A functional isoform of the human granulocyte/macrophage colony-stimulating factor receptor has an unusual cytoplasmic domain
(hematopoiesis/cytokine receptor/alternative splice)

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ABSTRACT The granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor (GMR) transduces a signal that results in the proliferation, differentiation, and functional activation of hematopoietic cells. This study sought to determine whether functional isoforms of the receptor exist that may be important in generating this diversity of cellular response. We have isolated a cDNA encoding an isoform of the low-affinity human GMR that is a product of alternative splicing of the GMR gene and results in a predicted 410-amino acid protein with a cytoplasmic domain that is rich in serine residues, a feature of regions critical in signal transduction for other receptors of the hematopoietin receptor superfamily. This receptor bound ligand and was functionally active when introduced into a murine factor-dependent cell line; mRNA transcripts representative of this isoform were coexpressed with those for a previously isolated 400-amino acid isoform of the GMR in normal hematopoietic and leukemic cells. In view of the recent isolation of a cDNA, designated GM-CSF RΔ, that confers high-affinity binding of GM-CSF in cotransfection experiments with the low-affinity receptor, we suggest that the previously isolated low-affinity receptor be designated GM-CSF Rα1 and the one described in this report be designated GM-CSF Rα2.

Hematopoietic development is regulated by a family of growth factors that mediate cell proliferation, differentiation, and function. One of these, granulocyte/macrophage colony-stimulating factor (GM-CSF), induces the proliferation and differentiation of granulocyte and macrophage progenitors and, in combination with erythropoietin, supports the development of erythroid progenitors in vitro (1, 2). Mature neutrophils, eosinophils, and monocytes become functionally activated following exposure to GM-CSF (2). These effects on hematopoietic cells have been confirmed in vivo (3–6) and are mediated following binding of the growth factor to a specific cell surface receptor (the GMR) (7–9). High- and low-affinity GMRS are expressed in low abundance on hematopoietic cells (50–500 per cell), and cross-linking studies suggest that they have more than one subunit (7–13). Furthermore, at 4°C cells that express both the GMR and the interleukin 3 (IL-3) receptor show cross-competition; GM-CSF competes for binding of IL-3 to its receptor and vice versa (11–16). This raises the possibility that the GMR and the IL-3 receptor share a common subunit. A cDNA clone encoding a GMR that binds human GM-CSF with low affinity has been isolated from a human placental library (17) and the gene for this receptor has been mapped to the X-Y pseudoautosomal region (18). Although this GMR is functional in that it confers human GM-CSF responsiveness on a factor-dependent cell line (19), the cytoplasmic domain is relatively short, and the 54 amino acids within this domain do not contain motifs known to be associated with signal transduction. It also lacks the serine-rich domains shown to be critical in receptor function for two members of the hematopoietin receptor superfamily (17, 20–22); the β chain of the interleukin 2 receptor (23) and the erythropoietin receptor (24). Therefore it is likely that additional proteins associate with this low-affinity GMR to mediate signal transduction and convert it to a receptor that binds with high affinity. Indeed, a cDNA clone has recently been isolated that does not bind GM-CSF itself but confers high-affinity binding when cotransfected with the low-affinity GMR (25). These data, combined with the knowledge that GM-CSF can exert pleiotropic effects on hematopoietic cells, led us to consider whether isoforms of the GMR may exist as one of the means to generate the complexity of cellular responses observed.

MATERIALS AND METHODS

Construction and Screening of the cDNA Library. Poly(A)* RNA from the cell line M-07 (26) was converted to double-stranded cDNA using oligo(dT) primers. A CDNA library was then constructed in the expression vector pMT 21, a derivative of pMT 2 (27). This library was screened on nitrocellulose filters (28) with a probe derived by the polymerase chain reaction (PCR) from a pool of 100,000 M-07 cDNAs and oligonucleotides complementary to the extracellular and 3′ untranslated regions of the published GMR sequence [ref. 17, GMR.A (proposed nomenclature, GM-CSF Ra1); see Fig. 1A]. The probe was 32P-labeled using random hexanucleotide primers, and hybridization was performed at 65°C in 4× SSC (1× SSC, 150 mM sodium chloride/15 mM sodium citrate, pH 7.4)/5× Denhardt’s reagent/0.5% SDS containing salmon sperm DNA at 100 μg/ml. Filters were washed at 65°C in 2× SSC/0.1% SDS. The clone that hybridized to this probe [GMR.B (proposed nomenclature, GM-CSF Ra2)] was sequenced using the dideoxynucleotide chain-termination method (29). The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64445).

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; GMR, GM-CSF receptor; IL-3, interleukin 3.

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The cDNA for GMR.A had been isolated from a AZAP human placental cDNA library (Stratagene) by hybridization of clones with a full-length GMR.B DNA probe. The identity of the clone representative of GMR.A was confirmed by restriction enzyme digestion and partial sequencing. Hybridization and washing conditions for screening the genomic and plasmid cDNA libraries were the same as those for the M-07 cDNA library. Genomic clones were mapped by digestion with a variety of restriction enzymes and hybridization with probes specific for regions of the GMR. A genomic DNA fragment that hybridized to sequences unique to GMR.B was subcloned and sequenced.

**Southern Blotting.** Human genomic DNA was digested with *Bam*HI, *Bgl*II, *Hind*III, and *Xba*I, electrophoresed on a 0.6% agarose gel, transferred to a nylon membrane, and separately probed with DNA probes containing sequences of the common or unique sequences of GMR.B. Hybridization conditions were as above and the filter was washed at 65°C in 0.2X SSC/0.1% SDS.

**Isolation of RNA and Analysis by RNase Protection and by Northern Blotting.** Poly(A)+ RNA was selected on oligo(dT)-cellulose from total RNA prepared from cells by guanidinium isothiocyanate extraction followed by centrifugation through cesium chloride. RNase protection analysis was performed as described previously (30). Briefly, 15 μg of each poly(A)+ RNA was hybridized overnight at 52°C to an RNA probe prepared from a *Par*I fragment of GMR.B (see Fig. 2). RNase digestion was performed with RNase T1 (1.75 μg/ml) and RNase A (35 μg/ml) at 37°C for 30 min. The reaction was stopped with proteinase K (333 μg/ml) and SDS (0.3%). The protected products were electrophoresed on a 6% urea/acylamide gel and the autoradiograph was exposed at −70°C.

Northern blotting analysis was performed by electrophoresing poly(A)+ total RNA in a 1% denaturing agarose gel that was then transferred to nitrocellulose and probed with a DNA probe containing sequences of the cytoplasmic region of GMR.B. Hybridization was performed at 42°C in 50% formamide/5X SSPE (1X SSPE, 150 mM sodium chloride/10 mM monobasic sodium phosphate/1 mM EDTA, pH 7.4)/5X Denhardt's reagent/0.1% SDS containing salmon sperm DNA at 100 μg/ml. The filter was washed at 68°C in 0.2X SSC/0.1% SDS.

**Electroporation.** FDC-P1 cells (31) were coelectroporated with linearized constructs of GMR.B (50 μg) and the neo-resistance gene (10 μg), both in the expression vector pMT 21. Electroporation was performed in serum-free RPMI 1640 medium with conditions of 980 μF and 250 V. Cells were selected for growth first in G418 and then in human GM-CSF.

**Radiiodination of GM-CSF and Binding Studies.** Recombinant human GM-CSF (derived from *Escherichia coli*) was iodinated using the iodine monochloride method as published previously (32). This procedure resulted in 125I-labeled GM-CSF with a specific activity of 20,000–40,000 cpm/ng by self-displacement analysis and a biological activity of 80–100% in progenitor assays.

Binding experiments were performed as described previously (9). Prior to these studies, FDC-P1 cells that had been maintained in human GM-CSF were washed and incubated in cytokine-free medium for 6 hr. Binding assays were performed at 4°C overnight. Cells (7.5 × 10⁶) were incubated with 125I-labeled human GM-CSF (10 pM to 10 nM) in the absence or absence of a 100-fold molar excess of unlabeled human GM-CSF. Unbound radioactive material was removed by centrifuging the cells through a phthalate/oil mixture and the radioactivity of the cell pellet was determined with a gamma counter.

**Thymidine Incorporation Assays.** Parental FDC-P1 cells maintained in WEHI-3B-conditioned medium and FDC-P1 cells transfected with GMR.B and maintained in the presence of human GM-CSF were washed three times and then 2 × 10⁶ cells were plated in 200-μl cultures containing serial dilutions of human GM-CSF. The cells were incubated at 37°C for 20 hr, pulsed with 0.5 μCi of [³H]thymidine for 4 hr, and harvested onto glass fiber filters. Radioactivity was determined by liquid scintillation counting.

**RESULTS**

**Isolation and Characterization of cDNAs Encoding GMR.A.** A cDNA library was constructed from the human megakaryoblastic leukemia cell line M-07 (25), which expresses both GM-CSF and IL-3 receptors. This library was screened with a probe obtained by PCR amplification of a fragment predicted from the published GMR sequence (GMR.A) to be 449 base pair (bp) long. However, by restriction enzyme digestion, the PCR product appeared to lack approximately 100 bp between the Sac I and the EcoRI sites (Fig. 1A). Sequence analysis of the probe showed that a deletion of 97 bp had resulted in complete loss of sequences corresponding to the transmembrane domain of the receptor (data not shown). Examination of genomic clones showed that the deletion had occurred precisely at splice junctions (data not shown).

When this probe was used to screen 215,000 clones of the M-07 library, a single positive clone was isolated. This clone (GMR.B) was 1480 bp long and lacked a poly(A) tail. Molecular analysis of the GMR.B clone showed nucleotide identity between this cDNA and that of GMR.A in the extracellular, transmembrane, and proximal cytoplasmic domains but complete lack of similarity in the distal cytoplasmic region (Fig. 1A). The intracellular domain of this clone encodes 64 amino acids (compared with 34 amino acids for GMR.A) and it is rich in serine and proline residues (Fig. 1B). Of the 35 nonhomologous amino acids, seven are serine residues and six are proline residues. Comparison of the nucleotide and amino acid sequences of GMR.B with sequences available in GenBank (February 1990) showed no highly significant similarities.

**Southern Analysis and Characterization of Genomic Clones Encoding the GMR.** To ensure that the GMR.B cytoplasmic region was not an artifact of library construction, human DNA was digested with a variety of restriction enzymes and, after transfer to nitrocellulose, probed with sequences specific for the common or unusual regions of GMR.B. This analysis revealed that most bands are shared between the two probes and suggested that GMR.B is derived from the same gene as GMR.A (data not shown). To confirm this observation and determine the molecular mechanism by which GMR.A and GMR.B had arisen, genomic clones were isolated from a human genomic DNA library. One of the clones isolated hybridized to a probe that contained only the unusual sequences of GMR.B. Sequence analysis of a fragment that hybridized to this probe revealed the presence of splice donor and acceptor sites separated by intronic sequences at the point of divergence between GMR.A and GMR.B cDNAs (Fig. 1C). Further mapping and sequencing place the GMR.B exon 5' to sequences encoding the 3' end of GMR.A (data not shown).

The existence of alternatively spliced transcripts for the GMR gene prompted us to screen an additional 200,000 clones from the unamplified M-07 library as well as from a human placental library with a full-length GMR.B probe. We identified three positive clones from the M-07 library, two of which were identical to GMR.A and one of which was identical to GMR.B. One full-length clone isolated from the human placental library was identical to GMR.A (data not shown). Clones containing a transmembrane domain, as suggested by the original PCR probe, were not isolated from either library. However, similar deletions have been observed in other receptors of the hematopoietin family, and
secreted forms of such receptors that lack a transmembrane domain have been isolated (33-35). Another group has also recently reported the isolation, by PCR, of GMR cDNAs that lack 97 bp encoding the putative transmembrane domain (36).

Expression of GMR Isoforms in Normal Tissues and in M-07 Cells. We next determined whether mRNA species that represent GMR.B are present in normal cells or whether these transcripts are unique to the leukemic cell line M-07. To this end, RNase protection analysis was performed with a probe derived from GMR.B (Fig. 2). GMR.B transcripts should protect a 421-nucleotide (nt) fragment whereas GMR.A transcripts should protect a 230-nt fragment. Fig. 2 demonstrates the presence of transcripts for both of the receptor isoforms, not only in M-07 cells but also in human bone marrow and placenta. The T-lymphoma cell line Jurkat, which does not bind 125I-labeled human GM-CSF (37), lacked transcripts for both GMR isoforms. The relative abundances of the mRNAs showed a predominance of GMR.A, with levels of GMR.B ≈ 10-fold less as assessed by scanning densitometry. Prominent bands at 135 nt and 85 nt were present in M-07 cells, bone marrow, and placenta but not in GMR.B-transfected COS-1 cells. These protected fragments are distinct from other bands of <421 nt present in all lanes (considered to be cleavage products of larger species) and may represent additional receptor isoforms. We also note that the 85-nt protected fragment shown in the M-07, bone marrow, and placenta lanes is consistent with the existence of GMR transcripts that lack the 97-nt transmembrane region deleted in the PCR probe.

Binding and Growth Characteristics of FDC-P1 Cells That Stably Express GMR.B. To examine whether the cDNA for GMR.B encodes a functional receptor, stable lines of the murine factor-dependent cell line FDC-P1 (30), which expresses GMR.B, were established. Parental FDC-P1 cells proliferate in response to both murine IL-3 and murine GM-CSF but not to human GM-CSF. We reasoned that if the signal transduction components of the murine cell are able to interact with a human GMR, cells that express GMR.B might proliferate in response to human GM-CSF and confirm that the receptor is functional. FDC-P1 cells were electroporated with a cDNA encoding GMR.B and a separate construct containing the neomycin-resistance gene. A cloned cell line, selected for growth in G418 and then in human GM-CSF, was shown to contain mRNA transcripts for GMR.B (Fig. 3A).

FDC-P1 cells that expressed GMR.B bound 125I-labeled human GM-CSF with a $K_d$ of 4 nM (Fig. 3B), an affinity similar to that seen with the transiently transfected receptor in COS-1 cells. This affinity agrees with that observed for GMR.A (17). Scatchard analysis of the binding demonstrated the presence of approximately 77,000 sites per cell (Fig. 3B Inset). Cross-linking of the transfected receptor in COS-1 cells with 125I-labeled human GM-CSF and disuccinimidyl suberate demonstrated a broad band of approximate $M_r$, 100,000, consistent with ligand bound to a glycosylated receptor of $M_r$, 85,000 (data not shown).

In addition to binding GM-CSF, the receptor was capable of transducing a proliferative signal. The growth of transfected FDC-P1 cells was supported in a dose-dependent manner by human GM-CSF alone (Fig. 3C). Although a high concentration of ligand, consistent with a low binding affinity, was required for proliferation, these cells were capable of a maximal proliferative response to human GM-CSF equivalent to that achieved in the presence of murine IL-3 or murine GM-CSF. The cells maintained parental-type responses, in the picomolar range, to murine IL-3 and GM-CSF. In the same assay, M-07 cells (which express high-affinity GM-CSF receptors) exhibited a peak proliferative response to human GM-CSF in concentrations as low as 1 ng/ml (data not shown). GMR.B-transfected FDC-P1 cells remained factor-dependent for growth and could be maintained long-term (>6 months) in medium containing only human GM-CSF.

**DISCUSSION**

These results demonstrate the existence of a second protein that binds human GM-CSF and can transduce a signal. This receptor isoform is a result of alternative splicing of the GMR
gene. Its expression is not restricted to a leukemic cell line but is found in normal tissues and is likely to have functional significance. The isoform we describe has a cytoplasmic domain that is rich in serine and proline residues, similar to regions of the interleukin 2β chain and erythropoietin receptors that have been shown to be critical for signal transduction. A relative abundance of serine and proline residues has also been noted in the cytoplasmic regions of the receptors for IL-3, interleukin 4, and interleukin 7 (33, 34, 38), although a role for these amino acids in intracellular signaling via these receptors has yet to be firmly established.

In addition, this study suggests the existence of other GMR isoforms in addition to GMR.A and GMR.B. In particular, it is likely from our PCR analysis (and ref. 36) that a form of the GMR lacks the transmembrane domain and thus may occur as a soluble form. Furthermore, a human cDNA clone was recently isolated that does not bind GM-CSF itself but confers high-affinity binding on the low-affinity GMR when
cDNAs that encode both polypeptides are cotransfected into COS cells (25).

Evidence accumulating that alternative splicing is a mechanism by which receptor heterogeneity is generated, and recently three different mRNAs encoding the receptor for human granulocyte colony-stimulating factor have been identified (35). It is noteworthy that while one mRNA encoded a putative secreted receptor, another receptor class contained a 27-amino acid insertion within the cytoplasmic domain that did not alter the translational reading frame. It would seem that receptors with different cytoplasmic domains may be another general feature of the hematopoietin receptor superfamily.

GM-CSF is capable of inducing different responses in the immature and mature cells to which it binds. Although the functional significance of the existence of receptor isoforms is not yet understood, it is possible that differential expression of these isoforms plays a role in determining the nature of the cellular response. Future studies must focus on the respective functions of these isoforms, both separately and together, and how they interact with other components of the GM-CSF signal transduction pathway.

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