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Phosphatidylinositol 3′-Kinase Is Required for Growth of Mast Cells Expressing the Kit Catalytic Domain Mutant[1]

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ABSTRACT

The Kit receptor tyrosine kinase is critical for the growth and development of hematopoietic cells, germ cells, and the interstitial cells of Cajal. Gain-of-function mutations in codon 816 of the catalytic domain of human Kit [codon 814 of murine Kit (mKit)] are found in patients with mastocytosis, leukemia, and germ cell tumors. There are no drugs that inhibit the activity of Kit catalytic domain mutants to a greater extent than wild-type Kit. The objective of this study was to understand the biochemical mechanisms mediating mast cell transformation by this Kit mutant to identify molecular targets for pharmacological intervention. To this end, we examined signaling pathways activated in the murine mast cell line IC2 infected with either wild-type (IC2-mKit) or mutant mKit (IC2-mKit^{D814Y}). In this study, we show that mKit^{D814Y} is constitutively phosphorylated on tyrosine 719, and this likely results in constitutive association with activated phosphatidylinositol 3′-kinase (PI3K). *In vitro* growth of IC2-mKit^{D814Y} cells is more sensitive to inhibition of PI3K than SCF-induced growth of IC2-mKit cells. s.c. injection of IC2-mKit^{D814Y} in syngeneic mice results in mast cell tumors. To determine whether inhibition of PI3K could reduce mKit^{D814Y}-mediated tumorigenicity, mice were treated with 1.5 mg/kg wortmannin three times a week. Five weeks after injection of tumor cells, a 75% reduction in tumor weight was observed when wortmannin treatments were initiated 2 days after inoculation with tumor cells. A 66% reduction occurred when treatment was initiated 2 weeks after inoculation. Treatment with wortmannin increased necrosis in the tumors, and this was associated with apoptosis. Interestingly, there was no effect on tumor vasculature. Thus, PI3K is required for survival and growth of the IC2-mKit^{D814Y} mast cell line both *in vitro* and *in vivo*. These findings may provide insight into designing strategies for treatment of mastocytosis and other diseases associated with mutations in the Kit catalytic domain.

INTRODUCTION

Kit, a type III receptor tyrosine kinase, binds to ligand SCF[3] and initiates signals critical for the growth and development of mast cells, melanocytes, hematopoietic stem cells, and the interstitial cells of Cajal (1, 2). Gain-of-function mutations in Kit are associated with a number of cancers in humans (3–9). Substitution of tyrosine/valine/histidine for aspartic acid in codon 816 of the catalytic domain of the human gene (codon 814 of the murine gene) is found in patients with mastocytosis and mast cell leukemia, as well as some patients with acute myelogenous leukemia and germ cell tumors (3–8). Mutations in the Kit juxtamembrane region are found in patients with GISTs (9). STI 571 is a potent inhibitor of Kit juxtamembrane mutants *in vitro* and has shown remarkable promise in the treatment of GISTs (10, 11). In contrast, there are no drugs that inhibit the activity of Kit with mutations in the catalytic domain to a greater extent than wild-type Kit (12). This is important because partial sparing of wild-type Kit is required for normal hematopoiesis, gametogenesis, and intestinal function.

Recent work has shed some insight into the signaling pathways mediating transformation of hematopoietic cells by Kit$^{D816V/D814Y}$. Piao *et al.* (13) found that Shp-1, a negative regulator of Kit, is rapidly degraded in cells expressing mKit^{D814Y}. Expression of hKit^{D816H} in progenitor cells leads to constitutive activation of Stat3 and Stat1 (14). Furthermore, Stat3 contributes to tumorigenicity of this oncogene in fibroblast cells (15). Recently, we showed that tyrosine 721 is required for tumorigenicity of hKit^{D816V} in the MIHC progenitor cell line (16). Work from a variety of laboratories has shown that PI3K binds this tyrosine residue (16–20). However, other signaling components could associate with tyrosine 721 and contribute to transformation mediated by this oncogenic mutant.

Signaling through Kit is lineage specific. For example, Shp-1 and Rac2 have different functions in progenitor cells and mast cells (21–24). ERKs are activated in erythroleukemia cells that express hKit^{D816V}, but not in myeloid progenitor cell lines (14, 16, 25). Our earlier work focused on signaling events mediated by hKit^{D816V} in a hematopoietic progenitor cell line, but little is known about the mechanisms mediating mast cell transformation by this Kit mutant. Because many patients with mastocytosis have mutations in codon 816, identification of signaling components activated by this mutant in mast cells could lead to identification of pharmacologicals useful in the treatment of this disease (3–5, 26, 27). The aim of the present study was to directly assess the role of PI3K in transformation and tumorigenicity of a mast cell line by this oncogenic mKit mutant. These data support a critical role for PI3K in transformation of mast cells by mKit^{D814Y}. Furthermore, our findings suggest that the PI3K pathway could serve as a molecular target in designing treatment strategies for diseases associated with mutations in the catalytic domain of Kit.

MATERIALS AND METHODS

Cells. IC2 cells expressing wild-type mKit or mKit^{D814Y} have been described previously (28). The cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 1% PSG, 10% FCS (Hyclone, Salt Lake City, UT), and 10 ng/ml mIL-3. Cos-7 cells were maintained in DMEM (Invitrogen) containing 1% PSG and 10% FCS.

Mice and *in Vivo* Tumor Studies. Female 8–10-week-old DBA/2 mice (Animal Production Area, Frederick Cancer Research Facility, Frederick, MD) were maintained as described previously (29). These mice are syngeneic with the IC2 cell line (28). Mice received s.c. injection on bilateral flanks with a volume of 0.1 ml containing 2–5 × 10^6 IC2 cells infected with vector control, wild-type mKit, or the mKit^{D814Y} mutant. Mice were palpated weekly for development of tumors. IC2-mKit^{D814Y} cell-injected mice generally developed tumors within 3–4 weeks. Tumors were measured (length and width) using calipers, and tumor weight was calculated with the following formula: weight (mg) = [length (mm) × width^2 (mm)]/2. All tumor-bearing animals were monitored daily, and, consistent with the National Cancer Institute-Frederick Animal Care and Use Committee Guidelines for Neoplasia in Rodents, animals

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[3] The abbreviations used are: SCF, stem cell factor; mSCF, murine SCF; PI3K, phosphatidylinositol 3′-kinase; hKit, human Kit; mKit, murine Kit; GIST, gastrointestinal stromal cell tumor; Stat, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; mIL, murine interleukin; 1% PSG, 1% penicillin, 1% streptomycin, and 1% glutamine; MAP, mitogen-activated protein.

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showing signs of distress were euthanized immediately. To examine the effects of wortmannin on tumor progression, mice were gavaged three times a week with 1.5 mg/kg wortmannin or DMSO vehicle control. Treatments were initiated 2 days after injection of tumor cells into mice (early treatment) or 3 weeks after receipt of tumor cells (late treatment). After sacrifice of the animals, tumors were excised, placed in Carnoys fixative, and sent for histopathological analysis for H&E staining or terminal deoxynucleotidyl transferase-mediated nick end labeling analysis of tumors (Pathology Associates, Frederick, MD). To isolate cells for assessment of surface Kit expression, tumors were digested overnight at 4°C in a 0.017% (v/v) solution of trypsin and EDTA in PBS. A single cell suspension was prepared and analyzed using fluorescence-activated cell sorting as described previously (29). To make lysates for Western blotting, the tumors were minced and resuspended in lysis buffer and sonicated on ice. After this, they were incubated on ice for 30 min and precleared.

Cytokines, Antibodies, and Inhibitors. mSCF and mIL-3 were purchased from PeproTech (Rocky Hill, NJ). Antibodies specific for Kit and ERKs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing PI3K, Akt, phosphorylated Akt, and Kit phosphorylated on tyrosine 719/721 (indicating murine/human forms of Kit, respectively) were obtained from Cell Signaling Technology. Anti-phosphotyrosine monoclonal antibody 4G10 (anti-pTyr) was from Upstate Biotechnologies (Lake Placid, NY). Lyn antibody was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The PI3K inhibitor wortmannin was from Sigma (St. Louis, MO), whereas LY294002 and PP2 were from Calbiochem. Biotinylated rabbit/mouse/goat IgG and horseradish peroxidase-labeled streptavidin were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protein assay reagents were from Pierce (Rockford, IL). Enhanced chemiluminescence reagent (ECL) was from New England Nuclear Life Science Products (Boston, MA).

Cell Proliferation Assay. [^{3}H]Thymidine incorporation assays to analyze cell proliferation were performed as described previously (29). Cells were pretreated with PI3K inhibitors or Src family inhibitor PP2 for 30 min before the addition of growth factors or media control. Plates were incubated for 36 h, pulsed with [^{3}H]thymidine overnight, and harvested 48 h after drug treatment. The percentage of inhibition of growth after drug treatment was calculated by using untreated cells as control.

Immunoprecipitation and Immunobloting. Cells were washed twice with RPMI 1640 containing 1% PSG and resuspended in the same media at 10^7 cells/ml. Cells were incubated for 10 min at room temperature in the presence or absence of mSCF (100 ng/ml), pelleted, and lysed at 10^7 cells/ml in ice-cold lysis buffer [10 mM Tris base, 50 mM NaCl, 5 mM EDTA, 5 mM tetra sodium PP_i, 5 mM NaF, 5 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 0.1% bovine serum albumin (pH 7.6)]. Lysates were incubated on ice for 20 min and then centrifuged at 14,000 rpm for 30 min at 4°C. Cells treated with drugs were incubated for 20 min with drug and then incubated for 10 min with mSCF. Protein concentration of the clarified lysates was determined using BCA protein assay (Pierce). Lysate (600 μg of protein) was immunoprecipitated with the indicated antibody (1 μg/ml). The immunoprecipitates were resolved by SDS-PAGE and transferred onto Immobilon-P, followed by Western blotting using the indicated antibodies.

Kinase Assays. Kinase assays for Lyn were done on immunoprecipitates using antibodies specific for Lyn. Autophosphorylation was used to assess Lyn activity as described previously (29).

Electroporation/Lipid Transfection. IC2 cells were electroporated as described previously (29). Cotransfection studies with green fluorescent protein indicated that the efficiency of transfection of mast cells ranged from 15% to 20%. After transfection, cells were transferred to a 6-well plate and resuspended in RPMI 1640 containing 1% PSG, 20% FCS, and 10 ng/ml mIL-3. Cells were plated for proliferation assay 24 h after transfection, incubated with or without growth factors for 36 h, pulsed with [^{3}H]thymidine overnight, and harvested. The PI3K constructs used for transfections have been described previously (30). Cos-7 cells were tranfected using LipofectAMINE 2000 (Invitrogen) per the manufacturer's instructions. Forty-eight h after transfection, cells were incubated for 10 min in the presence or absence of SCF and lysed. The wild-type and hKit^{Y721F} constructs have been described previously (31) and were generously provided by Dr. Lars Rönnstrand.

RESULTS

Activated PI3K Is Constitutively Associated with mKit^{D814Y}. IC2 cells are a murine mast cell line that requires interleukin 3 for survival and growth. Infection of these cells with wild-type mKit results in SCF-induced growth, whereas expression of the mKit^{D814Y} mutant induces factor-independent growth and transformation (28, 32). As reported previously (13), the mKit^{D814Y} mutant is constitutively phosphorylated on tyrosine residues (Fig. 1*B*). An immediate consequence of Kit autophosphorylation is recruitment of PI3K to the receptor complex. Our group has demonstrated previously (16) that p85^{PI3K} is constitutively associated with hKit^{D816V} in a hematopoietic progenitor cell line. The same finding was observed in IC2-mKit^{D814Y} cells (data not shown), demonstrating that this association is not lineage specific. Consistent with these findings, increases in PI3K activity constitutively associated with mKit^{D814Y} were noted compared with the activity associated with unstimulated wild-type mKit (data not shown). These data confirm that activated PI3K is constitutively associated with mKit^{D814Y} in the IC2 mast cell line.

The PI3K Docking Site on mKit^{D814Y} Is Constitutively Phosphorylated. PI3K associates with tyrosine 719 of mKit or tyrosine 721 of hKit (16–20). Therefore, constitutive association of PI3K with mKit^{D814Y} could be mediated by constitutive phosphorylation of this tyrosine residue. Alternately, because this mutation alters autophosphorylation sites on mKit ^{D814Y}, association with PI3K could be mediated by a phosphorylated tyrosine unique to this mutant (13). To determine whether tyrosine 719 of mKit^{D814Y} is constitutively phosphorylated, we used an antibody specific for phosphorylated tyrosine 719. This corresponds to tyrosine 721 of hKit. Fig. 1*A* (*top panel*) demonstrates that this antibody recognizes phosphorylated wild-type

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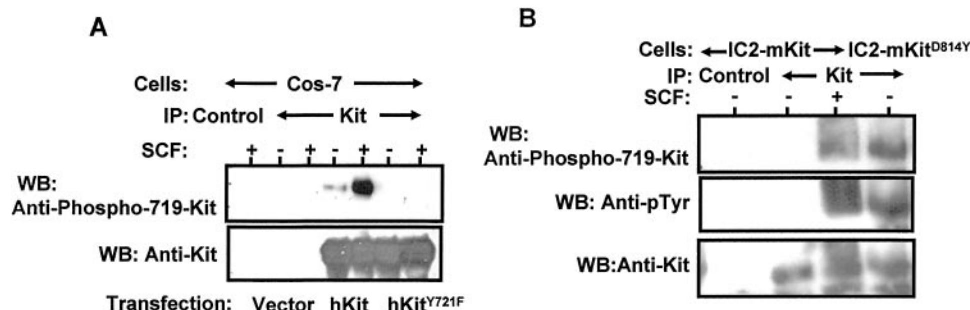

Fig. 1. Increased phosphorylation of the p85^{PI3K} docking site, tyrosine 719, on mKit^{D814Y}. Cos-7 cells were transiently transfected with vectors expressing either wild-type hKit (hKit), the hKit^{Y721F} mutant (hKit^{Y721F}), or empty vector, as indicated. The cells were harvested 48 h after transfection and incubated in the presence or absence of SCF. Kit immunoprecipitates were analyzed for phosphorylation using antibody specific to phosphorylated tyrosine 721 of hKit (corresponding to tyrosine 719 of mKit; *top panel* of *A*) or for expression of hKit protein (*bottom panel* of *A*). Kit immunoprecipitates from IC2 cells expressing wild-type mKit or the mKit^{D814Y} mutant were analyzed for tyrosine 719 phosphorylation. *B* shows the phosphorylation of mKit and mKit^{D814Y} on tyrosine 719 (anti-phospho-719-Kit, *top panel*) and total tyrosine (anti-pTyr, *middle panel*) as well as equivalent loading of Kit protein in each of the Kit immunoprecipitates (anti-Kit, *bottom panel*). The experiment shown is representative of three independent experiments.

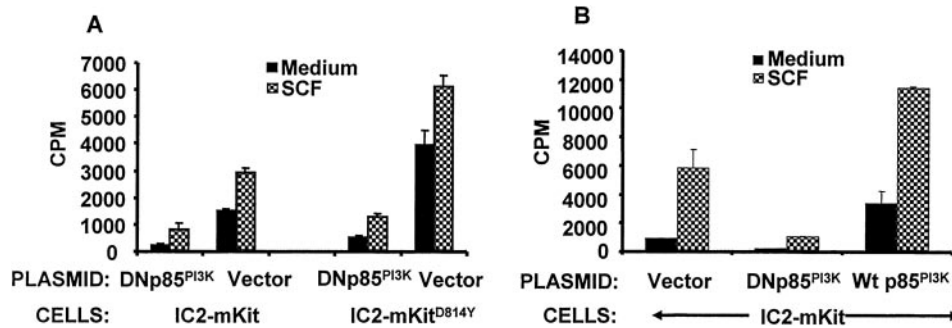

Fig. 2. Overexpression of DNp85^{PI3K} reduced factor-independent growth of IC2-mKit^{D814Y} cells, whereas overexpression of wild-type p85^{PI3K} (*Wt p85^{PI3K}*) enhanced SCF-induced growth in IC2-mKit cells. The indicated cells were transiently transfected with DNp85^{PI3K} or vector control using electroporation (*A*). Transfected cells were cultured for 24 h and then assessed for growth in the presence or absence of SCF for 36 h, followed by [^{3}H]thymidine incorporation overnight. IC2-mKit cells were next transfected with DNp85^{PI3K}, Wt p85^{PI3K}, or vector control as described in *A* and assessed for growth in the presence or absence of SCF (*B*). All data represent the mean ± SE (in cpm) of triplicate samples. The experiment shown is representative of three independent experiments.

hKit expressed in Cos-7 cells but does not recognize a hKit mutant with tyrosine 721 replaced by phenylalanine (hKit^{Y721F}). The *bottom panel* of Fig. 1*A* demonstrates that similar amounts of wild-type hKit and the hKit^{Y721F} mutant were expressed in the transfected Cos-7 cells. Furthermore, immunoblotting with antibody that recognizes multiple phosphotyrosine residues indicated that SCF did induce an increase in tyrosine phosphorylation of the hKit^{Y721F} mutant (data not shown). These data demonstrate the specificity of this antibody for phosphorylated tyrosine 721/719 of Kit.

We were next interested in determining whether tyrosine 719 of murine mKit^{D814Y} was constitutively phosphorylated. Whereas SCF was required to induce increases in phosphorylation of tyrosine 719 of wild-type mKit, this residue was constitutively phosphorylated in cells expressing the mKit^{D814Y} mutant (Fig. 1*B, top panel*). The *bottom panel* of Fig. 1*B* demonstrates that relatively similar amounts of Kit were present in the Kit immunoprecipitates. Although there is slightly less Kit protein in unstimulated wild-type cells, this does not account for the dramatic increase in phosphorylation of wild-type mKit after SCF treatment or the difference in the level of phosphorylation of the mKit catalytic domain mutant. These data demonstrate that tyrosine 719 of mKit^{D814Y} is constitutively phosphorylated. Phosphorylation of this residue likely promotes association of mKit^{D814Y} with p85^{PI3K} and contributes to activation of the PI3K pathway. This possibility is supported by our previous data demonstrating that tyrosine 721 is required for association of PI3K and the D816V hKit mutant (16).

PI3K Is Required for Factor-independent Growth of IC2 Cells Expressing mKit^{D814Y}. Whereas tyrosine 719/721 of mKit^{D814Y} is the only known binding site for PI3K, other signaling components could associate with this phosphotyrosyl residue and contribute to transformation mediated by this oncogenic mutant. To define the role of PI3K in transformation mediated by mKit^{D814Y}, independent of its capacity to associate with this mutant, we transiently transfected IC2 cells expressing wild-type or mutant mKit with a dominant negative p85^{PI3K} construct (DNp85) and assessed growth in the presence or absence of SCF. Immunoblotting with antibody specific for p85^{PI3K} demonstrated similar expression of all constructs in cells after transfection by electroporation (data not shown). Overexpression of the dominant negative p85^{PI3K} inhibited factor-independent growth of cells expressing mKit^{D814Y} by 87% and SCF-induced growth of cells expressing wild-type mKit or mKit^{D814Y} by approximately 80% (Fig. 2*A*).

To determine whether increased expression of PI3K is sufficient to induce factor-independent growth of IC2 cells, we transiently transfected IC2-mKit cells with wild-type or dominant negative p85^{PI3K}. Consistent with the data shown in Fig. 2*A*, DNp85^{PI3K} inhibited the growth of these cells even in the presence of SCF. In contrast, ectopic expression of wild-type p85^{PI3K} enhanced SCF-dependent growth of these cells. Some increase in factor-independent growth of IC2 cells expressing wild-type mKit was noted, albeit to a limited extent (Fig. 2*B*). These data demonstrate that PI3K is necessary but not sufficient for mast cell growth mediated by normal and mutant forms of mKit. However, the enhanced growth in response to SCF does indicate that PI3K interacted with signaling pathways activated by wild-type mKit. These results also demonstrate that inhibition of cellular growth by the dominant negative PI3K construct is specific and not the result of toxicity associated with transfecting the cells with plasmid DNA.

Other Signaling Pathways Activated by Wild-type mKit Are Not Maintained by the mKit^{D814Y} Mutant. Wild-type Kit activates multiple signaling pathways including PI3K, the Ras-Raf-MAP kinase pathway, and Src family kinases (33). Our previous studies have shown that the Src family member Lyn makes an important contribution to SCF-induced growth of mast cells (29). We therefore examined Lyn activity in IC2 cells expressing wild-type or mutant mKit. Stimulation of cells expressing wild-type mKit with SCF induced a modest increase in Lyn autophosphorylation. Although Fig. 3*A* demonstrates a small increase in Lyn activity in cells expressing mutant mKit compared with the baseline activity in cells expressing wild-type mKit, this was not observed consistently (Fig. 3*A*). Western blots of Lyn in each immunoprecipitate demonstrated that equivalent amounts of protein were used in this *in vitro* kinase assay (data not shown). To further examine the possible role of Lyn or other Src family members on growth mediated by mutant mKit, we compared the effect of the Src family inhibitor PP2 on growth mediated by mutant and wild-type mKit. Fig. 3*B* demonstrates no difference in the effect of PP2 on the growth of IC2-mKit^{D814Y} and IC2-mKit cells at concentrations ranging from 100 nM to 10 μM. Previous studies showed that 10 μM of this drug inhibited Lyn activity, but not mKit autophosphorylation (34). These data suggest that Lyn and other Src family members do not contribute to factor-independent growth mediated by mKit^{D814Y}.

We also have examined whether the Ras-Raf-MAP kinase pathway, another signaling cascade activated by wild-type Kit, was constitutively activated in IC2 cells expressing the mKit^{D814Y} mutant. Consistent with studies in myeloid progenitor cells, ERKs were not constitutively activated in IC2-mKit^{D814Y} cells (data not shown). These findings demonstrate that Src family members and the Ras/Raf/MAP kinase pathway are not constitutively activated in mast cells expressing the mKit mutant.

Inhibition of PI3K Reduces Growth Mediated by mKit^{D814Y} to a Greater Extent than SCF-induced Growth of IC2 Cells Expressing mKit. Our work has shown that activated PI3K is constitutively associated with mKit^{D814Y}, but this catalytic domain mutant does not maintain the activity of ERKs or of Lyn to a significant extent. These

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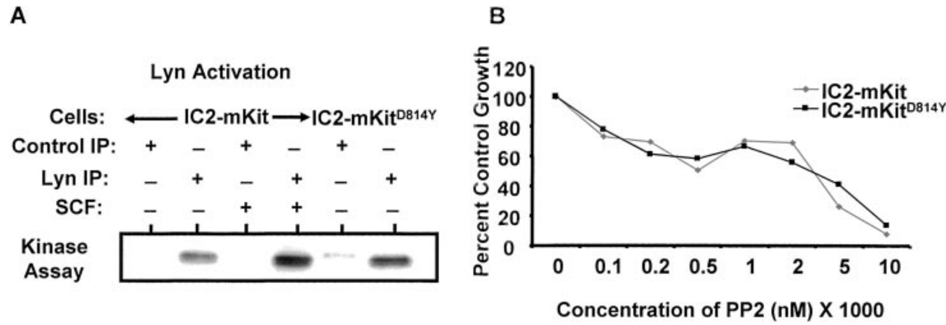

Fig. 3. Lyn is not constitutively active in IC2-mKit^{D814Y} cells. Lyn autophosphorylation activity was measured by *in vitro* kinase assay of immunoprecipitates using anti-Lyn antibody as described in "Materials and Methods" with lysates from IC2-mKit (stimulated with SCF or not) and IC2-mKit^{D814Y} cells (*A*). Kinase activity was visualized using autoradiography. Cells were incubated in the presence or absence of the indicated concentration of the Src family kinase inhibitor PP2 for 1 h, and growth in the presence (*IC2-mKit*) or absence (*IC2-mKit^{D814Y}*) of SCF was assessed using [^{3}H]thymidine incorporation (*B*). Data are expressed as the percentage of control cell growth and result from the means of triplicate samples. Results shown are representative of three independent experiments.

data suggest that this mutant relies more on the PI3K pathway than wild-type mKit. If this is so, then the growth of cells expressing mKit^{D814Y} should be more sensitive to inhibition of PI3K than SCF-induced growth of cells expressing wild-type mKit. To test this possibility, we used LY294002 and wortmannin, two PI3K inhibitors (Fig. 4, *A* and *B*). Both drugs reduced factor-independent and SCF-induced growth of cells expressing the mKit^{D814Y} mutant to a greater extent than SCF-induced growth of IC2 cells expressing wild-type mKit. Preferential inhibition of the growth of cells expressing mKit^{D814Y} was seen at concentrations as low as 20 nM wortmannin and 2 mM LY294002. These concentrations of drugs are known to specifically inhibit the PI3K pathway (35, 36). Statistical analysis using the Student's *t* test indicated that the difference between the effects of these drugs on growth of cells expressing mutant and wild-type mKit was significant (*P* for wortmannin treatment at 20 mM was 0.0367, and *P* for LY294002 treatment at 2 μM was 0.0043). Treatment with LY294002 inhibited SCF-induced phosphorylation of Akt and suggests that these effects are mediated through inhibition of the PI3K pathway (Fig. 4*C*). Furthermore, these findings support the possibility that the growth of cells expressing mKit^{D814Y} relies more on constitutive activation of the PI3K pathway than SCF-induced growth of cells expressing wild-type mKit.

Wortmannin Reduces Tumor Growth Induced by Injection of IC2-mKit^{D814Y} Cells into Mice. We next were interested in determining whether a drug that inhibits the PI3K pathway reduces the growth of IC2 cells expressing mKit^{D814Y} *in vivo*. Our hope was that by using a relatively low concentration of wortmannin, growth mediated by the oncogenic mKit mutant could be inhibited while partially sparing PI3K-mediated functions critical for survival, growth, and function of normal cells. Syngeneic mice that received s.c. injection with IC2-mKit^{D814Y} cells formed tumors (20 of 20 injection sites), whereas those that received injection with parental IC2 cells or cells expressing wild-type mKit did not (0 of 20 injection sites for each group). Histopathological assessment indicated that the tumor cells were similar to those injected into the mice, and the morphology of the tumor cells was consistent with mastocytoma (data not shown). Furthermore, cells isolated from excised tumors expressed similar levels of surface Kit as the IC2-mKit^{D814Y} cells (Fig. 5*A*). We next examined the effect of the PI3K inhibitor wortmannin on the growth of tumors mediated by IC2-mKit^{D814Y} cells in mice (Fig. 5*B*). Mice received s.c. injection with tumor cells, and treatment with wortmannin was initiated either 2 days (early treatment group) or 2 weeks (later treatment group) later. Wortmannin or the DMSO vehicle control was given three times a week by gavage. Thirty-six days after injection of tumor cells, there were decreases in tumor weights of mice treated with wortmannin in both the early and late treatment groups. This corresponded to 34 days of wortmannin treatment in the early group and 22 days of treatment in the late treatment group. In the late treatment group, a decrease of 66% in tumor weight was observed in mice given wortmannin compared with mice given vehicle control.

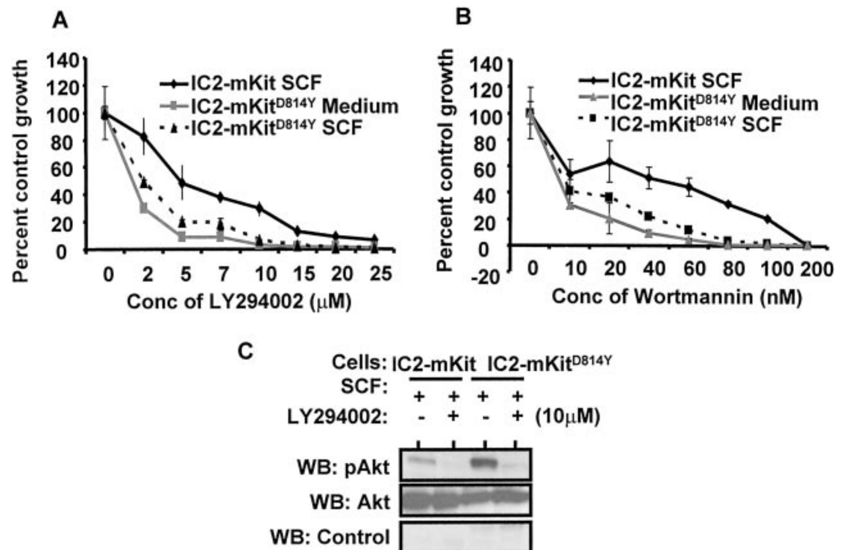

Fig. 4. Inhibition of PI3K reduces factor-independent growth of the mKit mutant to a greater extent than SCF-induced growth of cells expressing wild-type mKit. IC2-mKit and IC2-mKit^{D814Y} cells were pretreated for 30 min with PI3K inhibitors wortmannin (*A*) or LY294002 (*B*) at the indicated concentrations. Cells were then incubated for 36 h in the presence or absence of SCF, pulsed overnight with [^{3}H]thymidine, and harvested. Data are expressed as the percentage of control cell growth and represent the means of triplicate samples. IC2 cells expressing wild-type mKit or mKit^{D814Y}were pretreated with the indicated drug for 20 min and then incubated in the presence of SCF for 10 min. Cells were lysed, and whole cell lysates were resolved by SDS-PAGE, transferred to Immobilon-P, and immunoblotted (*WB*) with the antibodies indicated (*C*) to examine inhibition of Akt phosphorylation by the PI3K inhibitor. Results shown are representative of three independent experiments.

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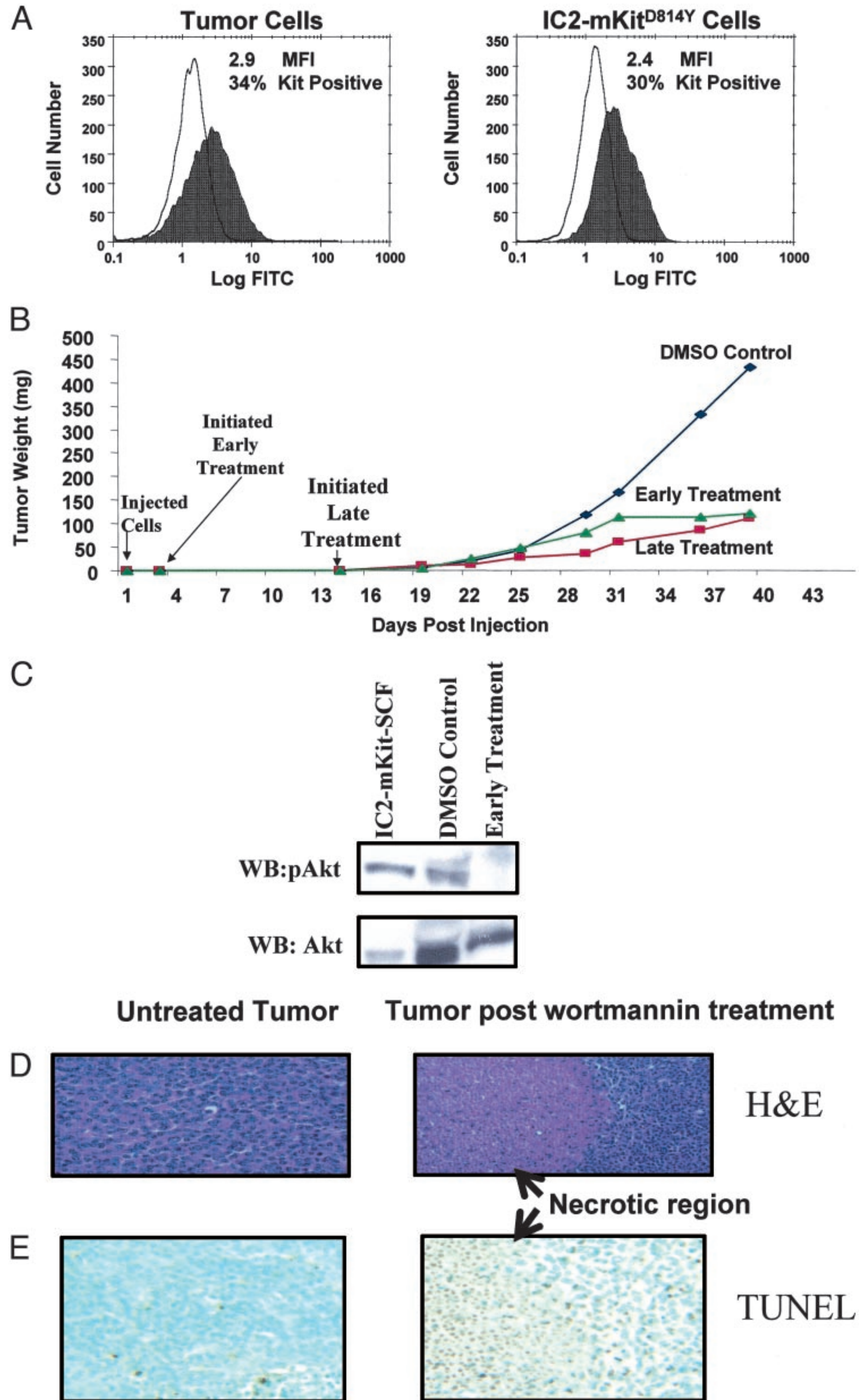

Fig. 5. Wortmannin slows the growth of tumors induced by injection of IC2-mKit^{D814Y} cells into mice. IC2-mKit^{D814Y} cells were injected s.c. in bilateral flanks of mice. To isolate cells for assessment of Kit expression, tumors were excised and digested overnight in trypsin, and fluorescence-activated cell-sorting analysis was performed on a single cell suspension using antibody specific for mKit (*A*). To assess the effects of wortmannin on the growth of tumors *in vivo*, mice (5 mice/group) received bilateral injection with IC2-mKit cells (10 injection sites/group) and were given 1.5 mg/kg wortmannin or vehicle control either 2 days (early treatment) or 3 weeks (late treatment) after injections. Wortmannin was given by gavage three times a week, and tumors were measured weekly. The data shown are representative of four independent experiments (*B*). To look at the level of phosphorylation of Akt after wortmannin treatment, tumors from the early treatment group and the DMSO-treated group were lysed, the lysates were resolved by SDS-PAGE and transferred onto Immobilon-P, and the blots were probed with the indicated antibodies (*C*). To compare the morphology of tumor cells from vehicle controls and wortmannin treatment groups, tumors were excised, fixed in Carnoys fixative, embedded in paraffin blocks, and stained with hematoxylin (*D*). The excised tumors were also stained for terminal deoxynucleotidyl transferase-mediated nick end labeling (*TUNEL*) activity (*E*) to look for apoptosis.

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The average tumor weights for these groups were 113 and 330 mg, respectively. Using Student's *t* test, we found that these numbers were significantly different ($P = .0002$). Initiation of wortmannin treatment early in development of tumors (2 days after injection with tumor cells) resulted in a 75% reduction in tumor weight. This corresponded to tumor weights from mice treated with wortmannin of 85 mg compared with a tumor weight of 330 mg from mice treated with the DMSO vehicle control. The difference in these numbers was also statistically significant ($P = 0.0005$). Thus, wortmannin, administered early or late in development of mastocytoma, significantly decreased the rate of growth of these tumors. To determine whether the reduction in tumor growth correlated with inhibition of the PI3K pathway

in vivo, we compared phosphorylation of Akt in tumors from mice that received vehicle control or wortmannin. Fig. 5*C* demonstrates a reduction in phosphorylated Akt in mice treated with wortmannin. These data suggest that the drug was inhibiting the PI3K pathway in the tumors. Interestingly, degeneration and necrosis associated with apoptosis were observed in tumors obtained from mice treated with wortmannin, but not the vehicle controls (Fig. 5, *D* and *E*). The necrosis was not due to inhibition of tumor angiogenesis (data not shown). None of the mice in any treatment groups showed toxicity due to the treatment procedure or the drug. These data support the hypothesis that that PI3K is required for survival and growth of the IC2 mast cell line expressing the mKit^{D814Y} mutant both *in vitro* and *in vivo*.

DISCUSSION

Numerous mutations in codon 816 of hKit (814 of mKit) lead to constitutive activation of this receptor tyrosine kinase and are oncogenic. Hematopoietic cells expressing this mutation are factor independent and tumorigenic (16, 25, 28, 32, 37–40). mKit^{D814Y} is constitutively phosphorylated on tyrosine residues and is degraded more rapidly than its wild-type counterpart (16, 28, 37, 39, 41). Mutations in codon 816 of humans are prevalent in patients with mastocytosis and mast cell leukemias (3–5, 8). Despite the fact that this mutation was identified a decade ago, surprisingly little is known about the mechanism of pathogenesis mediated by this oncogenic protein, particularly in mast cells.

Recent reports suggest that inhibitors specific for mutations associated with certain malignancies can be used in the treatment of these diseases. STI 571, an inhibitor of BCR-ABL, the platelet-derived growth factor receptor, and Kit, is being successfully used in treatment of patients in chronic phase of chronic myeloid leukemia (42, 43). This drug also shows remarkable promise in the treatment of GISTs containing activating mutations in the juxtamembrane region of Kit (11, 44, 45). Unfortunately, STI 571 does not inhibit the growth of cells expressing hKit^{D816V} (12, 46–48). These data suggest that this drug will not be suitable for treatment of mastocytosis, core factor binding leukemias, and germ cell tumors, all diseases associated with activating mutations in codon 816 of hKit. At present, no inhibitors have been identified that impair the activity of this Kit mutant to a greater extent than the activity of wild-type Kit. This is important because the normal function of wild-type Kit is important for hematopoiesis, gastrointestinal function, pigmentation, and reproduction.

Previous studies have demonstrated that Lyn, ERKs, and Janus kinase 2 contribute to but are not required for growth mediated by wild-type Kit (29, 49–51). Thus, multiple signaling components are activated by SCF, but these diverse pathways make overlapping contributions to biological responses. Whereas hKit^{D816V} associates with activated PI3K, it does not constitutively activate ERKs and Lyn (data not shown; Fig. 3; Ref. 16). These data, in conjunction with the increase in sensitivity of cells expressing mKit^{D814Y} to PI3K inhibitors, suggest that this mutant does not operate with the biochemical redundancy characteristic of wild-type Kit (Fig. 4). Thus, targeting pathways preferentially used by this mutant may be a feasible approach for impairing the growth of cells expressing this mutant while sparing, at least in part, the growth and/or function of cells expressing wild-type Kit.

Studies by Ning *et al.* (14, 15) indicate that Stat3 is activated by the hKit catalytic domain mutant in the megakaryoblastic cell line Mo7e. Consistent with these findings, Stat3 is constitutively phosphorylated in IC2 mast cell line expressing mKit^{D814Y}, and this is independent of PI3K (data not shown). Interestingly, both PI3K and Stat3 are necessary but not sufficient for growth of hematopoietic cells (Fig. 2; Refs. 14 and 15). These findings suggest that both Stat3 and PI3K may serve as molecular targets for impairing the growth of cells expressing mutant Kit. Whereas several PI3K inhibitors are currently available, a clinically feasible means to inhibit Stat3 is more problematic. Therefore, targeting the PI3K pathway is a more promising approach for preclinical studies in the immediate future.

Our previous work demonstrated that tyrosine 721 of the hKit^{D816V} mutant is required for tumorigenicity of a myeloid progenitor cell line (16). Because other signaling components could associate with tyrosine 721 of hKit (corresponding to tyrosine 719 of mKit), the role of PI3K in these events remained to be firmly established. We also showed that activated PI3K is constitutively associated with hKit^{D816V} in the MIHC progenitor cells (16). Because many signaling components play lineage-specific roles in stimulus-response coupling mechanisms (21–24), we have confirmed this finding in the mast cell lineage (data not shown). The present study extends these findings as follows: first, tyrosine 719 of mKit^{D814Y} is constitutively phosphorylated; and this likely mediates constitutive association of PI3K with the mKit catalytic domain mutant (Fig. 1). Second, PI3K plays a critical role in mast cell growth mediated by wild-type mKit and mKit^{D814Y}. This is illustrated by our results directly targeting PI3K with pharmacological inhibitors and ectopic expression of a dominant inhibitory PI3K mutant (Figs. 2 and 4). Results from studies using hKit receptor mutants incapable of associating with PI3K have been used to suggest that this lipid kinase is not critical for mast cell response to SCF. Thus, our data demonstrate a more global role for PI3K in Kit-mediated responses of mast cells than previously thought (20, 52–54). This is likely due to indirect activation of PI3K through signaling pathways independent of the PI3K docking site on Kit. This could be mediated by Src family members or the Gab family of adaptor proteins (55–57). Studies with low doses of PI3K inhibitors suggest that PI3K is particularly critical for the growth of cells expressing mutant mKit *in vitro* (Fig. 4, *A* and *B*). The similarity in degree of inhibition of growth mediated by wild-type and mutant mKit by dominant negative PI3K is likely due to apoptosis of cells transiently transfected with high levels of this inhibitory mutant (Fig. 2). In this regard, preferential inhibition of the growth of IC2 cells expressing mKit^{D814Y} was seen at low concentrations of PI3K inhibitors, whereas higher concentrations abrogated growth of cells expressing both wild-type mKit and mKit^{D814Y} (Fig. 4, *A* and *B*).

Results with a PI3K inhibitor also suggest that PI3K plays a critical role in growth mediated by the mKit mutant *in vivo*. Oral administration of wortmannin reduced the growth of mast cell tumors in syngenic mice and induced large regions of tumor necrosis, most probably due to apoptosis resulting from inhibition of PI3K (Fig. 5). Thus, molecular targeting of PI3K may be useful in the treatment of mast cell disease, as well as other diseases associated with the Kit catalytic domain mutation, such as some forms of acute myelogenous leukemia and germ cell tumors. Indeed, both wortmannin and LY294002 slow tumor growth in several animal models of cancer (58–60). Wortmannin has also been used successfully in the treatment of a murine model of Alzheimer's disease (61). However, it is important to note that inhibition of PI3K leads to reduction but not elimination of tumors (Fig. 5; Refs. 58–60). We are currently working to identify drugs to combine with low concentrations of PI3K inhibitor to eradicate tumors in this animal model of mastocytoma.

Although PI3K is clearly essential for survival, growth, and function of normal cells, our data suggest that growth mediated by the mKit catalytic domain mutant is particularly dependent on this pathway and may be inhibited by concentrations of wortmannin that are not lethal for normal physiological processes. In support of this possibility, no overt toxicity was observed in mice treated with wortmannin at concentrations of 1–1.5 mg/kg. These findings could be of

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particular importance because overexpression or activation of components of the PI3K pathway has been noted in multiple forms of cancers (62). Thus, this pathway may prove a molecular target in the treatment of a variety of cancers.

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