Single Proline Substitutions in Predicted α-Helices of Murine Granulocyte-Macrophage Colony-stimulating Factor Result in a Loss in Bioactivity and Altered Glycosylation*

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Contributions of α-helices to biological activity in murine granulocyte-macrophage colony-stimulating factor were analyzed using site-directed mutagenesis and protein expression in COS-1 cells. A series of single proline substitutions were made for residues within the four predicted α-helices as a means of disrupting local helical secondary structure. Mutations in three of the four helices resulted in marked reductions in bioactivity. Five mutants E21P, L56P, E60P, L63P, and L107P showed 10^−7−10^−10-fold reduction in bioactivity as well as hyperglycosylation. The same Pro substitutions made on non-N-glycosylated molecules had a similar loss in bioactivity implying that a Pro-induced structural change and not hyperglycosylation was responsible for the major decrease in bioactivity. Additional amino acid substitutions at these residues which conserved charge or hydrophobicity, or replaced the original residue with an Ala, verified that conformational changes in the protein structure were specifically due to steric constraints imposed by the Pro residue rather than loss of important side chain functions.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of a family of glycoproteins essential for regulating growth and differentiation of hematopoietic progenitor cells (1−3) as well as stimulating functional activation of mature cell populations (4, 5). Besides having similar and overlapping biological activity the colony-stimulating factors show synergistic effects when present together (6, 7). Taken with the fact that multiple colony-stimulating factors are active at picomolar concentrations, studies using purified native molecules were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; mG-CSF, murine granulocyte-macrophage colony-stimulating factor; hG-CSF, human granulocyte-macrophage colony-stimulating factor; DMEM, Dulbecco’s minimal Eagle’s medium; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 50% S.I., 50% stimulation index; mAb, monoclonal antibody.

recombinant factors became available for extensive characterization of activity in vitro and in vivo and have allowed structural analysis to ensue.

The protein sequences of GM-CSF from human (10), gibbon (11), murine (12, 13), and bovine (14) sources have been deduced from cloned cDNA sequences (Fig. 1). Despite considerable sequence homology among the proteins, including location of cysteine residues, species specificity of biological activity is still maintained. Human and murine proteins have the least homology (54% identical amino acids) and fail to cross-react (15). Detailed biophysical studies using purified proteins demonstrated a high similarity between the two molecules. The proteins exhibited similar peptide backbone conformations, physical characteristics, and conformational stability (16).

In the absence of structural information Parry et al. (17) using a series of predictive algorithms proposed the four α-helical bundle motif as a structural model for a family of related cytokines including hGM-CSF. A number of structure-function studies by other investigators fit the model suggested by Parry. Synthetic peptide analogues synthesized by Clark-Lewis et al. (18) identified minimal sequence necessary for detectable activity. They showed that neither the amino-terminal 15 residues nor the six carboxyl-terminal amino acids were critical for activity. Removal of additional amino acids from either end which include residues predicted to be in helix 1 or helix 4, significantly lowered activity. Shanafelt and Kastelein (19) used scanning deletion analysis to map four regions critical to activity in the murine protein. These regions aligned with the same residues proposed by Parry et al. as helical (Fig. 1). More recently, Kaushansky et al. (20) identified two distinct regions of hGM-CSF, residues Glu-21 to Asp-31 and Thr-78 to Thr-94, that are important for the GM-CSF bioactivity by assaying various hybrid molecules for species-specific activity. Additional receptor competition assays using these chimeric molecules suggest that the two regions comprise the active receptor-binding domain (20). These results were then extended further. Brown et al. (21) used the same chimeras to map the binding epitopes of two monoclonal antibodies that neutralize hGM-CSF activity. Their results confirmed that the two regions identified by Kaushansky et al. (20) were near each other in the folded conformation. They also identified specific residues critical to antibody binding which may also be involved with receptor interactions (21).

In this report we examined the contribution that each of the four predicted helices has on mGM-CSF activity. By making a series of Pro substitutions for residues located in the proposed helices our aim was to cause local disruptions of an individual helix. Mutant proteins were transiently ex-
pressed in COS-1 cells. Bioactivity of each mutant protein was assayed and compared to that of native mGM-CSF.

**MATERIALS AND METHODS**

**Bacterial Strains and Vectors**—The *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA) was used for the propagation and maintenance of all plasmid DNA. The VCS-M13 helper phage was used for helper phage rescue of single strand DNA used as template DNA in mutagenesis experiments.

A partial length cDNA containing the coding region for mGM-CSF was subcloned into the HincII site of pUC18. A HindIII-XbaI fragment from the pUC18 subclone was ligated into pRJB10B (22) which had been digested with HindIII-XbaI yielding pRJB-GM. pRJB-GM expresses the native human GM-CSF under the control of the rat CMV promoter and bovine growth hormone polyadenylation signal. When coupled with a SV40 origin placed upstream of the Rous promoter, this expression cassette is highly efficient in COS-1 cells. In addition, pRJB-GM has an M13 phase I origin that permits rapid production of single-stranded phagemid DNA (23) which can be used for single strand sequencing and site-directed mutagenesis.

**Mutagenesis**—Site-directed mutagenesis was performed using the protocol provided with the oligonucleotide-directed in vitro mutagenesis system V.2 (Amersham RPN.1523, Arlington Heights, IL) which is based on the method of Eckstein (24-28). Oligonucleotides, 22 nucleotides long, corresponding to mGM-CSF sequences incorporating the desired amino acid substitutions were made complementary to the template DNA and used as primers in the mutagenesis reactions. Bacteria were transformed with DNA from the final step of the mutagenesis reactions and cultured on agar plates. Plasmid DNA was isolated from three to five colonies, digested with diagnostic restriction enzymes to check vector integrity, and then sequenced using Sequenase (United States Biochemical, Cleveland, OH) protocol (27, 28). At least 200 nucleotides of GM-CSF-coding sequence was sequenced for all mutants and entire coding sequences were often determined. Confirmed mutants were prepared and plasmid DNA was twice transformed and finally amplified before transfection.

**Transfections and Metabolic Labeling**—COS-1 cells were transfected by the DEAE-dextran method (29) with modifications (30-33). Twenty h before transfection, cells were plated on 60-mm culture dishes at 2×10⁴ cells/dish in 4 ml of complete Dulbecco's minimal Eagle's medium (DMEM) high glucose (GIBCO) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT). After 20 h, the medium was aspirated and the cells washed once with 5 ml of Dulbecco's phosphate-buffered saline. One ml of complete DMEM supplemented with 10% Nu-serum (Collaborative Research, Lexington, MA) was added to the phos-ten μg of plasmid DNA (A = 0.2 absorbance unit) was mixed with 0.8 ml of serum-free DMEM containing 1 mg/ml DEAE-dextran and 250 μM chloroquine, the mixture was added to the dish and incubated at 37°C for 4 h. The transfection solution was aspirated and replaced by 2 ml of 10% dimethyl sulfoxide (Sigma) in Dulbecco's phosphate-buffered saline. After 5 min. After 5 min, the cells were washed once with 5 ml of phosphate-buffered saline and incubated in 5 ml of complete DMEM with 10% FCS for 24 h. The medium was then replaced with 2.5 ml of harvest media (methionine-free DMEM, 10% FCS, supplemented with methionine (Irvine Scientific, Santa Ana, CA) to a final concentration of 0.3 μg/ml and 0.1 μCi/ml [35S]Met (ICN Biomedicals, Costa Mesa, CA) with a specific activity of 100 μCi/μmol. Cells were harvested on glass fiber filters (Whatman, Clifton, NJ) with a multi-well cell harvester (Cambridge Technology, Cambridge, MA) and washed, air dried. [3H]Thymidine incorporation was measured using an LKB 1208 Rack-beta (Pharmacia LKB Biotechnology Inc.) scintillation counter. Values reported as counts/million are the mean of the triplicate for each dilution. Counts were performed on at least two separate transfection experiments.

**Immunoprecipitation**—COS transfection supernatants containing native or mutant GM-CSF metabolically labeled with [35S]Met were immunoprecipitated using either polyclonal rabbit antiserum or a purified rat monoclonal antibody mAb A2 as previously described (36, 37) with modifications. COS supernatants (0.5 ml) were clarified by centrifugation for 10 min then transferred to a fresh Eppendorf tube. Samples were incubated for two 1-h cycles with 100 μl of heat-inactivated formalin fixed Staph A protein (Iggsob, Enzyme Center, Malden, MA) suspended in wash buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.1% NaN3, 0.5% Nonidet P-40, 0.5% deoxycholic acid, 10% glycerol) to adsorb nonspecific proteins. Purified rat monoclonal antibody (mAb A2) (0.5 μg) was added and mixed 10 h followed by a second 10-h incubation with 10 μg of rabbit anti-rat IgG (Vector Laboratories, Burlington, CA). Alternatively, 5 μl of concentrated rabbit serum containing polyclonal antibody made against mGM-CSF was added to the preclreated COS-1 supernatant and mixed for 10 h. Samples were incubated with 200 μl of Iggsob for 5 h and then precipitated with Iggsob protein washed three times with wash buffer. Supernatants were suspended in 100 μl of denaturing buffer, and boiled for 3 min. After centrifugation, the proteins were separated by SDS-PAGE (15%) (38) and either exposed to XAR-5 film (Kodak, Rochester, NY) or a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). Immunoprecipitated using either polyclonal rabbit antisera or a purified rat monoclonal antibody (mAb A2) (0.5 μg) was added and mixed 10 h followed by a second 10-h incubation with 10 μg of rabbit anti-rat IgG (Vector Laboratories, Burlington, CA). Alternatively, 5 μl of concentrated rabbit serum containing polyclonal antibody made against mGM-CSF was added to the preclreated COS-1 supernatant and mixed for 10 h. Samples were incubated with 200 μl of Iggsob for 5 h and then precipitated with Iggsob protein washed three times with wash buffer. Supernatants were suspended in 100 μl of denaturing buffer, and boiled for 3 min. After centrifugation, the proteins were separated by SDS-PAGE (15%) (38) and either exposed to XAR-5 film (Kodak, Rochester, NY) or a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA).

**Determination of Relative Specific Activity**—The concentration (%) of native mGM-CSF COS supernatant that gave 50% maximal stimulation in the DA-3 proliferation assay was determined from the 16-point serial 3-fold dilution curve. Values for a and b were calculated for the equation $Y = aX^b$ using a least squares algorithm to fit the curve to the data from the GM-CSF COS supernatant titration. $Y_{max}$ was calculated from the background count as determined by the average value (cpm) for all dilutions of the mock transfection titration curve. $Y_{max}$ was the average count for all the dilutions of the GM-CSF COS transfection outside the linear region that define the upper limits of the titration curve. The 50% stimulation index (50% S.I.) equals $\frac{Y_{max} - Y_{baseline}}{Y_{baseline}}$ and the bioactivity equals 100 times the reciprocal of the (%) COS supernatant at 50% S.I. The least squares algorithm was then used to fit the curve to the mutant titration values and utilizing the 50% S.I. from the native GM-CSF curve the mutant protein bioactivity was calculated.

The amount of immunoreactive protein in the COS transfection supernatants are quantified by integrating the area x density of the signal from the autoradiogram using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA) or scanning an exposed screen using a phosphorimager (Molecular Dynamics). Protein concentrations (volume) were performed after subtracting the background protein concentration of the mock transfection.

**Specific activity** equals bioactivity/protein concentration. Relative specific activity is defined as specific activity mutant/specific activity GM-CSF and allows for comparison of mutant bioactivity from several COS transfection experiments.

It should be noted that the sensitivity of this quantification method of GM-CSF may not be as reliable as other immunological techniques (19, 39) and permits merely an estimate of specific activity. Since mAb A2 only recognizes the folded form of GM-CSF Western immunoblotting was not possible. Our method of immunoprecipitation and quantitation of quantitating protein concentrations has some unknown inaccuracies, but complete control experiments were done to insure that all of the labeled immunoreactive protein was measured (data not shown). Differences of 3-4-fold were occasionally seen, consequently we considered differences in relative specific activity of 10-fold or greater to be significant.

**RESULTS**

**Introduction of Proline Substitutions and Expression of Mutant Proteins**—Ten residues were initially selected for Pro substitution based on the position of the residue relative to the four helical regions predicted by Parry et al. (17). Addi-
Using the mGM-CSF-responsive cell line DA-3 were compared the propensity of the residue as determined by a number of regions predicted to be α-helices. Each graph includes substitutions that have on protein function, proliferation assays using the mGM-CSF cDNA inserted between the Rous sarcoma virus promoter and the bovine growth hormone polyadenylation signal. The GM-CSF cDNA fragment (37) which includes only 17 amino acids of the native secretory signal sequence starting from the second in-frame Met has been shown to be secreted efficiently in COS-1 cells, and the supernatants containing the mature protein were collected and analyzed as secreted nascent proteins.

**Structure-Function Studies of mGM-CSF**

Table I includes calculations of relative specific activity for the first series of Pro substitutions. The two mutations located in region A had distinct differences in bioactivity. Mutant E17P showed similar activity to that of native GM-CSF while the activity of mutant E21P was about 850-fold less active. Region B contained the three mutations E35P, E38P, and E43P, all of which had activity levels resembling that of the native molecule. The three mutations, L56P, E60P, and L63P, located in region C exhibited various degrees of reduced bioactivity ranging from 25- to 450-fold less activity than that of the native protein. Of the three mutants, L63P had the most profound effect on activity (450-fold) followed by E60P (50-fold) and L56P (25-fold). The two mutants in region D resembled those in region A in that one functioned like the native protein and the other had the lowest bioactivity of all the mutations. The titration curve of mutant A101P paralleled that of the native molecule while mutant L107P (5,500-fold) was unable to reach maximal stimulation even at the highest concentration tested.

Assessing the contribution of a single helix to bioactivity is most evident for regions B and C. In both cases all three mutants in each helix had similar effects on bioactivity and glycosylation. Additional studies using combinations of 2 Pro replacements supported the original findings. Double Pro mutations in region B showed only a minimal reduction (~10-fold) in bioactivity and no changes in glycosylation (data not shown). In addition, it appears that Pro substitution mutagenesis can be used either to define the boundaries of an α-helix or to determine the critical residues within a single helix. For example, two of the least active mutants E21P and L107P were located in regions A and D indicating the importance of these regions. While mutants E17P and A101P, located in the same regions, had no measurable effect on bioactivity suggesting that these residues are not critical for helix formation.

**Proline Substitutions Cause Variations in Glycosylation Patterns**—As shown in Fig. 4, no immunoreactive protein was found in the mock transfection, while native GM-CSF had three major bands ranging in molecular mass from 14.5 to 28 kDa. Experiments on both murine and human recombinant GM-CSF utilizing enzymatic digestion of carbohydrates (37, 43) as well as site-directed removal of glycosylation sites (44, 45) has assigned each band to a different glycosylation variant. The 28-kDa species represents proteins with two N-linked carbohydrates, the 21-kDa species has only one of the two possible N-linked carbohydrates attached and the lowest band with a molecular mass of 15 kDa has no N-linked carbohydrates.
Structure-Function Studies of mGM-CSF

GM-CSF BIOACTIVITY

Fig. 3. Titration of mutant protein activity as measured by proliferation of DA-3 cells. [3H]Thymidine incorporation was measured as a function of the concentration of COS transfection conditioned media (COS CM (%)) as a percentage of total well volume. Native mGM-CSF is indicated by the solid line without symbols and the mock transfection by the dotted line. Mutant proteins are identified by their native single-letter amino acid code, residue number, amino acid substitution and appear as solid lines with symbols. Mutant localized to specific regions appear together in the same graph.

glycosylation. It has also been suggested that O-linked glycosylation occurs, and this may be the reason for the difference between the molecular mass of 14.3 kDa predicted from gene structures and the larger apparent molecular mass encountered from recombinant protein made in mammalian expression systems (45). Proline substitutions that decreased activity of the protein (E21P, L56P, E60P, L63P, L107P) also showed hyperglycosylation when compared to the native molecule (Fig. 4 lanes 4, 8–10 and 12). It is possible that these Pro substitutions cause a conformational change which results in both decreased bioactivity and increased glycosylation. Alternatively, the mutations may cause increased glycosylation which in turn results in reduced bioactivity.

Hyperglycosylation Reflects a Change in Protein Conformation and Is Not the Major Cause of Decreased Bioactivity—A series of mutants was made to determine if loss in bioactivity was caused by a change in protein structure or steric hindrance due to additional carbohydrate groups (45). Proline substitutions that decreased activity of the protein (E21P, L56P, E60P, L63P, L107P) also showed hyperglycosylation when compared to the native molecule (Fig. 4 lanes 4, 8–10 and 12). It is possible that these Pro substitutions cause a conformational change which results in both decreased bioactivity and increased glycosylation. Alternatively, the mutations may cause increased glycosylation which in turn results in reduced bioactivity.

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Proline Substitutions Are the Major Cause for Decreased Bioactivity—Proline substitution at 5 residues measurably decreased the activity of the protein. Our concern was whether the differences in activity caused by the Pro substitutions were due to constraints imposed by the Pro residue itself or the inability of the protein to tolerate any amino acid changes at these sites. To test this, two additional amino acid substitutions were made for those 5 residues exhibiting loss of

(1) In the case of Asn-66 a Ser was inserted and for Asn-75 a Thr was used. The resulting double mutant referred to as 2N was confirmed as a single species of GM-CSF in COS transfection supernatants (Fig. 6, lane 3). The third substitution was either E21P, L56P, E60P, L63P or L107P made on the 2N mutant template. The activity of the mutants are shown in Fig. 5 and compared in Table II. As anticipated from previous studies (43–45), the activity of mutant 2N was at least as active as the native glycosylated form. Similarly the Pro substitutions made on the non-N-glycosylated form of the protein were more active than the glycosylated Pro substitution mutants. The non-N-glycosylated Pro substitutions, however, had the same relative effect on activity as they did in the glycosylated molecule, indicating that increased glycosylation is not the major cause for decreased bioactivity.
bioactivity and hyperglycosylation. For each residue a functionally similar amino acid, either in charge or hydrophobicity, was substituted. Native Glu residues were mutated to Asp residues and Leu residues were changed to Val residues. The chain function would yield the observed changes in bioactivity.

DA-3 proliferation assays of all three substitutions made for a single residue are compared to the native molecule in each of the 5 graphs in Fig. 7. In all cases the functional analogue had no effect on bioactivity demonstrating that similar side chains conserve function. Alanine substitutions also had no measurable effect on bioactivity except possibly mutant L63A which showed a consistent 5-10-fold decrease. With the present methods, differences of less than 10-fold are not considered significant; therefore L63A is considered to be similar to native bioactivity although more rigorous examination may verify some difference. The conclusion based on this series of mutations confirmed that diminished bioactivity of mutants E21P, L56P, E60P, L63P, and L107P is due to conformational changes induced by the Pro substitution.

Other Amino Acid Substitutions Can Alter Glycosylation and Not Change Significantly the Bioactivity of GM-CSF-In all cases the functional analogue substitution had no effects on N-linked glycosylation (Fig. 8). In contrast two Ala substitution mutants caused hyperglycosylation. Unexpectedly, both L63A and L107A were hyperglycosylated but still maintained full or near full bioactivity. The fact that two mutants exhibited hyperglycosylation but retained levels of bioactivity like the native protein was additional proof that hyperglycosylation was not the major cause for the decreased bioactivity of GM-CSF.

**Structure-Function Studies of mGM-CSF**

![Image of SDS-PAGE analysis](https://example.com/sdg-page.png)

**Fig. 4.** SDS-PAGE analysis of metabolically radiolabeled COS transfected supernatants. COS cells were transfected with either plasmid RJB-GM or various mutants and labeled with [35S]Met. Equal volumes of transfection supernatants were immunoprecipitated with rat anti mGM-CSF monoclonal antibody mAb A2 as described under "Materials and Methods." Precipitated samples were denatured in denaturing buffer, separated on a 15% SDS-PAGE gel and exposed to x-ray film.

![Image of GM-CSF BIOACTIVITY](https://example.com/gm-csf-bioactivity.png)

**Fig. 5.** Titration of mutant protein activity as measured by proliferation of DA-3 cells. [3H]Thymidine incorporation was measured as a function of the concentration of COS transfection conditioned media (COS CM (%)) as a percentage of total well volume. Native GM-CSF and mock transfection are indicated by the solid line without symbols and the dotted line, respectively. Mutant proteins are identified by their native single-letter amino acid code, residue number, amino acid substitution and appear as solid lines with symbols.

### Table I

| Sample | Protein concentration* | Bioactivity* | Specific activity* | RSA* | RSA* | RSA* |
|--------|------------------------|-------------|-------------------|------|------|------|
|        | volume | reciprocal titer |                 |      |      |      |
| GM-CSF | 9,247  | 8,258       | 0.89             | 1.0  | 1.0  | 1.0  |
| E17P  | 9,076  | 21,245      | 2.3              | 2.6  | 2.2  | 1.8  |
| E21P  | 10,287 | 9.7        | 9.4 × 10⁻⁴       | 1.1 × 10⁻³ | 1.6 × 10⁻³ | 8.5 × 10⁻⁴ |
| E35P  | 6,788  | 5,470       | 1.2              | 1.3  | 0.68 | 0.85 |
| E38P  | 12,029 | 13,864      | 1.2              | 1.3  | 1.5  | 1.1  |
| E43P  | 9,053  | 2,994       | 0.33             | 0.37 | 0.32 | 0.30 |
| L56P  | 14,799 | 108        | 7.3 × 10⁻³       | 8.2 × 10⁻³ | 2.7 × 10⁻² | 2.3 × 10⁻² |
| E60P  | 20,399 | 256        | 1.3 × 10⁻²       | 1.5 × 10⁻² | 5.4 × 10⁻² | 5.0 × 10⁻² |
| L63P  | 17,126 | 15         | 8.8 × 10⁻⁴       | 9.9 × 10⁻⁴ | 4.1 × 10⁻³ | 1.4 × 10⁻³ |
| A101P | 10,663 | 13,674     | 1.3              | 1.5  | 1.4  | N.T.* |
| L107P | 7,957  | 0.69        | 8.7 × 10⁻⁶       | 9.8 × 10⁻⁵ | 4.0 × 10⁻⁴ | 4.5 × 10⁻⁵ |

* Protein concentration of total immunoreactive protein (volume) was measured by integrating the entire signal (area × density) from the autoradiogram and subtracting the volume of the mock lane.

* Specific activity (SA) equals the bioactivity/protein concentration.

* Relative specific activity (RSA) equals SA mutant/SA. GM-CSF and was calculated from the data presented in Figs. 3 and 4 (using mAb A2).

* RSA was calculated from the same COS transfection as in RSA except protein concentration was determined from the immunoprecipitation using polyclonal antisera.

* RSA was calculated from a separate COS transfection experiment using polyclonal antisera for the immunoprecipitation.

* Not tested.
the mutants which have both hyperglycosylation and reduced bioactivity.

**DISCUSSION**

Protein crystallographic studies have revealed common structural features among proteins that neither resemble each other in sequence or function. One such recurring structural topology some proteins with high α-helical content have is the arrangement of helices to form a four α-helical bundle (46). In a study of 30 cytokines, Parry et al. (17) applying a series of protein prediction algorithms proposed that interleukins and colony-stimulating factors belong to a family of proteins that fit the four α-helical bundle motif. Specifically, a structural model was discussed for hGM-CSF that identified the four helices that comprise 54% of the molecule and the possible relative orientation of the helices based on disulfide bonds and connecting loop constraints. Of particular interest was the orientation of helices C and D due to the disulfide bond formed between Cys-51 and Cys-93. The GM-CSF molecule was the only cytokine predicted to have a parallel arrangement of two neighboring helices on the linear protein. In good agreement, Wingfield et al. (26) calculated α-helical content of 47% for both h- and mGM-CSF by measuring circular dichroic spectra. From the same experiment comparable CD spectra support the evidence from sequence alignment data that the two proteins have similar backbone conformation. Shanafelt and Kastelein (19) examined structure-function relationships by generating a large panel of three amino acid deletion mutants that extended along the entire length of the mGM-CSF. Their results identified four regions of the protein defined as critical to GM-CSF bioactivity. Our analysis of the Parry et al. predictions and the scanning deletion studies (see Fig. 1) indicated that the critical regions aligned with homologous sequences in the human counterpart, predicted to be α-helical, suggesting that helices play an important role in maintaining protein integrity and bioactivity. The fact that deletions were three amino acids, which corresponds to nearly a full turn of an α-helix, implied that merely shortening any one of the four helices had significant consequences on protein bioactivity. Our results dispute those of Shanafelt and Kastelein regarding helix 2. Our analysis suggests that helix 2 has a noncritical role in maintaining mGM-CSF bioactivity. This contradiction may simply reflect the differences between the two methods of probing mGM-CSF structure. The use of scanning deletion mutagenesis is likely to result in greater conformational changes in the molecule which are necessary to compensate for the gap left by the deletions.

The aim of our work was to investigate more closely the role of α-helices and compare the differential contributions that helices play in preserving mGM-CSF bioactivity. The approach used single amino acid substitutions as a means of minimizing gross structural perturbations caused by inserting or removing peptide fragments. Our strategy involved substituting Pro for residues localized to a particular predicted helix with the intention of disrupting that helix. Proline was selected as the replacement residue because it is regarded as sterically incompatible with helical secondary structure. Constraints due to the unusual Pro side chain characteristics limit peptide bond rotation and prevent H-bonding, both of which are important to α-helical conformations. Clearly, a single Pro insertion may not be capable of overcoming the cooperative interactions of the neighboring residues or stabilizing effects of adjacent helices. This has been seen in the interleukin 2 molecule where helix B accommodated a Pro by introducing a kink (47). Therefore, it is important to remember that all structural changes due to the Pro residue are theoretical. A true understanding of the alterations imposed by the

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**Fig. 6.** SDS-PAGE analysis of metabolically radiolabeled COS transfection supernatants. COS cells were transfected with either plasmid RJB-GM or various mutants and labeled with [35S]Met. Equal volumes of transfection supernatants were immunoprecipitated using the rat monoclonal antibody mAb A2 as described under “Materials and Methods.” Precipitated samples were boiled in denaturing buffer, separated on a 15% SDS-PAGE gel, and quantitated using a phosphorimager.

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**Table II**

| Sample | Protein concentration* | Bioactivity* | Specific activity* | RSA* | RSA* | RSA* | RSA* |
|--------|------------------------|-------------|-------------------|------|------|------|------|
|        | volume                 | reciprocal  |                   |      |      |      |      |
| GM-CSF | 32,365                 | 8,036       | 0.25              | 1.0  | 1.0  | 1.0  |
| 2N     | 27,389                 | 11,474      | 0.42              | 1.7  | 0.80 | 3.4  |
| 2N.E21P| 2,714                  | 17          | 6.3 × 10⁻³        | 2.5 × 10⁻²| 2.5 × 10⁻²| 4.0 × 10⁻²|
| 2N.L56P| 2,867                  | 15          | 5.2 × 10⁻³        | 2.1 × 10⁻²| 3.5 × 10⁻²| 2.3 × 10⁻²|
| 2N.E60P| 4,456                  | 235         | 5.3 × 10⁻²        | 0.21 | 0.30 | 0.19 |
| 2N.L63P| 6,081                  | 5.6         | 9.2 × 10⁻⁴        | 3.7 × 10⁻³| 3.3 × 10⁻³| 7.4 × 10⁻³|
| 2N.L107P| 2,576                 | 0.64        | 2.5 × 10⁻⁴        | 1.0 × 10⁻³| 1.0 × 10⁻³| 3.7 × 10⁻⁴|

*Protein concentration of total immunoreactive protein (volume) was measured by integrating the entire signal (area × density) from the autoradiogram and subtracting the volume of the mock lane.

Bioactivity equals 100 × the reciprocal of the % COS supernatant that gave 50% maximal response.

Specific activity (SA) equals the bioactivity/protein concentration.

Relative specific activity (RSA) equals SA mutant/SA mGM-CSF and was calculated from the data presented in Figs. 5 and 6 (using mAb A2).

RSA was calculated from a separate COS transfection experiment using mAb A2 for the immunoprecipitation.

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D. A. D. Parry, unpublished observations.
### Table III

Relative specific activity comparison of mGM-CSF and mutant proteins

| Sample  | Protein concentration | Bioactivity | Specific activity | RSA<sup>a</sup> | RSA<sup>b</sup> | RSA<sup>c</sup> |
|---------|-----------------------|-------------|------------------|-----------------|----------------|----------------|
|         | volume                | reciprocal  |                  |                 |                |                |
| GM-CSF  | 7,546                 | 5,260       | 0.70             | 1.0             | 1.0            | 1.0            |
| E21A    | 8,917                 | 3,687       | 0.41             | 0.59            | 1.0            | 0.60           |
| E21D    | 11,854                | 2,542       | 0.21             | 0.30            | 0.38           | 0.33           |
| L56A    | 8,845                 | 10,146      | 1.2              | 2.9             | 1.9            |                |
| L56V    | 9,019                 | 10,800      | 1.2              | 1.6             | 1.8            |                |
| E60A    | 10,896                | 6,720       | 0.62             | 0.89            | 2.3            | 1.3            |
| E60D    | 12,325                | 6,519       | 0.53             | 0.76            | 1.3            | 1.5            |
| L63A    | 12,770                | 1,196       | 0.09             | 0.13            | 0.28           | 0.14           |
| L63V    | 10,935                | 5,230       | 0.48             | 0.69            | 0.71           | 0.91           |
| L107A   | 8,247                 | 2,413       | 0.30             | 0.43            | 0.59           | 0.21           |
| L107V   | 11,357                | 8,183       | 0.72             | 1.0             | 1.3            | 0.87           |

<sup>a</sup> Protein concentration of total immunoreactive protein (volume) was measured by integrating the entire signal (area × density) from the autoradiogram and subtracting the volume of the mock lane.

<sup>b</sup> Bioactivity equals 100 × the reciprocal of the % COS supernatant that gave 50% maximal response.

<sup>c</sup> Specific activity (SA) equals the bioactivity/protein concentration.

<sup>d</sup> Relative specific activity (RSA) equals SA mutant/SA GM-CSF and was calculated from the data presented in Figs. 7 and 8 (using mAb A2).

<sup>e</sup> RSA was calculated from the same COS transfection as in RSA<sup>d</sup> except protein concentration was determined from the immunoprecipitation using polyclonal antisera.

<sup>f</sup> RSA was calculated from a separate COS transfection experiment using mAb A2 for the immunoprecipitation.

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**GM-CSF BIOACTIVITY**

**Fig. 7.** Titration of mutant protein activity as measured by proliferation of DA-3 cells. [3H]Thymidine incorporation was measured as a function of the concentration of COS transfection conditioned media (COS CM (%)) as a percentage of total well volume. Native mGM-CSF and mock transfection are indicated by the **solid line without symbols** and the **dotted line**, respectively. Mutant proteins are identified by their native single letter code amino acid, residue number, amino acid substitution and appear as **solid lines with symbols**. Each graph compares mutations for a single residue and includes the native protein as well as all three different amino acid substitutions.
substitutions can only be addressed by crystallographic data (48).

Since alterations in primary structure can lead to secondary structural changes which may affect bioactivity (e.g., glycosylation), it is important to determine the contribution of each structural change to bioactivity. Earlier experiments using hGM-CSF with mutated N-linked glycosylation sites (44, 45) or proteins which had the carbohydrates removed enzymatically (43) measured increased activity with decreasing N-linked glycosylation. Elegant biochemical studies using purified preparations of hGM-CSF from activated T-lymphocytes recently demonstrated that increased N-linked glycosylation decreased the specific activity of native protein and confirmed previous observations were not artifacts of mammalian expression systems. Cebon et al. (49) was able to attribute the lower activity to reduced receptor affinity. One of the most interesting findings from our experiments is the strong correlation between mutations that decreased bioactivity and changed N-linked glycosylation processing. Previous studies in this laboratory demonstrated the same phenomenon with a carboxyl-terminal deletion mutant (37). LaBranche et al. generated a deletion mutant that had over 10-fold reduced bioactivity and showed that the hyperglycosylation was N-linked. The major species of mGM-CSF has only 1 Asn residue glycosylated (Fig. 4, lane 2). Since the deletion mutant removed Cys-118 and therefore eliminated the second disulfide bond a more open protein conformation may have existed that was able to accommodate additional carbohydrate groups. Two mutants L63A and L107A support this idea. Both mutations removed hydrophobic side chains that, in the Parry model, are important for helix packing. The loss of hydrophobic interactions in the interior of the protein might result in a more relaxed conformation and allow greater exposure of both Asn residues permitting occupancy of both sites. Similar effects were seen with some Pro substitutions that disrupted critical helices and possibly helix packing. While hyperglycosylation does result in a minor (6-fold) decrease in bioactivity (49), other structural changes are necessary for a major loss in bioactivity. Assays of Pro substitutions made on non N-glycosylated molecules (Table II) showed that hyperglycosylation was not the major cause of bioactivity differences and indicated that hyperglycosylation represents a change in protein conformation. Thus, it is likely that specific Pro substitutions in mGM-CSF result in conformational changes which are reflected by both decreased bioactivity and hyper-glycosylation.

The present results with mGM-CSF and prior investigations of hGM-CSF suggest a close proximity of N-linked carbohydrate attachment sites to the active site. This is an important consideration because in the two species the carbohydrates are located in different regions of the molecule. The N-linked carbohydrates are attached at Asn-66 and Asn-75 in mGM-CSF and Asn-27 and Asn-37 in the human molecule (Fig. 1). Protein modeling by Kaushansky et al. (50), based on the predictions of Parry et al. and bond energy minimization analysis, placed these two regions next to each other in the folded form. Independent confirmation of this prediction comes from two different studies. Initially, Kau- shansky et al. (20) using human-mouse chimeric molecules demonstrated the importance of the regions Glu-21 to Asp-31 and Thr-78 to Thr-94 to the bioactivity of hGM-CSF. Both of these regions overlap with at least one of the N-linked glycosylation sites in either the human Asn-27 or the murine Asn-75 protein (Fig. 1). Using the same series of chimeras Brown et al. (21) mapped the binding epitopes of two mAbs that neutralize hGM-CSF activity. They showed that mAb 221 recognized residue Arg-24 and region His-83 to Thr-94 and that mAb 213 bound to the region Leu-77 to His-83. Together these experiments established the proximity of these two regions to each other and present a strong case for their involvement with receptor binding.

Methods for predicting secondary structure are constantly being refined. Improvements in accuracy can be attributed to both the increasing numbers of protein structures solved by x-ray crystallographers and the use of high powered computers to analyze large amounts of available structural information. These predictions can provide testable models for studies designed to identify secondary structural elements and examine their molecular interactions or contribution to function. Our study shows that proline insertion mutagenesis is a sensitive means to evaluate helix contribution to protein function. Beyond comparing different helices, our data suggest that this method may be gentle enough to probe subtle differences that can occur along the length of a single helix. This approach should be suited both for molecules whose helices have been identified through crystallographic data and for molecules where helices are predicted.
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