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Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation

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The receptor tyrosine kinase Kit plays critical roles in hematopoiesis, gametogenesis and melanogenesis. In mast cells, Kit receptor activation mediates several cellular responses including cell proliferation and suppression of apoptosis induced by growth factor deprivation and γ-irradiation. Kit receptor functions are mediated by kinase activation, receptor autophosphorylation and association with various signaling molecules. We have investigated the role of phosphatidylinositol 3′-kinase (PI 3-kinase) and Src kinases in Kit-mediated cell proliferation and suppression of apoptosis induced both by factor deprivation and irradiation in bone marrow-derived mast cells (BMMC). Analysis of Kit$^{-/-}$ BMMC expressing mutant Kit receptors and the use of pharmacological inhibitors revealed that both signaling pathways contribute to these Kit-mediated responses and that elimination of both pathways abolishes them. We demonstrate that the PI 3-kinase and Src kinase signaling pathways converge to activate Rac1 and JNK. Analysis of BMMC expressing wild-type and dominant-negative mutant forms of Rac1 and JNK revealed that the Rac1/JNK pathway is critical for Kit ligand (KL)-induced proliferation of mast cells but not for suppression of apoptosis. In addition, KL was shown to inhibit sustained activation of JNK induced by γ-irradiation and concomitant irradiation-induced apoptosis.

Keywords: JNK/Kit/phosphatidylinositol 3′-kinase/Rac1/Src kinase

Introduction

The c-*kit* proto-oncogene encodes a growth factor receptor with ligand-dependent tyrosine kinase activity, which is structurally related to the colony-stimulating factor-1 (CSF-1) and platelet-derived growth factor (PDGF) receptors (Besmer *et al*., 1986; Yarden *et al*., 1987; Qiu *et al*., 1988). Common features of this receptor tyrosine kinase subfamily are an extracellular domain with five immunoglobulin-like domains and a cytoplasmic domain consisting of a kinase domain which is interrupted by an insert of variable length, a juxtamembrane domain and a C-terminal domain. The Kit receptor and its ligand, Kit ligand (KL) or stem cell factor (SCF), are encoded at the murine *White Spotting* (*W*) and *Steel* (*Sl*) loci, respectively (Chabot *et al*., 1988; Geissler *et al*., 1988; Besmer, 1991). The phenotypes of mutations at the *W* locus imply essential functions of Kit in gametogenesis, melanogenesis and hematopoiesis in the developing and adult animal. The mutant phenotypes in the hematopoietic system include macrocytic anemia and lack of tissue mast cells. Mast cells are an important model for the study of Kit functions (Besmer, 1997). Mast cell progenitors in the bone marrow can be induced by interleukin-3 (IL-3) or KL to proliferate and differentiate into bone marrow-derived mast cells (BMMC). In BMMC, activation of the Kit receptor stimulates diverse cellular responses including proliferation (Nocka *et al*., 1990), survival (Yee *et al*., 1994), differentiation (Tsai *et al*., 1991), chemotaxis (Meininger *et al*., 1992), adhesion to a fibronectin matrix (Dastych and Metcalfe, 1994; Kinashi and Springer, 1994) and enhancement of serotonin and histamine release (Columbo *et al*., 1992; Ziegler *et al*., 1993). Mast cells from mice with Kit receptor mutations are defective in these responses.

KL binding to the Kit receptor mediates receptor dimerization, activation of its intrinsic tyrosine kinase activity and autophosphorylation (Besmer, 1997). The activated receptor then phosphorylates various substrates and associates with signaling molecules, thereby activating distinct signaling cascades. Molecules known to associate with the Kit receptor *in vivo* include the p85 subunit of phosphatidylinositol 3′-kinase (PI 3-kinase), phospholipase Cγ-1 (Reith *et al*., 1991; Rottapel *et al*., 1991), the Grb2 adaptor protein, the Src kinase (Blume-Jensen *et al*., 1994) and the tyrosine phosphatases SHP1 and SHP2 (Yi and Ihle, 1993). In addition, Kit receptor activation causes phosphorylation and activation of the Shc adaptor protein (Cutler *et al*., 1993), Ras (Duronio *et al*., 1992) and the Vav GDP/GTP exchange factor (Alai *et al*., 1992).

KL stimulation is important for the survival and proliferation of several cell types in the hematopoietic system, where it may function in combination with other growth factors. In mast cells, KL alone is sufficient to mediate proliferation in serum-free conditions (Yee *et al*., 1994) and to suppress apoptosis induced by growth factor deprivation and irradiation, in a dose-dependent manner (Yee *et al*., 1994). However, the molecular mechanisms by which KL and its receptor Kit mediate mitogenic and anti-apoptotic responses are still poorly defined. Recently, PI 3-kinase and its downstream effector protein kinase AKT (or PKB) were implicated as important mediators of survival of neuronal cells or fibroblasts (Dudek *et al*., 1997; Kennedy *et al*., 1997; Kulik *et al*., 1997). However, the significance of other downstream effectors of growth factor receptors, such as Ras and Src, in cell survival is contradictory. The Ras–MAPK pathway has been identified as an important signaling pathway for mitogenic responses to many growth factors (Marshall, 1995) and it

has also been suggested to play a role in the survival of cells (Xia *et al*., 1995). An activated form of Src was shown to be protective in fibroblasts exposed to UV irradiation (Kulik *et al*., 1997) but was not sufficient to mediate survival upon serum withdrawal (Kennedy *et al*., 1997). In contrast, in an avian B-cell line, the Src-like kinase Lyn mediates osmotic stress- and UV-C irradiation-induced apoptosis (Qin *et al*., 1997).

Investigations of receptor-mediated signaling mechanisms most often are studied in cell lines; however, cell lines may harbor mutations in tumor suppressor and/or other genes functioning in cell survival processes and this might complicate the interpretation of results. BMMC provide an excellent primary cell system to investigate the mechanisms by which KL mediates cell proliferation, suppression of apoptosis, cell adhesion and secretory enhancement. Introduction of normal Kit receptor into $Kit^{-/-}$ W^{sh}/W^{sh} BMMC restores KL-induced proliferation, survival and adhesion to fibronectin, as well as activation of PI 3-kinase, Ras and mitogen-activated protein kinase (MAPK) (Serve *et al*., 1995). Tyr719 is the only PI 3-kinase-binding site in the Kit receptor (Serve *et al*., 1994). Elimination of the PI 3-kinase-binding site in Kit by substitution of Tyr719 with phenylalanine impairs Kit-mediated adhesion, reduces the rate of proliferation (Serve *et al*., 1995) and abolishes Kit-mediated secretion (Vosseller *et al*., 1997). The activated Kit receptor also binds and phosphorylates Src (Blume-Jensen *et al*., 1994), but the residues mediating this interaction have not yet been established. In the structurally related PDGF β chain receptor (PDGFRβ), Src family tyrosine kinases $p60^{c\text{-}src}$, $p59^{fyn}$ and $p62^{c\text{-}yes}$ (Kypta *et al*., 1990) bind via tyrosines in the juxtamembrane domain, and a recent peptide binding study implies that the Fyn kinase associates with the phosphotyrosines 568 and 570 in the juxtamembrane domain of the human Kit receptor (Price *et al*., 1997).

In this report, we have studied the contributions of PI 3-kinase and Src kinase signaling pathways to the KL-mediated proliferative and cell survival responses in mast cells. Mutant Kit receptors lacking PI 3-kinase- and/or Src kinase-binding sites were expressed in $Kit^{-/-}$ BMMC isolated from W^{sh}/W^{sh} mice. We show that Tyr567 of the murine Kit receptor serves as a docking site for Fyn *in vivo*. By using PI 3-kinase- and Src kinase-binding site mutations in the Kit receptor and pharmacological inhibitors, we provide evidence that in mast cells both the PI 3-kinase and Src kinase signaling pathways contribute to Kit-mediated survival and proliferation, but that neither pathway alone is sufficient. Elimination of both pathways completely abolished Kit-mediated suppression of apoptosis and induction of proliferation. The PI 3-kinase and the Src kinase signaling pathways are shown to converge to activate the small G-protein Rac and protein kinase JNK (c-jun N-terminal kinase), and evidence for a critical role for Rac1 and JNK in Kit-mediated proliferation of mast cells is provided.

Results

Kit binds to the Src family kinase Fyn via Tyr567

Tyrosines 567 and 569 in the juxtamembrane domain of Kit are consensus sites for binding of Src family kinases, and they are homologous to tyrosines 579 and 581 of PDGFRβ (Mori *et al*., 1993). Myeloid 32D cells lack expression of endogenous Kit (Hu *et al*., 1995), but express Src kinase and the Src-related kinases Fyn and Lyn, which are expressed in the myeloid cells including mast cells (Lowell and Soriano, 1996). To determine whether Kit binds Src family kinases through residues 567 or 569, we expressed mutant Kit receptors Kit^{Y567F}, Kit^{Y569F} and $Kit^{Y567/569F}$ in 32D cells by retroviral infection. Binding of wild-type and mutant Kit receptors to the SH2 domain of Fyn was measured by co-immunoprecipitation from cell lysates (Figure 1A). Cells were stimulated with KL for 5 min, lysed and incubated with a fusion protein consisting of the Fyn SH2 domain fused to glutathione-*S*-transferase (GST) conjugated to agarose beads. Proteins bound to GST–Fyn SH2 were fractionated by SDS–PAGE and immunoblotted with anti-Kit antibody. The Kit^{Y569F} receptor bound to the Fyn SH2 domain with affinity similar to wild-type Kit, whereas Kit^{Y567F} and $Kit^{Y567/569F}$ did not co-precipitate with GST–Fyn SH2 (Figure 1A). An immunoblot of whole-cell lysates showed that all Kit receptor constructs were expressed at comparable levels (Figure 1A). Therefore, similarly to other receptor systems, it appears that Tyr567 of the Kit receptor serves as a docking site for the Src-like kinase Fyn. Whether other Src family members can also bind to tyrosines 567 or 569, or whether Tyr569 serves as a docking site for other molecule(s), is currently under investigation.

Mutant Kit receptors are expressed and autophosphorylated in mast cells

To study the contribution of PI 3-kinase and Src family kinase signaling pathways to Kit-mediated survival and proliferation, wild-type (Kit^{WT}) or mutant Kit receptors (Kit^{Y567F}, Kit^{Y719F} and $Kit^{Y567,719F}$) were stably expressed in $Kit^{-/-}$ BMMC using retroviral vector as a vehicle. BMMC lacking endogenous Kit expression were derived from the W^{sh}/W^{sh} mice (Serve *et al*., 1995). Kit expression at the cell surface was determined by fluorescence-activated cell sorting (FACS) analysis using an anti-Kit monoclonal antibody coupled to fluorescein isothiocyanate (FITC). The expression levels of Kit in infected cells were comparable with that of endogenous Kit in wild-type BMMC (Figure 1B). To characterize the autophosphorylation of the mutant Kit receptors upon stimulation with KL *in vivo*, Kit proteins were immunoprecipitated, fractionated by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibody. All mutant Kit receptors were autophosphorylated in response to KL in mast cells. However, tyrosine phosphorylation levels of the single mutant Kit receptors, Kit^{Y567F} and Kit^{Y719F}, and especially of the double mutant $Kit^{Y567/719F}$ receptor were somewhat reduced compared with that of wild-type Kit (Figure 1C). Despite the somewhat reduced autophosphorylation of Kit^{Y567F} receptor, its ability to bind the p85 subunit of PI 3-kinase was not affected, as demonstrated by co-precipitation of p85 with the activated mutant Kit receptors (Figure 1C).

To confirm the observation made in 32D cells that Kit binds to the Fyn kinase via Tyr567, we investigated the activation of the Fyn kinase in BMMC expressing mutant Kit receptors. Upon stimulation with KL, cells were lysed, Fyn proteins were immunoprecipitated and Fyn kinase activity was determined in an *in vitro* kinase assay using

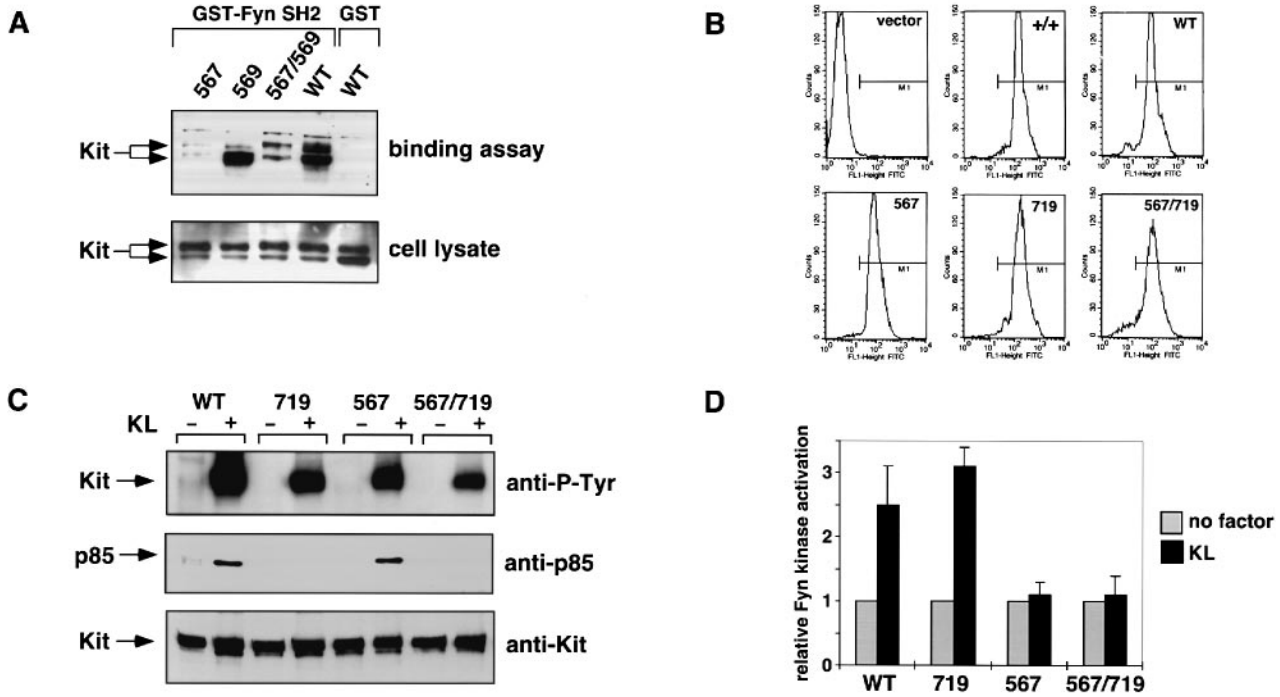

Fig. 1. Biochemical characterization of Kit receptor mutants. (**A**) Association of the Fyn SH2 domain with Kit. 32D cell transfectants expressing the Kit^{WT}, Kit^{567}, Kit^{569} and $Kit^{567/569}$ receptors were serum starved for 12 h and stimulated with KL for 10 min. Cell lysates were prepared and incubated with GST–Fyn SH2 agarose conjugates and blotted with anti-Kit antibody (upper panel). Kit protein levels in the whole cell lysates are shown in the lower panel. (**B**) Cell surface Kit expression in +/+ BMMC and W^{sh}/W^{sh} BMMC infected with the pGD Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ expression vectors and vector alone was determined using FITC-coupled anti-Kit antibody and analysis by FACS. (**C**) Phosphorylation of mutant Kit receptors in response to KL and association with the p85 subunit of PI 3-kinase. Cells were starved of growth factors for 12 h and then stimulated with KL for 5 min. Upper panel: Kit proteins were immunoprecipitated, fractionated by SDS–PAGE and blotted with anti-phosphotyrosine antibody. Middle panel: membranes were reblotted with the anti-p85 antibody. Lower panel: Kit protein levels are shown. (**D**) Activation of Fyn kinase by Kit receptor mutants. Cells were starved for 12 h in serum-free medium and then stimulated with KL for 5 min. *In vitro* kinase assay of Fyn was performed using a $p34^{cdc2}$-derived peptide as a substrate. The results represent the average of duplicates. Similar results were obtained in two separate experiments.

$p34^{cdc2}$-derived peptide as a substrate (Cheng *et al*., 1992) (Figure 1D). Wild-type Kit as well as Kit^{Y719F} activated Fyn kinase activity 2.5- to 3-fold, but both Kit^{Y567F} and $Kit^{Y567/719F}$ failed to activate Fyn, in agreement with the hypothesis that Kit binds and activates Src-like kinases via juxtamembrane Tyr567.

PI 3-kinase- and Src-binding site mutations (Y719F and Y567F) affect Kit-mediated proliferation and suppression of apoptosis

To determine whether PI 3-kinase and Src kinases are important mediators of Kit-induced mitogenesis in mast cells, we measured DNA synthesis in BMMC expressing wild-type or mutant Kit receptors incubated with KL, IL-3 (as an internal control for proliferation) or medium alone 24 h after factor addition. [^{3}H]Thymidine was added for 4 h and [^{3}H]thymidine incorporation was subsequently determined. W^{sh}/W^{sh} BMMC stably transduced with the wild-type Kit receptor showed a proliferative response similar to BMMC expressing endogenous Kit receptor (Figure 2A). Tyrosine to phenylalanine substitution at the Src-binding site (Kit^{Y567F}) and to a lesser extent at the PI 3-kinase-binding site (Kit^{Y719F}) reduced DNA synthesis in response to KL, whereas the double mutation ($Kit^{Y567,719F}$) completely abrogated it (Figure 2A). Analysis of cell-cycle progression of BMMC expressing mutant Kit receptors, performed by FACS after staining with propidium iodide, confirmed this notion (data not shown).

KL is an important survival factor for mast cells due to its ability to protect mast cells from irradiation- and growth factor deprivation-induced apoptosis. We were interested in determining whether PI 3-kinase and Src kinase activation play a role in Kit-mediated mast cell survival. To this end, we analyzed Kit-mediated survival of BMMC by measuring the extent of apoptosis, using a specific marker of apoptosis, AnnexinV.

First we determined whether KL-induced protection from growth factor deprivation-induced apoptosis of mast cells is affected by the three Kit receptor mutations, Kit^{Y719F}, Kit^{Y567F} and $Kit^{Y567,719F}$. Cells expressing wild-type or mutant Kit receptors were pre-incubated for 12 h in serum-free medium containing IL-3, starved of IL-3 for 1 h and subsequently incubated for 50 h in medium with and without addition of IL-3 or KL. Cells were harvested, stained with AnnexinV and scored for the percentage of apoptotic cells using FACS analysis. BMMC expressing Kit^{Y719F} or Kit^{Y567F} were partially defective in their ability to survive in the presence of KL, while cells expressing $Kit^{Y567,719F}$ receptors completely lacked responsiveness to KL as a survival factor (Figure 2B).

To investigate the effects of the Kit^{Y719F} and Kit^{Y567F} mutations on irradiation-induced apoptosis, cells were pre-

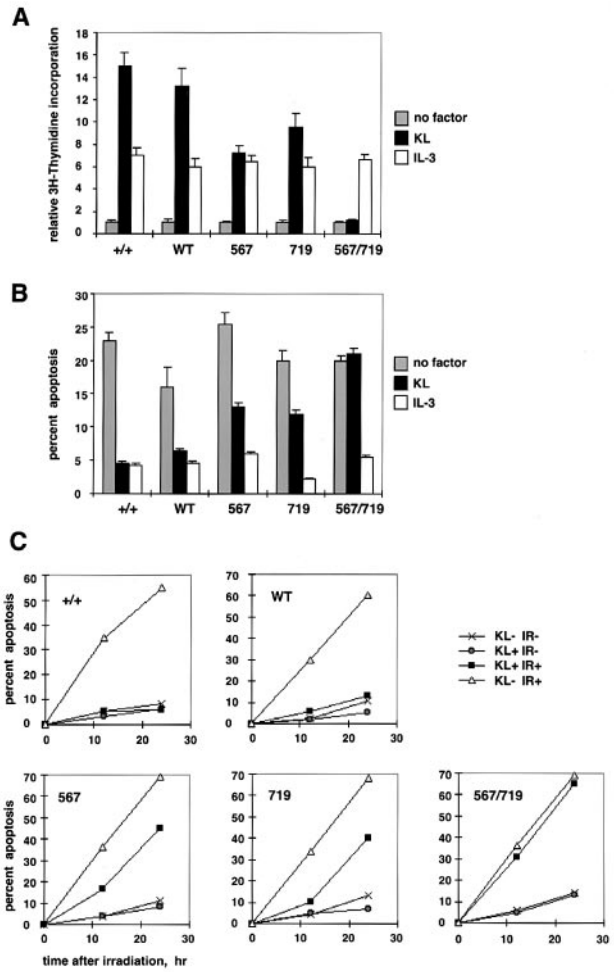

Fig. 2. Effect of Kit receptor mutations on DNA synthesis and suppression of apoptosis. (**A**) BMMC expressing Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ were stimulated with KL (200 ng/ml), IL-3 (20 ng/ml) or medium alone for 20 h; [^{3}H]thymidine (2.5 μCi/ml) was then added for 4 h. Cells were harvested on glass fiber filters and β-emission measured. Data were calculated relative to the values of cells stimulated by IL-3 and are expressed as means ± standard error of triplicate samples. (**B**) Suppression of growth factor deprivation-induced apoptosis by KL. BMMC expressing wild-type or mutant Kit receptors were incubated for 12 h in serum-free medium containing IL-3, starved of IL-3 for 1 h and then incubated for 50 h in medium with and without the addition of IL-3 or KL. Cells were collected, stained with AnnexinV and the percentage apoptosis was determined by FACS analysis. Similar results were obtained in three independent experiments. (**C**) Suppression by KL of irradiation-induced apoptosis. Cells were pre-treated similarly to (B), starved for 1 h without IL-3 and subjected to γ-irradiation (25 Gy). Cells were harvested after 12 and 24 h of incubation at 37°C, and analyzed as described in (B).

incubated for 12 h in serum-free medium containing IL-3, then starved for 1 h without IL-3 and subjected to γ-irradiation (25 Gy) or left untreated in the presence and absence of KL. Cells were harvested at the indicated times, stained with AnnexinV and scored for the percentage of apoptotic cells using FACS analysis. Similarly to suppression of growth factor deprivation-induced apoptosis, Kit^{Y567F}- and Kit^{Y719F}-expressing cells were partially deficient in suppression of irradiation-induced apoptosis by KL, while cells expressing the double mutant, $Kit^{Y567,719F}$, were completely inactive in this response (Figure 2C). Thus, similarly to Kit-induced proliferation

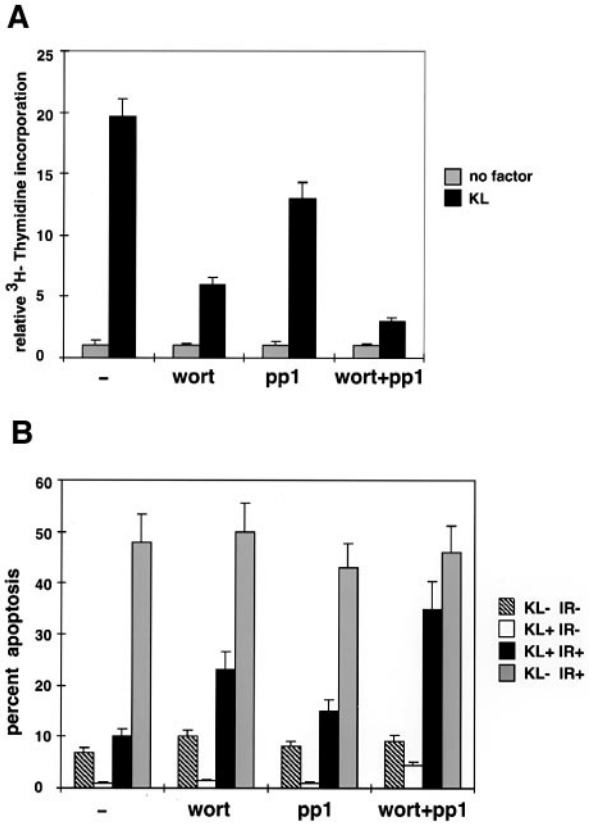

Fig. 3. KL-induced proliferation and suppression of apoptosis are impaired by pharmacological inhibitors of PI 3-kinase and Src kinases. (**A**) +/+ BMMC were treated as in Figure 2A, except that wortmannin (10^{-7} M) and pp1 (20 ng/ml) were added to the cells 1 h prior to the growth factors. (**B**) +/+ BMMC were treated as in Figure 2C, except that the inhibitors were added to the cells 3 h prior to irradiation.

of BMMC, Kit-mediated suppression of irradiation- and growth factor deprivation-induced apoptosis is dependent on activation of both PI 3-kinase and Src kinases. Elimination of both pathways abolishes these responses by Kit.

Pharmacological inhibitors of Src and PI 3-kinase affect Kit-mediated proliferation and suppression of apoptosis

In order to confirm the notion that the cellular effects of the Kit^{Y567F} and the Kit^{Y719F} mutations affect Src kinase and PI 3-kinase signaling, Kit-mediated proliferation and suppression of irradiation-induced apoptosis of mast cells expressing endogenous Kit were examined in the presence of inhibitors of PI 3-kinase (wortmannin) and the Src kinases (pp1). However, it was not feasible to determine the effects of these inhibitors on deprivation-induced apoptosis due to the inherent longer experimental times. DNA synthesis and irradiation-induced apoptosis were measured as described, except that the inhibitors were added 1 h prior to KL in proliferation assays, and 3 h prior to irradiation in apoptosis assays. Wortmannin as well as pp1 caused a partial reduction of KL-induced DNA synthesis and suppression of apoptosis, whereas the combination of inhibitors blocked both Kit-mediated responses almost completely (Figure 3A and B). This is in agreement with the results obtained with BMMC expressing Kit^{Y719F} and Kit^{Y567F} (Figure 2). Therefore,

even though we cannot exclude that other signaling intermediates bind to the phosphorylated tyrosines 567 and 719 of the Kit receptor, PI 3-kinase and Src kinases appear to be the major effectors of the observed cellular defects of these mutations.

KL suppresses irradiation-induced JNK activation

JNK has been implicated as an important mediator of apoptosis in certain cell systems (Xia *et al*., 1995; Kyriakis and Avruch, 1996; Verheij *et al*., 1996). Therefore, we were interested in investigating the possible involvement of JNK activation in irradiation-induced apoptosis of mast cells and its suppression by KL. Cells were pre-incubated for 12 h in serum-free medium containing IL-3, subsequently starved for 1 h without IL-3 and subjected to γ-irradiation or left untreated in the presence and absence of KL. In other cell systems, irradiation induces a slow and sustained activation of JNK, reaching maximal levels between 3 and 6 h after treatment (Chen *et al*., 1996). Therefore, irradiated cells were lysed at 1 and 4 h after irradiation, and an *in vitro* kinase assay using GST–Jun fusion protein as a substrate was performed. JNK activity was increased slightly at 1 h after irradiation but was activated much more strongly at 4 h (Figure 4A). When KL was added to the cells during irradiation, activation of JNK was inhibited completely at both time points (Figure 4A). KL alone did not cause a significant change in JNK activity at either 1 or 4 h after treatment.

Next, we tested whether irradiation-induced JNK activation may be suppressed by KL in BMMC expressing mutant Kit receptors. Irradiation-induced JNK activity was suppressed by KL to comparable levels in mast cells expressing endogenous Kit, Kit^{WT}, Kit^{Y719F} or Kit^{Y567F} (Figure 4B). In contrast, in cells expressing the $Kit^{Y567,719F}$ receptor, JNK activity induced by irradiation remained elevated in the presence of KL. The inability of the $Kit^{Y567,719F}$ receptor to suppress irradiation-induced JNK correlates with its inability to suppress irradiation-induced apoptosis. However, BMMC expressing the single mutants Kit^{Y567F} or Kit^{Y719F} were partially deficient in suppression of irradiation-induced apoptosis, while suppression of irradiation-induced JNK activation was complete. Therefore, these results imply that the role of PI 3-kinase and Src kinases in suppression of irradiation-induced apoptosis by KL might involve other events in addition to the suppression of irradiation-induced JNK activity.

Kit-mediated activation of MAPK is not affected by PI 3-kinase- and Src kinase-binding site mutations

The Ras–MAPK cascade is thought to be critical for mitogenesis in several distinct cell types (Marshall, 1995). Moreover, activation of MAPK has been implicated to play a role in protection of cells from apoptosis (Xia *et al*., 1995). To test whether Kit mutations which affect proliferation and survival would also affect the Ras–MAPK cascade, we determined Erk1 MAPK activation in response to KL. Cells were starved for 12 h in low serum medium, stimulated with KL for 5 min, lysed, immunoprecipitated with anti-Erk1 antibody and MAPK activation was determined employing an *in vitro* kinase assay with the myelin basic protein (MBP) peptide as a substrate. IL-3-induced MAPK activation was used as an internal control. All of the mutant receptors, including the

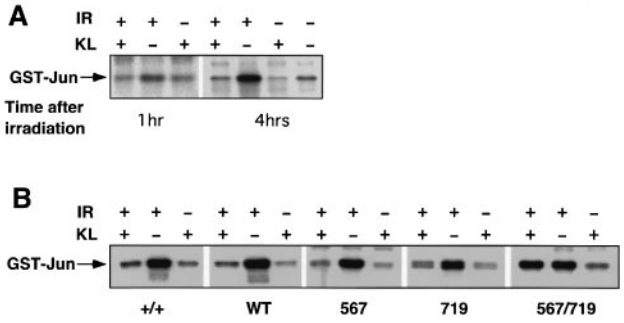

Fig. 4. KL suppresses irradiation-induced JNK activation in BMMC. (**A**) +/+ BMMC were pre-incubated with IL-3 in serum-free medium for 12 h, starved for 1 h without IL-3 and treated with γ-irradiation (25 Gy) or left untreated in the presence and absence of KL (200 ng/ml). After 1 and 4 h of incubation, cells were lysed, JNK proteins were immunoprecipitated and an *in vitro* kinase assay was performed using the GST–Jun fusion protein as a substrate. (**B**) BMMC expressing Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ were treated as described in (A) and *in vitro* kinase activity of JNK was determined after 4 h of incubation.

double mutant $Kit^{Y567,719F}$, were able to induce Erk1 activity 8- to 10-fold, comparable with Erk1 activation by wild-type Kit (Figure 5A). Similar results were obtained when Erk2 activation by Kit was determined (data not shown). These results suggest that Kit-mediated activation of Erk MAPK is not sufficient for induction of mitogenesis, but other events mediated through the PI 3-kinase and Src kinase pathways play a critical role in mitogenesis.

Next, we tested whether treatment of cells with inhibitors of PI 3-kinase (wortmannin) and the Src kinase (pp1) would affect the activation of MAPK by KL. BMMC were treated as described, except that inhibitors were added to cell cultures 1 h prior to stimulation with KL. Cells treated with the inhibitors alone or in combination activated Erk1 MAPK to the same extent as untreated controls (Figure 5B), in agreement with our findings with BMMC carrying mutant Kit receptors. Therefore, Kit-mediated activation of MAPK is not sufficient for induction of mitogenesis and suppression of apoptosis in BMMC, implying that other events mediated through the PI 3-kinase and Src kinase pathways play critical roles in these Kit-mediated responses.

KL induces rapid and transient activation of JNK in mast cells

A number of growth factors were reported to activate JNK (Minden *et al*., 1994; Logan *et al*., 1997). Moreover, this activation was dependent on PI 3-kinase (Logan *et al*., 1997). Therefore, we were interested in determining whether KL would stimulate JNK activity in mast cells and whether this activation is affected by Kit mutations. Similarly to some other growth factors, KL strongly induces JNK activation in BMMC, but with kinetics different from those of irradiation. KL-induced JNK activity was apparent already at 5 min after stimulation, reaching maximum levels between 15 and 20 min and then declining to background levels within 1 h (Figure 6A). In contrast, JNK activity was only marginally induced by irradiation 1 h after treatment, reaching maximum levels only 3–4 h post-irradiation (Figure 4A).

We next wanted to determine whether the Kit receptor mutations which affected KL-mediated proliferation and

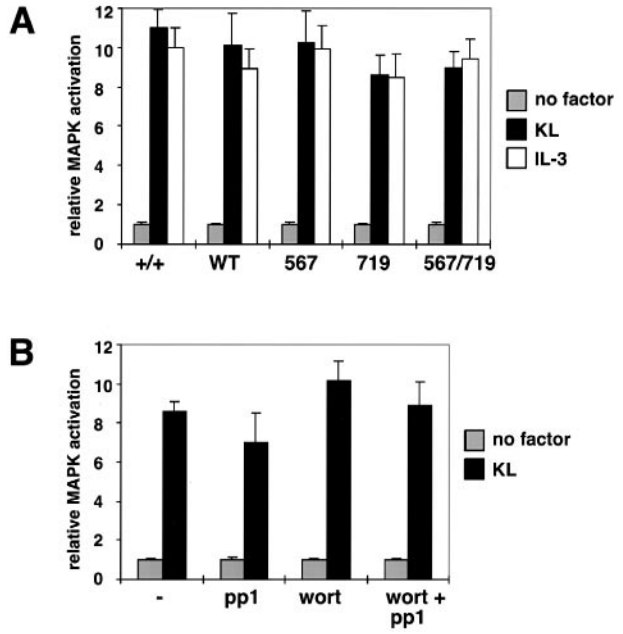

Fig. 5. Kit-mediated activation of MAPK is not affected by Kit mutations. (**A**) BMMC expressing Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ were starved in low (1%) serum medium for 12 h and then stimulated with KL (200 ng/ml) or IL-3 (20 ng/ml) for 5 min. MAPK (Erk1) was immunoprecipitated and an *in vitro* kinase assay was performed using the MBP peptide as a substrate. Data are presented as means ± standard error of three independent experiments. (**B**) Normal BMMC were treated and analyzed as described in (A), except that prior to stimulation with KL, cells were incubated with the Src inhibitor pp1 (20 ng/ml), the PI 3-kinase inhibitor wortmannin (10^{-7} M) or a combination of the two for 1 h.

suppression of apoptosis would also affect KL-induced JNK activation. Unlike KL-induced MAPK activity (Figure 5A), KL-induced JNK activity was strongly affected by both the Kit^{Y719F} and Kit^{Y567F} mutations: JNK activation is reduced from ~11- to 3-fold in BMMC expressing either the Kit^{Y719F} or Kit^{Y567F} receptors compared with those expressing the wild-type Kit receptor (Figure 6B). In BMMC expressing the double mutant $Kit^{Y567,719F}$ receptor, JNK activation by KL was completely lost (Figure 6B). Similar results were obtained using inhibitors of PI 3-kinase and Src kinases. The PI 3-kinase inhibitor wortmannin as well as the Src kinase inhibitor pp1 both reduced activation of JNK from 15- to 3-fold, while the two inhibitors together blocked KL-induced activation of JNK completely (Figure 6C). The correlation between the effects of Kit mutations on KL-induced proliferation and suppression of apoptosis and the activation of JNK indicates that JNK activity may be important for these responses.

Several JNK variants have been identified recently, including p46 JNK1 and p54 JNK2 (Gupta *et al.*, 1996; Kyriakis and Avruch, 1996). To determine whether irradiation and KL activate the same JNK isoforms, we made use of specific phospho-JNK antibodies, which recognize only activated JNK. Cells were treated with irradiation or KL as described before, then lysed, and JNK proteins were precipitated using polyclonal anti-JNK antibody which recognizes different JNK isoforms. The activated JNK species were identified by immunoblotting using anti-phospho-JNK antibodies (Figure 6D). Interestingly, KL induced both the p54 and p46 JNK species, while irradiation induced only p46 JNK. Thus, differential activation of JNK isoforms as well as different kinetics of JNK activation are observed in response to Kit activation and irradiation in BMMC.

BMMC expressing dominant-negative JNK2-APF are deficient in Kit-mediated mitogenesis

The correlation between the ability of various mutant Kit receptors to mediate mitogenic and survival responses and activation of JNK and Rac1 suggested that the JNK cascade may play important roles in these Kit-mediated responses. To test this hypothesis, we generated stably transfected BMMC expressing JNK1, JNK2 and inhibitory mutants of JNK, JNK1-APF and JNK2-APF. The cDNAs encoding the mutant JNK proteins have been described (Derijard *et al.*, 1994; Kallunki *et al.*, 1994). Recently, eight additional JNK isoforms have been identified, indicating that the previously described JNK1 and JNK2 cDNAs represent one of the four known alternatively spliced products of the JNK1 and JNK2 genes respectively, in particular JNK1α1 and JNK2α2 (Gupta *et al.*, 1996).

BMMC stably expressing JNK isoforms were generated by retroviral gene transfer as described in Materials and methods. Cultures obtained after selection with G418 displayed normal growth and survival characteristics, with the exception of BMMC expressing JNK2-APF and to a lesser degree JNK1-APF, which exhibited reduced growth and survival. The addition of both KL and IL-3 to the growth medium enabled us to maintain these cells in culture and, therefore, in all experiments cells were pretreated with these growth factors. Expression of the various JNK protein products in the BMMC transfectants is shown in Figure 7A. To confirm that the expression of the JNK-APF mutants in BMMC impairs KL-induced activation of JNK, JNK activity was determined *in vitro*. As expected, phosphorylation of the JNK substrate GST–Jun was diminished substantially in cells expressing JNK2-APF and to a lesser degree in cells expressing JNK1-APF (Figure 7B).

To determine whether the expression of wild-type or inhibitory JNKs affects KL-induced survival, the JNK transfectants were assayed for suppression of growth factor deprivation- and irradiation-induced apoptosis. Cells were pre-incubated for 12 h in serum-free medium containing both IL-3 and KL, starved of growth factors for 2 h and subjected either to starvation for 50 h or γ-irradiation as described above. All cultures successfully suppressed both growth factor deprivation- and irradiation-induced apoptosis in the presence of KL (Figure 7C and D). However, the analysis of KL-induced DNA synthesis in the JNK transfectants by using a [^{3}H]thymidine incorporation assay revealed that KL-mediated DNA synthesis was reduced in JNK1-APF-expressing BMMC and completely abrogated in cells expressing JNK2-APF (Figure 7E). Cell viability in these experiments was not reduced appreciably (data not shown). Interestingly, expression of neither the wild-type JNK proteins nor inhibitory mutants affected progression of apoptosis induced by irradiation or factor deprivation. These results suggest that JNK is critical for Kit-mediated proliferation of mast cells. Our results also suggest that the JNK may not be critical for suppression of apoptosis by KL.

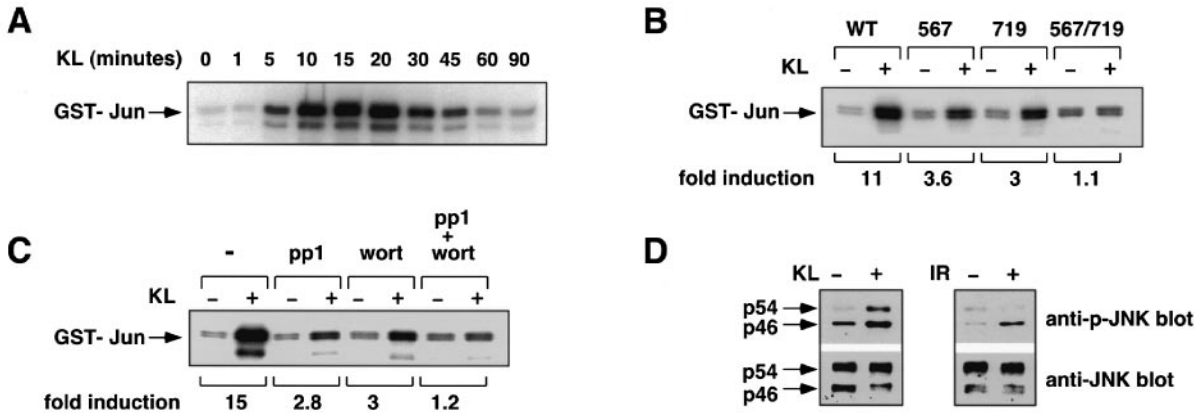

Fig. 6. KL induces rapid and transient activation of JNK in mast cells. (**A**) Time course of JNK activation by KL. Normal BMMC were starved in the serum-free medium for 12 h and then stimulated with KL (200 ng/ml) for the indicated times. JNK *in vitro* kinase assays were performed as described in Figure 4. (**B**) Decreased KL-induced JNK activation in BMMC expressing mutant Kit receptors. BMMC expressing Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ were treated as described in (A) and JNK activation was determined by an *in vitro* kinase assay. The relative phosphorylation was quantitated by using a phosphoimage analyzer (Fuji Mac Bas). (**C**) Effect of Src and PI 3-kinase inhibitors on KL-induced JNK activation. Normal BMMC were treated as in (A) except that prior to stimulation with KL, cells were incubated with the Src inhibitor pp1 (20 ng/ml), the PI 3-kinase inhibitor wortmannin (10^{-7} M) or with a combination of the two for 1 h. (**D**) Differential activation of JNK isoforms by KL and irradiation. Normal BMMC were treated as described in (A) for KL-induced JNK activation and as described in Figure 4 for the activation of JNK by irradiation. JNK proteins were immunoprecipitated using polyclonal anti-JNK1 antibody (recognizes several JNK isoforms) and blotted with anti-phospho-JNK antibody (upper panel). The JNK protein levels in the lysates were determined using anti-JNK antibody (lower panel).

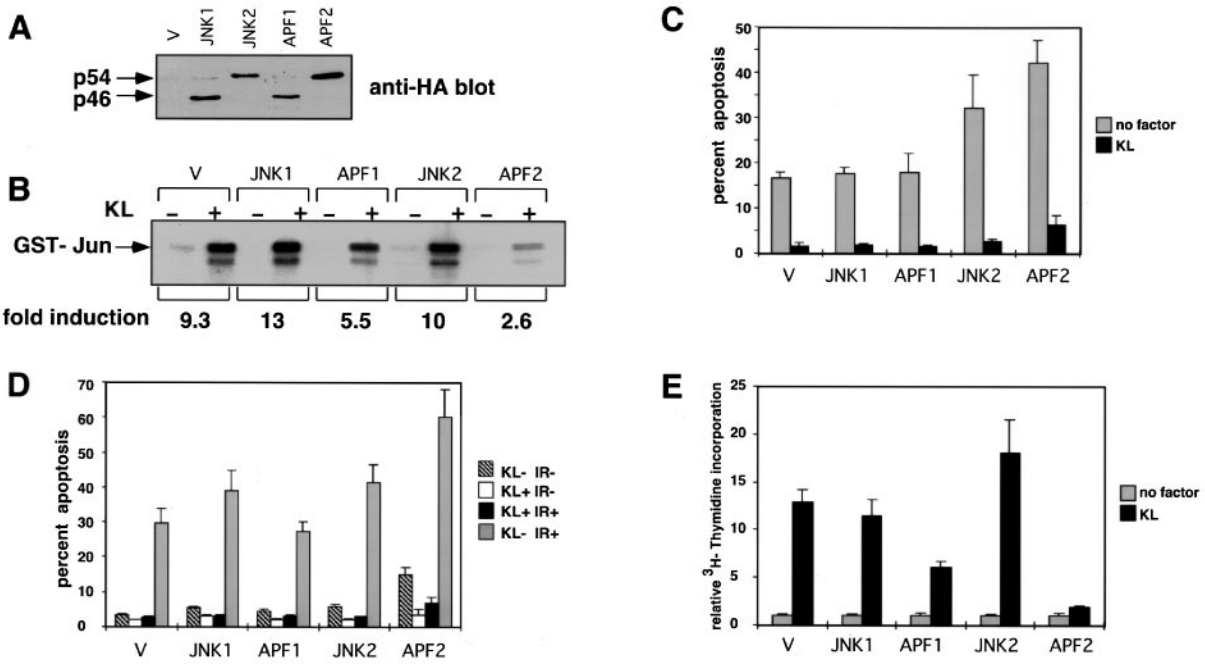

Fig. 7. Analysis of BMMC expressing wild-type and inhibitory versions of p46 JNK1, JNK1-APF, and p54 JNK2, JNK2-APF2. (**A**) Expression of HA-tagged JNK proteins in the BMMC transfectants. (**B**) Expression of inhibitory mutants of JNK reduces phosphorylation of c-Jun in BMMC. The JNK *in vitro* kinase assay was performed as described. (**C**) Expression of inhibitory mutants of JNK does not affect Kit-mediated suppression of deprivation-induced apoptosis in BMMC. BMMC expressing JNK1, JNK2, JNK1-APF and JNK2-APF were pre-treated in serum-free medium containing KL (200 ng/ml) and IL-3 (20 ng/ml) for 12 h, then starved for 2 h without factors and analyzed as described in Figure 2B. (**D**) Expression of JNK1-APF and JNK2-APF does not affect Kit-mediated suppression of irradiation-induced apoptosis in BMMC. BMMC expressing wild-type and mutant JNK proteins were pre-treated as in (A) and analyzed as described in Figure 2C. Cells were harvested 12 h after irradiation. (**E**) Expression of JNK1-APF and JNK2-APF impairs KL-induced cell cycle progression in BMMC. BMMC expressing wild-type and mutant JNK proteins were pre-treated as in (A) and [^{3}H]thymidine incorporation was determined as described in Figure 2A.

Activation of Rac1 by KL is mediated by both Src and PI 3-kinase signaling pathways

The Rho family GTPases, Rac1 and Cdc42, were shown to activate JNK (Coso *et al*., 1995; Minden *et al*., 1995). We sought, therefore, to determine whether Rac1 activation was involved in Kit-mediated regulation of JNK activity. Upon activation, Rac1 is translocated into the cytoskeletal, Triton X-100-insoluble, fraction of the cell (el Benna *et al*., 1994; Gulbins *et al*., 1996). To investigate the possibility that KL may activate Rac1 in mast cells, Triton X-100 solubility of Rac1 in mast cells was examined. Rac1 immunoblots of Triton X-100-insoluble fractions of mast cells expressing Kit^{WT} revealed a significant accumulation of Rac1 in the cytoskeletal fraction upon

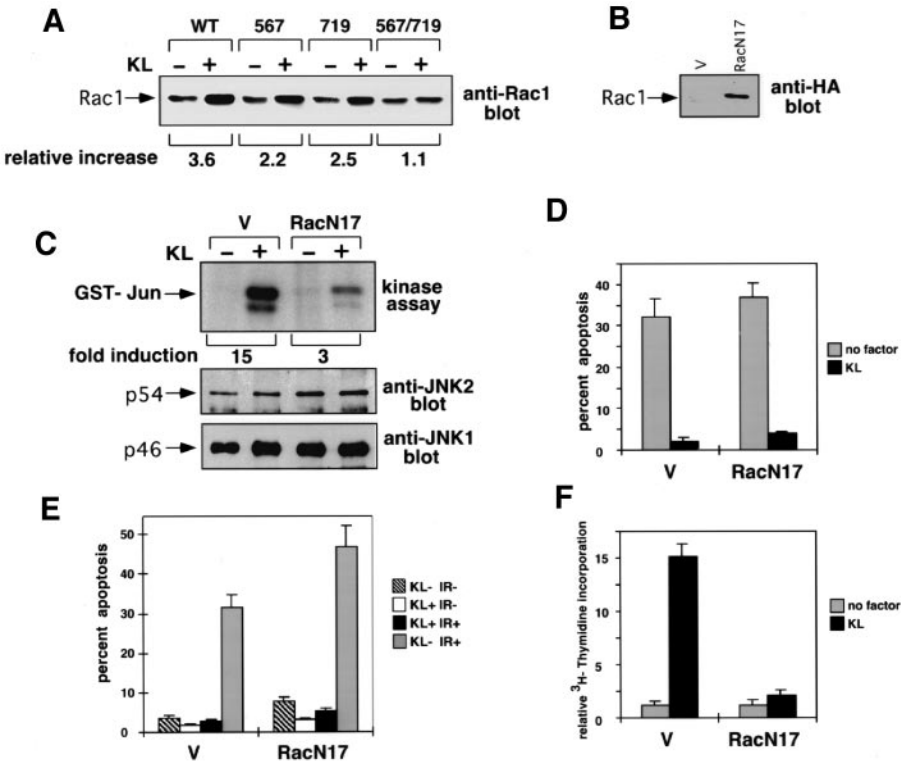

Fig. 8. Analysis of BMMC expressing dominant-negative mutant Rac1 (RacN17). (**A**) KL mediates the translocation of Rac1 into the cytoskeletal fraction. BMMC expressing Kit^{WT}, Kit^{567}, Kit^{719} and $Kit^{567/719}$ were starved in serum-free medium for 12 h and stimulated with KL (200 ng/ml) for 10 min. Cells were lysed in Triton X-100 lysis buffer, followed by ultracentrifugation of the lysates for 2 h. Triton X-100-insoluble pellets were resuspended in loading buffer, proteins were separated by SDS–PAGE and blotted with anti-Rac1 antibody. Relative amounts of Rac1 protein in the Triton X-100-insoluble fraction were quantitated using a phosphoimager. (**B**) Expression of HA-tagged RacN17 in BMMC transfectants. (**C**) Expression of RacN17 inhibits Kit-mediated activation of JNK in BMMC. BMMC expressing vector or RacN17 were stimulated with KL as described above, and a JNK *in vitro* kinase assay was performed. JNK1 (p46) and JNK2 (p54) protein levels in the immunoprecipitates are shown below. (**D** and **E**) Expression of RacN17 does not affect Kit-mediated suppression of deprivation- and irradiation-induced apoptosis in BMMC. BMMC expressing empty vector or RacN17 were analyzed as described in Figure 7C and D. (**F**) Expression of RacN17 abolishes KL-induced cell cycle progression in BMMC. BMMC expressing empty vector or RacN17 were analyzed as described in Figure 7E.

stimulation with KL (Figure 8A). The result that stimulation with KL causes translocation and possibly activation of Rac1 is in agreement with the previous observation that KL induces membrane ruffling in mast cells (Vosseller *et al*., 1997), a cytoskeletal rearrangement controlled by Rac1 (Ridley *et al*., 1992). In mast cells expressing the single mutant receptors Kit^{Y719F} or Kit^{Y567F}, Rac1 translocation was reduced, and in cells expressing the double mutant Kit receptor $Kit^{Y567,719F}$, Rac1 translocation was abolished (Figure 8A). In agreement with this observation, the PI 3-kinase inhibitor wortmannin and the Src inhibitor pp1 caused reduction of Rac1 translocation, while the combination of both inhibitors blocked Rac1 translocation (data not shown).

The apparent correlation between Rac1 and JNK activation in BMMC expressing mutant Kit receptors suggests that Rac1 may function in the same pathway as JNK in Kit signaling, similarly to what is observed in other cell systems and with other growth factors. To examine this hypothesis, we generated stably transfected BMMC expressing the dominant-negative form of Rac1, RacN17, using a retroviral vector as a vehicle. Cultures obtained after selection with G418 displayed reduced growth and survival compared with those transfected with the empty vector, similarly to BMMC expressing JNK2-APF. Expression of hemagglutinin (HA)-tagged RacN17 in BMMC was evaluated by anti-HA immunoblot analysis of cell lysates (Figure 8B).

Next we sought to determine whether expression of RacN17 affects KL-induced activation of JNK by measuring JNK activity *in vitro*. In cells expressing RacN17, activation of JNK by KL was significantly impaired (3-fold activation) compared with the activation seen in control cells expressing vehicle alone (15-fold induction) (Figure 8C). This result implies that Rac mediates Kit-induced JNK activation. To investigate this notion further, we established BMMC expressing the dominant-negative Rac mutation, RacN17, and investigated the proliferative and survival responses of these cells. Similarly to BMMC expressing inhibitory JNK-APF mutants, Kit-mediated suppression of both growth factor deprivation- as well as irradiation-induced apoptosis was normal in BMMC expressing dominant-negative Rac1 (Figure 8D and E). In contrast to the lack of an effect on the survival response, the proliferative response to KL was severely compromised in BMMC expressing RacN17 (Figure 8F). Taken together, these results implicate Rac1 in Kit-mediated activation of JNK and suggest that the Rac/JNK pathway is essential for the Kit-mediated mitogenic response in BMMC. Furthermore, these results imply that signaling events other than the Rac/JNK pathway are critical for Kit-mediated suppression of apoptosis.

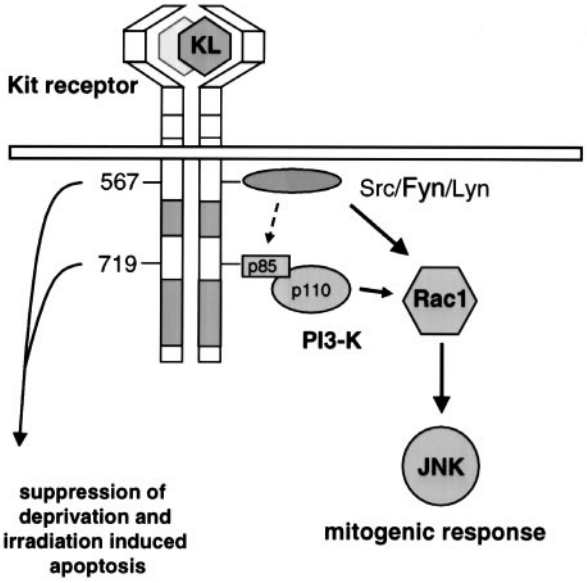

Fig. 9. Schematic representation of Kit-mediated PI 3-kinase and Src family kinase signaling in bone marrow-derived mast cells.

Discussion

Critical roles for PI 3-kinase and Src kinase signaling in Kit-mediated mitogenesis and suppression of apoptosis

In an attempt to understand the mechanisms by which KL suppresses apoptosis and induces proliferation in mast cells, we have analyzed the contributions of PI 3-kinase and Src family kinases to these responses. For this purpose, we have made use of Kit receptors with single amino acid mutations at critical tyrosine residues which serve as docking sites for PI 3-kinase (Serve *et al*., 1995) and for Src family kinases. Src kinase-binding site mutations were created based on the homology of Kit with other receptor tyrosine kinases (Mori *et al*., 1993). Substitution of Tyr567 with phenylalanine in the juxtamembrane domain of Kit abolished KL-induced binding of the Src family kinase Fyn, and consequently prevented activation of Fyn and presumably other Src-like kinases. Importantly, a primary cell system—BMMC—was used, in which Kit elicits several distinct cellular responses. BMMC lacking endogenous Kit expression isolated from W^{sh}/W^{sh} mice were reconstituted to express wild-type or mutant Kit receptors at levels comparable with the level of normal endogenous Kit expression. The use of primary cells as opposed to immortalized cell lines is particularly important for the investigation of mechanisms of cell survival induced by growth factors. Activation of both a Src-like kinase (such as Fyn) and PI 3-kinase was found to be essential for Kit-mediated survival and proliferation of mast cells, and inactivation of the two signaling pathways completely abolished both responses. Furthermore, we provide evidence that the PI 3-kinase and Src kinase signaling pathways converge to activate Rac and JNK, suggesting that these molecules play roles in the Kit-mediated mitogenic response as well as in suppression of apoptosis (Figure 9). The critical importance of Rac/JNK activation in Kit-mediated mast cell proliferation was demonstrated by using dominant-negative Rac and JNK mutants.

Our observation of a reduced KL-mediated survival and proliferation response in BMMC expressing Kit^{Y567F} as compared with cells expressing wild-type Kit is in apparent contrast to a report by Herbst *et al*. (1995) where deletion of Y567/V568, as found in feline v-Kit in an epidermal growth factor (EGF)–Kit receptor fusion, enhanced the mitogenic and transforming potential of the receptor in fibroblasts. In addition, recently, Kit^{Y567F}-expressing Ba/F3 cells were found to exhibit a hyperproliferative response to KL, possibly due to recruitment and activation of the phosphatase SHP-2 (Kozlovski *et al*., 1998). The reasons for this discrepancy are presently unclear, but two explanations come to mind: first, more than one signaling intermediate can bind to Tyr567 of Kit (Src kinases and SHP-2), and second, in different cellular contexts different signaling pathways are available and this will determine the functional consequences of the mutation. Importantly, our results obtained with Kit receptor mutants were corroborated by the use of pharmacological inhibitors of Src family kinases and PI 3-kinase. Taken together, these data support the notion that these molecules are critical for Kit-induced mitogenic and survival signaling in mast cells.

Activation of the Jun N-terminal kinase (JNK) by growth factors

JNKs are strongly activated by inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α), and by cellular stress including heat shock, UV and ionizing radiation (Kyriakis and Avruch, 1996; Verheij *et al*., 1996). However, significant activation of JNK has also been observed for a number of growth factors including EGF and nerve growth factor (NGF) (Minden *et al*., 1994), IL-3 (Nagata *et al*., 1997) and G-CSF (Rausch and Marshall, 1997), and for aggregation of high affinity FcεRI (Ishizuka *et al*., 1997). Members of the Rho family GTPases Rac1 and Cdc42 were shown to modulate JNK activity (Coso *et al*., 1995; Minden *et al*., 1995), and dominant-negative mutants of Rac1 blocked activation of JNK induced by an activated Met oncoprotein (Rodrigues *et al*., 1997) and Fas receptor ligation (Brenner *et al*., 1997). In addition, JNK activation mediated by growth factors is blocked by wortmannin and by a dominant-negative form of PI 3-kinase, suggesting that JNK is in a downstream effector pathway of PI 3-kinase (Logan *et al*., 1997). Similarly to other cell systems, we observed that in mast cells wortmannin strongly inhibited Kit-mediated JNK activation. However, in contrast to other studies, blocking of Kit-mediated PI 3-kinase activation by wortmannin or by mutation of the PI 3-kinase-binding site in the Kit receptor (Kit^{Y719F}) is not sufficient to abolish activation of JNK by KL. We found that the Src kinase pathway significantly contributes to Kit-mediated JNK activation, since the Src kinase inhibitor pp1 and a Src-binding site mutation in the Kit receptor (Kit^{Y567F}) impaired JNK activation by KL. Only inhibition of both pathways abolished JNK activation by KL. Therefore, these results provide new insight into regulation of JNK by growth factors, such as KL, suggesting that distinct independent signaling pathways link Kit receptor activation (or possibly activation of other receptor tyrosine kinases) with stimulation of JNK activity. Furthermore, these findings provide evidence that Src and PI 3-kinase signaling pathways converge at the level or upstream of

Rac1 since, similarly to JNK, Rac1 activation by Kit was abolished only by inhibition of both pathways (Figure 9).

Cross-talk between signaling mediated by the Rho family GTPases, PI 3-kinase and and Src family kinases has been reported. Src family kinases activate Rac via the guanine nucleotide exchange factor (GEF) Vav which induces GDP/GTP exchange activity of Rho family members, including Rac, both *in vitro* and *in vivo* (Crespo *et al*., 1997; Han *et al*., 1997; Teramoto *et al*., 1997). Activation of Vav by the Src family kinases is mediated by tyrosine phosphorylation within the Dbl homology domain (DH) of Vav (Crespo *et al*., 1997; Han *et al*., 1997). In addition, the Dbl-domain containing GEFs Vav and Sos was shown recently to be activated by phospholipid products generated by PI 3-kinase via the pleckstrin homology domain (PH) of Vav and Sos (Han *et al*., 1998; Nimnual *et al*., 1998). While activation of the Kit receptor results in rapid tyrosine phosphorylation of Vav, this response is abolished in BMMC expressing the Kit^{Y567F} Src-binding site mutation (unpublished observation), raising the possibility that reduced activation of Rac1 by Kit^{Y567F} may result from impaired Vav phosphorylation.

Cellular responses mediated by Rho family GTPases and JNK

In mammalian cells, Rho family GTPases, Rac, Rho and Cdc42, control various aspects of the regulation of cytoskeletal rearrangements (Hall, 1998). In addition, these molecules have been suggested to play a role in promoting cell growth and transformation (Van Aelst and D'Souza-Schorey, 1997). Injection of Rho, Rac or Cdc42 proteins into Swiss 3T3 fibroblasts facilitated cell cycle progression through G_1 and S-phase (Olson *et al*., 1995), and cells expressing activated forms of these GTPases displayed enhanced growth in low serum, anchorage-independent growth and caused tumor formation in nude mice (Khosravi-Far *et al*., 1995). In addition, Rac1 and Cdc42 have been reported to regulate the JNK and p38 cascades (Coso *et al*., 1995; Minden *et al*., 1995). The JNK cascade has been implicated in a number of cellular responses, including the immune response (Su *et al*., 1994; Ishizuka *et al*., 1997), oncogenic transformation (Raitano *et al*., 1995; Rodrigues *et al*., 1997) and promotion of apoptosis (Xia *et al*., 1995; Kyriakis and Avruch, 1996; Verheij *et al*., 1996). Here we demonstrate that in BMMC, Kit-mediated activation of the Rac1/JNK cascade, but not the MAPK cascade, correlates with Kit-mediated proliferation and survival. In mast cells expressing the Kit receptor mutant, $Kit^{Y567,719F}$, which is deficient in the activation of Rac1 and JNK but not of MAPK, KL fails to induce proliferation or to suppress apoptosis induced by irradiation and growth factor deprivation. Cells expressing the single site Kit receptor mutants, Kit^{Y567F} and Kit^{Y719F}, are partially impaired in Kit-induced proliferation and suppression of apoptosis. Importantly, stable expression of a dominant-negative mutant of JNK, JNK2α2-APF, and of Rac1, RacN17, in BMMC abolished the KL-mediated mitogenic response in these cells. However, neither of these inhibitory mutants affected Kit-mediated suppression of irradiation- and growth factor deprivation-induced apoptosis. These observations demonstrate that the Rac1/JNK pathway plays a critical role in mediating Kit-induced cell proliferation, while other mediators are involved in the anti-apoptotic response to Kit. This is in agreement with a recent observation that expression of JNK-specific dual specificity phosphatase inhibited IL-3-dependent proliferation of Ba/F3 cells (Smith *et al*., 1997). In addition, an essential role for JNK in Met oncogene-mediated transformation has been suggested (Rodrigues *et al*., 1997). In addition, the JNK signal transduction pathway inhibitor, JIP-1, abolished Bcr-Abl-induced transformation of pre-B cells, implicating the JNK pathway in pre-B cell transformation by Bcr-Abl (Dickens *et al*., 1997).

Specificity of signaling through JNK family kinases

The finding that KL-induced JNK activation is critical for Kit-mediated cell cycle progression in BMMC is in contrast to the observation that KL inhibits JNK activation induced by γ-irradiation in BMMC. Furthermore, inhibition of JNK activation correlates with suppression of irradiation-induced apoptosis by KL. Therefore, JNK may have both mitogenic and pro-apoptotic roles in BMMC. In small cell lung carcinoma cells (SCLC), Butterfield *et al*. (1997) observed differential effects of the JNK isoforms JNK1α1 and JNK2α2 on sensitivity to UV irradiation. Overexpression of the kinase-inactive mutant JNK1-APF increased resistance of SCLC cells to UV irradiation-induced apoptosis, while JNK2-APF did not have such an effect. Furthermore, in the renal IMCD-3 cells, expression of JNK2-APF but not JNK1-APF significantly decreased resistance to hypertonicity (Wojtaszek *et al*., 1998). In Ba/F3 cells, JNK activation, while being important for IL-3-dependent proliferation, was not required for induction of apoptosis by factor deprivation or ceramide treatment (Smith *et al*., 1997).

These observations suggest that JNK activation by different stimuli including growth factors and stress factors may mediate distinct cellular responses, and this may be determined by the cellular context. Specificity of different JNK activities may be achieved by several mechanisms. First, different JNK isoforms could have differential substrate specificity. Three genes, *JNK1*, *JNK2* and *JNK3*, give rise to 10 different JNK isoforms, presumably by alternative splicing (Gupta *et al*., 1996), raising the question of the functional roles of these isoforms. JNK proteins are known to interact with different downstream targets, including the transcription factors c-Jun, ATF2 (Livingstone *et al*., 1995; Gupta *et al*., 1996) and Elk-1 (Whitmarsh *et al*., 1995). Whereas activation of c-Jun and ATF2 is mediated by binding of JNK and subsequent phosphorylation, Elk-1 activation and phosphorylation do not require JNK binding (Gupta *et al*., 1996). Furthermore, the affinities of the binding interactions between JNK isoforms and c-Jun and ATF2 differ (Gupta *et al*., 1996).

Another possible basis for the specificity of JNK family kinases is differential spatial and temporal compartmentalization of JNKs activated either by stress factors or growth factors. It is conceivable that similarly to MAP kinases (Marshall, 1995), JNKs activated in a sustained fashion may produce different cellular responses from those produced by transiently activated JNKs. In conclusion, differences with regards to kinetics, compartmentalization and substrate specificity between different JNK isoforms may determine mitogenic, pro- and anti-apoptotic activities of JNKs. Therefore, comparative studies of the

various JNK isoforms should help to unravel the various roles of JNK in cellular physiology.

Materials and methods

Mast cell cultures and transfections

C57Bl/6J mice and homozygous mutant W^{sh}/W^{sh} mice were described previously (Serve *et al*., 1995). BMMC were produced by culturing bone marrow in RPMI 1640 supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 5.5×10^{-5} M β-mercaptoethanol, 0.075% sodium bicarbonate, 10% fetal bovine serum (RPMI complete) and 10% X63-derived IL-3-containing conditioned medium. Mutant Kit constructs were subcloned into the retroviral vector pGD and expressed in W^{sh}/W^{sh}-derived BMMC as described previously (Serve *et al*., 1995). Briefly, E86 packaging cells were transfected with the Kit constructs by electroporation. After selection with G418 (800 μg/ml), packaging cells were co-cultured for 24 h with 3- to 5-week-old W^{sh}/W^{sh} BMMC in virus-containing filtered conditioned medium supplemented with 8 μg/ml polybrene. Infectants were selected in 400 μg/ml G418 starting at 48 h. Kit cell surface expression of infected cells was monitored using anti-Kit monoclonal ACK2 antibody (1:60) coupled to FITC and FACS analysis. BMMC stably expressing JNK constructs were generated as described above, using +/+ BMMC. The HA-JNK1, HA-JNK2, HA-JNK1-APF and HA-JNK2-APF cDNAs cloned into the replication-defective retroviral vector pLNCX (Butterfield *et al*., 1997; Wojtaszek *et al*., 1998) were generously provided by Dr Lynn Heasley. The pCEV rac1N17 construct was generously provided by Dr Erich Gulbins (Gulbins *et al*., 1996). The HA-tagged version of Rac1N17 was created by PCR and cloned into the retroviral vector pLNCX.

Cytokines and antibodies

Recombinant murine KL was prepared as described previously (Yee *et al*., 1993); recombinant murine IL-3 was purchased from Biosource (Camarillo, CA). The monoclonal anti-Kit antibody (ACK2) was described before (Serve *et al*., 1995). Anti-phosphotyrosine antibody (PY-20) was purchased from Transduction Laboratories (Lexington, KY). Anti-Fyn antibody, anti-Kit polyclonal antibody, anti-Erk1 and GST–Fyn SH2 agarose conjugates, anti-JNK1 polyclonal antibody, cross-reacting with other JNK variants, anti-JNK1 monoclonal antibody, anti-phospho-JNK and anti-Vav antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Rac1 polyclonal antibody, anti-p85 monoclonal antibody and anti-JNK2 polyclonal antibody were purchased from UBI (Lake Placid, NY) and anti-HA monoclonal antibody from Boehringer Mannheim. Wortmannin was obtained from Sigma (St Louis, MO) and pp1 from Calbiochem (San Diego, CA).

Fyn kinase and binding assays

To determine Fyn kinase activity, 10^7 cells were lysed in the modified RIPA buffer [50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 50 μM $ZnCl_2$, 25 mM NaF, 20 mM β-glycerophosphate, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate]. Cleared lysates were normalized for protein content and incubated for 1.5 h with beads coupled with anti-Fyn antibody. Immunoprecipitates were washed twice with Triton-only lysis buffer (50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 mM sodium orthovanadate), twice with washing buffer (50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 0.1% Triton X-100) and once with the assay buffer (50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$). After washes, 20 μl of kinase buffer containing 1.5 mM $p34^{cdc2}$-derived substrate peptide (UBI, Lake Placid, NY), 200 μM ATP and 0.5 μCi/ml [γ-^{32}P]ATP were added to the beads, and the reaction was allowed to proceed for 15 min at 30°C. The reactions were placed on ice and stopped by spotting of 10 μl of supernatant on phosphocellulose paper. Each reaction was spotted in duplicate. Filters were washed in 0.85% phosphoric acid, and bound ^{32}P radioactivity was measured in a liquid scintillation counter.

For Fyn binding studies, 5×10^8 E86 cells were lysed in the modified RIPA buffer lacking SDS. Cleared lysates were normalized for protein content and incubated for 1.5 h with beads coupled to GST–Fyn SH2 fusion protein or beads coupled to GST alone. Beads were washed twice with Triton-only lysis buffer and twice with washing buffer, proteins were resuspended in the sample buffer, separated by SDS–PAGE and blotted with anti-Kit polyclonal antibody.

Proliferation assay

The proliferation assay was performed as described previously (Yee *et al*., 1994). Briefly, cells were starved of growth factors in RPMI complete for 12 h, 10^5 cells were seeded at 0.2 ml/well in triplicate in 96-well plates, followed by stimulation with KL (200 ng/ml) or IL-3 (20 ng/ml) for 24 h. Cytokines were used at concentrations which produced a maximal proliferative response (data not shown). After 20 h, 0.5 μCi of [^{3}H]thymidine was added for 4 h. Cells were harvested and ^{3}H radioactivity was measured in a liquid scintillation counter.

Determination of apoptosis: AnnexinV–FITC binding assay

For apoptosis assays, 10^6cells were seeded at 2 ml/well in 6-well plates. After treatment with apoptotic stimuli as described in the figure legends, cells were collected into FACS tubes, washed once with AnnexinV binding buffer [10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.5% bovine serum albumin (BSA)] and incubated for 30 min in 100 μl of binding buffer containing 10 μl of AnnexinV–FITC (Biosourse), in the dark. Cells were washed twice with the binding buffer without BSA, resuspended in phosphate-buffered saline (PBS) and analyzed by FACS. The fraction of green fluorescent cells represented the fraction of cells undergoing apoptosis.

MAPK and JNK in vitro kinase assays

A total of 10^7 cells were collected in lysis buffer (50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 50 μM $ZnCl_2$, 25 mM NaF, 20 mM β-glycerophosphate, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF and 1 mM sodium orthovanadate), cleared lysates were normalized for protein content and MAPK or JNK were immunoprecipitated with antibody-pre-coupled beads for 2 h at 4°C. The immunoprecipitates were washed three times in NP-40 buffer [1% NP-40, 2 mM sodium orthovanadate in PBS), once in LiCl buffer (10 mM Tris–HCl, 500 mM LiCl, 1 mM dithiothreitol (DTT)] and once in kinase buffer (12.5 mM MOPS pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT).

MAPK assays were done as follows: 20 μl of kinase buffer containing 2 mg/ml of MBP peptide (UBI, Lake Placid, NY), 200 μM ATP and 0.5 μCi/ml [γ-^{32}P]ATP were added to the beads and the reaction was allowed to proceed for 15 min at 30°C. The reactions were placed on ice and stopped by spotting of 10 μl of supernatant on phosphocellulose paper. Each reaction was spotted in duplicate. Filters were washed in 0.85% phosphoric acid and bound ^{32}P radioactivity was measured in a liquid scintillation counter.

JNK assays were done as follows: 30 μl of kinase buffer containing 4 mg/ml of GST–Jun-1–135 (Verheij *et al*., 1996), 25 μM ATP and 1 μCi/ml [γ-^{32}P]ATP were added to the beads and the reactions were incubated for 20 min at 30°C. Reactions were stopped by addition of sample buffer and proteins separated by SDS–PAGE.

Rac1 translocation assay

Activation of Rac proteins correlates with their translocation from the Triton X-100-soluble fraction to the Triton X-100-insoluble fraction (el Benna *et al*., 1994). To determine translocation of Rac1 upon KL stimulation, 10^7 cells were lysed in the hypotonic Triton X-100 lysis buffer (20 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 8% sucrose, 0.5% Triton X-100, 5 mM EGTA) supplemented with protease and phosphatase inhibitors, normalized for protein content and subjected to ultracentrifugation, 60 000 r.p.m. for 2 h. After centrifugation, the pellet was used as a Triton X-100-insoluble fraction. Pellets were resuspended in SDS-sample buffer, boiled for 10 min, and proteins were separated by SDS–PAGE and blotted with anti-Rac1 antibody.

Immunoprecipitation and Western blotting

Cells were lysed in Triton X-100 lysis buffer, cleared lysates were normalized for protein content and proteins were immunoprecipitated with beads coupled with polyclonal antibodies specific for Kit, JNK and Vav for 2 h at 4°C. 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% non-fat milk or 5% BSA for anti-phosphotyrosine blotting, overnight. Membranes were incubated for 1 h with the following antibodies: anti-Kit polyclonal antibody (1:500), anti-phosphotyrosine antibody (1:1000), anti-phospho-JNK antibody (1:50),

anti-Vav antibody (1:500), anti-Rac1 antibody (1:1000), anti-p85 antibody (1:500) and anti-HA antibody (1:500). Proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

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