Integrative Signaling by Minimal Erythropoietin Receptor Forms and c-Kit*

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Erythroid homeostasis depends critically upon erythropoietin (Epo) and stem cell factor cosignaling in late progenitor cells. Epo biore sponses are relayed efficiently by minimal receptor forms that retain a single Tyr-343 site for STAT5 binding, while forms that lack all cytoplasmic Tyr(P) sites activate JAK2 and the transcription of c-Myc plus presumed additional target genes. In FDCER cell lines, which express endogenous c-Kit, the signaling capacities of such minimal Epo receptor forms (ER-HY343 and ER-HY343F) have been dissected to reveal: 1) that Epo-dependent mitogenesis, survival, and bel-x gene expression via ER-HY343 depend upon the intactness of the Tyr-343 STAT5 binding site; 2) that ER-HY343-dependent bel-xl gene transcription is enhanced markedly via c-Kit; 3) that socs-3, pifup, dpp-1, and eacy-bp gene transcription is induced via ER-HY343, whereas dpp-1 and eacy-bp gene expression is also supported by ER-HY343F; 4) that ectopically expressed SOCS-3 suppresses proliferative signaling by not only ER-HY343 but also c-Kit; and 5) that in FDCER and primary erythroid cells, c-Kit appears to provide the primary route to MAPK activation. Thus, integration circuits exist in only select downstream pathways within Epo and stem cell factor receptor signaling.

Epo,1 the prime hormonal regulator of red cell development, initiates its effects by binding to receptor dimers on the surface of erythroid burst- and colony-forming units and activating the tethered Janus family kinase (JAK) 2 (1, 2). JAK2 then mediates the phosphorylation of eight cytoplasmic tyrosine sites within the Epo receptor, and via these sites, a complex set of Src homology 2 domain-encoding factors (and associated co-factors) are engaged. These include STAT5A and B; Grb2/mSOS/Raf/Ras; phosphatidylinositol 3-kinase, phospholipase-y1; and SHIP; Lyn, Syk, and Tec; SHPTP-1 and 2; the nucleotide exchange factors Vav and C3G (via Cbl); Cis and SOCS-3; and the adaptors She, Gab1, Gab2, CrkL, APS, and IRS-2 (reviewed by Wojchowski et al. (Ref. 3)). Although many of these are proto-oncogenic growth regulators, others are negative effectors whose action in terminating Epo-stimulated events is likewise crucial to regulated erythropoiesis. These include Cis, which appears to compete with STAT5 for binding at Tyr-343 (4, 5); SHPTP-1, which acts to dephosphorylate JAK2 (6), and the suppressor of cytokine signaling, SOCS-3, which also binds and inhibits JAK2 (7). In SOCS-3−/− mice, in fact, a fatal erythrocytosis is precipitated (8).

Despite the complexity of this signaling network, studies of tyrosine-mutated and -truncated Epo receptors in cell lines (9, 10), murine fetal liver (11, 12), and adult murine marrow and spleen (13) have established that signals necessary for Epo receptor-dependent erythroid development are supported by receptor forms retaining only a membrane-proximal box domain for JAK2 binding plus a single phosphotyrosine Tyr(P)-343 STAT5 binding site. Additionally, in JAK2−/− mice (14) (as in Epo receptor−/− mice (Ref. 2)), definitive erythropoiesis fails and lethal embryonic anemias are engendered. In at least certain systems, however, Epo receptor forms that lack all cytoplasmic Tyr(P) sites (yet activate JAK2) also have been reported to retain significant bioactivity (4), and among STAT5 a−/− and b−/− mice those which survive embryonic stress later sustain no apparent defects in adult erythropoiesis (14). Together, these observations raise basic questions concerning how JAK2, and possibly STAT5 plus SOCS-3, integrate key Epo receptor-derived signals. As evidenced by severe anemias in mice sustaining mutations in the receptor tyrosine kinase c-Kit or its ligand (15), SCF also is known to exert important effects on red cell production. Through colony-forming assays (11, 13, 16) and in vivo analyses (17), SCF has been shown to act in marked synergy with Epo on cotargeted progenitor cells. In fact, this has been proposed to involve the trans-phosphorylation of the Epo receptor by c-Kit (18). In the present investigation, FDCW2-derived cell lines (which express endogenous functional c-Kit) (19) have been used to dissect signaling events that are relayed via Tyr(P)-343-retaining versus Tyr(P)-deficient Epo receptor forms and c-Kit. Data reveal roles for Tyr(P)-343 in mediating bel-xL and socs-3 transcription, and in augmenting novel effects of c-Kit on Epo-stimulated bel-xl and cis transcription. In addition, SOCS-3 is shown to inhibit not only Epo receptor but also c-Kit-dependent proliferation; several new ER-HY343 (and ER-HY343F) target genes are identified; and a primary role for SCF in MAPK activation is described.

EXPERIMENTAL PROCEDURES

Epo Receptor, SOCS-3, Pin-1, and Bel-xl Expression Constructs—pMKwtER was prepared by the stepwise cloning of a wild type Epo receptor cDNA (20) to pSL1180 (Amersham Pharmacia Biotech) at SpeI and SalI sites, and to the dicistronic pMK10a vector (21) as a 1.8-kb SpeI to XbaI fragment. pMKER-HY343 is an Epo receptor form
cated at Ala-375 and was prepared by PCR using the primers 5'-GAT TCC TCA TCT CGC TGT TGC TGA TGA-3' and 5'-AAG CCTT CAT CCA TAG TCA GGT GAT CCA C-3'. This 436-base pair PCR product was cloned stepwise to pCR-ScriptSK(+)(Stratagene), to pLL1180wtER as a BglII to XhoI fragment, and to pMK1059, as a 1.5-kb SpeI to XhoI fragment for the ER-HY343 and HY343F receptor forms. A Ty3-SacI fragment, a Ty3-SacI and EcoRI fragment, or a Ty3-SacI and XbaI fragment, was ligated (to phenylalanine) by overlap extension using the primers 5'-GAT CGC GCC CCT ACT GGT AGC AGG CGC TGG GAG GTG ATC AGG CCC AGA CCT CCT TGT TAT AGA GTG-3' and 5'-GTA ACA CCT AGC TTC ATC CAT AGT CAC GGG TCC AC-3'. The resulting 158-base pair Ty3-SacI fragment of pXMwtER (30), SpeI to EcoRI fragment of pXMwtER (30), and XhoI to XbaI fragment of pXMwtER were ligated into pEF (26) extended 1.2-kb SpeI to XhoI fragment. All products were confirmed by sequencing. SOCS-3 was expressed using a pEF vector (22). For Pir-m and Bcl-xL expression, cDNAs (23, 24) were cloned as 1.2-kb EcoRI to NotI and 0.8-kb EcoRI fragments, respectively, to pEPNeo.

Cell Lines and Primary Erythroid Progenitor Cells—FDCW2(pMK)-wtER, -pMK1-ER/HY343, and -pMK1-ER/HY343F cells were maintained at 37°C, 7.5% CO2 in Opti-MEM I (Life Technologies, Inc.) and selection in G418 (0.9 mg/ml). The parental line, FDCW2, in a well expanded from marrow were pre-cultured for 10 h at 2 x 10^6 cells/ml in Opti-MEM I, 1% FBS, and 4% conditioned medium from WEHI-3B cells as a source of IL-3 (25), and prepared by electrotransfection (26) and maintained at 37°C, 7.5% CO2 in Opti-MEM I (Life Technologies, Inc.), 7% fetal bovine serum (FBS), and 4% conditioned medium from WEHI-3B cells and 5% murine IL-3 (25). Mice were injected subcutaneously at 10^6 cells/ml in Opti-MEM I, 1% FBS and 8 x 10^5 cells per mouse. Cells then were adjusted to 0°C, washed, and lysed in three volumes of 10 mM NaCl, 6 mM MgCl2, 1 mM dithiothreitol, 0.1 mM NaVO4, 0.1% Triton X-100, 10 mM Tris, pH 7.4, plus protease inhibitors. Supernatants were centrifuged (15 s at 8,000 x g) and resuspended in three volumes of 20% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM diethiothreitol, and 0.5 mM PMSF, plus protease inhibitors. Samples then were incubated on ice for 30 min, and centrifuged (10 min at 8,000 x g). Supernatants were assayed for protein (BCA assay, Pierce) and flash-frozen. EMSAs were performed using a GAS-like element from the β-casein promoter (TGGTCCTTG-GAATTT) as a probe (31). Extracts (25 μg/assay) were incubated for 10 min at 23°C with the above 32P-labeled GAS element in 4% Ficoll, 0.1 mM EDTA, 1 mM diethiothreitol, 12 mM HEPES, pH 7.9 (plus 2 μg of poly(dI-dC)/20-μl assay) prior to electrophoresis in 0.25× TBE.

FDCW2-TVA Cells and Retroviruses—For use in transduction experiments, FDCW2 cells were electrotransfected with pCDNA3.1-TVA50, selected in G418 (1 mg/ml) and cloned by limited dilution. Clones that demonstrated 90–100% transduction efficiency were selected and grown in the presence of Epo and/or SCF. 2

RESULTS

Signals Relayed via JAK2/STAT5 Versus JAK2-activating Epo Receptor Forms—Epo receptor (ER) forms used in the present investigation include the wtER, ER-HY343 in which a single cystosplasmic Tyr(343)-P(343) site is retained (and seven additional PY sites are deleted) within a minimal Epo receptor form truncated at Ala-375, and ER-HY343F in which this Tyr-343 site for STAT5 binding (within this truncated receptor form) is mutated (Fig. 1A). For expression in factor-dependent FDCW2 cells, a dicistronic vector (pMK1059) was used to obviate the use of Epo as a selection agent. As assayed by Northern blotting, each of the above Epo receptor forms was expressed at highly comparable levels in stably transfected, G418-selected FDCW2 cells (Fig. 1B). 2 32P-Epo binding assays of FDCER-wtER, FDCER-HY343, FDCER-HY343F and FDCER-wt cells also demonstrated Epo receptor densities to be uniformly on the order of 800–1000 receptors/cell. 2 In addition (and as shown below), each of these Epo receptor forms proved to support c-Myc expression at comparable rates. In FDCER cells, the requirement for Tyr-343 in STAT5 activation has not been demonstrated previously and this therefore also was assayed in initial experiments. Mitogenic activities of the above Epo receptor forms in FDCER cells next were assessed. wtER and ER-HY343 receptor forms supported Epo-induced [3H]Thymidine incorporation at comparable rates while ER-HY343F was essentially inactive (Fig. 1C). These results were confirmed in repeated experiments using polyclonal lines from independent transfections (as well as in direct viable cell counting assays) and strongly suggest that in this strictly hematopoietic growth factor-dependent model, Tyr-343 (and STAT5) target proliferative effectors. Finally, EMSAs using a β-casein promoter-derived GAS element and extracts from FDCER-HY343 versus ER-HY343F cells were performed and clearly confirmed an essential role for Tyr-343 in this response (Fig. 1D).

The abilities of ER-HY343 and ER-HY343F receptor forms to transduce Epo-induction of several known immediate response genes was next studied. As shown in Fig. 2, c-Myc transcripts were induced via HY343 and HY343F receptor forms at comparable efficiencies, whereas in FDCER-HY343F cells, Epo

2 T. J. Pircher, J. N. Geiger, C. P. Miller, D. Zhang, P. Gaines, and D. M. Wojcicki, unpublished results.
induction of cis and pim-1 transcripts was blocked (as was predicted by the loss of STAT5 activation). More notably, Epo signaling of bcl-xL gene transcription in FDCER-HY343F cells was discovered to also be inhibited sharply (i.e. 4-fold based on phosphorimaging). This result was confirmed in three independent experiments and is the first analysis to directly link Tyr-343 of the Epo receptor to endogenous bcl-xL gene transcription. Parallel effects were observed for bcl-2, but in FDC cell lines levels of bcl-2 transcript expression (and induction by either Epo or IL-3) were #10% of bcl-xL transcripts. Based on these results, whether exogenous Bcl-xL might rescue the Epo-dependent growth of FDCER-HY343F cells next was tested (Fig. 3). Cells were transfected with pEFNeo-Bcl-xL, and clones stably expressing exogenous Bcl-xL were identified (Fig. 3, right panel). In FDCER-HY343F-Bcl-xL cells, however, ectopic expression of Bcl-xL at elevated levels failed to affect growth (Fig. 3, left panel). As a second proto-oncogene whose Epo-induced expression likewise depends upon Tyr-343 (see above), pim-1's ability to rescue FDCER-HY343F-Pim-1 cell growth also was tested (Fig. 3, lower panels). Effects, however, were limited to relatively high level expressing clones in which a modest increase in background rates of [3H]dT incorporation was observed. Each ectopically expressed factor, however, did protect FDCER-HY343F cells from apoptosis due to cytokine withdrawal by as much as 3-fold, respectively (data not shown). These results are consistent with those of related in vivo studies (34, 35) and suggest the existence of important Tyr(P)-343-dependent effectors besides bcl-xL or pim-1.
analyzed using a cDNA expression arrays. Transcription of 90 min), and RNA was isolated. Differential cDNA expression was absence of cytokines. Cells then were exposed to Epo (20 units/ml for 90 min), and RNA was isolated. Differential cDNA expression was analyzed using a cDNA expression arrays. Transcription of 90 min), and RNA was isolated. Differential cDNA expression was absence of cytokines. Cells then were exposed to Epo (20 units/ml for 90 min), and RNA was isolated. Differential cDNA expression was

These results led us to investigate whether the above minimal Epo receptor forms might support the induction of additional response genes. Specifically, FDCER-HY343 and FDCER-HY343F cells were exposed to Epo (20 units/ml), [32P]cDNAs were prepared from poly(A)+-enriched RNA, and were hybridized to duplicate microarrays of known murine cDNAs. In independent hybridizations using [32P]cDNAs prepared from independent RNA samples, we identified four additional genes not previously known to be regulated via ER-HY343 or ER-HY343F, i.e. SOCS-3 (22), proliferation-associated protein I (pifap; p38–2G4) (36), dipeptidyl-peptidase I precursor (dpp-1) (37), and calcyclin-binding protein gene (cacy-bp) (38) (Fig. 4). Although each was induced via ER-HY343, dpp-1 and cacy-bp transcription also was activated efficiently via Tyr-343-independent routes in FDCER-HY343F cells. With the exception of c-Myc, these latter two genes are the first in the Epo receptor system to be shown to lie downstream of JAK2 per se. How PLFAP, DPP-1, and Cacy-bp might function in proliferative signaling is under investigation, while effects of SOCS-3 on not only Epo- but also SCF-dependent growth are described below. For SOCS-3, the possible role of STAT5 in regulating its transcription also was tested functionally in the following way. To allow for analyses of short term effects, FDCW2 cells first were transfected stably with the avian leukosis virus receptor, TVA. Next, avian retroviruses encoding the constitutively active form of STAT5A (STAT5A1*6) (or GFP as a control) were prepared and transduced into FDCW2-TVA cells. In these STAT5A1*6-transduced cells, levels of SOCS-3 transcripts were elevated following the withdrawal of IL-3 as determined by Northern blot and phosphorimaging (Fig. 5). Without correction for the estimated efficiency of transduction (~20% of cells; Fig. 5, lower panel), this effect was severalfold. Although this result does not demonstrate direct transcriptional activation of SOCS-3 by STAT5, it does provide function evidence in situ for STAT5’s involvement in this response pathway.

Integration of Epo Receptor and c-Kit Growth and Survival Signals—As mentioned in the Introduction, coactivation of the Epo receptor and c-Kit is essential for the expansion of progenitor cells at normal rates. Previously, we have shown that FDCW2 cells and derived sublines express endogenous, functional c-Kit (19). Combined effects of Epo and SCF on FCDCR cell responses therefore were investigated next (Fig. 6). With regard to proliferation, (and as predicted) (19), c-Kit synergized with Epo receptor forms in FCDCR-wt and FCDCR-HY343 cells (Fig. 6). More interestingly, c-Kit also synergized with ER-HY43F, and this result defines at least a basal Epo receptor (phosphotyrosine-independent mechanism of c-Kit cosignaling as compared with FCDCR-HY343 cells. Maximal effects of Epo plus SCF on proliferation in FCDCR-HY343F cells, however, were blunted somewhat as compared with FCDCR-HY343 cells. Whether Epo and SCF might possibly cotarget bcl-xL or alternate bcl-2-related genes next was assessed. Initially, RNase protection assays were used to inventory levels of Epo and/or SCF-dependent transcription of bcl-xL, bcl-w, bak, bax, bad, and bcl-2 (Fig. 7). These assays indicated that only bcl-xL and bcl-2 transcript levels were modulated significantly, and this was observed only in FCDCR-HY343 cells and only in the presence of Epo. In addition, in the presence of Epo plus SCF, a detectable increase in bcl-xL and bcl-2 transcription was observed. Based on these apparent effects on bcl-xL gene transcription, Northern blot analyses also were performed (Fig. 8). For bcl-xL transcripts in FCDCR-HY343 cells, marked synergistic effects (i.e. severalfold above additive values as estimated
by scanning and imaging) were observed. Additionally, cis was observed to be a clear target of Epo and SCF synergy, but such synergy was selective and SCF was not observed to significantly affect Epo-induced pim-1 expression, for example. SCF alone was a poor inducer of bcl-xL transcript accumulation, and did not detectably stimulate pim-1 or cis gene transcription. Thus, SCF specifically augments the transcription of select Epo response genes, and as one such target bcl-xL may contribute to the combined proliferative effects of Epo and SCF.

Like bcl-xL, cis, and pim-1, the expression of socs-3 also was shown (above) to be induced by ER-HY343 (but not ER-HY343F). Possible coordinate regulation of SOCS-3 expression by Epo plus SCF therefore was assessed by Northern blotting.

As shown in Fig. 9A, however, SCF proved to have little effect on Epo-dependent SOCS-3 expression in FDCER-HY343 cells. In an extended functional context, effects on Epo- and SCF-dependent growth of exogenously expressed SOC-3 were also assessed in stably transfected FDCER-HY343 cells (Fig. 9B). In clones ectopically expressing SOCS-3 at increased levels (and as predicted), Epo receptor-dependent growth was attenuated sharply. Importantly, 32P RT-PCR served to confirm that this was not due to possible differences in levels of ectopically expressed ER-HY343 receptors among clones (Fig. 9B). More remarkably, exogenous SOCS-3 also proved to significantly inhibit proliferative signaling via c-Kit (as well as the proliferation supported by Epo-plus-SCF) (Fig. 9C) while effects on IL-3-dependent growth were relatively minor (~20% attenuation). c-Kit therefore is revealed to comprise a lateral target for SOCS-3.

Finally, in an expanded population of human peripheral erythroid progenitor cells, Epo and SCF recently have been shown to cotarget ERKs (16), and in FDCER cell lines ERK activation therefore also was investigated. In FDCER-HY343 cells (Fig. 10A, top panel) ERKs (especially ERK2) were observed based on Western blotting with an antibody specific to
phosphorylated ERKs 1 and 2) to be activated efficiently by SCF, but much less so by Epo. FDCW2 cells expressing the wt Epo receptor also were assayed, and similar results were obtained. To test whether this bias in signaling perhaps reflected an unusual property of FDCER cells, Epo and SCF activation of ERKs also was assayed in primary erythroid cells expanded from marrow, and in splenocytes from mice treated with thioguanine. For each preparation, using transgenic mice expressing an EGF receptor-Epo receptor chimera from a GATA-1 gene-derived expression vector, we have shown previously that erythroid progenitor cells comprise \approx 50% of expanded populations (13, 39). As shown in Fig. 10A (center panels), ERKs were activated efficiently by SCF but once again much less so by Epo. For progenitor cells from spleen, this result also was confirmed in vitro kinase assays using myelin basic protein as a substrate (Fig. 10A, lower panel). Finally, to confirm Epo sensitivity in these erythroid progenitor cells, levels of cis transcript induction and STAT5 activation were assayed. As shown by Northern blotting and EMSA, respectively, Epo efficiently activated each response (Fig. 10B). Thus, the activation of ERKs in each of the above model systems is proposed to occur primarily via SCF/c-Kit rather than Epo-dependent routes.

DISCUSSION

Aspects of the present work that merit discussion include signaling defects associated with the mutation of a STAT5 binding site in the Epo receptor form ER-HY343, and mechanisms underlying the ability of this minimal Epo receptor form to synergize with c-Kit. These newly defined signaling routes are summarized in the model presented in Fig. 11. The efficient induction of bcl-x gene expression by ER-HY343 (but not HY343F) first is of physiological interest since, among Epo-regulated survival factors, Bcl-xL has been shown in several model systems to be affected most in its expression (24, 40, 41). However, relatively little is understood concerning underlying mechanisms, and conflicting results have been reported. In particular, a phosphotyrosine-deficient Epo receptor form Epo-R previously has been reported to support bcl-2 (and bcl-x) transcript expression as expressed in 32D cells, and in this system possible roles for STAT5 in this response pathway were largely discounted (42). In FDCW2 cells, similar results were obtained for at least certain sublines transfected with monocistronic ER-HY343F expression vectors and selected in Epo (19). However, responsive clones were rare, and proliferative responses were attenuated. In part, this provided the impetus for presently preparing FDCW2 cell lines stably expressing HY343 and HY343F receptors from bicistronic vectors. As shown in Figs. 1, 2, and 6, FDCER-HY343F cells failed to grow or survive in the presence of Epo yet efficiently supported c-myc transcript induction. Notably, this result is consistent with the reported inactivity of cytoplasmic Tyr(P)-deficient chimeric receptor forms as assayed for CFU-e forming activity in transduced fetal cells (12), with the limited activity of a Tyr(P)-deficient Epo receptor form as assayed in fetal liver cells from Epo receptor \(-/-\) mice (11), and with the loss in CFU-e
forming activity observed upon the mutation of Tyr-343 within a truncated prolactin receptor - Epo receptor chimera (12). Based on the suggestion that Tyr-343 and STAT5 therefore may comprise important transducing components, Socolovsky et al. (43) also recently evaluated embryonic erythropoiesis in mice deficient in STAT5A and STAT5B. Unlike surviving adult animals in which erythropoiesis appears normal (44), embryos displayed marked anemia and apoptosis appeared to be a frequent fate of erythroid progenitor cells. Furthermore, based on experiments in Ba/F3 and HCD-57 cells using a bcl-x promoter reporter construct plus dominant-negative and activated forms of STAT5, bcl-xL was proposed to comprise a STAT5 target gene (33, 34). Present findings are not only consistent with this model, but also provide a specific link between the Epo receptor STAT5 binding site Tyr(P)-343 and endogenous bcl-xL gene transcription.

The forced expression of Bcl-xL in FDCER-HY343F cells, however, did not rescue proliferative activity (even upon Epo activation of ER-HY343F). This result is consistent with studies of exogenously expressed Bcl-xL in fetal liver cells (34), and prompted the screening via arrays of known genes of additional ER-HY343 and/or ER-HY343F targeted genes. Four ER-HY343 response genes were identified, i.e. socs-3, plfap, dpp-1, and cacy-bp. Among these, socs-3 previously has been reported to be induced via the full-length Epo receptor (22). Beside this, Epo induction of socs-3 and plfap were observed to depend upon intactness of Tyr-343 and therefore are suggested to perhaps constitute STAT5 target genes. Interestingly, a human homologue of PLFAP, EBP-1 (ErbB-3-binding protein) recently has been shown to interact with ErbB-3 in a two-yeast hybrid screen (46), and ErbB-3 has been shown to interact with She/Grb-2 complexes in response to neuregulin (46). dpp-1 and cacy-bp, in contrast, were induced via both ERY343 and ERY343F and to our knowledge are the first target genes other than c-myc to be mapped downstream of JAK2-dependent, Epo receptor Tyr(P)-independent routes in the Epo receptor system. Interestingly, DPP-1 gene expression also has been discovered to be induced by IL-2 in primary human lymphocytes, and may act as a central coordinator of serine proteases in hematopoietic cells (47). cacy-bp previously was cloned from a mouse brain library using a cDNA probe deduced from the partial amino acid sequences of calcyclin (38). The sequence of this clone was novel, and based on its Ca<sup>2+</sup>-dependent interaction with calcyclin, Cacy-bp also has been proposed to function in signal transduction (47).

With regards to the capacities of the above Epo receptor forms to synergize with c-Kit, four novel effects are described. First, an Epo receptor Tyr(P)-independent mechanism of mitogenic synergy with c-Kit is dissected. Based both on the inactivity of directly related Epo receptor forms in other model systems (11, 12) and on a proposed mechanism for synergy wherein c-Kit acts to phosphorylate Epo receptor Tyr(P) sites (11), the observed ability of the Tyr(P)-deficient receptor form ER-HY343 to synergize with c-Kit in stimulating mitogenesis was somewhat unexpected. Mechanistically, studies by Sui et al. (16) argue that this is not a direct consequence of Epo receptor-associated JAK2 phosphorylation of c-Kit. Rather, it is speculated that other downstream JAK2-dependent events account for this effect, and these are the subjects of ongoing studies. Second, and in contrast, effects of SCF on bcl-x gene expression appear to depend upon Tyr-343 since this response is not induced by SCF in Epo-exposed FDCER-HY343F cells. Epo-activated STAT5 therefore is likely to be one possible target of SCF effectors. Although ectopically expressed c-Kit has been reported in certain systems to activate STAT5 (48), this possible direct mechanism was discounted in assays of STAT5 activation in FDCER cell lines and primary erythroid cells. Additionally, c-Kit previously has been shown in FDCER lines to fail to activate JAK2 (19). In other type 1 cytokine receptor systems, STAT activity has been shown to be enhanced via ERK-dependent S/T phosphorylation (49, 50) and such events therefore may underlie c-Kit effects on certain STAT5 target genes (including cis (Ref. 5)). Alternatively, it is possible that c-Kit targeted transcription factors might act in concert with STAT5, and in melanocytes the transcription factor Mi, for example, previously has been discovered as a specific target for c-Kit activated MAPKs (51). Third, it previously has been observed in human erythroid colony-forming cells (as expanded from peripheral BFU-e) that Epo and SCF act synergistically to stimulate ERKs (16). In FDCER cell lines, at least additive effects of SCF plus Epo on ERK activation were observed in certain experiments, yet ERK activation via c-Kit was a more potent effect in all model systems examined. In FDCW2 cell lines, this was not due to levels of receptor expression, as I<sup>25</sup>I ligand binding assays have shown c-Kit and Epo receptor forms to be expressed at similar low densities. Also, results were not attributable to distinct kinetics of Epo versus SCF signaling, since ERK activation by each was shown in extended time-course experiments to peak sharply at 5 min of cytokine exposure. Therefore, the more efficient coupling between c-Kit and ERKs also may underlie SCF and Epo's combined effects on progenitor cell expansion. Fourth and finally, evidence is provided that SOCS-3, as induced via an ER-HY343 (but not ER-HY343F nor c-Kit)-dependent pathway, acts to inhibit not only Epo receptor but also c-Kit induced proliferation. For at least certain SOCS factors, their discovery was based on an ability to bind JAKs (52–54) and interactions between both the NHL-terminal kinase inhibitory and Src homology 2 domains in SOCS-3 and the kinase domain in JAK2 have been described (55). Additional mechanisms of suppression also appear to exist, and in the growth hormone receptor system, JAK2 suppression by SOCS-3 is dependent on tyrosine phosphorylation of the growth hormone receptor (7, 56). By comparison, in the c-Kit system SOCS-1 has been shown to affect proliferation by binding competitively to Grb-2 (57). In repeated experiments in FDC cell lines and in primary erythroid cells, no significant induction of SOCS-1 expression by SCF or Epo was detected. This does not discount important suppressive effects, however, and this is illustrated by persis-
tent expression of SOCS-3 in Epo receptor<sup>−/−</sup> mice (8) as well as by the presently discovered suppression of c-Kit signaling by SOCS-3. Suppression of SCF/c-Kit-dependent proliferation in FDCER-HY343 cells, in fact, was almost as efficient as suppression of Epo-induced mitogenesis. IL-3-dependent proliferation, however, was relatively unaffected but this might be due to the maintenance of FDCER-HY343-SOCS-3 cells in IL-3. Since SCF dramatically enhances BFU-e and CFU-e production, however, was relatively unaffected but this might be due as by the presently discovered suppression of c-Kit signaling by SOCS-3.

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