Focus Issue: Cell biology meets cancer therapy

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Growing and dividing cells, including cancer cells, have high metabolic requirements because they must build new proteins, membranes, and nucleic acids. Drugs that interfere with cellular metabolism or macromolecule biosynthesis can trigger various types of cell stress pathways, such as those involving proteotoxic stress. The accumulation of unfolded proteins in the endoplasmic reticulum (ER) causes ER stress and induces the unfolded protein response (UPR). The accumulation of unfolded or misfolded proteins in the cytosol also triggers proteotoxic stress. Another cellular response to proteotoxic stress, which can result from temperature stress, oxidative stress, or nutrient deprivation as well as ER stress, is the integrated stress response (ISR). Whereas the UPR increases the abundance of protein chaperones that reside in the ER, the ISR inhibits most protein translation, with the exception of specific genes, many of which are regulated by the stress-activated transcription factor ATF4, a key mediator of the ISR. Prolonged proteotoxic stress can induce cell death.

Research by Kline et al. and Ishizawa et al. (see the Focus by Greer and Lipkowitz) revealed that ONC201, a drug in clinical trials, triggers cell death in both hematological cancers and cells derived from solid tumors by inducing a stress response with characteristics of the ISR. The pair of papers demonstrated that ONC201 induced the activation of ATF4, which increased the expression of a gene encoding a death receptor and its ligand in solid tumor cells and a protein that inhibited the growth-promoting kinase complex mTORC1 in lymphoma and leukemia cells. Fortunately, ONC201-triggered apoptosis did not require the activity of the tumor suppressor and transcription factor p53, which is often mutated in many cancers.

One way that cells can adapt to ER stress is by increasing a process called ER-associated degradation (ERAD) that eliminates the unfolded proteins by transporting them out of the ER and targeting them for proteasomal degradation. Singh et al. found that breast cancers positive for the growth factor receptor HER2 had increased ER stress signaling and were “addicted” to ERAD for survival. Because HER2 signaling promoted protein synthesis, the cells experienced severe proteotoxic stress when ERAD was blocked. Pharmacologically blocking this pathway caused cell death in HER2-positive cells, including those that were resistant to clinically used HER2 inhibitors.

Growth factor signaling stimulates the phosphoinositide 3-kinase (PI3K)–AKT pathway. Activation of this pathway in the presence of abundant nutrients stimulates the kinase activity of mTOR, a kinase that as part of the mTORC1 complex promotes protein translation by phosphorylating the translation inhibitor 4Ebp1. Intrinsic or acquired resistance of cancer cells limits the clinical usefulness of drugs that inhibit mTOR activity or signaling through the PI3K–AKT pathway. Hsieh et al. found that intrinsic differences in 4Ebp1 expression and protein synthesis rates were associated with the propensity of the cells to become tumorigenic and with resistance to therapies targeting mTOR in mice and those targeting PI3K in patients. The luminal epithelial cells that tended to become cancerous had high 4Ebp1 abundance and low protein synthesis rates. These data explain why drugs targeting this signaling axis have been ineffective in treating prostate cancer, despite data from experiments with cultured cells and xenografts showing drug resistance that was associated with loss of 4Ebp1 and high protein synthesis. This study highlights the importance of understanding the cell-specific properties of particular cancers to optimize treatment approaches.

Accumulation of damaged or improperly folded proteins in the cytosol can lead to the formation of protein aggregates, which can be eliminated through autophagy, a mechanism by which cellular membranes form around protein aggregates, forming autophagosomes that then fuse with lysosomes for degradation. However, if autophagy is compromised or insufficient, cells may experience proteotoxic stress that results in cell death. Indeed, Zhang et al. found that the clinically used drug verteporfin triggered the accumulation of toxic amounts of protein oligomers that selectively killed colorectal cancer cells in mice and cancer cells cultured under hypoxic and nutrient-deprived conditions. Normal cells in culture and in tumor-adjacent tissue sections from mice cleared these aggregates through autophagy and survived, indicating that in this model verteporfin produced tumor-selective proteotoxicity. These data not only suggest additional therapeutic applications for verteporfin but also indicate that drugs that can induce the rapid formation of protein aggregates may be effective when used in combination with drugs that inhibit autophagy or in cancers with limited capacity for autophagy.

Many proteins pass through the ER and Golgi before reaching the plasma membrane or being released from cells. This complex biosynthetic process enables cells to perform quality control on the newly synthesized proteins, control the rate of release or appearance of the proteins at the cell surface, and add posttranslational modifications (such as the addition of sugar moieties through the process of glycosylation). Lau et al. found that metastatic melanoma was associated with reduced abundance of the enzyme that mediated fucosylation. This type of sugar modification affects the interaction of growth factors and chemotactic molecules with their receptors, and attenuating fucosylation reduced melanoma cell adhesion and enhanced melanoma cell migration. Mice injected with melanoma tumor cells showed reduced tumor growth.
and metastasis when fucose was added to their drinking water. Thus, understanding how protein glycosylation is altered in cancer cells may enable appropriate dietary or pharmacological strategies to restore proper glycosylation and cellular behavior.

Transmembrane receptors that reach the plasma membrane do not stay there permanently, but instead undergo dynamic endocytosis and recycling back to the cell surface or to the lysosome for degradation. Ben-Chetrit et al. found that many patients with aggressive breast cancer have tumors with increased expression of SYNJ2, which encodes the lipid phosphatase synaptojanin 2. In response to epidermal growth factor (EGF), SYNJ2 localized to lamellipodia and invadopodia, which are cellular protrusions associated with invasive behavior and are the sites where EGF stimulates its receptor to promote cell migration. Knocking down SYNJ2 inhibited recycling of the EGF receptor to the cell surface and decreased the invasive behavior of cultured breast cancer cells. Breast cancer cells expressing a phosphatase-deficient mutant of SYNJ2 produced smaller, less metastatic tumors when xenografted into mice. Encouragingly, inhibitors of SYNJ2 that reduce cell invasion in 3D culture were identified in a chemical screen, which suggests that it may be possible to target SYNJ2 and thereby prevent metastasis in breast cancer patients.

In addition to their role in receptor endocytosis and trafficking, endosomes also serve as organizing sites for signaling complexes. The kinase BRAF is normally activated by growth factor signaling, and activating mutations in this kinase cause colorectal cancer. Margalef et al. found that the endosome-associated kinase TAK1 phosphorylated a proteolytic fragment of another kinase, IKKa, and BRAF-mutant colorectal cells required TAK1-mediated phosphorylation of the IKKa cleavage product for transformation and proliferation. Endosomes are acidic compartments, and inhibition of endosomal acidification blocked the phosphorylation of the IKKa cleavage product and induced cell death. This study reveals an unexpected connection between endosomes, proteins involved in the nuclear factor kB (NF-kB) pathway, and a mitogen-activated protein kinase pathway activated by BRAF. Furthermore, the data suggest that drugs that block endosomal acidification and that are currently used for malaria prevention or as antifungals could be repurposed to treat cancers associated with active BRAF mutations.

The studies highlighted here underscore the importance of not only finding compounds that can kill cancer cells or stop their proliferation in culture but also understanding how they work to identify the patients with cancers most likely to respond to a particular treatment. Furthermore, these articles are examples of using a molecular understanding of the signaling events associated with cellular stress responses to leverage the development of novel therapeutic approaches or combination therapies for more effective treatment.

Related Resources

Research Articles


Focus


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Despite the efforts of pharmaceutical companies to develop specific kinase modulators, few drugs targeting kinases have been completely successful in the clinic. This is primarily due to the conserved nature of kinases, especially in the catalytic domains. Consequently, many currently available inhibitors lack sufficient selectivity for effective clinical application. Kinases phosphorylate their substrates to modulate their activity. One of the important steps in the catalytic reaction of protein phosphorylation is the correct positioning of the target residue within the catalytic site. This positioning is mediated by several regions in the substrate binding site, which is typically a shallow crevice that has critical subpockets that anchor and orient the substrate. The structural characterization of this protein-protein interaction can aid in the elucidation of the roles of distinct kinases in different cellular processes, the identification of substrates, and the development of specific inhibitors. Because the region of the substrate that is recognized by the kinase can be part of a linear consensus motif or a nonlinear motif, advances in technology beyond simple linear sequence scanning for consensus motifs were needed. Cost-effective bioinformatics tools are already frequently used to predict kinase-substrate interactions for linear consensus motifs, and new tools based on the structural data of these interactions improve the accuracy of these predictions and enable the identification of phosphorylation sites within nonlinear motifs. In this Review, we revisit kinase-substrate interactions and discuss the various approaches that can be used to identify them and analyze their binding structures for targeted drug development.

The Catalytic Domain of Eukaryotic Protein Kinases

Typically, eukaryotic protein kinases are composed of nonconserved regulatory domains and a conserved catalytic core of ~250 amino acid residues that binds and anchors substrates and is responsible for catalysis (1). The catalytic domain consists of two lobes called N and C (also known as small and large lobes, respectively), named for their N- or C-terminal position, respectively, within the domain. The N-lobe consists of five-stranded, anti-parallel β sheets that are an essential part of the adenosine triphosphate (ATP) binding site, whereas the C-lobe is mostly helical (Fig. 1A). The active-site cleft, which contains the ATP binding site, lies between the two lobes (2). In an activated kinase, the lobes converge to form a deep cleft where the adenine ring of ATP binds such that the γ-phosphate is positioned at the outer edge where the transfer of the phosphoryl group occurs, whereas the adenosine moiety is buried in a hydrophobic region of the pocket (Fig. 1B). Adjacent to the ATP binding pocket is a shallow crevice called the substrate binding site (SBS) that anchors the substrate and correctly positions the phosphorylatable residue (2).

Catalysis is mediated by opening and closing of this active-site cleft. Substrates are anchored and positioned near this cleft so that the hydroxyl group of the phosphorylatable residue (termed P0) can accept the γ-phosphate. Flanking regions help stabilize the active kinase and are also essential for catalysis (1). Tyrosine kinases have a deeper cleft crevice around P0 than serine/threonine (Ser/Thr) kinases to better accommodate a bulky side chain (3).

An increase in the catalytic activity of kinases often leads to cancer (4); therefore, their activation must be tightly regulated, and several regulatory mechanisms maintain kinases inactive. One such mechanism is the intramolecular interactions that hide the catalytic domain in a kinase (5, 6). Then, to activate the kinase, conserved mechanisms involve movements in the activation loop and DFG (Asp-Phe-Gly) motif, exposing the ATP binding pocket (described further below). Other regulatory mechanisms are kinase-specific and involve blocking the SBS. For example, protein kinase C (PKC) presents a pseudosubstrate region in its regulatory domain that interacts with the SBS in the catalytic domain (5), aiding the maintenance of the inactive state of the kinase. This pseudosubstrate region is similar to PKC substrates except that the phosphorylatable residues Ser and Thr are substituted to Ala. For protein kinase A (PKA), a pseudosubstrate also helps maintain the kinase inactive; however, in this case, regulatory domains are encoded by separate genes (5). The SH2 and SH3 domains found in the regulatory domain of the nonreceptor tyrosine kinase SRC interact with the catalytic domain, thereby maintaining the kinase inactive. Dephosphorylation of a Tyr residue that interacts with the SH2 domain disrupts this intramolecular interaction and is an important step for SRC activation (6). Activation of the receptor-coupled tyrosine kinases typically occurs upon binding of a ligand and dimerization of the receptor, followed by conformational changes that make the catalytic domain available to interact with the substrates. Conformational changes are usually followed by a series of phosphorylations that lead to kinase activation (1).

Active kinases form what is called a regulatory hydrophobic spine (R-spine) that is assembled after the phosphorylation of the activation loop. This spine is composed of two residues from the N-lobe and two from the C-lobe (1). A catalytic spine (C-spine) is assembled upon ATP binding. Thus, kinase activation involves assembly of R- and C-spines, and inactivation involves disassembly of the R-spine. Once the R- and C-spines have been formed, kinases are termed “primed” for catalysis, and binding to scaffold proteins and substrates is enhanced (4). The activation loop, which in many kinases is the site of regulatory phosphorylations or interactions with activity modulators, shows considerable structural diversity.
The phosphorylation of the activation loop, which upon phosphorylation is released from the active site, thereby enabling substrate binding. Some kinases require more than one phosphorylation in the activation loop, and both auto- and heterophosphorylation of the activation loop may occur (4). At the N terminus of the activation loop, what is known as the DFG motif is found. In some kinases (such as ABL), this motif is flipped out in the inactive kinase (and referred to as “DFG out”) and flipped inward in the active kinase, exposing a hydrophobic (allosteric) pocket that can be a binding site for drugs (8). A “gatekeeper” residue helps regulate whether DFG is “in” or “out.” Mutations in the gatekeeper residue may lead to a constitutive activation of the kinase by changing the position of the activation loop and stabilizing the hydrophobic spine (9). The gatekeeper residue may also influence substrate specificity (10). Protein kinase activation has the objective of structurally positioning residues involved in catalysis and substrate binding that may be distant in the primary sequence.

Substrate Recognition by the SBS

Most substrates are anchored by binding to the C-lobe to facilitate phosphoryl group transfer. Furthermore, substrate anchoring is an important factor for determining kinase-substrate specificity and occurs mainly through electrostatic interactions. Variability between protein kinases is found in differences in charge and hydrophobicity of surface residues in the SBS of the catalytic domain, and this variability contributes to the specificity of kinase-substrate interactions (5). Analysis of the sequence surrounding the phosphorylated residue (referred to as P0) and use of synthetic peptides have shown that Ser/Thr kinases specifically recognize residues surrounding the P0 residue. These residues from the N to C terminus are named according to their positions relative to P0, namely P-3, P-2, P-1 and P1, P2, P3 (11). In an elegant study, Alexander and collaborators (13) used positional scanning-oriented peptide library screening (PS-OPLS) to independently test the position of each amino acid in a specific sequence and determine the consensus sequence preferably recognized by several mitotic kinases. These sequences were further validated as to their presence in the linear sequence of identified substrates. The authors demonstrated that despite overlapping localizations of the kinases, substrate recognition of specific motifs is particular to the different mitotic kinases, thus further strengthening the fact that kinases recognize specific consensus sequences (13).

There are five categories of Ser/Thr kinase substrates based on consensus recognition motifs formed by basic, prolyl, acidic, or hydrophobic residues or even previously phosphorylated seryl, threonyl, and tyrosyl residues (14). The active site interacts with four residues on either side of the phosphorylated P0 residue. More distant residues interact with regions outside the active site (3) but still may contribute to substrate specificity, as has been shown for the cGMP synthase kinase 3 (GSK3) (15). These studies suggested that anchoring residues other than the phosphorylatable residue are important to achieve kinase-substrate specificity and affinity (15).

Linear consensus motifs recognized by tyrosine kinases are still being determined. The search for linear consensus motifs has been widely used as a strategy to find kinase-specific substrates and is further discussed below. For example, a crystal structure of PKA [Protein Data Bank (PDB): 3FJQ] (16) interacting with a pseudosubstrate peptide containing a linear consensus motif for PKA formed by flanking basic amino acids ([K/R][K/R][S/T]) is depicted in Fig. 2A. However, substrates frequently do not contain linear consensus sequences; about 13% of the PKA substrates reported in PhosphoSitePlus database do not contain a linear consensus sequence (17). Mutations or deletions of regions far from the phosphorylatable residue in casein kinase 2 affect autophosphorylation (18), and mutation of noncontiguous regions in the casein kinase 2 substrate 46-kD mannose 6-phosphate receptor (MPR 46) also affected substrate phosphorylation (19). Mutation of a linear consensus motif for PKA does not prevent the cyclic adenosine monophosphate (cAMP)-dependent PKA phosphorylation of acetylcholinesterase, suggesting that PKA recognized nonlinear consensus motifs (20). Site-directed mutagenesis of the basic residues (Lys160 and Lys164) located far from a phosphorylated threonine (Thr253) in α-tubulin decreases phosphorylation of this residue by PKC, and structural analysis shows that these lysines form a PKC consensus phosphorylation site resembling a linear consensus named “structurally formed consensus motif” (Fig. 2B). Additionally, experimentally validated sites phosphorylated by PKA (17) deposited in the PhosphoSitePlus data bank that did not contain a linear consensus motif were modeled and suggested to also contain structurally formed consensus motifs (17).

The idea that phosphorylation occurs mainly in unstructured flexible regions has been proposed as an explanation for the lack of a linear consensus sites because unstructured regions could easily accommodate to the catalytic site (21), but this notion should be revisited. Recently, it was shown that 37% of phosphorylation sites reported occur in structured regions (22). Thus, the concept of a conformational motif seen only in structured proteins where noncontiguous residues come close to form conformations similar to the ones found in linear consensus sites can at least in part explain kinase interactions with substrates lacking linear consensus motifs. This perception confirms the importance of anchoring residues in determining substrate specificity and in catalysis itself and suggests that phosphorylation in structured, less flexible regions of proteins may also
have important functions and should be underscored. By taking tri-
dimensional structures into consideration, detection and validation of sub-
strates containing “conformationally formed consensus motifs” will aid in
developing a new software that tries to attribute specific kinases to sub-
strates or predict phosphorylation sites (as discussed below).

Substrate-Anchoring Domains in Kinases

Substrate recruitment by interactions with other regions of the kinase, mainly but not exclusively in the regulatory domain, has also been sug-
gested to be important for kinase-substrate interactions (23). Substrate
binding regions that are not in the active site (though often present in
the catalytic domain) are frequently called “docking domains” or docking
sites. Docking sites aid in substrate anchoring to and recognition specific-
ity by the kinase. In some cases, substrate docking may also have an al-
losteric effect (24). These docking sites in kinases often bind short peptide
motifs (such as SH2, SH3, PH, and C2 domains) (25) that mediate spe-
cific protein-protein interactions (PPIs). Besides the target substrate, these
interactions at docking sites often involve scaffold proteins or phospha-
tases. Interactions with scaffold proteins increase the local concentration of
substrates (discussed in more detail below), whereas interactions with phospha-
tases help sequester inactive kinases in the cytoplasm and may
compete with substrates for kinase binding (26). Sites other than the active
site that interact with substrates and are important for substrate recognition
and specificity are observed in many kinases, particularly members of the
mitogen-activated protein kinases (MAPKs), such as extracellular signal-
regulated kinases 1 and 2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK),
and ERK5.

The MAPK pathway is composed of (i) MAP kinase kinase kinase
(PDK1), (ii) MAP kinase kinase (MAP2K), and (iii) MAPK. Dual
phosphorylation of Tyr and Thr residues at the activation loop activates
MAPKs, and inactivation is mediated by several phosphatases. This fam-
ily of kinases contains common docking (CD) motifs, usually found in
close proximity of the catalytic site, that interact with D-motifs (also
known as Kim motifs, D boxes, or DEJL domains) that are found in their
specific substrates and phosphatases. D-motifs are 13 to 16 amino acids
long and composed of a cluster of positively charged residues surrounded
by hydrophobic residues (27), namely, (R/K)2–3–X2–6–UA–X–UB, where
U is any hydrophobic residue (29). Thus, interaction with the D-motif is
mainly hydrophobic and electrostatic. Recognition of the D-motif is one
of the factors determining substrate specificity for the kinases in the MAPK
signaling cascade, and most of the proteins interacting with MAPKs contain
D-motifs (28). Peptides derived from different MAPK substrates, including
the kinases MAPKAP (MAPK-activated protein) kinase 2 (MK2, a p38α
substrate), MAPK-interacting Ser/Thr protein kinase 1 (MNK1, an ERK1
and p38α substrate), and the transcription factor nuclear factor of activated
T cells, cytoplasmic 3 (Nfatc3, a JNK1 substrate that also interacts with
p38α), all contain linear D-motifs (Fig. 3A). Peptides containing D-motifs
can discriminate effectively between JNK and p38 but not between p38 and
ERK (28). In this case, besides the CD domain of the kinases, specificity
is also found in a site called ED that also contributes to substrate docking.
Swapping ED residues in ERK2 for ED residues in p38 changes the sub-
strate specificity of ERK2 to that of p38. Besides the above-listed sub-
strates, scaffold proteins [such as JNK-interacting protein 1 (Jip1)] (29)
and some phosphatases [such as dual specificity phosphatases (DUSPs),
Tyr and Ser/Thr phosphatases, hematopoietic Tyr phosphatase (HepT),
immune system–specific, striatum-enriched phosphatase (STEP), and
brain-specific, STEP-like PTP] interact with MAPKs (28) at a D-motif.
Substrate docking sites have also been described in Tyr kinases. For ex-
ample, in the C-terminal SRC kinase (CSK), a kinase that specifically

Fig. 2. Examples of protein kinases interacting with substrates with linear
and structurally formed consensus sites. (A) Crystal structure of PKA
(PDB: 3FJQ; gray) (16) interacting with a pseudosubstrate peptide that
presents the linear consensus (yellow). The red spots on the surface of
PKA highlight acidic residues that interact with the P-2 and P-3 positions
of the substrate (blue sticks, both arginines), which determine the PKA/
PKC consensus phosphorylation motif as [K/R][K/R]X[S/T]. The purple
on the substrate denotes the catalytic residue, and the orange mark

on the substrate denotes the position of the phosphorylated residue (P0).
(B) Model of PKC (gray) interacting with a known substrate (α-tubulin,
blue). Acidic residues that interact with the basic residues on the substrate
are depicted in the SBS (red). The phosphorylatable residue, Thr253
(orange) of α-tubulin is not found in a PKA/PKC phosphorylation motif.
Structural analyses of this substrate revealed that basic residues Lys163
and Lys164 (blue sticks) are close to Thr253, presenting a spatial conforma-
tion similar to the linear substrate.
phosphorylates SRC, mutations in the docking site of CSK decrease SRC phosphorylation but only partially affect general kinase activity (30). Likewise, cyclin-dependent kinases (CDKs) are activated by cyclins that recognize specific docking motifs in substrates. For example, substrates containing LP motifs (enriched in Leu and Pro residues) are preferentially recognized by the G1/S cyclin complex Cln1/2-CDK2, whereas RXL motifs are preferentially recognized by S-phase cyclins. A conserved region in Cln1/2 first described in yeast was shown to recognize the LP motif and aid in the phosphorylation of substrates with multiple phosphorylation sites. Disruption of substrate binding to the docking site delayed the transition between G1 and S phases (31). As has been shown for p38α, substrate docking not only ensures specificity but also has an allosteric effect on kinase activity, enhancing substrate phosphorylation and enabling phosphorylation even under low concentrations of ATP. ATP binding and substrate docking are cooperative; thus, previous ATP binding may assist the docking of substrates that otherwise have low affinities for their docking sites (24). Docking sites found in substrates and phosphatases generally are linear and short; however, the presence of a “conformational D-motif” has also been reported in MAPK phosphatase 5 (MKP5). Crystal structure analysis revealed that the binding of p38α to MKP5 is mediated by distinct helical regions in the phosphatase that come together to form the kinase binding domain (Fig. 3B) (32). These results further support the idea that structured regions are also important for the establishment of kinase interactions with its substrates and phosphatases because conformation can aid the binding of kinases to substrates (as discussed above).

One of the factors that controls signal transduction pathways is the balance between kinase and phosphatase activity toward a substrate. Regulation of these activities can be mediated by docking of the kinase or phosphatase to a specific substrate, which is the case of the retinoblastoma tumor suppressor protein (Rb). Upon mitogen stimulation, CDK phosphorylates Rb, thereby coordinating the initiation of S phase. The dephosphorylation of Rb by protein phosphatase 1 (PP1) is required for mitotic exit. Because the binding sites for the kinase and phosphatase occupy the same region on Rb, the kinase and phosphatase compete for the same binding site. This is a mechanism of regulating the antagonistic processes of Rb regulated by phosphorylation and dephosphorylation (33). Besides substrate docking sites within kinases, scaffold proteins that bind to kinases also help determine substrate and phosphatase specificity, in some cases enhancing substrate phosphorylation, as discussed below (34).

Substrate Scaffolding by Adaptor Proteins

Substrates are frequently found in low abundance in cells. Thus, cells have found other means to increase kinase-substrate interactions. In cells, scaffold proteins are essential components of signal transduction, increasing the kinase-substrate interactions and reaction kinetics. Scaffold proteins also contribute to substrate specificity and localization of kinases at different subcellular locations within cells (23, 35).

Substrate recruitment in cells is an essential step because it increases local concentration of the substrate and thus the frequency of proximity between kinase and substrate. The idea that recruitment mediated by scaffolds could substitute for the absence of linear consensus motifs in some substrates (23) should be revisited to include the fact that structurally formed consensus motifs could be present in substrates that do not contain linear motifs. Nevertheless, scaffolding is underscored, specifically within the context of the cell where, besides increasing the interaction with substrates in low concentrations, scaffold proteins position kinases and substrates within specific subcellular locations. Several reviews discuss scaffold proteins; here, we acknowledge that they are also key components of signal transduction pathways and are important for the recognition and interaction of the substrate by a specific kinase. Interactions between kinases and scaffold proteins have also been explored to develop more specific kinase modulators. One well-studied example is RACKs (receptors

Fig. 3. Examples of protein kinases interacting through the docking site. (A) Structure of protein kinase p38α (gray; PDB: 3GC7) (122) with the docking site highlighted (orange). Three peptides of substrates from different MAPks are overlapped to show small differences in physical interactions that determine specificity: in blue, a peptide from p38α substrate MK2 (PDB: 2OKR) (123); in red, a peptide from ERK1 substrate, MNK1 (PDB: 2Y9Q) (124); and in green, a peptide from JNK1 substrate, NFAT4 (PDB: 2XRW) (124). The peptide in pink is a spatial reference for the location of the SBS, showing the distance between these two key interaction sites in p38α. (B) Structure of p38α interacting with phosphatase MKP5 (pink) compared to the interaction with the linear peptide from a p38α substrate (blue), showing that the interaction on the docking site also presents the possibility of “structural consensus specificity,” which can be relevant for substrate specificity.
for activated C kinase) that help anchor active PKCs to specific subcellular locations and often promote the interaction of a specific PKC isoenzyme with its substrates (36). Peptide inhibitors derived from RACK binding sites in PKC inhibit isoenzyme-specific interactions between a specific PKC isoenzyme and its RACK, whereas peptide agonists promote these interactions (37). A second example is the A-kinase–anchoring proteins (AKAPs) that anchor inactive PKA to specific subcellular locations and promote substrate kinase interactions upon increases in cAMP concentrations. Inhibition of this interaction inhibits PKA signaling at distinct subcellular locations within cells (38).

Identification and Prediction of Specific Kinase Substrates

Biochemical methods

Even though kinases have been studied for several years, few physiological substrates, meaning proteins that would be phosphorylated by a specific kinase in cells, have been established. One of the reasons for this is the fact that kinase-substrate interactions are transient. Thus, several methods have been developed to detect kinase-specific substrates. Here, we describe some of the most recently used methods (Table 1).

With the development of more sensitive equipment, mass spectrometry is frequently coupled to other methods used to detect kinase-specific substrates (39). Among these are separation of proteins by two-dimensional gel electrophoresis stained with phosho-specific dyes (40) and analysis of more complex samples with, for example, stable isotope labeling of amino acids in cell culture (SILAC) (41, 42) combined with genetic overexpression or knockdown (43) or pharmacological manipulation [with small molecules (44) or peptides (45, 46)] of a specific kinase. However, an important limitation of mass spectrometry is that the signal of proteins that are present in low abundance is frequently suppressed in favor of those that are more abundant (47). The combination of subcellular fractionation and techniques of phosphopeptide enrichment has improved the detection of low-abundance proteins and phosphorylation sites (48).

Immunoprecipitation using kinase-specific antibodies combined with mass spectrometry is also applied to discover candidate substrates. However, kinase-substrate copurification is often difficult because of the transient nature of this interaction. After substrate phosphorylation, the negative charge of the added phosphate frequently repels the kinase, disrupting the association between the substrate and the kinase (49). To improve kinase-substrate communoprecipitation efficiency, many methods to covalently cross-link proteins have been developed (15, 50–53). Immunoprecipitation of substrates with specific antibodies that recognize proteins that contain a phosphorylated residue within a consensus sequence has also been used. For example, in detecting substrates of the kinase AKT in 3T3-L1 adipocytes stimulated with insulin (54), new AKT substrates that contain a Rap GAP (guanosine triphosphatase–activating protein) domain and two phosphorytyrosine binding (PTB) domains were identified (54). Although there has been much success using antibodies against phosphorylation motifs for screening of kinase substrates (54–57), the identification of a specific kinase responsible for a given phosphorylation event remains a major challenge because many kinases recognize similar consensus sequences. However, substrate promiscuity may occur also within cells because many kinases exhibit overlapping functions (3, 58). Because linear and structurally formed consensus sites are similar in conformations, antibodies against phosphorylation motifs may be able to detect both types of substrates in immunoprecipitation assays under native conditions.

An important drawback of immunoprecipitation assays is the frequent nonspecific binding of proteins to resins, and several strategies have been developed to eliminate the detection of these proteins. Therefore, it is recommended to cross-link the antibody to the beads used during immunoprecipitation assays to reduce contaminating the antibodies (59). Despite these difficulties found when using immunoprecipitation assays combined with mass spectrometry, this has still been a technique of choice to find substrates with the advantage of being able to detect substrates and signaling complexes.

An alternate assay called the kinase-interacting substrate screening (KISS) assay consists of binding a specific kinase to beads, which then interact with proteins in cell lysates. Bound proteins are subsequently digested with trypsin and enriched for phosphopeptides, which are then detected by mass spectrometry. Using this method, for example, 356 phosphorylation sites of 140 proteins were identified as candidate substrates for Rho-associated kinase (ROCK2), among which some of the substrates detected were validated and shown to interact with ROCK2 within the context of cells (60).

Another method called the yeast two-hybrid (Y2H) system is used to detect a physical association between a specific kinase and substrate within the context of cells. In the Y2H system, a DNA sequence including the coding region of the catalytic domain of a kinase is fused to the DNA binding domain of the transcription factor Gal4p (this serves as a bait protein). At the same time, a complementary DNA (cDNA) library is constructed to encode for putative substrates in fusion with a transcriptional

Table 1. Biochemical methods used to detect protein kinase substrates. Listed are various methods using biochemical approaches to predict substrates on the basis of linear and structural motifs.

<table>
<thead>
<tr>
<th>Biochemical methods</th>
<th>Arrangement of detected motif in detected substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-dimensional gel electrophoresis and mass spectrometry</td>
<td>Linear and structural motifs</td>
<td>(40)</td>
</tr>
<tr>
<td>Stable isotope labeling by amino acids in cell culture (SILAC)</td>
<td>Linear and structural motifs</td>
<td>(41, 42, 53, 128)</td>
</tr>
<tr>
<td>Immunoprecipitation and mass spectrometry (kinase-specific antibodies)</td>
<td>Linear and structural motifs</td>
<td>(129)</td>
</tr>
<tr>
<td>Immunoprecipitation and mass spectrometry (antibodies against phosphomotifs)</td>
<td>Linear and structural motifs</td>
<td>(54)</td>
</tr>
<tr>
<td>Yeast two-hybrid (Y2H) system</td>
<td>Linear and structural motifs</td>
<td>(61, 62)</td>
</tr>
<tr>
<td>Split-ubiquitin system (SUS)</td>
<td>Linear and structural motifs</td>
<td>(64, 65)</td>
</tr>
<tr>
<td>Bimolecular fluorescence complementation (BiFC)</td>
<td>Linear and structural motifs</td>
<td>(65, 66)</td>
</tr>
<tr>
<td>Kinase-interacting substrate screening (KISS)</td>
<td>Linear and structural motifs</td>
<td>(60)</td>
</tr>
<tr>
<td>Kinase assay linked with phosphoperoteomics (KALIP)</td>
<td>Linear motifs</td>
<td>(67)</td>
</tr>
<tr>
<td>Engineered kinases (chemical genetics)</td>
<td>Linear and structural motifs</td>
<td>(70, 71)</td>
</tr>
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activation domain in Gal4p (these serve as prey proteins). If the bait (kinase) and prey proteins (substrate) interact, they reconstitute a functional Gal4p that activates the expression of a reporter gene in a yeast strain (61, 62). Despite the use of a heterologous system, the Y2H system has the advantage of being able to detect PPIs within a cellular context. However, Y2H assays have some limitations, mainly associated with high frequency of false positives due to overexpression conditions of both kinases and putative substrates. In addition, phosphorylation is a transient event (as noted above), and consequently, the fast interaction between the substrate and the kinase is often not sufficient to activate the reporter gene. Despite these biases, the use of the Y2H system has successfully identified some protein kinase substrates (62, 63). One of the first papers showing the importance of the Y2H system in identifying PKC substrates was published by Staudinger and colleagues in 1995. The authors used the catalytic region of PKCα fused to the DNA binding domain of yeast Gal4 as bait to screen a mouse T cell cDNA library, in which cDNA was fused to the Gal4 activation domain. Using this approach, it was possible to identify the PKC substrate, protein interacting with PKCα 1 (PICK1). More recently, de Souza and colleagues used the Y2H approach to study the functions of the Ser/Thr kinase NEK7 and identified putative binding partners and new substrates. Immunoprecipitation assays combined with mass spectrometry analysis validated kinase-substrate interactions and the phosphorylation status of identified proteins. This analysis suggested that NEK7 is involved in key cell division processes and chromosome segregation (63).

Other methods developed from the Y2H system to detect PPIs have been successfully used to detect kinase substrates. In the split-ubiquitin system (SUS), interacting proteins are combined with the N- or C-terminal half of ubiquitin, and upon successful interaction, a complete ubiquitin moiety is reconstituted and coupled to a metabolic readout, such as degradation of the orotidine 5′-phosphate decarboxylase (UR3) fused to the C-terminal half of ubiquitin. Degradation of UR3 prevents the conversion of 5-fluoroorotic acid monohydrate (5-FOA) to the toxic compound 5-fluorouracil; thus, when there is a PPI, the cells survive in the presence of 5-FOA (64). Such an assay was used to detect substrates of CDKA in Arabidopsis (65). A variation of the SUS is the bimolecular fluorescent complementation [BifC; also called split yellow fluorescent protein (YFP)] assay (66). Pusch and collaborators used both the SUS and BifC systems to detect and validate in vivo substrates for the Arabidopsis CDKA. A few of the substrates were further validated to show that CDKA phosphorylates proteins that control the redox state by the cell cycle (65).

An assay described in 2012 by Xue and colleagues (67) uses an integrated proteomic strategy termed “kinase assay linked with phosphoproteomics” (KALIP); this assay combines a sensitive kinase reaction with endogenous kinase-dependent phosphoproteomics to identify direct substrates of protein kinases. This assay consists of preparing cell lysates, digesting the cellular proteins, and then dephosphorylating them. The proteins are then phosphorylated in vitro with a specific kinase, and phosphorylation sites are detected by mass spectrometry. KALIP may not be effective for kinases that require priming phosphorylation events (such as GSK3), additional interacting surfaces, or a docking site on the protein (such as in the case of CSK). In addition, because of loss of localization information when the cell is lysed, this approach cannot eliminate certain false positives in cases where substrates of other kinases have similar motifs as the kinase of interest. Furthermore, digestion may abolish certain motifs, and structurally formed phosphorylation sites will not be detected using this approach.

Metabolic labeling enabled the isolation of different subsets of the proteome containing posttranslational modifications (68). In 2007, Green and Pfum validated that several kinases can use γ-biotinylated ATP as a substrate, thus transferring phosphobiotin directly to the substrates (69). Although the range of kinases that can accept biotin-ATP as a substrate and the precise catalytic parameters for its use have not been determined, this approach holds promise as a generally accessible way to identify new kinase substrates through direct labeling (69).

Chemical biology approaches are also being successfully developed to find kinase-specific substrates. In 1997, Shokat and colleagues published an approach using [γ,32P]-labeled orthogonal ATP analogs containing sterically bulky groups in the adenosyl moiety. These analogs were used by analog-sensitive kinase generated by site-directed mutagenesis of the ATP binding site, thus generating substrates labeled with specific analogs, which were then detected by mass spectrometry (70). Because only the mutant kinase was active on these bulky ATP analogs, this approach allowed identification of substrates for only engineered kinase v-Src (70). The Shokat group later developed another set of ATP analogs by replacing the 12P-labeled phosphate with γ-thiophosphate and specifically enriched the thiophosphate-modified proteins using iodoacetyl agarose (71). This technique was then successfully used to identify >70 substrates of the CDK1–cyclin B complex (72). The disadvantage of this approach, however, is that some ATP analogs are not cell-permeable, and therefore, one cannot detect substrates within the cellular context. However, both linear and structurally formed phosphorylation sites can be detected, and the transient nature of kinase-substrate interactions would not hinder the detection of substrates because they are labeled with the ATP analogs.

Computational methods

Despite the improvement in techniques used to detect phosphorylated amino acid residues, credited largely to advances in mass spectrometry, it still has been difficult to find phosphorylation sites of low-abundance substrates within the cellular context. Ideally, predictive tools capable of finding kinase-specific substrates would be more cost-effective and could predict phosphorylation of low-abundance proteins. With an increasing number of validated substrates deposited in several phosphoprotein data banks, new computational approaches can be developed to more accurately predict kinase-specific substrates.

Predictive tools can be briefly summarized from a computational point of view as an artificial intelligence algorithm focused on finding recognizable patterns over samples of phosphorylation data (Table 2). Most of the available methods rely on the evaluation of the amino acid sequence, which is apparent in a review of computational methods for predicting eukaryotic phosphorylation sites, in which there are references for 29 methods that use only the primary structure of proteins, whereas only 10 use some kind of structural information (73).

The importance of using three-dimensional (3D) information in phosphorylation prediction studies is not a new concept. One of the first methods ever developed in this field, NetPhos (74), was already a first attempt to use structural data to predict phosphorylation sites. Blom et al. justify the NetPhos development, stating that “it is obvious that what the kinase actually recognizes is the three-dimensional structure of the polypeptide at the acceptor residue, and not the primary structure” (74). This method is based on a neural network fed with structural backbone information of phosphorylated sites. Although at the time this method presented an overall worse performance than sequence-based neural network methods, it was able to correctly predict sites that did not match the typical consensus, therefore generating negative predictions on sequence-based methods but including structural features that resembled some of the known phosphorylation sites. This method was later updated to deal with kinase specificity (75).

 Whereas NetPhos used a statistical learning algorithm, thus generating predictions on computer-generated parameters, Predkin (76) was developed totally based on structural analyses. On the basis of crystal structures


Table 2. Computational methods used to predict protein kinase substrates. Listed are various methods using computational tools to predict substrates on the basis of sequence or structure.

<table>
<thead>
<tr>
<th>Computational methods</th>
<th>Basis of the search space</th>
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<tbody>
<tr>
<td>NetPhos</td>
<td>Sequence</td>
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<td>Predkin</td>
<td>Sequence</td>
<td>7 residues</td>
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<td>NetphosK</td>
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<td>DISPHOS</td>
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<td>pkaPS</td>
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<td>80 residues</td>
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<td>NetworKIN</td>
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<td>Unnamed method</td>
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<td>9 residues</td>
<td>(81)</td>
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<tr>
<td>Predkin 2.0</td>
<td>Sequence</td>
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<td>Phos3D</td>
<td>Structure</td>
<td>2 to 10 Å</td>
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<td>Unnamed method</td>
<td>Structure</td>
<td>3 to 12 Å</td>
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of PKA, phosphorylase kinase (PHK), and CDK2 with bound substrate peptides, key residues for their interactions were determined, and a set of rules to determine specificity was generated. Predkin 2.0 (77) implemented a more robust approach, in which the substrate-determining residues are predicted by a scoring scheme on the basis of substrate-weighted matrices. In the DREAM (Dialogue on Reverse Engineering Assessment and Methods) challenge, an open science initiative aiming at promoting collaborative efforts joining computational and experimental biologists focused on methods to reverse engineer cellular networks from high-throughput data (78), Predkin had the best performance in specificity on the category of peptide recognition domain, in which participants had to determine the specificity of uncharacterized protein kinases and compare it with previously unknown experimental data (79).

An increase in the complexity of the newer versions of NetPhos and Predkin indicates new trends in the field. DISPHOS (disorder-enhanced phosphorylation predictor) (80) adds physical chemical parameters in a learning reinforcement algorithm. Plewczynski and collaborators used a structural fragment database on a support vector machine (SVM) method (81). pkaPS (82) uses biochemical properties of the amino acids for a highly specific method to predict PKA substrates. NetworKIN (83) combines sequence-based phosphorylation prediction with protein network interaction data.

Although all these methods brought innovative approaches and an interesting increase in prediction capabilities, they all share a central characteristic with the sequence-based methods: They limit the prospection around the phosphorylation site based on the primary structure. Some methods (such as Predkin) work with 7-residue long peptides (76), whereas others (such as pkaPS) (82) use a ≤81-residue window. This creates a limitation because the structure of the sequential residues may not be representative of the phosphorylation site environment, in which participants in the context. On the folded protein, the residues spatially flanking the phosphorylation site are not necessarily in proximity on the primary structure. The 3D structure of phosphorylation sites on proteins has been studied for some time. Phosho3D (84) is a public database of this kind of structures. The new version of this database, Phosho3D 2.0 (22), includes a 3D zone tool that compares information of the residue composition on the spatial surroundings of the phosphorylated residue (P0).

Prediction tools also incorporated this kind of information. Phos3D (85) evaluated a long list of physical-chemical characteristics of the region within 2 and 10 Å of P0. Using a statistical approach on an SVM algorithm, an improvement on the prediction capabilities was achieved, although the general conclusion was that most of the discriminatory effect was connected to the local one-dimensional sequence. Using a similar approach in a more recent work, Su and Lee (86) were able to outperform most of the sequence-based methods. The bioinformatics methods that use structural knowledge to predicted phosphorylation sites and the search space of each one of them are listed in Table 2.

Computational methods present a great set of advantages. They are inexpensive compared to experimental techniques, fast in that they are capable of analyzing whole genomes, and easily available to everyone. Innovative methods are flourishing. One such method is the Phosphorylation Set Enrichment Analysis (PSEA), an analog of Gene Set Enrichment Analysis (GSEA), used for analyses of DNA microarrays, which is adapted for the analysis of phosphorylated substrates (87). However, all the existing methods, especially the structure-based ones, can be further improved by the advances in the related fields and increased knowledge about the structural basis of kinase-substrate interactions.

Once the substrates are detected or predicted, they should be validated. 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 (67). In vitro phosphorylation frequently 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 (67). The substrates must interact with a specific kinase within the context of a living cell. Therefore, site-directed mutagenesis and phospho-specific antibodies are commonly used to confirm phosphorylation sites both in vitro and in vivo (17).

Perhaps because of the transient nature of kinase-substrate interactions, relatively few crystal structures of kinases with their respective substrates have been reported (68–98). Nuclear magnetic resonance (NMR) has also been used to map kinase-substrate interaction sites (99, 100). Improved techniques that can directly determine the structural nature of kinase-substrate interactions or increasing the affinity between the kinase and the substrate may lead to a better understanding of the structural nature of this interaction and the development of more specific kinase inhibitors.

The SBS as a Drug Targeting Site

More than 4500 lead compounds have been described as kinase inhibitors. Among these, 40 compounds were launched and 27 compounds are in clinical phase 3 (data obtained from Thomson Reuters Integrity, 2015). However, none of these target the SBS or interactions with substrates. Figure 4 depicts the different types of kinase inhibitors and how they overlap with each other.

Several of these compounds are ATP-competitive inhibitors and are called type 1 inhibitors. Because ATP binding sites are highly conserved...
Fig. 4. Different regions (mainly N-lobe) of the catalytic domain of protein kinases explored as drug binding sites. (A) The ATP pocket is targeted by type 1 inhibitors (PDB: 1FMO) (88). (B) A pocket formed in the DFG-out conformation is targeted by type 2 inhibitors, such as imatinib [PDB: 2HYY (125)], depicted in darker blue sticks. (C) Type 3 inhibitors target a hydrophobic pocket (but not the ATP binding region) released in DFG-out conformations. For example, depicted in darker red sticks within the red hydrophobic pocket is the non–ATP-competitive inhibitor N-[4-[(1S)-1,2-dihydroxyethyl](benzyl)-N-methyl-4-(phenylsulfamoyl)benzamide of human LIMK2 kinase domain (PDB: 4TPT) (126). (D) A pocket formed in the surface of the N-lobe of MEK1 binds the non–ATP-competitive inhibitor 2-[[3R-3,4-dihydroxybutyl]oxy]-4-fluoro-6-[[2-fluoro-4-iodophenyl]amino]benzamide (PDB: 4ARK) (127). (E) A shallow crevice and ATP binding pocket are occupied by an inhibitor formed by a synthetic peptide linked to thiophosphoric acid α-(adenosyl-phosphorophospho)-α-acetamidyl-diester, a typical type 4 inhibitor (magenta sticks; PDB: 1GAG) (117). (F) General view of all surfaces of pockets used by different inhibitor types. The reference structure (gray) is PDB: 2HYY (125).

among the different kinases (as discussed above), most ATP binding site competitive inhibitors are not very selective. Furthermore, intracellular concentrations of ATP may be high, which contributes to the low efficiency of this class of compounds. Both specificity and efficiency problems have been undertaken by the discovery of inhibitors that bind the inactive conformation of kinases (“DFG-out,” as discussed above), which is less conserved among the different kinases. Early examples of these compounds, called type 2 inhibitors, include imatinib (STI571), BIRB796, and sorafenib (BAY43-9006) (101). Furthermore, type 3 inhibitors bind the catalytic domain of the kinase close to the ATP binding site but do not interact with the hinge region. Rather, type 3 inhibitors can interact with the hydrophobic (allosteric) pocket generated by the DFG-out conformation (102). Type 4 inhibitors bind allosteric regions around the catalytic domain, and type 5 inhibitors are bivalent or bisubstrate compounds that at least in part would occupy the SBS and the ATP binding site (103).

The main strategy to overcome the issue of lack of specificity would be to explore allosteric sites or PPIs between kinases and substrates, scaffolds, and modulator proteins. Competitive inhibitors of substrate binding are difficult to develop because kinase-substrate interactions are usually found at shallow crevices, making it difficult to find small molecules that would directly compete for substrate binding.

The development of inhibitors of PPIs is not trivial. Such inhibitors frequently violate Lipinski’s “rule-of-five” (104), which consists of a group of experimental parameters important for the pharmacokinetics of the drug in the human body and help determine the drugability of a compound: (i) no more than 5 hydrogen bond donors (the total number of hydrogen-hydrogen and oxygen-hydrogen bonds), (ii) no more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms), (iii) a molecular weight of less than 500 daltons, and (iv) an octanol-water partition coefficient log P not greater than 5 (ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium) (105). Several molecules that do not follow these rules, for example, are larger than 500 daltons, effectively bind shallow pockets (106) and could be good PPIs. The development of inhibitors of PPIs is highly dependent on new chemical approaches that involve the combination of hits from screenings of fragment libraries (107) and generation of new libraries using diversity-oriented synthesis (a strategy aimed at improving compound synthesis frequently building compounds upon an original scaffold (107)) or even libraries of large macrocycles (ring-shaped molecules containing 12 or more ring atoms) (108). Drugability of these compounds that do not follow Lipinski’s rules (105) will have to be determined de novo because they do not have the same pharmacokinetic properties of the commonly used kinase inhibitors.

Peptides have proven to be good inhibitors of PPIs despite their well-known limitations (108). Pseudosubstrate peptides, which contain the consensus motifs recognized by a specific kinase but with an Ala substitution for the phosphorylatable residue, efficiently inhibit substrate phosphorylation [revised in (109)]. In another example, a peptide mimicking the docking site potently inhibited CSK-mediated phosphorylation...
of SRC but only moderately inhibited its general kinase activity, suggesting that inhibition of substrate docking sites may be a good strategy to develop specific kinase-substrate interaction inhibitors (30). The disadvantage of using peptides is that often their delivery to cells is difficult, despite the fact that several peptide delivery systems have been described, being the most popular attachment to cell-penetrating peptides (108). Peptide inhibitors of scaffold proteins have been shown to efficiently inhibit kinase activity; examples of these have been extensively discussed elsewhere (37, 110, 111).

Small molecules that are either substrate competitors or allosteric inhibitors have also been found. For example, benzothiazinone compounds are relatively specific substrate-competitive inhibitors and allosteric modulators of GSK3, a key enzyme involved in the regulation of glycogen metabolism and thus a target for diabetes (112).

The Ser/Thr kinase Polo-like kinase 1 (PLK1), which is involved in mitosis, has a substrate binding domain termed “polo-box domain” (PBD). Peptides that compete with PBD binding to substrates are selective PLK inhibitors, as are fragment-ligated inhibitory peptides (113). Recently, a selective PLK1 small-molecule inhibitor that blocks substrate binding to PBD was rationally developed using docking strategies and is relatively selective and effective both in vitro and in vivo (114). Screening assays generally find ATP-competitive inhibitors; therefore, there is a need to develop new assays to find allosteric or substrate-competitive inhibitors. For example, the substrate activity screening assay, which is based on the conversion of substrates into inhibitors, was developed to find a small-molecule competitor of the kinase c-Src that was effective both in vitro and in cells and acted synergistically with ATP-competitive inhibitors (115).

Assurance that a small molecule is in fact a substrate-competitive inhibitor may be difficult at times and some small molecules that were initially described as substrate-competitive inhibitors were later shown to have other targets or to in fact be ATP-competitive inhibitors. For an extensive review of small molecules that are substrate-competitive inhibitors, see Breen and Soellner (116).

To this end, bispecific type 5 inhibitors constitute a clear evolution. Structurally, they are bifunctional, occupying both niches or rather substrate and ATP binding sites (Fig. 4E) (117) [extensively revised (103)]. Usually, type 5 inhibitors are peptide/small-molecule hybrids (118); their druggability is still to be explored.

Future Perspectives for Clinical Development
Several studies demonstrate the power of interfering with PPI as a new frontier for drug development. Particularly, in the case of protein kinases, this may be a way of overcoming the problem of lack of specificity of kinase inhibitors. Peptides that interfere with PPIs involving kinases and scaffold proteins or other binding proteins are being developed (37, 110, 111) and may serve as lead compounds as more specific kinase inhibitors. At the substrate end, because structurally formed consensus sites require correct folding, one can envision developing inhibitors that bind to the substrate in a manner that interferes with this folding, thereby inhibiting its interaction with a kinase.

One of the reasons for the limited success of kinase inhibitors in clinical settings is the lack of specificity of these inhibitors. With the advancement in mass spectrometry, chemical biology techniques, and predictive tools, more kinase-specific substrates have been and will be detected. The detection of substrates, together with advancements in the area of NMR and crystallography aimed at mapping interaction sites, will be helpful to further understand the structural basis of the interaction between kinases and their substrates and to develop more accurate predictive tools.

To this end, one can envision the prospect that kinase and substrate mimetopes based on the SBS and neighboring regions may have several applications. For basic research and elucidating signal transduction cascades, these mimetopes can be competitive inhibitors used to detect substrates when coupled to phosphoproteomic approaches. Furthermore, key kinase-substrate interactions have been found to be important for certain diseases (119); thus, as a diagnostic or prognostic tool, we can use peptides derived from mimetopes to produce antibodies that can differ substrate-bound and unbound kinases.

There are very few structures of kinases bound to substrates; the elucidation of structures of new complexes and molecular modeling approaches will be essential for the design of mimetopes of kinase-bound states. For therapeutic purposes, mimetopes of either kinases or substrates can serve as drug leads to design mimetics that can specifically compete with the interaction between a depicted kinase and a specific substrate. In some cases, such as certain types of cancer (120) and Parkinson’s disease (121), in which increasing the catalytic activity of a kinase toward a specific substrate may be beneficial, understanding the nature of this interaction may help design allosteric activators.

Overall, the knowledge of PPI surfaces involved in kinase-substrate recognition and activation will certainly be prominent for basic research and development of more specific therapeutics for cancer and beyond.

REFERENCES AND NOTES
14

www.SCIENCESIGNALING.org 22 March 2016 Vol 9 Issue 420 re3


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CANCER IMMUNOLOGY

Lipocalin 2 from macrophages stimulated by tumor cell–derived sphingosine 1-phosphate promotes lymphangiogenesis and tumor metastasis

Michaela Jung et al. (Bernhard Brüne)

Tumor cell–derived factors skew macrophages toward a tumor-supporting phenotype associated with the secretion of protumorigenic mediators. Apoptosing tumor cells release sphingosine 1-phosphate (SIP), which stimulates the production of lipocalin 2 (LCN2) in tumor-associated macrophages and is associated with tumor metastasis. We explored the mechanism by which SIP induces LCN2 in macrophages and investigated how this contributed to tumor growth and metastasis. Knockdown of SIP receptor 1 (SIPR1) in primary human macrophages and experiments with bone marrow–derived macrophages from SIPR1-deficient mice showed that SIP signaled through SIPR1 to induce LCN2 expression. The LCN2 promoter contains a consensus sequence for signal transducer and activator of transcription 3 (STAT3), and deletion of the STAT3 recognition sequence reduced expression of an LCN2-controlled reporter gene. Conditioned medium from coculture experiments indicated that the release of LCN2 from macrophages induced tube formation and proliferation in cultures of primary human lymphatic endothelial cells in a manner dependent on the kinase PI3K and subsequent induction of the growth factor VEGFC, which functioned as an autocrine signal stimulating the receptor VEGFR3. Knockout of Lcn2 attenuated tumor-associated lymphangiogenesis and breast tumor metastasis both in the breast cancer model MMTV-PyMT mice and in mice bearing orthotopic wild-type tumors. Our findings indicate that macrophages respond to dying tumor cells by producing signals that promote lymphangiogenesis, which enables metastasis.

DEVELOPMENTAL DISORDERS

Dominant-negative Gα subunits are a mechanism of dysregulated heterotrimeric G protein signaling in human disease

Arthur Marivin et al. (Mikel Garcia-Marcos)

Auriculo-condylar syndrome (ACS), a rare condition that impairs craniofacial development, is caused by mutations in a G protein–coupled receptor (GPCR) signaling pathway. In mice, disruption of signaling by the endothelin type A receptor (ETAR), which is mediated by the G protein (heterotrimeric guanine nucleotide–binding protein) subunit Gαq/11 and subsequently phospholipase C (PLC), impairs neural crest cell differentiation that is required for normal craniofacial development. Some ACS patients have mutations in GNAI3, which encodes Gαi3, but it is unknown whether this G protein has a role within the ETAR pathway. We used a Xenopus model of vertebrate development, in vitro biochemistry, and biosensors of G protein activity in mammalian cells to systematically characterize the phenotype and function of all known ACS-associated Gαq3 mutants. We found that ACS-associated mutations in GNAI3 produce dominant-negative Gαq3 mutant proteins that couple to ETAR but cannot bind and hydrolyze guanosine triphosphate, resulting in the prevention of endothelin-mediated activation of Gαq3 and PLC. Thus, ACS is caused by functionally dominant-negative mutations in a heterotrimeric G protein subunit.
**NEUROSCIENCE**

**Augmented noncanonical BMP type II receptor signaling mediates the synaptic abnormality of fragile X syndrome**

Risa Kashima et al. (Akiko Hata)

Epigenetic silencing of fragile X mental retardation 1 (FMR1) causes fragile X syndrome (FXS), a common inherited form of intellectual disability and autism. FXS correlates with abnormal synapse and dendritic spine development, but the molecular link between the absence of the FMR1 product FMRP, an RNA binding protein, and the neuropathology is unclear. We found that the messenger RNA encoding bone morphogenetic protein type II receptor (BMPR2) is a target of FMRP. Depletion of FMRP increased BMPR2 abundance, especially that of the full-length isoform that bound and activated LIM domain kinase 1 (LIMK1), a component of the noncanonical BMP signal transduction pathway that stimulates actin reorganization to promote neurite outgrowth and synapse formation. Heterozygosity for BMPR2 rescued the morphological abnormalities in neurons both in Drosophila and in mouse models of FXS, as did the postnatal pharmacological inhibition of LIMK1 activity. Compared with postmortem prefrontal cortex tissue from healthy subjects, the amount of full-length BMPR2 and of a marker of LIMK1 activity was increased in this brain region from FXS patients. These findings suggest that increased BMPR2 signal transduction is linked to FXS and that the BMPR2-LIMK1 pathway is a putative therapeutic target in patients with FXS and possibly other forms of autism.

**PHYSIOLOGY**

**Enhanced responsiveness of GhαrQ343X rats to ghrelin results in enhanced adiposity without increased appetite**

Yacine Chebani et al. (Jacques Pantel)

The ability of the gut hormone ghrelin to promote positive energy balance is mediated by the growth hormone secretagogue receptor (GHSR). GHSR is a G protein–coupled receptor (GPCR) that is found centrally and peripherally and that can signal in a ligand-independent manner basally or when heterodimerized with other GPCRs. However, current Ghsr knockout models cannot dissect ghrelin-dependent and ghrelin-independent signaling, precluding assessment of the physiological importance of these signaling pathways. An animal model carrying a Ghsr mutation that preserves GHSR cell surface abundance, but selectively alters GHSR signaling, would be a useful tool to decipher GHSR signaling in vivo. We used rats with the GhsrQ343X mutation (GhsrM/M), which is predicted to delete the distal part of the GHSR carboxyl-terminal tail, a domain critical for the signal termination processes of receptor internalization and β-arrestin recruitment. In cells, the GHSR-Q343X mutant showed enhanced ligand-induced G protein–dependent signaling and blunted activity of processes involved in GPCR signal termination. GhsrM/M rats displayed enhanced responses to submaximal doses of ghrelin or GHSR agonist. Moreover, GhsrM/M rats had a more stable body weight under caloric restriction, a condition that increases endogenous ghrelin tone, whereas under standard housing conditions, GhsrM/M rats showed increased body weight and adiposity and reduced glucose tolerance. Overall, our data stress the physiological role of the distal domain of GHSR carboxyl terminus as a suppressor of ghrelin sensitivity, and we propose using the GhsrM/M rat as a physiological model of gain of function in Ghsr to identify treatments for obesity-related conditions.
**GPCR SIGNALING**

**Purinergic P2Y<sub>6</sub> receptors heterodimerize with angiotensin AT1 receptors to promote angiotensin II–induced hypertension**

Akiyuki Nishimura et al. (Motohiro Nishida)

The angiotensin (Ang) type 1 receptor (AT1R) promotes functional and structural integrity of the arterial wall to contribute to vascular homeostasis, but this receptor also promotes hypertension. In our investigation of how Ang II signals are converted by the AT1R from physiological to pathological outputs, we found that the purinergic P2Y<sub>6</sub> receptor (P2Y<sub>6</sub>R), an inflammation-inducible G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptor (GPCR), promoted Ang II–induced hypertension in mice. In mice, deletion of P2Y<sub>6</sub>R attenuated Ang II–induced increase in blood pressure, vascular remodeling, oxidative stress, and endothelial dysfunction. AT1R and P2Y<sub>6</sub>R formed stable heterodimers, which enhanced G protein–dependent vascular hypertrophy but reduced β-arrestin–dependent AT1R internalization. Pharmacological disruption of AT1R-P2Y<sub>6</sub>R heterodimers by the P2Y<sub>6</sub>R antagonist MRS2578 suppressed Ang II–induced hypertension in mice. Furthermore, P2Y<sub>6</sub>R abundance increased with age in vascular smooth muscle cells. The increased abundance of P2Y<sub>6</sub>R converted AT1R-stimulated signaling in vascular smooth muscle cells from β-arrestin–dependent proliferation to G protein–dependent hypertrophy. These results suggest that increased formation of AT1R-P2Y<sub>6</sub>R heterodimers with age may increase the likelihood of hypertension induced by Ang II.

**CELL BIOLOGY**

**Proximity biotinylation provides insight into the molecular composition of focal adhesions at the nanometer scale**

Jing-Ming Dong et al. (Brian Burke)

Focal adhesions are protein complexes that link metazoan cells to the extracellular matrix through the integrin family of transmembrane proteins. Integrins recruit many proteins to these complexes, referred to as the “adhesome.” We used proximity-dependent biotinylation (BioID) in U2OS osteosarcoma cells to label proteins within 15 to 25 nm of paxillin, a cytoplasmic focal adhesion protein, and kindlin-2, which directly binds β integrins. Using mass spectrometry analysis of the biotinylated proteins, we identified 27 known adhesome proteins and 8 previously unknown components close to paxillin. However, only seven of these proteins interacted directly with paxillin, one of which was the adaptor protein Kank2. The proteins in proximity to β integrin included 15 of the adhesion proteins identified in the paxillin BioID data set. BioID also correctly established kindlin-2 as a cell-cell junction protein. By focusing on this smaller data set, new partners for kindlin-2 were found, namely, the endocytosis-promoting proteins liprin β1 and EFR3A, but, contrary to previous reports, not the filamin-binding protein migfilin. A model adhesome based on both data sets suggests that focal adhesions contain fewer components than previously suspected and that paxillin lies away from the plasma membrane. These data not only illustrate the power of using BioID and stable isotope–labeled mass spectrometry to define macromolecular complexes but also enable the correct identification of therapeutic targets within the adhesome.