Functional and Biochemical Consequences of Abrogating the Activation of Multiple Diverse Early Signaling Pathways in Kit

ROLE FOR Src KINASE PATHWAY IN KIT-INDUCED COOPERATION WITH ERYTHROPOIETIN RECEPTOR*

Received for publication, July 15, 2002, and in revised form, December 9, 2002
Published, JBC Papers in Press, December 13, 2002, DOI 10.1074/jbc.M207068200

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Kit receptor tyrosine kinase and erythropoietin receptor (Epo-R) cooperate in regulating blood cell development. Mice that lack the expression of Kit or Epo-R die in utero of severe anemia. Stimulation of Kit by its ligand, stem cell factor activates several distinct early signaling pathways, including phospholipase Cγ, phosphatidylinositol 3-kinase, Src kinase, Grb2, and Grb7. The role of these pathways in Kit-induced growth, proliferation, or cooperation with Epo-R is not known. We demonstrate that inactivation of any one of these early signaling pathways in Kit significantly impairs growth and proliferation. However, inactivation of the Src pathway demonstrated the most profound defect. Combined stimulation with Epo also resulted in impaired cooperation between Src-defective Kit mutant and Epo-R and, to a lesser extent, with Kit mutants defective in the activation of phosphatidylinositol 3-kinase or Grb2. The impaired cooperation between the Src-defective Kit mutant and Epo-R was associated with reduced transphosphorylation of Epo-R and expression of c-Myc. Remarkably, restoration of only the Src pathway in a Kit receptor defective in the activation of all early signaling pathways demonstrated a 50% correction in proliferation in response to Kit stimulation and completely restored the cooperation with Epo-R. These data demonstrate an essential role for Src pathway in regulating growth, proliferation, and cooperation with Epo-R downstream from Kit.

Receptor tyrosine kinases (RTKs) trigger multitude of cellular events, including proliferation, survival, differentiation, and migration. In response to ligand-induced stimulation, RTKs undergo dimerization and autophosphorylation on several distinct cytoplasmic tyrosine residues (1-4). These phosphorylated tyrosine residues become binding sites for a variety of Src homology 2 domain-containing enzymes and adaptor proteins such as phospholipase Cγ, phosphatidylinositol 3-kinase p85 subunit (PI 3-kinase), Ras GTPase-activating protein, SHP2 phosphatase, Src kinases, Grb2, Grb7, and SHC (5). In this manner, the phosphorylated tyrosine residues initiate signal transduction via several distinct early signaling pathways. A major unresolved question in the field of RTK signaling is whether these diverse signaling pathways result in redundant or nonredundant biological functions. Recent studies utilizing the platelet-derived growth factor (PDGF) RTK have begun to address some of these issues in nonhematopoietic cells (6-8). However, relatively little is known about the biological consequence(s) of activation of diverse signaling pathways by RTKs in hematopoietic cells.

In hematopoietic cells, the RTK Kit plays an essential role in regulating proliferation, survival, differentiation, and migration of stem and progenitor cells (9-11). The proto-oncogene Kit encodes the receptor for stem cell factor (SCF) and belongs to the type III receptor tyrosine kinase subfamily (9-11). This family of cytokine receptors includes the macrophage colony-stimulating factor (M-CSF) receptor, the PDGF receptor, and the Flk-2/Flik-3 receptor (1). The structure of these receptors includes an extracellular domain with five Ig-like motifs, a single short membrane-spanning domain, and a cytoplasmic domain with tyrosine kinase activity (9-11). The kinase domain is separated by a kinase insert sequence that divides the kinase domain into an ATP binding region and phosphotransferase region (9-11). The product of the Kit gene is a transmembrane receptor composed of 976 amino acids (aa) with 519 extracellular aa, a transmembrane domain of 23 aa, and an intracellular tail of 433 aa (9-11). In addition to being expressed on hematopoietic cells, Kit is also expressed on cells of nonhematopoietic origin, including melanocytes, primordial germ cells, and interstitial cells of Cajal (9). In hematopoietic cells, Kit can synergize with other growth factor receptors to promote survival, proliferation, and differentiation of multiple hematopoietic lineages (9-11).

Intriguingly, the most profound phenotype due to the lack of Kit expression in mice is manifested in erythroid cells. Mutant mice that lack the expression of Kit (dominant white spotting, or W, mutants) demonstrate severe deficiencies in erythroid cell development (9, 12). Kit-deficient mice exhibit a severe reduction of colony-forming unit-erythroid (CFU-E) progenitors in the fetal liver and die of anemia around day 16 of gestation (9, 12). Epo-R-deficient mice also demonstrate a similar decrease in CFU-E progenitors and die of anemia between days 13 and 15 of gestation (13), suggesting that erythroid progenitors cannot survive, proliferate, or differentiate unless both Kit and the Epo-R signal transduction pathways are functional. Recent studies have suggested that Epo and Epo-R...
interactions may contribute to this process by preventing erythroid progenitors from undergoing apoptosis by activating Stat5 and subsequently inducing the expression of an anti-apoptotic protein, Bcl-xL (14). Consistent with these studies, mice deficient in the expression of Stat5 or Bcl-xL manifest a decrease in the number of erythroid progenitors due to enhanced apoptosis (14–17). However, the role of Kit in erythroid cell development alone or in combination with Epo-R is poorly understood. To this end, we have recently demonstrated an essential role for Kit in proliferation of erythroid progenitors (18).

Further, we and others have also demonstrated that Kit synergizes with Epo-R in enhancing proliferation and survival of erythroid progenitors (18–25). However, the role of early activating signal transduction pathways downstream from Kit in erythroid cell growth, proliferation, and cooperation with Epo-R is not known.

Activated Kit binds signaling molecules at specific tyrosine residues: PLC-γ at tyrosine 728 (26), PI 3-kinase at tyrosine 719 (28), Src class kinases at positions 567/569 (27, 29–31), Grb2 at tyrosine 702 (32), and Grb7 at tyrosine 934 (32). Other classes of signaling proteins have also been reported to bind activated Kit with unknown sequence specificity (33). Classes of signaling proteins have also been reported to bind activated Kit with unknown sequence specificity (33). Studies in multiple cell types have shown that Kit carrying tyrosine mutations to phenylalanine residues at the critical residues fail to bind the associated signaling molecules and consequently fail to activate these signaling pathways (10, 11).

In this study, we took advantage of the ability of Tyr → Phe mutations in Kit to block the activation of downstream signaling molecules to comprehensively investigate the effect of lack of activation of five early signaling pathways in Kit-induced growth, proliferation, and cooperation with Epo-R in a relevant cell type. Utilizing an erythroid progenitor cell line (G1E-ER2) that closely mimics primary proerythroblasts (15, 18, 38, 39) and primary fetal liver cells, we demonstrated that inactivation of any one of these five early signaling pathways in Kit significantly impairs growth and proliferation in response to Kit activation. The most profound defect was observed in Kit mutants impaired in the activation of the Src kinase pathway. Interestingly, when stimulated in combination with erythropoietin (Epo), the mutant of Kit deficient in the activation of Src kinase demonstrated a profound reduction in proliferation, which was associated with impaired transphosphorylation of Epo-R and expression of c-Myc. Remarkably, restoration of the Src kinase pathway in Kit, alone, in the absence of remaining four early signaling pathways restored the cooperation and transphosphorylation of Epo-R in response to Kit activation.

These results demonstrate an essential role for the Src signaling pathway in regulating growth, proliferation, and cooperation with Epo-R downstream from Kit.

**EXPERIMENTAL PROCEDURES**

**Cell Line—G1E-ER2 cells have been described previously (15). G1E-ER2 cells were grown in Iscove's modified Dulbecco's medium (Invitrogen) with 15% heat-inactivated fetal bovine serum (Fisher), recombinant Epo (2 units/ml) (Amgen, Thousand Oaks, CA), and recombinant rat SCF (50 ng/ml) (Amgen).**

**Construction of Wild Type (WT) and Mutant Chimeric Kit Receptors (CHR)—The CHR gene was constructed from DNA encoding aa 1–513 of the human M-CSF receptor and aa 528–977 of the murine Kit receptor joined at an EcoRI site. Plasmid containing the human full-length M-CSF receptor cDNA (a kind gift of Dr. Sherr, St. Jude's, Memphis, TN) was utilized. Forward (Not-I-containing) and reverse (EcoRI-containing) primers corresponding to the start site and transmembrane region of the M-CSF receptor were utilized to perform PCR on the extracellular domain of the M-CSF receptor. Forward (EcoRI-containing) and reverse (XhoI-containing) primers corresponding to the transmembrane and the stop site were used to perform PCR on the transmembrane and the cytoplasmic domain of the murine Kit receptor. The PCR product was digested and ligated into the Not-I and XhoI sites of pBluescript IIIC bicistronic retroviral expression vector (40). The sequence of the CHR was verified. To generate mutant CHRs, the Not1-XhoI WT CHR DNA fragment (2.9 kb) spanning the sites to be mutated was subcloned into Bluescript. The QuickChange site-directed mutagenesis kit (Stratagene) and primers containing the appropriate mutations were used to mutate tyrosine residues 567, 569, 702, 719, 728, and 745 in murine Kit receptor was verified by sequencing released from Bluescript and religated into the Not1-XhoI site of MIEG3 bicistronic retroviral vector. In some experiments, mutant CHR lacking all six tyrosine residues was used as a template to restore phenylalanine mutations at positions 567 and 569 back to tyrosine. Utilizing this bicistronic retroviral vector, we inserted the WT and the mutant CHR cDNAs upstream of the internal ribosome entry site (IRES) and the enhanced green fluorescence protein (EGFP) gene (see Fig. 1).

**Expression of WT and Mutant CHRs in G1E-ER2 Cells—To produce WT and mutant CHR viral supernatants for infection of G1E-ER2 cells, Phoenix ecotropic cells were transiently transfected with WT or the mutant CHR retroviral constructs using Lipofectamine Plus reagent (Invitrogen). Supernatants were collected 48 h after transfection, filtered through 0.45-μm membranes, and used. Cells were infected with 2 ml of virus supernatant in the presence of 8 μg/ml polybrene. Virus-infected cells were harvested 48 h later, sorted by a fluorescence-activated cell sorter, and expanded in culture. G1E-ER2 cells expressing similar levels of EGFP and M-CSF receptors were utilized to perform all of the experiments described in these studies.**

**Flow Cytometric Analysis—Phycocerythrin (PE)-conjugated secondary monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect the antibody to the extracellular domain of the...
Fig. 2. Expression, biochemical, and functional analysis of WT CHR. A, schematic of retroviral vectors MIEG3 and MIEG3-CHR. An improved Moloney leukemia stem cell virus-based bicistronic retroviral vector, MIEG3, expressing EGFP (x axis) and the M-CSF receptor (y axis) in G1E-ER2 cells transduced with the WT CHR and MIEG3-CHR. An improved Moloney leukemia stem cell virus-based bicistronic retroviral vector, MIEG3, expressing EGFP was utilized. WT and mutant CHRs were cloned in front of the internal ribosome entry site (IRES). LTR, long terminal repeat. B, expression of the WT CHR by flow cytometry. The left panel (y axis) demonstrates lack of M-CSF receptor expression in the parental (untransduced) G1E-ER2 cells stained with a PE-conjugated antibody against M-CSF receptor. The right panel demonstrates co-expression of both EGFP (x axis) and the M-CSF receptor (y axis) in G1E-ER2 cells transduced with the WT CHR. C, phosphorylation of WT CHR in response to M-CSF stimulation. Cells expressing endogenous Kit or WT CHR were stimulated for 10 min with either SCF (lane 2) or M-CSF (lane 4) or left unstimulated (lanes 1 and 3) and subjected to IP with an anti-Kit (lanes 1 and 2) or an anti-M-CSF (lanes 3 and 4) receptor antibody and subjected to Western blot analysis with an anti-phosphotyrosine antibody (top panel). D, analysis of M-CSF-induced proliferation in the absence and presence of Epo in G1E-ER2 cells expressing the WT CHR. Parental (A) or WT CHR (B) expressing G1E-ER2 cells were cultured in the presence of M-CSF or Epo or SCF or a combination of SCF, M-CSF, and Epo for 48 h. Proliferation was measured by thymidine incorporation assay. Bars denote the mean thymidine incorporation (cpm ± S.D.) of at least two independent experiments performed in replicates of six. *, p < 0.05 for Epo and M-CSF + Epo versus M-CSF and no growth factor (D, panel A). **, p < 0.05 for SCF versus Epo and M-CSF + Epo (D, panel A). ***, p < 0.05 for SCF + Epo versus SCF (D, panel A). #, p < 0.05 for M-CSF and SCF versus no growth factor (D, panel B). **, p < 0.05 for M-CSF + Epo and SCF + Epo versus SCF (D, panel B).

CHR (M-CSF receptor; 2-4A5; Santa Cruz Biotechnology). G1E-ER2 cells (1 × 10^6) expressing WT or mutant CHRs were incubated at 4°C for 30 min with 1 μg of the primary antibody. Cells were washed three times with phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma) and incubated with a secondary antibody for 30 min at 4°C, washed as above, and analyzed by a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA).

Effect of M-CSF and Epo on Proliferation and Survival of G1E-ER2 Cells—The effect of M-CSF and Epo on proliferation of G1E-ER2 cells was assessed by thymidine incorporation. 96-Well tissue culture plates were utilized for these studies. G1E-ER2 cells expressing WT or mutant CHRs were plated at 5 × 10^4 cells/well for 48 h, either in the absence or presence of M-CSF (50 ng/ml) and/or Epo (2 units/ml). Subsequently, 1.0 μCi of [3H]thymidine was added to each well for 6–8 h at 37°C. Cells were then harvested using an automated cell harvester (96-well harvester; Brandel, Gaithersburg, MD), and thymidine incorporation was determined in a scintillation counter. The effect of M-CSF and/or Epo on cell growth and viability was performed by plating 1 × 10^5 cells in a six-well tissue culture plate in replicates of three for 24 and 48 h, after which cells were subjected to trypan blue and counted under the microscope. Viable cells were scored at various time points.

Erythroid Colony Analysis—Fetal liver cells were harvested from day 12.5 WT embryos. Single cell suspensions were prepared and incubated with or without retrovirus expressing the WT CHR or CHR mutants containing tyrosine to phenylalanine mutations at positions 567 and 569 as described above. After infection, cells were plated in triplicate in α-methylcellulose (Stemcell Technologies, Vancouver, Canada) with Epo (2 units/ml) or Epo plus M-CSF (2 units/ml Epo and 100 ng/ml M-CSF). Benzidine-positive colonies were counted 2–3 days after plating.

Immunoprecipitation—Immunoprecipitations (IPs) were performed as previously described (41). Briefly, cells expressing either the WT or the mutant CHR deficient in the activation of Src kinases (CHR 567/569) or Src add-back (CHR 567/569B) CHR mutants were stimulated for the indicated times. Thereafter, cells were lysed in lysis buffer (10 mM K_2HPO_4, 1 mM EDTA, 50 mM EGTA, 10 mM MgCl_2, 1 mM Na_3VO_4, 50 mM β-glycerol phosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A (pH 7.2)). Lysates were clarified by centrifugation at 10,000 × g, 4°C for 30 min. IP was performed by incubating equivalent amounts of cell lysates with either anti-EpoR or an anti-Kit or anti-M-CSF receptor antibody overnight at 4°C (all from Santa Cruz Biotechnology). Protein A- or protein G-Sepharose beads (Amersham
Biosciences) were used to collect the antigen-antibody complexes. IPs were separated by SDS-PAGE, and proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). After blocking residual binding sites on the transfer membrane by incubating the membrane with 5% milk overnight, Western blot analysis using an anti-phosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) and Supersignal West Dura extended duration detection system (Pierce) was utilized according to the manufacturer’s instructions.

Western Blot Analysis—For Western blot analysis, $1 \times 10^5$ WT CHR and CHR 567/569 were plated in duplicate six-well tissue culture plates for 12 and 24 h at 37°C in the presence or absence of M-CSF (100 ng/ml) and/or Epo (2 units/ml). Thereafter, cells were harvested and lysed in lysis buffer as described above. An equal amount of protein was fractionated on a 10% SDS-PAGE gel and electrophoretically transferred to nitrocellulose membrane. Expression of c-Myc was determined by using an anti-c-Myc antibody (Santa Cruz Biotechnology). Expression of Bcl-xL was determined by using an anti-Bcl-xL antibody (Invitrogen). Activation of Stat5 was determined by utilizing a phosphospecific Stat5 antibody (Cell Signaling, Beverly, MA).

RESULTS

Construction of Wild Type and Mutant Chimeric Kit Receptors—To analyze the role of diverse early signaling pathways activated via Kit in response to SCF stimulation in erythroid cells, we utilized an erythroid progenitor cell line G1E-ER2 (15). These cells were utilized to specifically examine the role of Kit and Epo-R in the context of a cell type that is most affected due to mutations in Kit and Epo-R. We and others have previously shown that these cells mimic primary proerythroblasts
and respond to both SCF and Epo (15, 18). Since G1E-ER2 cells express endogenous Kit receptors, we constructed eight chimeric Kit receptors (CHRs) to bypass endogenous Kit receptors (Fig. 1). The CHR approach to investigate the role of a specific signaling pathway in nonhematopoietic cells downstream from a RTK, such as the PDGF receptor, has been previously described (6, 42). The M-CSF receptor and Kit belong to the same subfamily of RTKs but possess distinct ligand binding specificity (1). G1E-ER2 cells do not express endogenous M-CSFR (Fig. 2B, left panel) and show no response to M-CSF stimulation (Fig. 2D, panel A). Based on these observations, we cloned a cDNA encoding a protein consisting of the extracellular domain of the human M-CSFR and the transmembrane and the cytoplasmic domain of murine Kit (Fig. 1). This CHR is activated upon binding M-CSF but signals in a fashion similar to endogenous Kit receptor (Fig. 2, C and D (panel B)). Eight mutant chimeric receptor cDNAs were constructed, encoding tyrosine to phenylalanine mutations in the cytoplasmic domain of Kit. These mutant receptors cannot bind and activate signaling molecules (10, 11, 26–29, 31, 43). We mutated the binding sites for Src kinases at positions 567 and 569 of the Kit cytoplasmic domain, PI 3-kinase at position 719, PLC-γ at position 728, Grb2 at position 702, an additional mutation at position 745, a double mutant encoding tyrosine to phenylalanine mutations at positions 567 and 569, and a “naked” receptor that encodes tyrosine to phenylalanine mutations at all six positions (Fig. 1).

Expression and Function of Wild Type Chimeric Receptor—For expression, biochemical and functional analysis of the WT CHR, we cloned this receptor into a bicistronic retroviral vector MIEG3 (Fig. 2A) that expresses the EGFP via an internal ribosome entry site and generated viral supernatants as described under “Experimental Procedures.” We have previously reported the use of this vector in generating high transduction of hematopoietic cells (40). After infection of G1E-ER2 cells, EGFP-positive cells were sorted to homogeneity and utilized to perform functional and biochemical studies. Flow cytometry was utilized to examine the expression of M-CSFR in G1E-ER2 cells expressing the WT CHR. Fig. 2B (left panel) demonstrates complete lack of M-CSFR expression in parental untransduced G1E-ER2 cells stained with a PE-conjugated antibody against M-CSFR (y axis). Fig. 2B (right panel) demonstrates 100% co-expression of both EGFP and M-CSFR in G1E-ER2 cells transduced with the WT CHR.

To determine whether ligand-induced phosphorylation of the WT CHR is similar to endogenous Kit receptor, we starved the cells for 6 h of serum and growth factors, and stimulated them with either 100 ng/ml recombinant rat SCF or human M-CSF for 10 min, after which cells were lysed and subjected to immunoprecipitation using an anti-Kit antibody or an anti-M-CSFR antibody and subjected to Western blot analysis using an anti-phosphotyrosine antibody. As shown in Fig. 2C, stimulation of G1E-ER2 cells expressing the WT CHR with SCF (lane 2) or M-CSF (lane 4) resulted in comparable tyrosine phosphorylation, suggesting that the WT CHR biochemically behaves in a fashion similar to endogenous Kit receptor. The phosphorylation of various CHR mutants examined in the present study was also similar to WT CHR except for CHR mutants impaired in the activity of Src and PI 3-kinase pathway. These two mutant CHRs demonstrated a slight decrease in phosphorylation compared with WT CHR (data not shown).

Next, we analyzed the function of WT CHR by examining proliferation in response to M-CSF stimulation. Consistent with the lack of M-CSFR expression in G1E-ER2 cells shown earlier (Fig. 2B, left panel), the addition of M-CSF to parental untransduced G1E-ER2 cells did not induce proliferation and did not cooperate with Epo-R to enhance proliferation (Fig. 2D, panel A). In contrast, and as expected and previously shown (18), stimulation of G1E-ER2 cells with SCF resulted in significant proliferation, which was further augmented in the presence of Epo (Fig. 2D, panel A). Consistent with the expression and the biochemical observations noted above (Fig. 2, B and C), G1E-ER2 cells expressing the WT CHR demonstrated a similar increase in proliferation in response to M-CSF stimulation, as seen with SCF stimulation of endogenous Kit receptors (Fig. 2D, panel B). This increase in proliferation was further augmented in the presence of Epo, to levels observed in response to endogenous Kit activation with SCF and Epo (Fig. 2D, panel B). Collectively, these data demonstrate that the WT CHR appears to function in a fashion similar to the endogenous Kit receptor in G1E-ER2 cells.

Expression and Function of Mutant Chimeric Receptors—To determine the effect of abrogating the activation of diverse
early signaling pathways in Kit-induced proliferation/growth and cooperation with Epo-R, we expressed all eight CHR mutants in G1E-ER2 cells expressing various CHR mutants. G1E-ER2 cells expressing the indicated mutants were cultured in the presence of M-CSF or M-CSF plus Epo for 48 h. Proliferation was measured by thymidine incorporation assay. Bars denote the mean thymidine incorporation (cpm ± S.E.) of two independent experiments performed in replicates of six. *, p < 0.05 CHR mutants versus WT CHR. B, stimulation of Kit receptors impaired in the activation of Src kinases results in reduced CFU-Es. Fetal liver cells were harvested from day 12.5 WT embryos. Single cell suspensions were prepared and incubated with or without retrovirus expressing the WT CHR or CHR mutants containing tyrosine to phenylalanine mutations at positions 567 and 569 or 567 and 569 (double mutant). After infection, cells were plated in triplicate in a methylcellulose with Epo (2 units/ml) or Epo plus M-CSF (2 units/ml Epo plus 100 ng/ml M-CSF). Benzidine-positive colonies were counted 2–3 days after plating. C, impaired proliferation of G1E-ER2 cells in the presence of Src kinase inhibitor PP1. Parental G1E-ER2 cells were treated with the indicated concentrations of the Src kinase inhibitor PP1 and cultured in the presence of SCF or SCF plus Epo for 48 h. Bars denote the mean thymidine incorporation (cpm ± S.D.) of an independent experiment performed in replicates of six. *, p < 0.05 for SCF and SCF plus Epo (dimethyl sulfoxide (DMSO) or untreated versus SCF and Epo PP1 inhibitor).

Fig. 6. A, analysis of M-CSF and M-CSF plus Epo induced thymidine incorporation in G1E-ER2 cells expressing various CHR mutants. G1E-ER2 cells expressing the indicated mutants were cultured in the presence of M-CSF or M-CSF plus Epo for 48 h. Proliferation was measured by thymidine incorporation assay. Bars denote the mean thymidine incorporation (cpm ± S.E.) of two independent experiments performed in replicates of six. *, p < 0.05 CHR mutants versus WT CHR. B, stimulation of Kit receptors impaired in the activation of Src kinases results in reduced CFU-Es. Fetal liver cells were harvested from day 12.5 WT embryos. Single cell suspensions were prepared and incubated with or without retrovirus expressing the WT CHR or CHR mutants containing tyrosine to phenylalanine mutations at positions 567 and 569 or 567 and 569 (double mutant). After infection, cells were plated in triplicate in a methylcellulose with Epo (2 units/ml) or Epo plus M-CSF (2 units/ml Epo plus 100 ng/ml M-CSF). Benzidine-positive colonies were counted 2–3 days after plating. C, impaired proliferation of G1E-ER2 cells in the presence of Src kinase inhibitor PP1. Parental G1E-ER2 cells were treated with the indicated concentrations of the Src kinase inhibitor PP1 and cultured in the presence of SCF or SCF plus Epo for 48 h. Bars denote the mean thymidine incorporation (cpm ± S.D.) of an independent experiment performed in replicates of six. *, p < 0.05 for SCF and SCF plus Epo (dimethyl sulfoxide (DMSO) or untreated versus SCF and Epo PP1 inhibitor).
with Epo-R, we examined various Kit mutants for their ability to induce growth in the presence of both M-CSF and Epo. As shown in Fig. 5, and consistent with a role for Epo in maintaining the survival of erythroid progenitors, costimulation of G1E-ER2 cells expressing various Kit mutants with Epo and M-CSF maintained their growth and survival over a 2-day culture period. However, mutants of Kit impaired in the activation of Grb2 (Fig. 5, panel D), PI 3-kinase (Fig. 5, panel E), and the Y745F (Fig. 5, panel G) demonstrated an enhanced ability to cooperate with Epo-R in inducing growth over a 2-day culture period. A Kit mutant impaired in the activation of Src kinases demonstrated a complete lack of cooperation with Epo-R in inducing growth (Fig. 5, panel C). Similar results were obtained using a thymidine incorporation assay (Fig. 6A).

As shown in Fig. 6A, Kit mutants defective in the activation of Src and PI 3-kinase showed the most profound reduction in thymidine incorporation over 48 h in the presence of M-CSF alone (Fig. 6A, panels C and E). In contrast, and similar to the results shown in Fig. 4, panels D, F, and G, mutants of Kit defective in the activation of Grb2 (Fig. 6A, panel D), PLC-γ (Fig. 6A, panel F), and the Y745F (Fig. 6A, panel G) demonstrated only a modest decrease in proliferation in response to M-CSF stimulation. The naked receptor behaved in a fashion similar to the mutant of Kit defective in the activation of Src kinase (Fig. 6A, panel H). Further, similar to the results shown in Fig. 5, panel C, only the Src kinase-defective Kit mutants showed a profound decrease in thymidine incorporation in the presence of both Epo and M-CSF (Fig. 6A, panel C). Consistent with a role for Src kinases in mediating cooperation between Epo and SCF in G1E-ER2 cells, expression of the Src kinase-defective Kit mutants in primary fetal liver cells also resulted in a significant decrease in the number of CFU-E colonies

To further substantiate a role for Src kinases in Kit and Epo-R-induced cooperation, we treated G1E-ER2 cells with a Src kinase-specific inhibitor (PP1) and measured proliferation by thymidine incorporation in the presence of SCF and/or Epo over 48 h. As shown in Fig. 6C, a dose-dependent decrease in proliferation of G1E-ER2 cells was observed in PP1-treated cells both in the presence of SCF as well as in combination with Epo. Collectively, these results strongly implicate the Src kinase pathway in regulating both Kit- and Epo-R-mediated cooperation in erythroid cells.

Several previously described studies have demonstrated a physical association between Kit and Epo-R (19, 22, 41). Further, stimulation of Kit- and Epo-R-expressing cells with SCF alone has been shown to transphosphorylate Epo-R (19, 22). Wu et al. (21) have argued that Kit-induced phosphorylation of Epo-R is essential for the generation of CFU-Es. We hypothesized that the lack of cooperation between Src kinase-defective Kit mutant and Epo-R may in part be due to reduced transphosphorylation of the Epo-R in response to M-CSF stimulation. To test this, WT or 567/569 CHR-expressing G1E-ER2 cells were starved and stimulated for 10 min with M-CSF, subsequently the lysates were prepared and subjected to IP with an anti-Epo-R antibody, followed by Western blot analysis using an anti-phosphotyrosine antibody. As shown in Fig. 7A, and consistent with previously published results in HCD57 cells (19, 22), stimulation of G1E-ER2 cells expressing the WT CHR with M-CSF resulted in a slight increase in the transphosphorylation of Epo-R. However, only a modest increase in the phosphorylation of Epo-R was noted in G1E-ER2 cells expressing the Src kinase-defective Kit mutant (lane 4), suggesting that the reduced proliferation of G1E-ER2 cells expressing the 567/569 Kit receptor may in part be due to reduced transphosphorylation of the Epo-R in response to M-
Fig. 8. Restoration of the Src binding sites in the naked CHR restores cooperation between Kit and Epo-R. 

A, G1E-ER2 cells expressing the WT CHR (top left panel), naked CHR (top right panel), and 567/569 add-back CHR (top middle panel) were stained with a PE-conjugated antibody against the M-CSFR and analyzed by flow cytometry. Shown is the co-expression of EGFP (x axis) and the M-CSFR (y axis). G1E-ER2 cells expressing the WT CHR, naked CHR, and 567/569 add-back CHR were cultured in the presence of M-CSF or M-CSF plus Epo for 48 h. Proliferation was measured by thymidine incorporation assay. Bars denote the mean thymidine incorporation (cpm ± S.D.) of a representative experiment performed in replicates of six. Similar results were observed in two other experiments.

B, analysis of M-CSF-induced Epo-R transphosphorylation in G1E-ER2 cells expressing the WT (lanes 1 and 2), 567/569 (lanes 3 and 4), or the 567/569 B CHR (lanes 5 and 6). G1E-ER2 cells expressing the WT or the 567/569 mutant or the 567/569 add-back CHR were starved and stimulated with 100 ng/ml M-CSF (lanes 2, 4, and 6) for the indicated times. Lysates were subjected to IP with an anti-Epo-R antibody and Western blot analysis with an anti-phosphotyrosine antibody. The position of Epo-R is indicated.

(lane 4) as well as in the presence of Epo (lane 6), although expression of c-Myc in response to Epo alone was comparable between WT and the 567/569 Kit mutant (lanes 2 and 5). Further, Epo-induced Stat5 activation (Fig. 7C) and Bcl-xL expression (Fig. 7D) was also comparable between WT and the Src kinase-defective Kit mutant. Interestingly, c-Myc expression was also reduced in a P1 3-kinase-defective Kit mutant but not in other mutants of Kit (data not shown).

To determine whether restoring the Src kinase pathway in a Kit mutant lacking the activation of all five early signaling pathways (naked CHR) could restore Kit-induced proliferation and cooperation with Epo-R, including its transphosphorylation, we constructed and expressed a CHR mutant in which only the Src kinase pathway was restored by replacing the phenylalanines at positions 567 and 569 back to tyrosines (567/569B). Fig. 8A (top panel) demonstrates the expression of WT CHR (left panel), naked CHR (right panel) and CHR mutant in which the Src binding sites at positions 567 and 569 were restored to tyrosines (Src add-back mutant; middle panel). As shown in Fig. 8A (bottom left panel), stimulation of the Src kinase add-back mutant receptor with M-CSF alone, demonstrated a 50% restoration in proliferation, and completely restored the cooperation between Kit and Epo-R (Fig. 8A bottom right panel). Further, restoration of the Src binding sites in the naked mutant Kit receptor also restored the transphosphorylation of the Epo-R in response to M-CSF stimulation (Fig. 8B). These observations were further confirmed by expressing the WT, 567/569B and naked CHRs in 32D myeloid cell line that lack the expression of endogenous Kit receptors. Collectively, these results demonstrate that early activation of Src kinase pathway in Kit is necessary and sufficient to restore the cooperation between Kit and Epo-R in G1E-ER2 cells.

**DISCUSSION**

Kit is crucial for the development of erythroid progenitors, since mice that lack Kit or its ligand SCF exhibit significant reduction of fetal liver erythroid progenitors and die of anemia (9, 12). The survival and proliferation of erythroid progenitors also depends on Epo, suggesting that erythroid progenitors cannot proliferate and/or survive unless both Kit and the Epo-R signal transduction pathways are functional. We have recently demonstrated an essential role for SCF/Kit interactions in proliferation of proerythroblasts (18). Further, we have shown that Epo-R, in addition to cooperating with Kit in further augmenting the expansion of proerythroblasts, also maintains their survival by inducing the activation of Stat5 and the expression of Bcl-xL, a function Kit is incapable of performing (18). These results are in agreement with a previously described role for Epo and consistent with the manifestation of severe anemia in mice lacking the expression of Stat5 or Bcl-xL (14–16). Although a role for Epo-Stat5/Bcl-xL pathway has been well documented, and it is thought to be the major pathway in regulating the survival of erythroid progenitors, the nature of Kit-induced early signaling pathways in regulating erythroid cell proliferation alone or with Epo-R is not known.

Activation of Kit by its ligand, SCF, activates at least five early signaling pathways via binding to specific tyrosine residues in the intracellular domain of Kit. Whether these pathways play a redundant or a nonredundant role in erythroid cell expansion alone or in combination with Epo-R is not known. Utilizing a CHR approach and tyrosine to phenylalanine mutations in Kit, we have explored the importance of these early

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signaling pathways in Kit-induced expansion alone and in cooperation with Epo-R. The major conclusions of our study are as follows. 1) Tyrosine to phenylalanine mutations in the Kit intracellular domain required for activating the PI 3-kinase (position 719), PLC-γ (position 728), Grb2 (position 702), and position 745 pathways show a substantial reduction in proliferation upon Kit activation alone as well as in combination with Epo. However, tyrosine to phenylalanine mutations in Kit at positions 567/569, previously shown to bind Src kinase family members, are critical for Kit as well as Epo-R-induced proliferation of erythroid progenitors. 2) The lack of cooperation between a Kit mutant defective in the activation of the Src kinase pathway and Epo-R is associated with reduced transphosphorylation of the Epo-R and expression of c-Myc. 3) Remarkably, restoration of the Src binding sites in a Kit mutant stripped of all the other signaling pathways (naked receptor) restored 50% proliferation upon Kit activation alone and completely restored the cooperation with Epo-R, including its transphosphorylation.

The lack of a profound effect on proliferation in erythroid cells expressing Kit mutants defective in the activation of PLC-γ, PI 3-kinase, Grb2, and Y745F is intriguing. It is possible that in erythroid cells, these pathways play only a minor role in proliferation downstream from Kit. Consistent with this interpretation, mice carrying a mutation in the PI 3-kinase binding site (position 719) of Kit in vivo also do not manifest an erythroid phenotype (43, 46), although these animals do show a defect in mast cell proliferation and germ cell maturation in response to Kit activation (43, 46). Thus, early signaling pathways downstream from Kit may be differentially utilized in different cell lineages. Alternatively, since cooperation between Kit and Epo-R is absolutely essential for normal erythroid development in vivo, and since we demonstrate only a slight reduction in the proliferation of erythroid cells in response to co-stimulation with SCF and Epo in PI 3-kinase-defective Kit mutant, it is conceivable that mice carrying a mutation in the PI 3-kinase binding site of Kit in vivo may tolerate this mutation and hence a lack of an erythroid phenotype. Based on this interpretation and our results with the Src kinase Kit mutants, we hypothesize that other than the Src kinase-defective mutants, disrupting any of the other early signaling pathways in Kit, including the PLC-γ, Grb2, Grb7, or tyrosine Y745F would not result in an erythroid phenotype (anemia) in vivo, since at least in vitro, all of these mutations are well tolerated and do not manifest a profound phenotype upon co-stimulation with SCF and Epo.

The mechanism(s) by which Kit and Epo-R cooperate to regulate the expansion of committed erythroid progenitors is poorly understood (19–25). Thus far, two mechanisms of cooperation between Kit and Epo-R have been described in cell line models. Studies by Pircher et al. (24) and Sui et al. (23) have provided evidence that mitogen-activated protein kinase extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2 at least in part may function as downstream integrators of Epo-R and Kit signals (23, 24). Wu et al. (19) have shown that activation of Kit induces tyrosine phosphorylation of the Epo-R, and that Kit interacts with the Epo-R by physically associating with its cytoplasmic domain. Further, the ability of SCF to support proliferation of 32D cells expressing Kit requires co-expression of the Epo-R, demonstrating that at least one proliferative signal generated by Kit involves the Epo-R as a downstream signal transduction protein (19). Consistent with these findings, a functional interaction of activated Kit with the Epo-R was shown to be crucial for CFU-E generation (21). Specifically, erythroid progenitors from Epo-R−/− fetal livers, infected in vitro with a retrovirus expressing the wild type Epo-R, require the addition of both Epo and SCF to form CFU-E colonies (21). Therefore, it is likely that Kit activates Epo-R by phosphorylating tyrosine residues in its cytoplasmic domains, which allows for an efficient recruitment/binding of Src homology 2-containing signaling proteins, including the Src and the PI 3-kinases, resulting in further activation of downstream signaling proteins. Our data demonstrate that tyrosine residues 567 and 569 in Kit may play an essential role in regulating this process. Since erythroid cells expressing mutants of Kit lacking these two tyrosines demonstrate a significant reduction in Kit-induced phosphorylation of the Epo-R, which is associated with reduced proliferation and c-Myc expression. Remarkably, restoring these two residues back to tyrosines in the absence of other signaling pathways is sufficient to restore the phosphorylation of Epo-R and proliferation.

In fibroblasts, Src kinases play an essential role in regulating DNA synthesis by inducing the expression of c-Myc in response to PDGF stimulation (44, 45, 47). Studies have shown that neutralizing antibodies for Src kinase and dominant negative mutants for c-Src and Fyn inhibit DNA synthesis in response to PDGF, indicating that these kinases can act downstream from RTKs in a pathway that is required for proliferation. Remarkably, the expression of c-Myc was found to be sufficient to overcome the requirement for induction of DNA synthesis by RTKs (44, 45). Consistent with these observations, we demonstrate that Kit-induced activation of Src kinases also regulates the expression of c-Myc, which is associated with reduced proliferation in erythroid cells expressing Kit mutants defective in the activation of Src kinases. A similar decrease in the expression of c-Myc was also observed when G1E-ER2 cells were treated with the Src kinase inhibitor PP1 in the presence of SCF and Epo. Collectively, these results demonstrate that in addition to the above described mechanisms of cooperation between Kit and Epo-R, c-Myc at least in part may function as a downstream integrator of Epo-R and Kit signaling in erythroid cells.

The observation that only 50% correction in proliferation of erythroid cells was observed in cells expressing the Src add-back Kit mutant in response to M-CSF stimulation is intriguing and requires further investigation. It is possible that the Src kinase pathway alone is unable to activate all of the necessary signaling proteins essential for efficient Kit-induced proliferation, although Src kinases have been shown to regulate multiple signaling pathways, including the activation of the Ras pathway via Shc, the PI 3-kinase/Akt pathway via Cbl, and the Rac/c-Jun N-terminal kinase pathway via guanine exchange factors, such as Vav (29, 47, 48). Whether all of these signaling molecules are activated by Src kinases in response to Kit activation is currently being investigated. It is possible that only a few of these pathways are activated in Src add-back Kit mutants and that other pathways are necessary for complete rescue in proliferation. Alternatively, it is conceivable that all of the above pathways are activated in Src add-back Kit mutants, however at a significantly reduced level compared with wild type Kit receptor. Thus, it is possible that quantitative rather than qualitative differences in the activation of various signaling pathways exist between wild type and Src add-back Kit receptor. These possibilities are currently being investigated.

Acknowledgments—We thank Drs. Mervin Yoder, Wade Clapp, and Eddy Srour for critically reviewing the manuscript and members of our laboratories for useful discussions. We also thank Marsha Huppenkothen for assistance in preparation of the manuscript and expert administrative assistance.

B. L. Tan and R. Kapur, unpublished observation.
