tected sporadically, both during and between epidemics (46). Whether or by what mechanism these contrasting epidemiologies contribute to the differing recombination frequencies of GgB and GgC (Fig. 3) remains to be determined.

Evolution of EV71. It is well established that the longer-term genetic and antigenic diversification of human enteroviruses and the existence of serologically distinct EV types are key factors in their evolutionary success and ongoing ubiquitous presence in human populations. What is rather less clear is the underlying mechanisms and selection pressures involved in the generation of new serotypes, as this process has to date not been directly observed. In the case of EV71, it has been hypothesized that the evolution of capsid genes, particularly VP1, represents an equivalent, immunologically driven process of diversification (61, 63) and that the successive appearance of novel genogroups and subgenogroups is favored by an absence of preexisting immunity to them. The genetically diverse variants of EV71 may thus be precursors in their eventual further diversification into new serotypes in the future. This hypothesis is, however, not clearly supported by existing genetic and antigenic comparisons of EV71 genogroups (14, 24–26, 40, 61, 63). First, sequence divergence between genogroups and subgenogroups occurs overwhelmingly at silent sites, indicative of purifying or neutral evolutionary drift (14, 24, 61). Screening of large data sets of VP1 sequences provides little if any evidence for any sites in the capsid coding region being subjected to the positive selection that would typically be observed in sites under strong immunological pressure. Consistent with these analyses, there is little evidence from serological cross-neutralization experiments for genogroup- or subgenogroup-specific antibodies, nor indeed the existence of measurable antigenic diversity between EV71 isolates (24, 26, 28, 40, 63). However, infections with GgB variants induce higher levels of neutralizing antibodies than GgC, supporting an idea that the emergence of GgC may have been assisted by its intrinsically lower immunogenicity than GgB (40).

The alternative hypothesis is that the emergence and turnover of EV71 genogroups simply represent random processes of emergence and extinction of lineages without underlying natural selection, as proposed for other enteroviruses (2, 37, 50). Random fixation of individual variants with no fitness advantage within a population is indeed more likely when population sizes are small. Such conditions may occur during the periodic bottlenecks in a population size that inevitably occur in viruses that cause acute infections and show epidemic cycles of transmission. These instances of very rapid turnover and complete population replacements have been extensively documented, with a similar lack of evidence for antigenic replacement. However, as observed for EV71, such turnover is frequently associated with recombination events occurring during the founding of new evolutionary lineages. Understanding whether the partial or complete replacement of nonstructural gene regions associated with such recombination events provides a replicative or immunological selective advantage to the virus and thus drives the diversification of EV71 and other enteroviruses is a key unanswered question. Future biological and immunological investigation of both capsid region sequence change and recombination are clearly required if we are really to understand the evolution of enteroviruses and indeed of other nonenveloped RNA viruses.

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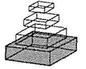

Cellular receptors for human enterovirus species A

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Human enterovirus species A (HEV-A) is one of the four species of HEV in the genus *Enterovirus* in the family *Picornaviridae*. Among HEV-A, coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) are the major causative agents of hand, foot, and mouth disease (HFMD). Some other types of HEV-A are commonly associated with herpangina. Although HFMD and herpangina due to HEV-A are common febrile diseases among infants and children, EV71 can cause various neurological diseases, such as aseptic meningitis and fatal encephalitis. Recently, two human transmembrane proteins, P-selectin glycoprotein ligand-1 (PSGL-1) and scavenger receptor class B, member 2 (SCARB2), were identified as functional receptors for EV71 and CVA16. In *in vitro* infection experiments using the prototype HEV-A strains, PSGL-1 and SCARB2 could be responsible for the specific receptors for EV71 and CVA16. However, the involvement of both receptors in the *in vitro* and *in vivo* infections of clinical isolates of HEV-A has not been clarified yet. To elucidate a diverse array of the clinical outcome of HEV-A-associated diseases, the identification and characterization of HEV-A receptors may provide useful information in understanding the HEV-A pathogenesis at a molecular level.

Keywords: human enterovirus species A, enterovirus 71, receptor, PSGL-1, SCARB2

INTRODUCTION

The genus *Enterovirus* within family *Picornaviridae*, non-enveloped viruses with a single-stranded RNA genome of positive polarity, is comprised of more than 100 serotypes (Pallansch and Roos, 2007). Human enteroviruses (HEVs) are presently classified into four species, HEV species A, B, C, and D (HEV-A, B, C, and D). At present, coxsackievirus A2 (CVA2), CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, CVA16, enterovirus 71 (EV71), EV76, EV89, EV90, EV91, and EV92 have been classified as HEV-A (Oberste et al., 2004, 2005, 2008). In addition, four types of simian enteroviruses are classified as HEV-A (Oberste et al., 2007). Although most enterovirus infections are asymptomatic, particular clinical manifestations are associated with specific types of enteroviruses (Pallansch and Roos, 2007). CVA2, CVA4, CVA5, CVA6, and CVA10 are commonly associated with herpangina. EV71 and CVA16 are major causative agents of hand, foot, and mouth disease (HFMD), a common febrile disease occurring mainly in young children, characterized by skin rash involving palms and soles, and ulcers on oral mucosa. Recently, HFMD outbreaks mainly due to CVA6 have been reported (Fujimoto et al., 2012). Clinical manifestations of HFMD caused by EV71, CVA6, and CVA16 are usually mild and self-limited. However, EV71 infection causes a diverse range of neurological diseases, such as aseptic meningitis, acute flaccid paralysis, brainstem encephalitis, and neurogenic pulmonary edema, and may result in long-term neurological sequelae, mainly in infants and young children (Alexander et al., 1994; McMinn, 2002; Modlin, 2007).

Recently, two human transmembrane proteins, P-selectin glycoprotein ligand-1 (PSGL-1; Nishimura et al., 2009) and scavenger receptor class B, member 2 (SCARB2; Yamayoshi et al., 2009), were identified as functional receptors for EV71 and CVA16 (Patel and Bergelson, 2009). In addition, annexin II (Yang et al., 2011), sialic acid (SA; Yang et al., 2009), and dendritic cell (DC)-specific ICAM3-grabbing non-integrin (DC-SIGN; Lin et al., 2009b) were found to be cellular factors involved in the early stages of EV71 infection. This review summarizes our current understanding of the EV71/CVA16 receptors and their role in HEV-A infection.

P-SELECTIN GLYCOPROTEIN LIGAND-1

Patients with severe EV71-associated encephalitis and neurological pulmonary edema showed a significant depletion of T cells and high levels of proinflammatory cytokines (Lin et al., 2003; Wang et al., 2003), suggesting the possible involvement of lymphocytes in EV71 infection and the immunopathogenesis. Therefore, we generated a cDNA library from Jurkat T cells and used it for expression cloning to identify a receptor on lymphocytes that specifically binds to EV71. Finally we identified PSGL-1 as a functional EV71 receptor on Jurkat T cells (Nishimura et al., 2009).

P-selectin glycoprotein ligand-1 is a sialomucin leukocyte membrane protein expressed as a homodimer of disulfide-linked subunits and it can bind to three different selectins (P, E, and L; Sako et al., 1993; Laszik et al., 1996; Somers et al., 2000). The tissue distribution of PSGL-1 is restricted to myeloid, lymphoid, and dendritic lineages, and platelets. PSGL-1 is also expressed on DCs of lymph nodes and macrophages in the intestinal mucosa (Laszik et al., 1996), which could be the primary sites of EV71 replication. PSGL-1 plays critical roles in the tethering and rolling of leukocytes for the recruitment of cells from blood vessels to the sites of acute inflammation upon stimulation by infection.

We found that some representative EV71 strains bind to PSGL-1 but other strains did not (Nishimura et al., 2009). According to their PSGL-1 binding capability, we classified the EV71 isolates as PSGL-1 binding strains (EV71-PB) and PSGL-1-non-binding strains (EV71-non-PB). The replication of EV71-PB in Jurkat T

cells was inhibited by anti-PSGL-1 monoclonal antibody (KPL1), indicating that EV71-PB replicated in Jurkat cells in a PSGL-1-dependent manner. On the other hand, EV71 replicated in non-leukocyte cells (such as RD cells) expressing little or no PSGL-1, and the replication was not affected by KPL1. Therefore we conclude that EV71 does not use PSGL-1 as the major cellular receptor on RD cells and other receptor(s), including SCARB2 or annexin II, may be responsible for EV71 infection in non-leukocyte cells expressing little or no PSGL-1.

Post-translational modifications of the N-terminal region of PSGL-1 contribute the efficient binding to selectins and chemokines (Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995; Liu et al., 1998; Hirata et al., 2004). In this region, there are a potential *O*-glycosylation residue (T57) and three potential tyrosine sulfation sites (Y46, Y48, and Y51). We demonstrated that tyrosine sulfation, not *O*-glycosylation, of the N-terminal region of PSGL-1 facilitates its binding to EV71-PB and viral replication in Jurkat T cells (Nishimura et al., 2010).

Coxsackievirus A16 is genetically and antigenically related to EV71 and is a major causative agent of HFMD as well as EV71 (Oberste et al., 2004). The inoculation of L-PSGL-1.1 cells, mouse L929 cells stably expressing human PSGL-1, with the prototype CVA16-G-10 strain induced faint cytopathic effects (CPE) (Nishimura et al., 2009). The replication of CVA16-G-10 was partially inhibited by KPL1 in L-PSGL-1.1 cells. This result indicated that the prototype CVA16 strain may use human PSGL-1 and another unidentified receptor(s) to infect L-PSGL-1.1 cells. CVA16-G-10 replication in Jurkat cells was not apparently inhibited by KPL1 (Nishimura et al., 2009), but significantly inhibited by a sulfation inhibitor, sodium chlorate (Nishimura et al., 2010). Therefore some sulfated molecules other than PSGL-1 might be involved in the replication of CVA16 in Jurkat cells. CVA16-G-10 would use unidentified receptor(s) to infect Jurkat T cells (Nishimura et al., 2009; Patel and Bergelson, 2009).

To investigate the PSGL-1-dependent replication phenotype of HEV-A, we tested the replication of 10 prototype HEV-A strains in L-PSGL-1.1 cells in the presence or absence of KPL1 (**Figure 1A**). On day 6 post-inoculation, there was no significant replication of CVA4, CVA5, CVA6, or CVA8 in L-bsd cells (blasticidin-resistant control L929 cells) or L-PSGL-1.1 cells. Although higher viral titers were found for CVA2 and CVA7 in L-PSGL-1.1 cells compared with those in L-bsd cells, replication was not affected by KPL1. These results suggest that CVA2 and CVA7 may infect to L-PSGL-1.1 cells in an alternative pathway via PSGL-1 or glycosylated PSGL-1, without the interaction between EV71-PB and the N-terminal region of PSGL-1 recognized by KPL1. We could not demonstrate any PSGL-1-dependent replication of the CVA3, CVA10, CVA12, and CVA14 strains in L-PSGL-1.1 cells, because they replicated even in PSGL-1 negative L-bsd cells as previously reported for certain HEV-A field isolates (Nadkarni and Deshpande, 2003; Yamayoshi et al., 2009).

Coxsackievirus A7 and CVA14 infection induced CPE in L-PSGL-1.1 cells, but not in L-bsd cells (**Table 1**). On the other hand, CVA7 and CVA14 induced CPE in L-Empty cells (puromycin-resistant control L929 cells; **Table 1**; Yamayoshi et al., 2009). The difference in the CPE induction by some HEV-A strains might be due to the maintenance or cultivation conditions of the mouse L929-derived cells regardless of the receptor expression of PSGL-1 or SCARB2. Some strains of HEV-A are able to infect mouse L929 cells regardless of expression of PSGL-1 or SCARB2 (Nadkarni and Deshpande, 2003; Yamayoshi et al., 2009). It is therefore impossible to determine receptor usage of HEV-A by simply investigating the susceptibility of mouse L929 cells expressing the putative cellular receptor. Receptor usage of HEV-A should be determined carefully by showing several lines of evidence such as acquisition of susceptibility by expressing a putative receptor in non-susceptible cells, loss of susceptibility by knocking down of the receptor in susceptible cells, and direct binding of the virus to the receptor, etc.

L-PSGL-1.1 cells did not support PSGL-1-dependent replication of the HEV-B and HEV-C strains (**Figure 1B**); however, the prototype EV70 strain (HEV-D) replicated in L-PSGL-1.1 cells more efficiently than in L-bsd cells. Although EV70 replication was not affected by KPL1 (**Figure 1B**), we cannot exclude the possibility that EV70 utilizes α2,3-linked SA, which could be a receptor for EV70 (Nokhbeh et al., 2005).

Recently we reported that that four out of five EV71-PB strains replicated poorly in L-PSGL-1.1 cells (Miyamura et al., 2011). We found that EV71 variants, which were propagated once in L-PSGL-1.1 cells, have several possible adaptive mutations, including a putative amino acid determinant of the adaptive phenotype in L-PSGL-1.1 cells at VP2-149 (Miyamura et al., 2011). The results suggest that adaptive mutations, along with a PB phenotype, may facilitate efficient PSGL-1-dependent replication of the EV71 variants in L-PSGL-1.1 cells. It is possible that HEV-A strains other than EV71 also require adaptive mutations for efficient replication in L-PSGL-1.1 cells.

SCAVENGER RECEPTOR CLASS B, MEMBER 2

Yamayoshi et al. (2009) identified SCARB2 (also known as lysosomal integral membrane protein II, or CD36b like-2) as an EV71 receptor on RD cells, widely used for isolation of EV71 from clinical specimens. They transfected EV71-non-susceptible mouse L929 cells with the genomic DNA of RD cells and selected two cell clones that were susceptible for EV71 infection. By a transcriptome analysis, SCARB2 was identified as an EV71 receptor on RD cells.

Scavenger receptor class B, member 2 is a heavily *N*-glycosylated type III transmembrane protein consists from 478 amino acids and belongs to the CD36 family of scavenger receptor proteins (Fujita et al., 1992; Calvo et al., 1995). SCARB2 has a N-terminal transmembrane domain, a ∼400 amino acid lumeral domain, a C-terminal transmembrane domain, and a C-terminal cytoplasmic tail of ∼20 amino acids (Fujita et al., 1992). SCARB2 involves in an enlargement of early endosomes and late endosomes/lysosomes and an impairment of endocytic membrane out of the enlarged compartments (Kuronita et al., 2002). SCARB2 deficiency caused ureteric pelvic junction obstruction, deafness, and peripheral neuropathy in mice (Gamp et al., 2003). SCARB2 is expressed ubiquitously in human tissues (Eskelinen et al., 2003); therefore, it might be involved in systemic EV71 infections (Yamayoshi et al., 2009).

Human SCARB2 has 10 potential *N*-glycosylation sites (Fujita et al., 1992). But the carbohydrate chains of human SCARB2 are not essential for the interaction between EV71 and human SCARB2 (Yamayoshi and Koike, 2011). Experiments using a

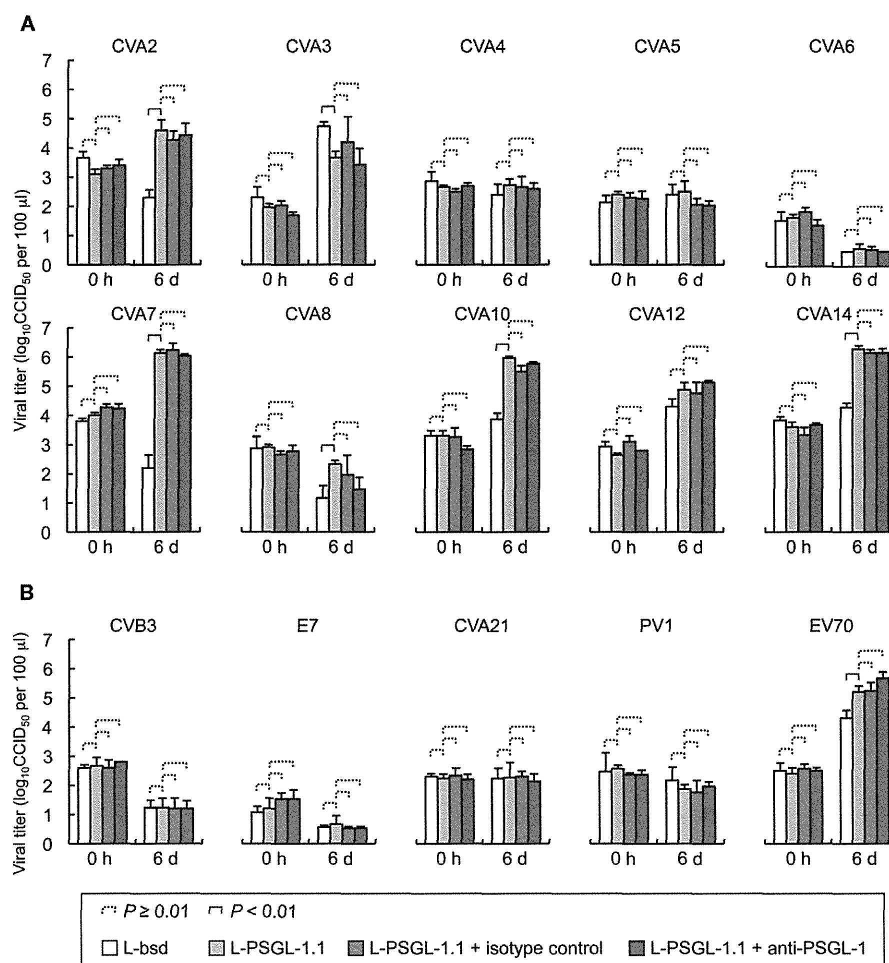

FIGURE 1 | Human enteroviruses replication in L-PSGL-1.1 cells. (A) Replication of the HEV-A strains (**Table 1**) in L-PSGL-1.1 cells in the presence or absence of anti-PSGL-1 mAb (KPL1) or an isotype control. Cells were inoculated with viruses at 10 $CCID_{50}$/cell for 1 h, washed, and incubated in the medium, as described previously (Nishimura et al., 2009). Cells were incubated at 34°C. For mAb inhibition, the cells were pretreated with 10 μg/ml mAb for 1 h, washed, and maintained in the medium with 10 μg/ml mAb. At the indicated time [just after infection (0 h) and 6 days postinfection (6 d)], the infected cells and supernatants were freeze-thawed and viral titers were determined by $CCID_{50}$ titration using RD cells. The titers are expressed as the mean and error bars indicate SD of triplicate analyses. The mean viral titers were compared using Student's *t*-test. *P* values < 0.01 were considered statistically significant. **(B)** Viral replication of HEV-B, C, and D in L-PSGL-1.1 cells. Replication of two HEV-B [CVB3-Nancy and echovirus 7 (E7)-Wallace] and two HEV-C strains [CVA21-Coe and poliovirus 1 (PV1)-Sabin 1], and one HEV-D (EV70-J670/71) strains in L-PSGL-1.1 cells in the presence or absence of KPL1 or an isotype control. The titers are expressed as the mean and error bars indicate SD of triplicate analyses. The mean viral titers were compared using Student's *t*-test. *P* values < 0.01 were considered statistically significant.

series of chimeric proteins between human and mouse SCARB2 identified that the amino acids 142–204 of human SCARB2 (encoded by human *SCARB2* exon 4) are responsible for EV71 binding and infection (Yamayoshi and Koike, 2011).

Mouse L929 cells expressing human SCARB2 in the presence of puromycin (L-SCARB2 cells) permitted the replication of all EV71 strains tested, including the non-PB strains (Yamayoshi et al., 2009). CVA16 induced CPE in L-SCARB2 cells, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, and CVA12 did not. CVA16 grew efficiently in L-SCARB2, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, and CVA12 did not (**Table 1**). Yamayoshi et al. (2009) concluded that CVA16 also infect L-SCARB2 cells in a SCARB2-dependent manner and that infection with most other HEV-A is not dependent upon SCARB2. CVA7, CVA10, and CVA14 induced CPE in both L-Empty cells and L-SCARB2 cells (Yamayoshi et al., 2009). They could not determine whether the CPE induced by these viruses were due to human SCARB2–mediated infection.

ANNEXIN II

Yang et al. (2011) identified annexin II as an EV71 VP1-binding protein on RD cells. Using a recombinant VP1 protein of EV71 fused with a calmodulin-binding peptide, they tried to identify VP1-binding proteins from the total cellular proteins of RD cells. A virus-overlay protein-binding assay followed by a mass spectrometry analysis identified annexin II as a VP1-binding protein.

Annexin II is a member of the annexin family – the multifunctional phospholipid-binding proteins. Annexin II on the surface of endothelial cells acts as a profibrinolytic coreceptor for both

Table 1 | Induction of CPE by the HEV-A strains.

Serotype	Strain	Accession No.	L-bsd[1]	L-PSGL-1.1	L-Empty[2,3]	L-SCARB2[3]
CVA2	Fleetwood	AY421760	–	–	–	–
CVA3	Olson	AY421761	–	–	–	–
CVA4	JR[4]	AB457644	–	–	–	–
CVA5	Swartz	AY421763	–	–	–	–
CVA6	Gdula	AY421764	–	–	–	–
CVA7	Parker	AY421765	–	+	+	+
CVA8	Donovan	AY421766	–	–	–	–
CVA10	Kowalik	AY421767	+	+	+	+
CVA12	Texas-12	AY421768	–	–	–	–
CVA14	G-14	AY421769	–	+	+	+
CVA16	G-10	U05876	–	+	–	+

[1]*Blasticidin-resistant L929 cells (a negative control for L-PSGL-1.1 cells).*
[2]*Puromycin-resistant L929 cells (a negative control for L-SCARB2 cells).*
[3]*Yamayoshi et al. (2009).*
[4]*Prototype CVA4 strain (high point) is unavailable from ATCC, therefore we used an in-house reference strain of CVA4, the JR strain.*

plasminogen and tissue plasminogen activator facilitating the generation of plasmin (Kim and Hajjar, 2002). The interaction to annexin II was specific to EV71; CVA16 did not bind to annexin II in the virus-overlay protein-binding assay (Yang et al., 2011).

SIALIC ACID

Sialic acid is usually found as terminal monosaccharides on the glycan chains of glycolipids and glycoproteins (Varki and Varki, 2007). Coxsackievirus A24 variant (CVA24v) uses SA-containing glycoconjugates as attachment receptors on corneal cells (Nilsson et al., 2008). Yang et al. (2009) hypothesized that SA would be important for EV71 infection, as the transmission route of EV71 and CVA24v is fecal-oral and/or droplet-aerosol route. EV71 infection to DLD-1 intestinal cells was inhibited by an *O*-glycan synthesis inhibitor, but not by an *N*-glycan synthesis inhibitor. Sialidase treatment decreased EV71 replication in DLD-1 cells. Furthermore, DLD-1 cells co-cultured with SA-linked galactose significantly reduced the EV71 infection. Thus Yang et al. (2009) concluded that SA-linked glycans are EV71 receptors on DLD-1 cells. Recently, Neu5Acα2,3Gal disaccharides on PSGL-1 were reported as a candidate receptor of CVA24v (Mistry et al., 2011). It is unknown whether other enteroviruses, including HEV-A, recognize SA-containing glycans as the entry receptors.

DENDRITIC CELL-SPECIFIC ICAM3-GRABBING NON-INTEGRIN

Dendritic cells play crucial roles in antiviral immunity by functioning as professional antigen-presenting cells to prime T cells and by secreting cytokines to modulate immune responses. In a mouse model of EV71 infection, DCs from the brains of EV71-infected, but not of uninfected, mice expressed viral antigen and primed T cells efficiently (Lin et al., 2009a). Lin et al. (2009b) reported that EV71 infection enhances mouse DCs to elicit protective immune response and also found that EV71 infects human immature DCs and that viral entry is partially inhibited by anti-DC-SIGN antibody. However, the direct interaction between EV71 and DC-SIGN is still unclear. It is essential to characterize the role of DC-SIGN and other receptors for EV71 in DCs for understanding the host immunological responses and immunopathogenesis of HEV-A including EV71.

CONCLUSION

Identification of PSGL-1 and SCARB2 as the cellular receptors for EV71 and CVA16 has advanced our understanding of the early stages of HEV-A infections at the molecular level. However, further experiments using clinical HEV-A isolates are necessary to clarify the general role of PSGL-1 and SCARB2 in HEV-A infection and their pathogenesis. Most of the prototype (laboratory-adapted) HEV-A strains other than EV71 and CVA16 may use unidentified receptor(s) to infect susceptible human cells such as RD cells. Characterization of the identified and unidentified HEV-A receptors is essential to understand the mechanism of HEV-A infection and development of a diverse array of the clinical outcomes of HEV-A-associated diseases.

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Human SCARB2-Dependent Infection by Coxsackievirus A7, A14, and A16 and Enterovirus 71

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Human enterovirus species A (HEV-A) consists of at least 16 members of different serotypes that are known to be the causative agents of hand, foot, and mouth disease (HFMD), herpangina, and other diseases, such as respiratory disease and polio-like flaccid paralysis. Enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) are the major causative agents of HFMD. CVA5, CVA6, CVA10, and CVA12 mainly cause herpangina or are occasionally involved with sporadic cases of HFMD. We have previously shown that human scavenger receptor class B, member 2 (SCARB2) is a cellular receptor for EV71 and CVA16. Using a large number of clinical isolates of HEV-A, we explored whether all clinical isolates of EV71 and other serotypes of HEV-A infected cells via SCARB2. We tested this possibility by infecting L-SCARB2 cells, which are L929 cells expressing human SCARB2, by infecting human RD cells that had been treated with small interfering RNAs for SCARB2 and by directly binding the viruses to a soluble SCARB2 protein. We showed that all 162 clinical isolates of EV71 propagated in L-SCARB2 cells, suggesting that SCARB2 is the critical receptor common to all EV71 strains. In addition, CVA7, CVA14, and CVA16, which are most closely related to each other, also utilized SCARB2 for infection. EV71, CVA14, and CVA16 are highly associated with HFMD, and EV71 and CVA7 are occasionally associated with neurological diseases, suggesting that SCARB2 plays important roles in the development of these diseases. In contrast, another group of viruses, such as CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, CVA10, and CVA12, which are relatively distant from the EV71 group, is associated mainly with herpangina. None of these clinical isolates infected via the SCARB2-dependent pathway. HEV-A viruses can be divided into at least two groups depending on the use of SCARB2, and the receptor usage plays an important role in developing the specific diseases for each group.

Human enteroviruses (HEVs) are one of the large families of human pathogens belonging to the *Picornaviridae*, and they can cause a variety of diseases, such as poliomyelitis, meningitis, acute flaccid paralysis, gastroenteritis, diarrhea, respiratory diseases, myocarditis, pancreatitis, hand, foot, and mouth disease (HFMD), and herpangina (40). HEVs are classified into species A (HEV-A) to species D (HEV-D) according to the similarity of the amino acid sequence of the capsid protein. HEV-A is composed of at least 16 members of different serotypes: coxsackievirus A2 (CVA2), CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, and CVA16 and enterovirus 71 (EV71), EV76, EV89, EV90, and EV91 (40). The prototype strain of EV71, strain BrCr, was isolated from a patient with neurological diseases between 1969 and 1972 (45). CVA7 prototype strain Parker was isolated in the United State in 1949 (10). CVA14 prototype strain G-14 and CVA16 prototype strain G-10 were isolated in the Republic of South Africa in 1950 and 1951, respectively (47). EV76 was isolated in France in 1991, and EV89, EV90, and EV91 were isolated in Bangladesh in 1999-2000 (35). Other prototype strains of HEV-A were isolated in the United States between 1947 and 1950 (10). These prototype strains were repeatedly subcultured using cultured cells or suckling mice. The members of HEV-A are increasing because of the isolation and characterization of new viruses (34, 36).

Members of HEV-A are known as causative agents of HFMD, herpangina, respiratory disease, meningitis, and polio-like flaccid paralysis (46). Phylogenetic analysis of HEV-A based on the capsid protein sequences revealed that these viruses were clustered into three major groups (35). Interestingly, there is an association between viral serotype and diseases. One of the groups consists of CVA7, CVA14, CVA16, and EV71 (called "the EV71 group" in this report). CVA16 and EV71 are the major causative agents of HFMD, which is characterized by fever and vesicular exanthema, mostly in the hands, feet, and oral mucosa (29). CVA7 and CVA14 infrequently cause sporadic cases of HFMD (1, 2, 41). Other HEV-As, such as CVA2, CVA4, CVA5, CVA6, and CVA10, which belong to another group ("the CVA2 group"), are mainly associated with herpangina in infants, which is also caused by coxsackie B viruses or echoviruses (9, 23, 28, 32, 50). It has been reported that CVA6 and CVA10 occasionally cause epidemic outbreaks of HFMD (6, 39). HFMD is usually mild, but neurological complications and even fatalities occur during HFMD outbreaks when the causative agent is EV71 (29). From 2008 to 2011, epidemic outbreaks of neurovirulent EV71 in China resulted in a total of approximately 3 million HFMD cases, including approximately

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1,500 fatal cases (http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohjbyfkzj/s2907/index.htm) (53). CVA7 is also neurotropic and can cause paralytic poliomyelitis (16, 17, 42). EV76, EV89, EV90, and EV91 were isolated from patients with gastroenteritis or polio-like acute flaccid paralysis and belong to the third group ("the EV76 group") (35). However, these viruses are not well characterized because there have been few reports and limited numbers of clinical cases.

Virus receptors play important roles in developing disease characteristics for each virus because they are the primary determinant of cell, tissue, and species tropism. Because the ability of the virus to bind to its receptor and the subsequent processes that lead to infection are determined by interactions between the viral proteins and the receptor, we thought that receptor usage in HEV-As might be closely related to the clinical symptoms caused by the viruses. So far, two molecules have been proposed as the receptors for EV71 and CVA16 but not for other viruses belonging to HEV-A. We previously reported that scavenger receptor class B, member 2 (SCARB2, also known as lysosomal integral membrane protein II or CD36b like-2) permits efficient infection of mouse L929 cells by EV71 (52). SCARB2 belongs to the CD36 family and has two transmembrane domains (13). Physiologically, it plays a role in the reorganization of the endosomal/lysosomal compartment (21) and works as the receptor for the mannose-6-phosphate-independent transport of β-glucocerebrosidase (β-GC) to the lysosome (5, 43). The expression of SCARB2 has been observed in monolayer culture cells of primate origin, which are susceptible to EV71 infection (52), and in almost all organs in humans (13). The SCARB2 region important for EV71 binding was mapped to amino acids 142 to 204 using a series of chimeric receptors of human and mouse SCARB2 (51). We established a cell line, L-SCARB2, that expresses human SCARB2 constitutively (52). It is possible to determine the SCARB2 dependency for infection by testing the infectivity of the viruses on L-SCARB2 cells and parental L929 cells. We tested the infectivities of 8 laboratory strains of EV71 in the L-SCARB2 cells. Infection of all of these strains of EV71 in L-SCARB2 cells but not in L929 cells was as efficient as that in the RD cells (52). P-selectin glycoprotein ligand 1 (PSGL-1, also known as selectin P ligand [SELPLG]), was identified as an EV71 receptor from human T cell leukemia Jurkat cells using the panning assay, which detected molecules having a strong binding affinity for EV71 particles (33). PSGL-1 is expressed primarily on leukocytes and is involved in leukocyte interactions with vascular endothelium (22). L929 cells expressing PSGL-1 were susceptible only to some EV71 strains (PSGL-1 binding strains) (33). Viral propagation and cytopathic effect (CPE) occurred much more slowly than in RD cells (33). However, it is not known whether SCARB2 can serve as a receptor for all EV71 strains. We therefore examined whether all EV71 strains infect via a SCARB2-dependent pathway. In addition, we found that the prototype strain of CVA16 also utilized SCARB2 (52). This result led us to the idea that SCARB2 is widely used as the receptor for the members of HEV-A that cause the same clinical symptoms. It was not possible, however, to determine whether prototype strains of CVA7, CVA10, and CVA14 infect cells via a SCARB2-dependent pathway using the above strategy because these three viruses propagated well in the parental L929 cells (52).

In this work, we hypothesized that SCARB2 is used as a common receptor for "the EV71 group" of HEV-As that mainly cause HFMD, and we conducted experiments to show the following: (i) that all clinical isolates of EV71 use SCARB2 as a receptor and (ii) that EV71, CVA7, CVA14, and CVA16, which are most closely related to each other and are highly associated with HFMD, also use SCARB2. Because the prototype strains of HEV-As have undergone a number of passages in cultured cells or suckling mice, they might have accumulated mutations that affect virus-receptor interaction. To overcome this potential problem, we collected a number of clinical isolates of HEV-As. We used L-SCARB2 cells to investigate SCARB2 dependency for HEV-A infection. In addition, we adopted the additional methods of small interfering RNA (siRNA) techniques to downregulate SCARB2 expression in RD cells to examine the SCARB2 dependency of infection and coimmunoprecipitation to examine direct virus-receptor binding.

MATERIALS AND METHODS

Ethics statement. All specimens were collected after the parents of the enrolled children had given oral or written informed consent. Demographic and clinical information was extracted from the patient record by the attending physician for each specimen. The anonymous samples and information were sent to Prefectural Institutes of Public Health and used for viral isolation. The use of isolated viral strains at Tokyo Metropolitan Institute of Medical Science was approved by the institutional committee for experiments of recombinant DNA and pathogens.

Cells. Human RD cells were cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 5% fetal bovine serum (FBS) and a penicillin-streptomycin solution (Invitrogen) (5% FBS-DMEM). L-Empty cells (52) and L-SCARB2 cells (52) were cultured in 5% FBS-DMEM supplemented with puromycin (4 $\mu g\ ml^{-1}$; Calbiochem).

Viruses. EV71 and CVs, which belong to HEV-A, are listed in Tables S1 and S2 in the supplemental material, with year, place of isolation, clinical diagnosis or symptoms, subgenogroup, and reference, if known. These strains were isolated between 1985 and 2010 at the Shimane Prefectural Institute of Public Health and Environmental Science, Aichi Prefectural Institute of Public Health, Yamagata Prefectural Institute of Public Health (30, 31), or Virus Research Center, Sendai Medical Center in Japan. Viruses were isolated from original clinical specimens using a variety of cell lines. The isolates were typed by a neutralization assay with virus-specific antisera or by sequences of the capsid region. Prototype viruses of CVA7 (strain Parker) and CVA14 (strain G14) were propagated in RD cells. EV71-GFP, which expresses green fluorescent protein (GFP) upon viral replication, was recovered from an infectious cDNA clone, pSVA-EV71-GFP (51, 52).

Virus inoculation. RD cells, L-Empty cells, and L-SCARB2 cells were infected with each virus listed in Tables S1 and S2 in the supplemental material. These cells were incubated at 37°C for a week to check for the appearance of a cytopathic effect (CPE).

Virus titration. Viral titers were determined by the microtitration method using RD cells or L-SCARB2 cells and are expressed as the 50% tissue culture infectious dose ($TCID_{50}$) according to the Reed-Muench method (44).

Sequence of EV71 VP1 for genotyping. Viral RNA was extracted from the infected cell culture supernatant using the QIAamp viral RNA minikit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed using the SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen) as follows: after 30 min of cDNA synthesis at 55°C and 2 min of denaturation at 94°C, samples were subjected to 40 cycles of amplification, consisting of 30 s at 94°C, 30 s at 42°C, and 1 min at 72°C, with a final additional extension step at 72°C for 5 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen) or a QIAquick gel extraction kit (Qiagen) and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730xl sequencer (Applied Biosystems). For PCR and sequencing analysis, primers 159 (sense, 5′-ACYATGAAAYTGTGCAAGG-3′, nucleotides 2387 to 2405 of EV71/SK-EV006/Malaysia/97; accession no. AB469182)

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TABLE 1 Oligonucleotide primers used for RT-PCR and sequencing

Primer	Sequence (5′–3′)[a]	Application(s)
EV146-600(+)	TTACCATATAGCTATTGGATTGG	PCR/SEQ[b]
EV146-2100(+)	ATGCTCATCGCCTACACCCCACC	PCR/SEQ
EV146-3400(-)	GCTGTCCTCCCATACAAGATTTGCCC	PCR/SEQ
EV146-4444(-)	GGTGTTTGCTCTTGAACTGC	PCR/SEQ
EV71-1050(+)	GGTTATGGTGAGTGGCCYTC	PCR/SEQ
EV71-1150(-)	CCCTTAGATGATTTYTCCCACA	PCR
EV71-1700(+)	TCTGAGTTTGCAGGTCTCAGRCAAGC	PCR/SEQ
EV71-1800(-)	GGGTGGRAGTTTGGTAGAATGGG	PCR
EV71-2100(+)	ATGCTCATAGCTTATACACCTCC	PCR
EV71-2200(-)	GTAATACCATGGATCAGCAACACTC ACTAC	PCR
EV71-2700(+)	GGAGAGATAGAYCTCCCTCTTGARGG	PCR
EV71-2800(-)	CGCATGTAGGTGAACAGCTCCAC	PCR/SEQ

[a] Y, C or T; R, A or G.
[b] SEQ, sequencing.

TABLE 2 Induction of CPE by EV71

Subgenogroup	No. of isolates	No. of isolates associated with CPE		
		RD cells	L-Empty cells	L-SCARB2 cells
B2	2	2	0	2
B4	18	18	0	18
B5	19	19	0	19
C1	12	12	0	12
C2	38	38	0	38
C4	73	73	0	73
Total	162	162	0	162

(7), 161 (sense, 5′-CTGGGACATAGAYATAACWGG-3′, nucleotides 2764 to 2784) (7), 162 (antisense, 5′-CCRGTAGGKGTRCACGCRAC-3′, nucleotides 2871 to 2852) (7), 16R-Y (antisense, 5′-GARAAACTGACTG GRTAGTG-3′, nucleotides 3561 to 3542), 159-190 (sense, 5′-AGCAACA CTCACTACAGAGC-3′, nucleotides 2231 to 2250), and 162-2 (antisense, 5′-CCGGTGGGCGTRCATGCAAC-3′, nucleotides 2871 to 2852) were used. The subgenogroup was determined by a phylogenetic tree constructed by the neighbor-joining method using the GENETYX software program, version 9.0.1 (Genetyx).

Sequence of the P1 region. Viral RNA was extracted from the infected cell culture supernatant using a QIAamp viral RNA minikit. The viral RNA was then transcribed into cDNA with ReverTra Ace (Toyobo) and a random primer. By using cDNA as a template, the P1 region was amplified by PCR using PfuUltra High Fidelity DNA polymerase (Agilent Technologies) or PfuUltra II Fusion HS DNA polymerase (Agilent Technologies). The PCR products were purified with a QIAquick PCR purification kit or a QIAquick gel extraction kit and then sequenced as described above. The sequence data were analyzed by GENETYX software. The primers used for PCR and sequencing analysis are listed in Table 1.

Phylogenetic tree based on P1 region of HEV-A. The phylogenetic tree of prototype strains of HEV-A was constructed using the neighbor-joining method with the GENETYX software program. The amino acid sequences of the P1 region of CVA2 (strain Freetwood; accession no. AY421760), CVA3 (Olson; AY421761), CVA4 (High Point; AY421762), CVA5 (Swartz; AY421763), CVA6 (Gdula; AY421764), CVA7 (Parker; AY421765), CVA8 (Donovan; AY421766), CVA10 (Kowalik; AY421767), CVA12 (Texas12; AY421768), CVA14 (G-14; AY421769), CVA16 (G-10; U05876), EV71 (BrCr; U22521), EV76 (10226; AY697458), EV89 (10359; AY697459), EV90 (10399; AY697460), EV91 (10406; AY697461), PV (Mahony; V01149) and EV70 (J670/71; EV70CG) were obtained from GenBank.

Coimmunoprecipitation assay. A coimmunoprecipitation assay was performed as described previously (51). Briefly, the indicated viruses were incubated with control Fc (Fc portion of human IgG) (3 μg; R&D systems) or human SCARB2-Fc (3 μg; R&D systems) and anti-human IgG (Fc specific)-agarose (Sigma) in 1 ml of 5% FBS-DMEM for 2 h at 4°C. The beads were then washed twice with 5% FBS-DMEM, suspended in SDS sample buffer, and incubated for 10 min at 95°C. After the beads were removed, the samples were loaded on 12% Mini-PROTEAN TGX precast gels (Bio-Rad), followed by Western blotting with the monoclonal antibody against VP2 (clone 422-8D-4C-4D; Millipore), a rabbit anti-CVA6 serum, a rabbit anti-CVA10 serum (27), or the anti-human IgG Fc_γ fragment-specific antibody (Jackson ImmunoResearch).

RNAi. RNA interference (RNAi) was performed using the following Accell siRNA sequences targeting the human SCARB2 coding region, GCAAU AUGAUUAAUGGAAC (#13), GUAUCGAGAAGAAAAUUGU (#14), CCCUUAUCCAUGUUUUCAG (#15), or UGGGUGUGUUCUUUG GUUU (#16) (Dharmacon), or using a control nontargeting siRNA (Dharmacon) according to the manufacturer's recommendations. Briefly, RD cells were treated with 1 μM siRNA in Accell siRNA delivery medium (Dharmacon) for 48 h. In some cases, RD cells were transfected with an empty vector or pCA-M(H4)-F (51) before siRNA treatment. These cells were harvested for Western blot analysis with a goat anti-SCARB2 antibody (R&D systems), a rabbit anti-FLAG antibody (Sigma), or a mouse anti-β-actin (ACTB) antibody (clone AC-74; Sigma) as an internal control or infected with the indicated viruses and incubated for another 24 h at 37°C. The cells infected with EV71-GFP were imaged with an IX70 microscope with a DP70 camera (Olympus) and analyzed using DP controller software (OLYMPUS). The cells infected with other viruses were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and strained with the monoclonal antibody against VP2, the rabbit anti-CVA6 serum, or the rabbit anti-CVA10 serum, followed by incubation with an Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) or an Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen). Images were acquired using the IX70 microscope. In some cases, viral titers in the cell culture were measured with RD cells.

RESULTS

All clinical isolates of EV71 induced CPE in L-SCARB2 cells. Eight laboratory strains of EV71, including the prototype strain BrCr, infected cells via a SCARB2-dependent pathway as described previously (52). To determine whether all EV71 isolates use SCARB2 for infection, we collected 162 clinical isolates of EV71 isolated between 1990 and 2010 in the Yamagata (30, 31), Miyagi, Aichi, and Shimane prefectures of Japan and identified their subgenogroups by their VP1 sequences (see Table S1 in the supplemental material). A total of 2, 18, 19, 12, 37 and 73 isolates were identified as subgenogroups B2, B4, B5, C1, C2, and C4, respectively. To confirm whether these isolates utilize SCARB2 for infection, each isolate was inoculated into L-SCARB2 cells, RD cells as a positive control, and L-Empty cells as a negative control. As a result of inoculation, all 162 isolates, irrespective of the subgenogroup, induced CPE in RD cells and L-SCARB2 cells but not in L-Empty cells (Table 2). However, a few isolates (approximately 10%) showed a low propagation efficiency in L-SCARB2 cells. To exclude the possibility that these viruses failed to use SCARB2 properly, we selected three strains as representative isolates from the isolates with a low growth phenotype (786-Yamagata [subgenogroup B4], 983-Yamagata [C2], and 75-Yamagata [C4]) and from the isolates with a high-growth phenotype (962-Yamagata [B4], 1002-Yamagata [C2], and 452-Yamagata [C4]). We compared the viral propagation in L-SCARB2 cells with that in RD cells by determining the viral titers of these 6 isolates using the microtitration method (Fig. 1A). The viral titers of the strains

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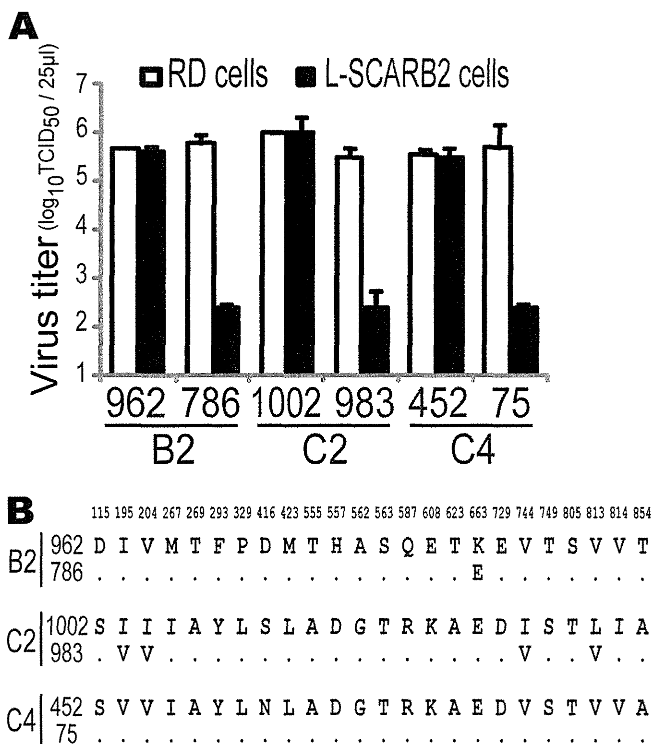

FIG 1 Comparisons of EV71 clinical isolates. (A) Viral titers of EV71 clinical isolates in RD cells and L-SCARB2 cells. Viral titers of clinical isolates of the EV71 strains 962-Yamagata (962), 786-Yamagata (786), 1002-Yamagata (1002), 983-Yamagata (983), 452-Yamagata (452), and 75-Yamagata (75) were determined with RD cells and L-SCARB2 cells. The data are shown as mean viral titers with SD ($n = 3$). (B) Comparison of amino acid sequences of the P1 region. Amino acid sequences of EV71 strains 962-Yamagata (subgenogroup B4), 1002-Yamagata (C2), and 452-Yamagata (C4) (upper lines) were compared with those of strains 786-Yamagata (B4), 983-Yamagata (C2), and 75-Yamagata (C4) (lower lines). Only amino acids divergent among the 6 isolates are shown.

962-Yamagata, 1002-Yamagata, and 452-Yamagata in L-SCARB2 cells were similar to those in RD cells, whereas the viral titers of the strains 786-Yamagata, 983-Yamagata, and 75-Yamagata in L-SCARB2 cells were approximately 1,000-fold lower than those in RD cells. We found a very small number of amino acid substitutions in the capsid region of these three viruses with the low-growth phenotype compared with those of the high-growth phenotype (Fig. 1B). Notably, there were no differences in the capsid region between strains 452-Yamagata and 75-Yamagata. We speculated that isolates of both growth phenotypes utilized human SCARB2 with similar efficiencies and that poor replication efficiency in the L-SCARB2 cells was due to the incompatibility with some host factors in mouse cells.

Evaluation of SCARB2-dependent infection of EV71 in RD cells. To confirm that these isolates were able to infect to human RD cells using SCARB2, we employed RNA interference (RNAi) technology to downregulate SCARB2 expression. First, using a recombinant EV71 strain, EV71-GFP, which was already characterized as a SCARB2-dependent virus (51, 52), we conducted an experiment to determine which of 4 kinds of small interfering RNAs (siRNAs) were able to downregulate SCARB2 expression and whether the downregulation of SCARB2 really inhibited EV71 infection. RD cells were treated with nontargeting siRNA or siRNAs against human SCARB2 (siSCARB2 #13, #14, #15, or #16) for 48 h, and the SCARB2 expression of these cells was evaluated by Western blotting (Fig. 2A). SCARB2 expression in RD cells treated with siSCARB2 #14 or #16 was appreciably or intermediately downregulated compared with that in mock-treated cells, evaluated by Western blotting. The RD cells were then infected with EV71-GFP, which expressed GFP upon infection, and imaged at 24 h postinfection (Fig. 2B). Compared with the mock treatment, the numbers of GFP-positive cells in the cell culture that had been treated with siSCARB2 #14 or #16 were greatly or slightly reduced, respectively, whereas those in the cell culture treated with nontargeting siRNA or siSCARB2 #13 or #15 were not affected. To quantify the microscopic observations, these cells (a total of 10,000 cells) were analyzed by fluorescence-activated cell sorting (FACS) to count the number of GFP-positive cells (Fig. 2C). There was no remarkable difference in the number of GFP-positive cells between the mock-treated cells (6,841 $\pm$ 17) and the cells treated with nontargeting siRNA (6,449 $\pm$ 26). The numbers of GFP-positive cells in siSCARB2 #13-, #14-, #15-, and #16-treated cells were 3,970 $\pm$ 15, 171 $\pm$ 7.0, 6,090 $\pm$ 68, and 2,453 $\pm$ 7.0, respectively (Fig. 2C). These results showed that siSCARB2 #14 and #16 were effective both for the downregulation of SCARB2 expression and for the inhibition of EV71-GFP infection. The results also indicated that the efficiency of EV71-GFP infection correlated with the expression level of SCARB2. We confirmed that these siSCARB2 siRNAs did not affect poliovirus infection of RD cells (data not shown).

To confirm the specificity of the inhibition of EV71 infection that was putatively achieved by the repression of SCARB2 expression, RD cells were transfected with either the empty plasmid or a plasmid encoding a human-mouse chimeric SCARB2, M(H4)-F (51), before treatment with siSCARB2 #14. M(H4)-F is a chimeric mouse Scarb2 wherein amino acids 142 to 204, the EV71 binding site, have been replaced with the corresponding sequence of human SCARB2. The chimera functions as an EV71 receptor to virtually the same extent as human SCARB2 and was able to escape downregulation by siSCARB2 #14 because siSCARB2 #14 did not match the mouse Scarb2 sequence perfectly. Endogenous SCARB2 expression repressed by siSCARB2 #14 treatment was restored by the exogenous expression of M(H4)-F but not by transfection with the empty plasmid (Fig. 2D). Similarly, the number of GFP-positive cells, reduced by treatment with siSCARB2 #14, was markedly restored by M(H4)-F expression (Fig. 2E). These results indicate that it is possible to evaluate SCARB2-dependent infection of RD cells using RNAi with siSCARB2 #14 and #16.

SCARB2-dependent infection of EV71 clinical isolates in RD cells. We tested the SCARB2-dependent infection of representative clinical isolates of EV71 with the siRNAs. RD cells treated with siSCARB2 #14 or #16 were infected with the 3 clinical isolates of EV71 shown in Fig. 1 that had a high- or low-growth phenotype in L-SCARB2 cells, fixed at 24 h postinfection, and then stained with an anti-VP2 monoclonal antibody to detect the EV71-infected cells (Fig. 3). Upon infection with 3 isolates of the high-growth phenotype in L-SCARB2 cells, VP2-positive cells in RD cell culture treated with siSCARB2 #14 or #16 were markedly reduced compared with those in mock-treated or nontargeting siRNA-treated cell culture. Similarly, when 3 isolates with the low-growth phenotype in L-SCARB2 cells were used, the number of VP2-positive cells in the RD cell culture treated with siSCARB2 #14 or

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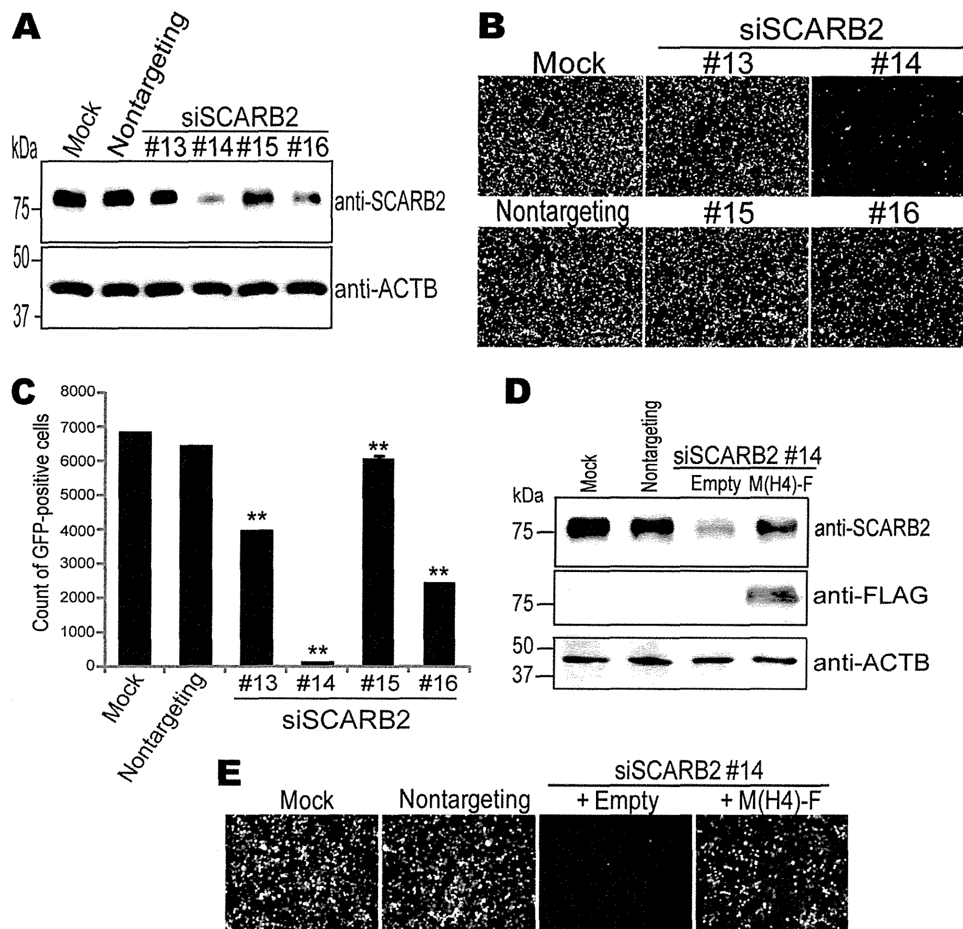

FIG 2 Downregulation of SCARB2 expression inhibited EV71-GFP infection. (A) Cell lysates were prepared from RD cells mock treated or treated with nontargeting siRNA or siSCARB2 #13, #14, #15, or #16 and were analyzed by Western blotting using an anti-SCARB2 antibody or an anti-ACTB (β-actin) antibody. (B and C) The siRNA-treated RD cells were infected with EV71-GFP and imaged via fluorescence microscopy at 24 h postinfection (B) and then analyzed by FACS to quantify the number of GFP-positive cells (C). A total of 10,000 cells were analyzed by FACS, and data are shown as mean counts with SD ($n = 3$). (D) SCARB2 expression was restored by exogenous M(H4)-F expression. Cell lysates were prepared from RD cells mock treated or treated with nontargeting siRNA or siSCARB2 #14, which were transfected with either empty vector or pCA-M(H4)-F and analyzed by Western blotting using an anti-SCARB2 antibody, an anti-FLAG antibody, or an anti-ACTB antibody. (E) M(H4)-F expression rescued the EV71-GFP infection that was inhibited by treatment with siSCARB2 #14. The siRNA-treated RD cells with/without plasmid transfection were infected with EV71-GFP. After 24 h, the cells were imaged via fluorescence microscopy.

#16 was reduced. The reduced infection of each clinical isolate with the high- or low-growth phenotype was restored by exogenous M(H4)-F expression (data not shown). These results show that at least 3 EV71 isolates that exhibited the low-growth phenotype in L-SCARB2 cells infect RD cells via the SCARB2-dependent pathway. The results suggest that all clinical isolates of EV71 are able to infect RD cells via a SCARB2-dependent pathway.

Binding of clinical isolates of EV71 to SCARB2. We then directly examined the binding of the 3 clinical isolates of EV71 with the high- or low-growth phenotype in L-SCARB2 cells to a soluble SCARB2 protein by using a coimmunoprecipitation assay. Briefly, each clinical isolate of EV71 used for Fig. 1 was mixed with 3 μg of control Fc or 3 μg of SCARB2-Fc together with anti-Fc-Agarose beads, and precipitated proteins were analyzed by Western blotting using the anti-VP2 or an anti-Fc antibody (Fig. 4). Similar amounts of control Fc and SCARB2-Fc were precipitated by anti-Fc-agarose beads (Fig. 4, lower panels). As expected, similar amounts of VP2 from each clinical isolate were detected in the SCARB2-Fc lane, and VP2 was not detected in the control Fc lane (Fig. 4, upper panels). These results indicate that all 6 clinical isolates of both growth phenotypes in L-SCARB2 cells bind to SCARB2 with similar efficiencies. Together, the data suggested that all clinical isolates of EV71 tested in this study are able to use SCARB2 as a receptor.

Correlation between viral serotypes and clinical symptoms. Next, to determine whether other members of HEV-As use SCARB2 as a receptor, we collected 9 to 22 clinical isolates of CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, CVA10, CVA12, CVA14, and 37 clinical isolates of CVA16 isolated between 1985 and 2010 in the Yamagata, Aichi, and Shimane prefectures of Japan (see Table S2 in the supplemental material). These viruses were isolated from patients with diseases, including HFMD, herpangina, acute upper respiratory infection, pharyngitis, and tonsillitis. We were unable to obtain any clinical isolates of CVA7, EV76, EV89, EV90, and EV91. The diseases caused by the clinical isolates belonging to each serotype are listed in Table S2 in the

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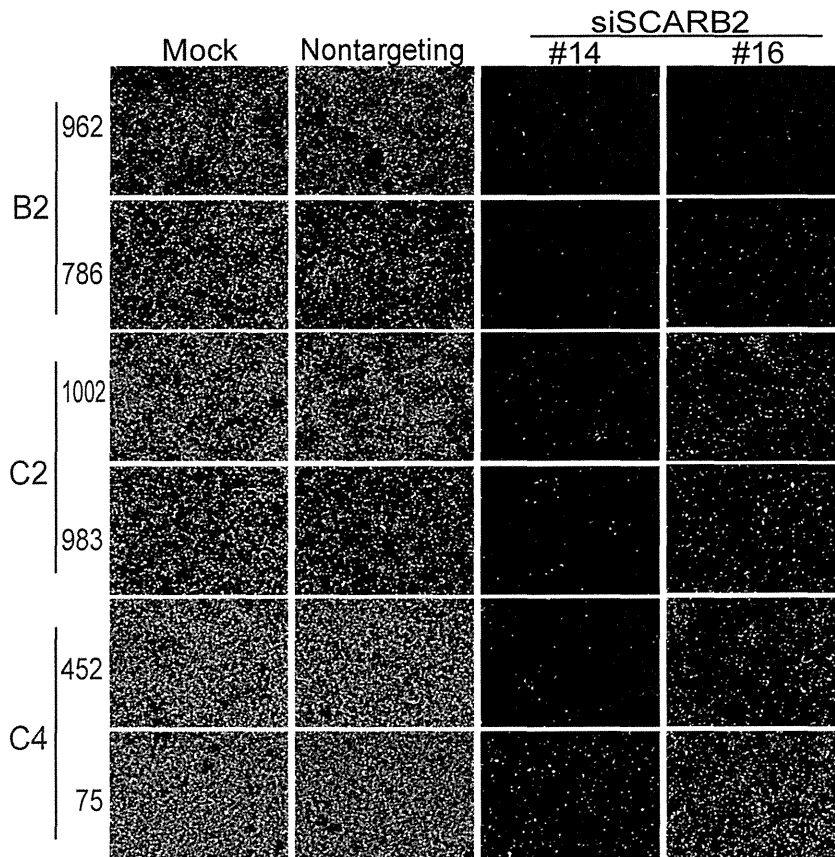

FIG 3 SCARB2-dependent infection of RD cells by EV71. siRNA-treated RD cells were infected with EV71 strains 962-Yamagata, 786-Yamagata, 1002-Yamagata, 983-Yamagata, 452-Yamagata, and 75-Yamagata. After 24 h, the cells were fixed and stained with monoclonal antibody against VP2.

supplemental material and summarized in Fig. 5 together with the phylogenetic tree of prototype strains of HEV-A based on the amino acid sequences of capsid proteins (37). The viruses were classified into three clusters in the phylogenetic tree: the EV71 group included CVA7, CVA14, CVA16, and EV71; the CVA2 group included CVA5, CVA12, CVA2, CVA4, CVA10, CVA6, CVA3, and CVA8; and the EV76 group included EV76, EV89, EV90, and EV91. In general, most enterovirus infections are

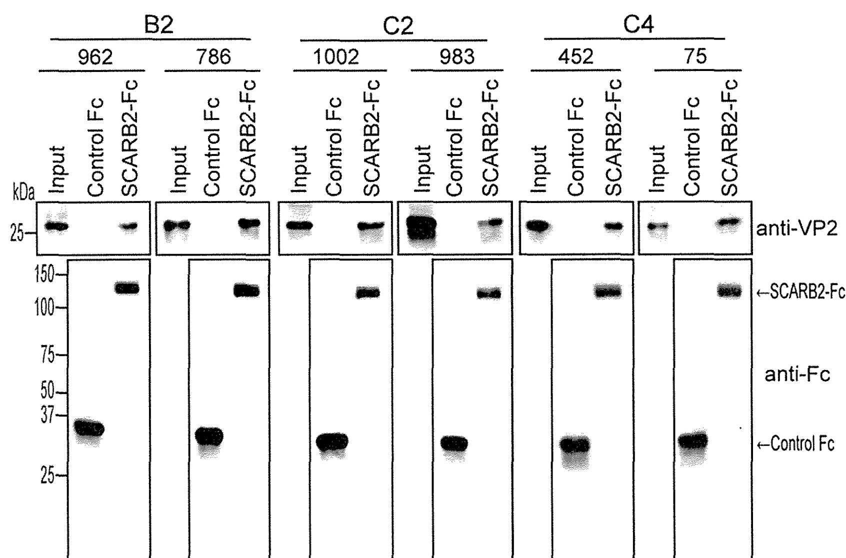

FIG 4 Binding of clinical isolates of EV71 to SCARB2-Fc. The EV71 strains 962-Yamagata, 786-Yamagata, 1002-Yamagata, 983-Yamagata, 452-Yamagata, and 75-Yamagata were incubated with control Fc (3 μg) or SCARB2-Fc (3 μg) bound to the anti-human Fc-agarose. Precipitated proteins were analyzed by Western blotting with monoclonal antibody against VP2 and anti-Fc antibody.

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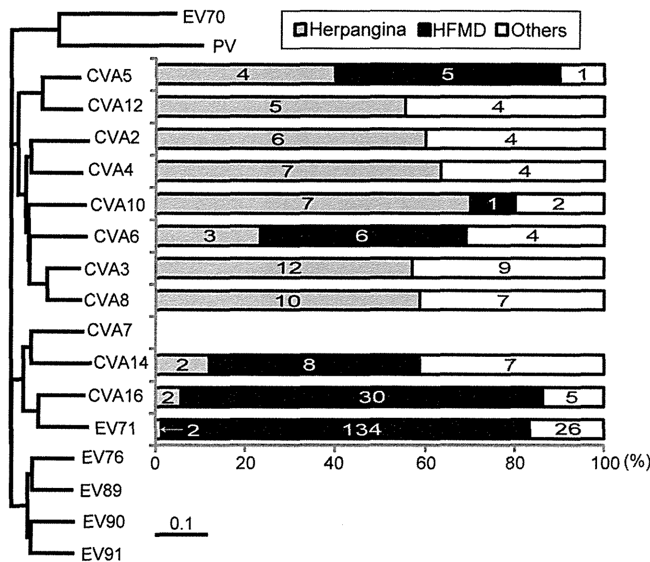

FIG 5 Association of viral serotypes and clinical symptoms. The phylogenetic tree was drawn based on the amino acid sequences of the capsid regions of the HEV-A prototype strains. PV and EV70 are defined as outgroups. The numbers of clinical isolates from patients with indicated diseases are based on data in Table S2 in the supplemental material.

asymptomatic, although the same receptors are presumably used in asymptomatic and symptomatic cases. In addition, many enteroviruses use different receptors but cause somewhat similar symptoms. However, in this case, we found an association between viral serotypes and clinical symptoms. The viruses in the EV71 group were highly associated with HFMD. They were isolated from patients with HFMD (79.3%) or herpangina (2.8%). In contrast, there is a tendency for the viruses in the CVA2 group to be associated with herpangina. More than 50% of CVA12, CVA2, CVA4, CVA3, and CVA8 isolates were obtained from patients with herpangina, and none were obtained from HFMD patients. CVA5, CVA6, and CVA10 were isolated mainly from herpangina patients, but some were from HFMD patients; CVA5 was isolated from patients with herpangina (4/10), HFMD (5/10), and other diseases (1/10); CVA6 was isolated from patients with herpangina (3/13), HFMD (6/13), and other diseases (4/13); and CVA10 was isolated from patients with herpangina (7/10), HFMD (1/10), and other diseases (2/10). The viruses in the CVA2 group were isolated from patients with herpangina (53.5%) and HFMD (11.9%). The relationships between the serotypes and the clinical symptoms or disease observed in our collected samples were similar to what has been previously reported elsewhere. Because we could not obtain any viruses belonging to the EV76 group, we focused on the EV71 group and the CVA2 group.

All clinical isolates of CVA14 and CVA16 propagate in L-SCARB2 cells. To confirm whether each isolate of the 156 HEV-A viruses listed in Table S2 in the supplemental material utilizes SCARB2 for infection, isolates were inoculated into L-SCARB2 cells, RD cells, and L-Empty cells. As a result of the inoculations, all 17 and 37 isolates of CVA14 and CVA16, respectively, induced CPE in both RD cells and L-SCARB2 cells but not in L-Empty cells (Table 3). All clinical isolates of CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, CVA10, and CVA12 produced CPE in RD cells but not in L-SCARB2 cells and L-Empty cells (Table 3). These results clearly indicated that all tested clinical isolates of CVA14 and CVA16 showed SCARB2-dependent infection, whereas the clinical isolates of CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, CVA10, and CVA12 did not. It is likely that HEV-A can be clustered at least into two groups depending on the use of SCARB2.

SCARB2-dependent infection of CVA7, CVA14, and CVA16 in RD cells. All clinical isolates of CVA14, CVA16, and EV71 showed SCARB2-dependent infection (Table 3). The prototype strains of CVA7, CVA14, CVA16, and EV71 are monophyletic based on the analysis of the amino acid sequences of capsid proteins (Fig. 5). We therefore hypothesized that CVA7 also utilizes SCARB2 as a receptor for infection and added the CVA7 prototype strain Parker to further analyses. We then evaluated the dependency on SCARB2 for infection of RD cells using siRNA techniques. RD cells treated with siSCARB2 #14 or #16 were infected with EV71 strains 452-Yamagata and 75-Yamagata as positive controls and CVA16 strain 2437-Yamagata, CVA14 strain 0006-Yamagata, CVA7 strain Parker, CVA6 strain 1547-Yamagata, or CVA10 strain 1788-Yamagata as a negative control at a multiplicity of infection (MOI) of 0.1 (Fig. 6A). Consistent with the results in Table 3, the viral titers of EV71s, CVA16, and CVA14 in siSCARB2 #14- or #16-treated cells were approximately 10- or 100-fold lower than those in mock or nontargeting siRNA-treated cells. The viral titer of CVA7 in RD cells treated with siSCARB2 #14 was significantly decreased, but the decrease was not evident in siSCARB2 #16-treated cells. siSCARB2 #14 and #16 did not affect the propagation of CVA6 and CVA10 in RD cells. These results showed that CVA14 and CVA16 utilize SCARB2 for infection, whereas CVA6 and CVA10 do not. The SCARB2 dependency of CVA7 was not apparent under this condition.

For further analysis of SCARB2 dependency for infection, we infected RD cells pretreated with siSCARB2s with the CVA7 strain Parker, the CVA14 strains G-14, 0006-Yamagata, and 0610-Yamagata, the CVA16 strains 1872-Yamagata, 1122-Yamagata, and 2437-Yamagata, the CVA6 strain 1547-Yamagata, and the CVA10 strain 1788-Yamagata and identified the infected cells with anti-VP2 antibody, anti-CVA6 antibody, or anti-CVA10 antibody (Fig. 6B). In CVA7-infected cells, the number of VP2-positive cells in RD cells treated with siSCARB2 #14 or #16 was obviously reduced compared with those of mock- or nontargeting siRNA-treated cells. The SCARB2 dependency of CVA7 infection was evident under this condition. The inhibition of viral infection by siSCARB2 treatment was similarly observed for each of the 3

TABLE 3 Induction of CPE by coxsackieviruses

Virus	No. of isolates	No. of isolates associated with CPE		
		RD cells	L-Empty cells	L-SCARB2 cells
CVA2	10	10	0	0
CVA3	22	22	0	0
CVA4	11	11	0	0
CVA5	10	10	0	0
CVA6	13	13	0	0
CVA8	17	17	0	0
CVA10	10	10	0	0
CVA12	9	9	0	0
CVA14	17	17	0	17
CVA16	37	37	0	37

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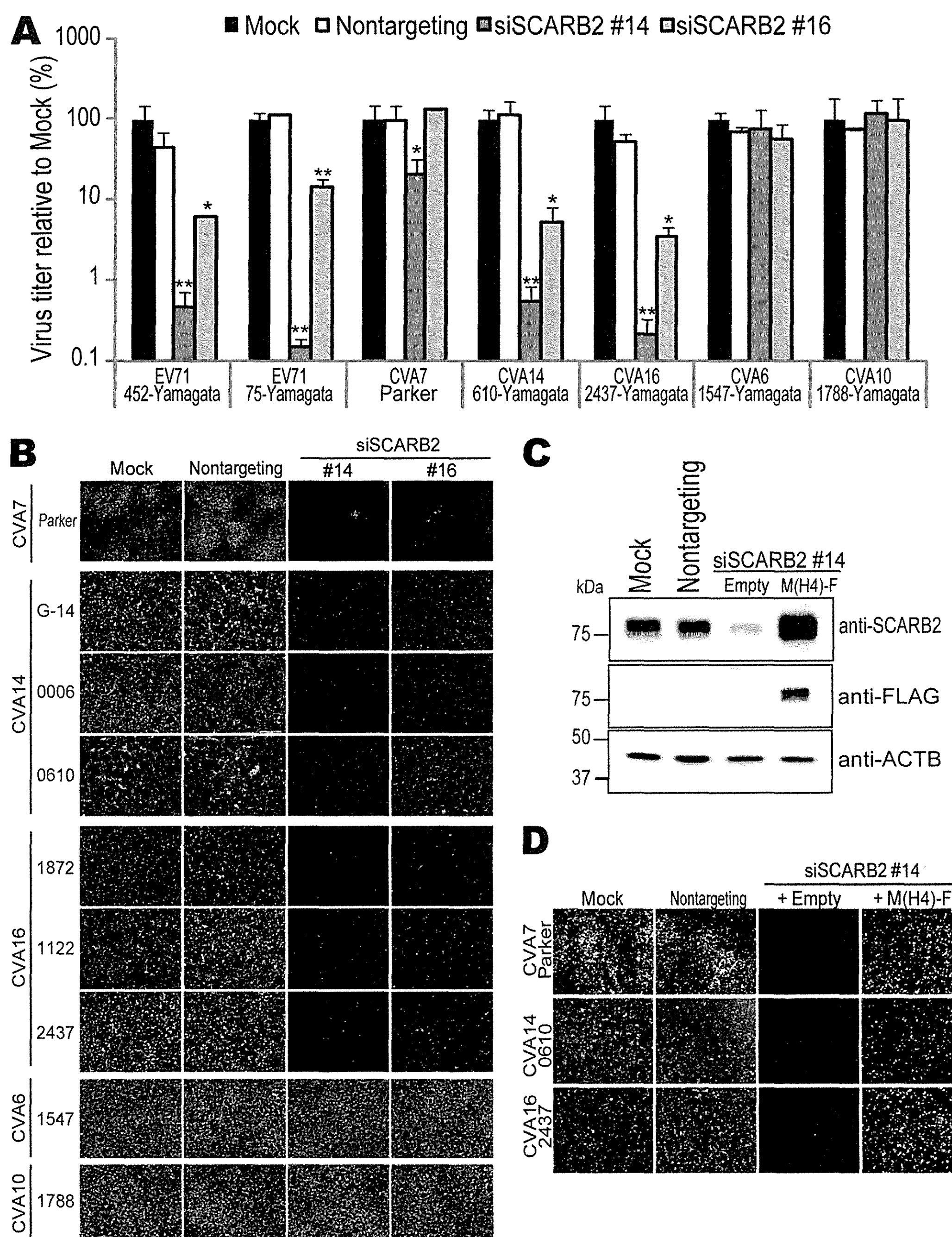

FIG 6 SCARB2-dependent infection of CVA7, CVA14, and CVA16. (A) Infection of CVA16 and CVA14 was inhibited by repression of SCARB2 expression. siRNA-treated RD cells were infected with the indicated viruses, and the viral titers were determined at 24 h postinfection. The data are shown as mean relative viral titers with SD ($n = 2$). Statistical significance was determined by Student's t test. (*, $P < 0.05$; **, $P < 0.01$) (B) Infection by CVA7, CVA14, and CVA16 was inhibited by downregulation of SCARB2. The siRNA-treated RD cells were infected with the CVA7 strain Parker, the CVA14 strains G-14, 0006-Yamagata, and 0610-Yamagata, the CVA16 strains 1872-Yamagata, 1122-Yamagata, and 2437-Yamagata, the CVA6 strain 1547-Yamagata, or the CVA10 strain 1788-Yamagata. After 24 h, the cells were fixed and stained with monoclonal antibody against VP2, an anti-CVA6 serum, or an anti-CVA10 serum. (C) Rescue of SCARB2 expression by exogenous M(H4)-F expression. Cell lysates were prepared from RD cells mock treated or treated with nontargeting siRNA or siSCARB2 #14, which were transfected with either empty vector or pCA-M(H4)-F and analyzed by Western blotting using anti-SCARB2 antibody, anti-FLAG antibody, or the -ACTB antibody. (D) M(H4)-F expression rescued the inhibition of CVA7, CVA14, and CVA16 infection. siRNA-treated RD cells with/without plasmid transfection were infected with CVA7 strain Parker, CVA14 strain 0610-Yamagata, and CVA16 strain 2437-Yamagata. After 24 h, the cells were fixed and stained with monoclonal antibody against VP2.

isolates of CVA14 and CVA16, whereas infection by CVA6 and CVA10 was not affected by siSCARB2 treatment.

To evaluate the specificity of siSCARB2, we transfected RD cells with pCA-M(H4)-F or the empty plasmid before treatment with siSCARB2 #14. The repression of endogenous SCARB2 expression by siSCARB2 #14 treatment was restored by exogenous expression of M(H4)-F but not by transfection of the empty plasmid (Fig. 6C). After infection with CVA7 strain Parker, CVA14

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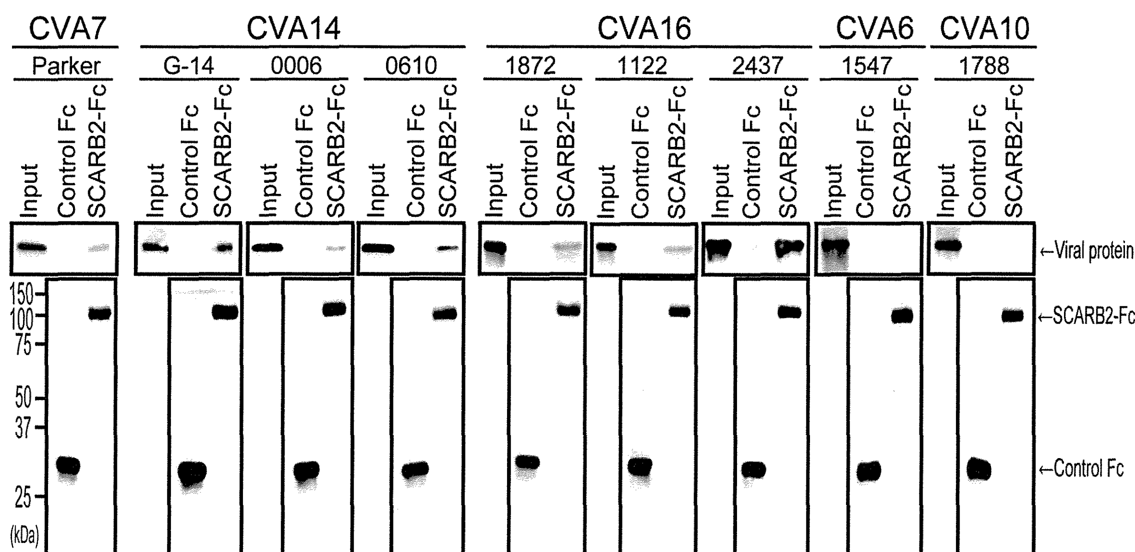

FIG 7 Binding of CVA7, CVA14, and CVA16 to SCARB2-Fc. CVA7 strain Parker, CVA14 strains G-14, 0006-Yamagata, and 0610-Yamagata, CVA16 strains 1872-Yamagata, 1122-Yamagata, and 2437-Yamagata, CVA6 strain 1547-Yamagata, and CVA10 strain 1788-Yamagata were incubated with control Fc (3 μg) or SCARB2-Fc (3 μg) bound to anti-human Fc-agarose. Bound viruses were analyzed by Western blotting with monoclonal antibody against VP2, anti-CVA6 serum, or anti-CVA10 serum.

strain 0610-Yamagata, or CVA16 strain 2437-Yamagata, a number of VP2-positive cells were observed (Fig. 6D). We found that the number of VP2-positive cells in the cells transfected with pCA-M(H4)-F was increased compared with that of the cells transfected with empty plasmid. These data showed that the reduction of the number of VP2-positive cells by siSCARB2 treatment is specifically caused by downregulation of SCARB2 expression, indicating that CVA7, CVA14, and CVA16 utilize SCARB2 for infection of RD cells.

Binding of CVA7, CVA14, and CVA16 to SCARB2. To confirm that CVA7, CVA14, and CVA16 bound directly to SCARB2, we conducted the coimmunoprecipitation assays shown in Fig. 4 using the isolates of CVA7, CVA14, CVA16, CVA6, and CVA10 used in Fig. 6B (Fig. 7). Similar amounts of control Fc and SCARB2-Fc were precipitated by anti-Fc-agarose beads (Fig. 7, lower panels). VP2 from CVA7, CVA14, or CVA16 was detected in each SCARB2-Fc lane (Fig. 7, upper panels), although the amounts of precipitated VP2 varied among the viruses. Viral protein of CVA6 or CVA10 was not detected in either the control Fc or SCARB2-Fc lanes. These results indicate that CVA7, CVA14, and CVA16 bind to SCARB2 specifically but that the binding efficiencies may not be equal among the virus strains.

DISCUSSION

In this study, we examined the relationship between the use of SCARB2 as the receptor and differences in diseases caused by the viruses. First, we investigated whether all EV71 clinical isolates used SCARB2 as a receptor. EV71 was genetically classified into 3 genogroups based on the VP1 nucleotide sequence: genogroups A, B, and C (7). Genogroup A contains a single member, prototype stain BrCr, and genogroups B and C are further subdivided into clusters: subgenogroups B1, B2, B3, B4, B5, C1, C2, C3, C4, and C5 (7). We collected a total of 162 clinical isolates of EV71 classified into subgenogroups B2, B4, B5, C1, C2, and C4. Viruses belonging to B1, B3, C3, and C5 were not included in this collection. We have shown that all 162 isolates of EV71 infected cells via a SCARB2-dependent pathway by testing the infectivity in L-SCARB2 cells. A few isolates (approximately 10%) propagated in L-SCARB2 cells less efficiently than other isolates. However, additional experiments of SCARB2 depletion in RD cells and a coimmunoprecipitation assay clearly showed that these isolates infected via a SCARB2-dependent pathway. These isolates with a low-growth phenotype in L-SCARB2 cells may have some incompatibility in the interaction of viral proteins/genome with the host factors (24–26, 48, 49) in mouse cells but not in the interaction with SCARB2. We have previously reported that EV71 strains BrCr/USA/70 (genogroup A), Nagoya/Japan/73 (subgenogroup B1), 258/Bulgaria/75 (subgenogroup B1), Hungary/78 (subgenogroup B1), and SK-EV006/Malaysia/97 (subgenogroup B3) infect cells via a SCARB2-dependent pathway (52). Although we have not tested the viruses belonging to subgenogroups C3 and C5, the data strongly suggested that EV71 universally utilizes SCARB2 as the receptor. On the contrary, PSGL-1 mediates infection of a subset of EV71 strains and is expressed primarily on leukocytes (22, 33). It is therefore unlikely that PSGL-1 is the receptor that plays a critical role in causing HFMD, but it may contribute to modulating EV71 pathogenicity for PSGL-1 binding strains under some circumstances. SCARB2 is widely expressed *in vivo* (13) and could be directly involved in systemic infection. Taken together, these results suggested that SCARB2 is the most probable candidate for the primary receptor and that it plays a critical role in EV71 infections.

Second, we also investigated whether other members of HEV-A used SCARB2 as a receptor. The results indicated that HEV-A viruses are divided into at least two groups: viruses whose infection is dependent on SCARB2 and viruses not dependent on SCARB2. The phylogenetic tree based on the amino acid sequences of capsid proteins revealed that HEV-A viruses diverged into three clusters, as described previously (37). We have explored the receptor usage of two of three groups. We

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showed that viruses in the EV71 group infect cells via a SCARB2-dependent pathway and viruses in the CVA2 group infect cells via a SCARB2-independent pathway. Each cluster roughly correlated with clinical outcomes; viruses in the EV71 group were mainly isolated from patients with HFMD, whereas viruses in the CVA2 group were generally isolated from patients with herpangina. Thus, the SCARB2-dependent viruses tend to cause HFMD, and the SCARB2-independent viruses tend to cause herpangina. There are some exceptions, however: CVA5, CVA6, and CVA10 have occasionally been isolated from patients with HFMD (3, 11, 14, 20, 38, 39). An outbreak of HFMD caused by CVA6 occurred in Japan in 2011. During this outbreak, clinicians reported that HFMD caused by CVA6 was different from typical HFMD; the exanthema caused by CVA6 was larger than the typical one and appeared in the thigh and abdomen as well as in hands and feet. It was also reported that onychomadesis was a characteristic feature in patients during the HFMD outbreak caused by CVA6 in Finland in 2008; parents and clinicians reported that their children shed fingernails and/or toenails within 1 to 2 months after HFMD (39). Although the clinical symptoms of HFMD caused by CVA5, CVA6, or CVA10 look similar to those caused by CVA16 and EV71, the molecular basis of the disease might be different from that for those caused by SCARB2-dependent viruses.

Among HEV-As, the viruses in the EV71 group infect cells via a SCARB2-dependent pathway, but the viruses in the CVA2 group do not. Among HEV-Bs, CVB1-6 uses coxsackie-adenovirus receptor (CAR), while other members of HEV-B do not (4). Similarly, only polioviruses 1 to 3 use CD155 as the receptor among the HEV-Cs (4). In contrast, major group human rhinoviruses (HRVs) that use intercellular cell adhesion molecule 1 (ICAM-1) as the receptor are present in both HRV-A and HRV-B, and minor group HRVs are all in HRV-A (4, 15). The monophyletic use of SCARB2 by CVA7, CVA14, CVA16, and EV71 is similar to the use of CAR by CVB1-6 and the use of CD155 by polioviruses 1 to 3.

CVA7, CVA14, CVA16, and EV71 share the same receptor and are frequently associated with HFMD. In addition, neurological disease caused by CVA7 and EV71 has been reported (16–18, 29, 42, 53). These data suggest that this group of viruses, which infect via a SCARB2-dependent pathway, is capable of invading the central nervous system (CNS) using this receptor. Supporting this idea, our preliminary experiments showed that SCARB2 is expressed in neurons in the CNS in humans, monkeys, and transgenic mice expressing human SCARB2 and that the adult transgenic mice showed encephalitis after infection with EV71. Although these viruses are able to utilize SCARB2 as a receptor, the occurrence of severe neurological disease caused by EV71 was quite low before recent outbreaks in the eastern Asian countries, and severe neurological diseases associated with CVA16 have not been reported. It is possible that the EV71 strains currently circulating in countries that suffer from severe EV71 outbreaks obtained an unidentified neurovirulence determinant(s) in the viral genome and became more neurovirulent than those that circulated previously. It is also possible that CVA16 has the potential to cause neurological disease, because it has been reported, in the case of poliovirus, that an increase in neurovirulence levels can be caused by point mutations or by genetic recombination between avirulent poliovirus vaccine strains and nonpolio enteroviruses (49, 50, 51, 52, 53, 54). Because CVA7, CVA14, CVA16, and EV71 utilize the same receptor and because SCARB2-dependent viruses sometimes cocirculate during an epidemic of HFMD (1, 12), these viruses might have a high potential to undergo an intertypic recombination by coinfection of a SCARB2-expressing cell *in vivo*. Indeed, it has been reported that intertypic recombination occurred between EV71 and CVA16 (8, 19, 54–56). Fortunately, neither severe neurological diseases caused by the intertypic recombinants between EV71 and CVA16 nor recombinant viruses of EV71 and CVA7 or CVA14 have been reported. These viruses might appear as an emerging infectious pathogen, however, and may have unexpectedly high virulence. Careful and continuous surveillance of this group of viruses is important for public health.

In summary, SCARB2 is used as a receptor for certain members of the HEV-A viruses and may play important roles in the pathogenesis of HFMD and neurological diseases. We are now investigating the precise interaction between EV71 and SCARB2 and generating a mouse model expressing human SCARB2. These further studies will help to elucidate the molecular basis of HFMD and the neurological disease caused by these viruses.

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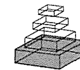

Scavenger receptor B2 as a receptor for hand, foot, and mouth disease and severe neurological diseases

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Enterovirus 71 (EV71) is one of the major causative agents of hand, foot, and mouth disease (HFMD). Infection with EV71 is occasionally associated with severe neurological diseases such as acute encephalitis, acute flaccid paralysis, and cardiopulmonary failure. Because cellular receptors for viruses play an important role in cell, tissue, and species tropism, it is important to identify and characterize the receptor molecule. Recently, cellular receptors and host factors that stimulate EV71 infection have been identified. Several lines of evidence suggest that scavenger receptor class B, member 2 (SCARB2) plays critical roles in efficient EV71 infection and the development of disease in humans. In this review, we will summarize the findings of recent studies on EV71 infection and on the roles of SCARB2.

Keywords: EV71, SCARB2, HFMD, neurological disease

INTRODUCTION

Human enteroviruses (HEVs) are a large family of human pathogens belonging to the Picornaviridae family, and these viruses can cause a variety of diseases. HEVs are classified into four groups, species A (HEV-A) to species D (HEV-D). HEV-A is composed of at least 16 members of different serotypes: Coxsackievirus (CV) A2, CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, CVA16, Enterovirus 71 (EV71), EV76, EV89, EV90, and EV91 (Pallansch and Roos, 2007). The number of isolated and characterized HEV-A viruses is continually increasing (Oberste et al., 2007, 2008). Members of HEV-A are known to be causative agents of hand, foot, and mouth disease (HFMD), herpangina, respiratory disease, meningitis, and polio-like flaccid paralysis (Pallansch and Roos, 2007). EV71 and CVA16 are the major causative agents of HFMD. HFMD is normally a mild disease, but HFMD caused by EV71 is sometimes associated with severe neurological diseases such as acute fatal encephalitis, polio-like acute flaccid paralysis, and neurogenic pulmonary edema (Schmidt et al., 1974; Chumakov et al., 1979a,b; Melnick, 1984; Ho et al., 1999; Chan et al., 2000). Recently, large outbreaks of EV71 associated with severe neurological diseases have occurred repeatedly in the Asia-Pacific region (Ho et al., 1999; Komatsu et al., 1999; Ahmad, 2000; Chan et al., 2000; McMinn et al., 2001a,b; Fujimoto et al., 2002; Wang et al., 2002; De et al., 2011). EV71 has become a serious public health concern (Qiu, 2008).

Virus infection is initiated by attachment of the virus to a cellular receptor at the surface of a susceptible cell. Cellular receptors for viruses therefore play important roles in determining the cell, tissue, and species tropism and pathogenicity of viruses (Bergelson, 2010). Thus, the identification and characterization of the cellular receptor for EV71 are important steps in the elucidation of the pathogenicity of EV71. Recently, several research groups identified cellular receptors and host factors that enhance EV71 infection (Nishimura et al., 2009; Yamayoshi et al., 2009; Yang et al., 2009, 2011; Han et al., 2010). Herein, we will summarize the importance of scavenger receptor class B, member 2 (SCARB2) as a receptor for EV71.

IDENTIFICATION OF SCARB2 AS A RECEPTOR FOR EV71

Monolayer cultures of primate cells such as RD cells and Vero cells are susceptible to EV71 strains, and these cells are often used for the isolation of EV71 from clinical specimens (Mizuta et al., 2005, 2009). Mouse cells, such as L929 cells, are generally not highly susceptible to EV71 infection. Only residual virus antigen-positive cells are observed, even if the mouse cells are infected at a high multiplicity of infection. This species-specific restriction of EV71 infection is due to the absence on mouse cells of the cellular receptor that permits viral entry into cells. Yamayoshi et al. (2009) employed a strategy to identify the human EV71 receptor; this strategy is outlined in **Figure 1**. These researchers transfected human genomic DNA into mouse L929 cells and succeeded in selecting two L929 cell lines, Ltr051 and Ltr246, that became susceptible to EV71 among approximately 70,000 transformed cells. The integrated human gene(s) are expected to encode the EV71 receptor.

One of the transformed cell lines, Ltr051, was highly susceptible to EV71, with an infection efficiency similar to that of RD cells, whereas the other cell line, Ltr246, was susceptible to EV71 but with a lower efficiency. By microarray analysis of the RNAs expressed in the transformant cells, it was shown that Ltr051 cells carried the gene for human *SCARB2*. L929 cells that expressed SCARB2 constitutively (L-SCARB2 cells) were susceptible to all EV71 strains tested, irrespective of the sub-genogroup. EV71 infection in RD cells was inhibited both by anti-SCARB2 antibodies and by soluble SCARB2. The human and monkey cell lines that are EV71-susceptible cells expressed SCARB2. These results suggest that SCARB2 plays a critical role in the EV71 infection pathway. In addition, Yamayoshi et al. (2009) found that CVA16 also uses SCARB2 as a receptor, suggesting that SCARB2 serves as a receptor for other HEV-As that cause HFMD.

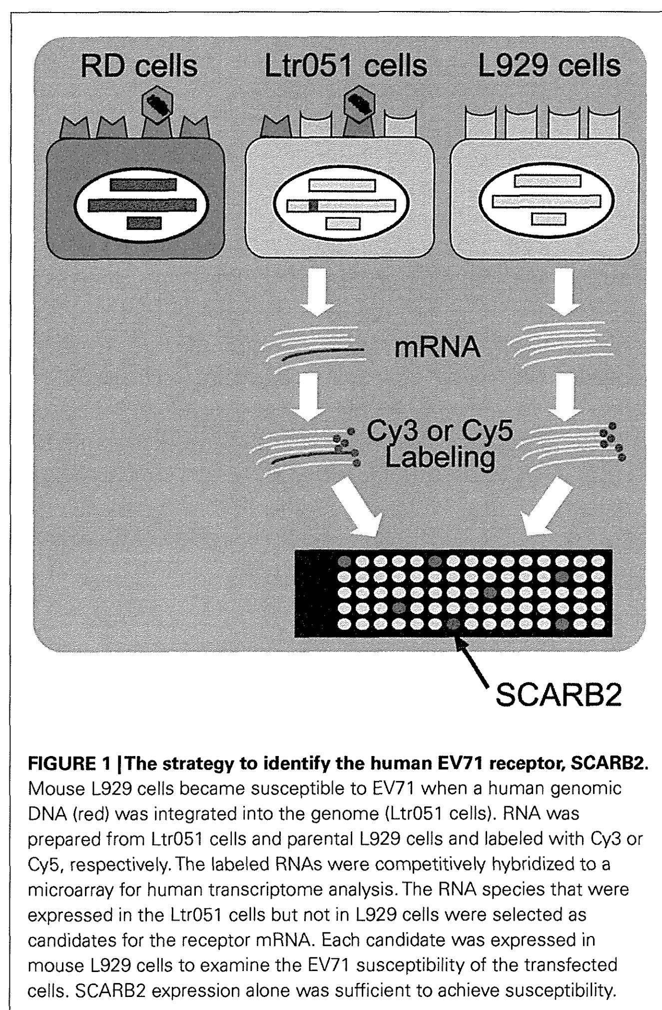

FIGURE 1 | The strategy to identify the human EV71 receptor, SCARB2. Mouse L929 cells became susceptible to EV71 when a human genomic DNA (red) was integrated into the genome (Ltr051 cells). RNA was prepared from Ltr051 cells and parental L929 cells and labeled with Cy3 or Cy5, respectively. The labeled RNAs were competitively hybridized to a microarray for human transcriptome analysis. The RNA species that were expressed in the Ltr051 cells but not in L929 cells were selected as candidates for the receptor mRNA. Each candidate was expressed in mouse L929 cells to examine the EV71 susceptibility of the transfected cells. SCARB2 expression alone was sufficient to achieve susceptibility.

The other cell line, Ltr246, is susceptible to a subset of EV71 strains but not all strains. Despite all of the efforts undertaken to identify the gene that is integrated in this cell line and that supports EV71 infection, this gene has not yet been identified. Other research groups have proposed that molecules act as receptors or other entities to enhance EV71 infection (see Mini Review by Nishimura and Shimizu, submitted). However, infection mediated by P-selectin glycoprotein ligand-1 (PSGL-1) or by an unknown molecule expressed in Ltr246 cells is not as efficient as infection mediated by SCARB2. Infection with EV71 in L929 cells expressing PSGL-1 was successful only for a subset of EV71 strains (Miyamura et al., 2011). SCARB2 seems to play the most important role in EV71 infection *in vitro* and *in vivo* because SCARB2 serves as a receptor for all EV71 strains and is expressed in the sites of EV71 replication *in vivo*.

STRUCTURE AND FUNCTION OF SCARB2

Scavenger receptor class B, member 2 (also known as Lysosomal Integral Membrane Protein II, LGP85 or CD36b like-2) is composed of 478 amino acids and belongs to the CD36 family, which includes CD36 and scavenger receptor B, member 1 (SR-BI and its splicing variant SR-BII; Calvo et al., 1995; Eskelinen et al., 2003). SCARB2 is one of the most abundant proteins in the lysosomal membrane and participates in membrane transportation and the reorganization of the endosomal/lysosomal compartment (Kuronita et al., 2002; Eskelinen et al., 2003; Blanz et al., 2010). SCARB2 shuttles between these compartments and the plasma membrane (**Figure 2B**, left; Eskelinen et al., 2003). SCARB2 is a type III double-transmembrane protein with a large extracellular domain (when it is present at the cell surface) and short cytoplasmic domains at the amino- and carboxy-terminus (**Figure 2A**; Calvo et al., 1995). SCARB2 is expressed in a variety of tissues, including neurons in the CNS. SCARB2 deficiency in mice causes ureteric pelvic junction obstruction, deafness, and peripheral neuropathy, and SCARB2 deficiency in humans causes action myoclonus–renal failure syndrome (AMRF; Gamp et al., 2003; Berkovic et al., 2008).

Mouse SCARB2 exhibits 85.8% amino acid identity with human SCARB2 (**Figure 2A**). Using chimeric mutants that include human and mouse SCARB2 sequences, Yamayoshi and Koike (2011) mapped the region that was important for efficient EV71 binding and infection. L929 cells expressing chimeras that carried amino acids 142–204 from the human sequence were susceptible to EV71, whereas chimeras that carried the mouse sequence in this region were not susceptible. It was shown that this region is also critical for binding to the virion. This region of the SCARB2 protein exhibits 76.2% amino acid identity between the human and mouse sequences. Removal of the carbohydrate moiety of the recombinant soluble SCARB2 protein by PNGase F treatment did not abolish the binding of the virus to the receptor, suggesting that the protein moiety of human SCARB2 plays a critical role in binding. Recently, Chen et al. (2012) identified critical residues required for human SCARB2 binding to EV71, which was comprised of residues 144–151 in a highly variable region among species. On the viral proteins, they showed that amino acids lined on the wall of the canyon (the EF loop of VP1) were important for SCARB2 binding and viral infectivity (Chen et al., 2012). To elucidate the mode of interaction of EV71 and SCARB2 more precisely, crystallographic analysis will be needed.

MECHANISM OF EV71 INFECTION IN RD CELLS

Hussain et al. (2011) have performed a screen of host factors required for EV71 entry into RD cells using an siRNA library. They found that the repression of genes associated with clathrin-mediated endocytosis, including AP2A1, ARRB1, CLTC, CLTCL1, SYNJ1, ARPC5, PAK1, ROCK1, and WASF1, resulted in significant inhibition of EV71 infection. They observed both co-localization of EV71 with clathrin using an immunofluorescence assay and virions in clathrin-coated pits by electron microscopy. EV71 entry into cells was inhibited when a dominant-negative mutant of Eps15, which binds to AP-2, was expressed and when cells were treated with drugs that selectively inhibit clathrin-dependent endocytosis (chlorpromazine and cytochalasin B). Entry was not inhibited by drugs that inhibit caveolae-dependent endocytosis and macropinocytosis. Hussain et al. also showed that EV71 infection was abolished when cells were treated with drugs that inhibit acidification of the endosome (Bafilomycin A1 and concanamycin A). Taken together, these results suggest that the mechanism of EV71 infection is that which is summarized in **Figure 2B** (right).