Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication

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Members of rhinovirus C (RV-C) species are more likely to cause wheezing illnesses and asthma exacerbations compared with other rhinoviruses. The cellular receptor for these viruses was heretofore unknown. We report here that expression of human cadherin-related family member 3 (CDHR3) enables the cells normally susceptible to RV-C infection to support both virus binding and replication. A coding single nucleotide polymorphism (rs6967330, C229Y) was previously linked to greater cell-surface expression of CDHR3 protein, and an increased risk of wheezing illnesses and hospitalizations for childhood asthma. Compared with wild-type CDHR3, cells transfected with the CDHR3-Y229 variant had about 10-fold increases in RV-C binding and progeny yields. We developed a transduced HeLa cell line (HeLa-EB) stably expressing CDHR3-Y229 that supports RV-C propagation in vitro. Modelling of CDHR3 structure identified potential binding sites that could impact the virus surface in regions that are highly conserved among all RV-C types. Our findings identify that the asthma susceptibility gene product CDHR3 mediates RV-C entry into host cells, and suggest that rs6967330 mutation could be a risk factor for RV-C wheezing illnesses.

rhinovirus C | CDHR3 | receptor

More than 160 types of rhinoviruses (RVs) are classified into three species (A, B, and C) of enteroviruses in the family Picornaviridae. Viruses belonging to the RV-C species were only discovered in 2006 (1, 2), some 50 y after the initial identification of other RVs (3), because these viruses are not detectable by standard tissue-culture techniques (4–7). RV-Cs are of special clinical interest because they can cause more severe illnesses requiring hospitalization in infants and children compared with the RV-A or RV-B, and are closely associated with acute exacerbations of asthma (8–10).

After multiple attempts to grow RV-C in established cell lines were unsuccessful, we described the use of organ culture of human sinus mucosa to propagate a few RV-C clinical isolates in vitro (11). Subsequently, we and others documented RV-C growth in cultures of epithelial cells isolated from airway tissue specimens that had then become rederivatized under air-liquid interface (ALI) conditions (12, 13). Notably, only fully differentiated ALI cultures supported RV-C replication, whereas undifferentiated airway epithelial cell monolayers did not. RV-C can only be produced using reverse genetics, as the full-length viral RNA transcripts synthesized in vitro are infectious when transfected into human cell lines (for example, HeLa, WsiL, and HEK293T) normally not susceptible to viral infection (11).

With these tools, it was possible to show that the RV-C receptor is distinct from the intercellular adhesion molecule 1 (ICAM-1) and low-density lipoprotein receptor (LDLR) family members used by the other species of RV (14, 15). However, the existing RV-C culture systems have relatively low throughput and are labor intensive. Failure to identify the cellular receptor and the inability to propagate RV-C in convenient cell lines has been a major obstacle to the study of virus-specific characteristics that could lead to effective antiviral strategies for this common and important respiratory pathogen. We now report that human cadherin-related family member 3 (CDHR3), a member of the cadherin family of transmembrane proteins, mediates RV-C entry into host cells, and an asthma-related mutation in this gene is associated with enhanced viral binding and increased progeny yields in vitro.

Results

In Silico Identification of Candidate RV-C Receptors. To obtain a working list of potential cellular receptors, we measured gene expression on microarray chips (Human Gene 1.0 ST Array, Affymetrix) in two series of experiments involving cells that were either susceptible or not susceptible to RV-C infection (Fig. 1 and Fig. S1). In one experimental series, susceptible cells included whole sinus mucosal tissue specimens (n = 5), epithelial cell suspension from sinus tissue, and nasal epithelium obtained via brushing, and unsusceptible cells, including monolayers of primary undifferentiated epithelial cells and transformed cell lines (n = 5, including HeLa). In a second experimental series, we compared three pairs of undifferentiated (monolayers) and fully differentiated (ALI) sinus epithelial cell cultures.

Significance

The rhinovirus C (RV-C) species was first identified in 2006 and is a major cause of acute respiratory illnesses in children and hospitalizations for exacerbations of asthma. In this study, we discovered that expression of human cadherin-related family member 3 (CDHR3), a transmembrane protein with yet unknown biochemical function, enables RV-C binding and replication in normally unsusceptible host cells. Intriguingly, we found that a coding SNP (rs6967330, C229Y) in CDHR3, previously linked to wheezing illnesses and hospitalizations for childhood asthma by genetic analysis, also mediates enhanced RV-C binding and increased progeny yields in vitro. Finally, using structural modeling, we identified potential binding sites in CDHR3 domains 1 and 2 interacting with viral capsid surface regions that are highly conserved among RV-C types.


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Gene Ontology terms

Plasma membrane

n = 66 genes

Receptor

n = 65 genes

Expression level:

RV-C susceptible > 7 log2 > RV-C unsusceptible

Plasma membrane

n = 38 genes

Receptor

n = 26 genes

12 genes
14 probe sets

3

Common

n = 8

Exp #1

Exp #2

Exp #1

Exp #2

CDHR3 Expression in HeLa Cells Enables RV-C Binding and Replication.

We next selected a subset of candidate genes [interleukin 5 receptor, alpha (IL5RA); chemokine (C-C motif) receptor-like 1 (CCL1); cadherin-related family member 3 (CDHR3); low density lipoprotein receptor class A domain containing 1 (LDLRAD1); calponin homology domain containing 2 (CHDC2); membrane-spanning 4-domains, subfamily A, member 8 (MS4A8)] for functional validation (Fig. 1B and Fig. S2). We transfected HeLa cells with plasmid DNAs encoding the identified genes under control of the CMV promoter. The cells were then exposed to a reporter virus (RV-C15-GFP) engineered to express GFP during replication (Fig. 2A) to facilitate the analysis. This reporter virus replicated well in susceptible ALI cultures of airway epithelial cells (Fig. S3). We repeatedly detected some GFP expression only in cells transfected with CDHR3-expressing cDNA (Fig. 2B).

To confirm that this result was not unique to the GFP-expressing virus, we then infected CDHR3-expressing HeLa cells with wild-type RV-C15. Relative to untransfected control cells, we again observed enhanced virus binding and evidence of vigorous replication (about 2-log increase in RV-C RNA compared with input) (Fig. 2C). Therefore, transfection-induced expression of CDHR3 was sufficient to convert HeLa cells normally susceptible to RV-C infection into targets that supported virus binding and subsequent replication.

Mutation in CDHR3 (Cys529→Tyr) Increases RV-C Binding and Progeny Yields.

CDHR3 is a related member of the cadherin family of transmembrane proteins. Typically, cadherins are involved in homologous cell adhesion processes that are important for epithelial polarity, cell-cell interactions, and tissue differentiation (6-18). Four alleles of CDHR3 gene are described, of which one representing a single nucleotide polymorphism (G→A) that converts residue cysteine to tyrosine at position 529 (Cys529→Tyr, rs6967330), was recently linked with a much greater risk of asthma hospitalizations and severe exacerbations in young children in a genome-wide association study (19). It has been shown that this point mutation in cadherin-repeat domain 5 near a calcium binding site leads to a marked increase in cell surface expression of the CDHR3 protein, presumably by altering the protein conformation.

We engineered this single amino acid change into CDHR3 cDNA, inserted the recombinant FLAG tag (DYKDDDDK) between the signal sequence and domain 1 to facilitate detection of CDHR3 surface expression, and then expressed it in HeLa cells. Fluorescent microscopy and Western blot analysis confirmed enhanced surface expression of Y529 (Fig. 2D), but also similar levels of overall cellular expression of C529 (wild-type) and Y529 (risk allele) variant (Fig. 2E). Notably, we found a higher (≥7-fold) number of cells expressing GFP after infection with reporter C15 virus (Fig. 2F) and about 10-fold greater RV-C15 binding and subsequent progeny yield, relative to the wild-type CDHR3 (Fig. 2G). The FLAG tag did not affect virus binding or replication (Fig. 2G). Parallel experiments with another human cell line (HEK293T) again showed that CDHR3 expression conferred susceptibility to RV-C infection (Fig. S4).
Development and Characterization of Transduced HeLa Cell Line Stably Expressing CDHR3-Y29Variant. To confirm RV-C multi-cycle replication and enable large-scale virus propagation, we established (by lentiviral transduction) a human epithelial cell line HeLa-E8 stably expressing CDHR3-Y29. CDHR3 is expressed in these cells as a GFP-fusion protein that is cotranslationally cleaved because of the presence of a 2A peptide derived from porcine teschovirus-1 to facilitate clonal selection of transduced cells. Similarly to transfected cells, RV-C15 efficiently replicated (about 2-log increase in viral RNA) in cells stably expressing CDHR3 (Fig. 3A). The phenomenon is not unique to the C15 virus type, because HeLa-E8 cells were also susceptible to C2 and C41 infection (Fig. 3A). Quantitative RT-PCR (RT-qPCR), Western blot analysis, and flow cytometry confirmed higher CDHR3 mRNA (over 400-fold increase) and protein expression in transduced HeLa-E8 cells compared with parental HeLa (control) cells (Fig. 3B and C).

The RV-C15 growth kinetics in HeLa-E8 monolayers was overall similar to that of representative clinical isolates of A and B species showing continuous increase in viral RNA over the time of infection (Fig. 3D). High expression levels of the ICAM-1 receptor used by the A16 and B52 strains to enter host cells might be responsible for about 1-log higher levels of their binding (2 h postinfection, hpi) and progeny yields (16–42 hpi), compared with that of C15. Analysis of the binding properties of fluorescently labeled RV-C15 (RV-C15-APC) by flow cytometry consistently showed increased binding to HeLa-E8 (22.1% of positively stained cells) compared with control HeLa (3.72%) cells (Fig. 3E).

An Extended Model of CDHR3 Structure. The CDHR3 protein sequence (885 aa) has a short signal peptide (19 aa) and six tandemly repeated cadherin domains (~100 aa each), followed by transmembrane (63 aa) and C-terminal cytoplasmic (150 aa) domains (Fig. 4A). The structure of CDHR3 has not yet been experimentally determined, but an approximation for domains 2–6, as modeled on the mouse N-cadherin ectodomain structure (PDB ID code 3Q2W), is described elsewhere (19). We extended this model to include CDHR3 domain 1 (Fig. S5), using I-Tasser (20) and Robetta (21) algorithms to template the domain 1 and 2 sequences on multiple linked-domain cadherin analogs (e.g., PDB ID codes 1FF5A, 1O5CA, and 2QVIA).

The consensus output was joined to the domains 2–6 model coordinates using the align function of PyMOL (Molecular Graphics System, v1.6). Multiple iterations of this process, specifying a range of templates, did not alter the general configuration of the final, full-domain structure (average RMSD: 1.632). The extended model (Fig. 4B) provides predictions of residues displayed on various repeat unit faces, including the Cys552→Tyr mutation at the domain 5–6 junction that defines the “risk” allele.

Docking the CDHR3 to a Two-Protomer Model of the RV-C15 Capsid. On cell surfaces or between cells, cadherins self-dimerize through reciprocal domain 1 pairings mediated by cysteine, tryptophan, or hydrophobic interactions (22). There can be multiple glycosylation sites dispersed along the ectodomain. The dimer format of CDHR3 is unknown, but there is a single tryptophan (Trp36) residue in domain 1. According to the prediction algorithms, there is also a single putative arsperagine-linked glycosylation site (Asn36) in domain 2. Trp36 and Asn36 position as solvent exposed on opposite sides of their respective domains (Fig. 4B). Assuming interactions similar to other picornaviruses, the RV-C are expected to access distal rather than proximal locations relative to the cellular membrane.

To test the feasibility of virus-receptor interactions, we docked the CDHR3 domains 1, 2, and 3 to a two-protomer model of RV-C15 capsid (23). Preliminary mutagenesis experiments suggested that Asn36, but not Trp36, was participating in virus binding; therefore, both queried algorithms (Gramm-X (24) and HADDOCK (25)) were given only two constraints: (i) CDHR3 Asn36 should be at or near the interface and (ii) the solvent exposed (outer) surface of the capsid proteins should be involved. The preferred complexes docked the receptor across the adjacent protomers, impacting the virus where capsid proteins VP1, -2, and -3 abut in a fivefold to twofold orientation (Fig. 4 C and D). In this orientation, Asn36 of domain 2 is buried in VP1 contacts and domain 1 extends across the twofold axis with VP1 and VP2 contacts,
Fig. 3. RV-C binding and replication in HeLa-E8 cells stably expressing CDHR3-Y259. (A) Binding (2 hpi) and replication (24 hpi) of RV-C clinical isolates in HeLa-E8 cells. (B) CDHR3 mRNA and protein expression in transduced (HeLa-E8) compared with control HeLa cells assessed by RT-qPCR and Western blot analysis. For mRNA expression, each bar represents five different RNA preparations (n &gt; 5) and data are presented as fold-change relative to control cells. For protein analysis, whole-cell lysates were probed with α-CDHR3 polyclonal antibodies. (C) Control and transduced (HeLa-E8) cells were fixed, permeabilized, stained with monoclonal anti-CDHR3 antibody (Abcam, ab56549), and analyzed by flow cytometry. Data for control and transduced cells were acquired separately and combined. Data are representative of three independent experiments. (D) Growth curve of RV-C15, RV-A16 and RV-B2 isolates in HeLa-E8 cells (n = 3, data are means ± SD). (E) Binding characteristics of fluorescently labeled RV-C15-APC in control and transduced (HeLa-E8) cells analyzed by flow cytometry. The percentages shown in the top two quadrants represent the percent of gated events that are negative for GFP fluorescence but positive for APC fluorescence (Left) or positive for both GFP and APC fluorescence (Right). Data are representative of three independent experiments.

whereas domain 3 is free of the virus and Trp56 is on a nondocked face (Fig. 4 D and E).

The RV-C as a species conserves &lt;75% capsid protein identity, but the RV-C15 structure model predicts a single shared receptor (23). The modeled CDHR3 footprint covers nearly every surface residue of RV-C15 that is conserved within the species (Fig. 4E). Moreover, although modeled as a monomer, this particular receptor orientation would easily accommodate domain 1-mediated dimer contacts across the twofold axis of the virus, or even other multimers around the fivefold.

Discussion

The cure for the common cold has been elusive, in part because of incomplete information on the molecular biology of RV-C, which is an important cause of both upper and lower respiratory illnesses as well as acute exacerbations of chronic childhood asthma. Toward this goal, we performed genome-wide gene-expression analysis of cells that were either susceptible or not susceptible to RV-C infection, selected and functionally validated a subset of candidate receptor genes by expression in HeLa cells followed by infection with GFP-expressing RV-C15, and established that human CDHR3 confers susceptibility to RV-C infection to normally unsusceptible cells. Furthermore, replication of multiple RV-C types was demonstrated in HeLa-E8 cells with stable expression of CDHR3-Y259. Finally, we modeled the complete CDHR3 structure, and identified a putative docking site for CDHR3 monomers and dimers to bind virus in a region with multiple residues conserved among all RV-C types. These findings have great potential utility for RV research. Cell lines such as HeLa-E8 will enable production of large quantities of virus that can be used to develop RV-C vaccines and to determine the actual, rather than modeled, virion crystal structure. In addition, the HeLa-E8 cells could be used to develop RV-C infectivity assays for antiviral drug screening, for detection of neutralizing antibody response to RV-C in clinical studies, and to grow RV-C clinical isolates from samples of respiratory fluids.

CDHR3 is a member of a cadherin family of transmembrane proteins with a yet unknown biological function that is highly expressed in human lung tissue (26), bronchial epithelium (19), and during mucociliary differentiation in human airway epithelial cells in vitro (27). Members of this family are responsible for communication between identical cells through calcium-dependent interactions. Cadherins on the same cell surface can self-associate into cis-dimers (lateral dimers) and cell adhesion is based on interactions between identical cadherins on neighboring cell surfaces to form transdimers (28). Our structure modeling of the CDHR3-virus complex identified a potential binding site for monomers and dimers in a region of the RV-C15 capsid that is highly conserved among different RV-C types. The docking exercise further suggests that receptor domains 1 and 2 may be key to virus interactions, and that glycosylation, particularly at Asn186, may be contributory. Such models of proteins and their interactions will help guide studies using mutational analysis to accurately map virus binding domains in CDHR3.

Notably, our findings demonstrate that coding SNP (rs6967330) in CDHR3, which was previously associated with wheezing illnesses and hospitalizations in childhood asthma by genetic analysis (19), is also associated with increased RV-C binding and progeny yields in vitro. If the same relationship is true in vivo, rs6967330 and the surface overexpression of CDHR3 could also be a specific risk factor for more severe RV-C illnesses. Another point to consider is that RV wheezing illnesses in infancy are a strong risk factor for subsequent childhood asthma and decreased lung function (29-31), but whether the viral infections serve a causal role is unknown (32). These findings suggest the possibility that the rs6967330 SNP could promote RV-C wheezing illnesses in infancy, which could in turn adversely affect the developing lung to increase the risk of asthma.

Expression of CDHR3 increased RV-C binding and enabled replication in normally unsusceptible host cells. These findings suggest that CDHR3 is a functional receptor for RV-C. One limitation of our results is that anti-CDHR3 antibodies to extracellular domains or recombinant CDHR3 (such as the CDHR3-Fc fusion protein) are not currently available to test for
Fig. 4. Structure modeling of RV-C15 binding to CDHR3 receptor. (A) Schematic map of the CDHR3 protein with indicated domains, predicted glycosylation sites, and engineered point mutation and FLAG tag. (B) Structural model of the complete CDHR3 protein. Domain 1 was added to the published model of domains 2–6 (19) after I-Tasser modeling relative to known cadherin structures. Key residues are highlighted. Gray spheres are interdomain calcium ions from PDB ID code 3Q2W. (C) Surface model of domains 1–3 docked to two RV-C15 receptors (model), projected onto pentamer coordinates. VP1 (blue), VP2 (green), and VP3 (red) are shown following standard colors. Model was then duplicated across twofold axis to show putative paired orientations. Triangle marks the icosahedral subunit. Biological subunit would include clockwise VP3. (D) Same as C, rotated x = 90°, y = 90°. (E) Similar to C with highlights (white) showing all RV-C15 surface amino acid residues conserved with >90% identity among all known RV-C isolates. See also Fig. S5.

direct interaction between the virus and putative receptor, which will be necessary to further prove its receptor function. Another point to consider is that our studies show some binding of RV-C to control HeLa cells. This low-level binding could be nonspecific, or could indicate that CDHR3 is expressed at a very low level in control HeLa cells. Alternatively, there could be a coreceptor for RV-C that interacts with CDHR3, or a cofactor that facilitates binding and entry. For example, bacterial surface polysaccharides stabilize polioviruses and facilitate cell attachment by increasing binding to the viral receptor (33, 34). In any case, experiments in our laboratory and elsewhere demonstrated that the RV-C interaction with native HeLa cells or other cell lines (e.g., A549) is not sufficient for efficient virus entry and replication (5, 6, 35).

In conclusion, no vaccines or effective antiviral drugs are currently available for RV (36, 37). Blocking interactions between major receptor group RVs and ICAM-1 inhibits infection, airway inflammation, and illness (38, 39), but is ineffective against replication of minor receptor group viruses or RV-C types. Anti-CDHR3 antibodies, soluble CDHR3, or short peptides specifically targeting virus-binding sites could serve as inhibitors of RV-C infection and provide a new therapeutic approach for RV-C-induced illnesses. Development of RV-C-specific antiviral drugs may be especially important for treatment of infants and children with the r6967330 asthma risk allele in CDHR3.

Materials and Methods

Expanded methods are presented in SI Materials and Methods.

Cell Cultures. Sinus epithelial tissue samples were obtained from residual surgical specimens from individuals undergoing sinus surgery and cultured as described previously (11). The protocol was approved by the University of Wisconsin-Madison Human Subjects Committee. Primary airway epithelial cells were cultured submerged (undifferentiated monolayers) or at the ALI (fully differentiated), as previously described (13, 40).

Viruses and Infection. Recombinant RV-C15, RV-C15-GFP, RV-C2, RV-C41, RV-A16, and RV-B52 were produced by transfecting viral RNA synthesized in vitro from linearized infectious cDNA clones into Wisl cells, as previously described (11, 40). Cells grown in 12-well plates (monolayers) or in Transwell polycarbonate inserts (0.4-μm pore size; Corning) (ALI cultures) were inoculated with RV at 103 PFU/well or well (unless other dose indicated), incubated for 2–4 h at 34 °C, and washed three times with PBS to remove unattached input virus.

RNA Extraction and RT-qPCR. Total RNA was extracted from sinus tissue or cultured cells using the RNeasy Mini kit (Qiagen). Viral RNA concentrations were determined by RT-qPCR using Power SYBR Green PCR mix (Life Technologies), as previously described (11). Relative expression of CDHR3 mRNA was determined by RT-qPCR using the CDHR3-qf and CDHR3-qr primers (Table S2).

Gene Expression Analysis. Total RNA samples were treated with RNase, labeled, fragmented and hybridized to Human Gene 1.0 ST arrays (Affymetrix). Raw data (CEL files) were uploaded into ArrayStar software v4.0 (DNASTAR) for normalization and statistical analysis. Gene-expression data have been deposited to Gene Expression Omnibus (GEO) database under accession no. GSE61396.

Expression Plasmids and Transfection. Plasmids for expression of receptor candidate genes CCR11 (Missouri S&T CDNA Resource Center), MS4A8 (OriGene), CHDC2, and CDHR3 (TransOmic) were purchased. ILSRA and LDR1AD1 ORFs were PCR-amplified from a DNA sample obtained from differentiated airway epithelial cells using the corresponding primers (Table S2). The mutation in domain 3 (C325Y) of CDHR3 was engineered by two-step PCR using the flanking (CDHR3-f and CDHR3-r) and internal
(CDHR3-C529Y-f and CDHR3-C529Y-g) primers. The plasmid DNA was prepared by Plasmid Maxi kit (Qiagen) and transfected into monolayers of HeLa or HEK293T cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions.

**Fluorescent Microscopy.** HeLa cells plated on glass coverslips were transfected with 1 µg of pCDHR3-FLAG DNA using Lipofectamine 2000 (Life Technologies) and fixed 24 h posttransfection. For detection of cell surface expression of CDHR3, nonpermeabilized fixed cells were washed (two times) with PBS, blocked, and then incubated with rabbit monodonal anti-FLAG primary antibody (Sigma, F2555). Cells were then washed (three times) and treated with Alexa Fluor 594 anti-rabbit antibody (Life Technologies). Next, for detection of total cellular CDHR3 expression, cells were permeabilized, washed (three times), reblocked, and stained with rabbit polyclonal anti-CDHR3 (Sigma, HPA012158). After wash (three times) with PBS, cells were treated with Alexa Fluor 488 anti-rabbit antibodies (Life Technologies).

**Generation of Stable HeLa Cell Line Expressing CDHR3.** The mutation in domain 5 (C529Y) of CDHR3 was engineered in lentiviral vector pLX304 containing wild-type CDHR3 sequence (TransOmic) by subcloning from pCDHR3-C529Y. We then added a 2A peptide sequence derived from porcine teschovirus-1 (41) and the GFP sequence to the 3'end of CDHR3 using synthetic gene fragments (gBlocks, Integrated DNA Technologies) to encode the CDHR3-GFP fusion protein, which is cotransfectionally cleaved to facilitate cloning and selection as described by direct fluorescent microscopy. The resulting plasmid, pLX304-CDHR3-C529Y-NP-GFP, was cotransfected with the mixture of packaging plasmids (psPAX2 and psMD2.G) into the 293T cells using Lipofectamine 2000 (Life Technologies) to produce lentivirus particles. HeLa cells were transduced, selected with blastidcin (5 µg/mL) and cloned by limiting dilution in 96-well plates, showing the highest RV-C replication levels (over 2-log), was selected for further experiments.

**Flow Cytometry.** Control or transduced cells grown in suspension were washed, stained with Ghost 780 (Tonbo) exclusion dye, fixed, and permeabilized. Cells were then blocked [10% (v/v)] in PBS, 0.05% Tween-20 in PBS, and reacted with anti-CDHR3 mAbs (Abcam, ab56549). After wash (three times) with PBS, cells were reacted with Alexa Fluor 647-conjugated donkey anti-mouse secondary antibody (Life Technologies), washed again (three times), and analyzed by flow cytometry.

**Fluorescent Labeling of RV-C15 and Virus Binding Assay.** The purified C15 virus was labeled with NHS ester fluorescent probe Dylight 650 (Thermo Scientific) following the manufacturer’s recommendations. Unincorporated dye was removed by fluorescent dye removal columns (Thermo Scientific). Monolayers of HeLa cells were trypsinized, incubated with labeled virus (multiplicity of infection of 10) for 1 h at 25 °C, washed (three times) before fixation with 2% (w/v) paraformaldehyde and analyzed by flow cytometry.

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