The Aberrant Localization of Oncogenic Kit Tyrosine Kinase Receptor Mutants Is Reversed on Specific Inhibitory Treatment

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Abstract
Kit is a cell surface type III tyrosine kinase (TK) receptor implicated in cell transformation through overexpression or oncogenic mutation. Two categories of Kit mutants displaying mutations either in the juxtamembrane intracellular domain (regulatory mutants) or in the catalytic domain (catalytic mutants) have been described. To explore the effect of Kit oncogenic mutations on its subcellular localization, we constructed enhanced green fluorescent protein (EGFP)-tagged human Kit chimeras harboring mutations either in the regulatory (V560G) or in the catalytic (D816V) domain. When expressed in Chinese hamster ovary cells, EGFP-tagged wild-type Kit was activated on stem cell factor stimulation, whereas both EGFP-tagged Kit mutants displayed a constitutive TK activity. Constitutively activated mutants exhibited a high-mannose-type N-glycosylation pattern and an intracellular localization, suggesting that these mutants induce downstream oncogenic signaling without the need to reach the cell surface. Inhibition of constitutive Kit TK activity with dasatinib induced a complex, mature N-glycosylation pattern identical to unstimulated wild-type Kit and resulted in the redistribution of the mutants to the plasma membrane. This relocalization was clearly correlated to the inhibition of TK activity because imatinib, a specific inhibitor of the V560G mutant, inactive on the catalytic D816V mutant, induced only the relocalization of the V560G mutant. These data show that on TK inhibition, the aberrant localization of Kit mutants can be fully reversed. Kit mutants are then exported and/or stabilized at the cell surface as inactive and fully N-glycosylated isoforms. (Mol Cancer Res 2009;7(9):1525–33)

Introduction
The c-Kit proto-oncogene encodes a 976-amino acid type III membrane tyrosine kinase (TK) receptor. Members of this subclass (platelet-derived growth factor receptors A and B, Flt-3, and the macrophage colony-stimulating factor receptor; ref. 1) share the same topology with five N-glycosylated immunoglobulin-like domains in the NH2-terminal extracellular domain (ECD) and a split intracellular COOH-terminal catalytic domain (see Fig. 1A) with separated ATP-binding and phosphotransferase domains. Kit ligand/stem cell factor (SCF) binding to Kit ECD has been shown to trigger receptor dimerization, autophosphorylation on well-defined intracellular tyrosine residues, and recruitment and/or phosphorylation of downstream signaling partners (see ref. 2 for review). Kit is physiologically expressed by hematopoietic progenitors, mature mast cells, Cajal cells, melanocytes, and germ cells. Kit activity has been found to be up-regulated in some neoplastic diseases through gain-of-function mutations [mastocytomas (>90%), gastrointestinal stromal tumors (GIST, >70%; refs. 3, 4), melanoma (>11%; ref. 5), sinonasal T-cell lymphomas (17%), seminomas/dysgerminomas (9%; ref. 5), and acute myeloid leukemias (1%; see ref. 6 for review)] or due to overactivity of the Kit/SCF axis involving Kit and/or SCF overexpression [small cell lung cancers (7), colorectal carcinomas (8), neuroblastomas (9), breast carcinomas (10), and gynecologic tumors (11)]. Kit activity is also increased in mastocytosis, a disabling inflammatory disease that includes a heterogeneous group of disorders characterized by abnormal growth and accumulation of neoplastic mast cells in multiple organs with indolent or aggressive clinical courses.

Kit gain-of-function mutations found in human neoplastic diseases can be classified in two main categories corresponding to distinct structural and functional locations (12). The catalytic mutant category displays point mutations located in the kinase domain, mainly in the activation loop (AL; residues 810-839). The D in position 816 (substitution by V being the more frequent) is, for instance, mutated in acute myeloid leukemias, germ cell tumors, and mast cell tumors and also in mastocytosis (13). The D816V mutation has been shown to keep the AL in an extended conformation corresponding to a constitutive phosphotransferase activity (14), but whether dimerization is required for this activity remains unclear (15, 16). The regulatory mutant category displays point mutations or short deletions in the juxtamembrane domain (residues 544 to 581), which is involved in the negative regulation of the wild-type (WT) receptor (2). In this category,
the V560G mutation, frequently found in GIST, was shown to induce constitutive dimerization responsible for permanent phosphorylation and activation of the receptor (15).

Oncogenic Kit addiction (i.e., the fact that continuous activity of oncogenic Kit is required for tumor maintenance), according to the general concept of oncogene addiction developed by Weinstein et al. (17, 18), has received clinical validation with the development of TK inhibitors targeting Kit activity, imatinib mesylate/Gleevec being the paradigm. Imatinib, first used as an inhibitor of Abl kinase in leukemias displaying Philadelphia chromosome Bcr-Abl fusion kinase (19), was also shown to be efficient on Kit (20). Indeed, imatinib has changed the clinical course of patients with advanced GIST involving juxtamembrane domain Kit mutants (21, 22). Unfortunately, most Kit AL mutations exhibited resistance to imatinib in the clinic (23, 24). Moreover, responsive patients were shown to develop resistance after long-term treatment. At the molecular level, the resistance was correlated with the occurrence of secondary mutations such as T670I (25, 26). To overcome imatinib limitations, other TK inhibitors have been developed, with some success. Sunitinib (25) was active on some secondary Kit mutants induced by imatinib, whereas dasatinib was active on both catalytic and regulatory mutants (27). It is nevertheless expected that these drugs, like imatinib, may induce the emergence of resistant secondary mutants.

Therapeutic targeting of oncogenic Kit inactivation can also be considered by using monoclonal antibodies (mAb) on the model of ErbB2/Neu or CD20-targeted therapies, which have proven their efficiency in breast cancer patients (28) and non–Hodgkin's lymphoma patients (29), respectively. To our knowledge, such a strategy has not yet been reported. To be a suitable target for a therapeutic mAb, it is mandatory that oncogenic Kit is expressed significantly at the cell surface because the therapeutic efficiency of mAbs is based on a functional effect obtained after target binding (natural ligand antagonistic effect and apoptosis induction) and/or on the recruitment of immune effectors through the Fc part of the mAb (30). Anti-Kit mAb inhibitory effect on Kit signaling could be obtained by inhibition of SCF binding to Kit (in the case of an overactivation of the Kit/SCF axis), inhibition of Kit dimerization, and/or induction of Kit degradation. The amount of accessible oncogenic Kit at the cell surface is likely to depend on its subcellular distribution and half-life (a result of the equilibrium between its synthesis and degradation). Studies on the D816V Kit mutant described a decreased global expression (15, 31, 32) compared with WT Kit, attributed to an increased degradation (33). Whether mutated Kit variants are expressed like WT Kit at the cell surface remains unclear. In this study, a set of enhanced green fluorescent protein (EGFP)-tagged WT or mutated Kit was constructed to address this question. We observed that point mutations either in the juxtamembrane domain (V560G) or in the catalytic domain (D816V) both induced constitutive activation of Kit, dramatic decrease of Kit expression at the cell surface together with modifications of the N-glycosylation pattern. We further showed that Kit inhibition with specific TK inhibitors induced its relocation from intracellular structures to the cell surface, suggesting a direct relationship between the ability of the compound to inhibit specifically the catalytic kinase activity and the relocation process.

Results

Construction and Validation of Fluorescent-Tagged WT and Mutant Kit

To investigate the consequences of Kit mutations found in human tumors on Kit subcellular distribution, we designed vectors for the expression of EGFP-tagged human Kit chimeras. Kit GNNK–isoform (missing the GNNK tetrapeptide, residues 510-513) was used in this study because it has been described as the form predominantly expressed in most tissues and with the strongest signaling activity (34-37). EGFP was inserted between the natural Kit signal peptide (which is eliminated during early steps of translation) and Kit ECD (Fig. 1; see Materials and Methods for details). This location for EGFP insertion was chosen because a similar construct, consisting in the fusion of a variant of EGFP with murine Kit, had been previously proven functional (with SCF-induced activation), whereas a fusion of EGFP at the COOH-terminal extremity of Kit had been found constitutively activated (38). In addition to the vector for the expression of WT Kit chimera (pEGFP-Kit WT; Fig. 1B), two EGFP-tagged mutant Kit expression vectors were constructed, pEGFP-Kit V560G and pEGFP-Kit D816V, to obtain information on the subcellular distribution of catalytic and regulatory mutants, respectively.

EGFP-Kit WT and mutant (V560G or D816V variants) allelic transient expression in Chinese hamster ovary (CHO) cells resulted in three isoforms (apparent molecular weight of 170, 150, and 130 kDa) and two isoforms (apparent molecular weight of 150 and 130 kDa), respectively, as detected by Western blot analysis (Fig. 2A). Anti-Kit ECD, anti-Kit intracellular domain (ICD), and anti-EGFP antibodies detected the same size molecular species, suggesting that the different bands corresponded to distinct posttranslational modifications (likely N-glycosylation; ref. 39) and not to protein degradation or alternative initiation of translation. The upper band (170 kDa), which corresponds to the expected size for a fully glycosylated membrane-bound Kit (145 kDa; ref. 1) fused to EGFP (30 kDa), was observed only for the EGFP-Kit WT allele, suggesting a differential N-glycosylation for the EGFP-Kit mutants. The lowest band (130 kDa), observed for the three alleles, is likely to correspond to immature proteins just after translation and elimination of the signal peptide (1190 residues), with no additional posttranslational modifications. Repeated transfections analyzed by Western blot (Fig. 2A and B; data not shown) showed that expression yield was higher for pEGFP-Kit WT than for pEGFP-Kit mutant plasmids, mutation D816V giving a lower signal than mutation V560G.

To check the functionality of the fusion constructs, the phosphorylation state of key tyrosine residues involved in downstream Kit signaling was tested by Western blot using Kit phospho-specific antibodies. As expected, phosphorylation of EGFP-Kit WT was entirely dependent on SCF. In the presence of ligand, the 170-kDa mature isoform (but not the other two isoforms) became highly phosphorylated on both Y823 (located in the AL) and Y721 (docking site for phosphatidylinositol 3-kinase, located in the kinase insert domain; Fig. 2B; refs. 40, 41). Although a strong signal was observed with the Kit phospho-specific antibodies, the fully mature isoform was barely detectable using an anti-Kit antibody as already

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observed in other studies using nontagged Kit (15, 24). This is likely a consequence of the internalization and degradation of the activated Kit receptor induced by SCF binding. EGFP-Kit oncogenic mutants were constitutively tyrosine phosphorylated (150 kDa band only), with no additional effect after SCF stimulation, EGFP-Kit D816V displaying a higher phosphorylation level than EGFP-Kit V560G with both Kit phospho-specific antibodies. These data showed that WT and mutant EGFP-Kit chimeras had an activation status similar to their nontagged counterparts (15, 23, 24) and could be used for further analysis.

Subcellular Localization of Kit Oncogenic Mutants Compared with WT Kit

Subcellular localization of the EGFP-Kit oncogenic mutants was first addressed by fluorescent-activated cell sorting (FACS) on transiently transfected CHO cells. Surface expression of EGFP-Kit variants was detected using a Kit ECD–specific antibody conjugated to allophycocyanin (APC), whereas total Kit expression was estimated by EGFP intrinsic fluorescent emission (Fig. 3, top). Total Kit content was also estimated on permeabilized cells by immunostaining using the Kit ECD–specific antibody (Fig. 3, bottom). For the EGFP-Kit WT allele, 34.9% of EGFP-positive cells expressed detectable level of Kit at the cell surface, whereas oncogenic EGFP-Kit-mutant cell surface expression was detected only in less than 6% of the EGFP-positive cells. The percentage of anti-Kit APC-positive cells increased only slightly on permeabilized cells for EGFP-Kit WT but dramatically for oncogenic EGFP-Kit variants, showing that EGFP-Kit mutants exhibited essentially an intracellular localization, whereas EGFP-Kit WT was located preferentially at the cell surface. A similar intracellular localization was observed in P-815 and FMA3 murine mastocytoma cell lines bearing a catalytic-type Kit mutation and a regulatory-type Kit mutation, respectively (data not shown; refs. 42, 43). Subcellular localization of the EGFP-Kit fusion constructs was also imaged by confocal microscopy (Fig. 4). EGFP direct detection and anti-Kit ECD–specific staining done on nonpermeabilized cells confirmed that mutant alleles were mainly expressed in intracellular vesicular structures, whereas WT Kit reached the cell surface. Altogether, these data suggest that despite an intracellular localization, Kit mutants are constitutively phosphorylated on Y721 and Y823 tyrosines.

Glycosylation State of Kit Oncogenic Mutants Compared with WT Kit

N-glycosylation is believed to occur on nine specific asparagine residues within the five NH2-terminal immunoglobulin domains of Kit (39). To determine the glycosylation state of oncogenic Kit mutant–activated isoform (i.e., intermediate 150-kDa isoform seen on Fig. 2A), we submitted Kit variants,
expressed transiently in CHO cells, to hydrolysis with specific endo-N-glycosidases. N-glycosylation is a posttranslational modification that begins early in the endoplasmic reticulum with the addition of a generic type of N-linked high-mannose oligosaccharide (44). In further processing, while progressing from the endoplasmic reticulum to the Golgi apparatus, proteins targeted to the cell surface have their common N-linked oligosaccharides modified, with, among other things, the addition of complex carbohydrate residues. Lysates of CHO cell expressing WT or oncogenic EGFP-Kit variants were treated with peptide N-glycosidase (PNGase F) or N-acetylglucosaminidase H (Endo H), which hydrolyses all N-linked carbohydrate residues or specifically N-linked high-mannose oligosaccharides, respectively, and analyzed by Western blot with an anti-Kit antibody (Fig. 5). The 150-kDa band observed for both WT and mutant isoforms was sensitive to Endo H hydrolysis, indicating a high-mannose-type glycosylation pattern. Conversely, the upper band observed only for EGFP-Kit WT (170 kDa) was Endo H resistant, indicating a complex, mature N-glycosylation pattern. These data show that activated (i.e., phosphorylated on Y568, Y721, and Y823) oncogenic Kit mutants are N-glycosylated with a high-mannose-type pattern, typical of an endoplasmic reticulum localization.

Effect of TK-Specific Inhibitors on Kit Mutant Localization

Specific TK inhibitors have been successfully developed for targeting oncogenic TKs, including Kit, for therapeutic purpose. We investigated the effect of TK inhibitor treatments on WT and mutant EGFP-Kit subcellular distribution using confocal microscopy and cytometry. Two drugs were tested: dasatinib, active on both catalytic (including D816V) and regulatory (including V560G) Kit mutants (27), and imatinib, active on Kit V560G (45) but not on Kit D816V (24). CHO cells expressing EGFP-Kit variants were treated with either imatinib or dasatinib. Kit cell surface levels were estimated by FACS using an anti-Kit ECD APC-conjugated antibody and normalized using EGFP natural fluorescence intensity. The ratio MFI_{APC}/MFI_{EGFP} is representative of the proportion of Kit expressed at the cell surface in each condition. Dasatinib affected significantly EGFP-Kit V560G and EGFP-Kit D816V cell distribution (MFI_{APC}/MFI_{EGFP} increased by a factor of 2 for both mutants) but was ineffective on EGFP-Kit WT. Volunteering activity was only on EGFP-Kit V560G (MFI_{APC}/MFI_{EGFP} increased by a factor of 2; Fig. 6A). Confocal analysis confirmed these observations with a clear cell surface detection of EGFP-Kit V560G when using imatinib and of both mutants when using dasatinib (Fig. 6B). Indeed, EGFP-Kit mutants were relocalized at the cell surface on specific TK inhibitor treatment.

Effect of TK-Specific Inhibitors on Kit Posttranslational Modifications

TK inhibitor–treated cells were also analyzed by Western blot for posttranslational modifications (Fig. 7). Drug-induced Kit mutant relocalization, as observed (Fig. 6), correlated with the detection of an additional 170 kDa band (the expected size for a complex, mature N-glycosylated isoform) compared with nontreated cells (only two isoforms detected). This “three bands profile” recovery was associated with a global increase of total Kit expression reaching levels similar to EGFP-Kit WT. The 170-kDa band was poorly phosphorylated on Y568, Y721, and Y823 tyrosines. Constitutive phosphorylation of Y568, Y721, and Y823 tyrosines of the 150-kDa high-mannose-type N-glycosylated isoform was also dramatically decreased compared with nontreated cells. Altogether, these data show that on inhibition of their TK activity, Kit mutants are exported and/or stabilized at the cell surface as inactive and fully N-glycosylated isoforms.
Discussion

We designed vectors for the expression of EGFP fused to the NH₂-terminal extremity of Kit to gain insight into the modifications induced by oncogenic Kit mutations on its intracellular trafficking. In addition to EGFP fused to WT human Kit, two variants were constructed, corresponding either to a regulatory-type mutant (mutation V560G, typical of GIST) or to a catalytic-type mutant (mutation D816V, found in mastocytomas). A similar construct consisting of enhanced yellow fluorescent protein–tagged murine WT Kit had been previously proven to be functional in simian COS7 cell line and in murine Ba/F3 hematopoietic cell line (38). This is the first comparative study that includes, besides human WT Kit, a regulatory and a catalytic Kit mutant using fluorescent-tagged Kit variants.

We observed that both mutant types were constitutively activated, displayed exclusively a high-mannose–type N-glycosylation pattern, and were located mainly in intracellular vesicular structures. These data suggest that these mutants are able to induce downstream oncogenic intracellular signaling without the need to reach the cell surface and that cell surface expression may not be mandatory for oncogenic Kit signaling. However, our data do not exclude that a limited amount of oncogenic Kit at the cell surface may be sufficient to transform cells. Nevertheless, our results parallel recent data provided by Xiang et al. (46) and Tabone-Eglinger et al. (45) on catalytic-type mutant D816V and on two different regulatory-type (mutated in Kit exon 11) mutants found in GIST, which showed that cell surface expression was not required for activation and oncogenic signal transduction. More specifically, GIST-type mutants stably expressed in NIH-3T3 cells displayed an intracellular localization even under lipid raft disruption, indicating that lower mutant surface expression was not due to a higher internalization of the receptor induced by Kit autoactivation but likely to a faster degradation as a consequence of intracellular activation (45). Intracellular localization of juxtamembrane Kit mutants was also detected on GIST biopsies (45, 47). It has already been observed that oncogenic Kit uses different signaling pathways than WT Kit, including Src kinase (48) or Fes kinase (49) pathways. This could be a consequence of a distinct localization, allowing the recruitment of distinct signaling partners, more than simply a consequence of a constitutive kinase activity due to activating mutations. Interestingly, activating mutations in other class III TK receptors, such as Flt-3 and platelet-derived growth factor receptor A, have been shown to exhibit an aberrant intracellular localization compared with their WT counterparts (50-52).

Whether intracellular dimerization is required for full oncogenic signaling needs also to be clarified. Using truncated forms of WT murine Kit and a D814V Kit mutant (corresponding to the human D816V Kit mutant), Tsujimura et al. (16) suggested that intracellular self-association of the murine Kit D814V mutant, independent of the extracellular SCF-induced dimerization site, was required for oncogenic activity. Kitayama et al. (15) have detected cell surface dimerization of the murine Kit V559G mutant (corresponding to the human V560G Kit mutant) but not of the murine Kit D814V mutant. The pEGFP-Kit vectors constructed for this study will allow to detect directly mutant intracellular dimerization. The EGFP cDNA will be replaced by cyan fluorescent protein or yellow fluorescent protein cDNAs and the

**FIGURE 3.** Analysis of Kit WT, Kit V560G, and Kit D816V cell surface expression by FACS. CHO cells were transfected with pEGFP-Kit WT, pEGFP-Kit V560G, or pEGFP-Kit D816V plasmids; trypsinized 24 h later; labeled with an APC-conjugated anti-Kit ECD antibody, either after membrane permeabilization with saponin treatment or not; and analyzed by FACS. Data represent percentage of Kit-positive cells among EGFP-positive cells. Oncogenic EGFP-Kit mutant expression was predominantly intracellular, whereas EGFP-Kit WT was clearly detected at the cell surface. These data are representative of three independent experiments. NP, nonpermeabilized; P, permeabilized; NC, negative control nontransfected cells.
corresponding fluorescent variant coexpressed in the same cell to detect dimerization by fluorescence resonance energy transfer. Extracellular SCF-induced Kit WT dimerization has already been observed with a fluorescence resonance energy transfer approach using anti-Kit fluorescent antibodies instead of fluorescent Tags (53). Moreover, pSigPep-EGFP 5′ c-Kit open reading frame (ORF) vector will be useful to obtain rapidly additional EGFP-tagged Kit mutants in a more extensive study, including other catalytic and regulatory mutants found in patients. Patients with oncogenic Kit usually coexpress both WT Kit and mutant Kit alleles (54). Nevertheless, Kit mutant studies (including ours) on cell models have all been done in a homozygotic context. Our model system will be useful to study the fate of individual Kit variants in a heterozygotic context.

In this study, we also compared the effect of two TK inhibitors, imatinib and dasatinib, on Kit mutant subcellular localization. We showed by flow cytometry, confocal microscopy, and Western blot that cell treatment with imatinib, a TK inhibitor active on Kit regulatory mutants, was associated with a re-distribution of the V560G chimera to the plasma membrane and a dramatic reduction of Y568, Y721, and Y823 phosphorylation, together with the acquisition of a complex, mature N-glycosylation pattern. These observations were not reproduced with the D816V catalytic-type mutant, which has been previously described as resistant to imatinib (24). Interestingly, treatment with dasatinib, a TK inhibitor active on both regulatory and catalytic mutants (27), induced the same modifications on both mutant categories. The partial (as opposed to total) inhibition of Kit D816V phosphorylation on Y residues with 1 μmol/L dasatinib (IC_{50} between 100 and 500 nmol/L, have been reported in other studies; refs. 27, 55) is probably due to high expression level combined to the high phosphorylation level of this mutant in our transient expression system. We conclude that efficient TK activity inhibition of Kit mutants reverses the aberrant subcellular location of Kit mutants and allows the export and/or stabilization of a fully mature and inactive isoform at the cell surface. They also raise the exciting possibility, however, that prior treatment with kinase inhibitors might be sufficient to drive mutant Kit to the surface and, hence, provide a more sensitive population of target cells for mAb-based intervention. If this is the case, it may provide a novel therapeutic approach not only for diseases involving activated c-Kit but also to those caused by activation of other type III TK receptors where transformation correlates with an intracellular localization of the oncogenic kinase. In support of the idea that TK inhibitors can be combined effectively with mAb intervention, Scultriti et al. (56) recently showed that lapatinib, an ErbB2 TK inhibitor, potentiated anti-ErbB2 trastuzumab-dependent cell cytotoxicity in vivo. In the case of oncogenic Kit targeting with mAbs, we expect that oncogenic Kit relocalization at the cell surface would increase mAb effect. Using a combination of Kit TK inhibitor, at subtoxic concentration, and an anti-Kit mAb could be an option to limit the emergence of drug-resistant tumors in patients.

Materials and Methods

Antibodies

For Western blot analysis, anti-Kit ECD (H300), anti-Kit ICD (C19), anti-EGFP and anti-Kit phosphotyrosine 568 (pKit-Y568)
antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-Kit phosphotyrosine 721 (pKit-Y721) and anti-Kit phosphotyrosine 823 (pKit-Y823) antibodies were from Invitrogen, and horseradish peroxidase–conjugated secondary antibody was from Dako. Anti-Kit mAb (K44.2; Sigma) and phycoerythrin (PE)–conjugated antibody (BD Biosciences) were used for confocal microscopy. The APC-conjugated anti-Kit mAb (clone 104D2; BD Biosciences), specific for Kit ECD, was used for flow cytometry analysis. Recombinant human SCF was obtained from ImmunoTools, and imatinib mesylate and dasatinib were kindly provided from Dr. L. Gros (AB Sciences).

c-Kit-EGFP Fusion Constructs

Human Kit signal peptide cDNA was amplified from a pBluescript plasmid encoding human Kit cDNA (GNNK–isoform) by PCR to insert Nhel (5′ end) and AgeI (3′ end) restriction sites. The resulting amplicon was Nhel/AgeI digested and cloned into pEGFP-N1 expression vector (Clontech). In this construct (pSigPep-EGFP-N1), Kit signal peptide sequence is in frame with EGFP cDNA. The Nhel/BsrGI insert (which contains Kit signal peptide in fusion with EGFP) was subcloned from pSigPep-EGFP-N1 into pEGFP-C1 vector (Clontech) backbone to obtain pSigPep-EGFP-C1. In this construct, there is a multiple cloning site located in 3′ of EGFP cDNA. c-Kit 5′ ORF cDNA, encoding Kit ECD, was amplified from the pBluescript c-Kit-GNNK–plasmid by PCR to insert BspE1 (5′ end) and EcoRI (3′ end) restriction sites. The resulting amplicon was cloned into pSigPep-EGFP-C1 to obtain pSigPep-EGFP 5′ c-Kit ORF. Finally, to obtain pEGFP-Kit WT (Fig. 1B), pEGFP-Kit V560G, and pEGFP-Kit D816V, EcoRI/KpnI inserts obtained from pBluescript c-Kit-GNNK–plasmid, or the corresponding constructs with V560G or D816V point mutation, respectively, were subcloned into pSigPep-EGFP 5′ c-Kit ORF. pEGFP-Kit WT, pEGFP-Kit V560G, and pEGFP-Kit D816V were checked by sequencing.

FIGURE 6. Effect of Kit mutant–specific TK inhibitor treatment on Kit WT, Kit V560G, and Kit D816V subcellular distribution. CHO cells were transfected with pEGFP-Kit WT, pEGFP-Kit V560G, or pEGFP-Kit D816V plasmids and, 24 h later, treated for 8 h with either 1 μmol/L imatinib or 1 μmol/L dasatinib or not treated. A. Freshly trypsinized (nonpermeabilized) cells were stained with an APC-conjugated anti-Kit ECD antibody and analyzed by FACS to evaluate Kit surface level. Histogram represents the MFI_{APC}/MFI_{EGFP} ratio (which is proportional to the cell surface expression relative to total expression) of the three fluorescent Kit chimeras in each condition. *, P < 0.05; **, P < 0.01, when compared with nontreated sample by t test, three independent experiments. B. Cells (grown on coverslips) were labeled and analyzed by confocal microscopy as in Fig. 4. Each set of four photomicrographs shows two representative individual cells analyzed for EGFP fluorescence (top of the set) and PE fluorescence (bottom of the set). These data show that mutant-specific TK inhibitor treatment drives Kit mutant relocalization at the cell surface.
with a primary antibody, washed, revealed with a horseradish peroxidase–conjugated secondary antibody, and visualized by enhanced chemiluminescence (GE Healthcare).

**N-Glycosidases Treatment**

Whole-cell lysates (80 μg) were treated with either PNGase F or Endo H N-glycosidases (both from Ozyme) for 2 h at 37°C according to the manufacturer’s instructions. The reactions were stopped with SDS-PAGE sample buffer (4×), and products were analyzed by immunoblotting as described above.

**Flow Cytometry**

Kit expression level at the cell surface was assayed by FACS. Briefly, transfected and treated CHO cells were harvested and washed twice in cold FACS buffer [PBS (pH 7.4), 1% FBS] before fixation with 4% (w/v) paraformaldehyde in PBS (BD Biosciences) for 10 min and incubation with an APC-conjugated anti-Kit antibody diluted in FACS buffer. Alternatively, for total Kit detection, cells were permeabilized after fixation using FACS buffer supplemented with 0.5% (w/v) saponin (10 min; Sigma) and APC-conjugated anti-Kit antibody was diluted in FACS-0.5% saponin. After two washes, cell fluorescence was analyzed by flow cytometry with a FACS Calibur (BD Biosciences), and mean fluorescence intensities (MFI) in FL1 channel (for EGFP fluorescence) and in FL4 channel (for APC fluorescence) were quantified using the Cell Quest Pro software (BD Biosciences). All steps were done at 4°C.

**Confocal Fluorescent Microscopy**

CHO cells were grown on coverslips before transfection and optional TK inhibitor treatment as mentioned above. For staining (all steps done at room temperature), cells were washed twice with PBS, fixed with PBS-4% paraformaldehyde for 1 h, and saturated with PBS-10% FBS for 20 min. Surface Kit expression was detected with anti-Kit (K44.2) mAb followed by PE-conjugated goat anti-mouse immunoglobulins. After washing, coverslips were mounted on glass slides in a drop of Fluoromount-G (Clinisciences). The fluorescence was examined under an LSM510 confocal microscope (Zeiss), where EGFP was excited at 488 nm (emission, 505-530 nm) and PE was excited at 543 nm (emission collected between 560 and 615 nm). At least 20 cells were observed in each condition.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


4. Andersson J, Sjogren H, Meis-Kindblom JM, Stenman G, Aman P, Kindblom LG. The complexity of Kit gene mutations and chromosome rearrangements and

**FIGURE 7.** Effect of Kit mutant–specific TK inhibitor treatment on Kit WT, Kit V560G, and Kit D816V posttranslational modifications. CHO cells were transfected and treated like in Fig. 6. Cell lysates (80 μg total protein) were analyzed by Western blot using anti-Kit ECD and anti-Kit phospho-tyrosine (pKit-Y823, pKit-Y721, and pKit-Y568) antibodies. Dasatinib inhibited EGFP-Kit V560G and EGFP-Kit D816V high-mannose–type N-glycosidolform (HM phosphorylation on Y823, Y721, and Y568). Imatinib had a similar effect but only on EGFP-Kit V560G. Effective inhibition of tyrosine phosphorylation (representative of Kit TK activity) correlates with the detection of a complex-type N-glycosidolform (CG) for both mutants. DG, completely deglycosylated form.
24. Frost MI, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKit is more sensitive to imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKit is resistant. Mol Cancer Ther 2002;1:1115–24.
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