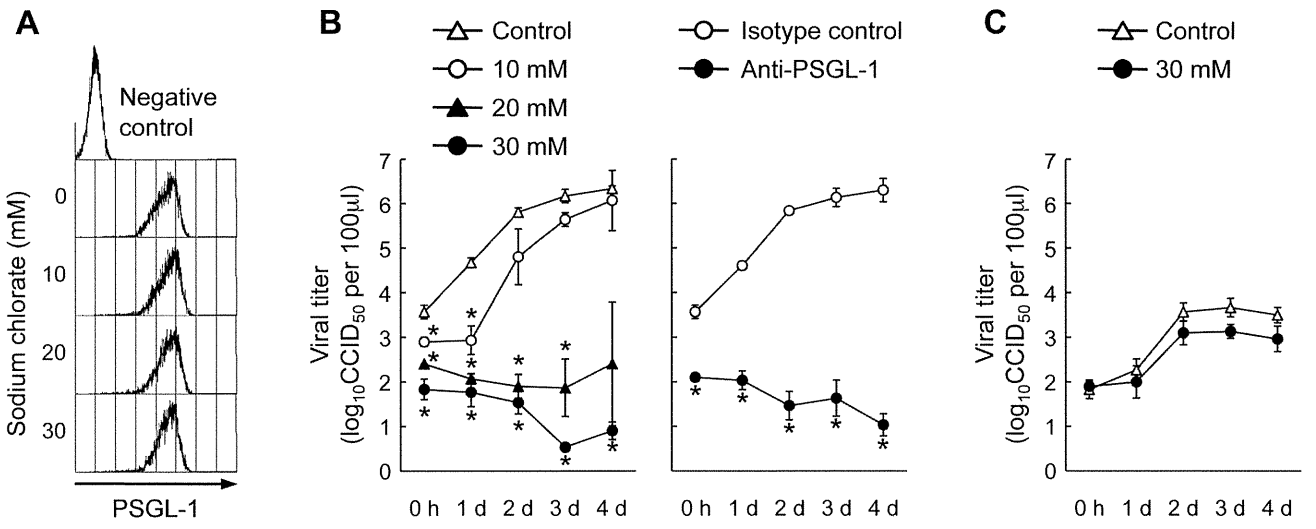

Figure 5. Effect of sodium chlorate on EV71-1095 replication in Jurkat T cells. (A) PSGL-1 expression on the Jurkat T cell surface, as measured by flow cytometry. As a negative control, Jurkat T cells maintained in the absence of sodium chlorate were stained with an isotype control antibody. (B) EV71-1095 growth kinetics in Jurkat T cells in the presence of sodium chlorate. Viral titers were determined at 0 h, 1 day, 2 days, 3 days, and 4 days after EV71-1095 inoculation in Jurkat T cells. As a control for inhibition of EV71 replication, EV71-1095 growth kinetics in Jurkat T cells in the presence of anti-PSGL-1 (KPL1) and control antibodies are shown. (C) EV71-02363 (EV71-non-PB) growth kinetics in Jurkat T cells in the presence of sodium chlorate. Viral titers are indicated as the mean ± S.D. of triplicate analyses. Asterisks indicate $P<0.01$ compared to those of the controls.
doi:10.1371/journal.ppat.1001174.g005

1095 and examined viral titers at 24 h posttransfection in the presence or absence of 30 mM sodium chlorate. Although infectious viruses were recovered in the presence of sodium chlorate, the mean viral titer in the presence of sodium chlorate was over 10 times lower than that of the control experiments (data not shown). Although sodium chlorate inhibited EV71-PB-binding to PSGL-1 expressing cells (Figs. 3C and 5B (0 h postinfection)), we could not rule out the possible involvement of the sodium chlorate treatment during the later stages of viral replication. Further studies are needed to elucidate the inhibitory mechanism of action of sodium chlorate in a receptor dependent or independent manner during different stages of viral replication of EV71.

Replication of the G-10 strain of coxsackievirus A16, which may use another unidentified receptor(s) to infect Jurkat T cells [5,24], was significantly inhibited by sodium chlorate (Fig. 6). This result suggests that some sulfated molecules other than PSGL-1 might be involved in the replication of coxsackievirus A16 in Jurkat T cells in a PSGL-1-independent manner.

Discussion

We have shown that tyrosine sulfation, but not *O*-glycosylation, of the N-terminal region of PSGL-1 is critical for EV71-PB binding to PSGL-1 and for virus entry and subsequent replication

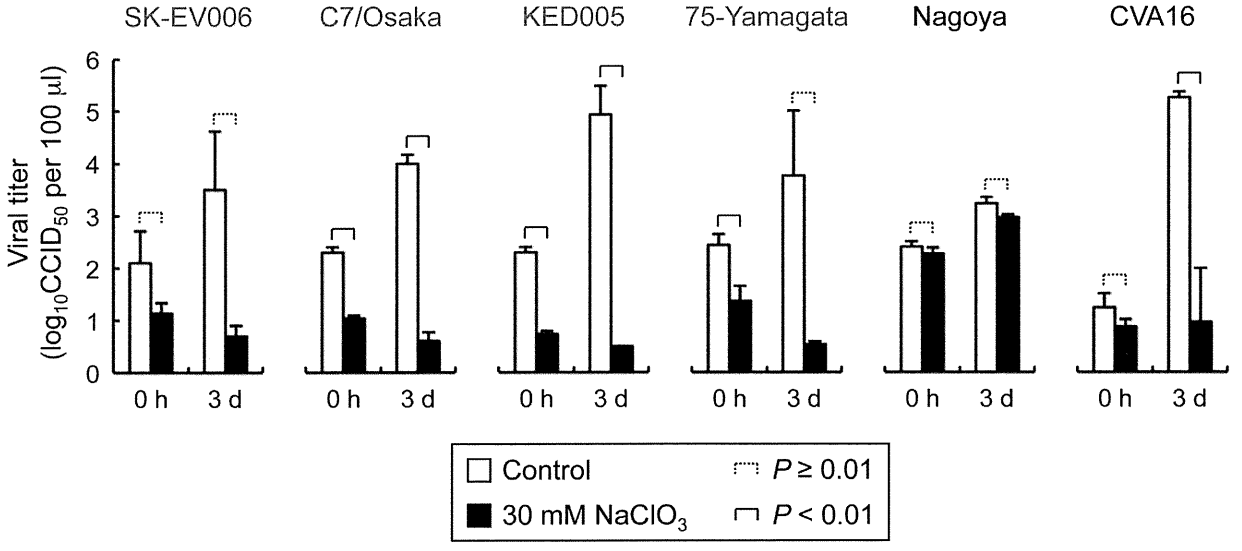

Figure 6. Replication of five EV71 strains and coxsackievirus A16 (CVA16) in Jurkat T cells in the presence of sodium chlorate. Viral replication was determined in Jurkat T cells incubated with 30 mM sodium chlorate. EV71-PB strains are indicated in red. Titers are expressed as the mean, and error bars indicate the S.D. of triplicate or quintuplicate (CVA16) analyses.
doi:10.1371/journal.ppat.1001174.g006

of EV71-PB in Jurkat T cells. First, unlike P-selectin-Fc, EV71-PB bound to a PSGL-1 mutant with an alanine substitution at the potential *O*-glycosylation site (T57) in a calcium-independent manner (Figs. 1 and S1). Second, removal of sialyl Lewis x by sialidase did not reduce PSGL-1 binding to EV71 (Fig. 2). Third, a sulfation inhibitor, sodium chlorate, significantly impaired EV71-PB binding to PSGL-1 in a dose-dependent manner (Figs. 3 and S1). Fourth, EV71-PB binding to PSGL-1 was inhibited when phenylalanine substitutions were made at one or more potential tyrosine sulfation sites in the N-terminal region of PSGL-1 (Figs. 4 and S1). Finally, PSGL-1-dependent viral replication of EV71-PB strains in Jurkat T cells, but not EV71-non-PB strains, was inhibited by sodium chlorate (Figs. 5 and 6).

Human PSGL-1 is one of the most characterized tyrosine sulfated proteins at the molecular level [11]. The involvement of *O*-glycans and sulfated tyrosines in the structural and functional basis of PSGL-1 binding to its natural ligands has been extensively studied, and distinct requirements for tyrosine sulfation for PSGL-1 binding to selectins have been elucidated. Among the three potential sulfated tyrosines of human PSGL-1, Y46 and Y51, but not Y48, are important for PSGL-1 binding to L-selectin along with a core-2 based *O*-glycan with sialyl Lewis x at T57 [22]. On the other hand, the crystal structure of the lectin and EGF domains of P-selectin co-complexed with the N-terminal domain of PSGL-1 revealed a critical involvement of sulfated tyrosines at Y48 and Y51 for direct molecular contact with P-selectin [11]. The corresponding interactions via sulfated tyrosines are not formed in E-selectin binding in the crystal structure of the PSGL-1–E-selectin complex [11]. Thus, tyrosine sulfation is critical for PSGL-1 binding to L- and P-selectins, but not to E-selectin [14]. In our study, we have shown that sulfated tyrosines at Y48 and Y51 play a critical role in PSGL-1 binding to EV71-PB. However, *O*-glycosylation at T57 and sialyl Lewis x moieties on the potential *O*-glycans of PSGL-1 were not required for the PSGL-1–EV71 interaction, suggesting distinct structural requirements between EV71 and P-selectin for PSGL-1 binding. To elucidate the structural basis of the PSGL-1–EV71 interaction, further studies will be needed to identify genetic determinants in EV71 capsid proteins required for PSGL-1 binding using both EV71-PB and non-PB strains.

Yang et al. [21] have recently reported that EV71 may use sialylated glycans as receptors for infection in intestinal DLD-1 cells. In our current study, we showed that potential *O*-glycans at T57 and sialic acids are not critical for binding to EV71-PB (Figs. 1 and 2). However, our study does not exclude possible contributions of sialic acids and other proteins with or without *O*-glycans on the cell surface of various cells during the course of EV71 replication in a PSGL-1-dependent or -independent manner [21,24,25].

In contrast to the structural requirements of *O*-glycans for PSGL-1 binding to selectins, all three sulfated tyrosines, but not *O*-glycans at T57, are required for PSGL-1 binding with the skin-associated chemokine, CCL27 [9]. PSGL-1 facilitates P-selectin-mediated T cell migration in the inflamed skin [26,27] and interacts with the chemokine CCL27 to regulate skin-homing T cells [9]. HFMD pathogenesis due to EV71 can be characterized as acute skin inflammation. Therefore, it is possible that binding of EV71-PB with PSGL-1-positive skin-homing T cells and/or Langerhans cells, and subsequent viral replication in those cells, may participate in HFMD pathogenesis and progression. The status of tyrosine sulfation of PSGL-1 on those cells may modulate cell migration and PSGL-1-dependent replication of EV71-PB in the inflamed skin.

An important role for tyrosine sulfation of a specific cellular receptor in viral entry and replication has been demonstrated for the first time in a co-receptor for HIV-1, CCR5 [18]. CCR5 is a functional receptor for macrophage inflammatory protein (MIP)-1α and MIP-1β, and is expressed on memory/effector T cells, macrophages, and immature dendritic cells [28]. The N-terminal region of CCR5 is highly modified by tyrosine sulfation and *O*-glycosylation, and sulfated tyrosines play critical roles in CCR5 interactions with chemokines [18]. Site-directed mutagenesis and treatment with sodium chlorate revealed that sulfation of tyrosine residues in the N-terminal region of CCR5 is required for efficient CCR5 binding to MIP-1α and MIP-1β, and to HIV-1 gp120-CD4 complexes, without affecting the expression of CCR5 [18]. Likewise, the efficacy of HIV-1 entry was significantly reduced in cells expressing CCR5 mutants with one or more phenylalanine substitutions at four potential tyrosine sulfated residues compared to that in cells expressing native CCR5 [18]. Tyrosine sulfation may be a common phenomenon in chemokine receptors expressed on immune cells such as leukocytes, platelets, and dendritic cells [16]. Therefore, tyrosine sulfation seems to regulate not only the migration of immune cells but also the infectivity of viruses.

Although the occurrence of severe EV71 infection with a number of fatal cases mainly in children continues to be a major public health threat in the Asia Pacific region, no vaccines or antiviral agents are currently available for EV71 [29]. Our data suggest that the virus-receptor interaction may be a promising target for potential antiviral agents. Thus, soluble PSGL-1 as one such agent may have an inhibitory effect on EV71-PB replication [5]. In our current study, we have demonstrated the possible involvement of tyrosine sulfation of PSGL-1 on EV71 entry into target cells, and accordingly, we showed the inhibitory effect of a tyrosine sulfation inhibitor on viral replication of EV71-PB strains in Jurkat T cells. Thus, the elucidation of the structural and functional basis of virus-receptor interactions will provide novel and unique antiviral approaches for the treatment of severe EV71-associated diseases.

Materials and Methods

Cells

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako) supplemented with 10% fetal calf serum (FCS). Jurkat T cells were maintained in RPMI medium (Sigma) supplemented with 10% FCS.

Viruses

All EV71 strains (Table 1) and the coxsackievirus A16 prototype strain (G-10) were propagated in RD or Vero cells. Because some of the strains produced diffuse plaques on RD cells, the viral titers were determined by a microtitration assay using 96-well plates and

Table 1. EV71 strains.

Strain (Subgenogroup)	PSGL-1 binding phenotype[1)]	Accession No.	Reference
SK-EV006 (B3)	PB	AB059819	[33]
C7/Osaka (B4)	PB	AB059818	[33]
KED005[2)] (C1)	PB		[33]
1095 (C2)	PB	AB059817	[30,34]
75-Yamagata (C4)	PB	AB177813	[35]
Nagoya (B1)	Non-PB	AB059813	[36]
02363 (C1)	Non-PB	AB115495	[34]

[1)]PB: PSGL-1–binding, Non-PB: PSGL-1–non-binding [5].
[2)]The VP1 nucleotide sequence of KED005 is identical to that of the 03784-MAA-97 strain (accession no. AY207612) isolated in Malaysia [37].
doi:10.1371/journal.ppat.1001174.t001

RD cells, as previously described [30]. Briefly, 10 wells were used for each viral dilution, and the viral titers were expressed as 50% cell culture infectious dose ($CCID_{50}$). For flow cytometry, we used concentrated viruses unless otherwise stated. To prepare virus concentrations, viruses were ultracentrifuged, and the amount of EV71 virions was measured.

Antibodies and recombinant proteins

The anti-EV71 monoclonal antibody (mAb) MA105 (mouse IgG_{2b}) was generated from mice immunized with EV71-1095 (Y. Tano et al., unpublished data) Immunization to mice, fusion, selection of hybridomas, and propagation of hybridomas in the ascite fluid of the mice, were outsourced to Nippon Biotest Laboratories Inc., Tokyo, Japan. The anti-human PSGL-1 mAb KPL1 and anti-sialyl Lewis x mAb CSLEX1 were purchased from BD Biosciences. Anti-human PSGL-1 mAb PL2 was purchased from Beckman–Coulter. Anti-sulfotyrosine mAb Sulfo-1C-A2 [31] was purchased from Millipore. For the negative control, mouse IgG_1 (MOPC-21) and IgG_{2a} (G155–178) were purchased from BioLegend and BD Biosciences, respectively. Recombinant P-selectin-Fc was purchased from R&D Systems.

Plasmids and mutagenesis

For directional cloning using a *Cpo*I recognition site [32], we introduced a *Cpo*I recognition-compatible (*San*DI) site into the pcDNA3.1(+) plasmid (Invitrogen). The *Bam*HI-*Eco*RI fragment of pcDNA3.1(+) was replaced with 5′-ggatccgggtcccggtaagaattc-3′ (*Bam*HI+*San*DI+gg+Stop+*Eco*RI) to produce pcDNA3.1SS. Human *FUT7* cDNA was amplified from Jurkat T cell cDNA with the primers FUT7-F1 (5′-atacggtccggccatgaataatgctgggcacggc-3′) and FUT7-R1 (5′-tgacggaccgtcaggcctgaaaccaaccct-3′). The *FUT7* ORF was sub-cloned into a *San*DI site in pcDNA3.1SS to produce pcDNA-FUT7. The sequence of the cloned *FUT7* ORF was identical to that of *FUT7* (NM_004479).

The primers used for mutagenesis/deletion are provided in Table S1. Briefly, cDNA of human *SELPLG* was cloned into pEF6-Flag-3S [5] to produce pEF-PSGL-1 [5]. Mutations and deletions were introduced into the N-terminal region of human PSGL-1 with PCR, and the mutated *SELPLG* cDNA was cloned into pEF6-Flag-3S.

Transfection of 293T cells

293T cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen), and DMEM medium was replaced with fresh medium 4 h after transfection. The cells were collected 24 h after transfection by pipetting, and were used for flow cytometry. For inhibition of tyrosine sulfation of PSGL-1, 293T cells were treated with 10–50 mM sodium chlorate in DMEM for 1 day. Four hours after transfection with pEF-PSGL-1, the medium was replaced with medium containing sodium chlorate, and the cells were further incubated for 20 h.

Flow cytometry

The cells were washed once with flow cytometry buffer (FC buffer; PBS(−) supplemented with 2 mM EDTA, 2% FCS, and 0.1% NaN_3) and incubated with the indicated mAb on ice for 30 min. After washing with FC buffer, the cells were incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen). To detect sialyl Lewis x, the cells were incubated with secondary antibodies conjugated with R-phycoerythrin (SouthernBiotech). To detect PSGL-1 by two-color flow cytometry, PL2 was labeled with a Zenon mouse IgG_1 R-phycoerythrin labeling kit (Invitrogen). To detect P-selectin-Fc binding, PBS(−) supplemented with 2 mM $CaCl_2$, 2% FCS, and 0.1% NaN_3 was used instead of FC buffer. The cells were washed and analyzed with FACSCalibur (Becton Dickenson).

EV71-binding assay by flow cytometry

293T cells (5×10^5) transfected with the indicated expression plasmid were washed once with FC buffer and incubated with the EV71-1095 preparation (1×10^7 $CCID_{50}$) supplemented with 0.1% NaN_3, or concentrated viruses (containing 0.5 μg of VP1 protein) per 50 μl FC buffer. The cells were washed and stained for 30 min on ice with Alexa Fluor 488-conjugated MA105.

Sialidase treatment of cells

Cells were processed as in the EV71-binding assay and flow cytometry described above. Prior to the addition of EV71, P-selectin-Fc, or mAb, cells (2.5×10^6) were incubated with 50 mU/ml of *Vibrio cholerae* sialidase (Roche) in 500 μl of DMEM supplemented with 2% FCS for 1 h at 37°C and then washed once.

Viral infection assays

Jurkat T cells (4×10^4 cells) were inoculated with viruses at 1 $CCID_{50}$/cell for 1 h on ice, washed, and incubated in medium (200 μl in a 48-well plate) at 34°C. For inhibition of tyrosine sulfation of PSGL-1, the cells were pretreated with 10–30 mM sodium chlorate in medium for more than 3 days, inoculated with viruses, washed, and maintained in medium supplemented with sodium chlorate. For mAb inhibition, the cells were pretreated with 10 μg/ml mAb for 1 h, washed, and maintained in medium with 10 μg/ml mAb. At the indicated time, the infected cells and supernatants were freeze-thawed, and viral titers were determined by $CCID_{50}$ titration in RD cells. All infection assays were carried out in triplicate unless otherwise stated, and the mean viral titers were compared using Student's *t*-test (two-tailed). P values<0.01 were considered statistically significant.

Supporting Information

Table S1 Substitution/deletion mutant primers.
Found at: doi:10.1371/journal.ppat.1001174.s001 (0.04 MB DOC)

Figure S1 Binding of four EV71-PB strains to 293T cells expressing PSGL-1. 293T cells were transfected with the indicated expression plasmids (wild-type PSGL-1, T57A, or FFF) and cultured in the absence (PSGL-1, T57A, and FFF) or presence (PSGL-1+$NaClO_3$) of 50 mM sodium chlorate. The transfectants were incubated with concentrated EV71 and used for the EV71 binding assay using flow cytometry. As a negative control, cells were incubated with concentrated supernatant from the RD cell culture (RD sup.). The percentage of cells bound to EV71 is indicated in the upper right quadrant.
Found at: doi:10.1371/journal.ppat.1001174.s002 (2.14 MB TIF)

Acknowledgments

The authors thank Dr. Nozomi Nishimura for preparing figures; Dr. Haruko Shirato for providing cells; Junko Wada for excellent technical assistance; Dr. Yuko Sasaki for helpful discussions; and Dr. Kevin Moore (Oklahoma Medical Research Foundation) for useful advice.

Author Contributions

Conceived and designed the experiments: YN. Performed the experiments: YN. Analyzed the data: YN TW HS. Contributed reagents/materials/analysis tools: YN HS. Wrote the paper: YN TW HS.

References

1. Alexander JP Jr., Baden L, Pallansch MA, Anderson LJ (1994) Enterovirus 71 infections and neurologic disease–United States, 1977–1991. J Infect Dis 169: 905–908.
2. McMinn PC (2002) An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev 26: 91–107.
3. Bible JM, Pantelidis P, Chan PKS, Tong CYW (2007) Genetic evolution of enterovirus 71: epidemiological and pathological implications. Rev Med Virol 17: 371–379.
4. Yang F, Ren L, Xiong Z, Li J, Xiao Y, et al. (2009) Enterovirus 71 outbreak in the People's Republic of China in 2008. J Clin Microbiol 47: 2351–2352.
5. Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, et al. (2009) Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat Med 15: 794–797.
6. Yamayoshi S, Yamashita Y, Li J, Hanagata N, Minowa T, et al. (2009) Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat Med 15: 798–801.
7. Eskelinen EL, Tanaka Y, Saftig P (2003) At the acidic edge: emerging functions for lysosomal membrane proteins. Trends Cell Biol 13: 137–145.
8. Laszik Z, Jansen PJ, Cummings RD, Tedder TF, McEver RP, et al. (1996) P-selectin glycoprotein ligand-1 is broadly expressed in cells of myeloid, lymphoid, and dendritic lineage and in some nonhematopoietic cells. Blood 88: 3010–3021.
9. Hirata T, Furukawa Y, Yang BG, Hieshima K, Fukuda M, et al. (2004) Human P-selectin glycoprotein ligand-1 (PSGL-1) interacts with the skin-associated chemokine CCL27 via sulfated tyrosines at the PSGL-1 amino terminus. J Biol Chem 279: 51775–51782.
10. Sako D, Chang XJ, Barone KM, Vachino G, White HM, et al. (1993) Expression cloning of a functional glycoprotein ligand for P-selectin. Cell 75: 1179–1186.
11. Somers WS, Tang J, Shaw GD, Camphausen RT (2000) Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe^X and PSGL-1. Cell 103: 467–479.
12. Liu WJ, Ramachandran V, Kang J, Kishimoto TK, Cummings RD, et al. (1998) Identification of N-terminal residues on P-selectin glycoprotein ligand-1 required for binding to P-selectin. J Biol Chem 273: 7078–7087.
13. Pouyani T, Seed B (1995) PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 83: 333–343.
14. Sako D, Comess KM, Barone KM, Camphausen RT, Cumming DA, et al. (1995) A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell 83: 323–331.
15. Wilkins PP, Moore KL, McEver RP, Cummings RD (1995) Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. J Biol Chem 270: 22677–22680.
16. Kehoe JW, Bertozzi CR (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. Chem Biol 7: R57–61.
17. Moore KL (2003) The biology and enzymology of protein tyrosine *O*-sulfation. J Biol Chem 278: 24243–24246.
18. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, et al. (1999) Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. Cell 96: 667–676.
19. Natsuka S, Gersten KM, Zenita K, Kannagi R, Lowe JB (1994) Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. J Biol Chem 269: 16789–16794.
20. Sasaki K, Kurata K, Funayama K, Nagata M, Watanabe E, et al. (1994) Expression cloning of a novel α1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. J Biol Chem 269: 14730–14737.
21. Yang B, Chuang H, Yang KD (2009) Sialylated glycans as receptor and inhibitor of enterovirus 71 infection to DLD-1 intestinal cells. Virol J 6: 141.
22. Bernimoulin MP, Zeng XL, Abbal C, Giraud S, Martinez M, et al. (2003) Molecular basis of leukocyte rolling on PSGL-1. Predominant role of core-2 *O*-glycans and of tyrosine sulfate residue 51. J Biol Chem 278: 37–47.
23. Leppänen A, White SP, Helin J, McEver RP, Cummings RD (2000) Binding of glycosulfopeptides to P-selectin requires stereospecific contributions of individual tyrosine sulfate and sugar residues. J Biol Chem 275: 39569–39578.
24. Patel KP, Bergelson JM (2009) Receptors identified for hand, foot and mouth virus. Nat Med 15: 728–729.
25. Lin YW, Wang SW, Tung YY, Chen SH (2009) Enterovirus 71 infection of human dendritic cells. Exp Biol Med (Maywood) 234: 1166–1173.
26. Borges E, Tietz W, Steegmaier M, Moll T, Hallmann R, et al. (1997) P-selectin glycoprotein ligand-1 (PSGL-1) on T helper 1 but not on T helper 2 cells binds to P-selectin and supports migration into inflamed skin. J Exp Med 185: 573–578.
27. Hirata T, Merrill-Skoloff G, Aab M, Yang J, Furie BC, et al. (2000) P-selectin glycoprotein ligand 1 (PSGL-1) is a physiological ligand for E-selectin in mediating T helper 1 lymphocyte migration. J Exp Med 192: 1669–1675.
28. Oppermann M (2004) Chemokine receptor CCR5: insights into structure, function, and regulation. Cell Signal 16: 1201–1210.
29. Modlin JF (2007) Enterovirus déjà vu. N Engl J Med 356: 1204–1205.
30. Nagata N, Shimizu H, Ami Y, Tano Y, Harashima A, et al. (2002) Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. J Med Virol 67: 207–216.
31. Kehoe JW, Velappan N, Walbolt M, Rasmussen J, King D, et al. (2006) Using phage display to select antibodies recognizing post-translational modifications independently of sequence context. Mol Cell Proteomics 5: 2350–2363.
32. Izumiya Y, Lin SF, Ellison T, Chen LY, Izumiya C, et al. (2003) Kaposi's sarcoma-associated herpesvirus K-bZIP is a coregulator of K-Rta: physical association and promoter-dependent transcriptional repression. J Virol 77: 1441–1451.
33. Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, et al. (1999) Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997–1998. Jpn J Infect Dis 52: 12–15.
34. Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, et al. (2004) Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. Pediatr Int 46: 231–235.
35. Mizuta K, Abiko C, Murata T, Matsuzaki Y, Itagaki T, et al. (2005) Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. J Clin Microbiol 43: 6171–6175.
36. Tagaya I, Tachibana K (1975) Epidemic of hand, foot and mouth disease in Japan, 1972–1973: difference in epidemiologic and virologic features from the previous one. Jpn J Med Sci Biol 28: 231–234.
37. Herrero LJ, Lee CSM, Hurrelbrink RJ, Chua BH, Chua KB, et al. (2003) Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997–2000. Arch Virol 148: 1369–1385.

Journal of General Virology (2011), 92, 287–291 DOI 10.1099/vir.0.022418-0

Short Communication

Adaptive mutations in the genomes of enterovirus 71 strains following infection of mouse cells expressing human P-selectin glycoprotein ligand-1

Kohei Miyamura,[1,2] Yorihiro Nishimura,[1] Masahiro Abo,[2] Takaji Wakita[1] and Hiroyuki Shimizu[1]

Correspondence
Hiroyuki Shimizu
hshimizu@nih.go.jp

[1]Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

[2]Department of Rehabilitation Medicine, Jikei University School of Medicine, 3-19-18 Nishishinbashi, Minato-ku, Tokyo 105-8471, Japan

We recently identified human P-selectin glycoprotein ligand-1 (PSGL-1) as a functional enterovirus 71 (EV71) receptor and demonstrated PSGL-1-dependent replication for some EV71 strains in human leukocytes. Here, we report that four out of five PSGL-1-binding strains of EV71 replicated poorly in mouse L929 cells stably expressing human PSGL-1 (L-PSGL-1 cells). Therefore, we compared the replication kinetics and entire genomic sequence of five original EV71 strains and the corresponding EV71 variants (EV71-LPS), which were propagated once in L-PSGL-1 cells. Direct sequence comparison of the entire genome of the original EV71 strains and EV71-LPS variants identified several possible adaptive mutations during the course of replication in L-PSGL-1 cells, including a putative determinant of the adaptive phenotype in L-PSGL-1 cells at VP2-149. The results suggest that an adaptive mutation, along with a PSGL-1-binding phenotype, may facilitate efficient PSGL-1-dependent replication of the EV71 strains in L-PSGL-1 cells.

Received 28 March 2010
Accepted 13 October 2010

Enterovirus 71 (EV71) is a small non-enveloped virus with a ssRNA genome of about 7500 nt, and is a major causative agent of hand, foot, and mouth disease. Hand, foot, and mouth disease is usually a mild and self-limiting febrile disease in children, but EV71 infection has been associated with various neurological diseases such as aseptic meningitis, polio-like paralysis and acute encephalitis with neurological pulmonary oedema, mainly in young children and infants (Chan *et al.*, 2000; Ho *et al.*, 1999; McMinn, 2002). Recently, EV71 outbreaks with a number of fatal cases have been reported throughout the world, particularly in the Asia–Pacific region, and the activity of EV71 infection remains a major public health threat (Yang *et al.*, 2009). Despite the importance of EV71, little is known about the pathogenesis of severe neurological diseases associated with EV71 at the molecular level.

Recently, we demonstrated that human P-selectin glycoprotein ligand-1 (PSGL-1) is one of the functional receptors for EV71 (Nishimura *et al.*, 2009). PSGL-1 is a type I sialomucin membrane protein expressed mainly on leukocytes (Laszik *et al.*, 1996; Sako *et al.*, 1993). PSGL-1 on leukocytes binds to selectins on the endothelium with high affinity, and the interaction between PSGL-1 and selectins mediates leukocyte rolling during the initial stage of inflammatory cell recruitment to inflamed tissues. On the other hand, Yamayoshi *et al.* (2009) identified scavenger receptor class B member (SCARB2) as another functional cellular receptor for EV71. SCARB2 is a type III transmembrane protein with double-membrane anchoring and cytoplasmic domains at N and C termini (Eskelinen *et al.*, 2003). Like the expression of PSGL-1, human SCARB2 expression enables normally non-susceptible mouse L929 cells to support viral replication and development of EV71 induced cytopathic effects (CPE) (Yamayoshi *et al.*, 2009). All EV71 strains and the prototype strain (G-10) of coxsackievirus A16 replicate in L929 cells expressing human SCARB2 and in SCARB2-positive RD cells in a SCARB2-dependent manner (Yamayoshi *et al.*, 2009). Previously examined EV71 strains can be classified as PSGL-1-binding and PSGL-1-non-binding strain (Nishimura *et al.*, 2009).

To investigate the replication of various PSGL-1-binding strains of EV71 in more detail, we established a mouse L929 cell line that highly and stably expresses human PSGL-1 on the cell surface (L-PSGL-1 cells) (Nishimura *et al.*, 2009). In the present study, we have shown that a single passage of the original EV71 strains in L-PSGL-1 cells induced one or more amino acid substitutions

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB550332–AB550341.

Supplementary figures are available with the online version of this paper.

022418 © 2011 SGM *Printed in Great Britain*

encoded in the EV71 genome, which may be associated with the adaptive phenotype in these cells, and that the substitution at VP2-K149I/M may be especially important.

We examined whether five PSGL-1-binding strains of EV71 (Table 1) induced CPE in L-PSGL-1 cells. The cells in 24-well tissue culture trays (1×10^5 cells) were infected with EV71 [1×10^6 50 % cell culture infectious dose ($CCID_{50}$)] for 1 h at 34 °C. The cells were washed and cultured for 4 days at 34 °C. L-PSGL-1 cells exhibited CPE 4 days after infection with the 1095 strain as reported previously (data not shown) (Nishimura *et al.*, 2009). However, four other strains (75-Yamagata, SK-EV006, C7/Osaka and KED005) did not induce significant CPE 4 days post-infection (data not shown). Because we could immunofluorescently detect a few cells that expressed the EV71 antigen in infected L-PSGL-1 cells (data not shown), we attempted to propagate original EV71 in L-PSGL-1 cells. The preparation of L-PSGL-1-adapted EV71 variants (EV71-LPS) is summarized in Supplementary Fig. S1 (available in JGV Online). L-PSGL-1 cells (1×10^6 cells) were inoculated with original EV71 (1×10^7 $CCID_{50}$) for 1 h at 34 °C in a 10 cm dish. Inoculated cells were then washed three times and incubated in 10 ml medium at 34 °C. We incubated the L-PSGL-1 cells until almost all cells showed CPE (about 10 days). Finally, we harvested the virus in the culture supernatant and named it EV71-LPS. When we infected L-PSGL-1 cells with EV71-LPS, all EV71 variants induced apparent CPE within 4 days after inoculation (data not shown). VP1 antigens were also detected in L-PSGL-1 cells infected with all EV71-LPS variants (data not shown). These observations indicated that EV71-LPS replicated well in L-PSGL-1 cells.

Next, we infected L-PSGL-1 cells with EV71-LPS in the presence of anti-human PSGL-1 mAb (KPL1; BD Biosciences), which blocks EV71 binding to PSGL-1, as described previously (Nishimura *et al.*, 2009). Briefly, the cells in 48-well tissue culture plates (4×10^4 cells per well) were infected with viruses of 4×10^4 $CCID_{50}$ for 1 h at 34 °C. We inoculated the cells with KED005 of 4×10^3 $CCID_{50}$, as we could not obtain sufficient viral titre for KED005-LPS2. For mAb inhibition, we pretreated the cells with 10 μg ml^{-1} mAb [KPL1 or isotype control (MOPC-21; BioLegend)] for 1 h prior to infection, washed the cells, and maintained them in medium with 10 μg ml^{-1} mAb. At 4 days post-infection, the infected cells and supernatants were freeze–thawed, and viral titres were determined by calculating $CCID_{50}$ with a microtitration assay in RD cells as described previously (Nagata *et al.*, 2002). All infection assays were carried out in triplicate, and the mean viral titres were compared using Student's *t*-test. *P*-values <0.01 were considered statistically significant. As a PSGL-1-negative control, we used L-bsd cells (L929 cells transfected with an empty plasmid and selected in the presence of blasticidin) (Nishimura *et al.*, 2009).

The original 1095 strain replicated in L-PSGL-1 cells, but not in L-bsd cells (Fig. 1), which do not express human PSGL-1, as reported previously (Nishimura *et al.*, 2009). The viral titres of the original 1095 strain at 0 h and 4 days post-infection were reduced in the presence of anti-PSGL-1 mAb, but not in the presence of an isotype control (Fig. 1). These results confirmed that the original 1095 strain replicated in a PSGL-1-dependent manner, as reported previously (Nishimura *et al.*, 2009). The viral titres of the other original EV71 strains in L-PSGL-1 cells were significantly higher than titres in L-bsd cells 0 h post-infection, and the viral titres were reduced by anti-PSGL-1 mAb (Fig. 1). These results indicate that original EV71 bound to L-PSGL-1 cells in a PSGL-1-dependent manner. However, the viral titre of original EV71, except the 1095 strain, remained low in L-PSGL-1 cells even after 4 days (Fig. 1). On the other hand, all EV71-LPS variants replicated well in L-PSGL-1 cells, but not in L-bsd cells. The replication of EV71-LPS variants was inhibited by anti-PSGL-1 mAb, but not by the isotype control (Fig. 1). These results indicated that EV71-LPS variants replicated in L-PSGL-1 cells in a PSGL-1-dependent manner.

To compare viral replication kinetics of the original EV71 and L-PSGL-1-adapted variant in human cells, we infected

Table 1. PSGL-1-binding EV71 strains

Strain (sub-genogroup)	Major symptom	Specimen	Cell*	Location	Year	GenBank accession no.		Reference
						Original	LPS variant	
1095 (C2)	HFMD†	Throat swab	RD	Japan	1997	AB550332	AB550333	Nagata *et al.* (2002); Shimizu *et al.* (2004)
SK-EV006 (B3)	Encephalitis (fatal)	Rectal swab	Vero	Malaysia	1997	AB550334	AB550335	Shimizu *et al.* (1999)
C7/Osaka (B4)	Encephalitis (fatal)	Stool	Vero	Japan	1997	AB550336	AB550337	Shimizu *et al.* (1999)
75-Yamagata (C4)	HFMD†	Nasopharyngeal swab	RD	Japan	2003	AB550338	AB550339	Mizuta *et al.* (2005)
KED005 (C1)	HFMD†	Stool	RD	Malaysia	1997	AB550340	AB550341	Shimizu *et al.* (1999)

*The cell line used to prepare the original EV71 strains in this study.
†Hand, foot, and mouth disease.

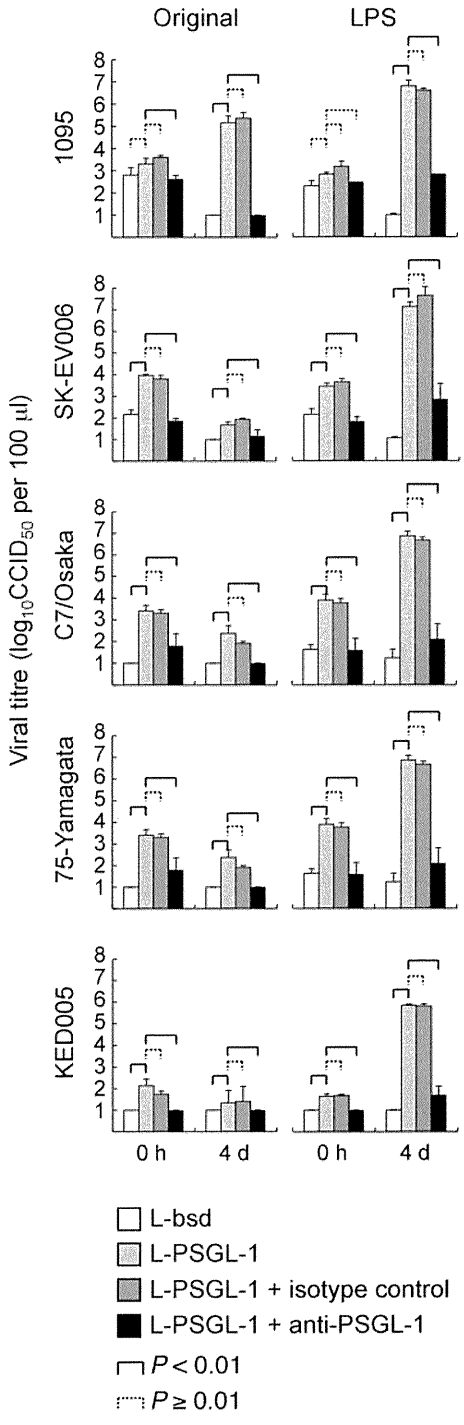

Fig. 1. EV71 replication in L-PSGL-1 cells in the presence of a PSGL-1-specific mAb. L-bsd cells were used as a PSGL-1-negative control.

PSGL-1-negative (RD), PSGL-1-positive (Jurkat) and human peripheral blood mononuclear cells (PBMC; purchased from Lonza Japan Ltd) with the original 1095 strain or 1095-LPS1 variant. RD (2.5×10^4 cells), Jurkat (4×10^4 cells) and human PBMC (2×10^5 cells) were inoculated with viruses of 2.5×10^4 (original 1095) or 2.5×10^3 (1095-LPS1) $CCID_{50}$ for 1 h at 37 °C (RD) or on ice (Jurkat or PBMC), washed and incubated in medium at 37 °C (RD) or 34 °C (Jurkat or PBMC). The original 1095 and 1095-LPS1 strains showed comparable replication kinetics in RD and Jurkat cells, and both of the EV71 strains did not grow well in human PBMC (Supplementary Fig. S2, available in JGV Online). We then tested the PSGL-1-dependent replication competence of other EV71-LPS variants in Jurkat cells, as described previously (Nishimura *et al.*, 2009). All five EV71-LPS variants replicated in Jurkat T cells in a PSGL-1-dependent manner, as demonstrated by the reduction of viral titres by anti-PSGL-1 mAb blockage at 3 days post-inoculation (Supplementary Fig. S3, available in JGV Online). These results indicate that the phenotypic difference between the original and L-PSGL-1-adapted EV71 strains was apparent in mouse L-PSGL-1 cells but not in human cells.

Finally, we examined mutations in the genomes of EV71 during the course of replication in L-PSGL-1 cells. We compared the complete nucleotide and deduced amino acid sequences between the original EV71 strains and the EV71-LPS variants. We extracted viral genomic RNA from the culture fluid of infected cells. We performed RT-PCR preparation of DNA fragments for DNA sequencing. The 5′ and 3′ ends of the viral genome were sequenced using the conventional RACE methods.

1095-LPS1 had two amino acid substitutions at VP2-K149M and VP3-Y29H compared with the original 1095 strain (Fig. 2). SK-EV006-LPS1 had four nucleotide mutations compared with the original SK-EV006 strain. Three out of the four mutations changed Y (C or T) to C or T in the 5′ non-translated region (NTR) and the region encoding non-structural proteins. The other mutation (A1398T) caused an amino acid substitution at VP2-K149I (Fig. 2). C7/Osaka-LPS1 and 75-Yamagata-LPS1 had only one amino acid substitution at VP2-K149 compared with the corresponding original EV71 strains (Fig. 2). Taken together, all four EV71-LPS1 variants had an amino acid substitution at VP2-K149I/M. Although the KED005-LPS2 variant did not have an amino acid substitution in VP2-K149, it had five amino acid substitutions in VP2 and VP1 (Fig. 2).

Previous studies have shown that the expression of specific cellular receptors for human enteroviruses on the cell surface of rodent cells allows effective viral replication similar to that in human susceptible cells expressing the corresponding viral receptors [human poliovirus receptor (Mendelsohn *et al.*, 1989), human intercellular adhesion molecule-1 (Shafren *et al.*, 1997), and human coxsackievirus and adenovirus receptor (Bergelson *et al.*, 1997)]. In the case of EV71, mouse L929 cells expressing human SCARB2 exhibit high susceptibility to EV71, which is comparable to human RD cells (Yamayoshi *et al.*, 2009). These results suggest that the efficacy of replication of enteroviruses, including EV71, mainly depends on the

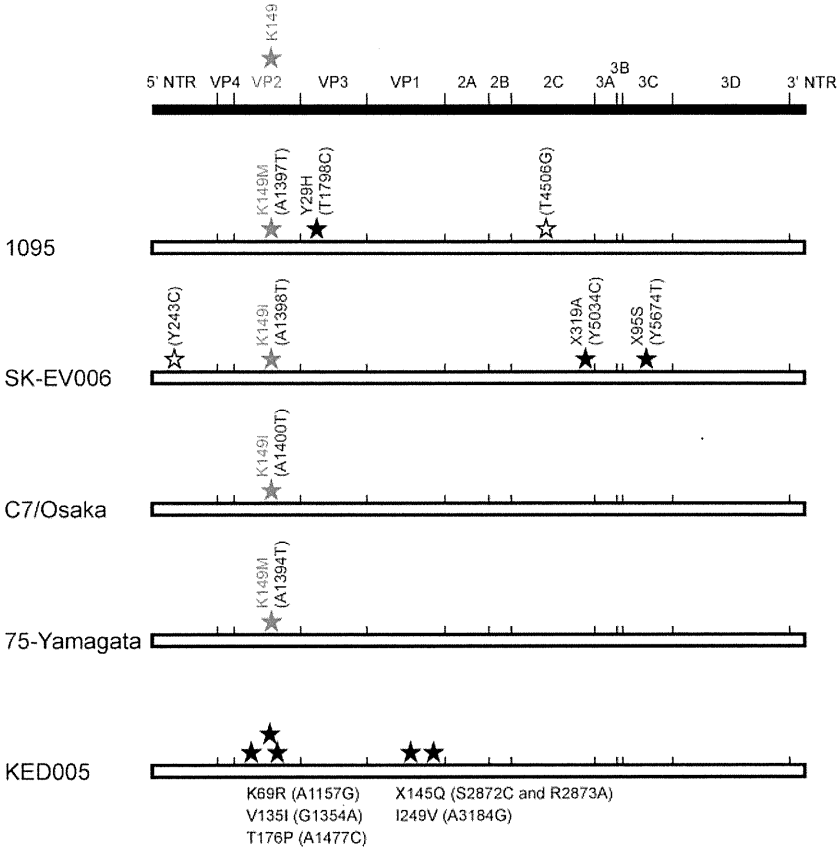

Fig. 2. Schematic of mutations identified in EV71-LPS1 (1095, SK-EV006, C7/Osaka and 75-Yamagata) and EV71-LPS2 (KED005) compared with the corresponding original EV71 strains. Amino acid substitutions observed in EV71-LPS are shown as black stars and VP2-149 are indicated as grey stars. A synonymous mutation and a mutation in the 5′ NTR are shown as white stars. The amino acid X indicates that its codon contains mixed nucleotides.

expression of specific receptors on the cell surface, even for rodent cell lines that are naturally non-permissive for enterovirus infection. In other words, after virus binding, entry and uncoating steps, which may be facilitated by virus-receptor interactions, rodent cells may support efficient viral replication of human enteroviruses.

On the other hand, not only the binding capability of the EV71 strains to PSGL-1, but also an adaptive mutation(s) in the capsid proteins was required for efficient viral replication of PSGL-1-binding strains of EV71 in L-PSGL-1 cells. A previous study using infectious molecular clones of EV71 revealed that a mutation at VP2-K149I is critical for efficient virus replication in Chinese hamster ovary cells (Chua *et al.*, 2008). In addition, one of the adaptive mutations was identified at VP2-K149 during the course of *in vivo* adaptation of EV71 by serial passaging of the virus in mouse brain (Wang *et al.*, 2004). Furthermore, a mouse-adapted EV71 variant (Nagoya-2876A strain) derived from a cDNA of the Nagoya strain of EV71 contains Ile at VP2-149 instead of the Lys residue that is conserved among other EV71 strains (Arita *et al.*, 2008). These results suggest that an amino acid substitution at VP2-K149 plays a critical role in the *in vitro* and *in vivo* adaptation of EV71 in rodent cells. Like human fibroblast cell lines, mouse L929 cells do not express detectable levels of mouse PSGL-1 (Thomas *et al.*, 2009). Therefore, mouse-adaptive mutations in the capsid proteins of EV71 may not be directly associated with a phenotypic change in EV71 variants with receptor-binding capability to mouse PSGL-1. For mouse-adapted poliovirus variants, some of the mouse adaptation determinants in the capsid proteins involve the efficacy of viral uncoating (Couderc *et al.*, 1996) and the others might be responsible for binding of the mutants to unidentified mouse receptor (Murray *et al.*, 1988). Likewise, it remains uncertain whether a mutation at VP2-K149 of EV71 is responsible for the change in tropism in a receptor-dependent or -independent manner.

Four out of five LPS variants, including 1095-LPS, contained an amino acid substitution at VP2-K149 after a single passage of the original EV71 strains in L-PSGL-1 cells. The VP2-K149 substitution confers only one amino acid difference between the original EV71 strains and the LPS variants of the C7/Osaka and 75-Yamagata strains, suggesting that the single amino acid at VP2-149 is a potential determinant for the adaptation phenotype of EV71 to L-PSGL-1 cells. For the KED005-LPS2 variant, an amino acid change at VP2-K149 was not identified, but instead, multiple amino acid substitutions (three in VP2 and two in VP1) were found after a second passage in L-PSGL-1 cells. Further analysis using infectious clones of EV71 will be required to elucidate the contribution of possible determinants for adaptation to mouse cells.

Mouse L929 cell lines expressing the human poliovirus receptor have played a critical role in laboratory diagnosis of polioviruses for global polio eradication (Hovi &

Stenvik, 1994; Pipkin *et al.*, 1993). Mouse cell lines expressing specific cellular receptors for EV71, PSGL-1 and SCARB2, may also be useful for receptor-specific isolation and identification of EV71 from clinical samples (Nishimura *et al.*, 2009; Yamayoshi *et al.*, 2009). However, as we have shown in this study, along with the PSGL-1-binding phenotype of EV71, the adaptation and selection bias among EV71 variants to grow in L-PSGL-1 cells should be carefully considered. Likewise, the mouse-adaptive phenotype of the EV71 strains and variants should also be taken into account when establishing transgenic mouse models carrying human receptors for EV71.

Acknowledgements

We are grateful to Junko Wada for excellent technical assistance. This work was supported by a Grant-in-Aid for Young Scientists (B, 21790452) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Y. N.). Y. N. and H. S. were supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases and a Grant-in-Aid for the Promotion of Polio Eradication from the Ministry of Health, Labour and Welfare, Japan.

References

Arita, M., Ami, Y., Wakita, T. & Shimizu, H. (2008). Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. *J Virol* **82**, 1787–1797.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg, R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323.

Chan, L. G., Parashar, U. D., Lye, M. S., Ong, F. G. L., Zaki, S. R., Alexander, J. P., Ho, K. K., Han, L. L., Pallansch, M. A. & other authors (2000). Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clin Infect Dis* **31**, 678–683.

Chua, B. H., Phuektes, P., Sanders, S. A., Nicholls, P. K. & McMinn, P. C. (2008). The molecular basis of mouse adaptation by human enterovirus 71. *J Gen Virol* **89**, 1622–1632.

Couderc, T., Delpeyroux, F., Le Blay, H. & Blondel, B. (1996). Mouse adaptation determinants of poliovirus type 1 enhance viral uncoating. *J Virol* **70**, 305–312.

Eskelinen, E. L., Tanaka, Y. & Saftig, P. (2003). At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol* **13**, 137–145.

Ho, M., Chen, E. R., Hsu, K. H., Twu, S. J., Chen, K. T., Tsai, S. F., Wang, J. R. & Shih, S. R. (1999). An epidemic of enterovirus 71 infection in Taiwan. *N Engl J Med* **341**, 929–935.

Hovi, T. & Stenvik, M. (1994). Selective isolation of poliovirus in recombinant murine cell line expressing the human poliovirus receptor gene. *J Clin Microbiol* **32**, 1366–1368.

Laszik, Z., Jansen, P. J., Cummings, R. D., Tedder, T. F., McEver, R. P. & Moore, K. L. (1996). P-selectin glycoprotein ligand-1 is broadly expressed in cells of myeloid, lymphoid, and dendritic lineage and in some nonhematopoietic cells. *Blood* **88**, 3010–3021.

McMinn, P. C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol Rev* **26**, 91–107.

Mendelsohn, C. L., Wimmer, E. & Racaniello, V. R. (1989). Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **56**, 855–865.

Mizuta, K., Abiko, C., Murata, T., Matsuzaki, Y., Itagaki, T., Sanjoh, K., Sakamoto, M., Hongo, S., Murayama, S. & Hayasaka, K. (2005). Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. *J Clin Microbiol* **43**, 6171–6175.

Murray, M. G., Bradley, J., Yang, X. F., Wimmer, E., Moss, E. G. & Racaniello, V. R. (1988). Poliovirus host range is determined by a short amino acid sequence in neutralization antigenic site I. *Science* **241**, 213–215.

Nagata, N., Shimizu, H., Ami, Y., Tano, Y., Harashima, A., Suzaki, Y., Sato, Y., Miyamura, T., Sata, T. & Iwasaki, T. (2002). Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. *J Med Virol* **67**, 207–216.

Nishimura, Y., Shimojima, M., Tano, Y., Miyamura, T., Wakita, T. & Shimizu, H. (2009). Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. *Nat Med* **15**, 794–797.

Pipkin, P. A., Wood, D. J., Racaniello, V. R. & Minor, P. D. (1993). Characterisation of L cells expressing the human poliovirus receptor for the specific detection of polioviruses *in vitro*. *J Virol Methods* **41**, 333–340.

Sako, D., Chang, X. J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J. & other authors (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* **75**, 1179–1186.

Shafren, D. R., Dorahy, D. J., Greive, S. J., Burns, G. F. & Barry, R. D. (1997). Mouse cells expressing human intercellular adhesion molecule-1 are susceptible to infection by coxsackievirus A21. *J Virol* **71**, 785–789.

Shimizu, H., Utama, A., Yoshii, K., Yoshida, H., Yoneyama, T., Sinniah, M., Yusof, M. A. B., Okuno, Y., Okabe, N. & other authors (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997–1998. *Jpn J Infect Dis* **52**, 12–15.

Shimizu, H., Utama, A., Onnimala, N., Li, C., Li-Bi, Z., Yu-Jie, M., Pongsuwanna, Y. & Miyamura, T. (2004). Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. *Pediatr Int* **46**, 231–235.

Thomas, G. M., Panicot-Dubois, L., Lacroix, R., Dignat-George, F., Lombardo, D. & Dubois, C. (2009). Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation *in vivo*. *J Exp Med* **206**, 1913–1927.

Wang, Y. F., Chou, C. T., Lei, H. Y., Liu, C. C., Wang, S. M., Yan, J. J., Su, I. J., Wang, J. R., Yeh, T. M. & other authors (2004). A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. *J Virol* **78**, 7916–7924.

Yamayoshi, S., Yamashita, Y., Li, J., Hanagata, N., Minowa, T., Takemura, T. & Koike, S. (2009). Scavenger receptor B2 is a cellular receptor for enterovirus 71. *Nat Med* **15**, 798–801.

Yang, F., Ren, L., Xiong, Z., Li, J., Xiao, Y., Zhao, R., He, Y., Bu, G., Zhou, S. & other authors (2009). Enterovirus 71 outbreak in the People's Republic of China in 2008. *J Clin Microbiol* **47**, 2351–2352.