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Rac Guanosine Triphosphatases Represent Integrating Molecular Therapeutic Targets for BCR-ABL-Induced Myeloproliferative Disease

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SUMMARY

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) initiated by expression of the p210-BCR-ABL fusion protein. We demonstrate in a murine model of p210-BCR-ABL-induced MPD that gene targeting of *Rac1* and *Rac2* significantly delays or abrogates disease development. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. We utilize NSC23766, a small molecule antagonist of Rac activation, to validate biochemically and functionally Rac as a molecular target in both a relevant animal model and in primary human CML cells in vitro and in a xenograft model in vivo, including in Imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an additional therapeutic target in p210-BCR-ABL-mediated MPD.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) initiated by malignant transformation of hematopoietic stem cells (HSC). The characterizing feature of this disease is the presence of the Philadelphia Chromosome [t(9;22)(q34;q11)], a somatic mutation in which the 3′ region of the Abelson leukemia virus (*ABL*) gene is fused to the 5′ region of the breakpoint cluster region (*BCR*) gene (Konopka et al., 1985; Shtivelman et al., 1985; Daley et al., 1990; Kelliher et al., 1990; Lugo et al., 1990). The p210 isoform of the resulting BCR-ABL fusion protein is necessary and sufficient for the development of CML (Daley et al., 1990). Expression of p210-BCR-ABL, a constitutively active tyrosine kinase, regulates a variety of signaling cascades, including Ras, extracellular-signal regulated kinase (ERK), Akt, c-Jun-activated kinase (JNK), p38, CrkL, signal transducer and activator of transcription 5 (STAT5), and nuclear factor-κB (NF-κB) (Ren, 2005); confers a proliferative advantage

SIGNIFICANCE

The introduction of tyrosine kinase inhibitors for the therapy of CML has extended the survival of CML patients by inducing long-term hematologic remissions. However, some proportion of CML patients demonstrate p210-BCR-ABL persistence at the molecular level and/or relapse with ABL kinase-inhibitor- resistant disease suggesting that inhibition of this kinase activity alone is not sufficient to eliminate all leukemic cells. Here, we show that the combined deficiency of Rac1 and Rac2 Rho GTPases significantly attenuates p210-BCR-ABL-induced proliferation in vitro and MPD in vivo and confirm Rac as a therapeutic target in p210-BCR-ABL disease using a first generation small molecule inhibitor.

to cells; and induces abnormal adhesion and migration of hematopoietic progenitor cells (Ramaraj et al., 2004; Zhao et al., 2001). p210-BCR-ABL expression appears to be directly responsible for the development of a transformed phenotype (Koschmieder et al., 2005).

Although allogeneic bone marrow (BM) transplantation is a curative treatment for CML, only ~25% of CML patients are eligible for this therapy (Goldman, 1997). Imatinib mesylate, an Abl kinase inhibitor, has been identified as an effective treatment option for p210-BCR-ABL-mediated leukemia (Druker et al., 1996). However, molecular remissions in response to Imatinib are not uniform and kinase domain mutations have been identified that are resistant to Imatinib therapy (Gorre et al., 2002; Lowenberg, 2003). Signaling proteins downstream of p210-BCR-ABL therefore may offer additional targets for treating p210-BCR-ABL-persistent and Imatinib-resistant disease.

The Rac subfamily of Rho guanosine triphosphatases (GTPases) plays an essential role in regulating hematopoiesis (Gu et al., 2003; Yang et al., 2001; Cancelas et al., 2005; Cancelas and Williams, 2006). Rho GTPases are Ras-like molecular switches that cycle between inactive, GDP-bound and active, GTP-bound states. In hematopoietic cells, Rac proteins integrate signals from growth factor, chemokine, and adhesion receptors to induce and coordinate a variety of cellular responses (Gu et al., 2003). The Rac subfamily is comprised of three highly homologous proteins: Rac1, Rac2, and Rac3. Rac2 is expressed specifically in hematopoietic cells, while Rac1 and Rac3 are ubiquitously expressed (Moll et al., 1991; Shirsat et al., 1990; Haataja et al., 1997). Although structurally similar, Rac1 and Rac2 share distinct as well as overlapping roles in the development and function of hematopoietic stem and progenitor cells (HSC/P). Rac1 is required for engraftment of HSC into the stem cell niche and regulates cell-cycle progression (Gu et al., 2003; Cancelas et al., 2005), whereas Rac2 is important for retention of HSC/P within the hematopoietic microenvironment (Yang et al., 2001) and regulates survival (Gu et al., 2003). Combinatorial expression of both proteins is necessary for normal HSC adhesion and migration and supports long-term hematopoiesis (Gu et al., 2003; Cancelas et al., 2005). Rac1 and Rac2 also regulate distinct aspects of cytoskeletal reorganization (Filippi et al., 2004). The role of Rac3 in hematopoiesis, which was initially cloned from a CML-derived cell line (Haataja et al., 1997), has not yet been fully defined.

Rac GTPases have been previously implicated in p210-BCR-ABL-mediated transformation (Sini et al., 2004; Renshaw et al., 1996; Skorski et al., 1998; Harnois et al., 2003; Burridge and Wennerberg, 2004; Schwartz, 2004), although the specific role(s) of individual Rac subfamily members in the development of disease in vivo have not been defined. Recent evidence also suggests that Rac3 plays a role in p190-BCR-ABL-mediated ALL, while Rac1 and Rac2 do not appear to be hyperactivated in these lymphoma lysates (Cho et al., 2005). This is of particular relevance, since p190-BCR-ABL differs from p210 in potentially important ways as it relates to RhoGTPases. For instance, while p210-BCR-ABL binds to and activates the Rho GTPases, apparently through the Dbl homology domain, p190-BCR-ABL, which lacks this domain, cannot bind to Rho GTPases but can still activate Rac1 and Cdc42 (Harnois et al., 2003) through activation of the guanine exchange factor (GEF) Vav1 by BCR-ABL (Bassermann et al., 2002). Rac GTPases have been shown to regulate signaling pathways that also are downstream of p210-BCR-ABL (Burridge and Wennerberg, 2004; Schwartz, 2004). Together, these data suggest that Rac GTPases may integrate multiple signaling components of p210-BCR-ABL-activated pathways.

We utilized a retroviral murine model in gene-targeted BM cells and analyzed primary human CML cells in vitro and in vivo in a xenograft model to investigate the importance of Rac GTPase activation in the development and progression of p210-BCR-ABL-mediated MPD. Here, we show that Rac GTPases are activated by p210-BCR-ABL, and the combined deficiency of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation in vitro and MPD in vivo. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. We utilize NSC23766, a small molecule antagonist of Rac activation, to biochemically and functionally validate Rac as a molecular target in both a relevant animal model and in primary human CML cells in vitro and in a xenograft model in vivo, including in Imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an additional therapeutic target in p210-BCR-ABL-mediated MPD.

RESULTS

Rac Is Hyperactivated in Chronic-Phase CML HSC/P

Recent studies in cell lines have suggested that Rho GTPases can be activated by p210-BCR-ABL in vitro and in vivo (Skorski et al., 1998; Harnois et al., 2003). Since the expression of p210-BCR-ABL in HSC appears to be sufficient to induce a transformation phenotype, we first analyzed whether Rac isoforms were hyperactivated in human chronic phase CML HSC/P. Activation of Rac was determined by p21-activated kinase (PAK)-binding domain (PBD) pull-down assays in isolated CD34+ cells from CML patients. We observed that Rac1, Rac2, and, to a lesser degree, Rac3 were hyperactivated in $CD34^+$ cells purified from peripheral blood of two CML patients at diagnosis (Figure 1A). To determine the effect of p210-BCR-ABL expression on activation of the Rac subfamily of Rho GTPases in a murine model of p210-BCR-ABL disease, we exogenously expressed p210-BCR-ABL in primary murine HSC/P. 5-fluorouracil (5-FU)-treated low-density BM (LDBM) cells were transduced with bicistronic vectors expressing enhanced green fluorescent protein (EGFP) either alone (empty vector, MIEG3) or with p210-BCR-ABL (MSCV-p210-BCR-ABL) (Hawley et al., 1993). Sorted, $EGFP^+$ cells were starved and then stimulated with stromal-derived factor-1α (SDF-1α), a chemokine

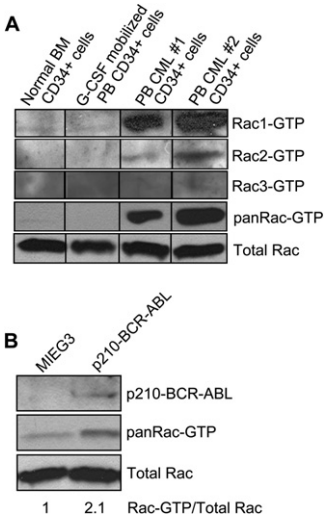

Figure 1. Rac GTPases Are Hyperactivated in Chronic-Phase Human CML HSC/P and Murine HSC/P Expressing p210-BCR-ABL

(A) Primary human CD34+ BM or G-CSF-mobilized peripheral blood cells and CD34+ chronic-phase CML peripheral blood cells (from two different CML patients) were starved for 1 hr and analyzed for Rac activation in a PAK-binding domain (PBD) pull-down assay. Samples were blotted and analyzed for Rac1-GTP, Rac2-GTP, Rac3-GTP, total Rac-GTP, and total Rac protein content.

(B) 5-FU-treated murine LDBM cells were transduced with either MIEG3 or MSCV-p210-BCR-ABL, bicistronic vectors expressing EGFP. The EGFP$^+$ cells were sorted, serum-starved for 6 hours, stimulated with 100 ng/ml SDF-1 for 5 minutes, lysed, and used in a PAK-binding domain (PBD) pull-down assay. The ratio of GTP-bound Rac to total Rac was determined by densitometry. The data represent one of three experiments with similar results.

known to induce migration of p210-BCR-ABL-expressing HSC/P (Zhao et al., 2001). Expression of p210-BCR-ABL in LDBM cells, confirmed by immunoblot, led to a >2-fold increase in GTP-bound Rac (Figure 1B) compared to MIEG3 transduced cells.

Rac1 and Rac2 Deficiency Significantly Attenuates p210-BCR-ABL-Mediated MPD In Vivo

To determine whether and which Rac GTPases are required for the development of p210-BCR-ABL-induced leukemogenesis in vivo, we utilized gene-targeted mice deficient in *Rac2* and with conditional (floxed) alleles of *Rac1* in a retroviral murine model of CML. 5-FU-treated Cre^{Tg+};*WT*, Cre^{Tg+};$Rac1^{flox/flox}$, Cre^{Tg+};$Rac2^{-/-}$, and Cre^{Tg+};$Rac1^{flox/flox}$;$Rac2^{-/-}$ LDBM cells were transduced with MIEG3 (control, empty vector) or MSCV-p210-BCR-ABL and sorted for EGFP$^+$ expression. Irradiated C57Bl/6 mice were transplanted with 50,000–75,000 of the EGFP$^+$ transduced cells together with 500,000 unmanipulated BM cells to assure the rescue of normal hematopoiesis in the postirradiation period. Ten days posttransplant, recipient mice were treated with polyI:C as previously described (Gu et al., 2003; Cancelas et al., 2005) to delete floxed *Rac1* genomic sequences (hereafter designated $Rac1^{\Delta/\Delta}$ mice). Recipient mice transplanted with MSCV-p210-BCR-ABL-transduced Cre^{Tg+};*WT* or Cre^{Tg+};$Rac1^{flox/flox}$ LDBM cells that were treated with PolyI:C uniformly developed CML-like MPD (leukocytosis, splenomegaly, pulmonary hemorrhage, and extensive liver infiltration with hematopoietic cells at necropsy) and died within forty days posttransplant (Figure 2A), consistent with the MPD phenotype, while all of the mice transplanted with MIEG3-transduced WT or $Rac1^{\Delta/\Delta}$ cells were still alive at day 100 posttransplant (Table S1). Strikingly, mice transplanted with p210-BCR-ABL-expressing Cre^{Tg+};$Rac1^{flox/flox}$;$Rac2^{-/-}$ cells that were treated with polyI:C to delete *Rac1* in the *Rac2* null background (designated $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice) showed significantly prolonged survival (Figure 2A; $p < 0.001$). Nearly 50% of these mice were still alive 100 days posttransplant. PCR analysis confirmed loss of exon 1 of *Rac1* in $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice treated with polyI:C (Figure 2B). Clonal analysis by linear amplification-mediated polymerase chain reaction (LAM-PCR) of BM cells from leukemic mice showed similar and oligoclonal reconstitution of p210-BCR-ABL-expressing wild-type, $Rac1^{\Delta/\Delta}$, $Rac2^{-/-}$ and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ cells (Figure 2C), suggesting that the delay in disease progression in these animals was not due to loss of p210-BCR-ABL vector integration and expression. Southern blot analysis confirmed the LAM-PCR study, showing 1–3 major clones/leukemia and no differences in the number of clones between genotypes (data not shown). All recipient mice maintained peripheral EGFP$^+$ cells throughout the study, confirming sustained engraftment of p210-BCR-ABL-expressing cells even in the absence of Rac1 and Rac2 (Figure 2D), a finding that is noteworthy due to our previous observations that hematopoietic engraftment of $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ HSC/Ps is rapidly lost in the absence of p210-BCR-ABL (Cancelas et al., 2005). Survival of mice transplanted with p210-BCR-ABL-transduced $Rac2^{-/-}$ cells was intermediate to $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice and significantly longer ($p < 0.001$) than the WT and $Rac1^{\Delta/\Delta}$ mice (Figure 2A).

We next confirmed that the increased survival seen in Rac-deficient, p210-BCR-ABL-expressing mice was not related to defective engraftment of Rac-deficient HSC. To analyze the homing and engraftment of p210-BCR-ABL-expressing cells in the presence or absence of the Rac GTPases, recipient mice were transplanted with PKH26-labeled WT and $Rac1^{flox/flox}$;$Rac2^{-/-}$ donor BM cells transduced with p210-BCR-ABL. The percentages of PKH26/EGFP-expressing cells in BM at 16 hr post-transplantation were similar between the WT and $Rac1^{flox/flox}$;$Rac2^{-/-}$ animals, suggesting unimpaired homing despite lack of Rac2 expression (Figure S1A), consistent with our previous findings in normal hematopoietic cells (Cancelas et al., 2005). In addition, there was an equivalent frequency of EGFP$^+$Lin$^-$Sca1$^+$c-Kit$^+$ cells observed in the BM of recipient mice transplanted with either WT or $Rac1^{flox/flox}$;$Rac2^{-/-}$ p210-BCR-ABL-expressing cells 18 days posttransplant after deletion of Rac1 sequences, suggesting that Rac-deficient HSC/P maintain an early graft as efficiently as wild-type cells in

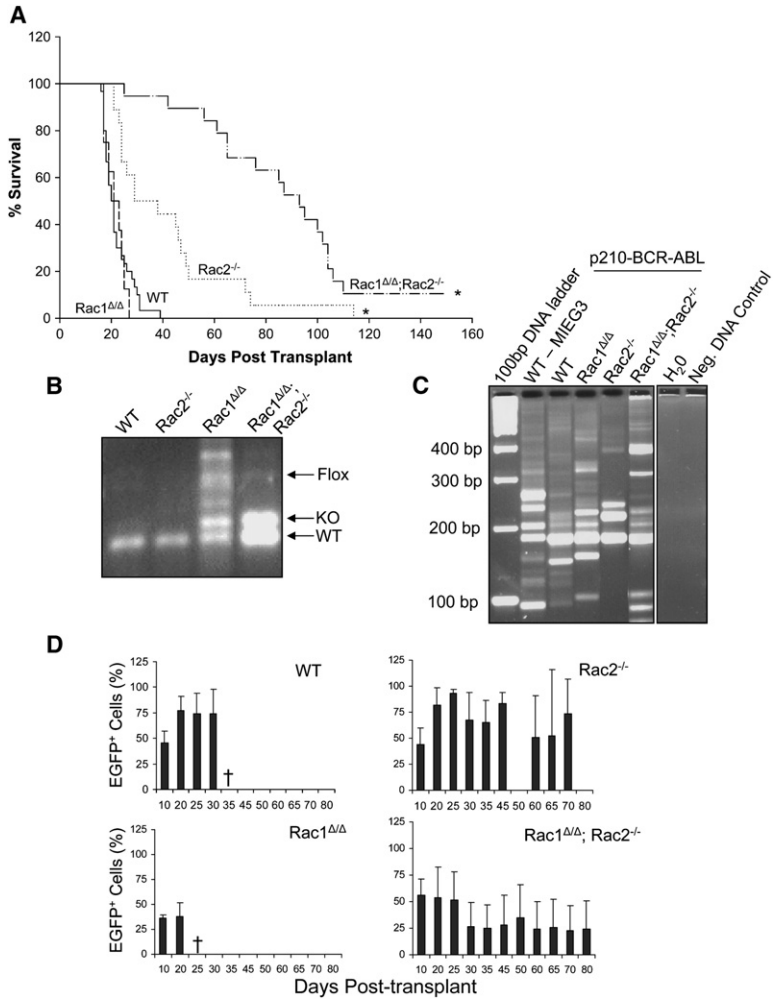

Figure 2. Rac GTPases Are Critical Regulators of p210-BCR-ABL-Mediated Leukemogenesis

(A) Kaplan-Meier survival curve of mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild-type and Rac-deficient cells. Mice demonstrating engraftment (monitored by the percentage of EGFP+ cells in the peripheral blood) of less than 1% at two consecutive time points were censored from the study. Genotypes are abbreviated in all figures as follows: WT, wild-type or flox allele at *Rac1* and *Rac2* loci, n = 30; $Rac2^{-/-}$, wild-type or flox allele at *Rac1* and null allele at *Rac2* locus, n = 18; $Rac1^{\Delta/\Delta}$, Cre-mediated null allele at *Rac1* locus and wild-type allele at Rac2 locus, n = 8; $Rac1^{\Delta/\Delta};Rac2^{-/-}$, Cre-mediated null allele at *Rac1* locus and null allele at *Rac2* locus, n = 19. *p < 0.001 (log P rank test) between MSCV-p210-BCR-ABL-expressing WT and $Rac1^{\Delta/\Delta}$ recipient mice and either BCR-ABL-transduced $Rac2^{-/-}$ or $Rac1^{\Delta/\Delta};Rac2^{-/-}$ groups.

(B) Representative PCR showing deletion of the Rac1 floxed gene in the peripheral blood of $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ recipient mice 70 days posttransplant, as visualized by the presence of the knockout (KO) band in the representative $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals. The presence of the wild-type (WT) allele in these mice does not signify expression of the $Rac1^{flox/flox}$ allele but rather represents contribution from unmanipulated BM cells coinjected with BCR-ABL-transduced cells. Thus, the efficiency of loss of exon 1 of *Rac1* can be determined by comparing the Flox band and the KO band in these samples.

(C) LAM-PCR amplifying retroviral vector insertion sites in p210-BCR-ABL-expressing BM cells from mice reconstituted with wild-type, $Rac1^{\Delta/\Delta}$, $Rac2^{-/-}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells. 3/3 leukemic $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals tested demonstrated oligoclonal integration patterns.

(D) Percentage of $EGFP^+$ cells in the peripheral blood of all surviving mice monitored over the course of the transplant. Data represent mean ± SD of all the mice included in Figure 2A.

the presence of p210-BCR-ABL (Figure S1B). In agreement with these data, there was no significant difference in expression of the hyaluronan receptor, CD44, which has recently been shown to play a specific and essential role in the homing and engraftment of p210-BCR-ABL-expressing leukemia-initiating cells (Krause et al., 2006) either in vitro (Figure S1C), using cotransduced (Cre-YFP and p210-BCR-ABL-EGFP) and sorted $Rac1^{\Delta/\Delta};Rac2^{-/-}$ LDBM cells, or in vivo (Figure S1D), from animals injected with p210-BCR-ABL-expressing $Rac1^{flox/flox};Rac2^{-/-}$ cells, compared to p210-BCR-ABL-expressing wild-type cells. Thus, these data suggest that prolonged survival of mice transplanted with p210-BCR-ABL-transduced Rac-deficient cells is not explained by reduced engraftment of leukemia-initiating stem cells.

Phenotype of MPD in the Rac-Deficient Mice

p210-BCR-ABL-expressing WT mice rapidly developed significant EGFP+ leukocytosis that persisted until death (Figures 2D and 3A). p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$ mice succumbed to splenomegaly and pulmonary hemorrhage (Table S1), consistent with the MPD phenotype. The majority of p210-BCR-ABL $Rac2^{-/-}$ mice showed gradual progression of leukocytosis and eventually died of MPD (Figure 3A; Table S2). Differential counts of the peripheral blood from p210-BCR-ABL-expressing WT and $Rac2^{-/-}$ mice ~30 days posttransplant demonstrated neutrophilia and the presence of immature granulocyte precursors and blasts in the peripheral blood (Figures 3B and 3C), consistent with the MPD previously described in this model (Daley et al., 1990; Kelliher et al., 1990). As expected, development of leukemia in these mice was accompanied by a predominance of $EGFP^+$ cells in the blood (Figure 2D), BM, and spleen (data not shown). $EGFP^+$ cells in the spleen and BM of all p210-BCR-ABL-transplanted WT, $Rac1^{\Delta/\Delta}$, and $Rac2^{-/-}$ recipient mice were uniformly $Gr\text{-}1^+/Mac\text{-}1^+$ (Table S2). In contrast to these mice, p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$

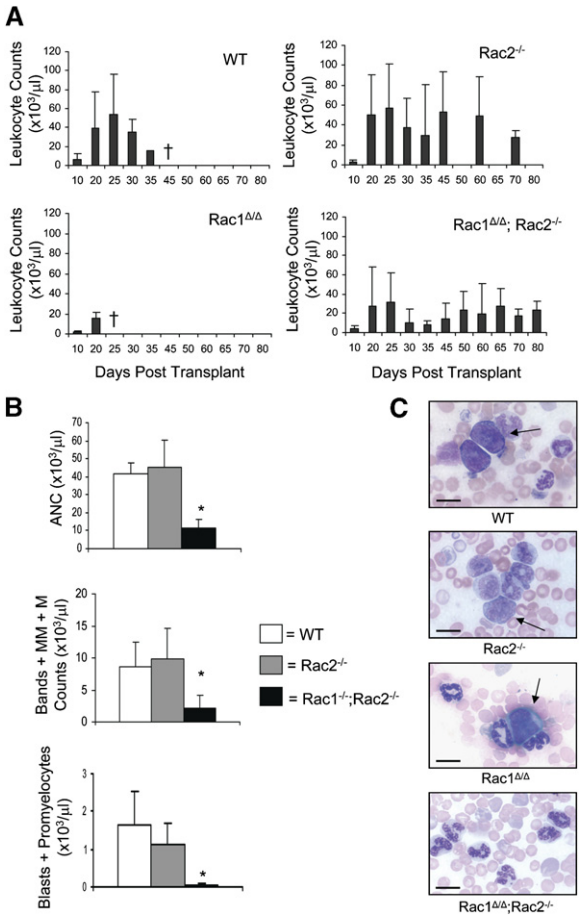

Figure 3. Deficiency of Rac1 and Rac2 Expression Significantly Delays or Appreciably Inhibits the Development of Leukocytosis in BCR-ABL-Expressing Recipient Mice

(A) Time course showing average leukocyte counts in the peripheral blood of recipient mice that were injected with either WT or Rac-deficient LDBM cells transduced with MSCV-p210-BCR-ABL. WT, n = 30; *Rac2*$^{-/-}$, n = 18; *Rac1*$^{\Delta/\Delta}$, n = 8; *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ n = 19. Data represent mean ± SD. Crosses represent time points at which no animals were surviving for analysis.

(B) Differential counts show decreased frequency of blasts and other immature myeloid progenitors in the peripheral blood of *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ BCR-ABL-transduced recipient mice approximately 30 days post-transplant, compared to the WT and *Rac2*$^{-/-}$ mice. Data represent mean ± SD. ANC, average neutrophil counts; MM, metamyelocytes; M, myelocytes. WT, n = 7; *Rac2*$^{-/-}$, n = 7; *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$, n = 8. *p < 0.05 between BCR-ABL-expressing WT and *Rac2*$^{-/-}$ mice and the BCR-ABL-transduced *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ group.

(C) Morphology of cells present in peripheral blood smears from representative BCR-ABL-transduced WT and Rac-deficient recipient mice approximately 30 days posttransplant. Myeloblasts (arrows) were apparent in all mice except for BCR-ABL-recipient *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice. Bars, 10 μm.

recipient mice showed normal peripheral blood morphology at ~30 days posttransplant in spite of significant chimerism with EGFP^{+} p210-BCR-ABL-expressing cells (Figures 2D, 3B, and 3C). The few p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice that developed early disease (arbitrarily defined as ≤69 days; 4/19 *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ mice) had either a myeloid (Gr-1^{+}/Mac-1^{+}, 67% of three animals tested) or lymphoid (B220^{+}, 33% of three animals tested) phenotype. One of the mice with a myeloid phenotype developed a solid tumor in the spine that showed high (86%) EGFP expression. The mice that succumbed to late disease (≥70 days; 15/19 *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ mice) had either a myeloid (Gr-1^{+}/Mac-1^{+}, 62% of 13 animals tested), lymphoid (B220^{+}, 23% of 13 animals tested), or bilineage (myeloid and lymphoid; 15% of 13 animals tested) phenotype. Additionally, two of the p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice with late onset disease developed solid tumors in the skull and brain, with histochemical and histological features consistent with CD68^{+} histiocytic sarcoma. Two of the p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice developed solid tumors in the spine with high (>70%) EGFP expression. One of the animals with a high percentage (40%) of CD3^{+} cells also presented with a tumor on the left kidney.

Activation of Signaling Cascades in Transformed Cells of p210-BCR-ABL-Expressing Mice

These data show that loss of Rac1 and Rac2 expression plays a key role in attenuation of the MPD phenotype, but suggest that late molecular events may overcome the loss of Rac1 and Rac2 function. To assess the status of Rac activation in p210-BCR-ABL-expressing WT, *Rac1*$^{\Delta/\Delta}$, *Rac2*$^{-/-}$, and *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice developing MPD, we performed PBD pull-down assays on splenocytes of diseased animals. Although *Rac1* and *Rac2* gene deletion was confirmed by PCR analysis (data not shown), active GTP-bound Rac as detected by a pan-Rac antibody was elevated in all p210-BCR-ABL-expressing leukemic mice tested (Figure 4A), suggesting that the third member of the Rac subfamily, Rac3, may be activated in p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ leukemic mice. Rac3 expression was confirmed in all leukemic animals tested by immunoblot (Figure 4B, lower panel) and PBD pull-down assays of splenocytes harvested from additional diseased animals showed enhanced GTP-bound Rac3 in p210-BCR-ABL-expressing leukemic mice, most clearly in the *Rac2*$^{-/-}$ and *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ samples (Figure 4B, upper panel), suggesting that Rac3 likely plays a key role in the eventual development of CML-like MPD.

Activation of Rac by p210-BCR-ABL in the WT mice was associated with increased baseline ERK, JNK, p38, Akt, STAT5, and CrkL phosphorylation (Figure 4C). These results are consistent with Rac signaling pathways previously implicated by us and others (Ren, 2005; Gu et al., 2003). Activation of ERK, JNK, p38, and CrkL was similar to WT mice in *Rac1*$^{\Delta/\Delta}$ mice, but was reduced in *Rac2*$^{-/-}$ and nearly completely abrogated in *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ splenocytes harvested from mice that developed MPD, despite continued activation of BCR-ABL in p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ splenocytes as determined by phospho-tyrosine immunoblots and increased activation of Rac3 (Figures 4B and 4D). These biochemical findings are strikingly in parallel with the survival curves of p210-BCR-ABL-expressing mice. Phosphorylation of Akt

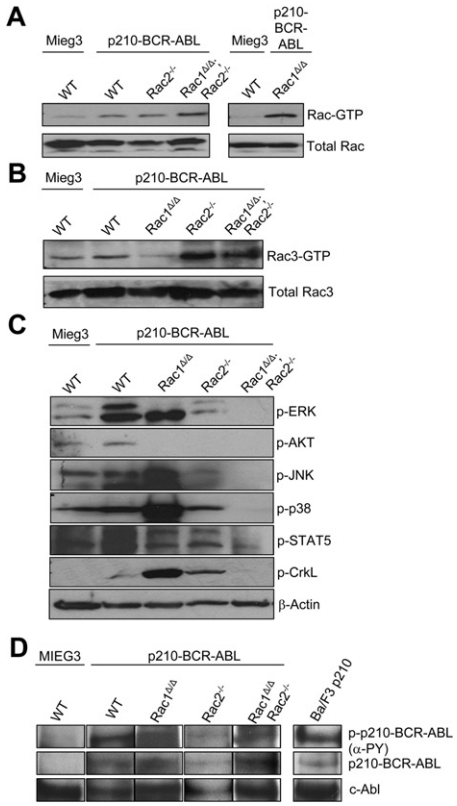

Figure 4. Rac GTPases Are Implicated in BCR-ABL-Mediated Activation of Multiple Signaling Cascades

(A) Representative example of Rac activation (PBD) pull-down assays performed on splenocytes harvested from MIEG3 and MSCV-p210-BCR-ABL-transduced WT and Rac-deficient mice developing MPD. Top panel (Rac-GTP) represents activated total Rac; lower panel represents total Rac protein expressed by immunoblot.

(B) Representative example of PBD pull-down assays of splenocytes harvested from leukemic animals to monitor Rac3 activation, using a Rac3-specific antibody.

(C) Representative examples of immunoblot analyses of splenocytes from BCR-ABL-expressing WT and Rac-deficient recipient mice developing MPD using phospho-antibodies specific to ERK, JNK, p38, Akt, CrkL, and STAT5. β-actin was used as a loading control. For each analysis, a minimum of three specimens from different mice were analyzed with similar results.

(D) Activation of p210-BCR-ABL in BM cells harvested from deceased or sacrificed leukemic mice. Phospho-p210-BCR-ABL expression is demonstrated in leukemic WT, *Rac1*$^{\Delta/\Delta}$, *Rac2*$^{-/-}$, and *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BM cells with a phospho-tyrosine antibody. A minimum of 3 samples in each genotype confirmed phosphorylation of p210-BCR-ABL in these samples. Expression of total p210-BCR-ABL and c-Abl was visualized using a c-Abl antibody. As a positive control, lysates from Ba/F3 cells stably expressing p210-BCR-ABL were analyzed.

was reduced in all of the Rac-deficient mice compared with WT mice. These data strongly suggest that activation of multiple signaling pathways by p210-BCR-ABL is dependent on Rac1 and Rac2. Interestingly, STAT5 phosphorylation was variably diminished in leukemic splenocytes even in the absence of Rac2 and more severely reduced but still detectable in the absence of both Rac1 and Rac2. p210-BCR-ABL, thus, may mediate activation of this pathway via induced Rac3, and/or STAT5 may be activated independently of Rac GTPases.

Rac Is a Molecular Target in p210-BCR-ABL-Expressing Cells

These results strongly support the hypothesis that p210-BCR-ABL signaling is dependent on Rac activation, suggesting that Rac GTPases may be unique molecular targets for CML therapy. We next used a genetic approach to determine whether deficiency of Rac1 and Rac2 influences p210-BCR-ABL-induced hyperproliferation of hematopoietic cells in vitro, a characteristic of this retroviral model of p210-BCR-ABL expression. LDBM cells were harvested from *Rac1*$^{flox/flox}$ and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ mice and cotransduced with MSCV-Cre-YFP and either MIEG3 or MSCV-p210-BCR-ABL. Deletion of *Rac1* in the Cre-YFP-expressing cells was confirmed by PCR analysis (data not shown). Proliferation of sorted EGFP$^+$ (*Rac1*$^{flox/flox}$) and EGFP$^+$/YFP$^+$ (*Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$) cells was determined by thymidine incorporation and cell counts. As shown in Figure 5A, p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ primary hematopoietic cells displayed significantly increased proliferation compared to MIEG3-transduced *Rac1*$^{flox/flox}$ cells. *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ cells exhibited significantly reduced p210-BCR-ABL-mediated hematopoietic cell proliferation, compared to p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ cells, suggesting that p210-BCR-ABL-mediated hyperproliferation of hematopoietic cells in vitro is dependent on activation of Rac1 and Rac2 and further validating Rac GTPases as key regulators of p210-BCR-ABL-mediated MPD.

To examine if pharmacologic inhibition of Rac in the presence of p210-BCR-ABL leads to attenuated cell proliferation, WT LDBM cells transduced with either MSCV-p210-BCR-ABL or MIEG3 and sorted for EGFP$^+$ expression were serum starved and incubated for 48 hr in the presence of increasing concentrations of NSC23766, a Rac-specific small molecule inhibitor that has been shown to inhibit Rac1 and Rac2 (Gu et al., 2003; Cancelas et al., 2005) and that we have found to inhibit Rac1, Rac2, and Rac3 in BCR-ABL-transduced LDBM cells (see Figure S2). NSC23766 does not inhibit RhoA or Cdc42 (Gao et al., 2004). Although inhibition of Rac activation with NSC23766 is reversible and less complete than genetic deletion, p210-BCR-ABL-induced proliferation was inhibited in a dose-dependent manner (Figure 5B, solid bars). No inhibition of MIEG3-transduced cells was detected at the same concentrations of inhibitor (Figure 5B, empty bars). Since we hypothesized that Rac3 was abnormally activated in splenocytes harvested from p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice developing late MPD, the effect of the Rac-specific inhibitor NSC23766 was tested in vitro on BM cells derived from these mice. The proliferation of EGFP$^+$ *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BM cells harvested from Rac1;Rac2-deficient mice with MPD was inhibited in a dose-dependent manner by the Rac inhibitor (Figure 5C), and immunoblotting confirmed

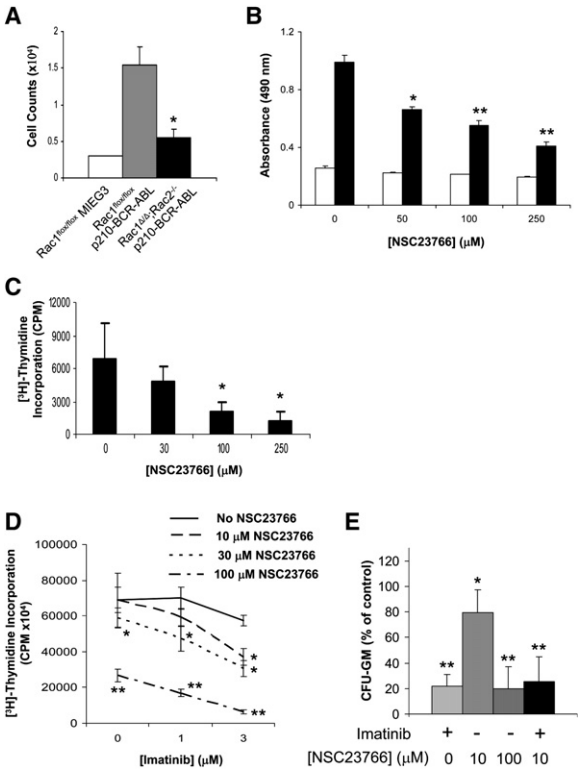

Figure 5. Loss of Rac Activation via Gene Deletion or by Treatment with NSC23766, a Rac-Specific Inhibitor, Diminishes the Proliferation of BCR-ABL-Expressing Murine and Human Cells

(A) 5-FU-treated *Rac1*$^{flox/flox}$ and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ HSC were transduced with MSCV-Cre-YFP to delete the *Rac1* floxed genomic sequence, together with MIEG3 or MSCV-p210-BCR-ABL. The EGFP^{+} and EGFP^{+}/YFP^{+} cells were sorted and plated. Cell proliferation was determined by cell counts 48 hr after the cells were plated. Data represent mean ± SD, n = 3 in each of two independent experiments. *p < 0.05 between the BCR-ABL-transduced *Rac1*$^{flox/flox}$ cells and the MIEG3-expressing *Rac1*$^{flox/flox}$ and BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ cells.

(B) 5-FU treated WT murine LDBM cells were transduced with either MIEG3 (white bars) or MSCV-p210-BCR-ABL (black bars), and the EGFP^{+} cells were sorted. Cells were then plated in the presence of increasing concentrations of NSC23766, and proliferation was determined 48 hr later by MTS assay. Data represent mean ± SD, n = 3 for three independent experiments. *p < 0.01, **p < 0.001 versus no drug.

(C) Effect of NSC23766 on BM cells harvested from BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice that developed MPD. Results are representative of mean ± SD from proliferation assays performed on three different *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice in triplicate. *p < 0.05 versus no drug.

(D) Effect of increasing concentrations of NSC23766 alone and in combination with Imatinib on the proliferation of Imatinib-resistant Ba/F3 p210-BCR-ABL-T315I cells. Data represent mean ± SD of four independent assays per sample done in duplicate. *p < 0.01; **p < 0.001.

(E) Analysis of CFU-GM formation from CML patients that developed myeloid blast crises in the presence of 1 μM Imatinib (n = 10 patients), NSC23766 (n = 10 patients), or a combination of Imatinib and NSC23766 (n = 5 patients). Data represent mean ± SD of the level of inhibition reached for each specific specimen. *p < 0.01; **p < 0.001.

inhibition of Rac3 activation in leukemic samples harvested from *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ animals (data not shown). These results further implicate Rac3 in the late development of MPD in the *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice.

To determine the effect of Rac inhibition on proliferation of an Imatinib-resistant p210-BCR-ABL mutant (La Rosee et al., 2002, 2004), Ba/F3 cells expressing the highly resistant T315I-p210-BCR-ABL mutant were incubated with Imatinib and/or NSC23766. As reported previously (Corbin et al., 2003), addition of up to 3 μM Imatinib alone had little effect on proliferation of these cells (less than 10% inhibition, Figure 5D). However, T315I-expressing cells treated with NSC23766 alone or in combination with Imatinib showed >90% inhibition of proliferation, similar to Ba/F3 cells expressing nonmutated p210-BCR-ABL (Figure 5D). Finally, the effect of NSC23766 on the growth of primary BM cells from CML patients in blast crisis was evaluated. Addition of 10–100 μM NSC23766 inhibited 20%–78% of blastic phase CML BM colony forming unit-granulocyte/macrophage (CFU-GM) colonies, a response similar to 1 μM Imatinib (Figure 5E). Similar inhibition was observed for CML blast phase erythroid progenitor cells (data not shown), while 100 μM NSC23766, but not lower doses, inhibited normal human progenitor BM cells in a fashion similar to 1 μM Imatinib (Figures S3A and S3B). These data demonstrate that Rac is essential for p210-BCR-ABL-induced proliferation of primary cells, and pharmacologic inhibition of Rac significantly reduces p210-BCR-ABL-mediated proliferation even in the presence of highly resistant kinase mutants.

To determine the effect of NSC23766 in vivo, mice were transplanted with p210-BCR-ABL-expressing murine HSC/P as described above and 10 days posttransplant, osmotic pumps containing NSC23766 (2 pumps/mouse, 100 μM NSC23766 per pump) or PBS (as controls) were surgically implanted subcutaneously to allow continuous infusion of the inhibitor. After 14 days, the pumps were replaced with fresh pumps and disease progression was monitored in mice exposed for up to 28 days. NSC23766-treated mice showed a significant increase in survival ($p < 0.01$) compared to control mice (Figure 6A). NSC23766 plasma levels were analyzed by HPLC and mass spectrometry and ranged from 1–3 μM (Table S3). This level of NSC23766 in control mice was associated with an expected doubling of the peripheral blood leukocyte count (Roberts et al., 1999) (Table S3), providing pharmacodynamic evidence of the presence of the inhibitor. Finally, CD34^{+} peripheral blood CML cells derived from leukapheresis products of two chronic-phase CML patients with significant leukocytosis (>300,000 WBC/mm^3) were transplanted into NOD/SCID mice and, after engraftment for 10 days, treated with NSC23766 by osmotic pumps for 14 days. As shown in Figure 6B, compared to the PBS-treated control mice, NSC23766 induced ~85% reduction in human CML by 17 days posttransplant. Taken together, these data indicate that NSC23766 impairs p210-BCR-ABL-induced leukemogenesis in primary murine and human cells in vivo and provides a rationale for targeting Rac proteins in this disease.

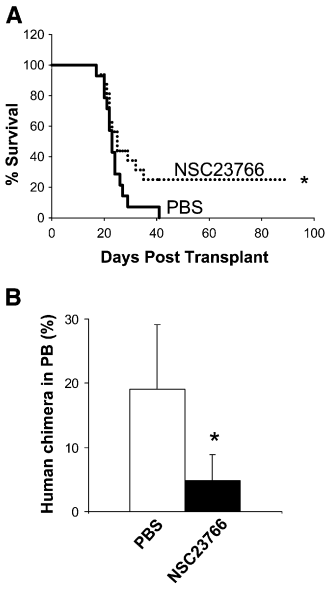

Figure 6. NSC23766 Significantly Delays the Development of MSCV-p210-BCR-ABL-Mediated Leukemogenesis in a Murine In Vivo Model and Inhibits Engraftment of Human Chronic Phase CML Cells in NOD/SCID Mice

(A) Survival curve of recipient mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild-type cells, then implanted with Alzet osmotic pumps containing either NSC23766 (2 pumps; 100 mM NSC23766 per pump; n = 16) or PBS (1 pump; n = 14) ten days post-transplant. Mice that died during surgery were censored from the study. *$p < 0.05$ (log P rank test) between PBS and NSC23766-treated groups. Data represent survival of pooled animals from two independent experiments with similar results.

(B) NOD/SCID mice were transplanted with CD34+ human CML peripheral blood cells from two newly diagnosed patients. Fifteen days posttransplant, Alzet osmotic pumps containing either NSC23766 (2 pumps, 75 mM NSC23766 per pump) or PBS (1 pump) were surgically implanted into the animals. Preimplant levels of human chimera in peripheral blood were 66.3 ± 30.3% and 76.3 ± 9.9%, respectively. Seventeen days postsurgery, animals were sacrificed and human chimerism in peripheral blood was analyzed. Data represent mean ± SD of pooled data from two independent experiments (n = 9 mice per group), *$p < 0.01$ (log P rank test).

DISCUSSION

Imatinib (Gleevec, also known as STI571 or CGP57148), an Abl kinase inhibitor that shows significant activity in all phases of CML and Ph-positive acute leukemias (Druker et al., 2002) by selective induction of apoptosis of p210-BCR-ABL-positive cells (Druker et al., 1996; Deininger et al., 1997; le Coutre et al., 1999), has provided an effective means of treatment in CML. The persistence of p210-BCR-ABL-positive HSC in Imatinib-treated patients suggests that inhibition of Abl kinase activity alone might not be sufficient to eliminate all leukemic stem cells.

We have previously shown that both Rac1 and Rac2 are essential for the regulation of multiple HSC functions with unique as well as overlapping roles, including proliferation, apoptosis, homing, and retention (Gu et al., 2003; Cancelas et al., 2005). These biological functions of Rac are associated with activation of multiple kinase signaling cascades, many of which are activated in CML blasts or in cell lines expressing p210-BCR-ABL. Thus, Rac proteins may integrate signals affecting survival/proliferation and cytoskeletal rearrangements leading to the motility and adhesion phenotypes reported in these cells. These observations led us to examine the requirement of Rac proteins in p210-BCR-ABL-mediated transformation.

In the studies reported here, significantly prolonged survival in vivo of p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice is apparent despite the observation by LAM-PCR and Southern blotting that all genotypes demonstrate oligoclonality. One to three retroviral integrations of the p210-BCR-ABL vector were observed in each genotype by Southern blot, a result not surprising based on the number of transduced cells injected into each animal and assuming the development of a HSC-initiated disease.

The increased survival of p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice correlates with nearly complete elimination of baseline hyperactivation of ERK, p38, JNK, Akt, and CrkL in BM cells from these mice. BCR-ABL *Rac1*$^{\Delta/\Delta}$/*Rac2*$^{-/-}$ mice that did develop MPD invariably appeared to induce Rac3 activation. The data supporting this conclusion include the absence of Rac1 and Rac2 genomic sequences in the EGFP^{+} peripheral blood cells of diseased animals, the increased Rac3 activity in splenocytes from these animals shown by pull-down assays, and the inhibition of proliferation in vitro of BM cells from *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ leukemic mice when incubated with NSC23766, a Rac-specific small molecule inhibitor (Gao et al., 2004). These data are consistent with previous reports of Rac3 activation in p190-BCR-ABL expressing malignant precursor B-lineage lymphoid cells (Cho et al., 2005).

p210-BCR-ABL-expressing *Rac2*$^{-/-}$ animals showed less reduction in the baseline activation of these pathways and less, although significant, prolongation of survival. p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$ mice behaved similarly to WT mice in this model. These data strongly suggest that each Rac protein plays a specific role in p210-BCR-ABL-mediated leukemogenesis, as we have noted in normal HSC/P functions (Gu et al., 2003; Cancelas et al., 2005). Alternately, these phenotypes could represent a combinatorial but variable decrease in total Rac activity. Studies are underway to determine the individual and combinatorial role(s) of each Rac protein, including Rac3, using *Rac3*$^{-/-}$ mice (Corbetta et al., 2005) bred into the *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ mouse line.

CrkL has been suggested in some studies to be a direct effector of the kinase domain of ABL. Thus, Rac activation in this model would not be expected to be dependent on CrkL activation. In that sense, our results are surprising but highly reproducible among leukemic animals. As CrkL activation has recently been reported to be dependent on a large multimeric protein complex that contains at least phosphoinositide-3 kinase (PI3K), docking protein 2 (DOK2), CrkL, Vav (a GEF responsible of Rac activation), and Rac (Nishihara et al., 2002; Sattler et al., 2001), we

suggest that the deficiency of Rac (Rac2 and especially the combined deficiency of Rac1 and Rac2) would also impair the activation of CrkL. The persistence of STAT5 signaling in $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells may also be important in at least two ways. First, we have previously shown that mice engrafted in a competitive repopulation assay lose contribution of $Rac1^{\Delta/\Delta};Rac2^{-/-}$ HSC/P shortly after Cre-mediated deletion of Rac1 sequences in the absence of Rac2 (Cancelas et al., 2005). Thus, persistent engraftment of p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells over 100 days suggests that p210-BCR-ABL may be providing important signals affecting HSC retention and function in the BM in this setting. In this regard, STAT5 is a reasonable downstream candidate. STAT5 has been previously implicated in both the p210-BCR-ABL transformation phenotype and in normal HSC/P proliferation (Bradley et al., 2002; Sillaber et al., 2000; Ye et al., 2006; Ilaria and Van Etten, 1996). Independently, JAK proteins, particularly JAK2, which are known to activate STATs, have been implicated in p210-BCR-ABL transformation (Xie et al., 2001; Wilson-Rawls et al., 1996). Indeed, we have seen inhibition of proliferation in vitro of BM cells harvested from p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$ mice that developed MPD using a JAK inhibitor (data not shown) (Thompson et al., 2002). Whether STAT5 activation in the absence of Rac1 and Rac2 is the result of direct signaling from p210-BCR-ABL via JAK (Xie et al., 2001) or the result of activation through Rac3 (or both) is yet to be determined.

In addition to the genetic data provided, our studies suggest that Rac proteins may prove to be useful molecular targets for pharmacologic intervention in human CML, particularly in p210-BCR-ABL-persistent and Imatinib-resistant disease. The combination of NSC23766 and Imatinib led to highly significant inhibition of proliferation of cells expressing the T315I mutant of p210-BCR-ABL. This first generation small molecule inhibitor of Rac is relatively nontoxic when administered chronically in vivo to mice (Cancelas et al., 2005) and appears to inhibit p210-BCR-ABL-induced leukemogenesis of primary murine and human cells in vivo.

Deregulated expression of Rac GTPases has previously been associated with several aspects of the leukemic phenotype. In particular, Rac1 has been shown to be an important downstream signaling component of p210-BCR-ABL (Skorski et al., 1998). p210-BCR-ABL-transduced Ba/F3 cells exhibit increased F-actin staining and an increased formation of filopodia and pseudopodia, reflecting elevated Rac1 activity (Salgia et al., 1997). In addition, a dominant-negative Rac1 mutant has been shown to inhibit p210-BCR-ABL-induced transformation in this cell line, and a signal cascade linking Abl kinase, phosphorylated Sos-1, and Rac-dependent phenotypes has been proposed (Sini et al., 2004). Harnois et al. reported that a stable complex could form between p210-BCR-ABL and multiple Rho GTPases and that Rac1, Rac2, Rho, and Cdc42 could be activated by p210-BCR-ABL, possibly through the Dbl homology domain of Bcr or activation of Vav as a guanine nucleotide exchange factor (GEF) that is associated with the complex (Harnois et al., 2003). However, these previous studies do not elucidate which specific Rac proteins are crucial in leukemic transformation and suffer from the lack of in vivo data on primary hematopoietic cells. The data presented here implicate Rac2, the combinatorial loss of both Rac1 and Rac2, and compensatory activation of Rac3 in leukemia development in p210-BCR-ABL disease. Our current data indicate that Rac GTPases are critical for p210-BCR-ABL-mediated transformation and, therefore, provide important targets for new therapy in CML.

EXPERIMENTAL PROCEDURES

Cell Lines

Stable Ba/F3 cell lines expressing full-length p210-BCR-ABL with the T315I point mutation and parental Ba/F3 cells were maintained as previously described (La Rosee et al., 2002).

Mice

The generation of C57Bl/6 Cre-transgenic ($Cre\text{-}^{Tg+}$);$Rac1^{flox/flox}$, $Cre^{Tg+};Rac1^{flox/flox};Rac2^{-/-}$, and $Cre^{Tg+};Rac2^{-/-}$ mice has been previously described (Gu et al., 2003; Cancelas et al., 2005). Animals used in these experiments were littermates. NOD/LtSz-scid/scid (NOD/SCID) mice, 5–6 weeks of age, were bred and housed under specific pathogen-free conditions in a laminar air flow unit and supplied with sterile food and drinking water containing doxycycline (6 mg doxycycline per gram of food, Bioserve Biotech, Laurel, MD) ad libitum. Housing, care, and all animal experimentation were done in conformity with protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Human Specimens

Normal BM and CML BM and PB were obtained through Institutional Review Board-approved protocols and donor informed consent from either Oregon Health Science University or Cincinnati Children's Hospital Medical Center. Material from therapeutic leukaphereses from two CML patients was submitted to CD34 selection by CliniMACS (Miltenyi Biotec Inc, Auburn, CA). Postselection purities were 99.6% and 99.7%. Interphase FISH showed that 98.6% and 99.4% of CD34+ cells carried the t(9,22) translocation, respectively.

Retroviral Vectors and Generation of Retroviral Stock

The retroviral vectors used have been described previously (Zhao et al., 2001; Gu et al., 2003; Williams et al., 2000). Generation of retroviral supernatants is described in Supplementary Experimental Procedures.

Bone Marrow Harvest, Transduction, and Transplantation

C57Bl/6 $Cre\text{-}^{Tg+}$;WT, $Cre\text{-}^{Tg+};Rac1^{flox/flox}$, $Cre^{Tg+}/Rac1^{flox/flox};Rac2^{-/-}$, and $Cre^{Tg+};Rac2^{-/-}$ mice were injected with 5-fluorouracil (5-FU, American Pharmaceutical Partners, Inc, Schaumburg, IL; 150 mg/kg i.p.) to enrich for HSC. BM cells were harvested as previously described (Gu et al., 2001) and stimulated with IMDM containing 2 mM L-glutamine, 10% FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 100 ng/ml AMP-4, 100 ng/ml recombinant human granulocyte-colony stimulating factor (G-CSF), and 100 ng/ml recombinant rat stem cell factor (rr-SCF, all Amgen, Thousand Oaks, CA) (cytokine-containing medium). Transduction of 5-FU-treated, LDBM cells was performed as previously described (Williams et al., 2000; Hanenberg et al., 1996). Two days posttransduction, $EGFP^+$ sorted cells were used either for transplantation or in vitro assays. For transplantation, a total of 50,000–75,000 EGFP-positive transduced BM cells were transplanted into lethally irradiated (1175 cGy, split-dose) 6–8 week old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) in the presence of 500,000 freshly isolated C57Bl/6 erythrocyte-lysed BM cells.

Hematological and Pathological Examination of Transplanted Mice

Animals were bled weekly starting ten days posttransplant. Complete blood counts and leukocyte differentials were determined and disease progression was monitored. Animals in which the percentage of $EGFP^+$ cells in the peripheral blood fell below 1% for two consecutive time points were censored from the study, due to loss of engraftment. While no WT and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals were removed from the study, four out of 12 $Rac1^{\Delta/\Delta}$ animals and 10 out 28 $Rac2^{-/-}$ animals were censored for this reason.

Homing and Engraftment Assays

See Supplemental Experimental Procedures online.

Generation of Rac1-Deficient Hematopoietic Cells

Cre-mediated recombination of floxed Rac1 sequences in hematopoietic cells was performed in vivo 10 days posttransplant as previously described (Cancelas et al., 2005; Mikkola et al., 2003). For in vitro deletion of the Rac1 floxed gene, LDBM cells were cotransduced with either MIEG3 and MSCV-Cre-YFP or MSCV-p210-BCR-ABL-EGFP and MSCV-Cre-YFP and were sorted for $EGFP^+/YFP^+$ expression.

In Vivo Administration of NSC23766

Ten days posttransplant, Alzet osmotic pumps (Model 2002, Durect, Cupertino, CA) containing either NSC23766 (2 pumps, 100 mM/pump) or PBS control at a flow rate of 0.5 μl/hr for 14 days were subcutaneously implanted into recipient mice. For NOD/SCID mice, mice were transplanted with a total of 10×10^6 $CD34^+$ cells per mouse and pumps were implanted on day +16 posttransplantation.

Proliferation Assays

Cell proliferation was assessed by either thymidine incorporation assays (transduced LDBM cells, $Rac1^{\Delta/\Delta};Rac2^{-/-}$ BM cells expressing p210-BCR-ABL, and Ba/F3-pSRC-T315I cells), cell counts (transduced LDBM cells and $Rac1^{\Delta/\Delta}/Rac2^{-/-}$ BM cells expressing p210-BCR-ABL), or MTS assays (transduced LDBM cells, Promega, Madison, WI), which are described in detail in Supplemental Experimental Procedures.

Rac Activation Assays

Splenocytes from transplanted mice or purified human CD34+ cells were processed for the preparation of protein extracts. The generation of protein extracts and Rac activation assays and electrophoresis are described in Supplemental Experimental Procedures.

Immunoblotting

Aliquots (25 μg) of protein extracts from the spleens of mice expressing either MIEG3 or MSCV-p210-BCR-ABL-EGFP were separated by electrophoresis on 7.5% or 12% SDS-PAGE gels under reducing conditions. Membranes were probed with antibodies specific for phospho-p42/p44 MAPK (1:1000), phospho-Akt (1:1000), phospho-p38 MAPK (1:1000), phospho-JNK (1:1000), phospho-CrkL (1:1000), phospho-STAT5 (1:1000), and β-actin (1:5000) (Cell Signaling) To examine phosphorylation of p210-BCR-ABL, BM cells from leukemic animals were lysed in high pH lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$ 10% glycerol, 20 mM Tris [pH 8.0]) supplemented with Complete protease inhibitor cocktail (Roshe Diagnostics, Indianapolis, IN) to which 1.25% of 10 M NaOH was added immediately before lysis. Cells were incubated on ice for 10 min, clarified at 12,000 × g for 3 min, and lysates were separated on a 7% SDS-PAGE gel. Membranes were probed with antiphosphotyrosine (4G10, Upstate, Charlottesville, VA) and anti β-actin antibodies. Blots were then incubated with a secondary antibody conjugated to HRP and directed against mouse or rabbit IgG (Cell Signaling Technology) and reactive proteins were visualized with LumiGLO (Upstate, Charlottesville, VA).

Flow Cytometric Analysis

Flow cytometric analysis is described in Supplemental Experimental Procedures.

Bone Marrow Hematopoietic Progenitor Assays

Colony-forming units-granulocyte/macrophage (CFU-GM) quantitation assays in methylcellulose have been previously described (Cancelas et al., 1998, 2005).

LAM-PCR

For clonality analysis of the p210-BCR-ABL insertion sites of leukemic animals, we performed LAM-PCR as previously described (Schmidt et al., 2003).

Statistical Analysis

Statistical analysis was performed using the unpaired Student's t test except for survival curves where the log P rank test was used. Values of $p < 0.05$ were considered significant.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental tables, and three supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/5/467/DC1/.

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