Correction

MICROBIOLOGY

The authors note that the author name Cornelis A. M. de Haan(Xander) should instead appear as Cornelis A. M. de Haan. The corrected author line appears below. The online version has been corrected.


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Enterovirus D68 receptor requirements unveiled by haploid genetics


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Enterovirus D68 (EV-D68) is an emerging pathogen that can cause severe respiratory disease and is associated with cases of paralysis, especially among children. Heretofore, information on host factor requirements for EV-D68 infection is scarce. Haploid genetic screening is a powerful tool to reveal factors involved in the entry of pathogens. We performed a genome-wide haploid screen with the EV-D68 prototype Fermon strain to obtain a comprehensive overview of cellular factors supporting EV-D68 infection. We identified and confirmed several genes involved in sialic acid (Sia) biology as being essential for infection. Sequence comparison of Sia-dependent and Sia-independent EV-D68 receptors showed that not only α2,3- and α2,6-linked Sia can be used as functional cellular EV-D68 receptors, but also that the screen did not reveal a specific protein receptor, suggesting that EV-D68 can use multiple redundant sialylated receptors. Upon testing recent clinical strains, we identified strains that showed a similar Sia dependency, whereas others could infect cells lacking surface Sia, indicating they can use an alternative, nonsialylated receptor. Nevertheless, these Sia-independent strains were still able to bind Sia on human erythrocytes, raising the possibility that these viruses can use multiple receptors. Sequence comparison of Sia-dependent and Sia-independent EV-D68 strains showed that many changes occurred near the canyon that might allow alternative receptor binding. Collectively, our findings provide insights into the identity of the EV-D68 receptor and suggest the possible existence of Sia-independent viruses, which are essential for understanding tropism and disease.

the genus Enterovirus of the family Picomaviridae contains many important pathogens for humans and animals. This genus consists of 12 species: four human enterovirus species (EV-A, EV-B, EV-C, and EV-D), five animal enterovirus species, and three human rhinovirus species. The best known human enterovirus is poliovirus (EV-C), the cause of poliomyelitis and acute flaccid paralysis. Other well-known enteroviruses are the coxsackieviruses (EV-B and EV-C)—which are the main cause of viral meningitis, conjunctivitis, myocarditis, and herpangina—and enterovirus A71, which causes hand-foot-and-mouth disease and is also associated with severe neurological disease, causing serious public health concerns in Southeast Asia (1).

Another emerging enterovirus that causes growing public health problems is enterovirus D68 (EV-D68, a member of the species EV-D). Unlike most enteroviruses, which are acid-resistant and multiply in the human gastrointestinal tract, EV-D68 is an acid-sensitive enterovirus (2) that replicates in the respiratory tract. EV-D68 was first isolated from children with respiratory infections in California in 1962 (3). It was long considered a rare pathogen, but the frequency of detecting EV-D68 during outbreaks of respiratory disease has increased (4, 5) and over the past decades, three clades of EV-D68 (A, B, and C) have emerged and spread worldwide (6, 7). EV-D68 infections mostly cause mild respiratory disease but can also result in severe bronchiolitis or pneumonia, especially among children (4, 5). In 2014, a nationwide EV-D68 outbreak in the United States was associated with severe respiratory disease and a cluster of acute flaccid myelitis and cranial nerve dysfunction in children, implicating EV-D68 as an emerging public health threat (8, 9).

Enteroviruses are small, nonenveloped viruses that contain a single-stranded RNA genome of positive polarity. To initiate infection, enteroviruses bind to specific receptors on host cells. To date, most known enterovirus receptors are cell surface proteins, many of which belong to the Ig superfamily or the integrin receptor family (10). A majority of these receptors bind to the “canyon,” a depression on the virion surface, thereby destabilizing virions and initiating uncoating (11). In EV-D68, the canyon is unusually shallow and narrow, possibly excluding use of large protein receptors (12). Both sensitivity of EV-D68 infection to neuraminidase (NA) treatment and hemagglutination assays point to the use of Sia as the receptor (13, 14). However, beside the role of a terminal Sia residue on the receptor, little is known about the type(s) of Sia that can be used by enterovirus D68 | haploid genetic screen | receptor | sialic acid

Significance

Enterovirus D68 (EV-D68) is an emerging pathogen that recently caused a large outbreak of severe respiratory disease in the United States and is associated with cases of paralysis. Little is known about EV-D68 host factor requirements. Here, using a genome-wide knockout approach, we identified several genes in sialic acid (Sia) biology as being essential for infection. We also showed that not only α2,6-linked Sia, which mainly occurs in the upper respiratory tract, but also α2,3-linked Sia, which mainly occurs in the lower respiratory tract, can serve as the receptor. Moreover, we identified recent EV-D68 isolates that can use an alternative, nonsialylated receptor. Our findings are essential for understanding tropism and pathogenesis of EV-D68 as well as the potential of using Sia-targeting inhibitors to treat EV-D68 infections.


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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT231897–KT231900).

1J.B. and H.J.T. contributed equally to this work.

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EV-D68 to infect cells, the composition of the underlying glycan, and whether specific sialylated proteins or glycolipids are required for infection.

Genome-wide genetic screening in human haploid cells is a powerful tool to reveal host factors involved in entry of various pathogens, including viruses (15, 16). In this study, we performed a haploid screen and demonstrate that genes involved in synthesis of sialylated glycans are essential for EV-D68 infection. Furthermore, we show that EV-D68 is able to use α2,6-linked as well as α2,3-linked Sia as a cellular receptor, and we provide the first insights into the composition of the underlying sugar backbone. Finally, we report the identification of recent EV-D68 isolates that can infect Sia-deficient cells, indicating that these strains can use an alternative receptor.

Results

Multiple Genes Involved in Sia Biology Determine Susceptibility of Cells to EV-D68 Infection. We performed a haploid genetic screen (17, 18) by infecting mutagenized human HAP1 cells with the EV-D68 prototype strain Fermon CA62-1. The screen identified nine genes involved in Sia biology (Fig. 1A and B and Fig. S1), seven of which were in the top 10. Among these nine hits are genes involved in the biosynthesis [UDP-GlcNAC-2-epimerase/ManAc kinase (GNE) and N-acetylneuraminic acid synthase (NANS)] and activation [cytidine monophosphate N-acetylneuraminic acid synthetase (CMAS)] of N-acetylneuraminic acid, the predominant form of Sia in humans. Other hits include transporters that transfer the activated sugars CMP-Sia and UDP-galactose [solute carrier family 5 member A1 and A2 (SLC35A1 and SLC35A2)] from the cytosol to the Golgi apparatus and four glycosyltransferases responsible for conjugation of N-acetylglucosamine (GlcNAC) to mannose residues in N-linked glycans [mannoside acetylglucosaminyltransferase 5 (MGAT5)], galactose [beta-1,4-galactosyltransferase 1 (B4GALT1)], and Sia, either via α2,3 linkage [ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (ST3GAL4)] or α2,6 linkage [ST6 beta-galactosamidase alpha-2,6-sialyltransferase 1 (ST6GAL1)]. Together, these data provide insights into the identity and composition of the EV-D68 receptor, pointing to an important role of α2,6- and α2,3-linked Sia on N-linked glycans in infection.

To confirm the results of the genetic screen, we used mutant cell lines lacking surface expression of Sia (SLC35A1KO and CMASKO) or having a defect in formation of α2,5- and/or α2,6-linked Sia (ST3GAL4KO/ST6GAL1KO and ST3GAL4/ST6GAL1DKO). The integrity of these mutant cell lines was confirmed by genetic analysis (Fig. S2A and B), lectin stainings (Fig. S2C), and infection with Sia-dependent and -independent control viruses. Analysis of the number of infected cells showed that SLC35A1KO and CMASKO cells were highly resistant to influenza A virus (IAV), whereas ST3GAL4/ST6GAL1DKO cells were partially resistant (Fig. 2A), consistent with the broad Sia specificity of IAV (19). In contrast, coxsackievirus-B3, which does not require Sia, could infect all cell lines (Fig. 2A and B), whereas equine rhinitis A virus, a picornavirus that requires α2,3-linked Sia (20), could infect ST6GAL1KO, but not ST3GAL4KO cells (Fig. 2C). Upon characterizing EV-D68 Fermon, we observed that infection was inhibited by NA treatment and almost completely blocked in unselected population are indicated (Fig. 2D) and low multiplicity of infection (moi) (Fig. 2B). Likewise, little, if any, production of progeny virus was observed in these mutant cell lines (Fig. 2C).

EV-D68 Can Use Both α2,6- and α2,3-Linked Sia to Infect Cells. Specificity for α2,3- or α2,6-linked Sia can greatly affect tissue tropism of respiratory viruses, as the Sia abundance varies between the upper (mainly α2,6-linked) and lower (mainly α2,3-linked) respiratory tract (21, 22). Identification of ST3GAL4 and ST6GAL1 suggested that both α2,6- and α2,3-linked Sia are used for infection. Indeed, ST6GAL1KO cells were less susceptible to EV-D68, whereas ST3GAL4KO cells were equally susceptible as wild-type cells (Fig. 2A–C), suggesting a preference of EV-D68 for α2,6-linked Sia. However, EV-D68 can also use α2,3-linked Sia as a receptor, as shown by the observation that ST3GAL4/ST6GAL1DKO cells were more resistant to infection than ST6GAL1DKO cells (Fig. 2A–C). Consistently, ST3GAL4/ST6GAL1DKO cells could be rendered more susceptible to EV-D68 by transfection of plasmid containing ST3GAL4 cDNA (and also ST6GAL1), but the effect was small due...
to the low transfection efficiency in HAP1 cells (Fig. 2D). Upon retroviral transduction with ST3GAL4 cDNA, however, high infection efficiency was observed (Fig. 2E).

Identification of ST3GAL4 in the genetic screen is likely due to heterogeneous expression of α2,3- and α2,6-linked Sia, which has been described in human airway epithelial cultures but also occurs in cultured cells, including HAP1 (Fig. S2E). Hence, knockout of ST3GAL4 in cells already expressing α2,6-linked Sia at reduced levels likely conferred resistance to infection. In summary, our data show that both α2,6- and α2,3-linked Sia can be used for infection by EV-D68.

Other EV-D Members Display a Similar Sia Preference Profile. We also investigated the Sia dependency of EV-D70 and EV-D94, two other members of the EV-D species. EV-D70 causes outbreaks of hemorrhagic conjunctivitis, which are often associated with neurological disorders (23). EV-D94 has been associated with acute flaccid paralysis (24), but information on the clinical relevance of this virus is scarce. EV-D70 was reported to be NA-sensitive (25), whereas any role of Sia in EV-D94 infection is unknown. Like EV-D68 Fermon, three clinical strains (670, 2042, and 2284) did not cause infection of different HAP1 cell lines. Like EV-D68, EV-D70 causes outbreaks of flaccid paralysis (24), but information on the clinical relevance of this virus is scarce. EV-D70 was reported to be NA-sensitive (25), whereas any role of Sia in EV-D94 infection is unknown. Like EV-D68 Fermon, three clinical strains (670, 2042, and 2284) did not cause infection of different HAP1 cell lines. Like EV-D68, EV-D70 causes outbreaks of flaccid paralysis (24), but information on the clinical relevance of this virus is scarce. EV-D70 was reported to be NA-sensitive (25), whereas any role of Sia in EV-D94 infection is unknown.
CPE (Fig. S3A). Strikingly, the other three strains (947, 1348, and 742) were able to replicate in Sia-deficient and NA-treated HAP1 cells (Fig. 3 A and B). Sia independence of these strains was not specific for HAP1 cells, as these viruses also efficiently infected NA-treated A549 and HeLa-R19 cells (Fig. S3C). In summary, these data demonstrate that several recent EV-D68 strains strongly depend on Sia, whereas other strains can infect cells in a Sia-independent manner, pointing toward the use of a nonsialylated receptor.

Sia-Independent EV-D68 Strains Retain Sia-Binding Capacity. A recent study showed that, in vitro, Sia-containing trisaccharides can bind to EV-D68, where the floor of the canyon would be in the major group rhinoviruses or polioviruses (26) (Fig. 4). To gain insight into the residues that allow Sia-independent infection and their location with respect to the Sia-binding site, we sequenced the capsid regions of the different EV-D68 strains. Amino acid sequence comparison showed that the genetically related Sia-dependent (670) and -independent (742 and 1348) strains in clade A differed at 10 positions, whereas the Sia-dependent (2042) and -independent (947) strains in clade B differed at seven positions (Fig. 4 and Tables S1 and S2). Although residues that differed between Sia-dependent and -independent strains in clade A show little overlap with those that were altered in clade B, some of the changed residues on the viral surface are near the Sia-binding site (26) (Fig. 4).

To investigate whether the amino acid substitutions that established an alternative receptor-binding site might have affected the Sia-binding capacity of these strains, we performed hemagglutination experiments with blood from nine different human donors. All EV-D68 isolates agglutinated human erythrocytes, although two Sia-independent strains (1348 and 742) agglutinated erythrocytes from only one donor (Table 1). Pretreatment of erythrocytes with NA prevented hemagglutination by EV-D68 strains but not by echovirus-7, which agglutinates erythrocytes by binding to its protein receptor, decay-accelerating factor (27). Remarkably, blood from three donors was not agglutinated by any of the EV-D68 strains, whereas hemagglutination titers of IAV were similar for all donors, indicating equal Sia expression levels. This variability suggests that EV-D68 does not merely bind any sialylated glycan but has a preference for specific sialylated glycan structures that are differentially expressed between individuals. No clear correlation between EV-D68 hemagglutination and ABO blood groups was observed. In summary, these data indicate that Sia-independent strains have retained their Sia-binding capacity, albeit to a lesser extent.

Discussion

In this study, we provided important insights into the identity/nature of the EV-D68 receptor. Using a genome-wide haploid screen, we identified genes involved in biosynthesis (GNE and NANS), activation (CMAS), transport (SLC35A1), and conjugation of Sia to glycans (ST3GAL4 and ST6GAL1) as factors required for EV-D68 infection. Using knockout cell lines and gene reconstitution, we have shown that EV-D68 can use both α2,6- and α2,3-linked Sia as a receptor to infect cells. This finding extends recent observations that both α2,6- and α2,3-linked Sia-containing trisaccharides can bind to the EV-D68 capsid and initiate virion uncoating in vitro (26). The observation that EV-D68 can use not only α2,6-linked Sia as a receptor but also α2,3-linked Sia, which resides mainly in the lower respiratory tract, may provide an explanation for its ability to cause severe lower respiratory tract infections.

Importantly, the screen did not identify a specific protein receptor, suggesting that EV-D68 can use multiple redundant receptors, given these are glycosylated with a suitable sialylated glycan. Our screen also provided insights into the preference of EV-D68 for specific sialylated glycans. Identification of glycosyltransferases responsible for conjugation of galactose (B4GALT1) and GlcNAc (MGATS) pointed to the importance of Sia–galactose–GlcNAc chains, consistent with the substrate specificity of ST3GAL4 and ST6GAL1. Furthermore, the identification of MGATS, which forms GlcNAc–β1,6-Man linkages, suggested that EV-D68 specifically recognizes N-linked glycans containing a β1,6-linked antenna. It should be noted that Sia–galactose–GlcNAc, although mainly expressed on N-linked glycans, also occurs on O-linked glycans and glycolipids and that we observed that HEK293S cells, which lack complex N-linked glycans (19), are susceptible to EV-D68. Further proof that EV-D68 does not merely bind any sialylated glycan but has a preference for specific glycans stems from our observation that erythrocytes of several donors could be agglutinated by IAV but not by EV-D68. More research is required to explore the glycan spectrum that can be bound by EV-D68.

Upon characterizing recent EV-D68 isolates, we identified strains that are able to infect Sia-deficient cells, implying that these viruses can use an alternative entry receptor. Genetic comparison of Sia-dependent and -independent EV-D68 strains within clades A and B revealed little overlap of residues determining Sia independence but pointed toward residues near the Sia-binding site as possible.
determinants for Sia (in)dependence. A similar scenario was described for Sia-dependent and -independent rotavirus strains, where only a few amino acid changes in the Sia-binding site could cause a receptor switch to a nonsialylated glycan (28). It remains to be investigated whether a single amino acid substitution or a combination thereof is required for the observed Sia-independent phenotype. Better understanding of the (combinations of) residues that facilitate Sia independence could ultimately allow prediction of receptor requirement based on sequence alignments.

It has been shown in vitro that Sia binds to the EV-D68 canyon at a unique site, compared with the glycan-binding sites in other picornaviruses (Fig. S4). In EV-D68, binding of Sia induces virion destabilization and pocket factor release, which is the first step in the uncoating process (26). We identified several EV-D68 strains that can use a nonsialylated receptor while retaining a Sia-binding capacity, albeit with different affinities. This indicates that the formation of an alternative receptor-binding site does not necessarily result in loss of the Sia-binding site and points to the possible existence of a dual receptor mechanism, where either Sia or a nonsialylated receptor can trigger similar structural conformational changes. However, it is unclear whether binding of Sia to the capsid of Sia-independent viruses still results in virion destabilization and pocket factor release as described for EV-D68 Fermon. Furthermore, more research is warranted to determine whether this alternative receptor is a protein or a sugar that lacks a terminal Sia moiety.

Although it remains to be established whether Sia-independent strains circulate in the human population, the occurrence of strains that use an alternative receptor could have an impact on tissue tropism and pathogenesis of EV-D68. Also, application of the sialidase DAS-181 (Fludase) (29), an investigational drug against influenza virus that was shown to inhibit EV-D68 (30), may be ineffective against Sia-independent EV-D68 strains. Hence, detailed insight into the interactions of EV-D68 with its receptor(s) is required to understand viral pathogenesis and to develop effective antiviral treatment.

Materials and Methods
Cells and Viruses. Information on viruses and cells used in this study is described in SI Materials and Methods.

Table 1. Hemagglutination titers (Log2) of EV-D68 strains on human erythrocytes from nine different donors

<table>
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<th>Donor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>1+NA</th>
<th>2+NA</th>
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<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
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<tr>
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<td>2</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>7</td>
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<tr>
<td>EV-D68 742</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>3</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>3</td>
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<tr>
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<td>9</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>9</td>
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<td>0</td>
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<tr>
<td>IAV WSN</td>
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<td>8</td>
<td>9</td>
<td>8</td>
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<td>7</td>
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<tr>
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ND, not determined.
Haploid Genetic Screen with EV-D68. HAP1 cells were gene-trap mutagenized as described previously (31). Following expansion, 10⁶ mutagenized cells were exposed to 6O-68 (yielding 414,290 unique gene-trap insertions mapped to genes) and a population of matched control cells of comparable complexity (495,679 unique gene-trap insertions mapped to genes) were aligned to the human genome not filtering for close reads (31). Subsequently, disruptive insertion sites (in sense orientation of the affected gene or mapping to exons) in significantly identified genes were compared in the two cell populations, and P values for enrichment were calculated using a Fisher’s exact test as described previously (31). Disruptive insertion sites in virus-selected and cells selected were plotted onto the RefSeq gene bodies for the following transcripts: NM_001497 (BAG4ALT), NM_018686 (CMAS), NM_001128227 (GNE), NM_002410 (MGTAS), NM_018946 (XANS), NM_006416 (SLC35A1), NM_005660 (SLC35A2), NM_006278 (ST3GAL4), and NM_173216.2 (ST6GALT).

Generation of Knockout Cells. ST3GAL4KO and SLC35A1KO HAP1 cells have been described (31). CMASCO cells were obtained from Haplogentic GmbH. The CRISPR-Cas9 system was used to generate ST6GAL4KO cells. The entire ST6GAL1 locus was excised (Fig. S2A), and subclones were analyzed by genotyping (Table S3). ST3GAL4ST6GAL4XKO cells were obtained by deleting an exonic region in ST3GAL4 from ST6GAL4XKO cells using CRISPR-Cas9.

Infectivity Assays. Cells were infected with virus for 1 h. After incubation for the indicated period, virus titers were determined by end-point dilution. Crystal violet staining was performed at 3 d postinfection. Where indicated, cells were pretreated with NA from Claudiiosterolperfnings (NEB) or from Arthrobacterurefaciens (Roche) in serum-free medium for 30 min.

Immunofluorescence Assays. Paraformaldehyde-fixed cells were stained using rabbit anti-caspase serum against EV-D68 Fermon (produced in house; 1:1,000) or a mouse monoclonal antibody against EV-B3 protein 3A (1:100) (32). For character- ization with lectins, cells were stained with fluorescein-labeled Sambucus nigrum lectin (Vector Laboratories; 1:1,000) and biotinylated Maackia amurensis lectin I (Vector Laboratories; 1:500). Cells were examined by confocal microscopy (Leica SPE-4) or standard fluorescence microscopy (EVOS FL cell imaging system).