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Slow-dissociation effect of common signaling subunit β on IL5 and GM-CSF receptor assembly

Tetsuya Ishino, Adrian E. Harrington, Meirav Zaks-Zilberman, Jeffery J. Scibek, Irwin Chaiken *

Drexel University College of Medicine, Department of Biochemistry and Molecular Biology, 245 North, 15th Street, Mail Stop 497, New College Building, Room 11102, Philadelphia, PA 19102-1192, USA

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Abstract

Receptor activation by IL5 and GM-CSF is a sequential process that depends on their interaction with a cytokine-specific subunit α and recruitment of a common signaling subunit β (βc). In order to elucidate the assembly dynamics of these receptor subunits, we performed kinetic interaction analysis of the cytokine–receptor complex formation by a surface plasmon resonance biosensor. Using the extracellular domains of receptor fused with C-terminal V5-tag, we developed an assay method to co-anchor α and βc subunits on the biosensor surface. We demonstrated that dissociation of the cytokine–receptor complexes was slower when both subunits were co-anchored on the biosensor surface than when α subunit alone was anchored. The slow-dissociation effect of βc had a similar impact on GM-CSF receptor stabilization to that of IL5. The effects were abolished by alanine replacement of either Tyr18 or Tyr344 residue in βc, which together constitute key parts of a cytokine binding epitope. The data argue that βc plays an important role in preventing the ligand–receptor complexes from rapidly dissociating. This slow-dissociation effect of βc explains how, when multiple βc cytokine receptor α subunits are present on the same cell surface, selective βc usage can be controlled by sequestration in stabilized cytokine–α–βc complexes.

Keywords: Interleukin-5; GM-CSF; Receptor; Interaction; Surface plasmon resonance

1. Introduction

Cytokines exert various biological activities through high affinity interactions with the extracellular regions of receptors on a target cell. These specific interactions initiate a series of events that ultimately result in a fully assembled complex of receptor subunits on the cell surface and intracellular signaling cascades within the cell. Although cytokine receptors vary in both composition and stoichiometry, the cytokine-triggered receptor subunit assembly is a common mechanism of transmitting information across the membrane and of stimulating intracellular signals [1].

Human interleukin-5 (IL5), interleukin-3 (IL3), and granulocyte–macrophage colony-stimulating factor (GM-CSF) are predominantly produced by activated T-lymphocytes and regulate myeloid cell development in haematopoiesis [2]. In particular, these cytokines stimulate eosinophil production, function, and survival, and therefore have been correlated with pathogenesis of diverse inflammatory diseases, such as asthma, gastrointestinal, and hypereosinophilic disorders, in which the eosinophil

Abbreviations: IL, interleukin; GM-CSF, granulocyte/macrophage-colony-stimulating factor; βc, common receptor β subunit; sβc, soluble form of βc; IL5Rα, interleukin 5 receptor α subunit; sIL5Rα, soluble form of IL5Rα; GMRα, GM-CSF receptor α subunit; sGMRα, soluble form of GMRα; CRM, cytokine recognition motif; FnIII, fibronectin type III; SPR, surface plasmon resonance; RU, resonance unit; PBS, phosphate buffered saline.

* Corresponding author. Fax: +1 215 762 4452.
E-mail address: Irwin.Chaiken@DrexelMed.edu (I. Chaiken).

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plays a major role [3–6]. The functions of eosinophils are primarily controlled by IL5, and to a lesser extent by IL3 and GM-CSF [7]. Genetically, these cytokines belong to the interleukin-4 (IL4) gene subfamily, and structurally, they belong to the short-chain cytokine subfamily of the four-helix bundle cytokine family [8]. At the molecular level, IL5, IL3, and GM-CSF mediate their biological effect via receptors that consist of two distinct subunits, a cytokine-specific α subunit and a common β subunit (βc) that transduces cell signaling [9–11]. The expression of α subunit for IL5 (IL5Rα) in humans is restricted to eosinophilic and basophilic lineages, whereas βc subunit and α subunits for IL3 and GM-CSF are expressed on various lineages including eosinophils, basophils, monocyte/macrophages, dendritic cells, and early haematopoietic progenitors [12]. Therefore, IL5, IL3, and GM-CSF elicit similar responses in eosinophils responsive to all three cytokines, and they even compete for binding to the same cell [13].

The signaling subunit βc shared by IL5, IL3, and GM-CSF is functionally analogous to gp130 and IL2 common receptor subunit γ, which are the common signaling subunits shared by various other cytokines responsible for immunological activities and haematopoiesis. These common subunits are known to act not only as signal transducers but also as affinity converters that enhance an initial cytokine–receptor complex into a higher-affinity state. Interestingly, IL5, IL3, and GM-CSF bind to their receptor subunits α with different affinities, while the binding affinities are increased up to a similar value in the presence of βc. Cellular binding assays have shown that βc can enhance the binding affinity 2- to 5-fold in the IL5 system [11,14], vs 20- to 100-fold for the GM-CSF case [9], and 500- to 1000-fold for the IL3 case [10,15]. In other words, the effects of βc on affinity enhancement vary depending on the cytokine–receptor systems: GM-CSF and IL3 bind to their α subunits with low affinities and high affinity complexes are formed in the presence of βc, whereas IL5 binds to IL5Rα alone with greater affinity and there is relatively smaller affinity enhancement by βc. While a growing body of evidence has accumulated to demonstrate the importance of common receptor subunits as signal-transducing machinery, very little is known about the mechanism of affinity enhancement.

Both α and βc subunits are members of the class I cytokine receptor superfamily, which is characterized by the presence of the so-called cytokine recognition motif (CRM) [16]. The CRM is composed of two fibronectin type III (FnIII) domains, each consisting of ~100 residues with four conserved cysteine residues in the first FnIII domain and a conserved WSXWS (X is any amino acid residue) sequence in the second FnIII domain. The extracellular region of α subunit is composed of three FnIII domains (D1, D2, and D3), of which D2D3 constitutes a CRM. In contrast, the extracellular region of βc is composed of four FnIII domains (D1, D2, D3, and D4), with both D1D2 and D3D4 corresponding to CRMs in the primary sequence [17]. The receptor subunits α for IL5, IL3, and GM-CSF share not only a high degree of amino acid sequence similarity but also the signaling subunit βc, and thus constitute a distinct subgroup (βc family) within the class I cytokine receptor superfamily. Extensive mutational analyses on the binding interfaces of cytokine–receptor complexes in βc family have been carried out over the past decade. In the case of IL5 receptor system, for instance, our recent data have shown that the ligand-binding epitope in IL5Rα comprises a cluster of negatively-charged residues (Asp55, Asp56, Glu58) from D1 domain and a cluster of positively-charged residues (Lys186, Arg188, Arg296) from D2 and D3 domains [18]. Although βc itself has no measurable affinity for cytokine in a bimolecular (cytokine–βc) complex [9–11,19,20], it is conceivable that IL5, IL3, and GM-CSF make direct contacts with βc in the high affinity trimolecular (cytokine–α–βc) complex. The ligand-binding epitope in βc appears to be composed of mostly aromatic residues from D1 (Tyr18, Phe82) and D4 (Tyr344, Tyr400) domains [21–24]. Crystallographic analysis has shown that the extracellular regions of βc exist as a domain-swapped dimer composed of two identical polypeptide chains [25], and that these binding residues are clustered in the interface of D1 and D4, domains from each polypeptide chain, that constitute a CRM in the three-dimensional structure [23,24].

Although mutational analyses and cellular binding assays have provided detailed insights into the static nature of the trimolecular cytokine–receptor interactions in βc family, the dynamic nature of receptor assembly and how this regulates the receptor activation switch have not yet been elucidated. In an attempt to address these questions, we studied interaction kinetics of cytokines with α and βc subunits by using a surface plasmon resonance (SPR) biosensor. We constructed soluble forms (extracellular domains) of IL5Rα and βc fused with C-terminal V5 peptide (GKPIPNPLLGLDST) tag and developed an anti-V5 antibody surface to anchor both subunits together in an oriented manner. The surface with both subunits had a higher affinity for IL5 binding than that with IL5Rα alone. Kinetic data analysis demonstrated that the higher binding affinity of the trimolecular complex formation was predominantly due to the slower dissociation. Furthermore, we constructed a soluble form of GM-CSF receptor α subunit (GMRα) fused with V5-tag and investigated the kinetics of interaction of GM-CSF with GMRα and βc. We found that the slow-dissociation effect had almost the same impact on the GM-CSF system as for the IL5 system. These data argue for a role of βc in the dynamics of receptor subunit assembly and activation.

2. Materials and methods

2.1. Materials

Human IL5 protein was prepared as previously described [20]. Human GM-CSF protein was purchased

from R&D systems Inc. (Minneapolis, MN). All the restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA). The anti-HIV gp120 monoclonal antibody (17b) was prepared as previously described [26], and the anti-βc monoclonal antibody (3D7) was purchased from BD Biosciences (San Jose, CA). All the synthetic oligonucleotide primers, anti-V5 antibody, *Drosophila* Schneider 2 (S2) cells, cell culture media, blasticidin S and L-glutamine solution (200 mM) were purchased from Invitrogen Inc. (Carlsbad, CA). For surface plasmon resonance measurements, the sensor chip CM5, surfactant P20, *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and 1 M ethanolamine (pH 8.5) were purchased from Biacore Inc. (Piscataway, NJ).

2.2. Construction of expression vectors and mutagenesis

The DNA fragment containing a soluble form (extracellular domain) of βc (sβc) was amplified from a full length human βc cDNA by iCycler Thermal Cycler (Bio-Rad Laboratories) with Pfu Turbo DNA polymerase (Stratagene). The PCR product was digested with Nco1 and Apa1 and ligated into the Nco1 and Apa1 sites of the *Drosophila* expression vector, pMT/Bip/V5-His plasmid (Invitrogen), yielding pMT-sβc-V5-His. In this construct, the gene for sβc was placed downstream of the *Drosophila* metallothionein promoter and the Bip secretion signal sequence. The C-terminus of sβc was linked to V5 peptide and hexahistidine tags. The expression vector for a soluble form of GM-CSF receptor α subunit (sGMRα) was constructed likewise, and denoted pMT-sGMRα-V5-His. The expression vector for a soluble form of IL5 receptor α subunit (sIL5Rα) was constructed previously [18].

Alanine substitutions were introduced into pMT-sβc-V5-His by in vitro site-directed mutagenesis. The mutant plasmids were amplified with synthetic oligonucleotide primers of the appropriate base changes by Pfu Turbo DNA polymerase, and the template plasmid was digested by the treatment of Dpn1 before transformation. All the plasmids created were verified by DNA sequencing.

2.3. Protein expression

For the transient expression of sβc, sIL5Rα, or sGMRα, *Drosophila* S2 cells were transfected with the vector pMT-sβc-V5-His, pMT-sIL5Rα-V5-His [18], or pMT-sGMRα-V5-His, respectively, using Cellfectin reagent (Invitrogen). Protein expression was induced by addition of 750 μM copper sulfate 3 days after transfection. Levels of expression were measured by western blot using anti-V5 antibody. The cell-free supernatant was collected by centrifugation and stored at −20 °C for subsequent binding analyses.

For the stable cell line expressing sβc, the vector pMT-sβc-V5-His, and the blasticidin-resistant vector pCoBlast (Invitrogen) were co-transfected into S2 cells using Cellfectin reagent. Blasticidin-resistant cells were selected as a stable polyclonal population and grown in *Drosophila* serum-free medium with 20 mM L-glutamine and 50 μg/mL blasticidin S. Cells were expanded to 1.0×10^7 cells/mL, and protein expression was induced by addition of 750 μM copper sulfate. The culture was harvested after 3 days and centrifuged at 1000x*g* for 15 min. Protein expression was verified by western blot using anti-βc antibody.

2.4. Purification of soluble βc protein

The cell-free supernatant including sβc protein was dialyzed against buffer A (50 mM Tris–HC1, pH 7.4, 50 mM NaCl) and loaded onto a HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with buffer A. Non-specific binding proteins were washed away with buffer B (50 mM Tris–HC1, pH 7.4, 25 mM imidazole, 300 mM NaCl), and the sβc protein was eluted with a linear gradient from buffer B to buffer C (50 mM Tris–HC1, pH 7.4, 300 mM imidazole). The pooled fraction was loaded onto Sephacryl-200 (26 × 600 mm, GE Healthcare) and eluted in phosphate buffered saline (PBS) buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). In this gel filtration, sβc eluted as a dimer. The resulting protein solution was concentrated and stored below −80 °C. The purity was evaluated by SDS–PAGE (4–20% linear gradient gel, Bio-Rad Laboratories). Protein quantitation was achieved by measuring the absorbance at 280 nm and calculating the concentration using the molar absorption coefficient ($\varepsilon_{280} = 100{,}380\ M^{-1}\,cm^{-1}$) according to Pace et al. [27].

2.5. Surface plasmon resonance interaction analysis

The kinetic interaction assay was performed by using a surface plasmon resonance (SPR) biosensor, Biacore 3000 (Biacore, Uppsala, Sweden). All the SPR experiments were conducted at 25 °C using PBS plus 0.005% P20 solution as a running buffer.

2.5.1. Immobilization

Anti-V5 antibody was immobilized on a CM5 sensor chip by the amine coupling method. A stock of protein solution (1.0 mg/mL) was diluted 20 times in 10 mM sodium acetate (pH 4.5) and injected onto a carboxymethylated dextran surface which had been pre-activated with a 35 μL injection of 1:1 mixture of 200 mM EDC and 50 mM NHS, followed by the injection of 1 M ethanolamine–HCl (pH 8.5). The amounts of immobilization were around 5000 resonance units (RU). Anti-gp120 antibody was immobilized similarly and used as a reference surface.

2.5.2. Binding assay

To anchor receptor proteins on the biosensor surface, the V5-tagged receptor subunits were injected onto the anti-V5 antibody surface. After washing the receptor surface by passing running buffer for 2 min, various concentrations of cytokine were injected over the receptor

surface for the measurement of cytokine–receptor interactions. To regenerate chip surfaces, both bound cytokine and anchored receptor were removed from the antibody surfaces by multiple injections of 10 mM glycine–HCl (pH 1.6). For this biosensor assay, all procedures were automated to create repetitive cycles of injection of receptor subunit (10 μL/min), cytokine (50 μL/min), and the regeneration buffer (100 μL/min).

2.5.3. Data analysis

The sensorgrams were analyzed using BIAevaluation (Biacore) and CLAMP software [28] which employ numerical integration and global fitting algorithms [29]. Prior to the analysis, the binding data were corrected for non-specific interaction by subtracting the reference surface data from the reaction surface data, and further corrected for buffer effect by subtracting the signal from buffer injections from those of protein sample injections. It has been observed that binding of IL5 to IL5Rα is compromised by mass transport effects [18]. Although a relatively fast flow rate (up to 100 μL/min) was used to minimize mass transport effects, they were not eliminated. Thus, the data obtained were globally fit using a simple 1:1 binding model combined with a two-compartment mass transport limitation model unless otherwise stated.

Equilibrium model (1)

$$\mathrm{IL5}_0 \underset{k_\mathrm{m}}{\overset{k_\mathrm{m}}{\leftrightarrows}} \mathrm{IL5}$$

$$\mathrm{IL5} + \mathrm{IL5R\alpha} \underset{k_\mathrm{off}}{\overset{k_\mathrm{on}}{\leftrightarrows}} \mathrm{IL5\text{–}IL5R\alpha}$$

In this model, $\mathrm{IL5}_0$ and IL5 represent the concentrations of IL5 protein in the bulk flow layer and the near surface layer, respectively, and k_m represents the transport rate constant between these two layers. IL5Rα is the immobilized species, and IL5–IL5Rα is the final complex that is counted as a response in the biosensor. The equilibrium dissociation constant (K_d) was calculated from the association and dissociation rate constants (k_on and k_off) as $K_\mathrm{d} = k_\mathrm{off}/k_\mathrm{on}$.

For the binding experiments using the mixed-receptor surface of both α and βc subunits, the interaction curves were analyzed using a trimolecular two-step reaction model as well as above simple 1:1 binding model. Since βc does not bind strongly to either IL5 or IL5Rα in a bimolecular complex [20], we assumed that IL5 binds to IL5Rα and the resulting complex recruits βc. The following equilibrium model was derived for these interactions.

Equilibrium model (2)

$$\mathrm{IL5}_0 \underset{k_\mathrm{m}}{\overset{k_\mathrm{m}}{\leftrightarrows}} \mathrm{IL5}$$

$$\mathrm{IL5} + \mathrm{IL5R\alpha} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathrm{IL5\text{–}IL5R\alpha}$$

$$\mathrm{IL5\text{–}IL5R\alpha} + \beta c \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} \mathrm{IL5\text{–}IL5R\alpha\text{–}\beta c}$$

In this model, IL5 binds to IL5Rα to form an intermediate complex (IL5–IL5Rα) similar to simple 1:1 binding, then this complex recruits βc on the surface to form a final complex (IL5–IL5Rα–βc). We use the symbols k_1 and k_{-1} to denote the association and dissociation rate constants for the first step, while k_2 and k_{-2} denote those for the second step. Individual kinetic parameters were obtained from at least three separate experiments. The kinetic binding data for GM-CSF and its receptor were analyzed in the same way.

3. Results

3.1. Binding of IL5 to individual receptor surfaces

We previously constructed a soluble form (extracellular region) of IL5Rα (sIL5Rα) fused with V5 peptide tag at its C-terminus to anchor the receptor by anti-V5 antibody on a surface resonance plasmon (SPR) biosensor. Using this assay method, we successfully observed the binding kinetics of the IL5–IL5Rα interaction and defined the epitope residues of IL5Rα for IL5 binding by alanine-scanning mutagenesis [18]. In the current work, we employed a similar approach to produce a soluble form of βc (sβc) and generated a multi-subunit receptor surface together with α subunits. The encoded protein was designed to contain C-terminal V5 and hexahistidine tags for display on the biosensor surface and purification of proteins, respectively (Fig. 1A). Protein was expressed in S2 cells by addition of copper sulfate and secreted into the culture media at approximately 50 nM. The molecular weight of the purified sβc was estimated by SDS–PAGE analysis to be 54 kDa under both reducing and non-reducing conditions (Fig. 1B), confirming that no intermolecular disulfide bond is formed [25,30]. The purified product was identified by western blot analysis using anti-βc antibody (Fig. 1B). Production and characterization of V5-tagged sIL5Rα were done as previously described [18,31].

Using the SPR biosensor, we first examined bimolecular binding of IL5 to individual receptor surfaces. V5-tagged protein was selectively anchored by anti-V5 antibody, providing an orientated surface for the subsequent quantitative ligand-binding assay. Fig. 2A shows the sensorgram and fit data for dose-dependent binding of IL5 to the sIL5Rα-anchored surface. The rate constants (k_on and k_off) were calculated by fitting the association and dissociation phases to a simple 1:1 binding model (Table 1). The rate constants and the equilibrium dissociation constant for the IL5–IL5Rα interaction were similar to the values reported previously [18]. We also assessed binding of IL5 to the sβc surface. As previously shown by biochemical and cellular binding assays [19,20], no significant binding was detected even when the concentration of IL5 was increased up to 2 μM (Fig. 2B).

3.2. Kinetics of IL5 binding to mixed-receptor surfaces

In order to define the functional role of βc in IL5 binding to receptor, we examined interaction kinetics of the tri-

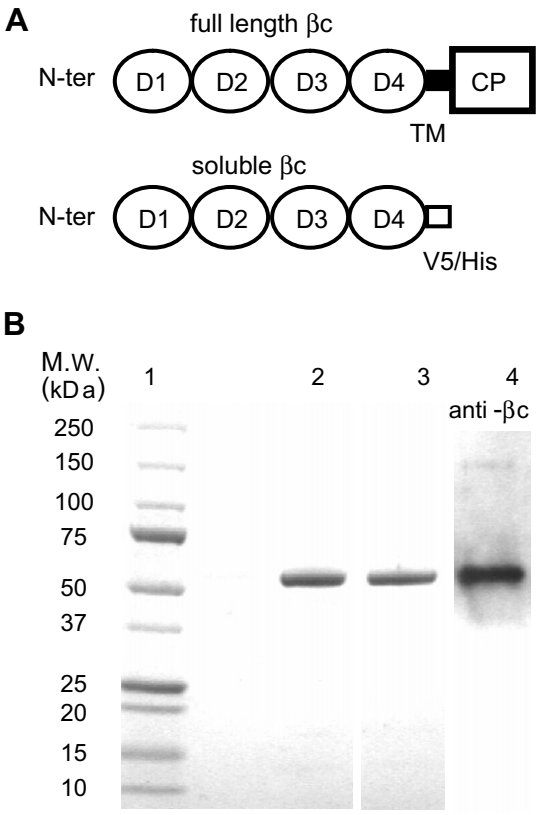

Fig. 1. Expression and purification of soluble βc subunit. (A) Comparison of soluble form of human βc (sβc) used in the V5-tag anchoring binding assay with full length human βc composed of an extracellular region (from N-terminus; D1, D2, D3, and D4), a transmembrane (TM) region and a cytoplasmic (CP) region. The soluble form of βc corresponds to the extracellular region. V5 peptide (GKPIPNPLLGLDST) and hexahistidine (HHHHHH) sequences (V5/His-tags) were fused at the C-terminal region, corresponding to TM region in the full length receptor subunit. (B) Coomassie blue-stained SDS–PAGE and western blotting analyses of sβc: lane 1, molecular marker (Precision Plus Protein Standard, Bio-Rad); lane 2, purified sβc (50 mM DTT); lane 3, purified sβc (non-reducing); lane 4, western blotting of purified sβc by anti-βc antibody.

molecular complex by injecting IL5 over the mixed-receptor surface comprised of sIL5Rα and sβc. Since IL5 does not bind on its own to βc (Fig. 2B), binding events observed from the mixed-receptor surface can be a composite of the bimolecular (IL5–IL5Rα) and the trimolecular (IL5–IL5Rα–βc) interactions. To minimize the contribution of the bimolecular interaction, a mixed surface with excess amounts of sβc was formed. We examined an optimum ratio of receptor subunits by varying amounts of sβc with a constant amount of sIL5Rα on the anti-V5 antibody surface. The sensorgrams were measured by injecting 10 nM of IL5 over the mixed-receptor surfaces of sIL5Rα and sβc in different ratios. We found that dissociation rates became slower with increasing amounts of sβc, indicating that IL5 is bound in a ternary complex with α and βc subunits, while association rate was little affected by the presence of sβc (Fig. 3A). Since there was no further difference in the measured dissociation rates between ratios of 1:3 and 1:9 (IL5Rα:βc), we reasoned that the observed sensorgrams at the ratio of 1:3 or more were mostly attributable to the trimolecular interaction. Thus, we chose 1:5 ratio of sIL5Rα to sβc for the subsequent trimolecular interaction experiments. Fig. 2C shows the sensorgram for dose-dependent binding of IL5 to the mixed-receptor surface. Since we immobilized high density of βc, it is possible that rebinding of IL5 to the mixed-receptor surface would occur in the dissociation phase, which should be eliminated for better model fitting of biosensor data. We examined the rebinding by injecting soluble IL5Rα in the dissociation phase and found that soluble IL5Rα had no effect on the rate of dissociation of IL5 from the mixed-receptor surface (data not shown), indicating that there is no significant rebinding in this system. Although biosensor analysis does not require information on the stoichiometry of receptor subunits on a biosensor surface to calculate kinetic parameters, it can be assumed that the complex is comprised of one cytokine, one α subunit and one pre-formed homodimer of βc (Fig. 2C; lower panel) as found by analytical ultracentrifugation [30]. We found that IL5 dissociates more slowly from the mixed-receptor surface of sIL5Rα and sβc (Fig. 2C) than that of sIL5Rα alone (Fig. 2A). For quantitative analysis of the trimolecular interaction, we tested two different models, one for simple 1:1 binding and a second for trimolecular two-step binding (see Section 2.5.3). Kinetic evaluation using the 1:1 model demonstrated that the mixed-receptor surface caused a slower association rate (3-fold) and a slower dissociation (6-fold), with resulting slightly higher binding affinity (2-fold) than the sIL5Rα surface (Table 1). The trimolecular two-step model failed to improve the fit. One possible reason for this is that, due to the proximity of α and βc subunits on the sensor surface, these subunits could be already engaged with each other. To test this possibility, IL5 pre-incubated with IL5Rα was injected over the mixed-receptor surface. We found that binding of IL5 to α and βc surface was largely reduced by addition of IL5Rα (Fig. 3B). The possible reasons for subunit pre-engagement on the sensor surface are discussed later. For the purposes of data analysis of the trimolecular interaction SPR sensorgrams, we used the simple 1:1 binding model to derive kinetic parameters.

3.3. Mutational effect on the IL5–IL5Rα–βc interaction

To verify that the effect of βc on interaction kinetics was due to specific βc interaction, we introduced site-directed mutations into a ligand-binding site in βc. Tyr18 and Tyr344 of βc (corresponding to Tyr15 and Tyr347 in the crystal structure [25]) have been found to be critical for IL5, IL3, and GM-CSF association into their respective trimolecular complexes [21–24]. These residues are located in a hypothetical ligand-binding interface of D1 and D4 domains (Fig. 4A). Structural integrity of these mutants was previously verified by measuring their cell surface expression with flow cytometry [23,24]. The two alanine-mutational variants, sβc(Y18A) and sβc(Y344A), were suc-

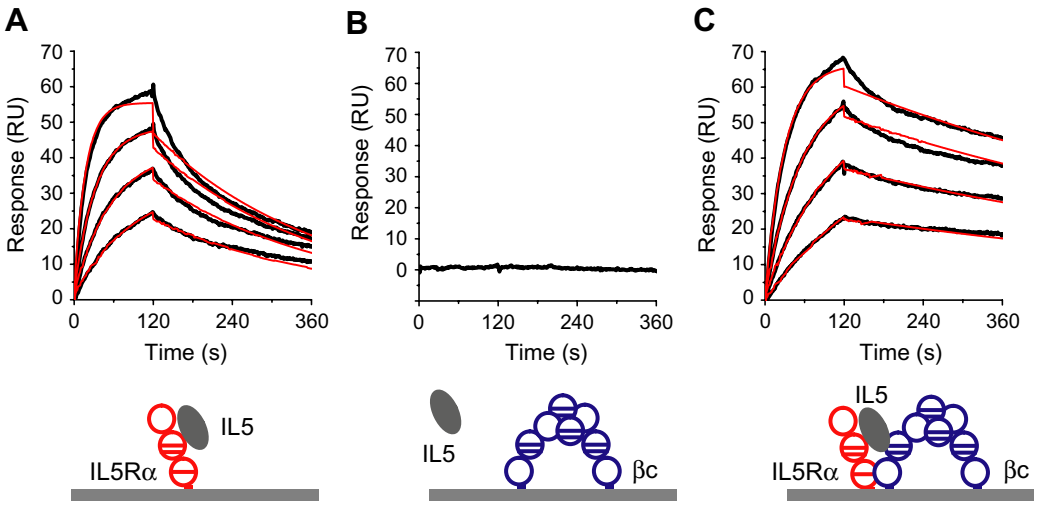

Fig. 2. Kinetic analysis of IL5 binding behavior on individual and mixed-receptor surfaces. (A) Overlay of real-time sensorgrams obtained from sequential injections of 5, 10, 20, and 40 nM IL5 over the sIL5Rα-anchored surface, followed by injection of running buffer at 120 s. The rate constants were calculated by globally fitting the association phase (0–120 s) and dissociation phase (120–360 s) to a model for 1:1 simple binding. *Black* and *red lines* show experimental and calculated sensorgrams, respectively. (B) A sensorgram from a single injection of 2 μM IL5 over the sβc-anchored surface. (C) Overlay of sensorgrams obtained from sequential injections of 5, 10, 20, and 40 nM IL5 over the mixed-receptor surface of sIL5Rα and sβc, followed by injection of running buffer. *Lower panels* show schematic diagrams of receptor components on the biosensor surface.

Table 1
Kinetic parameters of receptor binding of IL5 and GM-CSF

Receptor subunits	$k_{on} \times 10^{-6}$ (M^{-1} s^{-1})	$k_{off} \times 10^{3}$ (s^{-1})	$K_d \times 10^{9}$ (M)	K_d (cellular)[a] $\times 10^{9}$ (M)
IL5Rα	2.0 ± 0.2	6.1 ± 2	3.1 ± 0.5	1.0
IL5Rα + βc	0.76 ± 0.2	0.95 ± 0.2	1.3 ± 0.2	0.29
IL5Rα + βc(Y18A)	1.3 ± 0.4	4.0 ± 2	3.0 ± 0.5	—
IL5Rα + βc(Y344A)	1.1 ± 0.1	3.9 ± 0.8	3.6 ± 0.3	—
GMRα	0.11 ± 0.1	11 ± 0.4	100 ± 13	12
GMRα + βc	0.22 ± 0.09	1.5 ± 0.4	6.9 ± 0.1	0.13
GMRα + βc(Y18A)	0.082 ± 0.004	13 ± 3	140 ± 12	—
GMRα + βc(Y344A)	0.079 ± 0.003	8.8 ± 0.3	110 ± 6	—

Kinetic parameters were generated by using a model for 1:1 binding combined with mass transport limitation factor.
[a] Equilibrium dissociation constants determined as described [21] for cytokine binding to receptor subunits expressed on cells.

cessfully expressed in *Drosophila* S2 cells and secreted into the cell culture in amounts roughly equal to that achieved for wild type sβc protein. This expression of secreted soluble proteins was taken as further verification that these molecules were stably folded. Either sβc(Y18A) or sβc(Y344A) was anchored together with sIL5Rα on the anti-V5 antibody surface, and the above kinetic interaction assay was applied to these mutational variants (Fig. 4B). Both mutations resulted in kinetic parameters and binding affinity similar to those obtained for the IL5–IL5Rα interaction (Table 1), demonstrating that the effect of βc on the IL5–IL5Rα–βc interaction was greatly diminished by these mutations. We concluded that the kinetic effect of βc on trimolecular complex formation was due to the specific interaction between cytokines and βc.

3.4. Kinetics of interaction between GM-CSF and receptor subunits

The interaction of IL5 with IL5Rα is already high affinity in the absence of βc, unlike the interactions of IL3 and GM-CSF that become higher affinity only in the presence α and βc subunits together. We therefore sought to determine whether the kinetic effect of βc observed with IL5 also occurs for other βc cytokines such as GM-CSF and, if so, to what magnitude. We produced a soluble form (extracellular domain) of GM-CSF receptor α subunit (sGMRα) fused with V5-tag, and investigated interaction of GM-CSF with GMRα and βc (Fig. 5A). We first examined binding of GM-CSF to sGMRα in the absence of sβc (Fig. 5B). The kinetic parameters were calculated by using a simple 1:1 binding model (Table 1). The kinetic parameters for the interaction between GM-CSF and GMRα (Table 1) were similar to the values that were previously reported using an SPR biosensor [32]. We found that GM-CSF binds to GMRα with 30-fold lower binding affinity than IL5 binding to IL5Rα, mostly because of the slower association rate for GM-CSF. We optimized the ratio of receptor subunits on the biosensor surface for GM-CSF system. The slow-dissociation effect of βc also reached a plateau at 1:3 ratio of sGMRα to sβc (data not shown), and therefore we chose the 1:5 ratio as used for

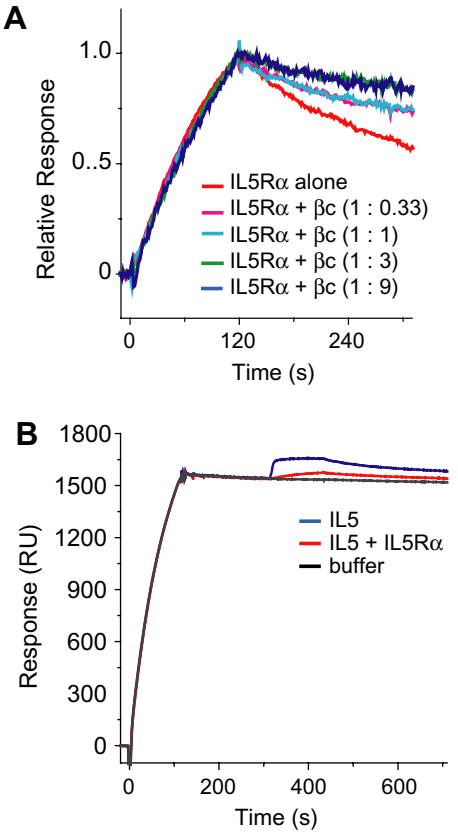

Fig. 3. Control binding experiments for SPR biosensor trimolecular assays. (A) Binding kinetics at different ratios of receptor subunits. Mixtures of different ratios of sIL5Rα and sβc were injected onto the anti-V5 antibody surface and then 10 nM of IL5 was injected over the mixed-receptor surfaces. The association (0–120 s) and dissociation (120–360 s) were measured and compared. Each sensorgram was normalized. (B) Inhibition of IL5 binding to the α and βc surface by IL5Rα. The overlay of real-time sensorgrams shows sequential injections of the mixture of soluble α and βc subunits (0 s) and running buffer (120 s), followed by injections (315 s) of buffer (*black*), IL5 (1 μM, *blue*) or IL5, and IL5Rα mixture (1 μM each, *red*).

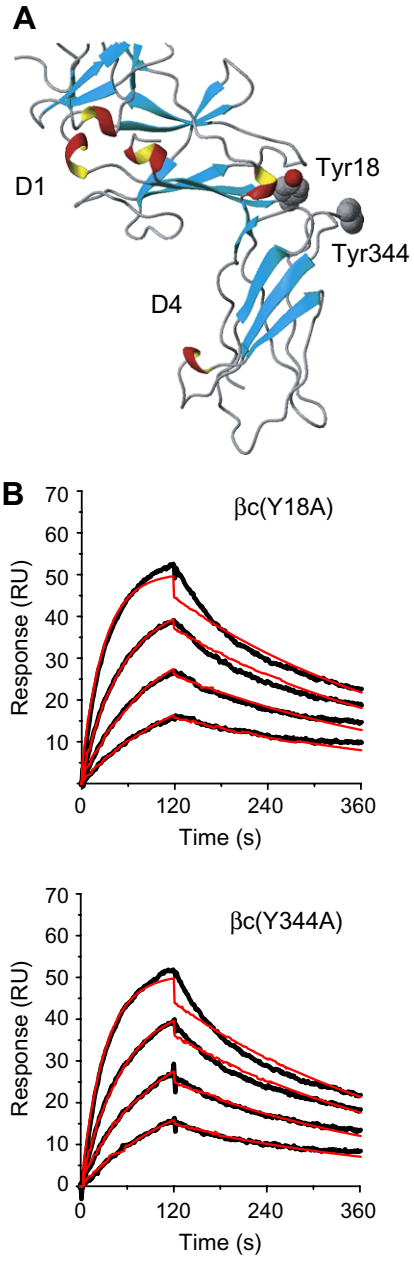

Fig. 4. Effect of βc mutation on IL5–IL5Rα–βc complex formation. (A) IL5 binding residues of βc are shown in CPK (Corey, Pauling, and Koltun) representation in the interface of D1 and D4 domains of the crystal structure of sβc (Protein Data Bank Accession Code 1gh7) [25] and labeled by residue type and number. The molecular graphics figure was prepared by the program MOLMOL [52]. (B) Kinetic analysis of βc mutational variants. Overlay of real-time sensorgrams shows sequential injections of 5, 10, 20, and 40 nM IL5 over the mixed-receptor surface of such βc mutational variants as sβc(Y18A) (*upper*) and sβc(Y344A) (*lower*) together with sIL5Rα, followed by injection of running buffer alone at 120 s. The rate constants were calculated by globally fitting the association (0–120 s) and dissociation (120–360 s) phases to a model for 1:1 simple binding. *Black* and *red lines* show experimental and calculated sensorgrams, respectively.

the IL5 system. Fig. 5C shows the sensorgram for dose-dependent binding of GM-CSF to the mixed-receptor surface. The binding affinity was 15-fold higher in the presence of sβc, due to the slower dissociation (Table 1). We also measured the binding of GM-CSF to sGMRα in the presence of sβc mutational variants, sβc(Y18A) and sβc(Y344A) (Fig. 5D and E), and found that the enhanced affinity was markedly reduced by these mutations. As expected, GM-CSF did not bind to the βc surface (Fig. 5F).

4. Discussion

4.1. Development of biosensor surface to investigate receptor assembly

The goal of this study was to elucidate the dynamic role of the signaling subunit βc in the receptor assembly process of IL5 and GM-CSF. In order to address this goal, we developed an analytical procedure that enables measurement of kinetics of interaction between cytokine and multiple receptor subunits on the surface of an SPR sensor chip.

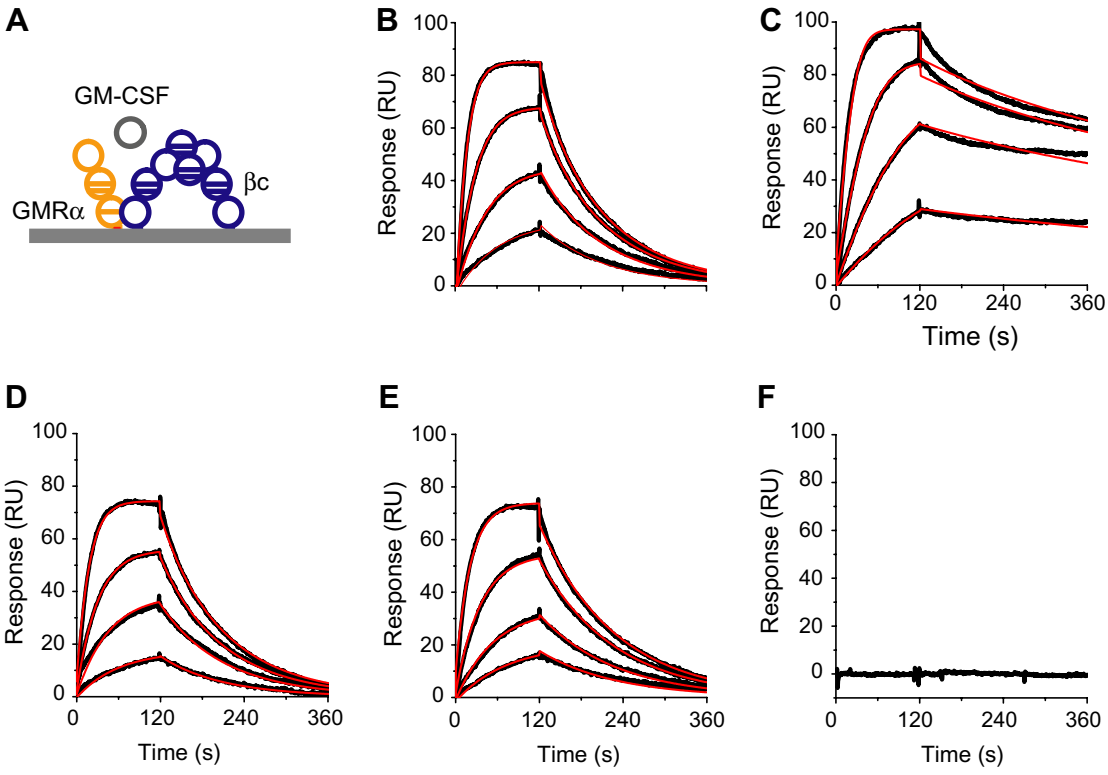

Fig. 5. Kinetic analysis of GM-CSF binding and effect of βc mutation. (A) Schematic diagrams show the configuration of the biosensor surfaces comprised of sGMRα and sβc. (B–E) Overlay of real-time sensorgrams from sequential injections of 50, 100, 200, and 400 nM GM-CSF over the sGMRα (B), sGMRα-sβc (C), sGMRα-sβc(Y18A) (D), and sGMRα-sβc(Y344A) (E) surfaces. The rate constants were calculated by globally fitting the association (0–120 s) and dissociation (120–360 s) phases to a 1:1 binding model. *Black* and *red lines* show experimental and calculated sensorgrams, respectively. (F) Real-time sensorgram from a single injection of 2 μM GM-CSF over the sβc-anchored surface.

Our previous biosensor analysis using soluble receptor showed that IL5–IL5Rα complex binds to the βc-immobilized surface with a low affinity, suggesting a sequential process of IL5–ILRα–βc complex formation [20]. While this configuration of assay was adequate to investigate the direct interaction of IL5–IL5Rα complex with βc, we sought to develop an assay method that cytokine binding to the α and β subunits in tandem on the biosensor surface. The gold surface on the SPR sensor chip used in this study is covered with a matrix of carboxymethylated dextran that forms a flexible hydrogel. The dextran matrix is known to be mobile enough to allow receptor subunits to assemble by addition of ligand [33,34]. Here, we introduced a new surface configuration, in which the soluble forms of α and βc were co-anchored through tight interaction between their C-terminal V5-tag and the anti-V5 antibody immobilized on the dextran surface (Fig. 6). We demonstrated that the surface with both α and βc subunits had a higher affinity for ligand binding than that with the α subunit alone, in both IL5 and GM-CSF systems (Figs. 2 and 5). This result is consistent with previous cell binding data [21] (Table 1), supporting the relevance of the biosensor assay configuration to binding events on the cell surface.

In the current study, we utilized simultaneous display of receptor subunits α and βc on an SPR sensor chip surface to enable evaluation of kinetic effects of tandem interaction of cytokine analyte with the receptor subunit combination. We observed that the dissociation rate of IL5 from the sensor surface decreased upon βc incorporation (vs the rate

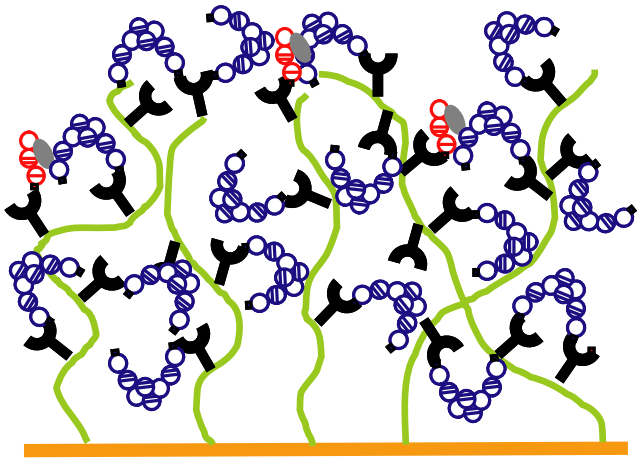

Fig. 6. Idealized scheme of interaction of IL5 with IL5Rα and βc on a dextran sensor chip surface. Soluble IL5Rα and excess amount of sβc are co-anchored through the tight interaction between their C-terminal V5-tag and anti-V5 antibody immobilized on the flexible carboxymethylated dextran surface. IL5, sIL5Rα, sβc, and anti-V5 antibody are shown as *grey*, *red blue*, and *black* shapes, respectively. The dextran and gold surface of biosensor are shown as *green* and *orange* lines, respectively.

with IL5Rα alone). The dissociation rate decrease was βc dose-dependent, and was maximal at a 1:3 ratio of α to βc (Fig. 3A). Hence, the observed binding from the mixed-receptor surface can be attributed mostly to the trimolecular complex formation in the condition we used (Fig. 6). Our simultaneous display method allowed for control of receptor subunit frequency. This was important for βc cytokines, as IL5 and GM-CSF do not bind to βc independently (Figs. 2B and 5F), and increasing the amount of βc in the mixed-receptor surface was needed to increase the relative frequency of the trimolecular (cytokine–α–βc) interaction vs the bimolecular (cytokine–α) process. The combination of V5-tagged recombinant technology and SPR biosensor analysis provides a useful tool to study kinetics of multiple receptor assembly, and can be applied to any ligand–receptor system without the need to optimize the regeneration condition. The V5-tag anchoring method has the advantage, vs direct covalent immobilization of the subunits that has been used for IL2-receptor system [34,35], that it allows optimizing the frequency of the receptor subunits on biosensor surface. This feature was not critical in the IL2 case, since IL2 interacts with finite affinity with each receptor subunit, but is less useful in the βc cytokine case since direct interactions of cytokines with βc alone are weak if they occur at all. Our method is similar to the previously described simultaneous display of receptor subunits on sensor chips coated with lipid bilayers. Using this latter method, interactions have been evaluated kinetically for interferon and receptor subunits simultaneously anchored through fused His tag to exposed lipid head groups containing immobilized metal chelate [36,37]. Of note, this latter method also enabled controlled variation of the ratios of receptor subunits on the sensor surface. Interestingly, the interferon study found that the ternary complex can be stabilized by fast re-association during the dissociation process, so-called kinetic stabilization, at a higher receptor surface concentrations. This result contrasts with our data for IL5 showing that the effect of increasing concentration of βc was saturated at a certain surface concentration (Fig. 3A). The difference likely occurs because IL5 does not directly bind to βc subunit alone with measurable affinity, while interferon binds measurably to each of the immobilized subunits. The lipid bilayer display method, as ours, also suffers the limitation that the receptors are not full length and are not integrated into bilayers through transmembrane domains. Interaction with controllable receptor assemblies integrated into lipid bilayers ultimately could be achieved by such methods as cell fluorescence resonance energy transfer [41], but this method currently will not enable kinetic analysis of the type achievable using optical biosensor methodology.

4.2. Biological implication of slow-dissociation effect of βc subunit

The βc subunit acts not only as a signal transducer but also as an affinity converter that enhances the binding affinity of βc cytokines (IL5, IL3, and GM-CSF) for their respective receptor complexes. Yet, how βc enhances the binding affinity of ligand–receptor complex has been unclear. In an effort to elucidate the mechanism and rationale of the affinity enhancement by βc, we investigated kinetics of either IL5 or GM-CSF binding to the individual (α or βc alone) and the mixed-receptor surfaces (α and βc together). Since formation and breakdown of protein complexes are controlled by such kinetic parameters as association and dissociation rate constants (k_{on} and k_{off}), these parameters are indispensable information for understanding the dynamic nature of ligand–receptor interaction. We found that the higher affinity of trimolecular vs bimolecular complex formation was predominantly due to the slower dissociation (smaller k_{off} value; Table 1), demonstrating the importance of βc-derived kinetic enhancement of cytokine–receptor binding affinity by decreasing the dissociation rate of cytokine–receptor complex. This slow-dissociation effect was abolished by alanine replacement of either Tyr18 or Tyr344, of βc, each of which constitutes a key part of a cytokine binding epitope (Figs. 4 and 5). From these data, we conclude that βc plays an important role in preventing ligand–receptor complexes from rapidly dissociating.

It has been believed that the receptor activation by βc cytokines is a sequential process that includes on the initial interaction of cytokine with a cytokine-specific α subunit, recruitment of βc into oligomeric receptor complexes, disulfide bond formation between α and βc subunits and the initiation of cytoplasmic phosphorylation events [38–40]. Subunits α and βc do not appear to preassemble in the absence of the ligand as judged from fluorescence resonance energy transfer studies on cell membranes [41]. Nonetheless, our SPR data infer that α and βc subunits could be physically associated or "pre-engaged" at least weakly on the biosensor surface. First, we found that IL5–IL5Rα complex does not bind efficiently to the mixed α and βc surface (Fig. 3B). Second, binding data from the mixed surface fit well to a simple 1:1 binding model, and no improvement of fit could be achieved with the trimolecular stepwise model. Third, the association rate was slower for the trimolecular complex of IL5 than for the bimolecular interaction (Table 1). Physical association of subunits could cause steric hindrance and consequent slow association of ligand binding to the receptor. Whether physical receptor subunit association on the sensor surface results from weak, biologically relevant interaction of α and βc or from non-specific crowding on the biosensor surface cannot be differentiated at present. In any case, the finding that βc prevents the ligand–receptor complex from rapid dissociation once formed is clear cut and argues for a functional role of βc after the trimolecular complex formation in the receptor activation process. The complex stabilized by βc might be important to entrap the cytokine to enable structural reorganizations such as conformational change and disulfide bond formation. Previously, cellular experiments using immunoprecipitation assay have shown that

a disulfide bond can be formed between α and βc subunits and that this is required for the receptor activation of βc cytokine receptors [42–44]. However, it has been shown that a simple mixture of purified α/βc subunits with ligand does not yield a disulfide-bonded receptor complex [30]. These data suggest that some redox facilitators on the cells may be involved in this inter-subunit disulfide formation. If so, one would imagine that the complex stabilized by βc might allow the facilitators to have enough time to access α and βc subunits and form the disulfide bond between them.

Cellular binding assays have shown that GM-CSF binds to GMRα with a relatively low affinity, and that higher-affinity complexes are formed in the presence of βc [9]. In contrast, IL5 binds to IL5Rα with a higher affinity, and only a relatively small affinity enhancement by βc has been observed in this case [11,14]. In our biosensor assay, we found an analogous tendency that efficiency of affinity conversion by βc is greater for GM-CSF receptor vs that for IL5. Kinetic evaluation demonstrated that the difference in the efficiency between IL5 and GM-CSF systems arises mostly from the difference in the association rates (Table 1). It should be noted that the dissociation rates of the trimolecular interaction of both IL5 and GM-CSF were very slow and nearly the same (Table 1). This suggests a similarity of the binding interfaces of βc for IL5 and GM-CSF, because the dissociation rate of protein–protein interaction can be attributed to processes that destabilize the binding interface of the final complex. We further demonstrated that the slow-dissociation effects of βc were abolished by alanine replacement of either Tyr18 or Tyr344 residue of βc in both IL5 and GM-CSF systems. Although these tyrosine residues are distant in the primary sequence, Tyr18 and Tyr344 are clustered in the interface of D1 and D4 domains (Fig. 4A). Since Tyr18 and Tyr344 are highly solvent-accessible (41% and 60%, respectively) and hence do not reside in the hydrophobic core of the protein, it has been assumed that these residues are involved in the direct interaction with the ligands as opposed to conformational stabilization of remote binding sites. The importance of a tyrosine cluster for cytokine recognition also has been observed in the IL4–IL4Rα interaction. The crystal structure of IL4–IL4Rα complex showed that Tyr13 and Tyr183 from D1 and D2 domains, which constitute the CRM of IL4Rα, make hydrogen-bond contacts with the side-chain carboxylate of Glu9 in the N-terminal helix of IL4 [45,46]. Interestingly, the key contact residues of IL5 and GM-CSF for βc recognition are also glutamic acid residues, which are Glu13 and Glu21 in the N-terminal helices of IL5 and GM-CSF, respectively [47,48]. Thus, it is tempting to speculate that the side chain of Glu13 of IL5 or Glu20 of GM-CSF might come into contact with the tyrosine cluster of βc in the trimolecular complexes. Although the detailed structural description of ligand–receptor complexes must await crystallographic determination of the complexes, our data strongly suggest that residues Tyr18 and Tyr344 of βc constitute a binding site for IL5 and GM-CSF that plays a critical role in the slow-dissociation of the cytokines.

Because IL5, IL3, and GM-CSF share a signaling subunit βc, they display broadly overlapping functions [49,50]. However, specificity in signal transduction can be achieved through several mechanisms [51]. Firstly, the specific receptor subunits α for these cytokines are differentially expressed in different haematopoietic cells. The expression of IL5Rα in humans is restricted to eosinophilic and basophilic lineages, whereas receptor α subunits for IL3 and GM-CSR are expressed on various lineages including eosinophils, basophils, monocyte/macrophages, dendritic cells, and early haematopoietic progenitors [12]. Although α subunits for IL5, IL3, and GM-CSF are all expressed in eosinophils and therefore these cytokines elicit similar responses on eosinophils [50], the biological effect of IL5 appears to have a greater quantitative impact on eosinophils than the others [7]. We found that IL5 binds to IL5Rα with a higher binding affinity due to the 20-fold faster association rate (larger k_{on} value) than GM-CSF (Table 1). In other words, the fast association can facilitate IL5Rα on the cell surface to capture the ligand. Lopez et al. have shown that IL5, IL3, and GM-CSF cross-compete for binding to the same cell [13]. For instance, GM-CSF cannot utilize βc on the cell once βc is utilized by IL5. In our study, we demonstrated that βc can stabilize the ligand–receptor complexes which dissociate rapidly in the absence of βc. This slow-dissociation effect of βc might argue for how βc is utilized on the cell surface for the specific biological responses: selective βc usage can be controlled by sequestration in the stabilized trimolecular cytokine–α–βc complexes when multiple receptor α subunits of βc cytokines are present on the eosinophils cell surface. The fast association of the IL5–IL5Rα interaction and the slow-dissociation effect of βc would provide an explanation for how IL5 can be more specific and dominant for activating eosinophils over the other competing βc cytokines.

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