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The amino-terminal helix of GM-CSF and IL-5 governs high affinity binding to their receptors

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Transduction of the biological effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5) requires the interaction of each cytokine with at least two cell surface receptor components, one of which is shared between these two cytokines. A strategy is presented that allowed us to identify receptor binding determinants in GM-CSF and IL-5. Mixed species (human and mouse) receptors were used to locate unique receptor binding domains on a series of human-mouse hybrid GM-CSF and IL-5 cytokines. Results show that the interaction of these two cytokines with the shared subunit of their high affinity receptor complexes is governed by a very small part of their peptide chains. The presence of a few key residues in the amino-terminal α-helix of each ligand is sufficient to confer specificity to the interaction. Comparison with other cytokines suggests that the amino-terminal helix of many of these proteins may contain the recognition element for the formation of high affinity binding sites with the α subunit of their multi-component receptors.
Key words: cytokines/mutagenesis/receptors/structure-function

Introduction

The biological effects of cytokines on cell growth and differentiation are mediated by specific cell surface molecules. These molecules transduce the binding of their cognate cytokines into cytoplasmic signals that eventually trigger a cascade of intracellular responses. GM-CSF is a cytokine that stimulates the development of various lineages of hemopoietic cells (Gough and Nicola, 1989; Metcalf, 1986). Hayashida *et al.* (1990) have shown that its functional receptor is composed of at least two subunits, GM-CSF receptor α (GM-Rα) and GM-CSF receptor β (GM-Rβ) (Chiba *et al.*, 1990; Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a). The α subunit binds GM-CSF with low affinity, whereas the β subunit does not measurably bind GM-CSF. Coexpression of GM-Rα and -Rβ leads to high affinity binding of GM-CSF and is required for signal transduction (Hayashida *et al.*, 1990). Recent evidence indicates that the GM-Rβ subunit is shared with the receptor systems of other cytokines (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a). High affinity binding of human interleukin-3 (IL-3), IL-5 and GM-CSF appears to require the common GM-Rβ subunit in combination with a receptor subunit that is unique for each of these cytokines (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Lopez *et al.*, 1990). In mouse, GM-CSF and IL-5 receptors share a common β subunit (AIC2B) (Devos *et al.*, 1991; Gorman *et al.*, 1990; Itoh *et al.*, 1990; Kitamura *et al.*, 1991a), while IL-3 incorporates the homologous AIC2A molecule in its high affinity receptor complex (Schreurs *et al.*, 1991). These findings provide an explanation for earlier observations indicating that these cytokines can compete with one another, either completely or partially (Budel *et al.*, 1990; Lopez *et al.*, 1990; Park *et al.*, 1989a,b).

Binding of GM-CSF to its functional receptor is followed by transduction of a signal to unknown cytoplasmic messengers. To understand the mechanism of signal transduction in a receptor system of such complexity, it is essential to identify the molecular nature of the cytokine-receptor interaction. Human and mouse GM-CSF are species specific; we have used this property to search for unique receptor binding domains in this protein hormone. To approach this problem, human-mouse GM-CSF hybrid proteins were analyzed in combination with mixed species human and mouse GM-CSF receptors. High affinity association (and a concomitant biological response) of mixed species α and β GM-CSF receptor chains is only expected to occur when hybrid GM-CSF cytokines present the correct species-specific receptor binding domains. This approach allowed us to identify the residues of the cytokine that interact with the β subunit of the receptor. These amino acids lie in the amino-terminal α-helix of GM-CSF.

Using a mouse(m)IL-5-human(h)GM-CSF hybrid we also show that mIL-5 interacts with GM-Rβ through residues in its amino-terminal α-helix. Comparative analysis of other receptor-cytokine pairs leads us to propose that the amino-terminal α-helix of many cytokines is the segment that is recognized by a component of their functional receptors.

Results

Mixed species receptors

The mGM-CSF dependent myeloid cell line NFS60 was stably transfected with a plasmid encoding the α subunit of the human GM-CSF receptor, and was named 3E6. Whereas NFS60 does not proliferate in response to hGM-CSF, 3E6 responds weakly to hGM-CSF. This presumably occurs through an interaction of the hGM-CSF-hGM-Rα complex with the mGM-Rβ component (Kitamura *et al.*, 1991a). However, no high affinity binding sites for hGM-CSF were observed on this cell line (data not shown). We had previously constructed a collection of human-mouse GM-CSF hybrid proteins (Shanafelt *et al.*, 1991). A comparison of the human and mouse GM-CSF amino acid sequences and a pictorial representation of the hybrid proteins are displayed in Figure 1. The responses of NFS60 and 3E6 to the series of amino-terminal mouse-carboxy-terminal human GM-CSF hybrids is shown in Figure 2a. The

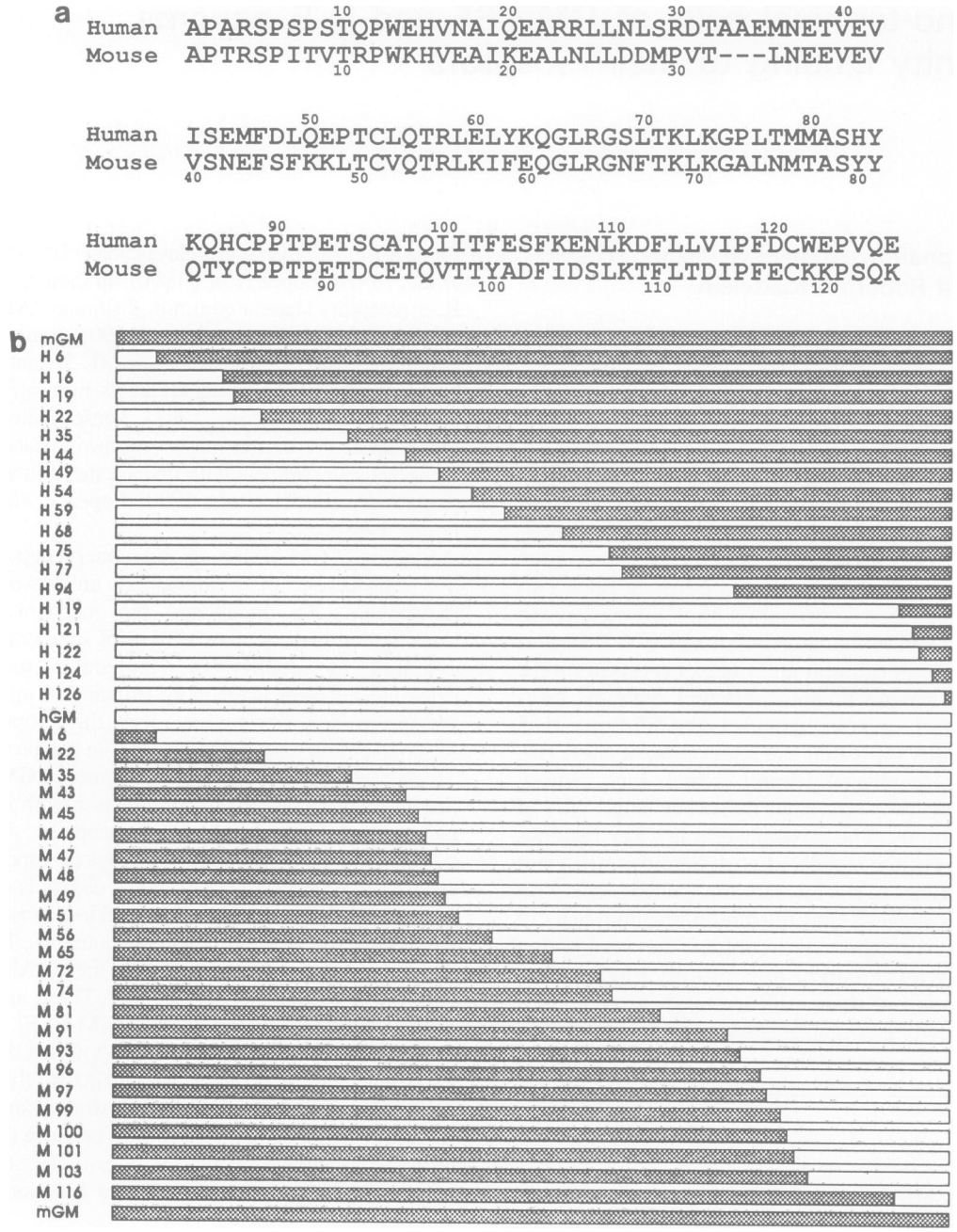

Fig. 1. Hybrid GM-CSF proteins. **a**, Amino acid seqences of human and mouse GM-CSF. Sequences are aligned for optimal homology. **b**, Pictorial representation of the hybrid GM-CSF proteins. Open bars correspond to hGM-CSF sequences, and shaded to mGM-CSF. The naming of the hybrid proteins follows the convention that either an M (mouse) or H (human) designates the species of origin of the N-terminal portion of the hybrid, where the number following indicates the amino acid at which the two polypeptide segments are fused.

carboxy-terminal 22 amino acids of mGM-CSF could be replaced with the corresponding human residues without affecting biological activity (Shanafelt *et al.*, 1991). Both 3E6 and NFS60 fully respond when activated with the hybrids M103 and M116, suggesting that these hybrids can bind with high affinity to mGM-Rα and mGM-Rβ. Hybrid ligands M56 to M101 show a reduced activity with both 3E6 and NFS60. We have no explanation for this reduction. However, most importantly, both NFS60 and 3E6 have an equivalent response to these hybrids. With hybrids M49 to M22 the responses of 3E6 and NFS60 are different. Whereas there is a minimal response of these hybrids on NFS60 cells, there is full biological activity to hybrids M49−M22 on 3E6 cells. Since the only difference between NSF60 and 3E6 is the presence of hGM-Rα, these hybrids seem to be interacting with this receptor in combination with the mGM-Rβ receptor to form high affinity binding sites capable of transducing the biological response. This is most clearly demonstrated with hybrid M22: M22 shows full biological activity on 3E6, yet it is completely inactive on NSF60. Apparently, the presence of only 22 N-terminal amino acids of mGM-CSF on hGM-CSF is sufficient to ensure high affinity binding. Replacement of additional mouse residues in hybrid M6 eliminates this ability; its activity is identical to hGM-CSF. This series of experiments identifies amino acid residues 7−22 as the region in mGM-CSF responsible for specific interaction with mGM-Rβ.

To examine the effect of these hybrids in the absence of

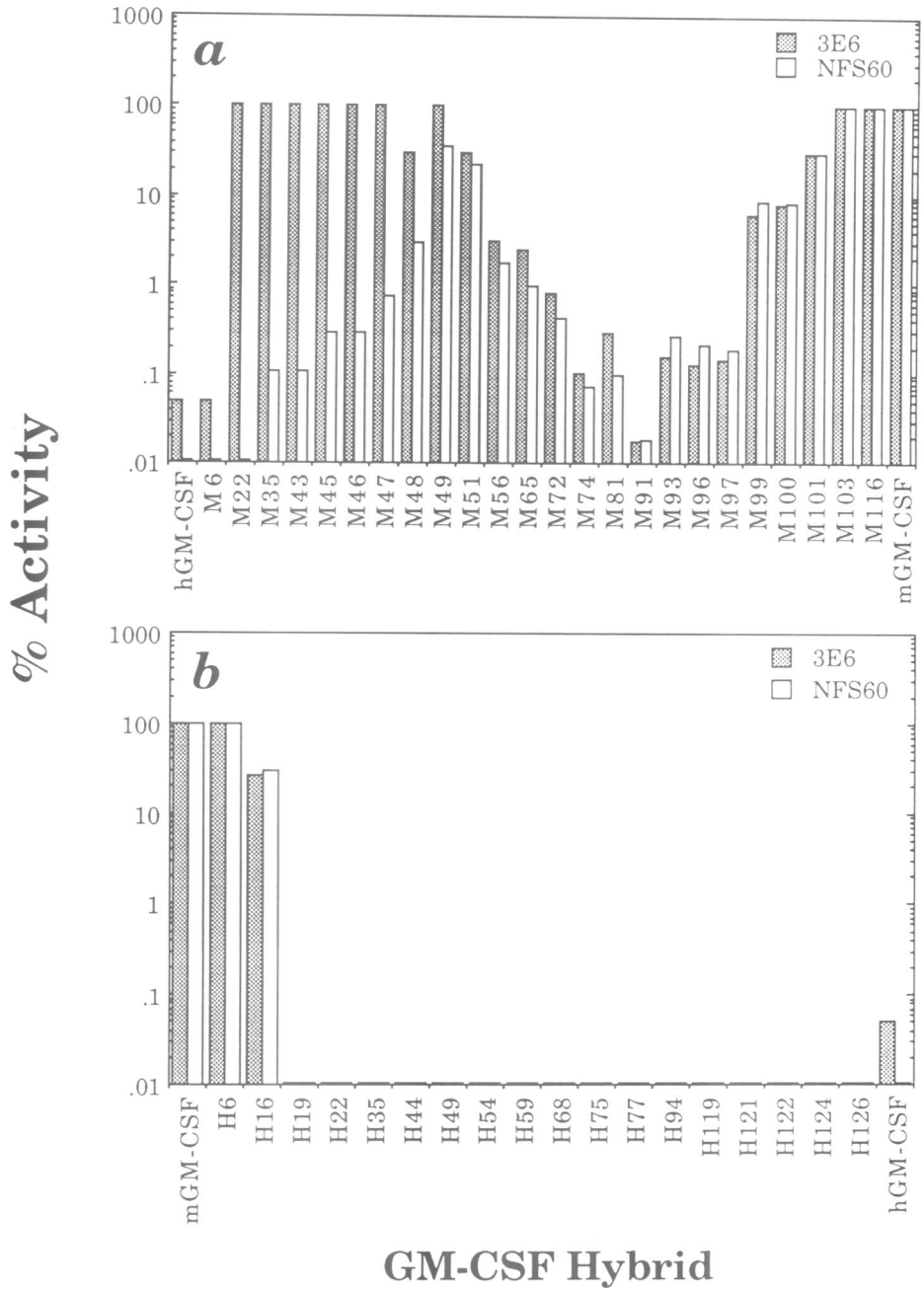

Fig. 2. Relative activity of GM-CSF hybrid proteins on NFS60 and 3E6 cells (NFS60 stably transfected with hGM-Rα). Results are expressed as % activity relative to that of wild-type mGM-CSF (see Materials and methods); hybrid proteins exhibiting >100% activity (M22, M35 and M43 to M47 on 3E6) are shown with activity equal to 100%. These particular hybrid proteins probably show greater than wild-type activity because of the higher expression of hGM-Rα compared with mGM-Rα on 3E6 (~10-fold). All active hybrid proteins (except M6, which appeared identical to hGM-CSF) had a maximal plateau response equivalent to mGM-CSF on 3E6). Open bars represent the response of NFS60 and shaded bars the response of 3E6. **a**, Response to amino-terminal mouse−carboxy-terminal human GM-CSF hybrids (M series). **b**, Response to amino-terminal human−carboxy-terminal mouse GM-CSF hybrids (H series).

mGM-Rα, we used an IL-2 dependent mouse T cell line that was stably transfected with hGM-Rα and mGM-Rβ [CTLL(hα−mβ)] (Kitamura *et al.*, 1991a). This cell line does not express any endogenous GM-CSF receptor subunits. Its response to the hybrids was identical to that of 3E6 with two exceptions: mGM-CSF, as expected, is inactive on the CTLL line, since no mGM-Rα is present; for the same reason, hybrids M103 and M116 which were active on 3E6, are now inactive (data not shown).

We have also described the response of NFS60 to a collection of amino-terminal human−carboxy-terminal mouse GM-CSF hybrids (H series) (Shanafelt *et al.*, 1991). Figure 2b shows the response of this series on 3E6 as compared with NFS60. Only two hybrids show activity, H6 (100%) and H16 (~30%). All other hybrids were inactive (<0.01%). Having determined the region of interaction of mGM-CSF with mGM-Rβ as residues 7−22, this result can now be understood. Only hybrids H6 and H16 still have an mGM-Rβ binding domain. This narrows down the number of residues of mGM-CSF critical for this interaction to amino acids 17−22. This region coincides precisely with that determined by scanning deletion analysis to be critical for biological activity (Shanafelt and Kastelein, 1989). Structurally this region is predicted to be part of an amino-

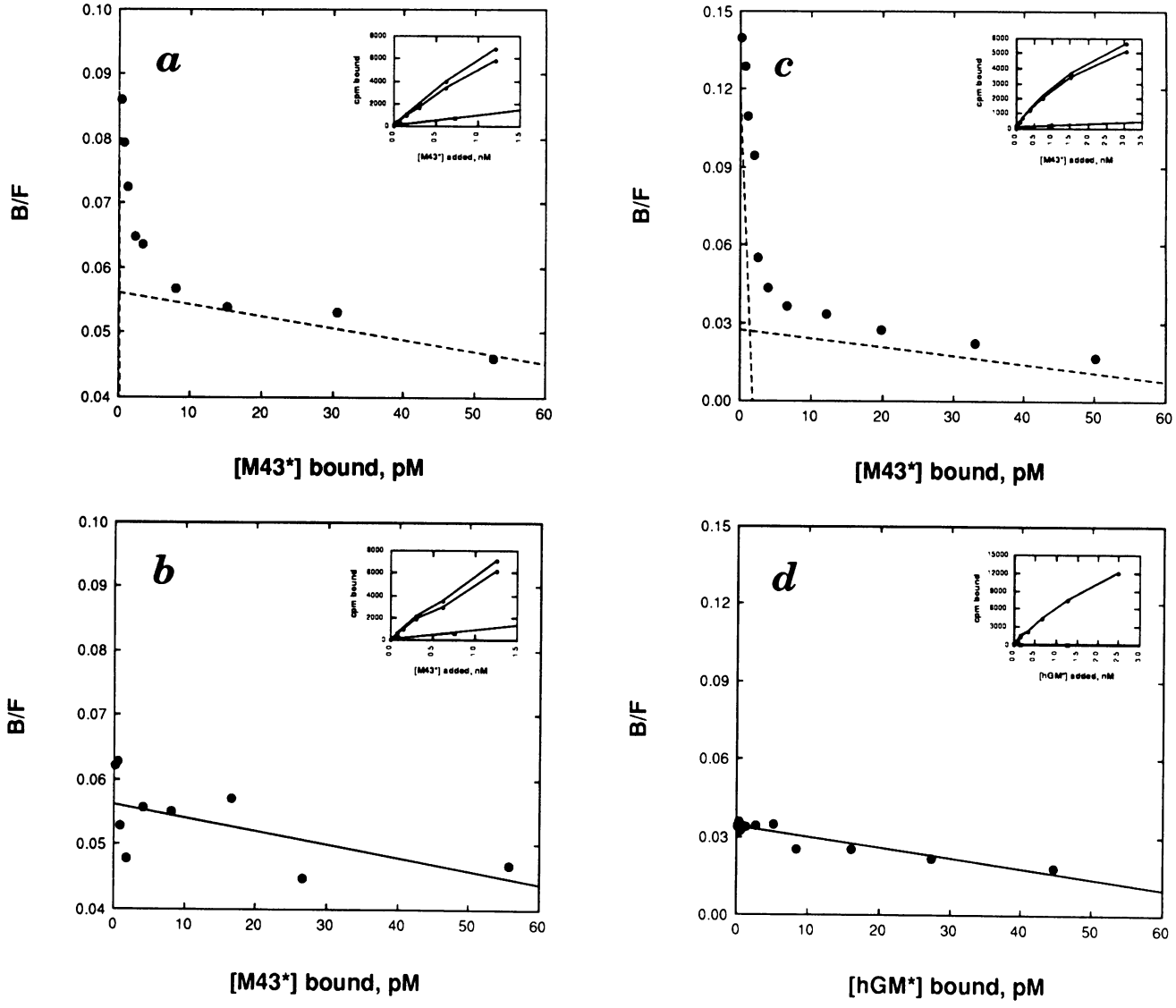

Fig. 3. Scatchard analysis of M43 and hGM-CSF binding to 3E6 and CTLL(hα−mβ) cells. Solid lines (single binding site) and dashed lines (two binding sites) represent best fit values obtained from the LIGAND program (Munson, 1983). Each point represents the mean of at least three sets of data. Insets display the equilibrium binding isotherm (total bound, specifically bound, and non-specifically bound [^{125}I]M43 or [^{125}I]hGM) for the respective Scatchard plot. **a**, [^{125}I]M43 binding to 3E6; **b**, [^{125}I]M43 + 500 nM unlabeled mGM-CSF binding to 3E6; **c**, [^{125}I]M43 binding to CTLL(hα−mβ); **d**, [^{125}I]hGM binding to CTLL(hα−mβ).

terminal α-helix stretching from residue 15 to 27 (Parry *et al.*, 1988). Our evidence suggests that this region functions as an α-helix, since proline substitutions destroy biological activity (Altmann *et al.*, 1991; A.B.Shanafelt and R.A.Kastelein, unpublished data).

Characterization of binding of M43

The biological response elicited by the active hybrids is most probably due to the formation of high affinity GM-CSF receptor complexes. To show the presence of high affinity sites, one of the fully active hybrids, M43, was purified, radio-labeled with ^{125}I, and used in binding experiments on 3E6 and CTLL(hα−mβ) (Figure 3). 3E6 expresses two classes of receptor for this ligand; these classes have differing affinities (Figure 3a; Table I). As expected, the M43 high affinity sites could be completely competed by mGM-CSF, since 3E6 still has the ability to form high affinity binding sites with mGM-Rα and -Rβ (Figure 3b). Scatchard analysis of M43 binding to CTLL(hα−mβ) also reveals high and low affinity binding sites with K_ds of 15 pM and 3.1 nM, respectively (Figure 3c; Table I). High affinity binding sites on CTLL(hα−mβ) are not seen with hGM-CSF as ligand (Figure 3d).

Table I. Equilibrium dissociation constants and binding site values for 3E6 and CTLL(hα−mβ)

Ligand	K_d[a]	Binding sites/cell[a]
3E6		
[^{125}I]M43	13 pM	59
	5.6 nM	38 000
[^{125}I]M43 + 500 nM mGM-CSF	4.8 nM	33 000
CTLL(hα−mβ)		
[^{125}I]M43	15 pM	2 200
	3.1 nM	100 000
[^{125}I]hGM-CSF	2.5 nM	100 000

[a]Mean values calculated from the LIGAND program (Munson, 1983).

Comparison of the amino acid sequences of mouse and human GM-CSF in the proposed β receptor binding region shows only two differences: Glu17 and Lys20 in mGM-CSF versus Asn17 and Gln20 in hGM-CSF (Figure 1a). Random substitution of Glu17 in mGM-CSF has only a modest effect on biological activity, indicating limited involvement of this residue in the interaction (data not shown). Strong evidence for the direct involvement of residue 20 was obtained when we replaced Gln20 in hGM-CSF by Lys, the mGM-CSF residue found at position 20. Whereas hGM-CSF elicits a minimal response with the 3E6 or the CTLL(hα−mβ) line, the hGM-CSF/Lys20 mutant has increased biological activity in both plateau and half-maximal response (Figure 4). Although this result identifies Lys20 as an important residue involved in the interaction with the mGM-Rβ, it is evident that other residues must also contribute; the hGM-CSF/Lys20 mutant is still ~30-fold lower in activity than mGM-CSF (Figure 4a).

mIL-5 interaction with mGM-Rβ

It has been suggested that in the mouse, the same β receptor subunit is present in the high affinity complexes that bind IL-5 and GM-CSF (Devos *et al.*, 1991; Kitamura *et al.*, 1991a). High affinity binding of these cytokines requires the interaction of each ligand with a unique ligand binding α subunit and the shared β subunit. We expected that, if mIL-5 forms high affinity binding sites with the mIL-5-Rα and mGM-Rβ, it would do so by using the same binding motif as mGM-CSF for binding to mGM-Rβ. We tested this possibility by generating a hybrid that consisted of amino-terminal mIL-5 and carboxy-terminal hGM-CSF residues. Specifically, we replaced amino acids 4−32 of hGM-CSF by amino acids 5−29 of mIL-5. This region of mIL-5 includes a predicted amino-terminal α-helix from residue 7 to 22 (Parry *et al.*, 1988). When this hybrid was tested on 3E6 it elicited a full biological response (Figure 4a). A similarly strong response was observed with the CTLL(hα−mβ) line (Figure 4b). When CTLL cells were used that expressed the mGM-Rβ homologue AIC2A together with hGM-Rα (Kitamura *et al.*, 1991a) instead of the mGM-Rβ subunit, no response of this hybrid was observed (data not shown). These results show that mIL-5 can use the same β receptor subunit as mGM-CSF. They also strongly suggest that receptor recognition by IL-5 follows the same principle underlying the mGM-CSF−mGM-Rβ interaction, i.e. the presentation of a few specific residues in the context of an α-helix. Sequence comparison shows that Lys20 and Glu21 in mGM-CSF are conserved in the proposed first helix of mIL-5 (Lys10 and Glu11; Table II). Moreover, the same pair of amino acids is generated in hGM-CSF when Gln20 is changed to Lys20, suggesting that besides Lys20, Glu21 may also be required for specific interaction with the mGM-Rβ subunit.

Discussion

Binding of GM-CSF to its functional receptor is a complex event that includes interaction with multiple receptor components. GM-CSF is able to bind with low affinity to the GM-Rα subunit (Gearing *et al.*, 1989). The GM-Rβ subunit does not by itself bind GM-CSF, but in conjunction with the GM-Rα subunit it forms the high affinity receptor. For a biological response to occur, GM-CSF has to interact

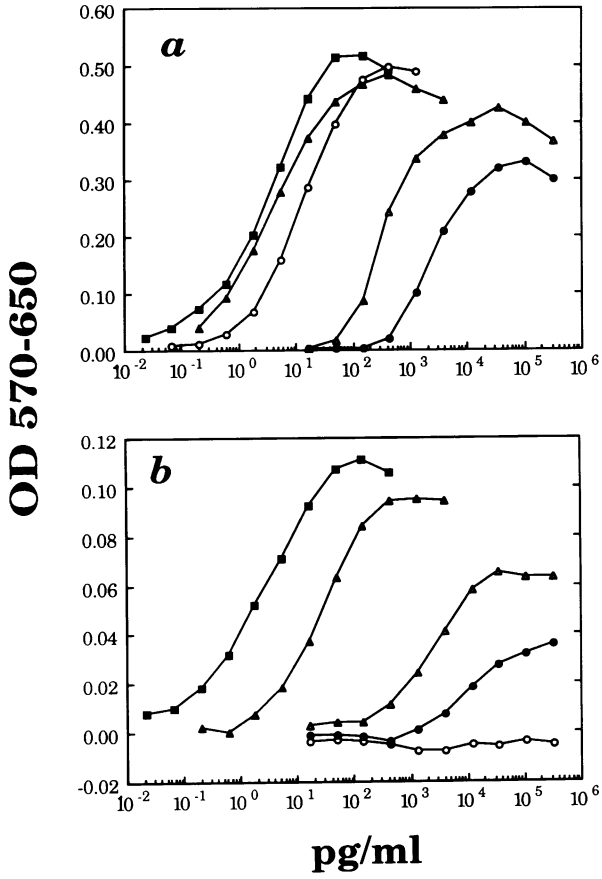

Fig. 4. Growth response of 3E6 and CTLL(hα−mβ) cell lines. **a**, response with 3E6 and **b**, response with CTLL(hα−mβ). Cells were incubated for 24 h in the presence of decreasing concentrations of mGM-CSF (○), hGM-CSF (●), M43 (■), mIL5−hGM-CSF hybrid (▲), and hGM-CSF/Lys20 (△).

with both the GM-Rα and the GM-Rβ receptor (Hayashida *et al.*, 1990). The work presented here shows that a small, discrete region in mGM-CSF is responsible for the interaction with the GM-Rβ. Structurally, this region is most likely presented to the β-receptor as an α-helix (Bazan, 1990a; Parry *et al.*, 1988) with at least Lys20 directly interacting with the receptor. Evidence for the existence of the same binding motif has recently been described for the IL-2 receptor system. Exhaustive mutational analysis of mIL-2 led to the identification of Asp34 as the only residue interacting directly with the IL-2-Rβ component (Zurawski *et al.*, 1990; Zurawski and Zurawski, 1989). The only other requirement for this interaction to occur was the presentation of Asp34 in the context of an α-helix. The similarities between this receptor system and the one described in this paper are remarkable. In both cases the interaction involves residue(s) in the amino-terminal α-helix of the cytokine and is targeted to a receptor component that by itself binds the ligand poorly (in the case of IL-2, Ringheim *et al.*, 1991) or not at all (in the case of GM-CSF, Gorman *et al.*, 1990; Hayashida *et al.*, 1990). A third example is the interaction of mIL-5 and mGM-Rβ. This is a biologically relevant interaction, since the GM-Rβ subunit seems to be required for high affinity IL-5 binding. Although we have not yet precisely identified the responsible residues within the mIL-5 amino-terminal helix, it is likely, based on the work presented here, that the interaction between IL-5 and its receptor follows the same general pattern.

Table II. Amino acid homology in predicted amino-terminal helices of cytokines

Cytokine	Proposed helix	Amino acid sequence[a]																
mIL-2[b]	31–39				L	L	M	**D**	L	Q	E	L	L					
hIL-2[b]	17–25				L	L	L	**D**	L	Q	M	I	L					
mIL-3	19–28			S	I	V	K	**E**	I	I	G	K	L					
hIL-3[b]	18–27			N	M	I	D	**E**	I	I	T	H	L					
mIL-4	8–17			N	H	L	R	**E**	I	I	G	I	L					
hIL-4	8–17			I	T	L	Q	**E**	I	I	K	T	L					
mIL-5[b]	7–22			T	V	V	K	**E**	T	L	T	Q	L	S	A	H	R	A	L
hIL-5	9–24			A	L	V	K	**E**	T	L	A	L	L	S	T	H	R	T	L
mIL-6	18–43			H	V	L	W	**E**	-	I	V	E	M	R	K	E			
hIL-6[b]	18–43			R	Y	I	L	**D**	G	I	S	A	L	R	K				
mIL-7	9–20			G	K	A	Y	**E**	S	V	L	M	I	S	I				
hIL-7	9–20			G	D	Q	Y	**E**	S	V	L	M	V	S	I				
mIL-9	7–22			W	G	I	R	**D**	T	N	Y	L	I	E	N	L	K	D	D
hIL-9	7–22			A	G	I	L	**D**	I	N	F	L	I	N	K	M	Q	E	D
mIL-10	21–31			H	M	L	L	**E**	L	R	T	A	F	S					
hIL-10	21–31			N	M	L	R	**D**	L	R	D	A	F	S					
vIL-10	9–19			Q	M	L	R	**D**	L	R	D	A	F	S					
hEPO[b]	4–28	Y	L	L	E	A	K	**E**	A	E	N	I	T	T	G				
mG-CSF	19–30	F	L	L	K	S	L	**E**	Q	V	R	K	I						
hG-CSF[b]	13–24	F	L	L	K	C	L	**E**	Q	V	R	K	I						
mGM-CSF	15–28	H	V	E	A	I	K	**E**	A	L	N	L	L	D	D				
hGM-CSF[b]	15–28	H	V	N	A	I	Q	**E**	A	R	R	L	L	N	L				

[a]Only the pertinent portion of each helix is shown.
[b]Predicted helices are from: for IL-2, Brandhuber *et al.* (1987) and Zurawski and Zurawski (1989); for hIL-3, Parry *et al.* (1988); for mIL-5, Parry *et al.* (1988); for hIL-6, Bazan (1990a); for hG-CSF, Parry *et al.* (1988); for hEPO, Bazan (1990a); for hGM-CSF. Parry *et al.* (1988). The location of the N-terminal helix in the other cytokines was based on comparable motifs from these secondary structure predictions.

The overall structural conservation of cytokines as four α-helical bundle proteins (Parry *et al.*, 1988) and the common receptor-cytokine binding motif described above hints at the existence of an amino-terminal recognition helix in many cytokines that exist as four α-helical bundle proteins. This recognition helix contains the primary and possibly the only specific site of interaction of the cytokine with one component of its functional receptor. In Table II, the sequences of proposed amino-terminal helices of a large number of cytokines are compared. There is an absolute conservation of a negatively charged residue (Glu/Asp) in each helix. Two other positions are conserved in relation to the negatively charged residue; if the charged residue is designated as position 0, residues at positions −2 and +5 are both hydrophobic. Upon examination of the spatial relationships in the helix, it is evident that the hydrophobic residues are located on one side of the helix separated by two helical turns, with the Glu/Asp residue in between these residues on the opposite side of the helix. While these conserved residues may define a core motif, other residues in the helix, such as Lys20 in mGM-CSF, possibly confer specificity to the interaction.

What residues of a cytokine receptor are targeted to interact with the proposed cytokine recognition helix? Cytokine receptors form a family of proteins with a number of conserved features (Bazan, 1990a,b). It is possible that one of these, the 'WSXWS' amino acid sequence in the extracellular domain near the transmembrane region, is the target region for this interaction. Recently, this region was implicated in a direct interaction between human growth hormone and the prolactin receptor, a member of this receptor family (Cunningham *et al.*, 1990). The binding was mediated by Zn^{2+} coordination of three residues in growth hormone, two of which are located in the amino-terminal helix of the protein, and a His residue in the immediate proximity of the 'WSXWS' sequence of the prolactin receptor. If the binding motif described here is directed towards a conserved feature of a receptor subunit, e.g. the 'WSXWS' motif, then this principle may not be restricted to cytokine-Rβ interaction alone. For example, both GM-Rα and -Rβ belong to this receptor family; therefore, it is possible that the same binding motif that governs interaction of GM-CSF and GM-Rβ also determines the interaction of GM-CSF with GM-Rα. There is some evidence which suggests that this is indeed the case. Critical regions identified in mGM-CSF coincide with predicted α-helices, emphasizing the importance of these regions (Shanafelt and Kastelein, 1989). Interestingly, inspection of the sequence of human and mouse GM-CSF, as well as of IL-5, shows that in the carboxy-terminal helix of these molecules the proposed core binding motif is present. This C-terminal region of IL-5 has been shown to affect both its biological activity and receptor binding characteristics (McKenzie *et al.*, 1991).

A key question remains. GM-CSF has no measurable affinity for the β subunit without GM-Rα, yet the equilibrium constant of the high affinity complex is in the picomolar range. It is likely that we have determined the specific ligand–receptor interaction that turns this complex into a high affinity receptor. However, other protein–protein associations must exist to account for this high affinity binding constant. It is not yet known how great the contribution of receptor–receptor interactions is to this process. The homology among cytokine receptors opens the interesting possibility that new receptor combinations could be formed that respond to appropriate hybrid ligands. Such hybrid ligands may be useful as antagonists since their potential to form high affinity complexes could be used to effectively compete for high affinity binding of the native ligand.

Materials and methods

Bacterial host strains and vectors

The *E.coli* K12 strain JM101 (Messing, 1983) was used as host for the propagation and maintenance of M13 DNA. CJ236 (Kunkel *et al.*, 1987)

was used to prepare uracil DNA for use in site-directed mutagenesis. AB1899 (Howard-Flanders *et al.*, 1964) was used as the host for expression of wild-type and mutant human and mouse GM-CSF proteins. Either pINIIIompH3 (Lundell *et al.*, 1990) or pOMPTH3 (a tetracycline resistant variant of pINIIIompH3) was used as the expression vector for all GM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E.coli* secretory expression system (Greenberg *et al.*, 1988).

Mutagenesis, recombinant DNA, and sequencing protocols

Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (1987). Individual clones were sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977) with modifications described in the Sequenase (United States Biochemical) protocol. M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs) for cloning into pINIIIompH3. Synthetic oligonucleotide overhangs corresponding to amino acid residues 5–29 of mIL-5 were ligated into the synthetic hGM-CSF gene (Shanafelt *et al.*, 1991) cleaved with *Bgl*II and *Sac*II. This construct replaced amino acid residues 5–32 of hGM-CSF with those of mIL-5 in the pINIIIompH3 expression vector. The generation of the human–mouse GM-CSF hybrids is described elsewhere (Shanafelt *et al.*, 1991).

Preparation and quantification of protein extracts

Expression and quantification of mutant proteins is described elsewhere (Shanafelt *et al.*, 1991). Briefly, all mutant proteins were produced in *E.coli* AB1899 and periplasmic extracts were prepared by osmotic shock (Shanafelt and Kastelein, 1989). When purified, protein prepared by osmotic shock behaved identically to the pure product (A.B.Shanafelt and R.A.Kastelein, unpublished results); the specific activity of osmotic shock extracts varied by $<10\%$ for a given mutant in multiple assays performed over several months time. The amount of mutant polypeptide produced was determined using quantitative immuno-slot blotting. The error in the calculated concentration of GM-CSF protein by this method was estimated to be 2-fold based on repetitive protein samples.

Transfection of mammalian cell lines

The low affinity hGM-CSF receptor (Gearing *et al.*, 1989) was stably transfected to the mGM-CSF dependent cell line NFS60 with the Lipofectin reagent (BRL) using the manufacturer's suggested protocol. The neomycin resistance gene was used as the selection marker. Stable transfectants were selected with G418 at 1 mg/ml; the clone used in this study had the strongest response to hGM-CSF and was designated 3E6. The generation of the CTLL(hα–mβ) cell line has been previously described (Kitamura *et al.*, 1991a).

Proliferation assays for human and mouse GM-CSF activity

Protein extracts were assayed using the mouse GM-CSF dependent myeloid leukemia cell line NFS60, 3E6 and CTLL(hα–mβ). Sample concentrations were adjusted to 108 000 pg/ml and titrated in quadruplicate to 1.8 pg/ml. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay described by Mosmann (1983) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices). The concentration of each mutant and wild-type GM-CSF that gave 50% maximum response was determined, and specific relative activity was calculated using the relationship:

$$\% \text{ Activity} = ([\text{wild-type}]_{1/2}/[\text{mutant}]_{1/2}) \times 100\%$$

where $[\text{wild-type}]_{1/2}$ and $[\text{mutant}]_{1/2}$ are the concentrations of wild-type and mutant GM-CSF proteins, respectively, that gave 50% maximum response in the NFS60 or 3E6 assays.

Purification and binding characterization of M43

M43, expressed in *E.coli*, was purified to homogeneity from periplasmic extracts by gel filtration through Superdex-75 (Pharmacia-LKB) followed by affinity chromatography using agarose-coupled (AminoLink, Pierce Chemical Co.) anti-mGM-CSF MAb 35E10 (kindly provided by M.Pearce). Purified M43 was radiolabeled with ^{125}I using the Bolton and Hunter reagent (ICN). Binding assays were performed as follows: 3E6 cells (maintained in mGM-CSF) were harvested and incubated with 1 ml ice cold 10 mM $NaPO_4$, 150 mM NaCl, pH 3.0 for 2 min, diluted to 50 ml with 10 mM $NaPO_4$, 150 mM NaCl, pH 7.0, centrifuged and finally resuspended in 1× Hanks' balanced salts solution (Gibco/BRL) containing 0.1% BSA, 0.02% NaN_3 and 10 mM HEPES, pH 7.5 (HBAH buffer). CTLL(hα–mβ) cells (maintained in mIL-2) were prepared without acid treatment. 1×10^6 (3E6) or 1×10^5 (CTLL(hα–mβ)) cells in 200 μl HBAH buffer were incubated with decreasing concentrations of [^{125}I]hGM-CSF (Amersham, sp. act. 947 Ci/mmol) or [^{125}I]M43 (sp. act. 400 Ci/mmol) at 4°C with continuous agitation for 4 h. Non-specific binding was determined by including unlabeled hGM-CSF or M43 as appropriate at a concentration of 1 μM. Cell bound radioactivity was separated from free ligand by centrifugation at 4°C (4 min, 12 000 *g*) through dioctylphthalate:dibutylphthalate (2:3), and bound and total radioactivity were measured with a Cobra 5010 γ-counter (Packard). The equilibrium binding data were analyzed using the LIGAND program (Munson, 1983).

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