Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1213–1217, February 1989
Biochemistry

Hematopoietic activity of granulocyte/macrophage colony-stimulating factor is dependent upon two distinct regions of the molecule: Functional analysis based upon the activities of interspecies hybrid growth factors

(hematopoiesis/amphiphilicity/structure–function relation)

KENNETH KAUSHANSKY, SARA G. SHOEMAKER, SHARON ALFARO, AND CHRIS BROWN

Division of Hematology RM-10, University of Washington, Seattle, WA 98195

Communicated by E. Donnall Thomas, November 28, 1988

ABSTRACT **Granulocyte/macrophage colony-stimulating factor (GM-CSF) is an acidic glycoprotein that stimulates hematopoiesis *in vitro* and *in vivo*. Despite a high degree of sequence homology, the GM-CSFs from human and murine sources fail to crossreact in their respective colony-forming assays. On the basis of this finding, a series of hybrid molecules containing various proportions of human- and murine-specific amino acid sequences were generated by recombinant DNA techniques and assayed for species-specific activity against human and murine marrow target cells. Two regions of GM-CSF, residues 38–48 and residues 95–111, were found to be critical for hematopoietic function. These regions are structurally characterized by an amphiphilic helix and by a disulfide-bonded loop, respectively, and are homologous in position in the human and murine growth factors. In addition, competition assays suggested that, together, these regions bind to the GM-CSF receptor.**

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is an acidic glycoprotein required for the survival, proliferation, and differentiation of hematopoietic progenitor cells in semisolid culture (1, 2) and has been shown to stimulate hematopoiesis *in vivo* (3, 4). The biological activity of GM-CSF is mediated by binding to specific cell surface receptors (5, 6). The dissociation constants for the human and murine growth factors are similar (≈100 pM) and are consistent with the concentrations that stimulate optimal colony growth *in vitro*.

cDNA clones for both human and murine GM-CSF have been analyzed, and the predicted amino acid sequences are 54% identical (7). The disulfide structure, which is critical for the function of GM-CSF, is completely conserved between the human and murine growth factors, and the carbohydrate modification pattern is similar. Despite these features, human GM-CSF (hGM-CSF) fails to stimulate murine progenitor or mature blood cells, and murine GM-CSF (mGM-CSF) fails to stimulate colony formation in cultures of human marrow progenitors (8). Recently, it was shown that removal of the carbohydrate side chains of hGM-CSF had no effect on the functions of the resultant polypeptides (9). In fact, elimination of the N-linked carbohydrate resulted in a 4- to 6-fold increase in the specific activity of the molecule, suggesting that the glycosylated asparagine residues might be located at or near the active site of the molecule. However, little else is known about the structural features responsible for the function of either mGM-CSF or hGM-CSF.

Our goal was to define the region(s) of the GM-CSF polypeptide required for biological activity. Toward this end, we took advantage of the failure of h- and mGM-CSF to crossreact in their respective colony-forming assays. Hybrid cDNA molecules were constructed that contained various lengths of the coding regions for h- and mGM-CSF. The chimeric cDNAs were expressed in COS cells, and the resultant recombinant hybrid growth factors were tested for biological activity in both human and murine colony-forming assays. By using this approach, two distinct regions of the molecule were found to be required for progenitor-cell proliferation. Receptor binding competition assays showed that both of these regions were required for binding to the GM-CSF receptor.

MATERIALS AND METHODS

Hybrid cDNA Constructions. A full-length cDNA for hGM-CSF was previously cloned in our laboratory (9), and the cDNA for mGM-CSF (10) was kindly provided by N. Gough (Walter and Eliza Hall Institute, Melbourne, Australia). Hybrid cDNA molecules were synthesized from discrete restriction fragments of the two cDNAs, by the addition of synthetic oligonucleotides to cDNA fragments, or by site-directed mutagenesis of hybrid or native cDNA molecules to introduce compatible restriction sites or altered coding sequence (Fig. 1).

Site-directed mutagenesis was performed in phage M13 as described (11), except that screening for the mutant plaques was accomplished by using 3 M tetramethylammonium chloride (12). To create human–murine hybrid cDNA following mutagenesis, individual h- and mGM-CSF cDNA fragments were subcloned into the mammalian expression vector pDX (9). After mutagenesis and/or assembly, the nucleotide sequence of the entire cDNA insert was confirmed by the method of Sanger *et al.* (13).

Hybrid Protein Expression. Hybrid cDNA expression vectors were transfected into COS cells by the calcium phosphate method (14). Cells were grown as described (9), and cultures were terminated after 3 days. To confirm the presence of chimeric GM-CSF-specific transcripts, Northern blot analysis was carried out (9). For use as hybridization probes, individual human (*Pst* I–*Nco* I; ref. 15) and murine (*Bam*HI–*Eco*RI; ref. 10) GM-CSF cDNA fragments were purified by gel electrophoresis and labeled to high specific activity with [α-^{32}P]dCTP by random priming with a kit from Boehringer Mannheim. The presence of GM-CSF-specific polypeptides was detected by Western blot analysis. For this, 10 μl of 20× concentrated conditioned medium was size-fractionated by $NaDodSO_4$/12% polyacrylamide gel electrophoresis (16). Protein was transferred to nitrocellulose (17) and probed with a rabbit polyvalent antiserum raised against

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; hGM-CSF, human GM-CSF; mGM-CSF, murine GM-CSF.

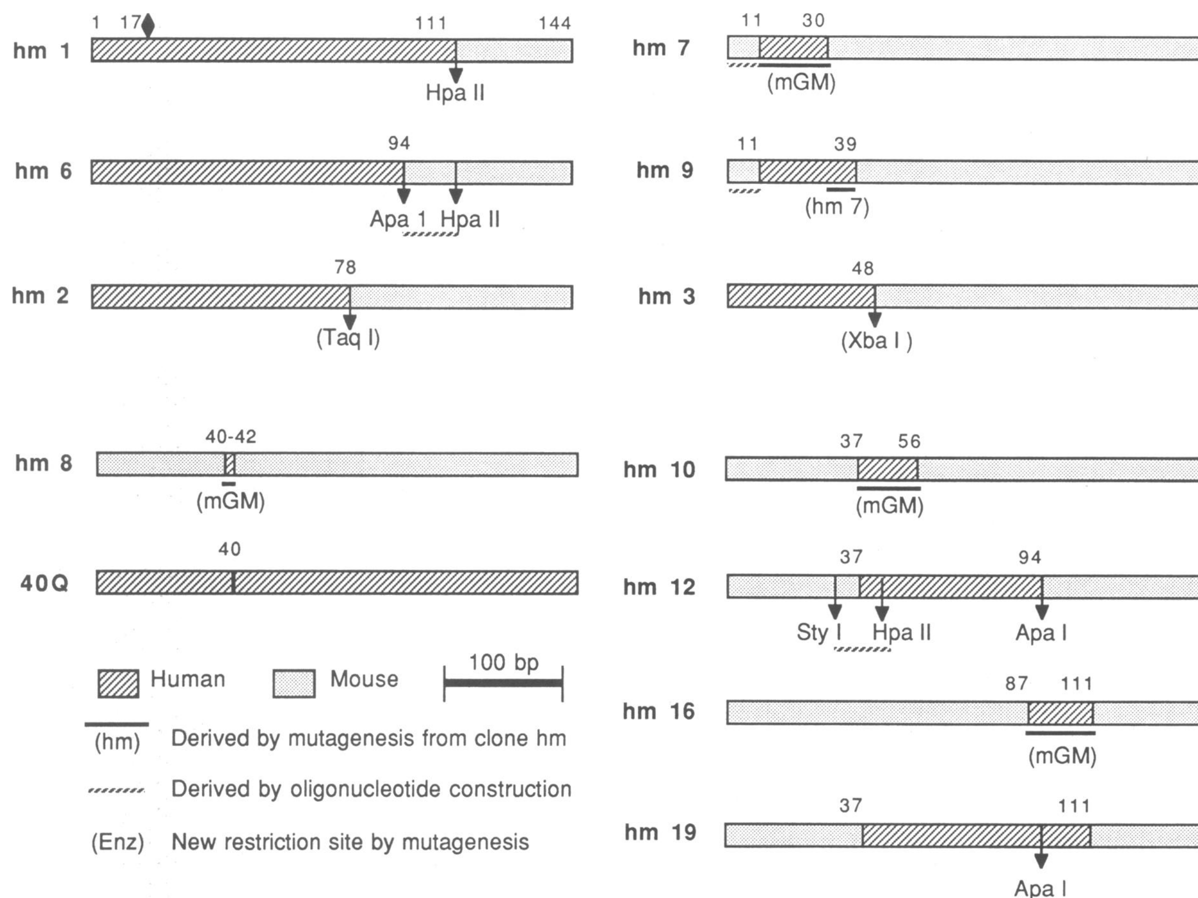


FIG. 1. Schematic representation of human–mouse hybrid constructions. Segments of cDNA derived from site-directed mutagenesis are underlined, along with the construct, in parentheses, from which it had been derived. Regions of the constructs derived from oligonucleotides are denoted by broken underlining. Useful restriction sites are marked, and sites that were created by mutagenesis of either the human or murine cDNA are in parentheses. The cleavage site of the secretory leader peptide is indicated by a diamond. To provide consistency in the numbering of murine and human residues (no. 1 = initiation methionine), the numbering of the mGM-CSF residues is adjusted to that of the human molecule. Since a three-amino acid gap must be introduced into mGM-CSF following residue 39 to allow for maximal alignment of the two sequences (7), murine residues beyond Ala-39 are numbered three residues higher than their true position. bp, Base pairs.

recombinant hGM-CSF. Immune complexes were visualized with an ^{125}I-labeled goat anti-rabbit IgG antiserum (Sigma) and autoradiography.

Determination of Relative Specific Activity. To determine the relative specific activities of several of the GM-CSF mutants, colony-forming assays and Western blot analysis were carried out. The relative specific activities of mutant forms of GM-CSF were calculated by comparing the functional activity obtained from colony-forming assays (18, 19) to the relative amount of immunoreactive protein, as described (9). Each determination was repeated at least three times.

hGM-CSF Receptor Competition Assay. Recombinant hGM-CSF was labeled with ^{125}I (Iodo-Beads, Pierce) and mixed first with a >100-fold excess of human–mouse hybrid protein and then with 2×10^6 HL-60 human promyelocytic leukemia cells in 100 μl of binding medium (RPMI 1640/1% serum albumin/25 mM Hepes, pH 7.4). After a 2-hr incubation at 23°C, the cell-associated ^{125}I-labeled hGM-CSF was separated from unbound label by centrifugation through 100 μl of prechilled 60:40 dibutyl/dinonyl phthalate oil. The results are expressed as the percent competition of binding and are compared to the competition produced by incubation with a 100-fold excess of unlabeled hGM-CSF.

RESULTS

Transcription of Human–Mouse Hybrid cDNA in Transfected Cells. To verify the hybrid nature of the cDNA constructs (Fig. 1), RNA was isolated from COS cells transfected with the hybrid GM-CSF expression vectors and was analyzed by Northern blotting. Identical blots were probed for h- or mGM-CSF-specific transcripts (Fig. 2). Constructs containing >100 bp of sequence from both the human and the murine cDNA (hm 2, hm 3, hm 6, hm 12, and hm 19) directed the expression of RNA that could be detected with both species-specific cDNA probes. Constructs containing <100 bp of the human cDNA [mGM (mGM-CSF), hm 7, hm 8, hm 9, hm 10, and hm 16] or of the murine cDNA [hGM (hGM-CSF) and hm 1] directed expression of RNA that could be detected only with the mGM-CSF- or the hGM-CSF-specific probe, respectively.

Functional Activity of the Human–Mouse Hybrid GM-CSF Proteins. Culture medium from COS cells transfected with the hybrid expression vectors was assayed in human and murine bone marrow colony-forming assays (18, 19). Table 1 lists several human–mouse constructs in order of progressive replacement of the C terminus of hGM-CSF by mGM-CSF-specific sequences (hm 1, hm 6, hm 2). As shown, hybrid GM-CSFs containing human residues 1–111 stimulated the production of GM colony formation from human, but not murine, marrow progenitor cells. In addition, when residues C-terminal to Leu-94 were replaced with murine-specific amino acids (hm 6), the activity of the conditioned medium was nearly eliminated, despite very high levels of transcription (Fig. 2) and hybrid protein production (Fig. 3). When residues from Leu-78 to Leu-94 were then further replaced by murine-specific sequence (hm 2), the hybrid

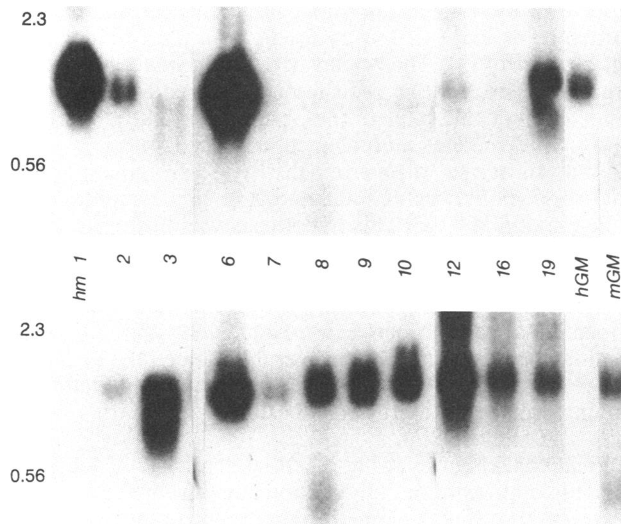


FIG. 2. Northern blot analysis of hybrid cDNA-directed RNA. Ten micrograms of whole cellular RNA isolated from COS cells transfected in different experiments with hm hybrid expression vectors was size-fractionated by formaldehyde/agarose gel electrophoresis and blotted onto nitrocellulose. Identical blots were analyzed with full-length hGM-CSF (*Upper*) and mGM-CSF (*Lower*) cDNA probes. Size markers (kilobases) are indicated at left.

protein was no longer active on human progenitor cells. As the presence of hm 2-specific protein was confirmed by Western blotting (Fig. 3), these data suggest that the region between Thr-95 and Thr-111 is critical to the function of hGM-CSF. In addition, as hm 2 does not stimulate murine progenitor-cell proliferation, these data further suggest that residues between the N terminus and Leu-78 are important for the function of mGM-CSF.

Table 1 also lists several of the human–mouse hybrids in order of progressive replacement of the N-terminal residues of mGM-CSF with hGM-CSF-specific sequences (hm 7, hm 9, hm 3). As shown, these hybrid proteins stimulated murine marrow progenitor cells unless residues between Arg-40 and Asp-48 are replaced with human sequence (hm 3). This suggests that these residues are critical for the function of

Table 1. Functional activity of human–murine hybrid proteins

Medium	Activity, units/ml	
	Human	Murine
hGM	11,900 ± 200	0
mGM	0	14,000 ± 2600
hm 1	4,100 ± 600	0
hm 6	45 ± 4	0
hm 2	0	0
hm 7	0	11,900 ± 1700
hm 9	0	2,400 ± 400
hm 3	0	0
hm 8	0	230 ± 0
hm 10	0	27 ± 3
hm 12	5 ± 2	0
hm 16	0	0
hm 19	10,100 ± 3000	0

Human–murine hybrids are listed in order of increasing replacement of the C-terminal residues of hGM-CSF with murine-specific residues. Culture medium conditioned for 3 days by COS cells transfected with each hm hybrid expression vector was assayed by serial dilution in human and murine marrow colony-forming assays. Fifty units of activity are defined by the dilution that produces half-maximal colony formation from 10^5 marrow cells, compared to saturating amounts of either h- or mGM-CSF. Values are the mean ± SEM of three separate experiments.

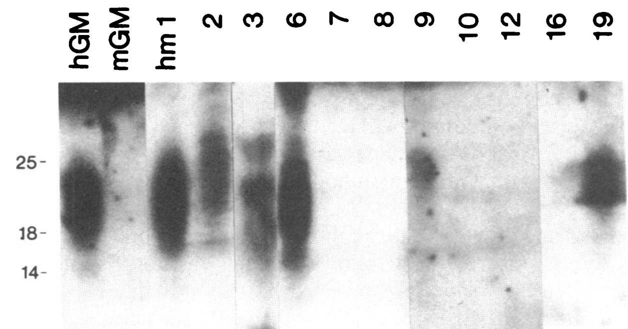
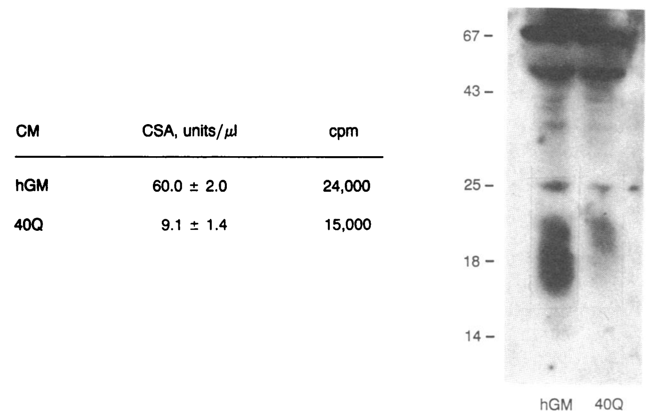


FIG. 3. Western blot analysis of hybrid proteins. Ten microliters of concentrated conditioned medium was size-fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and probed with a rabbit polyvalent antiserum raised against recombinant hGM-CSF and with an ^{125}I-labeled anti-rabbit IgG antiserum. Molecular weight ($M_r \times 10^{-3}$) markers are at left.

mGM-CSF. Again, however, despite the presence of hybrid protein as detected by Western blotting, hm 3 did not stimulate human marrow progenitors, suggesting that residues beyond Asp-48 are important to the function of hGM-CSF.

Additional mutants were analyzed by functional assays and Western blotting to determine the specific activities of these hybrid proteins relative to that of hGM-CSF or mGM-CSF. Mutant 40Q contains the native sequence for hGM-CSF except that Arg-40 has been replaced by glutamine. The specific activity of the 40Q polypeptide was 25% that of hGM-CSF (Fig. 4). To determine whether the same region is important in the function of mGM-CSF, insertional mutagenesis of this region of the mGM-CSF cDNA was performed. Mutant hm 8 contains a tripeptide inserted between residues 39 and 40. This recombinant growth factor was ≈2% as active as mGM-CSF (Table 1). Taken together, these data



CM	CSA, units/μl	cpm
hGM	60.0 ± 2.0	24,000
40Q	9.1 ± 1.4	15,000

FIG. 4. Relative specific activity of mutant 40Q. Ten microliters of concentrated conditioned medium (CM) from COS cells transiently expressing either native hGM-CSF or the mutant growth factor 40Q were assayed for colony-stimulating activity (CSA) and analyzed by Western blotting. (*Left*) Functional activity of the CM was determined by serial dilution in human marrow colony-forming assays, and the results represent the mean ± SEM for four experiments. (*Right*) The same volume of CM tested for functional activity was size-fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with an anti-hGM-CSF peptide (Glu-121 to Val-133; ref. 9) antiserum and then with an ^{125}I-labeled goat anti-rabbit IgG antiserum. After autoradiography, the regions containing immunoreactive protein were cut from the blot for measurement of radioactivity (cpm in *A*). As an equal volume of hGM CM contained 6.6-fold higher activity but only 1.6-fold higher levels of immunoreactive protein than 40Q CM, hGM-CSF has a specific activity 4-fold higher than that of the mutant.

suggest that position 40, or residues in the immediate region, are vital to the function of both h- and mGM-CSF.

Four additional mutants were developed to test the minimum sequence requirements for species-specific function. Hybrid hm 10 contains hGM-CSF sequence from Glu-38 to Thr-56 in place of the corresponding murine residues. This protein did not stimulate human or murine marrow progenitor cells (Table 1). Hybrid hm 12, which contains human-specific residues from Glu-38 through Leu-94, was a poor stimulator of human marrow progenitor cells and was not a stimulator of murine marrow progenitor cells. Hybrid hm 16, which contains hGM-CSF-specific residues only between positions 87 and 111, had no human or murine colony-stimulating activity. However, hybrid hm 19, which contains human-specific sequence between Glu-38 and Thr-111, was a potent stimulus for human progenitor cells. These results suggest that residues from both the N-terminal half and the C-terminal half of GM-CSF are necessary for the function of GM-CSF.

Hybrid Protein Competition Assays. To test whether the nonfunctional or poorly functional hybrid proteins hm 2, hm 3, hm 8, hm 10, and hm 16 could compete with hGM-CSF or mGM-CSF, concentrated COS cell supernatants containing these hybrid proteins were tested for their ability to alter colony formation induced by a half-maximally stimulating amount of hGM-CSF or mGM-CSF. No competition could be detected above that obtained with a culture supernatant of sham-transfected cells (data not shown). In addition, only those chimeric proteins that contain human GM-CSF-specific sequence between residues Glu-38 and Thr-111 could displace ^{125}I-labeled hGM-CSF from its cell surface receptor on HL-60 cells (Table 2). These data suggest that both of the regions identified by our functional analysis are required to form an active receptor-binding domain.

DISCUSSION

Precise mapping of the residues critical to the function of polypeptide hormones or cytokines should make it possible to design peptide fragments or protein analogues that have advantageous properties, such as increased potency, greater selectivity within the spectrum of characteristic biologic functions, or antagonism of growth-promoting activity. This approach has been applied to the study of parathyroid hormone (20), gonadotropin-releasing hormone (21), and interleukin 1 (22).

On the basis of the failure of h- and mGM-CSF to crossreact in assays of progenitor-cell proliferation, a strategy was developed to investigate the region(s) of each molecule required for function as a hematopoietic growth factor. Toward this end, a series of human–mouse hybrid GM-CSF molecules were produced and characterized.

Functional analysis of C-terminally hybrid proteins provided evidence that residues from Thr-95 to Thr-111 are critical to the function of GM-CSF. Recently, the disulfide structure of hGM-CSF was predicted by its homology to interleukin 2 (23). The region between residues 95 and 111 was predicted to lie in a disulfide-stabilized loop structure. This finding could explain the observation that reduction of GM-CSF eliminates all biological activity (24).

Table 2. HL-60 GM-CSF receptor competition assay

Competitor	% competition	Competitor	% competition
Sham	0	hm 6	7 ± 3
hGM	64 ± 3	hm 7	2 ± 2
mGM	0	hm 9	10 ± 7
hm 1	56 ± 4	hm 10	6 ± 3
hm 2	3 ± 3	hm 16	0
hm 3	4 ± 4	hm 19	62 ± 15

Cumulative results of HL-60 GM-CSF receptor competition assays. Results represent the mean ± SEM of four separate experiments expressed as the % competition for binding of ^{125}I-labeled hGM-CSF to HL-60 cells by a 100-fold excess of hm chimeric protein. Sham represents concentrated conditioned medium from COS cells transfected with an irrelevant expression vector.

Analysis of a set of N-terminally hybrid proteins suggested that residues between Glu-38 and Asp-48 are critical to the function of GM-CSF. This hypothesis was further tested by analyzing two additional mutants: 40Q, identical to hGM-CSF except for a single amino acid substitution (Arg → Gln) at position 40, and hm 8, identical to mGM-CSF except for a tripeptide insertion between residues 39 and 40. These modifications moderately to severely reduced the activity of the resultant growth factors. These experiments confirmed the importance of the residues in this region to the function of h- and mGM-CSF.

Final confirmation of the essential character of these two regions for progenitor cell function came from the construction of hybrids that contained either or both of the two regions essential for GM-CSF function. Only the hybrid molecule that contained hGM-CSF-specific residues between positions 38 and 48 and between positions 95 and 111 stimulated proliferation of human marrow progenitor cells.

Finally, a number of the hybrid molecules were used to compete with the native human and murine growth factors for progenitor cell proliferation and binding to the GM-CSF receptor. Overall, these experiments demonstrated that only hybrids containing hGM-CSF sequence from Glu-38 to Thr-111 could compete with the native molecule for binding to the cell surface receptor, suggesting that both the N-terminal and the C-terminal region identified by the functional analysis of hybrid proteins are critical for the binding of GM-CSF to its cell surface receptor.

Recently, the complete chemical synthesis of hGM-CSF and several truncated polypeptides was accomplished by Clark-Lewis *et al.* (23). Although the specific activities of these polypeptides were lower than that of native hGM-CSF by a factor of 70 to 90,000, these studies provided evidence that residues in both the N-terminal and the C-terminal half of the molecule are required for function. Using polypeptide fragments, these investigators found that deletion of residues between Glu-31 and Ile-36 reduced the activity of the polypeptide by a factor of 15. This analysis is similar to our comparison between chimeras hm 7 and hm 9, where a similar alteration reduced mGM-CSF activity by a factor of ≈5. However, Clark-Lewis *et al.* suggested that residues 33–35 (positions 16–18 of the mature polypeptide) are critical for biological activity. In contrast, our analysis suggests that residues C-terminal to this position may be critical for the function of hGM-CSF. Comparison of hm 9 to hm 3 suggests that alteration of residues between positions 40 and 48 lead to the complete elimination of mGM-CSF activity. Furthermore, hybrid hm 19, in which human residues from the N terminus of the protein to residue 37 have been replaced by murine-specific sequence, is fully active.

The use of chimeric molecules to define residues critical to the function of biologically active substances has proven useful for the analysis of other important cytokines. The receptor-binding domain of interferon α has been determined by a similar approach (25). Three amino acid residues were considered critical for interferon–receptor binding (26). All three of these residues were found to reside on the same face of an α-helix.

The active sites of a number of polypeptide hormones have been defined in terms of receptor binding or enzyme activation (27, 28). In many instances, the active-site region has characteristics of an amphiphilic α-helix (29). By the algorithm of Novotný and Auffrey (30), the three-dimensional structures of h- and mGM-CSF are predicted to have strong

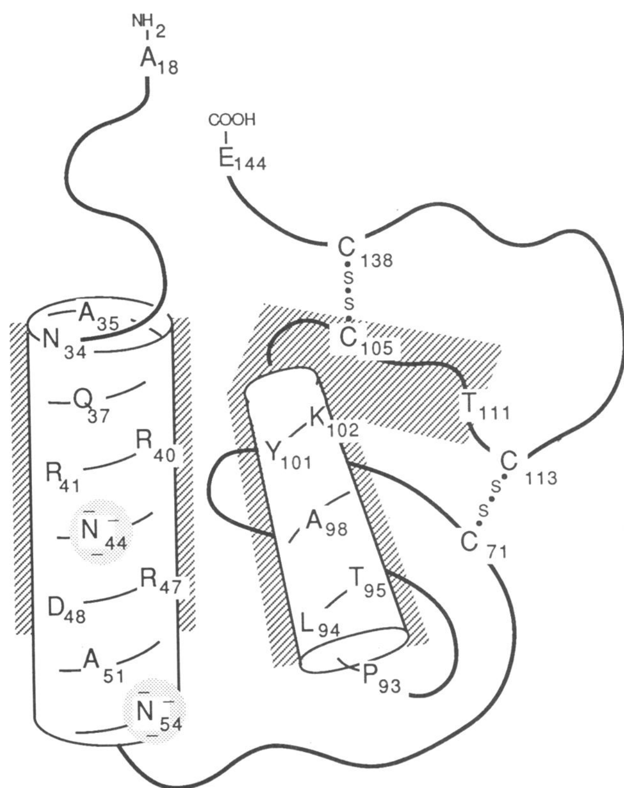


FIG. 5. Schematic model of hGM-CSF. The polypeptide structure of hGM-CSF is represented from the mature N-terminal residue (Ala-18) to the C terminus of the protein. Two of the regions predicted to be α-helical (30) are represented, and the regions identified by functional analysis to be critical to the biologic activity of GM-CSF are shaded. The two disulfide bonds (·S·S·) present in GM-CSF are shown, as is the acidic N-linked carbohydrate (stippled) present on Asn-44 and Asn-54. The hydrophilic face of the amphiphilic helix is shown. The relative positions of the two critical regions cannot be assigned with certainty.

tendencies to form α-helices in the regions between positions 34 and 67 and between positions 93 and 104. When analyzed by the methods of Kaiser and Kezdy (29), GM-CSF displays characteristics of an amphiphilic helix between Asn-34 and Asp-55, one of the two regions predicted to be involved in binding to the GM-CSF receptor. Fig. 5 depicts some of the tertiary structure suggested by these findings. As shown, the region of receptor binding identified by functional studies is also suggested to be a potential site for interaction with cell surface structures, by virtue of its amphiphilicity.

We thank Earl Davie, Fred Hagen, and John W. Adamson for many insightful discussions and for careful review of the manuscript. K.K. is the recipient of Physician-Scientist Award AM01304 from the National Institutes of Health. This work was supported in part by National Institutes of Health Grants CA31615 and AI22772.

1. Metcalf, D. (1986) *Blood* **67,** 257–267.
2. Clark, S. C. & Kamen, R. (1987) *Science* **236,** 1229–1237.
3. Donahue, R. E., Wang, E. A., Stone, D. K., Kamen, R., Wong, G. G., Sehgal, P. K., Nathan, D. G. & Clark, S. C. (1986) *Nature (London)* **321,** 872–875.
4. Groopman, J. E., Mitsuyasu, R. T., DeLeo, M. J., Oette, D. H. & Golde, D. W. (1987) *N. Engl. J. Med.* **317,** 593–598.
5. Walker, F. & Burgess, A. W. (1985) *EMBO J.* **4,** 933–939.
6. Gasson, J. C., Kaufman, S. E., Weisbart, R. H., Tomonaga, M. & Golde, D. W. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 669–673.
7. Miyatake, S., Otsuka, T., Yokota, T., Lee, F. & Arai, K. (1985) *EMBO J.* **4,** 2561–2568.
8. Kaushansky, K., Lin, N. & Adamson, J. W. (1988) *J. Clin. Invest.* **81,** 92–97.
9. Kaushansky, K., O'Hara, P. J., Hart, C. E., Forstrom, J. W. & Hagen, F. S. (1987) *Biochemistry* **26,** 4861–4867.
10. Gough, N. M., Metcalf, D., Gough, J., Grail, D. & Dunn, A. R. (1985) *EMBO J.* **4,** 645–653.
11. Zoller, M. J. & Smith, M. (1984) *DNA* **3,** 479–488.
12. Wood, W. I., Gitschier, J., Lasky, L. A. & Lawn, R. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1585–1588.
13. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
14. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52,** 456–467.
15. Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A. & Clark, S. C. (1985) *Science* **228,** 810–814.
16. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
18. Broudy, V. C., Kaushansky, K., Segal, G. M., Harlan, J. M. & Adamson, J. W. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7467–7471.
19. Kaushansky, K., Broudy, V. C., Harlan, J. M. & Adamson, J. W. (1988) *J. Immunol.* **141,** 3410–3415.
20. Rosenblatt, M. (1986) *N. Engl. J. Med.* **315,** 1004–1013.
21. Karten, M. J. & Rivier, J. E. (1986) *Endocr. Rev.* **7,** 44–66.
22. Palaszynski, E. W. (1987) *Biochem. Biophys. Res. Commun.* **147,** 204–211.
23. Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. E. & Kent, S. B. H. (1988) *J. Immunol.* **141,** 881–889.
24. Clark-Lewis, I. & Schrader, J. W. (1982) *J. Immunol.* **128,** 168–174.
25. Meister, A., Uze, G., Mogensen, K. E., Gresser, I., Tovey, M. G., Grutter, M. & Meyer, F. (1986) *J. Gen. Virol.* **67,** 1633–1643.
26. Weber, H., Valenzuela, D., Luijber, G., Gubler, M. & Weissman, C. (1987) *EMBO J.* **6,** 591–598.
27. Yokoyama, S., Fukushima, D., Kupferberg, J. P., Kezdy, F. J. & Kaiser, E. T. (1980) *J. Biol. Chem.* **255,** 7333–7339.
28. Moe, G. R., Miller, R. J. & Kaiser, E. T. (1983) *J. Am. Chem. Soc.* **105,** 4100–4102.
29. Kaiser, E. T. & Kezdy, F. J. (1984) *Science* **223,** 249–255.
30. Novotný, J. & Auffrey, C. (1984) *Nucleic Acids Res.* **12,** 243–255.