Generating Stable Chinese Hamster Ovary Cell Clones to Produce a Truncated SARS-CoV Spike Protein for Vaccine Development

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The spike (S) protein of the severe acute respiratory syndrome coronavirus (SARS-CoV) is important for vaccine development. S_{TR2} (an 88 kDa truncated SARS-CoV TW1 S protein carrying the S fragments S-74-253, S-294-739, and S-1129-1255) is capable of expressing a major form of glycoprotein as endo H-sensitive (~115 kDa) in CHO cells. To establish stable expressing cell clones, we transfected CHO/dhFr-cells with the amplifiable vectors ISID (IRES-driven dhfr) and ISIZ (SV40-driven dhfr) to select stepwise MTX, and observed enhanced ~115 kDa glycoform generation through gene amplification. Following stepwise MTX selection, we compared gene amplification levels between two vectors in engineered CHO cell chromosomes. These results confirm that the IRES-driven dhfr *promoter generates greater gene amplification, which in turn enhances S_{TR2} expression. Our results indicate that the ~115 kDa glycoform of S_{TR2} protein was capable of increasing after gene amplification. The S_{TR2} glycoform did not change between suspension and serum-free cultures, suggesting that the stable and amplified cell clones analyzed in this study have potential for producing homologous S_{TR2} on a large scale.* © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.,* 26: 1733–1740, 2010
Keywords: SARS-CoV, CHO cells expression, stable cell clone

Introduction

The SARS coronavirus (SARS-CoV) genome contains 5′-capped and 3′-polyadenylated positive-stranded RNA consisting of 29,740 bases that encode four structural proteins: (i) an envelope spike protein S [1,255 amino acid (aa)] that mediates receptor binding and virus fusion, (ii) a small E protein (76 aa) that assists with virus assembly, (iii) a nucleocapsid protein N (422 aa) that binds with and protects the virus RNA genome from damage, and (iv) a matrix protein M (221 aa) that interacts with S, N, and E and combines them to form a complete and infectious virus particle.[1–3]

The S protein of SARS-CoV is a major antigen that elicits neutralizing antibody response in hosts.[4,5] The S protein contains 1,255 aa, including (i) a signal peptide (SP) (1–12 aa) located at the N terminus, (ii) an extracellular domain (13–1,195 aa), (iii) a transmembrane domain (TM) (1,195–1,215 aa), and (iv) a cytoplasmic domain (CP).[6] The S protein can be cleaved into two subunits: a S1 subunit containing a receptor-binding domain (RBD) and a S2 subunit that mediates the fusion process via a fusion peptide (FP) with two helix regions (HR1 and HR2). Three domains (I, II, and III) have been mapped to elicit neutralizing antibodies where domain I (130–150 aa) is located in the N terminus of the S1 subunit; however, specifics of the neutralization mechanism remain unknown.[7] Domain II contains the 318–510 aa RBD, which can be blocked by neutralizing antibodies.[7,8] Domain III includes the 1,143–1,192 aa HR2, which can be neutralized by disturbing HR1-HR2 interaction.[9] The receptor-binding domain of the S protein is a primary target for subunit vaccine development.[8,10–12] According to one report, RBD protein expression in mammalian cells elicits a more potent neutralizing antibody response than RBD protein expression in *Escherichia coli* or insect cells.[10] In addition, glycosylation residues adjacent to the RBD protein have been shown to affect neutralizing antibody titers.[11] S protein RBD is considered a key function domain for receptor binding and contains neutralizing epitopes,[13] including one at the N terminus of the S protein and the other near HR2.[14] Residues 1,143–1,157 in HR2 have been identified as a 15-residue epitope that is recognized by neutralizing monoclonal antibodies.[9]

Intranasal or intramuscular inoculation with highly attenuated modified vaccinia virus Ankara (MVA) expressing full-length S proteins into BALB/c mice neutralizes antibody production and elicits protective immunity. Post-MVA-S

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Table 1. S Protein-Based Vaccines of SARS CoV

S Protein Fragments	Neutralizing Domains	Expression Host Cells	Note	Refs.
1–1,195 aa	I, II, III	Sf9	Ectodomain of S protein could induce highly neutralizing antibody reponse.	11
14–1,255 aa	I, II, III	Sf9	Neutralizing epitopes located at 12–327 aa, 318–510 aa (RBD), 528–635 aa	12
264–680 aa	II	*E. coli*	S protein (248–680 aa) fused with N protein (321–422 aa)	13
318–510 aa	II	293T	RBD fused with IgG1 Fc could induce highly neutralizing antibody response	6
318–510 aa	II	293T, Sf9, *E. coli*	rRBD in mammalian cells elicited stronger humoral immune responses	14
318–510 aa	II	293T	Deglycosylated rRBD could reduce the immune response	9
318–510 aa	II	CHO-K1	Stable cell clones (no *dhfr*-mediated gene amplification)	15
485–625 aa	II	*E. coli*	Neutralizing epitopes located at domain II (548–567 aa and 607–627 aa)	28
74–253 aa, 294–739 aa, 1,129–1,255 aa	I, II, III	CHO/dhFr−	Intron and exon splicing enhancers to increase S protein expression	20
74–253 aa, 294–739 aa, 1,129–1,255 aa	I, II, III	CHO/dhFr−	*dhfr*-mediated gene amplified stable cell clones	This work

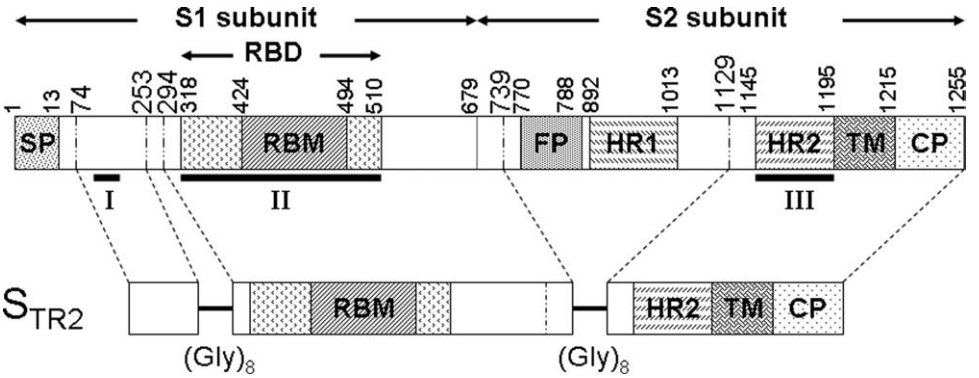

Figure 1. Schematic of the S protein.

The residue numbers of each region represent their positions in the S protein of SARS-CoV. CP, cytoplasm domain; FP, fusion peptide; HR, heptad repeat; RBD, receptor-binding domain; RBM, receptor-binding motif; SP, signal peptide; TM, transmembrane domain. The truncated form of the S protein (S_{TR2}) contains S fragments S74–253, S294–739, and S1129–1255, which are linked by 8-glycine linker. Three neutralizing domains were identified previously.

immunization, BALB/c mice show reduced SARS-CoV virus titer in their upper and lower respiratory tracts following challenges with the virus.[15] According to another report, a recombinant attenuated parainfluenza Type 3 vector expressing S instead of E, M, and N proteins induces neutralizing antibodies and provides protective immunity against SARS-CoV,[16] suggesting that S protein is the only protective antigen among all SARS-CoV structure proteins. In a third study, researchers intramuscularly injected a DNA vaccine containing codon-optimized truncated S protein (1–1,242) into BALB/c mice. They observed a strong T-cell response ($CD4^+$ and $CD8^+$ T cells against S protein), neutralized antibody production, and a greater than 10^6 reduction in virus replication in the lungs of vaccinated mice in response to SARS-CoV challenges.[17]

The SARS-CoV S protein is considered a primary target for subunit vaccine development.[6] Recent investigations using S proteins as vaccine candidates are listed in Table 1. A recombinant baculovirus-expressed S protein containing only the ecto-domain or a His-tagged full-length version has been found to elicit high titers of SARS-CoV neutralizing antibodies in mice.[4] S protein antigenicity is characterized by 38 monoclonal antibodies and three neutralizing epitopes (12–327 aa, 318–510 aa, and 528–635 aa).[5] A truncated S protein (248–680 aa) fused with an N protein fragment (321–422 aa) has been shown to provide post-challenge protection from virus replication in mice.[19] With the exception of the full-length S protein, the RBD region is the major target for developing protein subunit vaccines. Fusion proteins containing RBD and human IgG1 Fc can induce highly neutralizing antibody response.[8] When compared with recombinant RBD expressed in 293T cells, Sf9 cells, and *E. coli*, rRBD expressed in 293T cells is capable of eliciting stronger immune responses.[10] Furthermore, rRBD deglycosylated by PNGase F shows a lower neutralizing antibody response.[11] According to these findings, rRBD expressed in mammalian cells is (i) capable of correct folding and glycosylation and (ii) more antigenic. CHO cells have therefore been used to establish cell lines that stably express rRBD.[12]

We previously reported that a truncated form of the S protein (S_{TR2}) containing S fragments S74–253, S294–739, and S1129–1255 can be transiently expressed in CHO cells by using a 138 bp intron of pIRES (a bidirectional exon splicing enhancer) and a fibronectin EDA exon enhancer (Figure 1). The intron addition significantly enhanced S_{TR2} protein expression.[20] S_{TR2} protein contains RBD and two other neutralizing epitopes, suggesting that it is capable of inducing more neutralizing antibodies than the rRBD antigen alone.[21]

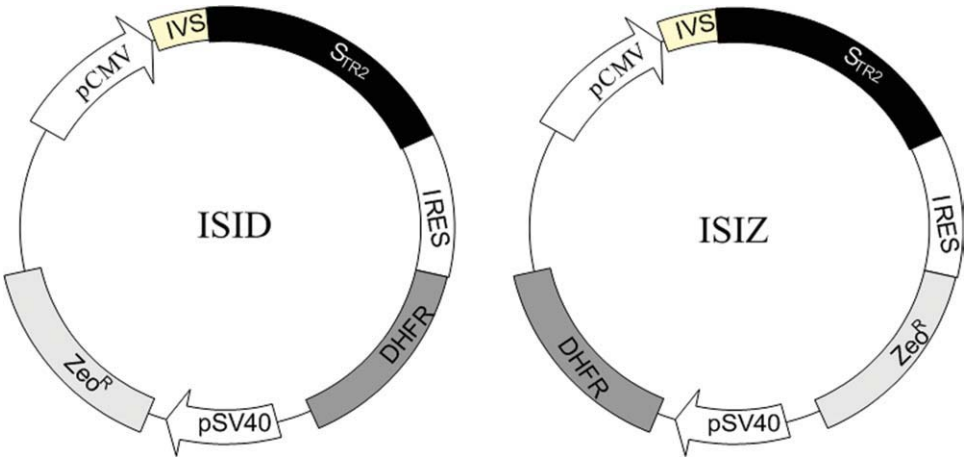

Figure 2. Construction of amplifiable vectors, ISID, and ISIZ.

The plasmids included zeocin-resistant gene (Zeo^r), *dhfr* gene, and S_{TR2} gene. The difference between the two vectors was that IRES driven the *dhfr* gene of pISID and SV40 driven the *dhfr* gene of pISIZ. IVS is an intron that enhances the expression of S_{TR2}.[20]

In this study, we obtained stable CHO cell clones using *dhfr*-directed gene amplification, because the weaker *dhfr* gene promoter generally results in more copies following MTX selection.[22] Accordingly, for gene amplification, we constructed two expression vectors, one containing the IRES-driven *dhfr* gene (ISID) and one containing the SV40-driven *dhfr* gene (ISIZ). We selected clones that expressed high-level S_{TR2} and analyzed differences in gene amplification and amplified gene stability between the two vectors, thereby generating stable CHO cell clones for subunit vaccine development against SARS-CoV.

Materials and Methods

Cell lines and media

A CHO/dhFr$^-$ (*dhfr* deficient) cell line named ATCC CRL-9096 was obtained from the Bioresource Collection and Research Center in Taiwan. CHO/dhFr$^-$ cells lacked DHFR protein and could not synthesize ribonucleosides (RNS) and deoxyribonucleosides (dRNS). Under nonselective conditions, CHO/dhFr$^-$ cells were maintained in minimum essential medium alpha medium (MEMα) with ribonucleosides (RNS) and deoxyribonucleosides (dRNS) (Invitrogen), supplemented with 10% fetal bovine serum. To amplify *dhfr*/S_{TR2}-expressing CHO cells, selection was performed in MEMα, supplemented with 10% dialyzed fetal bovine serum (DF) (Invitrogen) without RNS or dRNS. To observe *dhfr* amplified gene stability in host cell chromosomes, cells lines were maintained in EX-CELL® CHO DHFR$^-$ serum-free medium (Sigma) without serum. All growth media were supplemented with 100 units/mL penicillin/streptomycin (P/S) and the cells were maintained at 37°C with 5% CO_2.

Expression vector construction

pISID plasmid constructed to express S_{TR2}[20] was digested with XbaI and ApaI to remove the *dhfr* gene; a zeocin-resistant gene (Zeo^r) was inserted post-IRES. To remove the original Zeo^r, the plasmid was digested with AvrII and OJQBst1107I. A new *dhfr* gene was inserted following the SV40 promoter. The new construction was named pISIZ (Figure 2).

Western blotting

Protein samples were analyzed using 8% SDS-PAGE. 1×10^5 cells were added to 15 μL of loading buffer (only 5 μL if the mixed solution was already loaded) and run for 1–2 h at 150 V. Gel bands were electrically transferred onto nitrocellulose (NC) paper; blocking was performed in 5% skim milk. Rabbit antiserum containing polyclonal antibodies against the SARS-CoV spike protein was used to recognize S_{TR2} protein. Anti-mouse DHFR antibody (BD Biosciences) was used for DHFR western blotting.

Gene amplification of stable clones

We used lipofectamine 2000 reagent (Invitrogen, CA) to perform transfections to establish permanent clones. At 24 h post-transfection, cells from 24-well plates were subcultured in three duplicate wells in six-well plates. Medium in each well was replaced with MEMα without RNS and dRNS supplemented with 10% DF and 200 μg/mL Zeocin (Invitrogen). Following 2 weeks of selection, remaining cells were diluted and inoculated into 96-well plates for single clone selection. After 2 weeks of incubation at 37°C, single cell clones were selected by visual inspection under microscopy. Additional cell clone selection was performed by gradually increasing the concentration of MTX (Sigma), which was an inhibitor of DHFR protein, from 0.02–0.08 to 0.32–1.0 μM. Selection at each concentration occurred over a 2–3-week period.

Quantitative real-time PCR

Genomic DNA extraction from stable clones was performed using Qiagen DNeasy Tissue kits. As *dhfr* genes in mice (*Mus musculus*) and Chinese hamsters (*Cricetulus griseus*) are so similar, Taqman® probes were used in real-time PCR as a precaution against obtaining incorrect signals. Analyses were performed with an ABI PRISM 7500 sequence detection system; absolute DNA amounts were calculated using the ΔΔCt method. Primers and probes were obtained from Applied Biosystems (assay ID 293340). The standard curve for determining DNA amounts had a R^2 value ≥ 0.991.

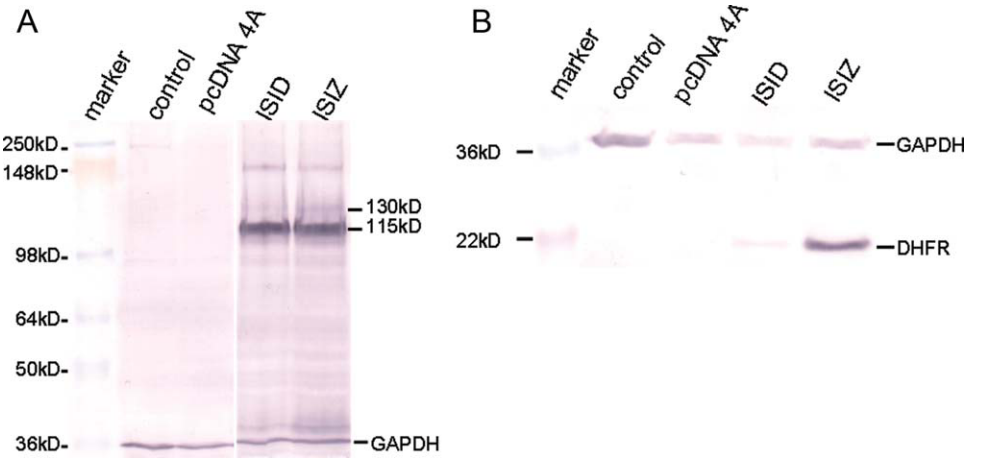

Figure 3. Western blot analysis of (A) S_{TR2} and (B) DHFR expression levels in CHO/dhFr$^-$ cells transfected with the control (no vector), pcDNA 4A (empty vector), ISID vector, and ISIZ vector.

GAPDH was as an internal control. The samples were obtained from cell lysates after 2-day transfection. The results were reproducible at least from three independent experiments.

Results

Expression vector construction and S_{TR2} transient expression in CHO cells

In an earlier study, we showed that S_{TR2} can be transiently expressed in CHO cells.[20] To obtain high-production and stable CHO cell clones, we performed *dhfr*-mediated gene amplification of S_{TR2}-expressing cells with incremental increases of MTX concentration. We used two vectors for co-amplified DHFR gene expression under two promoters: CMV (pCMV) and SV40 (pSV40) (Figure 2). The ISID vector contained both pCMV-driven S_{TR2} expression and IRES-driven DHFR expression. The ISIZ vector contained pCMV-driven S_{TR2} expression and pSV40-driven DHFR expression.

Plasmid DNA was transfected into CHO/dhFr$^-$ cells using Lipofectamine^{TM} 2000 (Invitrogen). Transfected cells were harvested at 48 h and centrifuged; cell pellets were collected and used for western blot analysis. We observed similar S_{TR2} expression levels in transfected CHO/dhFr$^-$ cells with either the ISID or ISIZ vector (Figure 3A). In contrast, DHFR expression in cells transfected with the ISIZ vector was approximately eightfold higher than in cells transfected with the ISID vector (Figure 3B). According to these results, the promoter strength of pSV40 (ISIZ vector) was significantly higher than that of IRES (ISID vector) for DHFR expression.

Stable cell clone selection through dhfr-mediated gene amplification

ISID-transfected and ISIZ-transfected cells were grown in selection medium (MEMα without RNS or dRNS + 10% dialyzed FBS + 200 μg/mL Zeocin) for 14 days to obtain stable CHO cell clones. Single cell cloning from a total of 48 stable clones was conducted and examined for the S_{TR2} expression by western blotting. Sixteen clones showing high-S_{TR2} expression from ISID-transfected cells and another sixteen clones from ISIZ-transfected cells were selected for *dhfr*-mediated gene amplification. These clones were passaged into six-well plates for MTX selection. MTX was a folate antagonist to inhibit DHFR protein. MTX concentration was gradually increased from 0.02–0.08 to 0.32–1.0 μM. To adapt the increasing concentration of MTX, the *dhfr* gene in the transfected cells would be amplified accompanying S_{TR2} gene amplification. The gene amplification process is shown in Figure 4. Stable clone selection at each MTX concentration required 2–3 weeks of effort, with recovery time generally ranging from 1.5 to 3 weeks, though longer in some cases. Cells required more time to recover at higher MTX concentrations; ISID cell clones needed more recovery time than ISIZ cell clones. Five ISIZ-transfected clones and three ISID-transfected clones survived following treatment with 1.0 μM MTX. Surviving cell clones at each MTX concentration were collected (in the same quantities as cells collected during MTX selection) and analyzed by western blotting for S_{TR2} expression. The results indicate that all ISID (Figures 5A and 6) and ISIZ (Figures 5B and 6) clones experienced increases in S_{TR2} expression during MTX selection. Although ISID clone survival rates were lower than for ISIZ clones (3/16 vs. 5/16), average S_{TR2} expression levels were higher in the ISID clones.

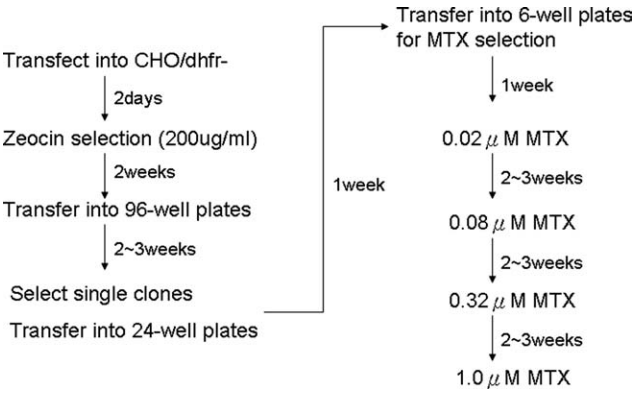

Figure 4. The flowchart of establishing amplified stable cell clones expressing recombinant proteins in CHO/dhfr$^-$ expression system.

S_{TR2} expression in stable cell clones after MTX removal

Cloned cells that recovered following treatment with 1.0 μM MTX were transferred to normal growth medium

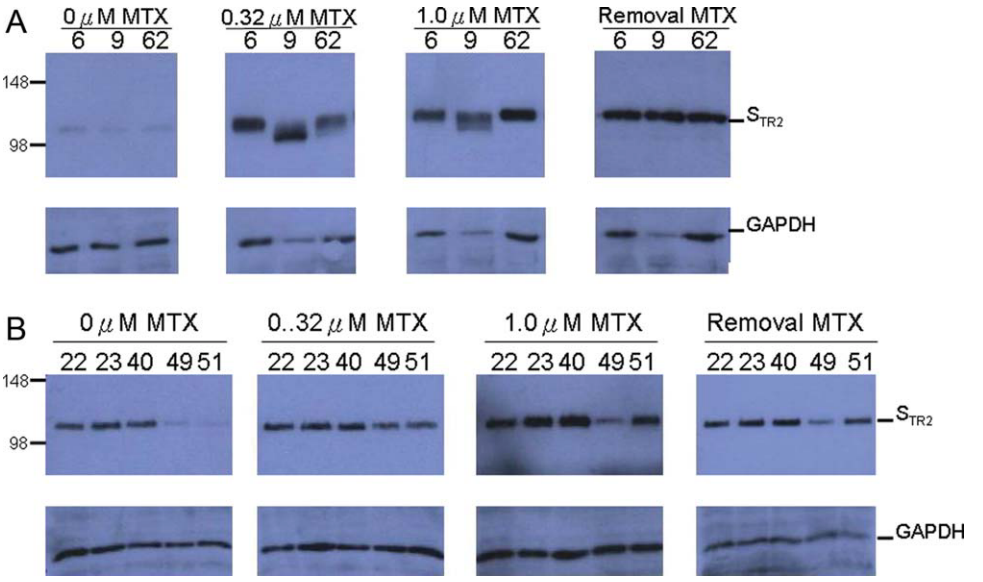

Figure 5. The S_{TR2} expression in the process of MTX selection.
(A) The cells transfected with ISID. (B) The cells transfected with ISIZ.

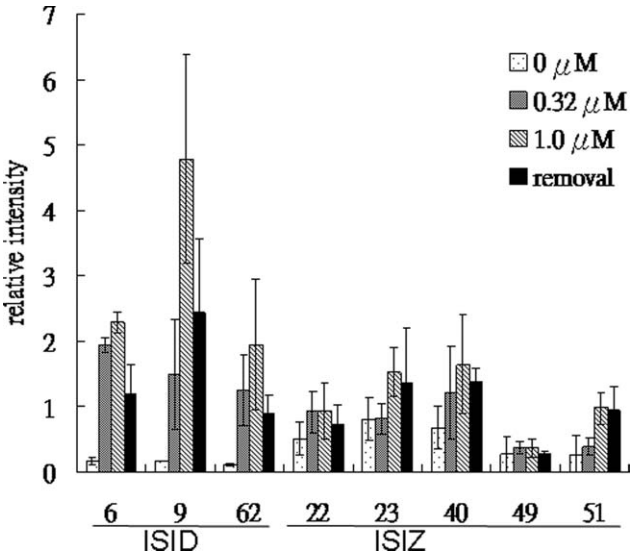

Figure 6. S_{TR2} expression of ISID and ISIZ clones comparison.
S_{TR2} expression of ISID clones (6, 9, and 62) was less than ISIZ clones (22, 23, 40, 49, and 51) before gene amplification. After MTX selection, ISID clones could express more S_{TR2} than ISIZ clones. With MTX stress removal, S_{TR2} expression of ISID clones decreased more than ISIZ clones. The relative intensity was analyzed by three repeats of western blotting ($N = 3$) with the mean ± standard deviations (error bars).

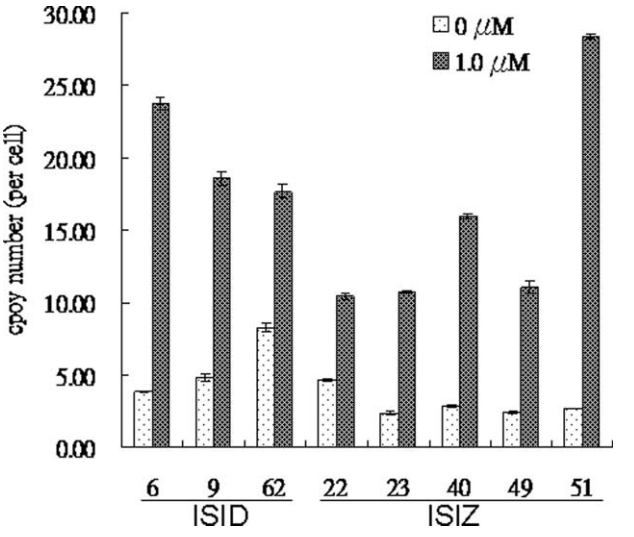

Figure 7. *dhfr* gene copy number comparison.
Following MTX selection, surviving clone cells before and after gene amplification were collected and subjected to real-time PCR. The *dhfr* copy number was determined from triplicate samples ($N = 3$) with mean ± standard deviation (error bars).

(MEMα with RNS and dRNS + 10% FBS) without MTX and cultivated for a minimum of 2 weeks, after which they were collected and analyzed for S_{TR2} expression by western blotting. As shown in Figures 5 and 6, S_{TR2} expression decreased in all ISID and ISIZ clones, a result of lower selection stress. In addition, the data indicate that S_{TR2} expression in ISID clones decreased more than in ISIZ clones. This result was compared with expression levels at a MTX concentration of 1 μM indicating S_{TR2} expression decreased by ~50% in the ISID clones but only 20% in the ISIZ clones. These cells were maintained for five passages within the 2-week cultures after MTX removal.

Gene copy number

Following MTX selection, surviving clone cells before and after gene amplification were collected and subjected to real-time PCR (ABI Prism 7500 Real time PCR system). As shown in Figure 7, *dhfr* gene copy numbers in ISID clones 6, 9, and 62 were greater than in ISIZ clones 22, 23, 40, 49, and 51 before MTX selection. After MTX selection, *dhfr* gene copy numbers in ISID clones were generally higher than in ISIZ clones except clone 51. It was confirmed that the copies of *dhfr* were correlated to the protein expression level by Western blot analysis of DHFR protein expression (Figure 8).

S_{TR2} expression in serum-free medium

We used two serum-free media to study cell growth curves: EX-CELL® CHO DHFR⁻ (Sigma) and CD CHO

(Invitrogen). CD CHO Medium is a chemically defined medium that contains no proteins, hydrolysates. EX-CELL® CHO DHFR⁻ medium is an animal component-free medium that contains recombinant human insulin, plant hydrolysates, and other organic compounds. The definition of robust cell growth was based on single passage assay. After adaption of the cells to serum-free media, the same amount of cells were seeded and maintained in each medium. After 3-day serum-free cultures, the amount of the cells was calculated to show better growth using EX-CELL® CHO DHFR⁻ medium compared with CD CHO medium (data not shown). As CHO cells grew more robustly in the EX-CELL® CHO DHFR⁻ medium, we chose it for subsequent analyses. ISID clones 6, 9, and 62 and ISIZ clones 22, 40, and 51 were individually suspended in 35 mL of EX-CELL® CHO DHFR⁻ medium. With the exception of 51, the cell densities of all clones increased within 5 days and decreased on day 6 (Figure 9A). Cells from each clone were collected on day 5 for western blot assays. Our results indicate no change in S_{TR2} glycoform (~115 kDa) in the serum-free medium, meaning that stable clones expressing S_{TR2} are capable of adapting to serum-free and suspension cultures without altering the post-translational modification process (Figure 9B).

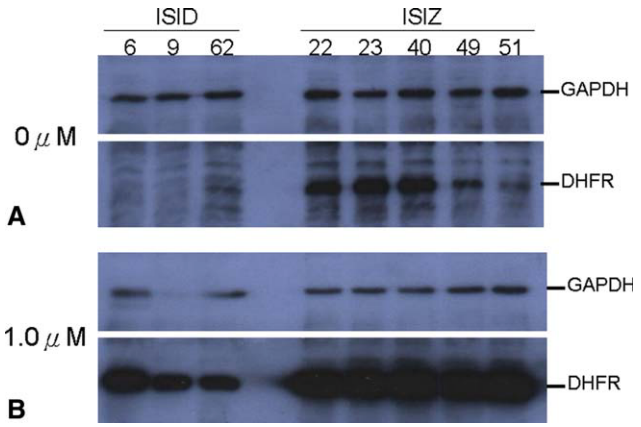

Figure 8. Western blot analysis of DHFR protein expression.

(A) Before MTX selection and (B) after 1 μM MTX selection. The expression of DHFR protein increased after gene amplification and the copies of *dhfr* were correlated to the protein expression level.

Discussion

Mammalian cells are the most commonly used host cells for biopharmaceutical production, because post-translational modification can improve their recombinant protein efficiency and stability. CHO cells were used as host cells in this study because they are capable of amplifying target genes following *dhfr* gene amplification, thus increasing productivity. Therefore, we used a CHO/dhFr⁻ cell expression system to establish stable amplified cell lines that were capable of producing proteins exhibiting proper folding, assembly, and post-translational modification.[22] We established several amplified cell clones that produced more recombinant S_{TR2} protein than unamplified cells, depending on the individual clone. The gene amplification process requires considerable time, meaning that highly variable antigens are not suitable for amplification. According to one recent study, the S proteins of SARS-CoV strains are very stable,[23] and therefore, generating stable CHO cell clones may be considered a good platform for developing SARS-CoV subunit vaccines.

To generate stable cell clones that stably expressed S_{TR2} following *dhfr*-mediated gene amplification via stepwise MTX selection, we found differences in the use of SV40-driven *dhfr* vs. IRES-driven *dhfr* promoters in our expression vector design. After MTX selection, the numbers of *dhfr* copies of both ISID and ISIZ clones increased at various levels, depending on the individual clone. With the exception of ISIZ clone 51, numbers of *dhfr* copies of ISID clones were generally greater than of ISIZ clones, and S_{TR2} expression in ISID clones was stronger than in ISIZ clones. The exception of ISIZ clone 51 may be due to the random integration into the genome at transcriptionally less active sites. These results confirm that the weaker *dhfr* gene promoter generates greater gene amplification, which in turn enhances S_{TR2} expression. According to one recent report, a weaker promoter driving *dhfr* gene expression can increase amplification range.[22] Another research team used a weak herpes simplex virus thymidine kinase (HSV-tk) promoter to construct a *dhfr*-amplifiable vector,[24] and a third used a novel approach using RNA interference to suppress *dhfr* gene expression.[18] It is possible to enhance gene amplification by combining RNAi and weak promoters, but doing so makes the process more difficult and requires even more time to establish stable and amplified cell clones.

S_{TR2} is a highly glycosylated protein with 11 putative N-linked glycosylation sites. The major form of S_{TR2}-

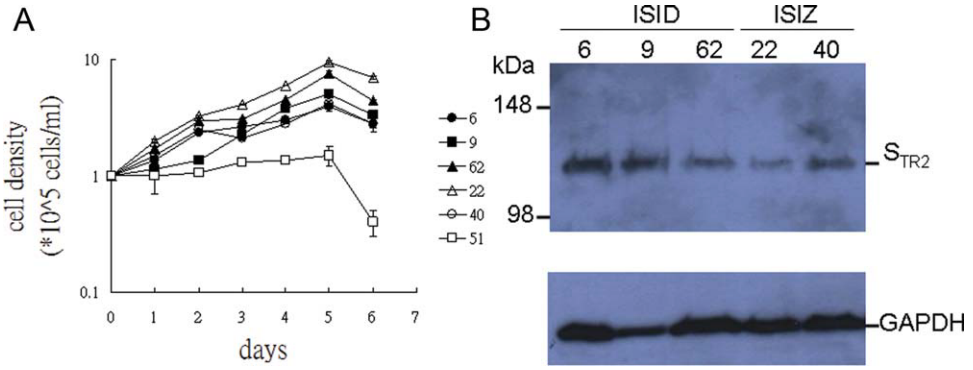

Figure 9. (A) Growth curve of the cell clones 6, 9, 62, 22, and 40 in the serum-free medium. (B) S_{TR2} expression in the EX-CELL® CHO DHFR⁻ (serum-free) medium.

Except clone 51, every clone could grow in the serum-free medium as shown in A. The glycoform of S_{TR2} expressed in the serum-free medium was unchanged (~115 kDa) as shown in B.

expressing CHO/dhFr$^-$ cells had molecular weights of ~115 kDa, which were sensitive to endo H treatment. A 130 kDa endo H-resistant glycoprotein was also detected during transient transfection; this glycoprotein showed a N-glycan pattern that favored Golgi complex processing[20] (Figure 3A). During the stable clone selection and gene amplification processes described in this report, S_{TR2} had variable glycoforms, molecular weights ranging from 88 to 115 kDa, and a 115 kDa (as opposed to 130 kDa) amplified form (Figure 5). During our experiment, we observed that the S_{TR2} glycoform depended on cell density in terms of adhesion culture. The molecular weight of S_{TR2} may be smaller than 115 kDa when cell confluence reaches 100%. Too high-cell density would result in increased ammonia in the medium, which comes from cellular metabolism or chemical decomposition of glutamine. It was reported that increased concentration of ammonia in the medium could increase heterogeneity of glycoproteins produced by CHO cells.[25–27]

Our results indicate that unlike the ~130 kDa glycoform, the ~115 kDa glycoform was capable of increasing after gene amplification (Figure 5). Therefore, we assumed that the CHO cell clones preferred ~115 kDa glycoform production. Moreover, the S_{TR2} glycoform did not change between suspension and serum-free cultures, suggesting that the stable and amplified cell clones analyzed in this study have potential for producing homologous S_{TR2} on a large scale.

Acknowledgments

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