

# Identification of residues involved in binding of IL5 to $\beta_{\text{com}}$ using $\beta_{\text{IL3}}$ and $\beta_{\text{com}}$ chimeras

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**Abstract** In mice there are two forms of the beta chain used in the IL3 receptor system,  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$ .  $\beta_{\text{com}}$  is used by the IL3, IL5 and GM-CSF receptors whereas  $\beta_{\text{IL3}}$  is only used in the IL3 receptor. In this work an assay was developed to identify residues of  $\beta_{\text{IL3}}$  that restrict IL5 activity. It was found that such residues reside within the 2nd CRM of the molecule. Furthermore, when residues in the  $\beta_{\text{IL3}}$  B'-C' loop were replaced with  $\beta_{\text{com}}$  sequence a form of  $\beta_{\text{IL3}}$  was produced that was able to respond to IL5. This region is also responsible for IL3 binding to  $\beta_{\text{IL3}}$  in the absence of alpha chain. It is therefore an important structural motif of  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  responsible for both ligand interaction and specificity.

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**Key words:** Interleukin 5; Interleukin 3; Cytokine receptor;  $\beta_{\text{com}}$ ;  $\beta_{\text{IL3}}$

## 1. Introduction

In humans, the receptor complexes for IL3, IL5 and GM-CSF are composed of a cytokine specific alpha chain and a shared beta chain ( $\beta_{\text{com}}$ ). The shared usage of  $\beta_{\text{com}}$  defines these cytokines as members of a subgroup of the cytokine family. They also share similarities in biological activity and structure. In particular, all three have a conserved glutamic acid within their first helix which acts as a contact point for  $\beta_{\text{com}}$  [1–4].  $\beta_{\text{com}}$  itself does not display any measurable binding for these cytokines in the absence of alpha chain. It does, however, provide an affinity conversion for the ligand/alpha chain complex. For IL3 the affinity is increased 500–1000-fold [5], GM-CSF 10–100-fold [6] and IL5 only 2–3-fold [7,8].

In mice there is an isoform of  $\beta_{\text{com}}$  that is specific for IL3 ( $\beta_{\text{IL3}}$ ). Unlike  $\beta_{\text{com}}$ ,  $\beta_{\text{IL3}}$  is able to bind IL3 in the absence of alpha chain. Despite these functional differences  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  share 91% sequence homology at the AA level (Fig. 1) [11].  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  belong to the class 1 cytokine receptor family [12]. The extracellular regions of  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  are predicted to comprise of two cytokine receptor modules (CRM) each of which is comprised of two fibronectin type III (FnIII) like domains. These FnIII like domains consist of eight antiparallel beta pleated sheets. The beta pleated sheets for the membrane distal FnIII like domain are commonly labeled A to H and those for the membrane proximal FnIII like domain A' to H'.

The low affinity conversion displayed by  $\beta_{\text{com}}$  for IL5 has restricted work identifying contact points between the two molecules. Previously such residues were identified as GM-CSF contact points and then confirmed later as IL5 contact points [9,10]. Although the glutamic acid in helix A provides a common contact point with  $\beta_{\text{com}}$ , the large difference in affinity conversion suggests that there must be contact points on  $\beta_{\text{com}}$  unique to each cytokine.

The structural requirements of  $\beta_{\text{IL3}}$  and  $\beta_{\text{com}}$  for ligand interaction are poorly understood. Chimeras of  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  were used by Wang et al. [13] to identify the areas of  $\beta_{\text{IL3}}$  which are able to bind IL3 in the absence of alpha chain. In addition contact regions for IL3, GM-CSF and IL5 on the human form of  $\beta_{\text{com}}$  have been identified by comparison to growth hormone receptor contact points [9,10].

This paper describes structure/function analyses of  $m\beta_{\text{com}}$  and  $m\beta_{\text{IL3}}$  using biological activity as a measure of receptor function. Chimeras of these two molecules were introduced into CTLL2 cells expressing IL5R $\alpha$  (CTLL2-hIL5R $\alpha$ ). Cells expressing beta chain were selected by virtue of their ability to proliferate in response to mIL3 and then tested for IL5 activity. It was found that mutation of  $\beta_{\text{IL3}}$  to  $\beta_{\text{com}}$  sequence in the region responsible for IL3 binding results in a beta chain that is able to respond to IL5. This is the first identification of residues on  $\beta_{\text{IL3}}$  that confer specificity to IL3. Furthermore, it is the first identification of residues on mouse  $\beta_{\text{com}}$  involved in IL5 interaction.

## 2. Materials and methods

### 2.1. Construction of chimeric of beta chains

cDNA encoding  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  in the vector pCDM8 were kindly donated by Dr Angel Lopez and Dr Joanne Woodcock (Adelaide, Australia). These were cloned into the mammalian expression vector pEE6 [14]. Chimeras of  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  were constructed using the common *Hind*III site located near the junction of CRM1 and CRM2 and the *Dra*III site located in the transmembrane region (Fig. 2). cDNA encoding the extracytoplasmic region of  $\beta_{\text{IL3}}$  which had been mutated from IPKY to MAYSF (residues 367 to 370) was a kind gift of Dr Atsushi Miyajima (Tokyo, Japan). This was cloned into pEE6 vector containing the intracytoplasmic region of  $\beta_{\text{com}}$  at the *Dra*III site within the transmembrane domain ( $\beta_{\text{IL3}}$ -MAYSF).

### 2.2. Production of bulk stable transfectants with CTLL2-hIL5R $\alpha$ cells

10<sup>6</sup> CTLL2-hIL5R $\alpha$  cells were electroporated (960  $\mu$ F, 260 V) with 15  $\mu$ g of pEE6 containing beta chain cDNA and 15  $\mu$ g of vector encoding the mIL3R $\alpha$  (pSut-1 [15]). Cells were allowed to recover from transfection in recovery medium (RPMI+10% FCS+200 U/ml hIL2+2  $\mu$ l/ml baculovirus supernatant containing mIL3) before being washed once (1100 rpm, 5 min) and resuspended in selection medium (RPMI+10% FCS+20  $\mu$ l/ml baculovirus supernatant containing mIL3). Once a healthy population of bulk stable transfectants was obtained the cells were tested for IL5 responsiveness.

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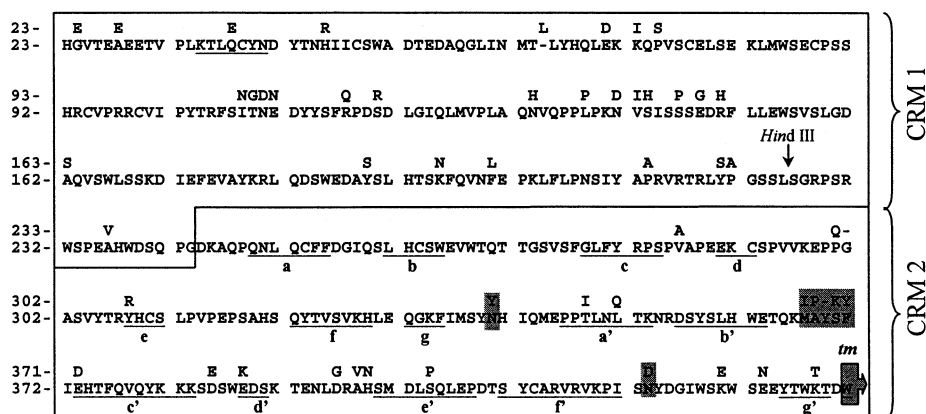


Fig. 1. Sequence comparison between the extracellular domains of  $m\beta_{IL3}$  (top) and  $m\beta_{com}$  (bottom). Only residues of  $m\beta_{IL3}$  differing from  $m\beta_{com}$  are displayed. Numbering is from the beginning of the leader sequence, however, only sequence of the mature peptide is displayed. The regions belonging to CRM1 and 2 are boxed and the predicted beta pleated sheet regions of CRM2 are underlined and labeled in lower case. The regions responsible for IL3 binding on  $\beta_{IL3}$  in the absence of IL3R $\alpha$  are highlighted [13]. The HindIII site used in chimera construction and the beginning of the transmembrane domain are labeled.

### 2.3. Testing for IL5, IL3 and IL2 responsiveness

Bulk cultures were washed three times in RPMI+10% FCS to remove residual IL3.  $1 \times 10^4$  cells in a 50  $\mu$ l volume were then added to microplate wells containing 50  $\mu$ l of test substrate and incubated at 37°C and 5% CO<sub>2</sub>. After 48 h, 10  $\mu$ l of RPMI+10% FCS containing 0.33  $\mu$ Ci of tritiated thymidine was added to each well and the cells incubated a further 4 h at 37°C and 5% CO<sub>2</sub>. The cells were then harvested onto glass fiber filters and level of tritiated thymidine incorporation determined on a Packard Matrix 9600 direct beta counter. For maximal proliferative response, 2500 ng/ml of mIL5 and 1/200 dilution of baculovirus supernatant containing mIL3 was used. ED<sub>50</sub> values for mIL3 and mIL5 were determined by fitting the data to the equation  $y = a0 + a1 / (1 + (x/a2)^{a3})$ .

## 3. Results

### 3.1. Responsiveness of cells expressing chimeras of $m\beta_{com}$ and $m\beta_{IL3}$ to mIL5

Cells expressing either  $m\beta_{com/IL3}$  or  $m\beta_{IL3/com}$  were able to respond to mIL3 (Fig. 3). In contrast, only cells expressing the  $m\beta_{IL3/com}$  chimera were able to respond to mIL5. This indicates that residues of  $m\beta_{IL3}$  restricting mIL5 activity are located within the membrane proximal CRM.

### 3.2. Activity of the $m\beta_{IL3}$ -MAYSF mutant

Within this CRM of  $m\beta_{IL3}$  reside the residues responsible for the ability of  $m\beta_{IL3}$  to bind to mIL3 in the absence of the mIL3R $\alpha$  [13]. In addition, this area aligns with a contact point for hIL5 on human  $\beta_{com}$  [9]. It therefore seemed possible that this region may also be responsible for the lack of IL5 activity displayed by  $\beta_{IL3}$ .

The  $\beta_{IL3}$ -MAYSF chimera encodes for  $\beta_{IL3}$  for the entire extracellular region except for residues 367, 368, 369 and 370 which were mutated from IPKY to MAYSF. Cells expressing this mutant were able to respond to mIL5, although this activity was low compared to cells expressing  $\beta_{com}$ .

### 3.3. Titrations of mIL5 and mIL3 on cells expressing beta chains

Titrations with mIL3 and mIL5 were performed on cells expressing chimeric and mutant beta chains to further assess the relative ability of these molecules to interact with these cytokines. All four forms of the beta chain had similar ED<sub>50</sub> values in response to mIL3 (Fig. 4). The ED<sub>50</sub> for cells expressing  $\beta_{IL3}$ -MAYSF was higher than that for cells expressing  $m\beta_{com}$  or  $m\beta_{IL3/com}$  indicating that this mutant had a reduced ability to interact with mIL5 (Fig. 5). It was demonstrated on three separate occasions that cells expressing  $\beta_{IL3}$ -MAYSF displayed an mIL5 ED<sub>50</sub> 3 to 4 times higher than that of cells expressing  $m\beta_{com}$  (data not shown).

## 4. Discussion

CTLL2 cells are a cytotoxic T-lymphocyte derived cell line which proliferate in the presence of IL2 and do not normally respond to IL3 or IL5 as they lack the receptor components. When the mIL3R $\alpha$  and either  $\beta_{com}$  or  $\beta_{IL3}$  are cloned into CTLL2 cells a line is produced that is capable of proliferating in response to IL3 [16]. Similarly, the cloning of the IL5R $\alpha$  and  $\beta_{com}$  into these cells produces an IL5 responsive cell line [17].

Here, CTLL2 cells into which had been cloned the hIL5R $\alpha$  were used to measure the function of  $\beta_{IL3}/\beta_{com}$  chimeras. Bulk stables expressing these chimeras were tested for their ability to respond to IL5. The human form of the IL5R $\alpha$  was chosen

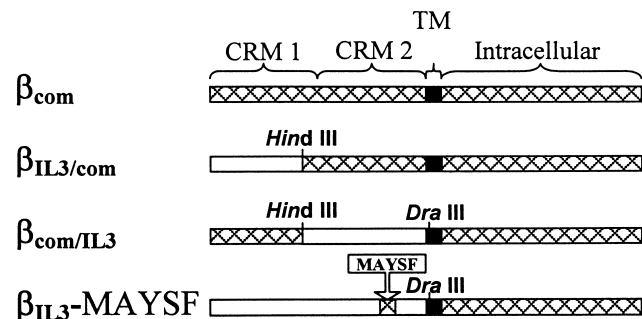


Fig. 2. Schematic representation of chimeras of  $\beta_{IL3}$  and  $\beta_{com}$ . Hatched areas represent  $\beta_{com}$  and clear areas represent  $\beta_{IL3}$  sequence. The approximate location of the MAYSF mutation to the  $\beta_{IL3}$ -MAYSF chimera, HindIII and DraIII sites used in cloning and the CRMs are also labeled.

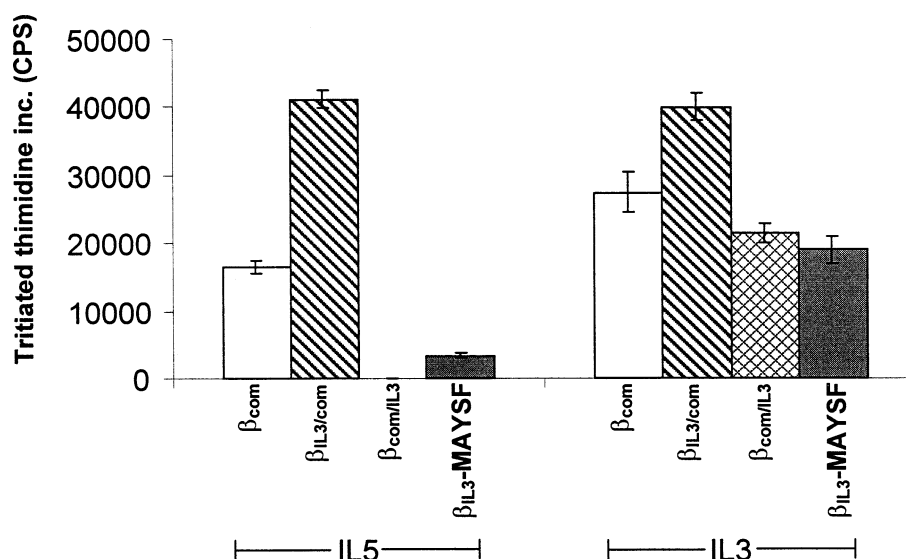


Fig. 3. Maximum response levels for cells expressing wild-type and chimeric beta chains. Values represent levels of tritiated thymidine incorporation as described in Section 2. Increasing the concentration above these levels did not result in any increase in activity. Murine IL5 was tested to a concentration of 22 125 ng/ml without any response from cells expressing  $m\beta_{IL3}$  or  $\beta_{com/IL3}$ . Error bars represent one standard deviation of triplicates. These results are representative of three separate experiments.

as it is able to form a functional receptor with  $m\beta_{com}$  [18]. In addition, unlike its murine counterpart, the  $hIL5R\alpha$  is able to interact with  $mIL5$  and  $hIL5$  with similar efficacy [19].

Residues within the B'-C' loop region of CRM2 of the  $\beta_{IL3}$  were identified which restrict IL5 activity. Mutation of these residues to the corresponding residues on  $\beta_{com}$  resulted in a molecule able to respond to IL5. Because the different stable transfectants varied in response to IL3, it was necessary to correct the IL5 response for the difference in proliferation capacity. This was done by expressing IL5 response as a percentage of the IL3 response (Table 1). This demonstrates that  $\beta_{IL3}$ -MAYSF is less able to be stimulated by IL5 than  $\beta_{com}$ . This reduction in activity is also reflected in the  $ED_{50}$  for these receptors. This demonstrates that there exist further regions of  $m\beta_{com}$  that are involved in  $mIL5$  interaction and lacked by

$m\beta_{IL3}$ . Such residues are likely to reside within CRM2 of the molecule as  $\beta_{IL3/com}$  responded normally to  $mIL5$ . Candidates include the two other less significant contact points for  $mIL3$  on  $m\beta_{IL3}$  identified by Wang et al. [13], Tyr-340 and Asp-422.

It should be noted that there may be variations between the cell lines produced in this work. Thus the ability to compare the IL5 activity of beta chain chimeras expressed on their surface is limited. Using  $mIL3$  activity as a control corrects for the majority of such variations. However,  $mIL3$  activity suffers from two variables that have no bearing on IL5 activity. Firstly,  $\beta_{IL3}$ -MAYSF has a higher affinity for IL3 than  $\beta_{IL3}$  as a result of two extra contact points at Tyr-340 and Asp-422 [13]. Secondly,  $mIL3R\alpha$  was introduced into CTLL2 ( $hIL5R\alpha$ ) cells at the same time as the beta chain. Therefore variations may exist in  $IL3R\alpha$  expression between cell lines. Using uncloned bulk cultures, as done here, minimizes but does not exclude these effects.

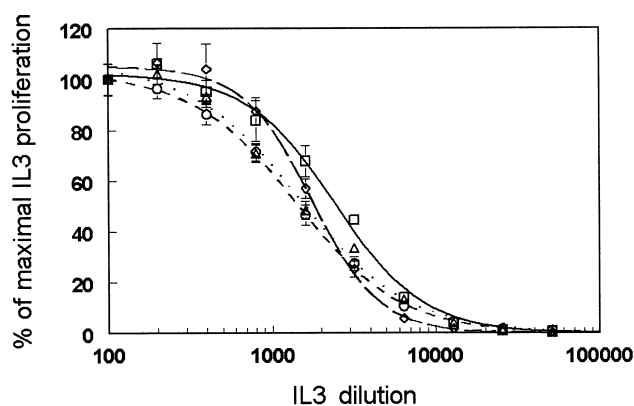


Fig. 4. Murine IL3 titration for cells expressing  $m\beta_{com}$  ( $\square$ , line),  $m\beta_{IL3/com}$  ( $\triangle$ , dotted line),  $m\beta_{com/IL3}$  ( $\diamond$ , dashed line, long dashes) and  $m\beta_{IL3}$ -MAYSF ( $\circ$ , dashed line, short dashes). Murine IL3 concentration is represented as a reciprocal dilution of baculovirus supernatant containing  $mIL3$ . Error bars represent one standard deviation of triplicates. Calculated  $ED_{50}$  values for these curves can be seen in Table 1.

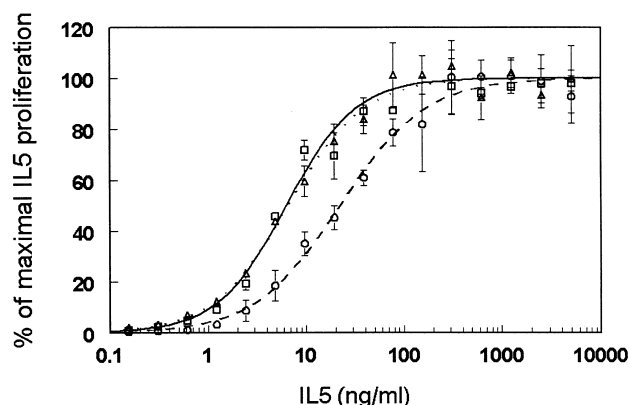


Fig. 5. Murine IL5 titration for cells expressing  $m\beta_{com}$  ( $\square$ , line),  $m\beta_{IL3/com}$  ( $\triangle$ , dotted line) and  $m\beta_{IL3}$ -MAYSF ( $\circ$ , dashed line). Error bars represent one standard deviation of triplicates. Calculated  $ED_{50}$  values for these curves can be seen in Table 1.

Table 1

Values representing the difference in activity between cell lines expressing various forms of beta chain in response to mIL5 and mIL3

Beta chain expressed	Cytokine	ED <sub>50</sub>	Max activity (10 <sup>3</sup> CPS)	% max activity (mIL5 vs. mIL3)
$\beta_{\text{com}}$	mIL5	6.3 ng/ml	16.6 ± 0.9	60%
	mIL3	2426	27.4 ± 2.9	
$\beta_{\text{IL3/com}}$	mIL5	6.6 ng/ml	45.1 ± 2.6	112%
	mIL3	1493	39.9 ± 2.0	
$\beta_{\text{com/IL3}}$	mIL5	n.a.	0.05 ± 0.01	0%
	mIL3	1770	21.4 ± 1.3	
$\beta_{\text{IL3-MAYSF}}$	mIL5	22.4 ng/ml	3.5 ± 0.2	11%
	mIL3	1436	19.3 ± 2.4	

CPS, counts per second of incorporated tritiated thymidine; n.a., not applicable.

ED<sub>50</sub> is expressed in ng/ml for mIL5 and as a reciprocal dilution of baculovirus supernatant containing mIL3. Maximum activity represents the highest level of tritiated thymidine incorporation in response to the respective cytokine. Errors for maximum activity represent one standard deviation of triplicates. Percentage maximum activity represents the maximum mIL5 activity as a percentage of the maximum mIL3 activity.

This work has identified a region of  $\beta_{\text{com}}$  required for mIL5 activity. The same region of  $\beta_{\text{IL3}}$  is responsible for mIL3 binding in the absence of the mIL3R $\alpha$  [13]. In addition this region aligns to contact points on human  $\beta_{\text{com}}$  for hIL5 and hGM-CSF but not hIL3 [9]. Thus, this region is an important structural motif of  $\beta_{\text{com}}$  and  $\beta_{\text{IL3}}$  that plays a role in both ligand interaction and specificity. By targeting this region, it may be possible to find compounds that alter the activity of one cytokine without effecting the others. This could be an important approach for drug discovery. Antagonists to IL5 are potential anti-asthma drugs, whereas upregulating IL3 and GM-CSF may be important in treating infectious diseases and recovery from irradiation.

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