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Analytical Biochemistry 366 (2007) 190–196
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

The use of hepatitis C virus NS3/4A and secreted alkaline phosphatase to quantitate cell–cell membrane fusion mediated by severe acute respiratory syndrome coronavirus S protein and the receptor angiotensin-converting enzyme 2

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Received 15 January 2007
Available online 27 April 2007

Abstract

The membrane fusion process mediated by severe acute respiratory syndrome coronavirus (SARS–CoV) S protein and its cellular receptor angiotensin-converting enzyme 2 (ACE2) had been reconstituted using two Chinese hamster ovary (CHO) cell lines that constitutively express these recombinant proteins separately. This system was applied to develop a quantitative measurement of cell–cell fusion using hepatitis C virus (HCV) NS3/4A protease and a secretion-blocked EGFP-4A/4B-SEAP (EGFP: enhanced green fluorescent protein; 4A/4B: a decapeptide substrate of NS3/4A protease; SEAP: secreted alkaline phosphatase) fusion gene. Both genes were transiently expressed in either of the CHO cell lines, followed by fusion treatment. Significant SEAP activity could be detected in the culture medium only after cell–cell fusion occurred. Cell–cell fusion provides an environment in which the protease encounters its substrate 4A/4B, thereby releasing SEAP from the fusion protein. In this study, we developed a simple, sensitive, and quantitative assay to study the membrane fusion process by measuring the extracellular activity of SEAP.

Keywords: SARS–CoV; ACE2; HCV; NS3/4A; EGFP; SEAP

Cellular membrane fusion is an essential process for cell physiology in a eukaryotic system. It is crucial for intracellular trafficking, cell secretion, exo- and endocytosis, and the entry of enveloped animal viruses. In cell biology, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[1] have been implicated in membrane fusion for more than a decade [1] and have been greatly elucidated. Correspondingly, enveloped viruses, such as human immunodeficiency, influenza, herpes, vaccinia, and hepatitis C viruses, have been widely studied for the mechanisms of their cellular entry. Each of these viruses can unload its genome into the host cytoplasm through the virus–cell membrane fusion process to initiate the viral replication cycle. The entry of severe acute respiratory syndrome coronavirus (SARS–CoV), a recently identified human coronavirus [2], is also a membrane fusion-dependent event mediated by the virion surface spike (S) protein and its cellular receptor angiotensin-converting enzyme 2

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[1] *Abbreviations used:* SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SARS–CoV, severe acute respiratory syndrome coronavirus; S, spike; ACE2, angiotensin-converting enzyme 2; CHO, Chinese hamster ovary; SEAP, secreted alkaline phosphatase; EGFP, enhanced green fluorescent protein; HCV, hepatitis C virus; NS, nonstructural; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NCBI, National Center for Biotechnology Information; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; IFA, immunofluorescence assay; FACS, fluorescence-activated cell sorting; RFP, red fluorescence protein.

0003-2697/$ - see front matter
doi:10.1016/j.ab.2007.04.031

(ACE2) [3,4]. As demonstrated in our previous study, the cellular surface expressions of both recombinant proteins can be used to mimic the membrane fusion process and provide a safe and simple assay for characterizing the abilities of anti-S monoclonal antibodies to block viral infection [5]. In the current study, we used noninfected Chinese hamster ovary (CHO) cells and further develop this system to allow accurate quantification of the cell–cell membrane fusion process.

As a highly sensitive reporter with low background from the endogenous alkaline phosphatase, secreted alkaline phosphatase (SEAP) secretion into the culture medium was used for quantitative analysis of membrane fusion without interfering cellular activity. The secretion could be blocked when SEAP was tagged with enhanced green fluorescent protein (EGFP) at the N terminus. A protease substrate linker between EGFP and SEAP was designed for releasing the phosphatase, whereas the linker (a decapeptide 4A/4B at the boundary of hepatitis C virus [HCV] 4A and 4B genes) is accessible to the protease. HCV is an enveloped, positive-stranded RNA virus; its genome encodes a unique polyprotein precursor that must be processed by proteases to produce individual viral proteins. HCV nonstructural protein 3 (NS3), a serine-type protease, and the cofactor NS4A [6] are required for the cleavage at junctions of NS proteins (except of NS2/3) within the polyprotein [7] and were used to unblock the tagged SEAP by cutting the linker 4A/4B. In this study, the quantitative method was developed by expressing the protease NS3/4A and the EGFP-tagged SEAP in different fusion partner CHO cells. When two CHO stable cell lines (CHO-SG and CHO-ACE2 are constitutively expressing either S or ACE2 fusion modulators) were transiently transfected, mixed, and treated with trypsin, the formation of syncytium was observed. The extracellular activity of SEAP could be measured due to the accessibility of NS3/4A protease to its recognition sequence.

Materials and methods

Cell lines for the cell–cell membrane fusion

Five stable cell lines (CHO-ACE2, CHO-SG, CHO-E2G, C2C12-SG, and C2C12-E2G) were established from the cell line CHO-K1 and C2C12 (American Type Culture Collection [ATCC]). C2C12, 293 and Cos7 (ATCC) cell lines were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 1 g L^{-1} glucose, 0.1 mg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, and 10% fetal bovine serum (FBS, HyClone), in addition to 1 mg ml^{-1} G418 (Gibco/BRL) for the stable cell lines. The constructions of CHO-SG and CHO-ACE2 were described previously [5,8]. The HCV E2 gene (the ectodomain of E2 protein from HCV polyprotein amino acid sequence 364–673) was amplified from the template HCV-S1 [9] (National Center for Biotechnology Information [NCBI] accession no. AF356827) with primers E2F (5′-GCAGATCTGCCACCatggtggggaactgggctaag-3′) and E2R (5′-GCatttaaatTCTGCCACTCTGTCGTAGAC-3′). The PCR fragment was double digested with *Bgl*II/*Swa*I and inserted into the plasmid pEGFP-N1-S-EGFP [8] with the same treatment. The ectodomain of S-EGFP was replaced with the ectodomain of E2 to produce E2-EGFP, and the cytoplasmic and transmembrane domains plus 20 amino acid residues in the outward juxtamembrane region of S protein were fused with the E2 protein. Stable cell lines of CHO-E2G, C2C12-E2G, and C2C12-SG were established by using lipofectamine (Invitrogen) as the transfection reagent and G418 for the selection. High-yield clones were selected by using a fluorescence microscope fitted with a fluorescein isothiocyanate (FITC) filter.

Quantitative constructs

Instead of making NS3/4A and GS (EGFP-4A/4B-SEAP) in a single plasmid [10], they were constructed in the *Kpn*I/*Not*I sites of pEGFP-N1 (Clontech) separately. The HCV NS3/4A was amplified from the template HCV-S1 [9] with primers NS3F (5′-GCGGTACCGCC ACC ATGgcgcctattacggcctactc-3′) and NS4AR (5′-GCGC GGCCGCTCATGAGCACTCCTCCATCTCATCG-3′). GS was amplified from three PCRs using EGFP and SEAP as templates and three sets of primers (see Supplementary Material). Mutations and modifications of GS for making GSK/R, GDS, and GDS/X were also PCR amplified using three sets of primers (see Supplementary Material). These PCR products (confirmed by sequencing) were double digested with *Bsr*GI and *Bam*HI for replacing the *Bsr*GI/*Bam*HI fragment of GS to construct GSK/R, GDS, and GDS/X.

Cell–cell fusion assay

Confluent CHO-ACE2 or GDS transient transfected CHO-ACE2 cells were dislodged with 0.04% EDTA and washed once with phosphate-buffered saline (PBS). Cell pellets were resuspended in serum-free culture medium and then overlaid (in a 1:1 ratio) on the confluent cell layer cultures of CHO-SG or NS3/4A transient transfected CHO-SG, CHO-E2G, C2C12-SG, or C2C12-E2G cells. After the cells were allowed to settle for 1 h, 1 μg ml^{-1} of porcine trypsin (JRH Bioscience) was added to initiate the membrane fusion process as described previously [5]. Then, 2 to 3 h later, the cells were observed under a microscope for syncytium formation.

Transfection, extracellular SEAP assay, and Western blot analysis

CHO cultures at 90% confluence were cotransfected as per the manufacturer's instructions (lipofectamine, Invitrogen) with the plasmid NS3/4A and plasmids (in a 1:1 ratio) of GS, GSK, GSR, GDS, GDSG, GDSE, or GDSR for SEAP releasing effect by the protease. Cos7, 293, and

CHO cells were also cotransfected with NS3/4A plasmid mixed with either GDS or GS or GDS alone for the SEAP activity. Furthermore, CHO-ACE2 was transfected with GDS, whereas CHO-SG and CHO-E2G (as a negative control) were transfected with NS3/4A. The cell–cell fusion assay was then performed with or without trypsin. SEAP assay was performed following the manufacturer's instructions (Clontech). The chemiluminescence was detected using a 96-well plate reader (1450 Microbeta Trilux, PerkinElmer). Media samples for SEAP assay collected from transient transfected cells and from quantitative cellular fusion processes were performed in triplicate (three individual experiments).

Transient transfected CHO cells were lysed with SDS–PAGE standard reducing sample buffer, and boiled lysates were then separated on 10% SDS–PAGE and transferred to nitrocellulose Hybond-C membrane (Amersham Biosciences) for Western blot analysis. Prestained molecular markers were purchased from Invitrogen. The membranes were blocked with 5% nonfat milk in PBST (PBS containing 0.2% Tween 20). Membranes were then treated with a primary monoclonal antibody specific to GFP (Roche), followed by a secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody (Pierce). Detection was based on chemiluminescence (SuperSignal West Pico Chemiluminescence Substrate, Pierce).

Results

Cell–cell membrane fusion mediated by the S protein and the receptor ACE2

The interaction between S protein of SARS–CoV and its cellular receptor ACE2 has been demonstrated to be essential for cellular binding [8] and membrane fusion [5]. Here two separate CHO stable cell lines established previously were used to reconstitute the cell–cell fusion process: CHO-ACE2 (which expresses the human ACE2) and CHO-SG (which expresses the SARS–CoV S protein). The surface expression of S on CHO-SG cells had been shown by nonpermeable immunofluorescence assay (IFA) using serum from a SARS convalescent patient, and the binding between CHO-SG and Vero E6 cells (ACE2 presenting cell line) was specifically blocked by either the serum from the SARS patient or a goat anti-ACE2 antibody [8]. The cellular surface expression of ACE2 on CHO-ACE2 cells was shown by fluorescence-activated cell sorting (FACS) analysis [5]. We also established three more cell lines, with C2C12-SG being used for confirming the cell–cell membrane fusion process that is mediated by S and ACE2 and being used as negative control cell lines CHO-E2G and C2C12-E2G. C2C12-SG was established in the same way as CHO-SG [8], using C2C12 cells as the transfection host. CHO-E2G and C2C12-E2G were established for expressing HCV E2 protein. The ACE2 was red fluorescence protein (RFP) tagged at the C terminus (see Supplementary Fig. 1), and the S and E2 were C-terminally tagged with EGFP (see Supplementary Figs. 1 and 2).

To determine whether cell–cell membrane fusion can occur, CHO-ACE2 cells were overlaid onto a monolayer of cells expressing either S or E2 and then were treated with trypsin to initiate the fusion process. Then, 2 or 3 h after trypsin treatment, cell–cell membrane fusion occurred between CHO-ACE2/CHO-SG cells as well as between CHO-ACE2/C2C12-SG cells (Figs. 1A and C). In contrast, CHO-ACE2/CHO-E2G and CHO-ACE2/C2C12-E2G cells were inert to the treatment (Figs. 1B and D). In this fusion process, both S and ACE2 are required and the trypsin treatment is empirically essential, in agreement with previous studies [5].

Gene constructions for the quantitative measurement

SEAP, the reporter, and HCV NS 3 and NS 4A (NS3/4A) [10,11] were used for quantitating the fusion process. To block the secretory character of SEAP, EGFP was tagged at its N terminus via a decapeptide linker (DEMEECASH) that corresponds to the NS3/4A protease cleavage site at the 4A/4B junction. The tagged SEAP could be unblocked and released extracellularly by the protease NS3/4A from the fusion encountered cells. Plasmids for the expression of NS3/4A or EGFP-4A/4B-SEAP (GS) were constructed (Fig. 2). To increase the accessibility of the 4A/4B linker to the NS3/4A proteases, mutations and modifications around the protease cleavage site were generated (Fig. 2). The fourth amino acid residue of the SEAP signal peptide was mutated to lysine (GSK) or arginine (GSR), and an octapeptide (DEDEDEDE) was intercalated in between EGFP and 4A/4B sequence for the construct GDS. Furthermore, the fourth amino acid residue of the SEAP signal peptide in GDS was mutated from leucine to glycine (GDSG), glutamic acid (GDSE), or arginine (GDSR).

Releasing blocked SEAP with NS3/4A protease

The amount of SEAP activity in the culture supernatants of CHO cells transiently transfected with GS as the negative control and NS3/4A cotransfected with GS, GSK, GSR, GDS, GDSG, GDSE or GDSR was measured 24 h after transfection (Fig. 3A). GS and its leucine mutants, GSK and GSR, all expressed near the basal level of extracellular SEAP activity, indicating that the 4A/4B linker might not be readily accessible to the protease, probably because it is too close to the hydrophobic core of the SEAP signal peptide or the hydrophobic region at the EGFP C terminus (Fig. 4). Indeed, when NS3/4A was cotransfected with GDS, a significant amount of SEAP activity that was detected in the culture supernatant was contributed by the extra octapeptide. Cotransfection of NS3/4A with the leucine mutation GDS derivatives, GDSG, GDSE, and GDSR, also yielded significant levels of extracellular SEAP activities, but the levels were lower

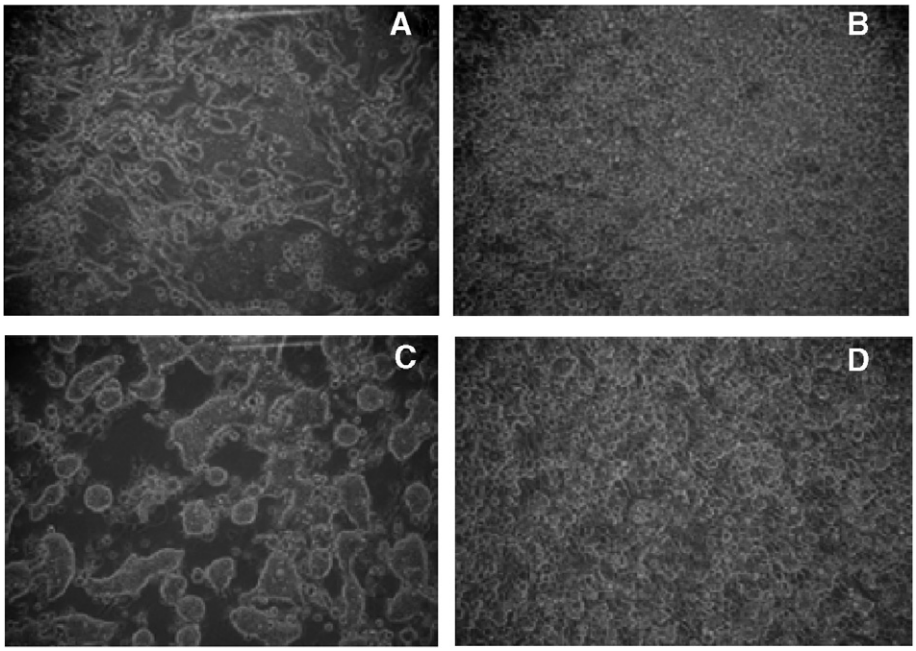

Fig. 1. Cell layers: CHO-SG (A), CHO-E2G (B), C2C12-SG (C), and C2C12-E2G (D) cells. CHO-ACE2 cells served as overlay cells for the fusion studies. The syncytium formation (panels A and C) was developed via the cellular fusion process between ACE2-expressing cells and S-expressing cells. E2-expressing cells (of both CHO and C2C12) were used as the negative controls. The magnification was 100×.

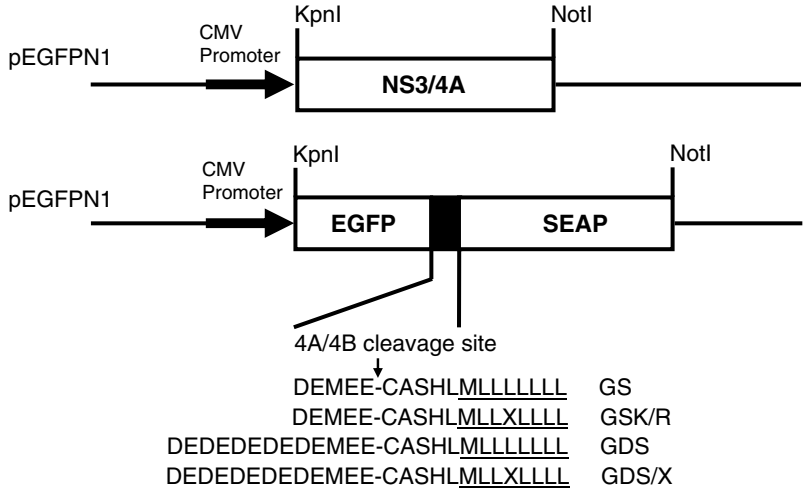

Fig. 2. Both NS3/4A and EGFP-4A/4B-SEAP (GS) genes were inserted into pEGFPN1 *Kpn*I/*Not*I sites driven by the CMV promoter. The decapeptide 4A/4B (DEMEECASH), a substrate of NS3/4A protease, was a linker to connect EGFP and SEAP. N-terminal sequences of SEAP are underlined. In GSK/R constructs, the fourth amino acid residue X indicates mutations from leucine to lysine or arginine. In GDS/X constructs, the X represents glycine, glutamic acid, and arginine. GDS construct was intercalated with an octapeptide (DEDEDEDE) upstream of 4A/4B.

than those with GDS. The cleavage activity of NS3/4A to GS or GDS was determined using Western blot analysis (Fig. 3B); the protease presented partial activity to GS, whereas the digestion to GDS was nearly completed. The expression levels of GS and GDS were also different, with the loading of GS samples in Fig. 3B being five times less than that of GDS samples.

The unblocking effect of GDS by the NS3/4A protease was tested in other high-transfection efficiency cell lines. In Fig. 5, the SEAP activity from GS or GDS cotransfected with NS3/4A or GDS alone was compared among CHO, 293, and Cos7 cells. SEAP released from GDS by NS3/4A in CHO cells was significantly greater than that in 293 and Cos7 cells. Therefore, CHO-ACE2 and CHO-SG cells expressing NS3/4A and GDS, respectively, were chosen for the development of a quantitative assay of the cell–cell membrane fusion process (see below).

Quantitation for the cellular fusion

For the membrane fusion experiment, CHO-ACE2 cells transfected with GDS were overlaid on cultures of

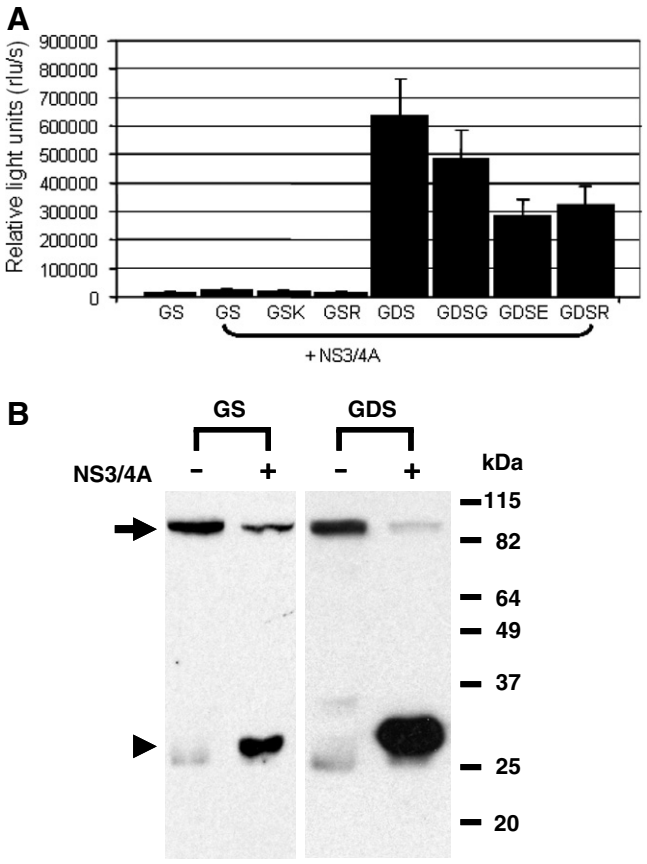

Fig. 3. Relative extracellular SEAP activity detected from the media of transfected cultures. (A) NS3/4A was cotransfected with GS, GSK, GSR, GDS, GDSG, GDSE, or GDSR into CHO cells, and GS alone served as the negative control. (B) Western blot was used to analyze the cleavages of GS and GDS by NS3/4A protease. Cell lysates of CHO cells transfected with GS or GDS and the cotransfection of NS3/4A with GS or GDS were separated on 10% SDS–PAGE and then processed with a primary monoclonal antibody specific to EGFP. The arrow indicates products from GS or GDS constructs containing EGFP. The arrowhead indicates NS3/4A-cleaved EGFP.

CHO-SG or CHO-E2G cells transfected with NS3/4A. A high level of extracellular SEAP was detected in the culture supernatant from the mixed cultures of CHO-ACE2 (+ GDS) and CHO-SG (+NS3/4A) after trypsin treatment (Fig. 6), indicating that the fusion between CHO-ACE2 and CHO-SG (see Fig. 1) allowed the NS3/4A protease to access and cleave the 4A/4B linker in GDS. As would be expected, there was no significant secretion of SEAP into the media from the mixed cultures of transfected CHO-ACE2 with either the negative control (NS3/4A transfected CHO-E2G) or NS3/4A transfected CHO-SG cells without trypsin treatment. This result indicates clearly that NS3/4A and GDS could be used for the quantitative measurement of cell–cell membrane fusion.

Discussion

Because SEAP in the GS construct was not significantly released by the protease, the cellular environment [10], such as membrane compartmentalization, may be a limiting factor. The sequence of GS was analyzed using the TMpred service (www.ch.embnet.org) for predicting possible transmembrane regions. The signal peptide (MLLLLLLLG LRLQLSLGI) of SEAP was strongly indicated as a transmembrane domain that may affect accessibility to the linker sequence for protease digestion. On the other hand, a range of 19 amino acid residues (219–237) at the EGFP C terminus was also suggested as a possible transmembrane domain; although EGFP is known as a cytoplasmic protein, its behavior may be different as in a fusion protein. To increase the accessibility of the linker to protease activity, we first tried to make a minimum modification by mutating one amino acid residue in the SEAP signal peptide to abolish the putative transmembrane domain while maintaining the signal peptide. An analysis of the 18 amino acid residues comprising the signal sequence using the program SignalP 3.0 (www.cbs.dtu.dk/services/SignalP) suggested changing the fourth residue, leucine, to either lysine (GSK) or arginine (GSR). However, these modifications did not increase the extracellular SEAP activity; in fact, they could only hinder the process by decreasing the secretion efficiency of GDSG, GDSE, and GDSR (Fig. 3A).

We then tried to increase the protease accessibility by inserting a hydrophilic peptide in between EGFP and 4A/4B while leaving the signal peptide intact. Because there are four negatively charged amino acid residues in 4A/4B, a negatively charged octapeptide was designed by composing Asp and Glu amino acid residues for limiting the interaction with 4A/4B and for making extra distance between 4A/4B and the hydrophobic region of EGFP. The Protean program in DNASTAR was used to compare the predicted hydrophilicity and surface probability of GS, GSK, and GDS (Fig. 4); the inserted octapeptide in GDS indeed increased the computed profiles for the region and possibly increased the protease accessibility. The result of cotransfected (GDS and NS3/4A) CHO cells supports our view of the accessibility; however, the octapeptide might not be the optimum. Some CHO-GDS stable clones still expressed a basal level of extracellular SEAP while they were transfected with NS3/4A (see Supplementary Fig. 3), and the secretion of SEAP from GDS and NS3/4A cotransfected 293 and Cos7 cells was insignificant. The parameters of the octapeptide could possibly be changed in length or modified with other hydrophilic amino acid residues. The cellular fusion between CHO-ACE2 and C2C12-SG cells is more drastic than that between CHO-ACE2 and CHO-SG cells, but the quantitation was limited by the low transfection efficiency of C2C12 cells. In addition, a promoter strong enough to drive the transient expression for either NS3/4A or GDS in C2C12 cells for the quantitative measurement is not available in our laboratory. We also established CHO-NS3/4A and CHO-GDS stable cell lines for the cell–cell membrane fusion quantitative studies, but the transient expressions of either S or ACE2 were not sufficient to perform the process significantly.

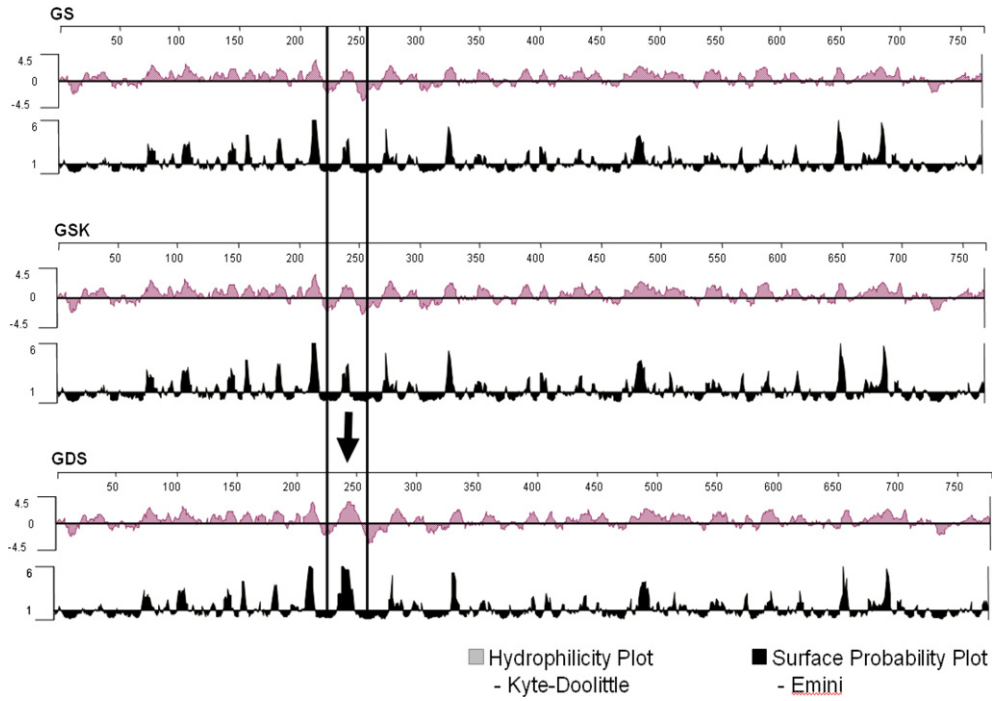

Fig. 4. Comparison of the hydrophilicity plots and surface probability plots of GS, GSK, and GDS. The arrow indicates the regions affected by the octapeptide in GDS.

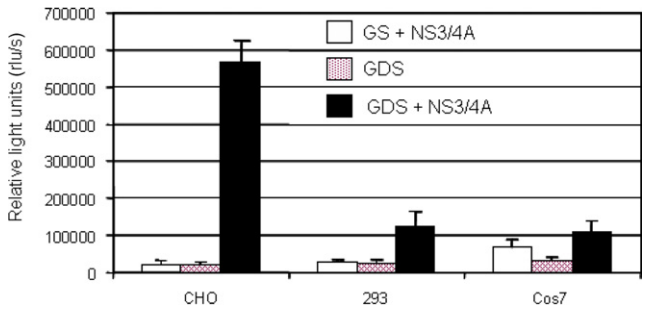

Fig. 5. Relative extracellular SEAP activity detected in the media from transfected CHO, 293, and Cos7 cells. Cells were transfected with GDS alone or were cotransfected with NS3/4A and GS or GDS. CHO cells cotransfected with NS3/4A and GDS produced the highest extracellular SEAP activity.

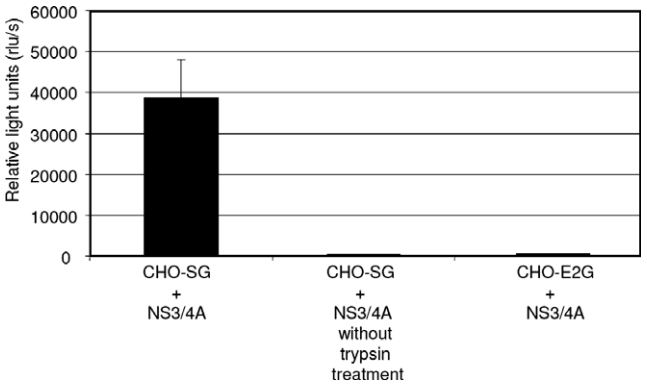

Fig. 6. CHO-SG and CHO-ACE2 cellular fusion quantitation. The media from cell–cell fusion cultures were analyzed for SEAP activity. GDS transfected CHO-ACE2 cells were processed for fusion with NS3/4A transfected CHO-SG or CHO-E2G cells. Cellular fusion was evident only between CHO-ACE2 and CHO-SG cells with trypsin treatment.

By making use of the protease activity of the HCV NS3 protein and designing a novel EGFP-4A/4B-SEAP substrate, GDS, we have developed a quantitative assay for the membrane fusion process between SARS–CoV S and its cellular receptor, ACE2. Such a quantitative system could be useful for analyzing the fine details of this important step in the SARS–CoV life cycle and could also provide a safe and simple assay for high-throughput screening to identify inhibitors that can stop the virus at this crucial step. Similarly, the assay could be adapted for the study of the membrane fusion process for other enveloped viruses.

It is estimated that 170 million people are chronically infected with HCV, and the development of effective HCV therapies is still a challenge due to the lack of adequate animal models and tissue culture systems for analysis and propagation of the virus. However, the development of a subgenomic HCV replicon system [12] has been critical in enabling preclinical evaluation of potential anti-HCV agents in support of drug discovery. The macrocyclic inhibitor BILN 2061 was identified using a substrate-based approach to designing inhibitors of the HCV NS3 active site [13], and subsequently identified inhibitors and NS3 mutants [13–16] all were based on the studies using the replicon system. The quantitative method using NS3/4A and GDS could be used as the alternative way to screen for HCV NS3 inhibitors in vivo [10,11] or to facilitate the studies of NS3 drug-resistant mutants by quantitative measurement. In this article, we have presented cell–cell

membrane fusion mediated by two stable CHO cell lines expressing recombinant SARS–CoV S protein and human ACE2 individually. Moreover, the fusion process was used as a model for developing a quantitative measurement of extracellular SEAP via the transient expressions of HCV NS3/4A protease and GDS (secretion blocked SEAP) from these cell lines separately.

Acknowledgments

We thank the Institute of Molecular and Cell Biology (IMCB) DNA Sequencing Facility for its help in sequencing. This project was supported by the Agency for Science and Technology (A*STAR), Singapore.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2007.04.031.

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