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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

Chih-Fong Chou *, Shuo Shen, Geetha Mahadevappa, Seng Gee Lim, Wanjin Hong, Yee-Joo Tan

Institute of Molecular and Cell Biology, Proteos, Singapore 138673

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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

* Corresponding author. Fax: +65 6779 1117.
E-mail address: mcbccf@imcb.a-star.edu.sg (C.-F. Chou).

[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.

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(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