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Identification and molecular cloning of a soluble human granulocyte–macrophage colony-stimulating factor receptor

(hematopoiesis/secreted receptors/cytokine receptor/PCR)

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ABSTRACT **Granulocyte–macrophage colony-stimulating factor (GM-CSF) plays an important role in hematopoiesis and host defense via interaction with specific cell-surface receptors in target tissues. We identified a truncated, soluble form of the low-affinity GM-CSF receptor (GMR) in choriocarcinoma cells. Low-affinity GMR cDNAs encoding both the membrane-bound and soluble receptors were obtained by PCR using primers corresponding to the published sequence. Clones encoding the soluble receptor were identical in sequence to the membrane-bound form but contained a 97-nucleotide internal deletion. The amino acid sequence of this deleted cDNA predicts a protein that lacks the 84 C-terminal amino acids of the membrane-bound receptor, including the transmembrane and cytoplasmic domains, and contains 16 different amino acids at its C terminus. Expression of the soluble GMR cDNA in murine ψ-AM cells as well as GM-CSF-dependent myeloid 32Dc13 cells produced a secreted protein that retained its capacity to bind GM-CSF in solution. RNase protection analysis indicates that this variant cDNA is derived from a naturally occurring mRNA. Soluble receptors have been identified for several other hematopoietin receptors and may be a general feature of this class. The striking similarity between the soluble form of the GMR and other hematopoietin receptors suggests that soluble binding proteins may play an important role in regulating the broad spectrum of biological responses mediated by these cytokines.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is one of a family of hematopoietic growth factors that stimulates the proliferation and differentiation of myeloid progenitor cells and enhances the function of mature myeloid effector cells (1). In addition to its action on cells of hematopoietic origin, GM-CSF has been shown to interact with several types of nonhematopoietic cells (2–5). The biological effects of GM-CSF are initiated by binding to specific cell-surface receptors (2, 6–8). High- and low-affinity forms of the GM-CSF receptor (GMR) have been described on both hematopoietic and nonhematopoietic cells and recent data have provided insight into the structural nature of these two forms. A cDNA clone encoding the low-affinity GMR was isolated (9) and shown to transduce a proliferative signal when transfected into a GM-CSF-dependent murine cell line (10). More recently, Hayashida *et al.* (11) have identified a cell-surface protein that has no intrinsic GM-CSF binding activity but can reconstitute a high-affinity form of the GMR if coexpressed with the low-affinity receptor. Thus, the GMR appears to be similar to the interleukin 2 (IL-2) and IL-6 receptors in that it is a multisubunit complex in which additional components contribute to high-affinity binding and signaling capacity.

While receptors are classically expressed as membrane-bound proteins, there are many cytokine receptors that are expressed as soluble binding proteins. In this report, we present the cloning of a human cDNA encoding a soluble form of the low-affinity GMR (sGMR).§ Our results indicate that this is a naturally occurring variant of the low-affinity GMR that may play an important role in regulating GM-CSF-mediated responses.

MATERIALS AND METHODS

Cell Lines and Reagents. BeWo and JAR choriocarcinoma cell lines were obtained from the American Type Culture Collection. ψ-AM cells were kindly provided by R. Mulligan (Massachusetts Institute of Technology) (12). The GM-CSF-dependent murine cell line 32D clone 3 (32Dc13) was a gift from J. Greenberger (University of Massachusetts) (13). All cells were grown in Iscove's medium supplemented with 10% calf serum and 2 mM glutamine.

Unlabeled recombinant GM-CSF purified from Chinese hamster ovary (CHO) cells was kindly provided by S. Clark (Genetics Institute, Cambridge, MA). ^{125}I-labeled GM-CSF (^{125}I-GM-CSF) was purchased from New England Nuclear/DuPont and was used for all GM-CSF binding experiments.

RNA Extraction and PCR Cloning. Total cellular RNA and polyadenylylated mRNA were isolated as described (14) and used to synthesize oligo(dT)-primed cDNA (15). Upon completion, cDNA reactions were diluted 1:5 with distilled water and used as a template for PCR. Two sets of overlapping primers were used for amplification: primers A, 5′-TTCTCTCTGACCAGCA (positions 131–147) and 5′-ACATGGTTCCTGAGTC (positions 660–676), which define a 530-base-pair (bp) 5′ fragment of the GMR; primers B, 5′-CGTGAATACTAGTCAAAGAGG (positions 439–459) and 5′-TTACTGGATCCTGGGATTACAGGCGTGA (positions 1494–1511), which define 1072 bp of 3′ sequences. PCR amplification was performed under the conditions recommended by the supplier (Perkin–Elmer) and used 40 cycles with 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C. Amplified DNA from the 530-bp fragment (primers A) was cloned into the *Sma* I site of Bluescript KS+ vector (Stratagene). The 1072-bp amplified DNA fragments (primers B) were cloned as *Kpn* I/*Bam*HI fragments using an internal *Kpn* I site (position 501) and a *Bam*HI site present in the PCR primer. Clones

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GMR, GM-CSF receptor; IL, interleukin; nt, nucleotide(s); sGMR, soluble GM-CSF receptor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M73832).

containing GMR sequences were verified by double-stranded DNA sequencing using T7 DNA polymerase (Pharmacia).

Expression of Recombinant GMR cDNAs. Clones comprising the entire coding region of the membrane-bound (GMR) and soluble (sGMR) receptors were constructed by ligating the different 3′ *Kpn* I/*Bam*HI fragments (primer B, PCR clones) with the same 5′ fragment (370-bp *Bam*HI/*Kpn* I fragment from the primer A PCR clone). These fragments were cloned into Bluescript KS+ and the retroviral vector LXSN (16).

Retroviral cDNA clones were transfected into the ψ-AM packaging cell line using calcium phosphate and stable transfectants selected by growth in G418 as described (14). Cell-free supernatants from stable transfectant clones were used to infect 32Dc13 cells. G418-resistant 32Dc13 clones represent pools taken from 10^5 infected cells. ψ-AM and 32Dc13 clones were assayed for GMR expression by RNA PCR as well as by binding to ^{125}I-GM-CSF.

Radioligands and Binding Assays. Binding of ^{125}I-GM-CSF to cells was performed as described (6) with incubation times of 30 min at 23°C. Binding of concentrated medium was similar to that described for solubilized membranes (2). Specific binding was determined as the difference between total bound counts and those blocked by competition with excess unlabeled GM-CSF.

RNase Protection Analysis. A PCR clone encoding the 3′ 1010-bp *Kpn* I/*Bam*HI fragment (from primer B PCR) of the membrane-bound GMR was digested with *Ase* I (position 960) and used as a template for *in vitro* transcription. Synthesis of the radioactively labeled antisense T7 RNA probe and RNase protection analysis were performed as described by the supplier (Promega).

RESULTS

Isolation and Characterization of Human GMR cDNAs. Poly(A)$^+$ RNA from the BeWo choriocarcinoma cell line was used to isolate cDNA clones encoding the low-affinity human GMR. Two sets of overlapping primers corresponding to the published sequence were used to amplify GMR-specific cDNA sequences with the RNA-directed PCR technique (Fig. 1*A*). A number of PCR-amplified clones were obtained that spanned either the 5′ or 3′ end of the GMR coding sequence. The nucleotide sequence of the cDNA clones isolated was identical to that reported by Gearing *et al.* (9). Several clones spanning the 3′ end of the GMR coding sequence (6 of 26 analyzed) contained a 97-nucleotide (nt) deletion (nt 1096–1192), which removed the entire transmembrane domain of the GMR (Fig. 1*B*). This deletion occurred within a repeated pentanucleotide sequence, GGTTC, making it difficult to precisely identify its boundaries. This variant cDNA (sGMR) predicts a protein product that encodes a truncated GMR, where 84 C-terminal amino acids of the GMR are replaced by 16 different amino acids (Fig. 1*C*). Furthermore, this protein should be secreted from the cell since it lacks a transmembrane domain but retains its signal sequence, suggesting that it encodes a soluble form of the GMR.

Expression of GMR cDNAs. To determine the GM-CSF binding properties of the full-length and truncated GMR cDNAs, the 5′ and 3′ cDNA clones were joined together and inserted into the LXSN retroviral vector. Both GMR-containing retroviral constructs were stably transfected into ψ-AM cells. Recombinant retroviruses from the ψ-AM cells were also used to transduce the GMR cDNAs into the murine GM-CSF-dependent 32Dc13 cell line. Cells transfected or transduced with the full-length GMR cDNA expressed a protein that specifically bound ^{125}I-GM-CSF at the cell surface (GMR; Fig. 2*A*). Receptors expressed by both the GMR ψ-AM and 32Dc13 cells exhibited a single class of binding protein with K_d values of 3–4 nM, and 82,500 and 8000 specific binding sites per cell, respectively (data not shown). These results are consistent with the binding data described for the placental-derived low-affinity human GMR transfected into COS-7 cells (9) and the murine GM-CSF-dependent myeloid cell line FDC-P1 (10).

Control ψ-AM and 32Dc13 cells (control; Fig. 2*A*) and cells transfected with the truncated GMR (sGMR; Fig. 2*A*) did not display significant cell-surface binding of ^{125}I-GM-CSF. High levels of sGMR RNA could be detected in cells transfected with the sGMR cDNA despite the lack of cell-associated binding (Fig. 2*A*). This result is consistent with the protein predicted from the sGMR cDNA, which terminates prior to the hydrophobic transmembrane domain and lacks the sequences responsible for anchoring it in the membrane. It was therefore expected that the expressed protein would be secreted from the cell and not associated with the cell surface. The low levels of cell-associated binding indicate

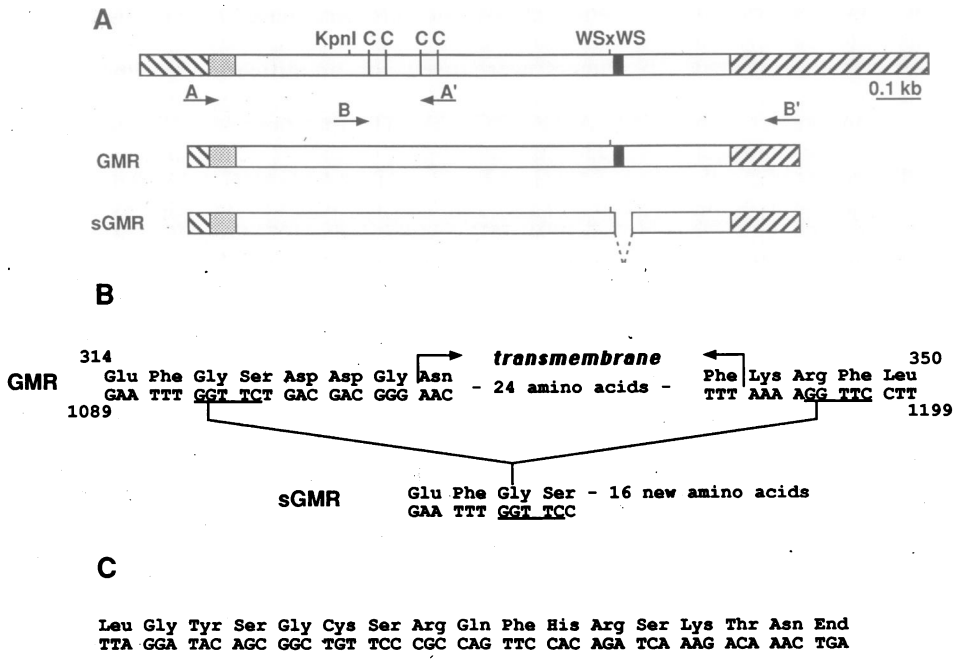

FIG. 1. Structure of low-affinity GMR cDNA clones. (*A*) Schematic diagram of cDNA sequence published by Gearing *et al.* (9) (*Upper*) and membrane-bound (GMR) and soluble (sGMR) cDNA clones derived by PCR. Primer pairs A and B are indicated. Open boxes, coding sequences; hatched boxes, untranslated sequences; stippled boxes, signal peptide sequences; solid box, transmembrane region. Conserved cysteine (C), WS × WS box, *Kpn* I site, and deleted sequences are also indicated. (*B*) Boundaries of sequences deleted and the corresponding translational reading frame are shown for each GMR cDNA (GMR and sGMR). (*C*) Predicted amino acid sequence of sGMR cDNA that occurs after deletion.

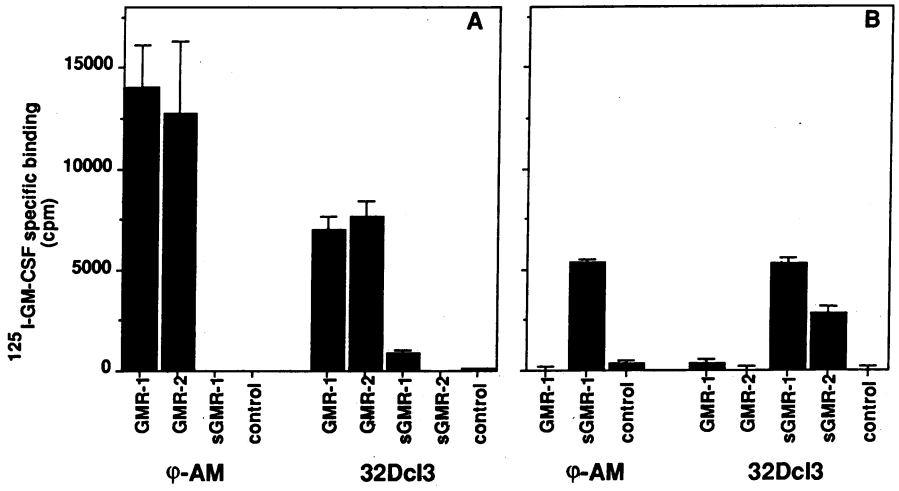

FIG. 2. GM-CSF binding characteristics of recombinant GMR cDNAs expressed by stable transfectants. (*A*) Specific binding of 2 nM ^{125}I-GM-CSF to 2.5 × 10^5 murine ψ-AM and GM-CSF-dependent 32Dc13 control cells and cell lines expressing either the membrane-bound (GMR-1 and GMR-2) or soluble form (sGMR-1 and sGMR-2) of the GMR cDNA. Specific binding is defined as the difference between total bound counts and those displaced by a 50-fold excess of unlabeled GM-CSF. For each cell line or conditioned medium analyzed, $n = 6$. (*B*) Specific binding of 1 nM ^{125}I-GM-CSF to 25 μl of 5-fold concentrated conditioned medium from control ψ-AM and GM-CSF-dependent 32Dc13 cells and from cells expressing the respective GMR cDNAs.

that the protein is efficiently secreted into the medium and that the 16 unique C-terminal amino acids are not sufficient to stably anchor the protein in the membrane.

To conclusively demonstrate the secretion of the recombinant protein by the sGMR-transfected cells, we analyzed the medium of transfected and untransfected cells for their ability to specifically bind ^{125}I-GM-CSF in solution (Fig. 2*B*). Only conditioned medium from cells transfected with the sGMR cDNA contained significant levels of ^{125}I-GM-CSF binding activity. In these samples, 50–70% of the total bound counts were displaced with a 50-fold excess of unlabeled GM-CSF. The level of specificity observed in this assay is similar to the specificity observed in the cell-associated binding experiments described above (data not shown). Detection of a soluble GM-CSF binding protein from cells expressing sGMR was reproducible by using several different preparations of conditioned medium and was observed in both the ψ-AM transfectants and in the transduced 32Dc13 cells. Although the sGMR appears to be a low-affinity GMR with a dissociation constant similar to the membrane-bound form, we have not yet been able to achieve saturable binding with concentrated conditioned medium and therefore cannot assign a definite dissociation constant to this protein. Nonetheless, these results demonstrate that the sGMR cDNA encodes a soluble form of the GMR that is efficiently secreted and that retains its ability to bind GM-CSF. In contrast to the sGMR-producing cells few, if any, GMRs were detected in medium from untransfected cells (control) or from cells transfected with the full-length GMR cDNA (GMR; Fig. 2*B*), suggesting that the membrane-bound receptor is not shed when expressed in this context.

Expression of sGMR mRNA. RNase protection analysis was used to demonstrate that the sGMR cDNA was not an artefact of PCR cloning and was present as a bona fide mRNA species in cells. A 602-nt antisense RNA probe was synthesized from one of the 3′ PCR clones encoding the membrane-bound GMR. This RNA probe contained 551-nt of GMR-specific sequences and contained the 97 nt that are deleted in the sGMR cDNA (Fig. 3*B*). Thus, the full-length GMR mRNA should protect a 551-nt RNase-resistant fragment, while the soluble GMR mRNA should yield two resistant products, one ≈317 nt and the other 136 nt. Total RNA from GMR- and sGMR-transfected ψ-AM cells (GMR-ψ2 and sGMR-ψ2) were used as positive controls and exclusively expressed the full-length or deleted mRNAs, respectively. The predominant transcripts protected by the transfected cell mRNAs yield the predicted 551-nt fragment in the GMR-ψ-AM RNA and the 317- and 136-nt fragment in sGMR-ψ-AM mRNA. The protected transcripts corresponding to the soluble GMR mRNA produced broader bands since there is a 5-bp repeat at the deletion boundary and thus protected fragments are produced that differ in size by several nucleotides.

Additional RNase-resistant products are also apparent and are presumably due to the protection of prematurely terminated transcripts contaminating the RNA probe. No RNase-resistant transcripts were observed in the RNA taken from

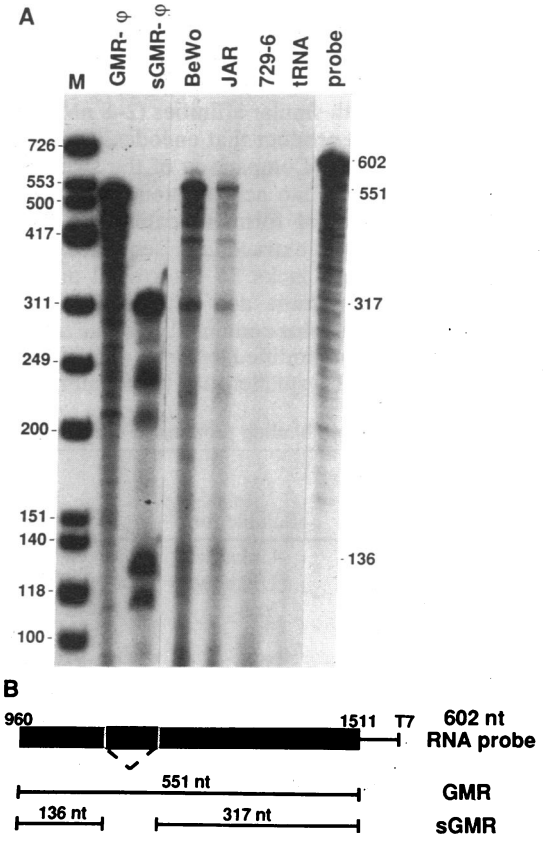

FIG. 3. RNase protection analysis. (*A*) Total RNA from BeWo, JAR, 729-6 cells, or ψ-AM cells transfected with the membrane-bound (lane GMR-ψ) or soluble (lane sGMR-ψ) GMR was hybridized to a 602-nt T7 RNA transcript. Samples were treated with RNase T2, precipitated, and analyzed on a 4% denaturing polyacrylamide gel. Sizes (nt) of protected fragments are indicated and were determined relative to the mobility of ^{32}P-labeled *Hin*fI-digested φX174 DNA (lane M). Undigested probe (lane probe) and digested probe (lane tRNA) are also shown. (*B*) Schematic diagram of T7 RNA transcript transcribed from PCR cDNA clone (primers B) containing membrane-bound GMR sequences (solid box) in Bluescript KS+. Predicted sizes of protected products corresponding to the membrane-bound (GMR) or soluble (sGMR) GMR mRNA are indicated.

cells that do not express GMRs—i.e., 729 B-cell RNA. Protected transcripts corresponding to both the full-length and soluble GMR are easily detected in BeWo RNA as well as in RNA extracted from JAR cells, another choriocarcinoma cell line. Thus, expression of the soluble GMR mRNA is not unique to BeWo cells.

Although there are several RNase-resistant fragments that may be due to premature termination of the probe, we cannot rule out the expression of other aberrant GMR RNAs in normal cells. The full-length and soluble transcripts, however, appear to be the two most prevalent GMR-related transcripts in BeWo and JAR cells. These results are in agreement with our PCR cloning data where these two forms of GMR mRNA were the only ones observed. We estimate that the soluble GMR mRNA constitutes ≈20% of the total GMR-related transcripts. The relative distribution of these mRNAs in BeWo cells is consistent with our cloning experiments as well as other PCR analysis (data not shown). These results suggest that the soluble form of the GMR is a naturally occurring molecule.

DISCUSSION

We used the published sequence of the low-affinity GMR to obtain PCR clones of the receptor in order to explore the mechanism of signal transduction induced by GM-CSF. Our analysis of the membrane-bound receptor indicates that it is identical in sequence to that reported by Gearing *et al.* (9) and when expressed in murine cells yields a recombinant protein that binds GM-CSF with similar affinities (2–8 nM). We also identified a GMR gene product that encodes a soluble form of the low-affinity GMR. Comparison of the structural features of the predicted amino acid sequence of this protein with the membrane-bound form indicates that they both retain identical signal and extracellular sequences but differ in that the soluble form lacks 84 C-terminal amino acids including the transmembrane and cytoplasmic region. In addition, the soluble receptor contains 16 unique amino acids predicting a mature, unmodified protein with a molecular mass of 36 kDa, ≈7.5 kDa smaller than the membrane-bound receptor. The soluble nature of this predicted protein was verified by expression in murine fibroblast and myeloid cells. The protein is efficiently secreted into the culture medium of the transfectants and retains its ability to specifically bind GM-CSF in solution. Indeed, all but 6 amino acids of the extracellular domain of the membrane-bound receptor are retained in this protein; most notable are the sites of potential glycosylation, the conserved cysteine residues, and the WS × WS box motif. These conserved regions define the GMR as a member of the hematopoietin receptor superfamily and may specify binding by helical cytokines (17, 18).

The sGMR appears to be a naturally occurring protein. Using both RNase protection and PCR (data not shown), we identified the sGMR mRNA in two choriocarcinoma cell lines, BeWo and JAR. Although the tissue distribution of this mRNA has yet to be determined, Ashworth and Kraft have recently reported a similar mRNA in placental tissue (19). Their results are consistent with our estimation of the relative abundance of the sGMR mRNA, ≈20% of the total GMR mRNA. We have also been able to detect a small amount of GM-CSF binding activity in highly concentrated conditioned medium from JAR cells. Although it is likely that this binding is due to secretion of the soluble GMR, we cannot rule out the release of membrane-bound receptors into the medium as a consequence of cell lysis. The development of antibodies that discriminate between the membrane-bound and soluble GMR will aid in characterizing the naturally occurring soluble GMR protein.

Soluble binding proteins have been identified for a number of cell-surface molecules, including receptors for nerve growth factor (20), CSF-1 (21), tumor necrosis factor (22, 23), IL-1 (24), IL-2 (25), epidermal growth factor (26), interferon γ (27, 28), and others (29–35). Several receptors of the hematopoietin superfamily also appear to be expressed in both membrane and soluble forms (29–33) (Table 1). The secreted receptors for human G-CSF (29), IL-7 (32), and murine IL-5 (31) are similar to the sGMR reported here in that all result from an internal deletion. Like the GMR, the 5′ boundary of the deletion is situated very near the transmembrane region and therefore does not affect the extracellular binding domain. The deletions

Table 1. Soluble binding proteins in the hematopoietin receptor superfamily

Hematopoietin receptor	Soluble form	Mechanism of secretion	Extracellular amino acids missing	Amino acids unique to soluble receptor
hIL-2β	Unknown	—	—	—
mIL-3II	Unknown	—	—	—
mIL-4	Yes	114-nt insertion ACC ↓ CAAGT . . . GATGG ↓ ACTTC	9	6
mIL-5	Yes	94-nt deletion TGG ↓ GAAA . . . AGT ↓ GTG	10	62
		179-nt deletion GTGG ↓ GAAA . . . GAG ↓ AAA	11	4
hIL-6	Yes	Unknown	—	—
hIL-7	Yes	94-nt deletion TCAG ↓ GG . . . AAAG ↓ GATT	4	27
hG-CSF	Yes	88-nt deletion CCCAG ↓ AG . . . TGCAG ↓ CCC	6	150
hGM-CSF-α	Yes	97-nt deletion TTG ↓ GTTCT . . . AAG ↓ GTTCC	3	16
hGM-CSF-β	Unknown	—	—	—
mEpo	Unknown	—	—	—
rGH	Yes	Replacement of 3′ sequences AAG ↓ AAA → AAG ↓ ATTT	3	17
rPRL	Unknown	—	—	—

Vertical arrows delineate boundary of deletion and are drawn to maximize splice consensus sites. Boldface type denotes consensus splice donor sequences. Underlined sequences indicate repeated sequences. h, Human; m, murine; r, rat; Epo, erythropoietin; GH, growth hormone; PRL, prolactin.

encompass most, if not all, of the transmembrane region and cause a shift in the translational reading frame such that all transmembrane and cytoplasmic C-terminal amino acids are removed. In each case, several amino acids unique to the soluble form are added. As would be predicted from the extracellular sequences expressed by these proteins, the soluble forms of IL-5, IL-7, and GMR are secreted and retain their ligand-binding capacity.

Although soluble receptors for growth hormone, IL-6, and IL-4 have been detected *in vivo* (27, 28, 34), their mechanism of secretion is unknown. The striking parallels in the deletions described above suggest that the receptors for IL-5, IL-7, G-CSF, and GM-CSF use alternative mRNA splicing as a mechanism to generate their soluble counterparts. Examination of the sequences situated directly 5′ to the deletion boundaries (Table 1) reveals sequences reminiscent of consensus splice donor sites ((C/A)AG). The extent of the deletion (≈95 nt) and the sequences encompassed (the entire transmembrane region) suggests that the deleted sequences constitute a single exon. Such a notion is consistent with the intron–exon organization of the transmembrane region of other membrane-bound molecules. Removal of the transmembrane domain by alternative splicing has been described in other systems and is best exemplified in IgM antibody secretion (35). Although alternative splicing seems to be the most likely mechanism, we cannot rule out the possibility that these deleted mRNAs arose from a gene locus distinct from that encoding the membrane-bound receptor.

The prevalence of soluble forms of hematopoietin receptors suggests that they are of biological importance. Soluble GM-CSF receptors may modulate GM-CSF-mediated responses by competing with the membrane-bound receptor for GM-CSF binding. It may act as a direct inhibitor of GMR function or in a feedback mechanism to turn off GM-CSF-stimulated responses. It is not known whether the β subunit of the GMR can influence these processes. Association of soluble receptors with other membrane-bound components has been documented for soluble IL-6 and epidermal growth factor receptors (36, 37). For example, the soluble IL-6 receptor can associate with gp130 to transduce a signal (36). The sGMR may be of clinical significance since it could play a role in regulating the differentiation and function of hematopoietic cells. Soluble GM-CSF receptors could also act to dampen inflammatory responses and therefore have therapeutic potential.

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