KIT Mutations Induce Intracellular Retention and Activation of an Immature Form of the KIT Protein in Gastrointestinal Stromal Tumors

Séverine Tabone-Eglinger,1 Frédéric Subra,5 Hiba El Sayadi,1 Laurent Alberti,1 Eric Tabone,2 Jean-Philippe Michot,2 Nathalie Théou-Anton,4 Antoinette Lemoine,4 Jean-Yves Blay,1,3 and Jean-François Emile4,6,7

Abstract

Purpose: Gastrointestinal stromal tumors (GIST) are frequently associated with gain-of-function mutations of KIT, which can be inhibited by imatinib both in vitro and in vivo. The survival of patients with GIST, following imatinib therapy, has been correlated with the nature of mutations but not with KIT expression.

Experimental Design: Subcellular localization, activation, and trafficking of the mature and the immature forms of KIT were investigated in GIST samples and in NIH3T3 cells infected with two different GIST-type exon 11—mutated human KIT cDNA.

Results: paranuclear dot expression of KIT was more frequent in GISTs with homozygous KIT mutations than in those with heterozygous (P = 0.01) or no mutations (P < 0.01). Activation of the immature 125 kDa form of KIT was detected in most GISTs with KIT mutations but not in GISTs without KIT mutations. In NIH3T3 cells, mutant KIT was mainly retained within endoplasmic reticulum and Golgi compartments in an immature constitutively phosphorylated form, whereas the wild-type KIT was expressed at the plasma membrane, in a mature nonphosphorylated form. Imatinib-induced inhibition of the phosphorylation of immature and mature mutant KIT proteins resulted in the restoration of KIT expression at the cell surface.

Conclusions: These results show that GIST-type KIT mutations induce an activation-dependent alteration of normal maturation and trafficking, resulting in the intracellular retention of the activated kinase within the cell. These observations likely account for the absence of correlation between response to imatinib and KIT expression using immunohistochemistry and may deserve to be investigated in other tyrosine kinase–activated tumors.

Gastrointestinal stromal tumors (GIST) are the most frequent sarcomas of the digestive tract and express KIT protein in >95% of cases (1). Gain-of-function mutations in the KIT gene, and more recently, in the PDGFR gene, have been shown to play a critical oncogenic role in GISTs (2–4). Imatinib mesylate, a KIT and PDGFR tyrosine kinase inhibitor, blocks the activation of both receptors in vitro and induces tumor control in >90% of patients with GIST (5–8). More than 80% of KIT mutations occur in exon 11 with a hotspot in the 550 to 561 codon region, whereas mutations in exon 9 and in exons 13 to 17 are observed in 10% to 15% and <1% of metastatic tumors, respectively (5, 9–13). Correlations between the nature of mutation (exon 11 versus others and sites of mutations within exon 11) and disease outcome prior to imatinib therapy has been reported in some but not all studies (9–12, 14–20). Conversely, response to imatinib therapy has consistently been influenced by the nature of KIT mutations: patients with exon 11 mutations were reported to have a better survival with imatinib than exon 9 or wild-type (WT) forms (5, 6). Of note, deletions involving the distal part of KIT exon 11 may be less favorable for response to imatinib (21). An intriguing observation is that immunohistochemical expression of the KIT protein in KIT-positive GISTs is not correlated with KIT protein activity in vivo (22), in contrast with observations in other models such as HER2 amplification in breast cancer (23).

In the present work, we observed that KIT mutations result in the modification of the protein subcellular localization. In GISTs, as well as in NIH3T3-infected cells, mutated forms of KIT were mainly expressed in an immature phosphorylated-activated form within the intracellular compartments and with limited expression at the cell surface in this model. Activation...
of the immature form of KIT was only detectable in cells expressing exon 11 KIT juxtamembrane mutations; imatinib-induced blockade of the activation of the immature KIT form restored the expression of the mature form at the cell surface.

Materials and Methods

Reagents and antibodies. Recombinant human SCF (rhSCF) used for the assays was purchased from R&D Systems. Chinese hamster ovary-KL, used for the culture, was a kind gift from P. Dubreuil (UMR 559, Centre de Recherche en Cancérologie, Marseille, France). Imatinib mesylate was kindly provided by Novartis. The rabbit polyclonal anti-KIT antibody (A4502), used for immunohistochemistry and Western blot, and the phycoerythrin-conjugated mouse monoclonal anti-KIT antibody (clone 1045D2) used for flow cytometry analysis were from Dako. The antiphosphotyrosine 823 of KIT (pY823KIT) and the antiphosphotyrosine 703 of KIT (pY703KIT), used for Western blot and immunofluorescence analysis, were purchased from Cellsigns. For immunohistochemistry assays, CD34 (clone QBEND mouse) was purchased from Immunotech, and anti-S100 protein (polyclonal rabbit) was from Dako. Anti–β-actin antibody (Sigma-Aldrich) and secondary horseradish peroxidase antibodies (Dako) were used in Western blotting experiments, whereas rabbit anti-calnexin, mouse anti-Golgin58K (Abcam), anti-rabbit biotin (Dako), streptavidin-Alexa 594, and anti-rabbit Alexa 647, and anti-mouse IgG1 Alexa 647 (Molecular Probes/Invitrogen) were used for immunofluorescence analysis.

Analysis of human GIST samples. The records of patients with GIST were retrieved from the files of Ambroise Paré University Hospital. Histology and immunohistochemistry of all cases were reviewed. Clinical records were reviewed as well, and no patients had received imatinib before surgical resection of the tumor. All the human samples were obtained by surgical resection done for therapeutic purposes and according to French ethical guidelines.

Mutations within exons 9, 11, 13, and 17 of KIT and within exons 12 and 18 of PDGFRA were detected as previously described (10, 24). Briefly, tumor DNA was extracted from either frozen or paraffin tumor samples after morphologic control. Screening of insertions and deletions within exons 9 and 11 of KIT and within exons 12 and 18 of PDGFRA was done by length analysis of PCR products (25), which allows the quantification of the mutant/wild-type ratio, and all samples were subjected to direct sequencing. Deletions or insertions within KIT exons 11 and 9 were considered as homozygous, when the fluorescence intensity of the mutant allele was at least three times higher than that of the wild-type allele. In all cases, histology confirmed that the analyzed sample contained >90% of tumor cells.

Immunohistochemistry was done on 4-μm-thick slides of paraffin-embedded GIST samples with anti-CD117 (1/300), anti-CD34 (1/2), and anti-S100 (1/200) antibodies. Slides were immersed for 20 min in a boiling antigen retrieval citrate buffer (pH 9.6), and staining was revealed with LSAB2 KIT (Dako). The cellular localization of KIT staining from 82 paraffin-embedded GIST samples was evaluated without knowledge of the mutational status.

Analysis of KIT expression and activation was done on 26 frozen GIST samples by Western blotting experiments. Samples were calibrated and mechanically homogenized (Mixer Mill MM 300) in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L orthovanadate, 10 mmol/L NaF, 1 mmol/L PMSF, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin, 10 IU/mL aprotinin, and 1% Triton X-100). Lysates were rocked for 30 min at 4°C, then centrifuged to remove insoluble material. Supernatant protein contents were determined using Bradford solution. The normalized soluble proteins (supernatant fraction) were directly analyzed by SDS-PAGE Western blotting with anti-KIT, anti-pY703KIT, anti-pY823KIT, and anti–β-actin antibodies.

Production of recombinant viruses and NIH3T3 infection. The full-length human KIT cDNA (GNNK isotom), and two mutants (KITdel557-558 and KITdel564-581) were excised from the pGem-Teasy plasmid through SalI and NotI restriction and inserted in oncoviral vectors plNCX (Clontech) and MigR1 (26) for wild-type and mutant forms, respectively. Both retroviral vectors included the same cytomegalovirus promoter. To produce viral stocks, the plasmids pLNXK KIT-vt and MIGR KIT-mutants were independently cotransfected with plasmid pMD.G (VS.V.G envelope) and pBR322 containing inserts Gag-PolGpt into 293T cells using the standard calcium phosphate procedure. Briefly, 293T cells (2.5 × 10⁶) were cotransfected with 15 μg of retrovectorial plasmid (pLNXK or MIGR1), 10 μg of pBR322, and 5 μg of pMD.G for 12 h. The next day, the medium containing the virus was harvested. Titers of produced retroviral vectors were determined after infection on NIH3T3 cells and either resistance to genetin (0.8-1 mg/mL) for plNCX, either green fluorescent protein expression for MigR1 (the expression of green fluorescent protein is regulated by an internal ribosome entry sequence). Infection of NIH3T3 cells were done at a multiplicity of infection of 0.5. Cells with the plNCX vector were selected with genetin for 7 to 10 days (maintained for the culture) before being isolated and amplified. Cells with the MigR1 vector were amplified for 48 h after infection and green fluorescent protein–positive cells were sorted.

Cell culture. NIH3T3-infected cells were cultivated in DMEM with 10% newborn calf serum (Life Technologies), 2% penicillin/streptomycin, and 50 ng/mL of Chinese hamster ovary–mouse SCF so that the growth of wild-type and mutant forms were equivalent. Chinese hamster ovary–mouse SCF was removed during serum starvation for the assays.

To study proliferation in low serum concentrations, cells were plated at 50,000 cells per well, then starved for 4 h before incubation with DMEM containing 1% serum for the times indicated. At each time point, cells were harvested, suspended in a precise volume of medium, and then counted with a FACS scan (Becton Dickinson). Results were expressed as mean of triplicate ± SD.

To perform soft agar assays, cells were plated at 10,000 in 1 mL of 0.33% agar (Difco) in DMEM + 10% newborn calf serum with or without rhSCF, over a 0.5% 1mL agar layer in triplicate in 35 mm dishes. After 3 weeks, plates were stained with 0.005% crystal violet and colonies were counted with Gene Tools software (Ozyme). Each condition was done in triplicate and results are given as the mean number of colonies.

mRNA analysis. The mRNA of NIH3T3 cells was extracted according to TRIzol method (Invitrogen). Then, 5 μg of RNA was submitted to reverse transcriptase (Super Script II, Invitrogen). Real-time PCR and subsequent calculations were done with the ABI Prism 7000 sequence detection system (Applied Biosystems).

Table 1. Comparison of KIT subcellular staining in three subsets of patients with GIST

<table>
<thead>
<tr>
<th>Total</th>
<th>Primary site</th>
<th>KIT staining of GIST cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastric</td>
<td>Small intestine</td>
</tr>
<tr>
<td>Homozygous KIT mutation</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>No mutation of KIT or PDGFRA</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Heterozygous KIT mutation</td>
<td>51</td>
<td>26</td>
</tr>
</tbody>
</table>

Human Cancer Biology
Primer and probes were described previously (24). The PCR mixture contained 1 × TaqMan Universal PCR Master Mix reagents (Applied Biosystems), 400 nmol/L of KIT forward and reverse primers, 200 nmol/L of probe, 0.25 µmol/L of amperase UNG, and 5 µg of KIT cDNA in a total volume of 50 µL. PCR conditions consisted of two steps of 5 min each at 55 °C and 5 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. TaqMan rRNA control reagents (Applied Biosystems), designed for ribosomal 18S RNA amplification, were used as references to normalize the results. Experiments were done in triplicate. Results were expressed as ratios of KIT to 18S PCR products (equivalency of the efficacy of the two PCRs was previously verified).

Protein analysis. NIH3T3 cells were grown to 80% confluence in complete medium, and then starved for 4 h before incubation with rhSCF or imatinib for the concentrations and times indicated. Cells were washed with cold PBS, and lysed directly in culture plates with 200 µL/10 cm² of lysis solution [10 mmol/L TRIS (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 1 mmol/L PMSF, 0.5 µg/mL leupeptin, 1 µg/mL pepstatin A, and 10 IU/mL aprotinin] for 30 min at 4 °C. The soluble protein (supernatant fraction) was either directly analyzed by SDS-PAGE Western blotting or first immunoprecipitated with the rabbit anti-KIT antibody.

For cell surface biotinylation of the protein, cells were labeled with Sulfo-NHS biotin (Perbio Science) according to the manufacturer's instructions before protein extraction, KIT immunoprecipitation, and Western blotting analysis.

Flow cytometry. NIH3T3 were grown to 80% confluence in complete DMEM, then serum-starved for 4 h before incubation or

![Fig.1. KIT perinuclear expression in GISTs with homozygous mutations and phosphorylation of the immature KIT form in GIST protein extracts. A, wild-type (arrow; 270 bp) and mutant alleles of KIT were detected by length analysis for fluorescent PCR products. These five GISTs had deletions within exon 11 of KIT, three of which were considered as homozygous (cases Y04-108, Y04-109, and Y04-210) because of the low amount of wild-type as compared with mutant alleles. B, KIT immunohistochemical staining of GIST on paraffin-embedded samples (top) and NIH3T3 embedded cells (bottom) disclosed a predominant Golgi-like pattern in GISTs with homozygous KIT mutations or in NIH3T3 KIT D6–infected cells (right) and a membrane and/or cytoplasmic pattern in GISTs without mutations or NIH3T3 WT KIT–infected cells (left). Bar, 20 µmol/L.

C, Western blot analysis of protein extracts of GIST frozen samples disclosed a variable KIT expression, with a main heavy (mature, 145 kDa) and a minor light (immature, 125 kDa) form. KIT phosphorylation (Tyr703 and Tyr823 residues) of both forms was analyzed in GISTs with homozygous or homozygous KIT mutations and GISTs without mutations of KIT and PDGFRA. Protein extracts of wild-type NIH3T3– and D6–infected cells were analyzed in each blot as a control of KIT expression and phosphorylation. β-Actin expression was analyzed as a loading control.](https://www.aacrjournals.org/)
not with SCF or imatinib. Cells were then harvested and resuspended in cold PBS before incubation with a phycoerythrin–conjugated anti-KIT antibody (Dako) for 15 min at 4°C. Cells were analyzed by flow cytometry on a FACS Scan (Becton Dickinson) and analyzed with Cell Quest Pro Software (Becton Dickinson). Results were expressed as mean of fluorescence intensity.

For analysis of total cell expression of KIT, cells were fixed in 4% PBS-paraformaldehyde for 15 min at 4°C before permeabilization with 5% PBS-bovine serum albumin/0.3% saponin for 15 min at room temperature, and then incubated with the phycoerythrin-conjugated anti-KIT antibody.

To inhibit KIT internalization via disruption of lipid rafts, cholesterol depletion of cell membranes was done using methyl-β-cyclodextrin (Sigma-Aldrich). Cells were serum-starved for 30 min, then incubated for 30 min with 30 mmol/L of methyl-β-cyclodextrin in DMEM without serum, and finally stimulated or not by 100 ng/mL of rhSCF for 20 min before staining with phycoerythrin-conjugated anti-KIT antibody.

**Immunocytochemistry and confocal microscopy in NIH3T3-infected cells.** Immunocytochemistry of NIH3T3-infected cells was done on pellets previously embedded with Shandon Cytoblock Cell Preparation System (Thermo Scientific) according to the manufacturer's instructions. Dewaxed paraffin sections were stained with anti-KIT antibody (Dako) and revealed by the Ultra View DAB Detection Kit (Ventana Medical System). Slides were observed at ×100 original magnification with Axioskop microscope (Zeiss), and then representative photographs were taken using Nikon Cool Pix camera (Nikon).

For confocal microscopy, cells were cultivated for 24 h onto polylysine-coated slides before fixation with 4% paraformaldehyde in PBS. Cells were then permeabilized with 0.1% Triton X-100, then stained with either anti-KIT, anti-pY703KIT, or anti-pY823 (revealed with anti-rabbit biotin plus streptavidin-Alexa 594) and/or anti-Golgin58K (revealed with anti-mouse IGG1 Alexa 647) or with rabbit anti-calnexin (revealed with anti-rabbit Alexa 647). Before incubation with anti-calnexin antibody, KIT or pY703 staining was fixed and blocked to avoid cross-binding with the other anti-rabbit antibody. Slides were mounted with Vectashield Hard-Set containing 4,6-diamidino-2-phenylindole for nuclear staining (Vector Laboratories/AbCys). Representative photographs were taken at original magnification (×63) with a Leica microscope TCS SP2. Colocalization analyses were done with WCIF ImageJ software8 according to the approach of Costes et al. (27).

**Statistical analysis.** For proliferation assays, the mean triplicate counts were compared among the different infected cell types using Student’s t test. All data are expressed as mean ± SD. The relevance of Golgi-like staining in GISTs was calculated with a χ² test.

**Results**

**Perinuclear expression of KIT in GISTs.** Cellular localization of KIT has previously been shown to be heterogeneous in GISTs, with either plasma membrane, diffuse cytoplasmic, and/or paranuclear dot-like patterns (i.e., “Golgi-like”). The slides of all GIST cases that were diagnosed with homozygous KIT mutations (n = 17) were compared with those of KIT-positive GISTs without KIT mutations (n = 14), and those of 51 GISTs with heterozygous KIT mutations (three times the number of cases with homozygous mutations, randomly chosen in our series; Table 1). We observed 11 of 51 point mutations, 3 of 51 insertions, and 37 of 51 deletions in the heterozygous group, and 16 of 17 exon 11 deletions (Fig. 1A) and 1 of 17 exon 9 insertions in the homozygous group. KIT was expressed (Fig. 1B) in the cytoplasm of 14 cases (82%) with homozygous mutations, 10 cases (71%) without mutations, and 32 cases (63%) with heterozygous mutations. Plasma membrane expression of KIT was observed in 5 (29%), 9 (64%), and 31 (61%) cases from the same group, respectively, and was therefore significantly less frequent in the first group (P = 0.05, χ² test). Paranuclear dot-like patterns were observed in 12 (70%), 2 (14%), and 18 (35%) cases (P < 0.01; χ² test); therefore, predominantly in GISTs with homozygous mutations, as compared with wild-type GISTs and to GISTs with heterozygous mutations.

The expression and activation of KIT extracted from GIST frozen samples was then analyzed using Western blot analyses.
Samples from GISTs without deletions (n = 11), with heterozygous deletions (n = 12; two point mutations, three insertions, and six deletions in exon 11; one insertion in exon 9), or with homozygous deletions (n = 3) of KIT gene were compared. The mature (145 kDa) form of KIT was phosphorylated in most of the 26 tumor samples. Importantly, the immature (125 kDa) form of KIT was also phosphorylated in GISTs with KIT mutations, but not (pY823) or weakly (pY703) in GISTs with wild-type KIT (Fig. 1C).

**Functional properties of NIH3T3 cells infected by human exon 11 KIT mutants or wild-type forms.** To evaluate the significance of the abnormal pattern of expression and activation of KIT in KIT-mutated GISTs, murine fibroblast cell lines (NIH3T3) were stably infected with oncoviral vectors containing either wild-type KIT (WT), mutated KIT11 DelWK557-558 (D6), or mutated KIT11 Del NGNNYYIYIDPTQPYDHKS64-KS81 (D54) human KIT cDNA, or with the empty vector (CT). The stably infected NIH3T3 cells, WT, D6, and D54 expressed the KIT protein in two forms: the mature and the immature proteins of 145 and the 125 kDa, respectively (Fig. 2A). D6 and D54 were gain-of-function mutations. Indeed, KIT was constitutively activated in cells infected with D6 and D54 oncoviral vectors, as shown by the phosphorylation of the Tyr823 residue in these cells in the absence of rhSCF, whereas WT KIT activation was weak in this condition. Upon stimulation with rhSCF, WT KIT was highly phosphorylated and its expression was decreased (Fig. 2A).

The oncogenic properties of KIT mutants were then compared (Fig. 2B): D6 and D54 mutants proliferated significantly more than wild-type or control NIH3T3 cells after 60 and 84 hours of culture in low serum concentrations (P < 0.05). We further tested the transforming potential of infected NIH3T3 cells by culturing them in soft agar (Fig. 2C; ref. 28). Only D6- and D54-infected NIH3T3 formed colonies in soft agar assays without the addition of rhSCF (mean[WT] = 0.00 ± 2.00; mean[D6] = 45.92 ± 11.34; mean[D54] = 29.00 ± 14.66).

**Subcellular expression of KIT mutants and WT KIT in NIH3T3 cells.** Using flow cytometry, the expression of KIT in D6, D54, and wild-type infected cells was observed at the cell membrane. In the absence of rhSCF, the WT KIT receptor was expressed at a high level. The constitutively activated D6 and D54 mutants were also detectable on cell surface, albeit at reduced levels as compared with the wild-type receptor (Fig. 3A).

To get more insight into the mechanisms of reduced KIT protein expression in D6- and D54-infected cells (Figs. 2A and 3C), KIT mRNA expression was compared in infected cells (Fig. 3B) using real-time PCR quantification. KIT/18S RNA ratios were similar in wild-type and mutant infected cells (range, 0.38-0.47), and was significantly higher than in control cells (which were negative for KIT expression).

The overall expression (i.e., plasma membrane and intracellular) of KIT was then analyzed by flow cytometry following cellular permeabilization (Fig. 3C). Although no differences were observed between intact and permeabilized wild-type infected cells, the expression of mutant KIT protein was higher in permeabilized cells than at the cell surface. Moreover, similar to the observations made in samples from GISTs with homozygous KIT mutations, KIT immunohistochemical expression was predominantly found in the perinuclear region in NIH3T3 cells infected with the mutated KIT gene, whereas it remained predominantly at the plasma membrane and diffusely in the cytoplasm in wild-type cells (Fig. 1B).

**SCF-dependent internalization and activation of KIT in wild-type but not in mutant-infected cells.** The cell surface expression of WT KIT was significantly decreased when WT KIT–infected cells were stimulated for 24 hours by rhSCF; the inhibition of KIT activation by 1 μmol/L of imatinib mainly restored the membrane expression of KIT (Fig. 4A). In constitutively activated KIT mutant–infected cells, additional stimulation with rhSCF did not significantly alter the membrane expression of the receptor stimulation. In contrast, the inhibition of KIT activation by imatinib, as shown further, was associated with an increased expression of the receptors at the cell surface (higher for D6 than for D54).

Methyl-β-cyclodextrin has been shown to inhibit the lipid raft pathway of KIT receptor internalization (29). Although methyl-β-cyclodextrin dramatically reduced the rhSCF-induced internalization of WT KIT, it had no effects on the surface expression of mutant KIT proteins, indicating that the weak membrane expression of the mutated KIT proteins was not due to an increased internalization (Fig. 4B).

**Specific phosphorylation of the immature form of the mutated KIT receptor.** The 145 and 125 kDa forms of KIT differ by...
glycosylation patterns (30): the 145 kDa protein corresponds to the maturation of the 125 kDa form within the Golgi apparatus, and complete maturation is requested for plasma cell membrane targeting.

To investigate whether the immature 125 kDa form was not expressed at the plasma membrane, the surface proteins of infected NIH3T3 cells were biotinylated and detected with Western blotting analysis after immunoprecipitation of both

---

**Fig. 4.** Regulation of KIT expression according to the kinase activity in the different infected cells. A, regulation of KIT cell surface expression after 24 h of stimulation by 100 ng/mL of rhSCF (SCF) and/or 1 μmol/L of imatinib (STI) was observed using flow cytometry in wild-type, D6, and D54 cells. B, effective KIT internalization was assessed using flow cytometry analysis of KIT cell surface expression with or without 30 mmol/L of methyl-β-cyclodextrin. Disruption of lipid raft for WT KIT-infected cells was followed or not by 20 min of stimulation with 100 ng/mL of rhSCF. Black arrows, modification of KIT expression under the effect of SCF with or without methyl-β-cyclodextrin.
immature and mature forms of KIT (Fig. 5A): only the mature 145 kDa form was expressed at the cell surface, whereas the intracellular immature 125 kDa form of the mutated KIT, which was phosphorylated on the Tyr703 (data not shown) and Tyr823 residues, was retained intracellularly.

Confocal microscopy confirmed the intracellular localization of the mutant KIT (Fig. 5B). Indeed, phosphorylated Tyr703 (Fig. 5B) and Tyr823 (data not shown) of KIT mutants were colocalized with the endoplasmic reticulum (as shown by coexpression of the specific calnexin) and Golgi apparatus (as shown by the coexpression of the specific Golgin58K) compartments.

The relation between activation and expression of mature and immature forms of KIT in NIH3T3 cells was then investigated using Western blotting. Both mature and immature forms of mutant KIT were phosphorylated on Tyr703 or Tyr823 residues (Figs. 2A, 5A and C). Their stimulation by rhSCF did not alter mature or immature form of KIT expression (Fig. 5C).

In contrast, prolonged inhibition of these mutants with 1 μmol/L of imatinib increased the expression of the mature form (Fig. 5C), and reduced the expression of the immature form, resulting in a pattern of KIT expression which is similar to unstimulated WT KIT. As we observed in the previous experiments, only the mature 145 kDa form of WT KIT was phosphorylated after rhSCF stimulation (Figs. 2A and 5A). Moreover, its activation was transient, as phosphorylation of Tyr703 and Tyr823 was weak after 24 hours of rhSCF stimulation (Fig. 5C). The prolonged stimulation also reduced the mature WT KIT form expression, but did not alter the expression of the immature form. Finally, the expression of the mature form was restored upon further addition of 1 μmol/L of imatinib (Fig. 5C).

**Discussion**

In the present work, we show that an immature form of GIST-type mutated KIT, but not of wild-type KIT, is activated.
and undergoes an abnormal subcellular trafficking in GISTs and in KIT-infected NIH3T3 cells.

In-frame deletions within exon 11 (especially codons 557-558) of KIT are present in more than half of GISTs (5, 9–13) and result in constitutive activation of KIT in these human tumors (3). Gain-of-function mutations are a major event in GIST oncogenesis, as shown by the high incidence of GISTs in patients with inherited KIT mutations (31–33), in transgenic mice (34, 35), and in studies reporting the major antitumor effects of treatments with the KIT inhibitor imatinib (8). Two forms of KIT protein are described in GISTs (4): the 125 kDa form is a precursor of the 145 kDa form (36) and lacks the complex glycosylation necessary for its cell surface targeting (30, 37). In accordance with the data reported in the literature, we found that KIT was activated in most of our GIST samples, as shown by phosphorylation of the mature (145 kDa) form of KIT. The results here further indicate that samples with homozygous KIT or heterozygous mutations on exon 11 (as well as one with exon 9), also presented an activation of the immature (125 kDa) form. These observations are consistent with the report of Duensing et al. who also observed the phosphorylation of the 125 kDa KIT form in some primary GISTs (38). Interestingly, in GISTs with KIT homozygous mutations, we observed a predominant paranuclear dot pattern of KIT on immunohistochemical staining, as compared with GISTs without mutations (P < 0.01) and those with heterozygous mutations (P = 0.01). Immunochemical features of KIT expression described in GISTs are variable: it can be diffuse or dot-like within the cytoplasm, as well as in the plasma membrane (22). The mechanisms underlying these observations were not known. Pauls et al. have observed that dot-like staining (also called “Golgi pattern”) of KIT was very frequently associated with mutations within the KIT gene (39). To evaluate the significance of the abnormal pattern of KIT expression and activation in KIT-mutated GISTs, we further investigated the KIT expression and maturation in NIH3T3-infected cells expressing either the wild-type or the juxtamembrane-mutated forms of human KIT.

The results we obtained with the NIH3T3 fibroblasts infected with WT KIT oncoviral vector are consistent with other models published until now. As in GISTs, the two forms of KIT were detected in NIH3T3-infected cells. The mature form of WT KIT, infected in NIH3T3 cells, was predominantly and mainly expressed at the cell surface. In fact, SCF stimulation of wild-type KIT receptor induces dimerization and activation of the mature (145 kDa) form but not of the immature (125 kDa) form (36). Moreover, previous studies have shown that the 125 kDa form is intracytoplasmic, retained within the endoplasmic reticulum or the Golgi compartments (30, 37). Finally, as already suggested by cholesterol depletion experiments (40), the internalization of the mature activated WT KIT form was shown to be lipid raft–dependent.

In contrast to the wild-type KIT, which was mainly expressed at the cell surface as a mature 145 kDa form, the mutated form was predominantly expressed within the endoplasmic reticulum and the Golgi compartments as the immature (125 kDa) form. As for GIST samples, activation of the immature form was exclusively detected in mutants. Several groups have also transfected murine cell lines with human KIT containing gain-of-function mutations. The 125 kDa form was either predominantly expressed (41, 42), as in the present study and in two others with deletion or mutation of codon 559, or was the only detected form as in two models with activating mutations of codon 816 (30, 37). Phosphorylation of the mature form in murine cell lines transfected by mutated but not wild-type alleles of KIT has also been observed by others (30, 42, 43). Debiec-Rychter et al. used cell lines transfected with a KIT mutant containing a deletion of codons 557 to 558, such as in the D6 model of the present work, but additionally associated with a mutation in exon 13 (T670I). They observed that the 125 kDa form was highly expressed and also slightly phosphorylated (44).

Several lines of evidence strongly suggest that the immature 125 kDa KIT protein plays an essential oncogenic role in NIH3T3 and in GIST, bypassing the normal posttranslational maturation and trafficking. First, the cell membrane expression of KIT was weaker in KIT mutant compared with WT KIT–infected cells, whereas KIT mRNA expression was similar in the two cases. This was not due to a higher internalization of the receptor induced by KIT autoactivation because assays of lipid raft disruption did not increase KIT expression at the cell surface. Furthermore, the immature 125 kDa form of KIT, although expressed under an activated form, was not found at the cell surface using a biotinylation assay. Finally, mutant KIT was predominantly expressed in the endoplasmic reticulum and Golgi compartments in an activated form using confocal microscopy. Using this model, we also showed that inhibition of KIT kinase activity with imatinib blocked mutant KIT phosphorylation, restored normal maturation of these proteins, and enabled its migration in the mature form to the cell surface, showing that abnormal maturation and trafficking of the mutated KIT protein was a result of its constitutive activation. Taken together, these data might suggest that activating mutations lead to a premature phosphorylation of KIT resulting in a premature regulation of the protein that decreases its cell surface expression. Furthermore, alteration of signaling pathways, often observed in GIST models (38, 43), might be a consequence of the endoplasmic reticulum/Golgi localization as it has been reported for the KIT kinase domain mutant (30) and needs to be further investigated.

Gain-of-function mutations of tyrosine kinase receptors have been shown to be major events in many human tumors (for review, see ref. 45). Here, we showed that gain-of-function mutations of KIT not only induced an autoactivation of the receptor, but also resulted in a blockade of normal KIT maturation and intracellular trafficking that resulted in an abnormal activation of its intracellular immature form. Similar results were recently published with the kinase domain mutant frequently found in mastocytosis, however, its relevance in the in vivo situation has not yet been shown (30). Moreover, abnormal maturation and trafficking of tyrosine kinase receptors has also been observed in other tyrosine kinase receptors such as CSF1R (46) and Flt3, which have been related to its tyrosine kinase activity (47). Interestingly, we observed that the KIT inhibitor imatinib partially relieved this blockade and restored KIT expression at the cell surface, suggesting that the constitutional activation of mutated KIT is responsible for the intracellular retention of the protein. Moreover, we previously showed that SCF was detectable in most GISTs, and might therefore contribute to its activation (48). Several
tyrosine kinase inhibitors are currently used to treat patients with cancer, and belong to two main types: antibodies directed against surface receptors and small specific inhibitors. The combination of both types of tyrosine kinase receptor inhibitors was activated within intracellular compartments of the cell, and thus, might escape the physiologic mechanisms of deactivation following ligand binding. Imatinib blocks this phenomenon, restoring normal cell membrane expression of a nonactivated immature form of the receptor. These observations may account for the lack of correlation between KIT expression at the cell surface and response to imatinib; their possible relevance to other tyrosine kinase inhibitors in different tumor models and tyrosine kinase inhibitor activities also needs to be explored. They also suggest that a cell-permeable inhibitor of KIT, such as imatinib, will likely be more efficient than a non–cell-permeable molecule. Whether these observations are relevant in other models of mutated activated tyrosine kinase receptors will need to be investigated.

Acknowledgments

We thank A. Puissieux, C. Caux, C. Menetrier Caux, I. Puissieux, M. Gobert, I. Le Mercier, and all members of “Équipe Cytokines et Cancers” and INSERM US90 for fruitful discussions; as well as J. Valladeau and CCG (confocal microscopy) and R. Saffroy (real-time PCR) for their technical advice.

References


3. Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-phenomenon, restoring normal cell membrane expression of a deactivation following ligand binding. Imatinib blocks this receptor was activated within intracellular compartments of the understanding of the biology of mutant KIT receptor and for combination of both types of tyrosine kinase receptor against surface receptors and small specific inhibitors. The tyrosine kinase inhibitors are currently used to treat patients with cancer, and belong to two main types: antibodies directed against surface receptors and small specific inhibitors. The combination of both types of tyrosine kinase receptor inhibitors was activated within intracellular compartments of the cell, and thus, might escape the physiologic mechanisms of deactivation following ligand binding. Imatinib blocks this phenomenon, restoring normal cell membrane expression of a nonactivated immature form of the receptor. These observations may account for the lack of correlation between KIT expression at the cell surface and response to imatinib; their possible relevance to other tyrosine kinase inhibitors in different tumor models and tyrosine kinase inhibitor activities also needs to be explored. They also suggest that a cell-permeable inhibitor of KIT, such as imatinib, will likely be more efficient than a non–cell-permeable molecule. Whether these observations are relevant in other models of mutated activated tyrosine kinase receptors will need to be investigated.


42. Vanderwinden JM, Wang D, Paternotte N, et al. Differences in signaling pathways and expression level of the phosphoinositide phosphatase SHIP1 between two oncogenic mutants of the receptor tyrosine kinase KIT. Cell Signal 2006;18:661–9.
KIT Mutations Induce Intracellular Retention and Activation of an Immature Form of the KIT Protein in Gastrointestinal Stromal Tumors

Séverine Tabone-Eglinger, Frédéric Subra, Hiba El Sayadi, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/8/2285

Cited articles
This article cites 50 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/8/2285.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/14/8/2285.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.