Developmental and Comparative Immunology 37 (2012) 354–362

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Molecular cloning of chicken interleukin-5 receptor α-chain and analysis of its binding specificity

Yuji Fukushima, Tomohiro Miyai, Manami Kumagae, Hiroyuki Horiuchi, Shuichi Furusawa *

Department of Immunobiology, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan

ARTICLE INFO

Article history:
Received 22 November 2011
Revised 21 February 2012
Accepted 24 February 2012
Available online 3 March 2012

Keywords:
Chicken
IL-5
IL-5Rα
KK34
Biacore

ABSTRACT

Interaction between interleukin (IL)-5 and its receptor (IL-5R) is important for the regulation of immunity against worm infections, allergic reactions and B cell response in mammals. In this study, we identified a full-length cDNA encoding chicken IL-5R α-chain (chIL-5Rα). The deduced amino acid sequence showed 41–43% identity to mammalian homologues. It has four well-conserved cysteines and a WSXWS motif in the extracellular region, and a PPXP motif in the cytoplasmic region. Quantitative RT-PCR analysis revealed that chIL-5Rα mRNA expression was markedly high in bone marrow and relatively high in spleen and lung. Recombinant proteins of soluble chIL-5Rα and cytokines (artificially produced chIL-5 (achIL-5) and another IL-5-like molecule KK34) were expressed by 293F cells to examine the cytokine-receptor interactions. Interaction assay using a Biacore biosensor showed that chIL-5Rα has the capability to bind with monomeric achIL-5, but not with KK34. In conclusion, chicken has an IL-5Rα homologue but KK34 does not complement the IL-5/IL-5R system.

1. Introduction

In mammals, interleukin (IL)-5 is a glycoprotein mainly secreted by stimulated T helper 2 (Th2) cells and mast cells (Kinashi et al., 1986; Plaut et al., 1989; Takatsu et al., 1980a,b). The IL-5 gene lies in a chromosome area close to the cytokines IL-3, IL-4, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Frazer et al., 1997), in so-called Th2 cytokine cluster. They all share a four α-helical bundle structure (Boulay and Paul, 1992; Eisenmesser et al., 2001) but IL-5 protein characteristically forms a disulfide-linked homodimer (Harada et al., 1987). It activates eosinophils and B cells by binding to the specific receptor (IL-5R) and has an effect on immunity against worm infections (Coffman et al., 1989; Finkelman et al., 1997), allergic reactions and B cell responses (Takatsu and Nakajima, 2008; Takatsu et al., 2009).

Mammalian IL-5R is a heterodimer consisting of a unique α-chain (IL-5Rα) and a common β-chain (βc). IL-5Rα specifically binds to IL-5 (Murata et al., 1992; Takaki et al., 1990), whereas βc, which is also present in receptors for IL-3 and GM-CSF (Takatsu, 1992), is predominantly responsible for signaling events. Janus kinase (JAK) 2 and JAK1 bind to IL-5Rα and βc in cytoplasm, respectively, and are activated following the cytokine stimulus. The pathway of JAK2/signal transducer and activator of transcription (STAT) 5 is essential for the IL-5-dependent signal transduction (Alam et al., 1995; Horikawa et al., 2001; Kagami et al., 2000). IL-5Rα contains four well-conserved cysteines and a typical sequence of tryptophan/serine (WSXWS motif) within the extracellular region, and so it belongs to the cytokine receptor class I family (Murata et al., 1992; Takaki et al., 1990). The cytoplasmic region between amino acid 346 and 387 of human IL-5Rα is responsible for the association with JAK2 (Ogata et al., 1998) and a proline-rich sequence (PPXP motif) in this region is essential for IL-5-mediated growth signal transduction (Takaki et al., 1994). IL-5Rα has been identified and characterized in mice (Takaki et al., 1990), humans (Murata et al., 1992), guinea pigs (Scott et al., 2000) and rats (Pierrot et al., 2001). Frog cDNA sequences, which show similarity to mammalian IL-5Rα, are available (GenBank ID: NM_001094351 and NM_001123462), but they have not yet been characterized.

Chickens are useful animals in both fundamental biology and livestock industry. Molecular analysis of their immunity is important with regard to protection from poultry diseases. Recently, various cytokine genes have been shown to exist in the chicken genome by bioinformatics approaches. The chicken Th2 cytokine cluster was identified by Avery et al. (2004). This cluster includes IL-3, IL-4, IL-5, IL-13 and GM-CSF genes, but IL-5 seems to be a pseudogene due to the lack of recognizable promoter and

Abbreviations: achIL-5, artificially produced chicken interleukin-5; βc, common β-chain; CDS, coding sequence; Ct, cycle threshold; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-5Rα, IL-5 receptor α-chain; JAK, Janus kinase; RACE, rapid amplification of cDNA ends; R_{max}, maximum RU value; RU, resonance units; SD, standard deviation; SPR, surface plasmon resonance; STAT, signal transducer and activator of transcription; Th2, T helper 2; TM, transmenbrane; UTR, untranslated region.

* Corresponding author. Tel./fax: +81 82 424 7967.
E-mail address: sfurusa@hiroshima-u.ac.jp (S. Furusawa).

0145-305X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2012.02.013

regulatory sequences. Around the same time, a novel cytokine-like transcript KK34 which is abundant in chicken γδ T cells was discovered (Koskela et al., 2004). The amino acid sequence was similar to mammalian IL-5 and the gene was present in the Th2 cytokine cluster, but not in a syntenic location. Little is currently known about its function. If KK34 is an alternative to chicken IL-5 (chIL-5), it should bind to chIL-5R. However, the chIL-5R has not been reported.

In this study, we identified a full-length cDNA encoding chIL-5Rα and analyzed the protein interaction with artificially produced chIL-5 (achIL-5) and KK34 to investigate their relations.

2. Materials and methods

2.1. Experimental animals and tissues

White leghorn H-B15 inbred female chickens were maintained at our animal facilities in accordance with the guidelines for animal experiments at Hiroshima University. Tissues (thymus, spleen, bone marrow, lung, liver, kidney, and ovary) were obtained from 1-month-old chickens.

2.2. Rapid amplification of cDNA ends (RACE) and sequence analysis

Spleen RNA was extracted using TRIzol reagent (Invitrogen), followed by treatment with RNase-free DNase I (Takara). Reverse transcription into first-strand cDNA was conducted using a GeneRacer kit (Invitrogen) according to the manufacturer's protocol. Gene-specific primers (GSP) (Table 1) were designed from the sequence of Gallus gallus similar to IL-5Rα mRNA (GenBank ID: XM_001235030). First amplification of the 5′ end was performed in a 25-μl reaction volume containing the cDNA equivalent to 63 ng of RNA, 1.25 U Ex taq polymerase (Takara), 200 μM each dNTP, 0.6 μM GeneRacer primer and 0.2 μM GSP under the following cycling conditions: 2 min at 94 °C, 5 cycles of 30 s at 94 °C and 2 min at 72 °C, 5 cycles of 30 s at 94 °C and 2 min at 70 °C, 20 cycles of 30 s at 94 °C, 30 s at 65 °C and 2 min at 68 °C, and final extension for 10 min at 68 °C. Second amplification was performed in a 25-μl reaction volume containing 0.5 μl of the first reaction product, 0.5 U Ex taq polymerase, 200 μM each dNTP, 0.2 μM GeneRacer primer and 0.2 μM GSP under the following cycling conditions: 2 min at 94 °C, 20 cycles of 10 s at 98 °C, 30 s at 70 °C and 2 min at 72 °C, and a final extension for 10 min at 72 °C. First and second amplification of the 3′ end was performed under the same conditions for the 5′ end, respectively. Second PCR products were cloned into a pGME T-Easy vector System (Promega) and JM109 competent cells were transformed. Bacterial colonies were screened by blue/white selection and insert DNA was amplified by PCR using M13–20 (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGC TATGCCATGATTAC-3′) primers. PCR products were treated with exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Promega), followed by direct sequencing using a Big Dye terminator v3.1 sequencing kit (Applied Biosystems). The nucleotide sequences are available under the following GenBank ID: AB618610 (isoform 1), AB618611 (isoform 2) and AB618612 (isoform 3).

The identified cDNA sequences were analyzed as follows. The exon–intron organization was determined by comparing the cDNA sequence with the chicken genomic sequence using BLAST (http://www.ncbi.nlm.nih.gov/Blast.cgi). Prediction of the signal peptide and transmembrane (TM) region were performed by SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/), respectively. Multiple sequence alignment and phylogenetic tree construction were performed using ClustalW at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html). The tree was constructed on the basis of amino acid differences (*p*-distance) with 1000 bootstrap trials, and was drawn using TreeView (Page, 1996). A sequence similarity search was performed using BLAST alignment.

2.3. Quantitative real-time PCR

Tissue RNA was extracted as described above and reverse transcribed into first-strand cDNA using Superscript III Reverse Transcriptase (Invitrogen) and oligo$(dT)_{15}$ (Roche). Primers used for the amplification of chIL-5Rα and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1. Real-time PCR was performed in a 15-μl reaction volume containing cDNA equivalent to 18 ng of RNA and 0.3 μM each primer using FastStart Universal SYBR Green Master (ROX; Roche) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification of chIL-5Rα and GAPDH was conducted three times. GAPDH was used to normalize PCR data. Relative mRNA levels of chIL-5Rα were calculated by the $2^{-\Delta\Delta\text{cycle threshold (Ct)}}$ method (Livak and Schmittgen, 2001).

2.4. Construction of expression vectors to produce recombinant proteins: soluble chIL-5Rα, soluble mouse IL-5Rα (mIL-5Rα), chIL-5, KK34 and mIL-5

Templates and primers used to amplify target genes were shown in Supplemental Table 1. PCR was conducted using KOD-Plus DNA polymerase (Toyobo). PCR products were cloned into pcDNA4/myc-His (B; Invitrogen) to produce soluble chIL-5Rα, soluble mIL-5Rα, chIL-5, KK34, and mIL-5 proteins with a myc-His tag, and chIL-5, KK34 and mIL-5 proteins without an epitope tag. The vector for non-tagged chIL-5 expression carries chIL-5 gene sequence including introns as described in Supplemental Table 1. It was also used to produce chIL-5 transcripts in cultured cells.

2.5. Cell culture and transfection

A chicken T cell line, RP1 (Nazerian et al., 1977), was kindly provided by Prof. K. Ikuta (the Research Institute for Microbial Disease,

Table 1
Primers used for RACE and real-time PCR.

Primer name	Sequence	Purpose
5-GSP1	5′-TGCTGGAAACGGTGAAGTGAGTAACAC-3′	First amplification in 5′ RACE
3-GSP1	5′-ACTCACTTCACCGTTTCCAGCACTG-3′	First amplification in 3′ RACE
5-GSP2	5′-TGGAGTTCTGCTGTCACCCAGTCACTG-3′	Second amplification in 5′ RACE
3-GSP2	5′-TGGCTTCCTGGCAAAGAGGCAC-3′	Second amplification in 3′ RACE
chIL5Rα-RT-F	5′-ACGGTCACGCTTATTGCTATAG-3′	Real-time PCR
chIL5Rα-RT-R	5′-TGGAAAGGGATCTCTGAACTTG-3′	Real-time PCR
chGAPDH-RT-F	5′-GGAAAGTCATCCCTGAGCTG-3′	Real-time PCR
chGAPDH-RT-R	5′-GGTCAACAACAGAGACATTGG-3′	Real-time PCR

Osaka University, Japan). The cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Irvine Scientific) at 38.5 °C in 5% CO_2. Two hundred and fifty microliters of RP1 cell suspension (containing 1.0×10^7 cells) was transfected with 25 μg of chIL-5 gene vector using Gene Pulser Xcell (Bio-Rad) at 550 V and 25 μF. Human embryonic kidney 293F cells (Invitrogen) were grown in FreeStyle 293 Expression medium (Invitrogen) at 37 °C under 8% CO_2. Transfection of 293F cells was conducted with all expression vectors described above using 293fectin transfection reagent (Invitrogen).

2.6. Sequencing of chIL-5 transcripts from transfected chIL-5 gene vectors

Three days after transfection, aliquots of chIL-5 gene-transfected RP1 cells and 293F cells were harvested and total RNA was extracted. Reverse transcription into first-strand cDNA was conducted using Superscript III Reverse Transcriptase and oligo$(dT)_{15}$. The CMV promoter-driven chIL-5 transcripts were amplified by PCR using chIL5F (5′-GCACATCAGGACCATGAGGAC-3′) and chIL5R-sm (5′-CCACTCTTGCATCTCTTGATAATTTTCG-3′) primers. PCR products were purified with NucleoSpin Extract II (Macherey–Nagel), and directly sequenced using a Big Dye terminator v3.1 sequencing kit. Nucleotide sequence of the transcript is available under the GenBank ID: AB618613.

2.7. Preparation of recombinant proteins

Each culture supernatant of transfected 293F cells was collected 72 h after transfection or from the stable transfectants. Myc-His-tagged chIL-5Rα and mIL-5Rα proteins were purified with Ni–NTA agarose (Qiagen) according to manufacturer's instructions. They were concentrated and transferred to HBS-EP buffer (GE Healthcare) by ultrafiltration using Nanosep 10 K omega centrifugal devices (Pall Life Sciences) for surface plasmon resonance (SPR) assay as described below. Aliquots of them were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5% gel and stained with Coomassie brilliant blue (CBB). On the other hand, recombinant cytokines without an epitope tag were directly concentrated and were transferred to HBS-EP buffer using Nanosep 10 K omega centrifugal devices. Various dilutions of the cytokine samples were used in the SPR assay. Aliquots of the cytokines were resolved by 18% SDS–PAGE and stained with a silver stain kit (Daiichi Pure Chemicals) according to manufacturer's protocol. Furthermore, recombinant cytokines with a myc-His tag in culture supernatants were resolved by 12.5% SDS–PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and incubated in blocking solution (5% nonfat dried milk in tris-buffered saline Tween-20 (TBST)) overnight at 4 °C. The membrane was incubated for 5 h with horseradish peroxidase (HRP)-conjugated mouse anti-c-myc antibodies (Abs) (Invitrogen) diluted 1/2000 in blocking solution and washed three times with TBST. The proteins were detected using enhanced chemiluminescence, immunoStar LD (Wako).

2.8. SPR assay

The interaction between the receptors and cytokines was analyzed by SPR assay using a Biacore 2000 apparatus (GE Healthcare). The CM5 sensor chip was activated with a 1:1 mixture of 400 mM N-ethyl-N-(3-dimethylaminopropyl) carbodiimide/100 mM N-hydroxysuccinimide, and mouse anti-c-myc Abs 9E10 (Sigma–Aldrich) diluted 1/150 in 10 mM acetate (pH 5.0) was injected onto the activated sensor surface to obtain an immobilization level of approximately 7000 or 9500 resonance units (RU). Reactive groups remaining on the surface were then inactivated with 1 M ethanolamine-HCl (pH 8.5). Myc-His-tagged receptors (40–70 μg/ml) diluted in HBS-EP buffer were captured by the immobilized Abs on flow cell 4 (Fc4). Concurrently, normal HBS-EP buffer was added into Fc3 as a control. A cytokine sample was then injected into both flow cells. After each cycle, 10 mM glycine–HCl (pH 1.5) was added in order to generate the chip surface. Conditions for repetitive injections were as follows: 5 μl/min for 1–6 min (receptors), 30 μl/min for 60 s (cytokines) followed by 60 s of dissociation in HBS-EP buffer, and 100 μl/min for 30 s (10 mM glycine–HCl). Sensorgrams were corrected for nonspecific binding by subtracting control data (Fc3) from the interaction data (Fc4). Furthermore, data for buffer injection was subtracted from those for cytokine sample injections in order to correct the dissociation of the captured receptors (Myszka, 2000). Data analysis was performed using BIAevaluation v3.1 software (GE Healthcare). Cytokine binding signals were measured at the end of injection.

3. Results

3.1. Identification of full-length cDNA encoding chIL-5Rα

On line database at the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov) was searched for the nucleotide sequence of chIL-5Rα, and the putative mRNA (GenBank ID: XM_001235030) consisting of eight exons was found on chromosome 12 (GenBank ID: NC_006099, nucleotides 18,717,202–18,721,953) (Fig. 1A). The chromosomal region also included genes for contactin 4, tRNA-nucleotidyltransferase and cereblon, which were conserved at least among human, mouse, rat and chicken (data not shown).

We next conducted 5′ and 3′ RACE to identify the full-length cDNA, and obtained three isoforms with different exon–intron organizations from one another and from the putative sequence (Fig. 1A). Exons 1–7 were shared by the three isoforms, but isoform 3 had an elongated exon 8 and no further exons. Isoforms 1 and 2 also shared exon 8 and 9, which encoded a TM domain, but the downstream differed. Isoform 2 had exon 10 but isoform 1 had another type of exon 10 and exon 11. The full-length sequences of isoform 1, 2 and 3 were 2495 bp, approximately 1656 bp (with low confidence at the 3′ end) and 1759 bp, respectively, and all the three were confirmed by Northern blot (Supplemental Fig. 1).

Nucleotide and deduced amino acid sequences of membrane-anchored type isoform 1 are shown in Fig. 1B. Isoform 1 mRNA consisted of a 336-bp 5′ untranslated region (UTR), a 1236-bp coding sequence (CDS) and a 923-bp 3′ UTR with two typical polyadenylation signals. The deduced 411 amino acid sequence included the 17 amino acid signal peptide at the *N*-terminus, and a TM domain was identified at residues 329–351. A WSXWS motif and four potential asparagine (N)-linked glycosylation sites were found in the extracellular region. A PPXP motif was present in the cytoplasmic region. Moreover, the amino acid sequence was compared with that of mammalian IL-5Rα using a multiple alignment and the unrooted phylogenetic tree (Fig. 2A and B). The alignment showed conservation of four cysteine residues that are characteristic of the class I cytokine receptor superfamily, besides the WSXWS and PPXP motifs. The amino acid sequence in the chicken was similar to that in mammals throughout the entire length. The phylogenetic tree divided the chicken from the clusters of mammals and frogs by a long branch, although the genetic distance between the chicken and mammalian cluster was slightly smaller. A BLAST search revealed that the amino acid sequence of chicken shared 41–43% identity and 57–60% similarity with mammalian IL-5Rα, and approximately 30% identity and 50% similarity with frog IL-5Rα.

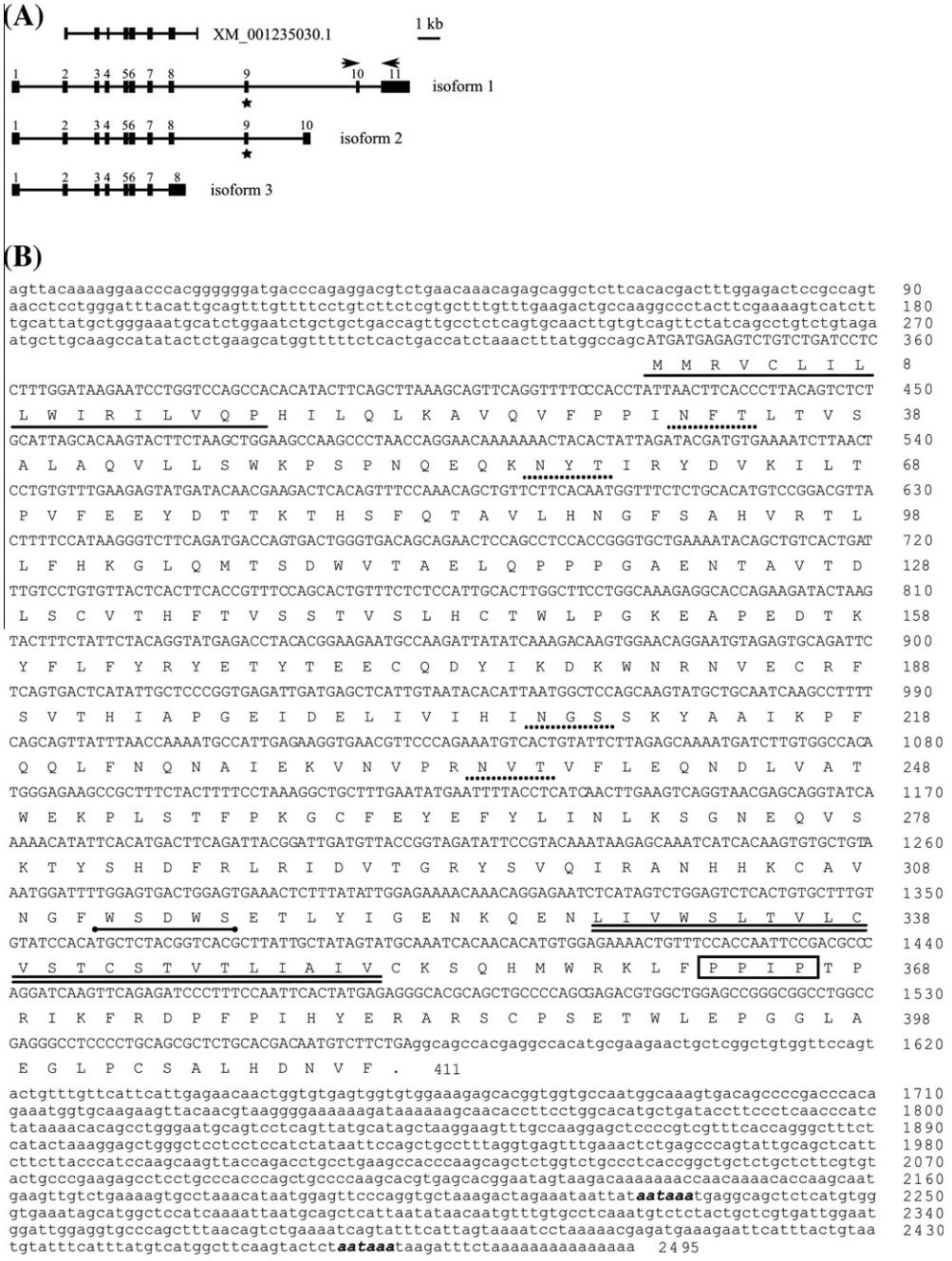

Fig. 1. Characteristics of chIL-5Rα gene structures, nucleotide and amino acid sequences. (A) Gene structures of putative and identified chIL-5Rα mRNA. Exons and introns are indicated by boxes and lines, respectively. The exons encoding the transmembrane region are indicated by star symbols. Scale bar represents 1 kb. Arrows indicate primers used for amplification of isoform 1 on real-time PCR (Fig. 3). (B) Nucleotide and deduced amino acid sequences of chIL-5Rα isoform 1. UTR is shown in lower case and CDS is shown in upper case. Stop codon (TGA) is indicated by a dot. Signal sequence is underlined. Four potential N-link glycosylation sites (NXS/T) are indicated by dotted lines. TM region is indicated by double lines. The WSXWS motif is indicated by a line with dots. Cytoplasmic PPXP motif is boxed. Typical polyadenylation signals are in bold italic.

Based on the gene synteny, conserved domains and high similarity, isoform 1 was regarded as an IL-5Rα orthologue in chickens. On the other hand, isoform 2 had shorter cytoplasmic region but it did not contain the PPXP motif. In addition, the protein domain search program did not detect a TM domain in isoform 3, and it therefore appeared to be soluble.

3.2. Expression analysis of chIL-5Rα mRNA

PCR primers were designed based on the sequences of exons 10 and 11 in the isoform 1, and thus does not amplify the other isoforms (Fig. 1A). Both lymphoid tissues (thymus, spleen and bone marrow) and non-lymphoid tissues (lung, kidney and ovary) were used in the measurement, as shown in Fig. 3. The mRNA expression was markedly high in bone marrow, and was relatively high in spleen and lung.

3.3. Production of recombinant proteins by culture cells

In order to investigate the interaction between chIL-5Rα and cytokines, recombinant proteins were produced by cultured cells. Initially, the soluble chIL-5Rα (Fig. 4A, extracellular region) with a myc-His epitope tag was expressed by 293F cells and purified from the culture supernatants. An aliquot was subjected to SDS–

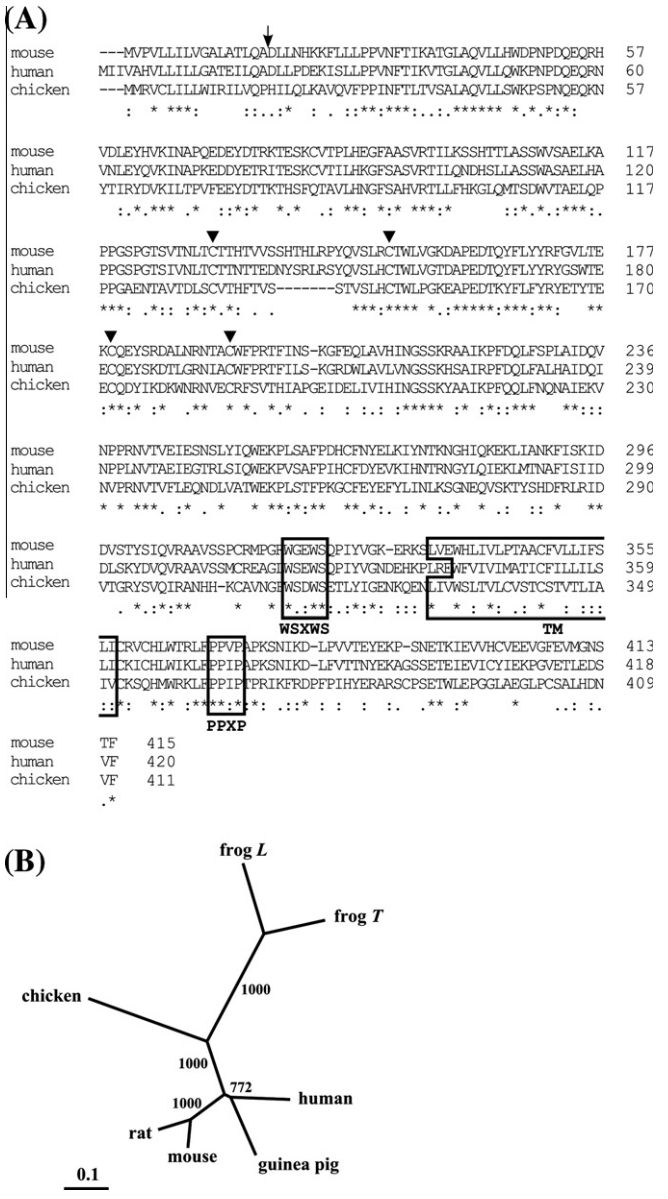

Fig. 2. Comparison of IL-5Rα amino acid sequences among vertebrates. GenBank ID for each sequence are as follows: NP_032396 (mouse), NP_000555 (human), NP_446097 (rat), NP_001166596 (guinea pig), NP_001087820 (frog *L*: *Xenopus laevis*) and NP_001116934 (frog *T*: *Xenopus tropicalis*). (A) Alignment of mouse, human and chicken IL-5Rα. Identical residues are indicated by asterisks, and strong and weak conservative changes are represented by colons and dots, respectively. Vertical arrow indicates the signal peptide cleavage site. Arrowheads mark the four cysteine residues characteristic of the class I cytokine receptor superfamily. WSXWS motif, TM region and PPXP motif are boxed. (B) Unrooted phylogenetic tree. Scale represents amino acid substitutions per site. Numbers are bootstrap values for 1000 replicates.

PAGE under reducing and non-reducing conditions (Fig. 4B). The apparent molecular mass was found to be 50 kDa under reducing conditions, although it was predicted to be 41 kDa from the amino acid sequence (Fig. 1B) and epitope tag. These findings suggest post-translational modification, for example, glycosylation, in the extracellular region of chIL-5Rα.

We next tried to produce recombinant chIL-5 and KK34 proteins but we couldn't obtain the evidence for chIL-5 mRNA as described by Avery et al. (2004). Accordingly, chIL-5 gene was expressed under a CMV promoter, which was termed achIL-5, and its transcripts in chicken T cell line RP1 and 293F cells were examined by RT-PCR (Fig. 5A). In both RP1 and 293F cells, the size of the PCR products (lanes 2 and 4) was smaller, approximately 0.4 kb, than that of the gene (lane 1), thus suggesting splicing of the intronic regions. The sequences of both mRNAs were identical, indicating that the splicing pattern did not differ between human and chicken cells. An alignment of the mRNA and chromosomal sequences indicated that all splice acceptor and donor sites conformed to the GT-AG rule (Breathnach and Chambon, 1981) but the sequence corresponding to exon 1 was shorter than the computer-assisted gene prediction (GenBank ID: NM_001007084) (Fig. 5B), resulting in a single amino acid substitution at residue 41 and a deletion at residues 42–51 without frameshift (Fig. 5C). In contrast, KK34 CDS was amplified from chicken spleen cDNA and cloned into an expression vector. The cloned sequence included five single nucleotide polymorphisms, two of which were silent mutations, and three of which resulted in amino acid substitutions at residues 132, 136 and 138 with strong similarity (Supplemental Fig. 2). Molecular mass of the mature achIL-5 and KK34 proteins was estimated to be 12.8 and 17.2 kDa, respectively, based on the deduced amino acid sequences. After transfection of 293F cells with the expression vectors, recombinant proteins without an epitope tag in culture supernatants were visualized by silver staining (Fig. 6A). The apparent molecular mass of achIL-5 protein was approximately 13 kDa under reducing conditions, as expected from the deduced amino acid sequences, and this was similar under non-reducing conditions. In contrast, KK34 protein migrated as a strong band at 27 kDa under reducing conditions and thus appeared to be subjected to glycosylation, but the band intensity was very weak under non-reducing conditions.

For further characterization, the recombinant cytokines with a myc-His epitope tag were produced by 293F cells and analyzed by Western blotting using anti-myc Abs, as shown in Fig. 6B. A single band of achIL-5 protein was detected under reducing and non-reducing conditions, and was at a similar size as the band on silver staining coupled with an epitope tag. On the other hand, KK34 protein was found as a single band under reducing conditions, but was detected in a higher molecular mass range as multiple bands under non-reducing conditions. This indicates the potential for polymerization through disulfide bonds. Briefly, achIL-5 and KK34 proteins were prepared and they appeared to exist as monomers and multimers, respectively, in contrast to homodimeric mammalian IL-5 (Fig. 6B).

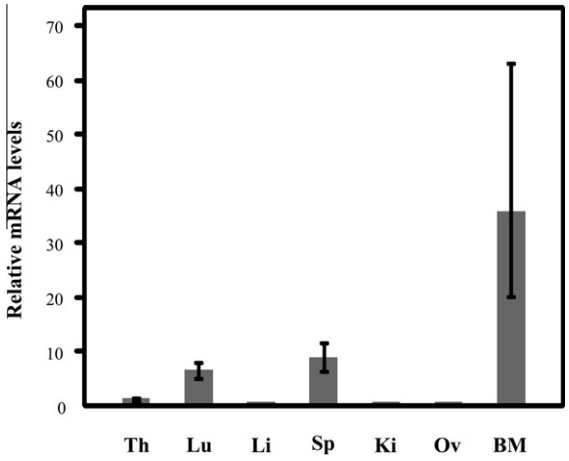

Fig. 3. Quantitative real-time PCR analysis of chIL-5Rα mRNA. Thymus (Th), lung (Lu), liver (Li), spleen (Sp), kidney (Ki), ovary (Ov) and bone marrow (BM) samples were measured. Expression levels were averaged from three PCR reactions and are shown relative to those in thymus. Standard deviation (SD) of ΔΔCt value was calculated from the three replicates of reactions. Error bars represent ranges of relative mRNA level ($2^{-\Delta\Delta Ct}$) determined using the ΔΔCt value ± SD (for details, see ABI PRISM 7700 User Bulletin No. 2). These data are representative of three independent experiments.

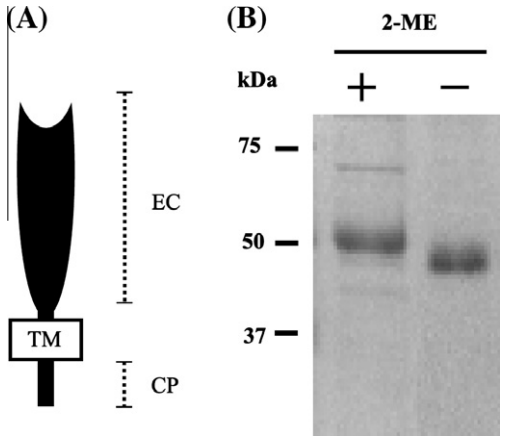

Fig. 4. Production of soluble chIL-5Rα protein. Extracellular region of chIL-5Rα associated with a myc-His tag is expressed by 239F cells. (A) Scheme of full-length chIL-5Rα protein. Extracellular (EC), TM and cytoplasmic (CP) regions are shown. (B) SDS–PAGE analysis. Protein (0.5 μg) was resolved by SDS–PAGE with Precision Plus Protein standard (Bio-Rad).

3.4. Interaction analysis by SPR assay

Interactions between recombinant receptors (chIL-5Rα and a control, mIL-5Rα) and cytokines (achIL-5, KK34 and a control, mIL-5) were analyzed. An overview of the assay is shown in Fig. 7A. Anti-myc Abs were immobilized on a sensor chip surface and soluble IL-5Rα was captured via the Abs. Cytokines were then injected onto the surface in order to measure interaction with the receptors.

Initially, highly (eightfold) concentrated cytokines were injected onto the chIL-5Rα-captured surface in order to obtain sufficient responses (Fig. 7B). Sensorgrams showed that achIL-5 bound to chIL-5Rα (513 RU) with a signal of 114 RU, but KK34 and mIL-5 did not bind. Moreover, various concentrations of achIL-5 sample were injected, and even a 1/16 dilution sample saturated chIL-5Rα (367 RU) at the end of injection, and yielded a response signal of 83 RU (Fig. 7C). Next, eightfold concentrated cytokines were injected onto the mIL-5Rα-captured surface in order to evaluate cross-reactivity (Fig. 7D). Sensorgrams showed that each signal for achIL-5 and KK34 was similar to those of the mock control and mIL-5 only bound to the surface. The results are summarized in Fig. 7E. These findings indicate that chIL-5Rα shows specific binding for monomeric achIL-5, and does not interact with KK34. Moreover, chIL-5Rα and achIL-5 do not show cross-reactivity with their mammalian counterparts.

4. Discussion

In mammals, IL-5R consists of two chains, IL-5Rα and βc. IL-5Rα specifically binds to IL-5 but βc is also a constituent of receptors for IL-3 and GM-CSF. Although investigation of IL-5R has been lacking in chickens, we believed that βc protein would be present because chGM-CSF activates chicken bone marrow cells, as described previously (Avery et al., 2004). In this study, we identified the full-length cDNA encoding chIL-5Rα. This is the first characterization of non-mammalian IL-5Rα.

Three types of chIL-5Rα cDNA were found; isoforms 1, 2 and 3 (Fig. 1A). Isoform 1 has the characteristic features of the class I cytokine receptor superfamily (four well-conserved cysteine residues and a WSXWS motif) and a PPXP motif (Figs. 1B and 2A). These observations imply capability of cytokine recognition and signal transduction. Isoform 2 has shorter cytoplasmic region but it lacks the PPXP motif. This structure indicates a lack of signal transduction, and therefore isoform 2 may be a decoy receptor. In addition, isoform 3 appears to be soluble. In mammals, soluble

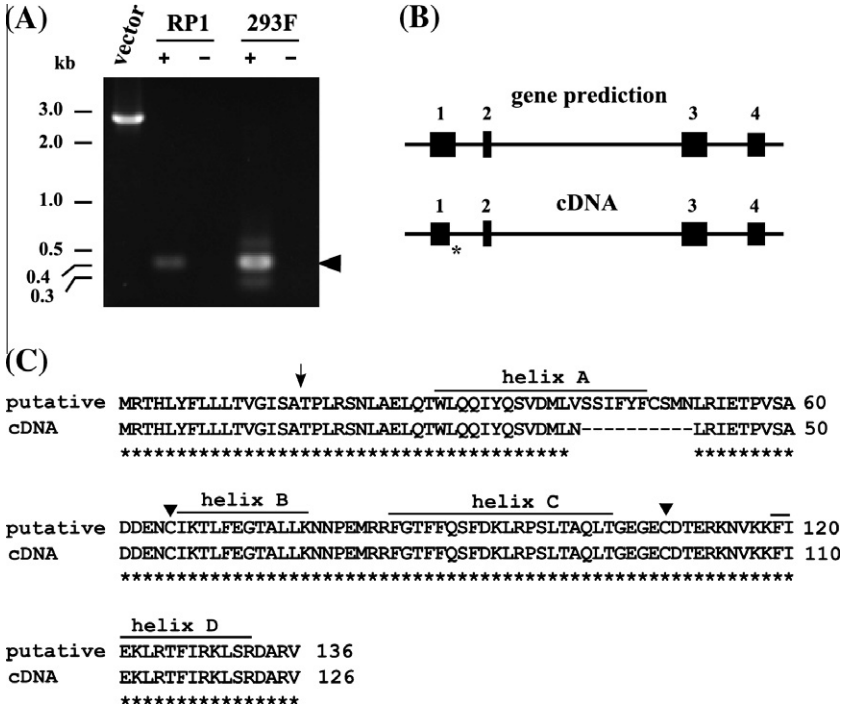

Fig. 5. CMV promoter-driven transcription from chIL-5 gene. (A) Transcription and splicing in RP1 and 293F cells. RP1 and 293F cells are transfected with chIL-5 gene vector (+) and mock vector (–). Amplified bands (arrowhead) indicate the transcribed and spliced products. The chIL-5 gene vector is also used as a control template in PCR (lane 1). (B) Exon–intron organization of chIL-5 gene. Exons and introns are indicated by boxes and lines, respectively. The upper gene comprises putative exons based on the computer-assisted gene prediction. The lower gene is the organization based on the chIL-5 cDNA sequence. Exon 1 in the lower gene is smaller (denoted by asterisk). (C) Alignment of amino acid sequences deduced from the putative exons and cDNA sequences. Vertical arrow indicates the signal peptide cleavage site. Arrowheads mark the two cysteine residues, which are important for formation of a dimeric structure (Sprang and Bazan, 1993). Four regions corresponding to the α-helices in human IL-5 (Avery et al., 2004) are indicated by horizontal lines.

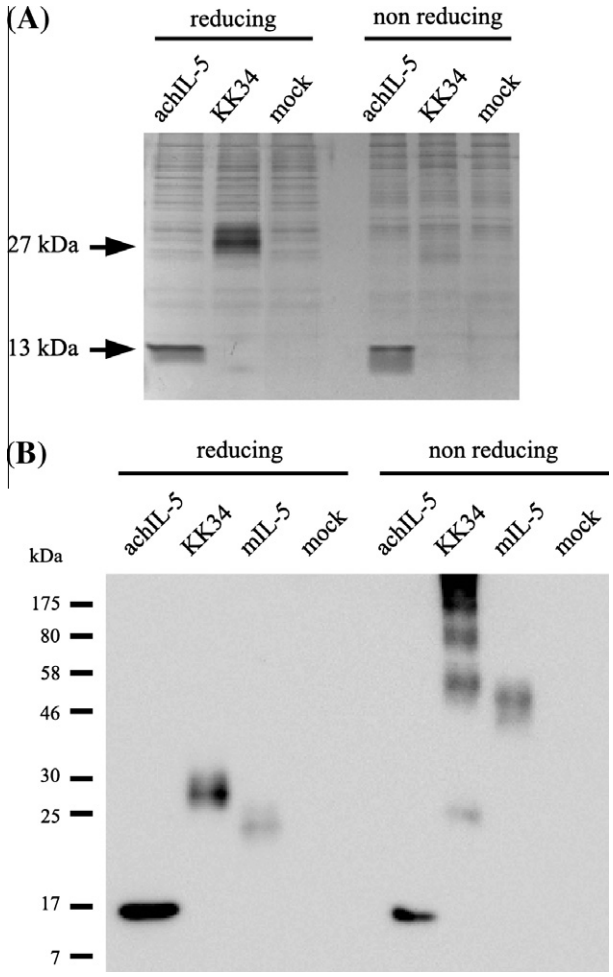

Fig. 6. Characterization of recombinant achIL-5 and KK34 proteins. (A) Silver staining. Culture supernatant including each cytokine without an epitope tag was resolved by SDS–PAGE. Precision Plus Protein standard is used as a molecular marker. Mock control is the culture supernatants of pcDNA4/myc-His-transfected cells. Apparent molecular masses are indicated with arrows. (B) Western blot analysis. Culture supernatant including each of the cytokines with a myc-His tag was resolved by SDS–PAGE. Prestained protein markers (New England Biolabs) were used as size markers.

IL-5Rα isoforms have been found and characterized (Monahan et al., 1997; Tavernier et al., 1991, 1992). The soluble IL-5Rα disrupts interaction between IL-5 and membrane-anchored IL-5Rα on the cell surface, thereby inhibiting signal transduction, inflammatory mediator release, and survival of IL-5R-bearing cells. Isoform 3 in the chicken may be the equivalent of soluble IL-5Rα in mammals.

IL-5Rα mRNA is strongly expressed by eosinophils and basophils in both mice and humans, and by mouse B-1 cells (Hitoshi et al., 1990; Takatsu et al., 2009). In our experiments, real-time PCR analysis showed chIL-5Rα mRNA was most abundant in bone marrow, and was relatively abundant in spleen and lung (Fig. 3). Eosinophils and basophils may also contribute to the high expression of IL-5Rα mRNA in chickens because bone marrow includes polymorphonuclear leukocytes at different stages of maturation. Although chicken spleen contains lymphocytes, further analysis is necessary to determine the expression level in chicken B cells and dependence on the cell subsets. In mammals, eotaxin is major eosinophil chemoattractants and its expression has been shown to be induced by IL-4 and IL-13 in murine lung (Gleich, 2000; Li et al., 1999), but chickens apparently lack eotaxins and their receptors (Kaiser, 2007). It is unclear whether there is another factor operating as an eosinophil

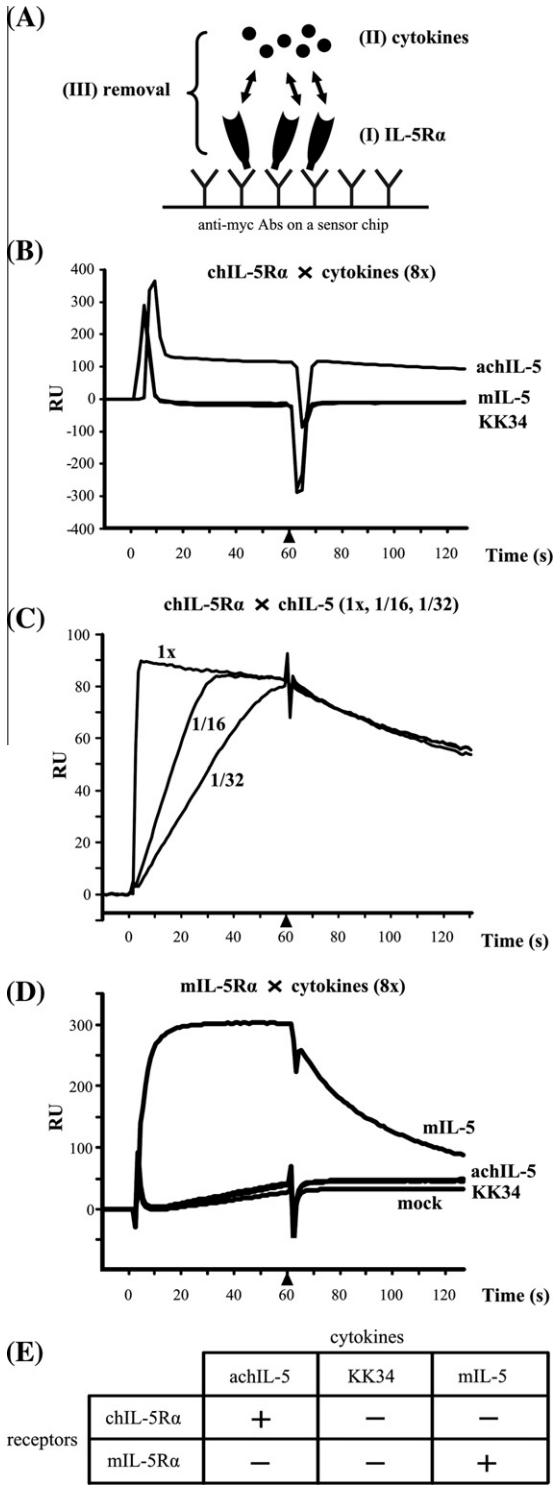

Fig. 7. Interaction analysis by SPR assay. (A) Overview of the assay. Anti-myc Abs are immobilized on the sensor chip surface. Initially, IL-5Rα proteins are captured by the Abs (I). Cytokines are then injected onto the surface (II). Surface-bound complexes are removed by 10 mM glycine–HCl (pH 1.5) to regenerate the sensor chip surface (III). (B) Interactions between chIL-5Rα and eightfold concentrated cytokines. Approximately 513 RU of the chIL-5Rα is captured at the end of cytokine injections (indicated by a triangle). (C) Interaction between chIL-5Rα and various dilutions of achIL-5. Approximately 367 RU of chIL-5Rα is captured at the end of cytokine injections. (D) Interaction between mIL-5Rα and eightfold concentrated cytokines. Approximately 557 RU of mIL-5Rα is captured at the end of cytokine injections. Mock control is the culture supernatants of pcDNA4/myc-His-transfected cells. (E) Brief summary of results. Plus and minus signs indicate binding and non-binding, respectively.

chemoattractant or other kinds of chIL-5Rα producing cells are present in the lung.

With the exception of IL-5, which is a dimer, mammalian cytokines in the Th2 cytokine cluster are monomeric proteins, although they seem to have evolved following ancient gene duplication events (Lee et al., 1989). Accordingly, conformational changes would have occurred in IL-5 during its evolution. It is therefore interesting that recombinant achIL-5 appears to be monomer (Fig. 6A and B). In general SPR assay, the binding signal is proportional to the molecular mass of the associated protein on the sensor chip surface. When the immobilized receptors are fully saturated with the cytokines, the maximum RU value (R_{max}) is theoretically estimated using the general formula R_{max} = (RU values of captured receptors)(molecular mass of cytokines)/(molecular mass of receptors). In our experiments, the saturating achIL-5 bound to 367 RU of chIL-5Rα (molecular mass: 50 kDa) with a binding signal of 83 RU (R_{max}). Accordingly, molecular mass of the associated achIL-5 was calculated to be 11 kDa, which supports the interaction with the monomeric molecule (Fig. 6A, 13 kDa). An artificial monomeric IL-5 was previously produced by inserting eight codons in loop 3 between helices C and D of human IL-5 and the protein retained its binding activity to its specific receptor with a 1:1 stoichiometry (Dickason and Huston, 1996; Li et al., 1997). As shown in Fig. 5C, CMV promoter-driven achIL-5 mRNA has three extra codons between helices C and D in comparison to human IL-5. Despite conservation of the two cysteine residues, which are important for formation of a dimeric structure, the achIL-5 protein exists in monomeric form and specifically binds to chIL-5Rα (Figs. 6A and 7B–E). Although chIL-5 seems to be a pseudogene due to the lack of regulatory elements, these findings support the notion that chickens possess an ancestral IL-5 gene sequence, which encodes a monomeric protein. Additional information on the genomic sequences in other vertebrates and subsequent protein analysis will provide more insight into cytokine evolution. As an artificial monomeric human IL-5 retains its bioactivity, all of the structural features necessary for its function are considered to be present within the monomeric protein (Dickason and Huston, 1996). Therefore, it is of interest as to whether achIL-5 protein (or a slightly modified protein) has any effect on chicken immunity because chIL-5R seems to have α-chain and βc.

Although KK34 appeared to be an IL-5-like transcript (Koskela et al., 2004), it exists in multiple forms (Fig. 6A and B) and does not interact with chIL-5Rα (Fig. 7B). As many as eleven cysteine residues are present among the 150 amino acids of KK34 protein, and these would contribute to the conformational properties (Supplemental Fig. 2). No orthologues of KK34 have been reported in other vertebrates, and its function remains uncertain. However, five single nucleotide polymorphisms were identified in the CDS, two of which were silent mutations, and three of which resulted in amino acid substitutions with strong similarity. This observation implies its importance in chicken, as these residues would no longer need to be conserved if KK34 protein had no function.

In conclusion, we demonstrated that chicken has an IL-5Rα homologue and an ancestral IL-5 gene sequence, which encodes a monomeric protein. Although the IL-5/IL-5R system appears not to work in chicken due to a lack of IL-5 mRNA expression, IL-5Rα retains the capability to interact with monomeric IL-5 protein. An IL-5-like cytokine KK34 does not complement the system.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2012.02.013.

References

Alam, R., Pazdrak, K., Stafford, S., Forsythe, P., 1995. The interleukin-5/receptor interaction activates Lyn and Jak2 tyrosine kinases and propagates signals via the Ras-Raf-1-MAP kinase and the Jak-STAT pathways in eosinophils. Int. Arch. Allergy Immunol. 107, 226–227.

Avery, S., Rothwell, L., Degen, W.D., Schijns, V.E., Young, J., Kaufman, J., Kaiser, P., 2004. Characterization of the first nonmammalian T2 cytokine gene cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokinelike transcript, KK34. Interferon Cytokine Res. 24, 600–610.

Boulay, J.L., Paul, W.E., 1992. The interleukin-4 family of lymphokines. Curr. Opin. Immunol. 4, 294–298.

Breathnach, R., Chambon, P., 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. 50, 349–383.

Cffman, R.L., Seymour, B.W., Hudak, S., Jackson, J., Rennick, D., 1989. Antibody to interleukin-5 inhibit helminth-induced eosinophilia in mice. Science 245, 308–310.

Dickason, R.R., Huston, D.P., 1996. Creation of a biologically active interleukin-5 monomer. Nature 379, 652–655.

Eisenmesser, E.Z., Horita, D.A., Altieri, A.S., Byrd, R.A., 2001. Solution structure of interleukin-13 and insights into receptor engagement. J. Mol. Biol. 310, 231–241.

Finkelman, F.D., Shea-Donohue, T., Goldhill, J., Sullivan, C.A., Morris, S.C., Madden, K.B., Gause, W.C., Urban Jr., J.F., 1997. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. Annu. Rev. Immunol. 15, 505–533.

Frazer, K.A., Ueda, Y., Zhu, Y., Gifford, V.R., Garofalo, M.R., Mohandas, N., Martin, C.H., Palazzolo, M.J., Cheng, J.F., Rubin, E.M., 1997. Computational and biological analysis of 680 kb of DNA sequence from the human 5q31 cytokine gene cluster region. Genome Res. 7, 495–512.

Gleich, G.J., 2000. Mechanisms of eosinophil-associated inflammation. J. Allergy Clin. Immunol. 105, 651–663.

Harada, N., Takahashi, T., Matsumoto, M., Kinashi, T., Ohara, J., Kikuchi, Y., Koyama, N., Severinson, E., Yaoita, Y., Honjo, T., Yamaguchi, N., Tominaga, A., Takatsu, K., 1987. Production of a monoclonal antibody useful in the molecular characterization of murine T-cell-replacing factor/B-cell growth factor II. Proc. Natl. Acad. Sci. USA 84, 4581–4585.

Hitoshi, Y., Yamaguchi, N., Mita, S., Takaki, S., Tominaga, A., Takatsu, K., 1990. Distribution of IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1(CD5)+ B cells. J. Immunol. 144, 4218–4225.

Horikawa, K., Kaku, H., Nakajima, H., Davey, H.W., Hennighausen, L., Iwamoto, I., Yasue, T., Kariyone, A., Takatsu, K., 2001. Essential role of Stat5 for IL-5-dependent IgH switch recombination in mouse B cells. J. Immunol. 167, 5018–5026.

Kagami, S., Nakajima, H., Kumano, K., Suzuki, K., Suto, A., Imada, K., Davey, H.W., Saito, Y., Takatsu, K., Leonard, W.J., Iwamoto, I., 2000. Both stat5a and stat5b are required for antigen-induced eosinophil and T-cell recruitment into the tissue. Blood 95, 1370–1377.

Kaiser, P., 2007. The avian immune genome-a glass half-full or half-empty? Cytogenet. Genome Res. 117, 221–230.

Kinashi, T., Harada, N., Severinson, E., Tanabe, T., Sideras, P., Konishi, M., Azuma, C., Tominaga, A., 1986. Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. Nature 324, 70–73.

Koskela, K., Kohonen, P., Salminen, H., Uchida, T., Buerstedde, J.M., Lassila, O., 2004. Identification of a novel cytokine-like transcript differentially expressed in avian gammadelta T cells. Immunogenetics 55, 845–854.

Lee, J.S., Campbell, H.D., Kozak, C.A., Young, I.G., 1989. The IL-4 and IL-5 genes are closely linked and are part of a cytokine gene cluster on mouse chromosome 11. Somatic Cell Mol. Genet. 15, 143–152.

Li, J., Cook, R., Doyle, M.L., Hensley, P., McNulty, D.E., Chaiken, I., 1997. Monomeric isomers of human interleukin 5 show that 1:1 receptor recruitment is sufficient for function. Proc. Natl. Acad. Sci. USA 94, 6694–6699.

Li, L., Xia, Y., Nguyen, A., Lai, Y.H., Feng, L., Mosmann, T.R., Lo, D., 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. J. Immunol. 162, 2477–2487.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Methods 25, 402–408.

Monahan, J., Siegel, N., Keith, R., Capron, M., Christine, L., Compton, R., Cusik, S., Hirsch, J., Huynh, M., Devine, C., Polazzi, J., Rangwala, S., Tsai, B., Partanova, J., 1997. Attenuation of IL-5-mediated signal transduction, eosinophil survival, and inflammatory mediator release by a soluble human IL-5 receptor. J. Immunol. 159, 4024–4034.

Murata, Y., Takaki, S., Migita, M., Kikuchi, Y., Tominaga, A., Takatsu, K., 1992. Molecular cloning and expression of the human interleukin 5 receptor. J. Exp. Med. 175, 341–351.

Myszka, D.G., 2000. Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with Biacore. Methods Enzymol. 323, 325–340.

Nazerian, K., Stephens, E.A., Sharma, J.M., Lee, L.E., Galities, M., Witter, R.L., 1977. A nonproducer T lymphoblastoid cell lines from Marek's disease transplantable tumor (JMV). Avian Dis. 21, 69–76.

Ogata, N., Kouro, T., Yamada, A., Koike, M., Hanai, N., Ishikawa, T., Takatsu, K., 1998. JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor α and βc subunit, respectively, and are activated upon IL-5 stimulation. Blood 91, 2264–2271.

Page, R.D.M., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
Pierrot, C., Bègue, A., Szpirer, C., Capron, A., Capron, M., Khalife, J., 2001. Cloning of the rat IL-5Ralpha gene: analysis of 5′-upstream region and expression by B cells. Biochem. Biophys. Res. Commun. 288, 328–339.
Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P., Paul, W.E., 1989. Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. Nature 339, 64–67.
Scott, C.W., Logsdon, N.J., Wilkins, D.E., Norris, T.E., Sobotka-Briner, C., Hubbs, S., Graham, A., 2000. Molecular cloning of the guinea-pig IL-5 receptor alpha and beta subunits and reconstitution of a high affinity receptor. Cytokine 12, 858–866.
Sprang, S.R., Bazan, J.F., 1993. Cytokine structural taxonomy and mechanisms of receptor engagement. Curr. Opin. Struct. Biol. 3, 815–827.
Takaki, S., Tominaga, A., Hitoshi, Y., Mita, S., Sonoda, E., Yamaguchi, N., Takatsu, K., 1990. Molecular cloning and expression of the murine interleukin-5 receptor. EMBO J. 9, 4367–4374.
Takaki, S., Kanazawa, H., Shiiba, M., Takatsu, K., 1994. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor α chain and its function in IL-5-mediated growth signal transduction. Mol. Cell. Biol. 14, 7404–7413.
Takatsu, K., 1992. Interleukin-5. Curr. Opin. Immunol. 4, 299–306.
Takatsu, K., Nakajima, H., 2008. IL-5 and eosinophilia. Curr. Opin. Immunol. 20, 288–294.
Takatsu, K., Tanaka, K., Tominaga, A., Kumahara, Y., Hamaoka, T., 1980a. Antigen-induced T cell-replacing factor (TRF). III. Establishment of T cell hybrid clone continuously producing TRF and functional analysis of released TRF. J. Immunol. 125, 2646–2653.
Takatsu, K., Tominaga, A., Hamaoka, T., 1980b. Antigen-induced T cell-replacing factor (TRF). I. Functional characterization of a TRP-producing helper T cell subset and studies on TRF production. J. Immunol. 124, 2414–2422.
Takatsu, K., Kouro, T., Nagai, Y., 2009. Interleukin 5 in the link between the innate and acquired immune response. Adv. Immunol. 101, 191–236.
Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W., Plaetinck, G., 1991. A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific α chain and a β chain shared with the receptor for GM-CSF. Cell 66, 1175–1184.
Tavernier, J., Tuypens, T., Plaetinck, G., Verhee, A., Fiers, W., Devos, R., 1992. Molecular basis of the membrane-anchored and two soluble isoforms of the human interleukin 5 receptor α subunit. Proc. Natl. Acad. Sci. USA 89, 7041–7045.