Development of a Single-cycle Infectious SARS-CoV-2 Virus Replicon Particle System for use in BSL2 Laboratories

Johnny Malicoat[a], Senthamizharasi Manivasagam[a], Sonia Zuñiga[b], Isabel Sola[b], Dianne McCabe[a], Lijun Rong[c], Stanley Perlman[a], Luis Enjuanes[b] and Balaji Manicassamy[a*]

[a] Department of Microbiology and Immunology, University of Iowa, Iowa City, IA, USA

[b] Coronavirus Laboratory, Departamento Biologia Molecular y Celular, Centro Nacional de Biotecnologia (CNB-CSIC), Madrid, Spain

[c] Department of Microbiology and Immunology, University of Illinois at Chicago, IL, USA

*Address correspondence to Balaji Manicassamy

Email: balaji-manicassamy@uiowa.edu

Running title: Single-cycle Infectious SARS-CoV-2 Replicon

Abstract

Research activities with infectious severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) are currently permitted only under biosafety level 3 (BSL3) containment. Here, we report the development of a single-cycle infectious SARS-CoV-2 virus replicon particle (VRP) system with a luciferase and green fluorescent protein (GFP) dual reporter that can be safely handled in BSL2 laboratories to study SARS-CoV-2 biology. The Spike (S) gene of SARS-CoV-2 encodes for the envelope glycoprotein, which is essential for mediating infection of new host cells. Through deletion and replacement of this essential S gene with a luciferase and GFP dual reporter, we have generated a conditional SARS-CoV-2 mutant (ΔS-VRP) that produces infectious particles only in cells expressing a viral envelope glycoprotein of choice. Interestingly, we observed more efficient production of infectious particles in cells expressing vesicular stomatitis virus (VSV) glycoprotein G (ΔS-VRP(G)) as compared to cells expressing other viral glycoproteins including S. We confirmed that infection from ΔS-VRP(G) is limited to a single round and can be neutralized by anti-VSV serum. In our studies with ΔS-VRP(G), we observed robust expression of both luciferase and GFP reporters in various human and murine cell types, demonstrating that a broad variety of cells can support intracellular replication of SARS-CoV-2. In addition, treatment of ΔS-VRP(G) infected cells with anti-CoV drugs remdesivir (nucleoside analog) or GC376 (CoV 3CL protease inhibitor) resulted in a robust decrease in both luciferase and GFP expression in a drug-dose and cell-type dependent manner. Taken together, we have developed a single-cycle infectious SARS-CoV-2 VRP system that serves as a versatile platform to study SARS-CoV-2 intracellular biology and to perform high throughput screening of antiviral drugs under BSL2 containment.

Importance

Due to the highly contagious nature of SARS-CoV-2 and the lack of immunity in the human population, research on SARS-CoV-2 has been restricted to biosafety level 3 laboratories. This has greatly limited participation of the broader scientific community in SARS-CoV-2 research and thus has hindered the development of vaccines and antiviral drugs. By deleting the essential Spike gene in the viral genome, we have developed a conditional mutant of SARS-CoV-2 with luciferase and fluorescent reporters, which can be safely used under biosafety level 2 conditions. Our single-cycle infectious SARS-CoV-2 virus replicon system can serve as a versatile platform to study SARS-CoV-2 intracellular biology and to perform high throughput screening of antiviral drugs under BSL2 containment.

Introduction

Due to the highly contagious nature of severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) and the lack of sufficient immunity in the population, research on SARS-CoV-2 is permitted only under BSL3 containment, which significantly limits SARS-CoV-2 research only to institutions with BSL3 infrastructure. In addition, due to physical limitations, high throughput screening of antiviral drugs can be impractical under BSL3 containment (1, 2). Fortunately, research with attenuated or conditional mutants of BSL3/BSL4 pathogens is allowed under lower containment upon demonstration of attenuation and safety (3). These include low pathogenic H5N1 (lacking the multibasic site in HA), an Ebola virus conditional mutant (VP30 deletion mutant), and Yersinia *pestis* conditional mutants, etc (4-6). Thus, to enable studies with SARS-CoV-2 under BSL2 containment, SARS-CoV-2 spike (S) pseudotyped HIV lentivirus particles or recombinant vesicular stomatitis virus (VSV) with the native G gene replaced with the S gene have been developed and can be used to study the viral entry process and to identify viral entry inhibitors (7-9). In addition, several SARS-CoV-2 replicon reporter systems that retain the minimal viral genes necessary for intracellular replication have been developed (10-12). Moreover, conditional deletion mutants of SARS-CoV-2 capable of replicating only in complemented cells expressing viral proteins have been reported (N gene or ORF3a/E genes deleted) (13, 14). A recent study reported a single cycle infectious particle system through co-expression of viral S, M, E and N proteins along with a luciferase reporter carrying cis-acting elements (packaging sequences) of SARS-CoV-2 in the 3'UTR (15). The generation of these SARS-CoV-2 tools has allowed us to safely study various aspects of SARS-CoV-2 biology under BSL2 containment.

Here, we report the development of a single-cycle infectious SARS-CoV-2 virus replicon particle system (ΔS-VRP) with a luciferase and green fluorescent protein (GFP) reporter. Using a bacterial artificial chromosome based reverse genetics system, we replaced the essential spike gene of SARS-CoV-2 with a luciferase (Luc) and GFP dual reporter (ΔS-Luc-GFP) (16). Co-transfection of the ΔS-Luc-GFP bacmid with a VSV-G expressing plasmid resulted in efficient production of infectious VRPs (ΔS-VRP(G)), which can be further amplified in VSV-G transfected cells. As expected, control vector transfected cells failed to produce any infectious ΔS-VRPs, demonstrating that VRP infection is restricted to a single cycle. ΔS-VRP(G) stocks produced from VSV-G expressing cells showed robust infection and expression of both

reporters in various murine and human cell lines, indicating that various cell types are permissive to SARS-CoV-2 replication. Importantly, treatment of ΔS-VRP(G) infected cells with antivirals Remdesivir (nucleoside analog) or GC376 (CoV 3CL protease inhibitor) resulted in a robust and drug-dose dependent decrease in both luciferase and GFP reporter activity, demonstrating that this platform can be useful for antiviral drug screening. Taken together, our studies demonstrate that this ΔS-VRP system with a dual reporter can serve as a versatile tool to investigate SARS-CoV-2 biology and perform antiviral drug screening under BSL2 containment.

Results

Design of a single-cycle infectious SARS-CoV-2 virus replicon particle system

To safely study SARS-CoV-2 biology under BSL2 containment, we deleted and replaced the essential viral spike (S) ORF with a tandem Gaussia luciferase and Neon Green GFP dual reporter separated by the porcine teschovirus 2A ribosome skipping signal under the control of the S gene transcription regulatory sequence (ΔS-Luc-GFP; Fig 1A). Similar to other viral replicons, the ΔS-Luc-GFP genome can undergo normal transcription and replication upon transfection into cells, yet is unable to produce infectious virus particles due to the lack of the S gene. As such, formation of infectious particles carrying the ΔS-Luc-GFP genome requires expression of the S protein or other viral glycoprotein(s) in producer cells. We rescued infectious VRPs by co-transfecting the ΔS-Luc-GFP bacmid with a VSV-G expression plasmid into a mixture of 293T/Huh7.5 cells (ΔS-VRP(G); Fig 1B). We observed a significant increase in luciferase activity in the supernatants and GFP expression in cells transfected with ΔS-Luc-GFP bacmid over time as compared to control vector transfected cells (Fig 1C-D). On day 6 post-transfection, the 293T/Huh7.5 cell mixture was transfected again with a VSV-G expression plasmid, which resulted in a significant increase in both luciferase activity and the numbers of GFP expressing cells. These data show that ΔS-Luc-GFP replicated efficiently in cells, and additional expression of VSV-G presumably increased the spread of ΔS-Luc-GFP genome to new cells. To produce ΔS-VRP(G) working stocks, supernatants from ΔS-VRP(G) rescue transfections were used as seed stocks to infect VSV-G expressing Huh7.5 cells. Next, we evaluated the infectivity of amplified ΔS-VRP(G) stocks by measuring the expression of viral nucleoprotein (N) and GFP in a human lung epithelial cell line (A549) expressing human angiotensin-converting enzyme 2 (hACE2) (A549-hACE2). At 18hpi, immunofluorescence

analysis showed GFP expression in N positive cells (Fig 1E). Furthermore, western blot analysis showed expression of N protein but not S protein in ΔS-VRP(G) infected cells (Fig 1F). Wild-type SARS-CoV-2 infected A549-hACE2 cell lysates were included as controls. Taken together, we have successfully established a SARS-CoV-2 VRP system with a dual reporter that can be easily propagated in VSV-G expressing cells.

ΔS-VRP(G) infection is restricted to a single cycle and neutralized by anti-VSV sera

Next, we compared the infectivity of ΔS-VRPs produced in Huh7.5 cells expressing glycoproteins from diverse viral families including SARS-CoV-2 S and observed more efficient production of infectious ΔS-VRPs only in cells expressing VSV-G (data not shown); hence, our subsequent experiments were performed with ΔS-VRP(G) particles. Next, to demonstrate that infection from ΔS-VRP(G) is restricted to a single round, we transferred the supernatants from ΔS-VRP(G) infected Huh7.5 cells (No VSV-G transfection; R2 sup) onto fresh Huh7.5 cells and observed no GFP or Luc expression. These results demonstrate that infection with ΔS-VRP(G) is limited to a single cycle (Fig 2A).

Next, to assess the reproducibility of the ΔS-VRP(G) system, we tested the infectivity of 3 independent preparations of ΔS-VRP(G) stocks in Huh7.5 cells and observed similar levels of luciferase activity across different preparations (Fig 2B). In addition, ΔS-VRP(G) preparations were stable during storage at -80C and retained infectivity at levels similar to fresh ΔS-VRP(G) preparations (Fig 2C). Moreover, we observed a dose dependent increase in the infectivity of ΔS-VRP(G) in Huh7.5 cells. Next, to demonstrate that ΔS-VRP(G) infection is solely mediated through the VSV-G glycoprotein, we tested if ΔS-VRP(G) infection can be inhibited in the presence of anti-VSV serum (Fig 2D-E). Infectivity of ΔS-VRP(G) in Huh7.5 cells was significantly reduced in the presence of anti-VSV serum as compared to control serum, indicating that ΔS-VRP(G) infection is mediated through the VSV-G protein. Taken together, we have developed a robust single-cycle infectious SARS-CoV-2 ΔS-VRP(G) dual reporter system that can be safely used under BSL2 containment.

ΔS-VRP(G) dual reporter system is a versatile tool to study SARS-CoV-2 biology in different cell types

As ΔS-VRP(G) infection of host cells is independent of SARS-CoV-2 host receptor ACE2 expression, we evaluated the robustness of the ΔS-VRP(G) dual reporter system in various cell types of human and murine origin (Fig 3A-D). We observed robust replication of ΔS-Luc-GFP in various cell types from different species including lung epithelial cells, kidney cells, monocytic cell lines, lymphocytes, macrophages and dendritic cells. These results indicate that several human and murine cell types are permissive to replication of SARS-CoV-2. Interestingly, we observed differences in the levels of replication in different cells types, indicating that cell specific host factors may regulate the intracellular replication steps of SARS-CoV-2.

Next, to investigate if the ΔS-VRP(G) dual reporter system can be useful for the assessment of host antiviral responses, we infected murine bone marrow derived macrophages (BMDM) and dendritic cells (BMDC), and measured expression of the viral N gene and various host antiviral genes. We observed robust induction of various antiviral genes including Ifnα, Ifnβ, Mx1, Isg15 and Tnfα in BMDMs and to a lesser extent in BMDCs (Fig 3E). Taken together, these results demonstrate that the ΔS-VRP(G) dual reporter system is a versatile tool to study SARS-CoV-2 biology as well as host immune responses in various cell types.

ΔS-VRP(G) dual reporter system can be valuable for antiviral drug screening

Next, to test if the ΔS-VRP(G) dual reporter system can be a useful platform for testing anti-SARS-CoV-2 drugs, we assessed the effects of well-known anti-CoV drugs Remdesivir and GC376 on ΔS-Luc-GFP replication (Fig 4). Huh7.5 or A549 cells infected with ΔS-VRP(G) were treated with different concentrations of Remdesivir or GC376 starting at 2hpi. At 18hpi, both luciferase activity in the supernatants and GFP expression in infected cells were compared between different treatment groups. In Huh7.5 cells, we observed a dose dependent decrease in luciferase activity along with a concomitant decrease in GFP expression in both Remdesivir and GC376 treatment groups as compared to DMSO treated cells (Fig 4A and 4C; Remdesivir IC50 26.7nM and GC376 IC50 17.3nM). We also observed a similar dose dependent inhibition of ΔS-Luc-GFP replication in A549 cells treated with either Remdesivir or GC376 (Fig 4B and 4D; Remdesivir 55.2nM and GC376 2300 nM); Interestingly, inhibition of ΔS Luc-GFP replication by Remdesivir and GC376 was less pronounced in A549 cells as compared to Huh7.5 cells, indicating cell type specific differences in the activity of antiviral drugs. These

results demonstrate that the ΔS-VRP(G) dual reporter system can be useful for rapid screening of antiviral drugs against SARS-CoV-2 under BSL2 containment. Taken together, we have successfully established a SARS-CoV-2 VRP dual reporter platform that allows for safe investigation of SARS-CoV-2 biology, host interactions, and antiviral responses, as well as for high throughput screening of anti-CoV drugs, under BSL2 containment.

Discussion

Studies with highly pathogenic viruses can be safely performed in biological laboratories with specialized biocontainment procedures. Through the generation of conditional replicating mutants or attenuated mutants, we can safely study some aspects of virus biology in standard BSL2 or BSL2+ facilities. Currently, research activities with SARS-CoV-2 are restricted to BSL3 facilities that mostly have limited capabilities. To overcome this limitation, we have developed a conditional mutant of SARS-CoV-2 through deletion and replacement of the essential viral S glycoprotein gene with a luciferase-GFP dual reporter, leaving the remaining SARS-CoV-2 genome intact. Through co-expression of VSV-G protein *in trans*, we have successfully generated single-cycle infectious SARS-CoV-2 replicon particles carrying the ΔS Luc-GFP genome. Various human and murine cells infected with ΔS-VRP(G) showed robust expression of both luciferase and GFP reporters, indicating that these cell lines are permissive to intracellular replication of SARS-CoV-2. In addition, as viral entry of ΔS-VRP(G) particles is mediated through the G protein, ΔS-VRP(G) can efficiently infect a variety of human and murine cell types lacking the SARS-CoV-2 ACE2 receptor. Importantly, the ΔS-VRP(G) dual reporter system showed robust responsiveness to treatment with Remdesivir or GC376, demonstrating that the ΔS-VRP(G) dual reporter system can be useful for high-through put screening of antiviral drugs against SARS-CoV-2.

Several groups have reported the development of SARS-CoV-2 replicon systems with either luciferase or GFP reporters, and the majority of these replicon systems require transfection of replicon DNA or RNA into cells, which may limit studies to cell types with higher transfection efficiency. In addition, there might be a time lag between nucleic acid transfection and optimal reporter expression. One advantage of our ΔS-VRP(G) dual reporter system is that large quantities of ΔS-VRP(G) can be easily amplified in VSV-G expressing Huh7.5 cells, and can be used to investigate SARS-CoV-2 biology in a variety of cell types. In addition to replicons,

conditional SARS-CoV-2 mutants with GFP or luciferase reporters have been developed. Ju, et al. generated a mutant lacking a portion of the essential N gene, thereby limiting replication to cells expressing N (14). Zhang, et al. generated a conditional mutant lacking the ORF3a/E genes, which restricted replication to cells stably expressing ORF3a and E proteins (13). As an additional safety measure, these authors elegantly modified all TRS sites to restrict the potential emergence of replication competent virus through recombination with wild-type SARS-CoV-2. Another advantage of our ΔS-VRP(G) system is that VSV-G mediated delivery of the ΔS-Luc-GFP genome occurs independently of the ACE2 receptor. Indeed, a vast majority of human and murine cell types supported ΔS-Luc-GFP replication, albeit with some differences in the levels of replication. It is possible that the observed variations in ΔS-Luc-GFP replication can be in part due to inherent differences in VSV-G mediated entry into various cell types. Finally, as the ΔS-VRP(G) system contains both luciferase and GFP reporters, we observed a strong co-relation between luciferase activity and GFP expression across different treatment groups in our studies with remdesivir and GC376. The presence of a dual reporter allows for rapid elimination of false positive hits that directly inhibit luciferase or interfere with GFP fluorescence during high-throughput antiviral drug screening.

In our studies, we observed robust luciferase and GFP expression in cells infected with ΔS-VRP(G) as compared to ΔS-VRP produced in cells expressing other viral glycoproteins including SARS-CoV-2 S (data not shown). We speculate that this is in part due to the higher efficiency of VSV-G protein in mediating viral entry as compared to other viral glycoproteins. It is not completely clear how VSV-G is incorporated onto ΔS-VRPs to mediate infection. VSV-G has been reported to be localized to intracellular compartments, such as the endoplasmic reticulum-golgi, as well as on the plasma membrane. As SARS-CoV-2 assembly and budding is thought to occur in the endoplasmic reticulum-golgi intermediate compartment (ERGIC), it is possible that intracellularly expressed VSV-G is incorporated onto VRP membranes during canonical SARS-CoV-2 budding in the ERGIC. In agreement with VSV-G dependent delivery of the ΔS Luc-GFP genome, ΔS-VRP(G) infection was neutralized in the presence of anti-VSV sera. Our future studies will determine if VSV-G incorporation onto ΔS-VRPs occurs in intracellular compartments.

In conclusion, we have developed a SARS-CoV-2 VRP platform with a dual reporter that can serve as a versatile tool to study SARS-CoV-2 host biology under BSL2 containment. Importantly, we observed robust and dose dependent changes in both luciferase and GFP expression upon treatment with anti-CoV drugs, demonstrating that this ΔS-VRP(G) platform can be suitable for safe high throughput screening of antivirals against SARS-CoV-2.

Materials and Methods

Biosafety statement

Studies with infectious SARS-CoV-2 and ΔS-VRP(G) viruses were approved by UIowa IBC and CCOM BSL3 Oversight Committee (Protocol # 210053). Initial validation and safety studies with ΔS-VRP(G) were performed in a BSL3 laboratory. After review of the safety data, both UIowa IBC and the NIH Office of Science Policy approved the use of ΔS-VRP(G) under BSL2+ containment at UIowa.

Ethics statement

All studies were performed in accordance with the principles described by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research. The protocol for isolating anti-VSV serum from mice was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa (Animal Protocol #1062127).

Anti-VSV serum

Antisera against VSV was produced in-house by immunizing C57BL/6J mice (6 weeks old) with $2x10^8$ PFU of live VSV-GFP virus via intramuscular route. On day 14, mice were boosted with the same dose of VSV-GFP virus. At 4 weeks post-immunization, serum was collected by cardiac puncture and used in neutralization experiments after heat inactivation.

Cell lines and primary cells

Human lung epithelial cells (A549), human embryonic kidney cells (HEK293T), hepatocellular carcinoma cell line (Huh7.5), mouse endothelial cell line (MS1), mouse lung epithelial cells (LA4), mouse macrophage cell line (Raw 264.7), and African green monkey kidney epithelial cells (Vero) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented wth 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000U/ml). Primary bone

marrow derived macrophages (BMDM) and dendritic cells (BMDC) were generated from bone marrows isolated from C57BL/6J mice (Jackson labs) by culturing in the presence of IL-4 and GMCSF or IL-4 and L929 conditioned media, respectively (17, 18).

Generation of ΔS-Luc-GFP bacmid

SARS-CoV-2 reverse genetics system based on the sequence of Wuhan-Hu-1/2019 isolate (Accession number: NC_045512) was designed and assembled into a pBeloBac11 vector as previously described for other coronaviruses (16). Our SARS-CoV-2 bacmid carries a unique engineered SanDI restriction site in the NSP15 gene and a naturally occurring unique BamHI restriction site near the 3' end of the S gene (70 nucleotides before ORF3a TRS). A gene fragment representing the dual reporter under S TRS was chemically synthesized as a 1.7kb gBlock fragment (Integrated DNA Technologies) in the following in-frame arrangement of reporters: S gene TRS, S signal peptide (MFVFLVLLPLVSSQC), Gaussia luciferase, porcine teschovirus 2A site (ATNFSLLKQAGDVEENPG⇓P), and Neon Green GFP reporter. The genomic sequence between the SanDI site and the S TRS was PCR amplified using PrimeStar Max (Clontech). Both fragments were combined together by fusion PCR and subsequently cloned into the SARS-CoV-2 bacmid cut with SanDI and BamHI enzymes using the HiFi assembly system (NEB). Assembly mixtures were transformed into DH10Bac and individual ΔS-Luc-GFP bacmid clones were identified by restriction digestion of bacmid DNA.

Rescue and amplification of ΔS-VRP(G)

To rescue recombinant ΔS-VRP(G) virus, a mixture of 293T cells/Huh7.5 cells in suspension ($1x10^6$ cells each cell type) were transfected with 4μg of ΔS-Luc-GFP bacmid and 1μg of VSV-G plasmid (Addgene #138479) using PEI transfection reagent (Polysciences; DNA:PEI ratio 1:4). After 5hrs post-transfection, the transfection mixture containing media was replaced with DMEM/2%FBS media. At 72-96hrs post-transfection, supernatants were collected and kept as ΔS-VRP(G) seed stocks. To amplify ΔS-VRP(G), Huh7.5 cells seeded in 150mm plates ($1.5x10^7$) were transfected with 40 μg of VSV-G plasmid using PEI reagent. At 5hrs post-transfection, the transfection mixture containing media was replaced with DMEM/10% FBS media. On the following day, 1ml of ΔS-VRP(G) seed stock was added to VSV-G transfected Huh7.5 cells and incubated for 2hrs. After washing with PBS, infected Huh7.5 cells were placed in 25ml of DMEM/2% FBS, and monitored for GFP expression and cytopathic effects

(CPE). At 48-72h post infection, supernatants were collected, clarified of debris, aliquoted and stored at -80C. These stocks were used to perform subsequent infection experiments.

ΔS-VRP(G) infection

Indicated cell types were seeded in 12-well (1-2x10^5 per well) or 6-well plates (5-8x10^5 per well) a day prior to infection and infected with 0.5ml or 1ml of ΔS-VRP(G) stock, respectively. After 2hr incubation, viral inoculum was removed and replaced with DMEM/2%FBS media, after two PBS washes. At 18hpi, luciferase activity in the supernatant and GFP expression in the cells were measured. For measurement of luciferase activity, 50ul of supernatant was mixed with 50ul of Renilla luciferase substrate (Promega) and immediately measured in a GloMax 20/20 single tube luminometer. GFP images were captured with a Nikon Eclipse microscope using a 20X objective at an exposure time of 600 mSec.

Remdesivir and GC376 inhibition

A549 or Huh7.5 cells seeded at a density of 1.5x10^5 per well (12-well plate) a day prior were infected with 0.5ml of ΔS-VRP(G) stock. After 2hpi, cells were washed with PBS and placed in DMEM/2%FBS media with the indicated amounts of drug. Measurement of luciferase activity and GFP expression were performed as described above at 18hpi.

Immunofluorescence

A day prior to infection, A549 cells were seeded onto glass coverslips at 1x10^5 cells/well in a 24-well plate in Opti-Modified Eagle Medium (Opti-MEM) and infected with 0.5ml of ΔS-VRP(G). After 2hrs, viral inoculum was replaced with Opti-MEM. At 18hpi, infected cells were washed twice with PBS and fixed with 4% Paraformaldehyde (Electron Microscopy Sciences) in PBS for 5 min at room temperature. After washing with PBS, fixed cell were permeabilized with 0.3% Triton X-100 in PBS for 5 min, washed with PBS and incubated in a blocking buffer consisting of 1% BSA, 0.5% fish gelatin (Sigma), and 0.01% Tween20 (Sigma) in PBS for 30 min. Permeabilized cells were incubated in blocking buffer with mouse anti-nucleoprotein antibody (1:1000 from Sino Biological) for 1hr followed by staining with goat anti-mouse Alexa647 secondary antibody for 30min. Coverslips were mounted onto microscopy slides with ProLong Gold Antifade Mountant with DAPI (Invitrogen). All incubations involving antibody staining were performed with gentle shaking at room temperature. Images were acquired on a

Leica DFC7000T microscope under 63x oil-immersion objective using Leica software and processed using ImageJ software.

Western blot analysis

A549 cells seeded in 6-well plates at $1x10^6$ cells/well were infected with 1ml of ΔS-VRP(G) and at 18hpi, cells were lysed in RIPA buffer and protein samples were separated on a 4-15% gradiatent SDS-PAGE gel (Bio-Rad). As controls, A549-hACE2 cells were infected with SARS-COV-2 (Wuhan-Hu-1/2019) at an MOI of 3 and lysed in RIPA buffer. Western blot analysis was performed following the transfer of proteins onto a nitrocellulose membrane using mouse anti-nucleoprotein or S antibody (1:1000 from Sino Biological) and goat α-mouse secondary antibody conjugated to horseradish peroxidase (#GENA931, Sigma).

Quantitative RT-PCR analysis

BMDM and BMDC seeded at a density of $2x10^6$ cells/well were infected with 2ml of ΔS-VRP(G) and at 18hpi, total RNA from infected cells were extracted using PureLink RNA extraction kit according to manufacturer's instructions. Residual genomic DNA contamination was removed by DNase I (Invitrogen Cat #12185010) treatment. cDNA was synthesized using Superscript IV Reverse Transcriptase (Invitrogen Cat #18090010) and Oligo d(T) (Invitrogen) and qPCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystem Cat #4368702) with technical duplicates and gene specific primers (19). 18S RNA was used as an endogenous house housekeeping gene to calculate delta delta cycle thresholds. qPCR primers are as follows - SARS-CoV-2 N forward: CAATGCTGCAATCGTGCTAC and reverse GTTGCGACTACGTGATGAGG; mouse Ifnα1 forward: TCAAAGGACTCATCTGCTGCTTG and reverse CCACCTGCTGCATCAGACAAC; mouse Ifnβ forward: CAGCTCCAAGAAAGGACGAAC and reverse: GGCAGTGTAACTCTTCTGCAT; Mx1 forward: GACCATAGGGGTCTTGACCAA and reverse: AGACTTGCTCTTTCTGAAAAGCC; Isg15 forward: GGTGTCCGTGACTAACTCCAT and reverse: TGGAAAGGGTAAGACCGTCCT: Tnfα forward: GACGTGGAACTGGCAGAAGAG and reverse: TTGGTGGTTTGTGAGTGTGAG; 18S forward: AAACGGCTACCACATCCAAG and reverse: CCTCCAATGGATCCTCGTTA. Results are represented as fold expression relative to mock samples.

Statistical analysis

Significance of data points was assessed using the unpaired Student's t-test. * denotes p-value <0.05 and ns denotes non-significant.

Acknowledgements

We would like to thank Drs. Lok-Yin Roy Wong and Ruangang Pan from the Perlman lab and Dr. Thomas Gallagher for their advice on coronavirus bacmid system.

Figure Legends

Fig 1. Development of single-cycle infectious SARS-CoV-2 replicon system with a dual reporter. (A) Schematic representation of SARS-CoV-2 and ΔS Luc-GFP SARS-CoV-2 genomes. The S gene in the SARS-CoV-2 genome was replaced with a luciferase and GFP dual reporter. (B) Generation and amplification of ΔS virus replicon particles. Left: 293T/Huh7.5 cell mixture was transfected with ΔS Luc-GFP bacmid and VSV-G plasmid. Right: Supernatants from ΔS-VRP(G) rescue transfections were amplified in Huh7.5 cells transfected with VSV-G plasmid. (C-D) Kinetics of luciferase and GFP expression during rescue transfection process. 293T/Huh7.5 cells were co-transfected with ΔS Luc-GFP bacmid and VSV-G plasmid, and at various days post-transfection, luciferase activity in the supernatants and GFP expression in the cell mixture were assessed. On day 6 post-transfection, 293T/Huh7.5 cells were again transfected with additional VSV-G plasmid. (C) Luciferase values and (D) GFP expression are shown at the indicated time points. Luciferase activity is shown for 3 independent Bac clones. GFP expression is shown for Bac clone #7. (E) Immunofluorescence imagining of GFP and nucleoprotein expression in ΔS-VRP(G) infected cells. A549-hACE2 cells were infected with ΔS-VRP(G) and at 18hpi, cells were stained with anti-N antibody and imaged. (F) Western blot analysis of nucleoprotein expression in ΔS-VRP(G) infected cells. A549-hACE2 cells were infected with ΔS-VRP(G) and at 18hpi, cell lysates were collected and analyzed for nucleoprotein and spike protein expression by western blot. Cell lysates from wild type SARS-CoV-2 infected cells were included as controls. β-actin levels are shown as loading controls.

Fig 2. Characterization of ΔS-VRP dual reporter system. (A) ΔS-VRP(G) infection is restricted to a single round. Huh7.5 cells were infected with ΔS-VRP(G) and at 2hpi, cells were washed and incubated in fresh media. At 48h, supernatants (Round 2 - R2 sup) were collected and added to new Huh7.5 cells. At 18hpi, luciferase activity in the supernatant was measured. (B) Comparison of infectivity of different ΔS-VRP(G) preparations. Huh7.5 cells were infected with 3 independent preparations of ΔS-VRP(G) and luciferase activity was measured at 18hpi. (C) Infectivity of ΔS-VRP(G) stored at -80C. Huh7.5 cells were infected with ΔS-VRP(G) stored at -80C or fresh preparations and luciferase activity was measured at 18hpi. (D-E) Neutralization of ΔS-VRP(G) infection by anti-VSV sera. Sera from Control (Cntrl) and VSV-infected mice were pre-incubated with ΔS-VRP(G) for 1 hour and subsequently incubated with

Huh7.5 cells for 2 hours. Luciferase and GFP expression were assessed at 18hpi. For panels B-E, luciferase activity in the supernatants was measured and is shown as relative light units (RLU). For panel G, luciferase activity was normalized to No Treatment control and is shown as percentage (%) of No Treatment control.

Fig 3. Permissiveness of human and murine cells to ΔS Luc-GFP replication. The indicated human and murine cells were infected with ΔS-VRP(G) and at 18hpi, luciferase activity and GFP expression were measured. (A-B) Luciferase expression in human and murine cell lines. (C-D) GFP expression in human and murine cell lines. (E-F) Primary BMDM and BMDC were infected with ΔS-VRP(G) and at 18hpi, expression of viral N mRNA and host antiviral genes were measured by qRT-PCR. (E) Viral N mRNA expression and (F) host antiviral gene expression.

Fig 4. ΔS-VRP(G) reporter system is suitable for antiviral drug screening. Huh7.5 or A549 cells were infected with ΔS-VRP(G) virus and at 2hpi, infected cells were treated with the indicated concentrations of Remdesivir or GC376 dissolved in DMSO. At 18hpi, GFP expression and luciferase activity were measured. (A-B) Assessment of effects of Remdesivir treatment on ΔS Luc-GFP replication in Huh7.5 and A549 cells. (C & D) Assessment of effects of GC376 treatment on ΔS Luc-GFP replication in Huh7.5 and A549 cells. Luciferase values are normalized to DMSO control and shown as % DMSO control.

References

1. Friedrich B, Scully C, Brannan J. 2011. Assessment of High-Throughput Screening (HTS) Methods for High-Consequence Pathogens. Journal of Bioterrorism & Biodefense 02.
2. Rasmussen L, Tigabu B, White EL, Bostwick R, Tower N, Bukreyev A, Rockx B, LeDuc JW, Noah JW. 2015. Adapting high-throughput screening methods and assays for biocontainment laboratories. Assay Drug Dev Technol 13:44-54.
3. Anonymous. Select Agents and Toxins Exclusions: Excluded Attenuated Strains of HHS Select Agents, Section 73.3 (e).
4. Kuroda M, Halfmann PJ, Hill-Batorski L, Ozawa M, Lopes TJS, Neumann G, Schoggins JW, Rice CM, Kawaoka Y. 2020. Identification of interferon-stimulated genes that attenuate Ebola virus infection. Nat Commun 11:2953.
5. Centers for Disease C, Prevention, Gangadharan D, Smith J, Weyant R. 2013. Biosafety Recommendations for Work with Influenza Viruses Containing a Hemagglutinin from the A/goose/Guangdong/1/96 Lineage. MMWR Recomm Rep 62:1-7.
6. Galvan EM, Nair MK, Chen H, Del Piero F, Schifferli DM. 2010. Biosafety level 2 model of pneumonic plague and protection studies with F1 and Psa. Infect Immun 78:3443-53.
7. Crawford KHD, Eguia R, Dingens AS, Loes AN, Malone KD, Wolf CR, Chu HY, Tortorici MA, Veesler D, Murphy M, Pettie D, King NP, Balazs AB, Bloom JD. 2020. Protocol and Reagents for Pseudotyping Lentiviral Particles with SARS-CoV-2 Spike Protein for Neutralization Assays. Viruses 12.
8. Dieterle ME, Haslwanter D, Bortz RH, 3rd, Wirchnianski AS, Lasso G, Vergnolle O, Abbasi SA, Fels JM, Laudermilch E, Florez C, Mengotto A, Kimmel D, Malonis RJ, Georgiev G, Quiroz J, Barnhill J, Pirofski LA, Daily JP, Dye JM, Lai JR, Herbert AS, Chandran K, Jangra RK. 2020. A Replication-Competent Vesicular Stomatitis Virus for Studies of SARS-CoV-2 Spike-Mediated Cell Entry and Its Inhibition. Cell Host Microbe 28:486-496 e6.
9. Case JB, Rothlauf PW, Chen RE, Liu Z, Zhao H, Kim AS, Bloyet LM, Zeng Q, Tahan S, Droit L, Ilagan MXG, Tartell MA, Amarasinghe G, Henderson JP, Miersch S, Ustav M, Sidhu S, Virgin HW, Wang D, Ding S, Corti D, Theel ES, Fremont DH, Diamond MS, Whelan SPJ. 2020. Neutralizing Antibody and Soluble ACE2 Inhibition of a Replication-Competent VSV-SARS-CoV-2 and a Clinical Isolate of SARS-CoV-2. Cell Host Microbe 28:475-485 e5.
10. Nguyen HT, Falzarano D, Gerdts V, Liu Q. 2021. Construction of a non-infectious SARS-CoV-2 replicon for antiviral drug testing and gene function studies. J Virol doi:10.1128/JVI.00687-21:JVI0068721.
11. He X, Quan S, Xu M, Rodriguez S, Goh SL, Wei J, Fridman A, Koeplinger KA, Carroll SS, Grobler JA, Espeseth AS, Olsen DB, Hazuda DJ, Wang D. 2021. Generation of SARS-CoV-2 reporter replicon for high-throughput antiviral screening and testing. Proc Natl Acad Sci U S A 118.
12. Luo Y, Yu F, Zhou M, Liu Y, Xia B, Zhang X, Liu J, Zhang J, Du Y, Li R, Wu L, Zhang X, Pan T, Guo D, Peng T, Zhang H. 2021. Engineering a Reliable and Convenient SARS-CoV-2 Replicon System for Analysis of Viral RNA Synthesis and Screening of Antiviral Inhibitors. mBio 12.
13. Zhang X, Liu Y, Liu J, Bailey AL, Plante KS, Plante JA, Zou J, Xia H, Bopp NE, Aguilar PV, Ren P, Menachery VD, Diamond MS, Weaver SC, Xie X, Shi PY. 2021. A trans-

complementation system for SARS-CoV-2 recapitulates authentic viral replication without virulence. Cell 184:2229-2238 e13.
14. Ju X, Zhu Y, Wang Y, Li J, Zhang J, Gong M, Ren W, Li S, Zhong J, Zhang L, Zhang QC, Zhang R, Ding Q. 2021. A novel cell culture system modeling the SARS-CoV-2 life cycle. PLoS Pathog 17:e1009439.
15. Syed AM, Taha TY, Tabata T, Chen IP, Ciling A, Khalid MM, Sreekumar B, Chen PY, Hayashi JM, Soczek KM, Ott M, Doudna JA. 2021. Rapid assessment of SARS-CoV-2 evolved variants using virus-like particles. Science doi:10.1126/science.abl6184:eabl6184.
16. Almazan F, Marquez-Jurado S, Nogales A, Enjuanes L. 2015. Engineering infectious cDNAs of coronavirus as bacterial artificial chromosomes. Methods Mol Biol 1282:135-52.
17. Kandasamy M, Suryawanshi A, Tundup S, Perez JT, Schmolke M, Manicassamy S, Manicassamy B. 2016. RIG-I Signaling Is Critical for Efficient Polyfunctional T Cell Responses during Influenza Virus Infection. PLoS pathogens 12:e1005754.
18. Schmolke M, Manicassamy B, Pena L, Sutton T, Hai R, Varga ZT, Hale BG, Steel J, Perez DR, Garcia-Sastre A. 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. PLoS pathogens 7:e1002186.
19. Han J, Perez JT, Chen C, Li Y, Benitez A, Kandasamy M, Lee Y, Andrade J, tenOever B, Manicassamy B. 2018. Genome-wide CRISPR/Cas9 Screen Identifies Host Factors Essential for Influenza Virus Replication. Cell Rep 23:596-607.