Protein tyrosine kinases: autoregulation and small-molecule inhibition
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Receptor and non-receptor protein tyrosine kinases (PTKs) are essential enzymes in cellular signaling processes that regulate cell growth, differentiation, migration and metabolism. The kinase activity of PTKs is tightly controlled through steric, autoregulatory mechanisms, as well as by the action of protein tyrosine phosphatases. Recent structural studies have revealed several modes of autoregulation governing the catalytic state of these enzymes. Ablcral catalytic activity of many PTKs, via mutation or overexpression, plays an important role in numerous pathological conditions, including cancer. Structural studies of the Abl tyrosine kinase domain in complex with the small-molecule inhibitor STI571 provide a molecular basis for understanding the specificity determinants of this highly successful drug used in the treatment of chronic myeloid leukemia.

Introduction
Tyrosine phosphorylation is an essential, regulated posttranslational modification utilized by multicellular organisms for cell-to-cell communication. This covalent modification occurs on select tyrosine residues in target proteins and is catalyzed by a family of enzymes known as protein tyrosine kinases (PTKs), which use ATP as a phosphate donor. PTKs belong to one of two families, the transmembrane receptor (type I) family or the non-receptor family, which are estimated to contain 58 and 32 members in the human genome, respectively [1•]. The receptor PTK family includes, among others, epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, fibroblast growth factor (FGF) receptor and the insulin receptor. Examples of the non-receptor family include Src, Abl, Fak and the Janus kinases. For appropriate subcellular localization, non-receptor PTKs typically contain modular domains that mediate protein–protein (e.g. Src homology 2 and 3 [SH2/SH3] domains) or protein–lipid interactions (e.g. pleckstrin homology [PH] domain), or are lipid-modified (e.g. myristoylated). In general, tyrosine autophosphorylation serves to stimulate the catalytic activity of PTKs and to generate docking sites for recruitment of substrate proteins. PTKs are critical components in signal transduction pathways that mediate cell proliferation, differentiation, migration and metabolism, and are active during organismal development and adult homeostasis. PTKs also play primary roles in the onset or progression of pathological conditions such as diabetic retinopathy, atherosclerosis and cancer.

Numerous PTKs have been identified as targets for anti-cancer therapy [1•,2]. ErbB2/Her2 is a member of the EGF receptor subfamily and is overexpressed in a subset of aggressive breast tumors [3]. A monoclonal antibody (Herceptin) directed against the extracellular domain of ErbB2 is currently used in the treatment of ErbB2-positive breast cancers [4], and two small-molecule inhibitors (Iressa, Tarceva) targeting the tyrosine kinase domain of ErbB2 are in late-stage clinical trials [5,6]. Because growing tumors require new blood vessel formation, the receptor PTKs for vascular endothelial growth factor (VEGFR1-2) and angiopoietin-1 (Tie2) are targets for inhibition [7]. Several naturally occurring mutations in the cytoplasmic juxtamembrane region of Kit, the receptor PTK for stem cell factor, have been linked to gastrointestinal stromal tumors [8]. As a final example, in chronic myeloid leukemia (CML), a chromosomal translocation (Philadelphia chromosome) results in the fusion of the bcr gene with the gene encoding the non-receptor PTK Abl, rendering a constitutively active Abl kinase. The Abl small-molecule inhibitor STI571 (also known as Gleevec/imatinib) has been highly effective in treating this cancer [9•]. Indeed, STI571 represents an important milestone in the development of targeted cancer therapeutics.

The first part of this review will focus on the structural mechanisms by which the catalytic activity of receptor and non-receptor PTKs is maintained at a low level prior to activation, typically initiated by extracellular stimuli. In the second part, the structural basis for inhibition of Abl by STI571 will be highlighted in the context of small-molecule inhibition of PTKs.

Protein tyrosine kinase structure and autoregulation
Receptor PTKs are cell-surface, transmembrane receptors possessing a multidomain extracellular portion that binds polypeptide ligands, a single-pass transmembrane helix, and a cytoplasmic portion containing a tyrosine kinase domain and regulatory sequences both N- and C-terminal to the kinase domain. The domain organization of non-receptor
PTKs exhibit considerable variability. Besides a tyrosine kinase domain, these proteins often possess several additional signaling domains, such as the SH2 domain (phosphotyrosine binding), the SH3 domain (polyproline binding) and the PH domain (phosphatidylinositol binding).

The tyrosine kinase domain of PTKs spans approximately 300 residues and adopts a two-domain architecture that is shared by the protein serine/threonine kinase family. It consists of an N-terminal lobe comprising a five-stranded β sheet and one α helix, and a larger C-terminal lobe that is mainly α-helical. ATP binds in the cleft between the two lobes and the tyrosine-containing segment of a protein substrate interacts with residues in the C-terminal lobe (Figure 1a). Several noncontiguous polypeptide segments of the kinase domain contribute to the formation of the active site, including the nucleotide-binding loop, β-strand 3 and α-helix C in the N-terminal lobe, and the catalytic and activation loops in the C-terminal lobe (Figure 1b).

Most PTKs are maintained in a low (basal) activity state through various autoregulatory mechanisms, all of which prevent the optimal configuration of the aforementioned polypeptide segments at the kinase active site. Although the conformation of the catalytic loop is remarkably similar from one PTK to the next, and between inactive and active states, other segments, in particular α-helix C and the activation loop, are often switch elements in intrasteric regulation. In general, activation of receptor PTKs is achieved through ligand binding to the extracellular domain, which stabilizes a dimeric (or higher order) receptor arrangement, facilitating trans-phosphorylation in the cytoplasmic domain. For non-receptor PTKs, the activation mechanisms are more complex, involving heterologous protein–protein interactions, as well as clustering to enable trans-phosphorylation.

**Autoregulatory mechanisms in receptor protein tyrosine kinases**

For many receptor and non-receptor PTKs, the activation loop, which serves as a platform for substrate binding and contributes an aspartic acid for Mg²⁺ ion coordination [10] (Figure 1b), is not properly configured in the basal state [11,12]. Trans-phosphorylation of one or more tyrosines in the activation loop leads to a repositioning of the activation loop and a concomitant increase in catalytic efficiency [10,13,14] (Figure 2a). The kinase activity of the insulin receptor is regulated almost exclusively through activation loop autophosphorylation (facilitated by insulin binding). In the unphosphorylated state, Tyr1162 in the activation loop acts as a pseudosubstrate inhibitor [15]. The crystal structure of the cytoplasmic domain of MuSK reveals that the activation loop plays a similar role in the autoregulation of this receptor PTK [16] (Figure 2b).

For a subset of receptor PTKs, including the ephrin receptors and the PDGF receptor subfamily, the cytoplasmic juxtamembrane region, between the transmembrane helix
and the kinase domain, has been implicated in the regulation of catalytic activity [8,17,18]. The juxtamembrane region of these receptor PTKs, each of which contains two tyrosine autophosphorylation sites in different sequence contexts, is thought to interact with the kinase domain to repress activity. A crystal structure of the tyrosine kinase domain and juxtamembrane region of the ephrin receptor EphB2 provides a structural basis for juxtamembrane autoinhibition in this subfamily of receptor PTKs [19]. In the structure, the juxtamembrane region interacts with both the N- and C-terminal kinase lobes, distorting α-helix C and preventing the activation loop from adopting its active configuration (Figure 2c).

Trans-phosphorylation of the two juxtamembrane tyrosines (via ligand-induced dimerization) is predicted to disrupt the inhibitory juxtamembrane–kinase interactions and, in addition, generate recruitment sites for SH2-containing proteins. Trans-phosphorylation of the activation loop on a single tyrosine would readily follow to complete the kinase activation process.

The juxtamembrane region involved in autoinhibition of EphB2 contains an α-helix, but lacks higher-order tertiary structure. A recent mutagenesis study [20] suggests that the juxtamembrane region of members of the PDGF receptor subfamily, including Kit, adopts the three-dimensional fold of a WW domain, a small, three-stranded structural motif that generally recognizes proline-containing ligands [21]. Interestingly, the second tryptophan (Trp593) of the putative WW domain in PDGF receptor-β marks the beginning of the kinase domain (as judged from PTK crystal structures) and is found in many non-receptor and receptor PTKs that do not possess a WW domain (e.g. Src [Trp260] and the insulin receptor [Trp989]). Structural studies will be needed to confirm whether the juxtamembrane region of PDGF receptors does indeed possess a WW-like domain and, if so, the mechanism by which it represses activity.

The segment C-terminal to the core kinase domain, which shows considerable variability in length and amino acid sequence in receptor PTKs, can also play a role in autoregulation. A crystal structure of the tyrosine kinase domain of Tie2 revealed that the C-terminal segment, which contains one or two tyrosine autophosphorylation sites, is stabilized through numerous interactions in a location near the substrate-binding site [22]. The autoinhibitory nature of this segment in Tie2 has been verified by biochemical studies of a C-terminal deletion mutant, which exhibits highly elevated kinase activity [23]. Again, ligand-facilitated trans-phosphorylation, this time in the C-terminal segment, would be predicted to destabilize the autoinhibitory conformation.

### Autoregulatory mechanisms in non-receptor protein tyrosine kinases

The Src family kinases are the prototypical non-receptor PTKs, and their structure has been studied extensively [13,24–29]. The concerted intramolecular interactions between the SH2 domain and the phosphorylated C-terminal tail, and between the SH3 domain and the SH2–kinase linker, prevent the kinase domain from adopting an active configuration [30] (Figure 3a). Engagement
of the SH2 and SH3 domains with phosphotyrosine- or polyproline-containing ligands, respectively, or dephosphorylation of the C-terminal tail, releases the constraints on the kinase domain, leading to trans-phosphorylation of the activation loop and a fully active enzyme.

Like Src family kinases, Abl possesses an SH3-SH2-kinase core configuration, yet lacks the C-terminal tyrosine phosphorylation site important for maintaining the repressed state of Src. This intrinsic difference, along with supporting experimental evidence, led to the hypothesis that Abl was kept in a downregulated state through a cellular inhibitory protein [31]. Although a crystal structure of the SH3, SH2 and kinase domains of Abl is not yet available, a recent biochemical study has demonstrated that, in fact, Abl is autoregulated through its unique N-terminal region [32••]. On the basis of mutagenesis data, a model has been proposed in which the N-terminal 80 residues contact both the SH3 domain and the N-terminal kinase lobe to stabilize a repressed state [32••] (Figure 3b). Lack of this N-terminal region in the Bcr–Abl fusion protein might contribute to its oncogenicity. The crystal structure of the isolated Abl kinase domain [33••] indicates that the unphosphorylated activation loop can adopt a pseudosubstrate inhibitory conformation similar to that of the unphosphorylated activation loop of the insulin receptor [15].

CSK, the non-receptor PTK that phosphorylates the C-terminal tail of Src, also possesses an SH3-SH2-kinase core, but lacks a tyrosine phosphorylation site in the activation loop (positive regulatory) as well as a phosphorylation site in the C-terminal region (negative regulatory). Not surprisingly, then, the mode of CSK autoregulation by the SH2 and SH3 domains is different from that of Src or Abl. Rather than repressing catalytic activity, biochemical studies indicate that the SH2 and SH3 domains, the latter in particular, are required for full activity [34]. In the crystal structure of full-length CSK [35••], the SH2 and SH3 domains are in completely different locations relative to the kinase domain compared to Src, and both domains interact with the N-terminal kinase lobe (Figure 3c). The SH2 domain, the SH3-SH2 linker and the SH2–kinase linker appear to stabilize directly the catalytically competent position of α-helix C and, indirectly, the activation loop, both of which are misaligned in the crystal structure of the isolated CSK kinase domain [36]. The binding of phosphotyrosine-containing sequences to the SH2 domain may assist in stabilizing the active position of α-helix C [35••].

**Figure 3**

Autoregulatory mechanisms in non-receptor PTKs. For each structure, the SH2 domain is colored blue, the SH3 domain orange and the kinase domain green. Disordered regions are represented by spheres. The catalytic loops were superimposed to orient the structures. (a) Crystal structure of Src [28]. The negative regulatory phosphotyrosine (pTyr527) in the C-terminal tail is shown. (b) Model structure of Abl [32••] based on Src. The N-terminal segment important for Abl autoinhibition is shown schematically in magenta, interacting with the SH3 domain and N-terminal kinase lobe. (c) Crystal structure of CSK [35••].

**Small-molecule inhibition of protein tyrosine kinases**

Small-molecule PTK inhibitors identified through screening of compound libraries are invariably competitive against ATP and not substrate protein/peptide. This is evidently a result of the deep ATP-binding cleft between the two kinase lobes and the relatively shallow surface on the C-terminal lobe to which a substrate peptide binds. Some recent efforts targeting the substrate-binding site of PTKs have been reported [37,38]. Despite a relatively high degree of sequence conservation in the ATP-binding cleft, sequence variations do exist that can be exploited by ATP-competitive compounds [39]. These variations are tolerated (evolutionarily) because ATP does not completely fill the cleft in the active configuration of protein kinases (less so in inactive configurations); one or more water molecules are present in crystal structures of active protein kinases.
kinases with bound ATP (or analog) [10,40,41]. Previous crystal structures of PTKs with bound ATP-competitive inhibitors have indicated how these compounds achieve specificity [27,29,36,42,43]. More recently, a crystal structure of a 4-anilinoquinazoline compound (Tarceva) bound to the EGF receptor kinase domain reveals how this subfamily of receptor PTKs can be selectively inhibited [44•].

The remarkable success of STI571 as a cancer drug can be attributed to the strong dependence of CML on Abl kinase activity and the relatively high degree of specificity of STI571 for Abl, although STI571 is also an effective inhibitor of PDGF receptor subfamily members, including Kit. Crystal structures of the complex between STI571 and Abl indicate how this specificity is achieved [33••,45••]. In the structure, STI571 is bound in the ATP-binding cleft of unphosphorylated (activation loop) Abl, making extensive contacts with residues lining the cleft and with peptide segments just outside the cleft (Figure 4). The nucleotide-binding loop undergoes a considerable conformational change upon binding STI571, placing Tyr253 of this loop in van der Waals contact with STI571, as well as with Phe382 in the activation loop. STI571 interacts with the N-terminal portion of the Abl activation loop — the conserved protein kinase DFG sequence — making hydrophobic contacts with Phe382, a hydrogen bond to the backbone of Asp381, and van der Waals contacts with Ala380 and Asp381 (Figure 4).

Although the interactions between STI571 and the activation loop are predominantly with residues of the conserved DFG sequence, selectivity for the Abl kinase is achieved in part through ‘conformational specificity’. That is, the activation loops of pre-activated (unphosphorylated) protein kinases have preferential conformations which differ among kinases, some of which are not stERICally compatible with the binding mode of STI571 to Abl. The extent to which the activation loop conformation in the STI571–Abl complex is induced by STI571 binding is not known — the activation loop follows a much different course when complexed with an inhibitor of the pyrido-pyrimidine class [45••] — but this conformation is probably less favorable in Src family kinases, inferred from the activation loop configurations in the crystal structures of Src [28] and Hck [29]. In fact, all of the Abl sidechains in contact with STI571 are identical in Src family kinases, save for the seemingly conservative substitution Tyr253→Phe, yet STI571 is a poor inhibitor of Src [46]. The finding that STI571 is a more potent inhibitor against unphosphorylated than phosphorylated Abl provides direct evidence that the activation loop conformation is a determinant of STI571 specificity [33••]. Recent mutagenesis data, however, indicate that the difference in the nucleotide-binding loop at Tyr253 (Phe278 in Src) does contribute to specificity; the IC_{50} of STI571 is ~70-fold higher for the Abl mutant Tyr253→Phe than for wild-type Abl [47•]. Evidently, the hydrogen bond between the hydroxyl group of Tyr253 and Asn322 is important for positioning of Tyr253 near STI571 (Figure 4).

Conclusions

Recent structural and biochemical studies of PTKs have revealed several autoregulatory mechanisms, in addition to activation loop phosphorylation, to which catalytic activity is subject. These include inhibition by the juxtamembrane and C-terminal regions in receptor PTKs, and by the SH2/SH3 domains and flanking regions in non-receptor PTKs. Future structural studies will inevitably uncover additional autoregulatory mechanisms for this diverse family of enzymes. The fundamental biochemical knowledge gleaned thus far is directly relevant to the therapeutic arena. The finding that CML patients who relapse during the blast crisis phase of the disease often harbor a Thr315→Ile mutation in Bcr–Abl [48], which makes them refractory to STI571, can be rationalized from the Abl–STI571 crystal structure [33••,45••,47•] (Figure 4). Therefore, structure-based design holds the promise of generating derivatives of STI571 capable of inhibiting mutant forms of Bcr–Abl, which would provide additional therapeutic agents to combat this cancer.
Acknowledgements
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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
**of outstanding interest

This is a comprehensive review of the mechanisms by which PTKs are deregulated in human cancers.


This structural study provides a second example (after the insulin receptor) of pseudosubstrate inhibition by the kinase activation loop and shows that a helix in the juxtamembrane region of MuSK is poised for protein-protein interactions, perhaps with an inhibitory protein.


This biochemical study provides evidence that phosphorylation of the juxtamembrane tyrosines in the Eph receptor EphB2 is critical for kinase activation. Mutant EphB2 receptors in which phenylalanine is substituted for tyrosine in the juxtamembrane region do not undergo kinase activation in vitro or in cells.

This is the first structural study to reveal the mechanism by which the juxtamembrane region of a receptor PTK can function to regulate kinase activity. In the crystal structure of the juxtamembrane region and kinase domain of the ephrin receptor EphB2, the juxtamembrane region kinks off helix C and hinders the activation loop from assuming its active configuration. Phosphorylation of two juxtamembrane tyrosines is predicted to disrupt these inhibitory interactions, leading to kinase activation.

This mutagenesis study, along with sequence comparisons, provides evidence that the juxtamembrane region of PDGFR receptor subfamily members contains a WW-like domain that inhibits kinase activity in the basal state. Activating mutations in the juxtamembrane region can be rationalized based on a model of the juxtamembrane region as a WW domain.


The structure of the Tie2 kinase domain shows that the C-terminal tail, which contains a site(s) of tyrosine phosphorylation, might partially interfere with substrate binding. In addition, the nucleotide-binding loop is positioned to occlude ATP binding.


This biochemical study provides evidence that Abl is autoregulated through interactions between its unique N-terminal region and the SH3 and tyrosine kinase domains. The previous consensus was that a cellular inhibitory protein was involved in maintaining the repressed state of Abl.
The structure of the tyrosine kinase domain of Abl in complex with a variant of the small-molecule inhibitor STI571 establishes the mode of inhibition of this clinically relevant inhibitor. This study provides an example of ‘conformational specificity’, in which interactions with conserved sidechains nevertheless contribute to specificity through differential positioning of the conserved residues. The unphosphorylated form of Abl is shown to be more susceptible to inhibition by STI571 than the phosphorylated, activated form.

This is the first report of a crystal structure of the EGF receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem 2002, in press. Crystal structures of the Abl kinase domain with bona fide STI571, as well as with an inhibitor of the pyrido-pyrimidine class, reveal different modes of interaction between the compounds and the unphosphorylated activation loop. The pyrido-pyrimidine compound inhibits both unphosphorylated and phosphorylated forms of Abl equally well, which might explain its tenfold higher potency over STI571.

Cytokine receptors are common targets for specific small-molecule inhibitors. J Biol Chem 2002, in press. This mutagenesis study tests the effects of various Abl mutations on inhibition by STI571 and ATP binding. The Thr315→Ile mutation found in relapsed CML patients shows a marked decrease in STI571 susceptibility without loss of ATP binding, as would be predicted. The study reveals additional mutations that confer STI571 insensitivity while maintaining Abl catalytic activity.

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