The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles.

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5   **efficient assembly, trafficking and release of virus-like particles.**

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Abstract

Production of virus-like particles (VLPs) constitutes a relevant and safe model to study molecular determinants of virion egress. The minimal requirement for assembly of VLPs for the coronavirus responsible for severe acute respiratory symdrome in humans (SARS-CoV) is still controversial. Recent studies have described that SARS-CoV VLP formation depends on either M and E or M and N proteins. Here we show that both E and N must be co-expressed with M for efficient production and release of VLPs by transfected Vero E6 cells. This result suggests that the mechanism of SARS-CoV assembly differs from other studied coronaviruses, which only require M and E for VLP formation. When co-expressed, the native envelope trimeric S glycoprotein is incorporated onto VLPs. Interestingly, when a fluorescent protein tag is added to the C-terminal end of N or S, but not M, the chimeric viral proteins can be assembled within VLPs and allow visualization of VLP production and trafficking in living cells by state of the art imaging technologies. Fluorescent VLPs will be used further to investigate the role of cellular machineries during SARS-CoV egress.

Introduction

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51

52   Coronaviruses are positive-sense RNA enveloped viruses that belong to the

53   *Coronaviridae* family in the Nidovirales order. These viruses are found in a wide

54   variety of animals and can cause respiratory and enteric disorders of diverse

55   severity (15, 18). In the past five years, several human and animal coronaviruses

56   have been discovered, including the highly pathogenic virus responsible for the

57   severe acute respiratory syndrome (SARS-CoV) (34, 58, 60, 64, 68, 69).

58   Coronavirus particles consist of a helical nucleocapsid structure, formed by the

59   association between nucleocapsid (N) phosphoproteins and the viral genomic

60   RNA, which is surrounded by a lipid bilayer where three or four types of structural

61   proteins are inserted: the spike (S), the membrane (M), the envelope (E) proteins

62   and, for some coronaviruses only, the hemagglutinin-esterase (HE) protein (see

63   for review: (12). Once sufficient amounts of new genomic RNA and structural

64   proteins have been produced, assembly of particles occurs. Assembly and release

65   of virions are the last stages of the virus life cycle.

66   The triple spanning membrane glycoprotein M drives assembly of coronavirus,

67   which bud into the lumen of the endoplasmic reticulum-Golgi intermediary

68   compartment (ERGIC) (32, 33, 62, 63). M is the most abundant envelope protein

69   that sorts viral components to be incorporated into virions. M oligomerisation,

70   mainly driven by its transmembrane domain, is believed to allow formation of a

71   lattice of M proteins at ERGIC membranes (14, 41). S and E membrane proteins

72   are integrated into the lattice through lateral interactions with M, whereas N and

73   viral RNA interact with M C-terminal domain, which is exposed to the cytosol (4, 8,

74   13, 19, 30, 36, 48, 54, 55). The coronavirus S protein, responsible for receptor

3

75 binding and membrane fusion, does not seem to have any major role in

76 coronavirus assembly. Recent studies show that E is a viroporin that forms ion

77 channels (46, 66, 67). Despite its minor incorporation into virion particles (7, 22,

78 40), the small E protein plays an important but not fully understood role in virus

79 morphogenesis and budding (20, 35, 70). Studies performed on coronaviruses,

80 including the SARS-CoV, demonstrate that depletion of the E gene from

81 coronavirus genome strongly diminish virus growth and particle formation (9, 16,

82 35, 37, 57). The N protein self-associates and encapsidates the RNA genome for

83 incorporation into budding virions through interactions with the M protein

84 independently of E and S proteins (24, 52, 53, 61). For SARS-CoV, the interaction

85 of N with M was described to be independent of viral RNA (25, 45).

86 Work on mouse hepatitis virus (MHV), bovine coronavirus (BcoV), infectious

87 bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV) has

88 established that E and M proteins are necessary and sufficient for assembly of

89 virus-like particles (VLPs), which share size and morphological features with real

90 viruses (1, 2, 7, 8, 38, 65). Nevertheless, the minimal requirement for assembly of

91 SARS-CoV VLPs is still controversial. Huang Y. and colleagues described

92 formation of VLPs in transfected human 293 renal epithelial cells that only required

93 co-expression of the M and N viral proteins (29). On the contrary, other studies

94 described that co-expressed M and E proteins were sufficient for release of

95 sedimentable particles from transfected mammalian cells (27) or insect cells, using

96 a baculovirus expression system (26, 50). A few groups have proposed

97 immunization with SARS-CoV VLPs as an effective vaccine strategy. VLPs

98 produced in insect cells or chimeric MHV/SARS-CoV VLPs produced in

99 mammalian cells were used in these studies (42, 44).

4

100   Our objective was to delineate the molecular mechanisms that regulate SARS-

101   CoV egress in mammalian cells. Here we demonstrate that whereas VLPs could

102   hardly be recovered from culture medium of cells co-expressing combinations of M

103   and E or M and N proteins, co-expression of both SARS-CoV E and N with M

104   allowed release of significant levels of VLPs within one day. When co-expressed,

105   the trimeric S protein was found in VLPs. Therefore, in apparent contrast to other

106   coronaviruses, SARS-CoV egress strongly depends on three structural proteins:

107   M, E and N. Addition of fluorescent tags to viral structural proteins allowed us to

108   visualize egress of fluorescent VLPs in living cells. Monitoring of VLPs egress in

109   living cells will be a powerful new tool to study the involvement of viral and cellular

110   factors during the late stages of the SARS-CoV life cycle.

111

112 **Materials and Methods**

113

114 ***Cells and culture conditions.*** The Vero E6 African Green Monkey kidney cell line

115 was cultured in Dulbecco's modified Eagle's medium supplemented with 10%

116 heat-inactivated foetal calf serum, 100 U of penicillin and 100   g of streptomycin

117 per ml, at 37°C with 5% CO_2.

118

119 ***Plasmid constructions.*** CDNAs coding for the SARS-CoV M, E, S and N

120 structural proteins were codon-optimized for mammalian cells and synthesized by

121 GeneArt (Regensburg, Germany). The M cDNA was amplified by PCR and sub-

122 cloned into the pIRES plasmid vector (BD Biosciences, San Jose, CA, USA)

123 between the Nhe I and EcoR I restriction sites of the upstream multiple cloning

124 site. The E cDNA was amplified by PCR and inserted either into the pcDNA3.1

125 plasmid vector between the Kpn I and Not I restriction sites or into the pIRES

126 plasmid vector between the Sal I and Not I restriction sites of the downstream

127 multiple cloning site. The S cDNA was amplified by PCR and inserted either into

128 the pcDNA3.1 plasmid vector between the Nhe I and Apa I restriction sites. The

129 pcDNA-Nflag plasmid was constructed from the original pCRScript-Nflag

130 (produced by GeneArt, Regensburg, Germany) using the Kpn I and Xho I

131 restriction sites. The Flag tag was in fusion with the 3' end of N cDNA and

132 separated from N cDNA by six nucleotides encoding two glycine residus. The

133 cDNAs coding for the enhanced yellow (eYFP), cyan (eCFP), green (eGFP)

134 fluorescent proteins and the monomeric red fluorescent protein (mRFP1, (5), were

135 amplified by PCR from plasmids purchased from Clontech Laboratories (Takara

136 Bio, Shiga, Japan) and subcloned into pcDNA3.1 or pIRES vectors. Then S, N or

6

137 M cDNAs were inserted in 5' of the fluorescent protein cDNAs using Cla I and Apa

138 I sites (S and N) or Xho I and Mlu I (M) restriction sites. The two fused cDNAs

139 were separated by two codons encoding glycines.

140

141 **Antibodies.** The M and E proteins were detected with rabbit polyclonal antibodies

142 raised against the C-terminal extremity of each protein. The rabbit polyclonal

143 antibody against the M C-terminal domain was purchased from ProSci

144 Incorporated (Poway, CA, USA). The rabbit polyclonal antibody against the E

145 protein was produced by Dr Nicolas Escriou (Institut Pasteur, Paris) using a C-

146 terminal peptide. The Flag tag was detected with the mouse monoclonal IgG1 M2

147 antibody purchased from Sigma-Aldrich. The mouse polyclonal antibody against

148 the S protein was obtained by immunizing mice with purified S trimers expressed

149 in mammalian cells as described previously (31). The mouse monoclonal antibody

150 against the N protein was a generous gift from Dr Chan KH (Department of

151 Microbiology, the University of Hong Kong) and produced as described in (56).

152

153 **Transient transfections and production of SARS-CoV VLPs.** Eight hundred

154 thousand cells were plated in 75 cm^2 dishes, incubated over-night and transfected

155 with plasmid constructs using the FuGENE 6 transfection reagent (Roche, Basel,

156 Switzerland), according to the manufacturer's instructions. Briefly, 54 μl of

157 FuGENE 6 transfection reagent were mixed with DMEM, incubated for five

158 minutes and then 6 μg of each plasmid were added. The FuGENE 6 / plasmid

159 mixture was incubated for 30 minutes at room temperature. Cell medium was

160 discarded and replaced with 3 ml of warm DMEM. The FuGENE 6 / plasmid

161 mixture was added to the cells. After 3 hours of incubation at 37°C, the medium

7

162 containing the transfection mixture was discarded and 10 ml of fresh medium was

163 added. Cells were incubated for 21 or 45 hours.

164

165 ***Purification of SARS-CoV VLPs.*** At 24 or 48 hours post-transfection, cell

166 medium was collected and cleared by centrifugation at low speed (1000g for 10

167 minutes) to pull-down cell debris. After passing through 0.45 μm filter, cleared cell

168 medium were then loaded on top of 20% sucrose cushions and ultra-centrifuged

169 for 3 hours at 28,000 rpm using a SW41 rotor (Belkman Coulter Inc, Fullerton, CA,

170 USA). VLP-containing pellets were resuspended in Tris HCl 50 mM NaCl 100 mM

171 EDTA 0.5 mM pH7.4 (TNE) buffer.

172

173 ***Western blot analysis of VLPs and cell lysates.*** For Western blot analysis of

174 purified VLPs, 15 μl of resuspended pellets from ultracentrifuged culture medium

175 were mixed with 5 μl lithium dodecyl sulfate (LDS)-containing loading buffer and

176 loaded on 4 to12 % polyacrylamide gels (NuPAGE Novex Bis-Tris Mini Gels,

177 Invitrogen, Carlsbad, CA, USA). Electrophoresis was performed with the NuPAGE

178 MOPS SDS Running Buffer from the same manufacturer. Alternatively,

179 resuspended pellets from 48 hours time points were loaded on top of 20 to 60%

180 sucrose gradients and ultracentrifuged for 3.5 hours at 26,700 rpm (27). Twenty

181 fractions were collected and analyzed by Western blot. For Western blot analysis

182 of cell lysates, cells were washed twice with PBS 1X at 24 or 48 hours post-

183 transfection, and lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl,

184 20 mM Tris-HCl pH7.5 and 1 mM EDTA for 15 minutes on ice with frequent

185 vortexing. Then lysates were cleared by centrifugation at 16100 g for 15 minutes

186 at 4°C and analyzed by Western blot. 15 μl of each lysate were mixed with 5 μl

8

187 LDS loading buffer and loaded on polyacrylamide gels. For detection of E but S,

188 samples were treated with dithiothreitol and heated at 95°C for 5 minutes before

189 migration on polyacrylamide gels. To detect both E and trimers of S from same

190 preparations, samples were split and either treated or not before loading of two

191 separate gels for E and S detection, respectively. Results for M and Nflag were

192 similar with or without treatment.

193

194 **Electron microscopy.** For transmission electron microscopy experiments,

195 transfected cells were harvested at 24 and 48 hours post-transfection. Cells were

196 detached using 10 mM EDTA, fixed in 2.5 % glutaraldehyde in cacodylate buffer

197 and post-fixed with 1% Osmium Tetroxide (OsO4) in Cacodylate buffer for 1 hour

198 at room temperature. Then cells were embedded in 2% agarose to form cell blocks

199 which were then dehydrated in graded series of ethanol and embedded in epoxy

200 resin. Ultrathin sections were stained for 45 minutes with 2% aqueous uranyl

201 acetate and for 30 minutes with Reynolds' lead citrate. For analysis of secreted

202 VLPs, VLPs were purified from cell medium by ultracentrifugation on 20% sucrose

203 cushion, separated on 20-60% discontinuous sucrose gradient and fraction 10 was

204 collected and then concentrated by ultracentrifugation in TNE buffer, pH 7.4 for

205 one hour at 28,000 rpm. 5µl of the VLP suspension, mixed with equal volume of

206 negative stain (2% aqueous uranyl acetate and 2% phosphotungstate solution, pH

207 7.0), was placed onto a formvar-carbon-coated copper grid for 2 minutes, excess

208 suspension were drained. The grids were viewed and photographed with a Philip

209 CM100 electron microscope at 80 kV.

210

211   ***Fluorescence microscopy.*** For fluorescence microscopy on fixed cells, Vero E6

212   cells were grown on glass coverslips, transfected and analyzed at 24 hours post-

213   transfection. Cells were washed with PBS, nuclei were stained with DAPI and

214   coverslips were mounted on glass slides for analysis. Fixed cells were visualized

215   under AxioObserver Z1 inverted motorized fluorescent microscope using the

216   ApoTome module and piloted through the Axiovision 4.6 software and images

217   were acquired through the MRm AxioCam high resolution CCD camera (Carl

218   Zeiss, Germany). For fluorescence microscopy on live cells, Vero E6 cells were

219   grown in glass-bottom culture dish (MatTech, USA), transfected and analyzed at

220   24 hours post-transfection. Cells were washed and medium was changed to

221   HBSS, Optimem pre-warmed culture medium. Widefield image acquisitions of live

222   cells were obtained using the system described above. Confocal acquisitions of

223   live cells were acquired at the Hong Kong University Core Imaging Facility using

224   an AxioObserver Z1 inverted motorized fluorescent microscope equipped with a

225   spinning disc confocal imaging system (UltraVIEW ERS, PerkinElmer, Shelton,

226   CT, USA). For brefeldin A (BFA) treatment, cells were incubated with 6 μg per ml

227   of BFA for indicated times. To release BFA effect, cells were washed three times

228   in PBS 1X and reincubated in normal medium for indicated times.

229

230   **Results**

231

232   **Efficient production and release of SARS-CoV VLPs are driven by co-**

233   **expression of M, N and E structural proteins.**

234   To determine the optimal conditions for efficient SARS-CoV VLP production, we

235   chose to co-express different combinations of structural proteins in the Vero E6

236   African green monkey cell line, which is permissive to SARS-CoV replication (34).

237   cDNAs encoding SARS-CoV structural proteins M, E and N were codon-optimized

238   for expression in mammalian cells. Understanding the importance to maintain a

239   low E/M ratio in transfected cells to ensure concomitant expression of E and M,

240   low incorporation of E into VLPs and prevent potential formation of E-containing

241   vesicles, we reasoned that the use of the pIRES bi-cistronic plasmid should be

242   appropriate. This plasmid presents the double advantage to ensure co-expression

243   of both cDNAs in transfected cell and allows a reduced rate of translation of the

244   downstream gene by utilizing a partially disabled internal ribosome entry site

245   (IRES) sequence. We therefore subcloned M upstream of E cDNA in the pIRES

246   vector. N was expressed from a pcDNA plasmid with a C-terminal Flag tag. Vero

247   E6 cells were either not transfected or transfected with pcDNA-E, pIRES-M or

248   pIRES-M-E alone, pIRES-M plus pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag

249   (Figure 1A). At 24 and 48 hours post-transfection culture medium and cells were

250   harvested. Culture medium was subjected to ultracentrifugation on a 20% sucrose

251   cushion to isolate VLPs and the SARS-CoV structural proteins assembled into

252   VLPs were analyzed by Western blot.

253   The M protein was readily detected at both 24 and 48 hours post-transfection in

254   lysates from Vero E6 cells transfected with pIRES-M, pIRES-M-E, pIRES-M plus

11

pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag (Figure 1A, upper panels). As

described previously, three major forms of M, which correspond to different

glycoforms, were detected on SDS-PAGE: two bands at ~ 18 and 28 KDa and a

smear at higher molecular sizes reflecting heterogeneity of glycosylation patterns

(51). As expected, higher levels of M were detected in cell lysates at 48 hours than

at 24 hours post-transfection. Whereas a 10 KDa protein corresponding to E could

be easily detected in cell lysates from cells transfected with pcDNA-E plasmid, its

expression was much lower in cells transfected with the pIRES-M-E bi-cistronic

vector. The Nflag protein was found in cell lysates from both pIRES-M plus

pcDNA-Nflag and pIRES-M-E plus pcDNA-Nflag transfected cells at similar levels.

A major band corresponding to a protein with an apparent molecular size of 45

KDa was detected.

Strikingly, the efficacy of VLP production was dramatically affected by the

combination of viral proteins co-expressed (Figure 1A, lower panels). Although M

was not detected in ultracentrifuged culture medium from pIRES-M, pIRES-M-E or

pIRES-M plus pcDNA-Nflag transfected cells at 24 hours post-transfection,

significant levels were found in preparations from pIRES-M-E plus pcDNA-Nflag

transfected cells. Similarly, the N protein was only detected in ultracentrifuged

culture medium from pIRES-M-E plus pcDNA-Nflag transfected cells at this early

time point. Signals for E were below the limit of detection at 24 hours and

detectable only at 48 hours. Finding low amounts of E in VLPs is in accordance

with the minor presence of this protein in coronavirus particles, despite its

important role for virion assembly and budding (9, 20, 37, 57, 65). In conclusion,

only when M, E and N proteins were co-expressed, VLPs that contained the M and

N proteins could be isolated from culture medium at 24 hours post-transfection.

280 At 48 hours post-transfection, E was found in ultracentrifuged culture medium from

281 cells transfected with the pcDNA-E plasmid alone. This is in agreement with

282 previously published data that describe secretion of E proteins independently of

283 other viral elements (7, 47, 50). At this time point, trace amounts of M were

284 detected in ultracentrifuged culture medium from pIRES-M and pIRES-M-E

285 transfected cells, whereas significant levels of M and N were found for pIRES-M

286 plus pcDNA-Nflag ultracentrifuged culture medium. Independent secretion of

287 SARS-CoV M proteins from Vero E6 cells as well as production of M-E VLPs was

288 described by Mortola and Roy (2004) at four days post-transfection. Production of

289 SARS-CoV M-N VLPs has already been reported by Huang Y. and colleagues in

290 transfected 293 renal epithelial cells at 63 hours post-transfection (29). Very

291 interestingly, in our conditions, significantly higher levels of M and N proteins were

292 found in purified VLPs from pIRES-M-E plus pcDNA-Nflag than pIRES-M plus

293 pcDNA-Nflag transfected cells at 48 hours post-transfection. We were also able to

294 detect E in these conditions, but not when cells were only transfected with the

295 pIRES-M-E vector.

296 In order to verify that inefficacy of production of M-E VLPs by cells transfected with

297 pIRES-M-E was not due to inefficient E expression, we performed similar

298 experiments but expressed E from a pCDNA-E plasmid and analyzed cells and

299 medium at 48 hours post-transfection (Figure 1B). As expected, higher levels of E

300 were detected in lysates of cells transfected with pIRES-M plus pCDNA-E and

301 pIRES-M plus pCDNA-E plus pCDNA-Nflag than pIRES-M-E and pIRES-M-E plus

302 pCDNA-Nflag, respectively (Figure 1B, left panel). Nevertheless, neither

303 production of M-E VLPs nor M-E-N VLPs were improved by increase of E

304 expression (Figure 1B, right panel). On the contrary, production of M-E-N VLPs

305   was enhanced when the bicistronic vector was used, as indicated by higher levels

306   of Nflag, M and E proteins secreted, and the ratio E(medium)/E(lysate) was

307   significantly higher in these conditions. This result suggests that expression of E

308   along with M from the pIRES-M-E bicistronic vector and in combination with N is

309   favorable to the production of SARS-CoV VLPs.

310   We then investigated co-sedimentation of secreted viral structural proteins by

311   performing sucrose gradient fractionation on ultracentrifuged cell medium. Cells

312   were either transfected by individual plasmids or combinations of plasmids as

313   described previously. In this experiment we used the individual plasmid for E

314   expression to verify potential presence of E-containing vesicles in medium when E

315   was expressed at higher levels. At 48 hours post-transfection, cleared cell medium

316   was ultracentrifuged on 20% sucrose cushion and pellets were resuspended in

317   TNE buffer. As controls, viral proteins contained in cell lysates and pellets from

318   ultracentrifuged medium were analyzed by Western blot (Figure 1C, left panel).

319   Pellets were loaded on top of 20-60% discontinuous sucrose gradients. After

320   another round of ultracentrifugation, 20 fractions were collected and analyzed by

321   Western blot (Figure 1C, right panels a to e). Results were consistent with the data

322   described in Figure 1 A and B. When M was expressed alone, very little amounts

323   of protein were found in cell medium and traces were detected in fraction 9 (Figure

324   1C, panel a). Coexpression with E or Nflag, allowed secretion of slightly higher

325   levels of M in cell medium and detection of M in fractions 7 to 11, with enrichment

326   in fraction 10 (Figure 1C, panel c) or 11 (Figure 1C, panel d), respectively. E

327   protein was not detectable in fractions from medium of either pCDNA-E or pIRES-

328   M plus pCDNA-E transfected cells (Figure 1C, panel b and c). Nflag cosedimented

329   with M when co-expressed (Figure 1C, panel d). As expected, greater levels of M,

14

330 E and Nflag proteins were found in medium of cells expressing all three viral

331 proteins (Figure 1C, panel e). M and Nflag were present in fractions 5 to 14 with

332 proportional levels. E was detected in fractions 8 to 10 where M and N proteins

333 were also enriched. Altogether, these results show that secreted viral structural

334 proteins cosediment in sucrose gradient and therefore strongly suggest that viral

335 proteins are associated into VLPs. Moreover, secretion of E-containing vesicles

336 does not seem to be a major phenomenon in Vero E6 cells expressing E alone or

337 in combination with other viral proteins in our system. Indeed, in all conditions,

338 levels of E in cell medium were systematically low and only detected in fractions

339 when co-expressed with M and N.

340 Altogether these data establish for the first time that all three M, E and N structural

341 SARS-CoV proteins are important for efficient production and release of SARS-

342 CoV VLPs.

343

344 **S is incorporated onto SARS-CoV M-E-N-containing VLPs.**

345 We then investigated whether the SARS-CoV S glycoprotein could be

346 incorporated onto secreted M-E-N VLPs. S cDNA was codon optimized and

347 subcloned in the pcDNA vector. Vero E6 cells were transfected with pIRES-M-E

348 plus pcDNA-Nflag plus pcDNA-S. Protein expression was verified in cell lysates at

349 48 hours post-transfection (data not shown). To monitor association of S onto M-

350 E-N VLPs, we performed sucrose fractionation on VLPs previously purified from

351 culture medium (Figure 2). All S, Nflag, M and E proteins were co-sedimented and

352 enriched in fractions 9 and 10 corresponding to ~40% sucrose. It is notable that S

353 was mainly detected in its native trimeric form in these fractions (540 KDa),

354 although we also detected low levels of monomers (180 KDa) and dimers (360

15

355   KDa), which may have resulted from sample treatment and SDS-PAGE conditions.

356   Higher molecular weight proteins, detected with anti-S antibodies, were found in

357   the lightest fractions 1 to 6. These forms could correspond to soluble, non-

358   incorporated forms of S proteins that have formed aggregates. We have previously

359   described formation of S aggregates in preparations of purified SARS-CoV S

360   trimers from mammalian cell lysates (31). This result confirms association of all

361   four SARS-CoV structural proteins into S-M-E-N VLPs that can be purified from

362   culture medium of transfected cells.

363

364   **VLPs bud into a perinuclear compartment and are transported within**

365   **vesicles to the plasma membrane.**

366   We next studied the subcellular localization of SARS-CoV VLPs by electron

367   microscopy (Figure 3). pIRES-M-E plus pcDNA-Nflag plus pcDNA-S co-

368   transfected Vero E6 cells were fixed at 24 and 48h post-transfection and ultrathin

369   sections were prepared for transmission electron microscopy (Figure 3A to G).

370   Large amounts of VLPs, with diameters ranging from 40 to 150 nm on sections,

371   were found within the cytoplasm of positive cells. Three main patterns of

372   subcellular localization were observed. First, VLPs were found within perinuclear

373   compartments, which had appearance of groups of vacuoles (Figure 3A, C and D),

374   or, alternatively but seldom observed, which presented morphological

375   characteristics of the endoplasmic reticulum (Figure 3B). Vacuoles were observed

376   at both 24 hours and 48 hours post-transfection with a diameter ranging from 300

377   nm to 1.5 μm on sections. Very interestingly, we detected several events of VLP

378   budding at vacuole membranes (Figure 3D). Although VLPs accumulated therein,

379   they were not highly compacted in this compartment. Moreover, VLPs appeared

16

380 pleiomorphic and heterogenous material was also observed (Figure 3D). Tubular

381 structures were seen within some VLP-containing vacuoles, mainly at 24 hours

382 post transfection (data not shown). Second, VLPs were observed in vesicles

383 scattered in the cytoplasm (Figure 3A, B, C and E). These vesicles had a diameter

384 ranging from 200 nm to 1 μm on sections and VLPs were more compacted inside.

385 They could be found at proximity of the perinuclear VLP-containing compartments

386 up to the cortical area. VLPs contained in these vesicles looked more

387 homogenous in size and shape. Third, VLPs were occasionally found at the cell

388 surface (Figure 3A, F and G). Although we could not observe obvious spike-like

389 structures surrounding the VLP envelope, spike-like protuberances were

390 occasionally detected (Figure 3G). Moreover, binding of VLPs to cell surface

391 suggests the presence of spikes and receptor recognition. Some cells, in which

392 very high amounts of VLPs were found, presented characteristics of apoptosis with

393 fragmented nucleus and disrupted membranes (data not shown). The cytoplasm of

394 these cells was filled up with VLPs, either free or within intracellular compartments.

395 The morphology of released VLPs was investigated further by electron microscopy

396 on negatively stained particles that were purified from cell medium (Figure 3F).

397 Round-shaped particles with a diameter of 80-100 nm were readily observed.

398 Again, we could not observe the typical corona of spikes around VLPs (21) but

399 globular structures protruding from VLPs were detected, which likely corresponded

400 to trimers of spikes. Altogether, our data suggest that SARS-CoV VLPs bud in an

401 intracellular compartment of Vero E6 cells and are efficiently transported within

402 vesicles to the plasma membrane where they are released. Shape and size of

403 secreted VLPs are in accordance with the morphological characteristics of the

17

404   SARS-CoV and therefore these VLPs should be a safe and appropriate model to

405   study assembly and release of SARS-CoV virions.

406

407   Assembly and release of VLPs that incorporate fluorescently-tagged

408   structural viral proteins.

409   In order to visualize in real time, the assembly, trafficking and release of SARS-

410   CoV VLPs, we engineered plasmid constructs that allow expression of viral

411   proteins in fusion with fluorescent proteins. The pIRES-MmRFP1-E, pcDNA-

412   NeCFP and pcDNA-SeYFP constructs were developed and expression of fusion

413   viral proteins was analyzed in individually transfected or co-transfected cells by

414   fluorescence microscopy (Figure 4). We did not include any construct coding for a

415   fluorescently tagged SARS-CoV E protein because it does not tolerate fluorescent

416   protein tags (C. Chan et al., unpublished data). All chimeric viral proteins could be

417   readily observed by fluorescent microscopy. In cells individually transfected with

418   the pIRES-MmRFP1-E, the MmRFP1 fusion protein was mainly present in a

419   perinuclear compartment, most likely the ERGIC/Golgi apparatus (Figure 4, panels

420   a,b). We also found MmRFP1 within the cytoplasm and occasionally at the plasma

421   membrane. As expected, the SeYFP protein was observed both in the

422   ERGIC/Golgi apparatus, and at the plasma membrane of individually transfected

423   cells (Figure 4, panels c,d). These expression patterns are similar to what we

424   observed previously for MeGFP and SeGFP fusion proteins at 15 hours post-

425   infection with Semliki Forest Virus expression vectors (51). In most of the cells,

426   individually expressed NeCFP protein formed bright cytosolic patches, suggesting

427   aggregation of the protein in the cytosol in absence of functional viral protein

428   partners (Figure 4 e,f). This pattern may reflect large inclusions of nucleocapsids,

429   which have been described to accumulate late in the infection of cells with HCoV

430   and MHV-JHM and SARS-CoV (6, 17, 23) or may constitute a cytosolic reservoir

431   of protein supposed to feed the viral budding system. Interestingly, when plasmids

432   were co-transfected, all three MmRFP1, SeYFP and NeCFP fluorescent proteins

433   presented similar intracellular distributions, co-localizing in the cytoplasm and at

434   the plasma membrane (Figure 4 panels g,i,k,m and h,j,l,n). When co-expressed

435   with MmRFP1, E and SeYFP, the subcellular distribution of the NeCFP protein

436   was dramatically changed and bright cytosolic patches were rarely found (Figure

437   4, panels e,f and k,l). Trafficking of NeCFP to the cell surface in co-transfected

438   cells suggests that interactions with other viral proteins have occurred leading to

439   NeCFP translocation. Nevertheless no VLPs were detected in medium from co-

440   transfected cells (data not shown). Altogether, these results suggest that although

441   MmRFP1, SeYFP, NeCFP and E are likely to interact when co-expressed in Vero

442   E6 cells, they are not released in the form of VLPs in cell medium.

443   We then reasoned that fluorescent VLPs could be produced by including only one

444   plasmid coding for one of the fluorescently tagged viral protein per co-transfection.

445   In these conditions, we investigated protein expression and release of VLPs by

446   Vero E6 cells at 48 hours post-transfection (Figure 5A). As positive control, we

447   monitored VLP release from pIRES-M-E, pcDNA-Nflag, pcDNA-S transfected

448   cells. As expected, S, Nflag, M and E proteins were detected in both cell lysate

449   and VLP preparation (Figure 5A, lane 2 and 7). Although the MmRFP1 fusion

450   protein was readily observed by fluorescent microscopy, it could not be detected

451   by Western blot. Most likely, the rabbit polyclonal antibody directed against the M

452   C-terminal domain cannot recognize its epitope when it is fused to the mRFP1

453   fluorescent tag. Although the E, Nflag and S proteins were detected on cell lysate

19

454 from pIRES-MmRFP1-E plus pcDNA-Nflag plus pcDNA-S transfected cells (Figure

455 5A, lane 3), no protein corresponding to purified VLPs were found in medium

456 (Figure 5A, lane 8). We concluded that fusion of the mRFP1 protein at the C-

457 terminal end of M inhibits VLP production. On the contrary, both pIRES-M-E plus

458 pcDNA-NeCFP plus pcDNA-S, as well as pIRES-M-E plus pcDNA-Nflag plus

459 pcDNA-SeYFP transfected cells were able to release VLPs in cell medium, as

460 indicated by the presence of viral proteins (figure 5A, lanes 9 and 10). Under these

461 conditions, the NeCFP protein was detected by a mouse monoclonal antibody

462 directed against N (56) and migrated to an apparent molecular size of 70 KDa.

463 To confirm that NeCFP or SeYFP viral proteins are correctly incorporated into

464 VLPs, we analyzed purified VLPs by fractionation on a 20-60% sucrose gradient

465 (Figure 5B). Both NeCFP and SeYFP co-sedimented with other viral proteins,

466 indicating that they were incorporated in purified VLPs (Figure 5B, upper and lower

467 panels, respectively). M-E-NeCFP-S VLPs were concentrated in fractions 10 and

468 11. The E protein was not detected, most likely because M-E-NeCFP-S VLPs

469 were less abundant in culture medium than M-E-Nflag-S VLPs for which E levels

470 were already low. M-E-Nflag-SeYFP VLPs were more efficiently produced and

471 concentrated in fractions 9 and 10 although high levels were also found in

472 fractions 6, 7 and 8. Considering the high levels of M and Nflag detected in these

473 fractions, incorporation of SeYFP seems less efficient than S (cf. Figure 2 with

474 Figure 5B, lower panel). Altogether, our results demonstrate that fluorescent VLPs

475 can be readily produced in Vero E6 transfected cells by incorporating either a

476 tagged N or S fusion protein.

477

Visualization of M-E-NeC/GFP-S VLP production and transport in living Vero E6 cells.

We then investigated formation and transport of fluorescent SARS-CoV VLPs in living transfected cells by fluorescent microscopy. Knowing that S is expressed all along the secretory pathway in both single- and co-transfected cells, we reasoned that N would be a better marker for monitoring of SARS-CoV VLPs assembly. Furthermore, having demonstrated that M, E and N are the crucial factors for assembly and egress of SARS-CoV VLPs, and anticipating that S-bearing VLPs could be internalized back into producer cells by ACE-2 driven endocytosis, we decided to omit S. Therefore, the pcDNA-NeCFP plasmid was either transfected alone or co-transfected with pIRES-M-E. Vero E6 cells were analyzed at 24 hours post-transfection. In most pcDNA-NeCFP transfected cells, eCFP signals were very bright and concentrated in large aggregates in the cytosol at the periphery of the nucleus (Figure 6A, panel a). Inversely and very interestingly, a different pattern was observed in most of cells co-transfected with pIRES-M-E and pcDNA-NeCFP. In these cells, eCFP fluorescence was more diffuse in cell cytosol: medium-size bright vesicles were concentrated in close proximity to the nucleus, smaller and dimmer vesicles were scattered in the cytoplasm and occasionally bright dots were enriched at the cell cortical area (Figure 6A, panels b). Few bright dots were also found outside cells in the surrounding medium. This difference in NeCFP distribution pattern, suggests that NeCFP assembles with co-expressed M and E viral proteins to form VLPs and traffics from the perinuclear assembly compartment to the cell surface where fluorescent VLPs are released into the medium.

502   We then further analyzed the trafficking dynamics of a fluorescently tagged N

503   protein, co-expressed with M and E envelope proteins in living Vero E6 cells. In

504   this aim, images were acquired using a spinning disc confocal microscope coupled

505   to a CCD camera suitable for high-speed and high-resolution imaging. In these

506   experiments we replaced the NeCFP by a NeGFP fusion protein, which is

507   excitable by the Argon laser the microscope was equipped with. We could

508   consistently identify three types of NeGFP containing vesicles, where fluorescent

509   signal intensities, sizes and movements differ (Figure 6B and corresponding

510   videos provided as supplementary data). First, the largest and brightest vesicles

511   were found close to the nucleus and were static. Second, smaller and dimmer

512   vesicles were trafficking actively, most of the time in a multidirectional way, making

513   transient interactions with other vesicles. Occasionally these vesicles moved in

514   rapid, unidirectional way and for longer distances, most likely along microtubules.

515   Third, some cells presented an accumulation of bright dots at the cortical area,

516   which may correspond to smaller secretory vesicles and released VLPs. Few

517   bright dots were found outside cells, which probably correspond to fluorescent

518   VLPs that have been released from producer cells. These results illustrate that

519   fusion of a fluorescent tag to the C-terminal end of the nucleocapsid viral protein

520   makes monitoring of SARS-CoV VLPs' egress possible.

521   We then investigated the effect of Brefeldin A (BFA), a fungal metabolite that has

522   multiple effects upon the organelles of the secretory pathway, including inhibition

523   of trafficking from ER to the Golgi apparatus, fusion of the cisternae of the Golgi

524   with the ER and fusion of the trans-Golgi network (TGN) with endosomes (59).

525   BFA has been used in previous study for analysis of viral protein transport and

526   virus assembly (10, 49). Time-lapse images of living Vero E6 cells transfected with

22

527   M-E plus NeGFP were acquired at 24 hours post-transfection (Figure 6C; for

528   video, see supplementary data). In a first time, cells were either non-treated (panel

529   a) or treated with 6 μg / ml BFA for 4 hours (panel d) or over-night (panel g). After

530   several minutes of acquisition in these conditions, medium was changed to either

531   BFA-containing medium (panels b and c) or normal medium (panels e, f and h, i),

532   respectively. New sequences of images were acquired and fluorescence

533   redistribution was analyzed. Upon BFA treatment we observed fusion of scattered

534   fluorescent vesicles into large cytosolic fluorescent clusters accumulating at the

535   center of the cell and diminution of puncta (panels a, b and c, supplementary

536   data). After treatment of cells for 4 hours with BFA, most of the fluorescence was

537   present in large bright patches surrounding the nucleus (panel d). Vesicles could

538   still be observed in the cytosol, although less numerous than in non-treated cells.

539   Following BFA wash, we could not observe any obvious reversibility of the

540   massive redistribution of fluorescence (compare panels d and e, panel f). After

541   over-night incubation with BFA, fluorescence was associated with large cytosolic

542   clusters and endoplasmic reticulum (panel g) and no drastic change was observed

543   after BFA wash and 1:30 hours recovery (panel h), although membrane dynamics

544   seem to increase at the edge on the reticular compartment where tubules

545   elongated from (panel h, arrow; supplementary data). Three hours recovery after

546   over-night BFA treatment partially restore trafficking of fluorescent vesicles in

547   transfected cells (panel i). The recovery process after BFA treatment was slow and

548   the dynamics could not be followed for several hours, as repetitive exposure to

549   laser induced apoptosis. Altogether, our results suggest 1/ that BFA induce

550   reorganization of NeGFP associated compartments and alters vesicle formation

551   and transport, 2/ that several hours of recovery after BFA wash are necessary to

552   restore dynamics of NeGFP associated compartment and vesicles trafficking.

553   Further studies are required in order to clarify the subcellular compartments VLPs

554   are associated with and delineate the dynamics of egress in association with

555   cellular structures.

556

Discussion

557

558

559   The minimal molecular requirement for efficient assembly and egress of SARS-

560   CoV virions is still controversial. Here we describe the development of a plasmid-

561   based transfection method for efficient production and release of SARS-CoV VLPs

562   in permissive Vero E6 green monkey kidney epithelial cells. In contrast to reports

563   on other coronaviruses, we demonstrate that all three M, E and N SARS-CoV

564   structural proteins are required for efficient assembly and release of VLPs by

565   transfected cells. When the S viral envelope glycoprotein is co-expressed with M,

566   N and E, trimers of S are incorporated onto VLPs. Of particular interest is the

567   finding that incorporation of a fluorescently tagged N protein into VLPs allows

568   visualization of transport of *de-novo* formed particles in producer cells. Fluorescent

569   VLPs constitute a new powerful model to study the mechanisms of SARS-CoV

570   egress in living cells and specific roles of cellular machineries by fluorescence

571   imaging techniques.

572   Other research groups have described formation of SARS-CoV VLPs in either

573   insect (26, 44, 50) or mammalian cells (27-29), using various expression systems.

574   Huang Y. and colleagues were the first to report generation of SARS-CoV VLPs in

575   human 293 renal epithelial cells (29). Human codon optimized genes encoding for

576   M, E, N and S proteins were subcloned into mammalian expression vector and

577   VLP formation was monitored by transmission electron microscopy on transfected

578   cells at 63 hours post-transfection. The authors show that, in their experimental

579   conditions, M and N are necessary and sufficient for formation of intracellular

580   VLPs, independently of E and S, but secretion of VLPs was not efficient.

581   Moreover, they described S as an important viral factor for maturation and egress

582 of VLPs from cells, but release of M-N-S VLPs in culture medium was still

583 inefficient. By contrast, in our system we detected sedimentable M and N proteins

584 in culture medium from M plus N expressing cells, suggesting that M-N VLPs can

585 form and be secreted (Figure 1). Furthermore, co-expression of E greatly boosted

586 levels of VLPs detected in culture medium (Figure 1) and addition of S did not

587 influence the rate of VLP production (Figure 2 and data not shown). In contrast,

588 Hsieh and colleagues have described that co-expressed E and M proteins are

589 released four days post-transfection in culture medium of Vero E6 cells in the

590 absence of S and N proteins. In their study, Vero E6 cells, previously infected with

591 a recombinant vaccinia virus harboring the T7 polymerase gene, were co-

592 transfected with plasmids encoding MycHis or V5His-tagged S, M, E and N

593 structural proteins. Nevertheless, sedimentable E and M proteins were also found

594 in culture medium even when they were individually expressed and very high

595 amounts of E were found in VLP preparations in comparison to other viral proteins,

596 suggesting formation of E-containing vesicles. Others have described that MHV

597 and IBV E protein expressed alone results in assembly of E-protein-containing

598 vesicles, with a density similar to that of VLPs (7, 47). We too did find

599 sedimentable SARS-CoV E proteins in culture medium from individually pcDNA-E

600 transfected cells, suggesting secretion of E vesicles, but we could not detect them

601 in any fraction after sucrose gradient fractionation, suggesting a low production

602 rate (Figure 1). We could also detect M-E VLPs at 48 hours post-transfection by

603 coexpressing both E and M proteins, albeit at very low levels (Figure 1A, B and C).

604 In our study, we took advantage of the pIRES bicistronic vector to ensure

605 concomitant expression of E and M and maintain a low expression level for E. We

606 show that use of this vector system, in combination with expression of N, ensures

26

607   slightly better levels of M-E-N VLPs in medium and great incorporation of E

608   (Figure 1B). Higher levels of E were expressed when an individual pcDNA-E

609   vector was used for E expression and resulted in increased E/M and E/N ratio in

610   cell medium (Figure 1B). We could not conclude whether this was due to higher

611   level of E incorporation into VLPs or to simultaneous release of E-containing

612   vesicles and VLPs. Analysis of viral protein in cell medium by sucrose gradient

613   fractionation revealed that E was only released at significant levels when co-

614   expressed with M and N proteins, with which it cosediments (Figure 1C, panels b,

615   c and e). We have observed efficient M-E-N-S VLP formation and release in 293T,

616   HeLa and Huh-7 human cell lines (data not shown). Consistently, formation and

617   release of M-E-N-S VLPs from 293T cells has been shown in another study

618   reported by Huang C. and colleagues, in which authors have used a pCAGGS

619   mammalian expression plasmid-based transfection system (28). Huang C. and

620   colleagues were able to generate approximately 1.3 μg of SARS-CoV VLPs from

621   2.10^7 293T cells (42). Recently, we have measured quantity of S incorporated into

622   envelope of M-E-N-S VLPs purified from the VLP-containing sucrose fractions in

623   our system. Approximately 1μg and 28 μg of S were present in VLP preparations

624   from 10^7 VeroE6 and 293T cells, respectively (data not shown).

625   We also investigated formation and secretion of SARS-CoV VLPs in transfected

626   cells by transmission electron microscopy and negative staining of purified

627   secreted particles (Figure 3). We were able to identify budding events in

628   cytoplasmic perinuclear compartments. VLP-containing vesicles were scattered

629   within the cytoplasm and found beneath the plasma membrane. Occasionally,

630   secreted VLPs were bound to the cell surface. The VLPs-associated

631   compartments that we observed in transfected Vero E6 cells share significant

632 similarities with the virions-containing compartments described in SARS-CoV

633 infected Vero E6 cells at 3-5 days post infection (23). VLPs were readily detected

634 in sedimented fractions from culture medium by negative staining and electron

635 microscopy (Figure 3H). Globular structures protruding from VLP envelope, and

636 which should correspond to spike peplomers, were occasionally detected, but we

637 could not identify any particle displaying a typical corona-like structure attesting for

638 optimal incorporation of spike trimers on virion envelope.

639 Interestingly, we found that a C-terminal Flag or eYFP tag affects levels of S

640 trimers incorporation onto VLPs (data not shown and Figure 5, respectively).

641 However Sflag and SeYFP proteins could still be incorporated into VLPs and tags

642 can be used as markers. Similar results had been obtained for MHV where S

643 sequence had been extended by fusion with a GFP fluorescent protein (3). These

644 data can be explained by several factors: the 30 KDa fluorescent protein may

645 cause geometrical constraints; M-S interactions, which are important for S

646 incorporation may be affected by the tag; S retention to the site of viral assembly

647 may be disturbed (43, 48). We also tried to produce VLPs containing the mRFP1

648 fluorescent protein fused to the C-terminal end of M (Figure 4 and 5A). Although

649 MmRFP1 was expressed in transfected cells, production of VLPs was abrogated.

650 The M endodomain is crucial for M-N, M-E and M-S interactions and VLP

651 formation (8, 11, 13, 19) and its fusion to a fluorescent protein tag may affect its

652 structure and/or availability for interaction with other partners. This hypothesis is

653 reinforced by the complete loss of recognition of the chimeric MmRFP1 by a rabbit

654 serum raised against a C-terminal peptide of M (Figure 5A, left panel).

655 Lastly, a fusion NeCFP protein could be expressed and assemble into VLPs with

656 M, E and S, although levels of VLPs detected in culture medium were significantly

657   reduced (Figure 5). Interestingly, whereas individually expressed NeCFP or

658   NeGFP were found in the cell cytosol, often accumulating in the perinuclear area,

659   the tagged N protein had a tendency to display a vesicular distribution pattern

660   when co-expressed with M and E (Figure 6). We analyzed both distribution and

661   trafficking of the NeGFP protein in transfected live cells by confocal microscopy

662   (Figure 6B). When cells were co-transfected with pIRES-M-E and pcDNA-NeGFP,

663   the NeGFP proteins were often found in static compartment near the nucleus, in

664   trafficking vesicles scattered in the cytosol and moving actively, making transient

665   interactions with other vesicles, and as small dot scattered at the cortical area

666   beneath the plasma membrane, often enriched in cell projections. Fluorescent

667   dots were also detected in the cell medium surrounding living cells. Subcellular

668   distribution of NeGFP is consistent with our data of transmission electron

669   microscopy. Most likely perinuclear static compartments and trafficking vesicles

670   identified by fluorescence microscopy correspond to perinuclear vacuoles, at

671   which membranes VLP budding events were observed, and to VLP-containing

672   vesicles found by electron microscopy, respectively. The fluorescent dots

673   observed at the cell cortical area may correspond to smaller secretory vesicles

674   containing fewer VLPs – vesicles containing only one VLP were found by electron

675   microscopy – and/or to released VLPs, which are bound to the cell surface.

676   Interestingly, transport of fluorescent vesicles was affected by the drug BFA, well

677   known to affect membrane transport in the secretory pathway (39, 59). BFA

678   treatment induced backward trafficking of fluorescent vesicles and fusion into

679   bright perinuclear clusters (Figure 6C panels a, b and c). Long lasting BFA

680   treatment resulted in suppression of vesicle trafficking and no fluorescent puncta

681   were found at plasma membrane (Figure 6C panel g) and recovery of vesicle

682   trafficking was restored after several hours in normal medium (Figure 6C panel i).

683   Altogether, our data demonstrate that M, E and N structural proteins are key

684   molecules for assembly and egress of the SARS-CoV. Production and analysis of

685   fluorescent M-E-NeGFP SARS-CoV VLPs in living cells allowed us to identify

686   three subcellular structures with different velocity characteristics along the

687   secretory pathway. Molecular and cellular determinants of SARS-CoV assembly

688   and egress will be investigated further using advanced fluorescence microscopy

689   techniques.

707   **References**

708

709   1.   **Baudoux, P., C. Carrat, L. Besnardeau, B. Charley, and H. Laude.** 1998.
710 Coronavirus pseudoparticles formed with recombinant M and E proteins induce
711 alpha interferon synthesis by leukocytes. J Virol **72:**8636-43.

712   2.   **Bos, E. C., W. Luytjes, H. V. van der Meulen, H. K. Koerten, and W. J.**
713 **Spaan.** 1996. The production of recombinant infectious DI-particles of a murine
714 coronavirus in the absence of helper virus. Virology **218:**52-60.

715   3.   **Bosch, B. J., C. A. de Haan, and P. J. Rottier.** 2004. Coronavirus spike
716 glycoprotein, extended at the carboxy terminus with green fluorescent protein, is
717 assembly competent. J Virol **78:**7369-78.

718   4.   **Bosch, B. J., C. A. de Haan, S. L. Smits, and P. J. Rottier.** 2005. Spike
719 protein assembly into the coronavirion: exploring the limits of its sequence
720 requirements. Virology **334:**306-18.

721   5.   **Campbell, R. E., O. Tour, A. E. Palmer, P. A. Steinbach, G. S. Baird, D.**
722 **A. Zacharias, and R. Y. Tsien.** 2002. A monomeric red fluorescent protein. Proc
723 Natl Acad Sci U S A **99:**7877-82.

724   6.   **Caul, E. O., and S. I. Egglestone.** 1977. Further studies on human enteric
725 coronaviruses. Arch Virol **54:**107-17.

726   7.   **Corse, E., and C. E. Machamer.** 2000. Infectious bronchitis virus E protein
727 is targeted to the Golgi complex and directs release of virus-like particles. J Virol
728 **74:**4319-26.

729   8.   **Corse, E., and C. E. Machamer.** 2003. The cytoplasmic tails of infectious
730 bronchitis virus E and M proteins mediate their interaction. Virology **312:**25-34.

32

731   9.   **Curtis, K. M., B. Yount, and R. S. Baric.** 2002. Heterologous gene

732   expression from transmissible gastroenteritis virus replicon particles. J Virol

733   **76:**1422-34.

734   10.   **Dasgupta, A., and D. W. Wilson.** 2001. Evaluation of the primary effect of

735   brefeldin A treatment upon herpes simplex virus assembly. J Gen Virol **82:**1561-7.

736   11.   **de Haan, C. A., L. Kuo, P. S. Masters, H. Vennema, and P. J. Rottier.**

737   1998. Coronavirus particle assembly: primary structure requirements of the

738   membrane protein. J Virol **72:**6838-50.

739   12.   **de Haan, C. A., and P. J. Rottier.** 2005. Molecular interactions in the

740   assembly of coronaviruses. Adv Virus Res **64:**165-230.

741   13.   **de Haan, C. A., M. Smeets, F. Vernooij, H. Vennema, and P. J. Rottier.**

742   1999. Mapping of the coronavirus membrane protein domains involved in

743   interaction with the spike protein. J Virol **73:**7441-52.

744   14.   **de Haan, C. A., H. Vennema, and P. J. Rottier.** 2000. Assembly of the

745   coronavirus envelope: homotypic interactions between the M proteins. J Virol

746   **74:**4967-78.

747   15.   **Decaro, N., V. Mari, C. Desario, M. Campolo, G. Elia, V. Martella, G.**

748   **Greco, F. Cirone, M. L. Colaianni, P. Cordioli, and C. Buonavoglia.** 2008.

749   Severe outbreak of bovine coronavirus infection in dairy cattle during the warmer

750   season. Vet Microbiol **126:**30-9.

751   16.   **DeDiego, M. L., E. Alvarez, F. Almazan, M. T. Rejas, E. Lamirande, A.**

752   **Roberts, W. J. Shieh, S. R. Zaki, K. Subbarao, and L. Enjuanes.** 2007. A

753   severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated

754   in vitro and in vivo. J Virol **81:**1701-13.

755 17. **Dubois-Dalcq, M. E., E. W. Doller, M. V. Haspel, and K. V. Holmes.**

756 1982. Cell tropism and expression of mouse hepatitis viruses (MHV) in mouse

757 spinal cord cultures. Virology **119:**317-31.

758 18. **Erles, K., C. Toomey, H. W. Brooks, and J. Brownlie.** 2003. Detection of

759 a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology

760 **310:**216-23.

761 19. **Escors, D., J. Ortego, H. Laude, and L. Enjuanes.** 2001. The membrane

762 M protein carboxy terminus binds to transmissible gastroenteritis coronavirus core

763 and contributes to core stability. J Virol **75:**1312-24.

764 20. **Fischer, F., C. F. Stegen, P. S. Masters, and W. A. Samsonoff.** 1998.

765 Analysis of constructed E gene mutants of mouse hepatitis virus confirms a pivotal

766 role for E protein in coronavirus assembly. J Virol **72:**7885-94.

767 21. **Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van**

768 **Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, and A. D.**

769 **Osterhaus.** 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature

770 **423:**240.

771 22. **Godet, M., R. L'Haridon, J. F. Vautherot, and H. Laude.** 1992. TGEV

772 corona virus ORF4 encodes a membrane protein that is incorporated into virions.

773 Virology **188:**666-75.

774 23. **Goldsmith, C. S., K. M. Tatti, T. G. Ksiazek, P. E. Rollin, J. A. Comer, W.**

775 **W. Lee, P. A. Rota, B. Bankamp, W. J. Bellini, and S. R. Zaki.** 2004.

776 Ultrastructural characterization of SARS coronavirus. Emerg Infect Dis **10:**320-6.

777 24. **He, R., F. Dobie, M. Ballantine, A. Leeson, Y. Li, N. Bastien, T. Cutts, A.**

778 **Andonov, J. Cao, T. F. Booth, F. A. Plummer, S. Tyler, L. Baker, and X. Li.**

779 2004. Analysis of multimerization of the SARS coronavirus nucleocapsid protein.

780 Biochem Biophys Res Commun **316:**476-83.

781 25. **He, R., A. Leeson, M. Ballantine, A. Andonov, L. Baker, F. Dobie, Y. Li,**

782 **N. Bastien, H. Feldmann, U. Strocher, S. Theriault, T. Cutts, J. Cao, T. F.**

783 **Booth, F. A. Plummer, S. Tyler, and X. Li.** 2004. Characterization of protein-

784 protein interactions between the nucleocapsid protein and membrane protein of

785 the SARS coronavirus. Virus Res **105:**121-5.

786 26. **Ho, Y., P. H. Lin, C. Y. Liu, S. P. Lee, and Y. C. Chao.** 2004. Assembly of

787 human severe acute respiratory syndrome coronavirus-like particles. Biochem

788 Biophys Res Commun **318:**833-8.

789 27. **Hsieh, P. K., S. C. Chang, C. C. Huang, T. T. Lee, C. W. Hsiao, Y. H.**

790 **Kou, I. Y. Chen, C. K. Chang, T. H. Huang, and M. F. Chang.** 2005. Assembly of

791 severe acute respiratory syndrome coronavirus RNA packaging signal into virus-

792 like particles is nucleocapsid dependent. J Virol **79:**13848-55.

793 28. **Huang, C., N. Ito, C. T. Tseng, and S. Makino.** 2006. Severe acute

794 respiratory syndrome coronavirus 7a accessory protein is a viral structural protein.

795 J Virol **80:**7287-94.

796 29. **Huang, Y., Z. Y. Yang, W. P. Kong, and G. J. Nabel.** 2004. Generation of

797 synthetic severe acute respiratory syndrome coronavirus pseudoparticles:

798 implications for assembly and vaccine production. J Virol **78:**12557-65.

799 30. **Hurst, K. R., L. Kuo, C. A. Koetzner, R. Ye, B. Hsue, and P. S. Masters.**

800 2005. A major determinant for membrane protein interaction localizes to the

801 carboxy-terminal domain of the mouse coronavirus nucleocapsid protein. J Virol

802 **79:**13285-97.

803   31.   **Kam, Y. W., F. Kien, A. Roberts, Y. C. Cheung, E. W. Lamirande, L.**

804   **Vogel, S. L. Chu, J. Tse, J. Guarner, S. Zaki, K. Subbarao, M. Peiris, B. Nal,**

805   **and R. Altmeyer.** 2006. Antibodies against trimeric S glycoprotein protect

806   hamsters against SARS-CoV challenge despite their capacity to mediate

807   FcgammaRII-dependent entry into B cells in vitro. Vaccine.

808   32.   **Klumperman, J., J. K. Locker, A. Meijer, M. C. Horzinek, H. J. Geuze,**

809   **and P. J. Rottier.** 1994. Coronavirus M proteins accumulate in the Golgi complex

810   beyond the site of virion budding. J Virol **68:**6523-34.

811   33.   **Krijnse-Locker, J., M. Ericsson, P. J. Rottier, and G. Griffiths.** 1994.

812   Characterization of the budding compartment of mouse hepatitis virus: evidence

813   that transport from the RER to the Golgi complex requires only one vesicular

814   transport step. J Cell Biol **124:**55-70.

815   34.   **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S.**

816   **Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A.**

817   **E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B.**

818   **Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini,**

819   **and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute

820   respiratory syndrome. N Engl J Med **348:**1953-66.

821   35.   **Kuo, L., K. R. Hurst, and P. S. Masters.** 2007. Exceptional flexibility in the

822   sequence requirements for coronavirus small envelope protein function. J Virol

823   **81:**2249-62.

824   36.   **Kuo, L., and P. S. Masters.** 2002. Genetic evidence for a structural

825   interaction between the carboxy termini of the membrane and nucleocapsid

826   proteins of mouse hepatitis virus. J Virol **76:**4987-99.

827   37.   **Kuo, L., and P. S. Masters.** 2003. The small envelope protein E is not

828   essential for murine coronavirus replication. J Virol **77:**4597-608.

829   38.   **Lim, K. P., and D. X. Liu.** 2001. The missing link in coronavirus assembly.

830   Retention of the avian coronavirus infectious bronchitis virus envelope protein in

831   the pre-Golgi compartments and physical interaction between the envelope and

832   membrane proteins. J Biol Chem **276:**17515-23.

833   39.   **Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D.**

834   **Klausner.** 1989. Rapid redistribution of Golgi proteins into the ER in cells treated

835   with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell **56:**801-13.

836   40.   **Liu, D. X., and S. C. Inglis.** 1991. Association of the infectious bronchitis

837   virus 3c protein with the virion envelope. Virology **185:**911-7.

838   41.   **Locker, J. K., D. J. Opstelten, M. Ericsson, M. C. Horzinek, and P. J.**

839   **Rottier.** 1995. Oligomerization of a trans-Golgi/trans-Golgi network retained

840   protein occurs in the Golgi complex and may be part of its retention. J Biol Chem

841   **270:**8815-21.

842   42.   **Lokugamage, K. G., N. Yoshikawa-Iwata, N. Ito, D. M. Watts, P. R.**

843   **Wyde, N. Wang, P. Newman, C. T. Kent Tseng, C. J. Peters, and S. Makino.**

844   2008. Chimeric coronavirus-like particles carrying severe acute respiratory

845   syndrome coronavirus (SCoV) S protein protect mice against challenge with

846   SCoV. Vaccine **26:**797-808.

847   43.   **Lontok, E., E. Corse, and C. E. Machamer.** 2004. Intracellular Targeting

848   Signals Contribute to Localization of Coronavirus Spike Proteins near the Virus

849   Assembly Site. J Virol **78:**5913-22.

850   44.   **Lu, X., Y. Chen, B. Bai, H. Hu, L. Tao, J. Yang, J. Chen, Z. Chen, Z. Hu,**

851   **and H. Wang.** 2007. Immune responses against severe acute respiratory

852 syndrome coronavirus induced by virus-like particles in mice. Immunology

853 **122:**496-502.

854 45.   **Luo, H., D. Wu, C. Shen, K. Chen, X. Shen, and H. Jiang.** 2006. Severe

855 acute respiratory syndrome coronavirus membrane protein interacts with

856 nucleocapsid protein mostly through their carboxyl termini by electrostatic

857 attraction. Int J Biochem Cell Biol **38:**589-99.

858 46.   **Madan, V., J. Garcia Mde, M. A. Sanz, and L. Carrasco.** 2005. Viroporin

859 activity of murine hepatitis virus E protein. FEBS Lett **579:**3607-12.

860 47.   **Maeda, J., A. Maeda, and S. Makino.** 1999. Release of coronavirus E

861 protein in membrane vesicles from virus-infected cells and E protein-expressing

862 cells. Virology **263:**265-72.

863 48.   **McBride, C. E., J. Li, and C. E. Machamer.** 2007. The cytoplasmic tail of

864 the severe acute respiratory syndrome coronavirus spike protein contains a novel

865 endoplasmic reticulum retrieval signal that binds COPI and promotes interaction

866 with membrane protein. J Virol **81:**2418-28.

867 49.   **Mirazimi, A., C. H. von Bonsdorff, and L. Svensson.** 1996. Effect of

868 brefeldin A on rotavirus assembly and oligosaccharide processing. Virology

869 **217:**554-63.

870 50.   **Mortola, E., and P. Roy.** 2004. Efficient assembly and release of SARS

871 coronavirus-like particles by a heterologous expression system. FEBS Lett

872 **576:**174-8.

873 51.   **Nal, B., C. Chan, F. Kien, L. Siu, J. Tse, K. Chu, J. Kam, I. Staropoli, B.

874 Crescenzo-Chaigne, N. Escriou, S. van der Werf, K. Y. Yuen, and R.

875 Altmeyer.** 2005. Differential maturation and subcellular localization of severe

876 acute respiratory syndrome coronavirus surface proteins S, M and E. J Gen Virol

877 **86:**1423-34.

878 52.   **Narayanan, K., K. H. Kim, and S. Makino.** 2003. Characterization of N

879 protein self-association in coronavirus ribonucleoprotein complexes. Virus Res

880 **98:**131-40.

881 53.   **Narayanan, K., A. Maeda, J. Maeda, and S. Makino.** 2000.

882 Characterization of the coronavirus M protein and nucleocapsid interaction in

883 infected cells. J Virol **74:**8127-34.

884 54.   **Narayanan, K., and S. Makino.** 2001. Characterization of nucleocapsid-M

885 protein interaction in murine coronavirus. Adv Exp Med Biol **494:**577-82.

886 55.   **Nguyen, V. P., and B. G. Hogue.** 1997. Protein interactions during

887 coronavirus assembly. J Virol **71:**9278-84.

888 56.   **Nicholls, J. M., J. Butany, L. L. Poon, K. H. Chan, S. L. Beh, S.

889 Poutanen, J. S. Peiris, and M. Wong.** 2006. Time course and cellular localization

890 of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. PLoS

891 Med **3:**e27.

892 57.   **Ortego, J., D. Escors, H. Laude, and L. Enjuanes.** 2002. Generation of a

893 replication-competent, propagation-deficient virus vector based on the

894 transmissible gastroenteritis coronavirus genome. J Virol **76:**11518-29.

895 58.   **Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J.

896 Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N.

897 Tsang, R. W. Yung, T. K. Ng, and K. Y. Yuen.** 2003. Coronavirus as a possible

898 cause of severe acute respiratory syndrome. Lancet **361:**1319-25.

899 59.   **Pelham, H. R.** 1991. Multiple targets for brefeldin A. Cell **67:**449-51.

900   60.   **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P.**

901   **Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A.**

902   **Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C.**

903   **Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B.**

904   **Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S.**

905   **Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and**

906   **W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with

907   severe acute respiratory syndrome. Science **300:**1394-9.

908   61.   **Schelle, B., N. Karl, B. Ludewig, S. G. Siddell, and V. Thiel.** 2006.

909   Nucleocapsid protein expression facilitates coronavirus replication. Adv Exp Med

910   Biol **581:**43-8.

911   62.   **Stertz, S., M. Reichelt, M. Spiegel, T. Kuri, L. Martinez-Sobrido, A.**

912   **Garcia-Sastre, F. Weber, and G. Kochs.** 2007. The intracellular sites of early

913   replication and budding of SARS-coronavirus. Virology **361:**304-15.

914   63.   **Tooze, J., S. Tooze, and G. Warren.** 1984. Replication of coronavirus

915   MHV-A59 in sac- cells: determination of the first site of budding of progeny virions.

916   Eur J Cell Biol **33:**281-93.

917   64.   **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J.**

918   **Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J.**

919   **Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus.

920   Nat Med **10:**368-73.

921   65.   **Vennema, H., G. J. Godeke, J. W. Rossen, W. F. Voorhout, M. C.**

922   **Horzinek, D. J. Opstelten, and P. J. Rottier.** 1996. Nucleocapsid-independent

923   assembly of coronavirus-like particles by co-expression of viral envelope protein

924   genes. Embo J **15:**2020-8.

40

925   66.   **Wilson, L., P. Gage, and G. Ewart.** 2006. Hexamethylene amiloride blocks

926   E protein ion channels and inhibits coronavirus replication. Virology **353:**294-306.

927   67.   **Wilson, L., C. McKinlay, P. Gage, and G. Ewart.** 2004. SARS coronavirus

928   E protein forms cation-selective ion channels. Virology **330:**322-31.

929   68.   **Woo, P. C., S. K. Lau, C. M. Chu, K. H. Chan, H. W. Tsoi, Y. Huang, B.**

930   **H. Wong, R. W. Poon, J. J. Cai, W. K. Luk, L. L. Poon, S. S. Wong, Y. Guan, J.**

931   **S. Peiris, and K. Y. Yuen.** 2005. Characterization and complete genome

932   sequence of a novel coronavirus, coronavirus HKU1, from patients with

933   pneumonia. J Virol **79:**884-95.

934   69.   **Woo, P. C., S. K. Lau, K. S. Li, R. W. Poon, B. H. Wong, H. W. Tsoi, B.**

935   **C. Yip, Y. Huang, K. H. Chan, and K. Y. Yuen.** 2006. Molecular diversity of

936   coronaviruses in bats. Virology **351:**180-7.

937   70.   **Ye, Y., and B. G. Hogue.** 2007. Role of the coronavirus E viroporin protein

938   transmembrane domain in virus assembly. J Virol **81:**3597-607.

939

Figure legends

Figure 1. Production of SARS-CoV VLPs by transfected Vero E6 cells. A.
Coexpression of M, E and N is necessary for efficient secretion of SARS-CoV
VLPs by Vero E6 cells at 24 and 48 hours post-transfection. Monolayers of Vero
E6 cells were transfected with plasmids driving the expression of SARS-CoV
structural proteins M, E and Flag-tagged N as specified on top of each lane.
Protein expression in cell lysates and in VLPs isolated from culture medium was
analyzed by Western blot at 24 and 48 hours post-transfection, as indicated below
corresponding panels. Samples were heat-denatured and reduced with
dithiothreitol before loading. The M and E proteins were detected with rabbit
polyclonal antibodies produced against the C-terminal extremity of each proteins.
The N protein was detected with the M2 monoclonal antibody recognizing the Flag
tag. Blots were exposed for 1 minute for signal detection except for detection of E
contained in pellets from ultracentrifuged culture medium (bottom panels), for
which blots were exposed for 10 minutes. The molecular mass (in kDa) and
migration of protein standards are shown between blots. **B.** Use of the bicistronic
pIRES-M-E vector restrains E expression level and favors production of M-E-N
VLPs. Vero E6 were transfected with indicated plasmid combinations and cell
lysates and medium were analyzed at 48 hours post-transfection as in A. To
ensure better detection of E, VLPs were concentrated four times more than in A.
Blots were exposed for 10 seconds for signal detection except for detection of E
contained in pellets from ultracentrifuged culture medium (right bottom panel) for
which the blot was exposed for 1 minute. **C.** Secreted viral structural proteins
cosediment in sucrose gradient. Three 75 cm^2 dishes of Vero E6 cells were

42

965 transfected with plasmids driving the expression of SARS-CoV structural proteins

966 M, E and Flag-tagged N either individually or in combination. Protein expression in

967 cell lysates and in pellets from culture medium ultracentrifuged on 20% sucrose

968 cushion was controlled by Western blot at 48 hours post-transfection (left panel).

969 Resuspended pellets from ultracentrifuged cell medium were then loaded on a 20-

970 60% discontinuous sucrose gradient and subjected to fractionation by

971 ultracentrifugation. Twenty fractions of 600 μl were collected (1-20, lightest to

972 heaviest). Nature of viral proteins associated with each fraction was determined by

973 Western blot (right panels, a to e). 15 μl of samples were heat-denatured and

974 reduced with dithiothreitol before loading. The molecular mass (in kDa) and

975 migration of protein standards are shown on the right side of the blots. Samples

976 from pIRES-M, pIRES-E plus pCDNA-E, pIRES-E plus pCDNA-E pluspcDNA-

977 Nflag and pcDNA-E, pIRES-M plus pcDNA-Nflag have been generated in two

978 separate experiments.

979 Ctrl: non-transfected cells; E: pCDNA-E; M: pIRES-M; M-E: pIRES-M-E; M +

980 Nflag: pIRES-M plus pcDNA-Nflag; M-E + Nflag: pIRES-M-E plus pcDNA-Nflag; M

981 + E: pIRES-M + pcDNA-E; M + E + Nflag: pIRES-M plus pcDNA-E plus pcDNA-

982 Nflag.

983

984 **Figure 2. S glycoprotein trimers are incorporated onto SARS-CoV M, E and**

985 **N-containing VLPs.** pcDNA-S plasmid was co-transfected with pIRES-M-E and

986 pcDNA-Nflag vectors. Culture medium was harvested at 48 hours post-

987 transfection, ultracentrifuged on 20% sucrose cushion and pellets were

988 resuspended in TNE buffer and ultracentrifuged on a 20-60% discontinuous

989 sucrose gradient. Twenty fractions were collected (1-20, lightest to heaviest) and

43

990 analyzed by Western blot. Samples were either heat-denatured and reduced with

991 dithiothreitol before loading for detection of Nflag, M and E or not heated and not

992 reduced for analysis of S. Blots were exposed for 10 seconds for signal detection,

993 except for E for which blots were exposed for 10 minutes. The highest levels of S,

994 M, N and E structural proteins were found in fractions 9 and 10 corresponding to

995 40% sucrose. S protein was detected with mouse polyclonal antibodies raised

996 against purified S trimers. Arrows indicate bands that correspond to trimeric and

997 monomeric forms of S. The molecular mass (in kDa) and migration of protein

998 standards are shown on the right side of the blots.

999

1000 **Figure 3. Structural analysis and intracellular distribution of SARS-CoV**

1001 **VLPs.** Vero E6 cells were co-transfected with pIRES-M-E, pcDNA-Nflag and

1002 pcDNA-S. At 24 and 48 hours post-transfection cells were fixed and ultrathin

1003 sections were analyzed by electron microscopy (panels A to G). **A.** VLPs were

1004 found in intracellular vacuoles (vc), in vesicles (vs) scattered in the cytosol and

1005 bound to the plasma membrane (pm). The arrow points VLPs attached to the cell

1006 surface. **B.** Large amount of VLPs within the lumen of the endoplasmic reticulum

1007 (er) and within a cytoplasmic vesicle. n: nucleus. **C.** Presence of VLPs within

1008 vacuoles and vesicles. Arrows point to small VLPs-containing vesicles beneath the

1009 plasma membrane. **D.** Magnification of a VLP-containing vacuole. Black arrows

1010 point to budding events. **E.** Compacted VLP-containing vesicles were found

1011 beneath the plasma membrane. **F.** VLPs bound to the surface of a producer cell.

1012 Two membrane-bound VLPs are shown by arrows. **G.** VLPs bound to a cell

1013 filopodia. Spike-like protuberances were visible on VLP surface (arrows). **H.**

1014 Electron microscopy images of negatively stained VLPs purified from cell medium

44

1015 at 48 hours post-transfection. A scale bar is indicated for each picture. A-D and F-

1016 G panels correspond to cells fixed at 48 hours post-transfection. Image E was

1017 taken from cells fixed 24 hours post-transfection. n: nucleus, er: endoplasmic

1018 reticulum, vc: vacuole, vs: vesicle, pm: plasma membrane.

1019

1020 **Figure 4. Expression and subcellular distribution of viral structural proteins**

1021 **tagged with fluorescent proteins.** Vero E6 cells grown on glass coverslips were

1022 either transfected with single plasmids (left panels) or co-transfected (right panels)

1023 with the three plasmids encoding the MmRFP1, E, SeYFP and NeCFP proteins. At

1024 24 hours post-transfection, cells were processed for nuclear staining with DAPI

1025 dye, fixed and analyzed under a fluorescent microscope equipped with an

1026 ApoTome device to acquire images of optical sections. a, b, g, h: MmRFP1; c, d, I,

1027 j: SeYFP; e, f, k, l: NeCFP; m, n: merged images. For all conditions, two

1028 representative images are shown side-by-side.

1029

1030 **Figure 5. Production of fluorescent VLPs by transfected Vero E6 cells. A.**

1031 Determination of optimal plasmid combinations for production of SARS-CoV

1032 fluorescent VLPs. Viral proteins from cell lysates (left panel) and sedimented VLPs

1033 from medium (right panel) were analyzed by Western blot. VLPs could be

1034 produced when either N or S (lanes 9 and 10) but not M (lane 8) were tagged with

1035 fluorescent proteins. **B.** Production of fluorescent VLPs and efficiency of

1036 incorporation of NeCFP and SeYFP fusion proteins into VLPs. Cells were co-

1037 transfected with the specified plasmid combinations (corresponding to lanes 9 and

1038 10 of panel A) and purified VLPs from medium were analyzed by sucrose gradient.

1039 Tagging S (lower blot) resulted in a greater yield of VLP production when

45

1040 compared to tagged N (upper blot). However, the eYFP tag greatly reduced S

1041 incorporation onto VLPs (lower blot). Arrows indicates spike trimers and

1042 monomers.

1043

1044 **Figure 6. Tracking of fluorescent SARS-CoV VLPs in living cells. A.** Widefield

1045 fluorescence microscopy images showing accumulation of fluorescent VLPs at the

1046 plasma membrane of pIRES-M-E plus pcDNA-NeCFP co-transfected cells (panels

1047 b), whereas a strong perinuclear staining was observed in Vero E6 expressing

1048 NeCFP alone (panel a). **B.** Confocal microscopy of living cells expressing M-E-

1049 NeGFP VLPs. Four categories of fluorescent signals were observed: a bright and

1050 static large perinuclear compartment (white encircling lines), smaller and dimmer

1051 actively trafficking vesicles (orange encircling lines), bright dots accumulating at

1052 the cell cortex (yellow encircling lines) and dots in the medium surrounding

1053 transfected cells (yellow dots). Videos are available as Supplemental Material. **C.**

1054 Treatment of transfected cells with brefeldin A (BFA) alters trafficking of

1055 fluorescent vesicles. Vero E6 cells were transfected with pIRES-M-E plus pcDNA-

1056 NeGFP plasmids. Cells were either non-treated (a) or treated with 6µg/ml of BFA

1057 for either 4 hours (d) or overnight (g). BFA was then added to non-treated cells

1058 and time-lapse acquisitions were performed. Panels b and c show the same cell

1059 than in panel a but after 25 and 70 minutes incubation with the drug. Alternatively,

1060 BFA was washed out and recovery after BFA treatment was analysed (panels e, f

1061 and h, i). Panels a, b and c, d and e, g and h show same cells at different time

1062 points. Video illustrating panels b, d, f, h and I are provided as supplementary

1063 data.

1064

A

anti-flag
anti-M
anti-E

anti-flag
anti-M
anti-E

Cell lysates   Medium

24h   48h

B

anti-flag
anti-M
anti-E

Cell lysates   Medium

48h

C

anti-flag
anti-M
anti-E

anti-flag
anti-M
anti-E

Cell lysates

Medium

M
anti-M   a

E
anti-E   b

M + E
anti-M
anti-E   c

M + Nflag
anti-flag
anti-M   d

M + E + Nflag
anti-flag
anti-M
anti-E   e

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 1

M-E + Nflag + S

anti-S

anti-flag

anti-M

anti-E

260
160
50
40
30
20
10

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 2

ACCEPTED

A — pm, vc, vs, vs, vs, vs, vs, vs, vc, vc, 1 μm

B — n, er, vs, pm, 1 μm

C — pm, vs, vc, vc, vs, 1μm

D — vc, 200nm

E — pm, vs, vs, 500nm

F — pm, 200nm

G — 200nm

H — 100nm

Figure 3

individual transfections

MmRFP1-E + SeYFP + NeCFP
co-transfection

a   b   g   h   MmRFP1

c   d   i   j   SeYFP

e   f   k   l   NeCFP

m   n   merge

Figure 4

A

	1	2	3	4	5	6	7	8	9	10
M-E	-	+	-	+	+	-	+	-	+	+
MmRFP1-E	-	-	+	-	-	-	-	+	-	-
Nflag	-	+	+	-	+	-	+	+	-	+
NeCFP	-	-	-	+	-	-	-	-	+	-
S	-	+	+	+	-	-	+	+	+	-
SeYFP	-	-	-	-	+	-	-	-	-	+

anti-S

anti-N

anti-flag

anti-M

anti-E

Cell Lysates   Medium

B

M-E + NeCFP + S

anti-S

anti-N

anti-M

anti-E

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

260
160
80
60
30
20
10

M-E + Nflag + SeYFP

anti-S

anti-flag

anti-M

anti-E

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

260
160
50
40
30
20
10

Figure 5

A NeCFP   M-E + NeCFP   **B** M-E + NeGFP

C   M-E + NeGFP

No BFA   No BFA + 25min BFA   No BFA + 70min BFA

4h BFA   4h BFA + 30min wash   4h BFA + 2h wash

O/N BFA + 5min wash   O/N BFA + 1h30 wash   O/N BFA + 3h wash

Figure 6