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) biosynthesis, transport, and conjugation to be essential for infection. Moreover, by using knockout cell lines and gene reconstitution, we showed that both $\alpha 2,6$ - and $\alpha 2,3$ -linked Sia can be used as functional cellular EV-D68 receptors. Importantly, 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.

enterovirus D68 | haploid genetic screen | receptor | sialic acid

The genus *Enterovirus* of the family *Picornaviridae* contains many important pathogens for humans and animals. This genus consists of 12 species: four human enterovirus species (*EV-A*, *-B*, *-C*, and *-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

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 $\alpha 2,6$ -linked Sia, which mainly occurs in the upper respiratory tract, but also $\alpha 2,3$ -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 to understand 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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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. [KT231897–KT231908](https://www.ncbi.nlm.nih.gov/nuclot/KT231897-KT231908)).

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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 α 2,6-linked as well as α 2,3-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. 1*A* 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 35 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 [β 1,4-galactosyltransferase 1 (*B4GALT1*), and Sia, either via α 2,3 linkage [ST3 β -galactoside α -2,3-sialyltransferase 4 (*ST3GAL4*)] or α 2,6 linkage [ST6 β -galactosamide α -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 α 2,6- and α 2,3-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 (*SLC35A1*^{KO} and *CMAS*^{KO}) or having a defect in formation of α 2,3- and/or α 2,6-linked Sia (*ST3GAL4*^{KO}, *ST6GAL1*^{KO}, and *ST3GAL4/ST6GAL1*^{DKO}). The integrity of these mutant cell lines was confirmed by genetic analysis (Fig. S2*A* and *B*), lectin stainings (Fig. S2*C*), and infection with Sia-dependent and -independent control viruses. Analysis of the number of infected cells showed that *SLC35A1*^{KO} and *CMAS*^{KO} cells were highly resistant to influenza A virus (IAV), whereas *ST3GAL4/ST6GAL1*^{DKO} cells were partially resistant (Fig. 2*A*), consistent with the broad Sia specificity of IAV (19). In contrast, coxsackievirus-B3, which does not require Sia, could infect all cell lines (Fig. 2*A* and *B*), whereas equine rhinitis A virus, a picornavirus that requires α 2,3-linked Sia (20), could infect *ST6GAL1*^{KO} but not *ST3GAL4*^{KO} cells (Fig. 2*C*). Upon characterizing EV-D68 Fermon, we observed that infection was inhibited by NA treatment and almost completely blocked in *CMAS*^{KO}, *SLC35A1*^{KO}, and *ST3GAL4/ST6GAL1*^{DKO} cells, both at high (Fig. 2*A* and Fig. S2*D*) and low multiplicity of infection (moi) (Fig. 2*B*). Likewise, little, if any, production of progeny virus was observed in these mutant cell lines (Fig. 2*C*).

EV-D68 Can Use Both α 2,6- and α 2,3-Linked Sia to Infect Cells. Specificity for α 2,3- or α 2,6-linked Sia can greatly affect tissue tropism of respiratory viruses, as the Sia abundance varies between the upper (mainly α 2,6-linked) and lower (mainly α 2,3-linked) respiratory tract (21, 22). Identification of *ST3GAL4* and *ST6GAL1* suggested that both α 2,6- and α 2,3-linked Sia are used for infection. Indeed, *ST6GAL1*^{KO} cells were less susceptible to EV-D68, whereas

ST3GAL4^{KO} cells were equally susceptible as wild-type cells (Fig. 2*A–C*), suggesting a preference of EV-D68 for α 2,6-linked Sia. However, EV-D68 can also use α 2,3-linked Sia as a receptor, as shown by the observation that *ST3GAL4/ST6GAL1*^{DKO} cells were more resistant to infection than *ST6GAL1*^{KO} cells (Fig. 2*A–C*). Consistently, *ST3GAL4/ST6GAL1*^{DKO} 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

Fig. 1. A haploid genetic screen for EV-D68 identifies genes involved in synthesis of sialylated glycans. (A) An overview of the different steps in synthesis of sialylated N-linked glycans. Significant hits related to Sia biology are shown in green. Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Sia, sialic acid. (B) RefSeq gene structures of *CMAS* and *SLC35A1* are shown with the transcriptional orientation pointing from left to right. Gene-trap insertions predicted to disrupt gene function (intronic insertions in sense orientation and insertions mapping to exons) are illustrated for HAP1 cells selected with EV-D68 (selected) and a cell population of similar complexity that had not been selected with EV-D68 (unselected). The FDR-corrected *P* values for the enrichment of disruptive gene-trap mutations in the EV-D68-selected population are indicated (Materials and Methods). For insertion plots of the other hits, see Fig. S1.

Fig. 4. A map of the EV-D68 surface residues. Surface representation of the complete EV-D68 capsid structure (12) (*Left*) and a close-up showing the Sia-binding site within the canyon (*Right*). The black triangle shows an icosahedral asymmetric unit of the EV-D68 Fermon structure. Residues are colored by radial distance (Å) to the virus center. The black dashed lines outline the canyon in the human rhinovirus 14 structure. Variable residues determining Sia (in)dependence of strains within clade A or clade B are highlighted by black and white contours, respectively. Note that residue 270 in VP1 (black and white contour) is variable both within clade A and clade B. Amino acids were numbered according to Liu et al., 2015 (12).

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 Feron. 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

[illegible]

ND, not determined.

Haploid Genetic Screen with EV-D68. HAP1 cells were gene-trap mutagenized as described previously (31). Following expansion, 10^8 mutagenized cells were exposed to EV-D68 Fermon (moi 3). After selection, surviving cells were expanded and used for genomic DNA isolation. Insertion sites identified in cells selected with EV-D68 (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 control cells were plotted onto the RefSeq gene bodies for the following transcripts: NM_001497 (*B4GALT1*), NM_018686 (*CMAS*), NM_001128227 (*GNE*), NM_002410 (*MGA75*), NM_018946 (*NANS*), NM_006416 (*SLC35A1*), NM_005660 (*SLC35A2*), NM_006278 (*ST3GAL4*), and NM_173216.2 (*ST6GAL1*).

Generation of Knockout Cells. *ST3GAL4*^{KO} and *SLC35A1*^{KO} HAP1 cells have been described (31). *CMAS*^{KO} cells were obtained from Haplogen GmbH. The CRISPR-Cas9 system was used to generate *ST6GAL1*^{KO} cells. The entire *ST6GAL1* locus was excised (Fig. S2A), and subclones were analyzed by genotyping (Table S3). *ST3GAL4/ST6GAL1*^{DKO} cells were obtained by deleting an exonic region in *ST3GAL4* from *ST6GAL1*^{KO} 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 *Clostridium perfringens* (NEB) or from *Arthrobacter ureafaciens* (Roche) in serum-free medium for 30 min.

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

The number of nuclei was quantified using ImageJ, and the number of infected cells was quantified visually.

Isolation and Sequencing of EV-D68 Strains from Clinical Specimen. Monolayers of tertiary monkey kidney cells (tMKs) or human rhabdomyosarcoma (RD) cells were incubated with 250 μ L EV-D68-positive clinical material (mixed nose and throat swabs) derived from patients with influenza-like illness or acute respiratory infection (5) and incubated at 34 °C until CPE was observed. EV-D68 strains 4311000670 (clade A; further referred to as 670) and 4311000742 (clade A; 742) were isolated on tMK cells, whereas strains 4310900947 (clade B; 947), 4310901348 (clade A; 1348), 4310902042 (clade B; 2042), and 4310902284 (clade C; 2284) were isolated on RD cells. Viruses were harvested by one freeze-thawing cycle at -80 °C and clarified by centrifugation. Subsequent passages of virus were done on tMK or RD cell monolayers. The full genome of the virus isolates was sequenced from passage 4 for strains 670 and 742 and from passage 2 of the remaining four strains. All infection experiments described in this study were performed with viruses that had undergone one or two more rounds of passage on RD cells. From these viruses, the 5'UTR and capsid region was sequenced. The latter sequences were used for amino acid comparisons shown in Tables S1 and S2. Detailed information on virus passages, sequences, and GenBank accession numbers is described in SI Materials and Methods. As required by Dutch legislation, surveillance studies have to be registered in the Personal Data Protection Act Register of the Personal Data Protection Commission. The influenza surveillance from which the clinical enterovirus D68 isolates were obtained is registered in this register and no further ethical approval was needed for this virologic study because only anonymized virus isolates were used.

Hemagglutination Assays. The hemagglutination assay was performed using standard methods. Briefly, twofold dilutions of the virus stocks were incubated with an erythrocyte suspension for 16 h at 4 °C.

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