Cloning of the chicken interleukin-13 receptor α2 gene and production of a specific monoclonal antibody

Miki Miyoshi[1], Hiroyuki Horiuchi[1,2*], Yuuji Fukushima[1], Haruo Matsuda[1,2] and Shuichi Furusawa[1,2]

[1]Laboratory of Immunobiology, Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan

[2]Hiroshima Prefectural Institute of Industrial Science and Technology, 3-10-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan

*Corresponding author. Address: Laboratory of Immunobiology, Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan. Tel/Fax: +81-824-24-7970

E-mail: hhori10@hiroshima-u.ac.jp

Abbreviations used: IL, interleukin; IL-13R, IL-13 receptor; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; NFκB, nuclear factor κB; IFN-γ, interferon-γ; GM-CSF, granulocyte-macrophage colony-stimulating factor; mAb, monoclonal antibody; IMDM, Iscove's modified Dulbecco's medium; SSH, suppression subtractive hybridization; PCR, polymerase chain reaction; EtBr,

ethidium bromide; DIG, digoxigenin; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; RT: room temperature; MBP, maltose binding protein; ELISA, enzyme-linked immunosorbent assay; ORF, open reading frame

Cloning of the chicken interleukin-13 receptor α2 gene and production of a specific monoclonal antibody

ABSTRACT

Mammalian interleukin-13 (IL-13) is an important regulatory T2 cytokine secreted by activated T lymphocytes. The IL-13 receptor (IL-13R) has two different chains, IL-13Rα1 and IL-13Rα2. Although the chicken *IL-13* gene is well characterized, little is known about IL-13Rs. We cloned a cDNA encoding the 380 amino acid pro-peptide of chicken IL-13Rα2 (chIL-13Rα2) and developed a monoclonal antibody (mAb), HU13-1, against it. The chIL-13Rα2 amino acid sequence showed 37-39% sequence identity with mammalian homologs. High levels of chIL-13Rα2 mRNA were expressed in liver, testis, ovary, brain, and lipopolysaccharide (LPS)-stimulated IN24 cells. HU13-1 specifically recognized recombinant chIL-13Rα2 in ELISAs, and western blots identified a 45-kDa glycoprotein or a 41-kDa non-glycosylated protein in LPS-stimulated IN24 cell lysates. LPS induced a gradual increase in HU13-1-positive IN24 cells over 20 h. These results indicate that mAb HU13-1 recognizes native chIL-13Rα2 and will be valuable for further studies of chicken IL-13Rs.

Keywords: chicken; interleukin-13 receptor; monoclonal antibody

INTRODUCTION

Interleukin-13 (IL-13) is a cytokine secreted by Th2 cells and some other cell types. It shares many, but not all, of its biological activities with IL-4, including the enhancement of B cell proliferative responses, the induction of immunoglobulin class switching to IgG4 and IgE, and anti-cytotoxic and inflammatory activities of monocytes [1-3]. Monocyte or macrophage-derived IL-13 enhances the expression of many members of the integrin family, including CD11b, CD11c, CD18, and CD29 [3], as well as MHC class II and CD23 [4]. In addition, IL-13 inhibits the production of pro-inflammatory mediators, including prostaglandins [5], reactive oxygen and nitrogen intermediates [6, 7], IL-1, IL-6, IL-8, tumor necrosis factor-α (TNF-α), and IL-12 [4], by monocytes and macrophages, through a mechanism that involves the suppression of nuclear factor κB (NFκB) [8]. Furthermore, IL-13 has recently been shown to play an essential role in protective immunity against nematodes, but to have detrimental effects on immune responses against schistosomiasis and in asthma [9-11].

IL-13 mediates its effects via a complex receptor system that includes the IL-4 receptor α chain (IL-4Rα) and two other cell surface proteins, IL-13Rα1 and IL-13Rα2 [12-15]. IL-13Rα1 binds IL-13 with low affinity. Co-expression of IL-13Rα1 and IL-4Rα results in the formation of a high-affinity receptor signaling complex [13, 14, 16], which is widely expressed on both lymphoid and non-lymphoid cells and can be activated by IL-4 [17]. In contrast to IL-13Rα1, IL-

13Rα2 selectively binds IL-13 with high affinity but its contribution to IL-13 signaling is unknown. The expression of IL-13Rα2 transcripts is restricted to the spleen and brain and, in contrast to IL-13Rα1, a soluble form of this receptor has been detected in mouse serum [18]. This has led to speculation that IL-13Rα2 is a decoy receptor such as the IL-1 type II receptor [19]. Recently, IL-13Rα2 was also shown to exist intracellularly, with large pools present in cultured monocytes, respiratory epithelial cells, primary respiratory epithelium, and primary human monocytes. This suggested that intracellular IL-13Rα2 is not restricted to a few cell types but rather is more widespread [20]. Furthermore, this intracellular pool was rapidly mobilized to the cell surface after cells were treated with interferon-γ (IFN-γ). However, these observations were made in mammals and it remains to be seen if the paradigm for IL-13 and IL-13Rs extends to non-mammalian species.

To date, no evidence has been cited for the existence of a T2 cytokine paradigm in the chicken. However, recently, a chicken T2 cytokine gene cluster has been identified using a genomics approach based on the conservation of synteny [21]. Sequencing of bacterial artificial chromosomes (BACs) showed that the chicken genome encodes genes for the homologs of mammalian IL-3, IL-4, IL-5, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [21]. These sequences represent the first T2 cytokines found in non-mammalian species. In addition, mRNA and recombinant protein expression was demonstrated for four of these genes (IL-3, IL-4, IL-13, and GM-CSF) [21].

Furthermore, recombinant chicken IL-4 (rchIL-4) and IL-13 (rchIL-13) were both shown to cause chicken B cell proliferation when co-stimulated with CD154 (CD40 ligand) in the form of a fusion protein [21]. However, the receptors for these T2 cytokines have not been characterized in the chicken. Such receptor identification and investigation of biochemical and cellular properties requires the cloning of the gene that encodes the protein and the development of appropriate analytical tools. To this end, we have initiated the cloning of chicken IL-13Rα2 and produced a specific monoclonal antibody (mAb) against it.

Materials and Methods

Animals and cell culture

Chicken tissue was obtained from partially inbred H-B15 White Leghorn chickens, originally kindly supplied by Dr. Vainio (Oulu University, Finland), and bred in our animal facilities. They were provided with feed and chlorinated water *ad libitum*.

The monocytic chicken leukemia cell line, IN24 [22], was grown in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS Lot No. AJL11137; HyClone, Logan, UT) in a 5% CO_2 incubator at 38.5 ^{o}C. For suppression subtractive hybridization (SSH), IN24 cells were grown to 80% confluence in 60-mm culture dishes (Becton Dickinson, Franklin Lakes, NJ), washed three times with serum-free

IMDM, and resuspended in 5 ml serum-free IMDM or serum-free IMDM containing 10 μg/ml lipopolysaccharide (LPS; *E. coli* O127: B8; DIFCO, Detroit, MI). The cells were cultured for 24 h and poly(A)$^+$ RNA was isolated at 4-h intervals. To analyze chIL-13Rα2 by western blotting, IN24 cells were cultured in serum-free IMDM with 10 μg/ml LPS and 10 μg/ml tunicamycin for 24 h.

Cloning of chicken IL-13Rα2

Total RNA was isolated from IN24 cells using ISOGEN-LS (Takara, Kyoto, Japan), and poly(A)$^+$ RNA was purified using Oligotex-dT30 Super (Nippon Roche, Tokyo, Japan). SSH was performed using the Clontech PCR-Select™ cDNA Subtraction kit (BD Biosciences Clontech, Shiga, Japan) according to the manufacturer's instructions, but with some modifications. Poly(A)$^+$ RNA (4 μg) from LPS-treated or control cells was used as tester RNA and driver RNA, respectively. The subtracted cDNA was diluted 1:400 prior to amplification by polymerase chain reaction (PCR) with a GeneAmp PCR system 9700 (Applied Biosystems Japan Ltd., Tokyo, Japan). Primary PCR was performed for 30 cycles, and secondary PCR for 15 cycles. The conditions of PCR followed the manufacturer's instructions of the Clontech PCR-Select™ cDNA Subtraction kit (BD Biosciences Clontech). A 3-μl sample of the secondary PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI).

This subtraction cDNA library was screened by the differential display

method using the Clontech PCR-Select™ Differential Screening kit (BD Biosciences Clontech) according to the manufacturer's recommendations, but with some modifications. Random clones were amplified by PCR for 23 cycles and 5-μl aliquots of the products were separated on 1.5% agarose gels, which were stained with ethidium bromide (EtBr). The PCR products including the cDNA inserts were denatured with NaOH, transferred to nylon membranes, and then baked for 2 h at 70 ºC. Probes for hybridization were prepared from the subtracted cDNA and labeled using a digoxigenin (DIG) DNA labeling kit (Roche Diagnostics, Tokyo, Japan). The membranes were hybridized with DIG-labeled probes according to the supplier's instructions. Selected positive clones were sequenced with an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator (Applied Biosystems). Rapid amplification of cDNA ends (RACE) was performed to obtain the 3' and 5' ends of the chIL-13Rα2 cDNA using the BD SMART RACE cDNA amplification kit (BD Biosciences Clontech), according to the manufacturer's instructions.

Phylogenetic analysis wae performed on the full-length amino acid sequences of the known IL-13 receptors and chicken IL-13Rα2, using the ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/), and the default parameters were used. A phylogenetic tree was generated by the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Reverse transcription-polymerase chain reaction (RT-PCR)

For expression analysis, 0.5 μg poly(A)$^+$ RNA, purified as described above, was reverse transcribed at 42 ºC using the SuperScript preamplification system (Invitrogen, Carlsbad, CA), in 20-μl reaction mixtures containing the oligo-$(dT)_{12-18}$ primer, as described by the manufacturer. cDNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Semi-quantitative PCR was carried out using the ABI PRISM 7700 sequence-detection system (Applied Biosystems) and SYBR Green PCR core reagents (Applied Biosystems). The cDNAs were normalized to chicken β-actin, as recommended by the manufacturer. Equal quantities of normalized cDNAs were used as templates in PCR reactions with one of the following chIL-13Rα2-specific primer sets: primer set 1: forward, 5'-AGCGGCCGCCTTTCGGGTCCCACTGC-3', reverse, 5'-CCCTCGAGGGCCAGGTTGTGAAGACG-3'; primer set 2: forward, 5'-CACCATCTCCAGAGCAAATCG-3', reverse, 5'-AAGCCCTCATCAGCACAGAAG-3'. The following primers were used to amplify β-actin: forward, 5'-CACCTTCCAGCAGATGTGGAT-3', reverse, 5'-GCAAATGCTTCTAAACCGGACT-3'. PCR amplification was carried out using 10 μM primers, 0.2 mM dNTP, 0.2 mM 1X Taq DNA polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 1.5 U AmpliTaq-Gold (Perkin Elmer, Wellesley, MA) in a final volume of 50 μl. The reaction was perfomed in a GeneAmp PCR System 9700 (Applied Biosystems) using the following

conditions: 95 ºC for 10 min, 94 ºC for 1 min (denaturation), 60 ºC for 1 min (annealing), and 72 ºC for 2 min (polymerization). After 25 amplification cycles in the linear range, the reaction mixture was held at 72 ºC for 10 min as a final extension step. The PCR products were analyzed on 1.5% agarose gels and visualized with EtBr.

Expression of chicken IL-13Rα2 in E. coli

The extracellular region of chIL-13Rα2 was amplified using the following primers: forward primer, 5'-CG*GAATTC*TCCTCCTCCCTCTGGCACACAGC-3'; reverse primer, 5'-CC*AAGCTT*TCATTACTTGACTTCTTTTCTGGAT-3' (Fig. 1). The PCR products were digested with *Eco* RI and *Hind* III, and subcloned into the pMAL-c2X plasmid (New England Biolabs, Ipswich, MA) containing the maltose binding protein (MBP) gene. *E. coli* BL21 transformed with the recombinant plasmid were grown to OD_{600} = 0.5 at 30 ºC and then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. After sonication, MBP-rchIL-13Rα2 was affinity purified on amylose resin (New England Biolabs) and eluted with 10 mM maltose, 200 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 7.4). Factor Xa (New England Biolabs) was added to cleave the MBP tail from the recombinant protein, which was then analyzed by 10% polyacrylamide gel electrophoresis (PAGE) and visualized by 0.25% coomassie brilliant blue R-250 staining

(Nacalai Tesque, Kyoto, Japan).

*Production of anti-ch*IL-*13Rα2 mAbs*

Six-week-old female BALB/c mice were immunized intraperitoneally with 50 μg MBP-rchIL-13Rα2 mixed with an equal volume of complete Freund's adjuvant. After three bi-weekly boosts with the same dose of immunogen in 0.1 ml PBS, mice with high serum titers of anti-rchIL-13Rα2 antibodies were identified using an enzyme-linked immunosorbent assay (ELISA) and western blotting. These mice were injected intravenously with 50 μg MBP-rchIL-13Rα2 in PBS and, three days later, their splenocytes were fused with SP2/0 Ag14 myeloma cells [23] using established methods [24]. Hybridomas secreting anti-rchIL-13Rα2 antibodies were screened by ELISA, and cloned by limiting dilution. The mouse immunoglobulin isotypes of the anti-rchIL-13Rα2 mAbs were determined using a mouse monoclonal antibody isotyping kit (Amersham Biosciences, Piscataway, NJ).

ELISAs

ELISAs were carried out in NUNC-immunomodule microplates (Nalge Nunc International, Rochester, NY), coated overnight at 4 °C with 50 μl/well PBS containing either 5 μg/ml MBP-rchIL-13Rα2 or MBP. The plates were blocked

with 200 μl/well 4% skim milk in PBS for 2 h at 37 °C. Aliquots (50 μl) of the supernatants from fusion wells or established hybridoma cultures were added to the wells and incubated for 1 h at 37 °C. After four washes in 0.05% Tween-20 in PBS (Tween-PBS), 50 μl/well alkaline phosphatase (ALP)-labeled goat anti-mouse Ig (KPL, Gaithersburg, MD), diluted 1:1000 in 0.1% skim milk in PBS, was added and incubated for 1 h at 37 °C. After eight washes with Tween-PBS, the ALP activity was developed with 100 μl/well 1mg/ml *p*-nitrophenyl phosphate in 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 100 mM glycine, pH 10.4 for 15 min at room temperature (RT). Absorbance was measured at 405 nm in a microplate reader (Model 680; Bio-Rad, Hercules, CA).

Western blotting with anti-rchIL-13Rα2 mAb

The rchIL-13Rα2 or detergent-solubilized IN24 lysates were separated by 10% or 12.5% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Bio-Rad). Membranes were incubated in blocking buffer (4% nonfat milk powder, 0.2% Tween 20, 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 25 mM EDTA) overnight at 4 °C. After three washes in Tween-PBS, membranes were incubated with hybridoma culture supernatants for 1 h at RT. After three washes as before, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig) (KPL), diluted 1:4000 in dilution buffer for 1 h at RT, washed three times, and incubated with ECL Plus Western

blotting detection reagent (Amersham Biosciences). Results were recorded using a LAS-3000 image reader (FUJIFILM, Tokyo, Japan).

Immunofluorescence

Culture medium was removed from IN24 cells, which were then washed three times with PBS and fixed in cold 4% paraformaldehyde for 30 min at RT. After washing with 10 mM glycine in PBS, the cells were incubated with 3% bovine serum albumin (BSA) in PBS for 15 min at RT, washed with PBS, then incubated with the anti- rchIL-13Rα2 mAb, HU13-1, diluted in 0.1% BSA-PBS, for 40 min at 37 ºC. The cells were washed, incubated in 1:100 Cy2-conjugated goat anti-mouse IgG (H-L) (Biomeda, Foster City, CA), in 1% BSA-PBS, for 45 min at RT, and washed again. The stained cells were observed with an inverted fluorescence microscope (Model IX71; Olympus, Tokyo, Japan).

RESULTS

Cloning chIL-13Rα2

SSH and differential display were used to isolate chicken IL-13Rα2 cDNA from the genes induced by LPS in the chicken monocytic cell line, IN24. Using cDNA from LPS-treated IN24 cells as tester and cDNA from untreated

cells as driver, we generated a subtracted cDNA population by SSH. From approximately 1000 clones in the original cDNA library screened by differential display, we identified 122 cDNA fragments that appeared to originate from differentially expressed genes. The nucleotide sequences of these cDNA fragments were analyzed using the BLAST homology search in the DDBJ/EMBL/GenBank databases [25]; one cDNA fragment showed approximately 40% identity to part of the mammalian IL-13Rα2.

The complete chIL-13Rα2 open reading frame (ORF) from which this fragment was derived was obtained by RACE cDNA amplification, and the 1,521-bp nucleotide sequence was then determined. The nucleotide sequence containing a complete 1,143-bp ORF and the deduced 380 amino acid sequence are shown in Fig. 1. Computer assisted analysis (http://www.cbs.dtu.dk/services/SignalP-2.0/ and http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) identified a 19 amino acid signal peptide at its N-terminus and a 23 amino acid transmembrane region (Fig. 1). This chIL-13Rα2 protein contained three asparagine glycosylation sites (NXS/T, ellipsoid) and a cytokine receptor motif, WSXWS/T. The consensus polyadenylation signal, AATAAA, was located at nucleotides 1481-1486.

The nucleotide sequence data reported in this paper have been filed in DDBJ/EMBL/GenBank under accession number AB243738.

Comparison of chicken and mammalian IL-13Rα2 sequences

The deduced amino acid sequence of chIL-13Rα2 was compared with that of human and mouse IL-13Rα2 using the Pileup command (GCG program; GCG sequence analysis software version 9.0, GCG Inc., USA). Alignment of the sequences, as shown in Fig. 2A, revealed that the putative chIL-13Rα2 protein contained the ten cysteine residues which are conserved in mammalian IL-13Rα2s. The chIL-13Rα2 precursor protein shared 37.1% and 38.9% identity and 77.4% and 77.7% similarity to human and mouse IL-13Rα2, respectively (Fig. 2B). Thus, as for other IL receptors, the degree of identity between chicken and mammalian IL-13Rα2s was found to be low [26-28].

To construct a tree depicting the relationship similarities between IL-13 α2 and the other IL-13R members, a phylogenetic tree was derived using the ClustalX program and the neighbor joining method. Confidence values for the phylogenetic tree were generated by bootstrapping, based on 1000 resampling replicates. Our cloned chIL-13Rα2 was shown to be a close relation of other IL-13Rα2 (Fig. 3).

Expression analysis

To determine the expression profile of chIL-13Rα2, we performed semi-quantitative or real-time PCR with cDNAs prepared from IN24 cells stimulated *in vitro* and from various chicken tissues. RNA levels were quantified

using real-time PCR and normalized to β-actin expression levels in the same tissue. The level of expression of chIL-13Rα2 mRNA was very low in untreated, control IN24 cells, but LPS treatment induced a gradual increase in its expression, which reached a maximum at 16 h (Fig. 4A).

The normalized expression levels of chIL-13Rα2 in various tissues, shown in Fig. 4B, are means from three independent experiments, expressed as tissue:kidney ratios, with the standard errors of the mean (SEM) shown. These real-time PCR experiments showed high levels of chIL-13Rα2 expression in brain, liver, ovary, and testis.

Production and characterization of mAbs to rchIL-13Rα2

We used a recombinant fusion protein, composed of the extracellular domain of chIL-13Rα2 fused to MBP, as the immunogen to raise mAbs. The hybridomas were screened by ELISA for reactivity with MBP-chIL-13Rα2 but not MBP, and this identified two hybridomas that were synthesizing anti-rchIL-13Rα2 antibodies (Fig. 5), which were then cloned by limiting dilution. The single resulting mAb was an IgG1 immunoglobulin and was named HU13-1.

Western blotting

Fig. 6A shows western blots of MBP-rchIL-13Rα2, before and after

treatment with Factor Xa, using mAb HU13-1. HU13-1 identified MBP-rchIL-13Rα2 as a band with an apparent molecular weight (MW) of approximately 80 kDa (lane 2), which is consistent with a MW of 43 kDa for MBP and a predicted MW of 37 kDa for the extracellular portion of rchIL-13Rα2. After treatment with Factor Xa to cleave MBP and rchIL-13Rα2, a 37-kDa polypeptide was identified by HU13-1 (lane 3). The protein bands labeled by HU13-1 with MWs of approximately 50 and 75 kDa are likely to be proteolytic fragments of MBP-rchIL-13Rα2. As expected, MBP was not labeled by HU13-1 (lane 1) and the secondary antibody used did not recognize MBP, MBP-rchIL-13Rα2, or the polypeptides formed after Factor Xa treatment (NC).

Fig. 6B shows western blots using lysates from IN24 cells treated with LPS and tunicamycin. LPS treatment induced a HU13-1-reactive band with an apparent MW of approximately 45 kDa (lane 5). When the IN24 cells had been cultured in the presence of tunicamycin, to inhibit glycosylation, the MW of the induced band was reduced to approximately 41 kDa (lane 6). No bands were labeled by mAb HU13-1 in IN24 lysates that had not been exposed to LPS (lane 4). To investigate the possible existence of a soluble form of chIL-13Rα2, concentrated samples of culture supernatants from LPS-treated IN24 cells were used for western blotting. However, no soluble form of chIL-13Rα2 was detected by mAb HU13-1 in these cultured supernatants (data not shown).

Immunofluorescence staining of IN24 cells with HU13-1

To confirm that mAb HU13-1 recognized native chicken IL-13Rα2, LPS-stimulated IN24 cells were labeled immunofluorescently. The cells were immunostained every 4 h, for 24 h after LPS was added to the cultures. As shown in Fig. 7, 4 h after the addition of LPS the IN24 cells retained their typical morphology, as spindle-shaped cells, but no HU13-1-positive cells were observed. By 8 h, the cells began to appear flattened and rounder and HU13-1-positive cells were visible. At 20 h, the majority of cells had changed shape and were labeled by mAb HU13-1. These results indicated that HU13-1 mAb recognized native chicken IL-13Rα2 on the surface of the cells and/or inside the cells.

DISCUSSION

This study describes the cloning and characterization of the chIL-13Rα2 gene and the production of the first mAb specific for chIL-13Rα2. We have cloned a 1,521-bp chIL-13Rα2 cDNA containing a complete 1,143-bp ORF and shown that the deduced amino acid sequence of chIL-13Rα2 has low identity with mammalian IL-13Rα2 sequences. We have shown that chIL-13Rα2 mRNA is strongly expressed in liver, testis, ovary, and brain and is induced by LPS in the IN24 monocytic cell line. We have produced the mAb HU13-1, shown that it specifically recognizes rchIL-13Rα2 in ELISAs and western blots and used

this mAb in western blots to show that native, glycosylated chIL-13Rα2 has a MW of approximately 45 kDa. MAb HU13-1 could also be used to identify native chIL-13Rα2 on or inside IN24 cells by immunofluorescence staining.

In chickens, it has only been possible to clone a few ILs based on homology with their mammalian counterparts, as they share only 20-40% amino acid sequence identity. More recently, as a result of expressed sequenced tag (EST) analysis and the availability of sequenced genomes, the rate at which chicken IL genes have been identified has increased tremendously [29, 30]. For similar reasons, very few chicken IL receptor genes have been cloned. For chicken cytokine research to progress in the future, cloned and characterized chicken IL receptor genes and analytical tools for studying their transcripts are needed. In particular, specific antibodies are needed, as they are powerful tools for analyzing receptor function and the sites at which receptors are expressed.

The cDNA sequence we cloned encoded a 380 amino acid propeptide of chIL-13Rα2. When this sequence was used in a BLAST search (http://www.ncbi.nih.gov/BLAST/), it was found to match a *Gallus gallus* mRNA (Accession number; XM_420209), which had been predicted by automated computational analysis from a genomic sequence. However, our cloned sequence covers only a small portion of the sequence obtained from this record (data not shown).

The chIL-13Rα2 precursor protein was 37.1% and 38.9% identical to human and mouse IL-13Rα2, respectively (Fig. 2B). This low degree of identity

between chicken and mammalian sequences has been seen with other chILs. In mammals, IL-13Rα2 transcripts have been found in the brain, spleen, liver, thymus, and testis. Similarly, chIL-13Rα2 transcripts have been found at high levels in the liver, testis, ovary, and brain [31, 32]. A distinctive aspect of this expression pattern is the high level of IL-13Rα2 mRNA in both chicken and human testis [31]. Since nothing is so far known about the role of IL-13 in the germ line, the expression of this receptor in testis is particularly interesting.

The expression of chIL-13Rα2 in IN24 cells was induced by the addition of LPS (Fig. 4A and Fig. 7). In mammals, IL-13 suppresses the production of inflammatory cytokines by monocytes and macrophages [5]. Our results therefore suggested that pro-inflammatory stimulation of the chicken monocytic cell line, IN24, with LPS might prepare the cells for a response to IL-13. Furthermore, the mAb HU13-1 that we developed, reacted with both glycosylated and unglycosylated chIL-13Rα2 (Fig. 6B and Fig. 7). This will make it a particularly useful reagent for future studies of the biological activity of chIL-13 and its receptor.

Mammalian IL-13 has diverse functions, affecting a wide variety of cell types that are relevant to the pathogenesis of allergic disorders [2-4, 9, 10]. This multiplicity of activities highlights the potent immunoregulatory role of IL-13. In human B cells in particular, human IL-13 has similar effects to IL-4, promoting B-cell proliferation, and promoting class switching to IgG4 and IgE, in the presence of CD40/CD40 ligand co-stimulation [33]. In addition, IL-13 has been

reported to activate mast cells and contribute to IgE priming of mast cells, given its role in promoting IgE synthesis [4]. However, chickens lack certain components of the IL-4 and IL-13 responses seen in mammals. For example, there is only a single chicken IgG equivalent, called IgY, and therefore no IgG sub-class switching occurs in chickens [34]. Chickens also lack IgE [34]. To study these intriguing differences between birds and mammals and why these kinds of differences occur during evolution, progress in the development of analytical tools is needed, to enable the characterization of chicken ILs and their receptors and increase the expertise available for further T2 interleukin research.

Acknowledgments

This research was supported by a Grant-in-Aid for Scientific Research (C) (No. 16580279) and a Cooperative Link of Unique Science and Technology for Economic Revitalization (CLUSTER) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Biotechnology Cluster in Central Hiroshima, Hiroshima Prefecture, Japan.

References

[1] Zurawski SM, Vega F Jr, Huyghe B, Zurawski G. Receptors for interleukin-13

and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J 1993; 12:2663-2670.

[2] Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci U S A 1993; 90:3730-3734.

[3] Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and Bcells, but not on T cells. Immunol Today 1994;15: 19-26.

[4] de Vries JE. The role of IL-13 and its receptor in allergy and inflammatory responses. J Allergy Clin Immunol 1998; 102:165-169.

[5] Endo T, Ogushi F, Sone S. LPS-dependent cyclooxygenase-2 induction in human monocytes is down-regulated by IL-13, but not by IFN-gamma. J Immunol 1996; 156:2240-2246.

[6] Doherty TM, Kastelein R, Menon S, Andrade S, Coffman RL. Modulation of murine macrophage function by IL-13. J Immunol 1993; 151:7151-7160.

[7] Sozzani P, Cambon C, Vita N, Seguelas MH, Caput D, Ferrara P, Pipy B. Interleukin-13 inhibits protein kinase C-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. J Biol Chem 1995; 270:5084-5088.

[8] Lentsch AB, Shanley TP, Sarma V, Ward PA. In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. J Clin Invest 1997; 100:2443-2448.

[9] Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science 1998; 282:2258-2261.

[10] Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998; 282:2261-2263.

[11] Chiaramonte MG, Schopf LR, Neben TY, Cheever AW, Donaldson DD, Wynn TA. IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by Schistosoma mansoni eggs. J Immunol 1999; 162:920-930.

[12] Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N, Ferrara P. Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. J Biol Chem 1996; 271:16921-16926.

[13] Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS, Leonard WJ. cDNA cloning and characterization of the human interleukin 13 receptor alpha chain. J Biol Chem 1996; 271:29265-29270.

[14] Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Willson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Natl Acad Sci U S A 1996; 93:497-501.

[15] Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, O'Hara RM Jr, Beier DR, Turner KJ, Wood CR, Collins M. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol 1998; 161:2317-2324.

[16] Murata T, Taguchi J, Puri RK. Interleukin-13 receptor alpha' but not alpha chain: a functional component of interleukin-4 receptors. Blood 1998; 91:3884-3891.

[17] Zurawski SM, Vega F Jr, Huyghe B, Zurawski G. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J 1993; 12:2663-2670.

[18] Zhang JG, Hilton DJ, Willson TA, McFarlane C, Roberts BA, Moritz RL, Simpson RJ, Alexander WS, Metcalf D, Nicola NA. Identification, purification, and characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned Il-13 receptor and Il-4 receptor alpha-chains. J Biol Chem 1997; 272:9474-9480.

[19] Colotta F, Orlando S, Fadlon EJ, Sozzani S, Matteucci C, Mantovani A. Chemoattractants induce rapid release of the interleukin 1 type II decoy receptor in human polymorphonuclear cells. J Exp Med 1995; 181:2181-2186.

[20] Daines MO, Hershey GK. A novel mechanism by which interferon-gamma can regulate interleukin (IL)-13 responses. Evidence for intracellular stores of IL-13 receptor alpha -2 and their rapid mobilization by interferon-gamma. J Biol Chem 2002; 277:10387-10393.

[21] Avery S, Rothwell L, Degen WD, Schijns VE, Young J, Kaufman J, Kaiser P. 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. J Interferon Cytokine Res 2004; 24:600-610.

[22] Inoue M, Sato A. Establishment and in vitro differentiation of a chicken monocytic leukemia cell line. Jpn J Vet Sci 1988; 50:648-653.

[23] Shulman M, Wilde CD, Kohler G. A better cell line for making hybridomas secreting specific antibodies. Nature 1978; 276:269-270.

[24] Galfre G, Howe SC, Milstein C, Butsher GW, Howard JC. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 1977; 266:550-552.

[25] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215:403-410.

[26] Li G, Lillehoj HS, Min W. Production and characterization of monoclonal antibodies reactive with the chicken interleukin-15 receptor alpha chain. Vet Immunol Immunopathol 2001; 82:215-27.

[27] Kawashima T, Hojyo S, Nishimichi N, Sato M, Aosasa M, Horiuchi H, Furusawa S, Matsuda H. Characterization and expression analysis of the chicken interleukin-11 receptor alpha chain. Dev Comp Immunol 2005; 29:349-359.

[28] Nishimichi N, Kawashima T, Hojyo S, Horiuchi H, Furusawa S, Matsuda H.

Characterization and expression analysis of a chicken interleukin-6 receptor alpha. Dev Comp Immunol 2006; 30:419-429.

[29] Kaiser P, Rothwell L, Avery S, Balu S. Evolution of the interleukins. Dev Comp Immunol 2004; 28:375-394.

[30] Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, Hughes S, Goodchild M, Morrell S, Watson M, Bumstead N, Kaufman J, Young JR. A genomic analysis of chicken cytokines and chemokines. J Interferon Cytokine Res 2005; 25:467-484.

[31] Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, O'Hara RM Jr, Beier DR, Turner KJ, Wood CR, Collins M. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol; 161:2317-23124.

[32] Wu AH, Low WC. Molecular cloning of the rat IL-13 alpha 2 receptor cDNA and its expression in rat tissues. J Neurooncol 2002; 59:99-105.

[33] Oettgen HC, Geha RS. IgE regulation and roles in asthma pathogenesis. J Allergy Clin Immunol 2001; 107:429-440.

[34] Higgins DA. Comparative immunology of avian species. In: Davison TF, Morris TR, Payne LN, editors. 24th Poultry Science Symposium on Poultry Immunology. Abingdon, UK: Carfax Publishing; 1996. p. 149-205.

Figure legends

Fig. 1. Nucleotide and predicted amino acid sequences of chIL-13Rα2. The numbers shown refer to the nucleotide sequences. The primers used for amplifying rchIL-13Rα2 are indicated by arrows. The termination codon (TGA) is marked with an asterisk, the putative signal peptide is underlined, and the putative transmembrane domain is indicated by double lines. Three potential N-glycosylation sites are circled, the WSXWT/S motif is indicated by a dashed line and the consensus polyadenylation signal is boxed.

Fig. 2. Sequence comparison of chicken, human and mouse IL-13Rα2. (A) Sequence alignment showing identical (*) and conserved (.) residues. Arrowheads indicate the ten conserved cysteine residues. The WSXWT/S motif is boxed. (B) Homology scores calculated for aligned sequences.

Fig. 3. Phylogenetic tree of mammalian, chicken and fish IL-13 receptors. Chicken IL-13Rα1 had been predicted from a chicken genomic sequence. The accession number of the sequences used are as follows: mouse IL-13Rα1 (NM_133990), rat IL-13Rα1 (AY044251), human IL-13Rα1 (NM_001560), chicken IL-13Rα1 (XM420218), mouse IL-13Rα2 (NM_008356), rat IL-13Rα2 (NM_133538), dog IL-13Rα2 (NM_001003075), human IL-13Rα2 (NM_000640), chicken IL-13Rα2 (AB243738), rainbow trout IL-

13Rα2 (AF361437), and outgroup sequence bastard halibut IL-8R (AB079600). The tree was constructed by the neighbor-joining method using the ClustalX and TreeView, and was bootstrapped 1000 times.

Fig. 4. Expression of chIL-13Rα2 mRNAs in LPS-stimulated IN24 cells and various chicken tissues. (A) Poly(A)$^+$RNA was isolated from untreated and LPS-treated IN24 cells and reverse transcribed. Equal quantities of cDNAs, normalized using chicken β-actin SYBR Green PCR, were used as templates in PCR reactions with primers specific for chIL-13Rα2. PCR products were separated on 1.5% agarose gels. (B) Real-time quantitative PCR was used to quantify the expression of chIL-13Rα2 in various tissues. The expression levels of chIL-13Rα2 were normalized to β-actin levels and tissue:kidney ratios were calculated from the mean results of three independent experiments. Error bars represent SEMs.

Fig. 5. ELISA to screen for rchIL-13Rα2-specific mAbs. Microplates were coated with equal concentrations of MBP-rchIL-13Rα2 (black bars) or MBP (white bars) and incubated with supernatants from five mAb-producing hybridomas, then with ALP-conjugated anti-mouse κ antibody, and finally with *p*-nitrophenyl phosphate substrate. Data shown are the mean absorbance at 405 nm of triplicate samples ±S.D. NC: negative control antibody.

Fig. 6. Binding of HU13-1 mAb to chIL-13Rα2 on western blots. (A) Western blot analysis with MBP (track 1), MBP-rchIL-13Rα2 produced in *E. coli* (track 2) and MBP-rchIL-13Rα2 produced in *E. coli* and cleaved with factor Xa (track 3) using mAb HU13-1. The relative mobility of protein markers is indicated on the left. Arrows indicate the positions of MBP-rchIL-13Rα2 (80 kDa) and rchIL-13Rα2 (37 kDa). NC: negative control antibody. (B) Western blot analysis with untreated IN24 cell lysate (track 4), LPS-treated IN24 cell lysate (track 5), and LPS-treated IN24 cell lysate, from cells cultured with tunicamycin (track 6), using mAb HU13-1. The relative mobility of protein markers is indicated on the left. Arrows indicate the major two positions (45 and 41 kDa).

Fig. 7. Induction of rchIL-13Rα2 by LPS in IN24 cells shown by immunofluorescent staining with mAb HU13-1. Bright-field (left panels) and dark-field (right panels) images are shown at 4-h intervals after the initiation of LPS treatment. Scale bar: 200 μm.

Fig.1. M. Miyoshi, et al

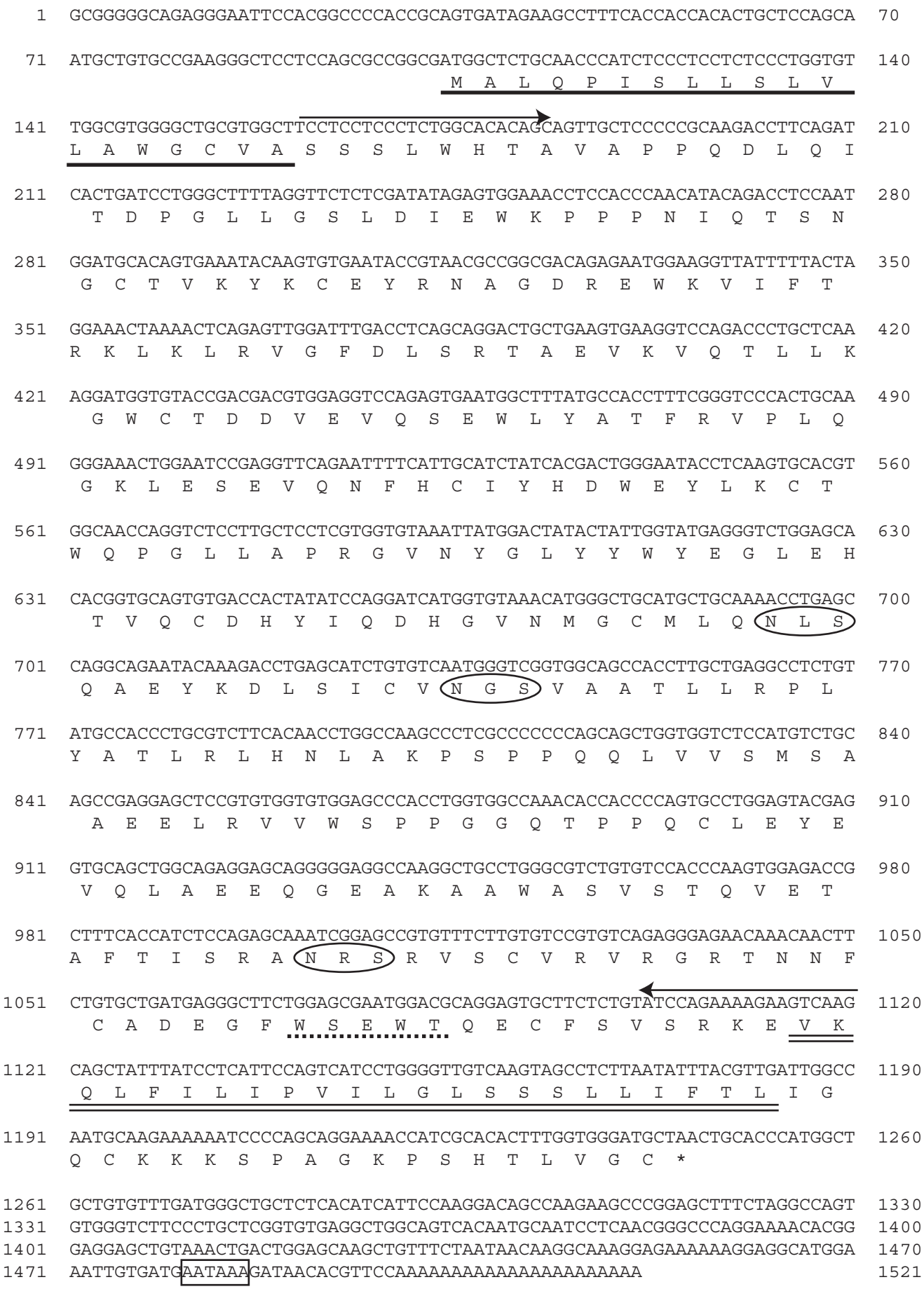

Fig.2. M. Miyoshi, et al

A

```
Human      1 MAFVCLAIGCLYTFLISTTFGCTSSSDTEIKVNPPQDFEIVDPGYLGYLYLQWQPPLSLD    60
Mouse      1 MAFV--HIRCLCFILLCTITGY----SLEIKVNPPQDFEILDPGLLGYLYLQWKPPVVIE    54
Chicken    1 ----MALQPISLLSLVLAWGCVASSSLWHTAVAPPQDLQITDPGLLGSLDIEWKPPPNIQ    56
               ....   . ..   *  .  .  ...  ...*.****..* ***.**.*...*.**  .

Human     61 HFKECTVEYELKYRNIGSETWKTIITKNLHYKDGFDLNKGIEAKIHTLLPWQCTNGSEVQ   120
Mouse     55 KFKGCTLEYELKYRNVDSDSWKTIITRNLIYKDGFDLNKGIEGKIRTHLSEHCTNGSEVQ   114
Chicken   57 TSNGCTVKYKCEYRNAGDREWKVIFTRKLKLRVGFDLSRTAEVKVQTLLKGWCTDDVEVQ   116
              ...**..*...*** ..  **.*.*..* ...****....* *. *.*   **...***

Human    121 SSWAETTYWISPQGIPETKVQDMDCVYYNWQYLLCSWKPGIGVLLDTNYNLFYWYEGLDH   180
Mouse    115 SPWIEASYGISDEGSLETKIQDMKCIYYNWQYLVCSWKPGKTVYSDTNYTMFFWYEGLDH   174
Chicken  117 SEWLYATFRVPLQGKLESEVQNFHCIYHDWEYLKCTWQPGLLAPRGVNYGLYYWYEGLEH   176
               * * .... .. .* .*...*.. *.*..*.** *.*.**  .  ..** ...*****.*

Human    181 ALQCVDYIKADGQNIGCRFPYLEASDYKDFYICVNGSSENKPIRSSYFTFQLQNIVKPLP   240
Mouse    175 ALQCADYLQHDEKNVGCKLSNLDSSDYKDFFICVNGSSKLEPIRSSYTVFQLQNIVKPLP   234
Chicken  177 TVQCDHYIQDHGVNMGCMLQNLSQAEYKDLSICVNGSVAATLLRPLYATLRLHNLAKPSP   236
               ..** .*.. .. * ** . .*  ..***. ******.   ..*..* ...*.*..**.*

Human    241 PVYLTFTRESSCEIKLKWSIPLGPIPARCFDYEIEIRED----DTTLVTATVENET-YTL   295
Mouse    235 PEFLHISVENSIDIRMKWSTPGGPIPPRCYTYEIVIRED----DISWESATDKNDM-KLK   289
Chicken  237 PQQLVVSMSAAEELRVVWSPPGGQTPPQCLEYEVQLAEEQGEAKAAWASVSTQVETAFTI   296
               *  *  . . . ... .** *.*..*..*  **. ..*.    .  . ...  ...  .

Human    296 KTTNETRQLCFVVRSKVNIYCSDDGIWSEWSDKQCWEGEDLSKKTLLRFWLPFGFILILV   355
Mouse    290 RRANESEDLCFFVRCKVNIYCADDGIWSEWSEEECWEGYTGPDSKII-FIVPVCLFFIFL   348
Chicken  297 SRANRSRVSCVRVRGRTNNFCADEGFWSEWTQ-ECFSVSRKEVKQLF-ILIPVILGLSSS   354
              ..*... .*. ** ..*..*.*.*.****.  .*...     . .  .  *. . ..

Human    356 IFVTGLLLRKPNTYPKMIPEFFCDT----------                          380
Mouse    349 LLLLCLIVEKEEPEPTLSLHVDLNKEVCAYEDTLC                          383
Chicken  355 LLIFTLIGQCKKKSPAGKPSHTLVGC---------                          380
               ..   *.  .    *   .   .
```

B

	Chicken	Human	Mouse
Chicken		37.1	38.9
Human	77.4		58.7
Mouse	77.7	85.8	

Identity (%)

Similarity (%)

Fig.3. Miyoshi et al

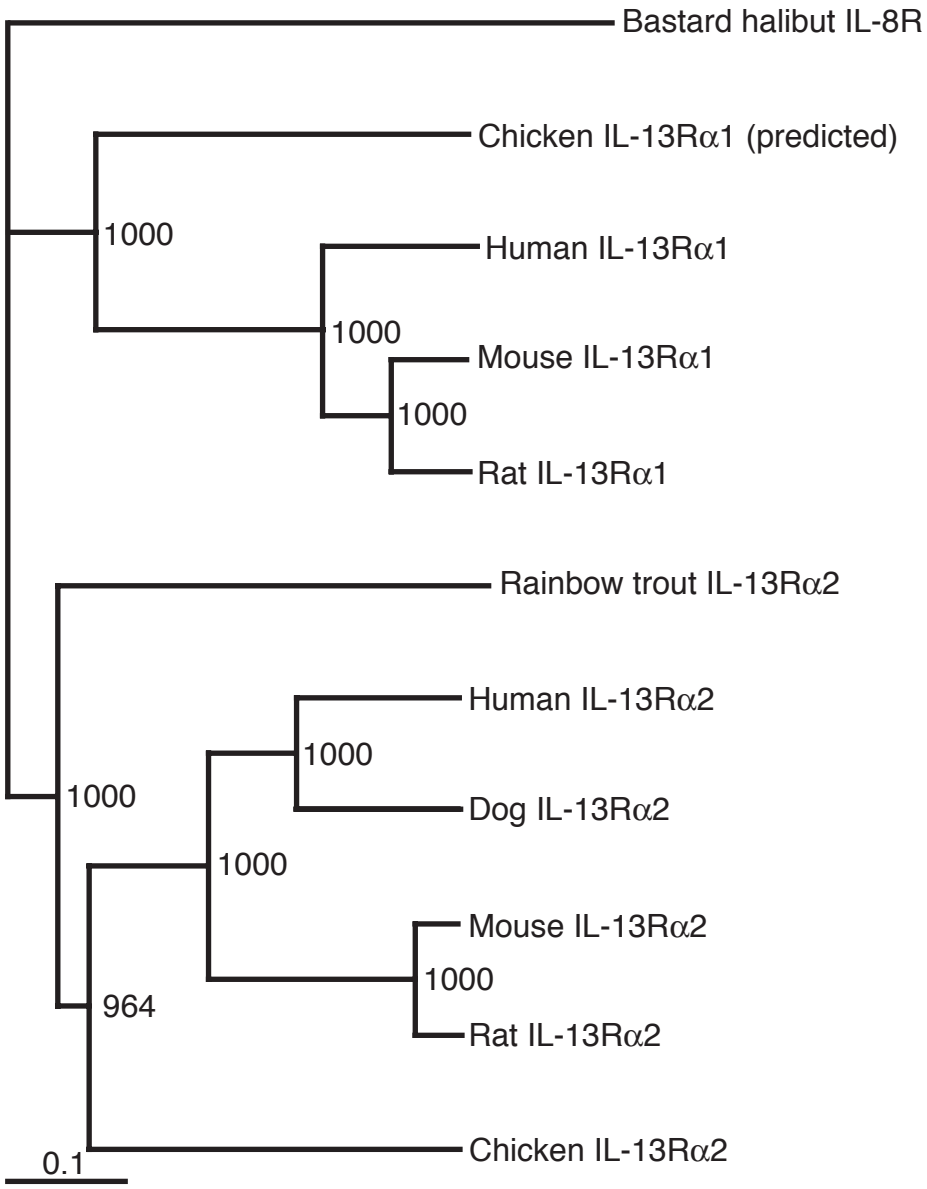

Fig. 4. Miyoshi, et al

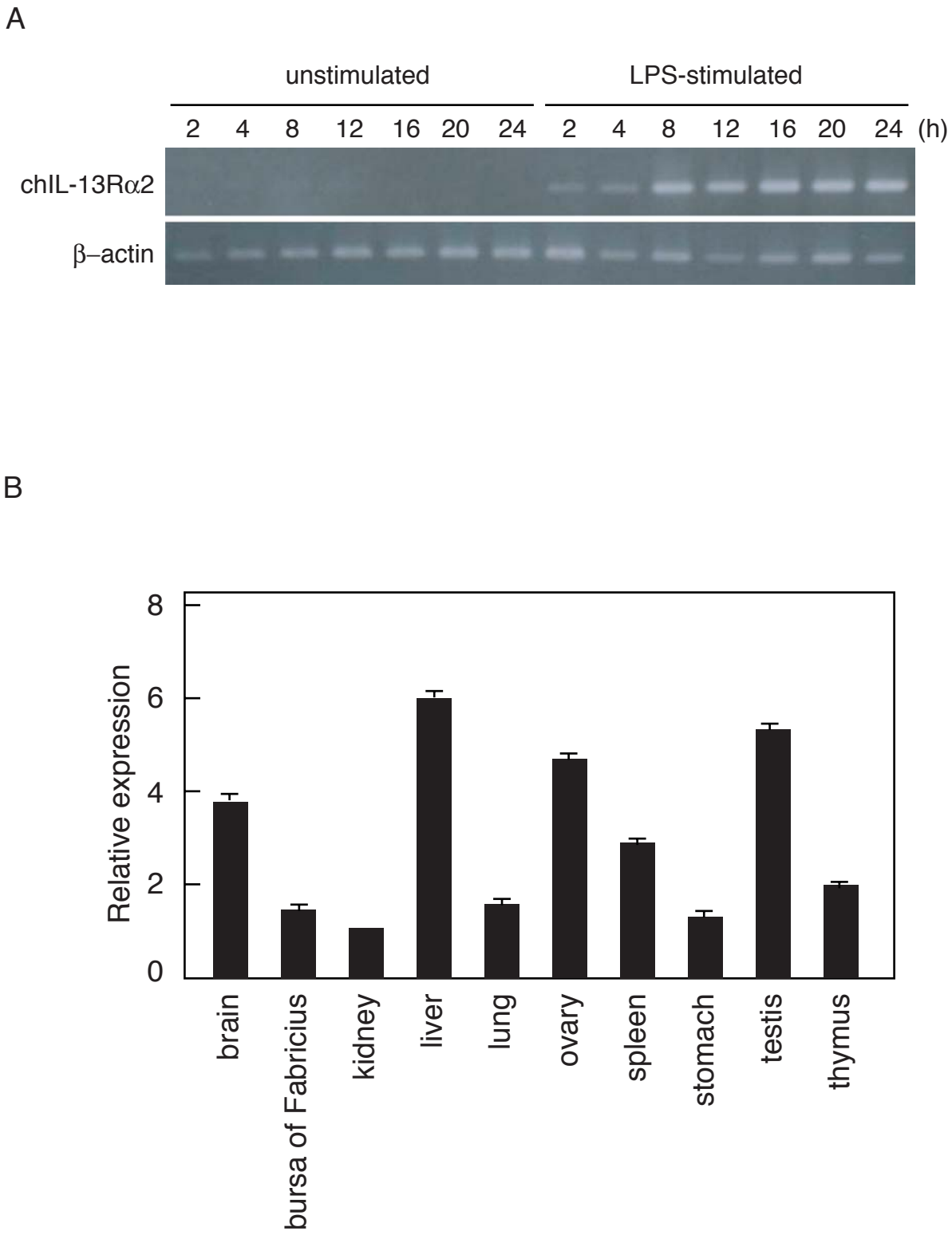

Fig. 5. Miyoshi et al.

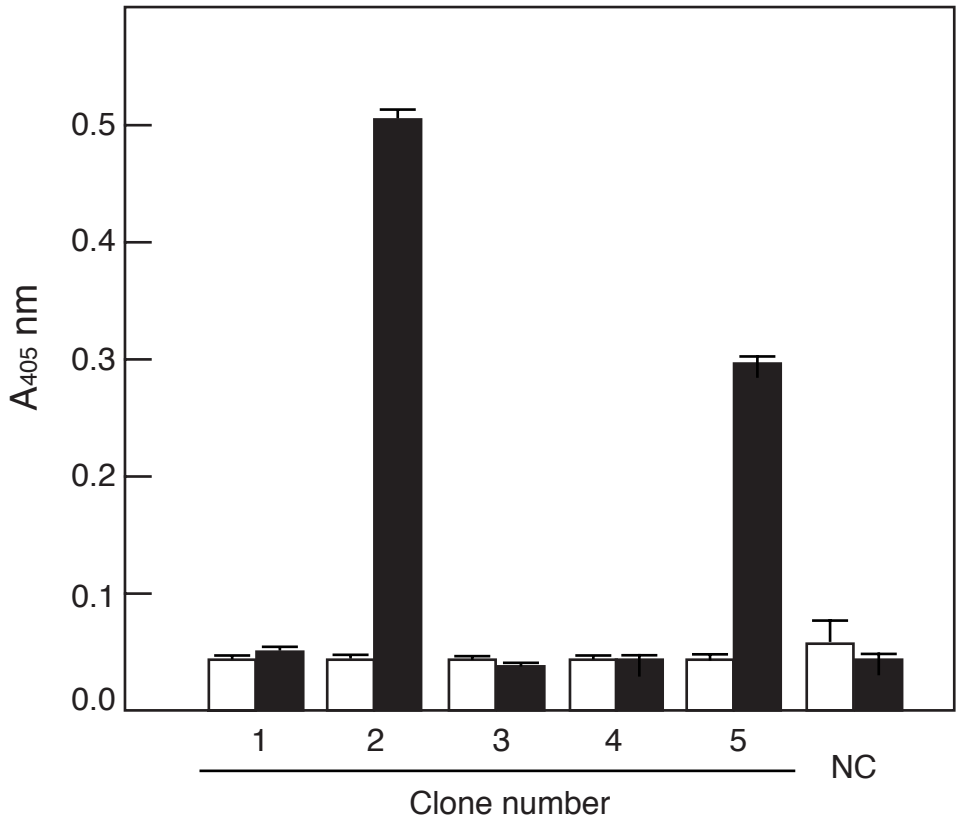

Fig. 6. Miyoshi et al.

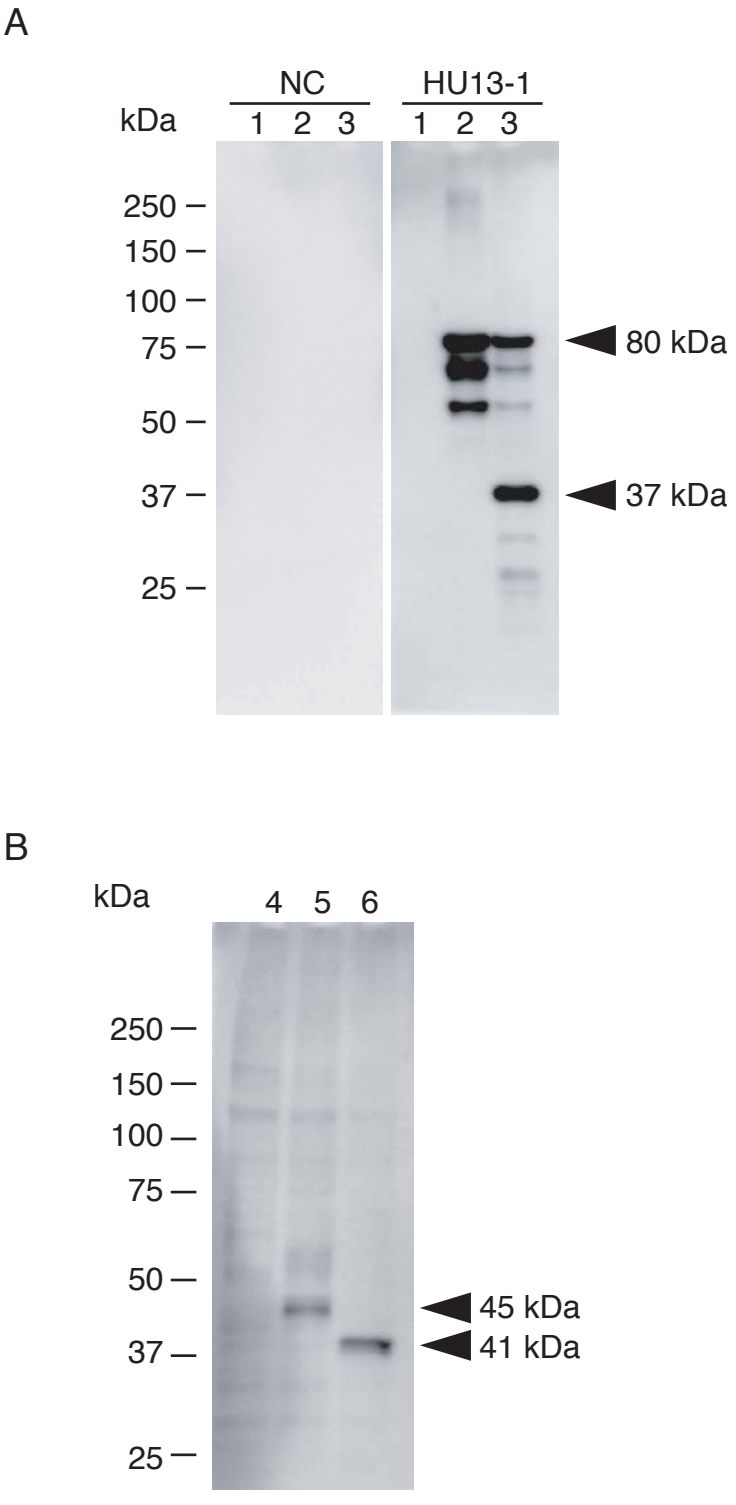

Fig. 7. Miyoshi et al.