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Breakthroughs and Views

Structure and regulation of Kit protein-tyrosine kinase—The stem cell factor receptor ☆

Robert Roskoski Jr. *

Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1100 Florida Avenue, New Orleans, LA 70119, USA

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Abstract

Signaling by stem cell factor and Kit, its receptor, play important roles in gametogenesis, hematopoiesis, mast cell development and function, and melanogenesis. Moreover, human and mouse embryonic stem cells express Kit transcripts. Stem cell factor exists as both a soluble and a membrane-bound glycoprotein while Kit is a glycoprotein receptor protein-tyrosine kinase. The complete absence of stem cell factor or Kit is lethal. Gain-of-function mutations of Kit are associated with several human neoplasms including acute myelogenous leukemia, gastrointestinal stromal tumors, mastocytomas, and nasal T-cell lymphomas. Binding of stem cell factor to Kit results in receptor dimerization and activation of protein kinase activity. The activated receptor becomes autophosphorylated at tyrosine residues that serve as docking sites for signal transduction molecules containing SH2 domains. Kit activates Akt, Src family kinases, phosphatidylinositol 3-kinase, phospholipase Cγ, and Ras/mitogen-activated protein kinases. Kit exists in active and inactive conformations as determined by X-ray crystallography. Kit consists of an extracellular domain, a transmembrane segment, a juxtamembrane domain, and a protein kinase domain that contains an insert of about 80 amino acid residues. The juxtamembrane domain inhibits enzyme activity in *cis* by maintaining the control αC-helix and the activation loop in their inactive conformations. The juxtamembrane domain also inhibits receptor dimerization. STI-571, a clinically effective targeted protein-tyrosine kinase inhibitor, binds to an inactive conformation of Kit. The majority of human gastrointestinal stromal tumors have Kit gain-of-function mutations in the juxtamembrane domain, and most people with these tumors respond to STI-571. STI-571 binds to Kit and Bcr-Abl (the oncoprotein of chronic myelogenous leukemia) at their ATP-binding sites.

Keywords: Abl; Activation loop; Acute myelogenous leukemia; Bcr-Abl; Cell signaling; Chronic myelogenous leukemia; Flt3; Dysgerminoma; Germ cells; Gleevec; Mast cells; Nasal T-cell lymphoma; Oncogene; Proto-oncogene; Seminoma; STI-571, Targeted cancer therapy

Protein kinases are enzymes that play a key regulatory role in nearly every aspect of cell biology [1]. They regulate apoptosis, cell cycle progression and proliferation, cytoskeletal rearrangement, differentiation, development, the immune response, motility, nervous system function, and transcription. Owing to the myriad actions of protein kinases, it is imperative that they be stringently regulated because aberrant activity of these enzymes leads to a variety of diseases including cancer, diabetes, and autoimmune, cardiovascular, inflammatory, and nervous disorders. Considerable effort has been expended to determine the physiological and pathological functions of protein kinase signal transduction pathways. Because mutations and dysregulation of protein kinases play causal roles in human disease, these enzymes represent attractive drug targets [2].

Protein kinases catalyze the following reaction:

$$\mathrm{MgATP^{1-}} + \text{protein-OH} \rightarrow \text{protein-OPO}_3^{2-} + \mathrm{MgADP} + \mathrm{H^+}$$

Based upon the nature of the phosphorylated –OH group, these enzymes are classified as protein-serine/threonine kinases and protein-tyrosine kinases. Manning et al. [3]

☆ *Abbreviations:* GIST, gastrointestinal stromal tumors; JM, juxtamembrane; PDGF, platelet-derived growth factor; pTyr, phosphotyrosine; PTB, phosphotyrosine binding; SCF, stem cell factor; SH2, Src homology 2; SH3, Src homology 3.

* Fax: +1 504 619 8775.
E-mail address: biocrr@lsuhsc.edu.

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identified 478 typical and 40 atypical protein kinase genes in humans (total 518) that correspond to about 2% of all human genes. The family includes 385 protein-serine/threonine kinases, 90 protein-tyrosine kinases, and 43 protein-tyrosine kinase-like molecules. Of the 90 protein-tyrosine kinases, a total of 58 are receptor and 32 are non-receptor in nature. The protein kinase family is the second largest enzyme family (after proteases) and the fifth largest gene family in humans [4].

Kit—the stem cell factor receptor

Kit is a type III receptor protein-tyrosine kinase [5] (see [6] for a description of type I through IX receptor protein-tyrosine kinases). The type III class also includes the platelet-derived growth factor (PDGF) receptor (α- and β-chains), the macrophage colony-stimulating-factor receptor (CSF-1), and the Fl cytokine receptor (Flt3). Receptor protein-tyrosine kinases all share the same topology: an extracellular ligand-binding domain, a single transmembrane segment, and a cytoplasmic kinase domain. The class III receptors are characterized by the presence of five immunoglobulin-like domains in their extracellular portion. Stem cell factor (SCF) binds to the second and third immunoglobulin domains while the fourth domain plays a role in receptor dimerization [7]. The structure of the class III receptors differs from that of other receptor tyrosyl kinases by the insertion of 70–100 amino acids near the middle of the kinase domain. In human Kit, the kinase insert is about 80 residues in length (Fig. 1); this domain undergoes phosphorylation and serves as a docking site for a few pivotal signal transduction proteins. The vascular endothelial growth factor receptor family contains seven immunoglobulin-like extracellular domains and a kinase insert like the PDGF receptor family [6].

Kit signaling is important in erythropoiesis, lymphopoiesis, mast cell development and function, megakaryopoiesis, gametogenesis, and melanogenesis [8]. Stem cells in both embryos and adults have the unique ability to balance self-renewal and differentiation such that mature cells necessary for the function of specific organ systems can be generated and replaced without depletion of the stem cell pool. The origin of the term stem cell factor referred initially to its role in survival, self-renewal, and differentiation of hematopoietic stem cells. However, recent work indicates that the pluripotent R1 mouse embryonic stem cell line expresses Kit transcripts and functional protein [9].

Using gene arrays and reverse-transcriptase polymerase chain reactions, Palmqvist et al. [9] found that Kit transcripts decrease to 20% of their initial value 72 h after the removal of leukemia inhibitory factor (LIF) as mouse embryonic stem cells lose their pluripotency and become differentiated. The authors suggest that differentiated cell types emerge later that re-express Kit. The R1 embryonic stem cell line does not express SCF, but irradiated mouse embryo fibroblasts that nourish the cell line in culture express SCF transcripts.

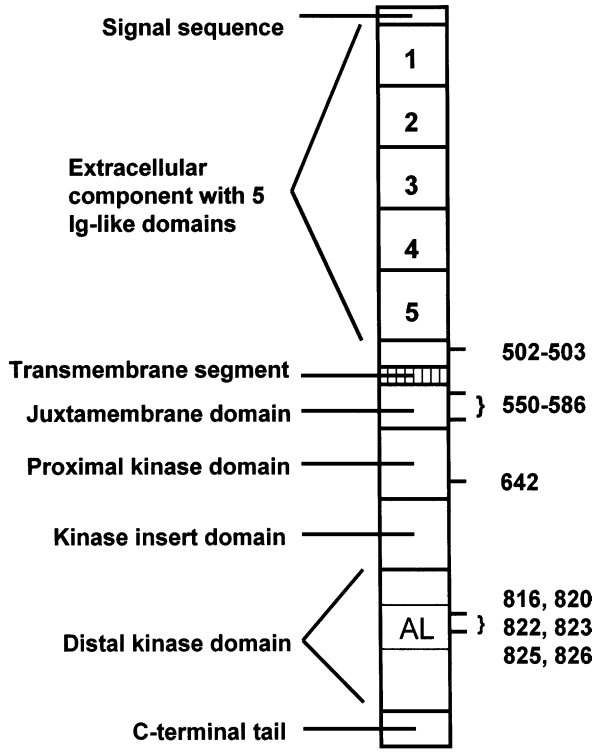

Fig. 1. Organization of Kit. The relative length of the domains is to scale. The location of Kit gain-of-function mutations is indicated by the residue numbers on the right hand side of the figure. Ig, immunoglobulin; AL, activation loop.

Stem cell factor and Kit signaling pathways

Binding of SCF to Kit leads to receptor dimerization and activation of protein kinase activity [10]. The receptor becomes autophosphorylated at tyrosine residues during activation; the resulting phosphotyrosine residues serve as docking sites for signal transduction molecules containing SH2 and phosphotyrosine-binding (PTB) domains. Activated Kit also catalyzes the phosphorylation of substrate proteins.

Kit has the potential to participate in multiple signal transduction pathways as a result of interacting with several enzymes and adaptor proteins [11]. The adaptor protein APS, Src family kinases, and Shp2 tyrosyl phosphatase bind to phosphotyrosine 568. Shp1 tyrosyl phosphatase and the adaptor protein Shc bind to phosphotyrosine 570. C-terminal Src kinase homologous kinase (Chk) and the adaptor Shc bind to both phosphotyrosines 568 and 570. These residues occur in the juxtamembrane domain of Kit. Three residues in the kinase insert domain are phosphorylated and attract: (a) the adaptor protein Grb2 (Tyr703), (b) phosphatidylinositol 3-kinase (Tyr721), and (c) phospholipase Cγ (Tyr730). Phosphotyrosine 900 in the distal kinase domain binds phosphatidylinositol 3-kinase that in turn binds the adaptor protein Crk. Phosphotyrosine 936, also in the distal kinase domain, binds the adaptor proteins APS, Grb2, and Grb7 [11].

The numerous Kit interactions cited above lead to activation of several signal transduction pathways. For example, phosphatidylinositol 3-kinase leads to the activation of Akt. Akt (protein kinase B), a protein-serine/threonine kinase, promotes cell survival [12]. One substrate of Akt is Bad (Bcl2 antagonist of cell death), a pro-apoptotic protein that promotes cell death. Following phosphorylation, Bad no longer promotes apoptosis. Activation of the phosphatidylinositol 3-kinase/Akt pathway may explain in part how activating mutations of Kit participate in neoplastic transformation. Other downstream effectors of Kit include the Ras/mitogen-activated protein kinases and the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathways [13].

Overview of Kit protein kinase structure

The Kit protein-tyrosine kinase domain has the characteristic bilobed architecture observed in all protein kinases (Fig. 2) [14,15]. Residues 582–671 make up the small N-terminal lobe of the kinase, and residues 678-953 make up the large C-terminal lobe with a hinge segment between them. The small lobe has a predominantly antiparallel β-sheet structure and is involved in anchoring and orienting ATP. It contains a glycine-rich (GAGAFG) ATP-phosphate-binding loop composed of residues 596–601. The large lobe is predominantly α-helical in nature. The large lobe is responsible for binding the peptide or protein substrate. Furthermore, part of the ATP-binding site occurs in the large lobe. As described for other protein kinases, the catalytic site of Kit kinase lies in the cleft between the small and large lobes [14,15].

The two lobes of protein kinases move relative to each other and can open or close the cleft [16,17]. The open form is necessary to allow access of ATP and release of ADP from the active site; the closed form is necessary to bring residues into the catalytically active state. Any process that blocks the interconversion of the open and closed forms of the cleft will be inhibitory. The juxtamembrane (JM) region consists of residues 544–581, which lies between the transmembrane helix (521–543) and the protein kinase domain (582–937). As noted below, the JM domain of Kit inhibits kinase activity, in part, by blocking the relative movement of the two lobes.

Hanks et al. [18] identified 12 subdomains with conserved amino acid residue signatures that constitute the catalytic core of protein kinases. Of these, the following three amino acids, which define a K/D/D (Lys/Asp/Asp) motif, illustrate the catalytic properties of Kit. Lys623 is an invariant residue of protein kinases that forms salt bridges with the β- and γ-phosphates of ATP. Asp792, the catalytic base, orients the tyrosyl group of the substrate protein in a catalytically competent state and may abstract a proton from tyrosine thereby facilitating its nucleophilic attack of the γ-phosphorus atom of MgATP. Asp810 is the first residue of the activation loop found in the large lobe. The activation loop of nearly all protein kinases, including Kit, begins with DFG (810–812) and ends with APE (837–839). Asp810 binds Mg^{2+}, which in turn coordinates the β- and γ-phosphate groups of ATP.

The two kinase lobes can adopt a range of relative orientations, opening or closing the active-site cleft [16]. Within each lobe is a polypeptide segment that has active and inactive conformations [16]. In the small lobe, this segment is the major α-helix. It is designated as the C α-helix or the αC-helix (where C refers to control). The αC-helix in some kinases rotates and translates with

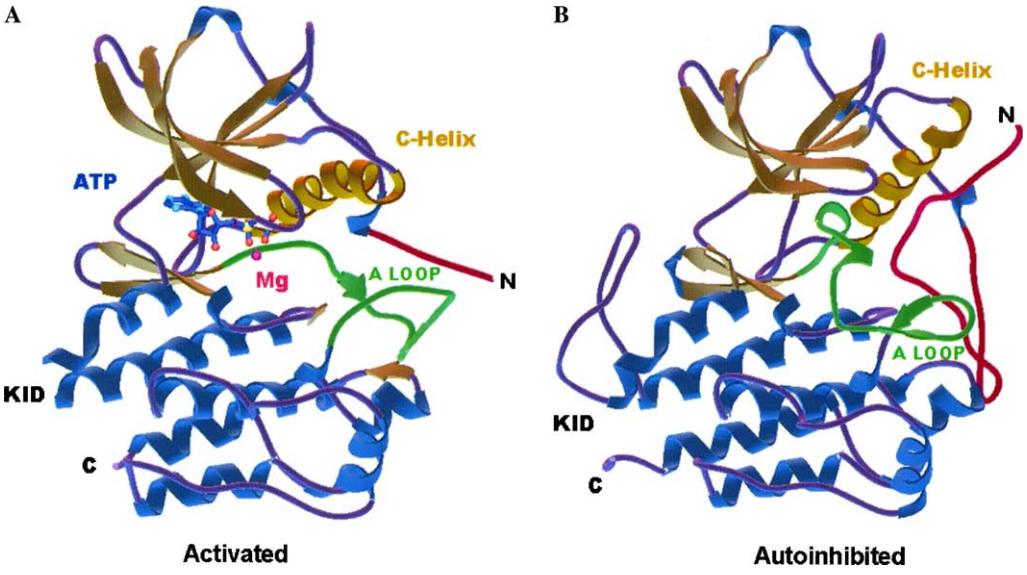

Fig. 2. Ribbon diagrams of the activated and autoinhibited forms of Kit showing the N- and C-termini. (A) The C (αC)-helix is in its productive conformation, and the A (activation) loop is in an extended conformation. The nucleotide is shown as the ball and stick model. (B) The autoinhibitory JM segment (red) inserts between the N- and C-lobes. The C (αC)-helix is in a dormant conformation, and the A (activation) loop is in a non-extended conformation. KID, kinase insert domain. The figure is reproduced from [15] with copyright permission from the *Journal of Biological Chemistry*.

respect to the rest of the lobe, making or breaking part of the catalytic site. In the large lobe, the activation loop adjusts to make or break part of the protein–substrate-binding site. In most kinases, including Kit, phosphorylation of the activation loop stabilizes the active conformation.

The catalytic loop surrounding the actual site of phosphotransfer is different for the protein-serine/threonine and protein-tyrosine kinases [19]. This loop is made up of RYDLKPEN in protein-serine/threonine kinases and HRDLAARN in protein-tyrosine kinases including Kit (His790-Asn797). Asp792 of Kit, which occurs in the catalytic loop, is the first D of the K/D/D signature sequence. The AAR sequence in the catalytic loop represents a receptor protein-tyrosine kinase signature, and RAA represents a non-receptor protein-tyrosine kinase signature. Important catalytic and regulatory human Kit residues are listed in Table 1.

Kit activation by stem cell factor

In the absence of SCF, which is the stimulatory Kit ligand, Kit exists in a monomeric dormant state. The general mechanism for activation of dormant receptor protein-tyrosine kinases involves binding of the appropriate ligand to the extracellular domain of two receptor monomers, bringing them together, and producing a receptor dimer. SCF exists as a non-covalent dimer, and this dimer binds to two Kit monomers thereby promoting Kit dimer formation [7]. For most receptor protein-tyrosine kinases, dimer formation is followed by transphosphorylation of 1–3 tyrosine residues that occur in the activation loop [16,17]. Such reactions stabilize the most active enzyme form.

Activation of Kit and the type III receptor protein-tyrosine kinases is more intricate than that of many other receptor kinases [20]. The JM segment of Kit is autoinhibitory, and this additional mechanism for maintaining a dormant enzyme state must be overcome. After dimerization, transphosphorylation of two tyrosine residues (568 and 570) in the autoinhibitory JM segment occurs [15]. As a result, the JM segment no longer immobilizes the small and large lobes in a static configuration. The activation loop is converted from a compact inactive conformation to an extended active conformation. Transphosphorylation of Tyr823 in the activation loop stabilizes the active form of the enzyme.

Table 1
Important amino acid residues in human Kit[a]

Residue or motif	
Signal sequence	1–22
Extracellular domain	23–520
Transmembrane segment	521–543
Juxtamembrane domain	544–581
JM phosphorylation sites	568, 570
Protein kinase domain	582–937
Glycine-rich nucleotide-binding loop	596–601
Phosphate-binding lysine	623
αC-Helix glutamate	640
Kinase insert domain	685–761
Kinase insert domain phosphorylation sites	703, 721, 730
Catalytic aspartate	792
Catalytic loop (HRDLAARN)	790–797
Activation loop beginning: DFG	810–812
Activation loop end: APE	837–839
Activation loop pTyr	823
C-terminal phosphorylation site	900, 936
Number of encoded amino acids	976

[a] Swiss-Prot Accession No. P10721.

Structure of active Kit

Mol et al. [14,15] determined the structure of an active and inactive conformation of the human Kit intracellular domain. The construct contained a truncated kinase insert. The active form of Kit was obtained by incubating the enzyme with MgATP to initiate the transphosphorylation reaction. Mass spectrographic analysis of the products revealed that Tyr568 and Tyr570 were the first residues to be phosphorylated. Thus, autophosphorylation of residues in the JM domain occurs before that of the activation loop Tyr823 [15]. This contrasts with the insulin and insulin-like growth factor receptors where activation loop phosphorylation occurs first [21]. However, it is unclear whether the initial transphosphorylation of the JM domain of Kit occurs in vivo. Mol et al. [15] found that Kit transphosphorylation occurs initially in the JM domain in vitro in a protein lacking the extracellular domain. Perhaps, a different result would be obtained with the complete protein or the complete protein in a cellular context.

The structure of Kit with ADP, Mg^{2+}, and the side chain of pTyr568 from an adjacent molecule bound at the active site is consistent with those of other active protein kinases [14]. The αC-helix and the activation loop are in active conformations. A glutamate residue from the αC-helix (Glu640) forms a salt bridge with Lys623 (the K of K/D/D) that bridges the α- and β-phosphates of ADP. Mg^{2+} binds to Asp810 (of the DFG sequence), Asn797, the phosphate of the phosphotyrosine residue that is a product of the transphosphorylation reaction, and the two phosphates of the ADP reaction product. Phe811 (of the DFG segment) exists in its active "on" [15] or "in" [22] conformation that allows the binding of the adenine of ADP. Most of the residues of the JM segment and the truncated kinase insert domain are disordered.

The activation loop of Kit is in its extended, or active, conformation even though Tyr823 is unphosphorylated. When the activation loop is in an extended conformation, the tyrosine residue is accessible and can be phosphorylated in *trans* by its dimer partner. The R group of Tyr823 is directed toward arginine 815 and 791. Phosphorylation of Tyr823 would further stabilize the extended conformation of the activation loop and maintain the enzyme in its active form [14,15].

Structure of inactive Kit

The Kit juxtamembrane domain inhibits kinase activity in *cis*. The JM segment of inactive Kit forms a V-shaped loop that inserts directly into the interface between the small and large lobes of the kinase (Figs. 2B and 3) [15]. This snapshot of the enzyme suggests that the JM domain has the potential to inhibit Kit by displacing the αC-helix, preventing the activation loop from assuming its extended and active conformation, and preventing the movement of the small and large lobes necessary for the binding and release of substrates.

Griffith et al. [24] determined the structure of Flt3, a type III protein-tyrosine kinase related to Kit. Flt3 is autoinhibited by the JM segment by a mechanism which is comparable to that observed for Kit. These investigators divided the JM segment into three topological components: from the N- to C-direction these are the JM-binding motif (JM-B), the JM switch (JM-S), and the JM zipper (JM-Z). The residues in human Kit that correspond to these components are 553–559 (JM-B), 560–571 (JM-S), and 572–581 (JM-Z); these are preceded by residues 544–552, the JM proximal segment (JM-P). These four components form a V-shaped structure that lies on the surface of the Kit kinase domain (Fig. 3).

The JM-B segment, which is nearly buried in Kit, interacts with nearly all structural components implicated in the activation–inactivation transitions of this enzyme. JM-B makes contacts with the glycine-rich nucleotide-binding loop, the activation loop, and the αC-helix. The JM-B segment constitutes a wedge that stabilizes the inactive kinase conformation by preventing the small lobe from rotating toward the large lobe to generate the activated kinase conformation. Moreover, JM-B prevents the extension of the compact, or non-extended, form of the activation loop from assuming its extended active state. The JM-S segment contains the two conserved tyrosine residues (568 and 570) that are the first to be transphosphorylated. The JM-Z is zippered up against the side of the N-lobe. This segment has the potential to move away (become unzippered) from the small lobe; in the active enzyme, the JM-Z segment assumes a disordered state.

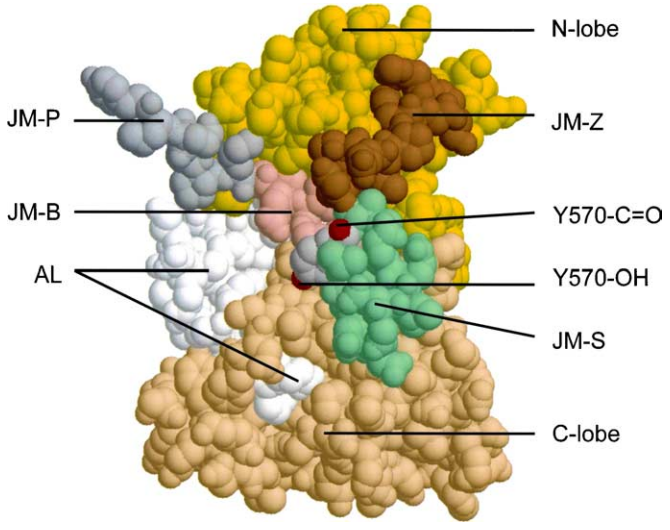

Fig. 3. A space-filling diagram of autoinhibited Kit. AL, activation loop; JM-B, juxtamembrane buried; JM-P, juxtamembrane proximal; JM-S, juxtamembrane switch; JM-Z, juxtamembrane zipper. Y570-C=O is the carbonyl oxygen of tyrosine 570. Y570-OH is the phenolic oxygen of tyrosine 570. In this view, tyrosine 568 is buried. Prepared from protein database file 1T45. pdb using Protein Explorer [23].

Several residues of the JM domain of inactive Kit form hydrophobic bonds with residues in both the small and large lobes. Moreover, Tyr553 of the JM segment forms hydrogen bonds with the side chains of buried and conserved Asp810 of the DFG segment and Glu640 of the αC-helix. In the inactive conformation, Phe811 (of the DFG segment) occurs in its inactive "off" [15] or "out" [22] conformation that prevents the binding of the adenine of ADP.

The JM domain of inactive Kit sterically blocks the activation loop from assuming an active conformation. In the autoinhibited state, the hydroxyl group of non-phosphorylated tyrosine 823 forms a hydrogen bond with the catalytic aspartate (Asp792) thereby preventing the binding of protein substrates to the active site. In addition, Asp810 of the DFG segment binds to the positively charged guanidinium group of Arg815 and not to Mg^{2+}.

Kit mutations and human neoplasms

Gain-of-function mutations occur in a percentage of human neoplasms including mastocytomas (>90%), gastrointestinal stromal tumors (>70%), sinonasal T-cell lymphomas (17%), seminomas/dysgerminomas (9%), and acute myelogenous leukemia (1%) [25]. Furthermore, autocrine or paracrine activation of Kit has been postulated in numerous other human malignancies including ovarian neoplasms and small-cell lung cancer [25,26]. Furthermore, a large number of human cancers express Kit. Activating Kit mutations occur in the extracellular, the JM, and the proximal and distal protein kinase domains (Fig. 1, Table 2).

Mastocytosis represents a spectrum of rare disorders that is characterized by mast cell hyperplasia that can involve the skin or a combination of organ systems [28]. Treatment, which includes histamine antagonists, is symptomatic. Mast cell leukemia is a uncommon disorder that is treated by various non-targeted chemotherapeutic regimens. Seminomas are neoplasms of male germ cells. Localized tumors are treated by surgical resection. Neoplasms that have spread to other organs are sensitive to radiation and to a combination of cisplatin and other chemotherapeutic agents. Most men with advanced cases can be cured [29]. Dysgerminomas are rare ovarian cancers. Surgical resection is the mainstay of treatment. In the case of recurrence or spread, dysgerminomas are sensitive to radiation therapy and combination chemotherapy [30]. Nasal T-cell lymphomas, which are rare, usually affect men in Asia. Treatment involves surgery, radiation therapy, and con-

Table 2
Oncogenic gain-of-function mutations in human Kit[a]

Residues	Tumor type	Location in Kit
501–502[b]	GIST	Extracellular domain
550–586	GIST, mast cell leukemia	JM segment
557	Germ-cell tumor	JM segment
559	T-cell lymphoma	JM segment
560	GIST, mastocytosis	JM segment
561	Mastocytosis, T-cell lymphoma	JM segment
642	GIST	Proximal kinase domain
816	Acute myelogenous leukemia, germ-cell tumor, mast cell leukemia, mastocytosis, T-cell lymphoma	Activation loop
820	Germ-cell tumor, GIST, mastocytosis	Activation loop
822	Germ-cell tumor, GIST	Activation loop
823[c]	Seminoma	Activation loop
825	T-cell lymphoma	Activation loop

[a] From [13] unless otherwise noted.
[b] Duplication of Ala501 and Tyr502.
[c] From [27].

ventional chemotherapy [31]. The majority of individuals with this disorder remain in remission with treatment.

Gastrointestinal stromal tumors (GIST) arise from the interstitial cells of Cajal; these cells play a role in intestinal motility. These tumors arise in the stomach (60%), small intestine (25%), rectum (5%), esophagus (2%), and a variety of other abdominal locations [32]. There are approximately 5000 new cases per year in the United States (compared with 175,000 new cases of lung cancer). These tumors range from benign to malignant with the potential for spread, or metastases, to the liver. The standard treatment for localized GIST is surgical removal. Recurrence after surgical resection is common, occurring in up to 90% of people with larger tumors, and long-term survival following recurrence is unusual. In people with recurrent disease, the results of surgery, radiation therapy, and general chemotherapy are poor. However, the outcomes of targeted Kit chemotherapy with STI-571, as described later, are encouraging.

In a pioneering study, Hirota et al. reported that gastrointestinal stromal tumors contain Kit mutations. These mutations involve most commonly the juxtamembrane domain [33]. Subsequent work involving greater numbers of samples indicated that JM domain mutations occur in about 67% of all cases of GIST [32]. These mutations involve residues 550–586 (Fig. 1). Although some of these mutations involve amino acid substitutions, most are deletions of 2–16 residues. The greatest frequency of mutation occurs at residues 557–559 in the critical JM-B segment. These gain-of-function mutations emphasize the autoinhibitory role of the JM domain noted previously. About 17% of mutations in GIST occur in the extracellular domain and involve a duplication of Ala501 and Tyr502. These mutations may disrupt an inhibitory dimerization motif. About 2% of the Kit mutations involve a Lys642Glu substitution in the αC loop of the small lobe. Another 2% of the Kit mutations involve residues 820 and 822 in the activation loop of the large lobe. The mutations at residues 642, 820, and 822 may stabilize the active conformation of Kit.

About 12% of the gastrointestinal stromal tumors lack a Kit mutation. About half of these samples contain mutations in the PDGFα receptor [34], also a member of the type III receptor protein-tyrosine kinases. The other half of the 12% lacks Kit and PDGFα receptor mutations. In contrast to Kit, the majority of PDGFα mutations occur within the activation loop, and a minority involves the JM domain.

Kit mutations in nasal T-cell lymphomas involve the JM domain (residues 559 and 561) and the activation loop (residues 816 and 825) [35]. Not enough cases have been examined to determine the relative frequency. The most common mutation in mast cell tumors occurs at residue 816; other mutations occur at residues 820 and 560. Residues 816 and 820 occur in the activation loop while residue 560 occurs within the JM domain [13]. The most common Kit mutation in germ-cell tumors occurs at residue 816 in the activation loop. Other mutations occur at residues 820 and 822 in the activation loop and residue 557 in the JM domain [13]. A Kit mutation at residue 816 has also been described in 1% of cases of acute myelogenous leukemia [25].

Kitayama et al. [36] examined the monomer–dimer transition in wild type, Val560Gly, and Asp816Val mutant mouse Kit (human Kit numbering). These two mutations lead to SCF-independent Kit activation. They prepared retroviral vectors corresponding to these proteins and introduced them into Ba/F3 cells. They found that the two mutants, but not the wild type enzyme, are constitutively tyrosine phosphorylated in the absence of SCF. This observation is consistent with a gain-of-function activating Kit mutation. Moreover, the extent of phosphorylation is greater in the activation loop mutant (residue 816) than in the JM mutant (residue 560). Ba/F_3 is a murine, interleukin 3-dependent, Kit negative pro-B lymphoid cell line.

Kitayama et al. [36] performed chemical cross-linking experiments using bis(sulfosuccinimidyl)suberate (BS^3), a bifunctional, water-soluble, membrane impermeable cross-linker that reacts with the Kit extracellular domain. In the absence of SCF, the JM mutant exists as a dimer while the activation loop mutant exists as a monomer. Receptor dimerization following ligand binding is considered to be the chief mechanism leading to receptor protein-tyrosine kinase activation [10]. The JM mutation in Kit leads to receptor dimerization with concomitant kinase activation. Besides autoinhibition, as described above, the JM segment inhibits Kit dimerization and subsequent kinase activation. When cells containing the Kit Asp816Val activation loop mutant are treated with SCF, receptor dimerization occurs. (See [15] for hypotheses regarding the biochemical basis for enzyme activation by the Asp816Val mutant.)

STI-571 targeted therapy of selected human neoplasms

STI-571 (Gleevec, imatinib) is an important and clinically useful protein-tyrosine kinase inhibitor (see [37] for an overview). In contrast to agents such as cisplatin, doxorubicin, and 5-fluorouracil, which are general cytotoxic compounds, STI-571 is a targeted cancer chemotherapeutic drug. This compound inhibits each of the following protein-tyrosine kinases: Abl, Bcr-Abl, Kit, and the PDGF receptor (α and β). Its clinical efficacy was established first in the treatment of chronic myelogenous leukemia. The Bcr-Abl oncoprotein is the consequence of the fusion of the *BCR* and *ABL* genes; this fusion results from the reciprocal translocation that forms the Philadelphia chromosome [37].

The Bcr-Abl oncoprotein exhibits elevated protein-tyrosine kinase activity, which is strongly implicated in the mechanism of development of chronic myelogenous leukemia. STI-571 is effective in the treatment of the stable phase of this disorder. The Abl protein kinase domain exists in an active and inactive conformation. STI-571 binds only to the inactive state of the enzyme as shown by X-ray crystallography [38]. The drug binds to a portion of the ATP-binding site and extends from there into an adjacent hydrophobic pocket [22]. STI-571 is a competitive inhibitor of Abl kinase with respect to ATP. Resistance to STI-571 is often the result of mutations in residues of the Bcr-Abl kinase that ordinarily bind to the drug.

STI-571 is effective in the treatment of a significant proportion of people with GIST [34]. About 85% of those individuals with mutations in the JM domain and about 45% of those with mutations of the extracellular domain have favorable responses. In contrast, a lesser percentage of people with mutations in the Kit kinase domain or those without a Kit mutation have a favorable response. STI-571 is also an inhibitor of the PDGFα receptor. However, gain-of-function mutations of this receptor involve the activation loop, and most of these mutants are resistant to STI-571.

Mast cell and germ-cell neoplasms that result chiefly from Kit activation loop mutants at residue 816 are resistant to STI-571. Mutations at residues 822 and 823 in the activation loop are sensitive to this drug [27], but these mutations are rare. Fortunately, germ-cell neoplasms respond to radiation treatment and combination chemotherapy. However, one long-term goal of many investigators is to develop targeted therapies for these disorders in order to minimize the toxic side effects of non-targeted therapy.

STI-571 binding to Kit

Mol et al. [15] determined the structure of the Kit/STI-571 complex by X-ray crystallography. They report that the drug enters the adenine-binding portion of the active site in the cleft between the small and large lobes; a portion of the drug extends into an adjacent hydrophobic pocket. STI-571 interacts with an inactive conformation by binding to the Phe811 "out" [15] or "off" [22] conformation; it is unable to bind to the active "in" or "on" conformation. The drug forms hydrogen bonds with the backbone amide of Cys673 in the Kit hinge region, the carboxyl side chain of Glu640 of the αC-helix, and the backbone amide of Asp810 of the DFG motif.

STI-571 is too large to fit in the cleft between the small and large lobes of the JM-autoinhibited enzyme. Although its binding resembles that observed with the Abl kinase, there is a readjustment of the DFG motif owing to a steric clash with the side chain of Phe811 of the DFG segment. Thus, STI-571 binds to an inactive conformation of Kit that differs somewhat from the JM-autoinhibited structure [15]. STI-571 is more effective in the treatment of GIST with the more common JM mutations, and it is less effective in the treatment of GIST with mutations in the activation loop. Presumably the activation loop mutations result in an overall active enzyme conformation, and STI-571 binds preferentially, if not exclusively, to inactive conformations [38].

During treatment, Bcl-Abl mutations occur that decrease the binding and effectiveness of STI-571 against chronic myelogenous leukemia [37]. Similar mutations occur in Kit during STI-571 treatment of GIST [39]. Mutations in Kit that confer resistance include a Thr670Ile mutation. Thr670 forms a hydrogen bond with STI-571 [15], but Ile670 cannot form such a bond. A similar Thr334Ile mutation occurs in Bcr-Abl in chronic myelogenous leukemia that confers drug resistance [37]. Besides forming a hydrogen bond with STI-571, threonine 670 of Kit and threonine 334 of Abl function as gatekeeper residues. A small gatekeeper (such as threonine) allows bulky aromatic substituents including the phenyl group of STI-571 to enter a hydrophobic pocket adjacent to the adenine-binding site of ATP [40]. In contrast, larger gatekeepers such as isoleucine restrict access to this hydrophobic pocket. (About 20% of human protein kinases have a threonine at this position [40]). Thus, substitution of an isoleucine for a threonine gatekeeper restricts access of STI-571 to the hydrophobic pocket and provides a residue that cannot function as an anchoring hydrogen bond donor.

Another mutation in Kit that confers resistance to STI-571 therapy in GIST is a Tyr823Asp mutation. Tyr823 is the activation loop phosphorylation site. Substitution of the negatively charged aspartate in this position presumably mimics the effect of tyrosine phosphorylation and leads to the formation of a more active enzyme that is not inhibited by STI-571. A third mutation that confers resistance is a Val654Ala substitution. Val654 forms hydrophobic bonds with STI-571 while Ala654 in the mutant protein cannot form these bonds.

Epilogue

Protein phosphorylation and dephosphorylation must be stringently regulated both in time and place. Most protein kinases occur physiologically in an inactive or less

active basal state. Non-receptor protein kinases such as Abl and Src are maintained in a basal state by inhibitory intramolecular interactions involving SH2 and SH3 domains [4,37]. Receptor protein kinases require a stimulatory ligand to convert them from a dormant to an active state. The ErbB/HER family of kinases are the receptors for a variety of growth factors including epidermal growth factor, heparin-binding epidermal growth factor, neuregulin/heregulin/neu differentiation factor, and transforming growth factor-α [41]. Ligand binding leads to receptor dimerization and activation of protein kinase activity. For the insulin receptor and non-receptor protein-tyrosine kinases, transphosphorylation of tyrosine residues in the activation loop leads to the fully activated state [20]. In contrast, transphosphorylation of tyrosine residues in the activation loop does not occur in the ErbB receptor family [41].

For members of the type III receptor protein-tyrosine kinase family, the JM domain imposes a significant constraint of kinase activity. Under basal conditions, the JM domain inhibits Kit receptor dimerization [36]. Furthermore, the JM domain locks the relative movement of the small and large lobes, and keeps the αC-helix and activation loop from assuming their active conformations. The importance of autoinhibition is emphasized by gain-of-function mutations of the JM domain of Kit that participate in neoplastic transformation. Following SCF binding and dimerization, transphosphorylation of two JM tyrosine residues leads to kinase activation. JM domain phosphorylation disrupts autoinhibition by both steric and electrostatic interactions; the JM domain is forced away from the small and large lobes, and allows receptor activation. In Kit, JM domain phosphorylation occurs before activation loop phosphorylation [14]. The regulation of Flt3 is similar [24]. Moreover, it is likely that the other type III receptor protein-tyrosine kinases exhibit analogous control mechanisms.

The phosphorylation of the human insulin receptor JM domain does not diminish insulin-induced kinase activation. Mutation of Tyr972 of the insulin receptor JM domain decreases the recruitment of signaling effectors, but it does not alter insulin activation [20]. In contrast, replacement of JM tyrosine residues in an ephrin receptor (EphB2) abolishes ligand stimulated activation [20]. Ephrins are membrane associated proteins that play a role in axon guidance, cell migration, and morphogenesis. Furthermore, the ephrin receptors represent the largest family of protein-tyrosine kinases [3]. Kimura et al. [42] prepared mouse Kit constructs with Tyr568Phe, Tyr570Phe, and both mutations. They found that the mutant receptors are autophosphorylated in response to SCF in human kidney 293 cells, indicating that substitution of the JM tyrosines does not lead to complete failure of kinase activation, as observed for the ephrin receptor. As expected, these mutations have been shown to decrease protein recruitment [11].

Mol et al. [15] determined the structure of the Kit/STI-571 complex. As was found for Abl [38], the structure shows that the compound is bound to an inactive Kit enzyme and occupies a portion of the ATP-binding site. However, this enzyme form is somewhat different than the JM-autoinhibited enzyme conformation. These workers suggest modifications of STI-571 that may improve binding affinity. STI-571 binds only to the inactive conformation of Kit and Abl. Another goal of protein-tyrosine kinase targeted therapy is the development of drugs that bind to the active conformation of enzymes that represent resistant enzyme forms [43].

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