

## Distinct Cytoplasmic Regions of the Human Granulocyte Colony-Stimulating Factor Receptor Involved in Induction of Proliferation and Maturation

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**The granulocyte colony-stimulating factor receptor (G-CSF-R) transduces signals important for the proliferation and maturation of myeloid progenitor cells. To identify functionally important regions in the cytoplasmic domain of the G-CSF-R, we compared the actions of the wild-type receptor, two mutants, and a natural splice variant in transfectants of the mouse pro-B cell line BAF3 and two myeloid cell lines, 32D and L-GM. A region of 55 amino acids adjacent to the transmembrane domain was found to be sufficient for generating a growth signal. The immediate downstream sequence of 30 amino acids substantially enhanced the growth signaling in the three cell lines. In contrast, the carboxy-terminal part of 98 amino acids strongly inhibited growth signaling in the two myeloid cell lines but not in BAF3 cells. Truncation of this region led to an inability of the G-CSF-R to transduce maturation signals in L-GM cells. An alternative carboxy tail present in a splice variant of the G-CSF-R also inhibited growth signaling, notably in both the myeloid cells and BAF3 cells, but appeared not to be involved in maturation.**

The formation of blood cells in the bone marrow is controlled by a regulatory network of hematopoietic growth factors (5, 23). One of these factors, granulocyte colony-stimulating factor (G-CSF) secreted by macrophages, fibroblasts, and endothelial cells, plays an essential role in the regulation of granulopoiesis and the maintenance of neutrophil levels in the peripheral blood. For instance, G-CSF not only is able to stimulate the proliferation of neutrophilic progenitor cells but also induces maturation of these progenitor cells towards neutrophilic granulocytes (8, 24, 32).

The murine and human receptors for G-CSF (G-CSF-R) consist of a single polypeptide with a molecular weight of 130,000 to 150,000 (10, 44). The high-affinity interaction between G-CSF and its receptor and subsequent activation of receptor signaling require the formation of homodimeric or oligomeric receptor complexes (10). The molecular cloning of the cDNAs encoding the murine and human G-CSF-R revealed that the murine and human G-CSF-R contain 812 and 813 amino acids, respectively, with a single transmembrane domain (12, 13, 21). The structure of the G-CSF-R shows significant homology to that of the interleukin-6 (IL-6) signal transducer gp130 (16, 38). Expression of the G-CSF-R cDNAs in murine hematopoietic cells leads to formation of high-affinity G-CSF binding sites on the cell surface and renders these cells responsive to G-CSF (11, 49). At least four different forms of the human G-CSF-R, resulting from alternative splicing of G-CSF receptor mRNA, have been cloned from human placenta and myeloid leukemia (U937) cells (13, 21).

On the basis of structural similarities, the G-CSF-R has been classified as a member of the superfamily of cytokine receptors (1, 6). The homologous structures shared by the members of the family consist of four conserved cysteine residues and a WSXWS motif in the extracellular region.

The cytoplasmic domain of the family is less conserved, but certain sequence similarities have been reported among the receptors for IL-3 to IL-7, G-CSF, granulocyte macrophage-CSF, erythropoietin (EPO), and the  $\beta$  chains of IL-2 and IL-3 (11, 29). Several studies have delineated functionally important regions in the cytoplasmic domain. For instance, a membrane-proximal region of the EPO receptor (EPO-R), IL-6 signal transducer gp130, and the  $\beta$  chains of IL-2 and IL-3 is essential for transmitting a growth signal (7, 15, 29, 39). In agreement with these findings, it has been established that the membrane-proximal region of the G-CSF-R is indispensable for growth signal transduction (11, 48). The cytoplasmic tail of the EPO-R has been shown to down-modulate the mitogenic response to EPO (7, 35). In contrast, the distal cytoplasmic region of the G-CSF-R has been reported to enhance growth signaling potential (11).

In this paper, we describe the identification of two cytoplasmic regions of the wild-type (WT) G-CSF-R, located distal to the box 1- and box 2-containing region, one that positively and one that negatively regulates the mitogenic response to G-CSF. We provide evidence showing that a distinct carboxy terminus present in a G-CSF-R splice variant also inhibits growth signaling. Further, we show that the negative regulatory region of the WT G-CSF-R but not of the splice variant is essential for G-CSF-induced neutrophilic maturation. These findings establish that distinct regions of the cytoplasmic domain of the G-CSF-R have a determinative role in G-CSF-induced proliferation and maturation of myeloid progenitor cells.

### MATERIALS AND METHODS

**Cell lines and culture.** The murine pro-B cell line BAF3 (34) was kindly provided by G. Plaetinck (Roche Research, Ghent, Belgium) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng of murine IL-3 per ml. Murine myeloid cell lines 32D (14) and

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L-GM (22) were provided by J. S. Greenberger (University of Massachusetts) and T. Honjo (Kyoto University Faculty of Medicine, Kyoto, Japan). 32D and L-GM cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 10% WEHI-3B cell conditioned medium (WEHI-CM) as a source of murine IL-3.

**G-CSF-R expression constructs.** For the construction of pLNCX-WT, the WT human G-CSF-R cDNA (13) (pHQ3, kindly provided by S. Nagata and R. Fukunaga) was excised from the pBluescript vector and inserted into the *HpaI* site of the retroviral expression vector pLNCX (25). For the construction of pLNCX-DC, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on total RNA isolated from normal granulocytes by using forward primer 5'TGTGATCATCGTGACTCCCTT3' (FW2) and reverse primer 5'CAAGATCTAGTTTACAATACTGAAG3' (RV2), and an 864-bp PCR fragment was subcloned into the pBluescript vector. A 248-bp stretch of the fragment was then cut out with *BsrFI* and *ClaI* and, together with the *HindIII-BsrFI* fragment obtained from pLNCX-WT, was inserted into *HindIII-ClaI*-cleaved pLNCX vector by triple ligation. Mutant DA was isolated from granulocytes of a patient with congenital neutropenia by RT-PCR using the same set of primers. The construction of the full-length DA mutant is essentially same as that described for the DC receptor. Mutant M1 was generated by PCR by introducing a termination codon at amino acid position 686 of the WT G-CSF-R. The PCR fragment was first ligated to the *HincII* site of the pBluescript vector and then excised from the plasmid by *HindIII* and *XhoI* digestion, blunt ended, and inserted into the *HpaI* site of pLNCX.

**DNA transfection.** The pLNCX expression constructs were linearized by *PvuI* digestion and transfected into BAF3, 32D, and L-GM cells by electroporation. Following gene transfer, cells were cultured in IL-3-containing medium for 48 h and then selected in G418 (Gibco-BRL, Breda, The Netherlands)-containing medium at concentrations of 1.5 (BAF3) or 0.8 (32D and L-GM) mg/ml. G418-resistant clones were expanded and tested for their capacities to specifically bind radiolabeled G-CSF. RT-PCR using primer sets FW2-RV1 (5'GTAGATCTTAGTCATGGGCTTATGG3'), FW2-RV2, and FW2-RV3 (5'TCTCAGGGCACCGGCTTCTTT3') was performed on the transfectants to test for possible incomplete transfer of sequences encoding the cytoplasmic domains.

**G-CSF binding assay.** Recombinant human G-CSF (Amgen, Thousand Oaks, Calif.) was radiiodinated by the method of Bolton and Hunter (2). G-CSF binding characteristics were determined as previously described (4). In brief, cells ( $10^6$ ) were incubated with serial dilutions of radiolabeled G-CSF ranging from 20 pM to 5 nM at 37°C for 1 h. Incubations in the presence of excess nonlabeled G-CSF were also performed to correct for nonspecific binding. Bound ligand was separated from free ligand by centrifugation (5 min at  $1,000 \times g$ ) through an ice-cold FCS layer. Receptor numbers and binding affinity were calculated as described previously (4).

**DNA synthesis and cell proliferation assays.** DNA synthesis was assayed by [ $^3\text{H}$ ]thymidine ([ $^3\text{H}$ ]TdR) uptake. Cells ( $10^4$ ) were incubated in triplicate in 100- $\mu\text{l}$  of 10% FCS-RPMI medium supplemented with titrated concentrations of G-CSF or WEHI-CM (20%) in 96-well plates for 24 h. Twelve hours before cell harvest, 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR (2 Ci/mM; Amersham) was added to each well. [ $^3\text{H}$ ]TdR incorporation was measured by liquid scintillation counting as described previously (40).

To quantify cell proliferation in suspension culture, cells were incubated in 25-ml culture flasks at an initial density of  $0.5 \times 10^5/\text{ml}$  (5 ml of culture) in medium supplemented with recombinant human G-CSF at the indicated concentrations or without growth factors. Culture medium was refreshed every 2 to 3 days, and the cell densities were adjusted to approximately  $0.5 \times 10^5$  to  $1 \times 10^5/\text{ml}$ . Viable cells were counted on the basis of trypan blue exclusion.

**Morphological examination.** Cells were collected every 2 to 3 days and spun onto glass slides. Cell morphology was examined after May-Grünwald-Giemsa staining.

## RESULTS

**Properties of the G-CSF-R variant and mutants.** The structures of the different forms of the human G-CSF-R relevant to this study are summarized in Fig. 1. DC represents an RT-PCR product obtained from normal granulocytes that is weakly expressed in comparison with the WT G-CSF-R (data not shown). Nucleotide sequencing revealed that DC is identical to the previously described splice variant D7, derived from placenta (21). From amino acid 727, DC is translated in an altered reading frame and contains 34 carboxy-terminal amino acids distinct from those of the WT G-CSF-R. DA, derived from granulocytes of a patient with congenital neutropenia, contains a C-to-T substitution at nucleotide position 2384 that introduces a TAG stop codon resulting in the truncation of 98 carboxy-terminal amino acids. No other alterations were found in this mutant by nucleotide sequencing. M1 contains only 55 amino acids in the cytoplasmic domain that includes the two subdomains called box 1 and box 2 (11, 29), with 3 additional amino acids downstream. The different forms of the G-CSF-R were introduced into the murine cell lines BAF3, 32D, and L-GM by electroporation. RT-PCR products derived from the transfectants by using primers that amplify the entire cytoplasmic domains all had the predicted sizes, indicating that no deletions in these regions had occurred during transfection and clone selection (Fig. 2).

**G-CSF binding to BAF3, 32D, and L-GM transfectants.** The numbers and dissociation constants of the G-CSF-R expressed by the different transfected cell lines were established following binding of  $^{125}\text{I}$ -radiolabeled G-CSF (Table 1). While the parental BAF3, 32D, and L-GM cells lacked specific G-CSF binding (data not shown), the different BAF3 transfectants (WT, DC, DA, and M1) expressed G-CSF binding sites with affinities ranging from 0.28 to 2.04 nM (Table 1), i.e., comparable to those of the G-CSF-R expressed on human peripheral blood neutrophils (4) and those of the G-CSF-R expressed on cell line transfectants (13, 21, 49). 32D/WT, 32D/DC, 32D/DA, and L-GM/M1 transfectants expressed G-CSF-R with similar average numbers and affinities. On the other hand, 32D/M1, L-GM/WT, L-GM/DC, and L-GM/DA transfectants expressed G-CSF-R at detectable but lower densities (less than 100 sites per cell), which did not allow estimation of binding affinities (Table 1). The reasons for these variations in surface expression levels of the G-CSF-R are unknown, but they may relate to differences in protein stability or transportation in the different cell line transfectants.

**Mitogenic signaling by different forms of G-CSF-R.** The abilities of different forms of the G-CSF-R to transduce a proliferative signal were first examined in [ $^3\text{H}$ ]TdR uptake assays. At least three independent clones of each construct were examined, giving comparable results. Parental BAF3, 32D, and L-GM cells or G418-resistant clones transfected

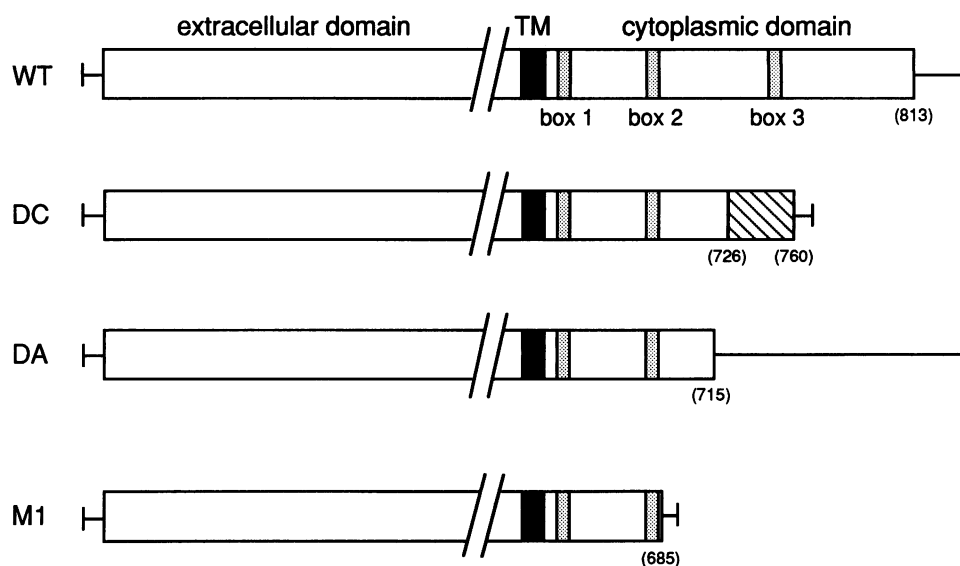


FIG. 1. Schematic diagram of the G-CSF-R cDNAs. Boxes 1, 2, and 3 denote subdomains conserved in some members of the cytokine receptor superfamily. The hatched box indicates the distinct cytoplasmic tail of the DC receptor from the corresponding region of the WT receptor (see text). Lines indicate noncoding regions. The numbers in parentheses mark amino acid positions. TM, transmembrane domain.

with the vector without G-CSF-R showed no response to G-CSF (Table 2). G-CSF induced DNA synthesis in BAF3 cells expressing the WT G-CSF-R to a level of approximately 40 to 50% of that induced by the optimal concentration of WEHI-CM (20%) as a source of murine IL-3 (Table 2 and Fig. 3A, BAF/WT). Deletion of 98 amino acids from the carboxy terminus did not negatively affect G-CSF-induced growth signaling in BAF3 cells (Table 2 and Fig. 3A, BAF/DA). However, further deletion of 30 amino acids significantly reduced the G-CSF sensitivity, resulting in a 100-fold increase in the G-CSF concentration (>100 ng/ml) required to elicit a maximal response (Fig. 3A, BAF/M1). BAF3 cells expressing the DC receptor, which contains 11 additional amino acids as compared with the DA receptor, followed by a distinct carboxy tail, also displayed a reduced responsiveness: the magnitude of maximal response was about 30 to 50% of that of BAF/WT cells (Table 2 and Fig. 3A, BAF/DC).

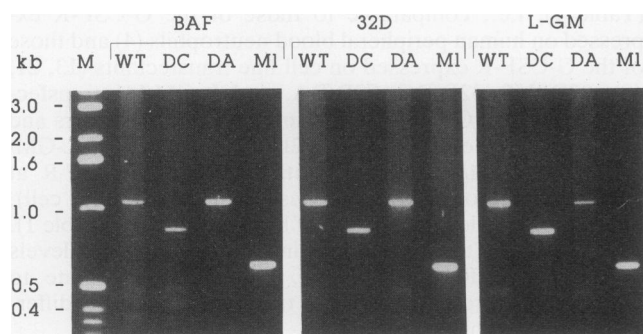


FIG. 2. RT-PCR analysis of the G-CSF-R cytoplasmic domain in BAF3, 32D, and L-GM transfectants. Primer sets used for amplification were FW2-RV1 for WT and DA, FW2-RV2 for DC, and FW2-RV3 for M1. For further details, see Materials and Methods. Expected sizes of PCR products: 1,106 bp for WT and DA, 864 bp for DC, and 630 bp for M1.

In contrast to the BAF transfectants, 32D cells expressing the WT G-CSF-R exhibited only a weak mitogenic response to G-CSF (Table 2 and Fig. 3B, 32D/WT). Similar responses to G-CSF were observed in 32D cells expressing the DC or the M1 receptor (Table 2 and Fig. 3B). The 32D/DA cells, however, showed a dramatic increase in responsiveness to G-CSF (Fig. 3B, 32D/DA). Additional titration experiments revealed that 32D/DA cells proliferated maximally at 0.1 ng of G-CSF per ml (data not shown).

L-GM cells transfected with the different forms of the G-CSF-R displayed a response pattern similar to that of 32D transfectants (Table 2 and Fig. 3C). L-GM cells expressing the WT G-CSF receptor showed a poor mitogenic response to G-CSF, as did L-GM subclones expressing the DC or the

TABLE 1. Binding properties of the G-CSF receptors on cell line transfectants<sup>a</sup>

Cell type	No. of sites/cell	$K_d$ (nM)
BAF/WT	3,030	2.04
BAF/DC	1,500	0.67
BAF/DA	2,560	0.28
BAF/M1	31,410	0.60
32D/WT	1,640	1.86
32D/DC	750	0.68
32D/DA	2,630	0.53
32D/M1	+ <sup>b</sup>	ND <sup>c</sup>
L-GM/WT	+	ND
L-GM/DC	+	ND
L-GM/DA	+	ND
L-GM/M1	1,290	1.84

<sup>a</sup> Parental BAF3, 32D, and L-GM cells did not show specific G-CSF binding.

<sup>b</sup> +, specific binding detectable but too low for an accurate estimation of receptor numbers and affinities.

<sup>c</sup> ND, not determined.

TABLE 2. Mitogenic responses of cell line transfectants in [<sup>3</sup>H]TdR uptake assay after 48 h of culture

Transfectant	dpm (mean ± SD) with:		
	No factor	G-CSF (10 ng/ml)	IL-3 (20% WEHI-CM)
BAF/Neo	33 ± 6	52 ± 20	46,298 ± 463
BAF/WT	35 ± 14	17,845 ± 393	36,833 ± 1,289
BAF/DA	45 ± 6	16,390 ± 344	41,772 ± 1,128
BAF/DC	20 ± 4	5,485 ± 527	19,673 ± 156
BAF/M1	17 ± 5	7,190 ± 360	31,021 ± 434
32D/Neo	33 ± 13	29 ± 20	24,452 ± 196
32D/WT	53 ± 21	954 ± 134	19,780 ± 692
32D/DA	76 ± 47	22,606 ± 633	18,312 ± 549
32D/DC	38 ± 7	1,256 ± 72	14,765 ± 221
32D/M1	149 ± 52	1,309 ± 94	27,554 ± 1,350
L-GM/Neo	40 ± 13	39 ± 8	2,968 ± 297
L-GM/WT	29 ± 7	383 ± 61	3,183 ± 267
L-GM/DA	43 ± 5	5,317 ± 393	3,293 ± 59
L-GM/DC	22 ± 4	441 ± 46	1,566 ± 49
L-GM/M1	22 ± 5	560 ± 50	1,369 ± 178

M1 receptor. L-GM/DA cells, on the other hand, showed a high rate of DNA synthesis even at low concentrations (0.1 ng/ml) of G-CSF.

**Cell proliferation of transfectants.** We next examined whether G-CSF could replace IL-3 as a stimulus for sustained proliferation of transfectants. As shown in Fig. 4A and B, BAF/WT and BAF/DA cells became G-CSF dependent and grew equally well in 1 and 100 ng of G-CSF per ml. Although BAF/DC and BAF/M1 cells could also grow continuously at 100 ng of G-CSF per ml with a reduced rate of proliferation (Fig. 4B), these cells could not survive at 1 ng of G-CSF per ml (Fig. 4A). Cells proliferating exponentially on G-CSF could be propagated for several months. The response of BAF/M1 cells indicates that the membrane-proximal region of 55 amino acids is sufficient for generating a growth signal. In addition, the data show that a region between amino acids 56 and 85 in the cytoplasmic domain enhances growth signaling. The C-terminal region of the DC receptor, which contains an additional 11 amino acids of the WT receptor and 34 different amino acids in comparison with

the DA mutant (Fig. 1), exerted a negative effect on growth signaling in BAF3 cells.

The 32D transfectants expressing the WT, DC, and M1 receptors died 4 days after transfer to G-CSF-containing medium (Fig. 4C). In contrast, 32D/DA cells proliferated continuously upon stimulation with G-CSF (Fig. 4C). Similarly, L-GM/DA cells could be cultured continuously in G-CSF-containing medium, whereas L-GM cells expressing the WT, DC, or M1 receptor gradually lost viability (Fig. 4D). Thus, the characteristics of the responses observed in 32D and L-GM subclones define a functional subdomain located in the distal carboxy-terminal region of the WT G-CSF-R that appears to suppress the growth signaling in myeloid cells. The altered carboxy terminus of the DC receptor also inhibits G-CSF-induced proliferation of the two myeloid cell lines.

**Induction of myeloid maturation by different forms of G-CSF-R.** A prominent feature of G-CSF is its capacity to induce neutrophilic differentiation. 32Dc13 and L-G cells, variant cell lines of 32D and L-GM cells, respectively, have previously been shown to mature into neutrophilic granulocytes in response to G-CSF (22, 45). We therefore examined whether L-GM and 32D cells expressing the different G-CSF receptor structures would mature towards neutrophilic granulocytes following G-CSF induction. L-GM/WT cells exhibited immature myeloblastic features when cultured in IL-3-containing medium (Fig. 5a). Upon transfer to G-CSF-containing medium, these cells gradually developed into mature neutrophilic granulocytes, showing an enlarged cytoplasm-to-nucleus ratio and lobulated nuclei. On day 9 of G-CSF induction, all cells displayed a morphology characteristic of mature neutrophils (Fig. 5b), similar to that of mature L-G cells (22). As shown in Fig. 5c and d, L-GM/DC and L-GM/DA cells did not mature towards granulocytes in G-CSF-containing medium. Identical results were obtained with L-GM/M1 transfectants (data not shown). Addition of different concentrations of murine granulocyte macrophage-CSF or murine IL-3 to the G-CSF-containing culture medium to prolong cell survival also failed to induce terminal maturation of L-GM/DC or L-GM/M1 cells (data not shown).

32D/WT cells, which were myeloblastic when cultured with IL-3 (Fig. 5e), did not mature upon transfer to G-CSF-containing medium. Instead, these cells manifested a pheno-

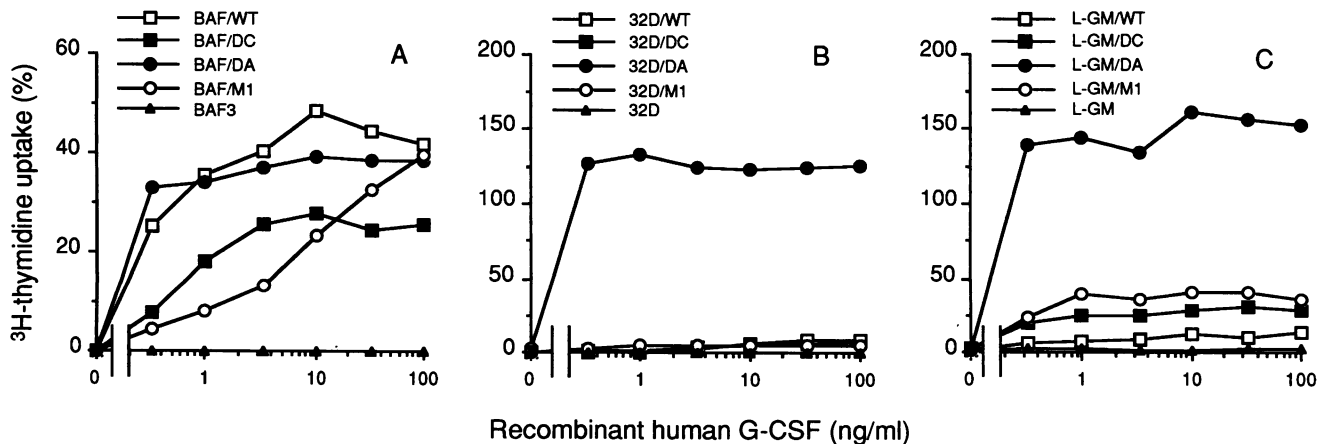


FIG. 3. G-CSF responsiveness of BAF3, 32D, and L-GM cells expressing different forms of the G-CSF-R. Cell proliferation was measured by the [<sup>3</sup>H]TdR uptake assay. Data represent the percentage of maximal response to IL-3 (20% WEHI-CM).

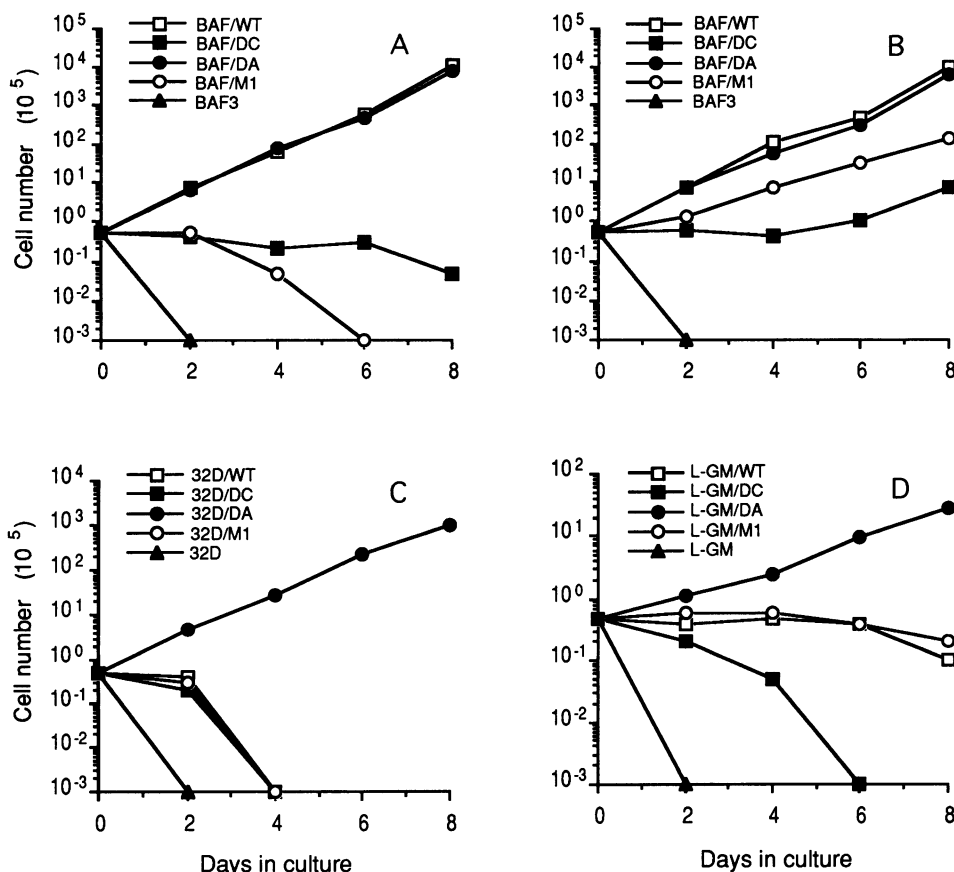


FIG. 4. G-CSF-dependent long-term growth of BAF3, 32D, and L-GM transfectants. Cells maintained in IL-3-containing medium were washed extensively and transferred to G-CSF-containing medium. Viable cell counts were determined at the indicated times. BAF3 subclones were cultured in medium containing 1 (A) and 100 (B) ng of G-CSF per ml. 32D (C) and L-GM (D) transfectants were cultured in 10 ng of G-CSF per ml, except for DA subclones (1 ng of G-CSF per ml).

type typical of apoptosis (i.e., condensation of nuclei) after culture in G-CSF-containing medium for 4 days (Fig. 5f). To exclude the possibility that these cells died from a lack of growth signaling before they could undergo myeloid maturation, cells were grown in G-CSF-containing medium supplemented with different concentrations of WEHI-CM (1, 2.5, 5, and 10%) to allow survival for up to 2 weeks, but still no maturation was noted (data not shown). Similarly, 32D/DC or 32D/M1 subclones were rapidly lost in cultures supplemented with G-CSF and failed to mature (Fig. 5g and data not shown). 32D/DA cells also showed no evidence of terminal myeloid maturation when maintained in G-CSF (Fig. 5h). These results show that the 32D cells used in this study were incapable of granulocytic maturation in response to G-CSF, suggesting a defect in the G-CSF-induced maturation signaling pathway in these cells.

#### DISCUSSION

In this study, we have delineated distinct functional regions in the cytoplasmic domain of the human G-CSF-R. We show here that a membrane-proximal region of 55 amino acids of the human G-CSF-R is sufficient to generate a mitogenic signal, consistent with a recent report by Ziegler et al. (48). This region contains two subdomains, termed box 1 and box 2, which are well conserved among some members of the cytokine receptor superfamily (11, 29). The impor-

tance of the box 1- and box 2-containing region for growth signal transduction has been demonstrated for several cytokine receptors (7, 15, 26, 29, 35, 39, 48). It is likely that this region is involved in activation of common or highly related signal transduction pathways. This notion is supported by several receptor transfection studies (7, 15, 29, 35, 48) and by the fact that some of the ligands for these receptors induce similar patterns of tyrosine phosphorylation (9, 27, 36).

The most prominent finding of this study is that the 98 carboxy-terminal amino acids of the WT G-CSF-R appeared to be involved in neutrophilic maturation. Little is known of the mechanisms by which hematopoietic growth factors control maturation. For instance, it has remained a matter of discussion whether activation of growth factor receptors truly drives maturation or simply supports the proliferation and survival of cells already programmed to mature along a certain hematopoietic lineage (18, 28, 33). With regard to the G-CSF-R, our results with the L-GM transfectants clearly favor the first possibility, because L-GM/WT cells developed into morphologically mature granulocytes when cultured in G-CSF, whereas L-GM/DA cells proliferated and survived in G-CSF without any evidence of maturation. This failure of L-GM/DA cells to mature was thus apparent in spite of their ability to proliferate in response to G-CSF. L-GM/DC and L-GM/M1 cells, which proliferated modestly

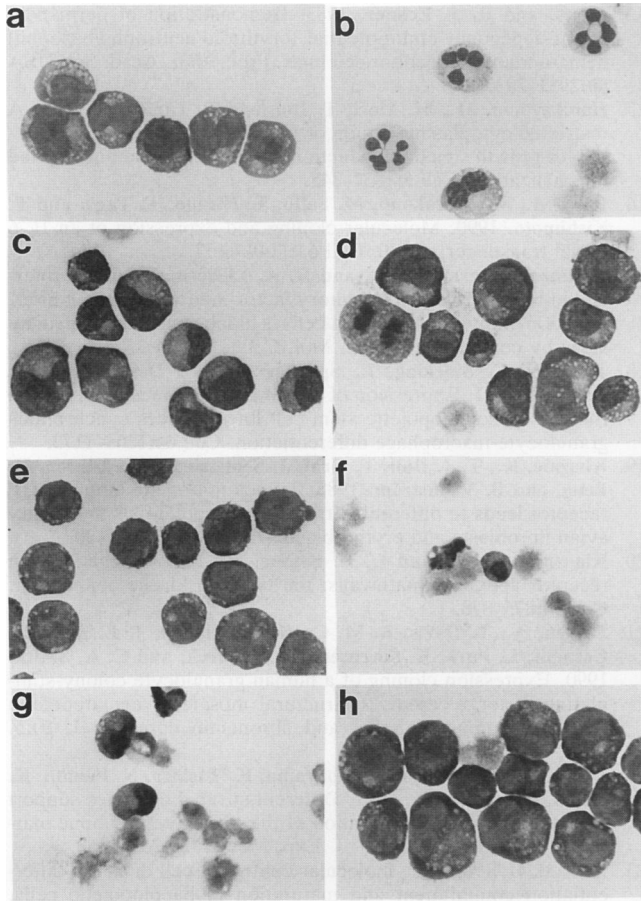


FIG. 5. Morphological analysis of 32D and L-GM transfectants. Shown are L-GM (a) and 32D (e) cells maintained in IL-3-containing medium (10% WEHI-CM); L-GM/WT (b), L-GM/DC (c), and L-GM/DA (d) cells cultured in medium containing 10 ng of G-CSF per ml; and 32D/WT (f), 32D/DC (g), and 32D/DA (h) cells in G-CSF-containing medium. May-Grünwald-Giemsa staining was used (original magnification,  $\times 63$ ).

in response to G-CSF, were also incapable of maturation. Our data therefore indicate that the carboxy-terminal part of the WT G-CSF-R contains a subdomain critical for maturation signaling that is required neither for proliferation nor for survival. A linkage between the carboxy-terminal truncation of the IL-2 gamma chain and T-cell maturation arrest in patients with X-linked severe combined immunodeficiency has recently been suggested (30). In G-CSF-R, a subdomain designated box 3 has been identified in this region, which is also present in the IL-6 signal transducer gp130 (11). Notably, IL-6 also stimulates granulocytic colony formation by normal mouse marrow cells (42) and was able to induce maturation of M1 leukemia cells (17, 41). Moreover, leukemia inhibitory factor and oncostatin M, which share gp130 for signal transduction, have also been reported to induce maturation of M1 cells (3). Thus, it appears likely that the box 3 subdomain may have a direct role in the induction of maturation, possibly through association with a specific cytoplasmic substrate.

The presence of the carboxy-terminal region in the WT G-CSF-R strongly inhibits the mitogenic signaling potential of the G-CSF-R in two myeloid cell lines, 32D and L-GM

cells (Fig. 3). In contrast, this negative effect on growth signaling was not evident in pro-B BAF3 cells, in which the WT G-CSF-R transduces a growth signal as strong as that of the DA mutant. Despite their enhanced sensitivity to G-CSF, 32D/DA and L-GM/DA cells remained fully factor dependent and died within 48 h upon withdrawal of IL-3 or G-CSF (data not shown).

In contrast to the L-GM transfectants, 32D cells expressing the WT G-CSF-R did not mature upon exposure to G-CSF. However, the cells responded only transiently to G-CSF in proliferation assays and could not be maintained in G-CSF-containing suspension culture. These findings suggest that the negative effect of the carboxy terminus of the WT G-CSF-R on proliferation and the induction of terminal maturation may be uncoupled and raise the possibility that separate domains within this region are involved in these functions. Alternatively, this could be explained by a lack of downstream molecules involved in the maturation signaling in the 32D cells.

It is still unclear by which mechanism proliferation signaling in myeloid cells is inhibited by the carboxy-terminal region of the WT G-CSF-R. Protein phosphorylation on tyrosine or serine/threonine residues has been shown to be frequently involved in the regulation of receptor function, both positively and negatively (20, 43). One possibility is that the down-regulation of the activity of the G-CSF-R is caused by receptor phosphorylation, as has been demonstrated for EPO-R (46). It is of note that, in contrast to our results with BAF3/WT transfectants, the negative regulatory region of the EPO-R also acted in BAF3 cells, suggesting that the carboxy-terminal inhibitory regions of the G-CSF-R and EPO-R act through different mechanisms. Alternatively, this region may contain a subdomain that specifically activates downstream molecules which transmit growth-inhibitory signals. In that case, these molecules would not be active in BAF3 cells. A negative effect of the carboxy-terminal sequence on growth signal transduction has been reported for several other growth factor receptors, including those of the receptor tyrosine kinase family (7, 19, 37, 47). Thus, negative regulation of proliferation signaling by the carboxy-terminal parts of receptors may be seen as a common feature of different growth factor receptor systems.

We have further shown that a region of 30 amino acids between residues 56 and 85 markedly augments the mitogenic signaling potential of the G-CSF-R. A similar region has been identified in the EPO-R and in the common  $\beta$  chain of IL-3, granulocyte macrophage-CSF, and IL-5 receptor (31, 39). It remains to be established whether this region is actively involved in transduction of a mitogenic signal or is important simply for a proper conformation that facilitates signaling from the box 1- and box 2-containing membrane-proximal regions. In this respect, it is relevant that Ziegler et al. have demonstrated that the region between amino acids 57 and 96 in the cytoplasmic domain of the G-CSF-R is required for the induction of acute-phase protein gene expression by G-CSF in hepatoma cell line transfectants (48). These data appear to suggest that this region indeed contains a functional subdomain.

Whether the DC splice variant has a physiological role in hematopoiesis is as yet unknown. We have shown that the DC variant receptor is able to transduce a mitogenic signal in the three murine cell lines, consistent with a previous report (49). However, the growth signal transduced by the DC receptor is significantly weaker than that transduced by the DA mutant, suggesting that the alternative carboxy tail also acts as a down-modulator of proliferation. Unlike the nega-



tive regulatory region of the WT G-CSF-R, the DC tail portion exerts its effect in both lymphoid and myeloid cells, indicating that it must act by a mechanism different from that of the WT G-CSF-R. Because the amino acid sequence in this portion is rather hydrophobic (21), one possibility is that the alternative tail folds towards the cell membrane, thereby hindering the association of growth-signaling molecules with other regions. Because the DC receptor is incompetent for maturation induction, it might act as a negative regulator of maturation at certain stages of myeloid development.

Our findings may have implications for studies dealing with the granulocytic maturation defects in certain hematological diseases, such as neutropenias and acute myeloid leukemia. The fact that G-CSF-R mutant DA was isolated from a patient with severe congenital neutropenia strongly suggests that defective G-CSF-R signaling had been involved in the course of the disease, a possibility that is currently being examined in detail.

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