficient spatial and temporal resolution with minimum perturbation to the signaling processes being studied. Analysis also needs to be at single-cell resolution, as variations in temporal responses and rapid events such as oscillations will likely be damped beyond detection in population analysis.

Imaging-based methods. High-resolution imaging of a particular phosphorylation in cells can be produced by imaging fluorescence resonance energy transfer (FRET) between a fluorescently conjugated protein of interest and a phosphospecific antibody conjugated with a suitable acceptor fluorophore. This has been achieved in several systems with good results. Particularly impressive work has been done with the epidermal growth factor (EGF) receptor[32]. The receptor is GFP-tagged and anti-phosphotyrosine antibodies conjugated with Cy3 are used to detect trans-phosphorylation of the receptor. Because of the precise distance-dependence of FRET, the results are superior to immunofluorescence assays using anti-phosphotyrosine antibodies and colocalization with the GFP signal. A substantial drawback of this technique is that either the Cy3-conjugated antibody has to be microinjected into cells or the cells need to be permeabilized and fixed; in the second case kinetic information is lost.

Translocation assays to measure signaling events in living cells are based on a phosphorylation-dependent translocation of a GFP-fusion protein such as protein kinase C (PKC)-GFP[33] or phosphospecific-binding domains or lipid-binding domains fused to GFP. These assays have been useful, but they are limited in scope and are usually of moderate resolution and sensitivity because they are based on a single-intensity measurement and the translocation is only partial. The use of total internal reflection fluorescence microscopy, which detects only the fluorescence very close to the cell membrane, can improve the signal-to-noise ratio of the technique[34].

Dye-conjugated forms of a protein can also be used to measure kinase activity in living cells. Solvation-sensitive dyes have been developed that markedly change fluorescence intensity according to the local physicochemical environment[35]. A dye can be covalently attached

BOX 2 PHOSPHOSPECIFIC ANTIBODIES AND BINDING DOMAINS

Phosphospecific antibodies, which detect protein phosphorylation in a sequence specific fashion, can detect specific and more general protein phosphorylation and form the basis of numerous commonly used techniques. Antibodies can be raised against phosphorylated peptides representing a specific site in a protein, or against a degenerate mix of phosphopeptides (for example, RxRxxpS/T for AKT), thus generating a broad range phosphospecific 'substrate' antiserum[51]. Purified antibodies raised against specific phosphorylation sites will typically recognize that particular phosphorylated site with greater affinity and specificity than an antiserum designed against a degenerate peptide mixture corresponding to the consensus phosphorylation site. Monoclonal or polyclonal antibodies against the phosphotyrosine moiety are exceptionally good; they are able to recognize phosphotyrosine with high affinity and specificity and provide an exceptionally convenient way to assay tyrosine phosphorylation in virtually any protein. Unfortunately, no equivalent high-affinity antibodies exist for phosphoserine and phosphothreonine, mainly because of the lower immunogenicity of the phophoserine and phosphothreonine side chains. The existing anti-phosphoserine and anti-phosphothreonine antibodies tend to recognize other phosphate monoesters as well and must be used with caution. These limitations are compounded in some approaches by the greater cellular abundance of phosphoserine and phosphothreonine than phosphotyrosine. An alternative to phosphospecific antibodies are phosphospecific binding domains such as SH2, PTB, BRCT, Polo box, 14-3-3, WW and FHA domains[52]. These bind with variable affinity (50 nM to 100 μM) to specific sequences in a phosphorylation-dependent manner. They have the potential advantage of being engineerable[53] and are easy to clone and thus can be conveniently used in a variety of molecular techniques. An AKT substrate has been isolated by means of a 14-3-3 protein[54] and a c-Abl substrate by means of an SH2 domain[55].

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to a protein close to the site of phosphorylation, and the phosphorylation of the protein can be detected on the basis of the normalized fluorescence of the molecule. A kinase of interest that is activated by phosphorylation (such as mitogen-activated protein kinase; MAPK) could be monitored with this technique. Typically, the signal-to-noise ratio of such images is very good and the technique can be applied to living cells after microinjection.

Genetically encoded biosensors. FRET-based biosensor proteins can reveal the localization of kinase activity in living cells in real time at high spatiotemporal resolution[36]. They consist of a substrate phosphorylation site designed for a particular kinase and a phosphospecific binding domain placed between CFP and YFP. When the substrate is phosphorylated b y a specific kinase, an intramolecular association with the phosphospecific binding domain occurs and the resultant change in conformation produces a change in the FRET efficiency of the construct, allowing real-time visualization of the localization and extent of the particular kinase activity. FRET-based biosensor proteins specific for protein kinase A, SRC, EGFR, ABL, PKC and AKT have recently been developed by Roger Tsien and co-workers, among others. For example, the PKA-biosensor AKAR1 consists of a PKA peptide substrate sequence and a 14-3-3 domain between CFP and YFP. When the substrate is phosphorylated by PKA, the 14-3-3 domain binds to the phosphorylated residue producing a change in conformation that results in increased FRET between the CFP donor and the YFP acceptor fluorophores. This can be measured as an increase in the ratio of yellow to cyan emissions, and the subcellular regions where the kinase is active can then be visualized. These reporters have numerous advantages over earlier methods[37]. The constructs are unimolecular, so unbalanced expression of the donor and acceptor fluorophore is not a problem, and ratio measurements can be made. The constructs are genetically encoded and can be used to generate transgenic cell lines or organisms that express the biosensor protein to study specific phosphorylation events *in vivo*.

These biosensors offer a means of investigating the dynamics of signal transduction that is not available by other methods. Progress in imaging technology and improvements in biosensors present great future possibilities. *C. elegans* and *Danio rerio*, in the translucent larval stage, might become excellent models for studying signaling processes in a relevant *in vivo* context[38]. Transgenic mice expressing a biosensor in a relevant tissue have been produced and successfully analyzed[39]. The advances in imaging technologies for mammalian specimens[40] may even one day progress to imaging phosphorylation networks in a whole mouse.

CONCLUSIONS

Our knowledge of phosphorylation-based signaling networks has advanced enormously in recent years (**Table 2**). Much therapeutic promise lies in gaining a more complete understanding, ideally at the level where detailed mathematical models of cell signaling can be produced. Broad-scale approaches, such as defining the kinome and the phosphoproteome, present an excellent foundation on which to build a description of cell signaling. Completion of the description of phosphorylation may prove to be difficult— no screen identifies all substrates, and it is likely that the more readily identified ones have been found first. Problem kinases or substrates will remain, such as those with very low specific activity, minimal phosphorylation site consensus, a requirement for adaptor proteins or very large size. However, the array of techniques described in this review is a powerful toolkit with which to enhance our understanding of cell signaling. Integration of techniques, such as bioinformatics with proteomics or structural analysis, is likely to improve our comprehension faster than any technique alone. Consideration of signaling networks as dynamic systems is crucial for a full understanding, and this requires techniques applicable to analysis in single living cells.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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