Hyperactivation of p21ras and the Hematopoietic-specific Rho GTPase, Rac2, Cooperate to Alter the Proliferation of Neurofibromin-deficient Mast Cells In Vivo and In Vitro

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Abstract
Mutations in the NF1 tumor suppressor gene cause neurofibromatosis type I (NF1), a disease characterized by the formation of cutaneous neurofibromas infiltrated with a high density of degranulating mast cells. A hallmark of cell lines generated from NF1 patients or Nf1-deficient mice is their propensity to hyperproliferate. Neurofibromin, the protein encoded by NF1, negatively regulates p21ras activity by accelerating the conversion of Ras-GTP to Ras-GDP. However, identification of alterations in specific p21ras effector pathways that control proliferation in NF1-deficient cells is incomplete and critical for understanding disease pathogenesis. Recent studies have suggested that the proliferative effects of p21ras may depend on signaling outputs from the small Rho GTPases, Rac and Rho, but the physiologic importance of these interactions in an animal disease model has not been established. Using a genetic intercross between Nf1/H11001/H11002 and Rac2/H11002/H11002 mice, we now provide genetic evidence to support a biochemical model where hyperactivation of the extracellular signal–regulated kinase (ERK) via the hematopoietic-specific Rho GTPase, Rac2, directly contributes to the hyperproliferation of Nf1-deficient mast cells in vitro and in vivo. Further, we demonstrate that Rac2 functions as mediator of cross-talk between phosphoinositide 3-kinase (PI-3K) and the classical p21ras-Raf-Mek-ERK pathway to confer a distinct proliferative advantage to Nf1-deficient mast cells. Thus, these studies identify Rac2 as a novel mediator of cross-talk between PI-3K and the p21ras-ERK pathway which functions to alter the cellular phenotype of a cell lineage involved in the pathologic complications of a common genetic disease.

Key words: Rac • Pak • PI3-K • cross-talk • neurofibromatosis type 1

Introduction
Mutations in the NF1 tumor suppressor gene cause neurofibromatosis type I (NF1),* a pandemic, autosomal dominant disorder with an incidence of 1 in 3,000. Neurofibromin, the protein encoded by NF1, functions as a GTPase-activating protein (GAP) for p21ras by accelerating the conversion of active p21ras-GTP to inactive p21ras-GDP (1, 2). Cutaneous neurofibromas are slow-growing tumors that are pathognomonic for NF1, and mast cells have been implicated in the pathogenesis of neurofibroma formation as well as other cutaneous tumors (3–7). Recently, we have...
shown that Nf1+/− mice have increased numbers of peritoneal mast cells compared with wild-type littermates (8). In addition, bone marrow–derived mast cells (BMMCs) cultured from Nf1+/− mice demonstrate increased proliferation relative to wild-type cells in response to stem cell factor (SCF), a known mitogen for mast cells (8). However, although these studies show that alterations in p21ras activity can alter mast cell fates in vitro and in vivo, little is known about the contribution of specific p21ras effector pathways to the hyperproliferation of Nf1+/− mast cells or other Nf1-deficient cell types.

Although a critical role for p21ras in controlling cellular proliferation has been clearly established, recent studies have focused on identifying specific p21ras effector pathways important for cell cycle progression. Several studies have suggested that the proliferative effects of p21ras may depend on signaling inputs from the small Rho GTPases, Rac and Rho (9). Like p21ras, the small Rho GTPases exist in an inactive (GDP-bound) and active (GTP-bound) conformation, and interact with target proteins in their GTP-bound state (9). However, although these studies have implicated cross-talk between p21ras and the Rho GTPases in controlling cellular proliferation, the mechanisms underlying these interactions are still relatively poorly defined (9). In addition, the physiological importance of signaling mechanisms that link p21ras and the Rho GTPases and its contribution to disease pathogenesis have not been established.

Previous studies have emphasized that loss of neurofibromin results in hyperactivation of the classical p21ras-Raf-Mek-extracellular signal–regulated kinase (ERK) signaling pathway in Nf1-deficient cells leading to hyperproliferation (10, 11). However, the contribution of Rho GTPases to this signaling cascade and to the Nf1 cellular phenotype have not been previously investigated in primary cells and in an animal disease model. Using Nf1+/− mice and BMMCs, we now provide pharmacologic, genetic, and biochemical data to support a model where hyperactivation of ERK we now provide pharmacologic, genetic, and biochemical data to support a model where hyperactivation of ERK

**Materials and Methods**

**Animals.** Nf1+/− mice were obtained from Tyler Jacks at the Massachusetts Institute of Technology (Cambridge, MA) in a C57BL/6.129 background and backcrossed for 13 generations into the C57BL/6J strain. Rac2−/− mice were backcrossed into a C57BL/6J as described previously (12). These studies were conducted with a protocol approved by the Indiana University Laboratory Animal Research Center. The Nf1 and Rac2 allele were genotyped as described previously (12–14). Multiple F0 founders were used to generate the four Nf1 and Rac2 genotypes used in these experiments as outlined. F0: Nf1+/--; +/+ × +/+; Rac2−/−. F1: Nf1+/--; Rac2+−/− × +/+; Rac2−/−. F2: Nf1+/--; Rac2+−/−, +/+; Rac2−/−, Nf1+/--; +/+ × +/+; +/+.

**Bone Marrow Mast Cell Culture and Proliferation Assays.** Six to nine BMMCs from each murine genotype were generated and used for proliferation and biochemistry assays. All experiments were conducted using at least three lines from each genotype. BMMCs were cultured as described previously (15) with minor modifications and homogeneity of BMMCs was determined by Giemsa staining. Aliquots of cells were also stained with alcian blue and safranin to confirm that they were mast cells. Furthermore, FACS® analysis revealed similar forward and side light scatter characteristics and the same percentage of c-kit expression in BMMCs of all murine genotypes (data not shown). Proliferation assays were performed as described previously (8). BMMCs from each genotype were deprived of growth factors for 24 h, and 3 × 105 cells were plated in 24-well dishes in 1 ml RPMI containing 1% glutamine (BioWhittaker), 1.5% HEPEs (BioWhittaker), 2% penicillin/streptomycin (BioWhittaker), and 25–100 ng/ml recombinant murine SCF (rmSCF; PeproTech) or no growth factors, as indicated, in a 37°C, 5% CO2, humidified incubator. In some experiments, LY294002 (Sigma-Aldrich), PD98059 (New England Biolabs, Inc.), or DMSO (Sigma-Aldrich) was added to cultures 6 h before harvest. In some experiments, LY294002, PD98059, or DMSO was added to the cultures 15 min to 1 h before addition of rmSCF. After 72 h, cells were counted using a hemocytometer. Cell viability was determined by a trypan blue exclusion assay. Assays were performed in triplicate.

**Ras and Rac2 Cooperate in Proliferation of Nf1-deficient Mast Cells**

For thymidine incorporation assays, BMMCs were deprived of growth factors for 24 h and 105 cells were plated in 96-well dishes in 200 μl of RPMI containing 1% glutamine, 10% fetal bovine serum (Hyclone Laboratories), and 25–100 ng/ml of rmSCF or no growth factors, as indicated, in a 37°C, 5% CO2, humidified incubator. In some experiments, LY294002 (Sigma-Aldrich), PD98059, or DMSO was added to the cultures 15 min to 1 h before addition of rmSCF. Cells were harvested on glass fiber filters (Packard Instrument Co.), and β emission was measured. Assays were performed in triplicate.

**Raf-1, Mek, ERK, Pak1, and Akt In Vitro Kinase Assays.** Activation of Raf-1, Mek, ERK, Pak1, and Akt kinases was determined by depriving BMMCs of serum and growth factors for 18–24 h and followed by stimulation with 10–100 ng/ml rmSCF for various amounts of time. Cells were washed twice with PBS containing 1 mM sodium orthovanadate and lysed in nonionic lysis buffer (20 mM Tris/HCl, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, and complete protease inhibitors (Amersham Pharmacia Biotech), as described previously (16). The protein lysates were equalized for protein concentration using the bichoninic acid (BCA) assay (Pierce Chemical Co.), and equal loading of protein in these assays was confirmed by Western blot. ERK and Pak1 kinase immunoprecipitations were carried out with an anti-ERK-2 (C-14) antibody (Santa Cruz Biotechnology, Inc.) and an anti-Pak1(C-19) antibody (Santa Cruz Biotechnology, Inc.), respectively. Raf-1 and Mek kinase immunoprecipitations were carried out with an anti-Raf-1 (C-12) antibody (Santa Cruz Biotechnology, Inc.) and an anti-Mek-1 (C-18) antibody (Santa Cruz Biotechnology, Inc.), respectively. Akt kinase immunoprecipitations were carried out
with an anti-Akt antibody (Cell Signaling Technology). The kinase complex assay was performed as described previously (16) and the following proteins were used as phosphorylation substrates: Elk-1 fusion protein (New England Biolabs, Inc.) for ERK kinase, histone 2B (Roche) for Akt kinase, myelin basic protein (Sigma-Aldrich) for Pak1 kinase, and recombinant Mek1 fused with GST at the NH2 terminus (Upstate Biotechnology) for Raf-1 kinase. For Mek, Raf-1, and ERK, kinase reactions were resolved on 10% Tris-glycine gels (Novex). For Pak1 and Akt, kinase reactions were resolved on 4–20% gradient Tris-glycine gels (Novex), and transferred to polyvinylidine difluoride (PVDF) filters. Gels and PVDF filters were dried and subjected to autoradiography. Densitometry of individual bands was conducted using NIH Image software.

Ras and Rac Activation Assays. BMMCs were deprived of serum and growth factors for 18–24 h and stimulated at various amounts of time with 10–100 ng/ml rmSCF. Ras and Rac activation was subsequently determined using Ras and Rac activation assay kits (Upstate Biotechnology) according to the manufacturer’s protocol and as described previously (17).

Immunoprecipitation and Western Blotting. BMMCs were lysed in nonionic lysis buffer as described previously (16). Cleared lysates were normalized for protein content using the BCA assay (Pierce Chemical Co.) and proteins were immunoprecipitated with protein A sepharose beads (Amersham Pharmacia Biotech) coupled with polyclonal antibodies for Raf-1 and Mek (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were washed three times in lysis buffer, resuspended in sample buffer, boiled for 5 min, subjected to SDS-PAGE, and transferred to nitrocellulose. The membranes were blocked in TBS–Tween containing 5% BSA overnight. Membranes were incubated for 1–3 h at room temperature with the following antibodies: anti-phospho-Mek (Upstate Biotechnology), anti-phospho-ERK (Transduction Laboratories). Proteins were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.).

Studies In Vivo. Adult Nf1+/−/+;+ Rac2−/−, Nf1+/−/−Rac2−/−, and wild-type mice received a continuous infusion of various doses of rmSCF or vehicle (PBS) from microosmotic pumps (Alzet) placed under the dorsal back skin. Osmotic pumps were removed, fixed in buffered formalin, and processed in paraffin-embedded sections. Specimens were stained with hematoxylin and eosin to assess routine histology and with Giemsa to identify mast cells. Cutaneous mast cells were quantitated in a blinded fashion by counting 2-mm² sections in proximity to the India ink stain.

For studies evaluating basal numbers of mast cells in the Nf1 and Rac intercrossed genotypes, 1-cm sections of ears were removed, fixed in buffered formalin, and processed in paraffin-embedded sections. Specimens were stained with hematoxylin and eosin to assess routine histology and Giemsa to identify mast cells. Cutaneous mast cells from four to five mice per experimental group were quantitated.

Results

Nf1+/− Mast Cells Have Increased p21ras Activity and Hyperactivation of Akt after Stimulation with SCF. SCF binding to its receptor, c-kit, causes a rapid increase in p21ras activity in primary BMMCs, and neurofibromin functions as a GAP for p21ras (1, 2, 15). However, it remains unclear whether heterozygosity of Nf1 alters p21ras activity in primary cells. To investigate whether heterozygosity at the Nf1 allele alters p21ras activity in mast cells, Nf1+/− and wild-type BMMCs were stimulated with SCF and assayed for changes in active p21ras-GTP levels. After stimulation of BMMCs, levels of GTP-bound p21ras in cellular lysates were determined by precipitating the active GTPase with a GST fusion of the p21ras binding domain of Raf-1 kinase in an effector pull-down assay. Even though Nf1+/− mast cells had detectable, but reduced, levels of neurofibromin as determined by Western blot (data not shown), Nf1+/− mast cells had higher basal and SCF-stimulated p21ras-GTP levels compared with wild-type cells (Fig. 1 A). Importantly, preincubation of both mast cell genotypes with PI-3K inhibitors, wortmannin or LY294002, did not alter p21ras activity (data not shown). Thus, these data position p21ras either in parallel or upstream of PI-3K in mast cells and demonstrate that heterozygosity at Nf1 alters p21ras activity in primary BMMCs.

Direct binding of the activated c-kit receptor tyrosine kinase to the SH2 domain of the p85α regulatory subunit of type Iα PI-3K is important for kinase activation after stimulation by its ligand, SCF (15). Importantly, in immortalized cell lines p21ras-GTP has previously been shown to bind to specific p110 catalytic subunits of type Iα PI-3K to augment kinase activity (18–20). To determine the effect of increased p21ras-GTP levels on PI-3K activity in Nf1+/− mast cells, we compared the activation of the serine/threonine kinase Akt, also known as Akt, in Nf1+/− and wild-type BMMCs after stimulation with SCF. Activation of Akt is PI-3K dependent and provides a sensitive measure for PI-3K activity (21). Nf1+/− BMMCs demonstrated greater basal and SCF-stimulated Akt activity compared with wild-type cells over a range of SCF concentrations from 10 ng/ml (Fig. 1 B) to 100 ng/ml (data not shown). In addition, inhibitors of PI-3K inhibited Akt activity in both mast cell genotypes (Fig. 1 B), confirming that p21ras contribution to Akt activation is dependent on activation of PI-3K. Taken together, these data demonstrate that increased p21ras activity in Nf1+/− mast cells is biochemically linked to increased activation of the PI-3K-Akt kinase pathway.

Hyperproliferation of Nf1+/− Mast Cells in Response to SCF Is Mediated through Cross-Cascade Activation of ERK from PI-3K. Proliferation of wild-type BMMCs in response to SCF is dependent on PI-3K activation (15). Although we have previously shown that Nf1+/− mast cells have increased proliferation in response to SCF, identification of p21ras effector pathways responsible for this phenotype remains unclear. To test whether SCF-induced hyperproliferation of Nf1+/− BMMCs is mediated through hyperactiva-
Given that PI-3K inhibitors significantly block the proliferation of both mast cell genotypes and Mek inhibitors partially inhibit proliferation, we hypothesized that PI-3K activity is biochemically linked to ERK activation in BMMCs. To test this hypothesis, we stimulated both mast cell genotypes with SCF which is consistent with hyperactivation of p21ras (8). While hyperactivation of the p21ras-PI-3K signaling pathway provides a simple model to account for the phenotype of Nf1+/- BMMCs, we next determined which specific downstream effectors of PI-3K were responsible for the hyperproliferation of Nf1+/- mast cells. Recent studies in immortalized cell lines have shown that signals from PI-3K converge on the p21ras-Raf-Mek-ERK pathway to activate ERK and promote transcription of genes that control mitogenesis (22–25). In addition, we have previously demonstrated that Nf1+/- BMMCs have elevated ERK activity relative to wild-type cells after stimulation with SCF which is consistent with hyperactivation of p21ras (8). To test whether ERK activity contributes to either wild-type or Nf1+/- BMMC proliferation, we performed proliferation assays as described previously with both mast cell genotypes stimulated with SCF or medium alone, in the presence or absence of the Mek inhibitor, PD98059.

The Mek inhibitor completely blocked ERK activation in both mast cell genotypes after stimulation with SCF (data not shown). In multiple independent experiments (n = 6), addition of PD98059 reduced the proliferative response of wild-type BMMCs by ∼10–15% and that of Nf1+/- BMMCs by 40–50% (Fig. 2 A). Importantly, whereas PD98059 caused a minimal reduction in wild-type mast cell proliferation, addition of PD98059 reduced the SCF-induced hyperproliferation of Nf1+/- mast cells to that of wild-type levels, establishing the importance of hyperactivation of ERK to the Nf1+/- proliferative phenotype (Fig. 2 A). Similar results were obtained when mast cell proliferation was assessed using thymidine incorporation assays as described previously with both mast cell genotypes stimulated with SCF or medium alone, in the presence or absence of the PI-3K inhibitor, PD98059.

Figure 1. Analysis of p21ras activity and Akt kinase activity of Nf1+/− and wild-type BMMCs stimulated with SCF. (A) p21ras activity in Nf1+/− and wild-type BMMCs. GTP-bound p21ras levels were determined by incubating cell lysates with Raf-1 p21ras binding domain (RBD) agarose beads, fractionating immunoprecipitates by SDS-PAGE, and probing with anti-p21ras antibody. Immunoblots, quantitative densitometry for p21ras-GTP levels, and Western blots for total p21 ras are shown. Data are representative of five independent experiments. (B) Akt kinase activity in Nf1+/− and wild-type BMMCs. After preincubation with 100 nM wortmannin or DMSO control for 15 min, Akt kinase activity was determined from Akt immunoprecipitates and an in vitro kinase assay was performed using histone 2B as a phosphorylation substrate. Autoradiography and quantitative densitometry of the phosphorylation of histone 2B and Western blots for total Akt are shown. Data are representative of four independent experiments. Similar inhibition of Akt kinase was observed in cells preincubated for 15 min in 25 μM of LY294002, a second PI-3K inhibitor (data not shown).
absence of a Mek or PI-3K inhibitor. (A) Effect of a PI-3K or Mek inhib-

ERK kinase activity of BMMCs in response to SCF in the presence or

Nf1 LY294002 or PD98059 by Student’s paired

SCF. After deprivation of growth factors for 24 h, 3

LY294002 or PD98059; 

Effect of heterozygosity of

Figure 2. Effect of heterozygosity of Nf1 on the proliferation and

ERK kinase activity of BMMCs in response to SCF in the presence or

absence of a Mek or PI-3K inhibitor. (A) Effect of a PI-3K or Mek inhibi-
tor on the proliferation of Nf1+/− wild-type BMMCs in response to

SCF. After deprivation of growth factors for 24 h, 3 × 10^5 cells/ml were

placed in triplicate in 24-well dishes in RPMI containing 1% glutamine,
10% fetal bovine serum, and 100 ng/ml of SCF in the presence or ab-
sence of either 50 μM PD98059 (Mek inhibitor), 10 μM LY294002
(PI-3K inhibitor), or DMSO control in a total volume of 1 ml as de-
scribed previously (8). After 72 h, viable cells were counted using a
hemocytometer and expressed as a percentage of input cells. *P < 0.01
for Nf1+/− vs. Nf1+/+; **P < 0.001 for Nf1+/− vs. Nf1+/+ with
LY294002 or PD98059; ***P < 0.001 for Nf1+/− vs. Nf1+/− with
LY294002 or PD98059 by Student’s paired t test. (B) ERK activity in
Nf1+/− and wild-type BMMCs after stimulation with SCF in the pre-

define or absence of 100 nM wortmannin or DMSO control. ERK activity

was determined by depriving cells of serum for 18–24 h, followed by
stimulation with 10 ng/ml of SCF for 2 and 5 min. In some experiments,
cells were preincubated for 15 min with 100 nM wortmannin, 25 μM
LY294002 (data not shown), or DMSO control before stimulation. Au-

toradiography and quantitative densitometry of the phosphorylation of
Elk-1 fusion protein by ERK kinase from lysates obtained from SCF-
stimulated BMMCs and Western blots for total ERK are shown. Data are
representative of five independent experiments.

Cross-Cascade Activation of ERK from PI-3K Occurs at the
Level of Mek and Raf-1 Kinase in Nf1+/− and Wild-Type
Mast Cells. In other experimental systems, cross-cascade
activation of ERK from PI-3K occurs at the level of either
Mek or Raf-1 kinase (22–26). To test whether cross-talk
between PI-3K and the p21ras-Raf-Mek-ERK pathway oc-
curs at the level of Mek, we stimulated Nf1+/− and wild-
type BMMCs with SCF in the presence or absence of a PI-
3K inhibitor and measured Mek activity. Nf1+/− BMMCs
demonstrated a greater increase in Mek activity from base-
line compared with wild-type cells, and Mek activity was
inhibited in the presence of PI-3K inhibitors in both mast
cell genotypes (Fig. 3 A).

Since Raf-1 functions as the immediate upstream effec-
tor of Mek by phosphorylating Mek Ser217/221 (27), we
stimulated Nf1+/− and wild-type mast cells with SCF in
the presence or absence of a PI-3K inhibitor and measured
Raf-1 kinase activity and phosphorylation of these residues
by immunoblot. Whereas wild-type BMMCs demon-
strated minimal Raf-1 kinase activation after stimulation
with 10 ng/ml of SCF, Nf1+/− BMMCs had a significant
increase in Raf-1 kinase activity from baseline in multiple
independent experiments (n = 5) which was completely
inhibited in the presence of the PI-3K inhibitor (Fig. 3 B).
In addition, Nf1+/− BMMCs had increased phosphoryla-
tion of Mek Ser217/221 which correlates with increased
Raf-1 activity in these cells (Fig. 3 C). Importantly, phos-
phorylation of these residues was also inhibited by a PI-3K
inhibitor in both mast cell genotypes (Fig. 3 C).

Taken together, as PI-3K inhibitors do not alter p21ras
activity (data not shown), these data suggest a biochemical
model where PI-3K activation of ERK occurs at the level
of either Raf-1 alone or at the level of both Raf-1 and Mek
in BMMCs after stimulation with SCF. Importantly,
increased Raf-1 and Mek activity observed in Nf1+/− mast
cells is consistent with the hypothesis that the hyperprolif-
erative phenotype of Nf1+/− BMMCs is directly related to
increased cross-talk from PI-3K to the p21ras-Raf-Mek-
ERK pathway.

Heterozygosity of Nf1 Results in Higher Basal and SCF-stimu-
ulated Rac and Pak1 Activity in BMMCs. Given the
importance of cross-talk between the PI-3K and the p21ras-
Raf-Mek-ERK pathways to the hyperproliferation of
Nf1+/− BMMCs, we performed experiments to identify
proteins responsible for cross-talk between the two path-
ways. Studies in hematopoietic cells have positioned the
small Rho GTPase, Rac, downstream of PI-3K, and some
experiments in immortalized cell lines have suggested that
Rac1 may function as a mediator of cross-talk between PI-
3K and the p21ras-Raf-Mek-ERK pathways (23, 28–32).
Given these prior experiments and our current observa-
Ras and Rac2 Cooperate in Proliferation of Nf1-deficient Mast Cells

...tions, we measured total Rac activity in both mast cell genotypes in response to SCF. After stimulation of BMMCs, levels of active, GTP-bound Rac in cellular lysates were determined by precipitating the active GTPase with a GST fusion of p21 activated kinase in an effector pull-down assay. Nf1/H11001/H11002 mast cells had higher basal and SCF-stimulated Rac-GTP levels compared with wild-type cells, providing evidence that p21ras is biochemically linked to Rac activation. (Fig. 4 A).

Pak1 is a well characterized downstream effector of Rac, and the catalytic activity of Pak1 is regulated in part by binding of Rac-GTP to the highly conserved NH2 terminus, known as the Cdc42/Rac interactive binding (CRIB) domain (35, 36). Importantly, Pak1 has been shown to mediate cross-talk between PI-3K and the p21ras-Raf-Mek-ERK pathway in immortalized cell lines by phosphorylating specific Raf-1 and Mek residues (22, 25). To test whether heterozygosity of Nf1 also altered Pak1 activity in BMMCs, we measured Pak1 activity in both mast cell genotypes in response to SCF. Whereas wild-type mast cells displayed minimal Pak1 activation, Nf1/H11001/H11002 BMMCs had dramatically increased basal Pak1 activity which increased modestly after stimulation with SCF, which is consistent with increased Rac activity (Fig. 4 B). Thus, these studies suggest that hyperactivation of the Rac and potentially the Pak1 pathway confer a distinct proliferative advantage to Nf1/H11001/H11002 BMMCs.

Hyperactivation of Rac2 Directly Contributes to the Hyperproliferation and Increased ERK Activity in Nf1/H11001/H11002 BMMCs in Response to SCF. Rac2 is a hematopoietic specific Rho GTPase, and Rac2/H11002 mice have multiple mast cell defects secondary to diminished Rac activity (12, 34). Importantly, other studies have recently positioned different isoforms of Rac downstream of PI3-K in hematopoietic cells (for a review, see reference 37). To test our hypothesis that hyperactivation of Rac2 pathway is directly responsible for the...
hyperproliferation of \textit{Nf1} \textit{H11001/H11002} BMMCs and to potentially identify which Rac protein may be involved in this signaling pathway, we intercrossed \textit{Rac2} \textit{H11002/H11002} and \textit{Nf1} \textit{H11001/H11002} mice, established BMMCs from the four F2 progeny (see Materials and Methods), and performed proliferation, Pak1, and ERK activity assays. To initially determine whether Pak1 was located downstream of Rac2 in \textit{Nf1} \textit{H11001/H11002} and wild-type mast cells, BMMCs harvested from the mice of the four F2 genotypes were stimulated with SCF and Pak1 activity was measured. Consistent with our initial observations, \textit{Nf1} \textit{H11001/H11002} Rac2 \textit{H11001/H11001} mast cells had higher basal and SCF induced Pak1 activity compared with wild-type cells (Fig. 5 A). However, \textit{Nf1} \textit{H11001/H11002} Rac2 \textit{H11002/H11002} and \textit{Nf1} \textit{H11001/H11002} Rac2 \textit{H11002/H11002} BMMCs had minimal Pak1 activation at baseline and in response to SCF (Fig. 5 A). Thus, these studies identify Pak1 as a downstream effector of Rac2 in both mast cell genotypes.

To assess the role of Rac2 activation on the proliferation of \textit{Nf1} \textit{H11001/H11002} mast cells, BMMCs harvested from mice of the four F2 genotypes were cultured in 10% fetal calf serum without added growth factors for 24 h before addition of SCF. Cell numbers were determined at the time SCF was added (day 0) and after 72 h in culture. As shown previously (8, 34), \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} BMMCs showed a greater proliferative response to SCF after 72 h in culture compared with wild-type cells, whereas \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} cells showed a slight decrease in proliferation (Fig. 5 B). However, \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} BMMCs had a 40–50% reduction in proliferation compared with \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} mast cells. Importantly, the proliferative responses of \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} BMMCs were similar to wild-type cells in multiple independent experiments (n = 6; Fig. 5 B). Thus, these experiments provide direct genetic evidence that the hyperproliferative phenotype of \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} mast cells is mediated through activation of the specific Rac2 isoform.

The reduction in proliferation of \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} BMMCs relative to \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} cells was similar to the reduction in proliferation observed with the addition of a Mek inhibitor (and subsequent inhibition of ERK activity) to the cultures of \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} mast cells. Given the similar results of these two experiments, we hypothesized that Rac2 was biochemically linked to ERK activation after stimulation of mast cells with SCF. To test this hypothesis, we compared ERK activity in the four F2 mast cell genotypes after stimulation with SCF. Consistent with prior observations, \textit{Nf1} \textit{H11001/Rac2} \textit{H11002} mast cells had higher...
ERK activity compared with wild-type cells (Fig. 5 C). However, \(Nf1^{+/+}Rac2^{-/-}\) BMBCMs had a 50% reduction in ERK activity compared with wild-type cells, providing genetic evidence that Rac2 is directly linked to ERK activation (Fig. 5 C). Consistent with the hypothesized role of hyperactivation of ERK to the proliferative phenotype of \(Nf1^{+/+}Rac2^{+/+}\) mast cells, \(Nf1^{+/+}Rac2^{-/-}\) mast cells had equivalent levels of ERK activity compared with wild-type cells in response to SCF (Fig. 5 C). Thus, these data provide further genetic confirmation that Rac2-mediated hyperactivation of ERK directly contributes to the hyperproliferation of \(Nf1^{+/+}\) mast cells in response to SCF.

Increased Accumulation of Mast Cells in \(Nf1^{+/+}\) Mice in Response to Local Cutaneous Administration of SCF Is Mediated through Rac2 In Vivo. Local cutaneous injection of SCF into wild-type mice results in a dramatic accumulation of mast cells at the site of injection (38, 39). Using 5-bromo-2′-deoxyuridine (BrdU) labeling in vivo, Tsi et al. demonstrated that accumulation of mast cells in this experimental model was closely linked to local cellular proliferation (39).

To test whether heterozygosity at \(Nf1\) alters accumulation of mast cells to SCF in vivo, slow release microosmotic pumps were filled with a range of concentrations of SCF or vehicle and placed into the dorsal back skin of either wild-type or \(Nf1^{--}\) mice. 7 d after placement, pumps were re-filled with either PD98059 (Mek inhibitor), LY294002 (PI-3K inhibitor), or DMSO control in a total volume of 1 ml as described previously. After 72 h, viable cells were counted using a hemocytometer and expressed as a percentage of input cells. *P < 0.01 for \(+/+\) vs. \(+/-\); \(Rac2^{-/-}\); \(+/-\) vs. \(+/+\); \(Rac2^{-/-}\) by Student’s paired t test. (C) Analysis of ERK activity in BMBCMs stimulated with SCF in the four F2 genotypes. ERK activity was determined after depriving cells of serum for 18–24 h, followed by stimulation with SCF for 2 min. Autoradiography and quantitative densitometry of the phosphorylation of myelin basic protein by Pak1 kinase from lysates obtained from SCF-stimulated BMBCMs and Western blots for total Pak1 are shown. Data are representative of five independent experiments.
type and $Nf1^{+/+}$ mice was observed at this dose (Fig. 6 C). Consistent with our in vitro proliferation results, the accumulation of mast cells in $Nf1^{+/+}$-$Rac2^{-/-}$ mice was greatly reduced compared with $Nf1^{+/+}$-$Rac2^{+/+}$ mice (Fig. 6 D). Importantly, no differences in mast cell accumulation were observed between wild-type and $Nf1^{+/+}$-$Rac2^{-/-}$ mice (Fig. 6 D). To determine whether loss of Rac2 alters endogenous cutaneous mast cell numbers in $Nf1^{+/+}$ mice, histologic sections from the ears of the 4 F2 genotypes were evaluated after giemsa staining ($n = 4–5$ mice/group). Cutaneous sections from the ear have been found in previous studies to be a sensitive site to detect genotypic differ-
Discussion

Although p21ras signaling outputs were originally described as operating in parallel with activation of PI-3K pathways, recent studies have suggested that receptor tyrosine kinase–mediated activation of ERK is potentially PI-3K dependent (9, 37). However, the majority of these studies were conducted in immortalized cell lines, and a clear example of the physiologic relevance of cross-talk between the p21ras-ERK and PI-3K pathways in an animal disease model has not been established previously. Multiple lineages of neurofibromin-deficient cells have a propensity to hyperproliferate in response to various growth factors which directly relates to the wide diversity of clinical manifestations observed in NF1 (41). Although neurofibromin has been shown to function as a GAP for p21ras to control cellular proliferation, lack of identification of alterations in specific p21ras effectors which control mitogenesis has limited our understanding of disease pathogenesis. In these experiments, we show that the hyperproliferation of Nf1+/− mast cells is directly related to increased cross-talk between the PI-3K and the p21ras-Raf-ERK pathway and identify Rac2 as a principal mediator of this aberrant signaling pathway (Fig. 7).

Numerous studies have shown that active p21ras-GTP can bind to multiple isoforms of the p110 catalytic subunits of type IA PI-3K to specifically augment kinase activity (18–20). However, in neurofibromin-deficient cells, most attention has been focused on modulation of the classical p21ras-Raf-Mek-ERK pathway to augment cellular proliferation. In primary mast cells, activation of PI-3K after c-kit binding to SCF is critical for controlling proliferation (15, 33). Our study shows that Nf1+/− mast cells have a twofold increase in proliferation in response to SCF compared with wild-type cells, and proliferation was completely reduced with PI-3K inhibitors, confirming the importance of PI-3K activation to the Nf1+/− cellular phenotype. Importantly, placement of cutaneous microsomatic pumps which released a continuous infusion of SCF resulted in a greater accumulation of mast cells at the site of insertion in Nf1+/− mice compared with wild-type mice. Others have previously shown that in vivo mast cell expansion in response to local injection of SCF occurs secondary to local proliferation (38, 39). Thus, our in vivo observations argue that the biochemical mechanisms identified in vitro for the Nf1+/− cell phenotype are valid and biologically operative in a more physiologic experimental system.

Figure 7. Schematic representation of the hypothesized biochemical pathway responsible for the hyperproliferative phenotype of Nf1+/− mast cells in response to SCF. Hatched arrows represent potential downstream effectors of Rac2.

Table I. Effects of Nf1 and Rac2 Genotypes on Cutaneous Mast Cell Numbers

| Genotype | Number of mast cells per mm² |
|----------|-----------------------------|
| +/+; +/+ | 69 ± 4.43                   |
| Nf1+/−; +/+ | 98 ± 10.32*                 |
| +/+; Rac2−/− | 63 ± 2.94b                  |
| Nf1+/−; Rac2−/− | 63 ± 4.96b                  |

Numbers of cutaneous mast cells were quantitated from ear biopsies. Results represent mean numbers of mast cells ± SEM from five animals in each genotype.

*P < 0.05 for comparison of Nf1+/−; +/+ vs. +/+; +/+.

**P < 0.01 for comparison of Nf1+/−; +/+ vs. +/+; Rac2−/− and Nf1+/−; Rac2−/− by Student’s t test.
SCF-stimulated proliferation of these cells. A Mek inhibitor reduces the proliferation of wild-type cells modestly (10–15%) in our studies. However, preincubation of Nf1+/− mast cells with a Mek inhibitor reduces proliferation much more significantly (40–50%), and importantly, diminishes the proliferative response to wild-type levels. Thus, in contrast to wild-type cells, hyperactivation of ERK from PI-3K provides a unique signal to confer a distinct proliferative advantage to Nf1+/− cells.

One interesting observation from these studies is that constitutive activation of multiple upstream effectors of ERK including p21ras and Rac does not consistently lead to constitutive activation of ERK. One potential explanation for this observation is that mitogen-activated protein kinase phosphatase (MKP), a phosphatase that specifically binds and dephosphorylates ERK, may be elevated in Nf1+/− cells (42, 43). Recently, Brondello et al. have argued that activation of ERK regulates MKP-1 protein expression through both an increase in the rate of transcription and in the rate of proteasome-mediated degradation of MKP-1 (42, 43). Further, these authors argue that upregulation of MKP-1 in response to activation of ERK may provide an important negative feedback mechanism to limit inappropriate or constitutive activation of ERK. Given the importance of ERK activity in regulating proliferation and potentially other functions in Nf1+/− mast cells, investigation of the basal activity of this phosphatase could be important in future studies. In addition, the regulation of ERK activity in vivo is likely to be far more complex. It also remains possible that constitutive activation of upstream effectors of ERK may circumvent normal feedback mechanisms and provide a subthreshold level for activation of signaling pathways necessary for proliferation. In this context, it is intriguing to note that administration of low dosages (2 μg/kg/day) of exogenous recombinant SCF to Nf1+/− mice results in profound increases in mast cell numbers while having no significant effect on wild-type controls.

Next, given the importance of the hyperactivation of ERK to the proliferative phenotype of Nf1+/− mast cells, we focused on identifying potential mediators of cross-talk between the PI-3K and p21ras-Raf-Mek-ERK pathways. Recent studies have suggested that the proliferative effects of p21ras are modulated at least in part through signaling inputs from the small Rho GTPase, Rac (9). p21-activated kinase1 (Pak1) has emerged as a molecule that can link p21ras and Rac signaling by converging on the p21ras-Raf-Mek-ERK pathway (22, 23, 25). ERK activation has been linked to the induction of cyclin D1 expression and progression through G1 of the cell cycle (44, 45). Using immortalized cell lines, Pak1 has been shown to increase signaling through ERK activation by phosphorylating specific residues on Raf-1 and Mek (22, 24, 25). Specifically, activated Pak1 can phosphorylate Mek1 at Ser298 to enhance the stable association between Raf-1 and Mek1 (22), and Pak1 can also phosphorylate Ser338 in the Raf-1 catalytic domain to enhance Raf-1 kinase activity (25). In these prior studies the net effect of increased activation of Mek and Raf-1 by Pak1 was increased ERK activation in response to different stimuli. Our data suggest that similar pathways are hyperactive in Nf1-deficient mast cells and function to increase ERK activity and proliferation in these cells. Pak1 activity and phosphorylation levels of Ser338 on Raf-1 and Ser298 on Mek1 (data not shown) in response to SCF stimulation were greatly increased in Nf1+/− mast cells compared with wild-type cells. Though our studies cannot eliminate the possibility that other effectors may contribute to cross-talk between PI-3K and the p21ras-Raf-Mek-ERK pathway, our data strongly suggest that hyperactivation of the Rac2 pathway directly accounts for the hyperproliferation of mast cells (Fig. 7). The elevated levels of Pak1 activity, as well as studies where expression of a dominant negative Pak1 cDNA reduced ERK activity in Nf1+/− mast cells to wild-type levels (unpublished results), suggest that Pak1 may serve as the effector molecule of Rac2 to enhance ERK activity in Nf1+/− mast cells. These findings are especially intriguing, as placement of a dominant negative Pak1 or a dominant negative Rac1 has been shown to reverse the transformation of a neurofibrosarcoma cell line taken from an individual with NF1 (28). However, as dominant negative constructs may potentially bind other upstream effectors of Pak1, future studies using Pak1 knockout mice will allow direct testing of this hypothesis. Given that different isoforms of Rac and Pak alter the biology of different neurofibromin-deficient cell lineages, further characterization of these pathways in Nf1+/− and Nf1+/− primary cells could provide insight into...
the early events leading to loss of heterozygosity and the subsequent malignant manifestations in NF1.

In summary, recent studies have emphasized an increasing appreciation of the complexity of interactions between signaling pathways which control specific cellular responses. Our data provide a clear example in an animal disease model of how aberrant signaling between two major pathways can specifically alter the phenotype of a cell lineage implicated in the pathogenesis of a common genetic disease. Further characterization of this pathway and identification of similar signaling mechanisms in other neurofibromin-deficient cells should provide biochemical insight into the complex pathogenesis of NF1.

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