Reconstitution of the Functional Receptors for Murine and Human Interleukin 5

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Summary

The murine interleukin 5 receptor (mIL-5R) is composed of two distinct subunits, α and β. The α subunit (mIL-5Rα) specifically binds IL-5 with low affinity. The β subunit (mIL-5Rβ) does not bind IL-5 by itself, but forms the high-affinity receptor with mIL-5Rα. mIL-5Rβ has been revealed to be the mIL-3R-like protein, AIC2B which is shared with receptors for IL-3 and granulocyte/macrophage colony-stimulating factor. We demonstrated here the reconstitution of the functional receptors for murine and human IL-5 on the mouse IL-2-dependent cell line, CTLL-2. CTLL-2 was transfected with the cDNAs for mIL-5Rα and/or AIC2B. Only CTLL-2 transfectant expressing both mIL-5Rα and AIC2B expressed the high-affinity receptor and proliferated in response to murine IL-5. Then CTLL-2 was transfected with the cDNAs for hIL-5Rα and/or KH97 (β), the human homologue of AIC2B. Though β did not contribute much to binding affinity of hIL-5R, only CTLL-2 transfectant expressing both hIL-5Rα and β proliferated in response to human IL-5. These results showed that the α subunit is indispensable in IL-5 signal transduction. We further investigated the function of IL-5-specific α subunit in transmitting IL-5 signals. Mutant mIL-5Rα, which lacks its whole cytoplasmic domain, was transfected into mouse IL-3-dependent cell line, FDC-P1 expressing AIC2B intrinsically. The resulting transfectant did not respond to IL-5, though the transfectant expressed the high-affinity IL-5R, indicating that the cytoplasmic portion of the α subunit also has some important role in IL-5-mediated signal transduction.

IL-5 is a glycoprotein produced by activated T cells and mast cells. IL-5 was originally identified as a cytokine that stimulates proliferation and differentiation of murine (m) activated B cells, and proved to regulate the production and function of some other hematopoietic cells, such as CD5+ B cells, eosinophils, and basophils (1). In humans, IL-5 acts mainly on eosinophils. IL-5 induces the production of eosinophils from bone marrow progenitors and works for survival and priming of eosinophils in vitro. IL-5 expression was observed in many diseases with eosinophilia, suggesting that IL-5 plays important roles in promoting production and function of eosinophils in vivo (2).

From the series of binding and crosslinking studies, we have shown that mIL-5 binds to a specific cell surface receptor (mIL-5R) with both high (Kd = 10–150 pM) and low affinity (Kd = 2–10 nM) and that two distinct membrane proteins, α and β, comprise the functional mIL-5R (3). The cloning of mIL-5Rα cDNA discloses molecular constitutions of mIL-5R (4). The mIL-5Rα alone binds IL-5 with low affinity. The mIL-5Rβ does not bind IL-5 by itself, however, it forms the high-affinity IL-5R in combination with the mIL-5Rα (4, 5). The mIL-5Rβ was then identified as AIC2B (6), the homologous protein of the low-affinity mIL-3R (AIC2A[7])(8, 9). The high-affinity mIL-5R is reconstituted on L cell transfectant coexpressing mIL-5Rα and AIC2B. Because the L cell transfectant does not respond to IL-5 in DNA synthesis, it remains, however, unclear whether the reconstituted high-affinity mIL-5R transduces the IL-5-mediated signal into cell interior.

In contrast to mIL-5R, there are controversial reports about human (h)IL-5R. In many cases, only a single class of hIL-5R with high affinity is detected on hIL-5-responsive cells, but its binding affinity to hIL-5 has been reported as various values (10–13). There is one report describing two kinds of hIL-5Rs on human erythroleukemic cell line, TF-1 (14). Some

1 Abbreviations used in this paper: h, human; m, murine; R, receptor.
we showed that the $\beta$ subunit has an important role not only
regulating the growth signal of IL-5.

The $\alpha$ subunit is the common $\beta$ sub unit of the receptors for raiL-3 and mGM-CSF (and that of AIC2A is mlL-3R$\alpha$) (19, 20). However, COS7 cells transfected with hlL-5Ro$\alpha$ cDNA binds hlL-5 with almost the same affinity compared to normal eosinophils (16). It remains an unsolved issue how putative hlL-5R$\alpha$ contributes to the binding of hlL-5 and transmitting signals.

In this report, we demonstrate the reconstitution of the functional receptors for mlL-5R in a mouse IL-2-dependent cell line, CTLL-2. In both mouse and humans, the functional IL-5R complex contains two different membrane proteins, $\alpha$ and $\beta$. hlL-5Ro$\beta$ is revealed to be the common molecule that is shared with hlL-3R and hGM-CSFR, and does not contribute much to increasing the binding affinity of hlL-5 in contrast to the mlL-5R system. In addition, using mutant mlL-5c which lacks the whole cytoplasmic domain, we showed that the $\alpha$ subunit has an important role not only in determining the binding specificity, but also in transmitting the growth signal of IL-5.

Materials and Methods

Cells and Reagents. A mouse IL-2-dependent CTLL-2 cell line was maintained in RPMI 1640 medium supplemented with 10% FCS and 50 $\mu$M 2-ME and 5% conditioned medium from Con A-stimulated rat spleen cells. A mouse IL-3-dependent FDC-P1 cell line was maintained in RPMI 1640 medium supplemented with 5% FCS, 50 $\mu$M 2-ME, and 5 U/ml of mlL-3. mlL-5 was prepared and purified using anti-mlL-5 mAb-coupled beads (3). Purified hlL-5 was obtained from NCI Research Institute.

Plasmid Construction. The cDNA fragments were inserted downstream of the Srα promoter in the expression vector, pME18, the derivative of pCEV4 (7) (Maruyama, K., and A. Miyajima, unpublished results). Either neomycin or hygromycin-resistant selection marker was added to the vectors. To construct the plasmid that expresses the truncated mutant mlL-5Ro$\alpha$, an Xhol-PstI fragment of mlL-5Ro$\alpha$ cDNA corresponding mostly to the extracellular and transmembrane portion was excised, and then ligated into the XhoI transmembrane portion was excised, and then ligated into the XhoI and NotI armed pME18 vector, to which the hygromycin-resistant gene had been added, by the use of asymmetric adopters (5'GAG-TGTGTTAGC and 5'GGCCGCTAACACTTCTGCA) that generate a new stop codon. Resulting plasmid-encoded mutant mlL-5Ro$\alpha$ terminated to the end of the transmembrane domain (Cys, position 361) in mlL-5Ro$\alpha$ [4].

Transfection. Cells were washed twice and resuspended in Hapes buffered saline (pH 7.2). 100$^7$ cells per 800 $\mu$l were then transfected with 50 $\mu$g of linearized plasmid DNA by electroporation by 350 V and 960 $\mu$F in a 0.4-cm gap cuvette using Gene Pulser (Bio-Rad Laboratories, Richmond, CA). Transfectants were selected in the medium containing either 400 $\mu$g/ml of G418 (geneticin; Sigma Chemical Co., St. Louis, MO) or 200-400 $\mu$g/ml of hygromycin (Wako Pure Chemical Industries, Osaka, Japan), depending on the selection marker on the plasmid DNA. The expression of the cDNA products was examined by flow cytometry analysis using mAbs against mlL-5Ro$\alpha$ (H7) (21), AIC2A or AIC2B (anti-Aic-2) (22), and and mK97 (CRS-1) (23), except for the hlL-5Ro$\alpha$ transfectants. The expression of hlL-5Ro$\alpha$ was verified by binding assay using $^{125}$I-hlL-5.

IL-5 Binding Assay. Purified hlL-5 was iodinated using IODOGEN (Pierce Chemical Co., Rockford, IL). mlL-5 were biosynthetically $^{35}$S-methionine labeled, as previously described (3). Specific activities of $^{125}$I-labeled hlL-5 and $^{35}$S-labeled mlL-5 were 5.5 x $10^9$ cpm/mmol and 4.7 x $10^9$ cpm/mmol, respectively. For binding assay, 5 x $10^5$ cells were incubated in the binding medium (RPMI 1640 medium containing 25 mM Hepes, pH 7.2 and 0.1% BSA) with increased concentrations of radiolabeled IL-5 at 4°C for 2 h. Cell-associated radioactivity was separated from free ligand by centrifugation through 3:2 dibutyrl/dioctyl phthalates and counted. Specific binding was defined as the difference between total and nonspecific binding obtained in the presence of 100-fold molar excess of unlabeled IL-5. Binding data were analyzed by Scatchard analysis using the EBDA and LIGAND computer programs (Elsevier-BIOSOFT, Cambridge, UK).

Chemical Crosslinking. Crosslinking experiments using mlL-5R transfectants were performed according to the method described previously (3). In brief, 5 x $10^5$ cells were incubated with radiolabeled IL-5, collected, and resuspended in 500 $\mu$l of HBSS containing 1 mM disuccinimidyl tartarate (DST; Pierce Chemical Co.). After incubation at 4°C for 30 min, cells were solubilized with lysis buffer (PBS containing 1% Triton X-100 and protease inhibitors). After removing insoluble fraction by centrifugation, cell lysates were subjected to SDS-PAGE (7.5% polyacrylamide) under reducing condition. Gel was fixed and dried, then analyzed by Fijix BAS2000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan). For hlL-5R transfectants, crosslinking was performed in PBS containing 200 $\mu$m disuccinimidyl suberate (DSS; Pierce Chemical Co.), instead of 1 mM DST (14).

Proliferation Assay. Transfectants were harvested and washed with HBSS, then inoculated into a 96-well microtiter plate at a concentration of 10$^4$-200 $\mu$l per well with various concentrations of m or mlL-5. The cells were pulse labeled with [H]$^3$Hthymidine (0.2 $\mu$Ci/well) during the last 6 h of a 48-h culture period, and incorporated [H]$^3$Hthymidine was measured by a liquid scintillation counter.

Results

Reconstitution of the Functional mlL-5R in IL-2-dependent CTLL-2 Cells. In a previous report, we reconstituted the high-affinity mlL-5R on fibroblastic L cells by coexpressing mlL-5Ro$\alpha$ and AIC2B, however, the transfectant does not respond to mlL-5 in DNA synthesis (8). To test the signal-transducing ability of recombinant mlL-5Rs, we introduced cDNAs of the mlL-5Ro$\alpha$ and/or AIC2B into mlL-2-dependent CTLL-2 cell line. The stable CTLL-2 transfectants expressing mlL-5Ro$\alpha$ alone (CTLL-m5Ro$\alpha$) or coexpressing mlL-5Ro$\alpha$ and AIC2A (CTLL-m5Ro$\alpha$-2A) bound mlL-5 with low affinity ($K_a = 2 - 5$ nM). Only the transfectant coexpressing mlL-5Ro$\alpha$ and AIC2B (CTLL-m5Ro$\alpha$-2B) bound mlL-5 not only with low but also with high affinity ($K_a = 10 - 60$ pM) (data not shown). These results were consistent with our previous observation obtained using L cell transfectants (8). Responsiveness of these CTLL-2 transfectants to mlL-5
Figure 1. IL-5-responsiveness of the CTLL-2 transfectants expressing mIL-5Rα, AIC2B, and AIC2A. Cells were incubated for 48 h in the presence of various concentrations of mIL-5 and incorporated [3H]thymidine was measured. The results are expressed as the percentage of incorporation of [3H]thymidine in the same cells incubated with 100 U/ml of hIL-2. Data are shown in mean ± SD of three independent transfectant clones. (●) CTLL-m5Rα (expressing mIL-5Rα), (□) CTLL-2B (expressing AIC2B), (■) CTLL-m5Rα-2A (expressing mIL-5Rα and AIC2A), and (○) CTLL-m5Rα-2B (expressing mIL-5Rα and AIC2B).

was then examined. As shown in Fig. 1, only CTLL-m5Rα-2B which expressed the high-affinity mIL-5R, responded to mIL-5 in DNA synthesis. mIL-5 induced a similar magnitude of proliferative response to that induced by IL-2. CTLL-m5Rα-2B could be maintained by mIL-5 in the absence of IL-2 for long periods of time (data not shown).

**Binding Characteristics of the hIL-5R Transfectants.** Next, we established the stable CTLL-2 transfectants expressing either hIL-5Rα and/or KH97 (βc). First, the hIL-5 binding characteristics of resulting transfectants were examined. CTLL-2 expressing hIL-5Rα alone (CTLL-h5Rc) bound hIL-5 with almost similar affinity (Kd = 650–720 pM) to that of normal eosinophils (Fig. 2, O) as we previously showed using COS7 cells (16). Transfectants expressing βc (CTLL-βc) did not bind hIL-5 (see Fig. 3, lanes 3 and 4).

The value of binding affinity of a transfectant coexpressing hIL-5Rα and βc (CTLL-h5Rα-βc) did not differ so much, but was slightly higher (Kd = 310–450 pM) (Fig. 2, ●) than that of CTLL-h5Rα. However, the interaction of βc with hIL-5Rα was confirmed by chemical crosslinking experiments. When CTLL-h5Rα was incubated with 125I-hIL-5 and crosslinked with DSS, an ~80-kD band corresponding to the complex of hIL-5Rα and hIL-5 was detected (Fig. 3, lane 2). In contrast, when CTLL-h5Rα-βc was crosslinked with 125I-hIL-5, a ~140-kD band corresponding to the complex of βc and hIL-5 was detected in addition to 80-kD band (Fig. 3, lane 6). Though βc did not greatly change the binding affinity of hIL-5Rα and hIL-5, it indeed existed in close proximity to hIL-5 when hIL-5Rα was expressed. It is highly suggestive that βc interacts with hIL-5Rα in binding to hIL-5.

**Reconstitution of the Functional hIL-5R.** To clarify whether βc is necessary for the signal transduction through IL-5R, the responsiveness of the transfectants was examined. As shown in Fig. 4, neither CTLL-h5Rα nor CTLL-βc respond to hIL-5. Only CTLL-h5Rα-βc responded to hIL-5 in DNA synthesis. Moreover, CTLL-h5Rα-βc acquired the ability to proliferate in response to hIL-5 without IL-2 for more than 2 mo (data not shown). From these results, it was confirmed that βc is indispensable for the functional hIL-5R. In murine IL-2–dependent cells, reconstituted hIL-5R could associate to murine cytoplasmic proteins that transduce growth signals.

**Consequences of the Deletion of the Cytoplasmic Domain of α Subunit.** From these transfection experiments, it was confirmed that the common β subunit shared among receptors for IL-5, IL-3, and GM-CSF, AIC2B in mice and βc (KH97) in humans, is indispensable to construct functional IL-5Rs.
Sharing of the signal-transducing molecule explains many overlapping biological activities of these cytokines, particularly on eosinophils. However, IL-5, IL-3, and GM-CSF induce different responses depending on cell types. We hypothesized that the signals specific for respective cytokines may exist and be mediated by the relevant α subunit, specific components to respective receptors. To examine the mIL-5Rα function, we constructed the mutant cDNA which lacks the whole cytoplasmic domain (Fig. 5 A), and transfected into mouse IL-3-dependent FDC-P1 cells endogenously expressing AIC2B. First, we examined interaction of mutant α subunit and AIC2B. As shown in Fig. 5 B, the high (Kd = 10 pM) and low-affinity (Kd = 2 nM) mIL-5Rs were reconstituted on FDC-P1 transfectants. The high-affinity receptor was detected on FDC-P1 transfectants not only at 4°C but also at 37°C (data not shown), indicating that mutant mIL-5Rα molecules were retained on cell surface and interacted with AIC2B. Though the number of high-affinity mIL-5R on mutant mIL-5Rα transfectants was smaller than that on intact mIL-5Rα transfectants, it was indicated that the cytoplasmic portion of mIL-5Rα is not indispensable to interact with AIC2B. This interaction was also confirmed by crosslinking experiment (Fig. 5 C). The ~75-kD complex of mutant mIL-5Rα and radio-labeled IL-5 and the ~150-kD complex of AIC2B and IL-5 were detected on the FDC-P1 transfectants expressing the mutant α subunit. We next investigated the IL-5 responsiveness of mutant mIL-5Rα transfectants (Fig. 6, D). FDC-P1 transfected with wild-type mIL-5Rα prolifer-
ated in response to mIL-5 as previously shown (4). In contrast, FDC-P1 transfectants expressing mutant mIL-5Rα did not respond to mIL-5. We obtained essentially the same result with CTLL-2 transfectants. CTLL-2 transfectants coexpressing mutant mIL-5Rα and AIC2B did not respond to mIL-5 (Fig. 6, O). The IL-5–mediated growth signal was never transmitted through the high-affinity IL-5R that consisted of mutant α and wild-type β subunit, indicating that the cytoplasmic domain of IL-5Rα has some important roles in transmitting IL-5 signals.

**Discussion**

We showed that both the α and β subunits are indispensable to form the functional IL-5R. hIL-5Rβ was revealed to be the same molecule to the common β subunit (βc) of receptors for hGM-CSF and hIL-3. In contrast to mIL-5R, or to receptors for hIL-3 and hGM-CSF, βc did not contribute much to hIL-5 binding affinity. We observed the same result using L cell or COS7 cell transfectants (data not shown), indicating that the same affinity of the reconstituted hIL-5R is independent of the nature of the parental cell line used for transfection. In other words, the hIL-5 binding is fully reconstituted by coexpressing hIL-5Rα and βc. Tavernier et al. (24) have also reported that βc is hIL-5Rβ. They isolated cDNAs encoding the soluble form of hIL-5Rα, and reported that COS cells expressing chimeric human–mouse IL-5Rα bind hIL-5 with low affinity (Kd = 1 nM) by itself, and that cotransfection of βc with the chimeric IL-5Rα leads to a fourfold increase (Kd = 250 pM) in binding affinity (24). The usage of the chimeric IL-5Rα may lead to somewhat different results from ours presented in this report. Recently, Tavernier et al. (25) reported that hIL-5 binding affinity of COS cells coexpressing the full-length hIL-5Rα and βc is only twofold higher than that of COS cells expressing hIL-5Rα alone. However, it is still not clear how the cross-competition of hIL-5 binding by hIL-3 or hGM-CSF occurs.

It is supposed that binding of hIL-5 is irrelevant to the existence of βc, as the binding affinity of α alone does not differ so much from that of α/β complex in contrast to hIL-3R and hGM-CSFR systems. The establishment of transfectant coexpressing βc and α subunits of hIL-5Rc, hIL-3Rc, and hGM-CSFR will answer this confusing question.

In addition to receptors for IL-5, IL-3, and GM-CSF, sharing of signal-transducing component has been demonstrated in receptors for IL-6, leukemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF), and oncostatin M (26). In the IL-6R system, the cytoplasmic and transmembrane domains of the ligand binding subunit (p80 IL-6R) are not required to transduce the growth signal, since the complex of the soluble form of p80 IL-6R and IL-6 associates with the signal-transducing subunit, gp130. There exist some differences between the IL-5R and receptor systems that use gp130 as a signal transducer. First, not only extracellular but also transmembrane regions of IL-5Rα are necessary to interact with β subunit. The anchoring of IL-5Rα is necessary for interacting with the β subunit, since the soluble form of mIL-5Rα inhibits binding of mIL-5 to IL-5–dependent cells (27) and does not induce proliferation of FDC-P1 even in the presence of IL-5 (Kikuchi, Y., S. Takaki, and K. Takatsu, unpublished data). Second, the cytoplasmic domain of IL-5Rα plays an important role in transducing the IL-5 signal. Mutant IL-5Rα, which lacks the whole cytoplasmic domain, did not transduce the IL-5–mediated growth signal, though the interaction to AIC2B was not completely impaired (Figs. 5 and 6). It is not clear at this time, how the α subunit functions in signal transduction. Each α subunit of IL-5R, IL-3R, and GM-CSFR has conserved region (RLPPFXV/PXXKX/PXXX/αxD) just underneath the transmembrane domain (4, 18). This region may be important to interact with the cytoplasmic domain of the common β subunit or with a certain common signal-transducing molecule. The elements of the COOH-terminal region of the respective α subunit diversified relatively from one another. The specific function of respective cytokine may depend on the structure of the distal part of the cytoplasmic portion of the α subunits. Or, each α subunit may simply support the function of the common β subunit by inducing conformational change or dimerization of the β subunit. CTLL-2 cells transfected with the chimeric receptor that consists of the extracellular domain of erythropoietin receptor (EPO-R) and the cytoplasmic domain of AIC2A, become responsive to EPO (28). Recent studies showed that EPO-R may be activated by homodimerization (29). If that is the case, homodimerization of the cytoplasmic domain of AIC2A may be sufficient to transmit the IL-3–mediated signal. From a high degree of homology between AIC2A and AIC2B (6), it is supposed that similar dimerization mechanism may be involved in IL-5–mediated signal transduction. However, this may only be the case in the signal transduction for cell growth. In other cases, such as signals for B cells to induce differentiation for Ig-secreting cells, the cytoplasmic domain of the α subunit may have some important roles in transmitting IL-5–specific signal. It is possible that there are several signal-transducing machineries that connect with the membrane receptors to the intracellular device, and that some machineries function only in a limited cell type.

A recent study (30) showed that the cytoplasmic region of βc has critical function in transducing growth signal. In addition, it was shown that the cytoplasmic region of hGM-CSFRα also has some roles in transmitting hGM-CSF–mediated signal (30). The transfectant of mIL-3–dependent Ba/F3 or mIL-2–dependent CTLL-2 expressing mutant hGM-CSFRα and βc does not respond to hGM-CSF at the beginning of culture, but adapts to response to hGM-CSF several days later (30). In the case of mIL-5Rα, the cytoplasmic domain was indispensable for transmitting signal, because the FDC-P1 transfectant expressing mutant IL-5Rα and the CTLL-2 transfectant coexpressing mutant IL-5Rα and AIC2B never became responsive to mIL-5. Even when the transfectants were cultured for several weeks in the presence of mIL-5, no IL-5–responsive transfectant grew up (data not shown). This discrepancy between hGM-CSFR and mIL-5 may suggest the functional difference of each α subunit. In addition, CTLL-2 transfectants expressing hIL-3Rα became responsive to hIL-3 because of the induction of the intrinsic AIC2B expression, whereas the transfectant expressing hGM-CSFRα
expression, whereas the transfectant expressing hGM-CSFRα did not induce any AIC2B expression (31). There is an interesting report (32) showing that IL-3 and GM-CSF induce self-renewal and differentiation activity, respectively, in different proportions in FDC-mix cells. These results may reflect the difference of signal-transmitting ability of each α subunit. Further studies using mutant receptors and reconstitution experiments in a variety of cell types will be required to dissolve the function of the common β subunit and ligand-specific α subunits in signal transduction.

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