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KIT associated intracellular tyrosines play an essential role in EpoR co-signaling

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Abstract

KIT and erythropoietin receptor (EpoR) mediated co-signaling is essential for normal erythroid cell expansion, however the intracellular signals that contribute to cooperative signaling are poorly understood. Here, we examined the role of intracellular tyrosine residues in KIT and EpoR cooperation by co-expressing tyrosine (Y) to phenylalanine (F) and deletion mutants of KIT and EpoR in 32D cells. Of the four EpoR mutants examined, only EpoR-Y343 induced proliferation to near wildtype EpoR levels. A modest increase in the growth was also observed in 32D cells expressing the EpoR-Y343F; however neither EpoR-W282R nor EpoR-F8 showed any increase in growth over baseline. Biochemical analysis revealed that EpoR-Y343 induced the activation of Stat5, PI-3Kinase/Akt and MAP kinase Erk1/2 to near wildtype EpoR levels, while the remaining mutants failed to activate any of these signals. Interestingly, none of the EpoR mutants cooperated with WT KIT, although EpoR-Y343 showed a modest increase in co-signaling. Loss of seven tyrosine residues in KIT (KIT-F7) completely abrogated EpoR induced co-signaling. Restoring the Src kinase binding sites in KIT-F7 alone or together with the PI3Kinase binding site restored KIT induced signals as well as co-signals with WT EpoR, although restoring the Src kinase binding sites along with the PLC- γ binding site repressed both KIT induced signaling as well as co-signaling with WT EpoR. Taken together, these results suggest that KIT and EpoR mediated co-signaling requires intracellular tyrosine residues and tyrosine residues that bind Src kinases in the KIT receptor appear to be sufficient for restoring both KIT signaling as well as co-signaling with EpoR. In contrast, restoration of the PLC- γ binding site in the context of Src binding sites appears to antagonize the positive signals induced via the Src kinase binding sites in the KIT receptor.

Key Words

KIT; EpoR; co-signaling

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1. Introduction

The survival, proliferation and differentiation of erythroid progenitors depends on signals emanating from KIT, a receptor tyrosine kinase, and the erythropoietin (Epo) receptor (EpoR), a member of the hematopoietin receptor family [1,2]. Mutant mice that lack the expression of KIT (dominant white spotting, or W, mutants) or EpoR demonstrate severe deficiencies in erythroid cell development [1,2]. 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 [1,3]. EpoR deficient mice also demonstrate a significant decrease in CFU-E progenitors and die of anemia between days 13 and 15 of gestation [2]. These data suggest that the growth and expansion of committed erythroid progenitors requires both the KIT and EpoR signal transduction pathways.

Although stem cell factor (SCF), the ligand for KIT has been shown to induce some growth and survival of erythroid progenitors; its response is profoundly amplified in combination with Epo. This type of cooperation between KIT and EpoR has been described in vitro with respect to the generation of blast forming unit (BFU)-erythroid (E) and colony forming unit (CFU)-E formation and in vivo upon co-administering SCF and Epo. However, the basis of this co-signaling is poorly understood. To this end, biochemical studies have provided evidence for physical association between KIT and EpoR via the box 2 region cytoplasmic domain of the EpoR [4]. Furthermore, co-stimulation of an erythroid progenitor cell line with SCF and Epo has been shown to tyrosine phosphorylate the EpoR [4]. Mechanistically, Wu et al. utilizing various truncation mutants of EpoR have shown that KIT stimulation by SCF does not activate the EpoR by inducing its dimerization, but by phosphorylating tyrosine residues in the cytoplasmic domain of the EpoR [5].

In contrast to the above studies supporting a role for tyrosine phosphorylation of EpoR by KIT as a potential mechanism of co-stimulation, studies utilizing a truncated EpoR suggest that tyrosine residues within the EpoR cytoplasmic tail may not be involved in the observed co-signaling between KIT and EpoR [6]. To this end, a truncated EpoR (Y343F) lacking all eight tyrosine residues in the EpoR was able to synergize with KIT, although to a lesser extent than the wildtype EpoR [7,8]. Further, under conditions of stress, mice expressing the truncated EpoR were significantly more susceptible to anemia than their littermate controls. Thus, although EpoR tyrosine-independent mechanism(s) could contribute to co-signaling, one cannot rule out the possibility that compensation by other signaling pathways in a truncated EpoR may contribute to this process, including molecules that negatively regulate EpoR signaling. Furthermore, the precise involvement of KIT specific tyrosines in EpoR induced co-signaling still remains poorly understood.

KIT is a member of the receptor tyrosine kinase (RTK) family and is encoded by the proto-oncogene KIT. The structure of KIT includes an extracellular domain with five immunoglobulin (Ig)-like motifs, a single short membrane spanning domain, and a cytoplasmic domain with tyrosine kinase activity. In response to ligand-induced stimulation KIT undergoes dimerization and autophosphorylates multiple tyrosine residues in their intracellular domain [9–12]. Activated KIT binds signaling molecules at specific tyrosine residues: phospholipase C- γ (PLC- γ) at tyrosine 728 [13,14], phosphatidylinositol 3-kinase (PI-3Kinase) at tyrosine 719 [15], Src family kinases at positions 567 and 569 [16–19], Grb2 at tyrosine 702 [20], and Grb7 at tyrosine 934 [20]. Through this interaction, the phosphorylated tyrosine residues initiate signal transduction via multiple distinct early signaling pathways. Studies of multiple cell types have shown that KIT molecules carrying tyrosine (Y) to phenylalanine (F) (Y→F) mutations at critical residues fail to bind the associated signaling molecules and consequently fail to activate these signaling pathways [21,22].

EpoR is a member of the class I cytokine family. Upon binding its ligand, the receptor dimerizes, enabling the formation of JAK2 kinase dimers which autophosphorylate. The active JAK2 then phosphorylates multiple tyrosine residues on the intracellular domain of the EpoR. Studies utilizing truncated and Y→F mutated murine EpoR have mapped the tyrosine binding sites for several src homology (SH)-2 adapter proteins and enzymes. Among these are Stat5a/b (Y343) and the p85 regulatory subunit of PI-3Kinase (Y479) [23]. However, the physiologic roles of the signaling cascades initiated via these proteins remain poorly understood. To this end, studies have implicated the Y343 and Y479 induced pathways to uniquely support erythroid growth, including the formation of CFU-E in vitro and the rescue of EpoR deficient phenotypes in vivo [6,23,24]. However, the precise role of these tyrosines in KIT and EpoR co-signaling is not fully understood.

In an effort to more clearly understand the relative importance of select tyrosine sites in KIT/EpoR co-signaling, we co-expressed tyrosine to phenylalanine mutants as well as deletion mutants of KIT and EpoR in 32D cells. We show that of the four mutant EpoRs examined, only EpoR-Y343 induced proliferation to near wildtype EpoR levels. Neither EpoR-Y343F nor EpoR-W282R or EpoR-F8 showed an increase in growth over baseline. Furthermore, only EpoR-Y343 induced the activation of Stat5, PI-3Kinase/Akt and MAP kinase Erk1/2 to near wildtype EpoR levels, while the remaining mutants failed to activate any of these signaling pathways. Additionally, only the WT-EpoR efficiently co-signaled with WT KIT. Loss of seven tyrosine residues in the intracellular domain of KIT receptor (KIT-F7) completely ameliorated EpoR induced co-signaling. Interestingly, restoring two phenylalanine residues back to tyrosines in KIT-F7 at tyrosines 567 and 569 was sufficient to completely restore KIT/EpoR co-signaling. Restoring the PI3Kinase binding site at tyrosine 719 in KIT-F7 together with the Src binding sites at tyrosine 567 and 569 also somewhat restored co-signaling, although restoring the Src binding sites along with the PLC- γ binding site repressed both KIT induced signaling as well as co-signaling with WT EpoR. Taken together, these results demonstrate that intracellular tyrosine residues in the distal region of KIT and EpoR play an essential role in regulating co-signaling and that Src and PI3Kinase binding sites appear to be sufficient for regulating this process.

2. Material and Methods

Cell lines

32D cells have been previously described [25].

Construction of wildtype and mutant KIT and EpoRs

The construction of wildtype and mutant KIT and EpoRs has been previously described [8, 26].

2.1 Expression of wildtype and mutant KIT and EpoRs in 32D cells

To produce wildtype and mutant KIT and EpoR encoding viral supernatants for infection of 32D cells, Phoenix ecotropic cells were transiently transfected with wildtype or various mutants of KIT and EpoR expressing retroviral constructs using the Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Supernatants were collected 48 h after transfection, filtered through 0.45 μ m pore size membranes, and used. Cells were infected with 2 ml of virus supernatant in the presence of 8 μ g of polybrene per ml. Virus infected cells were harvested 48 h later, sorted by fluorescence-activated cell sorter (FACS), and expanded in culture. 32D cells expressing similar levels of EGFP and KIT mutant receptors were reinfected with virus expressing EpoR mutants, selected in G418 (2 mg/ml) and cloned by limiting dilution. EpoR and KIT receptor double positive cells were sorted by FACS and expanded in culture in the presence of G418.

32D cells expressing similar level of KIT and EpoR mutants were utilized to perform all the experiments described in these studies.

2.2 Flow cytometric analysis

Phycoerythrin (PE)-conjugated secondary monoclonal antibody (mAb) (Santa Cruz, Biotechnology, Santa Cruz, CA) was used to detect the extracellular domain of the chimeric KIT (CHR) macrophage colony stimulating factor (M-CSF) receptor. 32D cells expressing (1×10^6) the wildtype or the mutant KIT CHRs were incubated at 4°C for 30 minutes with 1 µg of primary antibody. Cells were washed two times with phosphate buffered saline containing 0.1% bovine serum albumin (Sigma) and incubated with a secondary antibody for 30 minutes at 4°C, washed as above and analyzed by a fluorescence activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA). Phycoerythrin (PE) conjugated anti-human epidermal growth factor receptor (EGFR) monoclonal antibody (BD Pharmingen) was used to detect the extracellular domain of the human (h)EGFR for the chimeric EpoR [8,26].

2.3 Proliferation assays

The effect of M-CSF, hEGF or the combination of M-CSF and hEGF on the proliferation of 32D cells was analyzed by using thymidine incorporation assay. 96-well tissue culture plates were utilized for these experiments. 5×10^4 cells/well were plated for 48 hours, in the presence of 25 ng/ml of M-CSF or 5 ng/ml hEGF, or both. Subsequently, 1.0 µCi of ^3H -thymidine (Amersham) was added to each well for 6 to 8 hours at 37°C. Cells were then harvested using an automated cell harvester (96-well plate harvester, Brandel, Gaithersburg, MD) and thymidine incorporation was determined in a scintillation counter.

2.4 Western blot analysis

32D cells expressing various mutants of KIT and/or EpoR were starved of serum and growth factors for 6 to 10 hours. Starved cells were resuspended in Iscove modified Dulbecco medium (IMDM) and stimulated with hEGF or M-CSF or both. Thereafter, cells were harvested and lysed in cell lysis buffer (10 mM K_2HPO_4 , 1 mM EDTA, 5 mM EGTA, 10 mM MgCl_2 , 1 mM Na_2VO_4 , 50 mM β -glycerol phosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A containing 0.5% Triton X-100 pH 7.2). Equal amount of protein was fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. Activation of Akt, Erk1/2 and Stat5a/b was determined by using phospho-specific antibodies (Cell Signaling, Beverly, MA). Total Akt protein expression was determined by using antibodies against Akt (Cell Signaling, Beverly, MA).

3. Results

The goal of the present study was to quantitatively as well as qualitatively assess the role of intracellular tyrosine residues in KIT and EpoR co-signaling. This was achieved by examining several tyrosine to phenylalanine mutants of both KIT and EpoR as well as truncation mutants of EpoR and by co-expressing them in 32D cells. With respect to EpoR, we focused our studies on Y343-EpoR, Y343F-EpoR, W282R-EpoR and F8-EpoR mutants because of their inability to recruit and activate signaling molecules implicated in Epo signaling, including Stat5, JAK2 and various additional Src homology (SH)-2 containing enzymes and/or adaptor proteins (Fig. 1A) [27]. Likewise, various mutants of KIT lacking tyrosine residues known to recruit and activate Src family kinase, PI-3Kinase or PLC- γ were generated (Fig. 1B). Both KIT as well as EpoRs were constructed and expressed as chimeric molecules for the purpose of bypassing endogenous KIT and EpoRs in primary cells as well as cell lines as previously described [8, 26]. The chimeric KIT and EpoRs were generated by retaining the transmembrane and the intracellular domains of murine EpoR and KIT and the extracellular domain of the human (h)

epidermal growth factor (EGF) receptor and macrophage colony stimulating factor (M-CSF) receptor, respectively [8,26]. We have previously demonstrated that these chimeric receptors function in a manner similar to the wildtype KIT and EpoR and activate similar biochemical pathways as their wildtype counterparts [8,26]. Utilizing the wildtype chimeric EpoR and KIT as a template, various tyrosine residues in the cytoplasmic domain of EpoR and KIT were mutated to phenylalanines (Fig. 1A & 1B). Specifically, we constructed a KIT mutant receptor in which seven tyrosine residues were replaced with phenylalanines at positions that have been previously demonstrated to bind and activate distinct immediate early signaling pathways. This receptor was designated KIT-F7 (Fig. 1B). This receptor contains Y→F mutations at residues 567, 569, 702, 719, 728, 745, and 934 (Fig. 1B; numbers correspond to the amino acid position in the full-length murine KIT). These mutations prevent the activation of PI-3Kinase (position 719) [15], Src kinase family members (positions 567 and 569) [16,17,19,28], PLC-γ (position 728) [13,14], Grb2 (position 702) [20], and Grb7 (position 934) [20]. We also mutated an additional tyrosine residue at position 745. Utilizing the KIT-F7 receptor as a template, we constructed a series of add back KIT receptors in which phenylalanine residues at positions 567, 569, 719 and 728 were restored back to tyrosines either individually or in combination (Fig. 1B).

KIT and EpoR mutants were cloned into a murine stem cell virus (MSCV) based retroviral vector (Fig. 2). Specifically, KIT mutants were cloned into a bicistronic retroviral (also encodes an enhanced green fluorescence protein; EGFP) vector MIEG3 (Fig. 2). EpoR mutants were cloned into a separate MSCV based vector encoding the neomycin resistant gene driven by an internal phosphoglycerate kinase (PGK) promoter (Fig. 2). Viral supernatants were generated as described [26]. Viral supernatants were used to infect 32D cells and EGFP positive and G418 resistant cells were sorted to homogeneity and used to perform functional and biochemical assays. Flow cytometry was used to determine the co-expression of KIT and EpoR mutants. Fig. 3 demonstrates similar level of co-expression of KIT and EpoR mutants used during the course of this study.

To analyze the individual contribution of KIT associated tyrosines in EpoR cooperation and to determine how many and which tyrosine residues are required to regulate cooperative growth, several EpoR and KIT mutants were co-expressed in 32D cells. 32D cells lack the expression of KIT and EpoR and therefore make for an ideal model to investigate cooperation between these distinct receptors [4]. We first examined how 32D cells expressing various EpoR mutants proliferate in the absence of KIT stimulation. A thymidine incorporation assay was performed on cell lines expressing four distinct EpoR mutants (Fig. 4A). Since these were chimeric receptors, the function of EpoR mutants was assessed by stimulating the cells with hEGF. Two different concentrations of hEGF were used (i.e. 1 and 5 ng/ml). As seen in Fig. 4A, 32D cells expressing the WT-EpoR demonstrated the highest thymidine incorporation at both doses. Consistent with previously published results, a truncated EpoR mutant lacking all tyrosine residues (Y343-EpoR), but containing the JAK2 and Stat5 binding sites demonstrated a growth pattern similar to that seen in cells expressing the WT-EpoR, although in some experiments a slight reduction in proliferation of these cells was observed compared to WT controls. In contrast, a truncated EpoR mutant lacking all 8 tyrosines, including the binding site for Stat5 consistently demonstrated a 70% reduction in growth compared to WT EpoR expressing cells (Fig. 4A). Consistent with an essential role for JAK2 in regulating EpoR induced functions, an EpoR mutant lacking the JAK2 binding site (W282R-EpoR) did not support proliferation, nor did a mutant lacking all eight tyrosine residues in its cytoplasmic domain (EpoR-F8). As expected, stimulation of EpoR expressing cells with M-CSF (mediating the activation of KIT) did not induce discernable proliferation in these cells (Fig. 4A). To rule out the possibility that the modulation in the growth of 32D cells expressing various mutants of EpoR was not simply due to an overall defect in cytokine responsiveness in these cells, each EpoR/KIT co-expressing 32D cell line was stimulated with IL-3 alone (32D cells are an IL-3

dependent cell line) and thymidine incorporation was assessed. As seen in Fig. 4A, expression of mutant EpoRs in 32D cells did not alter the overall responsiveness of these cells to IL-3. Thus, the alteration in the growth of 32D cells expressing various mutants of EpoR is intrinsic to the receptors and not due to an overall defect in the proliferative ability of 32D cells expressing various mutant receptors.

We next sought to understand the biochemical basis for the lack of EpoR mutant responsiveness to hEGF stimulation in 32D cells expressing EpoR-Y343F, EpoR-W282R and EpoR-F8. Previous studies have suggested a critical role for PI3Kinase/Akt, Stat5 as well as the ERK MAP kinase pathway in regulating EpoR growth [8,29]. In line with these previous reports, we focused our biochemical analysis on the activation of the PI3Kinase/Akt, Stat5 as well as the ERK MAP kinase pathway in response to EpoR stimulation. As seen in Fig. 4B, stimulation of WT-EpoR resulted in the activation of Stat5, Akt and Erk1/2. Interestingly, stimulation of a truncated EpoR lacking 8 tyrosine residues (EpoR-Y343), but retaining the binding site for JAK2 and Stat5 also resulted in robust activation of Stat5, Akt and Erk1/2. In contrast, removal of the Stat5 binding site by mutating tyrosine 343 to phenylalanine in the Y343 truncated EpoR (EpoR-Y343F) completely abrogated the activation of Stat5, Akt as well as Erk1/2. Furthermore, an EpoR mutant lacking the binding site for JAK2 (EpoR-W282R) also did not activate Akt, Erk1/2 or Stat5 nor did an EpoR mutant lacking all eight tyrosine residues, but containing the JAK2 binding site (EpoR-F8).

Having established the ligand-dependent responsiveness of various EpoR mutants, we next examined the potential of 32D cells expressing various mutants of EpoR to cooperate with wildtype KIT. As seen in Fig. 5A, hEGF induced stimulation of 32D cells expressing various mutants of EpoR and WT KIT resulted in a proliferative profile similar to that observed in 32D cells expressing only the mutants of EpoR (Fig. 4A). In contrast, combined stimulation of these cells via the WT-KIT and WT EpoR resulted in a significant increase in proliferation (Fig. 5A). A modest increase in the growth of 32D cells expressing the WT KIT and Y343-EpoR was also observed, however none of the other EpoR mutants cooperated with WT-KIT, including Y343F, W282R or F8 (Fig. 5A). These results suggest that, although Y343-EpoR retains some potential to cooperate with WT-KIT; it is significantly less compared to WT-EpoRs potential to cooperate with WT-KIT. Furthermore, tyrosine residues in the cytoplasmic tail of EpoR are essential for KIT/EpoR mediated co-signaling.

To determine whether the tyrosine residues in the cytoplasmic domain of KIT are essential for KIT/EpoR co-signaling, we performed a proliferation assay in 32D cells expressing KIT-F7 along with various mutants of EpoR, including Y343, Y343F, W282R and F8. KIT-F7 lacks the binding sites for seven critical SH2 binding proteins, including Src kinases, p85 α regulatory subunit of class IA PI3Kinase, Grb2, Grb7, Ras-GAP as well as PLC- γ . Fig. 5B demonstrates no significant co-signaling between any of the four EpoR mutants and KIT-F7. Taken together, these results suggest that tyrosine residues in KIT are necessary for co-signaling with EpoR.

To determine whether qualitative or quantitative differences in the number of tyrosines in KIT affect KIT/EpoR co-signaling, we expressed and analyzed the function of three mutant KIT receptors (KIT-F7) in which phenylalanines at residues 567, 569, 719 and 728 were restored back to tyrosines in various combinations (Fig. 6). We chose to examine these four tyrosines because they have previously been implicated in activating the Src kinase pathways (tyrosine 567 & 569), PI3Kinase pathway (tyrosine 719) and PLC- γ pathway (tyrosine 728). All three pathways have been shown to contribute to KIT induced growth. As seen in Fig. 6, restoring the binding sites for Src kinases (Y567 & Y569) completely restored KIT induced growth to near wildtype levels at all concentrations of M-CSF with the exception of 5 ng/ml. Interestingly, although restoring the PI3Kinase binding site at tyrosine 719 along with the Src binding sites also rescued growth in these cells, but the rescue was somewhat less (in particular at 10 and

100 ng/ml of M-CSF, * $p < 0.05$) compared to the KIT-F7 receptor in which only the Src binding sites were restored (Y567/Y569-KIT; Fig. 6). To our surprise, restoring the PLC- γ (Y728) binding site along with the Src binding sites (Y567/Y569) showed minimal growth in response to M-CSF stimulation. These results suggest that restoring the PI3Kinase site (Y719) and to a larger extent the PLC- γ (Y728) binding site represses M-CSF induced rescue in growth observed in the KIT-F7 receptor consisting of only the Src binding sites (567 & 569).

We next assessed whether a similar growth profile is also observed when these mutant KIT receptors are co-expressed in cells expressing the WT EpoR. As seen in Fig. 7, restoring phenylalanine residues back to tyrosines at positions 567 and 569 in KIT-F7 completely rescued co-signaling between KIT and WT EpoR. In contrast, a mutant of KIT consisting of the Src binding sites (Y567 & Y569) as well as the PI3Kinase (Y719) binding site demonstrated only a modest but non significant co-signaling whereas a KIT mutant consisting of the Src binding sites and the PLC- γ binding site showed minimal co-signaling. These results suggest that restoring two tyrosine residues in a KIT receptor lacking the binding sites for seven SH2 containing adaptor proteins and/or enzymes is sufficient to restore KIT/EpoR co-signaling; however the presence of additional tyrosines, especially the PLC- γ binding site abrogates KIT/EpoR co-signaling. These results point to an essential role for PLC- γ in repressing the positive signals emanated via the tyrosine residues 567 and 569.

4. Discussion

Stem cell factor has long been known for its role in regulating the survival of hematopoietic stem and progenitor cells, although it is most commonly recognized for its ability to cooperate with other hematopoietic cytokines such as Epo, IL-3 and GM-CSF to augment stem and progenitor cell growth and survival. In spite of its long standing reputation as a co-stimulatory cytokine, the intracellular signals that contribute to cooperation between KIT and other cytokine receptors are poorly understood. To this end, recent studies have suggested that the physical interaction between KIT and other cytokine receptors could contribute to the cooperation by bringing the two receptors in physical proximity, thereby inducing receptor trans-phosphorylation and activation of downstream substrates. Although, this scenario has been studied to some extent utilizing the KIT/EpoR system, in other receptor systems, including the GM-CSF/SCF system, no evidence for trans-phosphorylation of KIT or the GM-CSF receptor has been reported, however studies have shown the formation of a complex between KIT and the GM-CSF receptor does take place in this system [30]. Thus, the precise role of intracellular tyrosines in KIT mediated co-signaling with cytokine receptors is still poorly understood.

Cooperation between KIT and cytokine receptors at the level of downstream kinases has also been reported. To this end, combined stimulation of cells with SCF and GM-CSF results in sustained activation of Mek1/2 and Erk1/2 compared to either cytokine alone [30]. Sustained activation of Erk1/2 is PI-3Kinase dependent in both the SCF/GM-CSF system as well as the SCF/Epo system [29,30]. Furthermore, PI-3Kinase is synergistically activated in response to combined stimulation by Epo and SCF compared with single cytokine. Likewise, association between KIT and IL-7 receptor in the cooperative activation of the JAK/STAT pathway in the absence of IL-7 has also been reported [31]. We show that KIT and EpoR associated intracellular tyrosines play an essential role in regulating cooperation via these two receptors. Consistent with previously published observations, our results show that co-activation of EpoR and KIT augments proliferation to levels significantly greater than those seen with either factor alone. Among the different EpoR mutants examined, we show that KIT induced cooperation is only modestly supported by EpoR-Y343. Neither Y343F-EpoR, nor W282R-EpoR or F8-EpoR showed an increase in growth over baseline or cooperated with the wildtype KIT receptor. Interestingly, only Y343-EpoR induced the activation of Stat5, PI-3Kinase/Akt and

MAP kinase Erk1/2 to near wildtype EpoR levels, while the remaining mutants failed to activate any of these pathways. Our results in 32D cells using the F8 EpoR are consistent with those of Longmore et al showing an essential role for intracellular tyrosine's in EpoR induced erythropoiesis in vivo. They showed an inefficient EpoR tyrosine independent pathway in supporting red cell development [32].

Our results suggest that the JAK2 binding site as well as tyrosines in the distal region of the EpoR are essential for co-signaling with KIT, as W282R-EpoR which lacks the binding site for JAK2 fails to cooperate with KIT. In addition, a mutant of EpoR lacking all 8 tyrosines in the distal region, but containing the JAK2 binding site also failed to cooperate with KIT. With respect to KIT mutants, our findings suggest that combination of different tyrosines in KIT can result in different functional outcomes with respect to co-signaling. We show that the presence of just the Src kinase binding sites in KIT-F7 is sufficient to restore KIT and EpoR mediated co-signaling. Whereas addition of PI3Kinase binding site along with the Src binding sites only modestly rescues co-signaling which is significantly ameliorated in cells expressing the PLC- γ binding site. These results suggest specificity with regard to tyrosines in KIT induced co-signaling with WT EpoR.

Although we observed increased activation of Stat5 in cells co-stimulated with SCF and Epo (data not shown), the precise role of Stat5 in erythroid cell expansion is controversial. Using retrovirally transduced mutants of EpoR into fetal liver cells from EpoR deficient mice, a transgenic approach, as well as a knock-in approach, studies thus far have implicated Y343 induced pathways to uniquely support erythroid progenitor growth via EpoR through the activation of Stat5. Y343 has been shown to primarily activate Stat5 and induce the expression of Bcl-xL. However, in some studies impaired binding of Stat5 in vivo did not lead to erythroid cell defects, while a marked reduction in the number of CFU-E progenitors as well as embryonic anemia due to Stat5 deficiency in mice has been reported [33]. In the context of a truncated EpoR that only contains tyrosine 343, mutation of tyrosine 343 to phenylalanine eliminates EpoRs ability to recruit Stat5. The functional consequences of this mutation are also controversial. Quelle et al.[34] reported no consequences of this mutation on EpoR function, while others have demonstrated reduced proliferation and differentiation. Our results in 32D cells suggest that this mutation significantly impairs Epo induced growth as well as activation of Stat5, Erk1/2 and Akt. Furthermore, this receptor lacks the ability to cooperate with KIT.

In summary, our results suggest an essential role for KIT associated intracellular tyrosines in regulating co-signaling downstream from KIT and EpoR. Our results suggest that co-signaling via KIT and EpoR is not dependent on the quantity of tyrosines in the intracellular domain of KIT but rather on the quality of signal in the distal region of the receptor. Therefore, the role of KIT associated tyrosines in EpoR mediated co-signaling is not likely to be redundant since two different combinations of four tyrosines appear to result in very different functional outcomes. Based on our results it is reasonable to conclude that KIT associated tyrosines 567/569, 728 and 719 are likely to induce distinct changes in gene expression when stimulated, in particular genes that regulate growth. Future studies looking at the changes in gene expression by these distinct set of receptors is likely to shed more information on the complex issue of co-signaling via the KIT and EpoR.

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A

B

Figure 1. A schematic of chimeric Epo and KIT mutant receptors

(A) The Epo chimeric receptor (CHR) consists of the ligand-binding domain of the human epidermal growth factor (hEGF) receptor. The transmembrane and the intracellular domain consist of the murine Epo-R. At the indicated residues Y→F changes were made as well as truncations in the Y343 and Y343F receptors. (B) The KIT CHR consists of the ligand-binding domain of the M-CSF receptor. The transmembrane and intracellular domains consist of the murine KIT receptor. The kinase domain is indicated. In the F7 KIT, Y at the indicated positions was changed to F. In the Y567/Y569-KIT, Y567/Y569/Y719-KIT and Y567/Y569/Y728-KIT, F at indicated positions were restored to Y.

Figure 2. A schematic describing the methodology used for co-expressing various mutants of KIT and EpoR in 32D cells

Retrovirus expressing either the KIT or the EpoR mutants were co-infected in 32D cells and EGFP positive cells were sorted to homogeneity and selected for neomycin resistance in the presence of G418 before performing functional and biochemical studies.

Figure 3. Co-expression of various KIT and EpoR mutants in 32D cells

100% EGFP positive/KIT positive cells were stained with an antibody against the extracellular domain of the hEGF receptor (X-axis). Shown are FACS generated histogram profiles of 32D cells expressing various mutants of EpoR along with WT-KIT (**A**), F7-KIT (**B**), Y567/Y569-KIT (**C**), Y567/Y569/Y728-KIT (**D**) and Y567/Y569/Y719-KIT (**E**). Open histograms indicate the background staining observed with isotype control antibody. Closed histograms indicate the expression of various EpoR mutants.

Figure 4. Growth and activation of Stat5, Erk1/2 MAP kinase and PI-3Kinase/Akt pathway in 32D cells expressing various mutants of EpoR

32D cells expressing the indicated EpoR mutants were growth factor starved and stimulated with hEGF for 48 h. Bars represent the mean thymidine incorporation ($\text{cpm} \pm \text{S.D.}$) $n=4$ (**A**). Lysates in **A** were subjected to western blot analysis using an anti-phospho-Stat5, Akt and MAP kinase Erk1/2 antibody. Total protein in each lane was assessed by stripping the blot and probing with an anti- β -actin antibody (**B**).

Figure 5. KIT mutant lacking seven tyrosine residues in its intracellular domain is impaired in its ability to co-signal with EpoR

32D cells expressing similar level of indicated EpoR mutants and WT KIT (**A**) or EpoR mutants and KIT receptor lacking 7 tyrosine residues (KIT-F7) (**B**) were starved and cultured in the presence of M-CSF, EGF or both for 48 h. Bars represent the mean thymidine incorporation (cpm $\pm$ S.D.). * $p < 0.05$. (n=4).

Figure 6. Mutants of KIT expressing three of the seven tyrosine residues rescue KIT induced growth to different degrees

32D cells expressing similar levels of WT KIT, Y567/Y569-KIT, Y567/Y569/Y728-KIT or Y567/Y569/Y719-KIT were starved and cultured in the presence of M-CSF for 48 h. Bars represent the mean thymidine incorporation (cpm $\pm$ S.D.). n=3. *p<0.05, 10 and 100 ng/ml M-CSF, Y567/Y569-KIT vs Y567/Y569/Y719-KIT.

Figure 7. A mutant of KIT expressing only two tyrosine residues can cooperate with WT EpoR
 32D cells co-expressing similar levels of WT EpoR along with Y567/Y569-KIT, Y567/Y569/Y728-KIT or Y567/Y569/Y719-KIT were starved and cultured in the presence of M-CSF, EGF or both cytokines for 48 h. Bars represent the mean thymidine incorporation (cpm $\pm$ S.D.). n=5.