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Structure-function analysis of interleukin-5 utilizing mouse/human chimeric molecules

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Interleukin-5 (IL5) is a T cell derived glycoprotein that stimulates eosinophil production and activation. In the mouse, but apparently not in the human, it is active on B cells. The murine and human IL5 polypeptides exhibit 70% sequence similarity and yet display distinct species-specific activity. Whilst mouse and human IL5 are equally active in human cell assays, human IL5 is 100-fold less active than murine IL5 in mouse cell assays. Two restriction sites were utilized to divide the human and mouse sequences into three fragments. Hybrid molecules consisting of all combinations of these fragments were constructed and expressed. In the human cell assays [using bone marrow or the erythroleukaemic cell line (TF-1)] all the hybrid proteins generated activity comparable to that of the human and mouse IL5. This implies that replacing different domains does not result in detrimental effects to the tertiary structure of the molecule. In the mouse cell assays [using bone marrow or the pro-B cell line (B13)] the hybrids clearly identified the importance of residues in the C terminus for biological activity. The changing of only eight residues in this region of human IL5, to those of mouse IL5, resulted in the hybrid producing biological activity comparable to mouse IL5. In addition, competition binding assays showed that this region probably interacts with the receptor.

Key words: B cells/eosinophils/hybrids/interleukin-5/structure-function

Introduction

Interleukin-5 (IL5) stimulates the production of eosinophils *in vitro* (Sanderson *et al.*, 1985, 1986; Lopez *et al.*, 1987) and has been shown to control the full pathway of eosinophil production *in vivo* (Dent *et al.*, 1990; Sher *et al.*, 1990). In the mouse, IL5 also has activity on B cells where it induces preactivated B cells to proliferate (Swain and Dutton, 1982; O'Garra *et al.*, 1986) and stimulates the production of IgM and IgG (O'Garra *et al.*, 1986). IL5 is also able to increase antigen specific antibody responses by B cells from antigen-primed mice (TRF activity) (Harada *et al.*, 1985; Takatsu *et al.*, 1988). A number of IL5-dependent mouse B cell lines have now been isolated (Mita *et al.*, 1989b; Rolink *et al.*, 1989). These provide a convenient assay for this factor. However, there is little or no evidence for an analogous activity on human B cells (Clutterbuck *et al.*, 1987), where a human erythroleukaemic cell line (TF-1) provides a simple assay (Kitamura *et al.*, 1989).

Both mouse IL5 (mIL5) and human IL5 (hIL5) have been characterized by cloning, sequencing and expression (Kinashi *et al.*, 1986; Campbell *et al.*, 1987, 1988). The mIL5 cDNA codes for a mature polypeptide of 113 amino acids (Mita *et al.*, 1989a), whilst the hIL5 cDNA encodes a mature polypeptide of 115 amino acids (Mita *et al.*, 1989a). Both have an M_r of ~13 000 (Sanderson, 1990). Recombinant material produced in mammalian cells has a native M_r in the range 35 000-60 000, which changes to 16 000-32 000 after treatment with a reducing agent (Takatsu *et al.*, 1988). Thus IL5 is unusual among the T cell-derived cytokines in being a disulphide-linked dimer. Recent investigations have identified that the two conserved cysteine residues cross-link the dimer in an anti-parallel arrangement (Minamitake *et al.*, 1990; McKenzie *et al.*, 1991). This dimerization is essential for the biological activity of the molecule. Monomeric mIL5 produced by mutating the cysteine residues to threonine were inactive on both the murine eosinophils and an IL5-dependent murine pro-B cell line (McKenzie *et al.*, 1991). The large M_r range is predominantly due to the heterogeneous addition of carbohydrate, which can be removed to leave a fully active molecule (Tominaga *et al.*, 1990). Apart from these observations little else is known about structural elements required for the biological function of either mIL5 or hIL5.

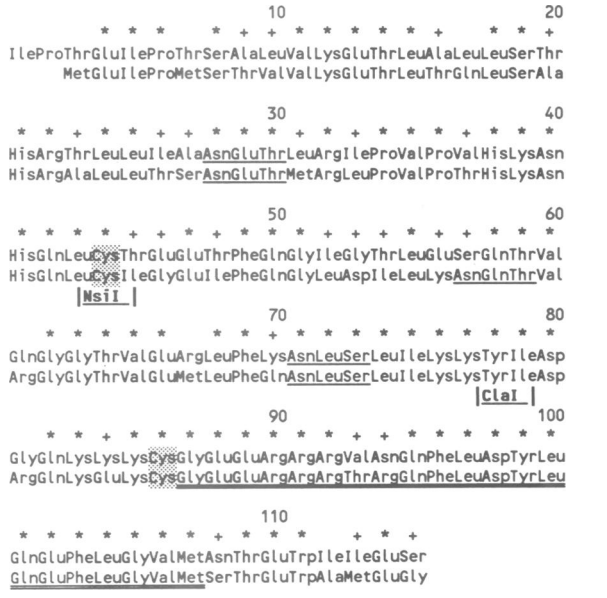

Fig. 1. Amino acid sequence alignment of human (top) and mouse IL5. The numbering system is based on the human sequence. '*' indicates identical amino acids, '+' indicates a conservative amino acid change. The predicted *N*-linked glycosylation sites are underlined. The region of sequence similarity to IL3, GM-CSF, IL4 and IL6 is underlined twice. The positions of the restriction sites used to construct the hybrid molecules are shown.

Although the human and mouse polypeptide sequences have 70% homology (Figure 1) they still display a considerable degree of species specificity. While both mIL5 and hIL5 have equivalent specific activities in human cell assays (Plaetinck *et al.*, 1990), mIL5 is substantially more active on murine cells, showing >50- to 100-fold more activity than hIL5 in the murine eosinophil differentiation assay and the murine B13 cell assay (Clutterbuck *et al.*, 1987; Plaetinck *et al.*, 1990).

Here we report the construction of a panel of human/mouse IL5 hybrids which have been utilized to define the regions of the IL5 molecule responsible for the species specificity, and to elucidate the area(s) important in receptor interaction. This system exploits both the high degree of amino acid homology between mouse and human IL5 and also the distinct cross-species reactivities displayed by these molecules. The chimeric cDNAs were expressed in COS cells, and the resultant hybrid IL5 molecules tested in IL5 assays utilizing both human and mouse cells. The equivalence of the activity of both human and mouse IL5 in the human cell assays provided a good control on the detrimental effects of replacing domains across species. The cross-species differences in activity observed on mouse cells identified an important role for the C-terminal region in the determination of both biological activity and receptor binding.

Results

An *NsiI* restriction site is present in the mouse cDNA sequence. This site was generated in a homologous position in the human molecule; this required the alteration of one amino acid, a conservative substitution of threonine to isoleucine—a change that did not alter the overall activity of the human construct (data not shown). A *ClaI* site was introduced into both the human and mouse cDNA without changing the amino acid sequence (Figure 1). The positions of the restriction sites allowed the molecule to be divided into approximately three equal portions; the N-terminal region of 44 amino acids (42 amino acids in the mouse), a central portion of 35 amino acids, and a C-terminal region of 36 amino acids (Figure 2). The six permutations of hybrid human/mouse constructs were made by substituting the relevant mouse region with that of human.

[^{35}S]Methionine-labelled proteins secreted from COS cells transfected with the hybrid IL5 constructs were analysed by SDS–PAGE and fluorography (Figure 3). The secreted IL5 molecules were in the M_r range of 32 000–43 000 due to the heterogeneous glycosylation associated with IL5 (Tominaga *et al.*, 1990; McKenzie *et al.*, 1991). The protein concentration of each preparation was determined by scanning the fluorograph across this region. Interestingly, constructs M/H/M, M/H/H and H/H/M migrated more rapidly than the others, and have the central portion of the human molecule in common, suggesting that these constructs are recognized differently during post-translational modification. This is not simply due to the absence of an *N*-linked glycosylation site in hIL5 as H/H/H did not show this migration pattern. In an attempt to clarify this point, transfected cells were cultured in the presence of tunicamycin, which inhibits *N*-linked glycosylation. The M_r of the molecules was reduced to 25 000 and 27 000 (Figure 4). Although the pattern seen in Figure 3 was abolished, another pattern emerged. In this case the higher M_r was seen with constructs containing the human C-terminal sequence and could be due to either incomplete blocking of *N*-linked glycosylation or *O*-linked glycosylation of this part of the human molecule or perhaps the existence of subtle structural differences.

Biological activity of the mouse/human hybrids

The COS cell supernatants were assayed in both mouse (murine eosinophil differentiation assay and B13 assay) and human (human eosinophil colony assay and TF-1 assay) IL5 assays. In each assay the native IL5 molecule was scored as 100% active (i.e. hIL5 is 100% active in the human cell assays, and mIL5 is 100% active in the murine cell assays). Figure 5 shows the relative specific activities generated in the TF-1 assay. No significant differences exist between the activities of the hybrids, or the human and mouse molecules in this assay. Similarly, when the samples were assayed in

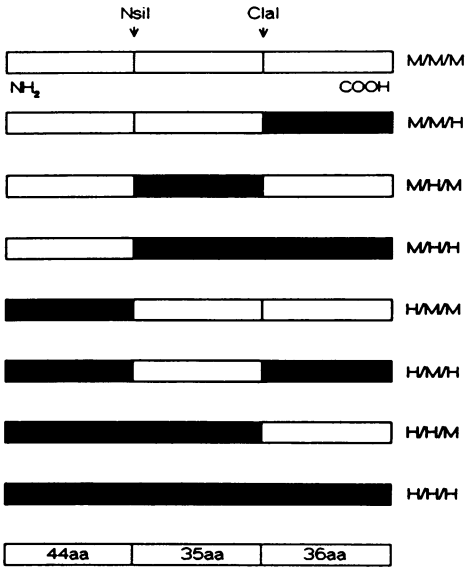

Fig. 2. Schematic diagram of the chimeric human/mouse polypeptides. Open boxes denote mouse derived sequence, shaded areas indicate sequence of human origin.

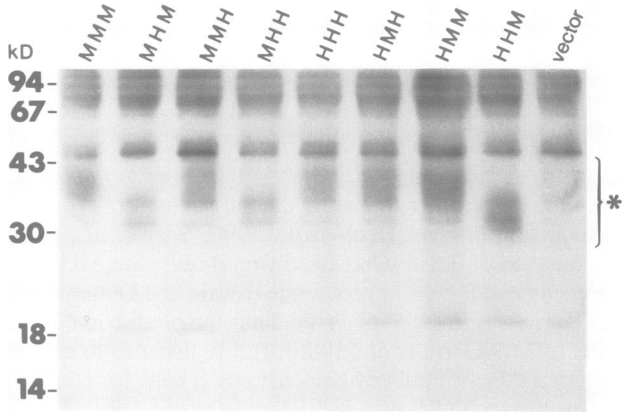

Fig. 3. Fluorograph of metabolically labelled supernatants from COS cells transfected with the hybrid IL5 constructs. All samples were electrophoresed under non-reducing conditions using an 11% SDS–PAGE gel. '*' indicates the wide range of M_r over which IL5 is produced.

the human eosinophil colony assay similar results were obtained, with the human and mouse native sequences having near equivalent activity (Figure 6). It would appear that the construction of the hybrid IL5 molecules does not result in detrimental effects on their functional activities.

When tested in the mouse eosinophil differentiation assay the human construct gave only 1–2% of the activity observed with the murine IL5 (Figure 7). The mutant proteins tended to display activity characteristics similar to either the human or the mouse native sequence. Thus, M/H/M, H/M/M and H/H/M all showed activity more typical to that of the mouse (>90% of that shown by the mouse sequence). In contrast, M/M/H, M/H/H and H/M/H all gave activity similar to that of the human (<15% of that obtained from mIL5). This observation was not restricted to the eosinophil assay; results obtained from the B13 cell line were consistent with those from the EDF assay (Figure 8), though in this assay system human IL5 was slightly more active (4–7% of the activity of the murine material), as were the human-like hybrids. It would appear that some element of the C-terminal region is required for the activity exhibited.

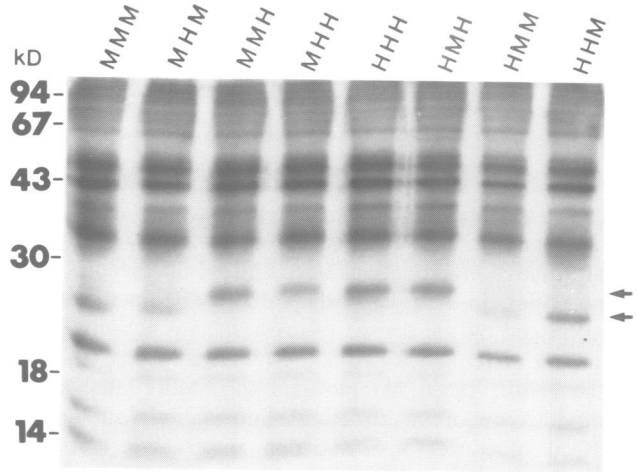

Fig. 4. Fluorograph of metabolically labelled supernatants from COS cells transfected with the hybrid IL5 constructs and grown in the presence of tunicamycin (5 μg/ml). All samples were electrophoresed under non-reducing conditions using an 11% SDS–PAGE gel. Arrows indicate slight difference in electrophoretic mobility between certain constructs.

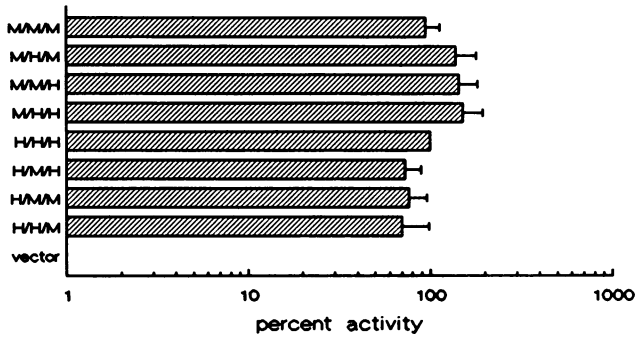

Fig. 5. Comparison of human TF-1 cell proliferation induced by mIL5, hIL5 and human/mouse IL5 hybrids. In this human cell assay human IL5 is 100% active. Each bar represents the mean and standard deviation of activity from supernatants from four separate electroporations.

Even when the first two-thirds of the molecule are mouse-derived, the addition of the C-terminal region of the human IL5 renders the molecule active at a level only equivalent to the native human IL5. The converse is also true. The biological activities displayed by the tunicamycin treated supernatants, in both the human and mouse assays, were directly comparable to those observed from the fully glycosylated constructs (data not shown). This suggests that *N*-linked glycosylation is not essential for biological activity.

Binding of mIL5 to B13 cells by [^{125}I]mIL5 was determined (Figure 9a). Non-specific binding was resolved in the presence of a 100-fold excess of unlabelled mIL5 and was never >10% of total binding. Saturable binding occurred at 1.5 nM. Transformation of these data by Scatchard analysis (Scatchard, 1949) identified two classes (high affinity and low affinity) of IL5 receptors (IL5-R) (Figure 9b). The high affinity receptor had an apparent dissociation constant (K_d) of 50–140 pM and the number of receptors per cell was determined to be 2650 ± 650. The low affinity binding site had a K_d of 2–4 nM and the number of these receptors per cell was 6500 ± 1500.

Competition binding analysis was used to investigate the ability of the hybrid constructs to compete with [^{125}I]mIL5 (25 pM) for the high affinity receptor (Figure 10). As expected the mIL5 competed at equimolar concentrations (50% competition at 1–2 ng/ml), whilst hIL5 was ~7- to

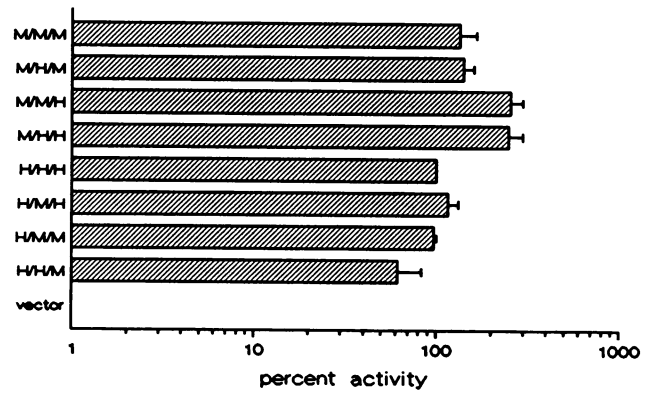

Fig. 6. Comparison of human eosinophil colony production by hIL5, mIL5 and human/mouse IL5 hybrids. In this human cell assay human IL5 is 100% active. Each bar represents the mean and standard deviation of activity from supernatants from two separate electroporations.

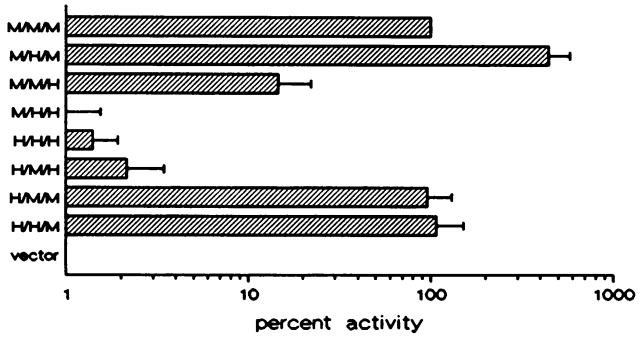

Fig. 7. Comparison of eosinophil differentiation activity induced by mIL5, hIL5 and human/mouse IL5 hybrids. In this murine cell assay mouse IL5 is 100% active. Each bar represents the mean and standard deviation of activity from supernatants from four separate electroporations.

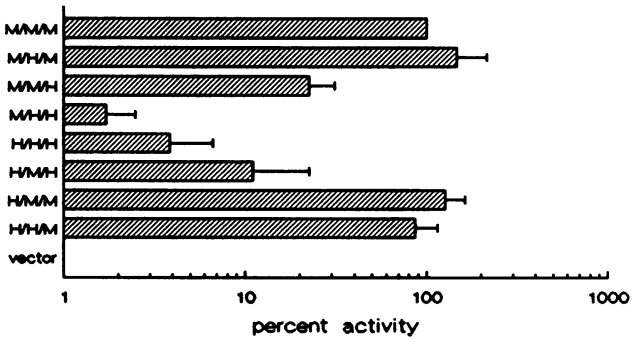

Fig. 8. Comparison of murine B13 cell proliferation induced by mIL5, hIL5 and mouse/human IL5 hybrids. In this mouse assay murine IL5 is 100% active. Each bar represents the mean and standard deviation of activity from supernatants from three separate electroporations.

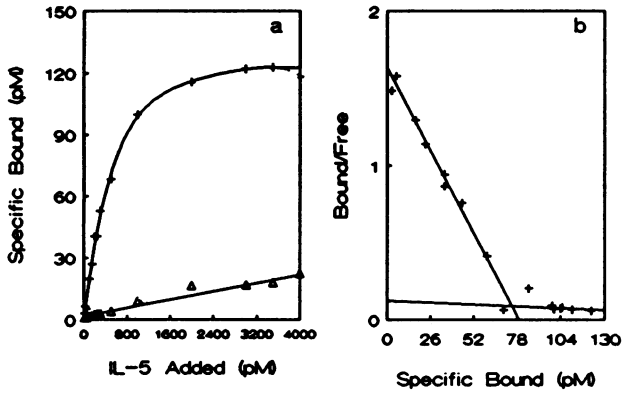

Fig. 9. Binding of [^{125}I]mIL5 to B13 cells. (**a**) Cells (5×10^6/reaction) were incubated with increasing concentrations of [^{125}I]mIL5 at 37°C for 30 min. '+' indicates total binding, △ non-specific binding. (**b**) Scatchard analysis of the binding data shown in (a).

10-fold less efficient at displacing the murine IL5 (50% competition at 15 ng/ml). The hybrid constructs competed for the murine IL5-R in a manner closely mirroring their biological activity. Hence, M/H/M and H/M/M were ≥100% active, closely following the competition curve of the mIL5, with 50% competition occurring at 2 ng/ml. As the biological activity of the constructs decreased so did their ability to compete for the IL5-R: H/H/M (50% competition 2–4 ng/ml); M/M/H (50% competition 11 ng/ml); H/M/H (50% competition 13 ng/ml) and M/H/H (50% inhibition >15 ng/ml). Once again the differences correlate to the presence of the species-specific C-terminal region.

Discussion

Structure–function analyses of cytokines have made use of a variety of techniques, often in the absence of three-dimensional structural determinations, to define regions of the molecules involved in the production of biological activity or receptor interaction. The aim has been to change amino acids to identify critical residues. Sequence deletions have been used in a number of studies (Gough *et al.*, 1987; Clark-Lewis *et al.*, 1988; Zurawski and Zurawski, 1988; Shanafelt and Kastelein, 1989; Ibanez *et al.*, 1990), but suffer from the inherent problem of also causing structural changes

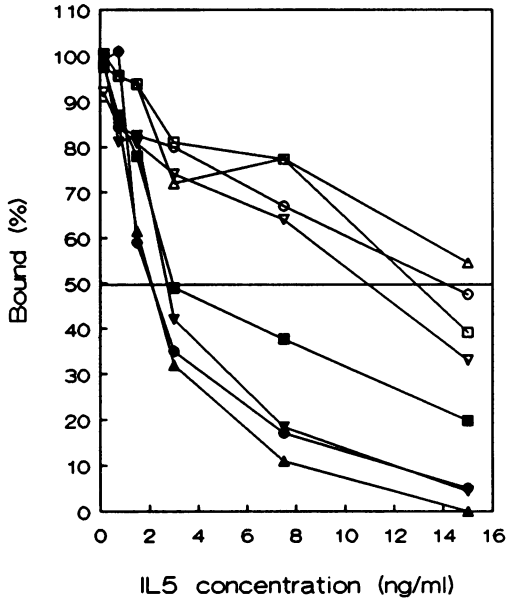

Fig. 10. Inhibition of binding of [^{125}I]mIL5 to murine B13 cells by mIL5, hIL5 or mouse/human IL5 hybrids. Cells were incubated with [^{125}I]mIL5 (750 pg/ml) and various concentrations of unlabelled IL5 constructs. M/M/M (▲), M/H/M (●), H/M/M (▼) and H/H/M (■) all show 50% inhibition in the range 2–4 ng/ml. H/H/H (○), M/M/H (▽), M/H/H (△) and H/M/H (□) all display 50% inhibition in the range 10–16 ng/ml.

to the whole molecule. These problems may also occur where structurally important amino acids are changed, either to alanine or glycine (Gething *et al.*, 1988). More informative investigations have utilized interspecies hybrid molecules. In these cases the value of the data is strongly influenced by the degree of cross-species activity and the amount of sequence homology (Kaushansky *et al.*, 1989; Lax *et al.*, 1989). Amino acid substitution analysis also provides important structural information, allowing the effects of conservative and non-conservative amino acid changes within a molecule to be assessed (Zurawski and Zurawski, 1989; Zurawski *et al.*, 1990).

We have utilized the differences in cross-species activity of mIL5 and hIL5 on mouse cells to identify regions responsible for this difference. The near identical activity of these molecules on human cells has been exploited as an internal control, in the belief that any amino acid changes that retain this equivalent activity are unlikely to cause deleterious conformational changes. A panel of human/mouse IL5 hybrid molecules was generated which displayed biological activity and binding kinetics similar to either the hIL5 protein or the mIL5 protein. Despite the changes made during the construction of the hybrids the biological activity exhibited by all of the constructs was not significantly altered in the human assays, suggesting that the 70% amino acid sequence similarity between the mouse and human molecules retains similar tertiary conformation, even when species domains are substituted. This is consistent with the secondary structural predictions based on the Robson–Garnier algorithm which indicate high structural conservation between the human and mouse IL5 polypeptides (Sanderson, 1990). The common factor leading to the species-specific phenotypes displayed against mouse cells is the presence of the mouse or human C-terminal region. Thus, replacing the

human C terminus with the mouse C terminus resulted in the hybrid displaying activity comparable to the murine sequence. When this type of exchange was performed the host sequence was changed only by eight amino acids [i.e. the differences between the host and donor—of these, six are conservative changes and only two are non-conservative substitutions (Figure 1)]. The resultant hybrid therefore contains 93% amino acid sequence similarity to the host, but its biological activity and binding kinetics are now donor specific. The converse replacement in the murine sequence results in a marked reduction in activity. While the competition binding data correlate with the activity results, the 10-fold differences in binding efficiency do not completely explain the 20- to 70-fold differences in bioactivity. These may be explained by signalling effects perhaps as a result of differential receptor association and occupation inducing a different magnitude of response.

The results from the human/mouse hybrid molecules certainly indicates that the C terminus is important in conferring the species specificity of IL5. Whilst this might be the result of an indirect interaction, the maintenance of activity on human cells, and the high sequence conservation in the M/M/H and H/H/M hybrids is consistent with this region being involved in direct interaction with the receptor. The analogous results obtained in the mouse EDF and mouse B cell assays suggest that the active site of the molecule is probably recognized in a comparable way by these cell types.

No significant homology has been identified between IL5 and any other proteins. However, by searching for similarity rather than homology to the IL5 sequence, using a matrix derived from evolutionary mutation (Dayhoff *et al.*, 1972), a small area of similarity is detected near the C-terminal end of IL5, IL3, GM-CSF, IL4 and IL6 (Sanderson *et al.*, 1988) (Figure 1). Within this group of growth factors IL3 and GM-CSF have been shown to interact with what appears to be a common receptor subunit, perhaps suggesting the existence of common structural elements between these polypeptides (Lopez *et al.*, 1989). Crosslinking studies using IL5 have identified at least two specifically crosslinked polypeptides, suggesting that IL5 associates with more than one polypeptide at the cell membrane (Rolink *et al.*, 1989; Mita *et al.*, 1989b). Recently, a cDNA encoding a mIL5-R has been cloned (Takaki *et al.*, 1990), which when transfected into COS cells only expressed a low affinity IL5 receptor. However, when it was transfected into an IL3 dependent cell line (IL5 unresponsive) the high affinity form of the receptor was also observed, suggesting the involvement of other receptor chains perhaps in common with other cytokines. It would appear therefore that IL5 may have sites which interact with at least two receptor chains. The region of apparent relatedness is encompassed by the C-terminal hybrids and contains only two amino acid changes between the mouse and human IL5 sequences.

Recent studies have identified IL5 as an antiparallel homodimer (Minamitake *et al.*, 1990; McKenzie *et al.*, 1991) and reported that the monomeric form is biologically inactive (McKenzie *et al.*, 1991) suggesting that dimer formation is essential for biological activity. These data suggest that IL5 may have two receptor binding sites located at the C terminus of each monomer, both of which may be required to stimulate a biological response, perhaps in a similar way to the signalling event of PDGF (Heldin *et al.*, 1989).

Materials and methods

Reagents

Restriction endonucleases and other DNA modifying enzymes were obtained from Northumbria Biologicals Limited (Cramlington, UK). [^{35}S]Methionine (29.6 TBq/mmol) was obtained from Amersham International plc (Amersham, UK).

Molecular biological manipulations were performed as outlined in Sambrook *et al.* (1989). PCR reactions were carried out in an 'intelligent' heating block (Cambio, Cambridge, UK) for one cycle consisting of 5 min at 93°C, 0.5 min at 40°C and 2 min at 72°C, followed by 30 cycles each consisting of 0.1 min at 93°C, 0.5 min at 40°C and 2 min at 72°C, and completed by 5 min at 72°C. Reactions were performed in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM of each deoxyribonucleotide, and 2.5 U *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, USA). Reactions (100 μl) were covered with mineral oil before thermal cycling.

Preparation of constructs

To generate the mouse/human chimeras, two restriction sites were introduced at homologous positions in both the human and mouse IL5 cDNA. Two human cDNA constructs were utilized, BBT/hIL5 (BBG 16) (British Biotechnology Ltd, Oxford, UK) which lacks the leader sequence, and the native hIL5 sequence (198–458). An *Nsi*I site was introduced at nucleotide position 180 (numbering from the initiation methionine of the native hIL5) using the oligonucleotide 5′-TTCTTCAaTGCAtAGTTGGT-3′ (the mutated nucleotides are in lower case). This mutation in the hIL5 cDNA changed Thr45 to Ile which is present at this position in mIL5 cDNA. The murine IL5 cDNA naturally contains an *Nsi*I site. A *Cla*I site was introduced at position 290 in the murine cDNA using the oligonucleotide 5′-TTTTGG-CGaTCgATGTATT-3′. The BBT/hIL5 sequence already contains a *Cla*I site at this position. Site-directed mutagenesis was performed on single-stranded M13mp19 clones containing either of the human sequences or the mouse sequence, using the method of Kunkel *et al.* (1987). After selection the mutant M13 phage plaques were verified by DNA sequence analysis using the dideoxy chain termination method (Sanger *et al.*, 1977). The mutated native murine cDNA (mIL5M) was isolated from the replicative forms of the phages as an *Eco*RI–*Bam*HI fragment and ligated into pUC19. The mutated BBT/hIL5 (BhIL5M) was isolated as a *Hin*dIII–*Bam*HI fragment and ligated into pUC18. The mutated native human IL5 cDNA was isolated from the single-stranded phage by PCR using two oligonucleotides 5′-ACGACGGCCAGTGAATTCCTGAAGACTTCA-3′ (to prime from the 5′ end and give an *Eco*RI restriction site) and 5′-GTCCTGT-CACCATGGATCCGCTCAGCCTCA-3′ (to prime from the 3′ end and give a *Bam*HI restriction site). The isolated fragment was ligated into pUC19. The pUC.M/H/M construct was generated by ligating the *Nsi*I–*Cla*I fragment (~100 bp) from BhIL5M into mIL5M from which the *Nsi*I–*Cla*I fragment was removed by agarose gel electrophoresis. The pUC.M/H/H and pUC.M/M/H constructs were prepared in a similar manner except that an *Nsi*I–*Bam*HI fragment was inserted to give the former and a *Cla*I–*Bam*HI fragment was ligated to give the latter. The pUC.H/M/H construct was generated by ligating the *Cla*I–*Bam*HI fragment from pUC.M/M/H into hIL5M from which the *Nsi*I–*Bam*HI was removed by agarose gel electrophoresis. The pUC.H/M/M and pUC.H/H/M were constructed in a similar manner except that an *Nsi*I–*Bam*HI fragment from mIL5M was inserted into hIL5M to give the former and an *Nsi*I–*Bam*HI fragment from pUC.M/H/M was inserted into hIL5M to give the latter. The correct fragment insertions were detected using unique restriction enzyme sites or by sequencing. Once constructed the coding region from each construct was isolated, using PCR to incorporate the relevant restriction sites at their 5′ and 3′ ends, and ligated into the COS cell expression vector pcEXV-3 (Miller *et al.*, 1985). As a control the hIL5M and mIL5M cDNA sequences used to construct the mutants were also cloned into the same vector. PCR reactions were performed as above except that the oligonucleotide 5′-ACGACGGCCAGTGAATTCCTGAAGACT-3′ (used to prime at the 5′ end and give an *Eco*RI restriction site) was combined with either 5′-CATAGATAGGTTGAATTCACTACAGGACAT-3′ (used to prime at the 3′ end of pUC.M/H/M, pUC.H/H/M and pUC.H/M/M and give an *Eco*RI restriction site) or 5′-GACTCTAGAGGAGAATTCTCAACTTTC-TATT-3′ (used to prime at the 3′ end of pUC.M/H/H, pUC.M/M/H and pUC.H/M/H and give an *Eco*RI restriction site).

Determination of relative specific activities

Monkey COS-1 cells were grown and transfected (Campbell *et al.*, 1988), using the vector pcEXV-3 as a control. Culture supernatants were harvested 3 days after transfection and the cells washed with methionine-free RPMI

+ 10% (v/v) dialysed fetal calf serum and then incubated for 2 h in this medium. The cells were then washed gently three times with methionine-free RPMI to remove the serum-containing medium and then incubated in methionine-free RPMI containing 4 MBq [^{35}S]methionine/ml. This culture supernatant was harvested 4 h later. In some experiments tunicamycin (5 μg/ml) was included in the medium when the radiolabelled methionine was added.

The proteins secreted into the supernatant were then analysed by SDS–PAGE and fluorography by standard procedures. The fluorographs were then scanned using a Chromoscan (Joyce Loebl, Dusseldorf, Germany) to determine the concentration of IL5 in each supernatant. The concentrations were then normalized to that of murine IL5 allowing the specific activity of each sample to be determined. Four separate electroporations and protein analyses were performed upon each construct.

Assays for biological activity

The eosinophil differentiation activity was assayed by incubating samples in microplates with bone marrow from *Mesocestoides corti* infected mice for 5 days (Strath *et al.*, 1990). Eosinophil numbers were determined indirectly by assaying for eosinophil peroxidase. Absorbance (A_{492}) was measured in a plate reader.

The murine B cell growth factor activity of each sample was assayed using the pro-B cell line B13 (Rolink *et al.*, 1989). Sample dilutions were incubated in microplates with B13 cells. Proliferation was determined by measuring, in a fluorescence plate reader, the conversion of methyl umbelliferol heptonate to methyl umbelliferol (Dotsika and Sanderson, 1987).

The human erythroleukaemic cell line TF-1 responds to several cytokines, including IL5 (Kitamura *et al.*, 1989). Sample titrations were incubated in microplates with TF-1 cells. Proliferation was determined using either MTT (Mosmann, 1983) or the uptake of tritiated thymidine.

The human eosinophil colony assays were performed in semi-solid agar containing human bone marrow cells. After 14 days the eosinophil colonies were stained with Luxol fast blue and counted (Strath *et al.*, 1990).

The activity, in units, from each assay is defined as the recriprocal of the dilution giving 50% of the maximal response in the assays.

Mouse IL5 receptor binding assays

Purified recombinant murine IL5 (Barry *et al.*, in preparation) was iodinated using a modified free iodine technique (Nicola and Metcalf, 1984). The specific activity of the ^{125}I-labelled mIL5 was determined to be 1.9×10^7 c.p.m./μg protein. The IL5 bioactivity of the iodinated sample was similar to that present before labelling.

Binding assays were performed using log-phase B13 cells growing in the presence of 3 U of mIL5. Before use the cells were washed three times in IL5-free medium and then incubated for 4 h in this medium to allow IL5/IL5-receptor complexes to be internalized. The cells were then washed twice in RPMI 1640 before being resuspended in binding medium (RPMI 1640, 0.5% BSA, 20 mM HEPES, pH 7.6) at 1×10^8 cells/ml. Iodinated IL5 samples were aliquoted into siliconized Eppendorf tubes with binding medium to a final volume of 150 μl. 50 μl of cells (5×10^6) were added, mixed, and incubated at 37°C for 30 min. The samples were mixed at 15 min intervals, and at the end of the incubation period were overlaid onto 100 μl of 84% silicone oil: 16% paraffin oil mixture in 500 μl microcentrifuge tubes. The tubes were spun at 1500 *g* for 3 min and cut just above the cell pellet. Cell associated and free radioactivity were determined by counting in a gamma counter (LKB Rackgamma), and specific binding calculated by subtraction of non-specific binding in the presence of 100-fold excess of unlabelled IL5. Duplicate data were transformed by Scatchard analysis (Scatchard, 1949) to determine receptor copy number and dissociation constants. Specific radioactivity of labelled IL5 was determined by competition of a fixed concentration of [^{125}I]IL5 (25 pM) with increasing amounts of unlabelled IL5. Competition binding analysis was performed using a fixed concentration of [^{125}I]mIL5 (25 pM) with increasing concentrations of cold mIL5, hIL5 and hybrid constructs being used to compete for the receptor. The results are expressed as the means of four individual values.

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