Inhibition of Spontaneous Receptor Phosphorylation by Residues in a Putative α-Helix in the KIT Intracellular Juxtamembrane Region*

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KIT receptor kinase activity is repressed, prior to stem cell factor binding, by unknown structural constraints. Using site-directed mutagenesis, we examined the role of KIT intracellular juxtamembrane residues Met-552 through Ile-563 in controlling receptor autophosphorylation. Alanine substitution for Tyr-553, Trp-557, Val-559, or Val-560, all sitting along the hydrophobic side of an amphipathic α-helix (Tyr-553–Ile-563) predicted by the Chou-Fasman algorithm, resulted in substantially increased spontaneous receptor phosphorylation, revealing inhibitory roles for these residues. Alanine substitution for other residues, most of which are on the hydrophilic side of the helix, caused no or slightly increased basal receptor phosphorylation. Converting Tyr-553 or Trp-557 to phenylalanine generated slight or no elevation, respectively, in basal KIT phosphorylation, indicating that the phenyl ring of Tyr-553 and the hydrophobicity of Trp-557 are critical for the inhibition. Although alanine substitution for Lys-558 had no effect on receptor phosphorylation, its substitution with proline produced high spontaneous receptor phosphorylation, suggesting that the predicted α-helical conformation is involved in the inhibition. A synthetic peptide comprising Tyr-553 through Ile-563 showed circular dichroism spectra characteristic of α-helix, supporting the structural prediction. Thus, the KIT intracellular juxtamembrane region contains important residues which, in a putative α-helical conformation, exert inhibitory control on the kinase activity of ligand-unoccupied receptor.

KIT, encoded by the protooncogene c-KIT (1, 2), is the receptor tyrosine kinase for stem cell factor (SCF)3 (3). KIT and the receptors for colony-stimulating factor 1 and platelet-derived growth factor define the receptor tyrosine kinase type III subfamily (1, 2, 4). These receptors have in common five immunoglobulin-like motifs in the extracellular domain and a bipartite kinase in the cytoplasmic portion. The current model for activation of receptor tyrosine kinases (4), which involves ligand-binding-induced receptor dimerization and autophosphorylation, is well exemplified in the case of KIT (5, 6). Molecular lesions that impair the kinase activity of KIT can lead to a variety of developmental disorders (7), while mutations that constitutively activate KIT (8–10) are associated with the pathogenesis of mastocytosis (10–12) and gastrointestinal stromal tumors (9). These activating mutations can transform cells in vitro and confer aggressive behavior to the cells in vivo (9, 13).

Prior to SCF binding, the kinase activity of KIT is kept in a repressed state. The structural basis for this repression is unknown. A number of in-frame deletion mutations in the c-KIT intracellular juxtamembrane coding region have recently been identified in situ in gastrointestinal stromal tumors and in mastocytomas and shown to cause SCF-independent receptor activation (9, 10). While these findings imply that this region is involved in negative control of the receptor kinase activity in the absence of SCF stimulation, the amino acid(s) that play inhibitory roles are not known. This is because deletion mutations are likely to result in conformational changes that are not specifically related to the eliminated residues but are necessary to compensate for the gap left by the ablation.

In this study we examined the role of a series of residues in the KIT intracellular juxtamembrane region in controlling receptor autophosphorylation. Our results reveal important residues in this region that exert inhibitory effects on the receptor kinase activity in the SCF naive state and demonstrate conformational requirements for these residues in repressing auto-activation of the receptor kinase.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human SCF, mouse monoclonal and rabbit polyclonal anti-human KIT antibody (Ab), and wild-type human full-length KIT cDNA were provided by Amgen (Thousand Oaks, CA). Mouse anti-phosphotyrosine (Tyr(P)) monoclonal Ab was purchased from Upstate Biotechnology (Lake Placid, NY). cDNA Construction and Transfection—Single residue substitutions were generated in human KIT cDNA in the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA) using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). COS cells (90% confluent in 10-cm plate) were transfected with 5 μg of plasmid using 15 μl of LipofectAMINE TM (Life Technologies, Gaithersburg, MD) in serum-free medium for 5 h. An equal volume of medium with 20% bovine calf serum was then added, and cells were incubated overnight, followed by 24-h culture in regular medium prior to receptor phosphorylation experiments.

Immunoprecipitation and Immunoblotting—For tyrosine phosphorylation assay, cells expressing either wild-type or mutant KITs were serum-starved for 18 h before incubation with, or without, SCF at 200 ng/ml for 10 min at 37 °C. Cells were harvested in lysis buffer containing 1 Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate. Centrifugation-clarified cell lysates were immunoprecipitated for 1.5 h at 4 °C with mouse

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§ The abbreviations used are: SCF, stem cell factor; Tyr(P), phosphotyrosine; Ab, antibody; CD, circular dichroism.
KIT Juxtamembrane Residues Inhibit Receptor Autoactivation

A Number of Juxtamembrane Residues Are Necessary for Inhibition of Spontaneous KIT Phosphorylation—To examine which amino acid(s) in the KIT intracellular juxtamembrane region may play inhibitory roles in control of the receptor kinase activity, we generated a series of mutant KITs with single alanine substitutions for residues Met-552 through Ile-563. This region covers most of the mutations identified in gastrointestinal stromal tumors (9) and in mastocytomas (10). A Predicted α-Helical Conformation Is Involved in Inhibition—The spontaneous phosphorylation pattern displayed by this series of mutant receptors is consistent with the corresponding residues being contained in an amphipathic α-helix (Tyr-553 through Ile-563) predicted by the Chou-Fasman algorithm (14). We assessed tyrosine phosphorylation of the mutated receptors in COS cells because these cells express neither KIT nor SCF (16). The Hydrophobicity of Trp-557 Is Important for Inhibition—To look more closely at the inhibitory role of Tyr-553, we mutated this tyrosine residue to phenyalanine. Removal of the hydroxyl group of Tyr-553 resulted in a slight increase (−2-fold) in basal receptor phosphorylation in comparison with wild-type receptor (Fig. 2). This result, together with the substantially increased spontaneous receptor phosphorylation caused by Y553A substitution (Fig. 1, lane 3), indicates that the phenyl ring of Tyr-553 exerts a major inhibitory effect, while its hydroxyl group plays a relatively minor inhibitory role. Whether this hydroxyl group interacts directly with another structural element or it is subject to phosphorylation is not known and awaits further study.
KIT activation. In the absence of the side chain of Val-555, which is critical for lack of autoactivation of the V555P mutant is most likely due to the inhibition, even though they both possess large hydrophobic side chains. To further test whether the predicted α-helical conformation is involved in the inhibition, we tried to disrupt the helical structure by introducing in this region proline residues that are sterically incompatible with α-helical conformation. We selected Val-555 and Lys-558 to be replaced by proline, since the alanine substitution assay had demonstrated that their side chains have no inhibitory effects, and they are located within the predicted helix. Therefore, any significantly increased basal receptor phosphorylation resulting from the proline substitution can be specifically ascribed to steric constraints imposed by proline rather than to loss of inhibitory side chain function. Substitution of proline for Val-555 led to a very high level of constitutive receptor phosphorylation (Fig. 3), as did substitution of alanine for Val-555 (Fig. 1, lane 10). The lack of autoactivation of the V555P mutant is most likely due to the absence of the side chain of Val-555, which is critical for KIT activation.

The Tyr-553–Ile-563 Fragment Forms an α-Helix in Solution—To examine more closely the predicted α-helical structure, we measured the CD spectra of an 11-residue peptide synthesized corresponding to Tyr-553 through Ile-563. This peptide displayed negative minima in ellipticity at 208 nm and 222 nm, which are characteristic of α-helical structure (15). The overall helical content of this peptide was 11% in aqueous solution and increased to 76% in the presence of trifluoroethanol (Fig. 4), which stabilizes the α-helical structure of peptides that have inherent propensity to be α-helical but are marginally stable in water (17, 18). The fact that this peptide can fold into an α-helix reinforces the prediction that an α-helical conformation of the juxtamembrane Tyr-553–Ile-563 region is involved in repression of spontaneous receptor phosphorylation.

The features of the inhibitory site, as we have shown, suggest that it interacts with another structural element for the inhibition. In light of the domain-domain interactions for repressing the kinase activity of the Src and Hck tyrosine kinases (19, 20), we speculate that the KIT juxtamembrane inhibitory site interacts with an epitope of the adjacent kinase domain and, in doing so, affects the kinase activity. Other mechanisms may also contribute to inhibition of KIT autoactivation and remain to be elucidated.

In summary, the present study identifies a number of residues in the KIT intracellular juxtamembrane region that exist in a putative α-helical conformation and exert inhibitory effects on the kinase activity of SCF-unoccupied receptor. These find-
ings provide a structural basis for understanding why multiple deletion and missense mutations in this region, which have been identified \textit{in situ} in gastrointestinal stromal tumors and mastocytomas, are able to cause constitutive activation of the receptor kinase (9, 10).

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