Molecular cloning and characterization of Duck CD25

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Abstract

The IL-2Rα chain (CD25, Tac) is an essential component of high affinity IL-2Rs, playing critical role for the immune specificity of antigen-activated T-cell clonal expansion. Up to now, no duck cytokine receptor has been described. Here, the cDNA segment of a duck cytokine receptor (duCD25), encoding a 226 aa precursor protein with a 20 aa signal peptide, was isolated. Then a novel mouse monoclonal antibody (mAb) was generated using the prokaryotically expressed duCD25 protein as immunogen. Using this mAb, the endogenous duCD25 molecule was localized on the surface of duck lymphocytes, and the duck IL-2-induced lymphocyte proliferation was further inhibited. Furthermore, flow cytometry analysis showed that duCD25 positive cells were upregulated in ducks infected with avian influenza virus (H9N2). Our findings confirm that duCD25 is a receptor of duck interleukin-2, and duCD25 positive cells play a potential role in H9N2 virus infection.

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1. Introduction

Interleukin 2 (IL-2) and IL-2 receptors are critical to regulate the magnitude and duration of the T-cell immune responses activated by antigen (Smith, 1989). The specific receptor of interleukin 2, CD25 (interleukin 2 receptor α, Tac), is a marker of activated and natural regulatory T-cells (Smith, 1984; Schwartz, 2005). The transient expression of CD25 (Cantrell and Smith, 1983), together with constitutively expressed receptor β, γ (Nishi et al., 1988; Takeshita et al., 1992), forms the high affinity receptor complex of IL-2, which guarantees the clonal expansion specificity of antigen-activated T-cells.

In the past, waterfowls were considered as a natural reservoir of avian influenza virus (AIV), playing an important role in the AIV ecology and propagation (Suzuki and Nei, 2002). Recent reports have confirmed that some of H5N1 isolates were highly pathogenic in ducks and wild aquatic birds (Chen et al., 2005; Sturm-Ramirez et al., 2004; Zhou et al., 2006). Calamitously, highly pathogenic avian influenza (HPAI, H5N1 and H7N7) has resulted in human infection and death (Claas et al., 1998; Fouchier et al., 2004; Subbarao et al., 1998). Therefore, understanding of duck immune system is very important to seek interspecies transmissions of AIV.

In this study, we cloned and identified the cDNA fragment of duck CD25, prepared a monoclonal antibody (mAb), and investigated its in vivo expression profiles in AIV-infected ducks. This is the first report on waterfowl cytokine receptor.
2. Materials and methods

2.1. Cells, virus and animals

Vero cells were maintained in MEM (Gibco BRL, USA), supplemented with 10% newborn calf serum. The A/Ck/JD/323/2001 (H9N2 virus) was stored in our laboratory. SPF Muscovy ducks were raised in a negative-pressure isolator in our laboratory. 4-week-old BALB/c mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. All laboratory animals and animal subjects used in this study have been approved by the Scientific Ethical Committee of the Zhejiang University, China.

2.2. cDNA screening

Duck splenic mononuclear cells (SMC) stimulated with Con A were prepared as described (Zhou et al., 2005). Total cellular RNA from the SMC blasts were extracted with Trizol reagent (Invitrogen, CA, USA), from which the total cDNA were reversely transcripted using a universal reverse primer [common downstream (CDS)-oligo (dT)] 5'-AAGCAGTGGTATCAACGCA-GAGTACTTTTTTTTTTTTTT TTTTTTTTTTTTTT-3' designed according to the 3'-RACE strategy. The potential duck homolog was screened from the total cDNA by annealing gradient PCR (56–68 °C), using synthesized serial upstream primer probes mapping to the cDNA of chicken IL-2 receptor α chain (Teng et al., 2006) and the specific downstream primer CDS (Fig. 1). Subsequently, the cDNA fragment, designated duck CD25 (duCD25) cDNA, was identified by sequencing. Further bioinformatics analysis was performed by following software: SignalP version 3.0 of SignalP World Wide Web server (BendtSEN et al., 2004), OMIGA2.0 (Oxford Molecular Ltd., Madison, USA), TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 Server (Julenius et al., 2005), Clustalw 1.8 (Thompson et al., 1994).

2.3. Prokaryotic expression of duck CD25 extracellular domain

Sequence encoding duCD25 extracellular domain was amplified by PCR from the above-mentioned duCD25 cDNA segment using the following primers: upstream primer 5'-GGGTACCGACGACGACGACGA-CAAGGGCAATGCCCACCTGTT-3' containing a KpnI site, and downstream primer 5'-CAAGCTTT-CAGGATGGATGAG CTGAACC-3' containing a HindIII site. Then the PCR product was digested with KpnI and HindIII, and inserted into the corresponding sites of the pET32a expression vector (Novagen, WI, USA). The recombinant plasmid (pET32a-duCD25) was transformed into Escherichia coli (E. coli) BL21 (DE3) strain. The recombinant duCD25 protein (rduCD25) expressed as a fusion protein carrying thioredoxin (Trx) and His tags, was analyzed by SDS-polyacrylamide gel electrophoresis and western blot with the anti-His mAb (Invitrogen) as described previously (Zhou et al., 2005), and was further purified by a nickel column under denaturing conditions following manufacturer’s instructions (Qiagen Inc., CA, USA).

2.4. Preparation of mAb against duCD25

SPF BALB/c mice were immunized subcutaneously with 50 μg of rduCD25 protein in complete Freund’s adjuvant (Sigma, MO, USA), 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 rduCD25. Spleen lymphocytes were fused with SP2/0 myeloma cells using standard procedures. Hybridoma secreting antibodies against duCD25 was screened and cloned by a limiting dilution technique. mAbs to duCD25 protein were further selected by indirect ELISA and western blot as described earlier (Zhou et al., 2005), using Trx, cell lysate of E. coli BL21 (DE3), rduCD25 and rchCD25 (Teng et al., 2006) as antigens. Subtype analysis of each mAb was performed using SBA Clonotyping™ System/HRP [Southern Biotechnology Associates Inc. (SBA), AL, USA].

2.5. Antibody reactivity to eukaryotic and endogenous duCD25 protein

To screen anti-duCD25 mAbs reacting with the natural duCD25 protein, the eukaryotically expressed duCD25 protein was initially used as antigen in immunocytochemistry assay. The open reading frame of duCD25 was subcloned into the pEGFP-C2 plasmid (BD Biosciences Clontech, CA, USA). Then the EGFP-tagged duCD25 plasmid was introduced into Vero cells by Lipofectamine 2000 reagent (Invitrogen). The transfected cells started to express duCD25 at 6 h post-transfection. After incubation for 20 h at 37 °C, the cells were then incubated with mAbs to duCD25 followed by HRP-labeled goat anti-rabbit/mouse-IgG
As described previously (Teng et al., 2006). Color development was carried out with 3-amino-9-ethylcarbazole (BD Pharmingen, CA, USA). Subsequently, to detect capability of anti-duCD25 mAbs recognizing endogenous duCD25 molecules, the Con A-stimulated duck SMC blasts were incubated with mAbs to duCD25 followed by FITC-labeled goat anti-rabbit/mouse-IgG (SBA) as described previously (Teng et al., 2006). Finally, to localize the expressed duCD25 molecules, the Con A-stimulated SMC blasts were prepared as mentioned in Section 2.7 in detail. Optical density (OD) values were measured at 570 nm (630 nm as a reference wavelength). SMC with only duIL-2 or RPMI 1640 medium, were used as positive and negative controls, respectively. Inhibition of cell proliferation was calculated using the following formula as previously described (Li et al., 2001): inhibition = (OD positive control – OD sample)/(OD positive control – OD negative control) × 100%.

2.7. Flow cytometry analysis of duck CD25 expression

Endogenous duCD25 expression was initially investigated in vitro in Con A-stimulation model. The Con A-stimulated SMC blasts were prepared as mentioned above. After removing dead cells by Histopaque-1077 (Sigma, USA), the concentration of live cells was adjusted to 5 × 10⁶ cells/ml. Duck SMC with no stimulation were used as negative control. In vivo kinetics of duCD25 expression was further determined using SMC of ducks inoculated avian influenza virus H9N2. Briefly, thirty-five SPF Muscovy ducks were housed in negative-pressure isolator cages with HEPA-filtered air, and inoculated intra-nasally (i.n.) with 10²⁵ EID₅₀/0.1 ml H9N2 virus in a 0.5-ml volume. After being infected with H9N2 virus for 24 h, SMC were isolated from five infected ducks every day until the 7th day post-infection. SMC from uninfected SPF ducks were used as negative control. The above-mentioned cells were incubated with mAbs against duCD25 or chicken CD25 (chCD25) (Teng et al., 2006) in PBS containing a final concentration of 5% SPF rabbit serum, and a secondary FITC-labeled goat antimouse-IgG (SBA) on ice, respectively. Then the cells were further counterstained with PE-labeled mouse anti-chicken CD4 (chCD4), or CD8 (chCD8) or monocyte/macrophage mAbs (SBA). Cells were analyzed by a Coulter XL flow cytometer (Beckman, CA, USA) with System II software. Cells incubated with normal mouse serum and FITC-conjugated mice IgG1 (SBA) were used as parallel controls.

3. Results

3.1. Isolation and characterization of the duCD25 cDNA

Initially a small cDNA fragment overlapping the stop codon was screened using upstream primer probes 1–10 and CDS-oligo (dT), respectively (Fig. 1). Only an approximate 0.74 kb cDNA fragment (Fragment I) was obtained from duck total cDNA using probes 2, 3 and 4 (data not shown), and this nucleotide segment shared over 69% nucleotide identity with the matching part of chicken CD25. A special reverse primer 11 (Fig. 1) matching Fragment I was further designed as a downstream primer. Then cDNA segments overlapping the start codon were screened by RT-PCR using upstream primers 12–23 (Fig. 1) and downstream primer 11. However, only a 0.75 kb cDNA fragment (Fragment II) was respectively obtained by forward primers 20–23 and reverse primer 11. Fragment II shared 74% identity with the matching part of chicken CD25. After splicing Fragments I and II, an 886 bp cDNA sequence (Genbank accession no. DQ299949) was obtained (duCD25 cDNA). The duCD25 cDNA contains an open reading frame (ORF), encoding a precursor protein consisting of 226 amino acid (aa) residues, with a signal peptide including 20 aa. The predicted
mature duCD25 protein contains an extracellular (181 aa) and a transmembrane (25 aa) domain, but no intracellular domain, with a theoretical molecular mass of 23.0 kDa and an isoelectric point value of 8.1. The extracellular domain of duCD25 molecule, with a theoretical molecular weight of 21 kDa, contains four potential N-linked glycosylation sites (Fig. 2) and multiple possible O-linked carbohydrate sites. Alignment analysis showed that duck CD25 molecule shared 61% aa identity with chCD25, 15–25% aa identities with mammalian CD25. Among the compared species, 23 conserved amino acid sites exist in mature CD25 molecules (Fig. 2). Especially, seven cysteines (Cys) in duCD25 were completely conserved in all the compared CD25 molecules, implying their important role in maintaining structure and function of CD25.

3.2. Prokaryotic expression and antibody preparation of recombinant duCD25 molecule

The pET32a-duCD25 vector was transformed into E. coli BL21 (DE3) strain, and the rduCD25 with Trx and His tags was expressed as insoluble inclusions after induced by a final concentration of 1 mM IPTG. As shown in Fig. 3A, the molecular mass of expressed rduCD25 protein was approximately 40 kDa, identical to its theoretical value [21 kDa + 19 kDa (Tag protein)]. Finally, the rduCD25 protein was purified using a nickel column under denaturing conditions. Fifteen hybridoma cell lines secreting anti-duCD25 antibodies were established. Western blot analysis demonstrated that all the fifteen anti-duCD25 mAbs were strongly recognized the rduCD25 protein (Fig. 3B). However, none of these antibodies showed any reaction with Trx, cell lysate of E. coli or rchCD25 antigen (data not shown). Furthermore, all the mAbs belong to IgG1 subtype, with a κ chain.

3.3. Antibodies recognizing eukaryotic and endogenous duCD25, and localization of duCD25 on SMC

To detect binding capability of mouse anti-rduCD25 mAbs to natural du25, the duCD25 fusing with a green fluorescent protein was expressed in Vero
cells and used as antigen. Immunocytochemistry assay showed that only five anti-rduCD25 mAbs could recognize the eukaryotically expressed duCD25 protein (data not shown). Subsequently, immunofluorescence analysis indicated that two mouse anti-rduCD25 mAbs (6F2, 3H11) could recognize the endogenous duCD25 expressed on duck SMC blasts (data not shown). Further confocal microscopy analysis demonstrated that the endogenous duCD25 mainly localized at cell membrane of duck SMC (Fig. 4).

3.4. Anti-duCD25 mAb inhibit duIL-2-stimulated SMC proliferation

In order to investigate the potential biological function of duCD25, inhibiting assay of duIL-2-induced T-cell proliferation was carried out. As shown in Fig. 5, the proliferation activity of duIL-2 protein was inhibited by anti-duCD25 mAb 6F2. However, the anti-duCD25 mAb 3H11 indicated no such inhibiting activity. These results showed that the anti-duCD25 mAb 6F2 blocked functional epitopes of natural duCD25 directly binding
duIL-2, confirming that duCD25 is a cell receptor of duck IL-2.

3.5. Frequency of duCD25+ cells in Con A-stimulated SMC

To analyze the effect of mitogen on duCD25 expression, duCD25+ cells in Con A-activated duck SMC were detected by FCM. Results indicated that a lower percentage of duCD25+ cells (<5%) existed in the unstimulated duck SMC (Fig. 6A). In contrast, the percentage of duCD25+ cells (>41%) remarkably increased after duck SMC were activated by Con A (Fig. 6B). These data demonstrated that only partial duck SMC expressed duCD25 molecules. In addition, neither duCD25+ SMC nor duCD25- SMC could be recognized by mouse anti-chCD25, chCD4, chCD8, chicken γδ T-cell or CD41/CD61 mAb, indicating that these mAbs recognizing chicken cell receptors do not cross-react with the counterparts in ducks.

3.6. Kinetics of splenic CD25-phenotype cells in ducks infected with H9N2 virus

To understand the profile of duCD25 expression in vivo, duCD25 positive SMC in H9N2 virus-infected ducks were detected by FCM. Results indicated that a lower percentage of duCD25+ cells (<5%) existed in the unstimulated duck SMC (Fig. 6A). In contrast, the percentage of duCD25+ cells (>41%) remarkably increased after duck SMC were activated by Con A (Fig. 6B). These data demonstrated that only partial duck SMC expressed duCD25 molecules. In addition, neither duCD25+ SMC nor duCD25- SMC could be recognized by mouse anti-chCD25, chCD4, chCD8, chicken γδ T-cell or CD41/CD61 mAb, indicating that these mAbs recognizing chicken cell receptors do not cross-react with the counterparts in ducks.
ducks were analyzed by FCM. As shown in Fig. 7, when compared with uninfected ducks, the frequency of duCD25+ SMC increased dramatically in the infected ducks within 24 h post-inoculation (p.i.), and reached a peak (15% ± 2.2%) at 2 days p.i. Then the frequency of duCD25+ cells began to decrease gradually at 3 days p.i. and returned to physiological levels at 6 days p.i. These data indicate that the duCD25+ cells play a potential important role during H9N2 virus infection.

4. Discussion

In the present experiments, an 886 bp duCD25 cDNA segment sharing 61% aa identity with chCD25 was successfully screened by the primer probes 2, 3, 4, 20, 21, 22 (Fig. 1), based on the sequence of chCD25 counterpart. The deduced mature protein of duCD25 contains a large extracellular domain, a transmembrane domain, and no cytoplasmic domain, which implies no binding sites for potential signal-regulating factors in duCD25. These properties were consistent with the results that signal transduction did not involve CD25 (Hatakeyama et al., 1986), and that CD25 was to deliver IL-2 to the signaling complex and act as a regulator of signal transduction (Stauber et al., 2006).

Interestingly, seven conserved Cys residues were revealed when comparing the CD25 aa sequences from 10 species. It was reasonable to hypothesize that these Cys play an important role in maintaining the CD25 structure and function, as site-specific mutagenesis in mammal indicated that any modification of these Cys residues greatly reduced the ability of CD25 to bind IL-2 (Rusk et al., 1988). Recent report demonstrated that the residues R-36 and L-42 in human CD25 were residue members resulting in the IL-2/CD25 interface (Stauber et al., 2006). Correspondingly, further studies are needed to investigate whether the conserved residues (R-35 and L-41, Fig. 2) in duCD25 molecule are crucial to the duIL-2/duCD25 interface.

In this study, we tried to functionally identify duCD25 molecule. With the facility of our cloned duCD25 cDNA, anti-duCD25 mAbs were prepared.
using the prokaryotically expressed duCD25 protein. Further experiments showed that the anti-duCD25 mAb could recognize the endogenous duCD25 molecules expressed on the surface of some duck SMC (Fig. 4), and partially inhibit the duIL-2-induced SMC proliferation in vitro (Fig. 5). Simultaneously, the mAbs to duCD25 showed no cross-reactivities with chicken and goose SMC (data not shown). These data confirm that duCD25 molecule is a membrane protein as a receptor involved in binding duck IL-2.

Subsequently, we analyzed in vitro and in vivo expression profiles of duCD25 molecules. FCM analysis showed a low proportion of CD25+ cells in the newly isolated SMC from SPF ducks, whereas an obvious up-regulation of duCD25 expression occurred in Con A-activated SMC and those of ducks infected with the H9N2 virus (Fig. 6 and Fig. 7), which was similar to the chCD25 expression profiles (Teng et al., 2006). However, after ducks were exposed to H9N2 AIV infection, the role of duCD25 molecules is currently unclear. Recently, several reports showed that Treg cells (CD4+CD25+) suppressed virus-specific CD8+ T-cell responses during acute and chronic viral infections (McGuirk and Mills, 2002; Truneh et al., 1985). Therefore, further studies should be focused on duck CD molecules and duck immune system.

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References


