Synergistic Growth of Stem Cell Factor and Granulocyte Macrophage Colony-stimulating Factor Involves Kinase-dependent and -independent Contributions from c-Kit*

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Stem cell factor (SCF) binds and activates the receptor tyrosine kinase c-Kit, and this interaction is critical for normal hematopoiesis. SCF also synergizes with a variety of growth factors, including those binding members of the cytokine receptor superfamily. The mechanisms mediating this synergy remain to be defined. The present study investigates both structural and biochemical cross-talk between c-Kit and the receptor for granulocyte macrophage colony-stimulating factor (GM-CSF). We have found that c-Kit forms a complex with the β-chain of the GM-CSF receptor, and this interaction involves the first part of the c-Kit kinase domain. Although inhibition of c-Kit kinase activity completely blocked SCF-induced proliferation, there was still greater than additive growth induced by SCF in combination with GM-CSF. In contrast, an inhibitory antibody against the extracellular domain of c-Kit (K-27) completely inhibited growth in response to SCF alone or in combination with GM-CSF. These results support a kinase-independent component of the synergistic growth induced by SCF and GM-CSF that may relate to interaction of these receptors. It is also clear that a significant part of the synergistic growth is dependent of c-Kit kinase activity. Although synergistic increases in phosphorylation of c-Kit and the β-chain of the GM-CSF receptor were not observed, SCF and GM-CSF in combination prolonged the duration of Erk1/2 phosphorylation in a phosphatidylinositol 3-kinase-dependent manner. Consistent with these findings, phosphatidylinositol 3-kinase is synergistically activated by SCF and GM-CSF together. Hence, c-Kit makes both kinase-independent and -dependent contributions to the proliferative synergy induced by SCF in combination with GM-CSF.

The mechanisms mediating hematopoiesis are not fully understood but require interactions between a wide array of cell surface receptors with growth factors and cytokines. Two receptor families important for hematopoiesis are the cytokine receptor superfamily and the receptor tyrosine kinase superfamily. Ligands for the cytokine receptors include granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin (Epo). The receptor for human GM-CSF is comprised of a GM-CSF-specific α-chain and a β-chain shared with the receptors for IL-3 and IL-5 (1, 2). Upon GM-CSF stimulation the α- and β-chains cluster together, bringing associated cytoplasmic tyrosine kinases close together resulting in activation and subsequent tyrosine phosphorylation of the β-chain. Stem cell factor (SCF) binds to the receptor tyrosine kinase c-Kit leading to its homodimerization and autophosphorylation (3). Phosphorylated tyrosine residues, in c-Kit and the β-chain, function as docking sites for signal transduction molecules with SH2 domains. The SH2 domain is a stretch of ~100 amino acids that binds phosphorylated tyrosine residues in an amino acid sequence context-dependent manner (4).

Previous studies have suggested that c-Kit may play a role in responses mediated by IL-3 and GM-CSF. Certain c-Kit inhibitory antibodies reduce colony formation induced by GM-CSF (5). Further, a decrease in IL-3-induced proliferation was seen after treatment of CD34+ bone marrow progenitors with anti-sense oligonucleotides against c-Kit (6). The A/J mice strain do not express the α-chain of the IL-3 receptor and hence do not respond well to IL-3 as a single factor (7, 8). However, these mice still respond synergistically to IL-3 in combination with SCF. It has been shown that the Epo receptor and c-Kit form a physical complex that allows trans-phosphorylation to occur, and this has been suggested to play an important role in erythroid colony formation (9, 10). Taken together these studies support a role for c-Kit in cytokine signaling. Thus, SCF synergizes with ligands for the cytokine receptor superfamily members (11). However, the molecular mechanisms underlying these events remain to be established. Because the in vivo effects of SCF are mediated primarily in combination with other factors, it is vital to have a detailed understanding of this process. Synergistic activation of early signal transduction pathways, including both the Erks and Stat proteins, has been reported (12–17). However, there are a lot of inconsistent results that may relate to variations in experimental procedures and/or the lineage and differentiation state of the cells used as model systems. Information concerning mechanisms mediating synergistic proliferation is valuable for designing means for optimal in vitro expansion of primitive hematopoietic stem and progenitor cells as well as for other therapeutic uses.

In the search for a molecular mechanism behind the synergy between SCF and GM-CSF we found that c-Kit and the β-chain form a complex. Several lines of evidence suggest this complex

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† The abbreviations used are: GM-CSF, granulocyte macrophage colony-stimulating factor; SCF, stem cell factor; IL-3, interleukin-3; IL-5, interleukin-5; Epo, erythropoietin; GST, glutathione S-transferase; MAP, mitogen-activated protein; PI, phosphatidylinositol; Mek, MAP kinase/ERK kinase; FCS, fetal calf serum.

BBD, Ras binding domain; Erk, extracellular signal-regulated kinase; Stat, signal transducers and activators of transcription; MAP, mitogen-activated protein; PI, phosphatidylinositol; Mek, MAP kinase/ERK kinase; FCS, fetal calf serum.
plays a structural role in synergy: 1) SCF in combination with GM-CSF induced greater than additive proliferation in the presence of a c-Kit kinase inhibitor, and 2) an antibody specific for a portion of the c-Kit extracellular domain reduced proliferation in response to SCF and GM-CSF, both alone and in combination. However, from our results it is clear that a large part of the synergy is dependent on c-Kit enzymatic activity. SCF and GM-CSF in combination induces a sustained phosphorylation of proteins in the MAP kinase pathway, and this correlated with synergistic activation of PI 3-kinase. In contrast, activation of the MAP kinases Erk1/2 by SCF and GM-CSF as single factors does not require PI 3-kinase activity. Pharmacological inhibition of Mek1/2 partially reduced synergistic proliferation. Thus, the Erk cascade contributes to the proliferative response by these factors. In conclusion, multiple mechanisms contribute to synergistic growth induced by SCF and GM-CSF.

EXPERIMENTAL PROCEDURES

Antibodies and Inhibitors—Antibodies specific for phosphorylated Mek1/2 (Cell Signaling Technology; Beverly, MA), Erk1/2 (Cell Signaling Technology), and c-Kit (sc-678) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies recognizing c-Kit (sc-168) and the GM-CSF receptor β-chain (sc-878) and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-Ras antibody (505-516) and GST-Raf-RBD (14-278) were from Upstate Biotechnologies (Lake Placid, NY). The PI 3-kinase inhibitor wortmannin was from Sigma, whereas LY294002, PD98059, and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein kinase wortmannin was from Sigma, whereas LY294002, PD98059, and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies and Inhibitors—Antibodies specific for phosphorylated Mek1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies recognizing c-Kit (sc-168) and the GM-CSF receptor β-chain (sc-878) and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-Ras antibody (505-516) and GST-Raf-RBD (14-278) were from Upstate Biotechnologies (Lake Placid, NY). The PI 3-kinase inhibitor wortmannin was from Sigma, whereas LY294002, PD98059, and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies and Inhibitors—Antibodies specific for phosphorylated Mek1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies recognizing c-Kit (sc-168) and the GM-CSF receptor β-chain (sc-878) and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-Ras antibody (505-516) and GST-Raf-RBD (14-278) were from Upstate Biotechnologies (Lake Placid, NY). The PI 3-kinase inhibitor wortmannin was from Sigma, whereas LY294002, PD98059, and phosphotyrosine (sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

SCF and GM-CSF-Mediated Synergy in MO7e Cells—We were interested in understanding the mechanism(s) mediating synergistic growth by SCF and GM-CSF. Previous work has shown that the megakaryoblastic cell line MO7e is a suitable model for studying synergy (19, 20). Fig. 1A illustrates the greater than additive titrated thymidine incorporation of these cells in response to GM-CSF and SCF combined, as compared with SCF alone. Next we investigated synergistic growth of MO7e cells in the presence of reagents that interfere with c-Kit function through two distinct mechanisms. One involved inhibition of c-Kit kinase activity, and the other targeted a structural domain in the c-Kit extracellular region with an antibody. Addition of a low molecular weight inhibitor targeting c-Kit kinase activity (AG1296) resulted in a complete inhibition of SCF-induced proliferation but had no significant effect on proliferation induced by GM-CSF (Fig. 1B). This contrasted the minimal effects of the Me3SO vehicle control on growth. Fig. 5 (see below)
illustrates that AG1296 completely blocked SCF-induced phosphorylation of c-Kit and has no effect of GM-CSF-induced phosphorylation of the \(/H9252\)-chain subunit of the receptor. Interestingly, SCF and GM-CSF still induced synergistic proliferation, albeit reduced, in the presence of this c-Kit inhibitor (Fig. 1B). This suggests a structural role for c-Kit in synergistic growth with GM-SCF, in addition to a kinase-dependent signaling function. To further investigate the possibility of a structural role for c-Kit in synergistic growth, we included an inhibitory antibody (K-27), specific for the fourth immunoglobulin-like domain in the extracellular region of c-Kit (21), in the proliferation assay. This resulted in complete inhibition of the SCF-induced DNA synthesis (Fig. 1B). Interestingly, this antibody also inhibited GM-CSF-induced proliferation (50–60% reduction), as well as blocked synergistic growth of SCF and GM-CSF in combination. An isotype-matched control antibody had minimal effect on growth. One model compatible with these data is that the K-27 antibody interferes with a physical complex between c-Kit and the \(\beta\)-chain of the GM-CSF receptor.

c-Kit Forms a Complex with the \(\beta\)-Chain of the GM-CSF Receptor—To determine if the \(\beta\)-chain of the GM-CSF receptor associates with c-Kit, we immunoprecipitated the \(\beta\)-chain from cells stimulated with SCF, GM-CSF, or the combination. The immunocomplex was resolved using SDS-PAGE and transferred to an Immobilon-P membrane. Fig. 2 (top panel) shows a phosphotyrosine immunoblot displaying bands with sizes corresponding to c-Kit (\(-160\) kDa) and the \(\beta\)-chain (\(-130\) kDa) in the \(\beta\)-chain immunoprecipitate. To confirm the identity of the phosphotyrosine bands, the membrane was reprobed with antibodies specific for c-Kit and \(\beta\)-chain (Fig. 2, middle panel and lower panel). This experiment shows that a fraction of the c-Kit population is constitutively associated with the \(\beta\)-chain, and within this complex c-Kit can be activated by SCF. Notably, the integrity of this complex was highly dependent on lysis condi-

FIG. 1. SCF combined with GM-CSF induces synergistic proliferation in MO7e cells. A, MO7e cells were incubated in RPMI supplemented with 10% FCS in the presence of SCF (50 ng/ml), GM-CSF (5 ng/ml), or both for 48 h followed by a 5-h \(\text{[3H]}\)thymidine pulse. B, \(\text{[3H]}\)thymidine incorporation assay of MO7e cells as described but in the presence of reagents that interfere with c-Kit signaling. Results are plotted as mean of triplicate samples. The results of a representative experiment of four are shown.

![Graph showing CPM for different conditions](image-url)
immunoprecipitation; phosphorylation of the Reciprocal Receptor—the c-Kit extracellular domain.

bilon-P membranes, immunoblotting with phosphotyrosine and subjected to immunoprecipitation with an anti-β-chain antibody, followed by 8% SDS-PAGE and transfer to an Immobilon-P membrane. After blocking, the membrane was probed with anti-phosphotyrosine, c-Kit, or β-chain antibodies. The results of a representative experiment of four are shown. C: SCF; G: GM-CSF; SG: SCF plus GM-CSF; Ip: immunoprecipitation; Ib: immunoblot; C: immunoprecipitation with species-matched control antibody.

To map the region of c-Kit involved in binding to the β-chain, we utilized GST fusion proteins of the different regions of the c-Kit intracellular domain. These include the juxtamembrane region (JXM, amino acids 544–577), the first part of the catalytic domain (K1, amino acids 578–685), the kinase insert (INS, amino acids 686–762), the second part of the catalytic domain (K2, amino acids 763–925), and the C-terminal tail (CT, amino acids 926–976). Because the association detected in the co-immunoprecipitation experiment was constitutive and, thus, independent of the phosphorylation status, we utilized unphosphorylated GST fusion proteins. Fig. 3A demonstrates that the β-chain co-immunoprecipitated most readily with the first part of the kinase domain. Equal loading of GST fusion proteins was verified by Ponceau S staining (Fig. 3B). This experiment indicates an intracellular mechanism for the interaction between c-Kit and the β-chain but does not exclude contributions from the c-Kit extracellular domain.

Stimulation with SCF or GM-CSF Does Not Result in Trans-phosphorylation of the Reciprocal Receptor—To investigate the possibility of trans-phosphorylation between c-Kit by kinases associated with the GM-CSF receptor, we stimulated MO7e cells for various periods of time with SCF, GM-CSF, or the combination. Cells were lysed, and c-Kit was immunoprecipitated and resolved using SDS-PAGE. After transfer to Immobilon-P membranes, immunoblotting with phosphotyrosine and c-Kit antibodies was performed. As can be seen in Fig. 4, no significant phosphorylation of c-Kit could be detected after GM-CSF treatment. Furthermore, stimulation with SCF together with GM-CSF did not enhance phosphorylation of c-Kit beyond that seen by SCF alone. These results do not exclude the possibility that trans-phosphorylation of c-Kit occurs at one or more specific tyrosine residues with such low stoichiometry that it is not detected by global phosphotyrosine immunoblotting. However, phosphospecific antibodies directed to tyrosine residues 703, 719, 823, and 936 in c-Kit did not reveal any significant differences in phosphorylation levels after stimulation with SCF alone or in combination with GM-CSF (see Fig. 4A and data not shown). This result is of importance because it contrasts the results for c-Kit and the Epo receptor where trans-phosphorylation has been observed and suggested to contribute to synergy (9).

To assess whether c-Kit contributed to GM-CSF-induced tyrosine phosphorylation of the β-chain, cells were pre-treated with a low molecular weight inhibitor against c-Kit (AG1296) before stimulation. c-Kit or the β-chain were immunoprecipitated, and proteins were separated by SDS-PAGE followed by transfer to Immobilon-P membranes and subsequent immunoblotting. Fig. 5A illustrates that preincubation with AG1296 completely blocks SCF-induced tyrosine phosphorylation of c-Kit. In contrast, tyrosine phosphorylation of the β-chain is not significantly affected by AG1296 (Fig. 5B). Thus, GM-CSF receptor β-chain tyrosine phosphorylation is not dependent on c-Kit kinase activity.

In summary, our findings demonstrate that c-Kit and the β-chain component of the GM-CSF receptor interact in vitro and in situ. This interaction is constitutive and does not lead to trans-phosphorylation of either receptor. Further, inhibition of c-Kit activity does not completely eliminate synergistic growth in response to SCF and GM-CSF. These data in combination with the striking effect of an antibody specific for the fourth immunoglobulin-like domain of c-Kit on synergy in response to SCF with GM-CSF support the possibility of kinase-independent mechanisms contributing to synergistic growth in response to SCF and GM-CSF.

The MAP Kinase Pathway Is Synergistically Activated by SCF in Combination with GM-CSF—Although the above data demonstrate that c-Kit makes kinase-independent contributions to synergy, treatment with an inhibitor of c-Kit (Fig. 1B) suggested kinase-dependent components as well. Accordingly, we next sought to define kinase-dependent components of synergistic growth. Figs. 4 and 5 demonstrate that neither c-Kit nor the β-chain component of the GM-CSF receptor was phosphorylated synergistically in response to both factors in combination. This finding is supported in the literature (22, 23). SCF activates a variety of different signaling pathways downstream of activated c-Kit (24). Consequently we next were interested in addressing whether one or more pathways downstream of c-Kit contributed to synergy. One pathway of particular interest is the Ras-MAP kinase cascade. There is conflicting data in the literature relating to whether the Ras-MAP kinase pathway is synergistically activated in response to SCF in combination with GM-CSF or IL-3. Work by O’Farrell et al. (12), Pearson et al. (13), and Lee et al. (15) suggested synergistic activation of this pathway in response to both factors. In contrast, in the studies of Hallek et al. (22) and Miyazawa et al. (25) this was not observed. In an attempt to resolve this controversy we examined the effect of SCF and GM-CSF on activation of the Ras-MAP kinase pathway. We first examined the role of c-Kit kinase activity in SCF-induced activation of Erk1/2. Indeed, studies with the c-Kit inhibitor demonstrate that activation of this pathway is dependent on c-Kit activity (data not shown). Phosphorylation of both Erk1/2 and Mek1/2 on residues Thr202/Tyr204 and Ser217/Thr221, respectively, closely correlates with activation of these kinases. Consequently we used phospho-specific antibodies directed against both these proteins. Both Mek1/2 and Erk1/2 were phosphorylated synergistically after concurrent stimulation with SCF and
GM-CSF (Fig. 6, B and C). Notably, the duration of both Mek1/2 and Erk1/2 phosphorylation is extended after stimulation with both factors. Ras is a key signaling component upstream of both Mek1/2 and Erk1/2. Therefore we next investigated if there was a synergistic increase in Ras activity in response to SCF and GM-CSF in combination. Fig. 6A shows no significant synergistic increase in Ras GTP loading after stimulation with SCF and GM-CSF compared with single factors. In some, but not all experiments, an increase in Ras GTP loading was noted in response to SCF combined with GM-CSF at later time points. These data suggest synergistic activation of the MAP kinase pathway occurs downstream of Ras and upstream of Mek1/2.

**PI 3-kinase Contributes to Synergistic Activation of MAP Kinases**—Previous studies have implicated PI 3-kinase in activation of Erk1/2 (14). How PI 3-kinase modulates Erk1/2 activation is not clear. One possibility is that the phospholipids generated by PI 3-kinase recruits PH domain-containing proteins like Gab2 to the cell membrane where they become phosphorylated and participate in activation of the Ras-MAP kinase pathway (26, 27). Another possibility is that activation of protein kinase Cz by PI 3-kinase lipid products leads to increased Raf-1 kinase activity, culminating in Erk1/2 activation (28, 29). Consequently we next investigated the role of PI 3-kinase in synergistic activation of MAP kinase by SCF in combination with GM-CSF. To this end, MO7e cells were stimulated for different periods of time in the presence or absence of the PI 3-kinase inhibitorwortmannin. Total cell lysates were prepared, separated with SDS-PAGE, and transferred to an Immobilon-P membrane. The membrane was then immunoblotted with phosho-Erk1/2 antibodies. Fig. 7 shows that the extended Erk1/2 phosphorylation in response to concurrent stimulation with SCF and GM-CSF is sensitive to PI 3-kinase inhibition. Similar results were obtained with LY294002, another inhibitor of PI 3-kinase (data not shown). As discussed above a possible mechanism for the PI 3-kinase-dependent and extended Erk1/2 phosphorylation by concurrent stimulation with SCF and GM-CSF could be enhanced phosphorylation of Gab2 due to increased membrane localization. However, we were unable to detect any differences in Gab2 phosphorylation after stimulation with SCF in combination with GM-CSF compared with SCF alone (data not shown). Similar results were obtained for other proteins analyzed, including c-Cbl, CrkII, CrkL, Dok-1, Gab-1, and Grb2 (data not shown).

PI 3-kinase can be activated directly by c-Kit through interaction of p85PI 3-kinasewith tyrosine 721 of human c-Kit or 719 of murine c-Kit. Alternately, PI 3-kinase can be activated through pathways independent of direct interaction with c-Kit. These include recruitment through other proteins such as c-Cbl and CrkL (30). PI 3-kinase can be activated by PI 3-kinase binding site on c-Kit or association of c-Kit with p85PI 3-kinase in response to both factors. In contrast, the enzymatic activity of PI 3-kinase after concurrent stimulation with SCF and GM-CSF was synergistically increased compared with single factors.
These data suggest that enhanced activation of PI 3-kinase contributes to synergistic activation of the Erk pathway in response to SCF and GM-CSF. Interestingly, this is in the absence of an increase in association of c-Kit and PI 3-kinase.

**Erks and PI 3-Kinase Contribute to Synergy**—To assess the functional significance of the Erk pathway in synergistic growth of SCF in combination with GM-CSF, we investigated the effect of PD98059, an inhibitor of Mek1/2. Fig. 9 illustrates that this small molecule inhibitor reduced SCF-induced proliferation by 80%, but had little effect on proliferation induced by GM-CSF. This finding is in agreement with the modest effect of GM-CSF on Mek1/2 and Erk1/2 phosphorylation. PD98059 inhibited synergistic growth in response to SCF and GM-CSF in combination by 40% compared with control cells (Fig. 9A).

Inhibition of PI 3-kinase by LY294002 resulted in a complete block in proliferation induced by SCF or GM-CSF (Fig. 9A). Further, proliferation induced by both factors together was dramatically reduced. Importantly, the cells were viable after the LY294002 or PD98059 treatment as judged by trypan blue exclusion (Fig. 9B). These data indicate that synergistic activation of Erk1/2 downstream of PI 3-kinase makes critical contributions to synergy mediated by SCF in combination with GM-CSF. However, it is also clear that PI 3-kinase has additional roles in proliferation induced by SCF and GM-CSF not related to Erk1/2 activation, because LY294002 completely blocked proliferation by these factors. This is in agreement with other studies showing that PI 3-kinase is essential for c-Kit-dependent growth and tumorigenesis (32–35).

**DISCUSSION**

SCF dramatically enhances growth when combined with other cytokines (11). SCF and GM-CSF induces synergistic proliferation in myeloid progenitor cells, including the megakaryoblastic cell line MO7e (19, 20). Although great progress has been made in understanding the mechanism of action of SCF as a single factor, the mechanisms mediating synergy with other growth factors are still poorly understood. Synergistic effects are of critical importance biologically, because hematopoietic stem cells and early progenitor cells require growth factors in combination for self-renewal and differentiation. Moreover, in the bone marrow microenvironment the physiological actions of SCF occur in combination with other growth factors and extracellular matrix proteins.

In this study we demonstrate that c-Kit forms a physical complex with the β-chain of the GM-CSF receptor, both in vivo by co-immunoprecipitation and in vitro by GST fusion protein pull-down assays (Figs. 2 and 3). This complex is constitutive and independent of phosphorylation of either receptor. Importantly, SCF can still induce tyrosine phosphorylation of c-Kit associated with the GM-CSF receptor. Because inhibition of the c-Kit kinase activity does not lead to a complete block of synergistic interaction between SCF and GM-CSF, part of the synergy observed between SCF and GM-CSF is probably due to a structural role for c-Kit (Fig. 1B). This idea is further sup-
ported by the fact that addition of a monoclonal antibody against c-Kit extracellular domain (K-27) abolishes the synergistic proliferation as well as significantly inhibits GM-CSF-induced proliferation. The K-27 antibody is specific for c-Kit, and the binding epitope has been mapped to immunoglobulin-like domain 4 (21). This finding parallels studies showing that c-Kit interacts with other transmembrane cell surface proteins such as the Epo receptor and TM4SF (9, 36). In these studies as well as in this work the interactions were highly sensitive to lysis conditions.

Physical interactions between different cell surface receptors may contribute to synergy through structural support, facilitation of receptor trans-phosphorylation, or activation of downstream signaling components. Indeed, trans-phosphorylation

**FIG. 6.** The MAP kinase pathway is synergistically activated by SCF in combination with GM-CSF. MO7e cells were stimulated with 50 ng/ml SCF, 5 ng/ml GM-CSF, or the combination as indicated. Total cell lysates were prepared, or a GST-Raf-RBD pull-down assay was performed (A), followed by SDS-PAGE and transferred to an Immobilon-P membrane. After blocking the membrane was probed with pan-Ras (A), phospho-Mek1/2 antibodies (B), or phospho-Erk1/2 antibodies (C). Equal loading was verified by immunoblotting of total Mek1/2 and Erk2, respectively. For the GST-Raf-RBD pull-down assay equal loading was confirmed by running a parallel gel with total cell lysates and pan-Ras immunoblotting. The results of a representative experiment of four are shown. PD, GST fusion protein pull-down assay; Ib, immunoblot; TCL, total cell lysate.

**FIG. 7.** Effect of PI 3-kinase inhibition on MAP kinase phosphorylation. MO7e cells were stimulated with 50 ng/ml SCF, 5 ng/ml GM-CSF, or the combination in the presence of 100 nM wortmannin as indicated. Total cell lysates were prepared and separated on 10% SDS-PAGE and transferred to an Immobilon-P membrane. After blocking the membrane was probed with anti-phospho-Erk1/2 antibodies. Equal loading was verified by total Erk2 immunoblotting. The results of a representative experiment of three are shown. TCL, total cell lysate; Ib, immunoblot.
FIG. 8. PI 3-kinase coupling to c-Kit after stimulation with SCF and GM-CSF as single factors or in combination. MO7e cells were stimulated with 50 ng/ml SCF, 5 ng/ml GM-CSF, or the combination as indicated. The cells were lysed and subjected to immunoprecipitation with an anti-c-Kit antibody (A) or an anti-p85 antibody (B). Immunocomplexes were separated 8% SDS-PAGE and transferred to an Immobilon-P membrane. After blocking, the membrane was immunoblotted as indicated. C shows the enzymatic activity of PI 3-kinase after stimulation with SCF, GM-CSF, or the combination. For each panel, the results of a representative experiment of three are shown. Ip, immunoprecipitation; Ib, immunoblot; C, indicates immunoprecipitation with species-matched control antibody.
occurs in the c-Kit-Epo receptor complex (9). Further, Src family kinases may play a role in this process (37). In contrast, we find no evidence for trans-phosphorylation between c-Kit and the GM-CSF receptor (Figs. 4 and 5). The function of this complex could be to modulate the rate of internalization and/or intracellular trafficking of the receptors. Alternately, it may co-localize the two receptors to facilitate activation of one or more downstream signaling pathways. One group has reported that association of c-Kit and the IL-7 receptor contributes to activation of the JAK/STAT pathway in the absence of IL-7 (38).

A number of groups have examined whether SCF induces synergistic activation of early signaling components when combined with other cytokines. Although there are conflicting reports regarding the effects of SCF and GM-CSF or IL-3 on tyrosine phosphorylation of c-Kit, most groups have not observed synergistic phosphorylation of either receptor component (22, 23). This is supported by findings in the present study (Figs. 4 and 5). However, downstream of receptor phosphorylation, several groups have suggested that SCF can synergistically activate MAP kinases in combination with IL-3, Epo, and GM-CSF (12–15). Lee et al. could show Erk1/2 was synergistically activated by SCF in combination with GM-CSF, but other MAP kinases such as stress-activated protein kinase/c-Jun N-terminal kinase and p38 were not. In contrast, others have not seen synergistic activation of Erk1/2 by SCF combined with other growth factors (22, 25).

Our work supports the possibility that synergistic activation of Erk1/2 contributes to growth mediated by SCF combined with GM-CSF. SCF combined with GM-CSF induced a more sustained phosphorylation of Mek1/2 and Erk1/2 than either factor alone (Fig. 6). The extended duration of Erk1/2 phosphorylation was sensitive to PI 3-kinase inhibition (Fig. 7). Notably, the Erk1/2 phosphorylation induced by single factors was independent of PI 3-kinase. Similarly, earlier studies have shown that PI 3-kinase plays an essential role in the synergistic activation of MAP kinase by SCF and Epo (14). Analysis of the PI 3-kinase binding site phosphorylation and p85$^{III}$-kinase

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**Fig. 9. Inhibition of the MAP kinase pathway or PI 3-kinase reduces proliferation.** A, MO7e cells were incubated in RPMI supplemented with 10% FCS in the presence of SCF (50 ng/ml), GM-CSF (5 ng/ml), or both for 48 h followed by a 5-h [3H]thymidine pulse. The PI 3-kinase inhibitor LY294002 or Mek1/2 inhibitor PD98059 was included as indicated. Results are plotted as mean of triplicate samples. B, MO7e cells were treated as in A but after the 48-h incubation, cell viability was analyzed by trypan-blue exclusion. Results are plotted as mean % viability of triplicate samples. The results of a representative experiment of three are shown.
association to c-Kit did not reveal any significant differences by stimulation with SCF alone or combined with GM-CSF (Fig. 8, A and B). However, PI 3-kinase was synergistically activated in response to combined stimulation compared with single factors (Fig. 8C). Interestingly, we detected an increase in the duration of Erk1/2 phosphorylation, not the magnitude. This is a possible explanation for the conflicting literature on synergistic activation of Erk1/2. In contrast, we do not see a reproducible synergistic increase in the Ras GTP loading in response to concurrent stimulation (Fig. 6A). These results suggest that synergy is induced at a level between Ras and Mek1/2. This is in concurrence with earlier data in the literature showing that Raf-1 antisense oligonucleotides abolish synergistic proliferation between SCF and IL-3 or GM-CSF in hematopoietic progenitor cells (39). Alternatively, it is possible that the prolonged phosphorylation of Mek1/2 and Erk1/2 by the combined stimulation is due to a synergistic inhibition or down-regulation of phosphatases that negatively regulates this pathway.

Because the synergistic phosphorylation of Erk1/2 is dependent on PI 3-kinase, we investigated the effect that inhibition of these pathways has on proliferation. We observed that inhibition of the MAP kinase pathway, using PD98059, partially inhibited the synergistic proliferation (Fig. 9A). Blocking the PI 3-kinase pathway using LY294002 completely blocked synergistic proliferation as well as proliferation induced by single factors (Fig. 9A). Interestingly, inhibition of PI 3-kinase blocked proliferation by single factors more potently than inhibition of the MAP kinase pathway and completely blocked synergistic proliferation (Fig. 9A). This points to a central role for PI 3-kinase in proliferation induced by SCF and GM-CSF alone or in combination, besides its effect on MAP kinase phosphorylation kinetics. This is supported by studies using mast cells from p85α-deficient mice, which display defects in many c-Kit-mediated functions, including reduced SCF-induced proliferation (34, 35).

Downstream of immediate early signaling pathways, it is likely that changes in gene expression culminate in synergistic growth. It is pathways that activated by SCF and the cytokine of interest converge and synergistically activate a common target molecule, e.g. transcription factors leading to synergistic induction of genes involved in survival, growth, or differentiation. This mechanism has been suggested for the synergistic effect of LIF and BMP2 on the differentiation of neuronal progenitor cells (40). Another possible mechanism for synergy is that one factor induces expression of the other growth factor and/or its receptor. There is evidence for this type of mechanism in the megakaryoblastic cell line MO7e, where SCF induces expression of GM-CSF (41).

In conclusion, this study demonstrates for the first time that c-Kit and the β-chain of the GM-CSF/IL-3/IL-5 receptor forms a physical complex. Further, SCF and GM-CSF induce synergistic phosphorylation of proteins in the Ras-MAP kinase pathway, and this correlates with activation of PI 3-kinase. The synergistic effect occurs downstream of Ras and upstream of Mek1/2 in conformity with studies from other groups showing that Raf-1 is critical for synergistic proliferation (39). Inhibition of the MAP kinase pathway partially inhibits synergistic proliferation. Blocking PI 3-kinase signaling potently inhibited DNA synthesis induced by SCF and GM-CSF alone or in combination suggesting an essential role for PI 3-kinase in proliferation induced by these factors. These data suggest that multiple mechanisms contribute to synergistic growth of SCF in combination with GM-CSF.
