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Critical Review Evolution of a Dynamic Molecular Switch

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Abstract

Eukaryotic protein kinases (EPKs) regulate almost every biological process and have evolved to be dynamic molecular switches; this is in stark contrast to metabolic enzymes, which have evolved to be efficient catalysts. In particular, the highly conserved active site of every EPK is dynamically and transiently assembled by a process that is highly regulated and unique for every protein kinase. We review here the essential features of the kinase core, focusing on the conserved motifs and residues that are embedded in every kinase. We explore, in particular, how the hydrophobic core architecture specifically drives the dynamic assembly of the regulatory spine and consequently the organization of the active site where the γ-phosphate of ATP is positioned by a convergence of conserved motifs including a conserved regulatory triad for transfer to a protein substrate. In conclusion, we show how the flanking N- and C-terminal tails often classified as intrinsically disordered regions, as well as flanking domains, contribute in a highly kinase-specific manner to the regulation of the conserved kinase core. Understanding this process as well as how one kinase activates another remains as two of the big challenges for the kinase signaling community.

ORIGINS OF THE KINOME

The concept of protein phosphorylation as a mechanism for regulating catalytic activity actually began with the pioneering biochemical work of Gerty Cori in the 1940s and 1950s, who was trying to understand the regulation of glycogen metabolism. She showed through elegant biochemistry that the active form of glycogen phosphorylase had one additional phosphate compared to the inactive form. What was missing was the concept of an enzyme that added the covalent phosphate (1–4). The foundation for the human kinome was

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subsequently built upon the seminal discovery of Krebs and Fischer who showed in 1959 that phosphorylase kinase (PhosK) upregulates the activity of glycogen phosphorylase by adding a single phosphate (5, 6). The human kinome that represents one of the largest gene families is built upon this concept of protein phosphorylation as a major mechanism for regulation in biology (7). The subsequent appreciation that so many diseases result from dysfunctional eukaryotic protein kinases (EPKs) has made these proteins major targets for drug discovery. In the first two decades, after the discovery of PhosK, protein phosphorylation focused mostly on the role of kinases in metabolism and in the regulation of glycogen metabolism and protein translation by second messengers such as cAMP, cGMP, calcium, and diacylglycerol, and was driven largely by pharmacologists, physiologists, and biochemists (8–11). The second protein kinase to be discovered by Walsh and Krebs, cAMP-dependent protein kinase (PKA), was originally found as a contaminant of PhosK preparation that functioned as an activator of PhosK. For this reason, PKA was initially named phosphorylase kinase kinase (12). So the concept of kinase cascades with one kinase activating another was revealed in those first two protein kinases. The importance of cascades was rapidly amplified later with the discovery of MAPK and MAPKK (13–15). Although we recognize how one kinase such as PKA phosphorylates and activates a protein substrate such as glycogen phosphorylase by adding a single phosphate near the N-terminus (16), the detailed molecular mechanisms showing how one kinase activates another are still a major challenge for the signaling community and for structural biologists (17).

From Glycogen Metabolism to Cancer

The discovery in the late 1970s that the oncoprotein, vSrc, had kinase activity (18) and that it phosphorylated tyrosine rather than Ser or Thr (19) added a new dimension to the world of protein phosphorylation. The comparison of vSrc with cSrc also reinforced the concept of dynamic switches that could be regulated by phosphorylation (20). These discoveries did not, however, prove that Src was an evolutionary ancestor of PKA. In contrast to the biochemists and pharmacologists, the cancer biologists also introduced the tools of molecular biology, in particular cloning. This was the early stages of cloning, and cSrc was quickly cloned in 1979 (21, 22) although the relevance of the Src sequence and its evolutionary relatedness to PKA and the other kinases was not apparent. This link was only revealed when PKA was sequenced in 1980 by classical and laborious protein chemistry techniques (23). The PKA sequence defined the chemical template for the enzyme family, and it was Margaret Dayhoff, a pioneer in the newly emerging field of bioinformatics, who immediately recognized that Src and PKA were evolutionary relatives (Fig. 1) (25) (indicating that the serine/threonine and tyrosine kinases are closely related, which at the time was somewhat surprising). This instantly added cancer biology, molecular biologists, and the whole field of genetics to the kinase world, and the sequence boundaries of the conserved kinase core that defines the kinome were quickly identified in one dimension.

Filling in the Kinome

PhosK, PKA, PKG, and MLCK were all sequenced manually in the early 1980s (26–28), but the magnitude of the kinome and its importance not only for biology but also for disease only became apparent with the advent of routine DNA cloning. The kinome expanded exponentially, and it quickly became clear that this was going to be a very large gene family.

The non-receptor tyrosine kinases were filled in within a few years, and in 1984 the EGFR sequence exemplifying a receptor tyrosine kinase was determined (29). The cloning of the insulin receptor was another milestone that linked to the early studies on glycogen synthesis and glucose metabolism where glucose uptake and glycogen synthesis was promoted by insulin in muscle and liver (30). The cloning of BCR-Abl (31) and RAF further confirmed the linkage of protein kinases to cancer (32). The discovery of PKC by Nishizuka (33) and its subsequent cloning (34) was another early addition to the EPK family. Ironically, because of its activation by tumor-promoting phorbol esters, it was predicted for many years that PKC was an oncogene (35). Only recently has Newton shown that in fact PKC is instead a tumor suppressor in most cells (36). Clearly, we may need to carefully reevaluate some of the early predictions!

Motifs Embedded in the Kinase Core

Dayhoff's alignment of the PKA and Src sequences not only defined the conserved kinase core (25) but also highlighted conserved residues that were scattered throughout the core. By 1988, by manually aligning just a handful of kinase sequences, Hanks, Quinn, and Hunter were able to define 12 distinct subdomains of the protein kinase core (24), and these continue to correlate extremely well with the elements of secondary structure that were later revealed by the structure (Fig. 1). Most branches of the kinome tree were represented in this first analysis, and by 1994 Hunter predicted 1,001 protein kinases (37). While the number of human kinases has settled down to under 600, when one considers the many splice variants, the actual number of kinases probably goes well beyond Hunter's early prediction.

DEFINING THE ACTIVE SITE

Many of our fundamental insights into protein kinase structure and function have come from the PKA catalytic (C)-subunit. The essential features of the active site, for example, were elucidated even before the sequence, let alone the structure, was known. The first essential residue was identified by affinity labeling using an ATP analog, fluorosulfonyl 5′-benzoyl adenosine (FSBA) that was designed by Roberta Colman (38). Zoller showed in 1979 that FSBA labeled a single lysine residue, and this led to the inhibition of kinase activity (39). ATP protected against covalent modification confirming its proximity to the ATP binding site. Mutation of this lysine in Src and Fps, two of the early site-directed mutagenesis experiments, led to inactivation confirming that this residue would likely be essential in all protein kinases (40, 41). This conserved Lys thus quickly became the standard to confirm the importance of kinase activity for biological function. Cross-linking with a hydrophobic carbodiimide subsequently identified two additional conserved residues that were predicted to be at the active site and close to Lys72—Asp184 and Glu91, and these predictions were subsequently confirmed by the structure (42, 43) (Fig. 2); however, how the dynamic assembly of the active kinase is regulated and how the dynamic features of the active kinase work together in a concerted way to catalyze the transfer of the phosphate are still being elucidated.

FROM SEQUENCE TO STRUCTURE

With the first protein kinase structure of the PKA C-subunit in 1991 (44), we went from a one-dimensional template to a three-dimensional folded molecule, and all of the sequence motifs and subdomains acquired functional significance. This structure of a fully active kinase defined a bilobal structure with an N-lobe rich in beta structure and a C-lobe dominated by helices and showed how the active site was created at the cleft between the two lobes (Fig. 2). With the subsequent structure of the ATP-bound C-subunit (45), all of the functional elements of the active site were defined including the two metal ions. We did not appreciate at the time how difficult it would be to crystallize other kinases in this active and fully closed conformation. With PKA we also had another advantage. Walsh had discovered earlier, as a contaminant of the early C-subunit purifications, a high-affinity pseudosubstrate inhibitor of the C-subunit, which he referred to as the heat-stable protein kinase inhibitor (PKI) (46). The inhibitor site was mapped to the first 25 residues of PKI (47, 48) and a peptide corresponding to PKI residues from 5 to 24 (PKI [5–24]) was well characterized by Walsh and Glass as a high-affinity inhibitory peptide (49). Cocrystallization of PKI [5–24] with the C-subunit (50) certainly facilitated the initial crystallization and confirmed perfectly the extensive peptide mapping studies that had been carried out by Walsh. What specific lessons did we learn from this structure?

In this structure, ATP was lodged in the cleft between the two lobes in a manner that was quite different from previous ATP binding sites (51). These structures also gave functional relevance to the regulatory triad and to the activation loop phosphate. The glycine-rich loop or G-loop, which corresponds to subdomain I, is a defined feature of every protein kinase and is distinct from the highly conserved P-loop, which is found in ATPases and metabolic kinases, as well as in dehydrogenases and GTPases, and the mechanistic differences are significant (Fig. 3). The G-loop buries the adenine ring under the first two strands of the five-stranded antiparallel beta sheet that defines the N-lobe. In contrast, the previously recognized P-loop buries the γ-phosphate of ATP under a beta strand followed by a helix (52, 53) while the adenine ring is on the surface. Thus, the two motifs, the G-loop and the P-loop, are fundamentally different and functionally distinct even though each motif has a strand, a loop that is rich in glycines, and an essential lysine. In PKA, the conserved lysine comes from β strand 3.

The structure also gave spatial and functional significance to the other conserved subdomains and in particular to the regulatory triad, K72, E91, and D184; in the structure, these three residues are clustered precisely around the ATP phosphates exactly as predicted from the affinity labeling (Fig. 2, left). ATP is tightly anchored between the N- and C-lobes, and most of the essential elements for binding ATP are embedded in the N-lobe. Two of the residues (Lys72 and Glu91) come from the N-lobe and one (Asp184) from the C-lobe (Fig. 1). The defining features of secondary structure in the N-lobe are the rigid beta sheet, the αC-helix, and the beta turn between αC and β4, and these are included in subdomains II–IV. The structure gives functional significance to these motifs that were originally defined exclusively based on the sequence alignment. While the G-loop is embedded in β1–β2, β3 carries the essential K72 and the αC-helix serves as a scaffold for E91 (Fig. 4).

Trapping ATP so precisely in the active site cleft was possible because PKI is a pseudosubstrate where the P-site Ser is replaced by Ala, ATP thus functions as an inhibitor in this complex rather than a substrate. As demonstrated by Walsh (56,57), the bindings of ATP and PKI [5–24] are both synergistically enhanced. The K_d value for ATP is approximately 25 μM in the absence of PKI compared to 60 nM in the presence of PKI, while the affinity of PKI [5–24] is increased from 100 nM in the absence of PKI to 1 nM in the presence of ATP. Only subsequently was it appreciated that the high binding affinity of both PKI and ATP is surprisingly highly dependent on the second metal ion (58, 59).

Another feature was embedded in this first structure, which represented a fully active and phosphorylated kinase, although we did not at the time entirely appreciate its significance nor did we appreciate how difficult it would be to trap this high-affinity state for other kinases. The activation loop was phosphorylated on Thr197 so it represents a fully active kinase, and this structure showed how this single phosphate integrates the entire molecule (60–62). In the absence of this, phosphate activity is severely diminished and the activation loop is disordered (63). Figure 2 (right) shows, in particular, how many of the subdomains are nucleated by this single phosphate. It is simply much more than an electrostatic node, in contrast to the single phosphate that is added to the N-terminus of glycogen phosphorylase (16). Only later did we come to appreciate the dynamic nature of the activation loop. It seemed deceptively simple in this first structure, but as we now know the assembly of the activation loop is highly dynamic and tightly regulated and most kinase structures that have been solved have a disordered activation loop. Likewise, the regulatory triad is also dynamically assembled, but this was also not appreciated from the first structures.

IMPORTANCE OF HYDROPHOBIC SPINES

Although we learned much from PKA, especially about catalysis and active kinases where we have trapped almost every step of the catalytic cycle in a crystal lattice (64, 65), it took many protein kinase structures to elucidate the mechanistic principles that are associated with the activation of protein kinases. In particular, is there a fundamental conserved mechanism for activation, and, if so, what are the features that drive this process? To do this, it was essential to compare many active and inactive kinases. Fortunately, because kinases are so important not only for biology but also for disease, the structural kinome began to fill in rapidly. Our first insights came from using a graph theory method, local spatial pattern (LSP) alignment, to spatially define conserved residues that were uniquely associated with all active kinases (66). From this analysis, there emerged a set of four noncontiguous hydrophobic residues (RS1, RS2, RS3, and RS4), spaced within the primary sequence, that had been largely ignored in the initial analysis of conserved sequence motifs (Fig. 5). These were defined as the regulatory spine (R-spine) because they were all aligned in a linear motif in every active kinase structure but typically broken in a variety of ways in inactive protein kinases. The dynamic assembly of the R-spine is the key element that drives the switch mechanism for the protein kinase family, and the aligned R-spine is now recognized to be the hallmark feature of every active kinase. Further analysis of the LSP alignments revealed another spine, which is referred to as the catalytic spine (C-spine) because it is completed by the binding of adenine ring of ATP (68). These two spines are both anchored to the highly

unusual hydrophobic αF-helix, which spans the C-lobe. These elements together constitute the hydrophobic core architecture of every active kinase (Fig. 4) (69, 70).

REVISITING THE CONSERVED MOTIFS

Although the original sequence alignment of kinase subdomains identified a set of conserved motifs in the kinase core (24), the concept of a dynamically assembled kinase active site and the contribution of conserved hydrophobic residues to the motifs that were embedded in the subdomains were largely ignored. If we reexamine the subdomains and the elements of secondary structure with our new knowledge of the contributions of hydrophobic residues, we obtain a more comprehensive portrait of the dynamic properties of the kinase and how these residues and subdomains contribute to both activation and catalysis. The two key C-spine residues in the N-lobe that cap the adenine ring, for example, are as highly conserved as the regulatory triad, however, their functional significance was not appreciated. Two key R-spine residues, RS3 and RS4, are also in the N-lobe. They are spatially conserved, as is their hydrophobicity, however, the sequence is not absolutely conserved. Their significance would thus be overlooked in a strict sequence alignment. How do the key hydrophobic spine residues correlate with the subdomains described in Fig. 1 and how do these residues contribute to the dynamic properties of the kinase? The subdomains of the N-lobe are shown in Fig. 5 while the structural elements of the N- and C-lobes are summarized in Figs. 6 and 7. In all figures, the important hydrophobic residues are also highlighted.

The five-stranded beta sheet in the N-lobe moves as a rigid body in response to the opening and closing of the catalytic cleft (54), and the key conserved functional residues that are embedded in this β sheet are firmly anchored to the surface of this rigid body that faces the active site cleft. Reexamining the subdomains of the N-lobe (Fig. 5) reveals that Ala70 is in β3 or subdomain II while Val57 in β2 (subdomain I) is part of the G-loop. These two residues are critical for capping the adenine ring (Figs. 4 and 6). What about the R-spine residues in the N-lobe? These are contained in subdomains III and IV, which contain two functionally important and well-defined elements of secondary structure, the αC-helix and the αC-β4 loop. RS4 (subdomain IV) is located in β4 and is also thus firmly anchored to the beta sheet. In contrast, RS3 (subdomain III) is part of the αC-helix that also serves as a scaffold for Glu91. Met120, also in the N-lobe, is the important "gatekeeper" residue that can be altered to accommodate specific ATP analogs (72). It is in β5 (subdomain V) immediately before the linker that joints the N- and C-lobes. This residue is one of three hydrophobic "shell" residues (Val104, Met118, and Met120) that bridge the R- and C-spines in the N-lobe (73).

Although the β sheet as a defining feature of the N-lobe was recognized in the first structure, the β structure in the C-lobe has been largely ignored since the helices and functional catalytic and Mg^{++} positioning loops are so dominant (Fig. 7). Furthermore, in contrast to the stable β sheet in the N-lobe, only three of the four β strands in the C-lobe (β6, β7, and β8) are rigidly anchored, while one (β9) is often dynamically assembled as part of the recruitment of the DFG motif. The contribution of conserved hydrophobic residues and the C- and R-spines to the motifs that are embedded in the C-lobe was simply not appreciated in the early structures.

In the C-lobe, there are two small beta sheets, β7/β8 that contribute to the C-spine and β6/β9 that are essential for positioning of the R-spine (Fig. 7). The catalytic loop, which harbors most of the residues that contribute directly to the transfer of the γ-phosphate such as Asp166 and Lys168, is flanked at its N-terminus by β6 and at its C-terminus by β7. The magnesium-positioning loop or Mg-loop that binds to the second metal ion and poises the γ-phosphate for transfer is flanked at its N-terminus by β8 and at its C-terminus by β9. Two of the β strands, β7 and β8, are enriched in C-spine residues and serve as the direct C-lobe cap for the adenine ring (Fig. 5). In contrast, the two key R-spine residues (RS1 and RS2) flank β6 and β9. Three of the four β strands are firmly anchored to the hydrophobic αF-helix as is the catalytic loop through Leu167. The hydrophobic residues that bridge β7 and β8 and the αF-helix constitute the C-spine (Fig. 4), while the Mg-loop is anchored to Asp220, another conserved residue is at the N-terminus of the αF-helix.

DYNAMIC ASSEMBLY OF THE CONSERVED REGULATORY TRIAD IS REGULATED

Unlike metabolic enzymes like the proteases and the eukaryotic-like kinases (ELKs), the EPKs are highly regulated proteins where their active sites are typically dynamically assembled. In some cases, like the tyrosine kinase-like (TKL) RAF family kinases, the kinase domain is locked into an inactive state by another domain. In other cases, like PKA and the CAM kinases, the active kinase is assembled by a highly regulated and often phosphorylation-mediated process and then the active site is shielded by a regulatory domain or regulatory protein and only exposed by the binding of a second messenger. In the original crystal structures of PKA, the dynamic features of the active site were obscured. We did not appreciate that the regulatory triad is highly dynamic and that for many kinases it is exceedingly difficult to trap the kinase in an active conformation. Furthermore, we did not appreciate that the assembly of the regulatory triad is inextricably linked to the hydrophobic core architecture, in particular, to the R-spine residues. While one of the regulatory triad residues, Lys72, is part of the stable β sheet in the N-lobe, the other two are anchored to R-spine residues. Glu91 is anchored in the αC-helix and RS3, while Asp184 is part of the DFG motif. The other two R-spine residues are both stably anchored, one (RS4) to the β sheet in the N-lobe (RS4) and the other (RS1) to the catalytic loop in the C-lobe. RS2 in the C-lobe is the DFG phenylalanine, and the position of the RS2 Phe is highly variable; there are many "DFG-out" configurations (Fig. 4). Often the kinase samples a large number of conformational states as part of the intrinsically disordered activation loop while sometimes it is extended out toward the surface of the cleft. In other cases, it can move into the central part of the kinase core and actually occupy the substrate binding site that is filled by a tyrosine in the substrate. The DFG motif delivers Asp184 to the regulatory triad, and the dynamic positioning of Asp184 is coupled to the assembly of the R-spine. In the N-lobe, RS4 in β4 is a stable part of the beta sheet whereas RS3 in the αC-helix moves with the inward and outward motions of the helix, which assumes "αC in" and "αC out" positions. The "αC in" conformation defines the active kinase. The position of this helix is highly variable in the inactive conformations of different kinases (Fig. 4). Thus, like RS2 in the DFG motif, it is also dynamically recruited to the active conformation in a manner that is dependent on the assembly of the R-spine. So the conserved regulatory triad is dynamically

assembled and is a feature of the active kinase only. The assembled active site cleft showing the contributions of the R- and C-spines is summarized in Fig. 8 and in the supplementary material movie.

MOLECULAR SWITCH MECHANISMS FOR ACTIVATION ARE EMBEDDED IN THE DFG MOTIF AND THE AC-HELIX

Evidence so far suggests that the protein kinases evolved from relatively efficient enzymes, the ELKs, to become relatively inefficient enzymes that are instead tightly regulated (55). The activation loop and the GHI (αG, αH, and αI) subdomain are unique features that distinguish the EPKs from the ELKs (74). Many protein kinases, in particular the protein tyrosine kinases and the protein tyrosine-like kinases such as BRAF, are constitutively locked into an inactive state in the absence of some signals. In most cases, it is a contiguous binding domain such as the Ras-binding domain in BRAF (75) or the phosphorylated C-terminal tail of Src, which, like Hck, binds to its own SH2 domain (76, 77). In the case of PKC, the assembled active site of the kinase core is shielded by the C2 domain, which is unleashed by binding to calcium (78). In all cases, the regulatory domains/motifs are predicted to stabilize the inhibited state by shielding the kinase domain. Unfortunately, we still know relatively little about most of these fully inhibited states, since it is experimentally easier to just work with the kinase domain alone. In addition, in most structures of the isolated kinase domains, the activation loop is disordered and not phosphorylated. In the case of PKA, it is binding to the regulatory subunit that inhibits the kinase activity and renders it dependent on cAMP (79,80). PKI is also a physiological inhibitor of the C-subunit. Both PKI and the RI subunits are pseudosubstrate inhibitors and, like PKI, high-affinity binding of RIα requires ATP and two metal ions. The synergistic high-affinity binding of both RIα and PKI is dependent almost entirely on the second metal ion (58). It is not clear how many other kinases utilize a pseudosubstrate mechanism, but PKC and PKG are two prominent examples. Whether they require ATP to stabilize the inactive state like PKI and RIα is not clear. In the case of the CAM kinases, it is the binding of calcium to an inhibitory domain that unleashes the catalytic activity (81). The position of the inhibitory domain in CAM kinase II, which is a dodecamer, is controlled by both calcium and phosphorylation (82) while a C-terminal hub domain mediates oligomerization (83). In some cases, it is not a fused domain but rather a small motif from a flanking intrinsically disordered region (IDR) that mediates inhibition or activation. P90Rsk is a twin-domain kinase that uses its intrinsically disordered tail and linker to recruit other kinases to the activation process. How transient is the active state? Probably it is much more transient than we appreciate. Does the activation/inactivation cycle in some cases happen with every turnover? These are some of the important questions that still need to be resolved. The dynamic nature of the EPKs, the role of interacting domains, and the lack of steady-state kinetics when the kinase is functioning in a cellular context make trapping the EPKs in an active conformation or in a stable inactive conformation extremely challenging in comparison to the relatively simple bacterial enzymes that are associated with metabolism and that utilize small molecules and do likely obey Michaelis–Menten kinetics in most cases. While one can obviously analyze protein kinases using Michaelis–Menten kinetics, in

cells the substrates are proteins and not small peptides that are in large excess compared to the kinase in *in vitro* assays.

ACTIVATION VERSUS OPENING AND CLOSING OF THE CATALYTIC CLEFT

Although the open conformation is often equated with an inactive state, it simply reflects the apo-form of the active kinase. The spines are not broken as a consequence of opening and closing of the cleft, and the beta sheet, which is fully exposed to solvent in the apo-conformation, is still stable. The N-lobe harbors four key residues, Lys72, Ala70, and Val57 in the beta sheet, and Glu91 in αC, that need to be engaged with the C-lobe in order for catalysis to occur (Fig. 5). The R-spine that includes RS4 and RS3 is already intact.

Binding of ATP engages the two lobes and commits the enzyme to catalysis, and this commitment is clearly captured by nuclear magnetic resonance (NMR) (84) although the full impact of synergistically engaging the entire hydrophobic architecture of the core can only be captured experimentally by labeling the hydrophobic side chains (67). The effect of ATP and the two Mg^{++} ions is also captured computationally by the community map analysis of the PKA C-subunit, which defines the kinase core based on correlated motions as a set of functionally integrated units that go beyond the elements of secondary and tertiary structures that are defined simply by the sequence alignments (85). The adenine ring of ATP is trapped specifically between Ala70 (β3) and Val57 (β2) in the N-lobe and Leu173 (β7) in the C-lobe, while the phosphates of ATP reach across to engage the functionally important residues that are positioned by the R-spine residues. Several hydrophobic residues in the rigid αC-β4 loop also make strategic contacts. Val104 is an essential shell residue that bridges directly to ATP and to the R-spine (73). Phe102 at the tip of the αC-β4 loop makes a strategic contact with the αE-helix that in turn interacts through His158 with Asp220 in the αF-helix. This residue is the only contact that stays anchored to the C-lobe in the open conformation (54). Otherwise, the two lobes behave as very independent entities, and this is reflected in all of the NMR studies (67, 84). The conserved lysine in the regulatory triad is also anchored to the β3 strand. The most mobile element of the N-lobe is the G-loop. While backbone amides from the G-loop contribute to the stability of the α- and β-phosphates of ATP, Phe54 contributes an important hydrophobic contact that helps to shield the γ-phosphate from water in the closed conformation. The key residues that are anchored to the β sheet in the N-lobe are highlighted in Fig. 4A. In Fig. 4B, one can see how these elements contribute to positioning of the ATP.

REGULATION OF THE KINASE CORE IS DRIVEN BY IDRs AND SMALL LINEAR MOTIFS

As we look beyond the conserved residues and motifs that are distributed throughout the N- and C-lobes, we find that it is the residues that flank the core regulating both the assembly of the R-spine and the tethering of substrates. These are typically associated with linkers including the C-terminal and N-terminal tails (C-tails/N-tails) that flank the core and with folded domains that are either part of the same polypeptide chain as the kinase domain or associated with other regulatory proteins such as the cyclins that regulate and activate the CDKs. Examples are shown in Fig. 9. The PKA C-subunit is somewhat unique in that the C-

and R-subunits are expressed as separate genes in contrast to PKG where the cyclic nucleotide-binding domains are fused to the kinase domain (26). In PKA, the kinase domain is regulated by the N- and C-terminal tails that wrap around both lobes of the core. These are the segments that stabilize the assembly of the active kinase. The C-tail in PKA is conserved in all AGC kinases and is highly regulated by phosphorylation (74, 87, 89). By looking only at the N-lobe, one sees not only how one surface of the beta sheet faces the active site cleft as discussed earlier but also that the other surface faces the N- and C-terminal tails that are only fully engaged with the kinase core in the closed conformation (65, 90, 91). Both tails are an essential part of the active C-subunit (REF-Robert). The domains that flank the various kinases are rich in motifs that recognize second messengers and in the case of receptors actually span the plasma membrane so that they directly link the extracellular and intracellular spaces. The MAPKs, like the PKA C-subunit, function as independent kinases with N- and C-terminal tails that regulate the conserved core and also provide docking sites for other proteins. The activation of p90Rsk, which actually drove the discovery of the MAPKs and the MAPKKs (17, 88), is an excellent example that shows how the intrinsically disordered tails and linkers with their embedded protein interaction motifs drive a dynamic activation process (Fig. 9). P90Rsk itself has two kinase domains, N-terminal and C-terminal kinase (NTK and CTK) domains, that belong to the AGC and CAMK kinome families, respectively. The C-terminal tail of the CTK has an inhibitory motif that docks to the active site cleft of the CTK as well as a docking site for ERK and a PDZ motif. Docking of ERK initiates the activation process, and the structure of dephosphorylated ERK bound to the CTK not only demonstrates the polyvalent nature of the C-tail but also provides one of the most convincing examples of how one kinase activates another (17, 88).

FUTURE CHALLENGES

The complexity of signaling in cells is complicated further by scaffolding proteins such as the A-kinase anchoring proteins that bind to several proteins such as phosphatases and cyclases in addition to the kinase, thereby creating dedicated signaling communities that are localized in close proximity to their substrates such as the tails and loops of channels, transporters, and receptors. As we move forward in trying to understand these large polyvalent complexes that are such an integral part of signaling in cells, we are poised to take advantage of the cryogenic electron microscopy (cryo-EM) revolution that is taking place in the structural biology community (92, 93). While X-ray crystallography, our primary structural biology tool in the past, has provided atomic resolution details of so many kinase structures, it is unforgiving to the flexible regions that flank the cores and has made it extremely difficult to obtain structures of full-length protein kinases. We see with PKA that structures of the individual R- and C-subunits provide only a partial portrait of the kinase (79). Allosteric regulation and the functional nonredundancy of the R-subunits in PKA, for example, are only revealed from full-length holoenzyme structures and even then the docking/dimerization modalities remain hidden due most likely to their flexibility (80, 94). Most kinase structures also represent only the kinase core with portions of the flanking regions. Recent advances in cryo-EM and cryogenic electron tomography (cryo-ET) open the door for exploring full-length kinases docked onto cellular partners (95). This will allow us not only to better appreciate domain dynamics, even at low resolution, but also high-

resolution features of the folded domains. Cryo-ET holds the further promise of providing us with a more comprehensive appreciation of how signaling is achieved within the context of the cell, not just with purified proteins where we attempt to accurately reconstruct the biological complexes. It is an unprecedented opportunity for the signaling community to partner with structural biologists, cell biologists, and computational chemists to dissect these processes across many scales.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AGC, PKA, PKG	PKC protein kinase family
BRAF	Serine/threonine protein kinase B-raf
CAMK	Calcium/calmodulin-dependent protein kinase
CDK	Cyclin-dependent protein kinase
DFG-motif	Conserved Asp-Phe-Gly sequence motif
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
GHI	Structural motif in protein kinases that contains αG, αH and αI helices
HRD	Conserved His-Arg-Asp sequence motif
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MLCK	Myosin light chain kinase

PDZ	Conserved structural motif originally found in Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1) and Zonula occludens-1 protein (zo-1).
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
RAF	Rapidly Accelerated Fibro6blastoma oncogene
RI	cAMP-dependent protein kinase type I regulatory subunit

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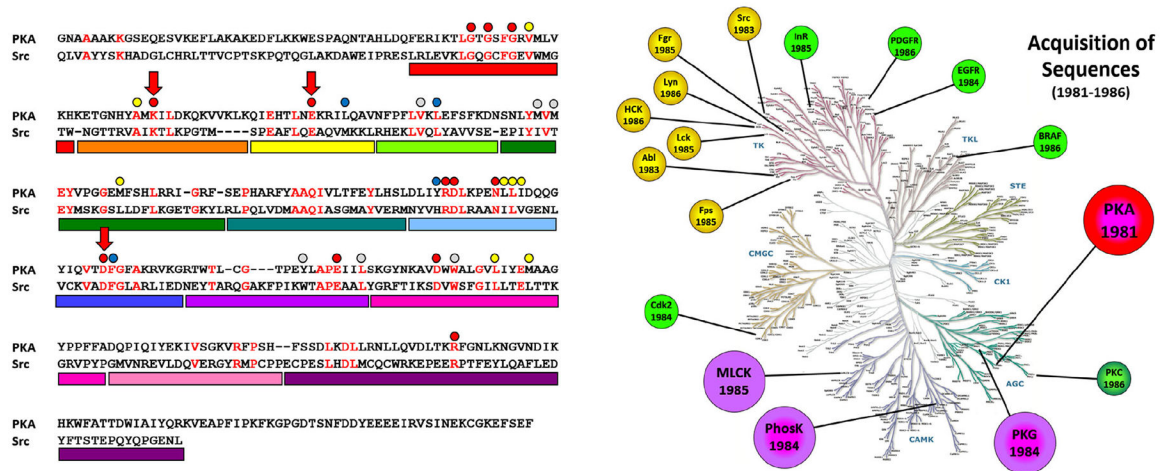
FIG 1.

Subdomains of Src and PKA define the boundaries of the conserved kinase core that is captured in the kinome. The sequence information that defines the eukaryotic protein kinase superfamily is captured in the human kinome tree on the right (7), which also highlights the early sequence information that came from classic protein sequencing (large pink circles) versus cloning (small circles), where yellow indicates Src family of nonreceptor tyrosine kinases and green are other early cloned kinases. The sequence alignment on the left confirmed that PKA and Src belonged to the same genetic family and also allowed Hunter (24) early on to identify conserved subdomains that extended throughout the kinase domain.
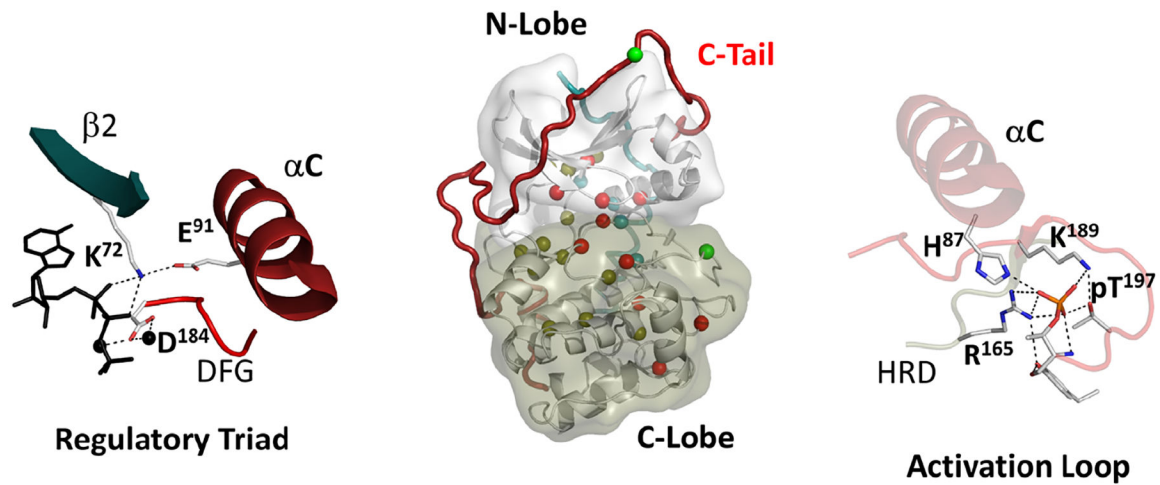
FIG 2.
Structure of the PKA catalytic subunit. The first protein kinase structure of the PKA C-subunit, highlighted in the middle panel, revealed the conserved protein kinase fold and provided a three-dimensional framework for the subdomains that were defined previously by the sequence alignments (24). The C-terminal tail is shown in red, the N-lobe is in white, and the C-lobe is in tan; red dots indicate the conserved residues identified by sequence alignment. On the left is the conserved regulatory triad that was predicted to be in close proximity to the phosphates of ATP. On the right is the phosphorylated activation segment showing how this phosphate, pThr197, nucleates many of the subdomains and motifs described in Fig. 1.
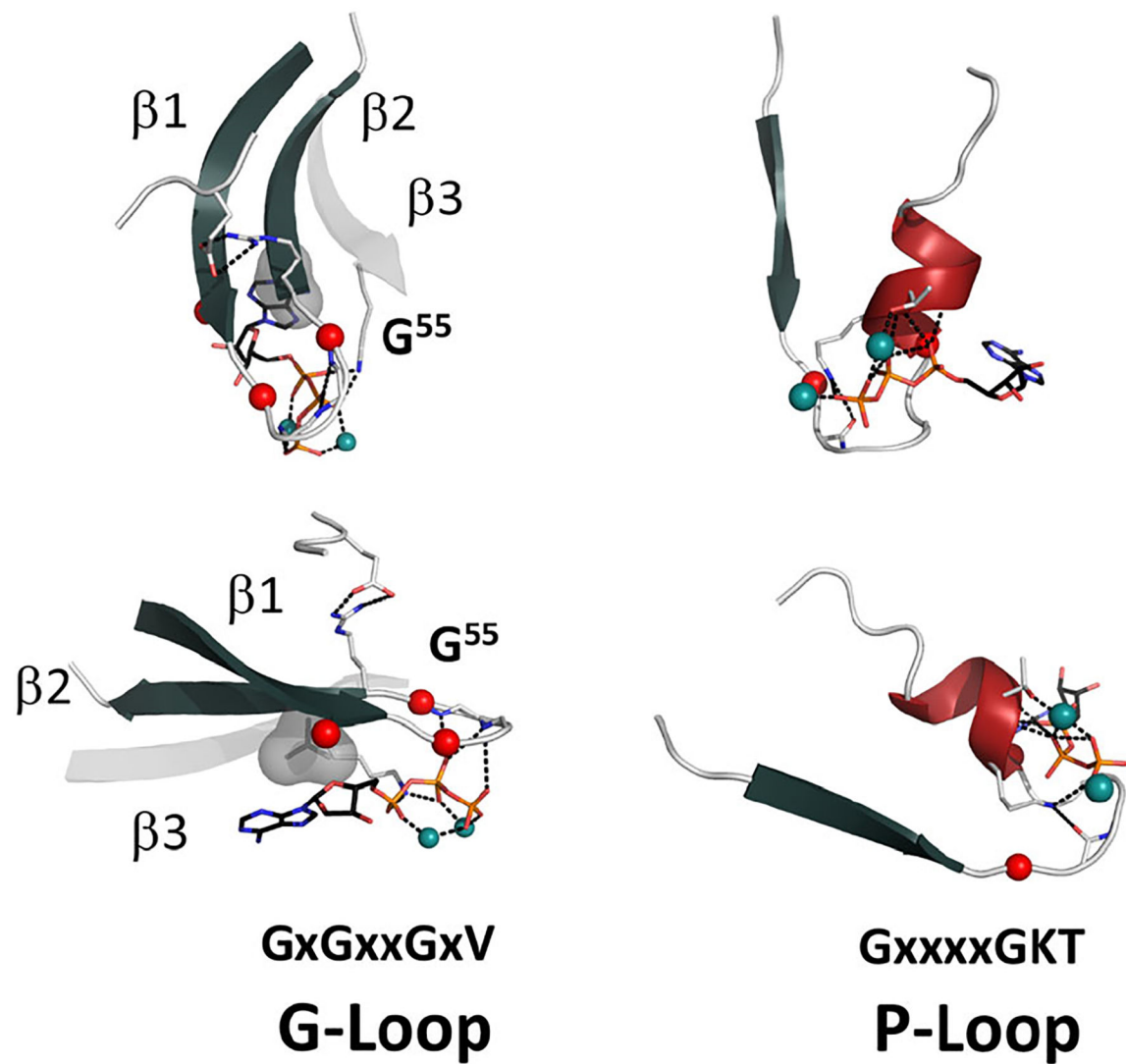
FIG 3.

Glycine-rich loop. The glycine-rich loop or G-loop (subdomain I) in protein kinases is distinct from the previously well-known P-loop that is found in ATPases, metabolic kinases, dehydrogenases, and G-proteins (52, 53). The G-loop buries the adenine ring of ATP under the first two β strands of the N-lobe and in addition to the three highly conserved glycines (Gly50, 52, and 55) contains Val57, which is also highly conserved. Val57 is part of the C-spine, and, in particular, contributes to the hydrophobic N-lobe cap for the adenine ring. Red dots indicate conserved glycines; teal dots indicate magnesium ions.
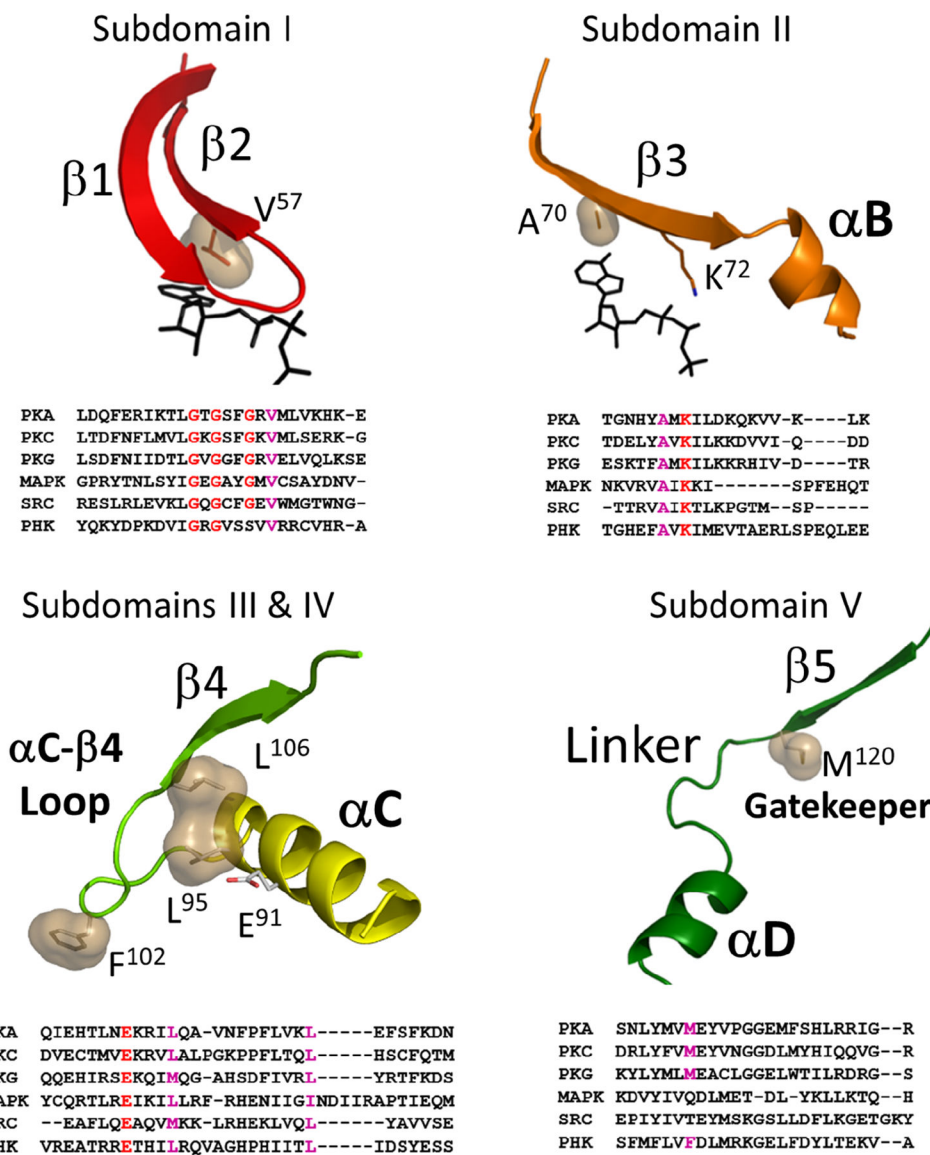
FIG 4.

Subdomains of the N-lobe. The first five subdomains (I–V) contribute to the N-lobe. The conserved motifs that are embedded in these subdomains include the G-loop (subdomain I), Lys72 in β3 (subdomain II), and Glu91 in the αC-helix (subdomain III). In addition, many of the conserved hydrophobic motifs that constitute the R- and C-spines are embedded in these subdomains of the N-lobe. These include two C-spine residues that cap the adenine ring of ATP—Val57 in β2 (subdomain I) and Ala70 in β3 (subdomain II) as well as two R-spine residues—Leu95 in the αC-helix (subdomain III) and Leu106 in β4 (subdomain IV). Three hydrophobic shell residues (SR1, SR2, and SR3) that span the R- and C-spines in the N-lobe are also part of the hydrophobic core architecture. These are localized to subdomains IV and V; SR3 is the gatekeeper residue that immediately precedes the linker that joins the N- and C-lobes. One additional residue, Phe102 in PKA, at the tip of the αC-β4 loop (subdomain IV) is conserved as a hydrophobic residue in most kinases and is the only
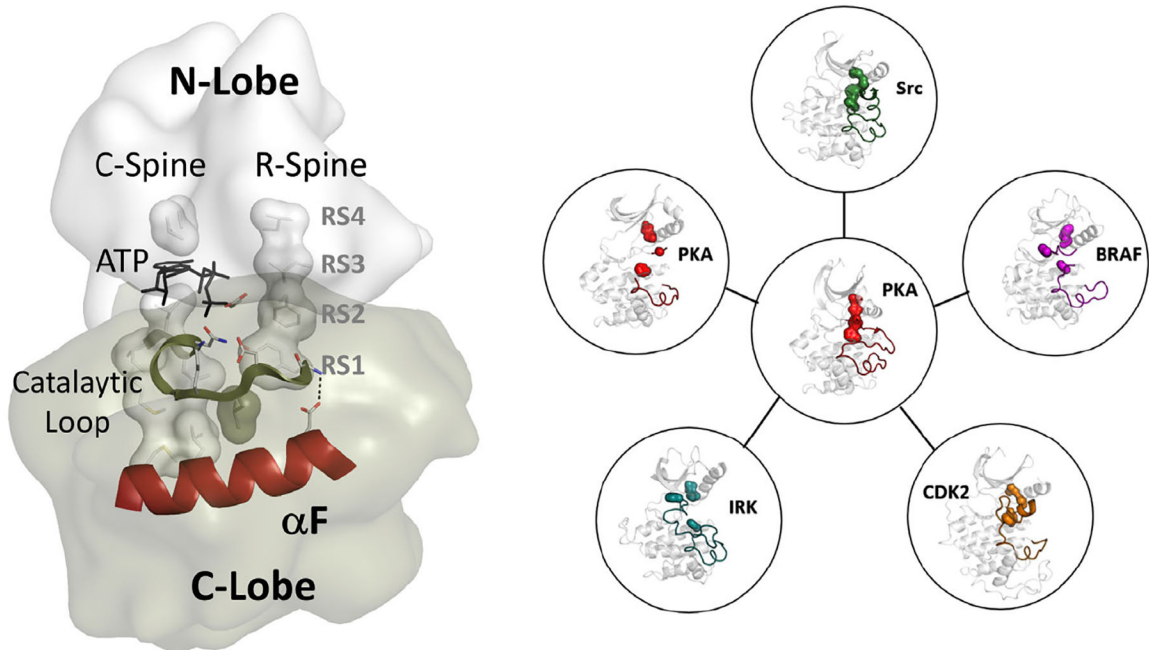
residue in the N-lobe that tracks with the C-lobe when the active kinase transitions between its open and closed conformations (54, 55).

FIG 5.
Two hydrophobic spines anchored to the αF-helix (left) define the architecture of the kinase core. Assembly of the R-spine encompasses the mechanisms that convert an inactive kinase into a fully active kinase, and this process varies with each kinase. The R-spine can assume many different conformations in inactive kinases (right) while the assembled R-spine is the hallmark signature motif of every active kinase. The C-spine is completed by the adenine ring of ATP, and the dynamic way in which the entire hydrophobic core is synergistically recruited to prepare the protein for transfer of the γ-phosphate is captured by nuclear magnetic resonance when the hydrophobic side chains are labeled (67).
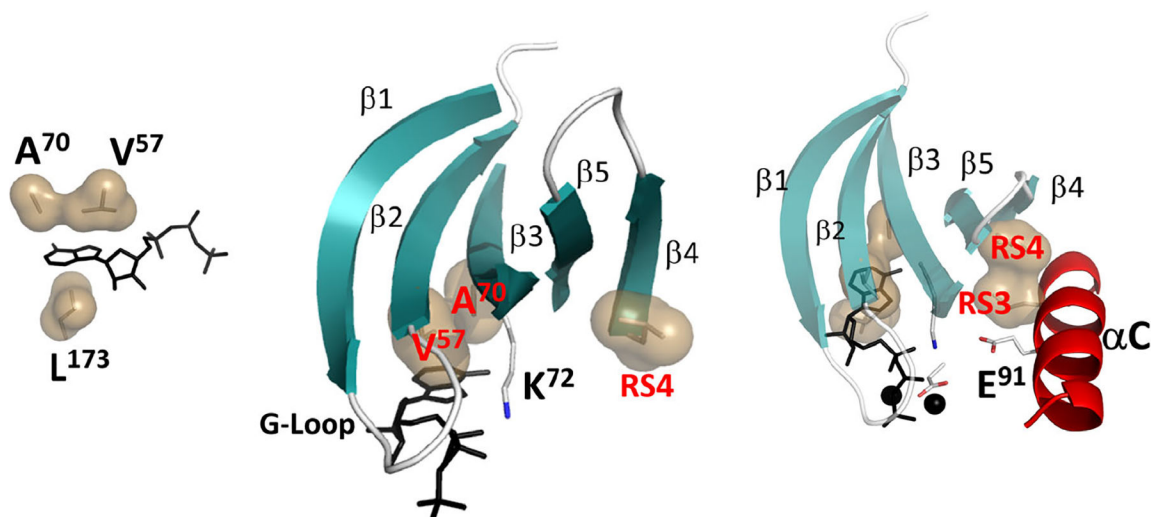
FIG 6.

Beta sheet of the N-lobe. Key motifs for docking the adenine ring and the phosphates of ATP are embedded in the five antiparallel stands of the β sheet in the N-lobe, which moves as a rigid body when the cleft opens and closes (54). These include Val57 and Ala70 that cap the adenine ring, Lys72 that binds to the α- and β-phosphates of ATP, and Leu106 (RS4), which is part of the R-spine. Glu91 in the αC-helix is recruited to Lys72 in the active kinase, and both contribute to opening and closing of the active site cleft (71). Leu95 (RS3) is also part of the αC-helix, and this motif is dynamically recruited as part of the assembly of the R-spine. The C-spine residues that cap the adenine ring from the N- and C-lobes are shown on the left, and binding of ATP in this way commits the core to catalysis (67).
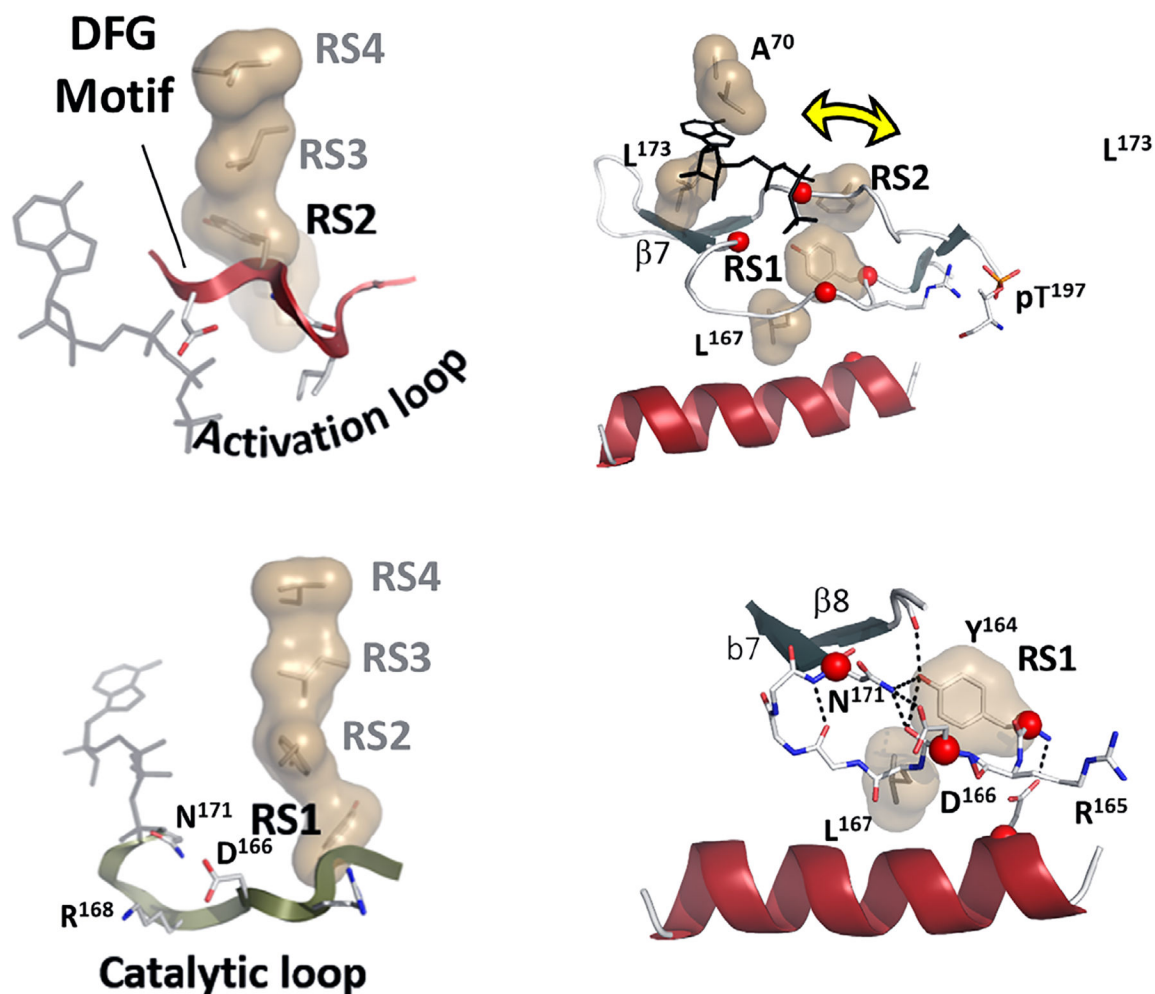
FIG 7.

Beta sheet of the C-lobe anchors the key catalytic features of the kinase core. The β sheets of the C-lobe consist of two segments each having two strands. β7 and β8 provide the capping residues for the adenine ring and are thus a key part of the C-spine. β7 follows the catalytic loop while β8 precedes the Mg⁺⁺ positioning loop (Mg-loop) that harbors the DFG motif. The β7 and β8 strands are firmly anchored to the hydrophobic αF-helix via the C-spine. β6 precedes the catalytic loop and is also firmly anchored to the αF-helix, as is the catalytic loop itself through Leu167. Its partner strand, β9, harbors the DFG motif and is highly dynamic, typically toggling between DFG-in and DFG-out states. Thus, in many inactive kinase structures the catalytic machinery is incomplete (bottom right). Phe185 in the DFG motif corresponds to RS3, while Tyr164 that is part of the HRD motif corresponds to RS1. Tyr164 reaches over to stabilize the catalytic loop and the interactions with β8 (right panel). Yellow arrow indicates R-spine residues that toggle between in (active) and out (inactive) conformations (see Fig. 4).
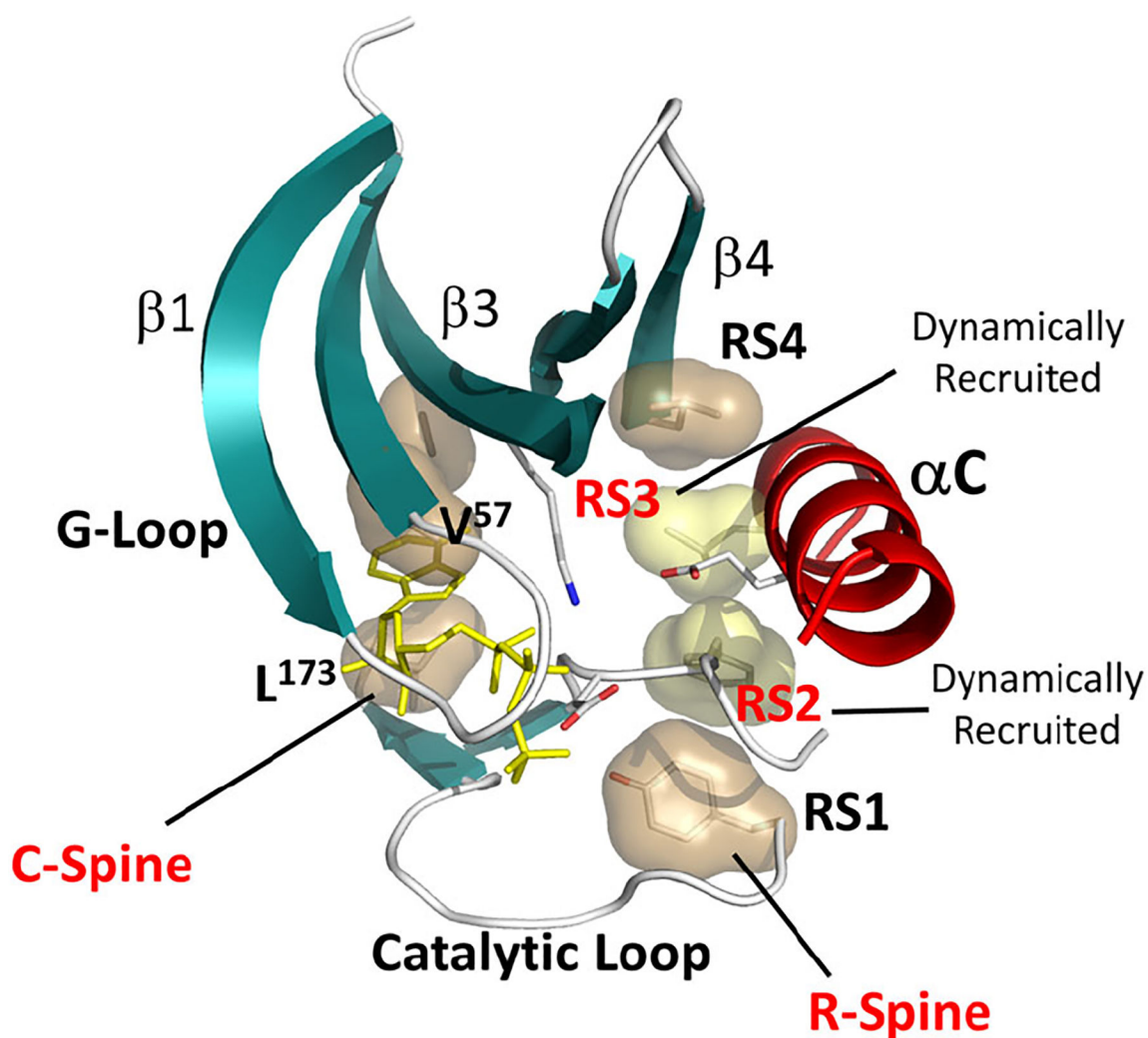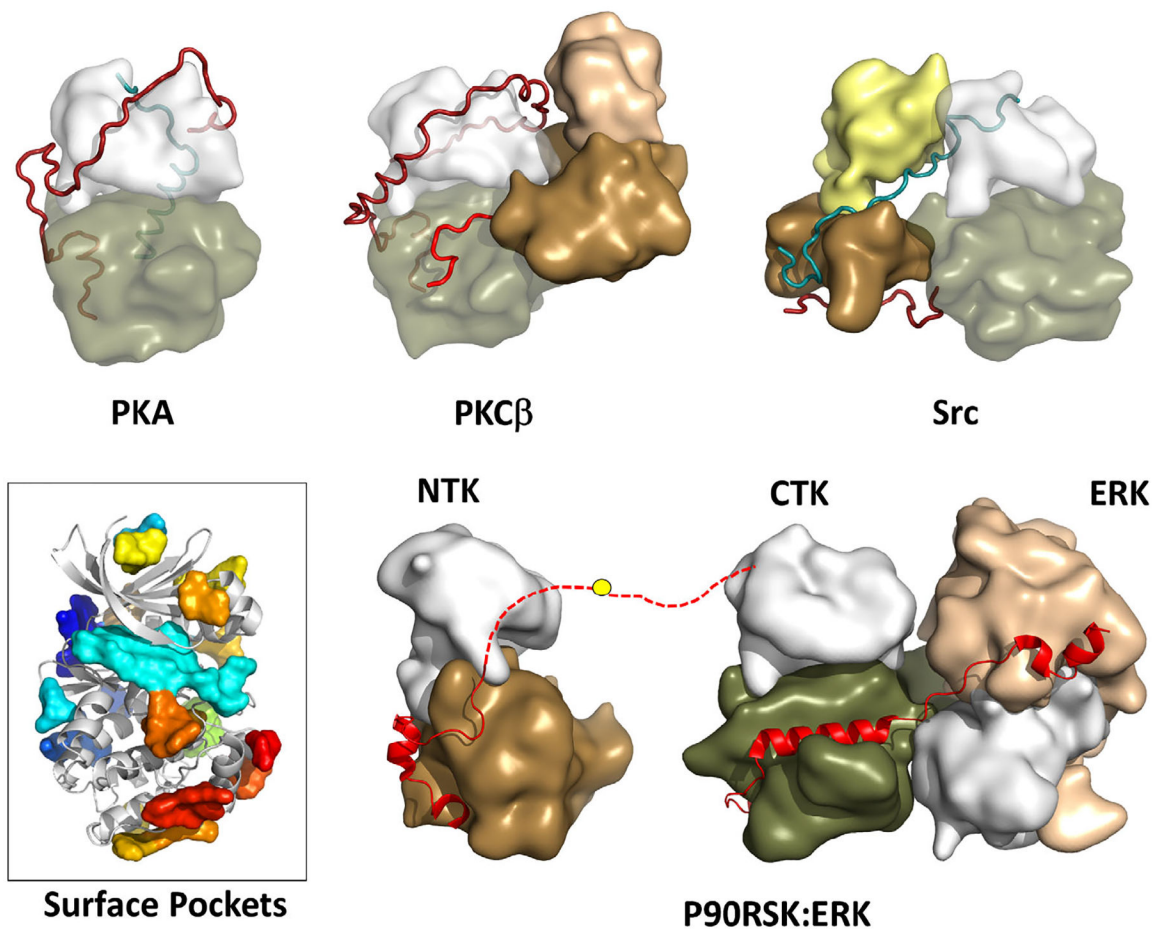
FIG 8.
Conserved motifs in N- and C-lobes define the active site cleft. The conserved motifs from the N- and C- lobe, described in Figs. 4, 6, and 7, that contribute to the assembly of the active site are summarized here. The movie demonstrates how the hydrophobic spines contribute to the organization of the active site (see supplementary material).

FIG 9.

Linkers, tails, and subdomains regulate the kinase core. On the bottom left is a model showing a set of spatially conserved pockets that decorate the surface of many protein kinases (86). In contrast to the core, the chemical nature of these pockets is not conserved and the mechanisms by which different motifs and domains occupy these surface pockets and thereby control the conformational state and activity of the core are dynamic and highly regulated. Each kinase is distinct and even small isoform differences can direct different dynamics. In the case of PKA, the N- and C-terminal tails wrap around and regulate the kinase core, and the structure and regulation by phosphorylation of the C-tail is a conserved feature of all AGC kinases (87). A PKC model shows how the C-2 domain docks onto the kinase core and locks it into an inactive state in the absence of calcium (78). In the case of Src, the SH2 and SH3 domains lock the kinase domain in an inhibited state due to the C-terminal pTyr, which binds to its own SH2 domain (76). P90Rsk is unusual in that it contains two kinase domains, an N-terminal kinase (NTK) and a C-terminal kinase (CTK), and is activated by ERK binding to its C-terminal tail (88). It demonstrates the remarkable ways in which intrinsically disordered regions that flank a kinase core can contribute to a dynamic activation mechanism (17).