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Dynamic Regulatory Features of the Protein Tyrosine Kinases

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Abstract

The SRC, ABL, TEC and CSK families of non-receptor tyrosine kinases (collectively the Src module kinases) mediate an array of cellular signaling processes and are therapeutic targets in many disease states. Crystal structures of Src module kinases provide valuable insights into the regulatory mechanisms that control activation and generate a framework from which drug discovery can advance. The conformational ensembles visited by these multidomain kinases in solution are also key features of the regulatory machinery controlling catalytic activity. Measurement of dynamic motions within kinases substantially augments information derived from crystal structures. In this review, we focus on a body of work that has transformed our understanding of non-receptor tyrosine kinase regulation from a static view to one that incorporates how fluctuations in conformational ensembles and dynamic motions influence activation status. Regulatory dynamic networks are often shared across and between kinase families while specific dynamic behavior distinguishes unique regulatory mechanisms for select kinases. Moreover, intrinsically dynamic regions of kinases likely play important regulatory roles that have only been partially explored. Since there is clear precedence that kinase inhibitors can exploit specific dynamic features, continued efforts to define conformational ensembles and dynamic allostery will be key to combating drug resistance and devising alternate treatments for kinase-associated diseases.

Introduction

At the end of the 1800s photographers had been producing still images for nearly a century. The advent of moving pictures at this time caused a dramatic shift in the viewer's experience - the actions of people and the motion of objects were depicted on screen forever changing the entertainment landscape. Protein crystallography may be considered the 'still photography' of structural biology. Detailed molecular insights are gained from the abundant protein crystal structures available to the scientific community. Measuring and simulating the dynamics of biological macromolecules (the moving pictures of biology) captured the attention of eminent scientists even as the very first crystal structures of biological molecules were solved. Martin Karplus (1) provides a view into the history of molecular dynamics and

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Author Contribution

The authors have all contributed to and approved the final version of the manuscript.

Competing interests

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experimental approaches to characterize macromolecular motions including how early efforts to simulate protein dynamics led to the realization that x-ray data captures information about internal motions (2–4). Today, in addition to x-ray crystallography, use of nuclear magnetic resonance (NMR) spectroscopy, hydrogen/deuterium exchange mass spectrometry (HDX-MS), small angle x-ray scattering (SAXS), single molecule studies, and imaging techniques are propelling biological macromolecular dynamics onto the big screen. As the intricate connections between dynamics, allostery, and structure are revealed, our view of protein function and regulation is changing in ways that mirror the cinematographic advances of the last century (5–15).

Protein Kinases – dynamics and drug discovery

Protein kinases direct the majority of cellular signaling pathways by phosphorylating target sequences on serine, threonine or tyrosine. Unregulated kinase activity is linked to many disease states and so these enzymes are prominent targets for pharmaceutical intervention (16–20). In the decades following the discovery of protein kinases, x-ray crystallography provided unprecedented information on the structural features that control kinase activity (Text Box 1). The dynamic features superimposed on the three-dimensional structures derived from crystallography are equally important in deciphering how kinases are regulated.

Kern and co-workers have extensively explored protein dynamics including the dynamic features of two non-receptor tyrosine kinases, SRC and ABL (21). By dissecting the evolutionary paths of these two related kinases, these authors elucidated the molecular determinants driving selectivity of the cancer drug Gleevec. Gleevec is highly selective for ABL over SRC despite the nearly identical crystal structures of the kinase/drug complexes (22). Kern demonstrates that the observed selectivity is due to differences in the conformational landscape or dynamic ensemble available to each kinase. Perhaps most interesting is the fact that evolution of these kinases occurred in the absence of the cancer drug and yet modern pharmaceutical approaches can clearly exploit specific conformational dynamics that are intrinsic features of all proteins. Understanding the conformational landscape of kinases is critical to accelerate drug development and tackle the increasing instances of drug resistance for this abundant class of signaling proteins. This review will focus on the emerging dynamic regulatory features of the non-receptor tyrosine kinases or “Src module” kinases from the SRC, ABL, TEC, and CSK families (Fig. 1a) (23). A large number of excellent studies have examined the dynamic behavior of a range of additional kinase families (13, 24–34).

The inactive Src module

The first structural insights into the Src module came in 1997 when the groups of Kuriyan, Harrison, and Eck published crystal structures of the SRC family kinases, HCK and c-SRC (35, 36). Unlike PKA and other serine/threonine kinases (Text Box 1), the regulatory regions and catalytic domain of the SRC kinases are present in a single polypeptide chain. In addition, the structures of the SRC kinases revealed that the regulatory SH3 and SH2 domains impinge on the distal side of the kinase domain providing allosteric control over the

catalytic site (Fig. 1b). SH3 sandwiches the SH2-kinase linker with the kinase domain N-lobe and the SH2 domain engages the phosphotyrosine (pY) site in the SRC C-terminal tail; an interaction that had been inferred by earlier work describing the molecular determinants of the transforming activity of v-SRC which lacks this pY site (37).

Numerous Src module structures have been solved since the 1997 debut of SRC and HCK including tyrosine kinases outside of the SRC family such as ABL, TEC, and CSK (38–40). The TEC and ABL kinases share the compact autoinhibited form of SRC but the regulatory apparatus of CSK differs substantially despite the common Src module domain organization (Text Box 2). Crystal structures of the Src module kinases in both active and inactive conformations have led to a set of 'rules' governing regulation of catalytic activity. These include the idea that kinase activation must be accompanied by release of one or both of the intramolecular contacts mediated by the SH3 and SH2 domains and (auto)phosphorylation of a conserved tyrosine in the activation loop drives the conformational shift (largely within the C-helix) to the catalytically competent kinase domain (reviewed in (23)).

Conformational dynamics and the kinase activation landscape

SH2 mediated regulation

Immediately following the first crystal structures of SRC and HCK, a body of work was produced that focused on dynamic coupling and crosstalk within the SRC SH3-SH2 segment (41–45). An overriding question centered around how the intramolecular SH2/pY tail interaction affects the molecular motions within SH3-SH2. Young *et al.* demonstrated that the connector between SH3 and SH2 dynamically couples these domains when SH2 is engaged with the phosphotyrosine tail. But either tail dephosphorylation or mutation in the connector region results in loss of allosteric communication (46). Recently, methyl side-chain dynamics measured by NMR relaxation were compared for pY-tail bound and unbound FYN SH2 and revealed dynamical changes (rigidification and increased motion) for SH2 domain residues clustered around the connector between SH3 and SH2 domains (47). In the assembled, autoinhibited state of SRC, this SH3-SH2 connector is adjacent to another key regulatory unit, the SH2-kinase linker. Thus, the allosteric changes induced by pY tail binding to SH2 likely modulate the autoinhibitory interface by dynamically altering connector and linker regions. Nevertheless, SRC kinases can autoactivate while retaining the SH2/phosphotyrosine tail interaction suggesting that this regulatory site is not a simple on/off switch that dictates activation status (48–50).

The TEC and ABL kinases lack the C-terminal regulatory tail yet the intramolecular interaction between SH2 domain and the kinase domain C-lobe is retained. The myristoylated N-terminus of ABL binds to a hydrophobic pocket in the kinase domain C-lobe stabilizing a bent conformation of the kinase domain I-helix to promote SH2 association (51, 52). Solution NMR data combined with SAXS analyses (see Text Box 3) have shown that apo ABL (lacking the N-terminal myristoylated tail) adopts the closed conformation visible in crystal structures of full-length ABL (53). This suggests that the I-helix adopts a range of conformations in solution that are compatible with SH2 association and the interaction between SH2 domain and kinase domain C-lobe does not require myristate binding. Ligand binding to the myristate pocket likely stabilizes the ABL C-lobe

for more efficient SH2 association via conformational selection. NMR data also indicate that inhibitor binding to the myristoyl pocket induces conformational exchange (microsecond to millisecond timescale motions) in the remote N-lobe while the SH3, SH2 and C-lobe remain relatively stable compared to the apo kinase (53). Interestingly, the ATP competitive inhibitor, imatinib, opens the autoinhibited ABL structure and subsequent myristate inhibitor binding dramatically shifts the equilibrium back to the closed, autoinhibited form with SH3 and SH2 domains engaged with the N- and C-lobes of the kinase domain, respectively (32, 53). These data support a preorganized, autoinhibited apo-ABL and suggest that ligand binding to the myristate pocket, while stabilizing the SH2/kinase interface, also propagates dynamical changes to the N-lobe that can overcome the open, active form of the kinase by allosterically stabilizing the interface involving SH3 and the SH2-kinase linker.

The Tec family contains neither the C-terminal phosphotyrosine tail of SRC nor the myristate pocket of ABL and so how the TEC family SH2 domain participates in autoinhibition has been difficult to deduce from sequence or structures of isolated domains. A model based on the recent crystallographic analysis of BTK (39) combined with NMR, HDX-MS, and biochemical assays (54) reveal a conserved acidic side chain (Asp656) at the end of the I-helix that mimics phosphotyrosine by forming a salt bridge with the conserved arginine in BTK SH2. Mutation of BTK Asp656 activates full-length kinase and HDX-MS shows the Asp656 mutation opens the SH3-SH2 interface (54). While the interaction between the C-lobe aspartate and SH2 is important for BTK regulation, the extent to which the TEC kinases adopt the autoinhibited closed conformation has been less clear. SAXS data support an open, extended domain arrangement (55) and the BTK crystal structure (39) is that of an extended domain swapped dimer rather than the compact autoinhibited form observed for SRC and ABL. In contrast, NMR analysis of full-length, apo BTK (54) supports conformational exchange between several states with the closed conformation being the predominant form in solution. Thus, for SRC, ABL and TEC kinases the SH2/C-lobe kinase interaction is a common feature yet the details and dynamics of this region differ substantially.

SH3 mediated regulation

The SH3 autoinhibitory interaction with the SH2-kinase linker region is structurally conserved among the various Src module kinases unlike the SH2 regulatory interactions. From a dynamics perspective however, HDX-MS has shown that SH3 domains from the SRC and TEC family exhibit an unexpectedly wide range of dynamic behavior (56, 57). The extent to which the different dynamic profiles of SH3 domains might influence regulation or substrate/adaptor interactions of the full-length SRC and TEC kinases in cells has yet to be fully understood and so our focus here will shift to examining the “other side” of the SH3/SH2-kinase linker interaction - the intramolecular contact between the SH2-kinase linker (bound to SH3) and the N-lobe of the kinase domain (58–61).

Comparing early structures of SRC SH3 bound to idealized proline rich peptides (62) to the structure of autoinhibited SRC with the SH3 domain bound to the suboptimal SH2-kinase linker shows significant structural deviation in the SH2-kinase linker compared to canonical SH3 ligand binding. Specifically, the Gly254-Leu255 dipeptide unit within the SH2-kinase

linker adopts a conformation that is distinct from canonical peptide ligands in this region. (Fig. 1c) Leu255 is oriented away from the SH3 domain in the autoinhibited SRC structure quite unlike the corresponding positively charged arginine in the peptide/SH3 complexes which points to a conserved aspartate (Asp99 in SRC) forming a stabilizing electrostatic contact (62). Leu255 in the SH2-kinase linker is therefore freed from the SH3 binding site and exerts regulatory control over the active site (63). Leucine 255 in autoinhibited SRC sits in the middle of a “hydrophobic stack” (58, 63) that coalesces the side chains of W286 and Y326 on the distal side of the SRC kinase domain N-lobe (Fig. 1d). These three hydrophobic residues are largely conserved across the ABL, TEC, and SRC families with the interesting exception of HCK that contains a histidine in place of the β 4 SRC Tyr326. Discussed in more detail below, the integrity of the hydrophobic stack is a crucial determinant of the autoinhibited Src module.

The sequence difference within HCK in the hydrophobic stack region is interesting in light of earlier work by Engen, Smithgall and co-workers showing that the kinases within the SRC family, in particular HCK, exhibit distinct susceptibilities to activation via SH3 domain displacement (64). HCK autophosphorylates more rapidly than SRC upon addition of proline-rich SH3 ligand suggesting that HCK autoinhibitory contacts are more dynamic than SRC. While more detailed dynamic studies are needed to confirm the role of the hydrophobic stack in tuning activation by SH3 displacement, comparing the crystal structures of SRC and HCK in this region (Fig. 1e) suggests that side chain contacts between residues of the SRC hydrophobic stack are maximized whereas the corresponding structure of HCK indicates considerably less efficient stacking interactions. Moreover, the non-conserved histidine at the bottom of the HCK hydrophobic stack may be in a protonated or deprotonated state (65) creating further potential deviations from the well aligned hydrophobic stack residues observed in SRC. Sequence differences in the SH2-kinase linker of the SRC family, in particular between splice variants of FYN, have also been tied to regulation of kinase activity (66, 67) and it is intriguing to consider that hydrophobic stack assembly may respond to differences in linker sequence and dynamics.

The active site and regulation of nucleotide binding

Using both SRC and the TEC family member, ITK, Leonard and co-workers (68) show that the autoinhibited conformations of these kinases (intact hydrophobic stack) preferentially bind ADP over ATP by more than an order of magnitude. The preference for ADP is retained when the activation loop tyrosine is phosphorylated or if the regulatory tail of SRC is removed. Mutation of SRC Leu255 in the SH2-kinase linker (disruption of the hydrophobic stack) without mutation of the regulatory tail tyrosine leads to a shift in nucleotide preference for ATP and dramatic activation of the SRC kinase activity compared to the extent of activation that occurs upon mutation of the C-terminal tail tyrosine (68). The allosteric effect mediated by loss of Leu255 in the SH2-kinase linker leads to a conformational shift in the C-helix and assembly of the R-spine creating a catalytically competent kinase (also reviewed in (23)). Further, it is noted that a direct contact between the hydrophobic stack residue Y326 on beta strand 4 and the gatekeeper residue on beta strand 5 (Thr338 in SRC) may serve to connect the hydrophobic stack motif to bound nucleotide (Fig. 1d). Likewise, for the TEC kinase ITK, which lacks the C-terminal tail

entirely, mutation of the conserved leucine in the SH2-linker (Leu351) activates ITK (68) supporting the idea that to the extent that the autoinhibited Src module is latched, the 'clasp' (Fig. 1d) may be localized to the hydrophobic interactions at the SH3/SH2-linker/kinase N-lobe interface (69). Furthermore, all Src module kinases share the hydrophobic stack but SRC family kinase activity rises only slightly upon deletion of the regulatory tail, the conformational ensemble of inactive SRC is not altered at all in the absence of the C-terminal tail, nor is the autoinhibited conformation of ABL completely lost in the absence of myristate (46, 49, 53, 68, 70–74).

It has long been established that mutation of the SRC regulatory tail tyrosine in cells results in constitutive activation and cellular transformation (37). How are the longstanding cellular observations reconciled with findings that the SRC pY regulatory tail may only partially affect overall activation status and other Src module kinases that lack the tail are tightly regulated? Perhaps the modest increase in SRC activity in the absence of the pY tail is sufficient to trigger cellular transformation. Alternatively, Leonard *et al.* (68) suggest that the SH2/pY interaction is more important for masking SRC SH2 mediated interactions with exogenous signaling partners than directly regulating catalytic activity due to constitutive membrane association. The authors cite work showing that membrane anchored BTK (normally cytosolic and lacking the pY tail) drives transforming activity (75) and C-terminal tail phosphorylation limits accessibility of exogenous ligands to the SRC SH2 domain (71). Nevertheless, it is noted that the sequence surrounding pY in the C-terminal tail of SRC family kinases does not conform to the optimal SH2 target sequence (76) suggesting that if the pY tail serves as a direct inhibitor it is also tuned for release.

Activation loop dynamics

Underscoring differences between closely related kinases, the catalytic activity of one SRC family member, FGR, seems to have evolved unfettered by SH3 and SH2 mediated regulation (77). Despite having all the hallmarks of a regulated kinase, the activation loop of FGR is phosphorylated regardless of the phosphorylation status of the regulatory tail and FGR activity is not increased by binding of peptides to the SH3 or SH2 domains. Constitutive activity is attributed to observations that overexpression of wild type FGR occurs in acute myeloid leukemia cells and FGR knockdown reduces cell growth (78). Characterization of FGR by HDX-MS suggests that, despite the unconstrained catalytic function, FGR adopts the compact autoinhibited structure of other SRC family members – and yet the SH3 and SH2 regulatory features are effectively bypassed (77). The authors of this work show that sequence differences between FGR and other Src family members in the activation loop are responsible, at least in part, for the high level of FGR kinase activity. Mutation of three FGR residues, Asn-Pro-Cys, just C-terminal to the tyrosine phosphorylation site to the Thr-Ala-Arg sequence derived from all other Src kinases reduced autophosphorylation of FGR by 50% and similarly reduced FGR transforming activity. Interestingly, sequence differences between FGR and other Src family members in the activation loop extend beyond this motif; in particular it is interesting to note that otherwise conserved glutamates at SRC position 305 (at the N-terminal end of the C-helix) and position 412 (C-terminal to the DFG motif) are instead lysine in FGR. This sequence difference raises the possibility that electrostatic repulsion between these two longer,

positively charged residues in the inactive state of FGR might promote a conformational shift of the C-helix and $\beta 3$ - αC loop toward the active state.

Sequence differences between the TEC family kinases BTK and ITK are also linked to differences in activation loop dynamics that increase phosphorylation and hence the activity of BTK compared to the closely related ITK (79). The minimal amino acid differences responsible for imparting distinct activation loop dynamics to BTK versus ITK are buried at the N- and C-termini of the activation segment instead of along the face of the activation loop as observed for FGR. The nature of the BTK/ITK sequence differences are somewhat unremarkable except for the presence of a proline residue in the more dynamic, more highly phosphorylated BTK activation loop – reminiscent of the proline in the constitutively active FGR. These systems suggest that intrinsic motions and conformational sampling drives phosphorylation on the activation loop tyrosine thereby controlling catalytic activity, in some cases overriding regulatory domain involvement. Moreover, what appear as subtle or even conserved sequence differences can have a functionally significant dynamical impact.

Long range dynamics induced by activation loop phosphorylation

In an impressive NMR study examining dynamics across the entire kinase domain, Seeliger and coworkers have measured the response to SRC activation loop phosphorylation, conformationally selective inhibitors, and peptide substrate binding (80). We limit our discussion here to the role of activation loop phosphorylation in affecting long-range dynamical changes. The authors compare backbone amide resonance intensities for phosphorylated versus unphosphorylated SRC kinase domain. Decreased intensity, or peak broadening, upon phosphorylation suggests increased dynamics/conformational exchange in the milli- to microsecond range while an increase in peak intensity is consistent with rigidification or decreased conformational exchange. The most pronounced changes resulting from activation loop phosphorylation segregate into two discrete regions within the SRC kinase domain (Fig. 2a).

Phosphorylation induced increases in dynamics are restricted to regions that control negative regulation (distal side of the C-lobe). In contrast, phosphorylation dampens kinase domain motions on the milli- to microsecond timescale in regions that are involved in catalysis and substrate recognition (Fig. 2a). These data support a model in which autophosphorylation triggers dynamical changes in autoinhibitory regions surrounding the C-terminal tail and C-lobe contacts to the SH2 domain and/or CSK (81) possibly resulting in further conformational shifts toward the active state. Increased dynamics in these autoinhibitory regions are balanced by phosphorylation induced rigidification in regions linked to the catalytic cycle and substrate docking (Fig. 2a). Decreased dynamics in the N-lobe ($\beta 3$ - αC and glycine rich loop), the strand preceding DFG, the activation segment and the G-helix suggest that activation loop phosphorylation might pre-organize specific regions of the kinase domain for substrate recognition and catalytic turnover. The precise dynamical changes and long-range communication uncovered by the elegant use of NMR provide valuable insight into how Src module kinase dynamics are modulated to shift the conformational ensemble between inactive and active states.

Flanking the Src module: intrinsically disordered regions and internal activating segments

The SRC, TEC and ABL kinases each contain N-terminal sequences that differ substantially between families (Fig. 1a). For SRC, the region spanning the amino-terminus to the SH3 domain contains the myristylation site and a poorly conserved region termed the ‘unique’ domain. Elegant solution studies by Pons and co-workers (82–85) provide data derived from SAXS and NMR paramagnetic relaxation enhancement to support a partially preorganized, dynamic structure for the unique domain of several SRC kinases. The findings also support a role for the SH3 domain in limiting the conformational ensemble of the unique region. There is evidence that the unique region and its structural preferences, even if “fuzzy”, are functionally important and perhaps the differences between the SRC unique regions permit tuning of each kinase to specific cellular cues (83). The recent report detailing NMR resonance assignments for the unique domain of HCK (86) promises continued activity in this area to ultimately define the role of these highly dynamic sequences in the SRC family.

In contrast to the intrinsically disordered structure at the amino-terminus of the SRC kinases, the TEC family are distinguished by the Pleckstrin Homology (PH)/Tec Homology (TH) domains (Fig. 1a). The PH domain has been well characterized as a phosphoinositol binding domain that mediates transient membrane association of the TEC kinases following cellular stimulation. Recent crystal structures (39) and solution NMR and HDX-MS data (54) show that the BTK PH domain also serves a negative regulatory role much like the intramolecular regulatory function of the SH3 and SH2 domains. Crystallography and solution studies reveal different binding sites for the PH domain on the BTK kinase domain perhaps indicating the dynamic nature of the multidomain protein during the transition from inactive to active kinase.

The TEC family kinases contain a proline rich region that is sandwiched between the PHTH and SH3 domains and conforms to the classical SH3 domain ligand sequence (87). Solution NMR work has demonstrated that the proline rich regions of BTK, ITK and TXK all associate with the neighboring SH3 domain in both intra- and intermolecular fashion (88–91). The TEC kinases share the proline-rich region with ABL, in which a PXXP motif is located in the N-terminal cap sequence. For both kinases, the proline motif serves as an internal activating device (Fig. 2b,c); the PXXP sequences compete with the SH2-kinase linker region in ABL and BTK for binding to the SH3 domain, thereby destabilizing the autoinhibited form to facilitate activation of the full-length kinases (54, 74). Interestingly, the TEC and ABL kinases have been described as graded switches rather than binary on/off switches during signaling (74, 92). The dynamic plasticity of these multidomain kinases may be driven by regions outside of the Src module cassette that can respond to the intracellular environment to tune the activity of the catalytic domain accordingly. Whether the unique domain of SRC also serves an activating role by triggering SH3 domain displacement is not yet clear although binding of a proline-rich peptide to the LYN SH3 domain abolishes the specific interaction between the unique domain and the SH3 domain (84).

Conclusions

Extracting information about protein dynamics clearly enriches the information provided by x-ray crystallography. Abundant experimental evidence demonstrates the coordinated dynamical motions control conformational preferences and hence the catalytic activity of individual kinases. We are experiencing an exciting time for basic science as the tools to measure dynamic allostery have matured and, while not discussed in this review, computational methods are equally valuable in deciphering how motion controls function. This wealth of insight into kinase dynamics extends well beyond the Src module kinases and is influencing broader thinking about controlling kinase function during disease. Continued efforts to decipher molecular motions across a range of timescales promises to significantly expand the therapeutic repertoire aimed at protein kinases.

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Abbreviations

NMR	nuclear magnetic resonance
HDX-MS	hydrogen/deuterium exchange mass spectrometry
SAXS	small angle x-ray scattering
SRC	proto-oncogene c-SRC
v-SRC	oncogenic gene product of Rous Sarcoma virus
ABL	Abelson murine leukemia viral oncogene homolog 1
TEC	tyrosine kinase expressed in hepatocellular carcinoma
CSK	C-terminal Src kinase
HCK	hemopoietic cell kinase
PKA	Protein Kinase A
BTK	Bruton's tyrosine kinase
ITK	interleukin-2-inducible T-cell kinase
TXK	TXK tyrosine kinase
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
LYN	Lck/Yes novel tyrosine kinase
SH3	Src Homology 3

SH2	Src homology 2
pY	phosphotyrosine
PH	pleckstrin homology
TH	Tec homology
C-terminal	carboxy terminal
C-lobe	carboxy lobe
N-terminal	amino terminal
N-lobe	amino lobe
ATP	adenosine triphosphate
ADP	adenosine diphosphate
Asp	aspartate
Gly	glycine
Leu	leucine
Trp	tryptophan
Tyr	tyrosine
Thr	threonine
R-spine	regulatory spine
DFG	aspartate-phenylalanine-glycine
PXXP	proline-X-X-proline

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Perspectives

- The important role of dynamics in controlling kinase activation is underscored by observations that small molecule inhibitors exploit different dynamic profiles of otherwise closely related kinases. Thus, advancing knowledge of kinase regulatory mechanisms at the level of dynamics continues to be necessary to devise more effective treatments and overcome drug resistance.
- Current solution methods such as NMR, HDX-MS and SAXS analyses are enhancing the information available from x-ray crystal structures. The conformational ensembles present in solution and the shifts in the equilibria of these ensembles that dictate activation or autoinhibition are being defined at ever increasing levels of spatial and temporal detail. Moreover, these methods are being applied to regions of protein kinases that have been recalcitrant to crystallography providing valuable insight into the regulatory function of disordered regions and internal activating motifs.
- Future efforts will continue to push the boundaries of dynamics measurements as size capabilities increase and newer methodologies such as cryo-electron microscopy are applied more broadly to large, physiologically relevant kinase complexes.

TEXT BOX 1**Protein kinase discovery and kinase domain architecture**

Current insights into the biochemistry and structural details of the protein kinase family began sixty years ago, when Krebs, Graves, and Fischer carried out the first purification of a protein kinase, phosphorylase kinase (93–96). In 1968, Walsh, Perkins and Krebs (97) isolated another serine/threonine kinase, adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinase or Protein Kinase A (PKA). The field had to wait until 1991 for the first glimpse of the kinase domain architecture when Knighton, Zheng, Ten Eyck, Ashford, Xuong, Taylor and Sowadski published the first structures of the catalytic subunit of PKA (98, 99). In the time since these structures were solved, the regulatory motifs and catalytic apparatus of the bi-lobal kinase domain, including the regulatory and catalytic spines, the DFG motif, the C-helix, and the activation loop (see Figure), have come into sharp focus greatly advancing our understanding of the structural transitions between active and inactive enzyme (100–103). Ten years after PKA was isolated, the first tyrosine kinase, the avian sarcoma virus *src* gene product, pp60^{src}, was purified by Erikson, Collett, Erikson, and Purcio (104). An explosion of information about a larger number of kinases ensued (105) and today we realize the human kinome (106) controls an enormous number of reversible phosphosites in human cells (107).

Protein kinase domain architecture. Key motifs that control the conformational transition between inactive and active kinase domain are shown. The study of protein dynamics is augmenting the view of kinase regulation derived from crystallography.

TEXT BOX 2**CSK regulation – the non-conforming Src module kinase**

C-terminal SRC Kinase (CSK) and CSK homologous kinase (CHK) negatively regulate SRC kinases by phosphorylating the tyrosine residue in the SRC C-terminal tail (108, 109). CSK is ubiquitously expressed while CHK is expressed primarily in neural and hematopoietic cells. CSK achieves exquisite specificity toward its SRC substrate via the highly conserved glutamines flanking the regulatory tyrosine in the SRC tail and through contacts between the CSK kinase domain and the α H/ α I loop & I-helix of SRC (110–112). While CSK shares the SH3, SH2 and kinase domain of Src module kinases, its regulation is entirely distinct (113). Similar to the TEC family, CSK lacks the SRC family C-terminal regulatory tail sequence and does not contain a myristylation site at the N-terminus. Perhaps the most profound difference between the primary sequence of CSK and other Src molecule kinases is the absence of the activation loop tyrosine autophosphorylation site and the three preceding residues common to SRC, TEC and ABL kinases. This shortened activation loop prevents peptide substrate docking and ensures specificity toward the SRC C-terminal tail (81, 112, 114).

The dynamic equilibrium between inactive and active CSK is also strikingly different from that of the other Src module kinases. In the inactive CSK structure, the SH3 and SH2 domains assemble only onto the N-lobe of the kinase domain and the ligand binding pockets of these regulatory domains are accessible to exogenous ligands instead of shielded by autoinhibitory interactions (40). The unique specificity of CSK and restricted cellular localization (115) may mean that, unlike the other Src module kinases, accessible SH3 and SH2 binding sites do not lead to spurious phosphorylation events in the cell. In both active and inactive CSK, the SH2-kinase linker and the SH3-SH2 linker (or connector) transverse the kinase domain N-lobe creating distinct contacts depending on the activation state. The largest conformational shift upon activation is movement of the CSK SH2 domain (116, 117) but not the SH3 domain, indeed, solution NMR work has shown that the CSK SH3 and SH2 domain exhibit distinct dynamical interactions with the kinase domain (118). A review by *la et al.* describes the CSK active/inactive conformational transition in superb detail (81).

TEXT BOX 3**Visiting extended conformations in solution – the view derived from SAXS data**

Using SAXS data, Jennings and co-workers report that CSK adopts an ensemble of extended structures in solution and does not exclusively populate the assembled structure viewed by crystallography (119). A similar finding is reported for the TEC family kinase, BTK; SAXS data suggest that the domains of BTK adopt an extended conformation in solution (55). Interestingly, the crystal structure of the SH3-SH2-kinase fragment of BTK shows a domain swapped dimer (39) wherein all of the autoinhibitory contacts are intact but the overall BTK polypeptide chain is extended. The apparent expanded conformations of CSK and BTK in solution are in contrast to SAXS data for SRC (120); 85% of the SRC population in solution is consistent with the compact, autoinhibited conformation evident in the crystal structure. Perhaps the most thorough SAXS analyses of a Src module kinase are those of ABL, the compact autoinhibited form is evident for the wild type protein, drug binding to the active site affects domain arrangements, and studies of the SH2-kinase fragment show the more open, extended form associated with active kinase (38, 53, 121). Unexpectedly, additional studies showed that an activating mutation that abolishes myristate binding retains the assembled domain structure associated with the autoinhibited state (73). This finding is consistent with the notion that the SRC pY tail is not necessary to maintain the autoinhibited SRC conformation (68). Overall, the use of SAXS to interrogate conformational preferences in solution clearly underscores the dynamic nature of these multidomain kinases and their capacity to visit conformational ensembles that are not routinely evident through x-ray crystallography. In each case, however, it need be pointed out that the kinases are studied in isolation and that cellular binding partners might skew the conformational status of these proteins significantly.

Figure 1.

Src module kinases share the SH3-SH2-kinase cassette. (a) Domain organization of the SRC, TEC, ABL and CSK families. Regions flanking the Src module vary across kinase families. FRK and BRK are not included but are related to the SRC family. (b) Autoinhibited structure of the Src module. The structure shown is that of SRC (PDB: 2SRC), which unlike the TEC, ABL and CSK kinases contains the C-terminal phosphotyrosine tail (cyan). (c) Four SH3/proline-rich peptide complex structures (PDB: 1QWF, 1PRL, 1PRM, 1RLP) are superimposed onto the structure of autoinhibited SRC (PDB: 2SRC). The SH2-kinase linker

of autoinhibited SRC is in magenta and the proline rich peptides are in gray clustered around the magenta linker. Gly254 and Leu255 from the SH2-kinase linker are labeled (alpha carbons are shown with spheres). (d) The hydrophobic stack discussed in the text is shown; W286 (distal face of N-lobe), L255 (SH2-kinase linker), and Y326 (distal face of N-lobe). Bound nucleotide and the gatekeeper residue are labeled. (e) Two views of the SRC and HCK hydrophobic stacks.

Figure 2.

SRC activation loop phosphorylation and ABL/TEC conformational gymnastics. (a) NMR peak intensity data from Seeliger (80) is mapped onto the autoinhibited SRC structure. Blue indicates increased motions (decreased peak intensities) upon activation loop phosphorylation where the ratio of the peak intensity (I) for the phosphorylated SRC kinase domain/peak intensity (I) for the unphosphorylated SRC kinase domain is less than 0.5. Red indicates decreased milli- to microsecond timescale motions (increased peak intensities) upon activation loop phosphorylation where the ratio of the peak intensity (I) for the

phosphorylated SRC kinase domain/peak intensity (I) for the unphosphorylated SRC kinase domain is greater than 1.9. (b) Model showing the equilibrium between inactive and active ABL. (left) Inactive ABL: the structure of the ABL N-cap/C-cap-SH3-SH2-linker fragment (PDB: 6AMV) is shown in surface rendering for the SH3 and SH2 domains and the SH2-kinase linker is magenta (occupying the SH3 binding pocket) and the PXXP motif is unstructured. The kinase domain is indicated schematically and shows the absence of the Lys/Glu salt bridge and the inactive conformation of the C-helix and activation loop. (right) Active ABL: the ABL N-cap/C-cap-SH3-SH2-linker fragment (PDB: 6AMW) shows the alternative extended conformation where the internal PXXP motif displaces the SH2-kinase linker from the SH3 domain leading to active kinase. The Lys/Glu salt bridge forms following the conformational transition of the C-helix and the activation loop no longer adopts the closed autoinhibited conformation (dotted line indicates absence of electron density for this mobile loop). (c) Comparison of the intramolecular PXXP/SK3 complexes in ABL (PDB: 6AMW, top) and ITK (PDB: 1AWJ, bottom). In both the PXXP motif displaces the SH2-linker from the SK3 domain shifting the conformational preference toward the active state.