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Expression and fusogenic activity of SARS CoV-2 Spike protein displayed in the

HSV-1 Virion.

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12   **Abstract**

13

14   Severe acute respiratory syndrome coronavirus (SARS-CoV) is a zoonotic pathogen that

15   can cause severe respiratory disease in humans. The new SARS-CoV-2 is the cause of

16   the current global pandemic termed coronavirus disease 2019 (COVID-19) that has

17   resulted in many millions of deaths world-wide. The virus is a member of the

18   Betacoronavirus family, its genome is a positive strand RNA molecule that encodes for

19   many genes which are required for virus genome replication as well as for structural

20   proteins that are required for virion assembly and maturation. A key determinant of this

21   virus is the Spike (S) protein embedded in the virion membrane and mediates attachment

22   of the virus to the receptor (ACE2). This protein also is required for cell-cell fusion

23   (syncytia) that is an important pathogenic determinant. We have developed a

24   pseudotyped herpes simplex virus type 1 (HSV-1) recombinant virus expressing S protein

25   in the virion envelop. This virus has also been modified to express a Venus fluorescent

26   protein fusion to VP16, a virion protein of HSV-1. The virus expressing Spike can enter

27   cells and generates large multi-nucleated syncytia which are evident by the Venus

28   fluorescence. The HSV-1 recombinant virus is genetically stable and virus amplification

29   can be easily done by infecting cells. This recombinant virus provides a reproducible

30   platform for Spike function analysis and thus adds to the repertoire of pseudotyped

31   viruses expressing Spike.

32

35 **Impact Statement**

36 The isolation of a pseudotyped herpes simplex virus type 1 (HSV-1) virus using the

37 Spike protein is new and innovative. This virus can be used to study entry and fusion

38 events mediated by the S protein as well as test antibodies for their ability to neutralize

39 this particle. In addition, these virions can be used for screening antibody specificity

40 using the S protein displayed in its natural membrane bound conformation.

41   Coronaviruses are enveloped, non-segmented, positive-sense RNA viruses that carry a

42   ~30,000 nucleotide genome [1-4]. The spherical structural particle of the virus is about

43   80-125 nm in diameter and the virus envelop is embedded with three membrane proteins:

44   the spike (S) protein that gives the virus the "corona" structure [5], and the envelope (E)

45   and membrane (M) proteins [1, 2]. The virus membrane envelops the RNA genome which

46   is encapsidated by the nucleocapsid (N) protein. The spike protein engages the

47   angiotensin converting enzyme-2 (ACE2) receptor [6, 7] and acts to fuse viral and cellular

48   membranes during entry [8, 9]. The E protein plays roles in virus assembly and budding

49   but also has other roles. The M protein is the most abundant envelop protein and

50   coordinates virus assembly and budding through protein-protein interactions with the

51   other virion components [10, 11]. The N protein with the viral genome form the

52   ribonucleoprotein core and has been shown to be involved in viral RNA synthesis,

53   transcriptional regulation of genomic RNA, translation of viral proteins, and budding [3].

54   The Spike protein is the structural protein responsible for the crown-like shape of the CoV

55   viral particles because it forms a trimeric complex. The 1255 aa long protein (~185 kD

56   glycosylated polypeptide) is a class-I viral fusion protein [12] and contributes to the cell

57   receptor binding, tissue tropism and pathogenesis. The Spike protein is cleaved by host

58   cell proteases at the S1/S2 cleavage site [5, 13-15]. Following cleavage, also known as

59   priming, the protein is divided into an N-terminal S1-ectodomain that recognizes the

60   cognate cell surface receptor and a C-terminal S2-membrane-anchored protein involved

61   in viral entry by membrane fusion. The S1-protein contains a conserved Receptor Binding

62   Domain (RBD), which recognizes the angiotensin-converting enzyme 2 (ACE2) receptor

63   [16]. Cell-cell fusion resulting in syncytia formation (multi-nucleated cell) is a characteristic

64   property of the Spike protein [17-19]. Syncytia formation also likely contributes to the

65   pathology of the disease as observed by the presence of multinucleate pneumocytes in

66   patients with advanced disease [20-25]. The biological processes that define Spike

67   protein in the virion binding to the cell (entry) and Spike protein in infected cell membrane

68   fusing with uninfected cells (syncytia formation) are similar in that they both require

69   binding to ACE2 receptor and proteolytic activation to expose the fusion peptide [18, 26-

70   29]. However, differences between these two processes are evident and the means to

71   inhibit them may similarly differ [30, 31]. Cell-cell transmission of the virus is also a means

72   by which the virus can evade some neutralizing antibodies [31, 32].

73

74   Our goal was to leverage our expertise in membrane protein display and self-assembly

75   of virion structures to develop and create tractable models to study this highly pathogenic

76   virus. Our first goal was to use herpes simplex virus type 1 (HSV-1) to express and display

77   the membrane proteins of SARS-CoV-2. Use of HSV-1 to display Spike protein could be

78   useful for investigation for serology, monoclonal antibody screening/specificity testing and

79   as pseudotyped HSV-1 virions that can be used safely to examine entry inhibition and

80   virus neutralization. The pseudotyped virus is also genetically stable, does not have to

81   be re-made each time by transfection methods and can be used safely in a BSL2 level

82   facility.

83

84   Previously, we have expressed and displayed membrane proteins to provide novel

85   platforms and tools for investigation of their functional activities using the Virion Display

86   (VirD) method [33-35]. HSV-1 produces large spherical virions displaying hundreds of

87   copies of envelop proteins. Our aim was to engineer this virus to express human

88   membrane proteins during the virus productive cycle and incorporate the human proteins

89   into the virion during the assembly process. This was achieved by cloning the membrane

90   protein gene in place of the glycoprotein B gene of HSV-1 (UL27) such that the expression

91   of the human membrane protein is driven by the gB (UL27) promoter. Because the gene

92   is now expressed as a "viral" gene it was subsequently incorporated into the virion

93   envelop during virus assembly. The expression of the human membrane proteins in

94   infected cells, at the cell surface and in purified virions, was in the correct transmembrane

95   orientation, and the proteins are biochemically functional [33, 34]. Subsequently, we

96   engineered the HSV-1 genome to be Gateway compatible by inserting the Gateway

97   selection cassette in the UL27 gene locus. This locus encodes glycoprotein B of HSV-1

98   which is the major mediator of cell fusion. Membrane protein ORFs can be cloned into

99   this site using standard Gateway cloning methods [34].

100

101   We obtained a codon optimized Spike protein ORF from BEI resources (WuHan strain).

102   Plasmid pCAGGS was used as a template to PCR amplify the ORF using Q5 (NEB)

103   polymerase. The primers contained the attB recombination sequences compatible with

104   Gateway cloning. A BP reaction was performed using BP clonase (Invitrogen) and

105   pDONR221. Transformants were screened for the recombined ORF and four clones were

106   sequenced. These four validated clones were used to transfer the Spike ORF into the

107   HSV-1 strain KOS bacmid using LR clonase (Invitrogen) to derive four recombinant

108   viruses S1- S4. The HSV-1 bacmid carries a Gateway cassette such that the expression

109   of the cloned membrane protein is driven by the gB (UL27) promoter and the C-terminus

110   is tagged with a V5 epitope sequence so we can monitor the expression of the protein.

111   Gateway cloning methodology has been described in more detail in Syu *et al*. [34].

112   Reconstitution of infectivity of the HSV-1 bacmid was performed as previously described

113   using the gB complementing cell line, D87 [34]. D87 is a Vero cell line that expresses gB

114   upon superinfection. All cell lines and virus stocks were prepared as described by Desai

115   *et al.* [36].

116

117   To examine expression of the Spike protein, Vero cells (5 x 10^5) were infected at a

118   multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell. The infected cells were

119   harvested at 24 hour post-infection and protein lysates prepared in Laemmli buffer.

120   Proteins were analyzed on 4-12% NuPage gels (Invitrogen) and transferred to

121   nitrocellulose membranes using the iBlot instrument (Invitrogen) as previously described

122   [37]. The blots were reacted with mouse anti-V5 antibody (Invitrogen). Abundant

123   quantities of the Spike protein were observed in the lysates of all four isolates (Fig. 1).

124   The Spike protein is 1255 amino acids long and is predicted to have a molecular weight

125   of approximately 185 kD (glycosylated). We also observed a proteolytic cleaved product

126   that is V5 reactive.

127

128   To examine whether the Spike protein was incorporated into HSV-1 virions, Vero cells (1

129   x 10^7) were infected with the recombinant viruses at an MOI of 10 PFU/cell. The

130   supernatants of the infected cells were collected at 24 hours post-infection and layered

131   onto a 20% sucrose cushion. This was centrifuged at 39 K for 20 minutes in a Beckman

132   SW41 rotor. The virion pellet was resuspended in Laemmli buffer and analyzed by

133   immunoblot as described above. Immunoblot data show the Spike protein is incorporated

134   into HSV-1 extracellular virions (Fig. 2). Both in the infected cells and more so in the virion

135   we observed a cleavage product that is V5 tagged (Fig 2). We assume this is the S2

136   polypeptide and it has a mobility around 90 kD. SARS-CoV-2 Spike protein is

137   proteolytically cleaved first by furin at the S1/S2 site and subsequently at the S' position

138   by serine protease 2, TMPRSS2 [38, 39]. In Vero cells, furin is likely the major processing

139   enzyme although endogenous levels of TMPRSS2 may also cleave the Spike protein

140   [14]. Our analysis cannot resolve that with the HSV-1 Spike protein expression seen in

141   Fig. 2, however, the cleavage that we observed was functional for syncytia formation.

142   Mutations in the polybasic cleavage site have been observed upon passage of the virus

143   in culture (Vero cells) and there is debate about role of the furin cleavage site for infectivity

144   [28, 40-42].

145

146   We also recombined into the S3 virus, a VP16-Venus marker which allows one to follow

147   HSV-1 virus entry and replication [43]. VP16 is a major virion (tegument) protein of HSV-

148   1. VP16 localizes to nuclear puncta and then subsequently also at cytoplasmic sites

149   during the infectious cycle. Recombination was done by co-infecting D87 cells with the

150   S3 virus and a virus that expresses VP16-Venus as well as mutations in UL16 and UL21

151   that are lethal [43]. The virus from the co-infected cells was plaqued on D87 cells. Plaques

152   that formed on D87 cells that were also fluorescent were purified further. Subsequently

153   an S3 virus expressing VP16-Venus was amplified and used in the experiments below.

154

155 In the recombinant HSV-1 virus, the cloned gene is in the UL27 locus which encodes

156 glycoprotein B (gB) [35]. This HSV-1 recombinant cannot replicate because of the loss of

157 the essential UL27 gene [44, 45]. Glycoprotein B of HSV-1 is the major fusogen for HSV-

158 1 and facilitates fusion of virion and cell membranes following receptor binding [46, 47].

159 Thus, we also investigated whether the SARS Spike protein can provide this function to

160 HSV-1 virions that cannot fuse and enter, essentially pseudotyping HSV-1. We took

161 virions that lack gB but now have Spike protein in their membranes and infected Vero

162 cells. We observed VP16-Venus fluorescence in single cells indicative of virus entry.

163 These initial fluorescent puncta progressed to large syncytia as time passed (Fig. 3A).

164 Syncytia formation in cells infected by these viruses indicated cell-cell fusion mediated by

165 S protein, was also functional in our assay.

166

167 We compared different cell lines to examine syncytium formation, Fig. 3B. Vero cells are

168 typically used for propagation and plaquing of the HSV-1 Spike virus. We also used two

169 cell lines transduced with the ACE2 expression vector, HEK-293T and a human lung cell

170 line A549 [48]. Vero and Vero E6 cells were the best cell lines for visualization of cell-cell

171 fusion. What was astonishing is the size of the syncytia formed in these cells. Therefore,

172 this was a fortuitous tag that visually illuminates the syncytium.

173

174 It has been reported that the HIV protease inhibitor, nelfinavir can inhibit cell fusion

175 mediated by the Spike protein [49]. We also tested this using the recombinant virus S3-

176 Venus. At concentrations of 15 and 20 uM, nelfinavir inhibited cell fusion mediated by the

177 Spike protein (data not shown).

178

179 The major goals of this investigation was to establish a pseudovirus system for SARS

180 CoV-2 that offers a safe tool to study virus entry and membrane fusion. The isolation of a

181 pseudotyped HSV-1 virus using the Spike protein is innovative. Futhermore, this reagent

182 provides a powerful platform to elucidate antibody responses, functional activities, identify

183 neutralizing monoclonal antibodies and therapeutic approaches. We have already

184 demonstrated we can generate S protein displaying virions and they have biological

185 activity in that they support virus entry and extensive cell-cell fusion. In addition, these

186 virions can be used for screening antibody specificity using the S protein displayed in its

187 natural membrane bound conformation. While the Knipe Lab made an HSV-1

188 recombinant expressing S protein, theirs was a replication defective mutant and thus does

189 not produce virions [50]. Their goals were to investigate host responses to S protein

190 expressed in different human cell lines.The pseudotyped virus is also genetically stable,

191 does not have to be re-made each time by transfection methods, unlike the lentivirus

192 based methods, and can be used safely in a BSL2 facility.

193

Conflicts of interest

The author declares that there are no conflicts of interest.

205 **Figure Legends**

206

207 **Fig. 1.** Expression of SARS-CoV-2 Spike protein in the HSV-1 KOS strain. The Spike

208 protein encoding gene was cloned into the UL27 locus of HSV-1. The ORF is fused at the

209 C-terminus with a V5 epitope tag. Four isolates were purified and amplified for

210 investigation. Cells were infected with all four viruses and harvested 24h after infection.

211 The protein lysates were examined for Spike protein expression using immunoblot

212 methods and V5 antibody. Protein with the correct mobility was detected in all four lysates.

213 In addition, a cleavage product was also present in each lysate and of approximately 90

214 kD in size. Protein standards are in the left lane.

215

216 **Fig. 2.** Incorporation of the SARS-CoV-2 Spike protein in HSV-1 virions. Virions in the

217 extracellular media were collected and purified through a sucrose cushion. The virions

218 were then analyzed by immunoblot methods using V5 antibody. Infected cell lysates were

219 included as positive controls. S2, S3 and S4 virions all contained Spike protein Wild-type

220 (WT) virions do not. A cleavage product (arrow) with a mobility of approximately 90 kD

221 was also present in the virions. Protein standards are in the left lane.

222

223 **Fig. 3.** Panel A. HSV-1 fluorescent tagged virus pseudotyped with Spike protein. A VP16-

224 Venus fluorescent fusion protein tag was incorporated into the genome of the S3 HSV-1

225 virus. Virus was plaqued on the complementing D87 cell line and visualized by a

226 fluorescence microscope. This virus following infection of Vero cells formed extensive

227 syncytia indicative of cell-cell fusion. Panel B. Syncytia formation in different cell lines.

228   Cells were infected with HSV-1 pseudotyped with Spike protein. Imaging was done by

229   fluorescence microscopy using a Zoe microscope (BioRad) using the X10 objective lens.

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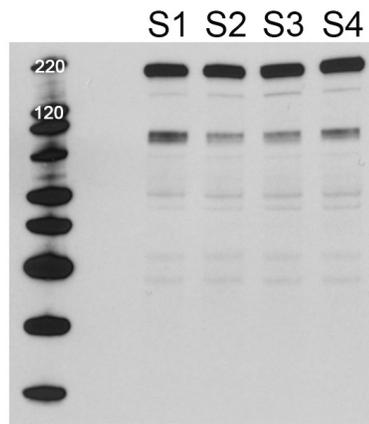
Fig. 1. Expression of SARS-CoV-2 Spike protein in the HSV-1 KOS strain. The Spike protein encoding gene was cloned into the UL27 locus of HSV-1. The ORF is fused at the C-terminus with a V5 epitope tag. Four isolates were purified and amplified for investigation. Cells were infected with all four viruses and harvested 24h after infection. The protein lysates were examined for Spike protein expression using immunoblot methods and V5 antibody. Protein with the correct mobility was detected in all four lysates. In addition, a cleavage product was also present in each lysate and of approximately 90 kD in size. Protein standards are in the left lane.
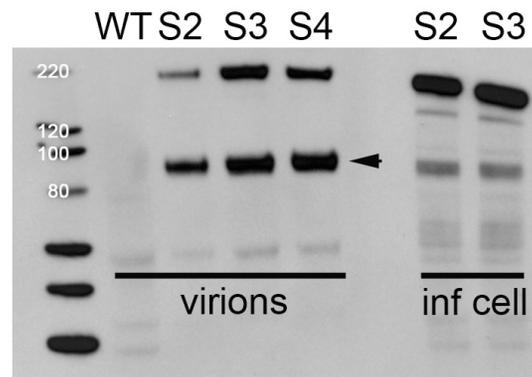
Fig. 2. Incorporation of the SARS-CoV-2 Spike protein in HSV-1 virions. Virions in the extracellular media were collected and purified through a sucrose cushion. The virions were then analyzed by immunoblot methods using V5 antibody. Infected cell lysates were included as positive controls. S2, S3 and S4 virions all contained Spike protein Wild-type (WT) virions do not. A cleavage product (arrow) with a mobility of approximately 90 kD was also present in the virions. Protein standards are in the left lane.
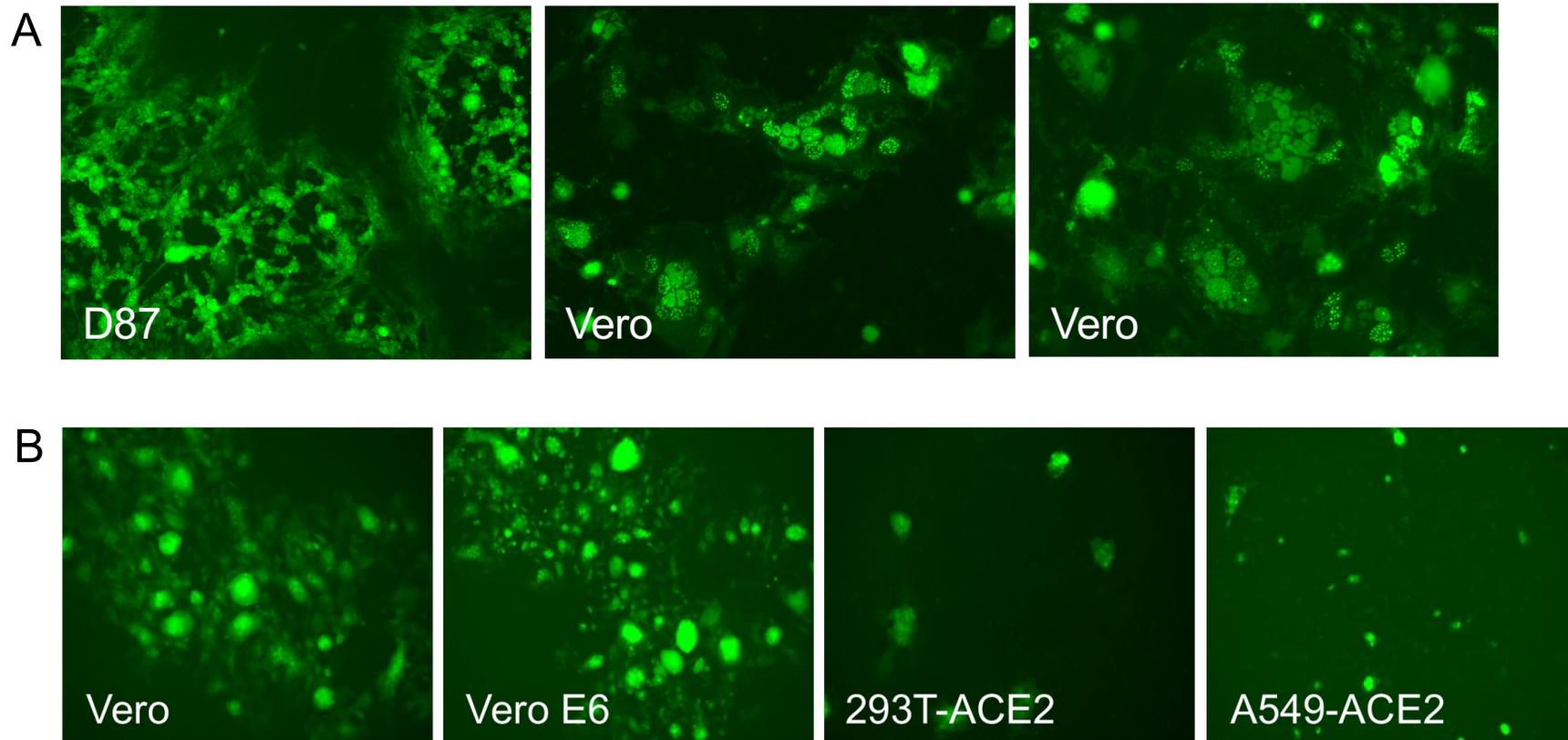
Fig. 3. Panel A. HSV-1 fluorescent tagged virus pseudotyped with Spike protein. A VP16-Venus fluorescent fusion protein tag was incorporated into the genome of the S3 HSV-1 virus. Virus was plaqued on the complementing D87 cell line and visualized by a fluorescence microscope. This virus following infection of Vero cells formed extensive syncytia indicative of cell-cell fusion. Panel B. Syncytia formation in different cell lines. Cells were infected with HSV-1 pseudotyped with Spike protein. Imaging was done by fluorescence microscopy using a Zoe microscope (BioRad) using the X10 objective lens.