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H.　知的財産権の出願・登録状況

該当無し

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Functional Comparison of SCARB2 and PSGL1 as Receptors for Enterovirus 71

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Human scavenger receptor class B, member 2 (SCARB2), and P-selectin glycoprotein ligand-1 (PSGL1) have been identified to be the cellular receptors for enterovirus 71 (EV71). We compared the EV71 infection efficiencies of mouse L cells that expressed SCARB2 (L-SCARB2) and PSGL1 (L-PSGL1) and the abilities of SCARB2 and PSGL1 to bind to the virus. L-SCARB2 cells bound a reduced amount of EV71 compared to L-PSGL1 cells. However, EV71 could infect L-SCARB2 cells more efficiently than L-PSGL1 cells. The results suggested that the difference in the binding capacities of the two receptors was not the sole determinant of the infection efficiency and that SCARB2 plays an essential role after attaching to virions. Therefore, we examined the viral entry into L-SCARB2 cells and L-PSGL1 cells by immunofluorescence microscopy. In both cells, we detected internalized EV71 virions that colocalized with an early endosome marker. We then performed a sucrose density gradient centrifugation analysis to evaluate viral uncoating. After incubating the EV71 virion with L-SCARB2 cells or soluble SCARB2 under acidic conditions below pH 6.0, we observed that part of the native virion was converted into an empty capsid that lacked both genomic RNA and VP4 capsid proteins. The results suggested that the uncoating of EV71 requires both SCARB2 and an acidic environment and occurs after the internalization of the virus-receptor complex into endosomes. However, the empty capsid formation was not observed after incubation with L-PSGL1 cells or soluble PSGL1 under any of the tested pH conditions. These results indicated that SCARB2 is capable of viral binding, viral internalization, and viral uncoating and that the low infection efficiency of L-PSGL1 cells is due to the inability of PSGL1 to induce viral uncoating. The characterization of SCARB2 as an uncoating receptor greatly contributes to the understanding of the early steps of EV71 infection.

Enterovirus 71 (EV71) belongs to the genus *Enterovirus* within the family *Picornaviridae* (1). The virus contains positive-sense RNA surrounded by an icosahedral capsid assembled from 60 copies of the four structural proteins VP1, VP2, VP3, and VP4 (2–4). VP1, VP2, and VP3 create a canyon on the viral surface (3, 4) that is the site of attachment to the cellular receptor on many enteroviruses (5). The first report of EV71 isolation was in patients with neurological diseases, including fatal encephalitis and aseptic meningitis, in California from 1969 to 1972 (6). Later studies reported that EV71 was a causative agent of hand, foot, and mouth disease (HFMD) in young children and infants (7, 8). The clinical symptoms of HFMD due to EV71 are generally mild and self-limiting; however, EV71 occasionally causes severe neurological diseases, such as brainstem encephalitis and acute flaccid paralysis (9). Recently, epidemic outbreaks of neurovirulent EV71 have been reported mainly in Southeast and East Asia, including Taiwan, Malaysia, Singapore, Japan, and China (10–15). From 2008 to 2011, the epidemic outbreaks of EV71 in China resulted in approximately 1,900 fatal cases (16). In 2011, the epidemic in Vietnam resulted in 98 fatal cases (http://www.wpro.who.int/vietnam/media_center/press_releases/hfmd_pr.htm).

Two molecules—human scavenger receptor class B, member 2 (SCARB2; also known as lysosomal integral membrane protein II or CD36b like-2) (17), and human P-selectin glycoprotein ligand-1 (PSGL1; also known as selectin P ligand) (18)—were reported to be the cellular receptors for EV71. SCARB2 belongs to the CD36 family and has two transmembrane domains (19). Physiologically, SCARB2 works as the receptor for β-glucocerebrosidase (β-GC) transport from the endoplasmic reticulum to the lysosome (20, 21) and plays an important role in the maintenance of lysosomes (19). Mouse cells become susceptible to all tested EV71 strains when they express human SCARB2 (17, 22). The binding of SCARB2 to EV71 occurs within the luminal domain of SCARB2 at amino acids 142 to 204 (23), and amino acids 144 to 151 were demonstrated to be particularly important (24). The EF loop region of VP1, which lines the wall of the canyon on the viral surface, was found to be important for binding to SCARB2 (24). EV71 infection via the SCARB2-dependent pathway was inhibited by a small interfering RNA (siRNA) treatment against the molecules that are involved in the clathrin-dependent endocytic pathway and by inhibitors of endosomal acidification (25, 26). In addition to EV71, coxsackievirus A7 (CVA7), CVA14, and CVA16 have utilized SCARB2 as a receptor for infection (17, 22). PSGL1 is a sialomucin leukocyte membrane protein that is expressed as a homodimer of disulfide-linked subunits and can bind to three different selectins (P, E, and L) (27–29). Physiologically, PSGL1 is expressed on myeloid cells and stimulated T lymphocytes (30) and plays a critical role in the tethering and rolling of leukocytes for the recruitment of these cells from blood vessels into inflamed tissues (30). Several EV71 strains (PSGL1-binding strain EV71-PB) bind to PSGL1, but other strains (PSGL1-nonbinding strain EV71-non-PB) do not (18). The binding of EV71-PB to PSGL1 requires tyrosine sulfations at the N-terminal region of PSGL1 (31). Mouse cells that express PSGL1 are susceptible only to EV71-PB and CVA16 (18). However, the transgenic expression of PSGL1 in mice failed to confer susceptibility to EV71 *in vivo* (32).

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The mode of infection of picornaviruses varies for each type, and each viral receptor plays a distinct role (33, 34). Poliovirus (PV) receptor (PVR; also known as CD155) and intercellular adhesion molecule 1 (ICAM-1) are cellular receptors for PV and the major-group human rhinoviruses (HRVs), respectively (35–39). These receptors play roles in viral attachment, internalization, and uncoating (33, 34). The αvβ1, αvβ3, αvβ6, αvβ8, and α5β1 integrins are the receptors for foot-and-mouth-disease virus (FMDV) (40), and members of the low-density-lipoprotein-receptor (LDL-R) family are the receptors for the minor-group HRVs (41). These receptors have roles in viral attachment and internalization but not in uncoating (33, 34). Several strains of coxsackievirus B1 (CVB1), CVB3, and CVB5 have two receptors, coxsackievirus and adenovirus receptor (CAR) (42, 43) and decay-accelerating factor (DAF). CAR plays roles in viral attachment, internalization, and uncoating, just like PVR and ICAM-1 in PV and major-group HRV infections, respectively. DAF can bind to the virus but cannot initiate uncoating (33, 44–46). The expression of CAR is sufficient for the establishment of infection in most cultured cells. However, in polarized epithelial cells, CAR is not located on the apical surface. DAF provides an initial attachment site on the apical surface, and DAF-mediated signals permit the virus to interact with CAR in the tight junctions (47).

Conformational alterations for the uncoating of picornaviruses require their respective viral receptor and/or acidic conditions. The uncoating of PV, CVB, and the major-group HRVs is triggered by their receptors (34, 46, 48, 49). After the binding of the receptor to the virion, the native virion releases VP4 and becomes an A particle at a neutral pH. After A-particle formation, the A particle releases viral genomic RNA and becomes an empty capsid. Another typical conformational alteration of the minor-group HRVs and aphthoviruses, such as FMDV and equine rhinitis A virus (ERAV), is triggered by the acidic environment of endosomes (34, 50, 51). Under an acidic pH, the native virion is converted either into an A particle and then an empty capsid (32) or into an empty capsid and then pentameric subunits for viral RNA release (50, 52). The uncoating of HRV type 16 (HRV16) and HRV14 requires both ICAM-1 and low pH (53).

Studies have reported that both EV71 receptors, SCARB2 and PSGL1, can promote EV71 infection by acting as viral attachment factors (17, 18). Recently, Chen et al. evaluated the role of SCABR2 and PSGL1 during uncoating, and they observed a shift in the viral RNA peak to a more slowly sedimenting fraction in sucrose density gradient centrifugation after the virion was incubated with SCARB2 at an acidic pH; this was not observed after incubation with PSGL1 (24). However, the precise roles of the two receptors and the mode of uncoating have not yet been reported. In this study, we compared the infection efficiency of EV71 via the SCARB2-dependent pathway with the infection efficiency via the PSGL1-dependent pathway. Additionally, we evaluated the binding of EV71 to SCARB2 and PSGL1 and the internalization of EV71 via these receptors. We also evaluated the process of the conformational alteration of EV71 by these receptors.

MATERIALS AND METHODS

Cells. Rhabdomyosarcoma (RD) cells, Vero cells, and L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 5% fetal bovine serum (FBS) and a penicillin-streptomycin solution (Life Technologies) (5% FBS–DMEM). L cells expressing SCARB2 (L-SCARB2 cells) and L cells transfected with empty pCAGGS-PUR vector (L-Empty cells) (17) were cultured in 5% FBS–DMEM supplemented with puromycin (4 μg ml^{-1}; Calbiochem).

Viruses. The EV71 strains SK-EV006/Malaysia/97 (SK-EV006; genogroup B), BrCr/USA/70 (BrCr; genogroup A), 1095/Japan/97 (1095; genogroup C), and EV71-GFP, which expresses green fluorescent protein (GFP) upon viral replication, were used in this study (17, 23, 54). Unless otherwise noted, we used EV71 strain SK-EV006 as a representative strain of EV71. The poliovirus strain Mahoney was recovered from the infectious cDNA clone pOM (55).

Plasmids. The cDNA fragment of PSGL1 was amplified from RD cells by reverse transcription (RT)-PCR with the primers PSGL1-Eco(+) (CA GAATTCACCATGCCTCTGCAACTCCTCC) and PSGL1-Xho(−) (CA CACTCGAGCTAAGGGAGGAAGCTGTG), and the PCR product was inserted into pCAGGS-PUR (56). The resulting construct was designated pCA-PSGL1.

Establishment of L-PSGL1 cells. L929 cells were transfected with pCA-PSGL1. After 24 h, the cells were passaged with 5% FBS–DMEM containing 5 μg ml^{-1} puromycin for selection. A total of 5 independent clones of the PSGL1-expressing transformant were tested for susceptibility to EV71-GFP. All of the PSGL1-expressing transformants were susceptible to EV71-GFP at similar levels. We selected a representative clone from these 5 clones to be the L cells expressing PSGL1 (L-PSGL1 cells).

Flow cytometry. We performed flow cytometry as previously reported (17, 57), with some modifications. Briefly, we resuspended L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells in phosphate-buffered saline [PBS(−)] (per liter, 8.00 g NaCl, 1.15 g Na_2HPO_4, 0.20 g KCl, 0.20 g KH_2PO_4 [pH 7.4]) containing 0.02% EDTA, stained them with anti-human LIMPII/SR-B2 goat polyclonal antibody (R&D Systems) or anti-human CD162 mouse monoclonal antibody (clone KPL-1; BD Pharmingen) followed by Alexa Fluor 488 donkey anti-goat IgG (H+L) or Alexa Fluor 488 donkey anti-mouse IgG (H+L) (Life Technologies), respectively, and then analyzed the cells on a FACSCalibur flow cytometer (Becton, Dickinson and Company) with CellQuest Pro software (Becton, Dickinson).

Single-round infection assay. L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells were infected with EV71-GFP and incubated for 24 h at 37°C. Images of the cells were acquired using an IX70 microscope (Olympus) and a DP70 charge-coupled-device camera (Olympus) and were analyzed using DP controller software (Olympus). These infected cells were detached and fixed with 4% paraformaldehyde (PFA). The cells were then analyzed with a FACSCalibur flow cytometer using CellQuest Pro software. The amount of EV71-GFP was chosen so that infected virus produces approximately 1,000 GFP-positive cells in RD cells under the same conditions.

Viral growth kinetics. RD cells, L929 cells, L-SCARB2 cells, and L-PSGL1 cells were infected with EV71 at a multiplicity of infection (MOI) of 0.01 or 10. After incubation for 1 h at 37°C, the inoculum was removed and the cells were washed twice with 5% FBS–DMEM. The infected cells were maintained in 5% FBS–DMEM for 0, 6, 12, 24, 36, or 48 h at 37°C and then collected. The samples were freeze-thawed 3 times before use. The viral titers were determined with Vero cells and were expressed as the 50% tissue culture infectious dose ($TCID_{50}$) according to the Reed-Muench method (58). After 24 h, the cells that were infected at an MOI of 0.01 or 10 were imaged.

Virus purification. RD cells were infected with EV71 at an MOI of 5. The cells and media were frozen at 18 h postinfection. After thawing, the cell debris was removed by centrifugation in an NA-4HS rotor (Tomy) at 10,000 rpm for 20 min at 4°C. The supernatant was layered onto a 1.25-g/ml and 1.48-g/ml CsCl discontinuous step gradient in PBS(−). The empty (E) particles and native virions (filled [F] particles) were collected from a fraction on the 1.25-g/ml CsCl gradient and a fraction between the 1.25-g/ml and 1.48-g/ml CsCl gradients, respectively. The E particles and F particles were diluted with PBS(−) containing 0.1% bovine serum albumin (BSA) and pelleted by ultracentrifugation in an SW32Ti rotor (Beckman) at 32,000 rpm for 2 h at 15°C. Each pellet was suspended in

PBS(−) containing 0.1% BSA and 1% SDS. Each supernatant was layered onto a 15 to 30% sucrose density gradient in PBS(−) containing 0.1% BSA and centrifuged in an SW41Ti rotor (Beckman) at 39,000 rpm for 2 h at 4°C. After fractionation (1 ml/fraction), the fractions that included the E particles and F particles were collected and pelleted by ultracentrifugation in the SW41Ti rotor at 39,000 rpm for 2 h at 4°C. The pellets were suspended in PBS(−) containing 0.1% BSA and used as purified E and F particles.

To prepare [^{35}S]methionine- and [^{35}S]cysteine-labeled EV71 strains SK-EV006, BrCr, and 1095 and poliovirus, RD cells were infected with EV71 or poliovirus at an MOI of 5. At 3 h postinfection, the medium was changed to methionine- and cysteine-free DMEM (Sigma) containing [^{35}S]methionine and [^{35}S]cysteine (EasyTag Express^{35}S protein labeling mix, [^{35}S]-; PerkinElmer). The labeled virus was purified as described above.

Coimmunoprecipitation assay. A coimmunoprecipitation assay was performed as previously described (23). Briefly, the purified F particles were incubated with control Fc (Fc portion of human IgG; 3 μg; R&D Systems), SCARB2-Fc (0.1, 0.3, 1, or 3 μg; R&D Systems) or PSGL1-Fc (0.1, 0.3, 1, or 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. Then, the beads were 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 onto 12% Mini-Protean TGX precast gels (Bio-Rad), followed by Western blotting with anti-EV71 serum (kindly provided by H. Shimizu, NIID, Japan) (54) or protein staining using an Oriole fluorescent gel stain (Bio-Rad).

Virus attachment assay. L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells were mixed with various amounts of purified [^{35}S]methionine- and [^{35}S]cysteine-labeled F particles and incubated for 2 h at 4°C to allow viral attachment to the cell surface. After two washes with PBS(−) containing 0.1% BSA, the virus bound to the cells was recovered with 0.5% SDS solution, and the radioactivity was measured using Insta-Gel Plus liquid scintillation cocktail (PerkinElmer) and an LSC-6100 liquid scintillation counter (Aloka). The radioactivity of the virus that specifically bound to the cells was calculated by subtracting the counts obtained for L-Empty cells from the counts obtained for L-SCARB2 cells or L-PSGL1 cells.

Observation of internalized EV71 in L-SCARB2 cells and L-PSGL1 cells by indirect immunofluorescence. A total of 2.8×10^4 cells of L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells were seeded onto 8-well CultureSlides (BD). Two days after seeding, purified EV71 F particles were added at an MOI of 300 at 4°C to allow viral attachment to the cell surface without entry. The cells were then shifted to 37°C (designated time zero). The cells were washed with PBS(−), fixed in PBS(−) containing 4% PFA for 5 or 15 min, and washed four times with cold PBS(−) before immunofluorescence processing. The fixed cells were incubated with PBS(−) containing 0.05% saponin and 5% BSA fraction V (Sigma) to permeate cells and block nonspecific reactions. The fixed cells were incubated overnight at 4°C with the following primary antibodies: a 1:1,000 dilution of the anti-EV71 serum that had been adsorbed with fixed L929 cells and a 1:300 dilution of anti-early endosome antigen 1 (EEA1) goat antibodies (N-19; Santa Cruz Biotechnology). After being washed with PBS(−), the cells were then incubated with the secondary antibodies (Alexa Fluor 488 or 568 donkey anti-rabbit or -goat IgG [H+L]; Life Technologies) for 90 min at room temperature. After another PBS(−) wash, the cells were mounted in Vectashield with DAPI (4′,6-diamidino-2-phenylindole) mounting medium (Vector Laboratories). The cells were imaged under a laser-scanning microscope (TCS SP2; Leica Microsystems). The maximum projection was created using Imaris software (Carl Zeiss, Inc.).

Quantification of viral RNA by real-time RT-PCR. Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen), and real-time RT-PCR was performed as previously described (59, 60), with several modifications. The viral RNA was assayed in a 20-μl reaction mixture containing 2 μl of viral RNA using a one-step SYBR PrimeScript Plus RT-PCR kit (TaKaRa) with the primers EQ-1 (ACATGGTGTGAAGAG TCTATTGAGCT) and EQ-2 (CCAAAGTAGTCGGTTCCGC). The mixtures were subjected to real-time RT-PCR using a LightCycler 480 system II (Roche) with a reverse transcription step at 42°C for 5 min, followed by 45 cycles at 95°C for 5 s and at 60°C for 20 s. The plasmid pSVA-EV71 (17) was used as a control for the quantification of the copy number.

Conformation alteration assay *in vitro*. [^{35}S]methionine- and [^{35}S]cysteine-labeled EV71 F particles from strain SK-EV006, BrCr, or 1095 (10,000 cpm) were incubated with control Fc (1 μg), SCARB2-Fc (1 μg), or PSGL1-Fc (1 μg) in PBS(−) (pH 7.4) containing 0.1% BSA for 1 h at 4°C. After a pH shift from pH 7.4 to the indicated pH by the addition of 9 volumes of PBS(−) (at the indicated pH) containing 0.1% BSA, the mixtures were incubated for 0, 0.5, 1, 2, or 3 h at 37°C, sonicated for 5 min using a Bioruptor UCD-200TM apparatus (Cosmo Bio), layered onto a 15 to 30% sucrose density gradient in PBS(−) (pH 7.4) containing 0.1% BSA, and centrifuged in the SW41Ti rotor at 39,000 rpm for 2 h at 4°C. After fractionation, the radioactivity of each fraction (0.6 ml) was measured using Insta-Gel Plus and an LSC-6100 liquid scintillation counter. In several cases, the fractions were pooled and ultracentrifuged in the SW32Ti rotor at 32,000 rpm for 2 or 3 h at 4°C. The pellets were suspended in PBS(−) including 0.1% BSA and used to quantify viral RNA or for autoradiography on a 10 to 20% Tricine gel (Life Technologies), a BAS-IP MS 2040 phosphorimaging plates (Fujifilm), and a BAS-2500 image scanner (Fujifilm). The EV71 empty capsid was obtained by heating the F particles for 15 min at 56°C. The condition is previously described as a way to generate PV empty capsids (61). The polioviruses that were incubated with 1 μg of PVR-His (R&D systems) or 1 μg of control Fc for 1 h at 37°C were used as markers of the native virion, A particle, and empty capsid (48).

Conformation alteration assay *in vivo*. [^{35}S]methionine- and [^{35}S]cysteine-labeled EV71 F particles (5×10^5 cpm) were incubated with 2×10^6 L-SCARB2 cells or L-PSGL1 cells in PBS(−) (pH 7.4) containing 0.1% BSA for 2 h at 4°C. After being washed twice with PBS(−) (pH 7.4) containing 0.1% BSA, the cells were incubated for 0.5, 1, or 2 h at 37°C in 5% FBS–DMEM, lysed with PBS(−) containing 1% Triton X-100, sonicated for 5 min using a Bioruptor UCD-200TM apparatus, and centrifuged at 20,000 × *g* for 1 min at 4°C. The supernatants (approximately 6×10^3 and 1×10^4 cpm for L-SCARB2- and L-PSGL1-associated virus, respectively) were layered onto a 15 to 30% sucrose density gradient in PBS(−) containing 0.1% BSA and centrifuged in the SW41Ti rotor at 39,000 rpm for 2 h at 4°C. After fractionation, the radioactivity of each fraction (0.6 ml) was measured using Insta-Gel Plus and an LSC-6100 liquid scintillation counter.

RESULTS

Comparison of EV71 infections in L-SCARB2 cells and L-PSGL1 cells. To compare the efficiency of EV71 infection in L929 cells that expressed SCARB2 and PSGL1, we established L-SCARB2 cells and L-PSGL1 cells that constitutively expressed human SCARB2 and PSGL1, respectively. Then, we confirmed the cell surface expression of human SCARB2 and human PSGL1 on L-SCARB2 cells and L-PSGL1 cells by flow cytometry (Fig. 1A). Human SCARB2 and human PSGL1 were detected on the surfaces of their respective cell lines. We therefore infected L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells with an equal amount of EV71-GFP. EV71-GFP, which has capsid proteins that are identical to those of the EV71-PB strain SK-EV006/Malaysia/97 (SK-EV006), can enter cells via the SCARB2-dependent pathway (17, 22) and the PSGL1-dependent pathway (18). EV71-GFP requires a longer time for a single round of replication than the wild-type EV71 strain SK-EV006 and does not effectively spread within 24 h (23). The infected cells were observed at 24 h postinfection (Fig. 1B). GFP-positive cells were not found in the L-Empty cells. A large number of GFP-positive cells were detected in L-SCARB2 cells, whereas a small number of GFP-positive cells

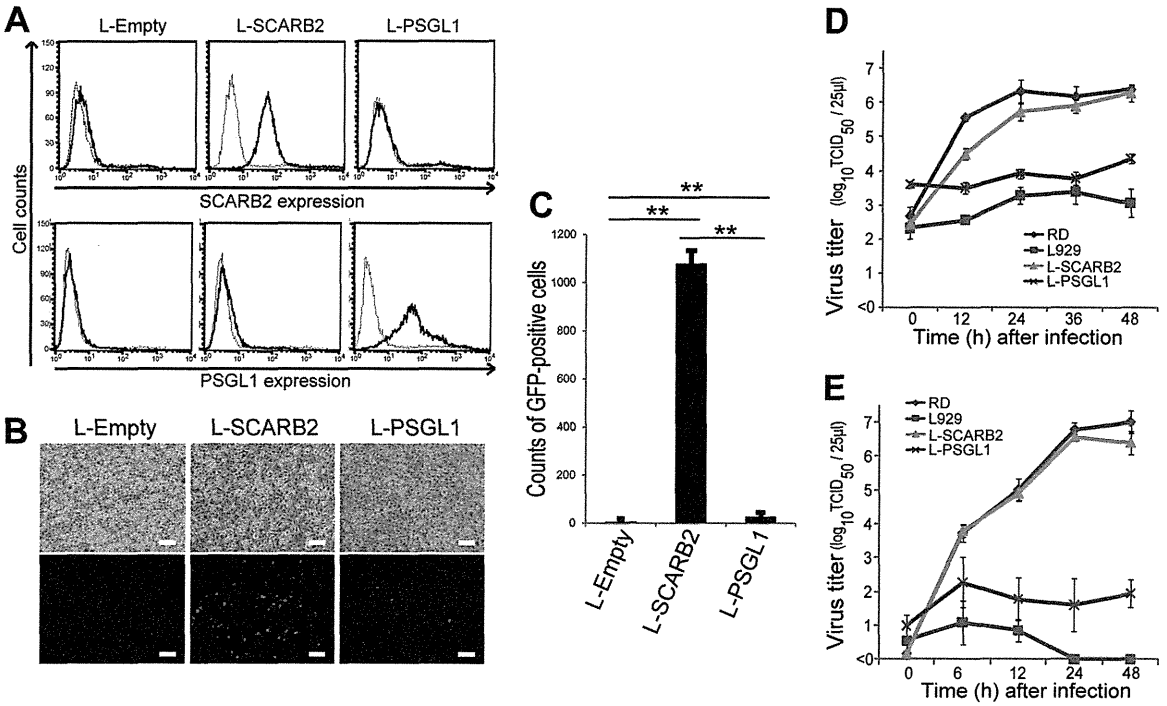

FIG 1 Efficiency of EV71 infection in L-SCARB2 cells and L-PSGL1 cells. (A) Expression of human SCARB2 (top) and PSGL1 (bottom) on the cell surface. L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells were stained with an anti-SCARB2 antibody (solid lines), a normal goat IgG (dotted lines), an anti-PSGL1 antibody (solid lines), or a normal mouse IgG (dotted lines) and analyzed by flow cytometry. (B and C) EV71-GFP infection of L-SCARB2 cells and L-PSGL1 cells. L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells were infected with EV71-GFP. These cells were imaged with a fluorescence microscope (B) and analyzed by flow cytometry (C). The flow cytometry data are shown as the mean counts with the SDs ($n = 3$). **, $P < 0.01$ according to Student's t test. (D and E) RD cells, L929 cells, L-SCARB2 cells, and L-PSGL1 cells were infected with EV71 at MOIs of 10 (D) and 0.01 (E), and the viral titers were determined at 0, 12, 24, 36, and 48 h and at 0, 6, 12, 24, and 48 h after infection, respectively. The data are shown as the mean viral titers with the SDs ($n = 3$).

were detected in L-PSGL1 cells. According to the flow cytometric analysis, the number of GFP-positive L-SCARB2 and L-PSGL1 cells was significantly greater than that of the L-Empty cells ($P = 0.0000044$ and $P = 0.0090$, respectively). The number of GFP-positive cells was significantly greater in the L-SCARB2 cells than in the L-PSGL1 cells ($P = 0.0000049$) (Fig. 1C). These results indicate that SCARB2 and PSGL1 mediate EV71-GFP infection in L929 cells and that the infection efficiency via the two receptors is significantly different.

To validate the results that were obtained in the experiments using EV71-GFP, we examined cytopathic effect (CPE) induction and viral spread using wild-type EV71. Using L-Empty cells, L-SCARB2 cells, and L-PSGL1 cells, the growth kinetics of EV71 were evaluated in infections at MOIs of 10 and 0.01 (Fig. 1D and E). EV71 grew in L-SCARB2 cells as efficiently as in RD cells (Fig. 1D and E). The viral growth in L-PSGL1 cells was better than that in L929 cells. However, the growth in L-PSGL1 cells was less efficient than that in L-SCARB2 cells and RD cells (Fig. 1D and E). The viral titer at the last time point in the L-SCARB2 cells was approximately 100-fold or 10,000-fold higher than the titer that was obtained in L-PSGL1 cells. EV71 propagated minimally in the negative-control L929 cells. At 24 h postinfection with an MOI of 10, we observed a clear CPE in all of the L-SCARB2 cells and the RD cells; however, only a small fraction of the L-PSGL1 cells showed a CPE (data not shown). In addition, a clear CPE was observed in L-SCARB2 cells at 24 h postinfection at an MOI of 0.01 but not in L-PSGL1 cells (data not shown). These results indicate that EV71 infects and spreads more efficiently via SCARB2 than PSGL1.

Purification of EV71 native virion F particles. It has been reported that EV71 in cell culture medium is a mixture of noninfectious empty (E) particles and infectious filled (F) particles (62). E particles are composed of VP0 (VP4-VP2), VP1, and VP3 and have no infectivity, whereas F particles are composed of VP1, VP2, VP3, VP4, and viral genomic RNA and are infective (62). To evaluate the interaction between EV71 and the receptors, we purified F particles as described in Materials and Methods. Fractions 8 to 10 (Fig. 2A, left) and fractions 3 to 5 (Fig. 2A, right) of the 15 to 30% sucrose gradient ultracentrifugation contained E particles and F particles, respectively. We then confirmed the purity and the protein components of the E and F particles by autoradiography (Fig. 2B) or Western blotting using anti-VP2 and anti-EV71 antibodies (Fig. 2C). The deduced molecular masses of VP0, VP1, VP2, VP3, and VP4 are 35, 33, 28, 27, and 7 kDa, respectively. The autoradiogram suggested that the purified E particles were composed of VP0, VP1, and VP3, whereas the purified F particles were composed of VP1, VP2, VP3, and VP4 (Fig. 2B). In agreement with the results presented above, Western blotting using the anti-VP2 antibody detected both the 35-kDa VP0 and the 28-kDa VP2 in the prepurified sample (Fig. 2C, lane Pre), while only VP0 or VP2 was detected in the purified E particles or F particles, respectively (Fig. 2C, lanes E particles and F particles). These data demonstrate that we successfully purified the native virion F particles without contamination with the E particles.