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Detailed analysis of the IL-5–IL-5Rα interaction: characterization of crucial residues on the ligand and the receptor

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The receptor for interleukin-5 (IL-5) is composed of two different subunits. The IL-5 receptor α (IL-5Rα) is required for ligand-specific binding while association with the β-chain results in increased binding affinity. Murine IL-5 (mIL-5) has similar activity on human and murine cells, whereas human IL-5 (hIL-5) has marginal activity on murine cells. We found that the combined substitution of K84 and N108 on hIL-5 by their respective murine counterpart yields a molecule which is as potent as mIL-5 for growth stimulation of a murine cell line. Since the unidirectional species specificity is due only to the interaction with the IL-5Rα subunit, we have used chimeric IL-5Rα molecules to define regions of hIL-5Rα involved in species-specific hIL-5 ligand binding. We found that this property is largely determined by the NH_2-terminal module of hIL-5Rα, and detailed analysis defined D56 and to a lesser extent E58 as important for binding. Moreover, two additional residues, D55 and Y57, were identified by alanine scanning mutagenesis within the same region. Based on the observed homology between the NH_2-terminal module and the membrane proximal (WSXWS-containing) module of hIL-5Rα we located this stretch of four amino acid residues (D55, D56, Y57 and E58) in the loop region that connects the C and D β-strands on the proposed tertiary structure of the NH_2-terminal module. Finally, by comparison with residues involved in ligand binding on the elucidated structure of the growth hormone–growth hormone receptor complex, residue R188 on hIL-5Rα was identified as contributing to ligand interaction.

Key words: interleukin-5/interleukin-5 receptor/ligand binding/mutagenesis/species specificity

Introduction

Interleukin-5 (IL-5) is a cytokine secreted mainly by T lymphocytes (TH_2 cells) during allergic immune responses (Walker *et al.*, 1991). It plays a crucial role in the differentiation, expansion and maturation of eosinophils (for review see Sanderson *et al.*, 1992). Only in the murine system, IL-5 also exerts activities on the B lymphocyte lineage (for review see Clutterbuck *et al.*, 1987; Takatsu *et al.*, 1988). Murine IL-5 (mIL-5) has similar biological activity in mouse as in human IL-5 bioassays. In contrast, human IL-5 (hIL-5) shows more species restriction and has a very low specific activity on mouse IL-5-responsive cells (Tavernier *et al.*, 1989; Mckenzie *et al.*, 1991). Previously it was demonstrated by Mckenzie *et al.* (1991) that the C-terminal part of the hIL-5 molecule is involved in the observed species specificity. Here we show more precisely that residues K84 and N108 are responsible for this phenomenon. Mutating these residues of hIL-5 to their mouse counterpart generates a molecule that is as potent in a mouse bioassay as mIL-5.

The human as well as the murine receptors for IL-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) are composed of two different subunits: a cytokine-specific α-chain, which binds ligand with low affinity and a common β-chain (for human referred to as βc; for mice as AIC2B) which by itself has no detectable binding activity but converts the low-affinity receptors to high-affinity forms (Hayashida *et al.*, 1990; Devos *et al.*, 1991; Kitamura *et al.*, 1991; Takaki *et al.*, 1991; Tavernier *et al.*, 1991; Hara *et al.*, 1992; Murata *et al.*, 1992; Park *et al.*, 1992) and which is necessary for signal transduction (Sakamaki *et al.*, 1992; Sato *et al.*, 1993). This common β-chain provides a molecular basis for the overlapping activities of IL-5, IL-3 and GM-CSF on eosinophils (Lopez *et al.*, 1991).

Apart from ligand binding, the α receptor subunits are also required for signal transduction (Sakamaki *et al.*, 1992; Takaki *et al.*, 1993; Weiss *et al.*, 1993; Cornelis *et al.*, submitted), suggesting that these ligand-specific receptor chains may be responsible for transmission of more specific cytokine signals.

Structurally the α and β receptor subunits of IL-3, IL-5 and GM-CSF belong to the cytokine receptor superfamily which is characterized by an ~200 amino acid extracellular motif, the hematopoietin domain. This domain constitutes two FN-III-like modules, each predicted to contain seven antiparallel β-strands arranged in two sheets (Bazan, 1990). This prediction is to a large extent confirmed in the case of the growth hormone receptor (GHR), the three dimensional structure of which has been elucidated (de Vos *et al.*, 1992). Four cysteine residues, involved in two Cys bridges in the first module, and a WSXWS sequence in the second module, are the most typical conserved features. The extracellular portions of AIC2B and βc consist of two repeats of the hematopoietin domain (Gorman *et al.*, 1990; Hayashida *et al.*, 1990), whereas the extracellular parts of IL-5Rα, GM-CSFRα and IL-3Rα contain one copy and an additional NH_2-terminal extension of 90–100 amino acid residues (Gearing *et al.*, 1989; Takaki *et al.*, 1990; Kitamura *et al.*, 1991; Tavernier *et al.*,

Table I. Eight not-conserved amino acid residues in the C-terminal region of human IL-5 with their corresponding mouse residues

Position	Human residue	Mouse residue
81[a]	Gly	Arg
84	Lys	Glu
93	Val	Thr
94	Asn	Arg
108	Asn	Ser
112	Ile	Ala
113	Ile	Met
115	Ser	Gly

[a]Numbering is based on the human sequence.

1991, 1992; Hara *et al.*, 1992; Park *et al.*, 1992). For hIL-5Rα we noticed that this latter region and the WSXWS module share several conserved structural motifs (Tuypens *et al.*, 1992; see below), suggesting that it adopts a similar tertiary structure.

Based on binding data of the structurally related GHR (de Vos *et al.*, 1992) and IL-6R (Yawata *et al.*, 1993), it has been suggested that the hematopoietin domains on the α receptor subunits of IL-3, IL-5 and GM-CSF contain the entire ligand-binding pocket (Goodall *et al.*, 1993). However, here we demonstrate that a sequence of four residues, D55, D56, Y57 and E58, located in the NH_2-terminal region of the extracellular portion of hIL-5Rα, is crucial for interaction with hIL-5.

Results

Identification of residues on hIL-5 responsible for species specificity

McKenzie *et al.* (1991) demonstrated that the C-terminal region of hIL-5 is responsible for its species-specific behaviour. A human/mouse molecule with a C-terminal region (C-terminal 36 residues, position 79–115) which originates from the mouse sequence exerts similar biological activities as native mIL-5. In hIL-5 only eight residues are not conserved between human and mouse within this region (Table I). In order to determine the crucial amino acid(s), we mutated each of the eight residues to their corresponding mouse counterpart. The single point mutants were expressed in Sf9 cells and quantitated as described (Tavernier *et al.*, 1994). Two mutants could not be analysed: G81R which is unstable at 37°C, and V93T which was only poorly expressed (not shown).

Figure 1 demonstrates the observed biological activities of K84E, N94R, N108S, I112A, I113M and S115G in a human and a mouse proliferation assay. For this we used respectively hIL-5-dependent FDC-P1-CA1 cells, a cell line derived from FDC-P1 by introducing the hIL-5Rα subunit and mIL-5-dependent B13 cells. Both cell lines contain high affinity IL-5 receptors made up of the endogenous mouse β-chain (AIC2B) and the human (FDC-P1-CA1) or mouse (B13) α-chain. On FDC-P1-CA1 cells, hIL-5 and mIL-5 have equivalent specific activities (Figure 1A) while B13 cells are at least 100-fold less sensitive to hIL-5 (Figure 1B) (0.1 ng/ml mIL-5 and 10 ng/ml hIL-5 to achieve half maximal stimulation, Figure 1C). This observation demonstrates that the species specificity of hIL-5 is determined by the α subunit and suggests that

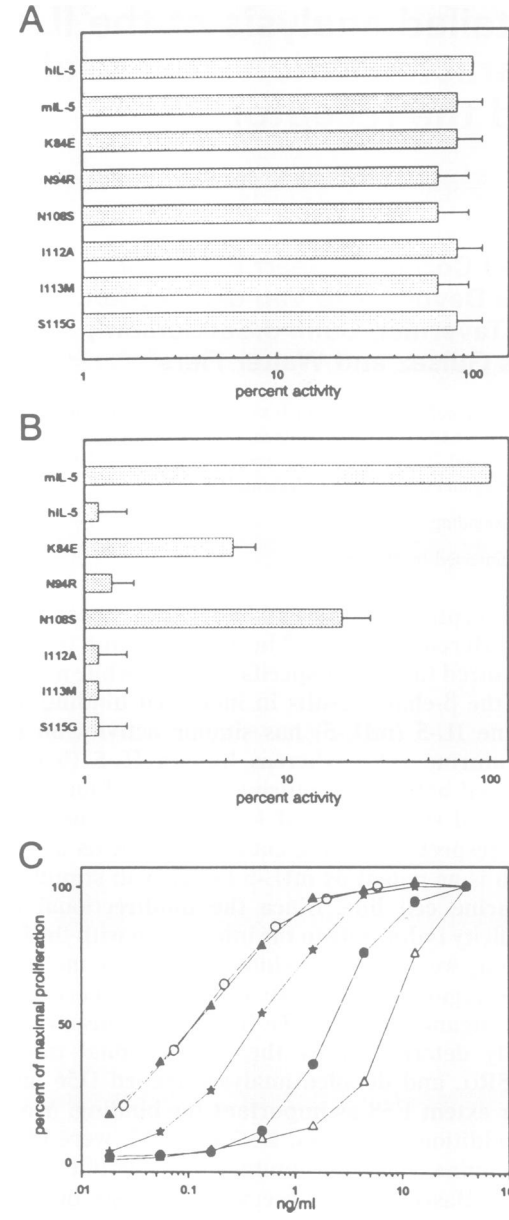

Fig. 1. Specific activities of hIL-5, mIL-5 and hIL-5 mutants in a human and a mouse proliferation assay. Comparison of human FDC-P1-CA1 cell proliferation (**A**) and mouse B13 cell proliferation (**B**) induced by hIL-5, mIL-5 and hIL-5 mutants. hIL-5 and mIL-5 are 100% active in the human and mouse assay, respectively. All data represent the average of two or more independent experiments. (**C**) Proliferative responses of B13-cells to hIL-5 (△), mIL-5 (○), hIL-5-K84E (●), hIL-5-N108S (★) and hIL-5-K84E/N108S (▲).

the murine β-chain (AIC2B) does not discriminate between murine and human IL-5. Figure 1A shows that all six tested chimeric molecules stimulated the proliferation of FDC-P1-CA1 cells to a similar extent compared with human and mIL-5. However, on the murine B13-cell line, K84E and N108S showed an enhanced responsiveness compared with hIL-5, while the other four mutants displayed specific activities similar to hIL-5 (Figure 1B). The specific activity of K84E was improved by ~5-fold, whereas that of N108S was improved by ~20-fold (Figure 1B) (2 ng/ml K84E and 0.5 ng/ml N108S in comparison with 10 ng/ml hIL-5 to achieve half maximal stimulation, Figure 1C). Combination of both individual mutations in

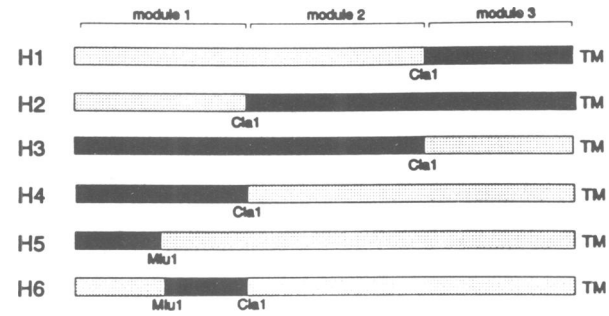

Fig. 2. Schematic diagram of the extracellular regions of the chimeric human/mouse polypeptides. The three predicted FN-III-like modules are indicated. Shaded and filled boxes denote human and mouse derived sequences, respectively. Introduced restriction sites used to generate the chimeric polypeptides are shown. TM, transmembrane region.

the double mutant K84E/N108S resulted in a mutant molecule which is as active on B13 cells as mIL-5 (Figure 1C).

A sequence of residues D55, D56, Y57, E58 located in the NH_2-terminal module of hIL-5Rα is involved in ligand interaction

The above results indicated that the species-specific property of hIL-5 is only restricted to the interaction with the α-chain. In order to identify residues involved in species-specific ligand interaction on hIL-5Rα we constructed a panel of human/mouse hybrids as shown in Figure 2. To avoid major conformational changes in the chimeric receptor molecules, the regions to be exchanged were chosen according to the proposed structural model of the extracellular part of IL-5Rα (Tuypens *et al.*, 1992). This part of the receptor is predicted to consist of three FN-III-like modules, two of them forming the hematopoietin domain and an additional NH_2-terminal module. The introduced *Cla*I restriction sites in hybrids H1, H2, H3 and H4 are located in the linker regions in between two modules (positions 108 and 216). The *Mlu*I site in hybrids H5 and H6 is created in between two predicted β-sheets of the first module (position 59). The six hybrid cDNAs were transfected in COS1 cells and [^{125}I]hIL-5 and [^{125}I]mIL-5 binding was evaluated. First, H1, H2, H3 and H4 were analysed. Figure 3A shows that these chimeric receptors bound [^{125}I]mIL-5 as efficiently as the native mouse receptor, indicating appropriate folding and expression of the hybrids, whereas [^{125}I]hIL-5 binding was substantially decreased (Figure 3B). In order to calculate the dissociation constants (K_d) of [^{125}I]hIL-5 binding on the transfectants by Scatchard analysis, the same cells were incubated with a serial dilution of [^{125}I]hIL-5 (Table II). The decreased binding affinities on H1 (K_d = 12 nM) and H4 (K_d: 50–100) compared with the native receptor (K_d = 1 nM) showed that both the C-terminal and the NH_2-terminal module contribute to species-specific ligand binding. From the K_d values measured on H2 and H3, we deduced that the second module is also involved. H2 and H3 showed a lower binding affinity for hIL-5 compared with H1 and H4, respectively. Since both H3 and H4 demonstrated the strongest reductions (Figure 3B, Table II) we concluded that the species specificity is mostly dependent on the NH_2-terminal module.

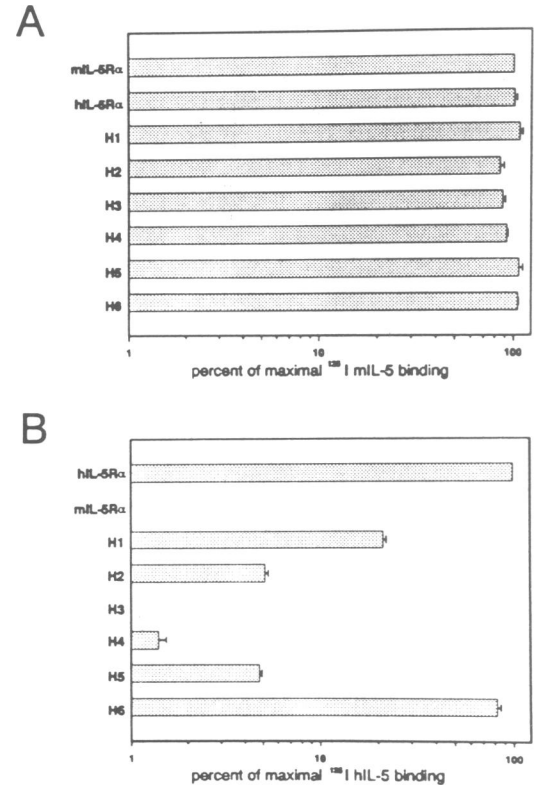

Fig. 3. [^{125}I]IL-5 binding on human/mouse hybrids of IL-5Rα. COS1 transfectants were incubated with [^{125}I]mIL-5 (**A**) or with [^{125}I]hIL-5 (**B**) at a concentration of 5 nM in the absence (total binding) or presence (non-specific binding) of excess unlabeled protein for 1 h at 4°C. 100% specific binding activity (total–non-specific) corresponds to [^{125}I]mIL-5 binding on mouse IL-5Rα in (A) and to [^{125}I]hIL-5 binding on human IL-5Rα in (B). All data represent the average of two or more independent experiments.

Table II. Equilibrium dissociation constants (K_d) of [^{125}I]hIL-5 binding on human/mouse chimeric IL-5 receptors and alanine substitution mutant IL-5 receptors with decreased hIL-5 binding affinity

Transfectant	K_d (nM)	Transfectant	K_d (nM)
Human IL-5Rα	1	D56E	40
		E58D	2
H1	12	D56E/E58D	50
H2	50		
H3	>100	D55A	[a]–
H4	50–100	D56A	50–100
H5	50	Y57A	[a]–
H6	2	E58A	4
		R188A	>100

COS1 cells were transfected with wild type or mutant IL-5Rα. Receptor binding assays were performed as described in Material and methods.
[a]No specific binding was detected.

In order to narrow down this region we introduced an *Mlu*I restriction site in H5 and H6 as shown in Figure 2. Only on the hybrid where the first half corresponds to the mouse sequence (H5) was the binding of hIL-5 decreased substantially compared with the wild type receptor, indicating that this region contains residues important for hIL-5 binding (Figure 3B, Table II). To localize these, all the amino acid residues in the first half of the NH_2-terminal module (from position 1–59) of hIL-5Rα different from

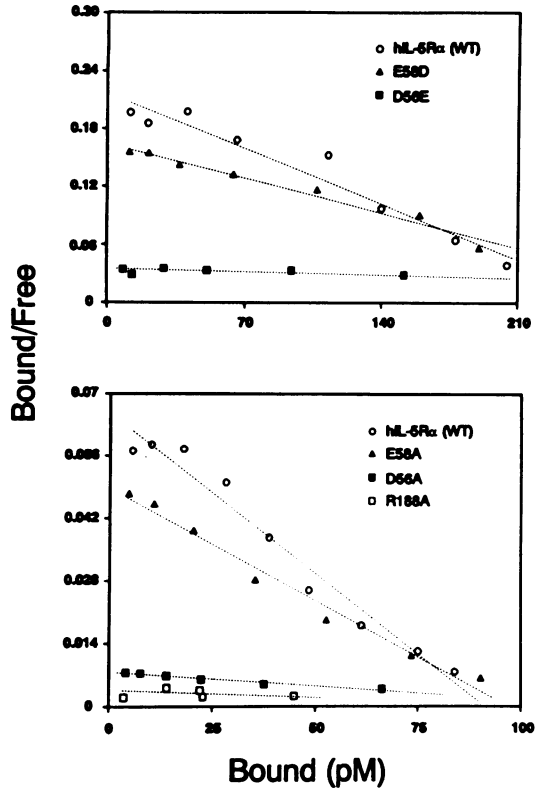

Fig. 4. Binding characteristics of mutant IL-5Rα expressed on COS1 transfectants. Scatchard plot analysis of [^{125}I]hIL-5 on COS1 cells transfected with the cDNAs encoding the interspecies mutants (upper) and with the cDNAs encoding the alanine mutants (lower). These binding data are representative for several independently performed experiments. The respective calculated affinities (K_d values) are listed in Table II.

those of mIL-5Rα were replaced with their corresponding mouse residues. Figure 5A displays the amino acid sequence of the NH_2-terminal module of hIL-5Rα (hIL-5R mod.1). Differing mouse residues are written above. Fourteen inter-species mutants were generated (P4N, D5H, E6K, I8F, S9L, V20A, Q29H K31D, N40H, N42D, Q46H, K53Q, D56E and E58D), transfected into COS1 cells and examined for [^{125}I]mIL-5 and [^{125}I]hIL-5 binding. Most of these mutants bound hIL-5 with an affinity similar to the native receptor (not shown). However, as shown in Figure 4 and Table II, the conservative replacement of residue D56, aspartic acid to glutamic acid, had a dramatic effect on hIL-5 binding (K_d = 40 nM). This means that the strongly reduced binding affinities of H4 and H5 for hIL-5 can be simulated to a large extent by one conservative mutation, D56E (Table II). Another conservative mutation, E58D, resulted in a 2-fold decrease in hIL-5 binding affinity (Figure 4 and Table II). This minor reduction has consistently been observed throughout several independent experiments. The combination of both mutations D56E and E58D in the double mutant D56E/E58D had an effect similar to the single mutation D56E (Table II).

Next, an alanine scanning mutagenesis was carried out across the NH_2-terminal module to search for other residues important for ligand binding in this region. In Figure 5A all amino acids which were individually substituted are underlined. The mutant cDNAs were transfected into COS1 cells and analysed for [^{125}I]hIL-5 binding. The mutants with a reduced hIL-5-binding affinity are listed in Table II with their respective K_d values. Scatchard plots of some of them are shown in Figure 4. We observed that the alanine substitutions of the two residues involved in species specificity, D56A and E58A, had a stronger reducing effect on the affinity for hIL-5 compared with the inter-species mutants (Figure 4 and Table II). In addition, we found that the mutations D55A, Y57A, H86A and H99A completely abolished hIL-5 binding. As a control for the expression and structural integrity of the mutant receptors, the COS1 transfectants were tested for staining by flow fluorocytometry with several FITC-labeled monoclonal anti-hIL-5Rα antibodies (not shown). The same results were obtained with the different antibodies. Cells transfected with H86A or H99A were not stained, suggesting both receptor mutants were not expressed at the surface of the transfected COS1 cells (not shown). Figure 6 shows the cytofluorometric analysis of D55A, D56A, Y57A and E58A with α16, a non-neutralizing antibody which recognizes the native receptor as well as a deletion mutant lacking the NH_2-terminal module, and demonstrates that the four receptor mutants were as well expressed as the native receptor. This suggests that a stretch of four adjacent residues (D55, D56, Y57 and E58) is involved in the interaction with hIL-5.

Identification of a critical residue in the second module of hIL-5Rα, based on the structure of the GHR–GH complex

Members of the cytokine receptor family share conserved structural motifs in the extracellular portion (Bazan, 1990). With an appropriate alignment to the amino acid sequence of GHR one can hypothesize regions on related receptors which may be involved in ligand interaction based on the binding data of the three-dimensional structure of the GH–GHR complex. Putative binding determinants in the second and third module of hIL-5Rα were localized in this way and five such residues were selected and substituted by alanine or glycine in three single mutants and one double mutant: Q130A, R188A, H214G/A215G and I247A. According to our alignment, these residues correspond to R43, W104, D126, E127 and W169, respectively, shown to be involved in ligand interaction on the GHR (de Vos *et al.*, 1992).

The five mutant cDNAs were transfected into COS1 cells and Scatchard analysis of [^{125}I]hIL-5 binding was performed as before. As shown in Figure 4 and Table II, mutant R188A showed a strongly reduced hIL-5 binding affinity, while the other mutations had no effect (not shown). Cell surface expression of the receptor mutant R188A was confirmed by FACS analysis with the anti-hIL-5Rα antibody α16 (Figure 6). R188 corresponds to W104 of GHR (Figure 5B), which is localized in a segment which forms a loop joining the E and F β-strands of the NH_2-terminal module (de Vos *et al.*, 1992).

Discussion

hIL-5 is species restricted in its binding and biological activities: it has a very weak affinity for the mouse α receptor and marginal specific activity in mouse bioassays. Mckenzie *et al.* (1991) demonstrated that the observed

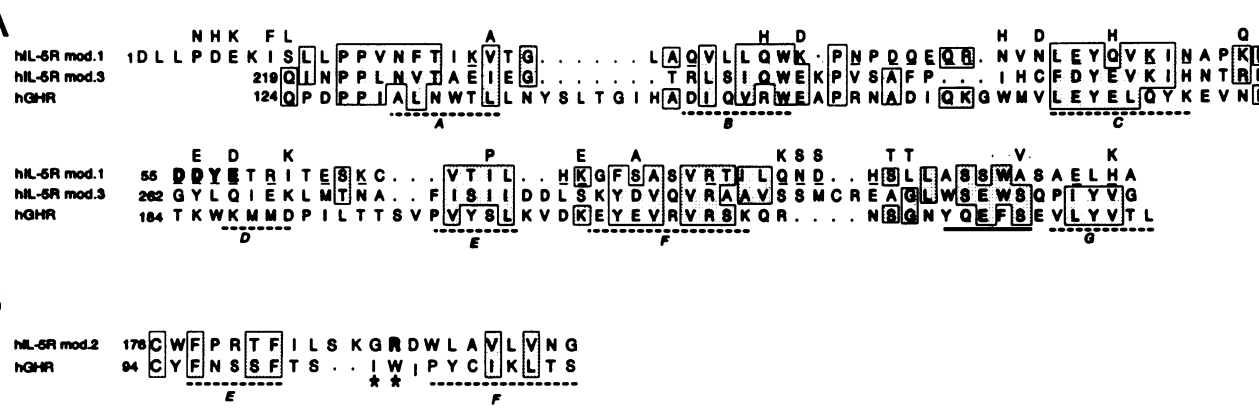

Fig. 5. Sequence alignment of hIL-5Rα with GHR and localization of residues which interact with hIL-5. (**A**) Alignment of the NH_2-terminal module of hIL-5Rα to the WSXWS containing module of hIL-5Rα and to the membrane proximal module of GHR. Gaps introduced to enhance the alignment are indicated by dots, the approximate locations of the β-strands are underlined with dashes (Bazan, 1990) and labelled A–G. Regions of conservation are shaded and amino acids are numbered at the left. The typical WSXWS sequence is double underlined. The performed human/mouse substitutions in the NH_2-terminal module can be deduced from the Figure: amino acid residues of mouse IL-5Rα different from human are written above the human sequence. Residues mutated to alanine are underlined. The stretch of four residues, D55, D56, Y57 and E58, found to be involved in hIL-5 interaction is shown in bold. (**B**) Sequence alignment of residues in the predicted loop between the E and F β-strands within the proposed structural models of hGHR and hIL-5Rα. Residues in this region shown to participate in ligand binding by GHR are indicated by asterisks (de Vos *et al.*, 1992). R188, shown to be involved in hIL-5 binding is written in bold. Amino acids are numbered at the left, regions of conservation are shaded and gaps introduced to enhance the alignment are indicated by dots.

species specificity is determined by the C-terminal region of hIL-5. In the present study we identified more precisely the residues responsible for this property. We observed that replacement of the residues K84 and N108 in hIL-5 by their mouse counterpart (E and S, respectively) is sufficient to produce a mutant with a specific activity on mouse cells similar to mouse IL-5. This suggests that the corresponding mouse residues, E and S, are important for interaction of mIL-5 with mIL-5Rα. According to Tavernier *et al.* (1994) however, K84 is not involved in the interaction of hIL-5 with hIL-5Rα. Apparently hIL-5 and mIL-5 do not bind with exactly the same corresponding residues to their respective α-chains. N108 was not evaluated for receptor interaction but, interestingly, is located adjacent to a stretch of four residues T109, E110, W111 and I112 shown to engage hIL-5Rα (Tavernier *et al.*, 1994). On the recently elucidated three-dimensional structure of hIL-5 (Milburn *et al.*, 1993; Tavernier *et al.*, 1994), both K84 and N108 represent a C-terminal residue of helix C and helix D, respectively.

The IL-3Rα, GM-CSFRα and IL-5Rα form a subfamily within the cytokine receptor superfamily on a structural as well as on a functional basis. Each contains in its extracellular portion one copy of the hematopoietin domain and an additional NH_2-terminal region of 90–100 amino acid residues. Each binds specifically to its cognate ligand and associates with a shared β-chain which functions as an affinity converter and signal transducer. Previously, we reported on the observed homology between the NH_2-terminal region and the WSXWS-module of hIL-5Rα, and proposed that the NH_2-terminal region originated as a duplication of the latter module (Tuypens *et al.*, 1992). In Figure 5A we show an alignment of these two modules (hIL-5R mod.1; hIL-5R mod.3), together with the membrane proximal module of GHR. The alignment of the NH_2-terminal module and the WSXWS-containing module of hIL-5Rα revealed a similarity of 58% and an identity of 29%. Hence, we presume that the additional NH_2-terminal region adopts a similar folding as predicted for the membrane proximal region and suggest that the extracellular part of IL-5Rα contains three FN-III-like modules, two of them forming the hematopoietin domain and an extra NH_2-terminal module.

As an approach to understand the interaction between hIL-5 and hIL-5Rα we have constructed chimeric human/mouse IL-5Rα molecules and evaluated them for hIL-5 binding. This experimental strategy was based on the observation that species specificity of hIL-5 is restricted to the interaction with the α-chain. We could demonstrate that this property is mostly determined by the NH_2-terminal module of hIL-5Rα. A detailed analysis of this receptor region defined D56, and to a lesser extent E58, as responsible for species-specific ligand binding. Alanine substitution of a number of residues within the same module resulted in the identification of two additional residues involved in hIL-5 interaction: D55 and Y57. The sequence of four crucial amino acid residues D55, D56, Y57 and E58 is, accordingly to our model, located on the non-conserved loop joining the third (C) and fourth (D) β-strand (Figure 5A). Possibly this loop region forms a ligand-binding surface.

Points of interaction on hIL-5 with the α and β receptor subunits have been proposed in a detailed structure–function analysis (Tavernier *et al.*, 1994). Three stretches of amino acid residues: H38, K39, H41; E89, R90, R91 and T109, E110, W111, I112 were shown to be involved in the interaction with hIL-5Rα. R90, R91 and R92 form a sequence of three exposed, positively charged residues (Milburn *et al.*, 1993). Possibly this sequence in hIL-5 forms a site of electrostatic interaction with the negatively charged sequence D55, D56, Y57, E58 on hIL-5Rα. However, the observation that a conservative mutation D56E (and to a lesser extent E58D) had such a drastic effect on hIL-5 binding suggests that these residues might also be important for the correct folding of this receptor region.

The importance of the first module of hIL-5Rα for binding is consistent with several other observations. First,

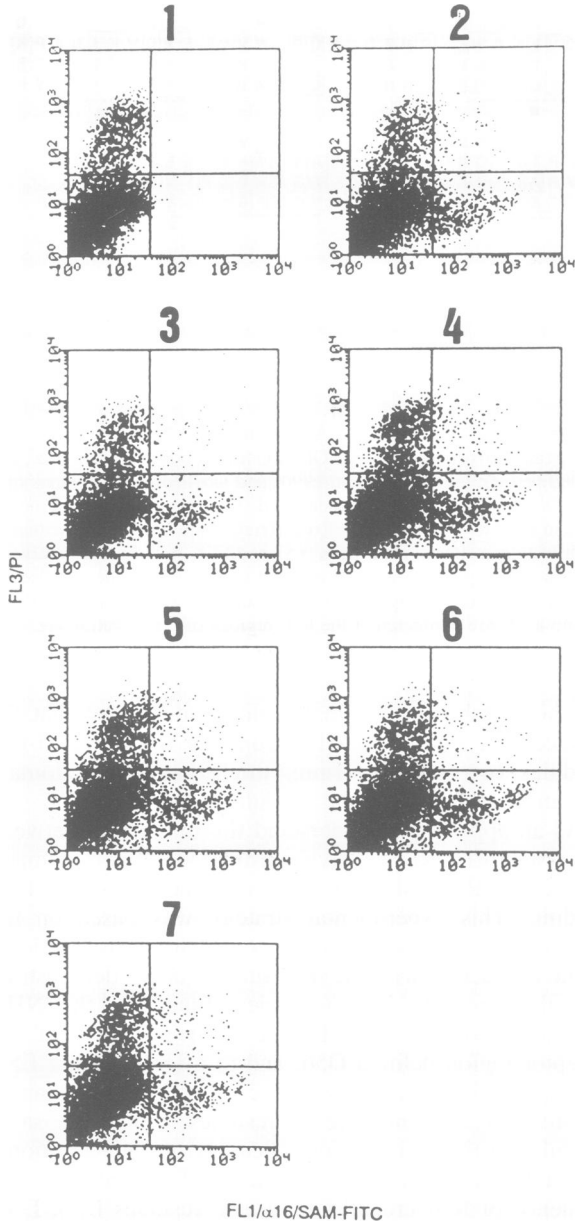

Fig. 6. Cytofluorometric analysis of expression of wild type and mutant IL-5Rα on COS1 transfectants. Cells were incubated successively with anti hIL-5Rα monoclonal antibody (α16) and an FITC-conjugated mouse anti-sheep monoclonal antibody (SAM-FITC). Fluorescence with SAM-FITC and PI is shown, respectively, on the *x*-axis and *y*-axis. Lower left window: living, non-IL-5Rα expressing cells; lower right window: living, IL-5Rα expressing cells; two upper windows: dead cells. The transfection efficiency was ~10%.
1, untransfected cells; 2, hIL-5Rα; 3, D55A; 4, D56A; 5, Y57A; 6, E58A and 7, R188A.

we reported that isothiazolone derivatives inhibit IL-5 binding on hIL-5Rα by a covalent modification of a free cysteine at position 66 present in the NH_2-terminal module of the receptor (Devos *et al.*, 1994). Secondly, an IL-5Rα deletion mutant lacking the first module does not bind IL-5 at all. This mutant is normally expressed and stained by several anti-hIL-5Rα monoclonal antibodies suggesting that this deletion does not introduce gross conformational changes (S.Cornelis, unpublished).

The binding affinity of the hIL-5Rα, hIL-3Rα and hGM-CSFRα chains for their cognate ligand varies considerably. hIL-5Rα shows the highest affinity for its ligand, K_d = 0.8–1 nM, in comparison with K_d = 2–8 nM for hGM-CSFRα (Gearing *et al.*, 1989) and K_d = 60–180 nM for hIL-3Rα (Kitamura *et al.*, 1991). Possibly, these differences in affinity binding are a reflection of the relative contribution of the NH_2-terminal module in ligand binding. In this respect, it is worth mentioning that the homology between the NH_2-terminal module and the WSXWS-containing module is much more pronounced for hIL-5Rα than for hGM-CSFRα and hIL-3Rα (Tuypens *et al.*, 1992; Goodall *et al.*, 1993).

Mutational analysis of the IL-6R (Yawata *et al.*, 1993) and the elucidated three-dimensional structure of the GHR–GH complex (de Vos *et al.*, 1992) showed that corresponding regions of these structurally related receptor proteins are essential for ligand binding. GHR residues which form the binding site for GH are clustered in the V-shaped crevice between the two modules, formed by loops connecting the β-strands (de Vos *et al.*, 1992). Analogous regions on the IL-6R were shown to participate in IL-6 binding (Yawata *et al.*, 1993). In order to indicate candidate regions which engage hIL-5 on the hematopoietin domain of hIL-5Rα, we aligned the amino acid sequence of the second and third module of the α subunit to GHR and targeted five residues which align with residues on GHR known to contact GH. We found that R188 in hIL-5Rα, corresponding to residue W104 in GHR, is important for IL-5 interaction. This residue is localized in a region which forms a loop joining the E and F β-strands of the NH_2-terminal β-barrel structure.

Like D56 and E58, R188 is not conserved between human and mouse IL-5Rα (murine counterpart: F). Substitution of these three residues individually, severely reduced hIL-5 binding, yet did not affect mIL-5 binding (not shown). Perhaps this observation explains why mIL-5 can bind to both human and mouse α receptor chains. Possibly mIL-5 only interacts with conserved residues, in contrast to hIL-5 which needs in addition some essential non-conserved contact points for efficient binding.

Molecular confirmation of this mechanism of the interaction of IL-5 and IL-5Rα will require detailed insight into the three-dimensional structures of each of the two molecules and of the complex of IL-5–IL-5Rα.

Materials and methods

Construction of human/mouse chimeras of IL-5Rα

Both hIL-5Rα and mIL-5Rα were cloned in the pSV-SPORT1 vector (BRL-Gibco, Gaithersburg). The mouse/human chimeras were constructed via three fragment ligation: two human or mouse IL-5-receptor α-specific fragments were ligated in *KpnI*/*NotI* opened pSV-SPORT1 vector. The receptor-specific fragments were obtained by PCR using a receptor-specific primer and a vector-specific primer. Identical vector-specific primers were used for the six constructions: a sense primer (position 350): 5′-GTGACACTATAGAAGGTA-3′ and an antisense primer (position 450): 5′-GTACGTAAGCTTGGATCCTC-3′. PCR reactions were carried out in a heating block (Biometra, Trio thermoblock) for 30 cycles each consisting of 1 min at 92°C, 2 min at 45°C and 2 min at 72°C. Reactions (100 μl) were performed with 2 U VENT polymerase (Biolabs) according to manufacturer instructions. For the construction of H4 for example, the first module of the mouse receptor was amplified in a first PCR reaction (PCR1) and the second and third module of the extracellular region + the TM-domain + the cytoplasmic domain of the human receptor was amplified in a second PCR reaction (PCR2). The primers used in PCR1 were the vector-specific sense primer (primer 11) and an antisense mIL-5Rα-specific primer (primer 12) introducing a

*Cla*I-site at position 675: ^{695}ACAAGTTAAATTCGTAAtCGAtGTTCC-AGGAGATCCTGGTGG-3'^{654} (the mutated nucleotides are in lower case). The primers used in PCR2 were the sense hIL-5Rα-specific primer (primer 21) introducing a *Cla*I site at position 631: ^{610}5'-CCACCAGGGTCTCCTGGAACaTCgATTGTGAATTTAACTTGC^{650}-3' and the vector-specific antisense primer (primer 22). The produced fragment in PCR1 was digested with *Kpn*I/*Cla*I resulting in a *Kpn*I–*Cla*I restriction fragment. The fragment produced in PCR2 was digested with *Cla*I + *Not*I resulting in a *Cla*I–*Not*I restriction fragment. The whole construct was generated by ligating the two restiction fragments into the *Kpn*I/*Not*I opened pSV-SPORT1 vector.

H1, H2, H3, H5 and H6 were constructed in a similar way. For H5 and H6, instead of *Cla*I, an *Mlu*I restriction site was introduced.

Construct pSV-SPORT1-H1

PCR1: template: pSV-SPORT1-hIL-5Rα primer 12: ^{972}5'-AGG-ATTTATTTGATCgATGGCGTGAAGGGCAAA-3'^{940}

PCR2: template: pSV-SPORT1-mIL-5Rα primer 21: ^{984}5'-TTCAGTCC-ACTTGCCATcGAtCAAGTGAATCCT-3'^{1016}

Construct pSV-SPORT1-H2

PCR1: template: pSV-SPORT1-hIL-5Rα primer 12: ^{650}5'-GCAA-GTTAAATTCACAATcGAtGTTCCAGGAGACCCTGG-3'^{613}

PCR2: template: pSV-SPORT1-mIL-5Rα primer 21: ^{654}5'-CCACCAGG-ATCTCCTGGAACaTCGaTTACGAATTTACTTGT-3'^{695}

Construct pSV-SPORT1-H3

PCR1: template: pSV-SPORT1-mIL-5Rα primer 12: ^{1019}5'-TGGAGG-ATTCACTTGaTCgATGGCAAGTGGACTGAA-3'^{984}

PCR2: template: pSV-SPORT1-hIL-5Rα primer 21: ^{943}5'-GCCCTTCA-CGCCATcGATCAAATAAATCCT-3'^{972}

Construct pSV-SPORT1-H5

PCR1: template: pSV-SPORT1-mIL-5Rα primer 12: ^{545}5'-GCTTTCA-GTCTTaCgcGTATCATATTCGTC-3'^{516}

PCR2: template: pSV-SPORT1-hIL-5Rα primer 21: ^{472}5'-GATGAC-TATGAAACgcGtATCACTGAAAGC-3'^{501}

Construct pSV-SPORT1-H6

PCR1: template: pSV-SPORT1-hIL-5Rα primer 12: ^{501}5'-GCTTTCA-GTGATaCgcGTTTCATAGTCATC-3'^{472}

PCR2: template: pSV-SPORT1-H4 primer 21: ^{516}5'-GACGAATATG-ATACgcGtAAGACTGAAAGC-3'^{545}

Site-directed mutagenesis of IL-5Rα and IL-5

Site-directed mutagenesis in hIL-5Rα and hIL-5 was carried out by a restriction–selection procedure using a commercially available kit (Transformer, Clontech, Palo Alto; Deng and Nickoloff, 1992). Briefly, this method involves simultaneous annealing of two oligonucleotide primers to one strand of the denatured double-stranded plasmid (pSV-SPORT1-hIL-5Rα, pVL941-hIL-5). One primer introduces the desired mutation. The second primer mutates a restriction site unique to the plasmid for the purpose of selection. The pSV-SPORT1-specific selection primer converts a unique *Sma*I restriction site into a new, unique *Nru*I site (5'-CCGGAATTCCCGtcgCGAGATCCATTGTG-3'). The pVL941 specific selection primer converts a unique *Nde*I restriction site into a new, unique *Nco*I site (5'-CTGAGAGTGCACCCATggGGGTGTG-AAATA-3').

After DNA elongation and ligation the mixture of mutated and unmutated DNAs are transformed into a *mutS Escherichia coli* strain. Since the parental DNA is sensitive to *Sma*I cleavage (for pSV-SPORT1) or to *Nde*I cleavage (for pVL941) it will be linearized, rendering it at least 100-fold less efficient in transformation of bacterial cells.

All the mutations were verified by DNA sequence analysis using the dideoxy chain termination method (Sanger *et al.*, 1977).

Expression of the hIL-5Rα mutants and hIL-5 binding analysis

All IL-5 receptor mutant cDNAs were transiently transfected in COS1 cells using DEAE–dextran in the presence of chloroquine as described in Sambrook *et al.* (1989). The expression of the human/mouse chimeras and human/mouse point mutants was examined by [^{125}I]mIL-5 binding as described (Devos *et al.*, 1990). Cell surface expression of the alanine substitution mutants was determined by flow fluorocytometry: ~3×10^5 transfected COS1 cells were incubated with a mouse monoclonal antibody against hIL-5Rα (α16) for 2 h at 4°C. Cells were washed twice and incubated with a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Amersham) for 45 min at 4°C. Samples were also stained with propidium iodide (PI) in order to discriminate between dead and living cells. Detection was performed by a Facssort (Becton Dickinson).

To determine the hIL-5 binding affinity of the different hIL-5Rα mutants, saturation binding assays with [^{125}I]hIL-5 were carried out as described (Plaetinck *et al.*, 1990; Tavernier *et al.*, 1991).

For the calculation of the dissociation constants (K_d) Scatchard transformations of the saturation binding data were generated by using the curve-fitting program EBDA–LIGAND (McPherson, 1985).

Expression of hIL-5 mutants and determination of their specific activities in human and mouse proliferation assays

Plasmid DNAs harbouring the mutant IL-5 genes were introduced in the AcNPV genome by cotransfection with linearized baculovirus cDNA in Sf9 cells (Baculogold, Pharmingen). Expression and quantification of the mutant molecules were determined by two different ELISAs as described (Tavernier *et al.*, 1994). The specific activities of mIL-5, hIL-5 and different mutants were compared in a mouse and a human proliferation assay: the mIL-5-dependent pro-B cell line B13 (Rolink *et al.*, 1989), and the hIL-5-dependent FDC-P1-CA1 cell line. The latter was obtained by introducing the hIL-5Rα subunit into FDC-P1 cells (Tavernier *et al.*, 1994). Cells (1×10^3) were cultured in the presence or absence of increased concentrations of mIL-5, hIL-5 and IL-5 mutants in a 96-well microculture plate for 72 h at 37°C. The cells were pulse labeled with 0.5 μCi of [^{3}H]thymidine for 4 h prior to harvest. Incorporated radioactivity was measured using Topcount microplate scintillation counter (Packard, Canberra). The activity is defined as the reciprocal of the dilution giving 50% of the maximal response in the proliferation assays.

Alignment of the hIL-5Rα and hGHR sequence

The alignment of the NH_2-terminal module and the WSXWS-containing module of hIL-5Rα was largely obtained by the program 'bestfit' of the Wisconsin Sequence Analysis Package. Small adjustments were made on the basis of the predictions of Bazan (1990).

The alignment with the membrane proximal module of GHR was constructed manually based on the predictions of Bazan (1990) and the structure of GHR (de Vos *et al.*, 1992).

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