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AUTOINHIBITORY MECHANISMS IN RECEPTOR TYROSINE KINASES

Stevan R. Hubbard

Skirball Institute of Biomolecular Medicine and Department of Pharmacology, New York University School of Medicine, New York, NY 10016

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1. ABSTRACT

Receptor tyrosine kinases (RTKs) are single-pass transmembrane receptors that possess intrinsic tyrosine kinase catalytic activity in their cytoplasmic domains. RTKs are critical components in signal transduction pathways involved in cellular proliferation, differentiation, migration, and metabolism. This large protein family includes the receptors for many growth factors and for insulin. Ligand binding to the extracellular portion of these receptors results in receptor dimerization, which facilitates *trans*-autophosphorylation of specific tyrosine residues in the cytoplasmic portion. The phosphotyrosine residues enhance receptor catalytic activity and/or provide docking sites for downstream signaling proteins. Because of the critical roles played by RTKs in cellular signaling processes, their catalytic activity is normally under tight control by intrinsic regulatory mechanisms as well as by protein tyrosine phosphatases. This review will focus on the autoinhibitory mechanisms that modulate RTK catalytic activity.

2. INTRODUCTION

2.1 Roles of RTKs in cellular signaling

Receptor tyrosine kinases (RTKs) are transmembrane glycoproteins that transduce extracellular signals to intracellular responses affecting proliferation, differentiation, migration, and metabolism (1). The RTK family includes the receptors for many growth factors such as epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), platelet-derived growth factor (PDGF), the fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF), and also includes the receptor for insulin. RTKs are essential for embryonic development and for adult tissue maintenance and regeneration, and also play important roles in pathological conditions such as diabetic retinopathy, atherosclerosis, and cancer. A related family of proteins, the non-receptor tyrosine kinases (NRTKs), which includes Src, Abl and the Janus kinases, lacks the extracellular and transmembrane portions of RTKs and instead possesses a diverse set of modular signaling domains (2).

An example of the roles of RTKs in organismal development and also in pathology is the vascular system (3). During vasculogenesis, the first stage of vascular development, epithelial cells differentiate as a consequence of VEGF signaling through one of its RTKs, VEGF receptor 2, to form a crude network of interconnected vessels. During angiogenesis, the second stage of vascular development, the vessels are remodeled and extended, and non-endothelial support cells are recruited to the maturing vasculature. Angiogenesis requires the activation of VEGF receptor 1 by VEGF, and in addition, requires the RTK Tie2 and its ligand angiopoietin 1. The ligand-RTK pair ephrinB2 and EphB4 have also been implicated in angiogenesis, in the demarcation of arteries and veins (4). The VEGF receptors are also critically important in tumor neovascularization, making these RTKs prime targets for therapeutic intervention (5).

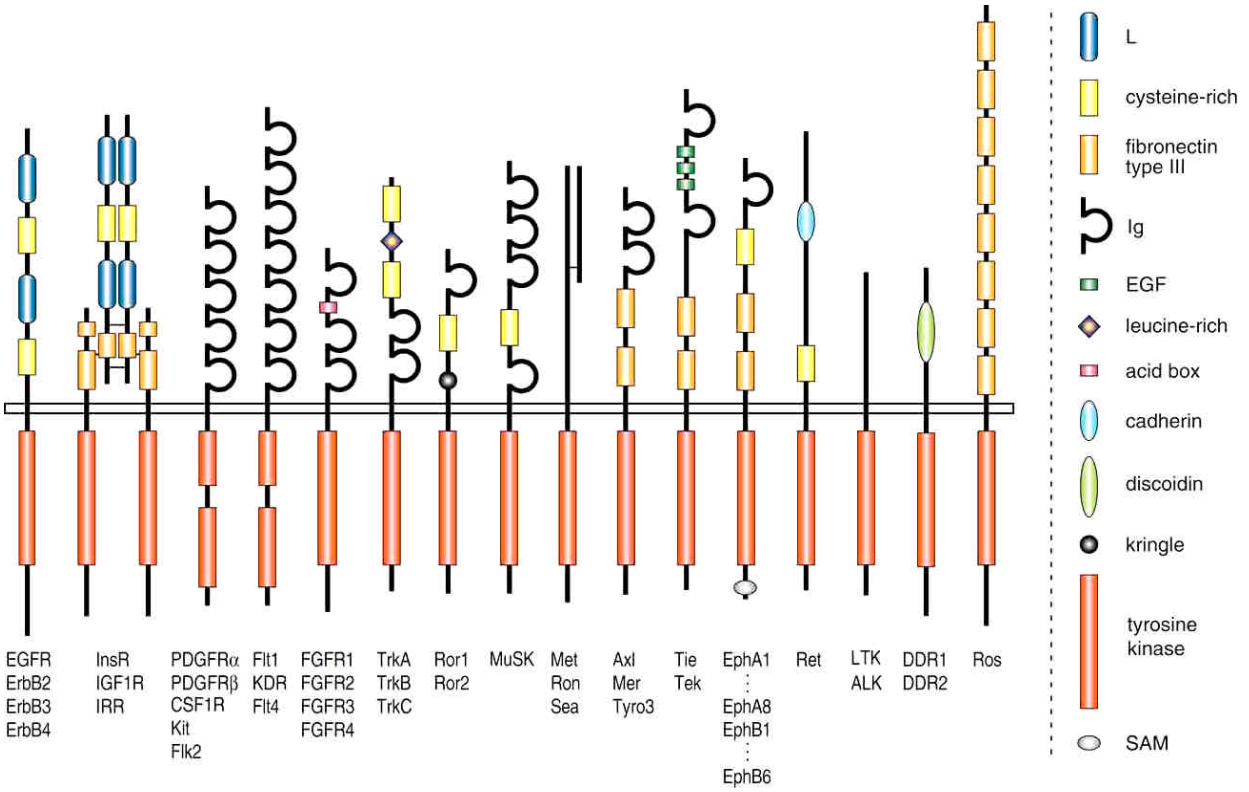

Figure 1. Architecture and domain organization in the RTK family. The extracellular portion of the receptors is on top and the cytoplasmic portion is on bottom. The legend for the domain types is on the right side. Some tyrosine kinase domains (*e.g.*, of the PDGF receptor subfamily) contain a large insertion, which is represented as a break in the rectangular kinase symbol. The lengths of the receptors are only approximately to scale.

2.2 Overall RTK architecture

RTKs consist of an extracellular portion which binds polypeptide ligands, a transmembrane helix, and a cytoplasmic portion which possesses tyrosine kinase activity (figure 1). The reaction catalyzed by RTKs (and NRTKs) is the transfer of the γ phosphate of adenosine triphosphate (ATP) to the side-chain hydroxyl group of tyrosine residues in protein substrates. Nearly all RTKs are present at the cell surface as a single polypeptide chain and are monomeric in the absence of ligand. Exceptions include the Met subfamily and the insulin receptor subfamily. Met, the receptor for hepatocyte growth factor, consists of a short extracellular alpha chain disulfide linked to a membrane-spanning beta chain. The insulin and IGF1 receptors consist of two large extracellular alpha chains which are disulfide linked to two membrane-spanning beta chains. The two alpha chains are also disulfide linked, yielding an $alpha_2beta_2$ heterotetramer. The polypeptide ligands that bind to and activate RTKs are mostly soluble, with the exception of the ephrins, the ligands for the Eph receptor family, which either span the plasma membrane or are tethered to it *via* a glycosylphosphatidylinositol (GPI) linkage (6,7).

The extracellular portion of RTKs consists of a wide array of modular domains including immunoglobin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains and EGF-like domains (figure 1). Typically, only a subset of domains within the ectodomain of an RTK are involved in ligand binding. The function of the remaining domains is not known in many cases, but for some RTKs may modulate ligand binding (discussed below). In contrast to the extracellular portion, the domain organization in the cytoplasmic portion of RTKs is simpler, consisting of a juxtamembrane region (carboxy-terminal to the transmembrane helix) followed by the tyrosine kinase catalytic domain and then a carboxy-terminal region. Members of the PDGF receptor subfamily contain a large insertion of approximately 100 residues in the tyrosine kinase domain. The kinase insert and the juxtamembrane and carboxy-terminal regions vary in length and sequence among RTKs and contain regulatory tyrosine and serine/threonine phosphorylation sites, which are phosphorylated by the receptors themselves (autophosphorylation) or by heterologous protein kinases.

2.3. General mechanism of RTK activation

Activation of an RTK is initiated through binding of its cognate ligand, which stabilizes a (non-covalent) dimeric form of the receptor. Dimerization facilitates *trans*-autophosphorylation (between receptors) of tyrosine residues in the two juxtaposed cytoplasmic domains of the dimer (figure 2).

Autophosphorylation serves two distinct functions in the receptor activation process: (i) stimulation of receptor catalytic (tyrosine kinase) activity, through

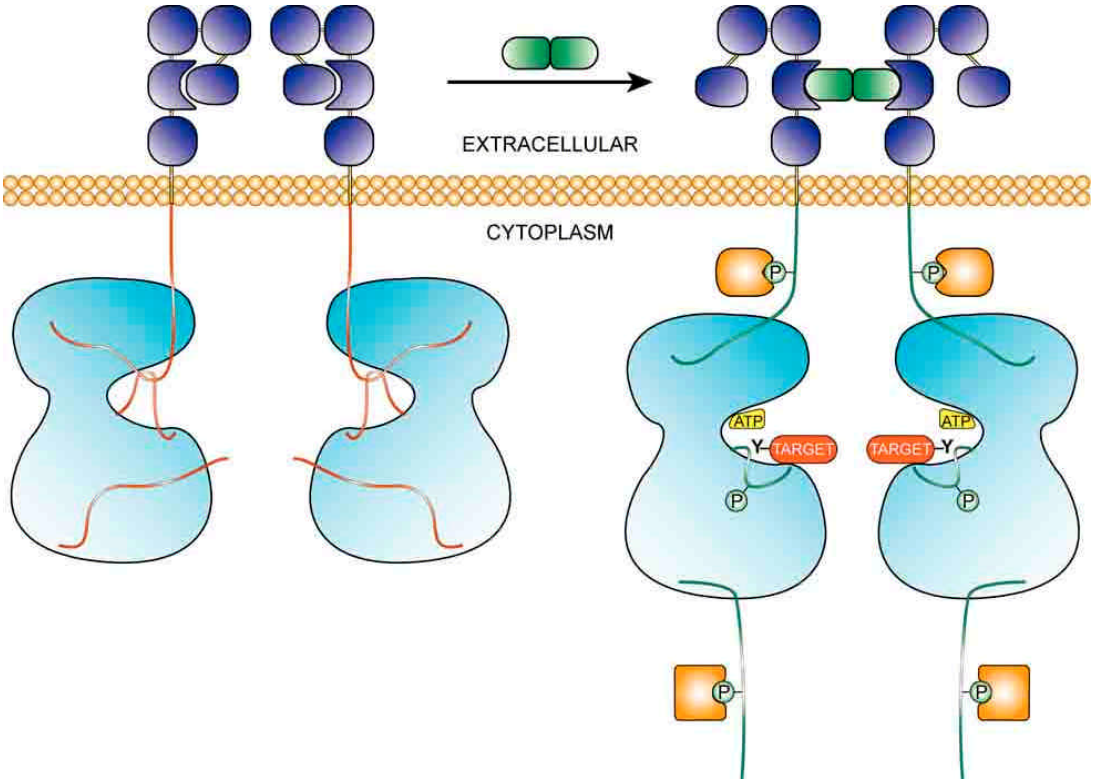

Figure 2. Mechanisms of RTK autoinhibition and activation. *(Left)* In the ligand-free (basal) state, various segments of the cytoplasmic domain, such as the activation loop and the juxtamembrane region, interfere with substrate binding or distort the active site cleft in the tyrosine kinase domain (blue). In addition, domains in the extracellular portion (purple) may mask the ligand-binding site. *(Right)* Upon stimulation with ligand (green), a receptor dimer is stabilized which provides sufficient opportunity for *trans*-autophosphorylation of tyrosines in the activation loop, juxtamembrane region and carboxy-terminal tail. Autophosphorylation relieves autoinhibition, allowing access to the active site of ATP (yellow) and protein targets (red), and creates binding sites for downstream signaling proteins (orange) containing phosphotyrosine-recognition modules.

stabilization of a proper active site configuration, and (ii) creation of binding sites for downstream signaling proteins, in which phosphotyrosine residues within specific sequence contexts serve as recruitment sites for proteins containing phosphotyrosine-recognition modules, such as the Src homology 2 (SH2) domain or the phosphotyrosine-binding (PTB) domain (8). In general, both functions are required for RTK activation.

The activation of TrkA, the high-affinity receptor for nerve growth factor (NGF), will serve to illustrate these concepts. NGF is a non-covalent dimeric ligand which binds to the Ig-like domains of the TrkA ectodomain (figure 1), stabilizing a dimeric arrangement of the two receptor molecules. Autophosphorylation ensues at three tyrosines in the activation loop of the kinase domain, at a tyrosine in the juxtamembrane region, and at a tyrosine in the carboxy-terminal tail. Autophosphorylation of the activation loop tyrosines is important for enhancing receptor catalytic activity (9,10). Autophosphorylation of the juxtamembrane tyrosine creates a binding site for the PTB domain of Shc (11-13), facilitating Shc phosphorylation by TrkA, recruitment of Grb2 and Ras activation. The autophosphorylation site in the carboxy-terminal tail of TrkA recruits phospholipase C gamma *via* an SH2 domain, leading to its phosphorylation and activation (14).

3. DISCUSSION

3.1. Requirement for autoinhibition in RTKs

Because *trans*-autophosphorylation of tyrosine residues is necessary for generating recruitment sites, a receptor with a fully active (non-inhibited) kinase domain would still require an encounter with another receptor to become activated. Therefore, why is suppression of RTK catalytic activity, *via* autoinhibition, necessary? Under certain circumstances – overexpression of wild-type receptors or activating mutations – ligand-independent collisions within the plane of the plasma membrane are sufficient to produce *trans*-autophosphorylation of monomeric RTKs. The activating mutations (discussed in more detail below) fall into two classes: those that facilitate ligand-independent dimerization and those that stimulate the intrinsic catalytic activity within a monomeric receptor. The latter class of mutations suggests that autoinhibition of catalytic activity in wild-type RTKs is necessary to limit the extent of ligand-independent autophosphorylation. Although RTK autoinhibition is clearly important, most RTKs can be activated non-specifically (in the absence of

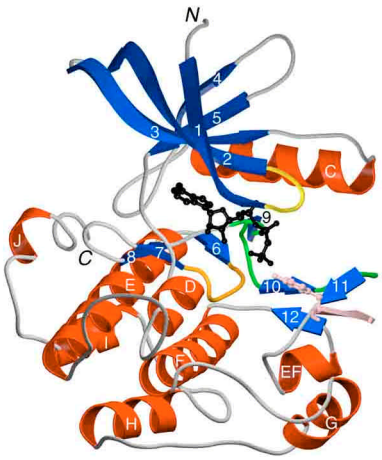

Figure 3. Ribbon diagram of the tyrosine kinase domain of the insulin receptor (26). The alpha helices (lettered) are shown in red, the beta strands (numbered) in blue, the nucleotide-binding loop in yellow, the catalytic loop in orange, the activation loop in green, the ATP analog (AMP-PNP) in black, and the tyrosine-containing peptide substrate in pink. The amino- and carboxy-termini are denoted by *N* and *C*.

ligand) by protein tyrosine phosphatase inhibitors such as vanadate, demonstrating that autoinhibition alone is not sufficient to maintain receptor quiescence.

As mentioned above, receptor dimerization (or possibly higher-order oligomerization) stabilizes a receptor complex in which a sufficiently high local concentration of enzyme (receptor) and substrate (other receptor) exists to promote *trans*-autophosphorylation at the various sites in the receptors. The following sections will focus on the autoinhibitory mechanisms by which various polypeptide regions within an unstimulated RTK act to suppress catalytic activity (figure 2). It seems plausible that the mechanism for a particular RTK has been fine tuned to be strong enough to limit autophosphorylation in the absence of ligand, yet weak enough to permit autophosphorylation of receptors that have been juxtaposed through ligand binding.

3.2. Role of the activation loop in RTK regulation

The tyrosine kinase domain of RTKs consists of two major subdomains, an amino-terminal lobe with five anti-parallel beta strands and one alpha helix, and a carboxy-terminal lobe composed of eight alpha helices and three pairs of anti-parallel beta strands (figure 3). ATP binds in the cleft between the two lobes, and the tyrosine-containing peptide segment of the protein substrate binds to the carboxy-terminal lobe. RTKs contain between one and three tyrosines in the kinase activation loop, a stretch of approximately 22 amino acids that begins with a protein kinase-conserved DFG sequence and ends with a tyrosine kinase-conserved proline residue. Autophosphorylation of activation loop tyrosines has been shown to be essential for stimulation of catalytic activity for RTKs such as the insulin receptor (15), the IGF1 receptor (16), FGF receptor 1 (17), Met (18), Ros (19), Nyk (20), TrkA (9), and TrkB (21). A notable exception to stimulation of catalytic activity through activation loop autophosphorylation is the EGF receptor. Although a tyrosine in the activation loop is conserved in this subfamily, substitution with phenylalanine has no measurable effect on the signaling properties of the EGF receptor (22).

Crystallographic studies of the tyrosine kinase domains of various RTKs and NRTKs, together with biochemical studies, have provided an in-depth understanding of the autoinhibitory properties of the unphosphorylated activation loop, and of the mechanism by which autophosphorylation of activation loop tyrosines stimulates catalytic activity. The general theme that has emerged from these studies is that in the absence of phosphorylation, the activation loop is not properly configured for catalysis. Phosphorylation of the activation loop stabilizes a conformation in which (i) the active site is accessible to both substrates, MgATP and tyrosine-containing peptide, and (ii) residues in the activation loop important for catalysis (DFG sequence) and for peptide binding (end of the activation loop) are optimally positioned.

The tyrosine kinase domain that has been best characterized structurally is from the insulin receptor. The activation loop of the insulin receptor kinase (IRK) contains three tyrosine autophosphorylation sites. In the crystal structure of unphosphorylated (low activity) IRK (23), one of the activation loop tyrosines, Tyr1162, is bound in the active site, hydrogen bonded to an aspartic acid and arginine (figure 4A), two conserved residues that reside in the so-called catalytic loop of protein kinases. In addition to Tyr1162, the adjacent residues Asp1161 and Tyr1163 also participate in hydrogen-bonding interactions that contribute to stabilization of this activation loop conformation. In this conformation, Tyr1162 is in position to be phosphorylated in *cis* (by its own kinase domain), but the beginning of the activation loop (including the DFG residues) blocks binding of ATP (figure 4A), and therefore *cis*-autophosphorylation is prevented. In fact, the activation loop is too short to permit simultaneous binding of Tyr1162 in the active site and ATP in the nucleotide-binding cleft.

In vitro solution studies of IRK indicate that in the absence of ATP, the predominant conformation of the activation loop is one in which the active site is occluded (24), consistent with that observed in the crystal structure. However, in the presence of millimolar quantities of ATP present in cells, the activation loop is in equilibrium between conformations which occlude the active site and those which do not (24), *i.e.*, ATP competes with the beginning of the activation loop for binding in the cleft between the two kinase lobes. Steady-state kinetic measurements for the unphosphorylated and phosphorylated states of IRK show that upon autophosphorylation the K_m values for both ATP and peptide substrate decrease and k_{cat} increases (25). Thus, the structural and biochemical data indicate that prior to autophosphorylation the activation loop of IRK serves as an autoinhibitory segment by competing with ATP (*via* the

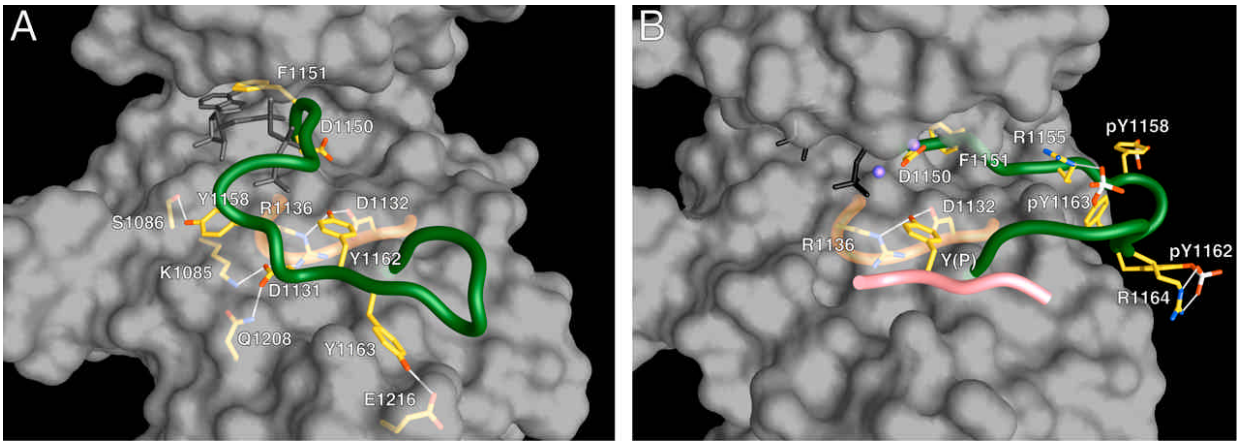

Figure 4. Regulation of catalytic activity through activation loop autophosphorylation. The insulin receptor kinase domain (IRK) is shown in a semi-transparent molecular surface representation except for residues in the activation loop (green). The catalytic loop is colored orange. Carbon atoms are yellow, oxygen atoms are red, and nitrogen atoms are blue. Hydrogen bonds are indicated by solid white lines. (A) Autoinhibitory conformation of the unphosphorylated activation loop of IRK (23). The ATP analog (gray) from the ternary IRK structure (26) is superimposed in the ATP-binding cleft. Asp1150 and Phe1151 are residues in the conserved DFG motif at the beginning of the activation loop. (B) Re-positioning of the activation loop of IRK upon autophosphorylation. The ATP analog is colored black, the active site Mg^{2+} ions are colored violet, and the tyrosine-containing [Y(P)] peptide substrate is shown in pink. Much of the ATP analog is masked by the nucleotide-binding loop of the amino-terminal lobe.

DFG residues) and protein substrates (*via* Tyr1162) for the active site.

Autophosphorylation of the activation loop (in *trans*) results in stabilization of an activation loop conformation that is optimal for catalysis. In the crystal structure of the tris-phosphorylated form of IRK (26), two of the three phosphotyrosines are engaged in interactions that stabilize the conformation of the activation loop. Phosphotyrosine 1162 (pTyr1162) is salt bridged to Arg1164, and pTyr1163 is salt bridged to a conserved arginine (Arg1155) after the DFG motif in the activation loop (figure 4B). A comparison of the tris-phosphorylated IRK structure with the structures of mono-phosphorylated PKA (27), LCK (28), and CDK2 (29) indicates that pTyr1163 is the key phosphotyrosine for stabilization of the IRK activation loop. Consistent with this, Tyr1163 is the last of the three activation loop tyrosines to be autophosphorylated (30). The first phosphotyrosine in the IRK activation loop, pTyr1158, does not engage in interactions with other kinase residues and appears to function as a docking site for downstream signaling proteins (31,32). Two short beta-strand interactions between the activation loop and other carboxy-terminal lobe segments (beta6/beta9 and beta10/beta12, figure 3) also contribute to stabilization.

Is activation loop autoinhibition as observed for IRK general for RTKs? From crystal structures of the kinase domains of several other RTKs, it appears that the conformation of the unphosphorylated activation loop is in general suboptimal for catalysis, but the particular way in which it represses activity varies. In the structure of the kinase domain of FGF receptor 1, the ATP-binding site is not occluded by the activation loop, but the end of the activation loop is not positioned to bind peptide substrate (33). In the structure of the Tie2/Tek kinase domain, the activation loop does not interfere with ATP or peptide binding, but the nucleotide-binding loop in the amino-terminal lobe (between beta1 and beta2, figure 3) is observed to block ATP binding (34), which is probably a consequence of the mis-positioning of the DFG residues in the unphosphorylated activation loop. In addition, the segment carboxy-terminal to the kinase domain, which contains two tyrosine autophosphorylation sites, feeds back towards the active site and may interfere with peptide substrate binding. The conformation of the unphosphorylated activation loop in MuSK, an RTK involved in formation of the neuromuscular synapse, resembles that of IRK, with the tyrosine corresponding to Tyr1162 (Tyr754) bound in the active site and the DFG residues obstructing the ATP binding site (J.H. Till, M. Becerra-Fernandez, A. Watty, T.A. Neubert, S.J. Burden & S.R. Hubbard, unpublished).

The sequence determinants that govern the conformation of the basal-state (unphosphorylated) activation loop are not well understood. Interestingly, in IRK and MuSK, the two examples in which an activation loop tyrosine acts as a pseudo-substrate, the activation loop contains three autophosphorylation sites. At least one glycine in the turn between beta-strands 10 and 11 may also be important for this particular activation loop conformation. It should be noted that in the various structures of tyrosine kinase domains, the middle portion of the unphosphorylated activation loop is often either disordered or the atoms possess high temperature factors, indicating that in solution an equilibrium exists between multiple conformations. Therefore, a careful evaluation of the interactions thought to stabilize a particular

conformational state, as well as an assessment of the effects of lattice contacts, are necessary before designation of an autoinhibitory mechanism.

While the unphosphorylated activation loop of RTKs exhibits a variety of conformations, the conformation of the phosphorylated activation loop is expected to look quite similar. The mono-phosphorylated activation loop of LCK (28), a Src family NRTK, follows a course similar to that of the tris-phosphorylated IRK activation loop (26), despite being shorter by one residue. A recently determined crystal structure of the IGF1 receptor kinase domain shows a nearly identical conformation of the tris-phosphorylated activation loop as in IRK (35), to be expected from the high degree of sequence conservation between these two receptors. Surprisingly, the mono-phosphorylated activation loop of VEGF receptor 2 (KDR) is observed to adopt a conformation similar to the unphosphorylated FGF receptor 1 kinase (36), *i.e.*, one in which the peptide binding site is obstructed. KDR has two autophosphorylation sites in the activation loop, and although the crystallized protein was phosphorylated on Tyr1059 (the equivalent of Tyr1163 in IRK), it may be that phosphorylation of the other tyrosine in the activation loop, Tyr1054 (equivalent of Tyr1158 in IRK), is necessary for complete stabilization of the active conformation.

3.3. Role of the juxtamembrane region in RTK regulation

As discussed above, the positioning of the unphosphorylated and autophosphorylated activation loop is one of the fundamental mechanisms by which RTK catalytic activity is regulated. Biochemical studies indicate that for a subset of RTKs, the juxtamembrane region (amino-terminal to the kinase domain) provides an additional mechanism for regulation of catalytic activity. For example, substitution of two tyrosines with phenylalanine in the juxtamembrane region of PDGF receptor beta substantially reduces autophosphorylation of the numerous other sites in the receptor, which are readily autophosphorylated in the wild-type receptor (37,38). As an another example, the majority of Eph family RTKs contain within their juxtamembrane sequence a conserved motif that includes a pair of tyrosine autophosphorylation sites. Mutagenesis studies of these two tyrosines show that autophosphorylation of these tyrosines, particularly the second tyrosine, is required for stimulation of catalytic activity (39). Finally, in MuSK, mutation of a juxtamembrane tyrosine in an NPXY motif impairs autophosphorylation of the three activation loop tyrosines (40). These observations are consistent with the juxtamembrane region serving a similar autoinhibitory role as the activation loop, *i.e.*, the unphosphorylated region impairs substrate binding or imposes constraints on catalytic residues, and *trans*-autophosphorylation of tyrosines in this region releases these constraints.

A recent crystal structure of the kinase domain and juxtamembrane region of EphB2 provides a structural basis for autoinhibition by the juxtamembrane region (41). In the structure, the unphosphorylated juxtamembrane region interacts with and perturbs alpha-helix C in the amino-terminal lobe, distorting the active site, and also makes contact with the carboxy-terminal lobe. Autophosphorylation of the two juxtamembrane tyrosines is believed to destabilize the inhibitory conformation through electrostatic and steric effects. The juxtamembrane tyrosines in the ephrin receptors and PDGF receptor beta actually have dual roles: an autoinhibitory role prior to autophosphorylation and a recruitment role, of SH2 domain-containing proteins, after autophosphorylation. Whether the mechanism of autoinhibition by the juxtamembrane region of the ephrin receptors and PDGF receptor beta is similar remains to be determined. Based on the different sequence motifs in which the juxtamembrane tyrosines are located, and the diversity previously observed for activation loop autoinhibition, it is probable that the detailed mechanism by which basal-level kinase activity is maintained by the juxtamembrane region will vary.

3.4. Other autoinhibitory mechanisms

As detailed above, the principal mechanisms of autoinhibition in RTKs are through positioning of the kinase activation loop and juxtamembrane region in the cytoplasmic portion of the receptor to repress catalytic activity. It has been known for some time that the extracellular portion of RTKs can exert an inhibitory effect. The v-erbB oncogene product retains the transmembrane helix and cytoplasmic domain of EGF receptor family members but lacks a majority of the ectodomain (42). Constitutive activation is a consequence of an enhanced ability of the transmembrane helix, sterically accessible in the truncated receptor, to self-associate and facilitate *trans*-autophosphorylation.

Ectodomain autoinhibition in normal cellular signaling has been described for the FGF receptor family. The extracellular portion of members of this family (four mammalian receptors) comprises three Ig-like domains (figure 1). FGF ligands bind to Ig-like domains 2 and 3 (43,44). Included in the linker segment between Ig-like domains 1 and 2 is a stretch of approximately eight acidic residues known as the acid box. It has been proposed that Ig-like domain 1 and the acid box serve autoinhibitory functions, suppressing binding of FGFs and heparin sulfate proteoglycans, respectively, to the receptor (43). Biological evidence for this autoinhibitory role comes from studies showing that a deletion mutant of FGF receptor 1 lacking Ig-like domain 1 and the acid box has a higher affinity for FGF1 and heparin than the full-length receptor (45). Moreover, a correlation has been observed between the progression of pancreatic and brain tumors and the expression of alternatively spliced FGF receptors lacking Ig-like domain 1 and the acid box, which would be predicted to have a stronger signaling capability (46,47).

The existing paradigm for RTK activation is that ligand binding to monomeric receptors stabilizes a dimeric receptor configuration in which *trans*-autophosphorylation can take place. Cytokine receptors, such as the erythropoietin (Epo) receptor, are structurally related to RTKs, but utilize cytoplasmically associated Janus kinases (Jaks) rather than an intrinsic tyrosine kinase domain for catalytic activity. Recent studies of the Epo receptor

provide evidence that ligand-free receptors exist as non-productive dimers which rearrange into active signaling dimers upon ligand binding, allowing *trans*-phosphorylation of the associated Jaks (48,49). Although x-ray crystallographic studies implicate the extracellular domain of the Epo receptor in mediating non-productive dimer formation (50), biochemical studies with full-length receptors in cells suggest that the transmembrane helix is the element most responsible for stabilizing preformed dimers (4749).

These results for the Epo receptor suggest that a subset of RTKs may operate in a similar fashion. Thus far, the only acknowledged cases of preformed RTK dimers in the absence of ligand are the insulin and IGF1 receptors, which are covalently assembled heterotetramers (figure 1). Here, in the absence of ligand, structural elements in the extracellular portion, and perhaps in the transmembrane and cytoplasmic portions, maintain a basal-state configuration of the cytoplasmic domains in which trans-autophosphorylation is suppressed. Binding of insulin/IGF1 is thought to induce a translation or rotation of the transmembrane helices that facilitates trans-autophosphorylation of the cytoplasmic domains (51).

3.5. Gain-of-function mutations in RTKs

A number of point mutations in the extracellular, transmembrane and cytoplasmic domains of various RTKs confer constitutive activity and lead to disease states. The molecular mechanisms by which constitutive activation arises through mutation is understood for a subset of cases but poorly understood in other cases. A discussion of these mutations in light of the autoinhibitory mechanisms just addressed is valuable for appraising our state of knowledge of RTK autoinhibition.

Numerous gain-of-function point mutations have been mapped in FGF receptor 3, which cause various human skeletal dysplasias, depending on the particular mutation and the level of ligand-independent activation, from relatively mild forms of craniosynostosis to neonatal lethal thanatophoric dysplasia (52). Several mutations in the extracellular domain of FGF receptor 3 create an odd number of cysteine residues, either by mutation to cysteine or by mutation of a disulfide-forming cysteine to another amino acid, resulting in disulfide-linked receptor dimerization and trans-autophosphorylation of the kinase domains. Aberrant disulfide pairing of this type also occurs via mutation in the ectodomain of Ret, the receptor for glial-derived neurotrophic factor, resulting in a dominantly inherited cancer syndrome, multiple endocrine neoplasia type 2A (53).

Point mutations in the kinase domain of FGF receptor 3 are also activating, but the mechanism is not through dimer formation. The gain-of-function mutation Asn540→Lys gives rise to hypochondroplasia, a type of dwarfism (54). In the structure of the kinase domain of FGF receptor 1 (FGFR1K) (33), the side chain of the equivalent residue, Asn546, makes two hydrogen bonds to the main chain of His541. Asn546 is located just before beta-strand 4, and His541 is in the loop between alpha-helix C and beta-strand 4 (figure 3), which is anchored in the carboxy-terminal lobe of the kinase. Asn546, through its hydrogen-bonding interactions, appears to restrict the relative movement of the amino-terminal lobe with respect to the carboxy-terminal lobe. Releasing this restriction (through mutation) may increase catalytic activity by facilitating a more optimal positioning of the DFG residues in the beginning of the activation loop, which are mis-aligned in the absence of activation loop phosphorylation. A monomeric receptor with enhanced catalytic activity would increase the probability that a ligand-independent collision between two receptors would result in a trans-autophosphorylation event.

A strongly activating mutation in the FGF receptor 3 kinase domain replaces Lys650 with glutamic acid, which leads to thanatophoric dysplasia (55). Lys650 is located in the activation loop, two residues carboxy-terminal to the tandem autophosphorylation sites in the FGF receptor family (corresponding to Tyr1162 and Tyr1163 in IRK). In the crystal structure of unphosphorylated FGFR1K, this portion of the activation loop is highly flexible. One would predict that this mutation shifts the conformation of the activation loop to a more active configuration, and it well may, yet crystallographic analysis of the Lys656→Glu FGFR1K mutant failed to reveal any differences in activation loop conformation between the mutant and wild-type kinase (M. Mohammadi & S.R. Hubbard, unpublished).

Gain-of-function point mutations in the activation loops of Met and Kit have been described which give rise to papillary renal carcinoma and mast cell leukemia, respectively (56,57). One mutation in common in the two RTKs is the substitution of an aspartic acid that is four residues carboxy-terminal to the DFG motif (Asp1246 in Met, Asp816 in Kit). There is some question as to whether the Asp816 mutation in Kit is activating due to enhancement of cytoplasmic dimer formation (58,59) or by a dimerization-independent mechanism (57,60). Because substitution of this aspartic acid with several different amino acids (e.g., valine and asparagine) is activating, one would assume that it is the loss of aspartic acid at this position rather than the gain of the substituted amino acid that is responsible for the increase in catalytic activity. Yet in the structures of unphosphorylated IRK and FGFR1K, the corresponding aspartic acid does not participate in any interactions that would suggest a role in stabilization of an autoinhibitory activation loop.

Mutations in the juxtamembrane region of Kit are also activating and lead to gastrointestinal stromal tumors (61). Here again, whether constitutive activation is the result of enhanced dimerization or some other mechanism is not clear. Cross-linking studies indicate that a Val560→Gly substitution in the juxtamembrane region increases ligand-independent dimerization (60). Alternatively, an amphipathic helix is predicted in the juxtamembrane region of Kit (62), which could interact with the kinase domain to repress catalytic activity prior to autophosphorylation of nearby tyrosines. Disruption of this

putative helix through deletion or point mutation could relieve constraints on the kinase domain.

Finally, a missense mutation in the Ret tyrosine kinase domain just after the activation loop, Met918→Thr, is causative for multiple endocrine neoplasia type 2B (63). The mechanism by which this mutation results in constitutive receptor activation is novel, as it appears to involve both an increase in catalytic activity and also an alteration in substrate specificity (64,65). Interestingly, methionine is conserved at this position in RTKs and threonine/serine is conserved in NRTKs. As viewed in the crystal structure of IRK (26), this methionine (Met1176 in IRK) occupies a critical spatial position, making contacts with a conserved hydrophobic residue at the end of the activation loop and also forming part of the hydrophobic binding site for the P+3 residue of the substrate peptide.

4. PERSPECTIVE

Structural and biochemical studies of RTKs have revealed many of the autoregulatory mechanisms by which RTK activity is controlled. This basic biochemical knowledge has potential application in the therapeutic arena. RTKs have been implicated in the onset and/or progression of various proliferative diseases such as cancer, diabetic retinopathy, atherosclerosis, and psoriasis (66), and over the last few years a large effort has been undertaken to identify, mainly through screening of chemical libraries, small molecules that are potent and specific inhibitors of various tyrosine kinases. From crystal structures of tyrosine kinases with bound inhibitors (67-72), it is becoming increasingly clear that in addition to specificity arising from sequence differences in the ATP-binding cleft, another source of specificity exists that derives from differences in autoinhibitory mechanisms.

In this second type of specificity, an inhibitor interacts with conserved residues residing in a segment whose conformation is not conserved, *e.g.*, the DFG sequence in the unphosphorylated activation loop. The best example of "conformational specificity" is inhibition of Abl by STI-571, an ATP-competitive small molecule. In the crystal structure of the Abl kinase domain with STI-571 bound (72), all of the residues observed to interact with the inhibitor are conserved between Abl and Src, yet STI-571 is a potent inhibitor of Abl but not Src. The conformation of the unphosphorylated activation loop of Abl places the DFG residues in position to interact favorably with STI-571, whereas the positioning of these residues in the activation loop of Src (71,73) would interfere sterically with binding of the compound. The remarkable success of STI-571 in patients with chronic myelogenous leukemia (74) validates the continuing efforts to understand, in molecular detail, the various autoinhibitory mechanisms to which RTKs are subject.

5. ACKNOWLEDGMENTS

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Send correspondence to: Dr. Stevan Hubbard, Skirball Institute of Biomolecular Medicine, NYU School of Medicine, 540 First Avenue, New York, NY 10012, Tel.: 212-263-8938, Fax: 212-263-8951, E-mail: hubbard@saturn.med.nyu.edu