Host protein CD63 promotes viral RNA replication by interacting with human astrovirus non-structural protein nsP1a/4

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Abstract

Human astrovirus non-structural protein nsP1a/4, located at the C-terminal end of nsP1a, is thought to be involved in regulating RNA replication. Here, we show that host protein CD63 interacts with the nsP1a protein. Further research showed that the large loop (LEL) domain of CD63 also interacts with nsP1a/4. Confocal microscopy showed that nsP1a/4 protein and CD63 co-localized in the cytoplasm of co-transfected cells. Co-localization of nsP1a/4 and CD63 was also observed in HAstV-1-infected cells. Overexpression of CD63 promoted replication of HAstV-1, whereas knockdown of CD63 reduced production of HAstV-1 viral progeny. These results suggest that CD63 plays a critical role in HAstV-1 replication, and provide an avenue to further understanding the interactions between host and virus proteins during replication and pathogenesis of HAstV.

INTRODUCTION

Viruses are obligate intracellular pathogens that utilize host cell machinery to infect cells, replicate and then exit the cells prior to another round of infection. Human astroviruses (HAstVs) are non-enveloped, single-stranded, positive-sense RNA viruses belonging to the family Astroviridae [1]. To date, eight serotypes of human astrovirus have been defined (HAstV-1 to 8) [2]. Two new clades of astroviruses have been identified in human stool samples, with VA1-VA5 and BF43 [3-8] in the first clade and MLB1, MLB2 and MLB3 in the second [4, 6, 9]. HAstVs, causative agents of viral diarrhoea, were detected in adults and children with or without diarrhoea [10-12].

The astrovirus genome has three open reading frames (ORFs). ORF1a and 1b encode two non-structural polyproteins (nsPs) while ORF2 encodes the capsid protein [13]. The nsP1a polyprotein transcribed from ORF1a is involved in viral transcription and replication. When HAstV infects a host cell, nsP1a is cleaved into at least four products, named nsP1a/1, nsP1a/2, nsP1a/3 (protease) and nsP1a/4. The nsP1a/4 protein is located at the C-terminal end of nsP1a. Several domains within nsP1a/4 have been identified: two coiled-coil regions, a death domain, a nuclear localization signal, a putative viral genome-linked protein and a hyper-variable region [14-19]. nsP1a/4 plays key roles in regulating RNA replication [20, 21]. To better understand the roles of nsP1a and nsP1a/4 during HAstV infection and replication, we characterized host proteins that interact with nsP1a or nsP1a/4 by utilizing a yeast two-hybrid approach. We identified at least 13 host proteins that bound to nsP1a. Preliminary experiments showed that among these, the CD63 protein has an effect on HAstV-1 replication.

CD63, a member of the tetraspan transmembrane protein family, is a four-span membrane protein widely distributed among multicellular organisms and known to be associated with virus functions including adhesion, fusion and trafficking [22, 23]. Tetraspanins, a large superfamily of cell surface membrane proteins characterized by four transmembrane domains, have the unique ability to form a network of protein–protein interactions by associating with multiple membrane proteins that play roles in several infectious diseases [24-26]. Increasing evidence suggests that intracellular...
pathogens, especially viruses, ‘hijack’ tetraspanins when entering, traversing and exiting cells during the course of infection. Several studies report that tetraspanins including CD151 [27], CD81 [28–30], CD82 [31], CD9 [32] and CD63 [33, 34] are key players in the life cycle of many viruses.

In this study, interactions between nsP1a/4 and CD63 were identified by co-immunoprecipitation and glutathione-S-transferase (GST) pull-down assays. Cytoplasmic co-localization of these two proteins was visualized by confocal microscopy. The replication of HAstV-1 was markedly affected by forced CD63 expression or downregulation in Caco-2 cells. These findings indicate that CD63 plays an important role in the HAstV life cycle.

METHODS

Cell culture and virus infection
HEK293T and Caco-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GibCO, UK) containing 10% fetal calf serum (GIBCO, UK). HEK293T cells were transfected with a HAstV-1 cDNA plasmid (pCAG-AVIC) that drives HAstV-1 cDNA expression from the CAG promoter [35]. Cell culture supernatants were collected 48 h after transfection and used as a source of virus to infect Caco-2 cells. The virus was activated by exposure to 200 µg ml⁻¹ porcine trypsin (Sigma-Aldrich, China) for 1 h at 37°C before introduction into cells. Virus titres in Caco-2 cell cultures were measured by fluorescent focus assay, as described previously [36]. Briefly, Caco-2 cells were infected with HAstV-1. After 24 h, cells were washed with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature with 4% paraformaldehyde. Cells were permeabilized for 15 min at room temperature with 0.05% Triton X-100 in PBS and then blocked for 1 h with 1% bovine serum albumin (fraction V; GibCO). Anti-serum raised against the nsP1a/4 astrovirus protein was used as the primary antibody [37]. A secondary antibody conjugated to Alexafluor (goat anti-rabbit immunoglobulin G-Alexa488) was used for detection. Virus infectivity was determined by counting infected cells. The results are reported as fluorescent focus units per millilitre (FFU ml⁻¹) and represent the arithmetic mean ± SEM of three independent experiments.

Construction and expression of plasmids
The HAstV-1 nsP1a protein (GenBank accession number: AGX15183.1) and proteins nsP1a/1, nsP1a/2, nsP1a/3 and nsP1a/4 were amplified using the primers listed in the Table and then cloned into the pGBK7 vector (Clontech USA, Mountain View, CA) to generate a BD-bait plasmid for the Y2H system. To generate N-terminal HA-tagged nsP1a and nsP1a/4 expression constructs, the nsP1a or nsP1a/4 frames were amplified using the primers listed in Table 1. Total RNA was extracted from HAstV-1 obtained from Caco-2 cells and nsP1a was amplified by RT-PCR. The PCR products were cloned into the PEF-HA PGK Hygr vector to create PEF-HA-nsP1a and PEF-HA-nsP1a/4 plasmids via the in-fusion HD Cloning method (TaKaRa Bio, Dalian, China). The expression plasmids pGEX-6P-1-nsP1a and pGEX-6P-1-nsP1a/4 were generated by insertion of nsP1a or nsP1a/4 cDNA between the BamHI and Xho I sites of pGEX-6P-1. CD63 (GenBank accession number: NM_00140034.1) contains four transmembrane domains, a short extracellular loop (SEL), a long extracellular loop (LEL), a very short intracellular loop, a cytoplasmic N-terminal and C-terminal tails. The expression plasmids pET-29a-CD63-SEL, pET-29a-CD63-SEL, pET-29a-CD63-N and pET-29a-CD63-C were generated by insertion of cDNA between the Xho I and BamH I sites of pET-29. To generate the N-terminal Flag-tagged CD63 or CD63-SEL expression constructs, CD63 or CD63-SEL PCR products were cloned into the pcDNA3.1(+) vector to create pcDNA3.1-3flag-CD63 and pcDNA3.1-3flag-CD63-SEL, respectively, using the in-fusion HD cloning method (TaKaRa Bio).

Expression and purification of recombinant proteins
Proteins nsP1a/nsP1a/4, CD63-SEL and CD63-SEL and terminal proteins CD63-C and CD63-N were expressed and purified using the same protocol. Briefly, the recombinant plasmids were transformed into competent Escherichia coli BL21 (DE3) cells, which were then grown overnight in 5 ml Luria–Bertani broth. Incubation was continued until the optical density (OD₆₀₀) reached 0.6–0.8. Protein expression was induced by the addition of 0.1–1.0 mM IPTG at 25–37°C with shaking at 220 r.p.m. Expression was analysed by SDS-PAGE. Supernatant containing the fusion protein was purified using Glutathione Sepharose 4B (GE Healthcare Bio-Sciences, Little Chalfont, UK), according to the manufacturer’s instructions. Purity of recombinant proteins nsP1a, nsP1a/4 and SEL was confirmed by western blotting.

Plasmid transfection
HEK293T cells were transfected with plasmids containing recombinant DNA using the Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s protocol. Briefly, cells were grown to approximately 90% confluence on 6-well palates. The culture medium was replaced by serum-free medium containing the desired plasmid and Lipofectamine 2000. The cells were incubated for 48 h at 37°C in 5% CO₂ and then harvested prior to western blot analysis to confirm protein expression.

Antibodies
An anti-Flag murine IgG1 monoclonal antibody, an anti-HA murine IgG1 monoclonal antibody, an anti-GST-Tag mouse polyclonal antibody and an anti-His-tag mouse polyclonal antibody (Sigma-Aldrich, China) were used for immunofluorescence, confocal microscopy and the GST pull-down assay, respectively. An anti-CD63 murine IgG monoclonal antibody (Abcam Trading, Shanghai, China) was used to detect endogenous CD63 by confocal microscopy. Horseradish peroxidase-labelled goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology, Shanghai, China) was used for western blotting. An Alexa Fluor
488-conjugated anti-mouse IgG antibody and an Alexa Fluor 594-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) were used as secondary antibodies for immunofluorescence and confocal microscopy, respectively. An anti-actin antibody (ZSGB-BIO, Beijing, China) was used for western blotting. A polyclonal antibody specific for the nsP1a/4 protein of HAstV-1 was prepared in a previous study [37].

**Isolation of nsP1a-interacting cDNA clones using the Y2H technique**

A full-length expression, high-quality, efficient, three-frame cDNA library derived from Caco-2 cells was constructed for a previous study [38]. The AY2H media set (Clontech, TaKaRa Biomedical Technology, Dalian, China) was used to identify nsP1a binding factors according to the manufacturer’s instructions. In brief, AH109 and Y187 cells were transformed with the bait (pGBKT7-nsP1a) and prey (Caco-2 cDNA library) constructs in accordance with the yeast transformation protocol (Clontech). A clone of the bait transformant was mated with a clone of the prey transformant and grown at 30°C overnight in 1 ml yeast extract peptone dextrose broth. The mated clones were selected on SD medium lacking tryptophan and leucine to ensure successful mating. Finally, the interacting partners were screened on SD medium lacking tryptophan (-W), leucine (-L), adenine (-A) and histidine (-H). Plasmids pGBKT7-53 and pGADT7-T, encoding the interacting protein pair P53 and Simian virus 40 (SV40) large T antigens, respectively, were used as positive controls. Plasmids pGBKT7-Lam and pGADT7-T, encoding the non-interacting protein partner and SV40 large T antigen, respectively, served as negative controls.

NsP1a or 1a/4 interacts with CD63 in yeast cells. HAstV-1 nsP1a, nsP1a/1, nsP1a/2, nsP1a/3 and nsP1a/4 bait plasmids were fused with the GAL4-binding domain (BD), and CD63 was fused with the GAL4 activation domain (AD). All plasmids were used to transform yeast strain AH109 in pairwise combinations. Transformants expressing both bait and prey plasmids were transformed with the bait and prey plasmid constructs, respectively, according to the yeast transformation protocol (Clontech). Protein interactions were selected by growth on medium lacking tryptophan and leucine (SD-W-L), and evaluated by growth on medium lacking tryptophan, leucine, histidine and adenine (SD-W-L-A).

**Western blotting**

Protein samples were separated on 12% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with...
anti-HA (1:5000), anti-Flag (1:5000), anti-His (1:2000) and anti-CD63 (1:1000) antibodies, rinsed with PBST and incubated with HRP-labelled goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (1:3000; Santa Cruz Biotechnology). Proteins were visualized using the ECL prime western blotting detection reagent (Beyotime Biotechnology, Beijing, China).

**GST pull-down assays**

GST-nsP1a or GST-nsP1a/4 protein was purified using a gravity-flow GST-Sefinose Resin (Sangon Biotech, Shanghai, China) column according to the manufacturer’s instructions and then detected by western blotting. His-tagged recombinant human CD63 protein was obtained from Thermo-Fisher Scientific Sino Biological (China). Expression and purification of the domains CD63-LEL, CD63-SEL, CD63-C and CD63-N were performed using the same method as for nsP1a and nsP1a/4. The Pierce GST Protein Interaction Pull-Down Kit (Thermo Fisher Scientific) was used to identify the interaction between nsP1a or nsP1a/4 and domains CD63 or CD63. In brief, GST-nsP1a or GST-nsP1a/4 bait protein samples were bound to glutathione agarose, followed by washing four times with wash solution (25 mM Tris–HCl, 0.15 M NaCl, pH 7.2). Pull-Down Lysis Buffer was incubated with recombinantHis-tagged domain CD63 or CD63 at 4 °C for at least 1 h with gentle shaking. The eluted proteins were detected by SDS-PAGE followed by western blot analysis with anti-GST and anti-His antibodies.

**Co-immunoprecipitation of nsP1a/4 and CD63-LEL**

HEK293T cells were transfected with expression plasmids PEF-HA-nsP1a/4 and CD63-LEL. Following transfection for 48 h, cells were lysed in NP-40 lysis buffer containing protease inhibitors. Antibodies specific for Flag or HA were bound separately and cross-linked to Protein G Dynabeads (Novagen) using the reagents provided in the Pierce Crosslink Co-immunoprecipitation Kit (Pierce). Approximately 1–5 mg of untransfected or transfected cell lysates were then mixed with different sets of antibody-coupled beads and washed. Eluted proteins were analysed by western blotting using anti-Flag or anti-HA antibodies.

**Immunofluorescence and confocal microscopy**

HEK293T cells were co-transfected with PEF-HA-nsP1a/4 and plasmid pcDNA3.1-3flag-CD63 or pcDNA3.1-3flag-CD63-LEL. Simultaneously, sub-confluent monolayers of Caco-2 cells were grown on coverslips in 6-well plates and infected with HAstV-1 at a multiplicity of infection (MOI) of 1. After 48 h, the cells were washed with 1 ml PBS and fixed for 10 min at room temperature with 4% paraformaldehyde. After additional washes in PBS, anti-Flag and anti-HA antibodies were applied at a dilution of 1:1000. Virus-infected cells were then exposed to antibodies anti-CD63 and anti-nsP1a/4. For double-labelling, cells were incubated with both antibodies together. After washing three times with PBS, the cells were incubated with the appropriate secondary antibody (Alexa Fluor 488-conjugated anti-mouse IgG antibody or Alexa Fluor 594-conjugated anti-rabbit IgG antibody) for 1 h at room temperature. Next, the coverslips were washed three times with PBS, counterstained for 5 min at room temperature with the nuclear stain DAPI (Life Technologies, Beijing, China), washed three times and then examined using a confocal microscope. Quantitative analysis was performed using CoLocalizer Pro (CoLocalization Research Software). Background signals were corrected in Auto mode by selecting an image pattern preset at ‘Average Contrast and Fluorescence’. Pearson’s correlation coefficient (PCC) and an overlap coefficient according to Manders (MOC) were calculated [39].

**CD63 overexpression and knockdown**

The primers used to construct the plasmids containing shRNA used for knockdown of CD63 and those used for overexpression of CD63 are listed in Table 1. Two pairs of shRNAs targeting human CD63 and a negative control shRNA were cloned into the hU6-MCS-CMV-GFP-SV40-Neomycin vector (Shanghai Genechem) to generate CD63-shRNA-1, CD63-shRNA-2 and CD63-NC. HEK293T cells were transfected with the constructs along with pcDNA3.1-3flag-CD63 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol.

**Real-time RT-PCR**

The target CD63 mRNA was quantified by real-time RT-PCR using the primers listed in Table 1. Total RNA was extracted from HEK293T cells using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Next, cDNA was reverse-transcribed from 1 µg total RNA using PrimeScript Reverse Transcriptase (TaKaRa Bio, Dalian, China). Quantitative real-time RT-PCR was performed using the SYBR PrimeScript RT-kit (TaKaRa Bio) according to the manufacturer’s instructions. Expression of viral RNA and GAPDH transcripts was analysed using the Threshold cycle (Ct) average value of triplicates method. Relative expression of viral RNA and GAPDH was calculated using the 2-ΔΔCT method. Confidence intervals were calculated from ΔΔCT values. Results were analysed using Bio-Rad IQTM5 optical system software. Data are presented as mean ±SD. Differences between groups were examined using Student’s t-test. P<0.05 was the threshold adopted for statistical significance.

**RESULTS**

A yeast two-hybrid screen with nsP1a and a Caco-2 cDNA library identified CD63 as a host protein that interacts with nsP1a

We investigated the interaction between HAstV nsP1a and host proteins using a protein–protein overlay assay. We amplified the nsP1a gene by PCR and inserted the resulting PCR product (2781 bp) into the yeast two-hybrid (Y2H) bait vector pGBK7T7 to generate a C-terminal fusion with a Gal4 DNA-BD. When introduced
alone into yeast Y2H Gold cells, the Gal4 (BD)-nsP1a bait construct was not toxic and did not auto-activate the Y2H reporter gene, indicating that the construct was suitable for use in a Y2H screen.

Next, we screened a human Caco-2 cDNA library using an Y2H assay with Gal4 (BD)-nsP1a as bait. Approximately 2.5×10^5 independent clones were assayed for histidine prototrophy and β-galactosidase activity. We identified 13 positive bait-prey interactions. Sequence and bioinformatics analysis of the 13 positive prey plasmids, listed according to their initial positive-interaction identification number, indicated that they represented 13 different human cDNAs. Next, we measured the strength of the interaction between the nsP1a bait and each of the 13 human prey proteins in yeast using a qualitative growth assay (Fig. 1a–c) and quantitative β-galactosidase activity assay (Fig. 1d). Bioinformatic analysis of the human proteins confirmed that these proteins were *Homo sapiens* fibronectin 1 (FN1), *Homo sapiens* NADH:ubiquinone oxidoreductase subunit A9 (NDUFA9), *Homo sapiens* exosome component 8 (EXOSC8), *Homo sapiens* RNA polymerase II subunit G (POLR2G), *Homo sapiens* peroxiredoxin 3 (PRDX3), *Homo sapiens* RNA-binding protein, mRNA processing factor (RBPMS), *Homo sapiens* lactate dehydrogenase A (LDHA), *Homo sapiens* ring finger protein 7 (RNF7), *Homo sapiens* Ras-converting CAAX endopeptidase 1 (RCE1), *Homo sapiens* cathepsin C (CTSC), *Homo sapiens* A-kinase-anchoring protein 1 (AKAP1), *Homo sapiens* chromosome 11 open reading frame 1 (C11orf1) and *Homo sapiens* chromosome 11 open reading frame 1 (C11orf1) and *Homo sapiens* CD63 molecule (CD63). Because decreased CD63 protein expression showed an effect on HAstV-1 replication, we selected tetraspantrans membrane protein CD63 (GenBank accession number: NM_001040034.1) for further study.

### nsP1a and nsP1a/4 proteins interact with CD63 in yeast cells

The nsP1a polyprotein is cleaved into at least four products, namely nsP1a/1, nsP1a/2, nsP1a/3 (protease) and nsP1a/4, when HAstV infects a host cell. We performed GAL4 yeast two-hybrid assays by co-transforming yeast cells with the bait constructs expressing nsP1a, nsP1a/1, nsP1a/2, nsP1a/3 and nsP1a/4 fused to GAL4 DNA-BD (Fig. 2a), together with a prey construct expressing GAL4 AD fused to CD63. To confirm nsP1a–CD63 interactions in yeast, we set up a growth experiment on SD plates in the absence of tryptophan and leucine (SD-W-L) or in the absence of tryptophan, leucine, histidine and adenine (SD-W-L-H-A) in the two hybrid systems, respectively. In this assay, yeast growth on SD medium is supported only when the two hybrid proteins interact and induce transcription from the reporter gene (Fig. 2b). We found that yeast was able to grow on media with AD-CD63 and BD-nsP1a or nsP1a/4 co-expression (Fig. 2c). Together, these results indicate that nsP1a/nsP1a/4 and CD63 interact with each other in yeast cells.

### Confirmation of the interaction between nsP1a and CD63

To verify and extend the binding data obtained from the yeast two-hybrid assay, we next performed immunoprecipitation experiments. We co-expressed nsP1a-HA and CD63-Flag in HEK293T cells; nsP1a-HA was expressed as a control. Lysates from HEK293T transfected with nsP1a-HA and CD63-Flag were immunoprecipitated with an anti-HA Affinity Gel (Sigma, St. Louis, MO, USA). The proteins in the complexes were analysed by western blotting with an anti-Flag antibody. CD63 protein was detected in immunoprecipitates obtained with the anti-Flag antibody. No proteins were detected in control lysates from

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Fig. 1. Analysis of putatively positive colonies of Y2H Gold cells. Y2H Gold clones containing the cDNA library were grown on dropout medium lacking tryptophan and leucine (SD-W-L). Yeast cells containing the bait (HAstV nsP1a) and prey plasmids (Caco-2 cell cDNA library) were screened for interactions on synthetic dropout media. Thirteen host proteins that bound to nsP1a were obtained. (a) Screen for interactions on SD-W-L medium. (b) Screen for interactions on synthetic dropout medium lacking tryptophan, leucine and histidine (SD-W-L-H). (c) Screen for interactions on synthetic dropout medium lacking tryptophan, leucine, histidine and adenine (SD-W-L-H-A). (d) Confirmation of positive interactions on SD-Trp-Leu-X-Gal medium. Co-transformation with pGADT7-T and pGBK7-T-Lam was used as a negative control (–). Co-transformation with pGADT7-T and pGBK7-T-Lam was used as a positive control (+). Co-transformation with pGBK7-nsP1a and pGADT7 was used as an auto-activation control (*).
cells transfected with a single nsP1a-HA plasmid. Both nsP1a (90 KDa) and CD63 (30 KDa) were detected in lysates from co-transfected cells (Fig. 3a).

Although the immunoprecipitation results showed that nsP1a interacted with CD63, it was not clear whether nsP1a interacted directly with CD63. Therefore, the interaction between nsP1a and CD63 was examined further in a GST pull-down assay.

Recombinant full-length nsP1a-GST fusion protein was expressed and purified from bacteria. Next, nsP1a-GST was immobilized on glutathione agarose and CD63-His was added to examine binding. A bacterial GST-containing lysate was used as a negative control for nsP1a-GST binding. The proteins in the complexes were analysed by western blotting with anti-GST or anti-His antibodies. As demonstrated in Fig. 3(b), nsP1a-GST (116 KDa) was detected in the nsP1a-GST/CD63-His complex. None of the proteins

**Fig. 2.** HAstV nsP1a and nsP1a/4 interact with CD63 in yeast cells. Bait plasmids HAstV nsP1a, nsP1a/1, nsP1a/2, nsP1a/3 and nsP1a/4 were fused with a GAL4-binding domain (BD), and CD63 was fused with a GAL4-activation domain (AD). All plasmids were used to transform yeast strain AH109 in pairwise combinations. Transformants expressing both bait and prey plasmids were selected by growth on medium lacking tryptophan and leucine (SD-W-L). Protein interactions were evaluated by growth on medium lacking tryptophan, leucine, histidine and adenine (SD-W-L-H-A). (a) Schematic representation of the HAstV nsP1a protein and four cleaved proteins. (b) Yeast cells expressing all vector combinations and co-expression plasmids were grown on basic (SD-W-L) medium in darkness for 3 days. (c) Yeast cells expressing all vector combinations and co-expression plasmids were grown on selective medium (SD-W-L-H-A) in darkness for 3 days.
were detected in the GST negative control. Hence, the GST pull-down assay confirmed that nsP1a interacts directly with CD63.

The C-terminal region of the nsP1a protein (nsP1a/4) interacts with the CD63 large loop domain

The CD63-LEL protein was detected in immunoprecipitates obtained with the anti-Flag antibody. No proteins were detected in control lysates from cells transfected with a single nsP1a/4 HA plasmid. Both nsP1a/4 (40 KDa) and CD63-LEL (11 KDa) were detected in lysates from co-transfected cells (Fig. 4a). Both nsP1a/4-GST (66 kDa) and CD63-LEL (11 KDa) were detected in an nsP1a/4-GST/CD63-LEL complex. None of the proteins was detected in the GST negative control. CD63-SEL, CD63-N terminal and CD63-C terminal proteins did not interact with nsP1a/4 (Fig. 4b).

CD63 or CD63-LEL co-localizes with nsP1a/4 in host cells

To examine co-localization of protein CD63 with nsP1a/4, we co-transfected HEK293 T cells with plasmids expressing HA-nsP1a/4 (red) and Flag-CD63-LEL (green). Both HA-nsP1a/4 protein and Flag-CD63-LEL were distributed within the cytoplasm (Fig. 5b), resulting in a yellow signal (overlap coefficient according to Manders=0.90 (P<0.05). This finding confirms that CD63 and the CD63 LEL domain co-localize with nsP1a/4 in HEK293 T cells.

To confirm that CD63–nsP1a/4 interaction occurs during HAstV-1 infection of host cells, we examined localization of endogenous CD63 and nsP1a/4 during infection of Caco-2 cells using double-label immunofluorescence and microscopy. The results indicated clear co-localization of CD63 (green) and nsP1a/4 (red) proteins at 24 h post-infection, resulting in a yellow overlap signal (overlap coefficient according to Manders=0.602 (P<0.05). Endogenous CD63, which localizes with nsP1a/4 during viral infection, was distributed within the cell cytoplasm (Fig. 5c).

HAstV infection increases expression of CD63

To determine whether HAstV-1 infection affects the expression of CD63, we infected Caco-2 cells with HAstV-1 (MOI=2). We collected lysates from mock-infected (negative control) and HAstV-infected cells at 24 h and 48 h post-infection and measured the expression of CD63 mRNA and protein using real-time RT-PCR (Fig. 6a) and western blotting (Fig. 6b), respectively. The results showed that CD63 mRNA and protein expression was higher in HAstV-infected cells than in mock-infected cells at 48 h post-infection, suggesting that CD63 plays an important role in HAstV-1 infection. To assess the role of CD63 in HAstV...
replication, we utilized a CD63-overexpression plasmid and a shRNA plasmid targeting CD63, and then examined virus yield in cells overexpressing or lacking CD63. CD63 expression in transfected cells at 48 h was assessed by real-time PCR (Fig. 6c) and western blotting (Fig. 6d), respectively. The results showed that the virus titre was increased twofold in CD63-overexpressing cells when compared to the control cells ($P<0.01$) and decreased tenfold in CD63 knockdown cells compared to the control cells ($P<0.01$). Furthermore, Caco-2 cells were infected (MOI=1) with HAstV-1 and the viral titre in the cell culture was measured at 48 h post-infection using Fluorescent Focus assay. A considerable reduction (−2 log) in the virus titre was observed in CD63 knockdown cells compared with control cells. In contrast,
the virus titre was higher (~1.4 log) in CD63-overexpressing cells than in negative control cells (Fig. 6e). These results indicated that CD63 plays an important role in HAstV replication.

**DISCUSSION**

Replication of positive-sense RNA viruses frequently depends on a large number of cellular proteins [40]. For
example, astrovirus replication and assembly are linked to fatty acid synthesis, ATP biosynthesis and cellular lipid metabolism [41]. In the case of HAstV, cellular proteins related to lipid metabolism are expressed in subcellular fractions in which the viral RdRp (RNA-dependent RNA polymerase) is present [41]. RNA interference with synthesis of

Fig. 6. HAstV-1 infection affects CD63 expression. Caco-2 cells were uninfected (Mock) or infected with HAstV-1 (MOI=2) for 24 and 48 h. Expression of CD63 mRNA and protein is shown in (a) and (b). Overexpression of CD63 mediated by eukaryotic expression vector pcDNA3.1 (+)-3flag (CD63-overexpression), and knockdown of CD63 expression (CD63-shRNA-1, CD63-shRNA-2) mediated by shRNA. Expression of CD63 mRNA and protein is shown in (c) and (d). CD63-NC was transfected with a CD63-negative shRNA plasmid. (e) CD63 knockdown correlates with reduced HAstV-1 infection. Cells overexpressing or lacking CD63 were infected with HAstV-1 (MOI=1) for 48 h. The cells and media were collected concomitantly and the viral titre was determined by fluorescent focus assay. Data are expressed in terms of log focus-forming units (FFU) ml⁻¹ and represent the arithmetic mean±standard deviation from triplicate samples. Each experiment was performed twice. **P<0.001 and *P<0.01.
these proteins reduces the production of viral RNAs, but the host proteins that contribute to successful virus infection have not been identified. It is suggested that the human astrovirus non-structural C-terminal nsP1a protein (nsP1a/4) co-localizes with the endoplasmic reticulum, viral RNA and then interacts with the viral polymerase [21].

Although the function of HAstV nsP1a remains unclear, nsP1a and nsP1a/4 are thought to be involved in many processes, including genome replication, apoptosis induction and capsid maturation [13, 16]. To better understand the role of nsP1a during viral replication, we analysed the interaction between nsP1a and host proteins. We found that either HAstV nsP1a or nsP1a/4 interact with the host protein CD63.

CD63 was the first tetraspanin to be characterized [22]. CD63 binds many different proteins, either directly or indirectly; these include integrins [42, 43], other tetraspanins [44, 45], cell surface receptors [46] and kinases [47]. Viruses are obligate intracellular pathogens and so must utilize host cell machinery to complete their life cycle. Viruses incorporate many host components, including tetraspan transmembrane proteins [26], and therefore it is highly likely that tetraspanins play an important role in the life cycle of viruses. Here, we sought to explore the nsP1a–CD63 interaction and examined the important role played by CD63 protein in HAstV infection. We confirmed direct interaction between nsP1a and CD63. Further analyses showed that the nsP1aC-terminal non-structural protein nsP1a/4 binds to the CD63 LEL domain. Tetraspanin proteins play roles in the life cycle of human immunodeficiency virus (HIV) [48–50], hepatitis C virus (HCV) [51, 52], human papillomavirus (HPV) [53, 54], human T-cell lymphotrophic virus (HTLV) [55], and porcine reproductive and respiratory syndrome virus (PPRSV) [56]. CD63 proteins interact with virus proteins [57, 58]. For example, in a rotavirus (RV) model, CD63 interacted with the rotavirus VP6 protein, played a role in the release of membrane vesicles (MV) and modulated viral immunity [59]. Rotavirus VP6 was also co-immunoprecipitated with CD63 from stool samples derived from a subgroup of children infected with RV [59, 60]. In addition, CD63 co-localized with the Gag protein in HIV-1-producing cells and collaborated with other tetraspanin proteins [48–50]. CD63 also plays an essential role in HIV-1 replication in macrophages [61], and knockdown CD63 inhibited HIV-1 virion production. In a human papillomavirus (HPV) model, CD63 co-localized with HPV 18 and HPV 31 L1 proteins [53]. Depletion of CD63 inhibits HPV infection of cell lines. These data demonstrate that CD63 plays a role in infection of epithelial cells by various viruses.

Here, we constructed CD63-knockdown and CD63-overexpressing cell lines to determine whether CD63 expression affects HAstV-1 replication. The results showed that knocking down CD63 inhibits HAstV-1 virion production. However, the molecular mechanism underlying the role of CD63 in HAstV-1 replication remains unclear. A previous study suggested that nsP1a/4 is involved in replication of viral RNA, and that it may be necessary for efficient formation of the viral RNA replication complex via direct interaction with viral RNA or other proteins within the complex [20].

In conclusion, the data presented herein demonstrate that the C-terminal end of the human astrovirus nsP1a polyprotein interacts with the host protein CD63. Overexpression of CD63 increases viral infection, whereas knockdown of CD63 decreases virus infection. Further studies will explore the molecular basis of the interaction between nsP1a/4 and
CD63. We aim to clarify the mechanisms underlying the role of CD63 during virus replication, and to confirm that the CD63–nsP1a/4–viral RNA– RdRp replication complex in the endoplasmic reticulum membrane promotes virus replication through the PI3K and ERK1/2 signalling pathways.

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Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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