Influence of intron and exon splicing enhancers on mammalian cell expression of a truncated spike protein of SARS-CoV and its implication for subunit vaccine development

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Abstract

The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is important for vaccine development. A truncated S protein of the TW1 strain, S TR2 (88 kDa), carrying three S fragments (S74–253, S294–739, and S1129–1255) was investigated to study the influences of intron and exon splicing enhancers to improve S TR2 protein expression in mammalian cells. Our results showed that S TR2 protein expression with the use of an 138 base-pair intron addition increased by 1.9-, 2.5-, and 4.1-fold in Vero E6, QRl-293A cells, and CHO/dhFr− cells (dihydrofolate reductase [dhfr] gene deficient CHO cells), respectively. Using the exon splicing enhancers, including a bidirectional splicing enhancer (BSE) or an exon splicing enhancer derived from the EDA alternative exon of the fibronectin gene (EDA ESE), were also found to increase S TR2 protein expression in CHO/dhFr− cells by 1.7- and 2.6-fold. Nevertheless, combination of the intron and the exon splicing enhancers resulted in suppressing the intron-enhancing e S TR2 protein expression in in CHO/dhFr− cells. Our studies also demonstrated the S TR2 protein was mainly as the Endo H-sensitive glycoprotein (115 kDa) expressed in Vero E6, QRl-293A, and CHO/dhFr− cells. However, only a minor form of the Endo H-resistant glycoproteins (∼ 130 kDa) was detected in CHO/dhFr− cells. Taken together, our results indicated that intron had a better enhancing effect on S TR2 protein expression than exon splicing enhancers, and the expression of ∼ 130 kDa S TR2 glycoprotein was enhanced by the intron addition into the expression vector construct. Results of the present study can provide an optimal strategy to enhance SARS-CoV S protein expression in mammalian cells and may contribute to the development of SARS-CoV subunit vaccine.

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1. Introduction

Severe acute respiratory syndrome (SARS) is an infectious viral disease that caused by a newly emerged coronavirus (SARS-CoV) [1–3]. SARS-CoV is a large RNA virus that contains four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S protein is responsible for receptor binding and membrane fusion, and is the most important vaccine target. The S protein can be divided into putative S1 (residues 17–680) and S2 (residues 727–1195) domain by sequence alignment [4]. S1 is presumably a globular domain that binds to ACE-2 through the receptor binding domain (residues 318–510) while S2 domain contains fusion peptide and heptad repeat motifs that mediate membrane fusion [4,5].

The SARS-CoV S protein is a highly glycosylated protein, with 23 putative N-linked glycosylation sites [6]. The predicted size of S protein is around 139 kDa, but it is glycosylated to ∼ 170 kDa in ER and further processed into ∼ 180 kDa in Golgi complex [5]. The presence of N-linked glycans on S protein is necessary for rabbit antiserum recognition which suggested the importance of N-linked glycosylation on correct S protein structure formation [5].
The SARS-CoV S protein has been the main target for SARS-CoV vaccine development. As previously reported, intranasal or intramuscular inoculation of highly attenuated modified vaccinia virus Ankara (MVA) that expressed full-length S protein could induce neutralization antibody production and elicit protective immunity in BALB/c mice [7]. Another report had indicated that recombinant attenuated parainfluenza type 3 virus that expressed S instead of E, M, and N protein could induce neutralizing antibody and provide protective immunity against SARS-CoV challenging, which suggested that S was the only significant protective antigen among all SARS-CoV structure proteins [8]. Moreover, the SARS-CoV S protein fragments: residues 318–510 (receptor-binding domain) [9], residues 485–625 [10], and residues 803–828 that fused with thioredoxin (Trx) or glutathione-transferase (GST) [11] were found to induce neutralizing antibody production in immunized animals. These results suggested that the S protein is an excellent vaccine target, since it could induce strong neutralizing antibody response and provide protection against virus challenging in immunized animals.

As the intron and its splicing out was known to enhance each step of RNA metabolism, including transcription, poly(A) tail addition, mRNA exportation, translation, and translated protein stability [12], hence we first studied the intron-enhancing effect on S TR2 protein expression. On the other hand, the exon splicing enhancers (ESEs) are known to be specific binding sequences for Serine/Arginine-rich proteins (SR proteins) that relate to the control of alternative splicing [13]. It has been demonstrated that some SR proteins, after binding to ESE, could assist in the open reading frame of STR2 were generated. Three DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using IGκ-A (138 bp intron of pIRES (Clontech)) that obtained sequence of EDA ESE added either in front, after, or between Igκ-BSE and Igκ/EDA as forward primer, and SIG-BSE-r, GGATCCGTCACCTTCTCCTTCCTTGAGAACTCCTGAGA or SIG-EDA-r, GGATCCGTCACCTTCTCCTTGAGAACTCCTGAGA as reverse primer, and SIG-BSE-r, GGATCCGTCACCTTCTCCTTGAGAACTCCTGAGA or SIG-EDA-r, GGATCCGTCACCTTCTCCTTGAGAACTCCTGAGA as reverse primer. Igκ-BSE and Igκ/EDA were ligated into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ. Two DNA fragments containing Igκ leader sequence following by bidirectional splicing enhancer (BSE) (GGAGGAGAGGA TGAAGAG) (Igκ-BSE) or EDA ESE (GAAGAAGAC) (Igκ/EDA) was obtained by PCR using SIG-f, GGTTAGCGTGTAAGATCAAGTTA CAAG and IVS-r: GGTGGAACCTTGAGAGAAAGGCAAAAGT was inserted into pS by Apfl and KpnI sites to generate pSκ.

2. Materials and methods

2.1. Cell lines and media

QBI-293A cells (QIAGEN) were grown in minimum essential medium (MEM) (Invitrogen) supplemented with 10% horse serum (FBS) (Invitrogen). Vero E6 cells (ATCC CRL-1586) were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen). CHO/dhFR- (dhfr deficient) cells (ATCC CRL-9096) were maintained in minimum essential medium alpha medium (MEMa) with ribonucleosides and deoxyribonucleosides (Invitrogen), supplemented with 10% FBS. All growth mediums were supplemented with 100 units/ml penicillin/streptomycin (PS). Cell lines were routinely maintained at 37 °C with 5% CO2.
in pS by Afl1 and Kpfl to generate pEIS, pEES, and pEIES. PCR fragment with lps leader sequence proceeding with EDA ESE sequence (EDA-lps) was also obtained by PCR using EDA-SIG-f: GCTTAAGGAGAAGACGCC-CAAAGTGAGACAC and SIG-r: GGGATCGGT-CACACGGTTGGAC as primers. The DNA fragment EDA-lps was ligated into pS by Afl1 and BamH1 sites to generate pES.

2.3. Transient expression in mammalian cells

For transient expression experiments, CHO/dhFr−, VeroE6, and QBI-293A cells was transfected by ExGen 500 above.

2.4. Western blot analysis

After separating on SDS-polycrylamide gel (8 or 10%), proteins were electroblotted onto a nitrocellulose membrane (Millipore). R8-AE01, rabbit serum raised against SARS-CoV S, M, and E peptides, was used as primary antibody (1:1000 dilution). The alkaline phosphatase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch) (1:5000 dilution) was used as secondary antibody in the detection.

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction from transfected cells was performed using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was synthesized by Oligo(dT) primers and Superscript II RT (Invitrogen). Real-time RT-PCR was conducted by using SYBR Green (Applied Biosystem) analysis where 200 ng cDNA of each sample was used as template. The transcripts containing vector pS and pIS vectors per 6 × 104 cells. Actinomycin D (100 μM/mL final, Calbiochem) was added to the medium 48 h post-transfection. At each specified time, the total RNA was extracted using Tri-zol reagent and analyzed by real-time RT-PCR as described above.

2.7. Transcriptional elongation rate

Elongation rate of transcription was determined by using HeLaScribe nuclear extract in vitro transcription system (Promega). The in vitro reaction was initiated by adding ribonucleotides and terminated using Stop Solution according to the manufacturer’s instructions. And then, total RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and reverse transcribed as cDNA using specific reverse primers for region I and region II, and analyzed by real-time PCR. The amounts of RNA transcript containing region I or region II separately detected by real-time RT-PCR with respect to time were plotted as the transcription curves. The time difference of the initial appearance of RNA between region I and region II was obtained from the transcription curves. The elongate rate (nucleotide/s) was calculated as the number of the time difference of the initial appearance of RNA divided by the nucleotide distance between region I and region II.

2.8. PNGase F and Endo H digestion

The cell lysates were diluted in 0.5% SDS and 1% β-mercaptoethanol and denatured at 100 °C for 10 min. PNGase F (New England BioLab) digestion was carried out in 50 mM sodium phosphate (pH 7.5), 1% NP-40 at 37 °C for 1 h. Endo H digestion (New England BioLab) was performed in 50 mM sodium citrate (pH 5.5) at 37 °C for 1 h. Enzyme treated samples were analyzed by 8% SDS-PAGE and Western blotting.

3. Results

3.1. Construction and expression of SARS-CoV SΔΔ and SΔΔΔ in mammalian cells

Three different constructs of SARS-CoV TW1 strain S protein were expressed in CHO cells: (i) the full-length cDNA (SΔΔΔ; pIS) and (ii) the cDNA of a longer truncated form (SΔΔΔ, pIS) (iii) the cDNA of a shorter truncated form (SΔΔΔ, pIS) (Fig. 1). The SΔΔΔ construct contains four S protein fragments: D1 (574–253), D2 (S249–739), D3 (S713–1113), and TM (S1129–1255) while SΔΔΔ construct lacks of D3 fragment. The results showed that SΔΔΔ instead of SΔΔΔ and SΔΔΔ could be expressed in CHO/dhFr− cells, the SΔΔΔ protein was recognized by rabbit anti-SARS-CoV polyclonal serum.
Fig. 1. Construction of pS FL and pS vectors. S FL consisted of full length spike protein with C-terminal HSV and His tag for antibody recognition. S TR2 started with an Ig/H926 signal sequence that was followed by three S protein fragments D1, D2, and TM. (R8-AE01), giving a protein band around 115 kDa in Western blots (Fig. 2).

3.2. The effects of intron addition on S TR2 expression

In order to investigate the influence of intron addition on S TR2 expression in mammalian cells, pIS was constructed by adding an 138 bp intron in the 5′-UTR of S TR2 (Fig. 3A), and both pS and pIS were transiently transfected into three mammalian cell lines: CHO/dhFr− cells, Vero E6 cells, and 293 cells (Fig. 3B). Cell lines transfected with pIS construct had higher S TR2 expression than those transfected with pS construct (Fig. 3B). The intensity of intron enhancing effect in the three cell lines was about 4.1-, 1.9-, and 2.5-fold in CHO/dhFr− cells, Vero E6 cells, and QBI-293A cells, respectively (Fig. 3C). S TR2 was mainly expressed as ∼115 kDa protein in the three cell lines (Fig. 3B). However, a weak ∼130 kDa band could also be observed in CHO/dhFr− cells that transfected with pIS (Fig. 3B).

To further investigate the intron-dependent enhancement in S TR2 expression, total RNA transcript, in vivo RNA stability, and the RNA elongation rate were measured for the intron-containing (pIS) and non-intron containing (pS) constructs. Total RNA transcripts obtained from the pIS-transfected CHO/dhFr− cells were 30% higher than that from the pS-transfected CHO/dhFr− cells (Fig. 4A). Therefore, the intron-dependent S TR2 expression in mammalian cells correlated with a higher level of RNA transcript accumulation in vivo. To further investigate the mechanism(s) of how the intron possess the ability to enhance RNA transcript accumulation, we further measured the in vivo RNA stability between the pIS-transfected and pS-transfected CHO/dhFr− cells. After treating with actinomycin D, the total RNA transcripts were extracted at each specified time to measure RNA decay quantified by real-time RT-PCR analysis. The results indicated that the RNA transcripts obtained from pIS-transfected CHO/dhFr− cells as compared to that from pS-transfected CHO/dhFr− cells exhibited similar rates for RNA decay (Fig. 4B). These two RNA transcripts exhibited half-lives of ∼7 h. Furthermore, the difference of the initial appearance time between region I and region II for the intron-containing construct was 28.3 s (41.7 s for region II and 13.4 s for region I) (pIS construct in Fig. 4C). The difference of the initial appearance time between region I and region II for pS construct was 31.3 s (36.2 s for region II and 4.9 s for region I) (Fig. 4C). The corresponding RNA elongation rate, as calculated as the difference of initial appearance time divided by the nucleotide distance (885 nucleotide residues between region I and region II), was 31.3 nucleotide/s for the intron-containing construct and 28.3 nucleotide/s for the...
The influence of intron addition on STR2 protein expression. (A) The 138 bp intron obtained from pIRES was inserted into pS vector within the 5′-UTR of STR2 gene to generate pIS. (B) Western blot analysis of STR2 protein expression in pS and pIS transient transfected cell lysate. (C) GelPro 3.0 quantification result of ∼115 kDa band on Western blot result.

Therefore, a higher elongation rate was observed for the intron-containing construct, indicating that a higher RNA elongation rate accounts for, or at least in part, the increased RNA transcript accumulation for the intron-dependent STR2 protein expression in mammalian cells.

3.3. The effects of exon splicing enhancer addition on STR2 expression

The enhancing effects of two chosen exon splicing enhancers, EDA ESE and BSE, on the mammalian cell expression of STR2 protein were investigated. Either EDA ESE or BSE was first inserted into the open reading frame of STR2 gene, right behind the Igκ signal sequence (pSBS and pSES). A 138 bp intron was further added in the 5′-UTR of STR2 to generate pSBS and pSES (Fig. 5A). Expression vectors of pS, pIS, pSBS, pSES, pISBS, and pISES were transfected into CHO/dhFr− cell and the expression of STR2 proteins were analyzed by Western blotting (Fig. 5B). The results indicated that BSE or EDA ESE alone was able to enhance STR2 protein expression without the use of intron. The enhancing effect of EDA ESE was 1.5-fold better than
Fig. 4. The influence of intron on RNA transcript. (A) Quantification of total RNA levels from CHO/dhFr− cells transfected by pS and pIS vectors. The amounts of RNA transcript of S TR2 were normalized to β-actin RNA and the averages of three independent experiments were plotted; error bars represent standard deviation. (B) Time course for S TR2 RNA decay in CHO/dhFr− cells transfected by pS and pIS vectors in the presence of actinomycin D. Data presented in percentage of the total S TR2 RNA. (C) Transcription curves of pIS and pS vectors using HeLa cell extract transcription system.

Fig. 5. The influence of ESE on S TR2 protein expression. (A) Construction of pSBS, pSES, pISBS, and pISES vectors. (B) Both pSBS and pSES vectors have higher S TR2 expression than pS while pISBS and pISES have lower S TR2 expression than pIS. (C) GelPro 3.0 quantification result of ~115 kDa band on Western blot result.
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Fig. 6. The influence of 5′-UTR EDA ESE on STR2 protein expression. (A) Construction of pEIES, pEIS, pIES, and pES expression vectors. (B) Cells transfected with pES have higher STR2 expression level when compared with pS, whereas neither pEIS, pIES, or pEIES have higher STR2 protein level than pS. (C) GelPro 3.0 quantification result of ∼115 kDa band on Western blot result.

BSE as quantified on the relative intensity of ∼115 kDa protein band (Fig. 5C). However, when either BSE or EDA ESE was coupling with an intron (pISBS and pISES), the STR2 expression levels were slightly lower than pS (Fig. 5C). Approximately 38% (pISBS) and 27% (pISES) reduction in STR2 expression was observed when compared to the construct only containing the 138 bp intron (pIS) (Fig. 5C). Therefore, BSE and EDA ESE could not work with the 138 bp intron to enhance STR2 expression.

To further investigate whether the combination of intron and ESE may work differently as ESE located in 5′-UTR instead of ORF, EDA ESE was added either in front (pEIS), after (pIES), or on both sides of the 138 bp intron (pEIES) in 5′-UTR of STR2 (Fig. 6A). At the mean time, a plasmid with only EDA ESE placed in the 5′-UTR of STR2 (pES) was used as a control (Fig. 6A). Expression vectors of pS, pIS, pEIS, pIES, pEIES, and pES were transient transfected into CHO/dhFr− cells, and the STR2 protein expression efficiency of each construct was determined by Western blot analysis (Fig. 6B).

Similar to the result of inserting EDA ESE in the STR2 open reading frame, EDA ESE sequence alone (pES) resulted in ∼2.8-fold increase in ∼115 kDa STR2 expression as compared to pS (Fig. 6B and C). However, the ∼115 kDa STR2 expression by pEIS, pIES, and pEIES were approximately 30%, 25%, and 40% lower than pS (Fig. 6C). The results suggested that neither of these constructs with ESE have better enhancing effect in than STR2 protein expression than adding intron alone.

3.4. The glycosylation pattern of STR2 within CHO/dhFr− cells

The predicted molecular weight of STR2 was 88.56 kDa, however, when pIS was transient transfected into CHO/dhFr− cells, the major expressed STR2 was at molecular weight around ∼115 kDa, whereas minor amount of ∼130 kDa STR2 glycoproteins could also be detected (Fig. 7). Since the STR2 protein sequence contains 15 putative N-link glycosylation sites, the increase in STR2 protein molecular weight might result from the addition of N-glycan during post-translational modification processes. In order to biochemically demonstrate the glycosylation pattern of STR2 expressed in CHO cells, peptide N-glycosidase F (PNGase F) and Endo H treatment were used for the following investigation. When pIS transfected cell lysate was treated with PNGase F, both ∼115 and ∼130 kDa bands were digested into a single ∼88 kDa band, which represented the predicted unglycosylated molecular weight of STR2 protein (Fig. 7). This indicated that the ∼115 and ∼130 kDa proteins were indeed glycoproteins. Additionally, when the pIS transfected cell lysate was digested with Endo
not due to the in vivo RNA stability (Fig. 4B) but from the increase of RNA elongation rate (Fig. 4C). Although other mechanisms may also account for the increased RNA transcript by intron addition such as pre-mRNA or mature mRNA stabilization, poly(A) tail addition, nucleocytoplasmic translocation, translation efficiency [12], our present study has demonstrated that the increase of RNA elongation rate accounted for, or at least in parts, the intron-dependent S_TR2 protein expression in mammalian cells.

4. Discussion

Although the S gene of SARS-CoV contains no introns, the expression of its truncated form, S_TR2, in mammalian cells was greatly enhanced by the intron addition (Fig. 3B and C). This finding consists with a previous report, which stated that the expression of naturally intronless gene, such as c-jun, could be enhanced by intron addition [19]. Intron and its spliced out processes are highly related to every step of RNA metabolism: transcription, poly(A) tail addition, mRNA translocation into cytoplasm, and translation, and intron works to improve each above-mentioned step [12]. We discovered that, no matter it was CHO/dhFr− cells, Vero E6 cells, or 293 cells, an 138 bp intron from pIRES vector produced in stable cell clones.

Fig. 7. The N-linked glycosylation pattern of S_TR2 protein. (A) The glycosylation pattern of S_TR2 protein in pIRES transient transfected cells. The major ∼115 kDa band was Endo-H-sensitive while the minor ∼130 kDa was Endo-H-resistant. (B) PNGase F and Endo H digestion of S_TR2 protein that band remain unaffected (Fig. 7). Therefore, S_TR2 was mainly produced in stable cell clones.

H. the ∼115 kDa band was disappeared while the ∼130 kDa band remain unaffected (Fig. 7). Therefore, S_TR2 was mainly expressed as Endo H-sensitive form (∼110 kDa) while a minor part of Endo H-resistant form could also be produced.

The S_TR2 protein, with 15 putative N-linked glycosylation sites in its sequence, is a highly glycosylated protein. Notably, the used of an intron could result in the production of a higher glycosylated S_TR2 (∼130 kDa) protein in CHO/dhFr− but not in 293 or Vero E6 cells (Fig. 3B). It might be that intron did not directly lead to the formation of ∼130 kDa S_TR2 protein, instead, higher S_TR2 protein production in CHO/dhFr− cell might spontaneously leads to the production of ∼130 kDa S_TR2 protein. The higher ∼130 kDa S_TR2 protein expression efficiency in CHO/dhFr− cells among others might result from the difference in endoplasmic reticulum quality control strictness in the three cell lines.

When exon splicing enhancers (ESE), such as BSE or EDA ESE, was placed right behind the lgsx signal sequence of S_TR2, the results showed 1.7-fold (BSE) or 2.6-fold (EDA ESE) increase of S_TR2 expression (Fig. 5C). When the position of EDA ESE was changed into the 5′-UTR of S_TR2 gene, 2.8-fold increase of S_TR2 expression can be observed (Fig. 6C). The EDA ESE was reported to increase luciferase expression by 3.7-fold through its interaction with SF2/ASF, a shuttling SR protein that capable of recruiting ribosome to mRNA [16]. It was also reported that the shuttling SR proteins, such as SF2/ASF, act as transportation adaptors and assist mRNA exportation into cytoplasm by interacting with TAP/NXF1 protein [15]. Both BSE and EDA ESE were reported to interact with shuttling SR proteins such as 9G8 and SF2/ASF, and the interaction might lead to the observed S_TR2 protein expression enhancing effect [17,20]. Better S_TR2 protein expression enhancing efficiency of EDA ESE (2.6-fold) than BSE (1.7-fold) was observed (Fig. 5C). It is possible that the different enhancing ability of EDA ESE and BSE is due to the different binding preference to different SR proteins, such as 9G8, SF2/ASF, and SC35, of the two sequences.

The result indicated that although BSE and EDA ESE themselves had enhancing effect, the S_TR2 expression vector coupling BSE or EDA ESE with an intron resulted in reduced S_TR2 expression compared to the construct with intron alone. The suppression of intron enhancing effect was unaffected by the position of EDA ESE in ORF or 5′-UTR of S_TR2 gene. Since the presence of BSE in adenovirus E1 A mRNA was found to activate the usage of a weak 3′-splice site [17]. Other ESEs such as drosophila doublesex (dxe) repeat element (dxRE) was also reported to activate the recognition of a weak, sex-specific 3′-splice site [21]. Therefore, it was possible that the presence of BSE or EDA ESE might activate certain cryptic splice sites within S_TR2 sequence, and therefore resulted in reduced S_TR2 expression due to aberrant splicing.

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The S_TR2 protein, with 15 putative N-linked glycosylation sites in its sequence, is a highly glycosylated protein.
The majority of mammalian cell expressed S TR2 protein has molecular weight around 115 kDa, and the ~115 kDa glycoproteins were sensitive to Endo H treatment (Fig. 7). Surprisingly, a minor band represented ~130 kDa glycoprotein, which was found to be resistant to Endo H treatment, was also detected in CHO/dhFR cells, whereas transient transfection of intron containing construct, indicating a different N-glycan pattern that favor Golgi complex processing (Fig. 7). The main question is why did the major glycosylation form of S TR2 protein lie in ~115 kDa instead of ~130 kDa? One of the possible answers might be the extensive quality control process of nascent protein in ER [22]. Enzyme or chaperones in ER would recognize the properties of non-native protein, such as abnormal exposure of hydrophobic regions, unpaired cysteine residues, and the tendency of aggregation, and result in the ER retention of a given protein [22]. Although the truncated nature of S TR2 renders it easily expressed than its full-length counterpart, linking different S protein fragment together might result in the disruption of native structure or creation of new structures that cannot be approved by ER quality control system.

Nevertheless, discovering of Endo H-resistant S TR2 suggested that it is possible to improve S TR2 glycosylation into more complex state. It has been reported that lowering the temperature during protein production can increase correct folding and secretion [23]. Furthermore, small molecular chemical chaperons have been discovered, such as glycerol, dimethylsulphoxide and trimethylamine N-oxide [24,25], which can carry out nonspecific, folding-promoting effect by stabilize native or native-like structure and preventing protein aggregation [22]. It will be quite interesting to know whether those molecules are able to increase glycosylation complexity of S TR2 proteins that produced by amplified cell clones. Moreover, culture conditions such as the availability of glucose and glutamine [26,27], the concentration of ammonia [28], the supply of oxygen [29], and the percentage of serum [30] can also affect the complexity of attached N-linked glycans on produced recombinant proteins. Therefore, optimization of the culture condition for stable CHO clones can also be carried out to improve the production of fully glycosylated S TR2 proteins.

The S TR2 protein contains receptor binding region and residue 485–625 of SARS-CoV S protein that was known to induce neutralizing antibody production in experimental animals, and is therefore an appropriate vaccine candidate. This study provides several possible ways for improving truncated S protein, S TR2 production in mammalian cell and may contribute to the development of successful mammalian cell based recombinant SARS-CoV vaccine.

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