The EMBO Journal vol.11 no.3 pp.909-916, 1992

# Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding

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Communicated by J.Tooze

**The functional role of the predicted first α-helix of human granulocyte-macrophage colony-stimulating factor (GM-CSF) was analysed by site-directed mutagenesis and multiple biological and receptor binding assays. Initial deletion mutagenesis pointed to residues 20 and 21 being critical. Substitution mutagenesis showed that by altering Gln20 to Ala full GM-CSF activity was retained but that by altering Glu21 for Ala GM-CSF activity and high affinity receptor binding were decreased. Substitution of different amino acids for Glu21 showed that there was a hierarchy in the ability to stimulate the various biological activities of GM-CSF with the order of potency being Asp21 > Ser21 > Ala21 > Gln21 > Lys21 = Arg21. To distinguish whether position 21 was important for GM-CSF binding to high or low affinity receptors, GM-CSF (Arg21) was used as a competitor for [$^{125}$I]GM-CSF binding to monocytes that express both types of receptor. GM-CSF (Arg21) exhibited a greatly reduced capacity to compete for binding to high affinity receptors, however, it competed fully for [$^{125}$I]GM-CSF binding to low affinity receptors. Furthermore, GM-CSF (Arg21) was equipotent with wild-type GM-CSF in binding to the cloned low affinity α-chain of the GM-CSF receptor. These results show that (i) this position is critical for high affinity but not for low affinity GM-CSF receptor binding thus defining two functional parts of the GM-CSF molecule; (ii) position 21 of GM-CSF is critical for multiple functions of GM-CSF; and (iii) stimulation of proliferation and mature cell function by GM-CSF are mediated through high affinity receptors.**

*Key words:* growth factors/haemopoiesis/mutagenesis/structure-function/receptors

## Introduction

Human (h) granulocyte-macrophage (GM) colony-stimulating factor (CSF) is a multi-potential growth factor capable of stimulating several haemopoietic cell lineages such as the neutrophilic, eosinophilic, monocytic and megakaryocytic series (Sieff *et al.*, 1985; Metcalf *et al.*, 1986). In addition GM-CSF is also able to stimulate the function of the differentiated progeny enhancing the effector functions of neutrophils and eosinophils (Vadas *et al.*, 1983; Gasson *et al.*, 1984; Lopez *et al.*, 1986) and the capacity of monocytes to kill tumour cells (Grabstein *et al.*, 1986) and adhere to various surfaces (Gamble *et al.*, 1989; Elliott *et al.*, 1990). Because of this pleiotropic effect GM-CSF has been used *in vivo* where it has been shown to increase the granulocyte counts in AIDS patients (Groopman *et al.*, 1987), accelerate bone marrow reconstitution following chemotherapy (Antman *et al.*, 1988) and enhance the effector function of circulating neutrophils (Baldwin *et al.*, 1988). In addition to normal haemopoietic cells, certain tumour cell lines have also been shown to respond to GM-CSF by proliferating *in vitro* (Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

The human GM-CSF receptor has now been cloned and shown to comprise at least a binding (α) chain that binds GM-CSF with low affinity (Gearing *et al.*, 1989) and a second (β) chain that does not seem to bind GM-CSF by itself but which allows the formation of a high affinity receptor when co-expressed with the α-chain (Hayashida *et al.*, 1990). The functions mediated by each chain of the GM-CSF receptor are not yet known.

Despite the multiple *in vitro* and *in vivo* studies with GM-CSF, little is known about regions of the molecule essential for activity and in particular whether different regions participate in binding to high and low affinity receptors and their relationship to function. Using a chemical-synthesis approach we have previously shown that the 14 most N-terminal and the six most C-terminal residues of GM-CSF are not required for function, and importantly that the 14-24 region in the first predicted α-helix of GM-CSF is essential for bioactivity (Clark-Lewis *et al.*, 1988). Similar results were obtained using human-mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991) and monoclonal anti-GM-CSF antibodies which blocked activity (Brown *et al.*, 1990). The latter two approaches identified, in addition, a second region in GM-CSF between residues 88-96 important for activity.

We have now used site-directed mutagenesis to study in more detail the predicted first α-helix of GM-CSF and, in particular, focussed on the hydrophilic residues Gln at position 20 and Glu at position 21. Our results show that residue Glu21 is critical for the full biological activity of GM-CSF. Significantly, substitution of Glu21 with Arg impaired the binding to high affinity but not to low affinity GM-CSF receptors, thus linking high affinity binding to the various functions examined and suggesting that Glu21 may be involved in binding to the β-subunit of the GM-CSF receptor.

## Results

### *Mutagenesis of Gln20 and Glu21 of human GM-CSF*

Initial experiments designed to examine the effect of N-terminal deletions on GM-CSF activity revealed that deletion of residues 1-24, 7-24 and 14-24 caused loss of GM-CSF activity (data not shown). We then focused on residues

Gln20 and Glu21 predicted to constitute a hydrophilic face of the GM-CSF molecule (Parry *et al.*, 1991). Deletion of residues 20–21 resulted in the complete loss of GM-CSF ability to stimulate bone marrow colony formation, the neutrophil respiratory burst and antibody-dependent cytotoxicity (ADCC), eosinophil-mediated antibody-dependent cytotoxicity (ADCC), and in the ability to compete for [$^{125}$I]GM-CSF high affinity binding to human neutrophils. In order to ascertain the importance of positions 20 and 21 for the biological activities of GM-CSF less severe modifications were carried out by introducing the non-polar residue alanine at both these positions. Experiments using transiently transfected COS cells showed that GM-CSF(Ala20) had the same potency as wild-type (WT) GM-CSF at stimulating day 14 GM bone marrow colonies (100.3% potency, not different to WT GM-CSF $P = 0.9$, $n = 6$), neutrophil ADCC (116.9%, $n = 6$, $P = 0.45$) and superoxide anion ($O_2^-$) generation (104.3%, $n = 12$, $P = 0.65$) and eosinophil ADCC (94.7%, $n = 4$, $P = 0.76$). In contrast GM-CSF(Ala21) was less potent than WT GM-CSF at stimulating neutrophil ADCC (24.2%, $n = 8$, $P < 0.001$) and $O_2^-$ generation (28.9%, $n = 12$, $P < 0.001$), and eosinophil ADCC (21.0%, $n = 4$, $P < 0.001$). The double substitution GM-CSF(Ala20 Ala21) was also less potent than WT GM-CSF at stimulating neutrophil ADCC (17.0%, $n = 6$, $P < 0.01$) and $O_2^-$ generation (7.5%, $n = 11$, $P < 0.001$). In addition, GM-CSF(Ala21) and GM-CSF(Ala20 Ala21) failed to stimulate 50% of WT GM-CSF day 14 GM colonies at the highest concentrations tested (100 ng/ml).

### *Single amino acid substitutions of Glu21*

Having identified residue 21 as important for several biological properties of GM-CSF, a series of single substitutions were carried out to replace Glu21 with amino acids of different hydrophilicity and polarity. The amino acids introduced were aspartic acid (hydrophilic, acidic), glutamine (hydrophobic, neutral), serine (hydrophilic, neutral), alanine (hydrophobic, neutral), arginine (hydrophilic, basic) and lysine (hydrophilic, basic).

Examination of these GM-CSF mutants for their ability to stimulate the proliferation of leukaemic cells showed that the substitution of Glu for Asp reduced activity by 4-fold, for Ser by 30-fold, for Ala by 80-fold and for Gln by 100-fold. Substitutions with Lys and Arg yielded GM-CSF mutants with no activity up to a concentration of 10 ng/ml (Figure 1A). A titration of these GM-CSF mutants on stimulation of monocyte adherence showed similar results, with the acidic residue Asp affecting GM-CSF activity the least and the basic residues Arg and Lys virtually abolishing GM-CSF activity up to a concentration of 10 ng/ml (Figure 1B). Essentially the same pattern was observed in the stimulation of neutrophil $O_2^-$ generation (data not shown).

Competition binding experiments on neutrophil high affinity GM-CSF receptors mirrored the hierarchy of the biological data. Using 70 pM [$^{125}$I]GM-CSF and the different GM-CSF proteins at 30-fold excess the levels of competition were 87.9% for WT GM-CSF, 26.1% for Asp21, 23.0% for Ser21, 18.3% for Ala21, 16.6% for Gln21, 14.6% for Arg21 and 7.4% for Lys21.

### *Purification and biological activities of GM-CSF with substitutions at positions 20 and 21*

To study in more detail the relevance of positions 20 and 21, GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were purified by affinity chromatography and reversed phase HPLC from supernatants of transfected CHO cells. After quantitation by amino acid analysis the GM-CSF mutants were visualized by Western blot analysis (Figure 2A) and silver staining (Figure 2B) to confirm purity and integrity. The purified GM-CSF mutants showed similar molecular weight heterogeneity, hence degree of glycosylation, to the wild-type GM-CSF (Figure 2A and B).

These purified mutants stimulated the [$^3$H]thymidine incorporation of leukaemic cells with different potencies. While GM-CSF(Ala20) was equipotent to the wild-type GM-CSF, GM-CSF(Ala21) was 30-fold, GM-CSF(Ala20, Ala21) 100-fold and GM-CSF(Arg21) and GM-CSF(Ala20,

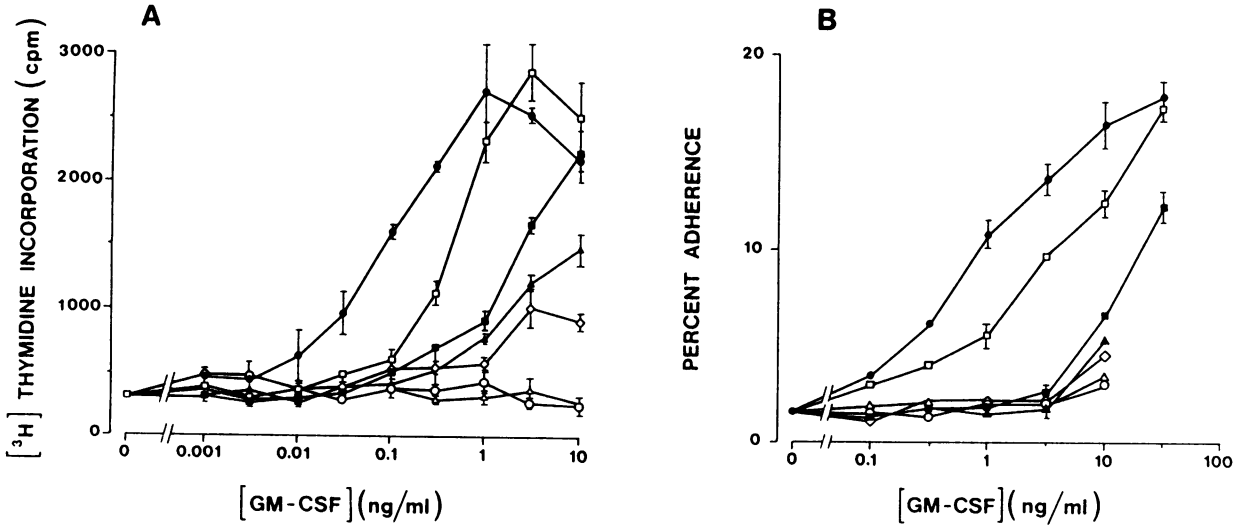


**Fig. 1.** Titration of GM-CSF mutants containing different residues at position 21 for their ability to stimulate the incorporation of [$^3$H]thymidine into chronic myeloid leukaemia cells (**A**) and to stimulate monocyte adherence (**B**). Wild-type (WT) GM-CSF (●) as well as GM-CSF(Ala21) (▲), GM-CSF(Arg21) (△), GM-CSF(Lys21) (○), GM-CSF(Gln21) (◇), GM-CSF(Ser21) (■) and GM-CSF(Asp21) (□) were tested at the concentrations shown. Representative experiments are shown with the bars spanning the SEM.

Arg21) 200-fold less potent than wild-type GM-CSF respectively (Figure 3A).

Stimulation of mature cell function showed a similar hierarchy and relative potencies in the stimulation of monocyte adherence and neutrophil $O_2^-$ production. On monocyte adherence GM-CSF(Ala21) was 30-fold less potent than wild-type GM-CSF and GM-CSF(Ala20), while GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were ~100-, 200- and 200-fold less potent respectively (Figure 3B). On neutrophil $O_2^-$ production GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21), GM-CSF(Ala20, Arg21) were 10-, 100-, 200- and 200-fold less potent than wild-type GM-CSF respectively (Figure 4A).

In competition binding experiments on the neutrophil high affinity GM-CSF receptor, GM-CSF(Ala20) was 3-fold, GM-CSF(Ala21) 40-fold, and GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) >100-fold less effective than wild-type GM-CSF at competing for [$^{125}$I]GM-CSF binding (Figure 4B).

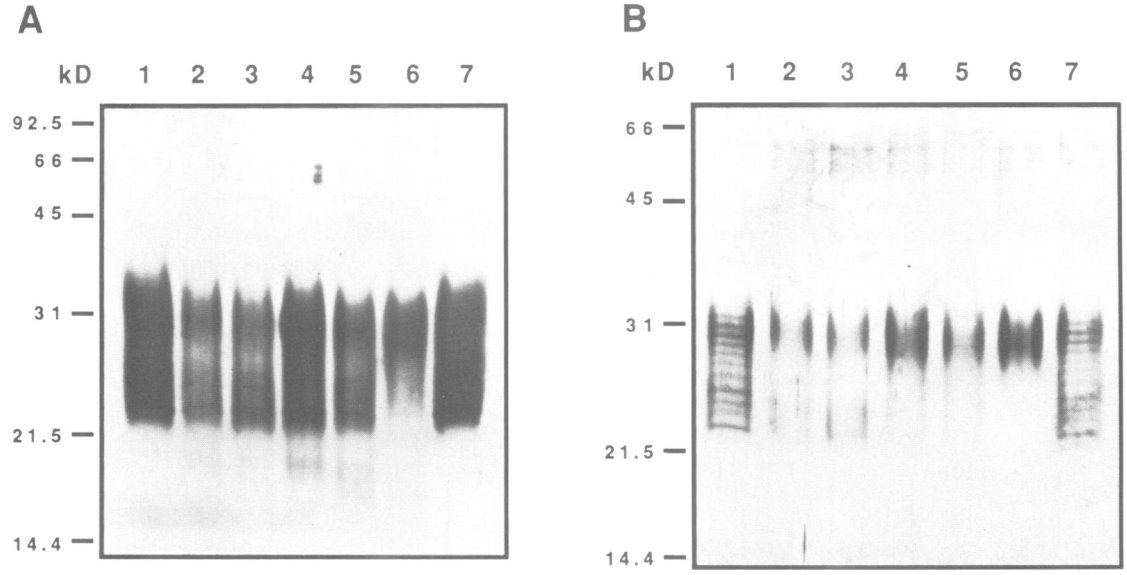


**Fig. 2.** Western blot analysis (**A**) and silver staining (**B**) of GM-CSF mutants after purification by affinity chromatography and HPLC. After quantitation by amino acid analysis 200 ng of protein was loaded per track and electrophoresed on a 12.5% polyacrylamide–SDS gel. In (A) GM-CSF protein was visualized by using a sheep antibody to human GM-CSF followed by a biotinylated rabbit anti-sheep antibody and developing the reaction with diaminobenzidine. In (B) the same amount of purified GM-CSF protein was visualized by silver staining. The different lanes contain WT GM-CSF (lane 1), GM-CSF(Arg21) (lane 2), GM-CSF(Ala20, Arg21) (lane 3), GM-CSF(Ala20) (lane 4), GM-CSF(Ala21) (lane 5), GM-CSF(Ala20, Ala21) (lane 6) and WT GM-CSF (lane 7).

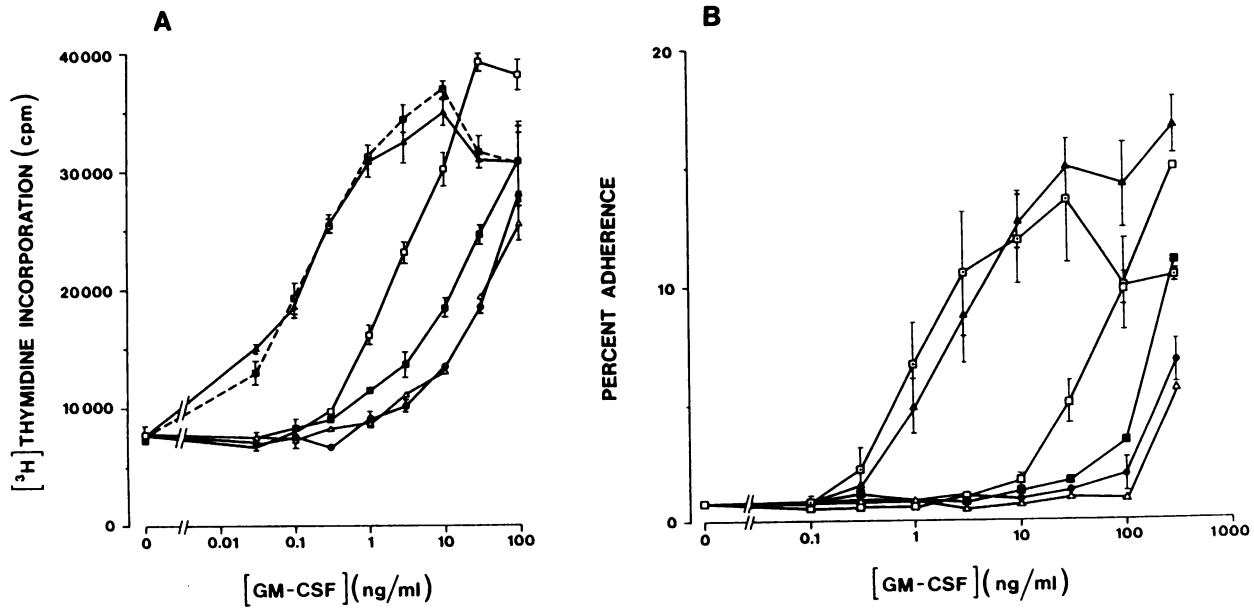


**Fig. 3.** Titration of purified GM-CSF proteins, WT GM-CSF (□), GM-CSF(Ala20) (▲), GM-CSF(Ala21) (□), GM-CSF(Arg21) (●), GM-CSF(Ala20 Ala21) (■) and GM-CSF(Ala20, Arg21) (△), for their ability to stimulate the [$^3$H]thymidine incorporation in chronic myeloid leukaemic cells (**A**) and to stimulate monocyte adherence (**B**). Representative experiments are shown with the bars spanning the SEM.

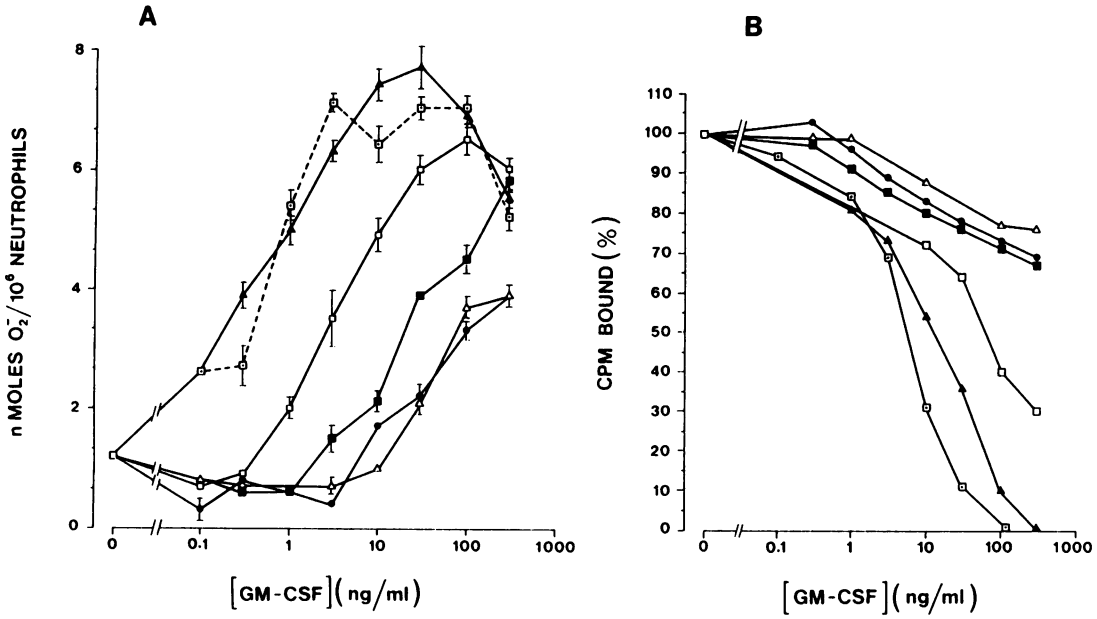


**Fig. 4.** Comparison of purified GM-CSF mutants for their ability to stimulate neutrophil superoxide production (**A**), and to compete for the binding of yeast-derived $^{125}$I-labelled GM-CSF to the high affinity GM-CSF receptors of neutrophils (**B**). The GM-CSF mutants used were as described for Figure 3. Representative experiments are shown with the bars spanning the SEM.

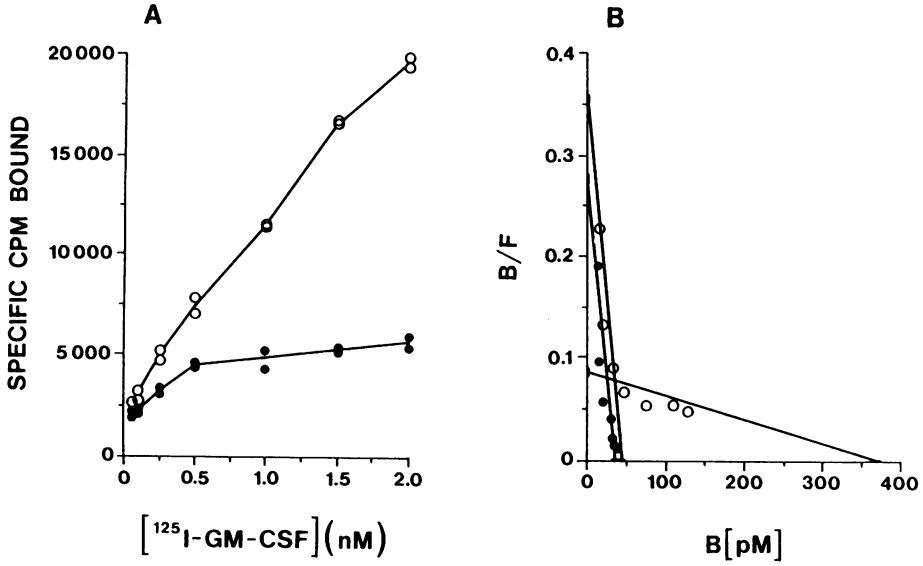


**Fig. 5.** Competition of yeast-derived [$^{125}$I]GM-CSF binding to monocytes by GM-CSF(Arg21). (**A**) Binding curve with different concentrations of [$^{125}$I]GM-CSF in the presence of 100-fold excess GM-CSF (○) or 35-fold excess purified GM-CSF(Arg21) (●). Specific counts for each duplicate determination are shown. (**B**) Scatchard analyses of the experiment in (A) showing the competition in the presence of GM-CSF (○) or GM-CSF(Arg21) (●). The mean values are plotted. Incubation was for 16 h at 4°C.

### *Differential binding of GM-CSF(Arg21) to high and low affinity GM-CSF receptors*

Although the experiments described above showed that Glu21 was important for several biological activities of GM-CSF and for high affinity binding, they did not distinguish whether binding to the low affinity receptor was also affected and as such could not discriminate which type of receptor was responsible for function. To address this question we selected the GM-CSF(Arg21) mutant which had been shown to be one of the weakest GM-CSF analogues. Purified GM-CSF(Arg21) was tested for its ability to inhibit GM-CSF binding to high and low affinity receptors by using human monocytes which express both types of receptor (Elliott *et al.*, 1989). A binding curve using increasing concentrations of [$^{125}$I]GM-CSF was performed in the presence of 35-fold excess purified GM-CSF(Arg21). This experiment revealed that GM-CSF(Arg21) slightly inhibited the binding of low concentrations of [$^{125}$I]GM-CSF (high affinity binding) and strongly inhibited the binding of high concentrations of [$^{125}$I]GM-CSF (low affinity binding) (Figure 5A). The percentage levels of inhibition by GM-CSF(Arg21) for each concentration of [$^{125}$I]GM-CSF were 13.7% (50 pM), 26.2% (100 pM), 35.6% (250 pM), 37.2% (500 pM), 58.4% (1 nM), 68.7% (1.5 nM) and 71.2% (2 nM). Scatchard transformation of these data showed that GM-CSF(Arg21) eliminated GM-CSF low affinity binding

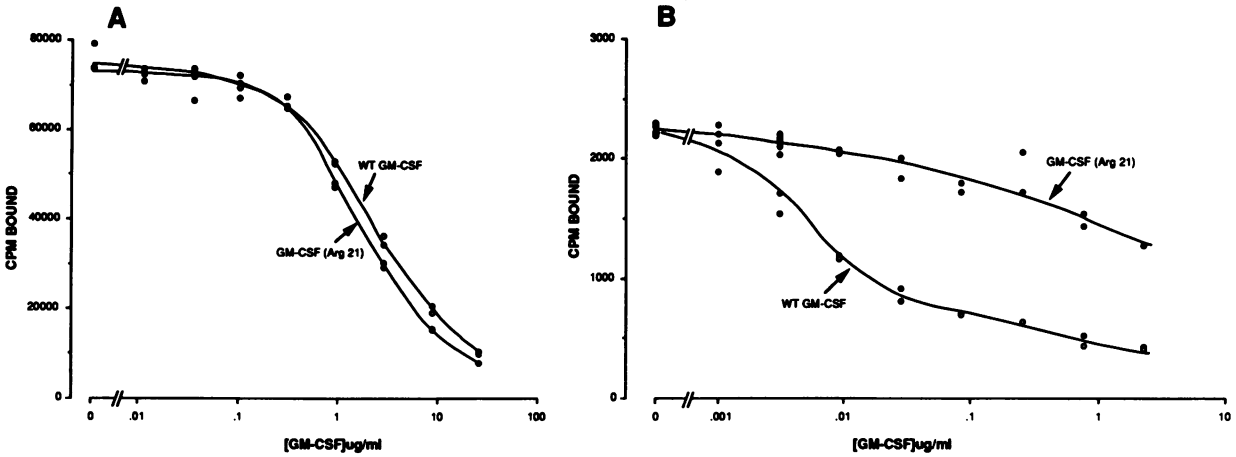


**Fig. 6.** Competition of *E.coli*-derived [$^{125}$I]GM-CSF binding to (**A**) COS cells transfected with the low affinity α-chain of the human GM-CSF receptor, and to (**B**) purified human neutrophils by increasing concentrations of (○) WT GM-CSF or (●) GM-CSF(Arg21). Binding was for 3 h at 4°C to (A) 1 × $10^6$ transfected COS cells with 6 nM [$^{125}$I]GM-CSF or (B) 1.5 × $10^6$ neutrophils with 2 nM [$^{125}$I]GM-CSF.

while slightly decreasing the number of high affinity sites (Figure 5B).

To confirm the ability of GM-CSF(Arg21) to distinguish between high and low affinity receptors experiments were carried out using COS cells transfected with the GM-CSF low affinity α-chain of the GM-CSF receptor (Gearing *et al.*, 1989) and neutrophils as controls. The results showed that the binding of GM-CSF(Arg21) to low affinity GM-CSF receptors was indistinguishable from that of wild-type GM-CSF (Figure 6A) and that GM-CSF(Arg21) was 300-fold less potent than wild-type GM-CSF at competing for neutrophil high affinity receptors (Figure 6B).

## Discussion

We have performed a structure−function analysis of human GM-CSF by using site-directed mutagenesis and multiple biological and binding assays. Our results show that residue 21 of GM-CSF is important for GM-CSF function including proliferation, differentiation and mature cell function. We also report that residue 21 is implicated in binding to high affinity as opposed to low affinity GM-CSF receptors thus linking high affinity binding with the multiple functions examined. Significantly, substitution of Glu21 with Arg greatly decreased binding to high affinity receptors but retained the capacity to bind to low affinity receptors strongly suggesting that residue 21 is involved in binding to the β-chain of the GM-CSF receptor.

Residue Gln20 appeared not to be critical in that GM-CSF(Ala20) exhibited the same biological activity as the native GM-CSF. This is in agreement with other experiments in which a mouse−human hybrid GM-CSF molecule containing a mouse sequence between amino acids 1−20 was found to be fully active (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), thus showing that a change from Gln20 to the mouse equivalent Lys20 does not alter GM-CSF activity. However, the finding that GM-CSF(Ala20) had a slightly impaired capacity to compete for high affinity binding (Figure 4B) and the fact that GM-CSF(Ala20, Ala21) was less active than either GM-CSF(Ala20) or GM-CSF(Ala21) suggest some contribution by Gln20 to the activities of GM-CSF.

In contrast to Gln20, substitution of Glu21 by amino acids of different hydrophilicity and polarity significantly affected the potency of GM-CSF. Biological analysis of these mutants showed that the substitution of an acidic (Glu) for another acidic (Asp) residue caused the least change of activity while substitution by basic residues (Arg, Lys) caused the biggest reduction in GM-CSF activities. The other substitutions, either hydrophilic uncharged or hydrophobic, exhibited intermediate potency.

These series of substitutions suggest that Glu at position 21 is critical for GM-CSF activity and its acidic nature is important for binding to the high affinity GM-CSF receptor or in maintaining an appropriate tertiary configuration. In view of these results it is tempting to speculate that Glu21 is part of a GM-CSF binding site or close to residues involved in it, a notion supported by predictive conformational studies suggesting that Glu21 lies in the external face of the first α-helix (Parry *et al.*, 1991). However, despite the fact that single point mutations can be more revealing than antibody molecules (which are six times the size of GM-CSF) or deletion mutants, the possibility cannot be ruled out that the mutations we made have induced a conformational change in GM-CSF. Nevertheless the fact that GM-CSF(Arg21) was equipotent to WT GM-CSF in competing for low affinity binding would argue against the latter possibility.

An important finding with these GM-CSF mutants was the demonstration that all the activities of GM-CSF tested were affected. Examination of GM-CSF mutant-mediated stimulation of cell proliferation showed the same hierarchy as for stimulation of neutrophil and monocyte function. In competition binding experiments the order in which these mutants inhibited the binding of [$^{125}$I]GM-CSF to the high affinity receptor of neutrophils paralleled their bioactivity.

Whilst these studies focused on the 14−24 region of GM-CSF there seem to be other regions in the GM-CSF molecule also important for activity. The fact that a chemically synthesized GM-CSF peptide spanning residues 1−53 was not sufficient for activity (Clark-Lewis *et al.*, 1988) suggests a second region of importance in the C-terminus. This region appears to extend between residues 77 and 94 as shown with human−mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), and inhibitory monoclonal antibodies that recognize this area (Brown *et al.*, 1990; Nice *et al.*, 1990). In addition the involvement of regions

40−77 and 110−127 has also been suggested based on inhibitory studies using monoclonal antibodies (Nice *et al.*, 1990).

Although mainly two regions of GM-CSF, encompassing residues 14−24 and 77−94, appear to be involved in binding and bioactivity they both need to be preserved for full activity. Neither the peptides 1−53 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990), 54−127 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990) nor 86−93 (Nice *et al.*, 1990) expressed agonistic or antagonistic activity when used separately. These results suggest that either both regions are adjacent in the tertiary structure, forming a single binding site, or that they bind separately to distinct binding sites of the GM-CSF receptor. Our results with GM-CSF(Arg21) showing impairment of binding to high but not low affinity receptors (Figures 5 and 6) argues for the presence of two binding sites and suggests that Glu21 is involved in binding to the β-chain (or a third member of the GM-CSF−receptor complex) but not to the α-chain (Figure 7).

We note that GM-CSF belongs to a family of related cytokines with similar predicted tertiary configuration (Parry *et al.*, 1988) and whose receptors also belong to a common family (Gearing *et al.*, 1989; Bazan 1990). Our model with GM-CSF (Figure 7) may apply to other molecule members of this family, in particular IL-3 and IL-5, both of which have a glutamic acid at positions 22 and 10 respectively which are predicted to lie in the external face of the first α-helix (Parry *et al.*, 1991). Appropriate changes in this region may alter the binding and function of these molecules in a similar way, particularly as the β-chain of the GM-CSF receptor appears to be part of the high affinity IL-3 and IL-5 receptors (Lopez *et al.*, 1990, 1991; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991).

An important implication of the differential binding to high and low affinity GM-CSF receptors by GM-CSF(Arg21) is that such a mutant, despite having greatly reduced potency at stimulating high affinity receptor-mediated functions, would be expected to fully occupy and activate low affinity receptor-mediated functions. The low affinity receptor may mediate proliferation signals in some cases as suggested by the relatively high concentrations of GM-CSF required for stimulation of [$^3$H]thymidine incorporation in monocytes (Elliott *et al.*, 1989) and in particular by the ability of nM but not pM concentrations of human GM-CSF to stimulate the proliferation of mouse FDCP1 cells transfected with the low affinity human GM-CSF receptor α-chain (Metcalf *et al.*, 1990). However, in a similar system but which used instead transfected CTLL cells, the α-chain alone was insufficient to allow proliferation (Kitamura *et al.*, 1991a) and evidence has been presented that interaction with the β-chain across species is required for signalling even though high affinity binding is not observed.

The latter experiment emphasizes a potential therapeutic use for molecules similar to GM-CSF(Arg21), namely their capacity to act as antagonists in situations where the low affinity α-chain provides binding but is incapable of generating cellular signals. Since binding to the α-chain as well as the β-chain is required for high affinity binding and signalling, GM-CSF(Arg21) may pave the way for the engineering of mutants that have totally lost their ability to interact with the high affinity receptor but which by retaining their ability to bind to the α-chain can antagonize the effect of native GM-CSF. Such antagonists, either as monomers or dimers, could have useful clinical applications in situations where the presence of GM-CSF can exacerbate inflammation (Koyanagi *et al.*, 1988) or lead to tumour cell growth (Baldwin *et al.*, 1989; Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

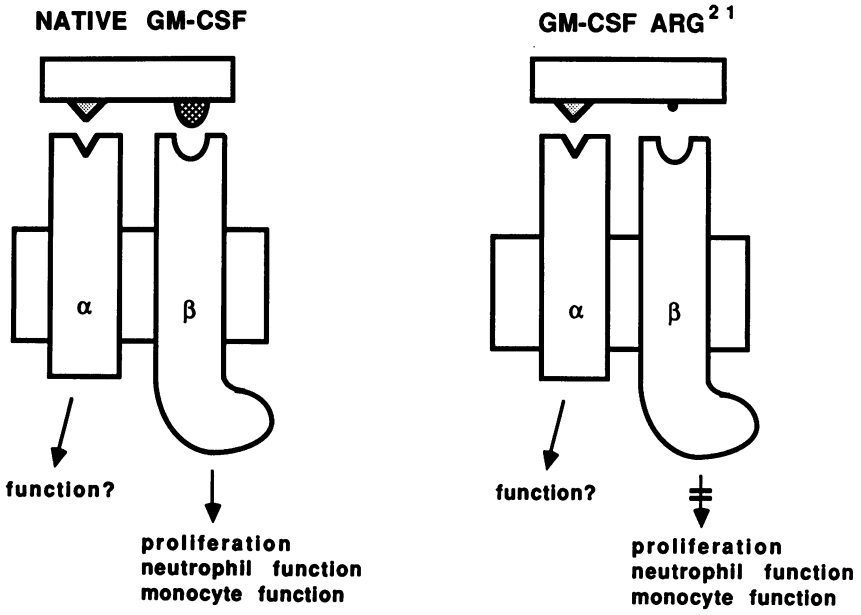


**Fig. 7.** Proposed model of GM-CSF interaction with the α- and β-chains of the GM-CSF receptor to accommodate the biological and binding data. On the left, binding to both the α- and β-chain of the GM-CSF receptor is shown. The triangular symbol in GM-CSF signifies a distinct structure (probably contributed to by helix D and the loop joining helices C and D) binding the α-chain, and the semi-circular structure, the critical contribution of Glu21 to the β-chain binding. The complex gives high affinity binding and full function. On the right, GM-CSF(Arg21) mutant is shown with an intact interaction with the α-chain (thus giving low affinity binding) but impaired interaction with the β-chain. The possibility that the α-chain by itself is able to stimulate some function in some cells is also indicated on this diagram.

## Materials and methods

### Site-directed mutagenesis and expression vector constructs

Site-directed mutagenesis of a human GM-CSF cDNA clone kindly provided by Dr S.Clark (Genetics Institute, Cambridge, MA), was performed in phage M13 as described (Zoller and Smith, 1984) with mutant plaques being screened by the 3 M tetramethyl ammonium chloride procedure (Wood *et al.*, 1985). The presence of the correct mutation was confirmed by chain termination sequencing (Sanger *et al.*, 1977) using a Sequenase Kit (United States Biochemical, Cleveland, OH). The mutant GM-CSF cDNAs were then excised from the M13 clones RF DNA and subcloned into the transient mammalian expression vector pJL4 (Gough *et al.*, 1985). Several mutant GM-CSF cDNAs were subsequently subcloned from the pJGM constructs into the neomycin selectable, mammalian expression vector pRSVN.07 (gift from Dr A.Robbins, Department of Biochemistry, University of Adelaide). All plasmid constructs were sequenced at the site of the mutation prior to transfection (Chen and Seeburg, 1985).

### Transfection of GM-CSF and its analogues

*Transient transfections in COS cells.* COS cells were grown to 50–70% confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 20 mM HEPES, penicillin, gentamicin and supplemented with 10% fetal calf serum (FCS). DNA constructs were introduced into COS cells by electroporation using a Bio-Rad Gene Pulser (Chu *et al.*, 1987). For each transfection 20 μg of DNA, 25 μg sonicated salmon sperm DNA and 50 μl FCS were mixed with $5 \times 10^6$ COS cells in 0.5 ml of 20 mM HEPES-buffered saline containing 6 mM glucose. After a 24 h incubation the medium was replaced with FCS-free DMEM and incubated for a further 72 h before the conditioned medium was harvested and assayed for GM-CSF protein.

*Permanent transfections in CHO cells.* CHO cells were grown to ~80% confluence in Hams F12 nutrient mixture containing penicillin, gentamicin and supplemented with 10% FCS. DNA constructs were introduced into CHO cells by electroporation after mixing 10 μg of pRSVNGM plasmid DNA with $5 \times 10^4$ CHO cells. 24–48 h after transfection selective F12 media containing geneticin (Gibco Laboratories) was added to the cells. Maximally expressing individual geneticin-resistant CHO colonies were selected and used to produce GM-CSF or mutant analogues.

### Visualization of mutant GM-CSF protein

GM-CSF-containing COS cell supernatants and purified GM-CSF protein was size-fractionated by SDS–12.5% PAGE (Laemmli, 1970). For Western blot analysis, protein was transferred to nitrocellulose as described (Towbin *et al.*, 1979). Filters were probed with a sheep anti-GM-CSF (gift from Dr S.Clark, Genetics Institute, Cambridge, MA) followed by a second layer of biotinylated rabbit anti-sheep IgG. After a further incubation with an avidin-biotinylated horseradish peroxidase conjugate, the complex was visualized using a diaminobenzidine substrate solution. For silver staining, the method of Morrissey (1981) was used.

### Quantitation and purification of GM-CSF protein

The amount of GM-CSF protein present in COS cell supernatants was quantitated by a radioimmunoassay (RIA). Some mutants were selected for purification and quantitation by amino acid analysis.

*RIA.* A competitive RIA was developed using $^{125}$I-labelled human GM-CSF and a polyclonal sheep anti-GM-CSF serum (gift from Dr S.Clark, Genetics Institute, Cambridge, MA). GM-CSF modified by the addition of an extra tyrosine in the N-terminus (gift from Dr L.S.Park, Immunex Corp., Seattle, WA) was labelled as described below. COS cell supernatants (50 μl) were incubated with sheep anti-GM-CSF serum (50 μl of 1:40,000 dilution). After 4 h incubation at 4°C, 0.1 ng of [$^{125}$I]GM-CSF was added for a further 16 h before adding 100 μl of reconstituted anti-sheep Immunobead reagent (Bio-Rad Laboratories, Richmond, CA) for 4 h. The mixtures were then washed twice with phosphate-buffered saline (PBS), the pellet resuspended in 200 μl of PBS and transferred to 3DT tubes for counting in a gamma-counter (Packard Instrument Company, Meriden, CT). The amount of GM-CSF protein was calculated from a standard curve constructed with known amounts of GM-CSF.

*Protein purification and amino acid analysis.* GM-CSF protein in the supernatants of CHO cell lines permanently transfected with selected GM-CSF mutant cDNA was purified using an affinity column containing the monoclonal antibody LMM111 attached to Sepharose beads (Cebon *et al.*, 1988). Further purification was achieved by reversed phase HPLC and the resulting GM-CSF protein quantitated by amino acid analysis as described (Cebon *et al.*, 1990). This procedure also calculated the GM-CSF mutant preparations to be >99% pure.

### Stimulation of haemopoietic cell proliferation

Two types of assay were performed.

*Colony assay.* This assay measured the clonal proliferation and differentiation of bone marrow progenitor cells in semi-solid agar and was carried out as described (Lopez *et al.*, 1988).

*Proliferation of chronic myeloid leukaemic (CML) cells.* Primary CML cells from one patient were selected for their ability to incorporate [$^3$H]thymidine in response to GM-CSF. This assay was performed as described (Lopez *et al.*, 1988).

### Functional activation of human granulocytes and monocytes

*Antibody-dependent cell-mediated cytotoxicity (ADCC) assay.* Neutrophils and eosinophils were purified on Metrizamide (Nyegaard, Oslo) and tested against antibody-coated, $^{51}$Cr-labelled P815 cells coupled with trinitrophenyl as previously described (Vadas *et al.*, 1983).

*Superoxide anion production assay.* This was carried out as described previously (Lopez *et al.*, 1986).

*Monocyte adherence.* Monocytes were purified from the peripheral blood of normal donors obtained from the Adelaide Red Cross Transfusion Service, as previously described (Elliott *et al.*, 1990). Stimulation of monocyte adhesion by GM-CSF was measured by an isotopic method essentially as described (Elliott *et al.*, 1990). In each assay the concentration of GM-CSF protein giving 50% maximal response was determined and the relative potency calculated by dividing the concentration of WT GM-CSF giving 50% stimulation by the concentration of mutant GM-CSF protein giving 50% stimulation and multiplying by 100. In some experiments the geometric mean from several experiments was calculated and statistical significance between values from different mutants determined by a paired *t* test.

### Radioreceptor assay

*Radioiodination of GM-CSF.* Yeast-derived human GM-CSF (gift from Dr L.Park, Immunex Corporation, Seattle, WA), or *Escherichia coli*-derived human GM-CSF was radioiodinated by the ICl method (Contreras *et al.*, 1983). Iodinated protein was separated from free $^{125}$I by chromatography on a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS containing 0.02% Tween 20, and stored at 4°C for up to 4 weeks. Before use, the iodinated protein was purified from Tween and non-protein-associated radioactivity by cation exchange chromatography on a 0.3 ml CM-Sepharose CL-6B column (Pharmacia) and stored at 4°C for up to 5 days. The radiolabelled GM-CSF retained >90% biological activity as judged from titration curves using non-iodinated GM-CSF as controls.

*Competition binding assays.* Competition for binding to high affinity receptors used freshly purified neutrophils which express only this type of receptor (Gasson *et al.*, 1986). The cells were suspended in binding medium consisting of RPMI 1640 supplemented with 20 mmol/l HEPES, 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Typically, equal volumes (50 μl) of $4 \times 10^6$ neutrophils, 70 pM iodinated GM-CSF, and different concentrations of GM-CSF and GM-CSF analogues were mixed in siliconized glass tubes for 3 h at 4°C. Competition for binding to high and low affinity GM-CSF receptors used human monocytes which express both types of receptor (Elliott *et al.*, 1989). Equal volumes (50 μl) of cells ($2 \times 10^6$), iodinated GM-CSF at different concentrations and 100-fold excess wild-type GM-CSF to establish non-specific binding, or 35-fold excess GM-CSF(Arg21) were mixed together for 16 h at 4°C before centrifugation of the cells over a cushion of FCS as above. Specific counts were determined by first subtracting the counts obtained in the presence of excess wild-type GM-CSF. Competition for binding to the low affinity GM-CSF receptor was performed using COS cells transiently transfected with a GM-CSF receptor cDNA clone as described (Gearing *et al.*, 1989). To $10^6$ COS cells 6 nM of *E.coli*-derived [$^{125}$I]GM-CSF were added in the presence of different concentrations of wild-type GM-CSF or GM-CSF(Arg21) for 3 h at 4°C. In each case cell suspensions were overlaid on 0.2 ml FCS at 4°C, centrifuged in a Beckman Microfuge 12, and the tip of each tube containing the visible cell pellet cut off and counted in a gamma counter. In the case of monocytes the results are expressed in the form of equilibrium binding data and Scatchard transformation of these data as described (Scatchard, 1949).

## Acknowledgments

We would like to thank Mrs Mari Walker and Barbara Meakins for excellent secretarial assistance, S.Beltrame, J.Eglinton, J.Halsall and P.Dyson for

excellent technical assistance, and Drs Jonathan Cebon and Richard Simpson for help with the purification and quantitation of GM-CSF mutants. This work was supported by grants from NH and MRC of Australia and by NIH grants CA 45822 and CA 22556.

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*Received on September 26, 1991; revised on December 16, 1991*

## Note added in proof

Shanafelt *et al.* [*EMBO J.*, **10**, 4105–4112 (1991)] have recently shown that the amino terminal helix of GM-CSF governs high affinity binding.