The Cytoplasmic Domain of Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF) Receptor α Subunit Is Essential for Both GM-CSF-mediated Growth and Differentiation

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates differentiation, survival, and proliferation of colony-forming unit-granulocyte-macrophage progenitor cells. The biologic actions of GM-CSF are mediated by binding to a specific receptor consisting of two chains designated as α and β subunits. We have demonstrated that the murine FDC-P1-derived cell line WT-19 transfected with the human GM-CSF receptor α and β subunits (GM-CSFRα and β) can be induced to differentiate by the addition of human GM-CSF (hGM-CSF). By expressing a series of GM-CSFRα mutants in WT19 cells, we have determined the amino acid domains of the GM-CSFRα cytoplasmic domain that regulate cell differentiation, proliferation, and survival. We found that the membrane proximal proline-rich domain and adjacent 16 residues are essential for both hGM-CSF-dependent cell proliferation and differentiation. In contrast, the C-terminal region of the GM-CSFRα cytoplasmic domain was not necessary for cell differentiation mediated by hGM-CSF, but the removal of this region severely impaired the ability of hGM-CSF to support cell survival. While the activation of JAK2, Shc, Erk, and STAT5 proteins correlated with hGM-CSF-mediated cell growth, cellular differentiation occurred in the absence of activation of these signal transduction pathways.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 22-kDa glycoprotein, which is secreted by activated T cells, endothelial cells, fibroblasts, mast cells, B cells, and macrophages (1–4). GM-CSF plays an important role in promoting differentiation, survival, and proliferation of colony-forming unit-granulocyte-macrophage progenitor cells as well as enhancing the function of mature neutrophils, monocytes, and eosinophils (5, 6) and stimulating burst promoting activity for burst-forming units, erythroid (7, 8). GM-CSF causes a major cytoskeletal reorganization in plasma cells and hairy cells, resulting in the inhibition of motility and loss of adhesion to cellular and matrix ligands (9).

The biologic actions of GM-CSF are mediated by binding to a specific receptor consisting of α and β subunits, both of which are members of the type-I cytokine receptor family (10, 11). The α subunit binds GM-CSF with low affinity (10). A soluble form of human GM-CSF receptor α subunit (GM-CSFRα) has also been identified, whose function in vivo is unclear (12, 13). While the β subunit does not bind GM-CSF by itself, it forms a high affinity receptor in combination with the α subunit (11). The β chain is called the common β chain (βc) because it is shared by interleukin 3 (IL3) and interleukin 5 (IL5) receptors (14, 15). Although the cytoplasmic domain of GM-CSFRα is only 54 amino acids, we and others have demonstrated that the GM-CSFRα cytoplasmic domain is necessary for GM-CSF-induced cell proliferation (16–18). Several studies showed that the cytoplasmic domain of the human GM-CSF receptor β chain (GM-CSFRβ) is also essential for the mitogenic signal (18, 19). However, because of the lack of adequate biologic model cell systems, the role of GM-CSFR α and β subunits in GM-CSF-induced cell differentiation has not been clearly demonstrated.

The present study defines the role of GM-CSFRα in GM-CSF-mediated differentiation by studying WT19 cells, an FDC-P1-derived cell line that uniformly differentiates toward the monocytic lineage in response to murine GM-CSF (mGM-CSF), but grows and does not differentiate in the presence of murine IL3 (mIL3) (20, 21). We find that when the wild type human GM-CSFR α and β subunits are both transfected into WT19 cells, these cells respond to the addition of human GM-CSF (hGM-CSF) by undergoing differentiation. To identify the residues of GM-CSFRα cytoplasmic domain necessary for the induction of cell differentiation, WT19 cell lines were established which express mutated cytoplasmic domains of the α subunit along with the wild type β subunit. The ability of GM-CSF to support cell survival of WT19 correlated with the tyrosine phosphorylation of Jak2, STAT5, Shc, and extracellular signal-regulated kinases (ERKs). However, the induction of differentiation in the cells containing the 18-amino acid deletion of the C-terminal region occurred without the detectable tyrosine phosphorylation of these four signaling molecules. Our results suggest that cell survival and differentiation are controlled by different signal transduction pathways regulated by varying portions of the GM-CSFRα.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—WT19 is a cell line established from a mouse factor-dependent myeloid cell line, FDC-P1 (a generous gift from Dr.
Larry Rohrschneider, Fred Hutchins Cancer Research Center, Seat- 
tle, WA). The cell line was cultured in RPMI 1640 medium (Life Tech-
nologies, Invitrogen), supplemented with 10% FBS (Fetal Bovine Sera), 
10% WEHI-3B conditioned medium containing mIL3.

Reagents—Recombinant hGM-CSF was purchased from Immunex 
Corp. (Seattle, WA). Recombinant mGM-CSF and mIL3 were obtained 
from Genzyme (Cambridge, MA).

Site-directed Mutagenesis and Construction of Expression Plas-
mids—The human GM-CSFRβ cDNA was removed from the plasmid 
pKH97 (a gift from Dr. A. Miyajima, DNAX Research Institute, Palo 
 Alto, CA), and the 2.9-kilobase pair fragment was ligated into pCEP4 
(Invitrogen), which contains a hygromycin selection marker giving the 
plasmid pCPE4-GM-CSFRβ. The 1.3-kilobase pair human GM-CSFR 
receptor α chain cDNA was removed from pCDEM vector (Invitrogen) and ligated into the pDNA3 vector (Invitrogen) giving the plasmid pCMV-GM-CSFRα.

To construct GM-CSFRα mutants, GM-CSFRα cDNA was cloned into 
M13 mp19. To site-directed mutagenesis was carried out using a kit 
from Amersham, using oligonucleotides: GGAGACCAGCCTATCCTAGG 
AACC (ter1), CTTTCCCTTCTCATAGTTAAGCTT (ter3), CCCCATGGATCTGACCAACTGGCGG (P360G), TGGAACTGGACCGAACAGC (P357G), TCTGTTCGTCAGTGTTAAC (del2), GTCTTTGATCTGTATCCTAAGGAACC (del3), TGAACTGGACAGGACAG (P358G), TCTGTTGCTGATGGAAGATCAGAGC (P359G), CTTTGATCTGACCAACTGGCGG (P360G).
The mutated cDNAs were isolated and ligated into pDNA3 vector. The 
structure of the constructs was confirmed by restriction enzyme map-
ning and DNA sequence analysis.

Isolations of WT19 Transfectants—pCEP4-GM-CSFRα was intro-
duced into WT19 cells by electroporation at 260 V, 975 microfarads 
using a Bio-Rad Gene Pulser, and transfectants isolated using hygro-
mycin (0.4 mg/ml). A clone termed WT19 β1 expressing GM-CSFRβ 
was used for the transfection of pCMV-GM-CSFRα wild-type or mutant 
subunits. Resistant clones containing the α subunit were then isolated 
using G418 selection (0.4 mg/ml). Resulting clones were screened by 
flow cytometry using anti-human GM-CSFRα monoclonal antibody, 
and three to five positive clones from each construct were expanded for 
future studies.

MTS Cell Proliferation Assay—5,000 cells were incubated in 100 μl 
of RPMI 1640 containing 10% FBS and various concentrations of hGM-
CSF for 14 h at 37 °C in a humidified 5% CO2 atmosphere. 20 μl of 
freshly prepared combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-
thoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/p henyl-
methanesulfonil fluoride (MTS/PM5) solution (Promega, Madison, WI) was 
delivered to each well. After an additional 4 h of incubation at 37 °C, 
the conversion of MTS into the aqueous soluble formazan was measured 
at an absorbance of 490 nm.

Antibodies—Polyclonal anti-human GM-CSFRα anti-sera was pre-
pared using a glutathione S-transferase fusion protein containing 
plasmid pKH97 (a gift from Dr. A. Miyajima, DNAX Research Institute, Palo 
Alto, CA). The anti-phosphotyrosine monoca-
lonal antibody (4G10) was purchased from Upstate Biotechnology Inc. 
(Lake Placid, NY). The anti-Sher polyclonal antibody and the anti-
STAT1 polyclonal antibody were obtained from Signal Transduction 
Laboratories (Lexington, KY). The anti-STAT3 and STAT5 polyclonal 
bodies were purchased from Santa Cruz Biotechnology, Inc. (Santa 
Cruz, CA). The STAT5 antibody (sc-335) is specific for both STAT5a 
and STAT5b.

Immunoprecipitation—Immunoprecipitation was performed as 
described previously (22). Briefly, cells were incubated in RPMI 
1640, 10% FCS, 20 μm Heps for 4 h at 37 °C, and incubated in binding 
buffer (RPMI 1640 + 2% bovine serum albumin, 20 μm Heps) contain-
ing varying concentrations of 125I-hGM-CSF (NEN Life Science Prod-
ucts) for 30 min at 37 °C. To measure nonspecific binding, 100-fold 
extcess of cold hGM-CSF was added. The cells were centrifuged 
through a phe- 
lathyl oil layer (2.25, dioctyl phthalate/di-n-butyl phthalate). The 
radioactivity of the cell pellet was counted in a γ counter. The binding 
data were subjected to Scatchard analysis.

DNA Fragmentation Analysis—Before lysis, cells were incubated for 
the indicated time periods in medium supplemented with hGM-CSF (10 
ng/ml) followed by a wash in PBS. The cells were then incubated in 
cell lysis buffer (10 μl EDTA, 50 μl Tris-Cl, pH 8, 0.5% SDS, 0.5 mg/ml 
Protease K) for 14 h at 50 °C. After an additional 3-h incubation with 
the addition of 20 μg/ml RNase A, the genomic DNA was extracted with 
phenol-chloroform and precipitated with ethanol. DNA fragments were 
visualized after 1.8% agarose gel electrophoresis by ethidium bromide 
staining.

RESULTS

mGM-CSF Induces Rapid Monocytic Differentiation of WT19 
Cells Which Is Reversible after Removal of the Factor—WT19 
cells (20) growing in mIL-3 demonstrated a myeloblastic mor-
phology including rounded nuclei, fine chromatin, and thin and 
basophilic cytoplasm. In response to mGM-CSF, the cells dem-
strated monocytic characteristics: an indented nucleus with 
shortened chromatin, increased cytoplasm containing a 
variable number of vacuoles, and larger total cell size. Cells 
treated with mGM-CSF also became positive for nonspecific 
esterase and acid phosphatase (Table I). To quantitate the 
number of cells undergoing differentiation after the addition of 
mGM-CSF or mIL3, surface marker changes were evaluated by 
FACS analysis. WT19 cells growing in mIL3 showed weak 
surface expression of F4/80 and Mac3 (Fig. 1A), both of which are monocytic specific markers (23–25). When the WT19 cells 
were incubated with mGM-CSF, the expression of both these markers 
was significantly increased, suggesting that the cells

| Morphology | Surface markers | Staining |
|------------|-----------------|----------|
| F4/80      | Mac1            | Mac3     | Non-specific esterase | Specific esterase | Acid phosphatase |
| +          | +               | +        | +                     | +               | +               |

Table I: Differentiation markers of WT19 cells after mIL3 and mGM-CSF stimulation
differentiated toward monocytic lineage (Fig. 1A). As demonstrated by FACS analysis, cell size and granularity also increased as evidenced by an increase in both forward scattergram (FSC) and side scattergram (SSC) (Fig. 1A). These characteristics were stable for at least 14 days (Fig. 1A). Differentiated cells continued to divide, and the cell number increased. Increases in both F4/80 expression and cell granularity were evident within 1–2 days after the addition of mGM-CSF (Fig. 1B). Washing out the mGM-CSF and replacing it with mIL3 caused the F4/80 expression to decrease to background levels within 3 days (Fig. 1C), suggesting that mGM-CSF-induced monocyte/macrophage differentiation of WT19 cells is a reversible phenomenon. In addition, mGM-CSF-induced cell differentiation of WT19 in the presence of mIL3, suggesting mGM-CSF-mediated differentiation signal is dominant over mIL3 (data not shown).

hGM-CSF-induced Differentiation of WT19 Cells Transfected with Human GM-CSF Receptors—To examine the ability of hGM-CSF to mediate the differentiation of WT19 cells, cells were transfected with an expression plasmid encoding human GM-CSFR β subunit containing a hygromycin resistance selectable marker. A clone expressing high levels of GM-CSFR β, WT19 β clone 1, was then transfected with the G418-selectable expression plasmid encoding GM-CSFRα wild type subunit, and the cells were further selected in G418. Treatment of these doubly transfected cells with hGM-CSF induced differentiation of WT19 cells as measured by changes in F4/80 and Mac3 surface markers (Fig. 2). This hGM-CSF-induce differentiation was also found to be reversible upon removal of human hGM-CSF (data not shown).

Expression of Human GM-CSFRα Mutants in WT19 Cells—We have demonstrated that the cytoplasmic domain of the α subunit regulates growth of factor-dependent hematopoietic cells (16, 17). Specific residues of the α subunit are highly conserved among growth factor receptors (Fig. 3B). As hGM-CSF is capable of inducing the differentiation of WT19 cells, it is possible to evaluate the role of the cytoplasmic domain in GM-CSF-mediated cell growth, survival, and differentiation. A series of expression plasmids encoding deletion and substitution mutants of GM-CSFRα (Fig. 3A) were created; ter1 mutant

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**FIG. 1.** mGM-CSF induced rapid, reversible monocytic differentiation of WT19 cells. A and B, WT19 cells were washed with factor-free medium and then were placed in medium containing either 10 ng/ml mIL3 or mGM-CSF. Forward (FSC) and side scatter (SSC) parameters of the cells were examined by flow cytometry. The cell surface expression of monocyte specific F4/80 and Mac3 was examined by flow cytometry after staining with the appropriate monoclonal and fluorescein isothiocyanate (FITC)-labeled secondary antibodies. The control WT19 cells were stained with the secondary antibody alone. C, WT19 cells maintained in medium containing 10 ng/ml mGM-CSF were washed in factor-free medium and switched to medium containing mIL3 for the indicated number of days. F4/80 expression was analyzed by FACS analysis.
lacks the entire cytoplasmic domain except for the membrane-proximal 5 amino acids; del1 has an internal deletion of 15 amino acid residues corresponding to the proline-rich box 1 region, which is well conserved among the type I cytokine receptor family; del2 has an internal 16-amino acid deletion adjacent to the box 1 region; del3 has an 8-amino acid deletion within the box 1 region removing the proline-rich domain (PPVP) (Fig. 3B); ter3 has a deletion of the C-terminal 18 amino acid residues; and three individual amino acid substitutions have mutations in the well conserved proline-rich domain: proline 357, 358, or 360 (Fig. 3A).

These GM-CSFRα expression plasmids were transfected into WT19 β clone 1, and the G418-resistant clones were isolated. The levels of expression of these mutants were analyzed by staining with a specific anti-GM-CSFRα monoclonal antibody followed by flow cytometric analysis with a FACScan (Fig. 4). All these mutants were confirmed to express βc equally well by immunoblotting using specific anti-GM-CSFRβ antibody (data not shown). Each transfectant was examined for hGM-CSF binding using 125I-labeled hGM-CSF, and the results of high affinity binding profiles were shown in Table II. All mutant transfectants including ter1 clones bind hGM-CSF with high affinity and K_d values suggesting that there are equivalent numbers of receptors with similar affinity. These results suggested that the mutations introduced in the cytoplasmic domain of GM-CSFRα did not affect the interactions of GM-CSFRα with the ligand or GM-CSFRβ.

Multiple Domains of GM-CSFRα Are Necessary for GM-CSF-induced Protein-tyrosine Phosphorylation of p52Shc, ERKs, JAK2, and STAT5—It is well established that tyrosine phosphorylation of an array of cytoplasmic proteins is critical for cytokine signal transduction. Accordingly, we analyzed the spectrum of substrates tyrosine-phosphorylated by addition of hGM-CSF to WT19 cells expressing various GM-CSFRα mutants.

Several hematopoietic cytokines including GM-CSF induce p52 Shc tyrosine phosphorylation, which correlates with their ability to activate Ras (26–30). GM-CSFRα transfectants were assayed for hGM-CSF-induced Shc tyrosine phosphorylation by anti-Shc immunoprecipitation followed by anti-phosphotyrosine immunoblotting. As shown in Fig. 5A, increased p52 Shc tyrosine phosphorylation was detected only in wild type and P357G GM-CSFRα transfectants but not in any other transfectants (data not shown). Activated Ras through a cascade of protein kinases stimulates phosphorylation of ERKs (31). hGM-CSF induced phosphorylation of both p44ERK1 and p42ERK2 in wild type and P357G cells, but not in any other transfectants (data not shown).

GM-CSF addition to cells activates JAK2, which leads to the
tyrosine phosphorylation and activation of STAT5 (32–34). Tyrosine phosphorylation of JAK2 and STAT5 was induced by GM-CSF in wild type and P357G mutants (Fig. 5, B and C). On shorter exposure, tyrosine phosphorylation of two STAT5 isoforms (STAT5a and b) was observed. A slight decrease in JAK2 and STAT5 phosphorylation seen in Fig. 5 in the P357G transfectants was not constantly reproducible. In the other GM-CSFRα mutant cell lines, GM-CSF did not induce detectable JAK2 or STAT5 tyrosine phosphorylation. Activation of STAT5 in wild-type and P357G GM-CSFRα transfectants was also detected by gel shift assay using γ-interferon-activated site of the IRF-1 promoter and anti-STAT5 antibody (data not shown). These data indicated that the same regions of GM-CSFRα that are essential for GM-CSF-induced Shc-ERK phosphorylation are also essential for the induction of JAK2 and STAT5 tyrosine phosphorylation.

**Protooncogene Expression in GM-CSFRα Transfectants—**

GM-CSF has been shown to induce rapid expression of a number of protooncogenes, including c-fos, c-jun, and c-myc (29, 35). In wild-type GM-CSFRα and the P357G transfectants, expression of c-fos, c-jun, and c-myc mRNAs was rapidly induced upon hGM-CSF stimulation (Fig. 6). In contrast, the expression of c-fos and c-jun mRNA was not induced in the other α subunit mutants except ter3. In ter3 mutant receptor cell lines, hGM-CSF was capable of inducing c-jun but not c-fos mRNA. In contrast, induction of c-myc mRNA expression was repeatedly observed in both the wild type and all of the mutant clones, indicating that hGM-CSF is able to induce c-myc mRNA in the absence of GM-CSFRα cytoplasmic domain and that all of the mutant receptors are capable of signaling.

**The Cytoplasmic Domain of GM-CSFRα Is Critical for GM-CSF-mediated Cell Proliferation—**

We next examined hGM-CSF-induced cell proliferation of WT19 transfectants expressing α chain mutants. As shown in Fig. 7, both the wild type GM-CSFRα transfectant and the P357G mutant proliferated upon addition of hGM-CSF to the medium. ter1, del1, del2, del3, P358G, and P360G did not show any proliferative response to hGM-CSF, suggesting that some residues of the α chain cytoplasmic domain is indispensable for hGM-CSF-mediated growth signal transduction. These studies confirm our earlier findings about the role of GM-CSFRα cytoplasmic domain in promoting growth of BaF/3 cells (16).

Treatment of ter3 clones that lack the C-terminal 18 amino acid residues of α chain with hGM-CSF did not lead to an increase in the cell numbers. Instead, the cells died, but more slowly than ter1 clones (Fig. 7). In MTS cell proliferation assays, ter3 clones clearly showed hGM-CSF-mediated cell proliferation signal, although it was weaker than wild type or P357G clones (Fig. 8). Cell death in factor-dependent cells is known to occur through apoptotic mechanisms. To examine if apoptosis occurred in ter3 cell number in the presence of hGM-CSF, genomic DNA was isolated from α chain transfectants incubated with hGM-CSF and DNA fragmentation was analyzed by agarose gel electrophoresis (Fig. 9). ter3 showed detectable DNA fragmentation characteristic of apoptosis by 9 h

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**Fig. 3. GM-CSFRα cytoplasmic domain mutants.** A, deleted amino acid residues are indicated as dashes. Amino acid substitutions are indicated by underlines and boldface type. B, comparison of the amino acid sequences conserved among the cytokine receptor family. The proline-rich regions of various cytokine receptor cytoplasmic domains are shown. Conserved prolines are boxed.

**Fig. 4. Flow cytometric analysis of GM-CSFRα expression on WT19 transfectants.** Cells were stained with either anti-GM-CSFRα monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG or with FITC-conjugated anti-mouse IgG alone.
and showed pronounced apoptosis by 24 h after withdrawal of mIL3 and the addition of hGM-CSF. These results suggest that ter3 mutants that are able to transduce a cell proliferation signal by GM-CSF have severely impaired anti-apoptotic signaling.

**hGM-CSF-induced Monocytic Differentiation of WT19 Cells Expressing hGM-CSF Receptors**—Next we analyzed hGM-CSF-induced differentiation of WT19 cells expressing human GM-CSFRα. All the α subunit transfectants examined retained the ability to differentiate when mGM-CSF was added to the medium (data not shown). Because several transfectants died within 24 h after the withdrawal of mIL3, cell lines were treated with hGM-CSF in the presence of mIL3. After incubation with hGM-CSF for 3 days, the cells were examined for monocytic differentiation by morphology, F4/80, and Mac3 surface expression. As shown in Fig. 10, after 3 days of incubation with GM-CSF, cells transfected with either the wild type GM-CSFRα or P357G showed characteristic morphology of monocytic lineage: larger cell sizes, fine-stranded nuclear appearance, and a variable number of cytoplasmic vacuoles. Both the morphologic and cell surfaces changes induced by hGM-CSF were identical to those induced by mGM-CSF treatment. They also showed increased surface expression of P4/80 and Mac3 (Fig. 2). None of the clones containing ter1, del1, del2, del3, P358G, or P360G was able to differentiate when incubated with hGM-CSF (Figs. 2 and 10). In contrast, all ter3 clones that were derived (five independent clones) differentiated as well as wild type clones in response to hGM-CSF (Figs. 2 and 10).

**DISCUSSION**

The GM-CSF receptor signals by ligand-mediated heterodimerization of GM-CSFRα and GM-CSFRβ. Although the cytoplasmic domain of the GM-CSFRα is only 54 amino acids, this short region of the receptor is necessary for GM-CSF-induced cell proliferation (16–18). In the present study, we have compared the role of GM-CSFRα cytoplasmic domain in GM-CSF-mediated cell proliferation, survival, and differentiation. This analysis was made possible by our use of the WT19 cell line, which grows but does not differentiate in mIL-3. In comparison, mGM-CSF induces differentiation, but does not stop the growth of these cells. Our studies suggest that the

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### TABLE II

**Binding profiles of GM-CSF on WT19 transfectants**

| High affinity receptor | Kd | No. of binding sites/cell |
|------------------------|----|--------------------------|
| Wild type              | 120 pM | 930–1,600               |
| ter1                   | 56 pM  | 600–1,100               |
| del1                   | 110 pM | 500–1,850               |
| del2                   | 102 pM | 500–1,600               |
| del3                   | 67 pM  | 970–1,500               |
| ter3                   | 50 pM  | 1,000–2,000             |
| P357G                  | 105 pM | 1,200–1,600             |
| P358G                  | 87 pM  | 800–1,600               |
| P360G                  | 73 pM  | 400–900                 |

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**Fig. 5. hGM-CSF-induced tyrosine phosphorylation of signaling molecules in WT19 transfectants.** WT19 cells expressing the wild-type GM-CSFRβ subunit and the indicated GM-CSFRα construct were incubated with or without 10 ng/ml hGM-CSF for 5 min. The cell lysates were immunoprecipitated with the appropriate antibody, and the immunoprecipitates were run on 8% SDS-PAGE. The Western blot was then carried out. A, tyrosine phosphorylation of Shc in response to hGM-CSF in WT19 transfectants. Cell extracts were immunoprecipitated with anti-Shc antibody, and the Western blot was probed with anti-phosphotyrosine antibody (upper panel) or anti-Shc antibody (lower panel). B, tyrosine phosphorylation of Jak2 in response to hGM-CSF in WT19 transfectants. Lysates were immunoprecipitated with anti-Jak2 antibody, and the immunoblot was probed with anti-phosphotyrosine antibody (upper panel) or anti-Jak2 antibody (lower panel). C, hGM-CSF-dependent phosphorylation of STAT5. Cell extracts were immunoprecipitated with anti-STAT5 antibody and immunoblotted with an anti-phosphotyrosine antibody (upper panel) or anti-STAT5 antibody (lower panel).
mGM-CSF-mediated differentiation process is reversible upon removal of mGM-CSF. The WT19 cells are equally capable of responding to both murine and human GM-CSF when transfected with the wild type human GM-CSF receptor subunits. By using a mutant GM-CSFRα lacking most of the cytoplasmic domain, we have shown that the cytoplasmic domain of GM-CSFRα is essential for both hGM-CSF-dependent cell differentiation and proliferation. This mutant was still able to interact with the β-chain to form a high affinity receptor complex (Table II), suggesting that the cytoplasmic domain of GM-CSFRα is not necessary for receptor dimerization. The GM-CSFRα cytoplasmic domain was necessary for the phosphorylation of signaling molecules, JAK2, STAT5, Shc, and ERKs and the induction of c-fos and c-jun mRNA expression. Weak activation of STAT1 and STAT3 by GM-CSF have recently been reported in polymorphonuclear leukocytes (36). However, we could not detect the activation of these STATs in any of the transfectants in response to GM-CSF (data not shown). Deletion of the intracytoplasmic domain did not abolish c-myc mRNA induction after GM-CSF stimulation. Similar results were observed by us using BaF/3 cells (16).

Using other cell systems and varying approaches other laboratories have suggested that the internal portion of the GM-CSFRα may not have a major role in regulating receptor function. For example, using a chimera of the extracellular domain of the erythropoietin receptor and the intracellular domain of the murine IL-3 receptor β chain (AIC2A), the addition of erythropoietin to the receptor was able to stimulate cell growth (37). Another study demonstrated that a chimera comprising the extracellular region of GM-CSFRα and the intracellular domain of the hβc can also transduce signals (38). These data suggest that dimerization of the β-chain is important. However, they do not necessarily exclude the possibility that the GM-CSFRα is an important dimerization partner, and there are no physiologic data demonstrating that two β chains dimerize to initiate signaling in normal cells. Both the α and β subunits have proline-rich regions close to the plasma membrane, and both of these regions are important for receptor function. In addition, we have shown that deletion of the α internal segment blocks both growth and differentiation.

By using deletion mutants of GM-CSFRα, we demonstrate that the membrane proximal proline-rich region and the adjacent 15 amino acids of the α subunit are indispensable for both cell proliferation and differentiation (Figs. 2, 7, and 10). The proline-rich region of GM-CSFRα contains a Pro-X-Pro sequence that exists in the membrane-proximal box1 region of many other members of cytokine receptors (Fig. 3B). Mutation of this domain in the IL6 receptor gp130 protein (39) and in the granulocyte colony-stimulating factor receptor (40) eliminated receptor activity. We have here shown that similar mutations (P357G, P360G) also result in a receptor that is unable to mediate proliferation, differentiation, or other signaling events. Proline 357 could be part of a Pro-X-Pro motif, such as has also been found in cytokine receptors and SH3-binding proteins (41). This proline appears to be dispensable, however, since the P357G mutant was able to fully support proliferation and differentiation.

The region downstream from the proline-rich domain was also indispensable for hGM-CSF-dependent transduction of cell growth, survival, and differentiation signals. These 15 amino acid residues, which include aspartic acid 368, are conserved in IL5 receptor α, prolactin receptor, growth hormone receptor, and IL2 receptor γ-chain. Our studies show that tyrosine phosphorylation of JAK2 and STAT5 is inhibited by the deletion of this region, demonstrating that the proline-rich domain alone is not sufficient for the GM-CSF-induced activation of the JAK2 signal transduction pathway. Similar results have been obtained in other systems. Deletion of 6 amino acids of the region downstream of proline-rich domain of IL5 receptor α, including the conserved aspartic acid, abolished IL5-induced JAK2 activa-
vation (42), while mutation of the region immediately down-
stream of box1 region in the erythropoietin (43) and IL6 recep-
tors (39) blocked the activation of JAK2. Interestingly, our data about proline mutations in the box1 region were different from the recently published study on the IL-5 receptor \( \alpha \)-chain. In the previous report, the existence of any one of the three proline residues was adequate for IL-5-
mediated cell proliferation signal (42). The difference between these findings could be secondary to differences in sequence in the \( \alpha \)-chains (PPVPQI, GM-CSF receptor; PPIPAP, IL-5 recep-
tor), or, possibly the difference in results could be due to the divergence in amino acid residues adjacent to the proline-rich domain.

The C-terminal deletion of GM-CSFR \( \alpha \)-Chain Mediates Cell Differentiation

FIG. 7. Growth response of transfectants and parental WT19 cells. Cells were incubated in 10 ng/ml of either mIL3 or hGM-CSF. At the times indicated, the number of viable cells in each culture was determined by trypan blue staining. The results were expressed relative to the cell number on day 0 of this experiment. Three independent clones of each cell type were assayed yielding similar results. The average value of the three clones was shown for mIL3 responses.

FIG. 8. MTS proliferation assay of three WT19 transfectants and parental WT19 cells. Cells (5,000 cells/each) were incubated in RPMI 1640 containing 10% FBS and various concentrations of GM-CSF for 14 h at 37 °C. After MTS/PMS solution was added, cells were incubated for 4 h at 37 °C. The conversion of MTS was measured by the amount of 490 nm absorbance. Error bars from triplicate experiments are also shown.

FIG. 9. The effects of C-terminal deletion on the anti-apoptotic function of hGM-CSF. Three WT19 transfectants were washed with factor-free medium and incubated in medium containing 10 ng/ml hGM-CSF for indicated time. Total cellular DNA was isolated and analyzed by 1.8% agarose gel electrophoresis. Sizes of DNA markers are indicated.

GM-CSFR \( \alpha \)-Chain Mediates Cell Differentiation

44). Shc, when tyrosine-phosphorylated, binds to SH2 domains of Grb2, which leads to the recruitment of Sos, a guanine nucleotide exchange factor for Ras, to the plasma membrane (45). Tyrosine phosphorylation of Shc is thought to play an important role in GM-CSF-mediated activation of Ras through this mechanism (27). In ter3 transfectants, tyrosine phospho-
rylation of Shc was not detectable in response to hGM-CSF stimulation (Fig. 5A). hGM-CSF-induced activation of Ras-
ERK pathway appeared to be impaired in these transfectants, since hGM-CSF-mediated ERK phosphorylation and c-fos in-
duction, which are downstream events regulated by Ras activa-
tion (46, 47), could not be detected (Fig. 6). Our findings are compatible with a previous report that Ras activation is neces-
sary for anti-apoptotic effect by GM-CSF, but not essential for GM-CSF-mediated DNA synthesis (27).

Our results demonstrate that ter3 transfectants differenti-
ated as well as wild type transfectants in response to hGM-
CSF. The ter3 clones are capable of stimulating increases in c-myc and to a lesser extent c-jun, but do not cause the phos-
phorylation of Jak2, STAT, and Shc, suggesting that activation of these proteins is not necessary for differentiation. The find-
ings that ter3 cells die of apoptosis while they were capable of differentiation in hGM-CSF suggests that the pathways con-
trolling cell survival and differentiation can be separated and are controlled by different portions of the GM-CSFR. hGM-
CSF-mediated tyrosine phosphorylation of JAK2 and STAT5 could not be detected in ter3 clones (Fig. 5, B and C), suggesting that these pathways may be important for the inhibition of apoptosis. We have recently demonstrated that the expression

FIG. 10. Growth response of transfectants and parental WT19 cells. Cells were incubated in 10 ng/ml of either mIL3 or hGM-CSF. At the times indicated, the number of viable cells in each culture was determined by trypan blue staining. The results were expressed relative to the cell number on day 0 of this experiment. Three independent clones of each cell type were assayed yielding similar results. The average value of the three clones was shown for mIL3 responses.
of a chimeric protein of CD16 and Jak2 is capable of preventing cell death, implying that part of the function of the Jak/STAT pathway could be to inhibit apoptosis (51).

In contrast to other signal transduction pathways, both c-myc and c-jun were induced in ter3 transfectants by hGM-CSF to similar levels to that seen in wild type transfectants, suggesting that c-myc and c-jun can be induced without the activation of either ERKs or JAK2. In a recent report, it was shown that the transient expression of the dominant negative form of JAK2 inhibited hGM-CSF-induced transcription of a reporter plasmid containing the c-myc promoter, suggesting that JAK2 is essential for c-myc mRNA induction by hGM-CSF (48). It is possible that inhibition of c-myc promoter was caused by a nonspecific effect of the overexpression of dominant-negative JAK2, although further experiments will be needed to clarify this possibility. c-jun overexpression induces monocytic differentiation of the WEHI-3B (49) and U937 cells (50), while c-fos overexpression did not have similar biologic effects (49). However, it is unlikely that c-jun alone is responsible for hGM-CSF-mediated cell differentiation, as c-jun mRNA expression was equivalently induced by mIL3, which has no effect on the differentiated phenotype of WT19 cells (data not shown).

In summary, specific regions of the intracytoplasmic domain of the α subunit play an essential role in hGM-CSF-mediated cell proliferation, survival, and differentiation, while the signal transduction pathway which controls c-myc activation is independent of this subunit. Our results demonstrate that differentiation may occur in the absence of Shc, ERK, or JAK2 activation, suggesting that there are specific novel signal transduction pathways, yet to be determined, which control this process.

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