Developmental and Comparative Immunology 34 (2010) 258–263

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Identification of the functional interleukin-2 binding domain of the chicken common cytokine receptor gamma chain

Jianyou Gu [a,b], Qiaoyang Teng [a,b], Zhenyu Huang [a,b], Xizhen Ruan [a,b], Jiyong Zhou [a,b,*]

[a] Key Laboratory of Animal Epidemic Etiology & Immunological Prevention of Ministry of Agriculture, Zhejiang University, Hangzhou 310029, PR China
[b] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University, Hangzhou 310003, PR China

ARTICLE INFO

Article history:
Received 22 May 2009
Received in revised form 1 September 2009
Accepted 6 October 2009
Available online 24 October 2009

Keywords:
Chicken CD132
Functional domain
Tertiary structure

ABSTRACT

CD132 is the common gamma chain to a number of cytokine receptor complexes including that for IL-2. To identify the functional domain of chicken CD132 (chCD132), the cDNA of chCD132 was cloned, and a neutralizing monoclonal antibody, raised against a recombinant chCD132 protein, was identified by inhibition of IL-2-dependent proliferation of T cells. Flow cytometry analysis revealed that chCD132 molecules are expressed on the surface of splenic mononuclear cells. The functional domain of chCD132 that binds to chicken interleukin 2, $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$, was found through phage display and peptide-competitive ELISA, and its critical residue Q^{96} was further identified. A tertiary structure model shows that the functional domain is positioned at the elbow-like junction of N and C terminal fibronectin-III domains of chCD132. These data provide experimental evidence for elucidating the interaction between chCD132 and chIL-2.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

The common cytokine receptor γ chain (γc, CD132), belonging to the type I cytokine receptor superfamily, functions as a shared subunit of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [1–3]. CD132 mediates receptor internalization and signaling through the cytoplasmic tail [4]. Mutations of γc are the primary causes of human X-linked severe combined immunodeficiency, which is characterized by a failure in T and NK cell development [5]. Although γc does not detectably bind cytokines by itself, its recruitment into the IL-2, IL-4, or IL-7 receptor complex enhances ligand binding. This is more evident in the context of the IL-2 receptor (IL-2R), where βγ heterodimers bind IL-2 with 100-fold higher affinity than IL-2Rβ alone [6], and the IL-2 affinity of αβγ trimers is increased 10-fold over that of αβ complexes [1]. A similar but more modest 3–5-fold increase in affinity has also been observed in the IL-4R [7] and IL-7R [8] complexes.

Cytokines binding to type I cytokine receptors are typically folded as bundles of four alpha-helices. IL-2 is composed of helices A (S^6–N^{30}), A′ (N^{33}–L^{40}), B (L^{53}–C^{58}), B′ (L^{59}–A^{73}), C (P^{82}–K^{97}), D (I^{114}–S^{130}) [9]. The receptor subunits, including γc, are characterized by extracellular homology regions consisting of two fibronectin type III domains that are each composed of seven β-strands. The first domain (N domain) contains four conserved cysteine residues linked by disulfide bonds, whereas the second domain (C domain) contains a conserved WSXWS motif [10]. The N domain (T^{32}–K^{125}) and C domain (L^{129}–S^{226}) are disposed at an elbow angle of ~120°, and the interdomain ($L^{126}Q^{127}N^{128}$) adopts a 3_{10} helical conformation in γc [11]. A complex of IL-2 crystallized with the IL-2R ectodomain was recently described. The quaternary structure reveals that the IL-2/γc interface exhibits the smallest buried surface and the fewest hydrogen bonds in the complex and the interdomain region of γc contacts IL-2 helices A and D [11,12]. A second crystal structure of a γc ternary complex, IL-4Rα/γc/IL-4, has also been resolved [13]. The structurally equivalent side chains of IL-2 residues E^{15}, L^{19}, Q^{126}, and S^{130} and IL-4 residues Q^8, K^{12}, R^{121}, and S^{125} collectively line the complementary canyon on the A and D helices, making analogous interactions with γc. Cytokine-contacting residues on γc loops BC2 (N^{154}–C^{160}) and FG2 (N^{206}–P^{221}) maintain similar conformations in both the IL-2 and the IL-4 complexes, suggesting that cross-reactivity is not occurring through structural plasticity of the receptor. Rather, a rigid γc surface encounters extraordinarily complementary shape features on γc-cytokines, interactions that are further enhanced by specific hydrogen bonds peripheral to the canyon [13].

Since Sundick and Gill-Dixon [14] cloned the chicken IL-2 (chIL-2) gene, several different avian IL-2 genes have been cloned and

Abbreviations: γc, the common gamma chain receptor; hCD132, human γc; chCD132, chicken γc; IFA, indirect immuofluorescence assay; NCS, newborn calf serum; SMC, splenic mononuclear cell.

* Corresponding author at: Key Laboratory of Animal Epidemic Etiology & Immunological Prevention of Ministry of Agriculture, Zhejiang University, Hangzhou 310029, PR China. Tel.: +86 571 8697 1698; fax: +86 571 8697 1821.
E-mail address: jyzhou@zju.edu.cn (J. Zhou).

0145-305X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2009.10.003

characterized [15–18]. However, understanding of the chicken IL-2 receptor (chIL-2R) molecules has progressed slowly, and only chIL-2Rα and chIL-2Rγ genes have been reported [19,20]. Herein we report the first mapping of the chicken γc chain (chCD132) functional domain providing evidence for the location of critical sites required for binding chIL-2. This structure–function analysis was accomplished through the use of a blocking chCD132 chain-specific mAb probe and epitope mapping through phage display of a peptide library.

2. Materials and methods

2.1. Cells

Vero cells were maintained in minimum essential medium (MEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% newborn calf serum (NCS). Splenic mononuclear cells (SMCs) were prepared as previously described [19].

2.2. RNA isolation, RT-PCR and DNA sequencing

SMCs were cultured in 6-well plates at a final concentration of 1×10^7 cells/mL with 10 μg/mL Con A in RPMI 1640 medium supplemented with 10% NCS and incubated at 39 °C (below the physiological temperature 41 °C) for 24 h. The Con A-stimulated SMCs were collected by centrifugation for 5 min at 2500 r/min and at 4 °C. Total cellular RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA), from which the cDNAs were synthesized using an Oligo $(dT)_{18}$ primer. The nucleic acid sequence of chCD132 was then amplified using PCR with a pair of primers (upstream primer 5′-ATGGCGGTGCCCGGGGCTTC-3′ and downstream primer 5′-TCACGCTCCCACGTAGCTG-3′) designed for the chIL-2Rγ cDNA sequence (GenBank accession no. AJ419898). The PCR assay was carried out with conditions consisting of 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 60 °C for 45 s and DNA extension at 72 °C for 1 min. The amplified products were purified, sequenced and inserted into the pMD18-T vector (TaKaRa, Japan). The ectodomain of mature chCD132 protein was predicted by the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) and aligned with human CD132 (hCD132) ectodomain (GenBank accession no. NP_000197) by DNASTAR 5.0 software (DNASTAR Inc., Madison, WI).

2.3. Construction of model of tertiary structure of chCD132

A tertiary model of the chCD132 ectodomain was constructed using CPHmodels 2.0 Server (http://www.cbs.dtu.dk/services/CPHmodels/). CPHmodels 2.0 is an improved method for fold recognition/homology modeling, in which a large sequence database is iteratively searched to construct a sequence profile until a template can be matched to a database of proteins with known structure. Query and template sequences are subsequently aligned using a score based on profile–profile comparisons. The program finally constructs the tertiary structure model and provides the file with coordinates for modeling in pdb format. The crystal tertiary drawing of the hCD132 ectodomain was represented with X-ray diffraction as a control (PDB ID code 2ERJC), which was composed of two conserved fibronectin type III domains (N domain and C domain) [11].

2.4. Bacterial expression of recombinant chCD132 fusion protein

The nucleotide sequence encoding chCD132 ectodomain was amplified by PCR from the complete chCD132 sequence using the following primers: forward primer 5′-ATGAATTCGCATCCCC-CAGCCCCAAAG-3′ containing an EcoRI site and reverse primer 5′-CGGTCGACTCACGTGTGGATCCAGAACC-3′ containing a SalI site. The PCR product was digested with EcoRI and SalI and directly inserted into the corresponding sites of the pET28a expression vector (Novagen, Madison, WI). The resulting plasmid was designated as pET28a-chCD132. The recombinant chCD132 protein (rchCD132) was expressed as a fusion protein carrying a His tag by transforming pET28a-chCD132 into the *Escherichia coli* BL21 (DE3) strain. His-tagged rchCD132 protein was purified on a nickel column under denaturing conditions following the manufacturer's instructions (Qiagen Inc., Valencia, CA). The rchCD132 protein was further analyzed by SDS-PAGE and western blot with an anti-His mAb (Amersham, USA).

2.5. Production and purification of mAb to chCD132

Five SPF BALB/c mice were immunized subcutaneously with 50 μg of rchCD132 protein in complete Freund's adjuvant (Sigma) and boosted three times with an equivalent dose in incomplete Freund's adjuvant (Sigma). Two weeks later, the mice were injected intraperitoneally with a dose of 100 μg rchCD132. Splenic lymphocytes were fused with SP2/0 myeloma cells using a standard procedure. Hybridomas secreting antibodies against rchCD132 were screened and cloned by limiting dilution. Further selection and confirmation of mAbs specific to the chCD132 protein were performed by an indirect ELISA as described previously [17] using cell lysates of *E. coli* BL21 (DE3) and rchCD132 as antigens.

To analyze the reactivity of these mAbs against the natural chCD132 protein, the chCD132 ORF with a signal peptide sequence was subcloned into the pEGFP-C2 plasmid (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. The resulting plasmid, pEGFP-chCD132, was transfected into Vero cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 48 h at 37 °C, the cells were washed with PBS and fixed in a methanol–acetone mixture (1:1, v/v). Cells were then incubated with mAbs to rchCD132 followed by a secondary incubation with an HRP-labeled goat anti-mouse-IgG (1:5000, Southern Biotechnology Associates Inc., Birmingham, USA). Color development was carried out with 3-amino-9-ethylcarbazole (BD Pharmingen, San Diego, CA). Subsequently, the mAbs were purified from ascites fluid by caprylic acid/ammonium sulfate precipitation on a HiTrap Protein G HP column (Amersham Biosciences) connected to an ÄKTApurifier UPC-900 system (Amersham Biosciences) according to the manufacturers' instructions. Subtype analysis of each mAb was performed using the SBA Clonotyping System/HRP (Southern Biotechnology Associates Inc., Birmingham, AL).

To further analyze whether the isolated mAbs could recognize chCD132 expressed on SMCs, Con A-activated SMCs were stained with anti-chCD132 mAbs by an indirect immunofluorescence assay (IFA) as described previously [19].

2.6. Identification of functional mAbs to chCD132

The functional activity of the mAb generation against rchCD132 was assayed by the inhibition of chIL-2-stimulated T-cell proliferation [19]. Briefly, SMCs stimulated with Con A for 48 h were further incubated in RPMI 1640 medium containing 0.1 mol/L a-methyl-mannoside for 30 min. Dead cells were removed using a Lymphoprep (Shanghai Hengxin Chemicals Co. Ltd., Shanghai, China) after centrifugation. Serial twofold dilutions of anti-chCD132 mAbs at 10 μg/mL were mixed with an equal volume (100 μL) of viable Con A-activated SMCs (1.5×10^6 cells/mL), and the resulting mixture was seeded into 96-well plates. Following incubation at 41 °C for 1 h, 50 μL (1 μg/mL) of rchIL-2 protein was added to each well and incubated at 41 °C for 48 h. Cell

proliferation was measured by the WST-8 assay (Cell counting kit-8®, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. SMCs mixed with only rchIL-2 or RPMI 1640 medium were used as positive and negative controls, respectively. Inhibition of cell proliferation was calculated using the following formula: inhibition = $(OD_{positive\ control} - OD_{sample})/(OD_{positive\ control} - OD_{negative\ control}) \times 100\%$.

2.7. Kinetics analysis of chCD132 expression on Con A-stimulated SMCs

SMCs were cultured in 6-well plates at a final concentration of 1×10^7 cells/mL in RPMI 1640 medium supplemented with 10% NCS and 10 μg/mL Con A and incubated at 41 °C for the indicated times. Non-stimulated SMCs served as a parallel control. Both sets of SMCs were collected at the indicated time by centrifugation for 5 min at 2500 r/min. Dead cells were removed using Lymphoprep. Viable cells were identified and counted with the Trypan blue exclusion test. Flow cytometry analysis was carried out as follows. Viable cells (2×10^6 cells/sample) were coated for 30 min with 5% NCS in RPMI 1640 medium at 4 °C. After centrifugation for 1 min at 4000 r/min, the cells were resuspended in 100 μL of mAb to chCD132 at 60 μg/mL and incubated at 4 °C for 1 h. Cells were then washed twice with PBS and incubated on ice for 30 min with 100 μL FITC-labeled goat anti-mouse-IgG (1:400, Southern Biotechnology Associates Inc., Birmingham, USA). Cells were then washed twice with PBS and suspended in 500 μL stationary liquid (1% formaldehyde, 0.02% NaN_3, 2% glucose in PBS). Cells were analyzed using a Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Inc., Fullerton, CA) with MXP software. Cells incubated with unrelated mouse IgG1 were used as negative controls.

2.8. Epitope mapping using a phage display peptide library

Phage display library Ph.D.-12 (NEB, Hertfordshire, UK) was screened by panning according to the manufacturer's instructions. Briefly, 96-well microtiter plates (Nunc) were coated with a mAb to chCD132 at a concentration of 100 μg/mL in 0.1 mol/L $NaHCO_3$ (pH 8.6). The mAb-coated wells were blocked with 0.5% BSA in blocking buffer and washed with 0.5% TBST. For each panning cycle, 2×10^{11} PFU of phage were placed in the well and incubated for 1 h. Following the removal of nonbinding phages, bound phages were competitively eluted from the well with 500 μg/mL rchCD132. The recovered phages were amplified for a new cycle of panning by growing in *E. coli* ER2738. A total of three rounds of selection were performed, and the selected phages were purified from single colonies for DNA sequencing. Finally, selected phages were assayed for binding to a mAb of chCD132 by a capture ELISA according to the manufacturer's instructions. The amino acid sequences of peptides displayed on positively selected phage were aligned by the Clustal W method. The consensus mimotope motif was further aligned with the chCD132 sequence.

2.9. Competitive ELISA to identify the native epitope in chCD132

According to the result of the alignment of the mimotope and chCD132 sequences, pep^{82-101} (NGQTLEIPSNRMELQNLVKP) and one-point mutation ($Q^{96}A$) $pep^{82-101m}$ (NGQTLEIPSNRME-LANLVKP) were synthesized with a solid-phase peptide synthesis method using a Symphony Multiplex Peptide Synthesizer (ProteinTechnologies, Inc., USA). Peptide purities were greater than 90% as assessed by HPLC and mass spectrometry. Peptides were conjugated to the carrier protein BSA using the heterobifunctional cross-linker Sulfo-SMCC (Pierce, USA). Pep^{82-101} and $pep^{82-101m}$ were used as inhibitors to block the binding of mAb C10 to chCD132 in a competitive ELISA as described previously [21]. Briefly, microtiter plates were coated with 0.17 μmol/L rchCD132 in 0.1 mol/L $NaHCO_3$ (pH 8.6) buffer at 4 °C overnight. Diluted mAb C10 at 6.7 nmol/L in 5% skim milk was mixed with an equal volume of inhibitors diluted to 8 μmol/L and pre-incubated for 1 h at 37 °C. Subsequently, mixtures were transferred to the rchCD132-coated plates at 100 μL/well, and the plates were incubated at 37 °C for 1 h. After washing, HRP-labeled goat anti-mouse-IgG was added, and the color was developed by TMB. Each dilution was tested in triplicate. BSA and rchCD132 (8 μmol/L) were used as negative and positive controls, respectively.

3. Results

3.1. Sequence and structure of the chCD132 ectodomain

Sequencing results showed that our acquired cDNA sequence (GenBank accession no. DQ852357) is identical to the published chCD132 sequence [20]. The ectodomain of mature chCD132 protein is composed of 214 aa. To characterize the relationship between the ectodomain sequence and its three-dimensional structure, a model of the tertiary structure of the chCD132 ectodomain was constructed using the CPHmodels 2.0 software. Chain C from the crystal structure of the IL-4-IL-4Ra-common gamma ternary complex (PDB ID code 3BPLC) was used as the template [13]. The predicted tertiary structure of the chCD132 ectodomain (Fig. 1), similar to that of the hCD132 ectodomain, contains two conserved fibronectin type III domains (N domain and C domain). Each domain is composed of seven β-strands (A, B, C, C′, E, F, G). The interdomain ($L^{95}Q^{96}N^{97}$) connecting the two domains also adopts a 3_{10} helical conformation in chCD132.

3.2. Expression of rchCD132 protein and generation of mAbs

The pET28a-chCD132 plasmid was transformed into the *E. coli* BL21 (DE3) strain, and rchCD132 protein with a His tag was optimally expressed. The rchCD132 protein was purified with a nickel column under denaturing conditions. The molecular weight of rchCD132 was approximately 28 kDa. BALB/c mice were immunized with the purified rchCD132 protein. Six hybridoma cell lines secreting anti-chCD132 antibodies were established (data did not shown). Western blot assay demonstrated that all mAbs (IgG1) exhibited specific binding to rchCD132 expressed in

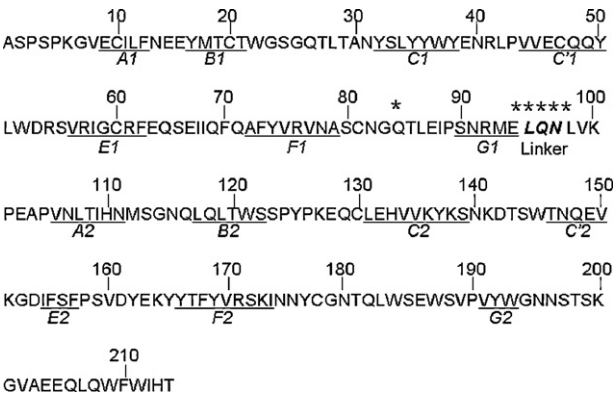

Fig. 1. Sequence of the chicken CD132 ectodomain. The sequence, first cloned by Min et al. [20], belongs to the type I cytokine receptor superfamily. Predicted β-strands are *underlined* and marked *A1* through *G1* for the N domain and *A2* through *G2* for the C domain. The interdomain is indicated by bold italic and marked Linker. Asterisks indicate the functional domain for binding chIL-2.

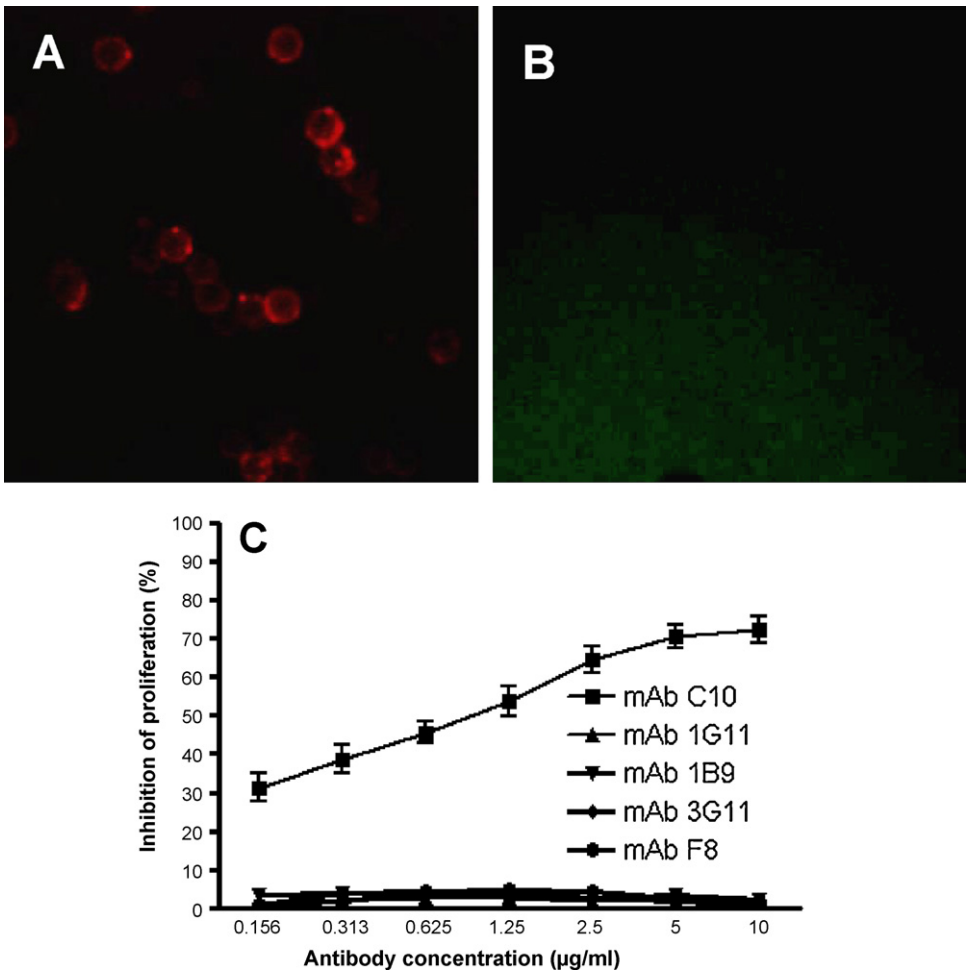

Fig. 2. Binding of anti-chCD132 mAbs to SMCs expressing chCD132 and inhibition of SMCs proliferation. (A) Anti-chCD132 mAb C10 binds chCD132 molecules on the cell surface of Con A-activated SMCs as visualized by an IFA (200×). (B) Anti-chCD132 mAb F8 used as the negative control of IFA. (C) Inhibition of chIL-2-stimulated SMCs proliferation. Cell proliferation was measured as described in Section 2. Values are expressed as the mean ± SD.

the prokaryotic *E. coli*. However, immunocytochemistry analysis showed that only five mAbs (1B9, 1G11, 3G11, C10 and F8) could specifically recognize the chCD132 protein expressed in eukaryotic Vero cells (data did not shown). Immunofluorescence analysis revealed that only mAb C10 exhibited strong binding of chCD132 on the surface of Con A-activated SMCs (Fig. 2A), while the others (such as mAb F8) did not bind chCD132 on the surface of Con A-activated SMCs (Fig. 2B). Furthermore, the mAbs were assayed for functional inhibition of rchIL-2-stimulated T-cell proliferation as described above. As shown in Fig. 2C, lymphocyte proliferation induced by rchIL-2 was only inhibited by mAb C10 with dose-dependent manner. These data demonstrate the functional activity of the anti-chCD132 mAb C10 and indicate that the chCD132 molecule is located on the cell membrane.

3.3. Kinetics of chCD132 expression on Con A-activated SMCs

Chicken SMCs were stimulated with Con A for 0, 4, 8, 16, 24, 32, 40 or 48 h, and chCD132 expression was identified by flow cytometry. As shown in Fig. 3, chCD132 protein was not detected prior to Con A stimulation (0 h), and only 3.04% of cells were gated as staining positive (% gated) for the chCD132 protein. Without Con A stimulation, the SMCs regulated the expression of chCD132 strictly at an average of 3.12% of cells gated positive during the 48 h culture in vitro. However, chCD132 expression on SMCs was gradually up-regulated as stimulation with Con A progressed. The cells gated positive was 18.25% at 40 h and 18.13% at 48 h, indicating that the expression remained constant between 40 and 48 h. These data demonstrate that the expression of the chCD132 protein can be up-regulated in SMCs following Con A stimulation.

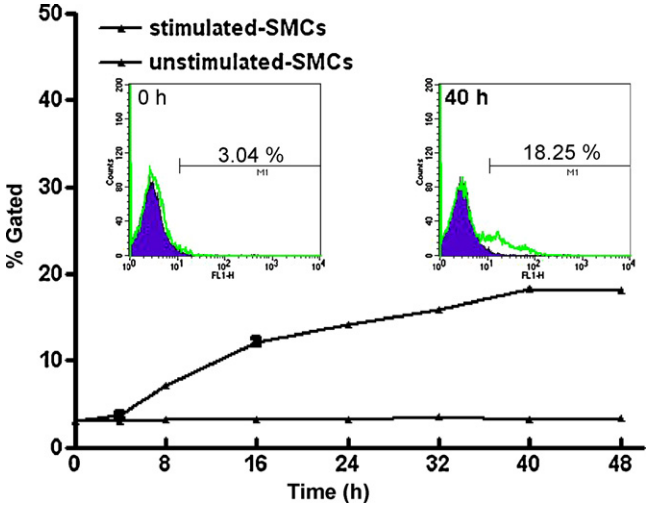

Fig. 3. Kinetics of chCD132 expression on the surface of Con A-stimulated SMCs. Con A-stimulated and unstimulated SMCs were incubated with anti-chCD132 mAb C10 and a secondary FITC-labeled goat anti-mouse IgG. SMCs incubated with unrelated mouse IgG1 served as negative controls.

Table 1
Alignment of sequences of peptides displayed on the phages isolated and their reactivity with mAb C10[a].

Phage clones	Phage-displayed peptide sequences	Frequency	OD450	
			mAb[b]	BSA[b]
PhaC10-1	VQGMLR**Q**DT**QNL**	1/8	0.873	0.054
PhaC10-2	TSDRD**HQNL**LAY	1/8	0.834	0.052
PhaC10-3	YNIH**EHQNL**RAV	1/8	0.835	0.059
PhaC10-4	II**QE**R**QN**IWTAP	1/8	0.882	0.058
PhaC10-5	ERSLW**QEHQN**FP	1/8	0.901	0.059
PhaC10-6	NNWDYP**QER**Q**N**I	1/8	0.743	0.066
PhaC10-7	A**QEHQNL**TWRPI	1/8	0.505	0.087
PhaC10-8	DR**Q**TW**QN**FMLIP	1/8	0.868	0.068
Consensus motif	**QEHQNL**			

[a] Alignments of phage-displayed peptide sequences by Clustal W were shown in boldface to indicate the consensus motif recognized by mAb.
[b] Binding of selected phages to mAb or BSA by a capture ELISA.

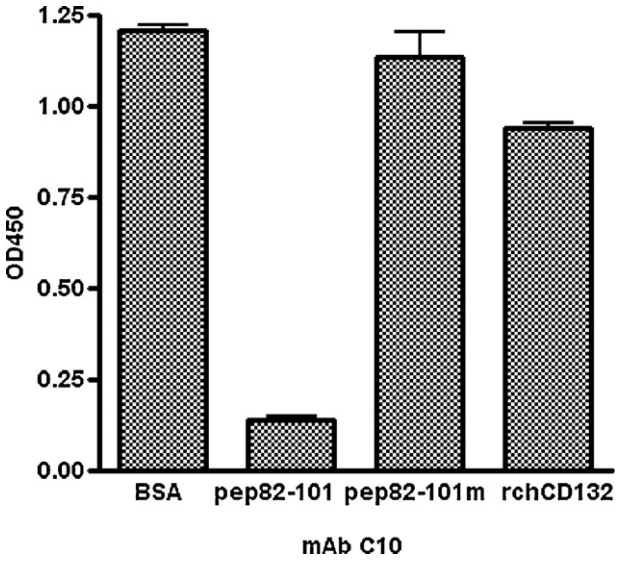

Fig. 4. Binding of mAb to synthesized native epitope peptide and rchCD132 protein. A competitive ELISA was carried out as described in Section 2.9. Synthesized peptides, pep^{82-101} and mutated $pep^{82-101m}$ at 8 μmol/L were used to competitively inhibit the binding of mAb C10 to rchCD132. BSA and rchCD132 alone at 8 μmol/L were included as negative and positive controls, respectively.

3.4. Mimotope motif of chCD132 selected by a phage display peptide library

To identify the functional binding epitope, the mimotope motif of the chCD132 molecule was selected through a phage-displayed peptide library utilizing the mAb C10. Panning results are summarized in Table 1. The mimotope motif was identified as QEHQNL by Clustal W. Aligned with chCD132, the putative native epitope of chCD132 was $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$, which is positioned in the synthetic peptide pep^{82-101} (NGQTLEIPSNRMELQNLVKP).

Competitive ELISA was performed to identify the putative native epitope and its critical amino acids that bind to the mAb at the antigen-binding site (Fig. 4). The binding of the mAb C10 to rchCD132 was completely blocked by pep^{82-101}, indicating that pep^{82-101} included the whole native functional epitope of the chCD132 protein. In the functional epitope $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$ of chCD132, the Q^{96} was aligned with the Q^{127} of hCD132. Previous research has showed that the Q^{127} of hCD132 is the critical residue for binding human IL-2 [12]. To analyze whether the Q^{96} of chCD132 is a critical residue for binding chIL-2, $pep^{82-101m}$ with the $Q^{96}A$ mutation was further synthesized. The result in Fig. 4 showed that $pep^{82-101m}$ had no ability to inhibit binding indicating that residue Q^{96} in chCD132 is a critical amino acid for functional epitope-binding to chIL-2. The data confirm that the native epitope, $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$, constitutes the intact conformational functional domain of chCD132, which aligns with the sequence $E^{115}K^{125}L^{126}Q^{127}N^{128}L^{129}$ in hCD132, and the Q^{96} of chCD132 is a critical residue for binding chIL-2.

3.5. Location of the functional domain in the tertiary structure of chCD132

To characterize the relationship between the domain function and its structure, we locate the function domain in the model of the tertiary structure of the chCD132 ectodomain. In the model, the functional domain of chCD132, $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$, was distributed between the C terminal of the N domain and the interdomain. Residue Q^{84} was positioned in the loop FG1 (S^{80}–P^{89}) of the N domain. Residues L^{95}, $Q^{96}$and N^{97} formed the 3_{10} helix and were situated in the elbow-like junction of the N domain and C domain (Figs. 1 and 5A). The aligned sequence $E^{115}K^{125}L^{126}Q^{127}N^{128}L^{129}$ in hCD132 was also positioned between the C terminal of the N domain and the interdomain (Fig. 5B).

4. Discussion

Using rchCD132 protein expressed in *E. coli*, we produced mAbs to chCD132. However, of these mAbs, only mAb C10 recognized CD132 molecules expressed naturally on the surface of chicken SMCs (Fig. 2A), confirming that the anti-chCD132 mAb is capable of binding an epitope on naturally expressed chCD132 molecules. Correspondingly, the mAb C10 inhibited in vitro SMCs proliferation induced by chIL-2 protein (Fig. 2C). These data suggest that there is

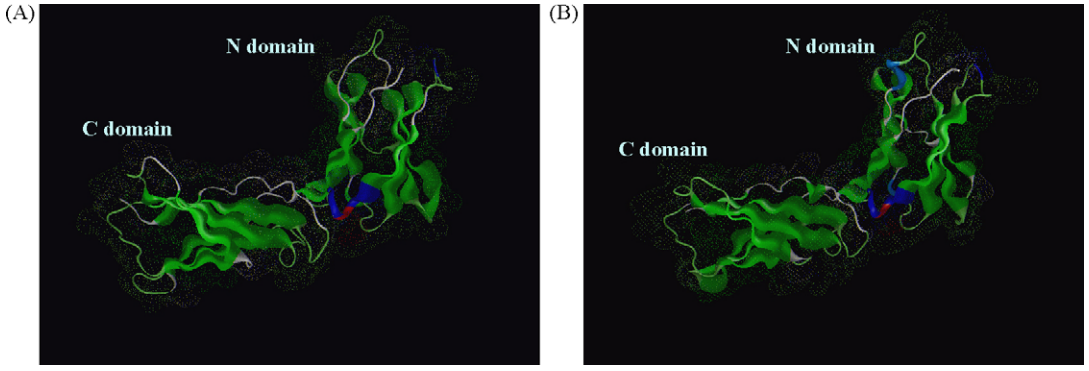

Fig. 5. Three-dimensional model localizes the functional domain on the chCD132 chain. (A) Chicken CD132 ectodomain; (B) Crystal structure drawing of human CD132 ectodomain. Beta sheets are colored green. The residues Q^{84}, E^{94}, L^{95}, N^{97}, and L^{98} in chCD132 and the aligned amino acids in hCD132 are colored blue, and the critical residue Q^{96} is colored red. The figures were produced using the program CPHmodels 2.0 in ribbon view from the same angle as SweetMollyGrace software.

a functional domain of the chCD132 molecule involved in binding chIL-2. By phage display and peptide synthesis techniques, the native conformational epitope $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$ was identified corresponding to the residues $E^{115}K^{125}L^{126}Q^{127}N^{128}L^{129}$ in hCD132. This epitope was confirmed through Clustal W analysis and peptide-competitive ELISA (Fig. 4). hCD132 binding sites for different cytokines overlap but are not identical. Mutagenesis of the cytokine-binding of hCD132 shows that Y^{103} of hCD132 is a key common ligand-interacting residue for IL-2, IL-4, IL-7 and IL-15 [22]. Three residues, I^{100}, L^{102} and L^{208}, of hCD132 dominate IL-4 binding but were not reported to be important for IL-2 and IL-7 binding [23,24]. Molecular mapping with neutralizing mAbs to hCD132 localizes critical residues to $N^{128}L^{129}V^{130}I^{131}P^{132}$ in the interdomain of hCD132 [22]. These data further support the evidence that epitope $Q^{84}E^{94}L^{95}Q^{96}N^{97}L^{98}$ is a critical functional domain for binding of chIL-2.

Molecular models for the interactions of CD132 with its cognate cytokines provide a conceptual framework for investigating the structural basis of the role of CD132 in ligand binding [11–13]. We used this information to explore the function domain in chCD132. The predicted tertiary structure of chCD132 ectodomain contains two typical fibronectin type III domains and an interdomain. Residue Q^{84} was positioned in the loop FG1 of the N Domain, which is far from the other residues of the binding epitope in the primary sequence. Residues L^{95}, Q^{96} and N^{97} formed a 3_{10} helix and are situated in the elbow-like junction of two domains (Figs. 1 and 5A). The aligned residues in hCD132 were also distributed between the C terminal of the N Domain and the interdomain (Fig. 5B). Recent crystallography studies confirm that the hCD132 binding sites on IL-2 and IL-4 exhibit apolar 'canyons' that receive the protruding elbow of hCD132 through near-ideal shape complementarity. Furthermore, hCD132 residue Y^{103} and the disulfide C^{160}–C^{209} engage the IL-2 canyon in a coupled manner as seen in the IL-4-hCD132 interface [13]. The interdomain region of hCD132 contacts IL-2 helices A and D. Around the canyon, there are only two specific hydrogen bonds in the IL-2/hCD132 complex: $T^{123\ IL\text{-}2}/Q^{127\gamma}$ and $Q^{126\ IL\text{-}2}/P^{207\gamma}$ [11,12]. Therefore, it is reasonable to postulate that residue Q^{96} of chCD132, corresponding to the Q^{127} of hCD132, is the specific site for binding chIL-2. Indeed, when the Q^{96} of pep^{82-101} was mutated to A^{96}, the resulting $pep^{82-101m}$ showed no ability to block mAb C10 from binding chCD132 (Fig. 4), which indicates that residue Q^{96} of chCD132 is a critical functional site for binding of chIL-2.

Continued studies to clarify the structure–function relationship of chCD132 with cognate ligands are now in progress. These studies aim to advance our understanding of chicken lymphocyte activation, the general immune response in chickens, and phylogenetic comparisons of mammalian and non-mammalian cytokines.

Acknowledgements

This work was supported by grants from National Science Foundation of China (Grant Nos. 30625030 and 30771589), and by the earmarked fund for Modern Agro-industry Technology Research System.

References

[1] Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, et al. Cloning of the gamma chain of the human IL-2 receptor. Science 1992;257:379–82.
[2] Sugamura K, Asao H, Kondo M, Tanaka N, Ishii N, Nakamura M, et al. The common gamma-chain for multiple cytokine receptors. Adv Immunol 1995;59:225–77.
[3] Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. J Immunol 2001;167:1–5.
[4] Nelson BH, Willerford DM. Biology of the interleukin-2 receptor. Adv Immunol 1998;70:1–81.
[5] Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 1993;73:147–57.
[6] Balasubramanian S, Chernov-Rogan T, Davis AM, Whitehorn E, Tate E, Bell MP, et al. Ligand binding kinetics of IL-2 and IL-15 to heteromers formed by extracellular domains of the three IL-2 receptor subunits. Int Immunol 1995;7:1839–49.
[7] Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science 1993;262:1880–3.
[8] Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science 1993;262:1877–80.
[9] Thanos CD, Randal M, Wells JA. Potent small-molecule binding to a dynamic hot spot on IL-2. J Am Chem Soc 2003;125:15280–1.
[10] Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci U S A 1990;87:6934–8.
[11] Stauber DJ, Debler EW, Horton PA, Smith KA, Wilson IA. Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. Proc Natl Acad Sci U S A 2006;103:2788–93.
[12] Wang X, Rickert M, Garcia KC. Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gamma c receptors. Science 2005;310:1159–63.
[13] LaPorte SL, Juo ZS, Vaclavikova J, Colf LA, Qi X, Heller NM, et al. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. Cell 2008;132:259–72.
[14] Sundick RS, Gill-Dixon C. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol 1997;159:720–5.
[15] Lawson S, Rothwell L, Kaiser P. Turkey and chicken interleukin-2 cross-react in in vitro proliferation assays despite limited amino acid sequence identity. J Interferon Cytokine Res 2000;20:161–70.
[16] Zhou JY, Wang JY, Chen JG, Wu JX, Gong H, Teng QY, et al. Cloning, in vitro expression and bioactivity of duck interleukin-2. Mol Immunol 2005;42:589–98.
[17] Zhou JY, Chen JG, Wang JY, Wu JX. Gong H. cDNA cloning and functional analysis of goose interleukin-2. Cytokine 2005;30:328–38.
[18] Chen JG, Chen WH, Zhou JY, Wang JY, Qi J, Zheng XJ, et al. In vitro expression and bioactivity of chicken interleukin-2. Sci Agric Sin 2005;38:1034–9.
[19] Teng QY, Zhou JY, Wu JJ, Guo JQ, Shen HG. Characterization of chicken interleukin 2 receptor alpha chain, a homolog to mammalian CD25. FEBS Lett 2006;580:4274–81.
[20] Min W, Lillehoj HS, Fetterer RH. Identification of an alternatively spliced isoform of the common cytokine receptor gamma chain in chickens. Biochem Biophys Res Commun 2002;299:321–7.
[21] Wang XN, Zhang GP, Zhou JY, Feng CH, Yang YY, Li QM, et al. Identification of neutralizing epitopes on the VP2 protein of infectious bursal disease virus by phage-displayed heptapeptide library screening and synthetic peptide mapping. Viral Immunol 2005;18:549–57.
[22] Raskin N, Jakubowski A, Sizing ID, Olson DL, Kalled SL, Hession CA, et al. Molecular mapping with functional antibodies localizes critical sites on the human IL receptor common gamma (gamma c) chain. J Immunol 1998;161:3474–83.
[23] Zhang JL, Buehner M, Sebald W. Functional epitope of common gamma chain for interleukin-4 binding. Eur J Biochem 2002;269:1490–9.
[24] Olosz F, Malek TR. Three loops of the common gamma chain ectodomain required for the binding of interleukin-2 and interleukin-7. J Biol Chem 2000;275:30100–5.