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Phosphorylation of the activation loop tyrosine 823 in c-Kit is crucial for cell survival and proliferation*

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*Running title: Phosphorylation of Y823 is crucial for c-Kit signaling

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Keywords: c-Kit; Activation loop; Y823, tyrosine phosphorylation, signaling

Background: Phosphorylation of Y823 in the A-loop of c-Kit is not required for kinase activity but might have other functions.

Results: Mutation of Y823 in c-Kit causes alterations in downstream signaling and a reduction in ligand-dependent cell survival and proliferation.

Conclusion: Phosphorylation of Y823 regulates c-Kit stability.

Significance: This provides a function for the activation loop tyrosine of c-Kit.

SUMMARY

The receptor tyrosine kinase c-Kit, also known as the stem cell factor receptor, plays a key role in several developmental processes. Activating mutations in c-Kit lead to alteration of these cellular processes and have been implicated in many human cancers such as gastrointestinal stromal tumors (GISTs), acute myeloid leukemia (AML), testicular seminomas and mastocytosis. Regulation of the catalytic activity of several kinases is known to be governed by phosphorylation of tyrosine residues in the activation loop of the kinase domain. However, in the case of c-Kit phosphorylation of Y823 has been demonstrated to be a late event that is not required for kinase activation. However, since phosphorylation of Y823 is a ligand-activated event, we sought to investigate the functional consequences of Y823 phosphorylation. By using a tyrosine to phenylalanine mutant of tyrosine 823 we investigated the impact of Y823 on c-Kit signaling. We here demonstrate that Y823 is crucial for cell survival and proliferation and mutation of Y823 to phenylalanine leads to decreased sustained phosphorylation and ubiquitination of c-Kit as compared to the wild-type receptor. Furthermore, the mutated receptor was upon ligand-stimulation quickly internalized and degraded. Phosphorylation of the E3 ubiquitin ligase, Cbl was transient followed by a substantial reduction in phosphorylation of downstream signaling molecules such as Akt, Erk, p38, Shc and Gab2. Thus, we propose that activation loop tyrosine 823 is crucial for activation of both the MAPK and PI3K pathways and that its disruption leads to a destabilization of the c-Kit receptor and decreased survival of cells.

c-Kit belongs to the family of type III receptor tyrosine kinases (RTKs) and is known for its critical role in hematopoiesis, pigmentation and reproduction. It is also important for survival, proliferation and differentiation of hematopoietic progenitor cells (1,2). Several activating mutations in c-Kit lead to a deregulation of the signaling cascades causing malignancies such as acute myeloid leukemia, gastrointestinal stromal tumors, testicular seminomas (3-6). Architecturally, c-Kit is comprised of five extracellular Immunoglobulin-like domains, a transmembrane domain, a juxtamembrane region, a kinase domain split into two parts by a kinase insert and a carboxyterminal tail. The activation loop is present in the C-terminal lobe of the kinase domain. Activation of the receptor is initiated by binding of its ligand, stem cell factor (SCF) which leads to receptor dimerization and transphosphorylation on specific tyrosine residues. These phosphorylated residues serve as docking sites for signal transduction molecules containing SH2 (Src homology 2) domains such
as Cbl, Gab2, Shc, SHP2. They get activated upon phosphorylation and/or binding to the receptor and mediate signaling downstream of the receptor that eventually leads to various cellular responses. Many receptor tyrosine kinases undergo monoubiquitination or polyubiquitination following ligand stimulation, which targets them for degradation in the lysosomes or proteasomes, respectively. Members of Cbl family of ubiquitin E3 ligases play a crucial role in ubiquitination of c-Kit (1,7,8).

In many tyrosine kinases phosphorylation of tyrosine residue(s) in the activation loop is a crucial early event that leads to activation of the kinase. Examples of this type of receptors include the insulin receptor and the FGFR1 (9,10). However, in the case of c-Kit the phosphorylation of tyrosine residues in the juxtamembrane region is the most important activating event. In the absence of phosphorylation of the juxtamembrane region, it is inserted into a cleft between the N-terminal and C-terminal lobes of the kinase domain. Thereby the so-called C-helix is disrupted and the DFG motif is prevented from establishing an active conformation. Upon phosphorylation of the tyrosine residues in the juxtamembrane region, it is released together with the activation loop from the active site, the C-helix can move into position in the active site and thereby correctly orient the DFG residues that are important for catalytic activity (11,12).

Kinetic studies on recombinant c-Kit in vitro using mass spectrometry revealed that phosphorylation of Y823 is a late event that was observed when 90% of the kinase was already phosphorylated (13). Mutation of Y823 to a phenylalanine residue did not impair kinase activity. Further, Mol et al. showed that in a crystal structure of unactivated enzyme Y823 is bound to the catalytic base D792 blocking the access of substrates to the catalytic site (11). Therefore, phosphorylation of Y823 may disengage the activation loop from its inhibitory state. Another possibility is that phosphorylation and dephosphorylation of Y823 stabilizes the receptor structure and downstream signaling without being directly involved in kinase activity. However, the role of Y823 and its effects on c-Kit signaling has not been studied until now.

In this study we have investigated the cellular and biochemical effects of mutating Y823 to phenylalanine (Y823F). We show that Y823 is crucial for cell survival and proliferation. Cells expressing the Y823F mutant of c-Kit showed much lower proliferation and survival as compared to cells expressing the wild-type receptor despite the fact that the kinase activity was intact. Furthermore, the Y823F mutant receptor was internalized and degraded much faster than the wild-type receptor. A reduction in phosphorylation of the adaptor proteins Cbl, Shc, and Gab2 was also seen. The PI3-kinase/Akt,Ras/Erk and p38 pathways were also affected, in that the phosphorylation of Akt, Erk and p38 was very transient and not sustained as in wild-type c-Kit. Taken together, this study adds a novel perspective towards understanding the role of the activation loop tyrosine in c-Kit that is related to downstream signaling rather than kinase activity.

**EXPERIMENTAL PROCEDURES**

Reagents and antibodies-Transfection reagent Lipofectamine 2000 was from Invitrogen and jetPEI was from Polyplus. Cycloheximide was purchased from Sigma. Human recombinant SCF and murine recombinant IL-3 (Interleukin-3) were obtained from ProSpec Tany Technogene (Rehovot, Israel). Rabbit polyclonal anti-c-Kit serum was raised against a synthetic peptide corresponding to the carboxyterminus of c-Kit and purified as described (14). The anti-Cbl antibodies have been described elsewhere (15). The phospho-tyrosine antibody 4G10 was purchased from Millipore and ubiquitin antibody from Covance Research Products. Antibodies against phospho-p38, p38 and Shc were from BD Transduction Laboratories. Phycoerythrin(PE)-labeled c-Kit antibody was from BD Biosciences. Anti-phospho-Akt antibody was from Epitomics. Polyclonal anti-Gab2, anti-Akt, anti-phospho-Erk, anti-Erk and horseradish peroxidase-coupled secondary anti-goat antibodies were purchased from Santa Cruz Biotechnology. Secondary Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Invitrogen. Cell culture-Ba/F3 cells and M07e cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin and 10 ng/ml recombinant murine interleukin-3 (IL-3) or 10 ng/ml recombinant human IL3, respectively. Kasumi-1 cells were cultivated in above medium lacking IL-3. Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/ml streptomycin and 100
units/ml penicillin was used to culture COS-1 and EcoPack cells.

Expression constructs- pcDNA3-c-Kit-WT, pMSCV-c-Kit-WT constructs were described previously (1,8). pcDNA3-c-Kit Y823F and pMSCV-c-Kit-Y823F constructs were generated by site-directed mutagenesis using QuikChange mutagenesis XL kit (Stratagene). All plasmids were verified by sequencing.

Transient and stable transfection- Transient transfection of COS1 cells was performed using transfection reagent, JetPEI according to the manufacturer's instructions. Transfected cells were incubated for about 30 h before they were serum-starved overnight. Cell stimulation, lysis and immunoprecipitation was performed essentially according to (16). Stable transfections were performed essentially as described (17). Cells expressing wild-type c-Kit or c-Kit Y823F were confirmed by flow cytometry.

Immunoprecipitation and Western blotting- Cell lysis, immunoprecipitation and Western blotting was performed as described (16). Immunodetection was performed by enhanced chemiluminescence using horseradish peroxidase substrate (Millipore Corporation, Billerica, MA, USA) and the signals were detected by a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Signal intensities were further quantified by Multi-Gauge software (Fujifilm).

In vitro kinase activity assays- COS-1 cells transfected with pCDNA3-cKit-WT and pcDNA3-c-Kit-Y823F plasmids were starved of serum overnight. Cells were stimulated with 100 ng/ml SCF ligand and cell lysates were starved for 4 hours at 37°C in RPMI-1640 medium lacking serum and cytokines. Cells were stimulated with 100 ng/ml SCF for the indicated periods of time. To assess internalization, cells were stained with a PE-labeled anti-c-Kit antibody and surface expression of c-Kit receptor was determined by flow cytometry. Alternatively, receptor internalization was verified by Western blotting. Freshly prepared solution of 0.2 mg/ml EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) dissolved in PBS was added to cells. The cells were then incubated on ice for 40 minutes to allow biotinylation of cell surface proteins. After incubation, excessive biotin was removed by washing once with cold PBS. In addition, cold 50 mM Tris was added to the cells to block excessive reactive biotin. After 5 min. of blocking, cells were lysed and processed for pulldown with immobilized Avidin agarose (Thermo Scientific). The supernatant obtained after centrifugation was subjected to immunoprecipitation with anti-c-Kit antibody. c-Kit from both fractions was detected by Western blotting.

Degradation experiments- Ba/F3 cells were incubated with 100 μg/ml of cycloheximide, starved followed by stimulation with SCF for the indicated periods of time. For protein degradation experiments, ligand stimulation was followed by lysis and immunoprecipitation with a c-Kit antibody. Cell lysates obtained after stimulation were subjected to immunoprecipitation followed by detection of c-Kit by Western blotting using an antibody against c-Kit.

Cell proliferation and survival assay- Ba/F3 cells were washed three times with RPMI-1640 medium and seeded in 24-well plates (70,000 cells/well). Cells were then incubated either with 100 ng/ml SCF, without cytokine or with 10 ng/ml IL-3 for 48 hours. Viable cells were counted using trypan blue exclusion method. Alternatively, cells were stained by Click-iT EdU Alexa 647 (Life Technologies) cell proliferation kit employing the manufacturer's protocol. Stained cells were then analyzed by flow cytometry (BD FACSCalibur). Apoptosis was measured using an Annexin-V, 7-Amino-actinomycin D (7-AAD) kit (BD Biosciences Pharmingen), according to the manufacturer's instructions; double negative (Annexin-V)/(7-AAD) cells represent viable cells.

Internalization experiment- For internalization assay, Ba/F3 cells were incubated with 100 μg/ml of cycloheximide and starved for 4 hours at 37°C in RPMI-1640 medium lacking serum and cytokines. Cells were stimulated with 100 ng/ml SCF for the indicated periods of time. To assess internalization, cells were stained with a PE-labeled anti-c-Kit antibody and surface expression of c-Kit receptor was determined by flow cytometry. Alternatively, receptor internalization was verified by Western blotting.
RESULTS

The activation loop Y823F mutation accelerates receptor phosphorylation. The kinase domain of c-Kit is known to interact with juxtamembrane (JM) domain to maintain the kinase in an inhibitory state. When four tyrosine residues of JM domain at positions 547, 553, 568 and 570 are phosphorylated upon ligand stimulation, the JM domain is released from the kinase domain and the substrate can interact with the catalytic site. In addition, the activation loop transits from the DFG-out to the DFG-in state in transition from the unactivated state to the activated state. Together these two changes make the kinase catalytically active (13). We wanted to assess the changes in kinase activity of the receptor if the activation loop tyrosine, Y823, was mutated. Therefore, we generated the Y823F mutant of c-Kit and stably transfected it into Ba/F3 cells. Ba/F3 is an immortalized murine bone marrow-derived pro-B-cell line which is dependent on IL-3 for its growth (20). We have used Ba/F3 cells in the current study because this cell line does not express c-Kit endogenously and therefore can be used to overexpress both wild-type and mutant forms of human c-Kit. We also performed experiments with wild-type c-Kit and Y823F c-Kit by transient transfections in Cos1 cells. Both cells lines were tested for cell surface expression of c-Kit by flow cytometry (Fig. 1A). In order to verify, that overexpression of c-Kit wt and c-Kit Y823F was comparable to endogenous expression of c-Kit, we used two leukemic cell lines, Mo7e and Kasumi as controls (Fig. 1B). We further observed that both the wild-type c-Kit as well as Y823F c-Kit are rapidly phosphorylated after stimulation with SCF. However, the onset of phosphorylation in the mutant was faster and stronger than in wild-type c-Kit, it rapidly declined in the Y823F mutant whereas wild-type c-Kit showed a more sustained response (Fig. 1C). This suggests that there is either a conformational change in the Y823F mutant or that the mutant c-Kit stability is altered.

Ubiquitination and degradation are more rapid in the Y823F mutant than in wild-type c-Kit. It has been previously shown that RTKs upon ligand stimulation undergo mono-ubiquitination or poly-ubiquitination that targets them to be internalized and degraded in the lysosomes or the proteasomes, respectively (1). Previous studies demonstrate that Cbl which belongs to a family of ubiquitin E3 ligases is crucial for ubiquitination of c-Kit (1,7,8). Furthermore, Cbl can either directly interact with c-Kit at Y568 and Y936 (1) or indirectly through the adapter protein Grb2 (8). This leads to mono-ubiquitination of c-Kit and targets it for degradation in lysosomes. We wanted to investigate whether mutation of Y823 in the activation loop of c-Kit affects the phosphorylation of Cbl which could result in changes in ubiquitination which in turn could affect receptor degradation. Cbl was immunoprecipitated from both wild-type and Y823F mutant c-Kit following ligand stimulation and extent of phosphorylation of Cbl was analyzed. The results clearly demonstrate that Cbl is phosphorylated already after 2 min. of SCF stimulation in both c-Kit wt and c-Kit-Y823F but the mutant is unable to sustain the phosphorylation (Fig. 2A). In order to assess the degree of ubiquitination, c-Kit was immunoprecipitated and Western blotting was performed using an antibody against ubiquitin. In agreement with the Cbl phosphorylation data, ubiquitination of c-Kit Y823F is very strong after 2 min. of SCF stimulation but then rapidly declines. In contrast, ubiquitination of wild-type c-Kit is weaker but increases over a longer period of time (Fig. 2B). Degradation of c-Kit was analyzed by starving Ba/F3 cells in presence of cycloheximide, an inhibitor of protein synthesis, for 4h followed by immunoprecipitation and detection using anti-c-Kit antibody. Degradation of c-Kit receptor was analysed for up to 30 min. of SCF stimulation. We observed that in wild-type c-Kit expressing cells, c-Kit is detectable for up to 30 min. whereas in cells expressing the Y823F mutant, c-Kit is degraded before 15 min. of SCF stimulation (Fig., 2C and D).

A change in activation loop Y823 to Y823F causes the receptor to internalize faster. It is known that together with JM domain, activation loop also plays a role in maintaining the receptor in an unactivated state. Phosphorylation of Y823 and of tyrosine residues in JM domain is crucial to maintain the receptor in an active state. Moreover, previous reports show that Cbl in addition to regulating degradation of RTKs also regulates their internalization. Thus, we analyzed the pattern of receptor internalization in response to ligand activation. Ba/F3 cells were serum-starved in presence of cycloheximide for 4h and stimulated with SCF for indicated periods of time. Cells were stained with a PE-labeled anti-c-Kit...
antibody and surface expression was determined by flow cytometry (Fig 3A). As an alternative method we verified the rate of of internalization of cell surface c-Kit compared with the total c-Kit by Western blotting (Fig. 3B and C). The data clearly demonstrate that the Y823F mutant of c-Kit is more rapidly internalized than wild-type c-Kit.

Mutation of activation loop tyrosine 823 has no effect on kinase activity. Activation of c-Kit by its ligand triggers activation of numerous downstream signal transduction molecules. Some of these molecules are tyrosine kinases by themselves, e. g. Src family kinases such as Lyn and the Fes kinase (21,22) and have been demonstrated to be able to phosphorylate c-Kit (23). Thus, the phosphorylation status of c-Kit in living cells could be influenced by other factors than the intrinsic kinase activity of c-Kit itself. Therefore we wanted to evaluate the kinase activity of wild-type c-Kit and the Y823F c-Kit mutant in an in vitro assay. To test whether activation loop tyrosine has an effect on kinase activity, both wildtype and Y823F mutant c-Kit were transiently transfected into Cos1 cells and stimulated with SCF. Immunoprecipitates of c-Kit from these cells were incubated with myelin basic protein (MBP) as an exogenous substrate and an in vitro kinase reaction was performed. No change in kinase activity was observed between the wild-type and the Y823F mutated c-Kit receptor (Fig. 4A and B) which is in agreement with previous reports (13).

Effect of Y823F mutation on phosphorylation of adapter proteins. Adaptor proteins such as Cbl, Shc, SHP2 and Gab2 are known to regulate the signal transduction through c-Kit either directly or through other adaptor proteins which in turn affect the downstream signaling pathways (24). We further analyzed the up-stream adapter proteins that affect cell proliferation and cell survival. SCF stimulated Ba/F3 cells were subjected to immunoprecipitation using specific antibodies and phosphorylation of each adaptor protein was detected using phospho-specific antibodies. We observed that phosphorylation of Cbl, Shc and Gab2 was reduced in cells expressing Y823F c-Kit, compared to cells expressing wild-type c-Kit, while SHP2 phosphorylation was not significantly affected by Y823F mutation (Fig., 6A and B).

Mutation of Y823 leads to a significant decrease in both cell survival and proliferation. We wanted to ascertain if the mutation of activation loop tyrosine in c-Kit had an effect on survival and proliferation of cells. The cells lacking Y823 showed a significant reduction in proliferation as confirmed by EdU incorporation in proliferating cells and trypan blue exclusion method (Fig., 7A and B). The effect of the Y823F mutation of c-Kit on cell survival was also examined by staining the cells with Annexin V and 7-Amino Actinomycin D and analyzing by flow cytometry. Ba/F3 cell expressing the Y823F mutant of c-Kit showed almost 60% reduction in cell survival compared to cells expressing wild-type c-Kit (Fig. 7C).

DISCUSSION

The activation loop of c-Kit spans about 20-25 amino acids in the C-lobe of kinase domain and together with the juxtamembrane domain maintains the kinase in an auto-inhibitory state. Studies indicate that two key processes are required for activation of kinase, one the release of JM domain that exposes the catalytic site to the substrate and second, the activation loop coming to the DFG-in state. We have observed that c-Kit phosphorylation is not hampered by the Y823F mutation. Y823 is the only tyrosine phosphorylation site in the activation loop and yet the previous structural studies point to its irrelevance in the kinase activation process. So what is Y823 doing in the activation loop? Does it have any role in downstream signaling through c-Kit receptor?
To date very little is known about the role of the activation loop tyrosines in other receptors other than in the regulation of kinase activity. In non-receptor tyrosine kinase, Syk phosphorylation of activation loop tyrosines is described crucial for propagation of signaling through the immunoreceptor but does not affect the kinase activity of Syk (25). In an early report from Server and co-workers (26), Y821 in murine c-Kit (homologous to Y823 in human c-Kit) was shown to be of importance for proliferation and survival in murine bone marrow-derived mast cells without affecting PI3-kinase, p21ras or Erk activation. However, the discrepancy with our data on Erk activation could be explained by the use of different cell systems. The EGFR Y845, analogous to Y823 in c-Kit, was demonstrated to be required in mitogenic pathways and as a mediator for an association between CoxII and EGFR crucial for cell survival (27,28). In both c-Kit Y821 and EGFR Y845, a phenylalanine mutant showed reduced cell survival. In PDGF receptor, activation loop Y857 was shown to be crucial for in-vitro kinase activity and for cell proliferation but did not affect internalization (29). In our study we show that Y823F affects downstream signaling pathways of c-Kit, it is fully dispensable for kinase activity and that the mutated receptor is internalized and degraded at a much accelerated rate compared to the wild-type receptor. Mutation of Y823 to Y823F further significantly reduced cell proliferation and survival. Thus, our study adds new perspectives to existing knowledge about the role of activation loop tyrosine (Y823) in c-Kit signaling.

Previous studies on the kinetics of phosphorylation of c-Kit have provided valuable information on the role of Y823 in c-Kit activation (13). However, those studies were performed on a recombinant intracellular fragment of c-Kit, and thus lacking the ligand-binding domain. Furthermore, in a cell free system the other tyrosine kinases and molecules that affect c-Kit signaling (such as Src and Fes) are lacking and their contribution to phosphorylation of the receptor is not seen. Phosphorylation, ubiquitination, and degradation experiments on ligand-induced c-Kit activation demonstrate that the Y823F mutant of c-Kit is able to transduce a phosphorylation signal but at a much accelerated rate and considerably more transient in its nature compared to wild-type c-Kit. Cbl, a ubiquitin E3 ligase is known to regulate ubiquitination and degradation of receptor tyrosine kinases. We observed that phosphorylation of Cbl increases when we increase the ligand stimulation time in wild type receptor whereas it decreases in Y823F. The effect on short lived ubiquitination and faster degradation could also be due to instant de-phosphorylation after a brief phosphorylation of Cbl. The mutant receptor is also internalized much faster than the wild type receptor. This could again be explained by the phosphorylation status of Cbl which is known to regulate internalization (1). Thus, the effects on ubiquitination, internalization and degradation could be the consequence of Cbl inactivation which can interact with c-Kit either directly or through activation of Src kinases. Further, phosphorylation of adaptor proteins such as Gab2 and Shc is also reduced in Y823F as compared to the wild type receptor. These molecules are downstream to SFK (Src Family Kinases) which directly interact with c-Kit (30,31). Gab2 and Shc are connected to Akt and Erk pathways downstream (21,24). Therefore a reduction in Akt and Erk1/2 phosphorylation could be explained by altered Shc and Gab2 signaling molecules. Reduced Akt and Erk1/2 phosphorylation reduce cell survival and cell proliferation which are linked to Akt and Erk pathways. Similar observations have been made in PDGFβ receptor where the activation loop Y857F mutation hampers complete activation of Akt and Erk (29). On the contrary, Y857F also led to reduced SHP2 activation which was not the case with Y823F in c-Kit. A reduction in SHP2 phosphorylation has been proposed to lead to reduction in Erk phosphorylation. Since SHP2 is not affected in our case, there are likely to be an alternate pathway affecting Erk signaling (29,32). One likely candidate for this is the adapter protein Shc, since we have previously shown that Src-phosphorylation of Shc is essential for the ability of c-Kit to mediate activation of Erk (21). Similarly, transient phosphorylation of p38 could also be caused by a reduction in Src-mediated Shc phosphorylation (33,34). Its role in cell migration has also been previously described (35). Thus, mutation in activation loop tyrosine, Y823 affects multiple signaling pathways as also described for Syk kinase where activation loop tyrosines are crucial for sustained downstream signaling without being involved in catalysis (36,37). Based on previous studies and our own current data, a perspective towards function of activation loop

tyrosine is that when the c-Kit receptor dimerizes and autophosphorylates, the potential tyrosine residues on JM domain are phosphorylated and release the auto-inhibitory state. Next is the phosphorylation of activation loop tyrosine that upon phosphorylation makes the loop in DFG-in state and locks the kinase from an unactivated state into an activated state. But when Y823 is mutated to F823, there is a conformational change and a de-stabilization of the kinase such that the activated state is no longer maintained. It could be due to this reason that signaling through the mutated receptor initiates normally but is not sustained due to a destabilized receptor which leads to a faster internalization, degradation and a marked reduction in cell viability and cell proliferation capacity. However, a change in conformation is suspected but it excludes the domain binding to kinase inhibitor, sunitinib. As proposed by DiNitto et al, the Y823F mutation could render the enzyme more flexible such that after activation it comes back to DFG-out unactivated state preferred by most kinase inhibitors.

It turns out that the effect of mutating Y823 is very much dependent on which amino acid replaces the tyrosine at position 823 is critical. DiNitto and coworkers demonstrated in their in vitro cell-free system that the Y823A and Y823D were devoid of kinase activity while the Y823F mutant has full activity (13). It is interesting that the Y823D mutation of c-Kit has been described in human testicular seminomas (38), malignant melanomas (39), gastrointestinal stromal tumors (40) and pediatric core-factor binding acute myeloid leukemia (41). The Y823D mutant was found to be constitutively active in living cells (38). This apparent discrepancy in results might be attributable to the fact that in the living cell c-Kit has access to accessory molecules, that is missing in a cell free system, that are necessary for its signaling. Furthermore, the Y823C (38) and Y823N (42) mutants have also been identified in tumors, but no data are available as to whether these mutations also are activating. Thus, the amino acid that substitutes the tyrosine residues is critical for signaling outcome. In one case it might lead to constitutive activation, in another case to modulation of signaling and in a third case to inactivation of the kinase.

In view of our results, Y823 appears to be a good target for cancer therapy. The Y823F makes the receptor not only more sensitive to therapeutic targets such as Sunitinib and Imatinib but also de-stabilizes the receptor such that activation of Akt, Erk1/2 and p38 is reduced. This in turn leads to a significant reduction in cell proliferation and cell survival. Thus, a therapy targeting Y823 such that its phosphorylation is prevented could, in combination with chemotherapy, provide an improved treatment option for tumors caused by c-Kit mutations. This study exemplifies that even when sequence of kinase domains is highly conserved across members of the RTK family, point mutations of various receptors play divergent roles in cellular outcomes and in the mechanism of signal transduction. This underlines the importance of investigating further point mutations in c-Kit and the mechanisms by which they influence cell signaling, to improve our current understanding of the association of receptor mutations with cancer prognosis.

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**FOOTNOTES**

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2The abbreviations used are: RTK, receptor tyrosine kinase; Mitogen-activated protein kinase; PI3K, Phosphoinositide 3-kinase; SCF, stem cell factor; SH2, Src-homology2; EGFR, Epidermal growth factor receptor; IL-3, Interleukin-3; 7-AAD, 7-Amino Actinomycin D; JM, Juxtamembrane; PE, Phycoerythrin.

**FIGURE LEGENDS**

**FIGURE 1:** Y823F mutation leads to enhanced and faster phosphorylation of c-Kit receptor. 
*A,* stably transfected Ba/F3 cells were labeled with phycoerythrin-conjugated anti-c-Kit antibodies or an isotype control and analyzed by flow cytometry. The *black peak* indicates cells labeled with the isotype control, and the *gray peak* corresponds to the cells labeled with anti-c-Kit antibody. B, Mo7e, and Kasumi cell lines expressing endogenous c-Kit and Ba/F3 cells expressing c-Kit and c-Kit/Y823F were lysed and subjected to immunoprecipitation with anti-c-Kit antibody. C-Kit expression levels were detected by Western blotting. C Ba/F3 cells were stably transfected with Ba/F3-c-Kit and Ba/F3-c-Kit/Y823F constructs. Cells were serum-starved overnight at 37°C and stimulated by SCF for indicated time periods. Cell lysates were prepared, immunoprecipitated (*IP*) with anti-c-Kit antibody...
and analyzed by Western blotting. Total receptor phosphorylation was detected using phosphotyrosine (pY) antibody and c-Kit was used as a loading control.

**Figure 2.** The Y823F mutant of c-Kit mediates increased phosphorylation of Cbl concomitant with increased ubiquitination and degradation of c-Kit. A, Ba/F3-c-Kit and Ba/F3-c-Kit/Y823F cells were serum-starved and stimulated with 100 ng/ml SCF for the indicated times. Cells were lysed, and lysates were immunoprecipitated (IP) with anti-Cbl antibody, followed by Western blotting with phosphotyrosine antibodies and with a c-Kit antibody. IB, immunoblot. B, Lysates from serum-starved Ba/F3-c-Kit and Ba/F3-c-Kit/Y823F cells were immunoprecipitated (IP) with anti-c-Kit antibody. Ubiquitination of receptor was detected using anti-Ub antibody. C, Receptor degradation was assessed by serum starvation of Ba/F3-c-Kit and Ba/F3-c-Kit/Y823F cells for 4h at 37°C in presence of 100μg/ml of cycloheximide. Cells were then stimulated with 100 ng/ml SCF for indicated periods of time and cells were immediately placed on ice. Cell lysates were prepared and were subjected to immunoprecipitation (IP) with c-Kit antibodies followed by immunodetection using c-Kit antibody. D, Signal intensities from two independent experiments were quantified using Multi-Gauge software to calculate the percentage of receptor degradation.

**Figure 3.** The Y823F mutation of the activation loop of c-Kit enhances receptor internalization. Ba/F3-c-Kit and Ba/F3-c-Kit/Y823F cells were serum-starved for 4h at 37°C in presence of cycloheximide and stimulated with 100 ng/ml SCF for the indicated times. A. Cells were transferred to ice followed by incubation with phycoerythrin conjugated anti-c-Kit antibody. The c-Kit surface expression level was analyzed by flow cytometry. Internalization of c-Kit was quantified at various time points compared with unstimulated cells. To determine mean fluorescence intensities (MFI) of wild-type receptor with mutated receptor, FloJo software was used. B. Cells were labeled with Sulfo-NHS-Biotin and incubated on ice for 40 minutes to allow biotinylation of cell surface proteins. Cells were lysed and processed for pulldown with immobilized Avidin. The supernatant obtained after centrifugation was subjected to immunoprecipitation with anti-c-Kit antibody. c-Kit from both fractions was detected by Western blot. C. Internalization of c-Kit was quantified at various time points and statistically analyzed using GraphPad Prism, ***, p < 0.001.

**Figure 4.** Y823F mutation in activation loop does not affect receptor kinase activity. Cos1-c-Kit-WT and Cos1-c-Kit/Y823F cells were serum-starved overnight and stimulated with 100 ng/ml SCF. Cell lysates were prepared and c-Kit was immunoprecipitated. A. Cell lysates were incubated with protein G beads, washed and subjected to in vitro kinase assay with γ32P-ATP and MBP as exogenous substrate. Phosphorylation signal was detected using L process software. c-Kit was detected by Western blotting. B. Calculation of relative MBP phosphorylation and statistical analysis was performed using GraphPad Prism, ns, not significant.

**Figure 5.** The Y823F mutation in c-Kit alters down-stream signaling. Ba/F3-c-Kit WT and Ba/F3-c-Kit/Y823F cells were serum-starved and treated with or without 100 ng/ml SCF for indicated periods of time. A, Total cell lysates were separated by SDS-PAGE, electrotransferred to Immobilon P membrane and probed with phospho-Akt antibody, phospho-Erk1/2 and phosphor p38 antibodies. Antibodies against Akt,Erk and p38 were used as loading controls. B, Signal intensities from three independent experiments were quantified using Multi-Gauge software to calculate the reduction and GraphPad Prism was used to calculate significance. ns, not significant, ***, p < 0.01.

**Figure 6.** Phosphorylation mutant Y823F negatively regulates activation of adaptor proteins. A, Ba/F3-c-Kit-WT and Ba/F3-c-Kit/Y823F cells were serum-starved and incubated in the presence or absence of SCF for indicated period of time. Cell lysates were subjected to immunoprecipitation with antibodies against Gab2, Shc, Cbl and SHP2 respectively. Proteins were separated by SDS-PAGE, electrotransferred to Immobilon P membranes and probed either with phosphospecific antibodies or general phosphotyrosine antibodies (pY). B, Signal intensities from three independent experiments were quantified with Multi-Gauge software and statistical analysis was done using GraphPad Prism.

**Figure 7.** Cells expressing the Y823F mutant of c-Kit display reduced cell proliferation and cell survival in response to SCF stimulation. Ba/F3-c-Kit WT and Ba/F3-c-Kit/Y823F cells were grown for 48 h in the presence or absence of SCF and IL-3. A, cells were incubated with EdU for 2 h, fixed,
labeled with Alexa Fluor 647 and analyzed by flow cytometry. B, Viable cells were counted by trypan blue exclusion method C, cells were labeled with annexin V and 7-aminoactinomycin D, and living cells were measured by flow cytometry. IL-3 was used as a positive control. Quantification of labeled cells was obtained with FloJo software and results from three independent experiments were statistically analysed using GraphPad Prism, ns, not significant, **p<0.01, ***, p < 0.001.
Figure 1

A. 

B. 

C. 

| SCF (min.) | WT | Y823F |
|-----------|----|-------|
| 0         | 2  | 5     |
| 10        | 20 |       |
| 20        |    |       |
Figure 2

A.

| SCF (min.) | WT | Y823F |
|------------|----|-------|
| 0 2 5 10 20 | 0 2 5 10 20 |

pCbl

Cbl

IP: Cbl

B.

| SCF (min.) | WT | Y823F |
|------------|----|-------|
| 0 2 5 10 20 | 0 2 5 10 20 |

Ub

c-Kit

IP: c-Kit

C.

| SCF (min.) | WT | Y823F |
|------------|----|-------|
| 0 2 5 15 30 | 0 2 5 15 30 |

WT

Y823F

IP: c-Kit

D.

% degradation

| SCF (min.) | WT | Y823F |
|------------|----|-------|
| 0 15 | 0 15 |

Bar graphs showing % degradation with SCF (min.) for WT and Y823F.
Figure 3

A.

![Graph showing SCF (min.) percent of Max for WT and Y823F over time](image)

B.

|       | WT |       | Y823F |
|-------|----|-------|-------|
|       | 0  | 2     | 5     | 15    | 30   | 0  | 2   | 5   | 15  | 30  |
| Surface c-Kit | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| Total c-Kit   | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| β-Actin      | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |

C.

![Graph showing % of Internalization vs SCF (min.)](image)
Figure 4

A.

MBP phosphorylation

| SCF (min.) | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 |
|------------|-----|-----|-----|-----|-----|
| WT         | -   | +   | -   | +   |     |
| Y823F      | -   | +   | -   | +   |     |

Relative MBP phosphorylation

B.

Relative MBP phosphorylation

| SCF (min.) | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 |
|------------|-----|-----|-----|-----|-----|
| WT         | +   | +   | -   | -   |     |
| Y823F      | -   | -   | +   | +   |     |

ns
Figure 5

A.

| SCF (min.) | WT | Y823F |
|------------|----|-------|
| 0          | 2  | 0     |
| 2          | 5  | 2     |
| 5          | 10 | 10    |
| 10         | 20 | 10    |

B.

Band intensity (%)

|        | WT | Y823F |
|--------|----|-------|
| pAkt   |    |       |
| Akt    |    |       |
| Erk1/2 |    |       |
| pErk1/2|    |       |
| P-p38  |    |       |
| p38    |    |       |

**ns,** **p < 0.01**
Figure 6

A.

B.
Figure 7

A. No cytokine, p=0.62

- WT: 11.69 ± 1.051 N=3
- Y823F: 10.77 ± 1.327 N=3

B. SCF, p=0.0001

- WT: 52.21 ± 0.8367 N=3
- Y823F: 23.10 ± 0.5781 N=3

C. Cell number x 10^4

- without SCF, IL3

D. % of cell survival

- no cytokine, SCF, IL3