Published in final edited form as:

Protein Expr Purif. 2015 January ; 105: 8–13. doi:10.1016/j.pep.2014.09.018.

Development of an Improved Mammalian Overexpression Method for Human CD62L

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Abstract

We have previously developed a glutamine synthetase (GS)-based mammalian recombinant protein expression system that is capable of producing 5 to 30 mg/L recombinant proteins. The over expression is based on multiple rounds of target gene amplification driven by methionine sulfoximine (MSX), an inhibitor of glutamine synthetase. However, like other stable mammalian over expression systems, a major shortcoming of the GS-based expression system is its lengthy turn-around time, typically taking 4–6 months to produce. To shorten the construction time, we replaced the multi-round target gene amplifications with single-round in situ amplifications, thereby shortening the cell line construction to 2 months. The single-round in situ amplification method resulted in highest recombinant CD62L expressing CHO cell lines producing ~5mg/L soluble CD62L, similar to those derived from the multi-round amplification and selection method. In addition, we developed a MSX resistance assay as an alternative to utilizing ELISA for evaluating the expression level of stable recombinant CHO cell lines.

Introduction

CD62L/L-selectin mediates rolling adhesion of leukocytes on vascular endothelial surfaces through binding to sialyl Lewis^xcarrying glycoproteins, such as P-selectin glycoprotein ligand-1 or mucins [1–3]. CD62L/L-selectin is a member of the selection family, which also includes the E- and P-selectins. All family members recognize O-glycans, but with different specificities and cellular origins. CD62L is primarily expressed on leukocytes and lymphocytes, and is critical for their homing to lymphoid organs and sites of inflammation. E- and P-selectins are expressed on the majority of endothelial cells to facilitate lymphocyte rolling adhesion. While the structures of E- and P-selectin in complex with their carbohydrate ligands have provided insight to their carbohydrate specificities and function [4,5], the structure of CD62L in complex with its carbohydrate ligands has not been resolved partly due to the lack of available recombinant protein for crystallization. To facilitate the structural studies of CD62L and its carbohydrate recognition, we constructed a recombinant

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CHO cell-based CD62L overexpression system using a glutamine synthetase (GS)-based plasmid expression vector for selection in glutamine deficient media [6,7]. Amplification of recombinant plasmid is achieved by increasing the concentration of a GS inhibitor, methionine sulfoximine (MSX), thereby requiring higher expression of the enzyme for survival. Similar to DHFR based mammalian recombinant protein overexpression systems [8,9], the glutamine synthetase (GS)-based system has been used successfully in large scale production of recombinant proteins for structural studies [10–13]. Despite their proven overexpression capability, both DHFR- and GS-based recombinant expression systems rely on multi-rounds of amplification of recombinant genes and thus takes 4–6 months to establish. This time-consuming procedure is a major short coming of stable mammalian recombinant protein expression systems compared to transient eukaryotic expression systems, such as baculovirus-based insect cell or HEK293T cell expression systems, which take about 3–6 weeks to establish. We present an improved glutamine synthetase-based overexpression method to generate stable recombinant CHO cell lines. The improved method replaces the multi-round amplification and selection procedure with a single round selection with increasing concentrations of methionine sulfoximine (MSX). The new method is less labor-intensive and shortens the turn-around time to about 2 months with the best expression clones comparable to those derived from repetitive amplifications. In principle, the improved streamlined procedure is also amenable for high throughput applications.

Materials and Methods

Reagents

DMEM/F12 medium, CHO-S-SFM II serum-free medium, fetal bovine serum (FBS), Lipofectamine 2000 and OPTI-MEM I transfection medium were purchased from Invitrogen, Inc. (Carlsbad, CA). Glutamine-free GMEM-S medium, GS Supplement, and methionine sulfoximine (MSX) were from Sigma-Aldrich (St. Louis, MO). Anti-CD62L antibodies and CD62L sandwich ELISA kit were from R & D systems (St. Louis, MO).

Cell lines and Plasmid constructs

The Chinese hamster ovary cell line CHO-lec3.2.8.1 was kindly provided by Dr. Pamela Stanley [14], and maintained in DMEM/F12 medium containing 5% FBS. CD62L transfected CHO cells were cultured in glutamine-free GMEM-S medium with GS supplement, 5% FBS and various amounts of MSX. Extracellular regions of human CD62L, residue 1–194, corresponding to the signal peptide, C-type lectin domain and the EGF domain, were cloned into a GS-based expression vector pcDNA-GS between BamH I and XhoI restriction sites [6,7]. A six-histidine tag was inserted at the C-terminal end of the EGF domain. The glycan deficient mutant CD62L was generated by mutating Asn 104 to Glu (N104E) using Quickchange mutagenesis (Qiagene).

Transfection of CHO-lec3.2.8.1 cells

CHO-lec3.2.8.1 cells were grown to near confluence in a T-25 flask prior to transfection. After medium exchange, CHO cells were transfected with 40–80 µg plasmid DNA with 20 µl Lipofectamine in 1 ml OPTI-MEM medium. After 2 days at 37°C, the transfected CHO cells were trypsinized, resuspended in 80 ml GMEM selection media containing 30µM

MSX. The transfected CHO cells were either plated into ten 72-well HLA Terasaki batch plates or distributed into four 384 well cell culture plates with 50 μ l medium in each well (Fig. 1). For the Terasaki batch plates-based method, stable CD62L expressing clones were selected, screened and amplified as previously described [6,7]. Briefly, stable CD62L expressing clones began to appear after incubating the transfected CHO cells at 37°C for 3–4 weeks. The clones were individually harvested from the wells of Terasaki dishes, transferred to 96 well plates and cultured with GMEM medium containing GS supplement, 5% FBS, and 30 μ M MSX. Upon confluence, the supernatants of individual clones in the 96 well plates were diluted 100-fold and screened for CD62L expression by ELISA using a concentration standard curve from the manufacturer. The highest CD62L expression CHO clones were selected to repeat the second and third round of amplifications in the presence of GMEM selection medium containing 150 and 500 μ M MSX.

For the 384 well-based method, the selection of MSX resistant clones and gene amplification were carried out in the 384-well plates by two subsequent increases in MSX concentrations from 30 μ M to 150 and 500 μ M in two weeks period of time. The 384-well plates were cultured for 2–3 weeks after reaching the final concentration of 500 μ M MSX and wells containing confluent colonies were trypsinized and transferred to 96-well plates for CD62L expression screening by ELISA.

Purification of recombinant CD62L

Harvested recombinant CD62L-containing CHO-SFM media was concentrated 10-fold, dialyzed against water for 12 hours and then loaded onto a Ni-NTA column, equilibrated with 10mM HEPES pH 7.2, 100mM NaCl, and 5mM CaCl₂. The bound protein was eluted with an initial step gradient to 40mM imidazole, and followed by a linear gradient to 300mM imidazole in the same buffer. Fractions containing recombinant CD62L were combined and concentrated to 5–10mg/mL using a Centricon, and treated with Endo H glycosidase in 50mM sodium acetate pH 5.5 using 500 units Endo H for 1–2mg of protein at 4°C for 2–3 days. The Endo H-treated CD62L was further purified through a Superdex 200 10/300 size exclusion column (GE Healthcare) in the same HEPES buffer. Fractions containing the soluble CD62L protein were concentrated to about 10mg/mL for further experiments.

BIAcore Binding experiments

Surface plasmon resonance measurements were performed using a BIAcore 3000 instrument (GE Healthcare). Recombinant CD62L N104E mutant expressed by the GS-system or purchased from R&D systems, Inc., or soluble DC-SIGN extracellular domain were immobilized onto carboxymethylated dextran (CM5) surface- based sensor chips by *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(NHS/EDC) crosslinking in sodium acetate buffers at pH 5.0 for CD62L or pH 4.5 for DC-SIGN. Immobilization levels were 3300, 1500, and 1000 RU for the purified N104E mutant, CD62L from R&D, and DC-SIGN, respectively. Binding assays were performed with 20nM DREG-56 or 2 μ M fucoidan in a buffer containing 10mM HEPES pH 7.2, 150mM NaCl, 0.005% P20, and 2mM CaCl₂.

Results

Generate stable recombinant CD62L expressing CHO cells through three-rounds of MSX selection and gene amplification

There are primarily two methods to establish stable CHO cell transfectants. One is to plate the transfected cells in 10-cm cell culture dishes and isolate colonies using cloning cylinders. The other is to plate the transfected cells in 96-well plates and use limiting dilutions to isolate single clones from confluent wells. Each has its own advantage. While using cloning cylinders to isolate clones saves time, the use of 96-well plates with limiting dilutions is less labor intensive and easy to perform. We used a 72-well HLA Terasaki batch plate method (Figure 1), a combination of batch cell culture with individual wells for single clone recovery. The initial transfection and selection in 30 μ M MSX produced 96 stable CHO cell clones. Of them, 70% of the clones expressed less than 50 μ g/l CD62L, 25% expressed between 50–100 μ g/l, 5% expressed above 100 μ g/l (Figure 2a). The two highest CD62L expressing clones yielded ~150–200 μ g/l CD62L. The top expression clones were expanded to between 10^6 – 10^7 cells and subjected to second and third round of selections under 150 and 500 μ M MSX concentrations, respectively. This resulted in a 6-fold expression amplification for the highest expression clone from the second round and additional 4-fold increase in CD62L expression from the third round of MSX selection (Figure 2b). After the final selection with 500 μ M MSX, the highest two clones, 28H9 and 28H13, produced approximately 5000 μ g/l of the recombinant protein (Figure 2c). The total increase in expression from the repeated MSX-mediated gene amplification is about 25-fold.

Generate stable CD62L expressing CHO cells through single round in situ amplifications

While the use of multi-round gene amplification to achieve overexpression of recombinant proteins is a well-established method, it is laborious and time consuming. Stable cell lines are obtained in each round of MSX selection, even though only the final stable cell lines are valuable for production. Thus, much of the effort spent in the multi-round amplification method is to establish stable intermediate cell lines that are different from the final high expression cells. In an effort to find a simple and faster procedure, we tested the use of 384 multiwell cell culture plates for selection of stable MSX resistant recombinant CD62L expression clones. The surface area of a 10-cm dish (56.7 cm^2) and that of a cloning ring (0.5 cm^2) determines the maximum number of resolvable clones to be ~100 in a single 10-cm dish, or 1000 from a single transfection plated in ten 10-cm dishes. To match the maximum number of 1000 resolvable clones, the transfected CHO cells were distributed in four 384-well plates for a total of 1536 wells. The use of large number of wells favors the recovery of single clones in each well. To avoid establishing intermediate stable expressing cell lines, the amplifications were carried out in the transfected four 384 well plates through sequential increases in MSX concentrations.

After three steps increase in MSX concentrations from 30 μ M to 150 and to 500 μ M, a total of 113 clones were recovered from the transfected 384-well plates. The recovery of 113 MSX resistant clones from 1536 transfection wells suggests they are likely single clones. The concentrations of recombinant CD62L present in the confluent culture supernatant of these clones were assayed by ELISA (Figure 3a). About 70% of the clones expressed less

than 500 μ g/l CD62L, 25% expressed between 500–3000 μ g/l CD62L. The two highest expressing clones, clone 41 and 100, expressed approximately 5000 μ g/l of the protein.

Generate stable recombinant CD62L N104E mutant expressing CHO cells

The single-round in situ amplification method was also used to generate stable CHO cells expressing a CD62L glycosylation mutant, N104E. A total of 80 clones were recovered from the transfected 384-well plates after 500 μ M MSX selection. While the majority of surviving clones expressed little CD62L, eight clones expressed CD62L at greater than 1000 μ g/l level and the highest two, clone 3I20 and 2G5 expressed ~5000 μ g/l, similar to the wild type CD62L (Figure 3b).

MSX resistance analysis of CD62L expression clones

ELISA offers a direct measure to the expression yields of stable CHO cell lines and is regarded as a gold standard for recombinant protein yield analysis. While a sandwich ELISA kit is commercially available for CD62L detection, the presence of suitable capture and detection antibodies, however, are not always available. Growth kinetics are not well-correlated with recombinant protein yield under selection conditions as high producers were often slow growers [13]. Here, we attempted to rank the top expression clones based on the survival of individual clones under increasing concentrations of MSX. This is based on the assumption that higher expression clones provide survival advantage under increasing MSX concentrations. Although all final CD62L expressing CHO cell lines were derived from 500 μ M MSX selection, their apparent CD62L expressions varied greatly, from less than 50 μ g/L to 5000 μ g/L. This suggests that the higher expressing clones may resist even greater concentrations of MSX. Eleven stable CD62L expressing CHO cell lines were seeded at approximately 500 cells/well into 48-well cell culture plates in GMEM selection medium with 100, 500, 1000 or 5000 μ M MSX, and cultured for 7 days before counting. The CD62L expression levels of the eleven clones were estimated by ELISA experiments to be high (clone s 28H9, 28H13, 41, 100), medium (51, 92, 102) and low (62,65, 79, 99). All clones exhibited decreased growth at higher concentrations of MSX, and none survived at 5000 μ M MSX (Figure 4a). Interestingly, the top five clones at 1000 μ M MSX were 28H9, 100, 41, 102 and 28H13, the good producers as determined by ELISA. The lowest group contained clones 99, 79, 65 and 92, that tend to be poor CD62L producers. If we define a MSX resistance index as the ratio between the number of live cells observed in 1000 versus 500 μ M MSX, this ratio reflects a clone's resistance to MSX concentration change rather than its growth kinetics (Figure 4b).

MSX resistance assays were also performed for the N104E mutant expressing clones. A majority of the clones survived 1 mM MSX culturing condition but failed to grow in media containing greater than 2mM MSX. Few clones, including 3I20, 1A6, 2G5 and 2G3, grew under the highest MSX concentration. Two of them, 3I20 and 2G5, are the highest CD62L expressing clones, suggesting the MSX resistance assay is an effective alternative to ELISA assays for ranking recombinant protein expression clones.

Purification of recombinant soluble CD62L

Highest recombinant CD62L expression clones were expanded into 6 triple-deck Nunc cell culture flasks (500 cm²) in GMEM selection media with 500 μ M MSX. Upon confluence, the media was replaced with CHO-S-SFM II serum-free media for the recombinant CD62L production. The cell culture supernatants were harvested every two days for three weeks. The harvested supernatants were combined, concentrated 10-fold, dialyzed against water for 12 hours before loading onto a Ni-NTA column. The recombinant CD62L was then eluted from the Ni-NTA column, digested with Endo H glycosidase to reduce glycosylation-associated heterogeneity, and further purified through a size exclusion column (Figure 5a). The fractions containing the recombinant CD62L were over 98% pure (Figure 5b). The purified recombinant CD62L and a commercial CD62L from R&D systems, Inc. bound similarly to a CD62L specific monoclonal antibody, Dreg 55, with 40 and 99 nM affinities, respectively (Figure 6a). Both bound to type III porcine submaxillary mucin (PSM-III) with 44 and 27 μ M affinities, respectively (Figure 6b), similar to previously reported [1]. Fucoidan [15], another known ligand of CD62L, bound to the recombinant CD62L of ours and that from R&D systems with similar affinities of 85 and 500 nM, respectively (Figure 6c). All these results show that the recombinant CD62L is fully functional.

Discussions

The multi-round drug-resistant driven method of recombinant gene amplification is the hallmark for the ability of both DHFR and GS-based recombinant protein expression systems to achieve high expression yield. The benefit of the second and third round amplifications is a 25-fold increase in CD62L expression, and is thus a very significant part of the overexpression strategy. However, the time-consuming multi-round selection and amplification procedure is a major disadvantage of generating stable mammalian recombinant cell lines compared to transient expression systems. Presumably, each round of MSX-mediated selection results in target gene amplification followed by their stable integration into CHO cell chromosomes. While significant amount of time spent is to establish stable intermediate cell lines at each MSX concentration, it is not clear, however, if chromosomal integrations at intermediate MSX concentrations are necessary for optimal gene amplification. In theory, the only stable CHO cells needed are at the final step of MSX selection and it may not be necessary to derive stable-expressing CHO cell lines through the intermediate amplification steps. In attempts to shorten the time required to establish a GS-based recombinant protein expression system, we replaced the three-round MSX selection and amplification method with a sequential increase in MSX concentrations during a single-round selection of clones, a method with consecutive MSX-driven gene amplifications that eliminates the establishment of intermediate stable transfectants. The improved method shortened the time required for generating stable recombinant protein expressing CHO cells from 4–6 months to about 2 months, approaching the turn-around time for transient recombinant protein expression systems. The highest CD62L expressing clones from the single-round method were ~5mg/L which is comparable to those from the three-round sequential selection and amplification method. The purified recombinant CD62L bound a functional antibody and its ligand PSGL-1 with expected affinities. We recently applied this method to express a recombinant extracellular domain of human BST2/Tetherin and our

preliminary data suggested the best clones produced between 1–10 mg/L of recombinant protein.

Acknowledgement

This research is supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Figure 1. Schematic comparison of stable CHO cell recombinant protein expression methods based on (left) multi-round sequential or (right) single-round in situ MSX-driven gene amplifications.

a

b

c

Figure 2.
Distribution of stable CD62L expressing clones from each round of MSX selection.

a

b

Figure 3. Distribution of stable recombinant CHO cell clones for CD62L wt (a) and N104E mutant (b) generated using the single-round in situ gene amplification method.

a

b

Figure 4. MSX resistant assay. (a) MSX dose-dependent survival of individual recombinant CD62L expressing clones. (b) MSX resistance index of each clone.

Figure 5.
Purification of recombinant CD62L. (a) Size exclusion chromatography profile with the peak corresponding to CD62L labeled. (b) Coomassie blue stained SDS gel of purified recombinant wildtype CD62L.

Figure 6. BIAcore binding of (a) an anti-CD62L antibody (DREG-55), (b) PSM-III, and (c) Fucoidan to purified recombinant CD62L. A recombinant soluble DC-SIGN, also a C-type lectin receptor, is included as a negative control.