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Expression and characterization of recombinant soluble porcine CD3 ectodomain molecules: Mapping the epitope of an anti-porcine CD3 monoclonal antibody 898H2-6-15

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ABSTRACT

The porcine CD3 specific monoclonal antibody 898H2-6-15 has been used in allo- and xeno-transplantation studies as a porcine CD3 marker and as an effective T cell depletion reagent when conjugated to the diphtheria toxin mutant, CRM9. A recombinant anti-porcine CD3 immuntoxin was recently developed using single-chain variable fragments (scFv) derived from 898H2-6-15. In this study, using published sequence data, we have expressed the porcine CD3 ectodomain molecules in *E. coli* through inclusion body isolation and *in vitro* refolding approach. The expressed and refolded porcine CD3 ectodomain molecules include CD3ε, CD3γ, CD3δ, CD3εγ heterodimer, CD3εδ heterodimer, CD3εγ single-chain fusion protein and CD3εδ single-chain fusion protein. These refolded porcine CD3 ectodomain molecules were purified with a strong anion exchange resin Poros 50HQ. ELISA analysis demonstrated that only the porcine CD3εγ ectodomain single-chain fusion protein can bind to the porcine CD3 specific monoclonal antibody 898H2-6-15. The availability of this porcine CD3εγ ectodomain single-chain fusion protein will allow screening for affinity matured variants of scFv derived from 898H2-6-15 to improve the recombinant anti-porcine CD3 immunotoxin. Porcine CD3εγ ectodomain single-chain fusion protein will also be a very useful reagent to study the soluble phase interaction between porcine CD3εγ and porcine CD3 antibodies such as 898H2-6-15.

1. Introduction

The TCR–CD3 complex consists of one clonotypic antigen-binding αβ or γδ TCR heterodimer and three conserved signal transducing modules: CD3εγ and CD3εδ heterodimers and a TCRζ homodimer. TCR–CD3 chains assemble into a minimal eight-subunit complex in the endoplasmic reticulum through a series of dimeric and trimeric interactions with a stoichiometry of one TCRαβ or TCRγδ heterodimer, one CD3εγ heterodimer, one CD3εδ heterodimer, and one TCRζ homodimer [1,11,13].

The anti-porcine CD3 monoclonal antibody 898H2-6-15 was generated by immunizing mice with porcine peripheral blood monoclonal cells [7]. It has been used as a porcine CD3 marker in our allo- and xeno-transplantation models as well as a T cell depletion reagent by conjugating with a diphtheria toxin binding mutant CRM9. We have demonstrated that the chemically conjugated anti-porcine CD3 immunotoxin is a very effective T cell depletion reagent in pigs [8] and this reagent has played a role in the maintenance of long-term hematopoietic stem cell transplants in the absence of graft versus host disease [3,4,6]. Recently using the single-chain variable fragment (scFv) derived from 898H2-6-15 monoclonal antibody (mAb) we have developed an anti-porcine CD3 recombinant immunotoxin [16]. Our interest to express the porcine CD3 molecules arose from the need to prepare a biotin-labeled porcine CD3 molecule to conduct the affinity maturation of this immunotoxin using a yeast display approach [2]. Our goal is to isolate the high affinity scFv (2-6-15) to improve the binding of our anti-porcine CD3 immunotoxin [15].

In this study, using the published sequence data we have synthesized and expressed the porcine CD3 ectodomain molecules in *Escherichia coli*. We isolated the inclusion bodies and refolded them *in vitro* [9]. We expressed and refolded following porcine CD3 ectodomain molecules: CD3ε, CD3γ, CD3δ, CD3εγ heterodimer, CD3εδ heterodimer, CD3εγ single-chain fusion protein and CD3εδ single-chain fusion protein. These refolded porcine CD3 ectodomain molecules were purified with a strong anion exchange resin Poros 50HQ. The binding reactivity to 898H2-6-15 mAb was analyzed

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by ELISA. The results demonstrated that only the porcine CD3εγ ectodomain single-chain fusion protein binds to the 898H2-6-15 mAb.

2. Materials and methods

2.1. Plasmid construction

We designed 8 overlapping PCR primers covering the entire ε, or γ, or δ ectodomain respectively (Table 1) based on the DNA sequence of porcine CD3 ectodomain molecules (GenBank ID: S82909 for porcine CD3ε, AB190229 for porcine CD3γ and NM_213775 for porcine CD3δ, [12]. The first sense primer contained a 5′ *Nde*I restriction site; therefore an N-terminal methionine residue was added to each of the porcine CD3 ectodomain chains. The next primer was antisense and overlapped by 21 bp at both ends. Alternating overlapping sense and antisense primers were continued until the ectodomain was covered. Six histadine (6x-His) residues were added to the C terminus of each construct to facilitate the purification. A stop codon was inserted after the last ectodomain amino acid codon followed by an *Eco*RI site. The synthesis was conducted by PCR with cloned pfu polymerase (Stratagene) to increase the fidelity. The PCR program is 95 °C for 5 min, 25 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, extension for another 10 min at 72 °C. In the 50 μl PCR reaction, 10 pmol was added for the 5′ and 3′ end primers, and 2 pmol was added for the rest of the primers. After running the agarose gel, the correct DNA band was cut out of a 1% agarose gel, extracted with QIAquik gel extraction kit (QIAGEN, 28704), digested with *Nde*I + *Eco*RI, and cloned in frame into pET17b. All of the plasmid clones were confirmed by DNA sequencing.

In order to build the porcine CD3 ectodomain εγ single-chain fusion construct (Fig. 1), the porcine CD3 ectodmain ε moiety was PCR amplified using primers Ep-NdeI + Epsilon R and the porcine CD3 ectodomain ε in pET17b was used as template. After running the agarose gel, the correct DNA band was cut out, extracted with QIAquik gel extraction kit, then digested with *Nde*I + *Bam*HI labeled as insert I. The porcine CD3 ectodomain γ moiety was PCR amplified using Gamma F1 + Gamma Rhis using the porcine CD3 ectodomain γ in pET17b as template. After running the agarose gel, the correct DNA band was cut out, extracted with QIAquik gel extraction kit, then digested with *Bam*HI + *Eco*RI as insert II. The insert I + insert II were cloned together into the *Nde*I–*Eco*RI digested pET17b and sequencing confirmed. The fusion construct was transformed into *E. coli* BL21 star (DE3) competent cell (Invitrogen). The porcine CD3 ectodomain εδ single-chain fusion construct was built with the same method as described above with the PCR primer Delta F replacing the Gamma F1 and the Delta Rhis replacing the Gamma Rhis. The porcine CD3 ectodomain δ in pET17b was used as template for amplifying the porcine CD3 ectodomain δ moiety.

2.2. E coli expression, inclusion body preparation and solubilization

Insoluble inclusion body protein was prepared using a protocol based on what is described by [5], with modifications. The characterized plasmid DNA was transformed into *E. coli* BL21 star (DE3). To prepare the seed culture, a single colony was inoculated into 25 ml LB containing 100 μg/ml ampicillin and cultured overnight at 37 °C with shaking at 250 rpm. The above seed culture was inoculated at 2.5% final concentration into 800 ml LB (in four 1 L flasks) containing 100 μg/ml ampicillin and cultured at 37 °C with shaking at 250 rpm until the OD600 reached 0.8–1.0. IPTG was added at 1 mM final concentration to induce the protein expression for 3 h at 37 °C with shaking at 250 rpm. The cells were harvested by centrifugation at 3000*g* for 10 min. The cell pellets were stored at –80 °C for later use.

The cell pellets from an 800 ml culture were suspended in 10 ml of 50 mM Tris HCl, pH 8.0, 25% sucrose, 1 mM EDTA, 0.1% sodium azide, 10 mM DTT. Lysozyme (1 mg/ml), DNase I (375 μg/ml), and 5 mM $MgCl_2$ was added. Lysis buffer was added at 2.5 ml per ml of the suspension containing 50 mM Tris HCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 100 mM NaCl, 0.1% sodium azide, 10 mM DTT, 10 mM EDTA, pH 8.0. The suspension was frozen and thawed for one cycle then 10 mM $MgCl_2$ was added for aiding DNase I activity. The cell debris was centrifuged at 13,000*g* at 4 °C for 50 min. The cell pellets were washed four times by centrifugation at 13,000*g* at 4 °C for 10 min with 50 mM Tris HCl, 0.5% (v/v) Triton X-100, 100 mM NaCl, 1 mM EDTA, 0.1% sodium azide and 1 mM DTT, pH 8.0. Then the inclusion bodies were suspended in 50 mM Tris HCl, 1 mM EDTA, 0.1% sodium azide, and 1 mM DTT, pH 8.0; centrifuged as above; pellets were then dissolved in 3 ml of 25 mM 2-(*N*-morpholino)ethanesulfonic acid, 8 M urea, 10 mM EDTA, and 0.1 mM DTT, pH 6.0 for 10 min at room temperature; centrifuged at 13,000 ×*g* at 4 °C for 10 min. The supernatant was stored at –80 °C. The protein concentration of the supernatant was determined with BCA protein assay kit using a BSA standard (Pierce, cat# 23225).

2.3. In vitro refolding of the porcine CD3 molecules

The inclusion body solution [ε (8 μM), γ (8 μM), δ (8 μM), ε (6 μM) + γ (18 μM), ε (6 μM) + δ (18 μM), εγ single-chain fusion protein (8 μM), εδ single-chain fusion protein (8 μM)] was directly injected into refolding buffer containing 100 mM Tris HCl, 1 M L-arginine–HCl, 2 mM EDTA, pH 8.2, 1 mM oxidized glutathione, 0.33 mM reduced glutathione using a 18 gauge needle with stirring in a cold room at 4 °C. The reaction was stirred for approximately 3 min to completely mix the protein. The reaction was then kept in a 10 °C cabinet for 48–72 h without stirring.

2.4. Purification of the refolded porcine CD3 molecules

Ni–Sepharose, fast flow 4 mL resin (GE Healthcare Cat. #17-5318-02) equilibrated with 10 column volumes (CV) of 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 5 mM imidazole was used for the first step purification. The refolded protein product was transferred into wet dialysis tubing with 3.5 kDa cutoff (Spectrum Laboratories, Inc. Cat# 132725), dialyzed for 24 h against 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 5 mM Imidazole with one complete buffer exchange and constant stirring. The dialyzed samples were loaded onto the equilibrated Ni–Sepharose column and the flow through was collected. The column was then washed with six CVs of 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 5 mM imidazole. Protein was eluted with six CVs of 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 100 mM imidazole then six CVs of 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 500 mM imidazole. Fractions containing the protein of interest were then dialyzed against 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 5% glycerol for 24 h with one complete buffer exchanged and constant stirring. Poros 50 HQ, strong anion exchange 10 mL resin , (Applied Biosystems, Cat# 1-2559-11) was used in a 0.7 cm dia × 1.5 cm high column equilibrated with 10 column volumes (CV) 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 5% glycerol (10 CVs) for the second purification. The dialyzed samples, 40–60 ml, were loaded onto the column. The column was washed with 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 5% glycerol (6 CVs), eluted with six CVs of 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 5% glycerol, with 150, then 200 mM NaCl. For each concentration of the NaCl, 6 fractions of 1 CV each were collected. The purified protein concentrations were determined by BCA protein assay kit using a BSA standard.