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Expression and purification of soluble murine CD40L monomers and polymers in yeast *Pichia pastoris*

Christina E. Hermanrud, Carrie L. Lucas, Megan Sykes, Christene A. Huang^{*}, and Zhirui Wang^{*}

Transplantation Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USAMGH-DF/HCC Recombinant Protein Expression and Purification Core, Boston, Massachusetts, USA

Abstract

The anti-murine CD40L monoclonal antibody MR1 has been widely used in immunology research to block the CD40-CD40L interaction for induction of transplantation tolerance and to abrogate autoimmune diseases. The availability of recombinant CD40L with high binding capacity for MR1 would provide a valuable immunological research tool. In this study, we constructed the single chain murine soluble CD40L monomer, dimer, trimer and successfully expressed them in yeast *Pichia pastoris* under the control of the alcohol oxidase promoter. The secreted single chain murine soluble CD40L monomers, dimers, and trimers were initially enriched through histidine tag capture by Ni-Sepharose 6 fast flow resin and further purified on a cation exchange resin. Purity reached more than 95% for the monomer and dimer forms and more than 90% for the trimer. Protein yield following purification was 16 mg/L for the monomer and dimer, and 8 mg/L for the trimer. ELISA analysis demonstrated that the CD40L dimers and trimers correctly folded in conformations exposing the MR1 antigenic determinant.

Keywords

Murine CD40L; *Pichia pastoris*; yeast expression; purification

Introduction

CD40 ligand (CD40L), or CD154, is a type II transmembrane glycoprotein that is primarily expressed on activated T cells and is a member of the tumor necrosis factor (TNF) family of molecules [1]. It binds to CD40, which is expressed mainly on antigen-presenting cells (APC). This interaction leads to many effects, depending on the target cell type. In general, CD40L plays the role of a co-stimulatory molecule and induces activation in APC [2].

Monoclonal antibody MR1 has been widely used in immunology research to block the CD40-CD40L interaction to induce transplantation tolerance and to abrogate the

^{*}Corresponding Authors: Zhirui Wang, Ph.D., Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School, MGH-East, Building 149-6117C, 13th Street, Boston, MA 02129, USA, Phone: +1-617-643-1957, Fax: +1-617-726-4067, zhirui.wang@tbr.c.mgh.harvard.edu. Christene A Huang, Ph.D., Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School, MGH-East, Building 149-9019, 13th Street, Boston, MA 02129, USA, Phone: +1-617-726-6505, Fax: +1-617-726-4067, christene.huang@tbr.c.mgh.harvard.edu.

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autoimmune disease [3]. Recombinant CD40L also has great potential for development as a protein drug to treat tumor and pathogen infection [4,5,6]. Our interest in developing the soluble murine CD40L arose from the need for murine CD40L-MR1 (anti-murine CD40L monoclonal antibody) immune complexes for use in our allogeneic bone marrow transplantation tolerance study [7,8,9]. We constructed, expressed and purified the soluble single chain murine CD40L monomer, dimer, and trimer. The binding ability for the monomer, dimer and trimer to MR1 were analyzed by ELISA.

Materials and Methods

Plasmid construction

Murine CD40L DNA was synthesized using the *Pichia pastoris* preferred codons [10]. Ten primers (Table 1) were designed to cover the full length of the murine CD40L soluble form (149 aa). There was about a 21 base overlap between any of the neighboring primers. Ten pmol of the first and the last primer, and two pmol for the rest of the primers were used. The PCR program was conducted at 95° C for 5 min, 25 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 1 min and an extension at 72° C for 10 min. The PCR products were analyzed with 1% agarose gel electrophoresis, and the band with the correct size was cut out and extracted with QIAquick Gel Extraction Kit. The synthesized and recovered DNA was digested using *XhoI* and *EcoRI* and cloned into pwPICZalpha [11] and designated as CD40L monomer construct after sequencing confirmation. To facilitate the downstream purification, six histidines (6xHis tag) were added at the C-terminus by PCR amplification using primers 40L1 and 40L10His. The PCR product was digested with *XhoI* + *EcoRI* and cloned back into pwPICZalpha and sequenced to generate CD40L monomer as final construct carrying 6xHis tag in its C-terminus.

In order to generate the soluble single chain murine CD40L dimer and trimer expression constructs (Fig. 1) the first CD40L was PCR amplified using sense primer CD40L1 carrying *XhoI* site, and antisense primer CD40L10B carrying *BamHI* and *EcoRI* sites. The PCR product was digested using *XhoI* + *EcoRI* and cloned into pwPICZalpha for sequencing confirmation. Then it was further digested using *BamHI* + *EcoRI*, resulting in a vector construct which contained the first CD40L. The second CD40L was amplified using sense primer CD40L1B carrying *XhoI* and *BglII* sites, and antisense primer CD40L10B carrying *BamHI* and *EcoRI* sites. The PCR product was digested using *XhoI* + *EcoRI* and cloned into pwPICZalpha for sequencing confirmation. *BglII* + *EcoRI* were used to cut out the second CD40L insert (*BglII*-CD40L-*EcoRI*) which were ligated into the *BamHI*/*EcoRI* digested vector pwPICZalpha-CD40L resulting in the construct containing two CD40L (*XhoI*-CD40L-*BamHI*/*BglII*-CD40L-*BamHI*-*EcoRI*). The third CD40L insert was prepared by PCR amplification using sense primer 40L1C carrying *XhoI* and *BamHI* sites, and antisense primer 40L10His carrying stop codon and *EcoRI* site. The PCR product was digested using *XhoI* + *EcoRI* and cloned into pwPICZalpha for sequencing confirmation. *BamHI* + *EcoRI* were used to cut out the third CD40L insert which were cloned into the pwPICZalpha-CD40L dimer vector to generate the CD40L trimer construct (*XhoI*-CD40L-*BamHI*/*BglII*-CD40L-*BamHI*/*BamHI*-CD40L-stop codon-*EcoRI*). The third CD40L insert was also cloned into pwPICZalpha-CD40L plasmid to generate the single chain CD40L dimer construct (*XhoI*-CD40L-*BamHI*/*BamHI*-CD40L-stop codon-*EcoRI*). The CD40L was tandem linked together by a (G₄S)₃ linker (Gly-Gly-Gly-Ser) in the dimer and trimer format.

Protein expression in *Pichia pastoris*

5–10 µg of the above constructed plasmid DNA was linearized by *SacI* digestion for 3h at 37° C, treated with Qiagen PCR purification kit, and transformed into *Pichia pastoris* strain X33 using the Gene Pulser MXcell Electroporation system (Bio-Rad). Cells were spread on

YPD agar plates (1% Bacto™ yeast extract, 2% Bacto™ peptone, 1.5% Bacto™ agar, 2% dextrose) containing 100 µg/ml of Zeocin and incubated at 30° C for 3–4 days. Six colonies were randomly picked and cultivated in small tubes containing 5 mL YPD (1% Bacto™ yeast extract, 2% Bacto™ peptone, 2% dextrose) at 30° C at 250 rpm for 24h as growth phase I, then in YPG (1% Bacto™ yeast extract, 2% Bacto™ peptone, 1% glycerol) at 30° C at 250 rpm for another 24h as growth phase II. The cultures were induced in 2 mL BMMYC (1% Bacto™ yeast extract, 2% Bacto™ peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids (MP), 4×10^{-5} % biotin, 0.5% methanol and 1% Bacto™ casamino acids) for 48h at 25° C at 225 rpm. 0.5% methanol was added in the beginning and end of the day to sustain the methanol level. The culture supernatants were analyzed using 12% NuPAGE SDS gel under non-reducing conditions.

One clone was selected and cultivated in shake-flasks (scaled up from the above described small tube expression) for downstream purification. 100 units/ml of penicillin and 100 µg/ml of streptomycin were added to suppress bacterial contamination. The supernatant was clarified by centrifugation at 3000 rpm at 4°C for 10 minutes prior to protein purification.

Protein Purification

Ni-Sepharose™ 6 Fast Flow was packed in a 5 cm x 20 cm XK50 column (GE healthcare Cat#18-1000-71) for the first step purification. The column was equilibrated with 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole (10 CV). The sample was prepared by adding 0.5 M NaCl, 20 mM sodium phosphate pH 7.4, and 5 mM imidazole and filtered through crepe fluted filter paper (VWR) and loaded onto the equilibrated column. The column was washed using 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole (5 CV). The bound proteins were eluted with 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 500 mM imidazole into eight fractions. The purification fractions were analyzed using 12% NuPAGE® Bis-Tris gel and stained with GelCode Blue stain reagent (Thermo Scientific). The fractions containing the protein of interest were pooled and dialyzed using a 3.5 kDa cut off Spectra/Por® membrane tubing (Spectrumlabs) against 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5% glycerol at 4° C. The dialysis buffer was replaced once.

Strong cation exchange resin Poros® 50 HS (Applied Biosystems) in a XK16/20 column (GE Healthcare) was used for the second step purification. The column was equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (10 CV). The above dialyzed sample was loaded onto the column followed by washing with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (8 CV). The bound protein was eluted with 50 and 100 mM sodium borate in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol into eight fractions, respectively. The purification fractions were analyzed using 12% NuPAGE SDS gel. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific).

Western blotting

Protein samples were separated by electrophoresis using NuPAGE 12% Bis-Tris Gel and the gel was electro-transferred at 35 V onto a nitrocellulose membrane filter paper using 1x NuPAGE® transfer buffer (Invitrogen). The membrane was thereafter blocked in 2% blotting grade blocker non-fat dry milk (Bio-Rad) in 1xPBS, 0.02% Tween 20 for 1 h with shaking and washed once with 1xPBS, pH7.4, 0.2% Tween 20 at room temperature with shaking. The murine CD40L monomer, dimer and trimer were detected with mouse anti-His monoclonal antibody (1:500) (Invitrogen) and rat anti-mouse IgG-HRP (1:1000) (Invitrogen) in 2% non-fat dry milk in 1xPBS, 0.02% Tween 20. Detection of the proteins was done by TMB membrane peroxidase substrate (KPL Cat#:50-77-02), and color development was stopped with dH₂O.

ELISA analysis for binding to anti-murine CD40L monoclonal antibody MR1

CD40L variants and BSA were coated to wells of a 96-well polystyrene microplate (Costar 3590) at a concentration of 34 nM in 100 μ L of 0.2M sodium carbonate-bicarbonate coating buffer at pH 9.4 (Pierce) by incubating at 4 degrees C overnight. As a positive control, anti-hamster IgG was coated to wells at a concentration of 17 nM (i.e., half the concentration of CD40L since the antibody is bivalent). Wells were then blocked with 300 μ L of SuperBlock Protein in 2.5 mM Tris, 0.15 M NaCl, pH 7.4 (Pierce) for at least 2 hrs at room temperature. Wells were then washed 3–6 times with a 0.1% Tween 20 solution at pH 7.4. Biotinylated hamster anti-mouse CD40L monoclonal antibody clone MR1 (National Cell Culture Center, Minneapolis, MN) was added in duplicate to appropriate wells at a concentration of 1, 0.5, 0.25, 0.125, 0.063, 0.031, or 0.016 μ g/mL in 100 μ L blocking solution with 0.1% Tween 20 and incubated at room temperature for 1–2 hours. After washing, 100 μ L of streptavidin-HRP at a concentration of 0.2 μ g/mL were added to the wells. The plate was incubated for 30 minutes at room temperature before washing and adding 100 μ L of TMB (tetramethylbenzidine/chromagen) substrate. The plates were covered and incubated in the dark at room temperature for 10–30 minutes before stopping the reaction with 100 μ L of 1 N HCl. Binding was determined by measuring absorbance at 450 nm within 30 minutes of stopping the reaction.

Results and Discussion

Plasmid constructs for the soluble murine CD40L monomers, dimers and trimers

As shown in Fig. 1, for purification purposes, a histidine tag was added to the sequence C-terminus in all of the monomer, dimer and trimer constructs. DNA sequence for the CD40L soluble form containing a codon-optimized stretch (112–260 aa) and a His-tag in the C-terminus was synthesized and cloned into the yeast strain *Pichia pastoris* expression vector pwPICZalpha (Fig. 1). Since the membrane-bound CD40L is trimeric [12,13], we hypothesized that the dimer and trimer would have higher affinity than the monomer to the anti-CD40L monoclonal antibody MR1. Therefore, we also developed the constructs for expression of the soluble CD40L dimers and trimers (Fig. 1). In the dimer and trimer constructs the neighbor soluble CD40L was tandem linked together to form a single chain fusion protein construct by (G₄S)₃ linkers. Since both Gly and Ser are neutral amino acids, we believe that their influence on the protein biological function would be minimal. This linker has been successfully used in two scFv (single chain fragment variable) based immunotoxins: A-dm-DT390 bis-scFv (UCHT1) and A-dm-DT390 scFvDb (C207) (11, 14, 15). All of the DNA constructs were confirmed by sequencing.

Expression and purification of the soluble recombinant murine CD40L monomers, dimers and trimers

The monomers, dimers and trimers of CD40L carrying a His-tag were expressed using a shake-flask system as described in Materials and Methods. The secreted monomers, dimers and trimers were captured directly by Ni-Sepharose 6 fast flow resin through their His-tags. The eluted fractions were pooled, concentrated on Centricon Plus-70 (5 kDa cut off) and dialyzed against 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% Glycerol to remove the salt excess. Based on the anticipated PI values of the conformers (9.69 for monomers, 9.72 for dimers, 9.73 for trimers), we opted for a strong cation exchange resin Poros 50 HS to carry out the second purification step. To this end, sodium borate was applied to separate the CD40L glycosylated yeast host protein and the aggregates [16]. As shown in 100 mM sodium borate elution fractions in Panels B of Figs. 2, 3 and 4, we obtained the pure CD40L monomer (17.2 kDa), dimer (34.5 kDa) and trimer (52 kDa) after the Poros 50 HS purification. The two bands detected in the 100 mM sodium borate elution fractions (Panels B of Figs. 2, 3, 4) are the N-glycosylated and non-glycosylated forms (data not shown). The

monomers, dimers and trimers were confirmed by Western Blotting analysis with an anti-His tag monoclonal antibody (Fig. 5). The recovery yield for the CD40L monomers and dimers was 16 mg/L with a purity of more than 95%. The yield for the trimers was 8 mg/L with a purity of more than 90%. Protein recovery yield was determined with a bicinchoninic acid (BCA) protein assay kit using BSA as standard (Thermo Scientific). Purity was determined by visible bands present on SDS gel (see 100 mM sodium borate elution fractions in Panels B of Figs. 2, 3, 4).

Structural integrity of the recombinant CD40L conformers

To assess whether the glycosylated CD40L products folded properly in solution, we examined the ability of CD40L monomers, dimers and trimers to expose a conformational determinant recognized by the anti-CD40L mAb (MR1). As shown in Fig. 6, soluble CD40L monomers bound poorly to MR1 even at high concentrations of antibody. In contrast, soluble single chain CD40L dimers and trimers showed a dose-dependent binding curve to MR1, indicating that this antigenic determinant was reconstituted on the CD40L polymeric forms. According to the CD40-CD40L interaction model proposed by Singh et al 1998 [17], the CD40L binding region to CD40 is folded by the CD40L dimer among the membrane-bound CD40L trimer. Since MR1 can block the CD40-CD40L interaction quite well, we speculate that the conformational binding region folded by the membrane-CD40L dimer for MR1 and for CD40 may overlap. It is possible that the CD40L monomer could not fold properly to form the required MR1 binding confirmation region, whereas the CD40L dimer and trimer both could. Based on this finding CD40L dimers or trimers would be required for CD40L-MR1 complex development.

Because the MR1 binding to soluble single chain CD40L dimers and trimers is very similar (Fig. 6), and the recovery yield and purity of CD40L dimers are likewise better than those of the trimers (see 100 mM sodium borate elution fractions in Panels B of Figs. 3 & 4), purified CD40L dimers were selected as final candidate reagents for future studies. We believe that the dimer form will be a very useful reagent in related immunology research fields. It has potential for development as a drug for tumor therapy and treatment of pathogen infection [4,5,6], as well as for a possible vaccine adjuvant [18,19]. It could also be used to study CD40-CD40L blockade for induction of transplantation tolerance and treatment of autoimmune diseases [3].

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Fig.1.
Schematic description of the soluble single chain murine CD40L monomer, dimer and trimer.

Fig. 2.
Purification for the soluble murine CD40L monomer: A) First step purification using Ni-Sepharose 6 fast flow resin. B) Second step purification using Poros 50HS.

Fig. 3.
Purification for the soluble single chain murine CD40L dimer: A) First step purification using Ni-Sepharose 6 fast flow resin. B) Second step purification using Poros 50HS.

Fig. 4. Purification for the soluble single chain murine CD40L trimer: A) First step purification using Ni-Sepharose 6 fast flow resin. B) Second step purification using Poros 50 HS.

Fig. 5. Western blot analysis of the soluble murine CD40L monomer, dimer and trimer with anti-his tag antibody.

Fig. 6. ELISA analysis of the binding between the soluble single chain murine CD40L monomer, dimer, trimer and anti-murine CD40L monoclonal antibody MR1.

Table 1

PCR primers to synthesize the codon-optimized soluble single chain CD40L monomer, dimer and trimer as well as the his tag in their C-terminus

40L1 (66 bases)
5' CCG CTC GAG AAG AGA GAG GCT GAA GCT ATG CAA AGA GGT GAC GAG GAC CCA CAA ATT GCT GCT CAC 3'
40L2 (66 bases)
5' CCA TTG CAA AAC AGA AGC AGC GTT AGA GTT AGC CTC AGA GAC AAC GTG AGC AGC AAT TTG TGG GTC 3'
40L3 (66 bases)
5' GCT GCT TCT GTT TTG CAA TGG GCT AAG AAG GGT TAC TAC ACT ATG AAG TCT AAC TTG GTT ATG TTG 3'
40L4 (66 bases)
5' GTA GTA CAA ACC CTC TCT CTT AAC AGT CAA TTG CTT ACC GTT CTC CAA CAT AAC CAA GTT AGA CTT 3'
40L5 (69 bases)
5' AAG AGA GAG GGT TTG TAC TAC GTT TAC ACT CAA GTT ACT TTC TGT TCT AAC AGA GAG CCA TCT TCC CAA 3'
40L6 (69 bases)
5' CTC AGA ACC GGA AGA TGG CTT CAA CCA CAA ACC AAC AAT GAA TGG TCT TTG GGA AGA TGG CTC TCT GTT 3'
40L7 (69 bases)
5' AAG CCA TCT TCC GGT TCT GAG AGA ATT TTG TTG AAG GCT GCT AAC ACT CAC TCT TCC TCT CAA TTG TGT 3'
40L8 (66 bases)
5' AGC TTG CAA CTC GAA AAC ACC ACC CAA GTG AAC AGA TTG TTG CTC ACA CAA TTG AGA GGA AGA GTG 3'
40L9 (69 bases)
5' GGT GTT TTC GAG TTG CAA GCT GGT GCT TCT GTT TTC GTT AAC GTT ACT GAG GCT TCT CAA GTT ATT CAC 3'
40L10 (69 bases)
5' CCG GAA TTC TTA CAA CTT CAA CAA ACC GAA GGA AGA GAA ACC AAC TCT GTG AAT AAC TTG AGA AGC CTC 3'
40L10His (54 bases)
5' CCG GAA TTC TTA GTG GTG GTG GTG GTG GTG CAA CTT CAA CAA ACC GAA GGA AGA 3'
40L1B (51 bases)
5' CCG CTC GAG AGA TCT GGT GGT GGT GGT TCT ATG CAA AGA GGT GAC GAG GAC 3'
40L1C (51 bases)
5' CCG CTC GAG GGA TCC GGT GGT GGT GGT TCT ATG CAA AGA GGT GAC GAG GAC 3'
40L10B (69 bases)
5' CCG GAA TTC CGC CGC GGA TCC ACC ACC ACC AGA ACC ACC ACC ACC CAA CTT CAA CAA ACC GAA GGA AGA 3'