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Membrane-associated and soluble granulocyte/macrophage-colony-stimulating factor receptor α subunits are independently regulated in HL-60 cells

(RNA splicing/polymerase chain reaction/hematopoiesis/mRNA stability/human leukemia cell lines)

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ABSTRACT **The effects of granulocyte/macrophage-colony-stimulating factor (GM-CSF) are mediated by interaction with its composite receptor (GMR), which consists of a unique α subunit (GMRα) and a β subunit (GMRβ) that is common to the receptors for GM-CSF, interleukin 3, and interleukin 5. GMRβ is required for high-affinity binding, cell proliferation, and protein phosphorylation but has no intrinsic GM-CSF-binding activity. GMRα in isolation binds to GM-CSF with low affinity and can signal for increased glucose uptake. In addition to the membrane-bound receptor (mGMRα), there is a naturally occurring soluble isoform (sGMRα) that is released free into the pericellular milieu. Analysis of genomic sequences reveals that the soluble GMRα isoform comes about by alternative mRNA splicing. To examine GMRα expression, we developed a quantitative reverse transcription–polymerase chain reaction assay based on serial dilutions of *in vitro* transcribed GMRα RNA. This assay provides a strict log–log measure of GMRα RNA expression, distinguishes transcripts related to the soluble and membrane-associated isoforms, and quantitatively detects 0.1 fg of GMRα-related mRNA. There was little or no GMRα expression in two human lymphoid cell lines and in the erythroblastic leukemia cell line K562, but all myeloid cell lines tested expressed both the membrane-associated and soluble isoforms of GMRα. Baseline level of expression of both isoforms varied >20-fold among the myeloid cell lines studied. Differentiation of HL-60 cells to neutrophils with dimethyl sulfoxide led to a 2-fold downregulation of sGMRα and a 20-fold upregulation of mGMRα. These differentiation-induced transcriptional changes were unrelated to changes in mRNA stability. These findings indicate that sGMRα is differentially expressed from mGMRα in human hematopoietic cells and that programmed downregulation of sGMRα may be important in myeloid maturation.**

Granulocyte/macrophage-colony-stimulating factor (GM-CSF) is a hematopoietic hormone with broad biological activities which include stimulation of proliferation and differentiation of hematopoietic progenitors and enhancement of the function of mature granulocytes and mononuclear phagocytes (1). The biological effects of GM-CSF are initiated at the cell surface by interaction with the GM-CSF receptor (GMR), which consists of two subunits, GMRα and GMRβ. The GMRα subunit is an 84-kDa transmembrane polypeptide which, in the absence of GMRβ, binds GM-CSF with low affinity (K_d = 2–6 nM) (2–4). GMRβ has no intrinsic ability to bind to GM-CSF but forms a high-affinity receptor in complex with GMRα with a K_d of 20–100 pM (5–8). In hematopoietic cells and cell lines, the effects of GM-CSF are mediated by a small number of high-affinity receptors, from <200 on myeloid progenitors to 700–1000 on mature polymorphonuclear neutrophils (9, 10). The low abundance of GMR-related transcripts has been an important obstacle to studies of GMRα expression.

Several isoforms of GMRα have been identified by analysis of GMRα-related cDNAs. One of these isoforms, molecularly cloned in our laboratory and elsewhere, encodes a GMRα-related protein that lacks the transmembrane amino acid sequences of the membrane-associated receptor (mGMRα) (11–13). We have shown that this isoform is a naturally occurring soluble GMR (sGMRα), which binds GM-CSF in solution at low affinity (13). Most receptors in the hematopoietin superfamily have soluble isoforms (14). Receptors for growth hormone, granulocyte-colony-stimulating factor (G-CSF), interleukin 4 (IL-4), IL-5, IL-6, IL-7, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and erythropoietin have been isolated either as soluble proteins or as alternatively processed forms that are predicted on the basis of their cDNA sequence to encode soluble receptors.

To investigate the expression of GMRα, we developed a reverse transcription–polymerase chain reaction (RT–PCR) assay that permits reliable quantitation of the expression of mGMRα- and sGMRα-related transcripts in human hematopoietic cells. Study of steady-state GMRα mRNA expression in human leukemia cell lines showed variable levels of sGMRα and mGMRα mRNA, suggesting that expression of the two isoforms may be separately regulated. In HL-60 human promyelocytic leukemia cells induced to differentiate along the granulocytic pathway, the ratio of mGMRα to sGMRα mRNA increased markedly, due to a prominent increase in mGMRα expression and a modest decline in sGMRα expression. Because the stability of these two mRNAs was unaffected during differentiation, the change in GMRα expression is most likely due to increased transcription of the GMRα gene with differential splicing to favor formation of the membrane-associated isoform.

MATERIALS AND METHODS

Cell Lines and Reagents. Human leukemia cell lines (KG1, KG1a, K562, MO7e, 729-6, HL-60, and MO-T) were maintained in Iscove's modified Dulbecco's medium (IMDM)

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Abbreviations: GM-CSF, granulocyte/macrophage-colony-stimulating factor; GMR, GM-CSF receptor; GMRα, GMR α subunit; sGMRα, soluble GMRα; mGMRα, membrane-bound GMRα; GMRβ, GMR β subunit; IL, interleukin; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcription–polymerase chain reaction.

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supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics (15–18). MO7e cells were maintained in the presence of 50 pM IL-3 (R & D Systems) or 500 pM GM-CSF (a gift from Amgen) as indicated (19). U-937 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (20). HL-60 cells were induced to differentiate into neutrophilic cells by adding dimethyl sulfoxide (DMSO; final concentration, 1.25%) or into macrophages with phorbol 12-myristate 13-acetate (0.1 μm; Sigma) (15, 21). DNA-dependent RNA synthesis was inhibited with actinomycin D (Sigma) at 5 μg/ml.

Genomic Organization. A genomic library from human polymorphonuclear leukocytes was constructed with a phage λ vector system (λ GEM-11, Promega) after partial digestion with *Sau*3A1 (New England Biolabs). Recombinant phage were screened with a *Pvu* II–*Eco*RI fragment of GMRα cDNA (nt 909–1287). Candidate phage were subcloned into pBluescript KS (Stratagene) and screened with specific oligomers corresponding to transmembrane (5′-ATTTATGTGCTCCTAATCGTGGGAACCCTTGTCTGTGGCA-3′, nt 1128–1167) and 3′ (5′-GGTTCCTTAGGATACAGCGGCTGTTCCCGCCAGTTCCACA-3′, nt 1192–1231) sequences of GMRα. Genomic sequences of selected subclones were determined with T7 DNA polymerase (Sequenase; United States Biochemical).

RNA Extraction and RT. Total cellular RNA was obtained by guanidinium thiocyanate extraction (RNAzol B; Biotecx Laboratories, Houston). For cDNA synthesis, 1 μg of cellular RNA in a 20-μl reaction volume was incubated in 10 mM Tris, pH 8.3/50 mM KCl/5 mM $MgCl_2$/1 mM dNTPs with 200 ng of random dN_6 and 20 units of avian myeloblastosis virus reverse transcriptase (XL; Life Sciences, St. Petersburg, FL) for 10 min at 23°C followed by 30 min at 42°C. Parallel reactions were performed in the absence of reverse transcriptase to control for the presence of contaminating DNA.

PCR. A cDNA aliquot in a volume of 50 μl in 10 mM Tris, pH 8.3/50 mM KCl/5 mM $MgCl_2$, 1 mM dNTPs with 10 units of *Taq* DNA polymerase (Boehringer Mannheim) and appropriate primers was incubated at 94°C for 1 min and 65°C for 2 min for 35 cycles. The primers were as follows: for GMRα, 5′-AGCCCAGAGCAAAACACA-3′ (nt 1009–1026) and 3′-CCATGCCATTCCTACACCCT-5′ (nt 1360–1379) (Fig. 1*A*); for GMRβ, 5′-CTACAAGCCCAGCCCAGATGC-3′ (nt 859–879) and 3′-ACCCGTAGATGCCACAGAAGC-5′ (nt 1390–1410). The 3′ primer was end-labeled with ^{32}P and each primer was present at 6 ng/μl. Products were electrophoresed in a 5% acrylamide gel at 100 V for 4 hr and assessed by autoradiography. Electrophoretic migration was consistent with the predicted size of the products (mGMRα, 370 bp; sGMRα, 273 bp; GMRβ, 572 bp). When appropriate, bands were cut out of the gel and quantitated by liquid scintillation counting or densitometry. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT–PCR product was used as an internal standard (22).

Preparation of GMRα RNA Standards. sGMRα and mGMRα cDNAs cloned in pBluescript KS(+) (Stratagene) were transcribed from the T7 promoter (Megascript, Ambion, Austin, TX). The RNA was exhaustively digested with DNase and quantitated spectrophotometrically and by the addition of trace levels of [α-^{32}P]UTP (New England Nuclear). mGMRα and sGMRα RNAs were mixed at a ratio of 10:1 and standard serial dilutions were prepared. In the standardization curves, RT–PCR of the diluted specimens was performed in the presence of 1 μg of RNA derived from 729-6 cells, a cell line that does not express GMRα (see *Results*).

RESULTS

Quantitative RT–PCR of GMRα RNA. Since GMR is expressed at low levels, analysis of GMR expression by Northern blot is difficult and discrimination between the soluble and membrane-associated isoforms requires the use of cumbersome RNase protection methods (13). sGMR arises as a result of alternative splicing of the same parent mRNA species that encodes the membrane-associated receptor (Fig. 1*A*). Our sequence analysis of the subcloned exons revealed that the transmembrane exon was 97 bp, the exon downstream was 82 bp (Fig. 1*B*), and each exon was flanked by consensus mRNA splice donor or acceptor sequence motifs. While the exon 3′ to the transmembrane exon does not encode the entire carboxyl terminus of mGMRα, it does encode the carboxyl terminus predicted for sGMRα, which, because of a frameshift gener-

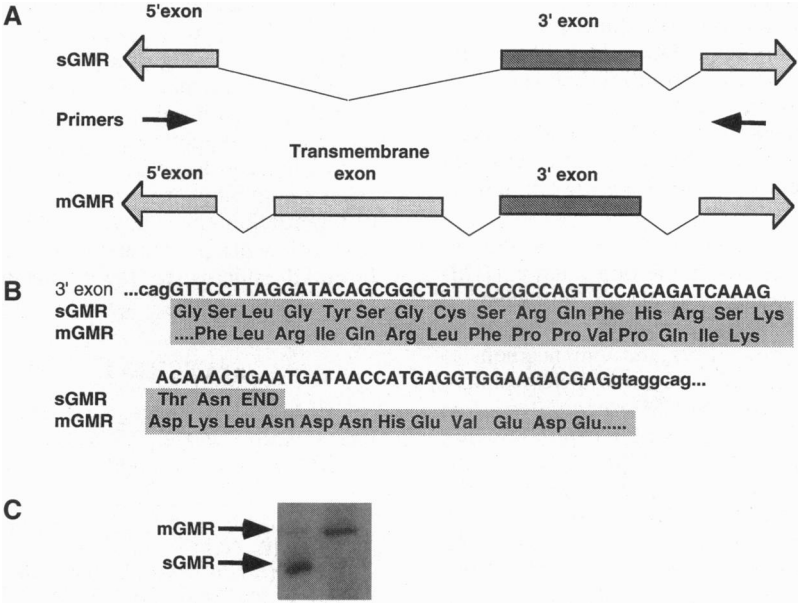

FIG. 1. (*A*) Genomic organization of GMRα in the region of the transmembrane exon and the position of GMRα PCR primers. (*B*) DNA sequence of the exon 3′ to the transmembrane exon with flanking consensus splice donor and acceptor sequences. Translational reading frames of the sGMRα and mGMRα gene products are indicated. (*C*) Electrophoretic migration of mGMRα and sGMRα PCR products derived from molecularly cloned cDNA plasmids.

ated by splicing to the upstream exon, terminates at nt 1250 of the membrane-associated receptor.

To study the expression of GMRα-related RNA in hematopoietic cells, we developed a quantitative RT–PCR method. Using primers that span the transmembrane exon, PCR clearly distinguished species corresponding to the membrane-associated and soluble receptors in molecularly cloned DNA (Fig. 1*C*). Quantitative standards of GMRα RNA were generated, and known quantities of GMRα RNA were mixed with total cellular RNA from 729-6 cells, a human B-cell line that does not express GMRα (see below). cDNA was prepared and, even after 35 cycles of PCR amplification, a strict log–log relationship between GMRα concentration and band intensity (or incorporated radioactivity) was observed (Fig. 2). With this dilution method, the lowest limit of detection was not defined, but it is <0.03 fg, or ≈50 copies of GMRα mRNA per 10^5 cells.

Expression of mGMRα and sGMRα RNA in Human Hematopoietic Cell Lines. To compare the level of expression of the soluble and membrane-associated forms of GMRα among different hematopoietic cells, we isolated total cellular RNA from the myeloid leukemia cell lines HL-60, KG1, KG1a, U-937, and MO7e (a cell line dependent on GM-CSF or IL-3 for growth), the erythroblastic leukemia cell line K562, the B-lymphoid cell line 729-6, and MO-T, a human T-lymphotropic virus type II-transformed T-lymphoblastic cell line (Figs. 3 and 4). cDNA was prepared from RNA derived from these cell lines in parallel with serial dilutions of GMRα RNA prepared as above. To ensure that RNA isolation and cDNA synthesis were equivalent among the samples, we measured the expression of the GAPDH gene, which is constitutively transcribed (22). GAPDH expression was similar in all samples except K562, which reproducibly expressed less GAPDH per microgram of total cellular RNA (Fig. 3). The expression of GMRα was then determined on another aliquot of cDNA by PCR. A standard curve was constructed with data derived from serial dilutions of *in vitro* transcribed GMRα RNA, and

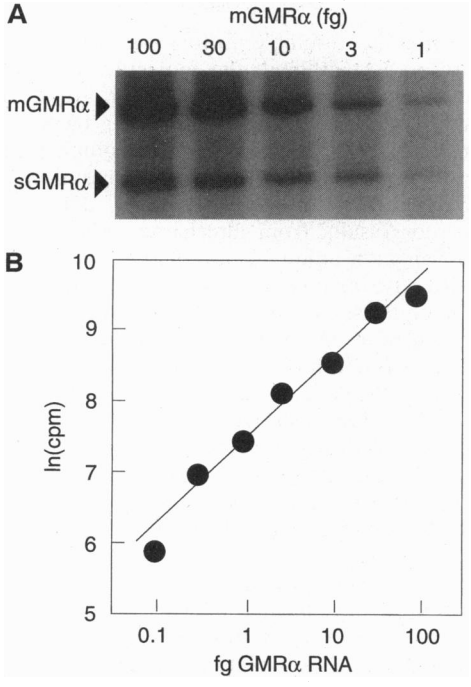

FIG. 2. RT–PCR of standardized GMRα concentrations. RNAs encoding the mGMRα or sGMRα were synthesized *in vitro* and quantitated. Serial dilutions of a mixture (mGMRα/sGMRα, 10:1) of these RNAs were added to 1 μg of total cellular RNA from 729-6 cells and subjected to RT–PCR. The products were separated by electrophoresis (*A*). The bands corresponding to each product were cut out and the incorporated radioactivity was plotted (*B*).

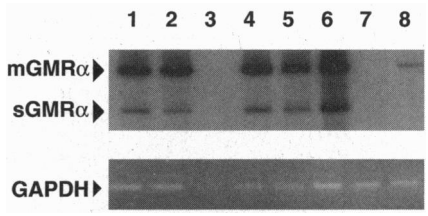

FIG. 3. GMRα expression in human hematopoietic cell lines. (*Upper*) Total cellular RNA was isolated from each cell line and subjected to RT–PCR in parallel with standardized serial dilutions of GMRα (data not shown). Lane 1, KG1; lane 2, KG1a; lane 3, K562; lane 4, MO7e cultured with IL-3; lane 5, MO7e cultured with GM-CSF; lane 6, U-937; lane 7, 729-6; lane 8, MO-T. (*Lower*) GAPDH expression from the corresponding cell lines was assessed by performing RT–PCR on a cDNA aliquot with GAPDH-specific primers. Ethidium bromide-stained products were analyzed in a 1.5% agarose gel. PCR was performed on all RNA samples in the absence of reverse transcriptase to ensure that there was no DNA contamination (data not shown).

GMRα mRNA levels from each cell line were determined by interpolation (Table 1). The lymphoid cell line 729-6 and the erythroblastic leukemia cell line K562 expressed no detectable GMRα RNA. MO-T, the T-lymphoblastic cell line, expressed trace levels of mGMRα. All of the myeloid leukemia cell lines tested expressed RNA that corresponded to mGMRα and to sGMRα. In the growth factor-dependent cell line MO7e, there was little difference in GMRα expression whether the cells were maintained in medium containing IL-3 or GM-CSF. In myeloid cell lines, the levels of mGMRα RNA varied considerably, from as little as 3 fg/μg of RNA in KG1a cells to as much as 57 fg/μg of RNA in U-937 cells. There was also considerable variation in sGMRα expression, from 0.1 fg/μg of RNA in KG1, KG1a, and MO7e cells to 5 fg/μg of RNA in HL-60 cells. Among these cell lines the ratio of sGMRα to mGMRα varied 30-fold, suggesting that sGMRα is differentially expressed.

Expression of GMRα in Differentiating HL-60 Cells. Myeloid cell lines were found to express sGMRα at variable levels, in absolute terms and relative to mGMRα. We therefore examined the hypothesis that the splicing event yielding sGMRα could be regulated independently of total GMRα gene transcription. To explore this concept within the context of cellular differentiation, we measured GMRα expression in

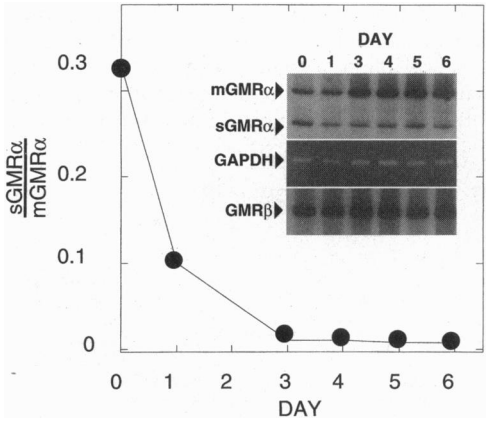

FIG. 4. Effect of granulocytic maturation on GMR expression. HL-60 cells were incubated for 6 days in the presence of 1.25% DMSO. Total cellular RNA was isolated each day and RT–PCR was performed with PCR primers specific for GMRα, GAPDH, and GMRβ as indicated (*Inset*). mGMRα RNA and sGMRα RNA were quantitated by interpolation onto a standard curve derived from RT–PCR of standardized GMRα RNA performed in parallel (data not shown). The ratio of sGMRα RNA to mGMRα RNA is plotted. Results are representative of three experiments.

Table 1. Expression of GMRα in human leukemia cell lines

Cell line	mRNA, fg (copies per 1000 cells)		
	mGMRα	sGMRα	sGMRα/mGMRα
KG1	11–19 (16–28)	0.1–0.3 (0.1–0.4)	0.01–0.02
KG1a	3–13 (4–20)	0.1–0.2 (0.1–0.3)	0.01–0.04
MO7e/IL-3	8–13 (12–20)	0.1–0.3 (0.1–0.4)	0.01–0.03
MO7e/GM-CSF	5–8 (8–12)	0.1–0.3 (0.1–0.4)	0.02–0.06
HL-60			
Promyelocytic	4–14 (6–21)	1.5–5 (2–8)	0.25–0.33
Granulocytic	287–530 (430–795)	1.0–2.5 (1–4)	0.005–0.007
U-937	32–57 (48–86)	1.4–2.6 (2–4)	0.04–0.05
K562	0	0	—
729-6	0	0	—
MO-T	<0.1	0	—

Results represent at least two experiments on independently isolated RNA specimens. The mRNA copy number reflects an approximate molecular mass of 500 kDa for GMRα mRNA (13) and isolation of 1 μg of total cellular RNA per 10^5 cells.

differentiating HL-60 cells. HL-60 cells were induced to undergo terminal differentiation to granulocytes by addition of 1.25% DMSO (15, 21). GMRα mRNA levels in these differentiating cells were measured by RT–PCR as described above. Over 6 days of maturation in DMSO, mGMRα mRNA increased by 20-fold (Fig. 4 and Table 1). This result is consistent with the finding that the number of GM-CSF binding sites increases 5- to 10-fold upon differentiation with DMSO and correlates with an increasing number of terminally differentiated neutrophilic cells (23). During the course of differentiation, however, the absolute level of sGMRα mRNA declined slightly. Thus the ratio of sGMRα mRNA to mGMRα mRNA decreased from 0.25–0.33 prior to the addition of DMSO to 0.005–0.007 on day 6 of treatment, and there was a preferential accumulation of mRNA encoding the membrane-associated isoform during granulocytic differentiation in HL-60 cells. A similar decline in the ratio of sGMRα to mGMRα was observed in HL-60 cells induced to mature along the macrophage lineage with 0.1 μM phorbol 12-myristate 13-acetate (data not shown), suggesting that preferential RNA splicing to form mGMRα is a common feature of terminal granulocytic and monocytic differentiation in HL-60 cells. To determine whether the expression of the GMRβ was also regulated in DMSO-treated HL-60 cells, cDNA aliquots were amplified with PCR primers specific for the GMRβ subunit, but there was no evidence of induction of GMRβ mRNA.

Stability of GMRα mRNA. To determine whether the differential accumulation of mGMRα mRNA in maturing HL-60 cells was due to a change in stability, HL-60 cells were treated with actinomycin D to arrest transcription before and after DMSO-induced granulocytic maturation. GMRα decay over a 2-hr time course was measured by RT–PCR prior to and after 4 days of DMSO incubation (Fig. 5). Although the initial amounts of mGMRα mRNA differed, corresponding with the increased accumulation of mGMRα mRNA during DMSO-induced differentiation, the decay curves of mGMRα and sGMRα mRNAs were similar to each other and did not differ appreciably over the course of DMSO-induced maturation. The half-life of GMRα mRNA decay was ≈1.5 hr. The short half-life of GMRα mRNA species may be explained by the presence of multiple mRNA destabilizing sequence motifs in the 3′ untranslated region (24–26). Since there was no evidence of altered turnover of either GMRα mRNA isoform during differentiation, the selective accumulation of mGMRα mRNA resulted from increased transcription of the GMRα gene and preferential splicing to form the mRNA species encoding the membrane-associated subunit.

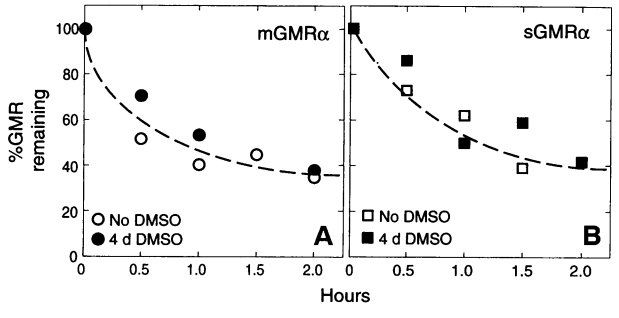

FIG. 5. Decay of GMRα in HL-60 cells. HL-60 cells prior to and after 4 days of DMSO incubation (○ and ●, respectively) were treated with actinomycin D at 5 μg/ml. The expression of mGMRα (*A*) and sGMRα (*B*) was measured by RT–PCR. Decay is expressed as percent of the initial value for each mRNA species under each culture condition as indicated. Results are representative of four experiments with untreated cells and two experiments with DMSO-treated cells.

DISCUSSION

The expression of a soluble isoform is common to many cytokine receptors, although the molecular mechanisms by which such soluble receptors are generated are diverse (14). For example, the soluble IL-6 receptor arises by proteolysis of the mature receptor after translation, the soluble receptor for ciliary neurotrophic factor arises by cleavage of an extracellular glycosyl-phosphatidylinositol anchor, and the soluble IL-5α receptor results from alternative splicing leading to inclusion of an exon unique to its soluble subunit. We found that sGMRα also results from alternative mRNA splicing, although, in contrast with the soluble IL-5 receptor, it comes about by a splice event that eliminates the transmembrane exon, causing a frameshift in the 3′ exon and termination of translation after the generation of 16 unique amino acids. The genomic organization of GMRα recently reported by Nakagawa *et al.* (27) is consistent with these results. The elements that govern the alternative RNA splicing of GMRα are unknown but probably include DNA sequence motifs within the flanking introns and RNA and protein components of the spliceosome (28–31).

We used the absence of the transmembrane exon in the sGMRα mRNA to develop an RT–PCR assay for the detection of the mRNAs encoding the soluble and membrane-associated isoforms of GMRα. By choosing PCR primers that span the transmembrane exon, we were able to distinguish the mRNAs that correspond to these two isoforms. Although some have considered that the exponential process of PCR does not lend itself to quantitation, more recent reports have applied PCR methodology quantitatively (32–35). The RT–PCR assay presented here provides a reliable log–log relationship between specific PCR product and standardized concentrations of *in*

vitro transcribed GMRα RNA and allows the derivation of a standard curve to quantitate experimental samples. The assay is extremely sensitive and can measure subfemtogram quantities of GMRα-related RNA.

Myeloid leukemia cell lines demonstrated considerable variation with regard to absolute and relative amounts of mGMRα and sGMRα expression. Moreover, in differentiating HL-60 cells, the ratio of mGMRα mRNA to sGMRα mRNA increased by >50-fold, suggesting that sGMRα expression is dynamic and, at least in part, regulated independently from mGMRα expression. Because the half-lives of mGMRα and sGMRα mRNAs are similar and are not affected by DMSO treatment in HL-60 cells, our data indicate that the differential expression we observed is a regulated process due to preferential mRNA splicing. In differentiating HL-60 cells, GMRβ expression is not affected by DMSO treatment, suggesting that the upregulation of mGMRα is critical for the observed increase in GM-CSF binding sites on the cell surface. Previous studies had suggested that the GMRβ subunit could be the limiting element in GM-CSF–receptor binding and, hence, in responsiveness to the ligand (5, 23).

Precisely regulated expression of a gene implies an important physiologic function for its product. In the case of the insulin receptor, alternatively spliced variants of the insulin receptor α chain have different affinities for insulin and their regulated expression alters the cellular response to ligand (36–38). Even more striking, alternative splicing of the *bek* gene gives rise to receptors with distinct ligand-binding properties—the keratinocyte growth factor receptor and the high-affinity receptor for basic and acidic fibroblast growth factors, FGFR-2 (39, 40). Because sGMRα binds to its ligand with low affinity, its biological function in the context of hematopoietic cells, which generally express high-affinity receptors for GM-CSF, has been unclear. We recently found that isolated mGMRα mediates increased glucose transport upon GM-CSF binding, a signaling pathway that is active in HL-60 cells (41). Thus, a secreted GMRα subunit could compete with mGMRα for ligand and antagonize the glucose-transport signaling pathway. The preferential induction of mGMRα during granulocytic differentiation further suggests an inhibitory role for sGMRα in myeloid maturation.

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