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Three residues in the common β chain of the human GM-CSF, IL-3 and IL-5 receptors are essential for GM-CSF and IL-5 but not IL-3 high affinity binding and interact with Glu21 of GM-CSF

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The β subunit (β_c) of the receptors for human granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5) is essential for high affinity ligand-binding and signal transduction. An important feature of this subunit is its common nature, being able to interact with GM-CSF, IL-3 and IL-5. Analogous common subunits have also been identified in other receptor systems including gp130 and the IL-2 receptor γ subunit. It is not clear how common receptor subunits bind multiple ligands. We have used site-directed mutagenesis and binding assays with radiolabelled GM-CSF, IL-3 and IL-5 to identify residues in the β_c subunit involved in affinity conversion for each ligand. Alanine substitutions in the region Tyr365–Ile368 in β_c showed that Tyr365, His367 and Ile368 were required for GM-CSF and IL-5 high affinity binding, whereas Glu366 was unimportant. In contrast, alanine substitutions of these residues only marginally reduced the conversion of IL-3 binding to high affinity by β_c. To identify likely contact points in GM-CSF involved in binding to the 365–368 β_c region we used the GM-CSF mutant eco E21R which is unable to interact with wild-type β_c whilst retaining full GM-CSF receptor α chain binding. Eco E21R exhibited greater binding affinity to receptor αβ complexes composed of mutant β chains Y365A, H367A and I368A than to those composed of wild-type β_c or mutant E366A. These results (i) identify the residues Tyr365, His367 and Ile368 as critical for affinity conversion by β_c, (ii) show that high affinity binding of GM-CSF and IL-5 can be dissociated from IL-3 and (iii) suggest that Tyr365, His367 and Ile368 in β_c interact with Glu21 of GM-CSF.

Key words: affinity conversion/cytokines/mutants/structure

Introduction

The human cytokines granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5) are important regulators of haemopoiesis and inflammation (reviewed in Metcalf, 1991; Lopez *et al.*, 1992b). The regulatory effects of these cytokines are mediated by high affinity cell surface receptors that are known to comprise at least an α chain and a β chain. The β chain (Hayashida *et al.*, 1990) and α chains of the GM-CSF (Gearing *et al.*, 1989), IL-3 (Kitamura *et al.*, 1991b) and IL-5 (Tavernier *et al.*, 1991; Murata *et al.*, 1992) receptors have been cloned and the receptor complexes reconstituted and characterized (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991). These experiments show that whilst both the α and β receptor subunits are required for signalling (Kitamura *et al.*, 1991a,1992; Takaki *et al.*, 1993) they subserve distinct functions in terms of binding. Thus the α chains are ligand specific and bind their cognate ligand with low affinity only ($K_d = 10^{-9}-10^{-7}$ M). On the other hand the β subunit is common (β_c) to the GM-CSF, IL-3 and IL-5 receptors and although it does not detectably bind ligand by itself, it acts as an affinity converter allowing high affinity (10^{-10} M) binding when co-expressed with an α chain.

The role of β_c in mediating affinity conversion and signal transduction by different ligands is similar to that of gp130 and the IL-2 receptor γ chain. Thus, gp130 provides high affinity binding for leukaemia inhibitory factor, IL-6, IL-11, oncostatin M and ciliary neurotrophic factor (Akira *et al.*, 1993) and is important for their function. Similarly, the γ chain of the IL-2 receptor increases the binding affinity of IL-2 (Takeshita *et al.*, 1992), IL-4 (Russell *et al.*, 1993) and IL-7 (Noguchi *et al.*, 1993) when co-expressed with the respective ligand specific receptor chains and contributes to signalling. It is not clear however, how a single subunit in all these receptor complexes can increase the binding affinity of several different ligands. Interestingly, the β_c subunit increases the affinity of GM-CSF, IL-3 and IL-5 binding to different degrees when co-expressed with the respective α chain. Thus β_c increases IL-5 binding affinity ~2- to 5-fold (Tavernier *et al.*, 1991, 1992), GM-CSF binding affinity 20- to 100-fold (Hayashida *et al.*, 1990) and IL-3 binding affinity 500- to 1000-fold (Kitamura *et al.*, 1991b, 1992) raising the possibility that more than one region is involved in conferring high affinity binding on GM-CSF, IL-3 and IL-5.

Structurally the β_c and α chains of the GM-CSF, IL-3 and IL-5 receptors belong to the family of cytokine receptors that includes receptors for growth hormone (GH), erythropoietin, IL-2, IL-4, IL-6 and IL-7 (D'Andrea *et al.*, 1989; Gearing *et al.*, 1989; Bazan, 1990). Members of this family share conserved structural motifs in the extracellular portion which can provide useful information when appropriately aligned. Growth hormone binds to a homodimeric receptor. The soluble form of this receptor, growth hormone binding protein (GHbp) has been crystallized in a complex with GH and the three dimensional structure elucidated (de Vos *et al.*, 1992). We have aligned the amino acid sequence of β_c to the GHbp and used

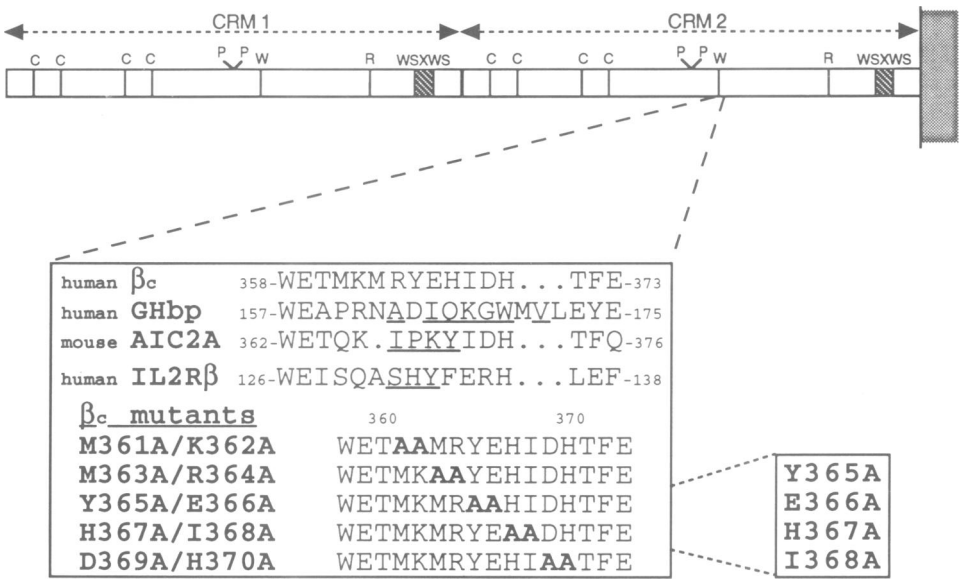

Fig. 1. Identification of residues in the human β chain important for high affinity GM-CSF, IL-3 and IL-5 binding by alanine scanning mutagenesis. Top: schematic representation of the extracellular domain of the human β chain showing two cytokine receptor modules (CRM1 and 2; Goodall *et al.*, 1993) and the conserved features of the cytokine receptor family (Bazan 1990). Bottom: the amino acid sequences corresponding to the proposed B′−C′ loop of CRM2 of the human β chain (β_c) (Hayashida *et al.*, 1990), GHbp (Leung *et al.*, 1987), AIC2A (Itoh *et al.*, 1990) and hIL2Rb (Imler *et al.*, 1992) are aligned, and previously identified regions that play a role in ligand-binding are underlined (de Vos *et al.*, 1992; Imler *et al.*, 1992; Wang *et al.*, 1992). Mutant forms of the human β chain which were made to identify ligand-binding sites are also shown either as double alanine substitutions (left) or single alanine substitutions (right).

information from the GH·$(GHbp)_2$ crystals to hypothesize regions in β_c which may be involved in ligand-binding (Goodall *et al.*, 1993). Of these regions we have targeted the region between residues 361 and 370 because it aligns with a region in GHbp (163–171) that is known to bind GH (de Vos *et al.*, 1992). This region also aligns with residues of the mouse IL-3 receptor β chain AIC2A, and the human IL-2 receptor β chain that are known to be involved in ligand-binding (Imler *et al.*, 1992; Wang *et al.*, 1992). In addition, recent studies (Lock *et al.*, 1994) showed that His367 of β_c is important for GM-CSF high affinity binding. It is not clear however, whether this region is involved in IL-3 and IL-5 as well as GM-CSF binding, nor the identity of complementary regions in the ligands involved.

A candidate for the common motif for the binding of human GM-CSF, IL-3 and IL-5 to β_c is represented by the conserved acidic residue Glu21 in GM-CSF, Glu22 in IL-3 and Glu13 in IL-5. The mutation of this Glu selectively impairs high affinity binding for human GM-CSF (Shanafelt and Kastelein, 1992; Lopez *et al.*, 1992a), IL-3 (Barry *et al.*, 1994) and IL-5 (unpublished observations). In particular, substitution of Glu21 of human GM-CSF for Arg and expression in *Escherichia coli* causes complete loss of high affinity binding as well as signalling, and results in a specific antagonist (Hercus *et al.*, 1994a). We have now used this molecule to test the hypothesis that the conserved Glu in GM-CSF, IL-3 and IL-5 interacts with the 361–370 region of β_c.

We show here, using alanine mutagenesis in conjunction with binding studies with all three known ligands, that residues Tyr365, His367 and Ile368 located in the membrane proximal domain of the extracellular portion of the β_c subunit are involved in high affinity binding. Importantly, these residues are essential for GM-CSF and IL-5 high affinity binding but not for IL-3, implying that an additional region may be involved in IL-3 high affinity binding and suggesting a structural explanation for the large effect of the β_c subunit in increasing IL-3 binding affinity. Finally, by using the GM-CSF mutant eco E21R which is deficient in β_c interaction (Hercus *et al.*, 1994a), we propose a model where the β_c region 365–368 interacts with Glu21 in the first helix of GM-CSF.

Results

Identification of residues of the human β chain critical for high affinity receptor formation; differential effects of alanine scanning mutants on GM-CSF, IL-5 and IL-3 high affinity receptor formation

In order to identify residues of β_c which may play a role in affinity conversion we focused on a region (358–373) which aligns with a region in the membrane proximal domain of GHbp that is involved in GH binding (de Vos *et al.*, 1992). The two subunits of GHbp bind GH in a non-equivalent manner and are therefore denoted GHbpI and GHbpII. The region 163–171 of GHbp is particularly attractive since it is involved in the binding of GH to GHbpII (de Vos *et al.*, 1992). In this respect GHbpII is functionally analogous to β_c in that GH does not bind to GHbpII unless it is already bound to GHbpI (Cunningham and Wells, 1989). In addition, the region 358–373 in β_c aligns with regions in the mouse IL-3 receptor AIC2A and the human IL-2 receptor β chain which have been shown to be important in ligand-binding (Figure 1) (Imler *et al.*, 1992; Wang *et al.*, 1992). This region of β_c is predicted to lie in or close to a loop between two β strands (Bazan, 1990).

Alanine scanning mutagenesis was carried out across

Table I. Dissociation constants for GM-CSF, IL-3 and IL-5 binding to COS cells expressing each receptor α chain alone and with wild-type or mutant receptor β chains

COS cells expressing:	Ligands									
	GM-CSF				IL-3			IL-5		
α chain only[a]	K_d = 2–12 nM				K_d = 20–100 nM			K_d = 0.8–1 nM		
	No. of sites detected	K_d site 1 (pM ± %)	K_d site 2 (nM ± %)	(No. expts)	No. of sites detected	K_d site 1 (pM ± %)	(No. expts)	K_d (pM ± %)		(No. expts)
+ Wild type β_c	2 $P < 0.001$	132 ± 27[b]	7 ± 33	(10)	2 $P < 0.001$	112 ± 18[c]	(10)	289 ± 6[d]		(4)
+ M361A/K362A	2 $P < 0.001$	142 ± 33 ns[e]	17 ± 30	(4)	2 $P < 0.001$	33 ± 35 ns	(2)	333 ± 16	ns	(2)
+ M363A/R364A	2 $P < 0.001$	220 ± 61 ns	6 ± 53	(4)	2 $P < 0.001$	104 ± 26 ns	(2)	367 ± 8	$P < 0.05$	(2)
+ Y365A/E366A	1	−[f]	8 ± 9	(10)	1[g]	118 ± 31 ns	(3)	649 ± 8	$P < 0.001$	(3)
+ H367A/I368A	1	–	5 ± 12	(7)	2 $P < 0.001$	531 ± 28 $P < 0.001$	(4)	966 ± 6	$P < 0.001$	(2)
+ D369A/H370A	2 $P < 0.001$	175 ± 36 ns	4 ± 59	(5)	2 $P < 0.001$	158 ± 23 ns	(2)	358 ± 8	$P < 0.05$	(2)

[a]Range of affinities observed for ligand binding to receptor α chain only.
[b]Binding data from separate experiments (expts) with GM-CSF were co-analysed and the affinity constants are shown ± the standard error (SE) expressed as a percentage (Munson and Rodbard, 1980).
[c]Analysis of data was performed as above except that given the extreme low affinity of IL-3 binding to IL-3R α chain, a constant value of 50 nM was used to allow estimation of high affinity (K_d site 1) sites.
[d]Analysis of data was performed as above. A single receptor class was detected with IL-5.
[e]Significant differences with *P* values or not significant (ns) differences between β_c mutants and wild type β_c.
[f]No high affinity binding sites were detected.
[g]Only high affinity binding was detected.

Table II. Dissociation constants for GM-CSF, IL-3 and IL-5 binding to COS cells expressing each receptor α chain alone and with wild-type or mutant receptor β chains

COS cells expressing:	Ligands									
	GM-CSF				IL-3			IL-5		
α chain only[a]	K_d = 2–12 nM				K_d = 20–100 nM			K_d = 0.8–1 nM		
	No. of sites detected	K_d site 1 (pM ± %)	K_d site 2 (nM ± %)	(No. expts)	No. of sites detected	K_d site 1 (pM ± %)	(No. expts)	K_d (pM ± %)		(No. expts)
+Wild type β_c	2 $P < 0.001$	132 ± 27	7 ± 33	(10)	2 $P < 0.001$	112 ± 18	(10)	289 ± 6		(4)
+Y365A	1	−[a]	4 ± 15	(4)	2 $P < 0.001$	490 ± 39 $P < 0.01$	(3)	836 ± 11	$P < 0.001$	(2)
+E366A	2 $P < 0.001$	191 ± 31 ns[b]	9 ± 21	(3)	2 $P < 0.001$	435 ± 34 $P < 0.01$	(3)	295 ± 12	ns	(2)
+H367A	1	–	8 ± 21	(4)	2 $P < 0.001$	476 ± 17 $P < 0.001$	(5)	455 ± 16	$P < 0.025$	(2)
+I368A	1	–	2 ± 19	(3)	1[c]	432 ± 27 $P < 0.001$	(4)	400 ± 11	$P < 0.025$	(2)

Binding experiments were analysed as in Table I.
[a]No high affinity binding sites were detected.
[b]Significant differences with *P* values or not significant differences (ns) between β_c mutants and wild type β_c.
[c]Only high affinity binding was detected.
[d]Range of affinities observed for ligand binding to receptor α chain only.

the region 361–370 in order to identify residues of the β chain required for affinity conversion and in particular their involvement with each ligand. Alanine substitutions were used to minimize conformational effects of mutation across this region (Cunningham and Wells, 1989). Initially, residues were substituted with alanines in pairs (Figure 1). The mutant β chains M361A/K362A, M363A/R364A, Y365A/E366A, H367A/I368A and D369A/H370A were co-expressed on COS cells with either the GM-CSF, IL-3 or IL-5 receptor α chain and binding of the appropriate ligand determined in saturation binding studies. In the absence of β_c the binding affinities of the transfected α chains alone ranged from 2 to 12 nM for GM-CSF, 20 to 100 nM for IL-3 and 0.8 to 1.0 nM for IL-5 (Tables I and II). These values are consistent with previously determined affinities (Gearing *et al.*, 1989; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991; Murata *et al.*, 1992). The β chain mutants M361A/K362A, M363A/R364A and D369A/H370A had no significant effect on GM-CSF or IL-3 high affinity binding (Figure 2 and Table I). However, the mutants Y365A/E366A and H367A/I368A had a profound effect on high affinity conversion by β_c (Figure 2 and Table I). Both mutants completely abolished GM-CSF high affinity binding. In contrast, the mutant Y365A/E366A did not significantly reduce IL-3 high affinity binding whilst the mutant H367A/I368A caused a reduction in high affinity from 110 pM (wild-type β_c) to 530 pM which was statistically significant (Table I). In the case of IL-5, the mutant M361A/K362A did not affect high affinity binding whilst the mutants M363A/R364A and D369A/H370A exhibited a small loss of IL-5 high affinity binding (Figure 2 and Table I). The mutants Y365A/E366A and H367A/I368A on the other hand caused the greatest loss of IL-5 high affinity binding (Figure 2 and Table I).

The loss of high affinity binding associated with mutant β chains Y365A/E366A and H367A/I368A was not due to lack of expression on the transfected cells. The cell

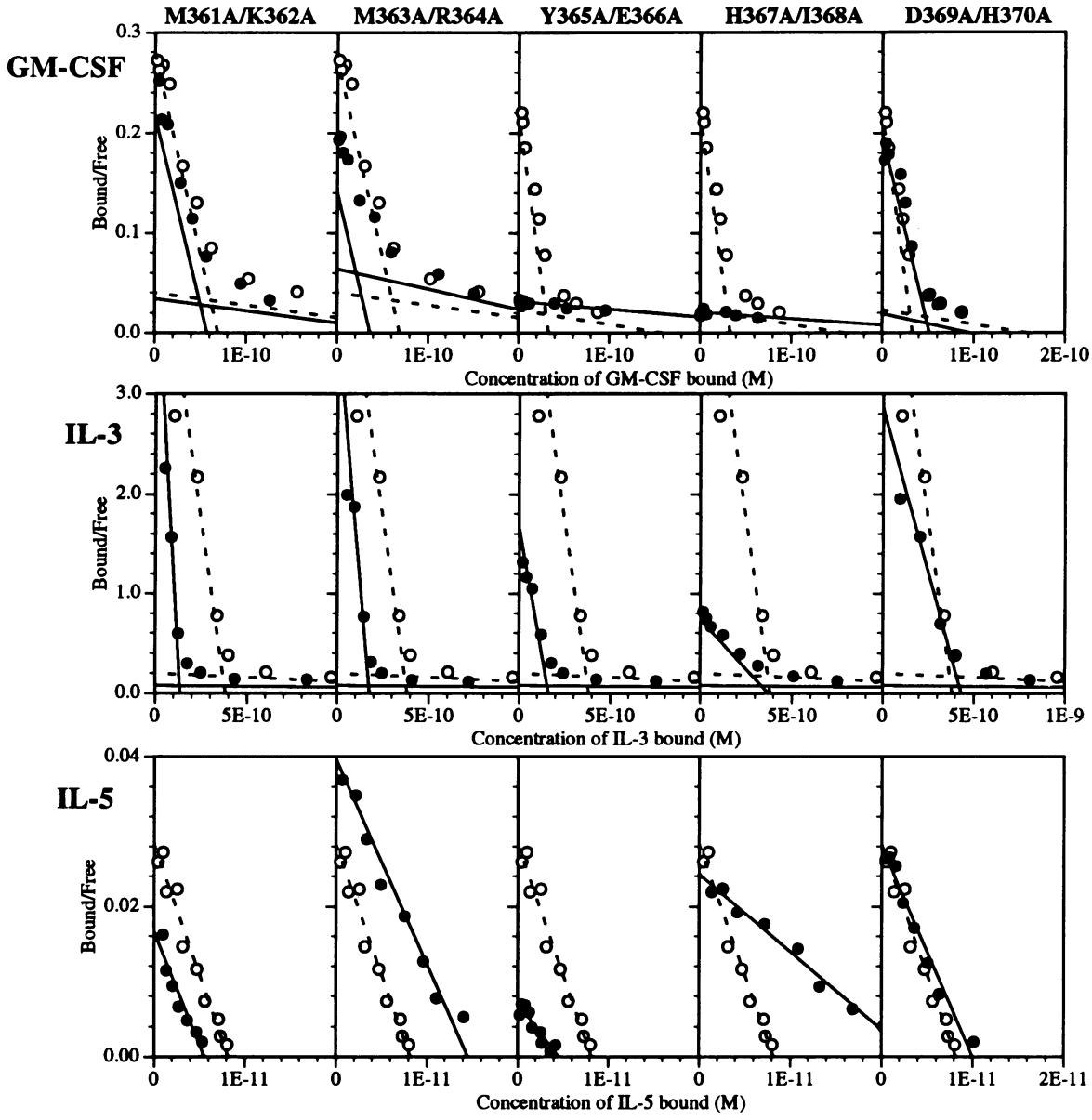

Fig. 2. Double alanine substitution in the β_c region 365–368 abolishes high affinity binding of GM-CSF and IL-5 but not of IL-3. Scatchard transformation of saturation binding studies with [^{125}I]GM-CSF (top), [^{125}I]IL-3 (middle) and [^{125}I]IL-5 (bottom) were carried out on COS cells expressing either the GM-CSFRα, IL-3Rα or IL-5Rα chains together with wild-type (○) or mutant (●) β_c. The range of ligand concentrations were; 10 pM−8 nM for [^{125}I]GM-CSF and [^{125}I]IL-3; 10 pM−5 nM for [^{125}I]IL-5. In each case specific binding was determined in the presence of 1 μM unlabelled ligand. Each point is the mean of two replicates using 1x10^6 COS cells. The broken line indicates the high and low affinity binding components using the wild-type β_c and the solid line represents the line of best fit for the different β_c mutants as determined using the LIGAND programme (Biosoft, Cambridge, UK). A representative experiment is shown and the K_d values derived from these and other similar experiments are shown in Table I.

surface expression of mutant β chains was monitored by flow cytometry using anti-β chain monoclonal antibodies 3D7 and 4F3 (see Materials and methods) and CRS-1 (Watanabe *et al.*, 1992). Antibodies 3D7 and 4F3 both specifically precipitate a protein of 120 kDa from permanently transfected surface-labelled CHO cells corresponding in size to the β chain (Figure 3A). Figure 3B shows a typical result where the anti-β chain antibody 4F3 stained COS cells transfected with mutant β chain H367A/I368A as well as cells transfected with wild-type β chain. Figure 3B also shows staining for GM-CSF receptor α chain expression on these transfectants with monoclonal antibody 8G6. In IL-5 studies surface expression of mutant β chains was confirmed by affinity cross-linking. Figure 3C shows the result of cross-linking [^{125}I]IL-5 to COS transfectants expressing IL-5 receptor α chain alone and with wild-type or mutant β chain Y365A/E366A. The protein bands detected by the irreversible cross-linking of [^{125}I]IL-5 correspond in size, after subtraction of the molecular weight of an IL-5 monomer (the gel was run under reducing conditions), to the IL-5 receptor α chain (60 kDa) and β_c (120 kDa).

In order to elucidate the contribution of individual residues to high affinity binding, single alanine substitu-

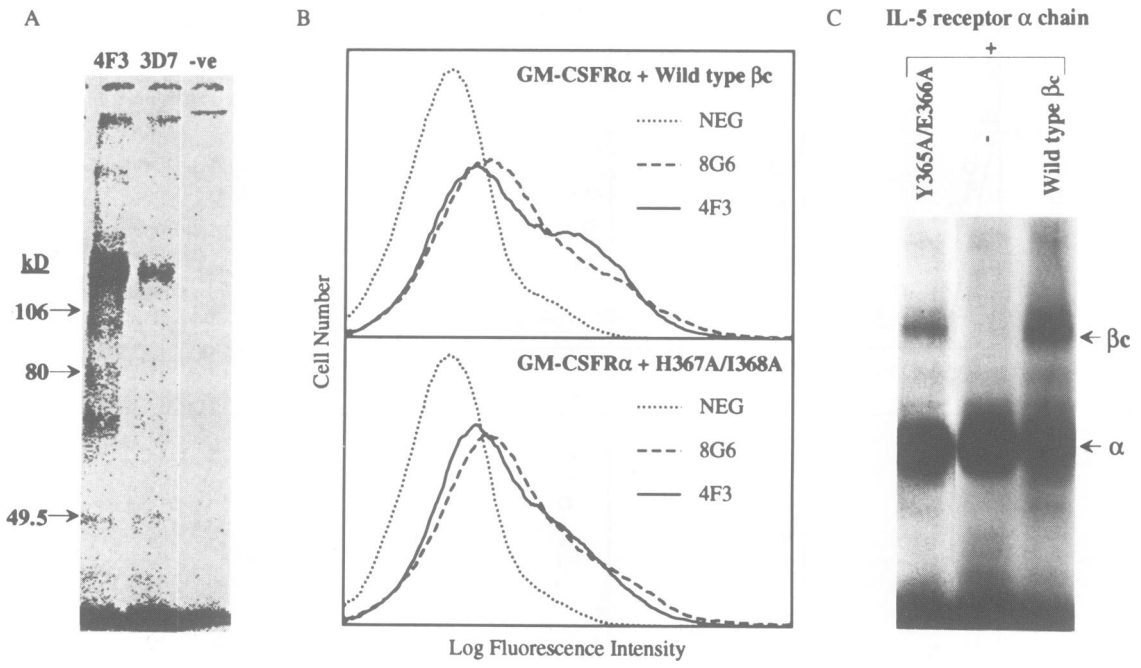

Fig. 3. Ligand-independent determination of β_c expression on transfected COS cells. (**A**) Characterization of anti-β_c (MAbs) by immunoprecipitation of radio-iodinated surface proteins from A8 CHO cells expressing β chain with MAbs 4F3 and 3D7 or no MAb (−ve). Briefly, 10^7 CHO cells were surface-labelled with I^{125} by the lactoperoxidase method and after washing, were incubated in lysis buffer containing protease inhibitors for 60 min at 4°C on a rotating mixer. The mixture was then centrifuged at 10 000 *g* for 15 min at 4°C and the lysate supernatant precleared with 100 µl anti-mouse Ig−Sepharose beads for 18 h at 4°C. Following centrifugation the lysate supernatant was mixed with 5 µg of MAb for 1 h at 4°C followed by 100 µl of anti-mouse Ig−Sepharose beads. The beads were then washed, boiled and the supernatants run on a 7.5% SDS−PAGE. The gel was visualized by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). (**B**) COS cells transfected with GM-CSF receptor α chain and wild-type or mutant β chain cDNAs were stained with anti-α (8G6) and anti-β chain (4F3) monoclonal antibodies and cell surface expression of these proteins detected by flow cytometry. (**C**) Chemical cross-linking of [^{125}I]IL-5 to COS cells expressing IL-5 receptor α chain alone and with wild-type or Y365A/E366A mutant β-chain.

tions were made at positions 365–368. In each case the effect of these substitutions was determined after cotransfection with either GM-CSF, IL-3 or IL-5 receptor α chain in saturation and competition binding studies. As above, cell surface expression of mutant β chains was confirmed by flow cytometry (data not shown). Results showed that alanine substitution of residues Tyr365, His367 and Ile368 led to the complete loss of high affinity GM-CSF binding, whereas alanine substitution of Glu366 had no significant effect on high affinity GM-CSF binding (Figure 4, Table II). In contrast to GM-CSF, the effects of individual substitutions at residues 365–368 on IL-3 high affinity binding were small, resulting in only a 4-fold decrease in affinity relative to the wild-type receptor in each case (Table II). Alanine substitutions at Tyr365, His367 and Ile368 caused significant reductions in IL-5 high affinity binding whereas substitution at Glu366 had no significant effect.

Partial restoration of binding of the GM-CSF analogue eco E21R to mutant β chains

We have previously shown that the GM-CSF analogue eco E21R is deficient in binding to β_c and signalling while retaining wild-type binding to the GM-CSF receptor α chain (Hercus *et al.*, 1994a). In order to ascertain whether the conserved Glu motif of GM-CSF, IL-3 and IL-5 is involved in direct interaction with the 365–368 region of the β chain we examined the ability of the eco E21R to compete for wild-type GM-CSF binding on mutant β chains. Competition binding studies were carried out on COS cells cotransfected with GM-CSF receptor α chain and wild-type or mutant β chain. Radio-iodinated wild-type GM-CSF binding was determined using 100 pM [^{125}I]GM-CSF in the presence of an increasing amount of eco E21R analogue. Characterization of eco E21R binding showed that this mutant was able to compete with wild-type GM-CSF with equal potency for binding to low affinity α chain receptors but exhibited a 100-fold reduction in its ability to compete for binding to high affinity αβ receptors (Figure 5A). Significantly, eco E21R competed more effectively on transfectants expressing β chains with alanine substitutions at position 365, 367 and 368 compared with the wild-type β chain or the β chain mutant E366A (Figure 5B). This suggests that the mutant β chains Y365A, H367A and I368A are able to interact with eco E21R and that the substitutions made in β_c complement in part the charge reversal substitution made in GM-CSF. Comparison of the effective dose for 50% competition (ED_{50}) exhibited by eco E21R showed that eco E21R bound to the GM-CSF receptor α chain plus Y365A, H367A and I368A complexes five to seven times better than to E366A or wild-type β_c complexes (Table III).

Discussion

We show here that the common β chain of the GM-CSF, IL-3 and IL-5 receptors contains three residues, Tyr365, His367 and Ile368 which are involved in GM-CSF and

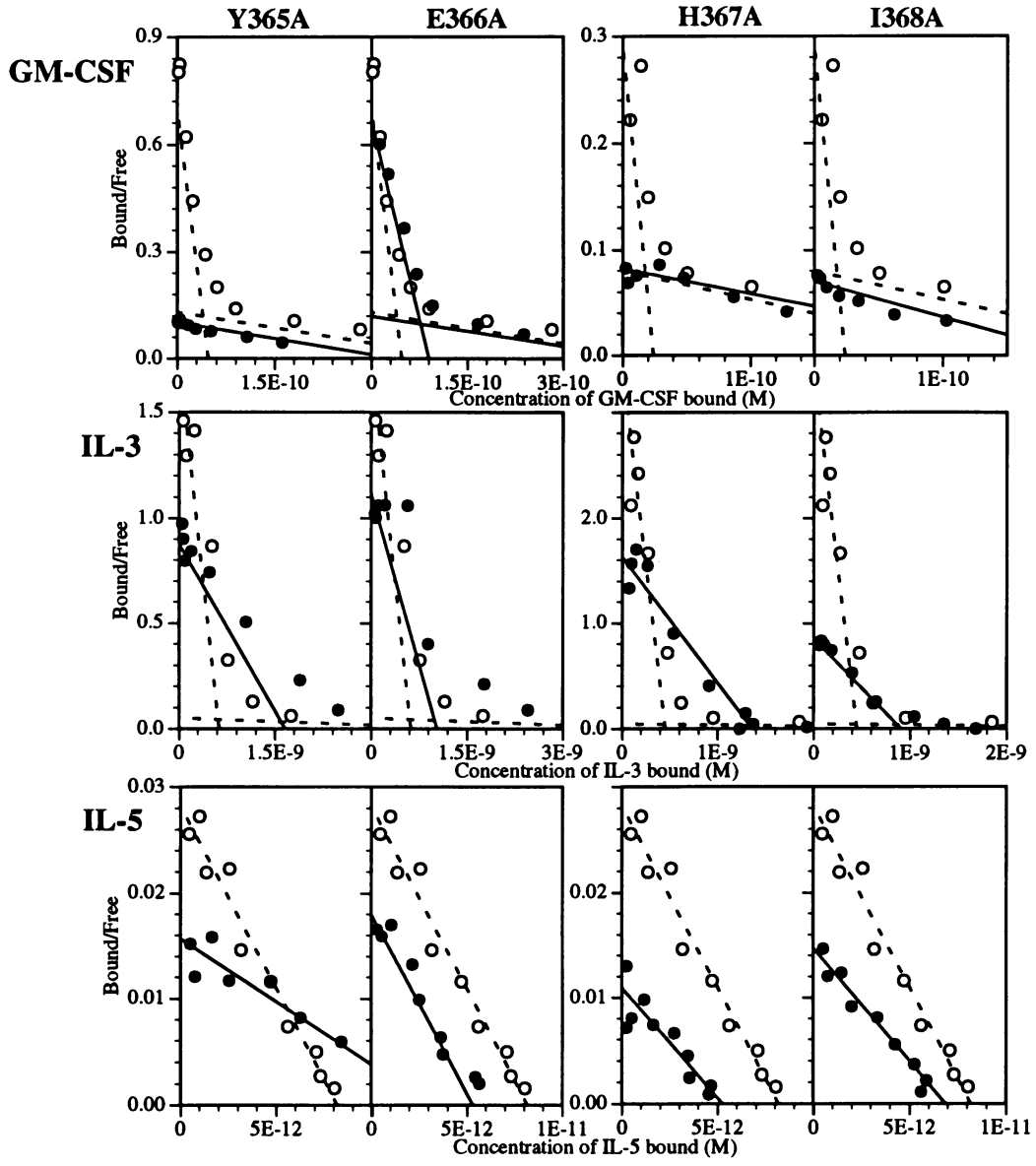

Fig. 4. The effect of single alanine substitution of residues Tyr365, Glu366, His367 and Ile368 of the human β chain on high affinity GM-CSF, IL-3 and IL-5 binding. Scatchard transformation of saturation binding studies with [^{125}I]GM-CSF, [^{125}I]IL-3 and [^{125}I]IL-5 were carried out as described in Figure 2. A representative experiment is shown and the derived K_d values of these and other experiments shown in Table II.

IL-5 high affinity binding. However, whilst alanine substitution of these three residues abolishes GM-CSF and IL-5 high affinity binding, they only marginally affect IL-3 high affinity binding. These findings raise the possibility that other common subunits of receptor complexes such as gp130 and the IL-2/IL-4/IL-7 receptor γ chain may also have common regions and unique regions recognized by different ligands. In addition, using the human GM-CSF antagonist eco E21R which has selectively lost its ability to bind to β_c and signal (Hercus *et al.*, 1994a) we show partial binding complementation on β_c mutants Y365A, H367A and I368A suggesting that the conserved Glu in the first helix of GM-CSF directly interacts with the 365–368 region of β_c.

By using alanine substitution mutagenesis we have defined the region 365–368 in β_c and, more specifically, Tyr365, His367 and Ile368 as being essential for GM-CSF high affinity binding. The region between Tyr365 and Ile368 aligns with a region in the second cytokine receptor domain of the GHbp (Goodall et *al.*, 1993) which has been shown to contact GH after GH binds to GHbpI (de Vos *et al.*, 1992) and with a region in AIC2A (Wang *et al.*, 1992) and IL-2R β (Imler *et al.*, 1992) important for mouse IL-3 and IL-2 binding respectively. By analogy with these molecules and from structural predictions (Bazan, 1990; Goodall *et al.*, 1993) this region is predicted to lie in the loop between the B′ and C′ β strands of the second cytokine receptor module (CRM) of β_c (Figure 6). This suggests that rather than forming a discrete contact point for interaction, this region forms a binding surface on which several residues contribute to ligand interaction. It is noteworthy that Glu366 does not appear to contribute to ligand interaction suggesting that the side chain of this residue is orientated away from the proposed binding surface.

The loss of affinity converting activity associated with

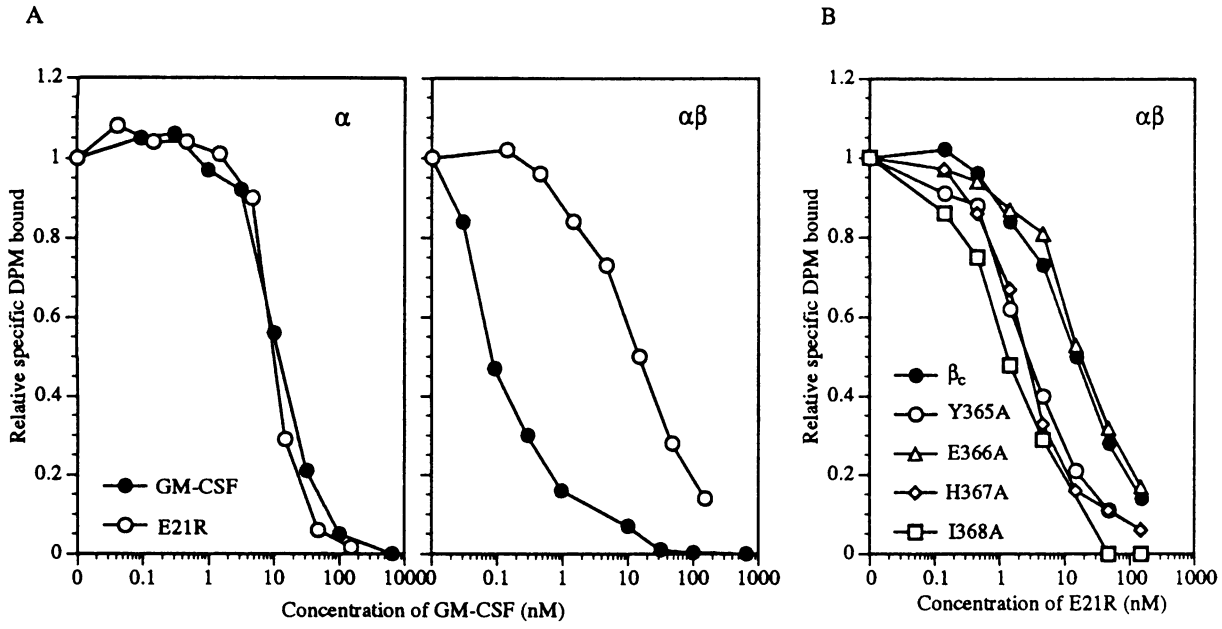

Fig. 5. Alanine substitution at Tyr365, His367 and Ile368 in β_c improves the ability of eco E21R to compete for wild-type [^{125}I]GM-CSF binding to COS cells expressing GM-CSF receptor α and mutant β chains. (**A**) Competition of eco E21R and wild-type GM-CSF binding for [^{125}I]GM-CSF at 100 pM on COS cells expressing GM-CSF receptor α chain alone (α) or α and wild-type β chains (αβ). (**B**) Competition of eco E21R for [^{125}I]GM-CSF binding at 100 pM on COS cells expressing GM-CSF receptor α chain and mutant or wild-type β chains.

Table III. The GM-CSF mutant E21R shows improved binding to COS cells co-expressing the GM-CSF receptor α chain and the β_c mutants Y365A, H367A and I368A

COS cells expressing α chain+	Experiment #1		Experiment #2		Fold improvement
	ED_{50}[a] (nM) ± SEM	Ratio mutant ED_{50}/Wt ED_{50}	ED_{50} (nM) ± SEM	Ratio mutant ED_{50}/Wt ED_{50}	
Wild-type β_c	13.93 ± 3.24	1	12.35 ± 2.45	1	1
Y365A	2.20 ± 0.32	0.16	3.06 ± 0.62	0.25	5.12
E366A	16.38 ± 3.75	1.18	24.40 ± 10.58	1.97	0.68
H367A	1.92 ± 0.49	0.14	1.97 ± 0.25	0.16	6.70
I368A	1.55 ± 0.36	0.11	2.93 ± 0.68	0.24	6.63

[a]Concentration of GM-CSF E21R causing 50% inhibition of wild-type [^{125}I]GM-CSF binding to COS cells co-expressing the GM-CSF receptor α chain and different β chain mutants as described in Materials and methods.

mutant β chains Y365A, H367A and I368A is neither the result of the lack of surface expression nor due to gross conformational changes as judged by the following criteria. First, transfected cells were all stained with monoclonal anti-β chain antibodies. Three different antibodies were used and the staining seen with each antibody was equivalent in all cases, confirming cell surface expression and suggesting that the mutations did not introduce gross conformational changes. Second, affinity cross-linking with [^{125}I]IL-5 to cells expressing receptor α and Y365A/E366A β_c which bind IL-5 with reduced affinity resulted in the cross-linking of two proteins which corresponded in size to the IL-5 receptor α and β chains. Third, mutant β chains which were deficient in affinity converting GM-CSF and IL-5 binding still conferred high affinity binding on IL-3.

In order to identify corresponding residues in GM-CSF likely to interact with the 365–368 region of β_c we focused on the first helix of GM-CSF which has been shown to be important for high affinity binding (Shanafelt *et al.*, 1991; Lopez *et al.*, 1992a). Of several GM-CSF helix A mutants we have produced, we used the GM-CSF mutant eco E21R because mutations at Glu21 lead to a selective loss of GM-CSF–β_c interaction (Lopez *et al.*, 1992a; Shanafelt and Kastelein, 1992) and Glu21 is the main residue in the first helix of human GM-CSF that interacts with β_c (Hercus *et al.*, 1994b). We found that the GM-CSF mutant eco E21R competed better for [^{125}I]GM-CSF binding on cells expressing the mutant β chains that affect high affinity GM-CSF binding, namely Y365A, H367A and I368A. No effect was observed on E366A, consistent with the lack of effect of this substitution in direct binding studies. These results raise the possibility that residues Tyr365, His367 and Ile368 interact directly with GM-CSF and more specifically with Glu21 (Figure 6). It is interesting to note that individual substitution of each residue, Tyr365, His367 and Ile368, is sufficient to abrogate GM-CSF high affinity binding. The mechanism by which these residues contribute to GM-CSF high affinity binding is not exactly known. It is possible that the mutations alter the overall structure of the high affinity binding site, however, this is unlikely in view of the fact that all the mutant receptors retained high affinity conversion for IL-3. This argues against severe structural

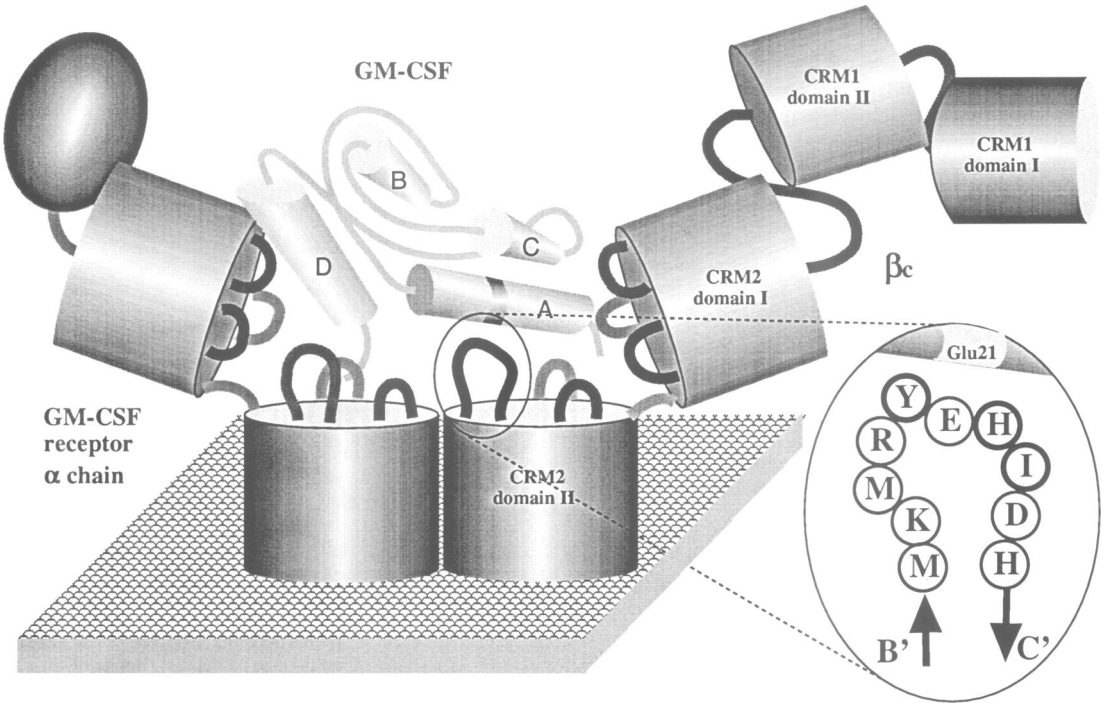

Fig. 6. Schematic diagram of the interaction between GM-CSF and the extracellular portions of the α and β chains of its receptor. GM-CSF is represented as a four helix bundle in which the helices are labelled A, B, C and D. The seven β strands of each receptor domain are viewed as barrels. The cytokine receptor modules (CRM1 and 2) are each comprised of two domains (I and II). The loops of domain II of CRM2 project towards helix A of GM-CSF. The insert shows the close positioning and putative interaction between Glu21 in helix A of GM-CSF and the B′−C′ loop on domain II of CRM2. Residues Tyr365 (Y), His367 (H) and Ile368 (I) which are predicted to be involved in interaction with GM-CSF Glu21 are outlined in bold circles. The scheme is based on a superposition of receptor sequences onto the homologous GHbp structure and the GM-CSF structure onto that of GH. In particular, residue Glu21 of GM-CSF is aligned with residue Arg19 of GH, a residue that interacts with the B′−C1 loop of GHbpII (de Vos *et al.*, 1992).

alterations. It is more likely however, that residues 365–368 act cooperatively to recognize helix A of GM-CSF. In this case the contribution of each residue to the ligand-binding determinant is dependent on the presence of the appropriate neighbouring residue. This cooperativity is also likely to explain the slight differences in affinity between the double and single mutants in IL-3 and IL-5 high affinity binding. Our results are consistent with the recent report of His367 of β_c being important for high affinity GM-CSF binding (Lock *et al.*, 1994). Interestingly, in contrast to our findings, Tyr365 was not found to be important in that study. This difference may be due to the nature of the substitution made; phenylalanine was substituted for Tyr365. This subsitution retains the hydrophobicity of this residue which may be important for its function. Residue Ile368 was not investigated in that paper (Lock *et al.*, 1994).

In the case of IL-5, the results were less clear than with GM-CSF and IL-3 due to the relatively small (2- to 5-fold) increase in binding provided by β_c over the IL-5 receptor α chain alone. Nevertheless, alanine substitutions revealed that Tyr365 was essential for affinity conversion by β_c. In contrast, the substitution of Glu366 alone did not reduce IL-5 binding and may have slightly attenuated the effect of Y365A in the double mutant. Individual alanine substitutions at positions 367 and 368 resulted in modest decreases in binding but together resulted in the abolition of detectable high affinity binding. Thus, as with GM-CSF binding, residues Tyr365, His367 and Ile368 are important for the high affinity binding of IL-5.

We found that in contrast to GM-CSF and IL-5, high affinity binding of IL-3 was not abolished by mutation of residues Tyr365, His367 and Ile368. In fact only a 4-fold reduction in affinity was observed (Figures 2 and 4 and Tables I and II) which is a relatively small effect when taking into account the ~500-fold increase in IL-3 binding affinity provided by β_c (Kitamura *et al.*, 1991b and Table II). This suggests that an additional or alternative region in β_c contributes to IL-3 high affinity binding which may explain why β_c is able to confer such a large increase in affinity on the IL-3 receptor α chain relative to GM-CSF and IL-5. This notion is supported by two other sets of evidence. First, whilst the GM-CSF eco E21R mutant has completely lost its ability to interact with β_c and signal, behaving as an antagonist (Hercus *et al.*, 1994a), the analogous IL-3 mutant eco E22R is a weak agonist (Barry *et al.*, 1994) suggesting residual interaction with β_c. Second, we found that the monoclonal antibody 4F3 partially inhibits IL-3 but not GM-CSF high affinity binding and stimulation of TF-1 cell proliferation (unpublished results). Alignment of β_c with the GHbp suggests that residues 309–315, 333–343 and 414–424 in the loops between β strands E and F, G and A′, and F′ and G′ respectively, may be involved and it would be of interest to test these hypotheses experimentally.

Finally, our results illustrate that GM-CSF and IL-5 high affinity binding can be dissociated from IL-3 high affinity binding by discrete mutations in β_c raising the possibility that differences in ligand specificity may also be found in other common receptor subunits such as

gp130 and the IL-2 receptor γ chain. The identification of ligand-binding regions and determination of their specificity in these common subunits may allow the selective blocking of individual cytokines or the simultaneous blocking of several cytokines with appropriately designed compounds.

Materials and methods

Mutagenesis of β chain and expression plasmid constructs

The cDNA for the human β chain was cloned by PCR from cDNA prepared from the KMT-2 cell line (Barry *et al.*, 1994). The 2.8 kb cDNA encoding the β chain was subcloned as an *Eco*RI–*Xba*I fragment into pSelect (Promega, Sydney, NSW). Mutations were introduced into the β chain cDNA using oligonucleotide-directed mutagenesis (Altered-sites, Promega). Using this method, a second oligonucleotide which restores ampicillin resistance to template DNA was used in combination with the mutagenic oligonucleotide and mutants selected for ampicillin resistance in a repair deficient strain of *Escherichia coli*. The mutagenic oligonucleotides used were 30mers and were synthesized on an Applied Biosystems 381A DNA Synthesizer (Burnwood, Victoria). The mutations were confirmed by nucleotide sequencing and the mutant β chain cDNAs subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen, San Diego, CA).

The IL-3 receptor α chain was cloned by PCR from KMT-2 cells (Barry *et al.*, 1994). The GM-CSF receptor α chain was kindly provided by Dr N.Nicola (Walter and Eliza Hall Institute, Melbourne, Victoria) and the IL-5 receptor α chain was obtained as described previously (Tavernier *et al.*, 1991). All the receptor α chains were cloned into the eukaryotic expression vector pCDM8 (Invitrogen) for transfection.

COS cells and DNA transfection

COS cells were maintained in RPMI-1640 medium supplemented with 5% v/v fetal calf serum (FCS). DNA constructs were introduced into COS cells by electroporation using a Bio-Rad Gene Pulser (North Ryde, NSW). Routinely 2×10^7 cells in 0.8 ml ice-cold PBS were co-transfected with 10 μg of GM-CSF or IL-3 receptor α chain DNA in pCDM8 and 25 mg of pcDNA1 vector containing the wild-type or mutated β chain cDNA at 500 μF and 300 V. For IL-5 studies cells were transfected using DEAE–dextran as described previously (Tavernier *et al.*, 1991). Binding assays and antibody staining were carried out on cells 2–3 days after transfection.

GM-CSF, IL-3 and IL-5

Recombinant human GM-CSF and IL-3 were produced in *E.coli*. Wild-type GM-CSF and the analogue eco E21R were expressed in the periplasmic space of *E.coli* from a synthetic human GM-CSF cDNA in the expression vector pIN-III-OmpH3 and purified as described elsewhere (Hercus *et al.*, 1994a). Recombinant IL-3 was expressed similarly from a native cDNA carrying the mutation F133Y to improve radio-iodination. The cDNA was cloned into the pFLAG vector (International Biotechnologies Inc., Newhaven, CT) which adds an octapeptide sequence to the N-terminus to facilitate affinity purification (Barry *et al.*, 1994). Recombinant human IL-5 was produced in Sf9 cells as detailed (Tavernier *et al.*, 1989).

Radiolabelling cytokines

Recombinant human GM-CSF and hIL-3 were radio-iodinated by the iodine monochloride method (Contreras *et al.*, 1983) to a specific activity of ~10 mCi/mg and 36 mCi/mg respectively. Iodinated protein was separated from iodide ions on a Sephadex G-25 column (Pharmacia, North Ryde, NSW), eluted with PBS containing 0.02% v/v Tween 20 and stored at 4°C for up to 4 weeks. Prior to use, radio-iodinated protein was buffer exchanged on a carboxymethyl–Sepharose CL-6B column loaded at pH 2.6 in 10 mM citrate-phosphate buffer and eluted in binding medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 0.5% w/v bovine serum albumin and 0.1% w/v sodium azide. Recombinant hIL-5 was iodinated with the Iodogen reagent (Pierce Chemicals, Rockford, IL), as described (Plaetinck *et al.*, 1990).

Binding assays

For binding studies, transfected COS cells were detached by treatment with RPMI containing 40 mM EDTA, 200 μg/ml chondroitin sulfate and 0.1% w/v sodium azide and resuspended in binding medium (as above). Typically binding assays were performed with $0.5–1\times10^6$ cells in 150 μl of binding medium containing radioligand in the presence or absence of unlabelled cytokine in a glass tube under shaking conditions at 24°C for 2–3 h. In saturation studies cells were incubated in the presence of increasing concentrations of radioligand. Routinely a concentration range of 10 pM–8 nM of [^{125}I]GM-CSF or [^{125}I]IL-3 and 10 pM–5 nM of [^{125}I]IL-5 was used. Non-specific binding was determined in assays in which high concentrations of radioligand were used by adding 1 μM of the unlabelled cytokine. The non-specific binding component for data points obtained at lower radioligand concentrations was obtained by interpolation. For competition studies cells were incubated with 100 pM radioligand in the presence of increasing concentration of competitor. After incubation cell-associated radioligand was separated from free radioligand by overlaying the cell suspension on a 0.2 ml cushion of FCS or phthalate oil (Plaetinck *et al.*, 1990) and centrifuging for 10 s at maximum speed in a micro-centrifuge. The visible cell pellet was removed by cutting and radioactivity was determined using the Cobra 5010 γ-counter (Packard, Meriden, CT). Dissociation constants were calculated using the EBDA and LIGAND programs (Munson and Rodbard, 1980; Biosoft, Cambridge, UK). Where indicated, multiple data files were co-analysed to obtain more accurate estimates. For GM-CSF and IL-3 data files were modelled on one-site and two-site fits and the better fit determined from the LIGAND program. Only where significantly preferred ($P < 0.05$) was a two-site model used to determine K_d values. In order to obtain more reliable estimates of the high affinity binding component of the IL-3 binding isotherms, a fixed value (50 nM) was introduced for the dissociation constant for the low affinity binding site. In the case of IL-5 only a single class of receptor affinity was identified in all binding experiments. Statistical significance between K_d values obtained with wild-type β and mutant β_c was determined using the non-paired *t*-test. ED_{50} values were obtained using the Fig.P program (Biosoft, Cambridge, UK).

Ligand-independent monitoring of receptor α and β chain expression

To monitor cell surface expression of transfected GM-CSF and IL-3 receptor α chains and mutant β chains in a ligand-independent manner we generated specific monoclonal antibodies (MAbs) and examined their binding to COS cell transfectants. Anti-β chain monoclonal antibodies 4F3 and 3D7 were raised in a mouse immunized with COS cells co-transfected with IL-3 receptor α chain and β chain. The splenocytes from this mouse were fused with NS-1 myeloma cells and the resulting hybridoma supernatants screened on CHO cells permanently expressing GM-CSF receptor α chains and β chains (A8s). Antibodies 4F3 and 3D7 both specifically precipitated a protein of 120 kDa from surface labelled A8 cells (Figure 3A). These two monoclonals and CRS-1 (Watanabe *et al.*, 1992), a rat monoclonal antibody reactive against the human β chain (kindly donated by Drs R.Kastelein and A.Miyajima, DNAX, Palo Alto, CA) were used to stain β chain transfected COS cells by flow cytometry. Anti-GM-CSFR and anti-IL-3R α chain monoclonal antibodies were obtained essentially in the same way except that hybridoma supernatants were screened on permanently transfected CHO cells; A9/C7 for GM-CSF receptor α chain and F6 for IL-3 receptor α chain (Korpelainen *et al.*, 1993). The appropriate anti-α chain monoclonal antibodies were used to stain COS transfectants for receptor α chain expression; 8G6 for GM-CSF receptor α chain and 6H6 for IL-3 receptor α chain. These antibodies both precipitated a cell surface protein of the appropriate size from the relevant CHO cell line (data not shown). In IL-5 studies cell surface expression of the IL-5 receptor α chain and mutant β chains was confirmed by affinity cross-linking (Plaetinck *et al.*, 1990).

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