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Molecular basis of a high affinity murine interleukin-5 receptor

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The mouse interleukin-5 receptor (mIL-5R) consists of two components one of which, the mIL-5R α-chain, binds mIL-5 with low affinity. Recently we demonstrated that monoclonal antibodies (Mabs) recognizing the second mIL-5R β-chain, immunoprecipitate a p130–140 protein doublet which corresponds to the mIL-3R and the mIL-3R-like protein, the latter chain for which so far no ligand has been identified. In this study we show that a high affinity mIL-5R can be reconstituted on COS1 cells by co-expression of the mIL-5R α-chain with the mIL-3R-like protein (β-chain). Cross-linking of ^{125}I-labeled mIL-5 to the COS1 cells co-transfected with both cDNAs revealed the same pattern as in B13 cells, i.e. two proteins of 60 and 130 kd which correspond to the low affinity mIL-5R α-chain and the mIL-3R-like protein, respectively. The dissociation rate of mIL-5 from this reconstituted high affinity site was lower than that of the low affinity site, whereas the association rate was unchanged. Nonetheless, the apparent dissociation constant (K_d) for this reconstituted receptor was still 10-fold higher than the K_d observed for B13 cells. Although the mIL-3R is >90% homologous to the mIL-3R-like protein, no increase in affinity for mIL-5 was detected on COS1 cells co-transfected with the cDNAs for the mIL-5R α-chain and the mIL-3R protein.

Key words: COS1 cell transfection/high affinity receptor/murine IL-5 receptor/murine IL-3 receptor/murine IL-3 receptor-like

Introduction

Recently, IL-5 has been identified as a key molecule controlling eosinophilopoiesis. IL-5 is responsible for the differentiation and growth of eosinophils in mice infected with helminths (Coffman *et al.*, 1989; Sher *et al.*, 1990; Yamaguchi *et al.*, 1990a), and transgenic mice expressing IL-5 develop eosinophilia (Dent *et al.*, 1990; Tominaga *et al.*, 1991). Studies on murine B cells (for review see Takatsu *et al.*, 1988) indicated that IL-5 also has an effect on the Ly-1$^+$ (CD5$^+$) B cells from autoimmune (Umland *et al.*, 1989) and normal mice (Wetzel, 1989). Moreover, accumulation of Ly-1$^+$ B cells was observed in IL-5 transgenic mice (Tominaga *et al.*, 1991). No IL-5-dependent pre-eosinophil cell lines have been described, and today mIL-5 is assayed on IL-5-dependent, Ly-1$^+$ B cell lines. These cell lines are derived from mouse bone marrow and can also proliferate in the presence of mIL-3 (Tominaga *et al.*, 1989; Tohyama *et al.*, 1990). To study the interaction between IL-5 and its receptor on these target cells we decided to isolate and characterize the components of the IL-5 receptor complex. High affinity (K_d = 50 pM) and low affinity (K_d = 5 nM) receptors for IL-5 have been detected on murine IL-5-dependent B cell lines (Mita *et al.*, 1989). Cross-linking experiments using radiolabeled mIL-5 revealed that the mIL-5R complex consists of at least two polypeptide chains. These results were supported by immunoprecipitation performed with Mabs directed against each of these proteins. A Mab recognizing a 60 kd protein completely blocked the binding of mIL-5 to IL-5-dependent cells (Yamaguchi *et al.*, 1990b), while Mabs immunoprecipitating a 130–140 kd protein doublet only partially blocked the binding of mIL-5 (Rolink *et al.*, 1989; J.Van der Heyden, in preparation). Interestingly, the high affinity IL-5R could be converted to a low affinity receptor by these latter Mabs (J.Van der Heyden, in preparation). These results gave evidence for a two chain IL-5R complex analogous to the IL-2R. Thus the low affinity IL-5R consists of a ligand-specific 60 kd protein (α-chain), while a p130–140 protein (β-chain) that does not bind mIL-5 by itself associates with the α-chain to generate a high affinity IL-5R. Recently cDNAs coding for two highly homologous proteins, mIL-3R- and mIL-3R-like protein, were isolated from a mouse IL-3-dependent cell line (Itoh *et al.*, 1990; Gorman *et al.*, 1990). The mIL-3R protein binds mIL-3 with low affinity (K_d = 17 nM), while the ligand for the mIL-3R-like protein has not been identified. Evidence from our laboratory indicated that the p130 and the p140 proteins recognized by the Mabs against the mIL-5R are identical to the mIL-3R- and mIL-3R-like protein respectively (Devos *et al.*, 1991). This led us to conclude that one or both of these proteins participates in the formation of the high affinity mIL-5R. Recently, Takaki *et al.* (1990) isolated a cDNA corresponding to the mIL-5R α-chain. These authors observed that this mIL-5R cDNA when transfected into mIL-3-dependent FDC-P1 cells could generate a high affinity mIL-5R. It was considered that the mIL-5R α-chain associates with (an) additional protein(s), present in FDC-P1 cells, resulting in the formation of a high affinity mIL-5R. In this report we identify such an additional protein as being identical to the mIL-3R-like protein.

Results

Isolation of cDNAs and expression of the murine low affinity IL-5R

Mabs recognizing the low affinity mouse IL-5R completely block the binding of mIL-5 to mIL-5-dependent murine pre-B cells, B13, and immunoprecipitate a 60 kd protein from B13

cell lysates (J.Van der Heyden, in preparation). Using these Mabs coupled to Hydrazide-Avidgel, the 60 kd protein was purified and internal amino acid sequences were generated (not shown). Oligonucleotides based on this sequence were then used to isolate cDNA clones from B13 poly(A)$^+$ RNA derived libraries constructed in the plasmid pCDM8 (Seed, 1987). The nucleotide sequences of the cDNA clones were in complete agreement with the recently published sequence for the mIL-5R cDNA isolated by Takaki *et al.*(1990). These cDNAs were then tested for expression in COS1 cells as measured in binding assays using radiolabeled Mabs. Some of these clones (cl-24,cl-26) expressed the antigen recognized by Mabs 3B1, 17D2, 23H4, 20H9 (J.Van der Heyden, data not shown) and were chosen for further studies. Figure 1A shows the saturation binding curve for mIL-5 binding to the COS1 cells transiently transfected with the mIL-5R α cDNA (cl-24). In agreement with the results of Takaki *et al.*(1990) only low affinity (K_d = 5 nM) binding was observed.

Identification of a high affinity mouse IL-5R

Mabs which partially block the binding of mIL-5 and mIL-3 to B13 cells were isolated (Rolink *et al.*, 1989; J.Van der Heyden, in preparation). These Mabs recognize a doublet protein of 130−140 kd, present in B13 cell lysate (Devos *et al.*, 1991), corresponding to the mIL-3R (Itoh *et al.*, 1990) and mIL-3R-like proteins (Gorman *et al.*, 1990). This led us to conclude that the high affinity mIL-5R and the high affinity mIL-3R as found on IL-5-dependent- and IL-3-dependent murine cell lines have a polypeptide in common, or that a very homologous protein is part of the mIL-5 receptor and mIL-3 receptor. To address the question of which protein—the mIL-3R, the mIL-3R-like, or both—can function as an additional component required for generating a high affinity mIL-5 receptor, we isolated cDNA clones corresponding to these proteins. These plasmid constructs in pCDM8 were then cotransfected in COS1 cells with the low affinity mIL-5R α-chain cDNA and the affinity of binding mIL-5 was measured. Cotransfection of COS1 cells with the mIL-5R α-chain cDNA and the mIL-3R-like cDNA resulted in the expression of high affinity (K_d = 500 pM) mIL-5 binding sites (Figure 1A and B). In accordance with Gorman *et al.* (1990), we found that COS1 cells transfected with the mIL-3R-like cDNA alone did not bind mIL-3 or mIL-5 (data not shown). In contrast, co-transfection of COS1 cells with the cDNAs corresponding to the mIL-5R α-chain and the mIL-3R did not result in the formation of high affinity mIL-5 binding sites (Figure 1B). As observed by Itoh *et al.* (1990), transfection with mIL-3R cDNA alone led to the expression of low affinity mIL-3 binding sites, proving that this plasmid was functional (not shown). The presence of Mabs 4F1 (J.Van der Heyden, in preparation) and R52 (Rolink *et al.*, 1989), which recognize the β-chain (mIL-3R-like) during the binding of mIL-5 on the COS1 cells transfected with both the α- and the β-chain cDNA, converted the high affinity IL-5R (K_d = 600 pM) to a low affinity IL-5R (K_d = 3 nM) (Figure 1C). This result shows that the mIL-3R-like protein together with the mIL-5R α-chain forms a high affinity site for mIL-5. Whether or not the mIL-5R α-chain/mIL-3R-like chain complex pre-exists or is induced by mIL-5 binding awaits further investigation. The apparent K_d obtained for this reconstituted mIL-5R on COS1 cells is 10-fold higher than the apparent K_d value obtained for the high affinity mIL-5R observed on B13 cells

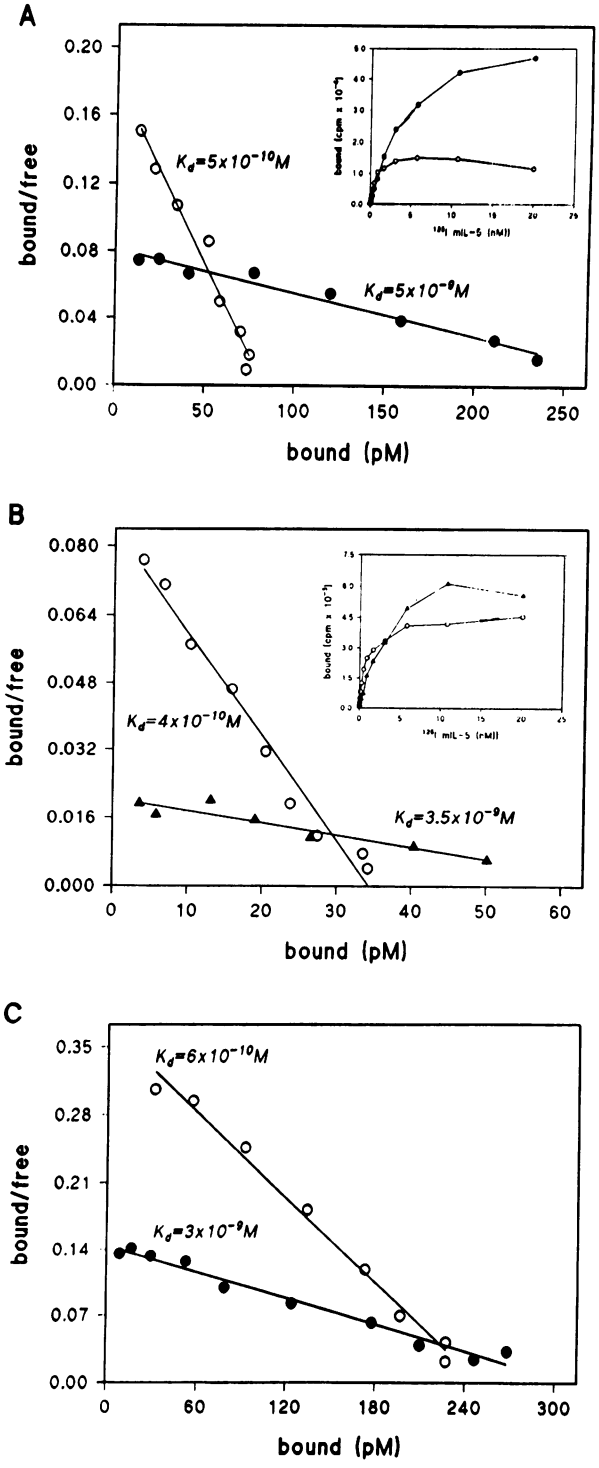

Fig. 1. Binding characteristics of mIL-5 receptors expressed on COS1 transfectants. (**A**) Scatchard plot analysis of [^{125}I]mIL-5 binding on COS1 cells transiently transfected with the mIL-5R α cDNA (●), and both the mIL-5R α cDNA and the mIL-3R-like cDNA (○). (**B**) COS1 cells transfected with both the mIL-5R α cDNA and the mIL-3R cDNA (▲); the mIL-5R α cDNA and the mIL-3R-like cDNA (○). The insets show the equilibrium binding profiles. (**C**) Effect of Mabs 4F1 and R52 on the binding of [^{125}I]mIL-5 to COS1 cells transfected with both the mIL-5R α cDNA and the mIL-3R-like cDNA. ●, Mabs added. ○, no Mabs added.

(K_d = 80 pM, Figure 2). The reason for this discrepancy in affinity might be a difference in secondary modification of the mIL-5R components expressed in COS1 cells versus

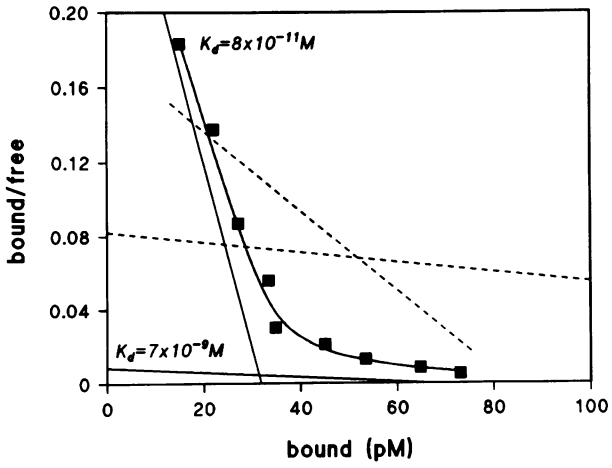

Fig. 2. Comparison of the affinity of mIL-5 receptors expressed on B13 cells and COS1 transfectants. 2×10^6 B13 cells were incubated for 30 min at 4°C with different concentrations of [^{125}I]mIL-5. The Scatchard analysis of the binding data (■) and computer derived low and high binding components are shown as solid lines. The Scatchard plots of mIL-5 binding on COS1 cells expressing the mIL-5R α-chain, and both the mIL-5R α-chain and the mIL-3R-like chain as displayed in Figure 1A are shown as dashed lines.

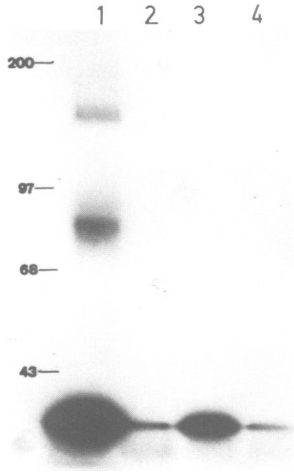

Fig. 3. Cross-linking of [^{125}I]mIL-5 to transfected COS1 cells. Untransfected COS1 cells (lane 4) and COS1 cells transfected with the mIL-5R α cDNA (lane 3), the mIL-3R-like cDNA (lane 2), the mIL-5R α- and the mIL-3R-like cDNA (lane 1) were incubated with [^{125}I]mIL-5 (5 nM). mIL-5 was cross-linked with BS^3 and analysed by SDS–PAGE as described in Materials and methods. Numbers at the left are the position of the pre-stained molecular weight markers in kd.

B13 cells, resulting in a decreased affinity of the ligand for its receptor. Alternatively, a third component involved in the formation of a high affinity mIL-5R cannot be ruled out. In contrast to B13 cells (Figure 2) and to COS1 cells transfected with the mIL-5R α-chain cDNA alone (Figure 1A), no low affinity mIL-5 binding sites could be detected on these co-transfected COS1 cells (Figure 1A and B). This could mean that on the latter cells both chains are expressed in equal number, while on B13 cells the β-chain is limiting.

As observed for mIL-5 dependent Ly-1$^+$ B cell lines (Mita *et al.*, 1989), both the α- and the β-chain when co-expressed in COS1 cells could be cross-linked to ^{125}I-

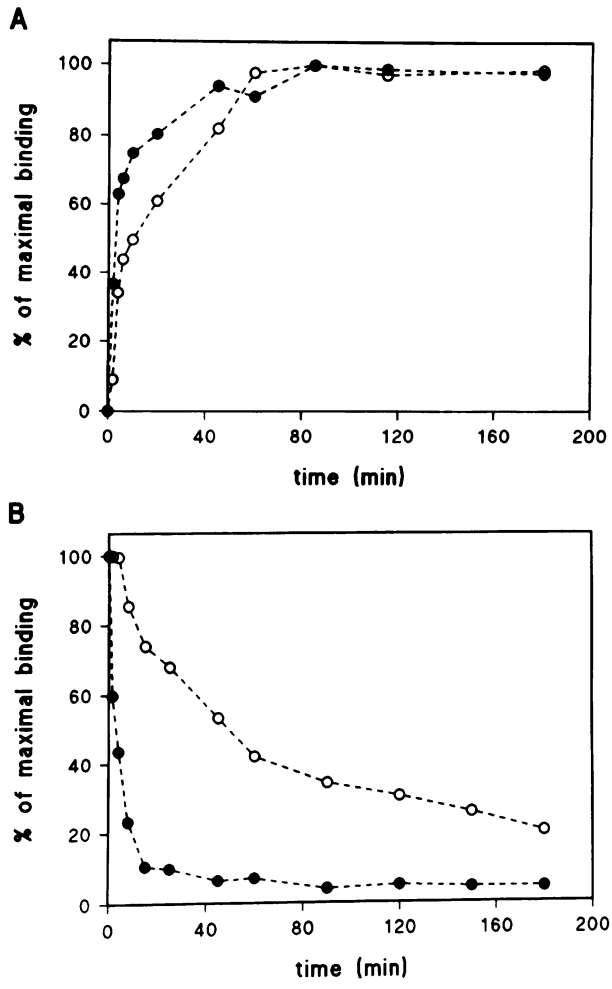

Fig. 4. Kinetics of binding of [^{125}I]mIL-5. (**A**) Association rate. Transfected COS1 cells were incubated with 0.5 nM [^{125}I]mIL-5 at 4°C for various times and the cell-bound radioactivity was measured. (**B**) Dissociation rate. A 200-fold excess of mIL-5 was added to transfected COS1 cells which were preincubated with 0.5 nM [^{125}I]mIL-5 for 3 h at 4°C, and the residual cell-bound radioactivity was measured at various times at 4°C. ●, COS1 cells transfected with the mIL-5R α cDNA. ○, COS1 cells transfected with both the mIL-5R α cDNA and the mIL-3R-like cDNA.

labeled mIL-5. Two cross-linked bands with molecular weights of 95 kd and 160 kd could be detected (Figure 3). Although the COS1 cells transfected with the α-chain cDNA alone bound at least the same amount of labeled mIL-5 relative to the COS1 cells transfected with both the α- and the β-chain cDNA (Figure 1A), no band was visible after cross-linking of [^{125}I]IL-5 to these cells. We observed that during the washes prior to cross-linking, >50% of the label was lost from the COS1 cells expressing the α-chain alone. When the binding kinetics of mIL-5 to COS1 cells expressing the mIL-5R α-chain were compared with the kinetics observed for COS1 cells expressing both the mIL-5R α-chain and the mIL-3R-like chain, it was evident that the high affinity binding caused by the β-chain was due to a decreased rate of dissociation. As shown in Figure 4A, the rate of binding of mIL-5 occurred with similar kinetics on both mIL-5R α cDNA transfected cells and α- and β-chain cDNA co-transfected cells. In contrast, the addition of unlabeled mIL-5 to COS1 cells preincubated with [^{125}I]mIL-5 led to the rapid release of radiolabeled mIL-5 from the mIL-5R

α-chain transfectant ($t_{1/2}$ <2 min), and a much slower release ($t_{1/2}$ >60 min) from the α−β co-transfectant (Figure 4B).

Discussion

The high affinity mIL-5R complex is composed of at least two molecules of which the mIL-5R α-chain can bind mIL-5 independently with low affinity. The second subunit (β-chain) does not bind mIL-5 but associates with the mIL-5R α-chain to form a high affinity mIL-5R complex. Our results show that this β-chain is identical to the mIL-3R-like protein previously described by Gorman *et al.*(1990). Surprisingly, the mIL-3R, which has >90% amino acid sequence identity with the mIL3R-like protein (Itoh *et al.*, 1990), is not able to increase the affinity of mIL-5 for its receptor. Takaki *et al.* (1990) observed a high affinity binding of mIL-5 to the mIL-3-dependent FDC-P1 cells transfected with the mIL-5R α-chain cDNA. This cell line then became responsive to mIL-5 for DNA synthesis. As FDC-P1 cells express the mIL-3R-like chain (Devos *et al.*, 1991), a high affinity mIL-5R could be reconstituted, supporting the above conclusions.

Murine IL-3 dependent cells bind mIL-3 with high affinity. As the mIL-3R protein binds mIL-3 with low affinity, an additional, as yet unidentified component might also be associated with the mIL-3R protein to form a high affinity mIL-3 receptor complex. It was shown by Gorman *et al.* (1990) that, unlike the mIL-3R protein, the mIL3-R-like protein does not bind mIL-3. Moreover, a Mab recognizing the mIL-3R and not the mIL-3R-like protein completely blocked the binding of mIL-3 to mIL-3-dependent cell lines, while the binding of mIL-5 to the mIL-5-dependent cells was unaffected (J.Van der Heyden, in preparation). This indicates that the mIL-3R chain is an essential component of the high affinity mIL-3R complex, but that the mIL-3R protein does not participate in the high affinity mIL-5R complex. Consistent with this model was our observation that mIL-3 and mIL-5 did not compete for each others binding on B13 cells (Devos *et al.*, 1991).

Recently, a high affinity receptor for human macrophage-granulocyte colony stimulating factor (hGM-CSF) could be reconstituted by co-expression of the low affinity hGM-CSFR α-chain (Gearing *et al.*, 1989) with a human homologue of the mIL-3R protein (Hayashida *et al.*, 1990). It is therefore possible that in the murine system the mIL-3R chain and/or the mIL-3R-like chain also associates with a mGM-CSF-specific membrane protein. This would mean that at least three different proteins, each specific for a cytokine ligand, form a high affinity receptor in association with an identical or highly homologous protein. These cytokine receptors would then be composed of two components in a manner analogous to the IL-2 receptor.

The post-receptor signal transduction pathway(s) induced by IL-5, IL-3 and GM-CSF is largely unknown. It has been shown that both IL-3 and GM-CSF stimulate protein tyrosine kinase activity resulting in the rapid phosphorylation of several membrane and cytoplasmic proteins (Morla *et al.*, 1988; Sorensen *et al.*, 1989a; Kanakura *et al.*, 1990). Both hIL-3 and hGM-CSF induce the phosphorylation of the proto-oncogene product c-raf1 in a factor-dependent myeloid cell line (Kanakura *et al.*, 1991), and mIL-3 was shown to induce the phosphorylation of c-raf1 in the murine cell line FDC-P1 (Carroll *et al.*, 1989). Recently, it has been shown that mIL-5- stimulated growth also leads to the tyrosine-phosphorylation of several proteins (Murata *et al.*, 1990). Since at least one component of the receptor of these lymphokines is common or homologous, it is quite likely that binding of these factors to their respective receptor transduces an identical or very similar signal. Neither the mIL-5R α-chain, nor the mIL-3R- and mIL-3R-like proteins have a structure typical of a tyrosine kinase. Isfort *et al.*(1988) have shown that mIL-3 binds to a 140 kd phosphotyrosine-containing membrane protein in FDC-P1 cells, and it was demonstrated by Sorensen *et al.*(1989b) that mIL-3 stimulates the specific tyrosine-phosphorylation of a 140 kd mIL-3R protein. It is therefore conceivable that this 140 kd protein is identical to the mIL-3R protein as described by Itoh *et al.*(1990). If this is correct, the mIL-3R and the mIL-3R-like proteins might become phosphorylated by a similar or identical tyrosine kinase. We have shown that a high affinity mIL-5R complex could be reconstituted by co-expression on COS1 cells of a mIL-5-specific α-chain together with the mIL-3R-like chain. Surprisingly, the affinity of this reconstituted receptor was still 10-fold lower than the affinity observed on mIL-5-dependent cells. This difference in affinity could be due to different post-translational modifications specific for COS1 cells. Alternatively, this difference in affinity could be explained by a tissue-specific component as was also observed for the IL-2R (Minamoto *et al.*, 1990). Further studies will be necessary to define the nature of this component and to find out its presumed relationship to tyrosine kinase activity.

Materials and methods

Cells and materials

Recombinant mIL-5 was produced in a baculovirus expression system as described (Tavernier *et al.*, 1989). The Mono-Q (Pharmacia) purified mIL-5 was labeled using the Bolton and Hunter reagent (Amersham Corp.) to a specific activity of 4−13 μCi/μg. Recombinant mouse IL-3, produced in a mouse myeloma cell line, was obtained from Dr Y.Furuichi (Nippon Roche Center, Kamakura, Japan) and labeled using Iodogen (Pierce Chemical Co.). B13 cells were grown in spinner flasks in the presence of mIL-5, and lysates were prepared as described (Devos *et al.*, 1991). Rat Mabs against the mIL-5R were prepared (J.Van der Heyden, in preparation), using B13 cells and a previously described immunization protocol (Rolink *et al.*, 1989). Bis(sulfosuccinimidyl)suberate (BS^3) was purchased from Pierce Chemical Co. Leupeptin, pepstatin A and *o*-phenanthroline were from Sigma.

Isolation of a mIL-5R α-chain cDNA

A mixture of three Mabs, 3B1, 17D2 and 20H9 (J.Van der Heyden, in preparation) directed against the mIL-5R α-chain, was covalently coupled to Hydrazide-Avidgel AX (Bioprobe Int. Inc., Tustin, CA) according to the guidelines of the manufacturer. Five milligrams of each Mab purified on protein G−Sepharose 4 Fast Flow (Pharmacia) was coupled per ml of gel. The frozen B13 cell lysates which were used for the affinity purification of the p130−140 protein (Devos *et al.*, 1991) were thawed, clarified by centrifugation and mixed overnight at 4°C with 2 ml of the gel. The mixture was then poured into a column, the gel washed and the bound proteins eluted with diethylamine as described (Devos *et al.*, 1991). After lyophilization the protein mixture was subjected to SDS−PAGE (10% acrylamide, 1.5 mm thickness). The gel was stained with 0.25% Coomassie brilliant blue in 10% acetic acid−30% methanol, destained and washed with distilled water. The 60 kd band was cut out from six minigels and the gel pieces collected in a 1 ml slot of a new concentration gel system. Proteins were electroeluted and concentrated (Rasmussen *et al.*, 1991), electrotransferred on an Immobilon-P membrane (Millipore Corp.), and detected by amido black staining. The protein spot was then excised and digested *in situ* with trypsin (Bauw *et al.*, 1989). Peptides which eluted from the blot were separated on a C4 reversed phase HPLC and some of them were selected for amino acid sequence analysis (470A gas phase sequenator equipped with an on-line 120A PTH-amino acid analyser; Applied Biosystems Inc.).

A unidirectional cDNA library was constructed in *Bst*XI−*Not*I opened pCDM8 DNA (In Vitrogen Inc.) using poly(A)$^+$ RNA derived from B13

cells, and a *Not*I primer adaptor (Promega). Plasmid DNA corresponding to 72 000 colonies in groups of 2000, were linearized with *Xho*I and after electrophoresis in a 1% agarose gel, transferred to a GeneScreen membrane (New England Nuclear, Dupont). Two oligonucleotides (32mers) were synthesized (Eurogentec S.A.) corresponding to the amino acid sequence Trp-Gly-Glu-Trp-Ser-Gln-Pro-Ile-Tyr-Val-Gly-Lys. DNA sequence oligo 1: 5'-CCIACG(A)TAT(A,G)ATIGGC(T)TGIGACCAC(T)TCICCCCA-3'; oligo 2: 5'-CCIACG(A)TAT(A,G)ATIGGC(T)TGA(G)CTCCAC(T)TC-ICCCCA-3'. The membrane containing the cDNA library was hybridized with the two 5'-^{32}P-labeled oligonucleotides using standard procedures (Sambrook *et al.*, 1989). Single plasmids in positive groups were isolated by colony hybridization. Insert DNA was analysed by digestion with restriction enzymes and DNA sequencing.

Isolation of mIL-3R and mIL-3R-like cDNAs

Membranes containing plasmid DNA corresponding to a B13 cell derived cDNA library in pCDM8 were hybridized with a labeled oligonucleotide corresponding to the 5' untranslated region (UR) of the mIL-3R/mIL-3R-like mRNA (Itoh *et al.*, 1990; Gorman *et al.*, 1990). Positive candidates were characterized by DNA sequencing and restriction enzyme analysis and shown to correspond to the mIL-3R-like cDNA. COS1 cells transiently transfected with these plasmids showed binding of the Mabs recognizing the p130–140 doublet (Devos *et al.*, 1991). A cDNA plasmid corresponding to the mIL-3R mRNA and which lacked a substantial part of the coding region was completed using a polymerase chain reaction (PCR) product specific for the mIL-3R mRNA. This PCR fragment was obtained from cDNA using a 5' UR forward oligonucleotide and a mIL-3R-specific reverse oligonucleotide (Gorman *et al.*, 1990). COS1 cells transiently transfected with this full length mIL-3R cDNA plasmid in pCDM8 showed binding of mIL-3, and of a Mab (25C9) which only recognizes the mIL-3R (J.Van der Heyden, in preparation).

Transfection in COS1 cells and binding studies

Subconfluent COS1 cells were seeded (1/2) 24 h prior to transfection using DEAE–dextran in the presence of chloroquine (Sambrook *et al.*, 1989), and incubated for 3 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (binding medium). Cells were then collected after treatment for 30 min at 37°C with PBS containing 0.5 mM EDTA and 0.02% sodium azide, resuspended at 1.5×10^5 cells per 0.3 ml binding medium containing 0.02% sodium azide and incubated with increasing concentrations of [^{125}I]mIL-5 at 4°C for 1 h. Cell-associated radioactivity was determined as described (Plaetinck *et al.*, 1990). Specific binding was calculated as the difference between the binding in the absence (total binding) and presence (non-specific binding) of 100-fold excess unlabeled mIL-5. Scatchard plot analysis of the binding data was performed using the LIGAND program (McPherson, 1985).

Cross-linking experiments

Transfected COS1 cells (1×10^6 cells) were cultured for 48 h and detached by washing once and incubation for 30 min at 37°C with phosphate buffered saline (PBS) containing 0.02% sodium azide and 0.5 mM EDTA. The cell pellet was suspended in 1 ml DMEM containing 10% FCS and [^{125}I]mIL-5 was added to a final concentration of 5 nM. After incubation for 1 h at 4°C, the cells were washed once with ice-cold balanced salt solution (BSS), BS^3 was added (1 mM final concentration) and the incubation continued for 30 min at 4°C. Next glycine was added (50 mM final concentration) and the cells were washed twice with BSS and lysed in 0.3 ml 1% Triton X-100 in BSS containing a cocktail of protease inhibitors (2 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonylfluoride, 10 μM leupeptin, 10 μM pepstatin A, 2 mM *o*-phenantroline, 200 kallikrein inhibitor units/ml). After centrifugation for 10 min at 4°C, the supernatant was analyzed on an SDS–7.5% acrylamide gel under non-reducing conditions.

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