The Structural Basis for Control of Eukaryotic Protein Kinases

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Abstract

Eukaryotic protein kinases are key regulators of cell processes. Comparison of the structures of protein kinase domains, both alone and in complexes, allows generalizations to be made about the mechanisms that regulate protein kinase activation. Protein kinases in the active state adopt a catalytically competent conformation upon binding of both the ATP and peptide substrates that has led to an understanding of the catalytic mechanism. Docking sites remote from the catalytic site are a key feature of several substrate recognition complexes. Mechanisms for kinase activation through phosphorylation, additional domains or subunits, by scaffolding proteins and by kinase dimerization are discussed.

Contents

INTRODUCTION

Most eukaryotic cellular processes and cell signaling pathways are regulated by protein phosphorylation (1, 2). Protein kinases in turn are regulated by inhibitory or activating protein partners, phosphorylation, cellular localization that limits availability of substrates and activators, protein degradation, and gene transcription. Deregulation of protein kinase activity through mutation to constitutively active forms, loss of negative regulators, and chromosomal rearrangements that lead to the formation of active fusion proteins are associated with a number of disorders. Protein kinases have become major targets for therapy, and protein kinase structures have had a significant impact on the development of selective and specific targeted therapies. An appraisal of these studies is outside the scope of this review, but a number of relevant, recent reviews are recommended (3–5).

Phosphorylation of protein substrates can have profound effects. Phosphorylation can result in enzyme activation, enzyme inhibition, the creation of recognition sites for recruitment of other proteins, and transitions in protein state from order to disorder or disorder to order (6). Reflecting the importance of kinases for eukaryotic cell signal transduction and metabolism, there are more than 518 human protein kinases (7, 8; and **http://kinase.com**) recognized through their conserved sequence motifs. These constitute the third most populous protein family and represent ~1.7% of the human genome. Of the total, 478 protein kinases are typical kinases, and 40 are atypical. The typical kinases are divided into those that phosphorylate serine or threonine residues (388 kinases) and those that phosphorylate tyrosine residues (90 kinases). Atypical kinases are proteins reported to have biochemical kinase activity but lack sequence similarity to the conventional eukaryotic kinases. By April 2011, 170 unique kinase domain structures from humans or closely related orthologs had been determined (9; and **http://www.thesgc.org/resources/kinases**).

A distinguishing feature of the protein kinase family is the different structures that they adopt between the active and inactive states. This family characteristic was first appreciated following the determination of the first protein kinase structures of protein kinase A (PKA) [Protein Data Bank (PDB) code 1ATP] (10) in the active conformation and cyclin-dependent protein kinase 2 (Cdk2) (PDB code 1HCK) (11) in an inactive conformation. Adoption of the active state occurs in response to specific signaling events, which are transduced via kinase-associated regulatory domains in *cis* or in *trans*, and/or by phosphorylation of the kinase domain. Structural details are now emerging on the importance of kinase scaffolding to kinase activation and substrate selection and on the role of pseudokinase domains in regulation. In this review, we summarize the results of structural studies on protein kinases that have provided insights into regulation and into the exquisite substrate specificity shown by protein kinases, which ensures fidelity in cell signaling.

THE PROTEIN KINASE FOLD

Serine/threonine- and tyrosine-specific protein kinases share a catalytic domain of ~290 residues in which the active site is sandwiched between an N-terminal lobe composed of a β-sheet and a single α-helix (the "C helix") and

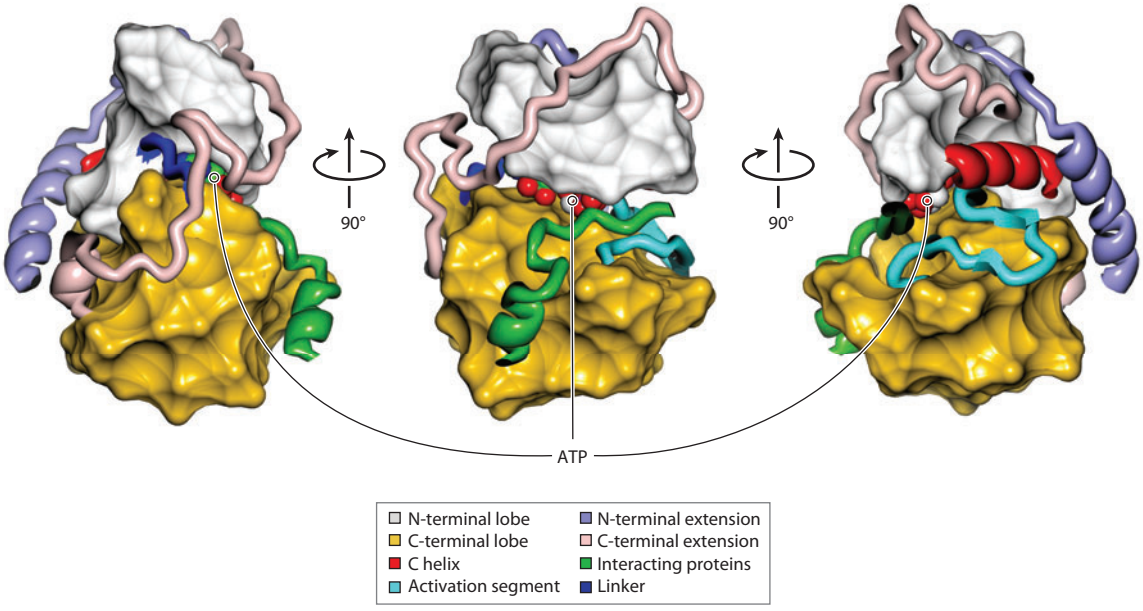


Figure 1

Architecture of a prototypical protein kinase. A highly reduced representation of the key structural and regulatory elements of a protein kinase, as exemplified by protein kinase A, Protein Data Bank (PDB) code 1ATP, illustrating the N-terminal lobe (*main chain surface representation*), C-terminal lobe (*main chain surface representation*), C helix (*ribbon representation*), activation segment (*ribbon representation*), N-terminal extension (*ribbon representation*), C-terminal extension (*ribbon representation*), and interacting proteins (here the proteinaceous inhibitor PKI, ribbon representation). Three consecutive views are related by a 90° rotation about a vertical axis. The same color scheme has been applied throughout the figures in this article. C helix is a secondary structural element named according to the nomenclature in Reference 10.

a larger C-terminal lobe, connected by a linker (**Figure 1**). The C-terminal lobe is predominantly α-helical and includes the activation segment, a region of 20–35 residues located between a conserved DFG (using the single-letter amino acid codes) motif and an APE motif, which is less conserved (**Figures 1** and **2*a***) (12, 13). In the active conformation, the C helix packs against the N-terminal lobe, and the aspartate of the DFG chelates an Mg^{2+} ion to orientate the ATP substrate (**Figures 1** and **2*b***). In the inactive conformation, this latter interaction is often disrupted, and the phenylalanine of the DFG motif is turned in toward the ATP site (**Figure 2*c***). In some kinases, the catalytic domain is flanked by N- and C-terminal extensions that may be involved in regulation (**Figures 1** and **2*a***).

Within the conserved ATP-binding pocket, the adenine ring forms specific hydrogen bonds between N1 and N6 and the peptide backbone of the hinge region, and nonpolar aliphatic groups line the pocket and provide van der Waals contacts to the purine moiety. The ribose O2′ and O3′ hydrogen bond to a glutamate side chain (E127) (residue numbers correspond to those of PKA unless otherwise indicated) and the main chain carbonyl oxygen of E170, respectively.

The triphosphate group points out of the adenosine pocket for transfer of the γ-phosphate to the peptide substrate. From the N-terminal lobe, a conserved glutamate residue within the C helix (E91) and a lysine located on β3 (K72) assist to optimally position the α- and β-phosphate groups. A second network of

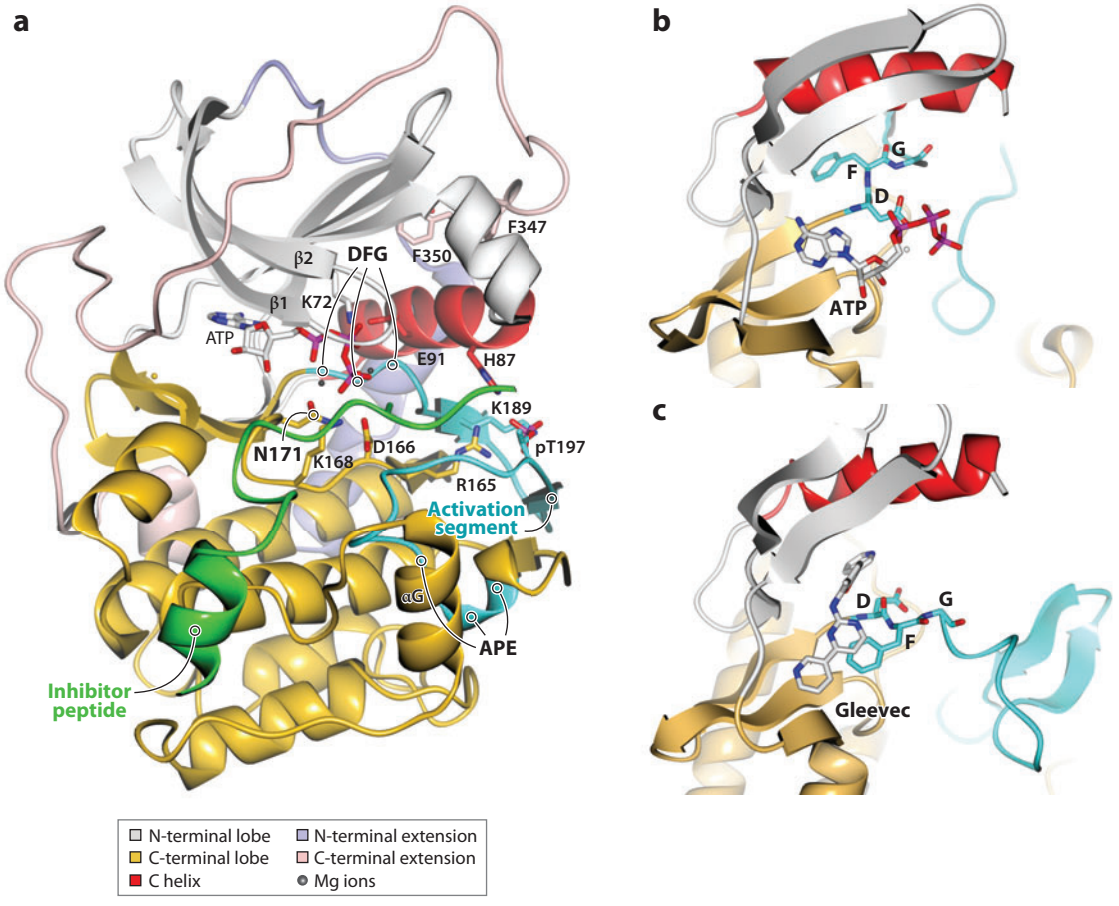


Figure 2

Active and inactive kinases. (*a*) A schematic representation of the conserved residues at the protein kinase A (PKA)-active catalytic site with ATP and substrate. The start of the activation segment is D184, the aspartate that chelates one of the Mg ions (*spheres*). Hydrogen bonds are not shown for simplicity, Protein Data Bank (PDB) code 1ATP. (*b*) Details of the C helix and DFG motif at the start of the activation segment for PKA in the active conformation. (*c*) Details of the C helix and DFG motif at the start of the activation segment for Abl tyrosine kinase complexed with Gleevec in the inactive conformation, PDB code 1IEP. Abbreviations: αG, a secondary structural element named according to the nomenclature in Reference 10; APE, a conserved eukaryotic protein kinase sequence motif at the end of the activation segment; DFG, a conserved eukaryotic protein kinase sequence motif at the start of the activation segment.

interactions to the α- and γ-phosphate groups mediated by a magnesium ion (Mg_2) bound between the aspartate (D184) of the DFG motif and an asparagine (N171) collectively ensure correct positioning required for ATP binding and catalysis. A second magnesium ion (Mg_1) is bound to D184 and the β- and γ-phosphate groups. Additional interactions between the ATP β- and γ-phosphate groups and the glycine-rich loop located between β1 and β2 further stabilize the ATP conformation (**Figure 2*a***).

The activation segment forms a crucial part of the substrate-binding site. In inactive kinases, the activation segment is often partially disordered. Adoption of the catalytically competent conformation to form the peptide-binding platform is triggered in many kinases by phosphorylation. As illustrated for PKA, the phosphothreonine 197 acts as an organizing

center and hydrogen bonds to the side chains of H87 from the C helix, R165 located immediately N-terminal to the catalytic aspartate, and K189 from the activation segment (**Figure 2*a***). The phosphate group promotes closure of the two lobes of the domain and the correct conformation of the activation segment for substrate binding. In other kinases [e.g., phosphorylase kinase (PhK), PDB code 1PHK; epidermal growth factor receptor (EGFR), PDB code 2GS2; cyclin-dependent kinase 5 (Cdk5), PDB code 1H4L], the activation segment does not require phosphorylation for activity and is able to adopt the correct conformation through other interactions. As more kinase structures emerge, atypical activation segments have been observed that include additional secondary structures (14, 15).

In all kinases, the substrate is oriented so that the hydroxyl is directed toward the catalytic aspartate (D166). In serine/threonine kinases, a lysine residue two residues away (K168) contacts the γ-phosphate and is poised to stabilize the developing local negative charge during catalysis. In tyrosine kinases, the stabilizing residue is four residues away and is an arginine to allow for the larger tyrosine residue.

In addition to the conserved residues that directly interact with the bound substrates, two additional chains or "spines" of conserved hydrophobic residues termed the catalytic and regulatory spines have been defined that traverse the N- and C-terminal lobes (16–18). These spines assemble as a response to changes within the catalytic cleft upon kinase activation and devolve those changes to the rest of the domain. The regulatory spine describes an assembly of interactions that is promoted by the conformation of the activation segment and is responsive to peptide binding, whereas the interactions that generate the catalytic spine include a number between residues of the N- and C-terminal lobes and the ATP adenine ring.

In contrast to the active kinases, which share a common catalytically competent conformation, the inactive kinases are structurally diverse (19). This diversity arises because no catalytic requirements constrain the fold when it is inactive, allowing the proliferation of different conformations that, nevertheless, share a number of common structural themes. These themes were first identified in Cdk2 and Src kinase (PDB code 1FMK) and further elaborated in the EGFR kinase and others. Analysis of these structures has provided insights into mechanisms for kinase activation that depend on the structures of the C helix and the activation segment (20).

CATALYTIC MECHANISM

Substrate Recognition

Protein kinases catalyze the transfer of a phosphoryl group from the γ-phosphate of ATP to the hydroxyl group of serine, threonine, or tyrosine residues in protein substrates, a process that may be summarized by the reaction scheme in Equation 1:

$$\text{Protein-OH} + \text{ATP}^{4-}.\text{Mg}^{2+} \rightarrow \text{Protein-O-PO}_3^{2-} + \text{ADP}^{3-}.\text{Mg}^{2+} + \text{H}^{+}.$$

Most protein kinases show specificity for the local region around the site of phosphorylation where certain residues are required for recognition. Examples are given in **Table 1**. Protein kinases normally phosphorylate sites that are in less well-ordered parts of the protein that are exposed on the surface (21). The preference for disordered regions allows the kinase to mold the region of the protein substrate to an extended conformation that fits the catalytic site and allows the localization of the specificity-determining residues to recognition pockets on the kinase (22). It may also allow those regions to act, upon phosphorylation, as specific interaction motifs capable of partnering with diverse proteins. In many kinases, specificity is also conferred by remote docking sites located either on the kinase at sites separate from the catalytic site [as in mitogen-activated protein kinases (MAPKs), for example, PDB code 2GPH, reviewed in Reference 23] or on separate domains or subunits as in the Cdk2/cyclin complexes (PDB codes 1H24, 1H26, 1H27, and 1H28) (24) or in Polo-like kinase 1 [(Plk1)

Table 1 Some protein kinases and their preferred substrate specificities

Name	Consensus sequence[a,b]
Serine and threonine kinases (abbreviation, if any)	
Cyclic AMP-dependent kinase (PKA)	-R-R-X-**S/T**-Φ
Protein kinase B (PKB) (Akt)	R-X-X-R-X-X-**S/T**-Φ
Phosphorylase kinase (PhK)	-R-X-X-**S/T**-Φ-R
Cyclin-dependent protein kinase 2 (Cdk2)	-**S/T**-P-X-K/R
Extracellular-regulated kinase 2 (ERK2)	-P-X-**S/T**-P
Polo-like kinase 1 (Plk1)[c]	-D/E/N-X-**S/T**-(Φ//not P)
Aurora B[c]	-R-R/K-**S/T**-(not P)
Tyrosine kinases (abbreviation)	
Insulin receptor kinase (Irk)	-D-**Y**-M-M
Cellular form of the Rous sarcoma virus transforming agent (c-Src)	-E-E-I-**Y**-X-X-F
C-terminal Src kinase (Csk)	-I-**Y**-M-F-F
Epidermal growth factor receptor kinase (EGFRK)	-E-E-E-**Y**-F

[a]The phosphorylated serine, threonine, and tyrosine residues are indicated in bold.
[b]Φ is a hydrophobic residue.
[c]Some kinases (e.g., Plk1 or Aurora B) discriminate against proline in the P+1 site (136).

PDB code 1Q4K], which has an N-terminal Polo box domain, which recognizes substrates that have been phosphorylated by Cdk1/cyclin B to dock the kinase on its substrate (25).

There are relatively few structural studies on protein kinases in complex with their protein substrate (**Table 2**). These studies have been achieved by crystallization in the presence of an inactive ATP analog or in PKA with an inhibitor peptide in which the residue to be phosphorylated has been substituted by alanine. The affinity of protein kinases for peptide substrates is weak (typically 2×10^{-4} M), requiring millimolar concentrations to saturate the kinase in crystallization studies, and this may partly explain why so few kinase/peptide substrate complexes have been cocrystallized. A comparison of recognition sites indicates that discrimination for serine/threonine or tyrosine kinases (**Figure 3**) is mostly achieved by a subelement of the activation segment (13). An inward orientation of the activation segment toward the catalytic site is observed for serine/threonine kinases, whereas an outward facing orientation, allowing a larger residue, is observed for tyrosine kinases.

Table 2 Protein kinase/peptide substrate complex structures

Protein kinase	Substrate[a]	References
PKA	Inhibitor peptide fragment 5–24	137, 138
PhK	R-Q-M-**S**-F-R-L	22
pCdk2/cyclin A	H-H-A-**S**-P-R-K	139
PKB	G-R-P-R-T-T-**S**-F-A-E	56
Irk	G-D-**Y**-M-N-M	71

[a]The phosphorylated serine, threonine, and tyrosine residues are indicated in bold.

There are three structures where the kinase has been cocrystallized with its intact protein substrate: ROCK-1 kinase with RhoE (PDB code 2V55) (26), RNA-dependent protein kinase (PKR) in complex with the α -subunit of the translation initiation factor eIF2 (PDB code 2A1A) (27), and C-terminal Src kinase in complex with the authentic, endogenous form of the Src kinase, c-Src (PDB code 3D7T) (**Figure 4**) (28). In these structures, the phospho-acceptor region was disordered, and so its position at the kinase catalytic site could not be determined. However, the location of the major part of the protein substrate was evident, and in the complexes for PKR and ROCK-1, despite quite different molecular partners, the protein substrate was located at

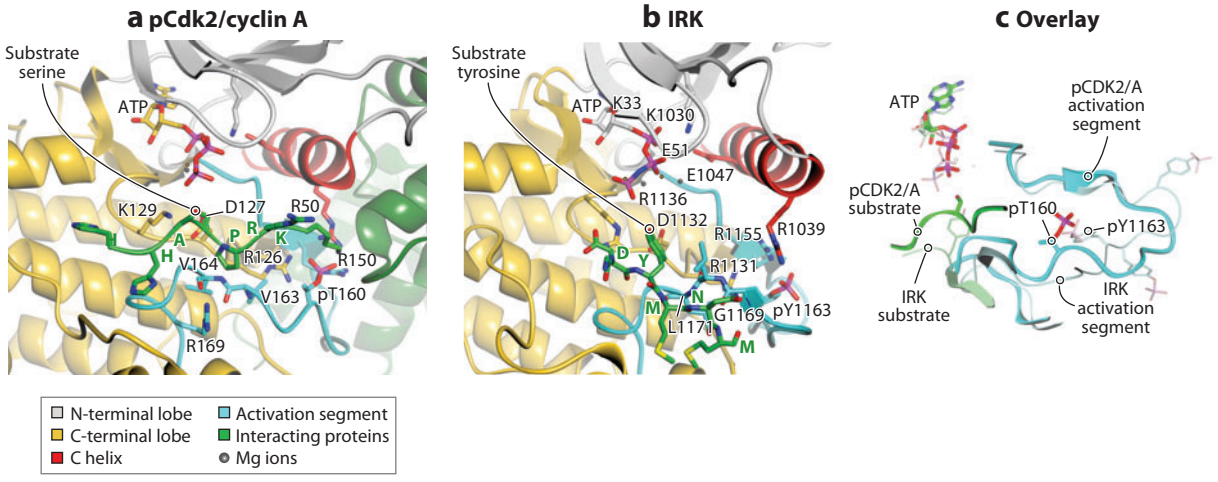


Figure 3

Protein kinase peptide substrates. (*a*) Human phospho-cyclin-dependent protein kinase 2 (pCdk2)/cyclin A in complex with the peptide substrate (HHASRK) in green. The catalytic aspartate, D127, is in hydrogen bond distance of the substrate serine hydroxyl, and K129 is poised to assist catalysis. The substrate is positioned by the docking of the adjacent proline of the serine-proline motif into a hydrophobic pocket created by a left-handed conformation of the activation segment residues V163 and V164, which is stabilized by R169. The residues interacting with phospho-T160 (pT160), which acts as an organizing center, are shown (PDB code 1QMZ). (*b*) Human insulin receptor kinase (IRK) in complex with the substrate peptide (GDYMNM) in green. The catalytic aspartate, D1132, is in hydrogen bond distance of the substrate tyrosine hydroxyl, and R1136 is poised to assist catalysis. The substrate is positioned by a short stretch of β-sheet between residues G1169 and L1171 of the IRK activation segment and the substrate. pY1163 acts as an organizing center. The γ-phosphate of ATP is misaligned for catalysis in this complex (PDB code 1IR3). (*c*) Overlay of the peptide-substrate complexes of pCdk2/cyclin A and IRK. For clarity, only the activation segment, peptide substrate, and ATP molecules are shown. The standard color scheme has been applied but structural elements of IRK are shown with decreased color saturation and with thinner bonds and ribbons.

a similar docking region comprising the kinase αG helix and part of the αF helix (**Figure 4*a*,*b***). Docking at this site placed the likely positions of the phospho-acceptor sites within reach of the kinase catalytic site. This docking region of the kinase is also used to locate the regulatory R subunit of PKA, which allows the R subunit to engage its inhibitory segment at the catalytic site of PKA (PDB code 1U7E) (29), and a similar region on phospho-Cdk2 is used to position the protein phosphatase KAP to allow the KAP catalytic site to reach phospho-T160 of Cdk2 (PDB code 1FQ1) (30).

The location of a substrate through a secondary, remote binding site allows the kinase to phosphorylate sites with suboptimal local sequences, in some cases increasing the apparent affinity for substrate by 1,000-fold (22, 27). The remote docking site allows a stable association, whereas the presence of the substrate at the catalytic site is transient. A recent study of the PKR-eIF2α complex suggests that substrate docking might fulfill a more active role in promoting kinase selectivity and prevent promiscuous substrate phosphorylation (31). Using mutant proteins designed following a comparison of the free and PKR-bound eIF2α structures, a model was proposed in which eIF2α binding to PKR elicits a conformational change in eIF2α that alters the accessibility and mobility of the PKR-phosphorylated residue (S51) enhancing its phosphorylation.

Kinase Catalysis

The phosphoryl transfer step is chemically simple and is dependent on the correct orientation of the two substrates, the γ-phosphate of ATP and the hydroxyl group of the serine, threonine, or tyrosine residue to be phosphorylated. The

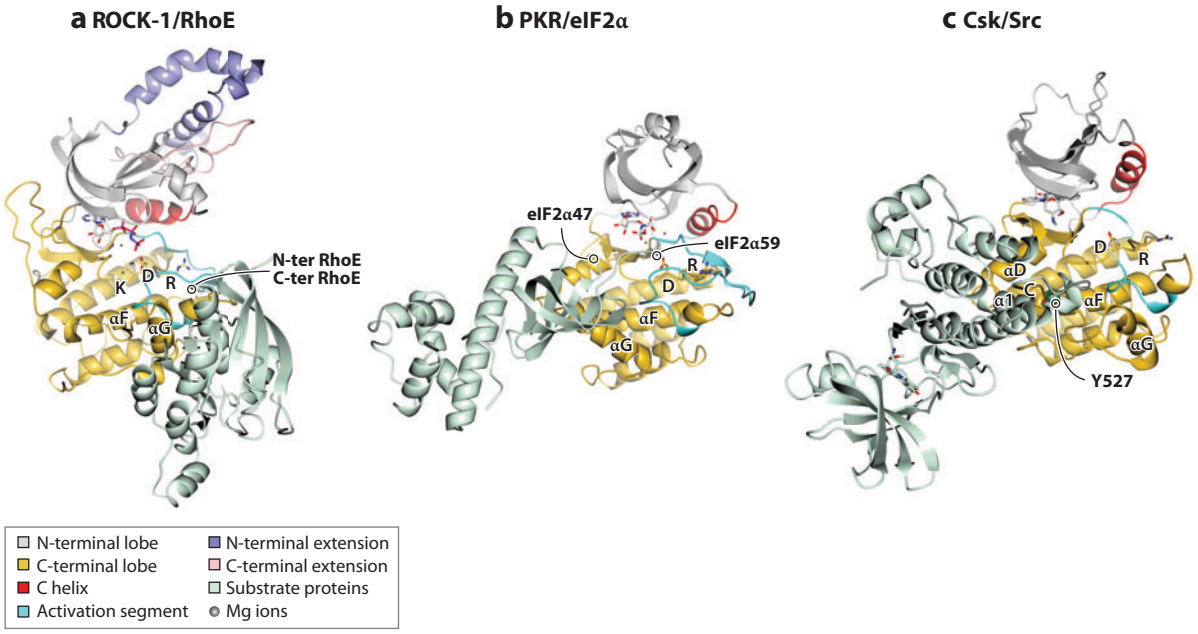


Figure 4

Protein kinase/protein substrate complexes. The ATP molecules and ATP-competitive inhibitors are shown bound, and the residues R and D mark the catalytic site, where the D is the catalytic aspartate. (*a*) ROCK-1 kinase in complex with RhoE substrate. The beginnings of the N-terminal (N-ter) and C-terminal (C-ter) regions of RhoE are marked. These regions contain the two and five ROCK-1 phosphorylation sites, respectively. They are disordered, but it is possible for them to reach the catalytic site some 20 Å away (PDB code 2V55). (*b*) RNA-dependent protein kinase (PKR) in complex with eukaryotic translation initiation factor 2α (eIF2α). The view is rotated ~15° about the vertical axis from the standard view for clarity and shows the docking of the β-sheet of eIF2α against the PKR G helix. S51, the site of phosphorylation on eIF2α, is in a disordered region between residues 47–59. However, it is positioned close enough to reach the catalytic site (PDB code 2A1A). (*c*) The kinase domains of C-terminal Src kinase (Csk) and Src in complex. Csk uses part of its D helix (αD) to bind Src through the region between helices H and I. The Csk activation segment is disordered. Src Y527, the site of phosphorylation, is 12 Å from the Csk catalytic site but could reach the site through conformational change. Staurosporine, a nonspecific kinase inhibitor, is bound at the ATP-binding sites of Csk and Src (PDB code 3D7T). The helices (αD–αG) within the C-terminal lobe are labeled according to the nomenclature originally used to describe the protein kinase A structure (PDB code 1ATP).

rate (k_{cat}/K_M) of a kinase-catalyzed transfer of phosphate to a serine residue is fast compared with the uncatalyzed reaction. For example, the phosphorylase kinase action on glycogen phosphorylase has $k_{cat} = 28\ s^{-1}$ and K_M (ATP) $= 7.0 \times 10^{-5}$ M, giving $k_{cat}/K_M = 4 \times 10^5\ s^{-1}M^{-1}$ (32). The rate of the uncatalyzed methanolysis of ATP^{2-}.Mg to methyl phosphate has $k_{cat}/K_M = 3.8 \times 10^{-9}\ s^{-1}M^{-1}$ (33). The enzyme catalyzed reaction demonstrates an extraordinary enhancement of $\sim 10^{14}$.

Kinetic and catalytic mechanisms of protein kinases were reviewed in detail in 2001 (34). Kinetic studies with ^{32}P-labeled ATP or radiolabeled peptide substrate with PKA indicate that both substrates have unrestricted access to the catalytic site and the binding of one does not exclude the other, although at high ATP concentrations, which are typical in the cell, there is a preference for ATP binding first (35). This is consistent with the arrangement of the ATP and substrate sites observed in the crystal structures, where the ATP site is partially shielded by the substrate peptide. With larger natural protein substrates, the ATP site could be even more shielded, and indeed, several kinases have

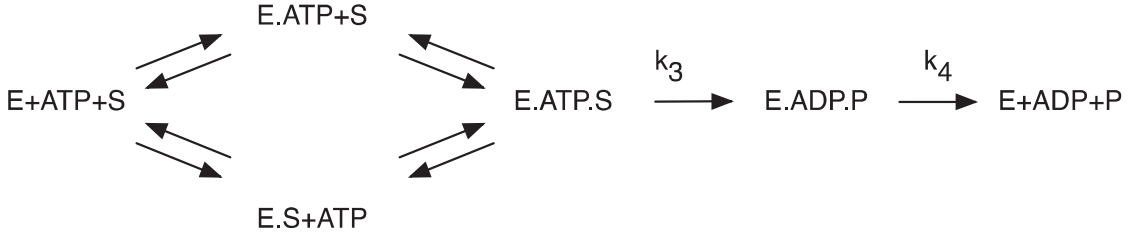
now been shown to demonstrate an ordered catalytic mechanism.

The kinase reaction proceeds with an in-line mechanism in which the attacking group (serine, threonine, or tyrosine OH) comes in opposite to the leaving group (phosphate ester oxygen), leading to inversion of configuration at the phosphorus. This geometry was supported by structural studies with PKA cocrystallized with a putative transition state analog (PDB code 1L3R) (36). One of the roles proposed for the bound magnesium ions is to stabilize the significant amount of negative charge that develops on the bridging oxygen as the reaction proceeds and thereby to aid departure of the leaving group, ADP (34). The transition state for the intermediate could be either dissociative or associative, as reviewed recently for phosphoryl transfer mechanisms for nonenzymatic and enzymatic reactions (37). In the dissociative mechanism, the reaction proceeds through a metaphosphate intermediate in which the bond to the leaving group is broken before the bond by the attacking group is made. In the associative mechanism, the reaction proceeds through a phosphorane pentavalent intermediate in which bond making occurs in advance or at the same time as bond breaking. Structural evidence from pCdk2/cyclin A crystallized with a putative transition state analog [PDB codes 3QHR, 3QHW (38), and 1GY3 (39)] supported the notion of the dissociative mechanism, whereas studies with PKA indicated that there may also be a small percentage (11%) of an associative mechanism (PDB code 1L3R) (36).

We may simplify the steps in the reaction as shown in Scheme 1 below:

where *E* represents the kinase, *S* and *P* are the protein substrate and product, respectively; k_3 is the rate constant for the catalytic step of the phosphoryl transfer; and k_4 is the rate constant for the dissociation of products. Kinetic studies in solvents of different viscosity that allowed binding and dissociation events to be distinguished from chemical catalytic steps showed that the catalytic step was fast ($k_3 \sim 300–500\ s^{-1}$), and the release of products relatively slow ($k_4 \sim 20–30\ s^{-1}$) (32, 35, 40, 41). Such measurements require careful interpretation and need to take account of possible conformational changes. In summary, once the substrates have been correctly oriented, the kinase chemical step is easy, and the rate-limiting step is the release of products, i.e., ADP and phosphorylated proteins.

A simplified representation of a kinase mechanism is shown in **Figure 5**. Kinase-catalyzed phosphoryl transfer can be envisaged as comprising three major steps: orientation of the substrates; nucleophilic attack by the substrate hydroxyl group, followed by general base catalysis from the catalytic aspartate; and subsequent general acid catalysis for the transfer of the proton (32). Support for the notion that deprotonation of the nucleophile by a catalytic base in the early stages of the reaction is not a rate-limiting step came from studies with the tyrosine kinase C-terminal Src kinase (42). A peptide containing the unnatural amino acid trifluorotyrosine showed similar efficiency as a substrate compared with the corresponding tyrosine-containing peptide despite a four-unit change in the phenolic pK_a. However, a residue with the ability to develop a negative charge at the correct separation is



Scheme 1

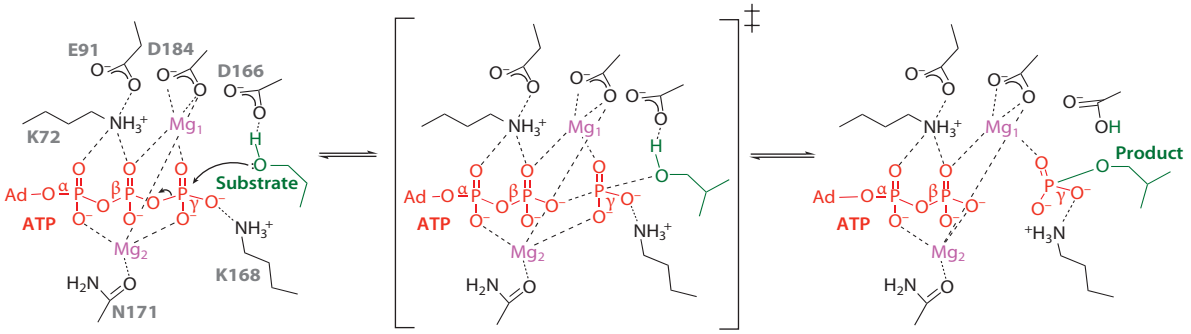


Figure 5

Schematic diagram of protein kinase catalytic mechanism. The reaction proceeds from the enzyme/substrate complex through the transition state (*center*) to the enzyme/product complex. Residue numbers for protein kinase A (PKA) are shown in the left panel. The OH group of the protein substrate is aligned so that the lone pair of electrons on the oxygen are directed in-line through the γ-phosphorus atom to the βγ-bridging oxygen of the bound ATP. The transition state involves a metaphosphate intermediate in which the bond breaking of the βγ-bridging oxygen of ATP is well advanced, while the incoming nucleophile bond making to the phosphorus is only just beginning. The negative charge on the γ-phosphate is compensated by the Mg ions and nearby lysine residue. As the reaction proceeds, the acidity of the substrate hydroxyl group increases, and its pK_a will become lower than the pK_a of the nearby catalytic aspartate, thus allowing transfer of a proton from the hydroxyl (normal pK_a ~12) to the aspartate (normal pK_a ~ 4.5). This proton is probably eventually transferred to the phosphate dianion of the product restoring the catalytic site aspartate to the carboxylate state. Abbreviations: Ad, adenosine; NH_3^+, represents the charged alternative form of the epsilon amino group of the side chain of K72 (labeled in the LHS panel).

important because mutation of the catalytic aspartate to asparagine, alanine, or glutamate results in a reduction of the k_{cat} by ~10^4 but little change in the K_M (32, 42).

MECHANISMS OF PROTEIN KINASE ACTIVATION

Activation by Accessory Proteins or Domains

The protein kinase fold is pliable and may be manipulated to an active or inactive conformation by extra domains or separate subunits. The cell cycle kinase Cdk2 is dependent on the association with a cyclin subunit for activity. The cyclin associates in the region of the C helix and promotes a rotation approximately about the axis of the helix (PDB code 1FIN) (43) so that an isoleucine from the PSTAIRE motif at the start of the C helix is buried in a hydrophobic pocket on the cyclin, and the glutamate of this motif contacts the β3 lysine to create an ion pair that is part of the ATP-binding site. In this conformation, the Cdk C helix hydrophobic residues are shielded by cyclin binding. In parallel with the shift of the C helix, a movement of the activation segment, starting at the DFG motif, takes the activation segment out of the catalytic site so that the threonine becomes accessible for phosphorylation and the aspartate of the DFG shifts to an internal site where it chelates the Mg^{2+} ion for ATP binding (**Figure 6*a***). As an example of a constitutively active kinase, the PhK catalytic subunit in isolation possesses a canonical amphipathic C helix that requires no further interactions to adopt the active conformation (22).

In contrast, the kinase domain of Src is held with the C helix in an inactive conformation by restraining interactions with its SH2 and SH3 domains that pack on the opposite side of the kinase and are not in direct contact with the C helix (PDB code 1FMK) (44, 45). When these restraints are removed, either through the SH2 and SH3 domains docking to recognition proteins or by phosphatase-mediated hydrolysis of the phosphorylated tyrosine that forms the SH2-docking site, the kinase is able to relax to its active conformation of the C helix (PDB code 1Y57) (**Figure 6*b***) (46).

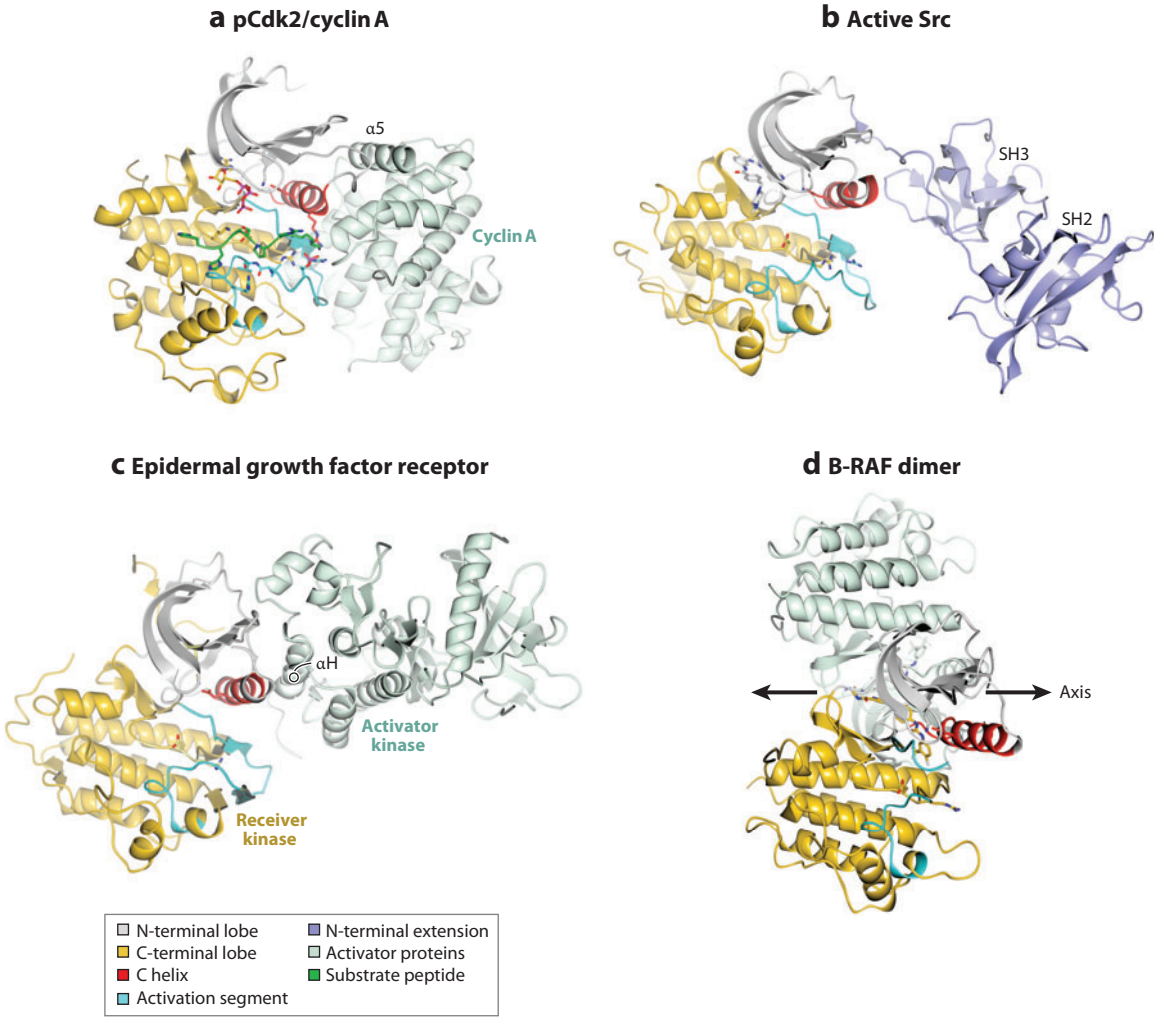


Figure 6

Stabilizing interactions for C helix conformations. (*a*) Human phospho-cyclin-dependent protein kinase 2 (pCdk2)/cyclin A. The cyclin H5 helix docks against the C helix to promote the active conformation (PDB code 1QMZ). (*b*) Human Src kinase in the active conformation. The SH2 and SH3 domains are liberated and no longer restrain the kinase in the inactive conformation (PDB code 1Y57), and may stabilize an active C-helix conformation. (*c*) Human epidermal growth factor receptor kinase. The receiver kinase (left) is activated by interaction between its C helix and the activator kinase H helix (*right*) (PDB code 2GS2). (*d*) Human B-RAF side-by-side dimer where the two monomers interact about a twofold axis (marked by the *double-headed arrow*) to promote an active conformation (PDB code 1UWH). Abbreviation: α5, the fifth α-helix of the N-terminal cyclin box fold of cyclin A2; αH, an α-helix structurally equivalent to the eighth helix of cyclic AMP-dependent protein kinase.

Kuriyan and colleagues (20) have illustrated the recurring theme of regions engaging the C helix hydrophobic patch to activate kinases. In PKA (**Figures 1** and **2*a***) (47) and extracellular signal-regulated kinase 2 (Erk2) (PDB code 1ERK) (48), C-terminal helical extensions wrap around the C helix; while in the p21-activated kinase 1 (PDB codes 1YHV and 1YHW) (49), an N-terminal helix performs this role; and in the protein kinases RET (PDB codes 2IVS and 2IVT) (50) and c-Kit (PDB code 1PKG) (51), an N-terminal region from the end of the

juxtamembrane region shields the C helix. In the TGFβ receptor kinase, part of the juxtamembrane sequence, known as the GS region, is held against the C helix in an inactive state by FKBP12, a small immunophilin protein. Inhibition is relieved by phosphorylation of the GS region creating a docking site for the Smad substrate (PDB code 1IAS) (52). The tyrosine kinase Fes utilizes its own SH2 domain to promote the active conformation of the Fes C helix (PDB code 3BKB) (53).

Among examples of stabilization of the C helix hydrophobic surface in *trans*, Aurora A kinase associates with the mitotic spindle assembly protein TPX2 to achieve activation (PDB code 1OL5) (54). TPX2 is located in a groove and interacts with the C helix in a similar manner to the flanking regions of PKA.

AGC kinases. The AGC group of kinases (7) comprises 60 members, including PKA (reviewed in Reference 55). For many of these kinases, activation involves phosphorylation not only on the activation segment but also on a hydrophobic motif (HM) located toward the C terminus with consensus sequence FXXF(S/T/D)Y. Phosphorylation of the HM promotes the intramolecular association of this region with an N-terminal lobe groove, where the extensive hydrophobic interactions from the two phenylalanines promote the active conformation of the C helix and lead to activation. Protein kinase B (PKB/Akt) is activated by phosphorylation on a serine residue (S473) in the HM by the kinase mammalian target of rapamycin complex 2 and by phosphorylation on the activation segment catalyzed by 3-phosphoinositide-dependent kinase-1 (PDK1) when both are colocalized at the plasma membrane (56). In PKA, the chain ends at the second phenylalanine and does not have the residue that is phosphorylated. However, its C-terminal region still folds against the N-terminal lobe to dock the two phenylalanines into a hydrophobic pocket in an identical manner to that achieved by those kinases activated by HM phosphorylation (**Figures 1** and **2*a***). Similar interactions occur within protein kinase C, where the HM is phosphorylated (PDB code 2I0E) (57), and within ROCK, where like PKA the HM is not phosphorylated (PDB code 2F2U) (58).

PDK1 is a key member of the AGC family and phosphorylates several AGC kinases on their activation segment. PDK1 autophosphorylates on the activation segment and is constitutively active, but activity for substrates is augmented by recognition through the phosphorylated HM motif. PDK1 does not possess an HM in its C-terminal region, but it does have the hydrophobic pocket in the N-terminal lobe (PDB code 1H1W) (59). Kinases such as S6K, SGK, and RSK are recognized by PDK1 through binding of their phosphorylated HM motifs to the PDK1 hydrophobic pocket.

AMP-activated kinase. AMP-activated kinase (AMPK) plays a homeostatic role in mammalian cells to maintain ATP levels by phosphorylating and inactivating acetyl-coenzyme A (CoA) carboxylase and HMG-CoA reductase, two rate-limiting enzymes in fatty acid and cholesterol biosynthesis, respectively (reviewed in Reference 60). It is a complex of three proteins in which the α-subunit is the catalytic kinase and the β- and γ-subunits play regulatory roles. The α-subunit requires phosphorylation within the activation segment at T172, the residue equivalent to T197 in PKA for activity. The γ-subunit has four cystathionine β-synthase motifs (sites 1–4), and each contains a potential nucleotide-binding site (61–63). Comparative studies suggest that the nucleotide-binding preference (ATP versus ADP versus AMP), affinity, and functional consequences of ligand binding at each site vary both between sites and at any comparable site across species (64).

Significant insights into how nucleotide binding to the γ-subunit allosterically regulates the activity of the α-subunit have been revealed by the determination of the structure of the regulatory core of the trimeric complex (composed of the γ-subunit and C-terminal fragments of the α- and β-subunits) bound to the kinase domain in which the activation segment is ordered as a result of phosphorylation on T172 (PDB code 2Y94) (**Figure 7**)

(65). First, the activation segment interacts with the C-terminal regions of the α- and β-subunits. As a result, the activation segment is constrained and not available to protein phosphatases, whose activity determines to a large extent the amount of phosphorylated AMPK (66). In at least one instance, the structure has shown that phosphatases require a mobile activation segment to dephosphorylate (30). Second, within the α-subunit, a motif known as the "α-hook" from the C-terminal extension regulatory segment docks into site 3 of the γ-subunit. Supported by both mutagenesis data and a comparison of this structure with previously determined structures of the γ-subunit bound to either ADP or Mg.ATP, the authors propose a switch model to explain the regulation of AMPK activity by nucleotides. In this model, the proportion of ADP and AMP bound within the γ-subunit at regulatory site 3 is read out by interactions of this site with the α-hook. Exchange of ADP or AMP by Mg.ATP leads to a steric clash with the α-hook, resulting in the dissociation of the hook and an increase in the flexibility of the linker to the kinase domain. In this form, the activation loop is no longer protected (see also Reference 67 for a supporting electron microscopy study).

AMPK and the AMPK-related protein kinases encode an additional regulatory region within the α-subunit, termed the autoinhibitory domain (AID) sequence C-terminal to the catalytic domain (residues 289–338 in human AMPK), which is largely unstructured in the above structure (**Figure 7**). However, in the structure of a *Schizosaccharomyces pombe* truncated α-subunit encoding the unphosphorylated kinase domain and the AID, the AID sequence is ordered into a three α-helical bundle with structural homology to the ubiquitin-associated (UBA) domain fold (PDB code 3H4J) (68). The AID binds to the kinase domain via helices αC and αE and the hinge sequence. This structure suggests that ordering of the AID sequence in the unphosphorylated AMPK structure could assist in maintaining the enzyme in its inactive state by stabilizing displacement of the C helix and possibly by eliciting more global changes to the kinase fold (68). How potential structural rearrangements within the AID sequence communicate with those that mediate allosteric regulation of the kinase domain by nucleotide binding to the regulatory core of the trimeric complex remains to be determined.

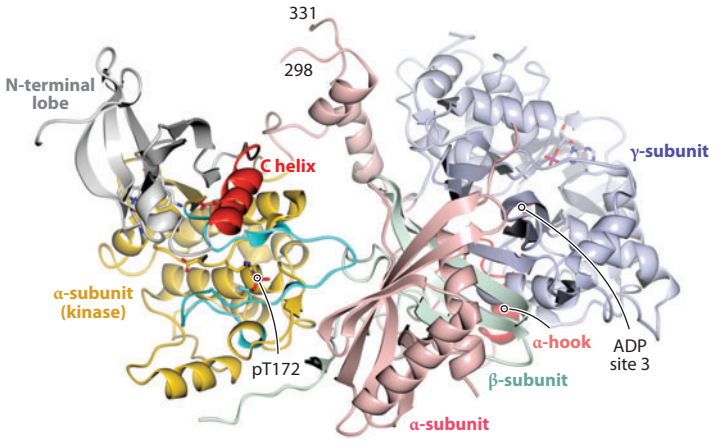


Figure 7

AMP-activated kinase (AMPK) regulation through activation segment contacts. The mammalian AMPK structure with the kinase (α-subunit) in the standard conformation showing phosphothreonine (pT) 172 on the activation segment and the glutamate/lysine pair within the ATP site, which also contains the inhibitor staurosporine. The C-terminal extension of the α-subunit (*pink*) interacts with the β-subunit (*pale green*) and the γ-subunit (*violet*), which contains the AMP/ADP activatory nucleotide-binding sites. The α-hook is in proximity to ADP bound at site 3 of the γ-subunit. The autoinhibitory region between residues 299–330 is disordered (PDB code 2Y94).

Dimerization and Activation

Many protein kinases dimerize as part of their activation mechanism, and dimerization can be regarded as a special case of kinase activation by accessory proteins or domains. In such cases, either both partners are activated by reciprocal phosphorylation or one partner (the activator kinase) activates the other (the receiver kinase) through an allosteric mechanism. Though activation subsequent to dimerization was first observed in members of the receptor tyrosine kinase subfamily, a number of examples of serine/threonine kinases that employ this mechanism have also been reported.

Binding of a ligand to the extracellular portion of a receptor tyrosine kinase results in activation of the protein kinase domain and subsequent downstream signaling. Almost exclusively, this requires receptor dimerization to permit phosphorylation and activation in *trans* (reviewed in References 12 and 69). The receptor complexes can be either preexisting, as is the case for the receptors for insulin and insulin-like growth factor 1, or assembled as a result of bivalent ligand binding (for example, the KIT and VEGF receptors). An inhibitory sequence is removed from the active site to permit rearrangement of the activation segment to form the peptide substrate-binding site. The inhibitory sequence can originate from different locations within the protein sequence, and subsequent to its removal, the structure of the rearranged activation segment is frequently secured by phosphorylation on a conserved threonine or tyrosine residue equivalent to T197 in PKA.

The receptor tyrosine kinases. Structures of the insulin receptor tyrosine kinase (IRK) domain were the first to delineate this mechanism of inhibition (PDB codes 1IRK, 1IR3) (70, 71). In this receptor, the inhibitory sequence originates from the activation segment. It contains three tyrosine residues and, in its inactive state, one (Y1162) occludes the ATP-binding site in *cis* by mimicking, in part, the interactions of the adenine ring of the ATP substrate. The resulting conformation of the activation segment also precludes protein substrate binding. Activation promotes autophosphorylation of the three activation segment tyrosine residues in *trans*, disrupting the inhibitory network of interactions within the ATP-binding site and stabilizing the activation segment in its peptide-binding conformation. Similar, but distinct, mechanisms involving the activation segment are also employed to inhibit the kinase domain of FGFR1 (PDB code 1CVS) (72) and the muscle-specific kinase (PDB domain 1LUF) (73). In the latter case, subsequent activation in *trans* is promoted by binding of the adaptor protein Dok7 (PDB code 3ML4) (74).

Subsequent structures of the inhibited forms of the intracellular domains of KIT (PDB code 1PKG) (51), EphB2 (PDB code 1JPA) (75), FLT3 (PDB code 1RJB) (76), and Tie2 (PDB code 1FVR) (77) revealed how their active sites could also be blocked by the binding of peptides derived from sequences outside the catalytic domain, the juxtamembrane region in the cases of KIT, Ephb2, and FLT3, and the C-terminal tail in the Tie2 structure (reviewed in Reference 78). Frequently, as mentioned above, additional sequences engage the C helix to ensure maintenance of the "off" state.

Serine/threonine protein kinases. Within the serine/threonine protein kinase family, dimerization-dependent phosphorylation of the activation segment in *trans* to promote kinase activation has also been reported, and a general molecular model has been proposed (reviewed in Reference 79). Studies of checkpoint kinase 2 (Chk2) generated the first structure and data in support of this model (PDB code 2CN5) (80, 81), but subsequent structure determinations of death-associated protein kinase 3 (PDB code 2J90), lymphocyte-originated kinase (PDB code 2J7T), and STE20-like kinase (PDB code 2J51) provided further support (82). Chk2 dimerizes after phosphorylation of T68 in the N-terminal serine-glutamine/threonine-glutamine cluster domain by the protein kinase Ataxia-telangiectasia mutated, which generates a ligand for the central fork head–associated domain of a second Chk2 molecule (reviewed in Reference 83). The crystal structure of the Chk2 kinase domain revealed an intimate dimer in which the activation segments exchange, forming interactions across the interface such that each sequence completes the active site of the reciprocal molecule by adopting the activation segment structure present in active kinase structures (**Figure 8*a***). It was proposed that small rearrangements within the activation segment could bring its two phosphorylated residues (T383 and T387) into a position in which phosphotransfer from the ATP bound to the other Chk2 molecule could occur.

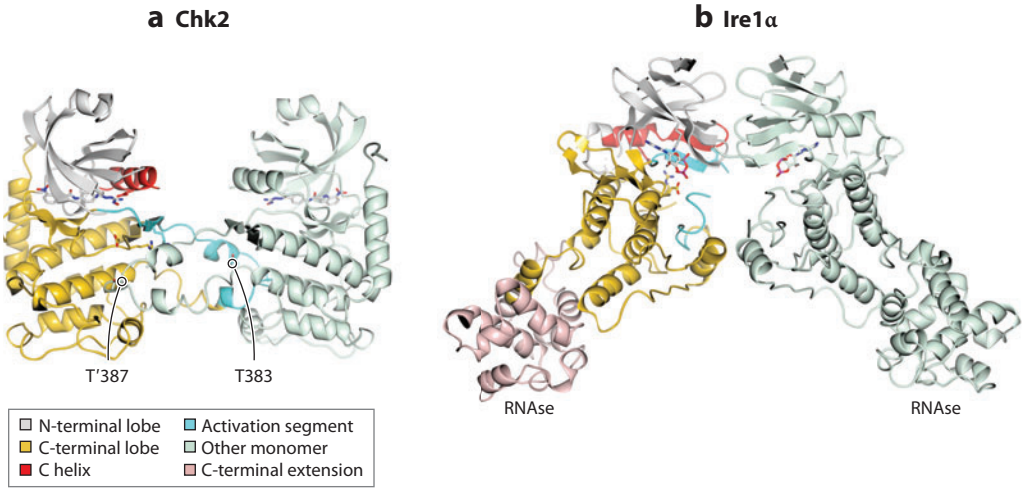


Figure 8

Dimerization through face-to-face activation segment exchange. (*a*) Human checkpoint kinase 2 in the presence of the inhibitor Pv1533. The sites of phosphorylation on the activation segment are T383 and T387. T383 is shown for one subunit and T'387 for the other subunit (PDB code 2XK9). (*b*) Human Ire1α in the presence of Mg.ADP in which the activation segments are disordered but are directed to the catalytic site of the other monomer for phosphorylation of S724 in the activation segment (PDB code 3P23).

Mechanistically, dimerization plays a role that is similar to a remote substrate-docking site in that, by optimally positioning the substrate, kinase-substrate affinity is enhanced, and phosphorylation at noncanonical sites is promoted.

A model for the mechanism of activation of Ire1 through dimerization has also recently been proposed on the basis of a series of Ire1 structures. Ire1 is a transmembrane serine/threonine kinase that is essential for the endoplasmic reticulum (ER) unfolded protein response (reviewed in Reference 84). The association of its luminal domain with the ER Hsp70 protein Bip maintains it in an inactive, monomeric state. However, accumulation of misfolded proteins in the ER activates the unfolded protein response, leading to disengagement of Bip from Ire1 and permitting Ire1 dimerization. A structure of an N-terminally truncated human Ire1α fragment encoding both the unphosphorylated kinase domain bound to Mg.ADP and the ribonuclease domain revealed a face-to-face orientation of the kinase domains (**Figure 8*b***) (PDB code 3P23) (85). This orientation is predicted to promote autophosphorylation and kinase activation in *trans*. Structures of the yeast Ire1 cytoplasmic domain in which the kinase domain is phosphorylated and as a result the RNase domain is proposed to be in its active conformation have revealed an alternative Ire1 dimeric structure in which the kinase domains are arranged back-to-back (PDB codes 2RIO, 3FBV, and 3LJ0) (86–88). Taken together, the structures could be reconciled to an Ire1 activation model in which engagement of the Ire1 ER luminal domains would promote kinase autophosphorylation in *trans*, rearranging the kinase domain into a catalytically active conformation that would then engender substantial rearrangement of the Ire1 dimer to its RNAase-active back-to-back form. Ire1 has been identified as a potential drug target to treat inflammation (84), and a detailed mechanistic understanding of Ire1 activation is required if it is going to be targeted effectively for therapy (3, 89).

The epidermal growth factor receptor kinase family. The EGFR kinase is unusual among the family of receptor tyrosine kinases in that it does not require phoshorylation of

Table 3 Key motifs that are not conserved in selected human pseudokinases[a]

Kinase	Glycine-rich loop[b]	β3 lysine	Catalytic aspartate, lysine, and magnesium-binding asparagine	Magnesium-binding activation segment aspartate-phenylalanine-glycine (DFG) motif	Crystal structure
Conventional active kinases					
PKA (for example)	**G**TG**S**F**G**	YAM**K**	YR**D**L**K**PE**N**	**D**FG	137, 138
Unconventional active kinases					
WNK1	**GRGS**FK	VAWC	HR**D**L**K**CD**N**	**D**LG	114
Titin	**GRGE**F**G**	YMA**K**	HF**D**IRPE**N**	EFG	115
CASK	**GKGP**FS	FAV**K**	HR**D**V**K**PHC	GFG	116
Kinases that regulate other kinases					
KSR1	**GQGRWG**	VAIR	HK**D**L**K**SK**N**	**D**FG	108
Her3	**GSGV**F**G**	VCI**K**	HRNLAAR**N**	**D**FG	92
STRADα	**GKGF**ED	VIVR	HRSV**K**ASH	GLR	118
JAK JH2	**GRGT**RI	VIL**K**	HGNVCTK**N**	**D**PG	
Scaffold proteins					
ILK	NENHS**G**	IVV**K**	RHALNSRS	**D**VK	123
VRK3	TRDNQ**G**	FSL**K**	HGNVTAE**N**	GFG	125

[a]Adapted from Reference 125.
[b]Key residues are in bold when conserved and underlined when not conserved.

the activation segment for full activity and its juxtamembrane sequence is required to activate, rather than to inhibit, the kinase domain. An elegant series of structures have delineated the role played by receptor dimerization, augmented by sequences outside the catalytic domain in regulating EGFR activity (reviewed in References 20 and 90). The EGFR family consists of four members, EGFR (ErbB1), HER2 (ErbB2, HER2/neu), HER3 (ErbB3), and HER4 (ErbB4), that can form both homo- and heterodimers. Notably, HER3 is regarded as pseudokinase as the key catalytic residues equivalent to E91 (in the C helix) and D166 (the proposed catalytic base) in PKA are replaced by a histidine and asparagine, respectively (**Table 3**). However, HER3 has been reported to autophosphorylate (91) and can act as an activator kinase when paired with a catalytically competent receiver kinase (92).

A comparison of the crystal structure of the inactive EGFR kinase domain (PDB code 3GOP) (93, 94) with the active structures of EGFR (PDB code 1M14) (95), HER2 (PDB code 3PP0) (96), and HER4 (PDB code 3BCE) (97) has revealed a conserved mechanism of allosteric activation in which one molecule (the receiver kinase) is remodeled and activated following formation of an asymmetric dimer with a second activator kinase (**Figure 6*c***) (98, 99). The conformational changes that accompany activation within the activated monomer and the character and location of the interface within the dimer are both reminiscent of the mechanism of CDK activation by cyclin binding (reviewed in Reference 20). However, the dimer formed between the kinase domains is not stable, and at least in vitro dimer formation requires the cytoplasmic sequence between the membrane and the start of the kinase domain (93, 94). This sequence is required

for receptor activation, and its mechanism has been elucidated by structural studies. After the transmembrane helix, the chain forms a helix that dimerizes across a receptor pair, and the sequence originating from the receiver kinase (now termed the juxtamembrane latch) binds to the C-terminal lobe of the activating subunit to stabilize the active asymmetric kinase dimer (93, 94).

RAF kinases. A second example of where kinase dimerization plays a crucial role in kinase activation and in which it now appears one of the monomers plays a scaffolding role and need not have catalytic activity is provided by the cytoplasmic serine/threonine kinase, RAF, and its close relative kinase suppressor of Ras (KSR) (100, 101). RAF, together with mitogen-activated protein kinase kinase (MEK) and the ERKs, comprises one of the evolutionarily conserved MAPK pathways that collectively signal to regulate cell growth, differentiation, and survival (reviewed in Reference 102).

The B-RAF kinase domain bound to the RAF inhibitor BAY43-9006 adopts an inactive structure (PDB code 1UWH) (103). Although some catalytically important residues were properly positioned (most notably the N-terminal glycine-rich loop, the catalytic loop, the lysine equivalent to K72, and the glutamate on the C helix equivalent to E91 in PKA, respectively), the B-RAF structure required movement of the DFG motif and activation segment to adopt the active structure seen in other protein kinases. Subsequent crystal structures of the RAF catalytic domain consistently revealed the same side-to-side dimer structure present in the original B-RAF crystal lattice (PDB codes 3C4C, 3C4D, 3C4E, and 3C4F) (104), (PDB code 3D4Q) (105), and (PDB code 2FB8) (106). It was noted that this dimer is mediated by interactions between the kinase N-terminal lobes, and from this observation, it was predicted that this arrangement retains the C helix in a position to support catalysis (**Figure 6*d***) (107). Collectively, these structures suggested that activation of RAF might, like the EGFR kinase, be mediated by an allosteric mechanism in which one kinase subunit acts as a scaffold (the activating kinase) to stabilize the active conformation of the other (the receiving kinase). The functional relevance of the crystallographically observed dimer structure was supported by a mutational study showing that alterations to residues predicted to be at the B-RAF/KSR interface affected the ability of KSR to activate B-RAF. Further experiments demonstrated that activation of B-RAF through formation of a KSR heterodimer was greater than activation subsequent to B-RAF homodimer formation (107). This result suggested that the mechanism of B-RAF activation by KSR might be twofold, firstly by allosterically activating the B-RAF kinase domain and subsequently by acting as a scaffolding protein to promote association of B-RAF with MEK.

The details of this model have subsequently been refined by the determination of the structure of a KSR2 kinase domain [KSR2(KD)]/MEK1 heterodimer (PDB code 2Y4I) (108). Within the crystal lattice, two heterodimers associate to form a tetramer through adjacent KSR2(KD) molecules. The KSR2(KD) and MEK1 face each other in the heterodimer complex, generating an interface that is composed of residues from their respective activation segments and αG helices. As a result, the activation segments are mutually constrained, and the KSR subunit is in an inactive conformation with the C helix displaced. The KSR2(KD) homodimer, however, forms a side-to-side association that is reminiscent of but distinct from that previously observed in the B-RAF homodimer structures. Only a subset of the intersubunit contacts is common to both structures. As a result the quaternary arrangements of the two complexes are different. An analysis of the residue conservation at the observed interfaces and the overall structures predicts that a KSR2(KD)/B-RAF heterodimer would resemble the B-RAF homodimer structure [as proposed (107)] and would be compatible with the KSR2(KD) αC helix only when it is in an active conformation.

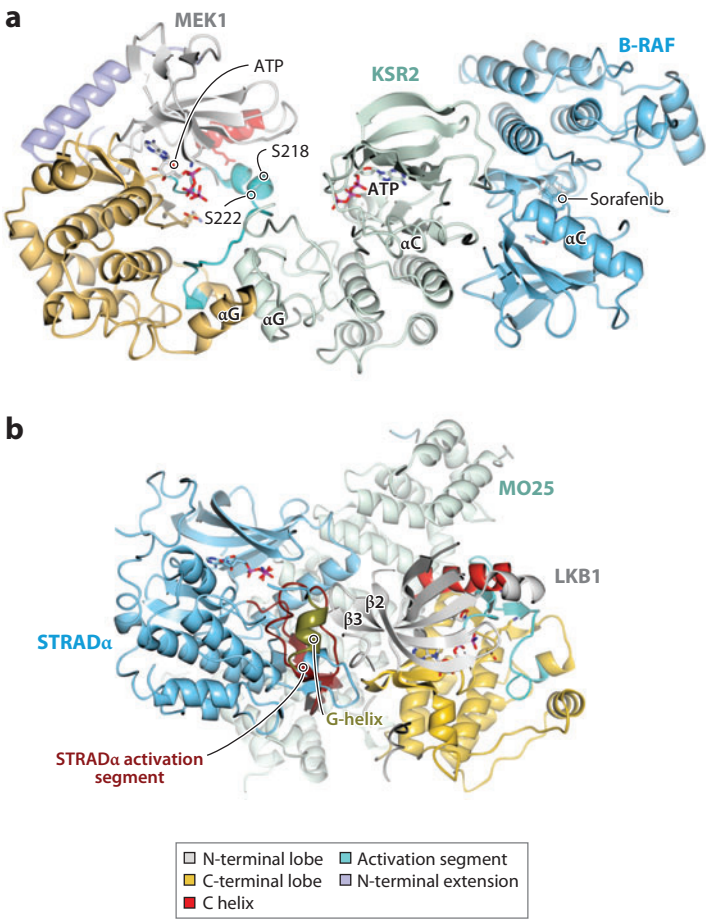


Figure 9

Pseudokinases exhibit different roles in the activation of their partner kinases. (*a*) The proposed trimeric assembly of human MEK1/KSR2/B-RAF kinases based on the structures of MEK1/KSR2 (PDB code 2Y4I) and dimeric B-RAF (PDB code 2UWH) in which MEK1, the target of regulation, is shown. A regulatory RAF is proposed to activate the pseudokinase KSR2 through the side-to-side dimer interface. In turn, KSR2 phosphorylates MEK1 in the N-terminal region through the face-to-face dimer interface. This results in a conformation in which an external catalytic RAF dimer phosphorylates MEK1 on the activation segment S218 and S222. (*b*) The human trimeric assembly of STRADα/MO25/LKB1. The pseudokinase STRADα (STE20-related adaptor kinase, *blue* in *standard orientation*) acts with MO25 (*pale green*) to activate the kinase LKB1 (*nonstandard view*). The STRADα activation segment (*dark red*), and the G helix (*olive green*) contact the β2-β3 loop and the C-terminal region of LKB1 while MO25 contacts the LKB1 C helix. Both STRADα and LKB1 have an active conformation, although STRADα has no catalytic activity (PDB code 2WTK).

Subsequent studies showed that addition of a B-RAF kinase-deficient mutant was able to promote KSR(KD)-dependent phosphorylation of MEK, supporting the model that the KSR(KD) does adopt an active conformation and that its observed low levels of activity are functionally significant within a physiological complex (108, 109). Taken together, the experimental data (107, 108) support a model in which the interactions between B-RAF, KSR(KD), and MEK in *cis* lead to an allosteric activation of KSR by B-RAF that promotes KSR phosphorylation of MEK, leading to a change in the accessibility of the MEK subunit to phosphorylation by an activating B-RAF molecule in *trans* (**Figure 9*a***). This chain of events results in MEK activation and subsequent downstream signaling. The importance of having a detailed mechanistic understanding of this pathway for the development of ATP-competitive inhibitors targeting B-RAF has been highlighted in recent studies (110, 111).

PSEUDOKINASES

Pseudokinases are defined by the lack of conservation of one or more of the catalytic site residues in the kinase core. The human phylogenetic kinome contains 48 pseudokinases distributed throughout the seven families (7, 112). These kinases are expressed, indicating that they are transcribed genes, but their function has been obscure. They are now recognized to be more than passive bystanders; some do exhibit activity, and others participate in signal transduction (reviewed in Reference 113). Some have been discussed above, including the roles of KSR in activation of RAF and HER3 as an activator of EGFR kinase.

The motifs that are changed in the pseudokinases include the glycine-rich loop, and the VAIK (β3 lysine), HRD (catalytic aspartate), and DFG motifs or combinations of these (**Table 3**) (112). The WNK [whose name is a contraction of with-no-K (Lys)] kinase lacks the K of the VAIK motif, but structural studies showed that a lysine residue in an adjacent region substituted for the missing lysine, and

the kinase is active (PDB code 3FPQ) (114). Similarly, in the giant protein titin that contains a kinase domain with DFG substituted by EFG, the structure is modulated so that the glutamate can perform a similar role to that of the aspartate (PDB code 1TKI) (115). Ca^{2+}/calmodulin-activated Ser/Thr kinase (CASK) catalyzes kinase activity in the absence of Mg^{2+}. The DFG motif is replaced by GFG, and CASK lacks the asparagine that contributes to Mg^{2+} binding at the catalytic site. Structural analysis showed that a histidine partially performs the role of neutralizing the charge on the phosphates of ATP (PDB code 3C0H) (116).

The LKB1/STRAD/MO25 complex demonstrates an activating role for a pseudokinase. The protein kinase LKB1 phosphorylates and activates AMPK and thereby couples the cell's function to energy supply (reviewed in Reference 117). LKB1 activity is regulated by the pseudokinase STE20-related adaptor kinase (STRADα) and the scaffolding protein MO25α through an allosteric mechanism. STRADα has a serine in place of the catalytic aspartate, the DFG motif is GLR, and the VAIK motif lysine is arginine (**Table 3**). Despite these alterations, STRADα can still bind ATP using the arginine in its GLR motif and a histidine to take the place of Mg^{2+}. STRADα presents an active kinase conformation when bound to ATP and to MO25α, where the scaffolding protein interacts with and orients the C helix, rather like cyclin in Cdk2 (see above). The heterotrimeric LKB1/STRADα/MO25α complex revealed an unusual allosteric mechanism of LKB1 activation (PDB code 2WTK) (118). MO25α contacts STRADα and the LKB1 C helix and activation segment, while STRADα uses its activation segment and G helix to contact LKB1 (**Figure 9*b***). The combined interactions result in an activated LKB1 in which the C helix is correctly oriented for ATP binding and the activation segment is ordered to accept the substrate. Neither STRADα nor MO25α alone are able to produce activation of LKB1.

A role for one kinase domain modulating another has also been demonstrated for the Janus tyrosine kinases (JAKs). JAKs together with STATs (signal transducers and activators of transcription) mediate cytokine receptor signaling to the nucleus. JAKs have a functional C-terminal kinase domain (JH1) and an N-terminal region consisting of a FERM domain and a pseudokinase domain (JH2) that binds to JH1 and in which the catalytic aspartate of the HRD motif is asparagine (119, 120). Despite this mutation, the JH2 domain is catalytically active and downregulates JAK2 activity by phosphorylating two negative regulatory sites, S523 and Y570 (121). A mutation in JH2 (V617F) depresses its activity, resulting in disregulated JAK2, and is present in patients with hematopoietic proliferation diseases. Structures of the JH1 kinase domain have been productive in the search for potent inhibitors for treatment of inflammatory diseases (PDB codes 3LXK and 3LXL) (122), but there is no structure as yet for the JH2-JH1 complex.

Several pseudokinases have a scaffolding role. Integrin-linked kinase (ILK) binds to the C-terminal tails of integrin and mediates integrin to actin regulation in cell-adhesion-dependent processes. ILK comprises an ankyrin repeat domain and a pseudokinase domain that has alterations in four critical regions: the glycine-rich loop, the catalytic aspartate, the next-but-one lysine, and the asparagine that chelates Mg_2 (**Table 3**). Structural studies of the kinase domain showed that it bound ATP, despite alterations in the glycine-rich loop, but had a degraded catalytic site that could not support catalysis (PDB codes 3KMU, 3KMW, and 3REP) (123, 124). The ILK kinase domain was demonstrated to bind to the α-parvin CH2 domain, a component of the signaling complex, using recognition sites on the G and EF helices, as observed for several kinase/substrate complexes (discussed above). When bound to parvin, the pseudokinase can still interact with integrin tails and recruit focal adhesion proteins.

VRK3 is a member of the vaccinia-related kinase family, which includes two active paralogs VRK1 and VRK2. VRK3 has alterations in three critical regions, rendering it

inactive as a kinase (**Table 3**) (PDB code 2JII) (125). However, the overall fold represents that of an active kinase. Further analysis indicated an "inverted" pattern of conservation in which portions of the molecular surface, but not the catalytic site, showed conservation. This result suggests that VRK3 may form interactions with other proteins that may explain its evolutionary retention.

FUTURE DIRECTIONS

Protein kinases have to be activated not only in response to appropriate signals but also at the right time and in the right place. Many individual protein kinases are dispatched to intracellular compartments by the presence of short localization sequences that can be provided in *cis* or in *trans*. However, the mechanisms that control the spatial and temporal integrity of other protein kinases are complex.

For example, multiple kinases can be tethered together via scaffolding proteins to ensure specificity and enhance activity. In certain cases, emerging evidence suggests that they play more active roles in signal transduction than had previously been anticipated (see above and reviewed in Reference 126). The *Saccharomyces cerevisiae* mating pathway was one of the first for which scaffold proteins were shown to play a dynamic role in modulating the activity of a signaling pathway and provides an indication of the complexity to be unraveled. *S. cerevisiae* Ste5 organizes the MAPK cascade composed of Ste11 (a MAPK kinase kinase), Ste7 (a MAPK kinase), and Fus3 (a MAPK) within the mating-type pathway (reviewed in Reference 127). However, in response to an alternative signal (nitrogen starvation), the filamentous growth pathway is activated in which Ste11 phosphorylates Ste7, which then phosphorylates an alternative MAPK Kss1 in a cascade that does not require Ste5. In an elegant study, Good and colleagues (128) demonstrated that, in addition to having a role in tethering Ste7 to Fus3, Ste5 also acts as a substrate-specific Ste7 cocatalyst, enhancing its activity toward Fus3 but leaving its activity toward Kss1 unchanged.

Many protein kinases exist either as domains within large multidomain proteins or as components of large macromolecular complexes. Both cases provide challenges for structure determination by X-ray crystallography. A combination of electron microscopy and other biophysical methods can generate lower-resolution models that are amenable to interpretation with structures determined by X-ray crystallography and verification by biochemical and cellular assays. As examples, models for the allosteric regulation of protein kinase C βII (129) and for the mechanism of activation of calcium-calmodulin-dependent protein kinase II within a dodecameric holoenzyme assembly have been recently established [PDB codes 3KK8, 3KK9, and 3KL8 (130); PDB codes 2WEL and 2UX0 (131); PDB code 3SOA (132)].

Very few structures are available for intact protein kinase-substrate complexes, reflecting the transient nature of their interactions. In some cases, gradients of kinase activity, determined by their anchoring within specialized regions within the cell, dictate substrate selection. The conversion of cell-based models, such as those that describe the role of the Aurora B gradient for successful execution of mitosis (133–135), to structural molecular models is a significant challenge for the future.

SUMMARY POINTS

1. Eukaryotic protein kinases have a common catalytic fold in which conformational elements, including the C helix and the activation segment, are key to correctly position catalytic residues. Catalysis is promoted by the precise alignment of ATP and the substrate OH group, by participation of a catalytic aspartate residue as an acid/base, and by a basic residue to stabilize the transition state.

2. Substrate recognition at the catalytic site involves specific residues in the region near the site of phosphorylation. Association between kinase and substrate is often low affinity, and greater stability is achieved through docking sites that are remote from the catalytic site.
3. Protein kinases are pliable, and different kinases may adopt distinct conformations in the inactive state. This diversity arises because there are no catalytic requirements to constrain the fold. Although distinct, they do share a number of common structural themes.
4. Mechanisms for achieving kinase activation vary but include phosphorylation on the activation segment, phosphorylation at other sites, removal of inhibitory sequences or subunits, and/or association of other domains or subunits. Engagement of the C helix hydrophobic patch by internal regions or by external subunits has emerged as a common theme for activation of some kinases. Protection of the activation segment by regulatory domains is another theme.
5. Kinase dimerization can be regarded as a special case of regulation by additional subunits. Mechanisms of dimerization include relocation of inhibitory sequences in certain receptor kinases, mutual strand exchange of activation segments to allow active phosphorylation of noncanonical sequences, and, as discovered in EGFR, a mechanism in which one kinase acts as a receiver and is allosterically activated by the other kinase acting as an activator.
6. Scaffolding proteins may play a dynamic and catalytic role in kinase activation and substrate selection as seen in a B-RAF/KSR/MEK complex.
7. Pseudokinases are more than inactive bystanders. Some do exhibit activity, and others participate as scaffolds to activate other kinases.

FUTURE ISSUES

1. The role of scaffolding proteins in determining kinase activity and substrate selection.
2. Integration of structural results from electron microscopy, X-ray crystallography and nuclear magnetic resonance approaches to understand the dynamics of the formation of complexes containing protein kinases.
3. The conversion of cell-based models describing kinase activity to detailed structural mechanistic models.

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