JVI Accepts, published online ahead of print on 3 September 2014
J. Virol. doi:10.1128/JVI.02078-14

Human coronavirus NL63 utilize heparan sulfate proteoglycans for attachment to target cells

Aleksandra Milewska[a], Mirosław Zarebski[b], Paulina Nowak[a], Karol Stozek[a], Jan Potempa[a,c], Krzysztof Pyrc[a,d,#]

[a] Microbiology Department, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland.

[b] Division of Cell Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland.

[c] Oral Health and Systemic Disease Research Group, School of Dentistry, University of Louisville, Louisville, KY, USA

[d] Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7, 30–387 Krakow, Poland

Word count:

1. Abstract: 152 + 114
2. Main text: 4 461

Corresponding author: Krzysztof Pyrc, Microbiology Department, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland; Phone number: +48 12 664 61 21; Fax: +48 12 664 69 02.
E-mail: k.a.pyrc@uj.edu.pl

ABSTRACT

Human coronavirus NL63 (HCoV-NL63) is an alphacoronavirus that was first identified in 2004 in the nasopharyngeal aspirate from a 7-month-old patient with a respiratory tract infection. Previous studies showed that HCoV-NL63 and the genetically distant SARS-CoV employ the same receptor for host cell entry, angiotensin converting enzyme 2 (ACE2), but it is largely unclear whether ACE2 interactions are sufficient to allow HCoV-NL63 binding to cells. The present study showed that directed expression of angiotensin-converting enzyme 2 (ACE2) on cells previously resistant to HCoV-NL63 renders them susceptible, showing that ACE2 protein acts as a functional receptor and its expression is required for infection. However, comparative analysis showed that directed expression or selective scission of the ACE2 protein had no measurable effect on virus adhesion. In contrast, binding of HCoV-NL63 to heparan sulfates was required for viral attachment and infection of target cells, showing that these molecules serve as attachment receptors for HCoV-NL63.

46 **IMPORTANCE**

47 ACE2 protein has been proposed as a receptor for HCoV-NL63 already in 2005, but the
48 in-depth analysis of early events during virus infection was not performed thus far. Here, we
49 show that the ACE2 protein is required for viral entry, but it is not the primary binding site on
50 the cell surface. Conducted research showed that heparan sulfate proteoglycans function as
51 adhesion molecules, increasing the virus density on cell surface and possibly facilitating
52 interaction between HCoV-NL63 and its receptor. Obtained results show that the initial
53 events during HCoV-NL63 infection are more complex than anticipated and newly described
54 interaction may be essential for understanding the infection process and, possibly, also assist
55 in the drug design.

56

57

58

59

60

61

62

63

64

65

66

67 **Keywords:** coronavirus, coronaviruses, virus, HCoV-NL63, attachment, receptor, ACE2,
68 angiotensin converting enzyme 2, heparan sulfate proteoglycans, heparan sulfate.

69

INTRODUCTION

Coronaviruses (CoVs) are enveloped positive-stranded RNA viruses with large genomes ranging in size from 27 to 32 kb. Six human coronaviruses have been identified to date, and four of them (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1) are thought to be responsible for ~30% of common cold cases (1). By contrast, infection with severe acute respiratory syndrome coronavirus (SARS-CoV) results in a serious respiratory tract infection, which in 2002-2003 season affected approximately 8 000 patients with a mortality rate of ~10% (2, 3). Similarly, the recently isolated Middle East respiratory syndrome coronavirus (MERS-CoV) causes life-threatening pneumonia and renal failure, with almost 300 fatal cases reported to date (4).

Human coronavirus NL63 was first identified in 2004 in the nasopharyngeal aspirate from a 7-month-old patient with a respiratory tract infection. The virus is distributed worldwide and causes respiratory infections of varying severity, with the most severe symptoms seen in children and immunocompromised patients (5-9).

Like other human coronaviruses, the HCoV-NL63 genome encodes a glycoprotein, called the spike (S) protein, which protrudes from the virion surface, thereby conferring the corona-like form (6, 10, 11). The S protein is the main mediator of viral entry and determines the host tropism of the coronavirus (12, 13). A study undertaken in 2005 used retroviral reporter pseudoviruses carrying the HCoV-NL63 spike protein to show that HCoV-NL63 engages the SARS-CoV receptor, angiotensin-converting enzyme 2 (ACE2), for infectious entry (14-16). ACE2 is a type I integral membrane protein abundantly expressed in tissues lining the respiratory tract. This carboxypeptidase cleaves angiotensin II and functions within the renin angiotensin system (RAS) important for maintaining lung homeostasis and blood pressure (17-19). Down-regulation of ACE2 protein levels may lead to the development of

acute respiratory distress syndrome. Thus, down-regulation of ACE2 expression in the lungs upon SARS-CoV infection is associated with viral pathogenesis (20-23).

HCoV-NL63 can be cultured in monkey epithelial cells lines that endogenously express ACE2 (e.g., LLC-Mk2, Vero E6, or Vero B4 cells), as well as in the human hepatoma cell line, Huh-7; this host preference is shared with SARS-CoV (24-26). Hofmann *et al.* (14) conducted a thorough analysis of the cellular tropism of these two human coronaviruses and found out that pseudovirions bearing the spike proteins of HCoV-NL63 (NL63-S) and SARS-CoV (SARS-S) showed a similar ability to infect target cells. However, some studies show that SARS-CoV S protein has a higher affinity for ACE2 than the HCoV-NL63 S protein (20, 27).

Even though the cellular receptor for the HCoV-NL63 was described, until present it was unknown whether it may serve as an adhesion factor and is sufficient to facilitate viral entry. Here, we show that directed expression of the ACE2 protein renders the cells permissive to HCoV-NL63 infection. Interestingly, the presence of the receptor protein seems not to correlate with the adhesion of virions to cell surface, hence suggesting presence of yet another factor important during early stages of infection. Subsequent analysis showed that heparan sulfate (HS) proteoglycans function as adhesion receptors for HCoV-NL63, complementing the action of the ACE2 protein. Assessment of viral replication dynamics clearly shows that the adhesion of HCoV-NL63 to heparin sulfate proteoglycans enhances viral infection.

MATERIALS AND METHODS

Cell culture

LLC-Mk2 cells (ATCC: CCL-7; *Macaca mulatta* kidney epithelial) were maintained in minimal essential medium (MEM; two parts Hanks' MEM and one part Earle's MEM; Life Technologies, Poland) supplemented with 3% heat-inactivated fetal bovine serum (Life Technologies, Poland), penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and ciprofloxacin (5 µg ml^{-1}). Human 293T (ATCC: CRL-3216; kidney epithelial), A549 (ATCC: CCL-185; lung carcinoma) were maintained in Dulbecco's MEM (Life Technologies, Poland) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Poland), penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), and ciprofloxacin (5 µg ml^{-1}). Cells were cultured at 37°C under 5% CO_2.

Isolation of nucleic acids and reverse transcription

HCoV-NL63 nucleic acids were isolated from cell culture supernatants using the Total RNA Mini-Preps Super Kit (Bio Basic, Canada), according to the manufacturer's instructions. Reverse transcription was carried out with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Poland), according to the manufacturer's instructions.

Cell lines expressing ACE2

293T cells (ATCC CRL-3216) were transfected with the pLKO.1-TRC-ACE2 plasmid using polyethylenimine (PEI; Sigma-Aldrich, Poland). The plasmid was based on the Addgene plasmid 10878 (28). At 24 h post-transfection, the cells were washed with sterile 1 × PBS and cultured at 37°C for 48 h in media supplemented with puromycin (2 µg ml^{-1}) at 37°C with 5% CO_2. Following selection, cells were passaged and the surviving clones were collected and analyzed as described below. ACE2-expressing ($ACE2^{+}$) cells were maintained

in Dulbecco's MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), ciprofloxacin (5 µg ml^{-1}) and puromycin (1 µg ml^{-1}).

ACE2-expressing A549 cells (A549_$ACE2^{+}$) were generated using retroviral vectors that were based on the Moloney Murine Leukemia Virus system. Briefly, Phoenix-Ampho cells (ATCC CRL-3213) were transfected with a pLNCX2 vector (Clontech, USA) encoding the ACE2 protein using PEI. At 24 h post-transfection the medium was refreshed and the cells were cultured for a further 24 h at 32°C. Subsequently, the vector-containing supernatants were harvested, aliquoted, and stored at −80°C.

A549_WT cells were cultured in six-well plates (TPP, Switzerland) and infected with 1 ml of generated retroviruses in the presence of polybrene (5 µg ml^{-1}, Sigma-Aldrich). After 24 h incubation at 37°C, the cells were cultured medium supplemented with G418 (BioShop, Canada; 5 mg ml^{-1}) and passaged for 3 weeks at 37°C. Surviving clones were recovered and analyzed as described below. A549_$ACE2^{+}$ cells were maintained in Dulbecco's MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), ciprofloxacin (5 µg ml^{-1}) and G418 (5 mg ml^{-1}).

Virus preparation, titration, and cell infection

The HCoV-NL63 stock (isolate Amsterdam 1) was generated by infecting monolayers of LLC-Mk2 cells. Cells were then lysed by two freeze-thaw cycles at 6 days post-infection (p.i.). The virus-containing liquid was aliquoted and stored at −80°C. A control LLC-Mk2 cell lysate from mock-infected cells was prepared in the same manner. The virus yield was assessed by titration on fully confluent LLC-Mk2 cells in 96-well plates, according to the method of Reed and Muench (29). Plates were incubated at 32°C for 6 days and the cytopathic effect (CPE) was scored by observation under an inverted microscope.

In subsequent experiments, fully confluent cells (293T_WT/ACE2^{+} and A549_WT/ACE2^{+}) in six-well plates (TPP) were exposed to HCoV-NL63 at a $TCID_{50}$ ml^{-1} of 5 000. HCoV-NL63-permissive LLC-Mk2 cells were infected with the virus at a $TCID_{50}$ ml^{-1} of 400. Following a 2 h incubation at 32°C, unbound viruses were removed by washing with sterile 1 × PBS and fresh medium was added to each well. Samples of cell culture supernatant were collected every 24 h for 6 days and analyzed by real-time PCR.

Quantitative PCR

The virus yield was determined using real-time PCR (7500 Fast Real-Time PCR machine; Life Technologies, Poland). Viral cDNA (2.5 µl per sample) was amplified in a 10 µl reaction mixture containing 1 × TaqMan Universal PCR Master Mix (Life Technologies, Poland), specific probes labeled with 6-carboxyfluorescein (FAM) and 6-carboxytertamethylrhodamine (TAMRA) (100 nM), and primers (450 nM each). The following primers were used for HCoV-NL63 amplification: sense, 5' – AAA CCT CGT TGG AAG CGT GT - 3'; antisense, 5' – CTG TGG AAA ACC TTT GGC ATC - 3', probe, 5' – FAM -ATG TTA TTC AGT GCT TTG GTC CTC GTG AT – TAMRA - 3'. Rox was used as the reference dye. The reaction conditions were as follows: 2 min at 50°C and 10 min at 92°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C.

Gradient purification of HCoV-NL63

The virus stock was concentrated 25-fold using centrifugal protein concentrators (Amicon Ultra, 10 kDa cut-off; Merck, Poland) and subsequently layered onto a 15% iodixanol solution in 1 × PBS (OptiPrep medium; Sigma-Aldrich, Poland). Following centrifugation at 175 000 × g for 3 h at 4°C (cushion), virus-containing fractions were layered onto a 10-20% iodixanol gradient (in 1 × PBS) and centrifuged at 175 000 × g for 18 h at 4°C.

Fractions collected from the gradient were analyzed by Western blotting, followed by detection of the HCoV-NL63 nucleocapsid protein. The resulting virus-containing fractions were aliquoted and stored at −80°C. The control cell lysate (mock) was prepared in the same manner as the virus stock.

Detection of sub-genomic mRNAs

Total nucleic acids were isolated from virus- and mock-infected cells 5 days p.i. using the Total RNA Mini-Preps Super Kit (Bio Basic, Canada), according to the manufacturer's instructions. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Poland), according to the manufacturer's instructions. Viral cDNA (3 µl) was amplified in a 20 µl reaction mixture containing 1 × Dream Taq Green PCR Master Mix and primers (each primer was used at 500 nM). The following primers were used to amplify HCoV-NL63 sub-genomic (sg) mRNA: common sense primer (leader sequence), 5' – TAA AGA ATT TTT CTA TCT ATA GAT AG – 3'; 1a/b polyprotein antisense, 5' – CAT CAA AGT CCT GAA GAA CAT AAT TG – 3'; spike antisense, 5' – ACT ACG GTG ATT ACC AAC ATC AAT ATA - 3'; spike (nested PCR) antisense, 5' – AGA GAT TAG CAT TAC TAT TAC ATG TG - 3'; ORF3 antisense, 5' – GCA CAT AGA CAA ATA GTG TCA ATA GT – 3'; envelope antisense, 5' – GCT ATT TGC ATA TAA TCT TGG TAA GC – 3'; membrane antisense, 5' – GAC CCA GTC CAC ATT AAA ATT GAC A – 3'; nucleocapsid antisense, 5' – CTT ATG AGG TCC AGT ACC TAG GTA AT – 3'. The conditions were as follows: 3 min at 95°C, 40 cycles (30 cycles for nested PCR) of 30 sec at 95°C, 30 sec at 47°C and 25 sec at 72°C, and then 5 min at 72°C and 10 min at 4°C. The PCR products were run on 1% agarose gels (1 × TAE buffer) and analyzed using Molecular Imaging Software (Kodak).

212 ***Western blot analysis***

213 Cells used for Western blot analysis were harvested at 5 days p.i. by scraping in ice-cold
214 1 × PBS. The cells were then centrifuged and resuspended in RIPA buffer (50 mM Tris,
215 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5) followed
216 by lysis in RIPA buffer for 30 min on ice. Subsequently, samples were centrifuged (10 min at
217 12 000 × g) and the pelleted cell debris was discarded. Total protein concentration of each
218 sample was quantified using the BCA method and the resulting supernatants were mixed with
219 sample buffer (0.5 M Tris pH 6.8, 10% SDS, 50 mg/ml DTT), boiled for 5 min, cooled on ice,
220 and separated on 10% polyacrylamide gels alongside dual color Page Ruler Pre-stained
221 Protein size markers (Thermo Scientific, Poland). The separated proteins were then
222 transferred onto a Westran S PVDF membrane (Whatman) by semi-dry blotting (Bio-Rad) for
223 1.5 h, 100 Volts in transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol at 4°C. The
224 membranes were then blocked by overnight incubation (at 4°C) in TBS-Tween (0.1%) buffer
225 supplemented with 5% skimmed milk (BioShop, Canada). A goat anti-human ACE2
226 ectodomain antibody (2 μg ml^{-1}; R&D Systems, USA) and horseradish peroxidase-labeled
227 rabbit anti-goat IgG (26 ng ml^{-1}; Dako, Denmark) were used to detect the ACE2 protein in
228 human cell lysates and cell supernatants. A mouse anti-HCoV-NL63-N protein antibody
229 (500 ng ml^{-1}; Ingenansa, Spain) and horseradish peroxidase-labeled rabbit anti-mouse IgG
230 (65 ng ml^{-1}; Dako, Denmark) were used to detect the HCoV-NL63 nucleocapsid protein. A
231 mouse anti-β-actin antibody (50 ng ml^{-1}; BD Biosciences, USA) and horseradish peroxidase-
232 labeled rabbit anti-mouse IgG (65 ng ml^{-1}; Dako, Denmark) were used for detection of β-
233 actin. All antibodies were diluted in 1% skimmed milk/TBS-Tween (0.1%). The signal was
234 developed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) and
235 visualized by exposing the membrane to an X-ray film (Kodak).
236

237 ***Flow cytometry***

238 A549-WT/ACE2^{+} and LLC-Mk2 cells were seeded in six-well plates (TPP,
239 Switzerland), cultured for 2 days at 37°C, and stimulated with PMA (phorbol 12-myristate
240 13-acetate; 1 µM; Sigma-Aldrich, Poland) for 1 h at 37°C. To examine HCoV-NL63
241 adhesion, cells were washed with 1 × PBS and incubated with iodixanol-concentrated
242 HCoV-NL63 or mock control for 2 h at 4°C. The cells were then washed with 1 × PBS, fixed
243 with 3% PFA, permeabilized with 0.1% Triton X-100 in 1 × PBS, and incubated for 1 h with
244 3% BSA/0.1% Tween 20 in 1 × PBS. To examine the HCoV-NL63 adhesion, cells were
245 mechanically detached from the plate surface and incubated for 2 h at room temperature with
246 a mouse anti-HCoV-NL63-N antibody (1 µg ml^{-1}; Ingenansa, Spain), followed by a 1 h
247 incubation with an Alexa Fluor 488-labeled goat anti-mouse antibody (2.5 µg ml^{-1}; Molecular
248 Probes). For ACE2 staining, cells were washed with 1 × PBS, scraped from the plates, and
249 incubated for 2 h at 4°C with goat anti-ACE2 ectodomain IgG (4 µg ml^{-1}; R&D Systems,
250 USA), followed by a 1 h incubation with an FITC-labeled rabbit anti-goat IgG antibody
251 (13 µg ml^{-1}; Dako, Denmark). Cells were then washed, resuspended in 1 × PBS and analyzed
252 by flow cytometry (FACSCalibur, Becton Dickinson). Data were analyzed using Cell Quest
253 software (Becton Dickinson).

254

255 ***Confocal microscopy***

256 LLC-Mk2 cells were seeded on coverslips in six-wells plates (TPP), cultured for 2 days
257 at 37°C and then stimulated with PMA (1 µM; Sigma-Aldrich, Poland) for 1 h at 37°C.
258 Subsequently, the cells were washed with 1 × PBS and incubated with iodixanol-concentrated
259 HCoV-NL63 or mock control for 2 h at 4°C. Cells were then washed with 1 × PBS, fixed
260 with 3% PFA, permeabilized with 0.1% Triton X-100 in 1 × PBS and incubated for 1 h with
261 5% BSA / 0.5% Tween 20 in 1 × PBS. To visualize HCoV-NL63 adhesion, cells were

incubated for 2 h at room temperature with mouse anti-NL63-N IgG (0.25 µg ml^{-1}; Ingenansa, Spain), followed by a 1 h incubation with Alexa Fluor 488-labeled goat anti-mouse IgG (2.5 µg ml^{-1}, Life Technologies, Poland). Nuclear DNA staining was performed with DAPI (0.1 µg ml^{-1}, Sigma-Aldrich, Poland). Immunostained cultures were mounted on glass slides in Vectashield medium (Vector Laboratories, UK). Fluorescent images were acquired under a Leica TCS SP5 II confocal microscope (Leica Microsystems GmbH, Mannheim, Germany). Images were acquired using Leica Application Suite Advanced Fluorescence LAS AF v. 2.2.1 (Leica Microsystems CMS GmbH), deconvolved with Huygens Essential package ver. 4.4 (Scientific Volume Imaging B.V.; The Netherlands) and processed using ImageJ 1.47v (National Institutes of Health, Bethesda, Maryland, USA). Viruses attached to the cell were quantified using 3D Object Counter ImageJ plugin (30) with the histogram threshold set to 80. Analysis was performed on z-stacks (step size: 0.13 µm) of at least 10 cells per sample.

Assessing the effects of neuraminidase on virus adherence

LLC-Mk2 cells were seeded in six-well plates (TPP, Switzerland), cultured for 2 days at 37°C, and incubated with type V neuraminidase (from *Clostridium perfringens*; 100-200 mU/ml; Sigma-Aldrich, Poland) for 1 h at 37°C. The adherence of iodixanol-concentrated HCoV-NL63 was examined as described above.

Assessing the effects of sugars and heparan sulfate on virus replication and adherence

LLC-Mk2 cells were seeded in six-well plates (TPP, Switzerland), cultured for 2 days at 37°C, and incubated with sugar monomers (50 mM; Sigma-Aldrich, Poland) or heparan sulfate - HS (Sigma-Aldrich, Poland) for 1 h at 37°C. Simultaneously, iodixanol-concentrated HCoV-NL63 was incubated with tested compounds for 1 h at 4°C, and virus adherence was examined as described above. To assess HCoV-NL63 replication, cells were washed with

287 1 × PBS and infected with virus pre-incubated with HS at a $TCID_{50}$ ml^{-1} of 100. Following a
288 2 h incubation at 32 °C, unbound virus was removed by washing with 1 × PBS and fresh
289 medium containing HS was added to each well. Samples of cell culture supernatant were
290 collected 6 days post-infection and analyzed in a real-time PCR assay.
291
292 **RESULTS**
293 ***Development of cell lines expressing the ACE2 protein***
294 Human cell lines stably expressing the ACE2 receptor (A549 and 293T) were
295 developed in-house. Expression and surface localization of the ACE2 protein were confirmed
296 by Western blotting (**Figure 1A**) and flow cytometry (**Figure 1B**), respectively.
297
298 ***ACE2 acts as a receptor for HCoV-NL63 and is sufficient to enable infectious entry***
299 Human cell lines expressing the ACE2 protein were used to determine whether surface
300 expression of ACE2 is sufficient for HCoV-NL63 entry. Both $ACE2^+$ and WT A549
301 and 293T cells were infected with HCoV-NL63 and cultured for 6 days at 32°C. Infection of
302 $A549\_ACE2^+$ cells resulted in clear CPE at 3 days p.i.; no CPE was observed in
303 HCoV-NL63-infected A549_WT cells and $293T\_WT/ACE2^+$ cells (**Figures 2A** and **2B**,
304 respectively**)**.
305 Despite the apparent lack of CPE in HCoV-NL63-infected $293T\_ACE2^+$ cells up to
306 7 days p. i., the virus replication was examined by Western blotting with antibodies specific
307 for the NL63-N protein. The results showed that viral protein was detectable in $293T\_ACE2^+$
308 and $A549\_ACE2^+$ cells, suggesting that expression of the ACE2 protein rendered these cell
309 lines permissive to infection by HCoV-NL63. No NL63-N protein was detected in WT cell
310 lines (**Figure 3).**

311 Coronaviruses employ discontinuous replication strategy to generate sg mRNAs during
312 minus strand synthesis; these mRNAs are then copied into plus strand mRNAs. Plus stranded
313 sg mRNA molecules are formed exclusively during virus replication and may therefore serve
314 as markers for an active infection. Thus, we next examined WT and ACE2$^+$ cells for the
315 presence of each HCoV-NL63 sg mRNAs after virus inoculation. As shown in **Figure 4A,**
316 HCoV-NL63 sg mRNAs were formed in A549 and 293T cell lines expressing the ACE2
317 protein. Five mRNAs encoding viral structural and accessory proteins (spike (S), ORF3
318 protein (ORF3), envelope (E), membrane (M) and nucleocapsid (N)) and genomic RNA were
319 present, indicating active virus replication. No replication was noted in WT cells. These
320 results confirm that ACE2 may act as a functional receptor for HCoV-NL63 virus.
321 Last but not least, viral replication kinetics was assessed by real-time PCR in cell lines
322 supporting HCoV-NL63 replication (**Figure 4B**). The results confirmed virus replication and
323 progeny production in A549_ACE2$^+$ cells; a steep rise in the number of viral copies in the
324 culture medium was observed already on day 3 p.i., corresponding in time with the first signs
325 of CPE. No CPE or significant increase in viral yield was observed in 293T_WT/ACE2$^+$.
326
327 ***Adhesion of HCoV-NL63 to mammalian cells***
328 Next, a set of experiments to determine whether ACE2 serves as an attachment factor
329 for HCoV-NL63 was performed. To address this, A549_WT and A549_ACE$^+$ cells were
330 incubated at 4°C with gradient-purified HCoV-NL63 and virus adhesion to the cell surface
331 was examined using flow cytometry. The virus bound to both cell lines, suggesting that a cell
332 surface molecule other than ACE2 must be responsible for adhesion (**Figure 5**).
333 Similarly, naturally permissive, normal and PMA-treated (31) LLC-Mk2 cells were
334 incubated at 4°C with gradient-purified HCoV-NL63 and virus adhesion to cell surface was
335 examined by flow cytometry. Even though PMA-mediated ACE2 scission inhibited

replication of HCoV-NL63 (**Figure 6A**), we observed no difference in virus attachment to normal and PMA-treated cells (**Figure 6B**). Likewise, decrease in cell surface ACE2 protein levels on LLC-Mk2 cells after PMA treatment was confirmed by flow cytometry (**Figure 6C**).

To confirm the flow cytometry results, we used confocal microscopy to examine HCoV-NL63 adhesion to PMA-stimulated and normal LLC-Mk2 cells. A representative image is presented in **Figure 6D**, which confirms that ACE2 shedding does not affect HCoV-NL63 binding to the cell surface.

Sialic acid or sugars moieties do not function as attachment receptor for HCoV-NL63

The results outlined above suggest that another molecule on the cell surface is responsible for virion attachment. Therefore, the role of sialic acid in virus adhesion was examined. To this end, HCoV-NL63 replication was analyzed in cells pre-incubated with *C. perfringens* type V neuraminidase, which shows a broad specificity for sialic acid-containing substrates (32). Flow cytometric analysis of HCoV-NL63 adhesion to LLC-Mk2 cells pre-incubated with neuraminidase showed no difference between control cells and cells lacking sialic acids and ACE2 (**Figure 7A-C**). To ensure that sialic acid was enzymatically removed, influenza virus was used as a positive control. As expected, a significant inhibition of virus replication on A549 cells was observed after neuraminidase treatment, as this common carbohydrate moiety represents a functional receptor for influenza viruses (data not shown).

Comparable experiments were undertaken to analyze whether lectins are responsible for HCoV-NL63 attachment to target cells. For this, several sugar monomers, such as D-(+)-galactose, D-(+)-mannose, D-(+)-N-acetylglucosamine, L-(-)-fucose (33-35) were used in virus adhesion experiments on LLC-Mk2 cells. Additionally, D-(+)-glucose, a carbohydrate

monomer, which does not constitute a ligand for known mammalian lectins, was included as a negative control. HCoV-NL63 adhesion to LLC-Mk2 in the presence of selected sugar moieties was analyzed using flow cytometry. No modulation of virus adhesion to cell surface was observed (**Figure 7D-H**).

Heparan sulfate inhibits virus attachment and entry

As HS proteoglycans are important for entry of several pathogens (36-50), a soluble HS was used to assess whether attachment of HCoV-NL63 is mediated by these molecules. Flow cytometric analysis demonstrated that in the presence of HS virus adhesion to LLC-Mk2 cells was fully inhibited, showing the role of this molecule in adhesion to susceptible cells and possibly also in cell entry (**Figure 8A**).

In order to analyze whether ACE2 protein participates in virus attachment process, additional experiments were performed. For this, virus adhesion was analyzed in the presence of HS (to avoid the masking effect) on LLC-Mk2 cells with surface expression of ACE2 (DMSO-treated) and ACE2-deprived (PMA-treated). Inhibition of virus-HS proteoglycans interaction resulted in lack of virus binding also on $ACE2^+$ cells; no difference between $ACE2^+$ and $ACE2^-$ was noted (**Figure 8A).** Flow cytometry results were further confirmed by confocal microscopy (**Figure 8B)**.

Subsequent analysis showed that pre-incubation of the virus with HS results in a dose-dependent decline virus replication (**Figure 9**). Taken together, obtained results show that HS proteoglycans act as HCoV-NL63 adhesion receptors and their presence is important for virus entry and replication.

DISCUSSION

Human coronavirus NL63 was first identified ~10 years ago. Since then, a number of research groups have studied this pathogen, resulting in the publication of a considerable number of papers about the virus' epidemiology and biology. Although at first glance HCoV-NL63 may simply be considered a close relative of HCoV-229E, the virus possesses several unique characteristics. The most striking is that it is the only α-coronavirus to use the ACE2 protein for cellular entry (similarly to SARS-CoV). Because these two pathogens use the same receptor, some wonder why SARS-CoV infection manifests as life-threatening acute respiratory syndrome, while HCoV-NL63 infection results in a common cold. One hypothesis presented by Glowacka *et al.* assumes that HCoV-NL63-S shows only low affinity for the ACE2 protein; therefore, its infection efficiency is sub-optimal (20). However, Wu *et al*. showed that the affinity of NL63-S for ACE2 is comparable with that of SARS-S (51). This discrepancy may result from the fact that Glowacka *et al.* used the complete S1 domain of HCoV-NL63-S, while Wu *et al.* used only the receptor-binding domain (RBD). Furthermore, in contrast to HCoV-NL63 infection, SARS-CoV infection results in a marked down-regulation of ACE2 expression on the cell surface, thereby disrupting RAS homeostasis; this in itself may cause severe lung injury (20). Dijkman *et al*. showed that ACE2 expression was down-regulated upon HCoV-NL63 infection, although the result was heavily dependent upon the infection efficiency (52). Based on these reports, one wonders whether ACE2 is actually the cellular receptor for HCoV-NL63. The surface plasmon studies previously published by Glowacka *et al.* and Wu *et al.* may suggest that NL63-S-RBD may interact with ACE2; however, another stimulus may be required to expose the RBD and enable its interaction with the ACE2. That would suggest similar strategy as one employed by HIV-1, where CD_4 binding by gp120 results in structural alteration of the viral protein, which enables gp120 binding to co-receptors and subsequent entry (53).

Here, we showed that directed expression of ACE2 on cells previously resistant to HCoV-NL63 infection renders them susceptible. Next, we examined whether viral adherence was dependent upon the level of ACE2 expression. Comparative analyses using gradient-purified virus, WT cells, and cells overexpressing ACE2 showed that although ACE2 protein is a pre-requisite for virus infection, it does not affect binding of virions to the cell surface. Also selective scission of the ACE2 protein from the cell surface does not affect the virus-cell interaction.

These observations are consistent with those reports showing that NL63-S protein has low affinity for ACE2, and suggest that another molecule/set of molecules may serve as attachment factors. In some β-coronaviruses, sialic acid may function as such a factor; however, we found that removing these surface molecules with neuraminidase had no effect on HCoV-NL63 replication or attachment. Similarly, soluble sugars that should hinder the interaction between the potential lectin-like domain and cellular glycoproteins did not affect virus binding (33-35).

It has been reported that some β and γ coronaviruses (SARS-CoV, culture adapted MHV, IBV) employ HS proteoglycans for adhesion or entry to susceptible cells (48-50). Therefore the adhesion of the virus was evaluated in the presence of HS – a soluble receptor analog. Apparently, this compound blocked the ability of HCoV-NL63 to bind to the cell surface of susceptible cell showing that HS proteoglycans are responsible for virus binding on cells. What is more, the presence of HS proteoglycans strongly enhances virus infection, showing the relevance of the observed phenomena.

One may, however, question whether ability of HS binding was not acquired due to cell culture adaptation, as described for other coronaviral species (49, 54, 55). Analysis of the S gene shows that despite *in vitro* propagation of the Amsterdam I strain, no new potential HS binding sites can be identified compared to clinical isolates (data not shown). It is possible,

however, that different HCoV-NL63 strains bind the HS with different affinity, what would explain the difficulty in acquiring new clinical isolates and late identification of the pathogen (56, 57).

In summary, we examined whether human ACE2 (the receptor for HCoV-NL63) also serves as an attachment factor. HCoV-NL63 adhered equally well to ACE2-expressing and non-expressing cells. These observations indicated the existence of an additional molecule involved in HCoV-NL63 attachment to target cells. Competition experiments using a range of soluble elements of cellular membrane-associated components revealed that HS proteoglycans constitute HCoV-NL63 adhesion receptors. Importantly, the interaction of the virus with HS proteoglycans is important not only for virus binding, but also for its replication.

ACKNOWLEDGEMENTS

This work was supported by a LIDER grant from the National Centre for Research and Development (Lider/27/55/L-2/10/2011), a grant from the Ministry of Science and Higher Education, Poland (Iuventus Plus grant IP2011 044371), and grants from the National Science Center (UMO-2012/07/E/NZ6/01712; UMO-2012/07/N/NZ6/02955). The Faculty of Biochemistry, Biophysics and Biotechnology at Jagiellonian University is a beneficiary of structural funds from the European Union (grant no: POIG.02.01.00-12-064/08 – "Molecular Biotechnology for Health").

Reference List

1. Fields BN, Knipe DM, Howley PM. 2013. Fields virology, 6th ed. Wolters Kluwer/Lippincott Williams & Wilkins Health, Philadelphia.
2. Peiris JS, Yuen KY, Osterhaus AD, Stohr K. 2003. The severe acute respiratory syndrome. The New England journal of medicine 349:2431-2441.
3. Stadler K, Masignani V, Eickmann M, Becker S, Abrignani S, Klenk HD, Rappuoli R. 2003. SARS--beginning to understand a new virus. Nature reviews. Microbiology 1:209-218.
4. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, Fouchier RA, Galiano M, Gorbalenya AE, Memish ZA, Perlman S, Poon LL, Snijder EJ, Stephens GM, Woo PC, Zaki AM, Zambon M, Ziebuhr J. 2013. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. Journal of virology 87:7790-7792.
5. Fouchier RA, Hartwig NG, Bestebroer TM, Niemeyer B, de Jong JC, Simon JH, Osterhaus AD. 2004. A previously undescribed coronavirus associated with respiratory disease in humans. Proceedings of the National Academy of Sciences of the United States of America 101:6212-6216.
6. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. Nature medicine 10:368-373.
7. Pyrc K, Berkhout B, van der Hoek L. 2007. The novel human coronaviruses NL63 and HKU1. Journal of virology 81:3051-3057.
8. van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, Petersen G, Forster J, Berkhout B, Uberla K. 2006. Human coronavirus NL63 infection is associated with croup. Advances in experimental medicine and biology 581:485-491.
9. van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, Petersen G, Forster J, Berkhout B, Uberla K. 2005. Croup is associated with the novel coronavirus NL63. PLoS medicine 2:e240.
10. Pyrc K, Jebbink MF, Berkhout B, van der Hoek L. 2004. Genome structure and transcriptional regulation of human coronavirus NL63. Virology journal 1:7.
11. Pyrc K, Dijkman R, Deng L, Jebbink MF, Ross HA, Berkhout B, van der Hoek L. 2006. Mosaic structure of human coronavirus NL63, one thousand years of evolution. Journal of molecular biology 364:964-973.
12. Gallagher TM, Buchmeier MJ. 2001. Coronavirus spike proteins in viral entry and pathogenesis. Virology 279:371-374.
13. Zheng Q, Deng Y, Liu J, van der Hoek L, Berkhout B, Lu M. 2006. Core structure of S2 from the human coronavirus NL63 spike glycoprotein. Biochemistry 45:15205-15215.
14. Hofmann H, Pyrc K, van der Hoek L, Geier M, Berkhout B, Pohlmann S. 2005. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proceedings of the National Academy of Sciences of the United States of America 102:7988-7993.
15. Hofmann H, Marzi A, Gramberg T, Geier M, Pyrc K, van der Hoek L, Berkhout B, Pohlmann S. 2006. Attachment factor and receptor engagement of SARS coronavirus and human coronavirus NL63. Advances in experimental medicine and biology 581:219-227.
16. Pohlmann S, Gramberg T, Wegele A, Pyrc K, van der Hoek L, Berkhout B, Hofmann H. 2006. Interaction between the spike protein of human coronavirus NL63 and its cellular receptor ACE2. Advances in experimental medicine and biology 581:281-284.
17. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. 2000. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circulation research 87:E1-9.

18. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. 2004. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. The Biochemical journal 383:45-51.
19. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. 2000. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. The Journal of biological chemistry 275:33238-33243.
20. Glowacka I, Bertram S, Herzog P, Pfefferle S, Steffen I, Muench MO, Simmons G, Hofmann H, Kuri T, Weber F, Eichler J, Drosten C, Pohlmann S. 2010. Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. Journal of virology 84:1198-1205.
21. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, Huan Y, Yang P, Zhang Y, Deng W, Bao L, Zhang B, Liu G, Wang Z, Chappell M, Liu Y, Zheng D, Leibbrandt A, Wada T, Slutsky AS, Liu D, Qin C, Jiang C, Penninger JM. 2005. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nature medicine 11:875-879.
22. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426:450-454.
23. Li W, Sui J, Huang IC, Kuhn JH, Radoshitzky SR, Marasco WA, Choe H, Farzan M. 2007. The S proteins of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2. Virology 367:367-374.
24. Hattermann K, Muller MA, Nitsche A, Wendt S, Donoso Mantke O, Niedrig M. 2005. Susceptibility of different eukaryotic cell lines to SARS-coronavirus. Archives of virology 150:1023-1031.
25. Hofmann H, Hattermann K, Marzi A, Gramberg T, Geier M, Krumbiegel M, Kuate S, Uberla K, Niedrig M, Pohlmann S. 2004. S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. Journal of virology 78:6134-6142.
26. Schildgen O, Jebbink MF, de Vries M, Pyrc K, Dijkman R, Simon A, Muller A, Kupfer B, van der Hoek L. 2006. Identification of cell lines permissive for human coronavirus NL63. Journal of virological methods 138:207-210.
27. Mathewson AC, Bishop A, Yao Y, Kemp F, Ren J, Chen H, Xu X, Berkhout B, van der Hoek L, Jones IM. 2008. Interaction of severe acute respiratory syndrome-coronavirus and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. The Journal of general virology 89:2741-2745.
28. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G, Piqani B, Eisenhaure TM, Luo B, Grenier JK, Carpenter AE, Foo SY, Stewart SA, Stockwell BR, Hacohen N, Hahn WC, Lander ES, Sabatini DM, Root DE. 2006. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124:1283-1298.
29. Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27:493-497.
30. Bolte S, Cordelieres FP. 2006. A guided tour into subcellular colocalization analysis in light microscopy. Journal of microscopy 224:213-232.
31. Lai ZW, Hanchapola I, Steer DL, Smith AI. 2011. Angiotensin-converting enzyme 2 ectodomain shedding cleavage-site identification: determinants and constraints. Biochemistry 50:5182-5194.
32. Rauvala H. 1979. Monomer-micelle transition of the ganglioside GM1 and the hydrolysis by Clostridium perfringens neuraminidase. European journal of biochemistry / FEBS 97:555-564.
33. Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. 2003. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. Journal of virology 77:12022-12032.

555 **34.** **Alvarez CP, Lasala F, Carrillo J, Muniz O, Corbi AL, Delgado R. 2002. C-type lectins DC-SIGN**
556 **and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. Journal of virology**
557 **76:6841-6844.**
558 **35.** **Zhang Y, Buckles E, Whittaker GR. 2012. Expression of the C-type lectins DC-SIGN or L-SIGN**
559 **alters host cell susceptibility for the avian coronavirus, infectious bronchitis virus.**
560 **Veterinary microbiology 157:285-293.**
561 **36.** **Sureau C, Salisse J. 2013. A conformational heparan sulfate binding site essential to**
562 **infectivity overlaps with the conserved hepatitis B virus a-determinant. Hepatology 57:985-**
563 **994.**
564 **37.** **Lamas Longarela O, Schmidt TT, Schoneweis K, Romeo R, Wedemeyer H, Urban S, Schulze**
565 **A. 2013. Proteoglycans act as cellular hepatitis delta virus attachment receptors. PloS one**
566 **8:e58340.**
567 **38.** **Kobayashi K, Kato K, Sugi T, Takemae H, Pandey K, Gong H, Tohya Y, Akashi H. 2010.**
568 **Plasmodium falciparum BAEBL binds to heparan sulfate proteoglycans on the human**
569 **erythrocyte surface. The Journal of biological chemistry 285:1716-1725.**
570 **39.** **Bucior I, Pielage JF, Engel JN. 2012. Pseudomonas aeruginosa pili and flagella mediate**
571 **distinct binding and signaling events at the apical and basolateral surface of airway**
572 **epithelium. PLoS pathogens 8:e1002616.**
573 **40.** **Norman MU, Moriarty TJ, Dresser AR, Millen B, Kubes P, Chaconas G. 2008. Molecular**
574 **mechanisms involved in vascular interactions of the Lyme disease pathogen in a living host.**
575 **PLoS pathogens 4:e1000169.**
576 **41.** **Lebrun P, Raze D, Fritzinger B, Wieruszeski JM, Biet F, Dose A, Carpentier M, Schwarzer D,**
577 **Allain F, Lippens G, Locht C. 2012. Differential contribution of the repeats to heparin**
578 **binding of HBHA, a major adhesin of Mycobacterium tuberculosis. PloS one 7:e32421.**
579 **42.** **Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RW, Zarski JP, Drouet E.**
580 **2002. Cellular glycosaminoglycans and low density lipoprotein receptor are involved in**
581 **hepatitis C virus adsorption. Journal of medical virology 68:206-215.**
582 **43.** **Kalia M, Chandra V, Rahman SA, Sehgal D, Jameel S. 2009. Heparan sulfate proteoglycans**
583 **are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral**
584 **infection. Journal of virology 83:12714-12724.**
585 **44.** **Cruz L, Meyers C. 2013. Differential dependence on host cell glycosaminoglycans for**
586 **infection of epithelial cells by high-risk HPV types. PloS one 8:e68379.**
587 **45.** **Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ,**
588 **Rosenberg RD, Spear PG. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes**
589 **simplex virus 1 entry. Cell 99:13-22.**
590 **46.** **Lambert S, Bouttier M, Vassy R, Seigneuret M, Petrow-Sadowski C, Janvier S, Heveker N,**
591 **Ruscetti FW, Perret G, Jones KS, Pique C. 2009. HTLV-1 uses HSPG and neuropilin-1 for**
592 **entry by molecular mimicry of VEGF165. Blood 113:5176-5185.**
593 **47.** **Patel M, Yanagishita M, Roderiquez G, Bou-Habib DC, Oravecz T, Hascall VC, Norcross MA.**
594 **1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines.**
595 **AIDS research and human retroviruses 9:167-174.**
596 **48.** **Watanabe R, Sawicki SG, Taguchi F. 2007. Heparan sulfate is a binding molecule but not a**
597 **receptor for CEACAM1-independent infection of murine coronavirus. Virology 366:16-22.**
598 **49.** **Madu IG, Chu VC, Lee H, Regan AD, Bauman BE, Whittaker GR. 2007. Heparan sulfate is a**
599 **selective attachment factor for the avian coronavirus infectious bronchitis virus Beaudette.**
600 **Avian diseases 51:45-51.**
601 **50.** **Lang J, Yang N, Deng J, Liu K, Yang P, Zhang G, Jiang C. 2011. Inhibition of SARS pseudovirus**
602 **cell entry by lactoferrin binding to heparan sulfate proteoglycans. PloS one 6:e23710.**
603 **51.** **Wu K, Chen L, Peng G, Zhou W, Pennell CA, Mansky LM, Geraghty RJ, Li F. 2011. A virus-**
604 **binding hot spot on human angiotensin-converting enzyme 2 is critical for binding of two**
605 **different coronaviruses. Journal of virology 85:5331-5337.**

52. Dijkman R, Jebbink MF, Deijs M, Milewska A, Pyrc K, Buelow E, van der Bijl A, van der Hoek L. 2012. Replication-dependent downregulation of cellular angiotensin-converting enzyme 2 protein expression by human coronavirus NL63. The Journal of general virology 93:1924-1929.
53. Wilen CB, Tilton JC, Doms RW. 2012. HIV: cell binding and entry. Cold Spring Harbor perspectives in medicine 2.
54. de Haan CA, Haijema BJ, Schellen P, Wichgers Schreur P, te Lintelo E, Vennema H, Rottier PJ. 2008. Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. Journal of virology 82:6078-6083.
55. de Haan CA, Li Z, te Lintelo E, Bosch BJ, Haijema BJ, Rottier PJ. 2005. Murine coronavirus with an extended host range uses heparan sulfate as an entry receptor. Journal of virology 79:14451-14456.
56. Dijkman R, Jebbink MF, Koekkoek SM, Deijs M, Jonsdottir HR, Molenkamp R, Ieven M, Goossens H, Thiel V, van der Hoek L. 2013. Isolation and characterization of current human coronavirus strains in primary human epithelial cell cultures reveal differences in target cell tropism. Journal of virology 87:6081-6090.
57. van der Hoek L, Pyrc K, Berkhout B. 2006. Human coronavirus NL63, a new respiratory virus. FEMS microbiology reviews 30:760-773.

FIGURE LEGENDS

Figure 1. Human cell lines overexpressing ACE2 protein. (A) Lysates of A549 +/- (A549_ACE2$^+$ and A549_WT) and 293T +/- (293T_ACE2$^+$ and 293T_WT) cells were tested for the presence of the ACE2 protein with Western blotting using antibodies specific to the ectodomain of the human ACE2 protein. „-" and „+" signs denote wild-type and ACE2 overexpressing cell lines, respectively. Concomitantly, the β-actin protein levels were assessed in each sample. Numbers on the left side represent molecular mass [kDa] assessed with a size marker. The results shown are representative of at least three independent experiments. **(B)** A549_ACE2$^+$, A549_WT, 293T_ACE2$^+$ and 293T_WT cells were tested for the surface expression of the ACE2 protein with flow cytometry using antibodies specific to the ectodomain of the human ACE2 protein. The results shown are representative of at least three independent experiments.

Figure 2. Cytopathic effect on A549_ACE2$^+$ cells during HCoV-NL63 infection. ACE2-overexpressing (ACE2$^+$) and wild type (WT) A549 and 293T cells were infected with HCoV-NL63 or inoculated with mock and cultured for 6 days. Cytopathic effect was observed only on HCoV-NL63 infected A549_ACE2$^+$ cells. Magnification: 200 ×. The results shown are representative of at least three independent experiments.

Figure 3. HCoV-NL63 nucleocapsid protein expression in ACE2$^+$ cells. ACE2$^+$ and WT 293T and A549 cells were infected with HCoV-NL63 (+) or mock (-). HCoV-NL63 nucleocapsid protein was detected 6 days p.i. in A549_ACE2$^+$ and 293T_ACE2$^+$ cell lysates, suggesting viral replication. No signal from the NL63-N protein was observed in mock-infected cells. Sample containing lysate of LLC-Mk2 cells infected with HCoV-NL63 was used as a positive control (PC). A position of 55 kDa a molecular mass marker is shown

653 on the left side. The results shown are representative of at least three independent
654 experiments.
655
656 **Figure 4. HCoV-NL63 replication in ACE2-overexpressing cells.** ACE2-overexpressing
657 and wild type cells were infected with HCoV-NL63 (+) or mock (-) and cultured for 6 days.
658 **(A)** Genomic RNA (1a) and a set of HCoV-NL63 sg mRNAs, including spike (S), ORF3,
659 envelope (E), membrane (M) and nucleocapsid (N), were detected in A549_ACE2$^+$ and
660 293T_ACE2$^+$ cells. No HCoV-NL63 sg mRNAs were detected in WT cells. LLC-Mk2 cells
661 infected with HCoV-NL63 (+) or mock (-) were used as controls. Positions of nt size markers
662 are shown on the left side of each panel. The results shown are representative of at least three
663 independent experiments. **(B)** HCoV-NL63 replication on A549 and 293T cells was evaluated
664 with real-time PCR. Marked increase in virus yield was observed on A549_ACE2$^+$ and, to
665 much lesser extent, on 293T_ACE2$^+$ cells. No increase in virus yield was observed on
666 HCoV-NL63-infected A549 and 293T WT cells. Data on virus replication are presented as
667 HCoV-NL63 RNA copies/ml. All assays were performed in triplicate and average values with
668 standard errors (error bars) are presented.
669
670 **Figure 5. Directed expression of the ACE2 protein on A549 cells does not alter HCoV-**
671 **NL63 adhesion.** Analysis of HCoV-NL63 adherence to ACE2-overexpressing (ACE2$^+$) or
672 wild type (WT) A549 cells was conducted with flow cytometry. The results shown are
673 representative of at least three independent experiments.
674
675 **Figure 6. Adherence of HCoV-NL63 to LLC-Mk2 cells deprived of the ACE2 protein.**
676 LLC-Mk2 cells were deprived of surface ACE2 protein by incubation with 1 μM PMA and
677 subsequently incubated with purified HCoV-NL63 or mock. DMSO-treated cells were used as

a control. **(A)** HCoV-NL63 replication on LLC-MK2 $ACE2^+$ and $ACE2^-$ cells was evaluated with real-time PCR. A significant decrease in viral replication was observed on LLC-MK2 cells pre-treated with PMA, compared to control cells. Data on virus replication are presented as HCoV-NL63 RNA copies/ml. **(B)** Analysis of HCoV-NL63 adherence to $ACE2^{+/-}$ LLC-MK2 cells. HCoV-NL63 was labelled with specific antibodies and virus adhesion was analyzed with flow cytometry. **(C)** A decrease in surface expression of the ACE2 protein on LLC-MK2 after PMA treatment was confirmed using flow cytometry. **(D)** HCoV-NL63 adhesion to $ACE2^{+/-}$ LLC-Mk2 cells was confirmed by confocal microscopy. LLC-MK2 cells were pre-treated with PMA **[PMA]** or DMSO **[DMSO]** and incubated with purified HCoV-NL63. HCoV-NL63 virions are presented in green, while the blue color denotes DNA. Each image is a single confocal plane (xy) with two orthogonal views (xz and yz) created by maximum projection of axial planes (thickness 0.7 µm). Scale: 10 µm. The results shown are representative of at least three independent experiments.

Figure 7. HCoV-NL63 adhesion to neuraminidase-treated cells and in the presence of sugar moieties. LLC-Mk2 cells were treated with DMSO **(A)**, 1 µM PMA **(B)** or 1 µM PMA and 200 mU/ml type V neuraminidase **(C)** and further incubated with purified HCoV-NL63 or mock. Virus adhesion was assessed also in the presence of 50 mM sugar monomers: galactose **(D)**, mannose **(E)**, N-acetylglucosamine **(F)**, fucose **(G)** or glucose **(H)**, as a negative control. Virus adhesion was analyzed with flow cytometry. The results shown are representative of at least three independent experiments.

Figure 8. HCoV-NL63 adhesion to $ACE2^+$/$ACE2^-$ cells in the presence of heparan sulfate. (A) Flow cytometry analysis of HCoV-NL63 adhesion. The ACE2 protein was removed from the surface of LLC-Mk2 cells by incubation with 1 µM PMA **($ACE2^-$)**, while

control cells were treated with DMSO **(ACE2^{+})**. Adhesion of HCoV-NL63 was assessed on ACE2$^{-/+}$ cells in the presence of 300 µg/ml HS or control PBS. **(B)** Confocal microscopy analysis of HCoV-NL63 adhesion. LLC-MK2 cells were stimulated with 1 µM PMA [**PMA**] or DMSO and incubated with purified HCoV-NL63 [**NL63**] in the presence or absence of heparan sulfate [**HS**]. [**NC**] denotes cells incubated with mock sample. HCoV-NL63 virions are presented in green, while the blue color denotes DNA. Each image is a single confocal plane (xy) with two orthogonal views (xz and yz) created by maximum projection of axial planes (thickness 4.8 µm). Scale: 5 µm. Bars represent the mean number of virions from 10 cells per sample ± standard error.

Figure 9. HCoV-NL63 replication in the presence of heparan sulfate. LLC-Mk2 cells were infected with HCoV-NL63 in the presence of increasing concentrations of HS or PBS. Virus replication in cell culture supernatants was evaluated using real-time PCR on day 4 p.i. Data on virus replication are presented as HCoV-NL63 RNA copies/ml. All assays were performed in triplicate and average values with standard errors (error bars) are presented. For all concentrations the decrease in virus yield is statistically significant (student's t-test; $p<0.05$).