The βc Component of the Granulocyte-Macrophage Colony–Stimulating Factor (GM-CSF)/Interleukin 3 (IL-3)/IL-5 Receptor Interacts with a Hybrid GM-CSF/Erythropoietin Receptor to Influence Proliferation and β-Globin mRNA Expression

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ABSTRACT

Background: The interaction of different members of the hematopoietic growth factor receptor family may be relevant to the increased proliferation and the failure of differentiation that characterizes the myeloid leukemias. We recently demonstrated that a chimeric receptor (GMER) that is composed of the extracellular and transmembrane domains of the human granulocyte-macrophage colony–stimulating factor (GM-CSF) receptor α-chain (GMRα) and the cytoplasmic domain of the murine erythropoietin receptor mEpoR binds hGM-CSF with low affinity (3 nM) and confers both proliferative and differentiation signals to stably transfected murine Ba/F3 cells.

Materials and Methods: To investigate whether the common β-subunit of the GM-CSF receptor (βc) can interact with GMER, either the entire β-subunit or a mutant, truncated β-subunit that completely lacks the cytoplasmic domain (βtr) was introduced into Ba/F3 cells that express GMER, and the binding of GM-CSF as well as proliferation and differentiation responses were measured.

Results: Scatchard analysis showed that both GMER + βc and GMER + βtr bound hGM-CSF with high affinity (K_d 40 pM to 65 pM). Proliferation assays showed that the maximum growth of cells expressing GMER + βc was identical to that of cells with GMER alone. However, proliferation of the cells that expressed GMER + βtr was reduced by 80–95% of GMER. Dose-response curves showed that the concentration of GM-CSF required for half-maximal growth was 0.5–5.0 pM for GMER + βc and 0.5–5 nM for GMER and GMER + βtr. The EpoR cytoplasmic domain of GMER also undergoes ligand-inducible tyrosine phosphorylation. However, the tyrosine phosphorylation did not correlate with growth in cells expressing βtr. Coexpression of βc with GMER in Ba/F3 cells grown in hGM-CSF markedly enhanced β-globin mRNA expression.

Conclusions: These results indicate that βc can transduce a unique signal in association with GMER to influence both proliferative and differentiation signal pathways.

INTRODUCTION

The process by which hematopoietic stem cells differentiate along one or another specific cell-lineage involves the action of a combination of several hematopoietic growth factors (HGFs). For example, erythroid progenitors require interleukin 3 (IL-3) or granulocyte-macrophage colony–stimulating factor (GM-CSF) and Steel factor during the early phases of their growth, while later during differentiation erythropoietin (Epo) is required for the developing erythroid cells to

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proliferate and mature into hemoglobin-containing erythrocytes (1,2). All of these HGFs act by binding to specific receptors on the cell surface. The GM-CSF receptor (GMR) is comprised of a unique α-chain (GMRα) (3,4) that binds its ligand with low affinity and a common β-chain (also shared with the IL-3 and IL-5 receptors) that does not bind ligand alone but is essential for high-affinity binding (5). The first step in cell activation is the ligand-induced heterodimerization of the GMRα and β-subunits (6). The cytoplasmic domains of both subunits appear to be essential for GM-CSF–stimulated proliferation (7), although the proliferative role of the cytoplasmic domain of GMRα is still uncertain (8).

To determine if the cytoplasmic region of an HGF receptor can transmit a specific proliferative and differentiative signal, we stably expressed a hybrid receptor, GMER (9), which consists of the extracellular and transmembrane regions of hGMRα and the cytoplasmic domain of the murine (m) EpoR into Ba/F3 cells (10), a murine IL-3–dependent cell with erythroid features (11). We found that these cells proliferated in hGM-CSF and expressed increased levels of cell surface glycophorin, an early erythroid marker (9). Control cells that expressed hGMRα + βc proliferated in response to GM-CSF but did not express glycophorin, thus establishing a role for the cytoplasmic region of the hybrid receptor in the glycophorin response. Since IL-3 and GM-CSF have a synergistic role with Epo in erythropoiesis, we stably expressed either βc or a cytoplasmically truncated βc (βtr) in Ba/F3 cells that contained GMER to determine if βc could affect GMER signaling. We show here that βc increases the binding affinity and proliferative response of GMER to hGM-CSF. In addition, coexpression of GMER with βc markedly increases β-globin mRNA in GMER cells stimulated with hGM-CSF. In contrast, despite high-affinity binding, expression of βtr acts as a dominant negative on cell proliferation without leading to increased β-globin mRNA levels.

MATERIALS AND METHODS

Cell Lines and Cultures

Ba/F3 wild-type cells (Ba/F3 wt) and Ba/F3 cells that express the mEpoR (Ba/F3-mEpo) were a gift of A. D'Andrea (Dana-Farber Cancer Institute, Boston, MA, U.S.A.) (12). GMER, a hybrid receptor consisting of the entire extracellular and transmembrane domain of the hGM-CSF receptor α-chain ligated to the complete cytoplasmic tail of the mEpoR, was transfected into Ba/F3 cells as previously described (1). Ba/F3 GMER cells were then electroporated with either βc or βtr along with the hygromycin resistance gene (8), or with βc or βtr in the pREP-4 vector (Invitrogen, San Diego, CA, U.S.A.) that carries the hygromycin resistance gene. These cells were maintained in the 800 μg/ml hygromycin (Calbiochem, La Jolla, CA, U.S.A.). Ba/F3 cells that express $GMR\alpha_2$ plus βc (Ba/F3-GMR + βc) were obtained as previously described (8). All of these murine cell lines are factor dependent and were grown in RPMI medium supplemented with glutamine and 10% fetal calf serum (FCS; Sigma Chemical Co., St. Louis, MO, U.S.A.). This medium was supplemented with purified recombinant mIL-3 (kindly provided by Steve Gillis, Immunex, Seattle, WA, U.S.A.) for the Ba/F3 wt cells and Ba/F3 cells transfected with GMER + βtr, 3 U/ml purified recombinant human Epo (R & D Systems) for Ba/F3-EpoR cells, or 1 nM purified recombinant Chinese hamster ovary (CHO) hGM-CSF (kindly provided by Steven Clark, Genetics Institute, Cambridge, MA, U.S.A.) for cells that expressed GMER, or 10 pM hGM-CSF for cells that expressed GMER + βc or GMRα + βc.

Immunoprecipitation

Equal numbers of parental Ba/F3 wt and transfected cells were washed and incubated in methionine- and cysteine-free RPMI medium supplemented with 2% FCS for 1 hr, after which 200 μCi/ml ^{35}S methionine/cysteine (New England Nuclear, Boston, MA, U.S.A.) was added and the cells were incubated for a further 5–6 hr at 37°C. The metabolically labeled cells were stimulated with mIL-3 or hGM-CSF at 1 nM for 10 min, washed three times with phosphate-buffered saline (PBS), containing phosphatase inhibitors (10 mM β glycerophosphate, 2 mM sodium pyrophosphate, 1 mM NaF, 1 mM EDTA, and 1 mM sodium orthovanadate, all from Sigma) and solubilized in lysis buffer that contained 1% NP40 (Sigma), 150 mM NaCl, 50 mM Tris, pH 8.0, with protease inhibitors (10 μg/ml leupeptin, 0.2 U/ml aprotinin, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride, all from Sigma) and phosphatase inhibitors (as above). After centrifugation (168,000 × *g* for 15 min at 4°C), the supernatants were incubated with monoclonal antibody to phosphotyrosine

(4G10, kindly provided by Brian Druker, Oregon Health Sciences University, Portland, OR, U.S.A.) overnight at 4°C. Protein A sepharose beads were added (25 μl packed volume), and after 1.5 hr at 4°C the beads were washed three times in lysis buffer. For the double immunoprecipitation experiments the bound proteins were eluted by boiling for 2 min in solubilization buffer (50 mM triethanolamine, 0.4% sodium dodecyl sulfate (SDS), 100 mM NaCl, 2 mM EDTA, and 2 mM mercaptoethanol) with the subsequent addition of iodoacetamide and Triton X-100, added to final concentrations of 10 μM and 10%, respectively. The eluates were then incubated with rabbit polyclonal antibody to the C terminus of βc for 4 hr at 4°C. This antibody specifically detects βc by immunoprecipitation and Western blot (13). Bound proteins were collected with protein A sepharose as above, eluted by boiling in Laemmli sample buffer that contained 5% β-mercaptoethanol, and subjected to SDS polyacrylamide gel electrophoresis (7–15% acrylamide gradient) and autoradiography after treatment with autoradiographic enhancer (New England Nuclear). Exposure times were 5–14 days. For immunoblotting, the primary antiphosphotyrosine/protein A beads were boiled in Laemmli sample buffer with 5% β-mercaptoethanol, and the eluates were subjected to PAGE, as above. The separated proteins were then transferred to nylon membrane (Hoefer, San Francisco, CA, U.S.A.), immunoblotted with a 1:50 dilution of rabbit polyclonal antibodies to the mEpoR C-terminus (14) (gift of Alan D'Andrea, DFCI), and washed, and the bound antibodies were detected with horseradish peroxidase secondary antibodies and enhanced chemiluminescence, as described by the manufacturers (ECL Western Blotting-Amersham, Arlington Heights, IL, U.S.A.).

GM-CSF Binding to Cells

Radioiodinated GM-CSF was purchased from New England Nuclear (DuPont Company, New England Nuclear Research Products, Wilmington, DE, U.S.A.). The ^{125}I-GM-CSF had a specific activity of 4.9×10^{18} cpm/mol. Before the binding of ^{125}I-GM-CSF, all factor-dependent cells were cultured for 24 hr or more in GM-CSF–free RPMI media containing 10% WEHI-conditioned media, followed by 6 hr in RPMI containing 10% FCS. The cells ($1–4 \times 10^6$) were washed and then resuspended in binding media RPMI with 4% FCS, 30 mM HEPES pH 7.4, 0.04% sodium azide that contained the indicated concentration of radiolabeled GM-CSF at 4°C for 18 hr. The cells were then centrifuged through 100% FCS with 0.04% sodium azide at 4°C and the cell pellets counted in a γ counter (Packard Instrument Company, Meriden, CT, U.S.A.). Nonspecific binding, which was never more than 25% of total binding, was determined by counting the pellet from cells that were incubated in 150-fold excess of unlabeled GM-CSF for 30 min before the addition of labeled factor. The binding data were subjected to Scatchard analysis using the LIGAND program (15).

Thymidine Incorporation

Ba/F3 wt cells and cells that expressed GMER or GMRα + βc or βtr were washed three times and plated at 3×10^4 cells/well in triplicate in 160 μl of the appropriate media in microtiter wells for 72 hr at 37°C in a 5% CO_2 incubator. Three hours and 15 min before harvesting, 1 μCi of [^{3}H]thymidine (New England Nuclear) was added to each well. Cells were harvested on filter paper by an automated harvester (Skatron, Tranby, Norway) and counted in a β counter (Wallac, Gaithersburg, MD, U.S.A.).

Northern Hybridization

RNA was prepared by guanidinium/CsCl method (16). Ten micrograms per lane were loaded on a 1% agarose-formaldehyde gel and RNA was separated by electrophoresis at 150 V for 3 hr. RNA was transferred by capillary transfer overnight using 20× SSC to Duralon (Stratagene, La Jolla, CA, U.S.A.) nylon membrane. RNA was cross-linked by UV using a Stratalinker (Stratagene) according to the manufacturer's instructions. DNA probes were labelled with ^{32}P using random primed labelling (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Blots were prehybridized for 30 min at 68°C in a hybridization oven using a commercial hybridization solution (Quik-hyb, Stratagene) and hybridized for 2 hr at 68°C. Blots were washed in 2× SSC/0.1% SDS at room temperature for a low-stringency wash, 0.1× SSC/0.1% SDS at 60°C for a high-stringency wash, and exposed to film.

RESULTS

Both βc and βtr Convert Binding of hGM-CSF in Ba/F3-GMER Cells from Low to High Affinity

Cell surface expression of GMER alone or with each of the β-subunits was characterized by

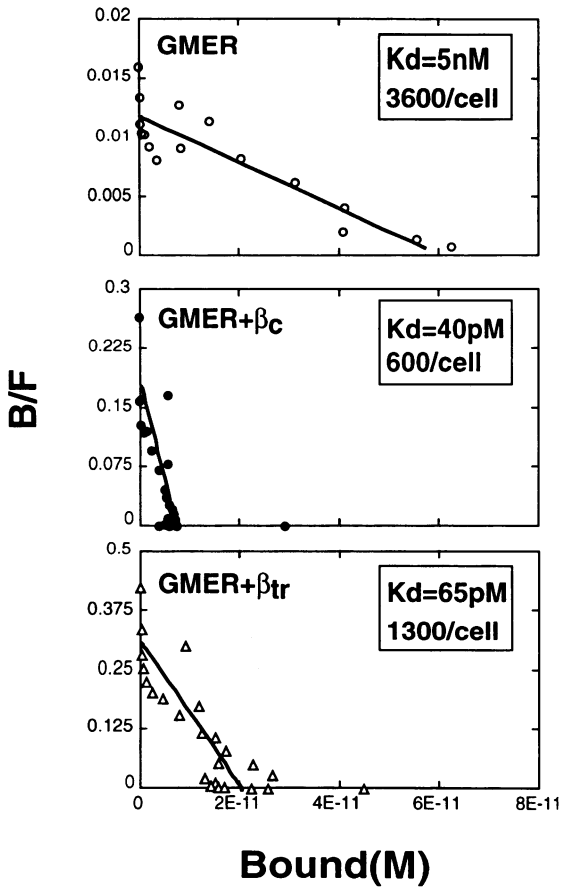

FIG. 1. Scatchard analysis of equilibrium binding of radiolabeled hGM-CSF to Ba/F3 cells that express GMER without or with βc or βtr

The upper panel shows low-affinity binding to Ba/F3-GMER, while the middle and lower panels show that βc or βtr can convert this binding to high affinity. The data are from one of two experiments.

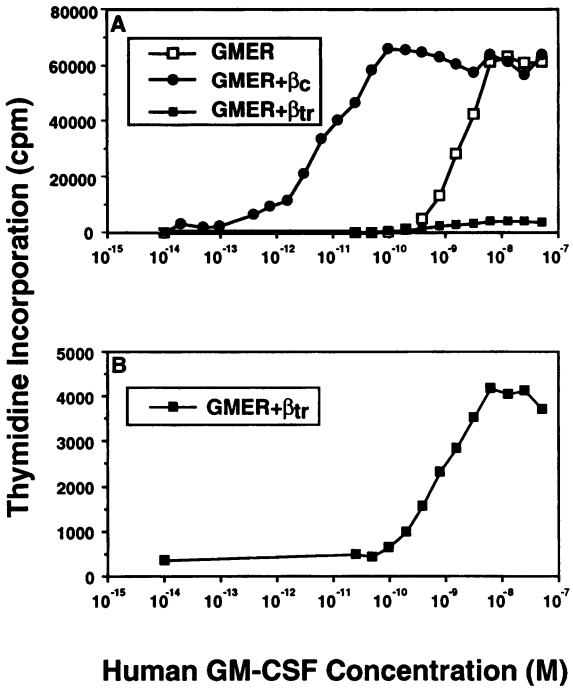

FIG. 2. βc confers a high-affinity hGM-CSF proliferative response on GMER, whereas βtr acts as a dominant negative

Tritiated thymidine incorporation assay of Ba/F3-GMER (□), Ba/F3-GMER + βc (●), and Ba/F3-GMER + βtr (■). The upper panel shows that Ba/F3-GMER + βc have the same maximum proliferation as Ba/F3-GMER but a 3-log increase in hGM-CSF sensitivity. Ba/F3-GMER + βtr proliferation is markedly inhibited; the upper plot is replotted with an expanded *y*-axis in the lower panel to show that the half-maximal proliferative response is similar to Ba/F3-GMER. The data are from one of three experiments.

binding assays with radiolabeled hGM-CSF. The results were analyzed by the LIGAND program and are presented in Fig. 1 as Scatchard plots. As illustrated in the top panel, Ba/F3-GMER showed low-affinity binding to hGM-CSF and a K_d of 5 nM, with 3600 binding sites per cell. Ba/F3-GMER + βc (middle panel) bound to hGM-CSF with a K_d of 40 pM and expressed 600 high-affinity receptors per cell. Ba/F3-GMER + βtr, shown in the bottom panel, bound GM-CSF with a K_d of 65 pM and 1300 high-affinity binding sites/cell. There was no significant difference in affinity between cells expressing GMER + βc and GMER + βtr. These data indicate that the majority of GMER were associated with βc as a high-affinity complex, since no low-affinity component was detected.

βc Confers a High-Affinity Proliferative Response while βtr Acts as a Dominant Negative

To examine whether coexpression of these two β-subunits affected GMER-induced proliferation, a growth assay was performed, as shown in Fig. 2. The closed circles in the top panel show that Ba/F3-GMER + βc cells responded to hGM-CSF and demonstrated the same growth rate as Ba/F3-GMER cell, shown as open squares. However, in contrast to Ba/F3-GMER cells, the half-maximal growth of these Ba/F3-GMER + βc was obtained at a 3 log lower concentration of hGM-CSF, identical to Ba/F3 cells that stably express hGMRα_2 + βc (not shown). In contrast, as shown by the closed squares, the maximum growth of Ba/F3-GMER + βtr was strikingly reduced to 3–20% of the maximum growth ob-

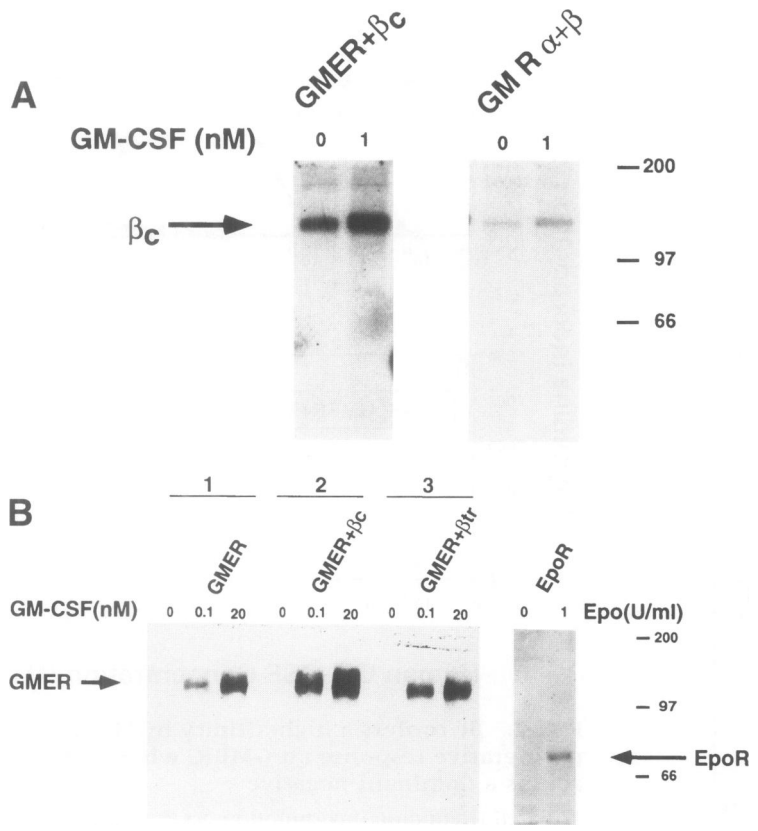

FIG. 3. Tyrosine phosphorylation of receptor subunits

(A) Double immunoprecipitation of Ba/F3-GMER + βc cells before (0) and after (1) hGM-CSF stimulation. Anti-phosphotyrosine immunoprecipitates were subjected to a second immunoprecipitation with rabbit polyclonal antibodies to the C terminus of βc. Background phosphorylation is increased after hGM-CSF stimulation, similar to control Ba/F3-GMRα + βc cells. (B) Immunoprecipitation/Western analysis of Ba/F3-GMER (1), Ba/F3-GMER + βc (2), and Ba/F3-GMER + βtr (3) before (0) and after (1) stimulation with 0.1 or 20 nM hGM-CSF. Cellular proteins were immunoprecipitated with 4G10 antibodies to phosphotyrosine, subjected to SDS polyacrylamide gel electrophoresis, and transferred to a nylon membrane. Western analysis was then carried out using antibodies to the C terminus of the mEpoR. A dose-related induction of phosphorylation was seen in each case, similar to control Ba/F3-EpoR cells stimulated with Epo, shown on the right. These data are from one of three representative experiments.

served with Ba/F3-GMER cells with or without full-length βc. In the bottom panel of Fig. 2, the expanded *y*-axis emphasizes that the half-maximal concentration of GM-CSF for Ba/F3-GMER + βtr is identical to that of Ba/F3-GMER alone, indicating that the observed growth was induced through the low-affinity GMER subunit. All three cell lines showed similar growth curves in response to murine IL-3 (not shown).

Receptor Tyrosine Phosphorylation Does Not Correlate with Cell Growth

To see whether a correlation exists between cell growth and the pattern of receptor phosphorylation, tyrosine phosphorylation of each of the receptor subunits was examined. Tyrosine phosphorylation of the β-subunit was measured by double immunoprecipitation with and without GM-CSF stimulation. Anti-phosphotyrosine immunoprecipitants were subjected to a second immunoprecipitation with antibodies to the C terminus of βc. An hGM-CSF–dependent increase in tyrosine phosphorylation of the βc-subunit in Ba/F3-GMER + βc was observed (Fig. 3A) and was indistinguishable from the induced tyrosine phosphorylation of βc in Ba/F3-GMRα + βc. To study phosphorylation of the EpoR cytoplasmic domain of GMER, the cells were stimulated with 0, 0.1, or 20 nM of hGM-CSF at 37°C for 10 min, immunoprecipitated first with anti-phosphotyrosine antibody and then blotted with an antibody to the C terminus of the EpoR. Figure 3B shows that the GMERs in all three cell lines were tyrosine-phosphorylated in response to hGM-CSF. This result is similar to the Epo-stimulated tyrosine phosphorylation of control cells that express wild-type EpoR, as shown on the right. Note that the cells with GMER + βtr did not show reduced intensity of phosphorylation, despite remarkably reduced proliferation. This suggests that there is no correlation between cell proliferation and induction of tyrosine phosphorylation of GMER and that the inhibition of proliferation induced by βtr occurs distal to the phosphorylation of tyrosine on GMER.

βc Increases β-Globin mRNA in Ba/F3-GMER Cells

To determine whether βc affects the differentiation function of GMER, we incubated cells in mIL-3, hGM-CSF, or a combination of both factors for 7 days before RNA extraction and North-

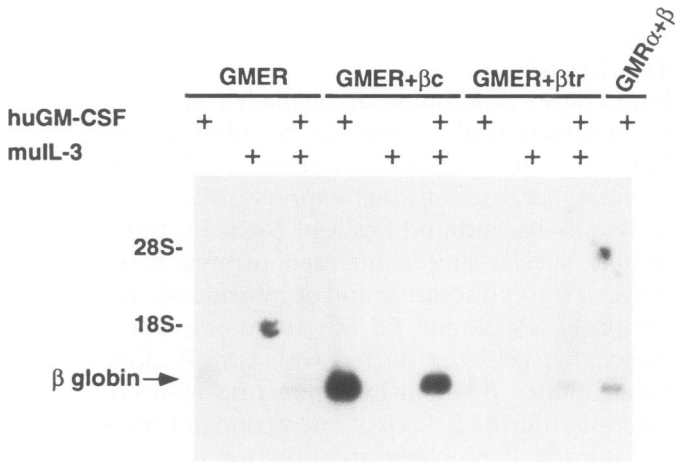

FIG. 4. Northern analysis of β-globin mRNA

Cells were cultured in hGM-CSF, mIL-3, or both, as indicated, and the RNA extracted. The samples were electrophoresed, transferred to nylon, and hybridized to a random-labeled β-globin probe. The greatest increase in β-globin mRNA was seen in GMER + βc cells stimulated with hGM-CSF; mIL-3 had no effect and appeared to down-modulate hGM-CSF–induced β-globin mRNA accumulation. Much lower levels of β-globin mRNA were seen with the GMER, GMER + βtr, and control GMRα + βc cells stimulated with hGM-CSF. The data represent one of two experiments.

ern blot analysis. Figure 4 shows that β-globin mRNA levels were markedly increased in cells that expressed GMER + βc after incubation in hGM-CSF. In contrast, the same cells incubated in mIL-3 showed no increase in β-globin mRNA. There was no increase in β-globin mRNA expression in GMER + βtr cells, demonstrating that the increase in β-globin message was mediated through the cytoplasmic domain of the β-chain and not via a protein that interacts with the extracytoplasmic or transmembrane portion of βc. Only slight levels of β-globin message were detected in cells transfected with GMRα + βc, indicating the importance of the cytoplasmic component of GMER in the differentiation signal.

DISCUSSION

The data reported here show, first, that βc converts the low-affinity binding and proliferation of a hybrid GMER receptor to high-affinity binding and proliferation. This result is analogous to the role of βc when coexpressed with the wild-type GMRα (5) or a truncated receptor (7). We also show that a complete cytoplasmic truncation mutant of the β-chain, βtr, while supporting high-affinity binding of GM-CSF to the GMER/β complex, greatly inhibits the proliferation of Ba/F3-GMER cells but does not affect the GM-CSF induced tyrosine phosphorylation of the EpoR cytoplasmic domain of GMER. Last, and surprisingly, βc also plays a role in the accumulation of β-globin in these GMER cells after incubation in hGM-CSF.

Studies with a constitutive active EpoR (17,18) and with a cytoplasmic truncated EpoR (19,20) suggest that dimerization may be important in EpoR signaling. Our observations that GMER-mediated proliferation is stimulated by GM-CSF in the low affinity dose range and that the truncated βc subunit acts as a dominant negative by inhibiting maximal proliferation, with no effect on the half-maximal GM-CSF concentration, are consistent with this dimerization hypothesis but do not prove it. We believe that the truncation mutant may interfere with the formation of GMER homodimers and does so efficiently, since the presence of βtr leads to high-affinity GM-CSF binding.

GM-CSF induced tyrosine phosphorylation of the Epo cytoplasmic domain of GMER in all three cell lines, GMER, GMER + βc, and GMER + βtr. The finding that tyrosine phosphorylation is unimpaired in GMER + βtr despite greatly reduced proliferation implies that receptor phosphorylation is not directly coupled to cell proliferation. This finding is consistent with the observation that a cytoplasmic truncation mutant of EpoR that lacks the 91 C-terminal amino acids, including seven of eight cytoplasmic tyrosines, is hypersensitive to Epo and that EpoR tyrosine phosphorylation has a negative regulatory role (12).

Ba/F3 cells transfected with mEpoR have been shown to have high levels of β-globin mRNA accumulation after growth in Epo (11), indicating that an the EpoR is important for this differentiation response. This result was not observed by Maruyama et al. (21), suggesting that Ba/F3 cells may exhibit variable responsiveness, possibly depending on receptor expression. However Maruyama et al. did demonstrate that mEpo-responsive TSA8 erythroleukemia cells transfected with an extracellular domain epidermal growth factor (EGF)/intracellular domain Epo hybrid receptor displayed EGF-stimulated growth and induction of β-globin protein syn-

thesis. The experiments reported here, however, suggest that the cytoplasmic domain of EpoR alone in GMER is insufficient for β-globin induction in Ba/F3 cells, but it can be made competent by introduction of a complete βc-subunit. Furthermore, the cytoplasmic domain of EpoR is essential, because cells that express GMRα + βc show only background levels of β-globin. Interestingly, species-specific interactions must occur between the extracellular and or transmembrane portion of GMER and βc, otherwise one would expect that cells transfected with GMER alone would induce β-globin by interacting with endogenous murine β. Lack of interaction of GMER and murine β probably accounts for the low-affinity binding of GM-CSF to GMER.

Could βc be important for differentiation in cells that express normal EpoR? It is difficult to test this idea in Ba/F3 cells because they express endogenous murine βc. There is published evidence to suggest that the EpoR may indeed associate with another protein. First, partially purified complexes of Epo and EpoR show an associated 130 kD protein that contains phosphotyrosine (pp 130) (22). Both the size and the ligand-induced tyrosine phosphorylation of pp 130 are consistent with its being βc, which is 134 kD and which becomes phosphorylated on tyrosine after GM-CSF or IL-3 binding. There is evidence, however, that this pp 130 kD protein is JAK2, a non-receptor tyrosine kinase that is phosphorylated on tyrosine after Epo stimulation and associates with the receptor (23). Recently, we were able to demonstrate associated βc in EpoR immunoprecipitation experiments (24). Second, it has been reported that hybrid receptors that comprise the extracellular domain of EpoR and the transmembrane and intracellular domains of the hIL-2R or mIL-3Rβ can induce β-globin gene expression in Ba/F3 cells (25). It is difficult to explain this induction of β-globin gene expression without invoking the action of a protein accessory to the extracellular domain of EpoR. An alternative explanation could be that some Ba/F3 clones express (or are induced to express) endogenous EpoR (20,26), and it is possible that the hybrid receptors associate with endogenous EpoR in the Ba/F3 cells and in the murine erythroleukemia cells tested in these studies. We are currently evaluating analogous chimeras of EpoR/βc and EpoR/GMRα in Ba/F3 and CTLL cells to test this possibility. Finally, observations by Hanazono et al. that Epo can induce tyrosine phosphorylation of βc suggest that EpoR and βc functionally interact (27).

In conclusion, we provide evidence that the extracellular and transmembrane domains of βc are sufficient to act as a high-affinity converter of a hybrid GMER receptor. The cytoplasmic domain of βc is necessary for high-affinity proliferation, and a truncated β-subunit greatly inhibits this response. Coexpression of GMER and βc leads to accumulation of β-globin transcripts, and this function depends on the erythropoietin receptor cytoplasmic domain of the hybrid. The βc appears to act as an accessory protein in this context. Whether it might fulfill the same role in normal Epo-responsive cells is the subject of current studies.

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