Dengue virus strain DEN2 16681 utilizes a specific glycochain of syndecan-2 proteoglycan as a receptor

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INTRODUCTION

Dengue virus (DENV) is one of the pathogenic arboviruses that circulate among humans and mosquitoes. Over 100 million people are infected annually with DENV in South-East Asia, Africa, South and Central America and Pacific Island countries. DENV has four serotypes (DEN1–4) and usually causes dengue fever (DF), characterized by transient fever, arthralgia and myalgia. Some patients also develop severe symptoms, such as dengue haemorrhagic fever or dengue shock syndrome (Halstead, 1988).

DENV infection starts with the bite of a carrier mosquito into human skin. Immature dendritic cells and Langerhans cells in the skin are thought to be the initial target cells for virus propagation (Wu et al., 2000). Monocytes and macrophages have also been reported to propagate the virus (Halstead et al., 1977). In addition, some B-cells, T-cells and bone-marrow cells are susceptible to DENV (Bielemfeldt-Ohmann, 1998; Bielemfeldt-Ohmann et al., 2001). These cells have been implicated not only in DENV proliferation, but also in pathogenesis (Halstead, 1989; Halstead et al., 1977; La Russa & Innis, 1995). Such susceptibility is presumably due to the expression of specific cellular receptors that facilitate virus entry.

Antibody-dependent enhancement (ADE) was originally reported as one of the mechanisms for DENV entry. ADE occurs during secondary infection of monocytes and macrophages by heterologous DENV (Halstead & O'Rourke, 1977; Halstead et al., 1977), wherein FcγR forms a complex with anti-DENV IgG and the DENV virion for internalization (Littaua et al., 1990; Schlesinger & Chapman, 1999). However, other host receptors for ADE-independent DENV entry have since been identified (Chen et al., 1997; Miller et al., 2008; Navarro-Sanchez et al., 2003).

Heparan sulfate (HS) was reported as a DENV receptor in non-human mammalian cells, human liver cells and progenitor monocyte cells (Bielemfeldt-Ohmann, 1998; Chen et al., 1997; Germi et al., 2002; Hung et al., 1999; Thepparit et al., 2004). It has also been demonstrated that DENV uses a secondary high-affinity receptor after binding to HS (Belting, 2003). HS is attached to HS proteoglycans (HSPGs); many HSPGs have been reported to date, including glypican 1–6 (GPC1–6), syndecan 1–4 (SDC1–4), betaglycans, CD44 and some other proteins that bind HS (Kramer & Yost, 2003). Although HSPGs are critical for elucidating the detailed pathway of HS-mediated DENV
entry, no specific HSPG has been recognized as a DENV receptor.

DC-SIGN (CD209) and DC-SIGNR (CD209R), which belong to the Ca\(^{2+}\)-dependent lectin (C-type lectin) family, have been reported as DENV receptors in human dendritic cells (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). Similar to DC-SIGN, C-type lectin mannos receptor has been identified as a receptor in macrophages (Miller et al., 2008). Recently, the monocyte-expressed C-type lectin CLEC5A was identified as a DENV-binding factor and inducer of inflammatory-cytokine signalling (Chen et al., 2008). In our present study, the DENV-susceptible K562 cell line was used to elucidate a mechanism of DEN2 16681 strain entry into target cells.

K562 cells differentiate spontaneously to early-stage monocytes, granulocytes and erythroid cells (Lozzio et al., 1981). K562 cells are known to propagate DENV (Littaua et al., 1990; Nakao et al., 1989) and to express Fc\(_r\) for ADE-dependent DENV entry (Littaua et al., 1990). An uncharacterized 100 kDa protein and glycosphingolipid (paragloboside, nLc4Cer) were also identified as DEN2-binding factors in K562 cells (Aoki et al., 2006; Rothwell et al., 1996).

The DEN2 16681 strain has been used in in vivo and in vitro studies involving a mouse model, a non-human primate model and permeability examination using human vascular endothelial (HUVEC) cells to elucidate DENV pathogenesis (Chen et al., 2007; Onlamoong et al., 2010; Srikiatkhachorn et al., 2007; Wu-Hsieh et al., 2009). The DEN2 16681 strain induced haemorrhage in mice more readily than a DEN2 strain isolated from a DF patient, and this was attributed to its faster rate of replication and enhanced level of viraemia (Wu-Hsieh et al., 2009).

In our present study, we established a K562-derived cell line that is highly susceptible to the DEN2 16681 strain, characterized a novel DENV receptor in this cell line and identified the S41 glycochain as an essential entry factor. Also, this is the first report that DEN2 16681 and some other DENV strains utilize the HSPG SDC2 as a receptor, and it seems that the choice of this receptor is associated with tissue-culture adaptation of DENV. Our findings could contribute to elucidation of the detailed HS-dependent DEN2 entry mechanism and understanding of HS adaptation, and to further study of the relation between pathogenesis and HS adaptation.

## RESULTS

### Cloned K562 cell lines acquired high DEN2 16681 strain susceptibility

The original K562-rcb0027 cell line was infected weakly by the DEN2 16681 strain, and was detected not to bind to this strain (Fig. 1a, b). In comparison, some K562 clones (clones 1–5), which were established by limiting dilution of the original K562 cell line, had enhanced DEN2 infection (Fig. 1a, b). DEN2 binding was increased in K562-clone1 to -4, but not in K562-clone5 (Fig. 1b). K562-clone3 had the highest DEN2 infection and binding, with about 70% of cells being infected by day 1 post-infection (p.i.) at an m.o.i. of 1 (Fig. 1a) and approximately 60% of the cells being infected...
positive for virus binding (Fig. 1b). The infection rate increased thereafter and was maintained at around 90 %, whereas <5 % of K562-rcb0027 cells were infected until day 3 p.i. (Fig. 1a). These data indicate that the K562 clone cell lines, especially K562-clone3, acquired high susceptibility to the DEN2 16681 strain. Infection by DEN2 correlated with its binding to the cloned K562 cells, except for K562-clone5. We therefore hypothesized that a virus entry receptor is highly expressed in the K562-clone1 to -4 cell lines.

**SDC2 is critical for DEN2 16681 strain susceptibility of K562-clone3 cells**

To obtain receptor candidates that may serve as a DEN2 16681 strain entry factor in K562-clone3 cells, expression levels of 2836 human genes of plasma-membrane proteins in the cells were compared comprehensively with those of K562-rcb0027 cells by microarray. Of the 2836 genes, 159 were exclusively upregulated in K562-clone3 cells. Specifically, 11 of 159 genes had significantly high expression in K562-clone3, and these were considered as DEN2 16681 strain receptor candidates (Supplementary Table S1, available in JGV Online).

To identify the receptor, a DEN2-binding assay was performed after treatment of K562-clone3 cells with small interfering RNAs (siRNAs) corresponding to the 11 genes (Supplementary Fig. S1, available in JGV Online). DEN2 binding was inhibited only by sdc2 siRNA treatment (Supplementary Fig. S1). SDC2 expression in K562-clone3 cells was reduced significantly at days 1 and 2 post-treatment with sdc2 siRNA (Fig. 2a). Compared with control (ctl) siRNA-treated cells, the DEN2-binding rate decreased by between 50 and 70 % after sdc2 siRNA treatment (Fig. 2b, c). Infectivity of DEN2 virus was also compared between ctl and sdc2 siRNA-treated cells. The sdc2 siRNA reduced DEN2 infectivity at days 1 and 2 p.i. (Fig. 2d, e). These results suggest that SDC2 is responsible for DEN2 16681 strain susceptibility in the K562-clone3 cell line. On the other hand, the HUVEC cell line, which has been reported to be infected by DENV in an HS-dependent manner (Dalrymple & Mackow, 2011), displayed slightly reduced DEN2 16681 infectivity and binding after sdc2 siRNA treatment; however, there were no significant differences (Supplementary Fig. S2, available in JGV Online).

To confirm the importance of SDC2 for DEN2 16681 strain susceptibility of K562 cells, an SDC2-expressing K562-rcb0027 stable cell line (K562-rcb0027/SDC2-WT) was established. The SDC2 knock-in cell line expressed SDC2 stably on its plasma membrane (Fig. 3a). K562-rcb0027/puro (negative control) and K562-rcb0027/SDC2-WT cells were infected with the DEN2 16681 strain. DEN2 susceptibility was increased significantly in K562-rcb0027/SDC2-WT cells (Fig. 3b). These data indicate that SDC2 expression is sufficient for augmenting the DEN2 16681 strain susceptibility of K562-rcb0027 cells.

**SDC2-attached S41-HS is implicated in the DEN2 16681 strain susceptibility of cells**

SDC2 is an HSPG and its bound HS can be expected to interact with DENV. The DEN2 16681 strain was treated with heparin prior to inoculation onto K562-clone3 cells to inhibit HS-mediated binding of virus particles to the cells. Heparin treatment almost completely inhibited the binding capacity of DEN2 in K562-clone3 cells (Fig. 4a).

K562 clone3 and Vero cells were treated with heparinase I and III, which cleave HS from HSPGs. The percentage of K562-clone3 cells with bound DEN2 was reduced slightly at 0.1–1.0 mU heparinase I treatment, whereas that of Vero cells with bound DEN2 was reduced slightly more by the same treatment. Heparinase III treatment at 0.1 mU resulted in a large reduction in the percentage of K562-clone3 cells with bound DEN2; however, in Vero cells the reduction was lower (Fig. 4b). These results indicate that DEN2 binding is mediated by heparinase I-resistant HS on SDC2.

SDC2 has four predicted HS-attachment sites at residues S41, S53, S55 and S57 (Bernfield et al., 1992). To clarify whether SDC2-attached HS is critical for DEN2 binding, two specific glycochain-knockout SDC2-expressing cell lines were established, namely K562-rcb0027/SDC-S41-HS(−) and K562-rcb0027/SDC2-S53/S55/S57-HS(−) (Fig. 5a).

Expression of SDC2 was confirmed on the plasma membrane of these cell lines (Fig. 5b). K562-rcb0027/SDC2-WT and K562-rcb0027/SDC2-S41/53/55/57-HS(−) cells showed an increase in DEN2-binding capacity, whereas K562-rcb0027/SDC2-S41-HS(−) cells did not show DEN2 binding (Fig. 5c, d). Similar to its binding capacity, DEN2 infectivity was not increased in K562-rcb0027/sdc2-s41a cells (Fig. 5e). These results demonstrate clearly that S41-attached HS of SDC2 is critical for SDC2-mediated infection.

**Expression level of sdc2 genes in cloned K562 cell lines**

Gene expression of sdc2 was checked further in the K562 clones and K562-rcb0027 cell line. The sdc2 gene was detected in K562-clone1 to -4 cells, but not in K562-clone5. K562-clone1 and K562-clone3 expressed sdc2 at a level similar to that in HUVEC cells, whereas K562-clone2 and K562-clone4 had much higher expression (Fig. 6). Correspondingly, these four sdc2-expressing K562 clone cells had high DEN2-binding capacity (Fig. 1b).

**SDC2-mediated susceptibility of SDC2 knock-in K562 cells is specific for certain DEN2 strains**

HS-sensitive and -insensitive DEN2 and DEN4 variants were demonstrated previously both in vivo and in vitro, and these variants were implicated in dengue disease severity (Añez et al., 2009; Prestwood et al., 2008). Thus, susceptibility to other DENV strains was examined in the SDC2 knock-in cell line (K562-rcb0027/SDC2-WT). K562-rcb0027/puro and K562-rcb0027/SDC2-WT cells were
infected with 19 primary DENV strains derived from patients in the Philippines, Thailand, Bangladesh and Vietnam, and four tissue culture-adapted DENV strains (DEN1 Hawaii, DEN2 NGB, DEN2 16681 and DEN4 no. 17 strains). Only the DEN2 16681 and DEN2 NGB strains showed high infectivity (Fig. 7), suggesting that SDC2 binding was strain-specific and that this specificity seems to be associated with tissue-culture adaptation.

**DISCUSSION**

Some articles have shown that HS is critical for DENV entry (Bielefeldt-Ohmann, 1998; Chen et al., 1997; Germi et al., 2002; Hung et al., 1999; Thepparit et al., 2004), but this is the first report that directly identifies SDC2 out of several HSPG candidates as a DEN2 receptor (Figs 2, 3). Furthermore, SDC2 S41–HS out of four HS glycochains studied was shown to be specifically critical for the DEN2 16681 strain (Fig. 5). This demonstrates that DEN2 infection mediated by HS can be regulated by a specific HS-attachment site. Also, this SDC2-mediated infection was demonstrated to be DEN2 16681 and NGB strain-specific (Fig. 7), which indicates that the SDC2-dependent pathway of infection is tissue culture-adapted DEN2 strain-specific.

K562 cells are known to express other HSPGs such as SDC3 and betaglycans (Saphire et al., 2001). This has been
confirmed in our microarray data from K562-rcb0027 cells (data not shown). Although these HSPGs also have HS, K562-rcb0027 cells did not show significant levels of DEN2 binding or infectivity (Fig. 1a, b). Hence, DEN2 susceptibility is not decided by the mere presence of HSPGs. In addition, the K562-clone3 cell line, the most DEN2-susceptible cell line (Fig. 1a, b), did not show the highest sdc2 gene expression level among K562 clone cell lines (Fig. 6), suggesting that some other factors in collaboration with SDC2 may be required for maximum infectivity and binding of DENV. This may be associated with a different effect on K562-clone3 and HUVEC cell lines after treatment of sdc2 siRNA (Fig. 2; Supplementary Fig. S2). Thus, the identification of SDC2 S41–HS as a DENV receptor could lead to the discovery of other collaborating host factors and to the elucidation of a more detailed mechanism for HS-mediated DENV entry.

The SDC2-bound HS glycans in K562-clone3 cells seem to be structurally different from the HS glycans of other cells. HS-mediated binding in other DEN2-susceptible cell lines is affected by heparinase I and III (Chen et al., 1997; Germi et al., 2002; Hilgard & Stockert, 2000). It has been reported that highly sulfated HS is modified in these cells, and that the HS-sulfation rate is crucial for DENV affinity of these cells (Chen et al., 1997). In contrast to these heparinase I-sensitive cell lines, including the Vero cell line, K562-clone3

Fig. 3. DEN2 susceptibility of SDC2 knock-in K562-rcb0027 cell lines. The sdc2 gene knock-in K562-rcb0027 cell line was established as described in Methods. (a) SDC2 expression in K562-rcb0027/puro and K562-rcb0027/SDC2-WT. These cells were treated with anti-SDC2 antibody (grey line) or goat IgG antibody control (filled histogram), then SDC2-positive cells were detected using anti-goat IgG–FITC treatment. (b) K562-rcb0027/puro cells (negative control; black bars) and K562-rcb0027/SDC2-WT cells (grey bars) were infected with DEN2 at an m.o.i. of 1. Percentage of infected cells was measured by flow cytometry at days 1, 2 and 3 p.i. The mean DEN2-positive value of the cells was obtained from duplicate samples.

Fig. 4. DENV binds heparinase I-resistant HS in the K562-clone3 cell line. (a) Effect of heparin treatment on DEN2 binding to K562-clone3 cells. DEN2 viruses were treated with or without 1.0 U heparin sodium. K562-clone3 cells were incubated with the heparin-treated or untreated viruses at an m.o.i. of 50. Percentage of DEN2-binding cells was measured by flow cytometry. The mean DEN2-positive value of the cells was obtained from duplicate samples. (b) Effect of heparinase treatment on DEN2 binding to K562-clone3 and Vero cells. These cells were treated with heparinase I or III at 0, 0.1, 0.5, 1.0 and 5.0 mU for 1 h on ice prior to virus inoculation. Percentage of DEN2-binding cells was measured by flow cytometry. The mean DEN2-positive value of the cells was obtained from duplicate samples. ■, Heparinase I/Vero; ▲, heparinase III/Vero; ●, heparinase I/K562-clone3; ○, heparinase III/K562-clone3.
Fig. 5. DEN2 binding in SDC2 mutant knock-in K562-rcb0027 cell lines. (a) SDC2 HS-attachment site and HS-deleted SDC2 mutant. SDC2 has HS glycochains on four predicted HS-attachment sites: Ser41, Ser53, Ser55 and Ser57. HS glycochains were deleted by S41A [SDC2-S41-HS(−)] and S53A/S55A/S57A [SDC2-S53/S55/S57-HS(−)] mutation, and these mutant-expressing K562-rcb0027 cell lines were established as described in Methods. (b) SDC2 expression in K562 knock-in cell lines. K562-rcb0027/puro, K562-rcb0027/SDC2-WT, K562-rcb0027/SDC2-S41-HS(−) and K562-rcb0027/SDC2-S53/S55/S57-HS(−) cells were treated with anti-SDC2 antibody or goat IgG antibody control, and SDC2-positive cells were then detected using anti-goat IgG–FITC treatment. (c, d) DEN2 binding in sdc2 knock-in K562 stable cell lines. DEN2 binding in K562-rcb0027/puro, K562-rcb0027/SDC2-WT, K562-rcb0027/SDC2-S41-HS(−) and K562-rcb0027/SDC2-S53/S55/S57-HS(−) cells was checked by flow cytometry. (c) Histogram data of DENV binding in K562-rcb0027 sdc2 or sdc2 mutant knock-in K562 cell lines. (d) Percentage of DEN2 binding in sdc2 or sdc2 mutant knock-in K562 cell lines. The mean DEN2-positive value of the cells was obtained from duplicate samples. (e) DEN2 infectivity in sdc2 or sdc2 mutant knock-in K562 cell lines. K562-rcb0027/puro, K562-rcb0027/SDC2-WT, K562-rcb0027/SDC2-S41-HS(−) and K562-rcb0027/SDC2-S53/S55/S57-HS(−) cells were infected with DEN2 at an m.o.i. of 1. Percentage of infected cells was measured by flow cytometry at day 1 p.i. The mean DEN2-positive value of the cells was obtained from duplicate samples.
and this specificity was also shown in knock-in K562-rcb0027/SDC2-WT cells (Fig. 7). In this report, 21 DENV strains, excluding the DEN2 16681 and DEN2 NGB strains, did not propagate well in the knock-in cells (Fig. 7). The DEN2 16681 and DEN2 NGB strains were passaged in green monkey kidney LLC-MK2 cells and mouse brain, respectively, several times (Kinney et al., 1997; Schlesinger & Frankel, 1952). SDC2 has been reported to be expressed in fibroblast and neuronal cells (Essner et al., 2006). There have been several reports that acquisition of an increased surface positive charge of flaviviruses during tissue culture enhanced virus entry into cells (Añez et al., 2009; Lee & Lobigs, 2002; Mandl et al., 2001; Prestwood et al., 2008). Thus, it is likely that, in our study, the viruses adapted to their host cells, acquired a higher positive charge on their surface and in turn gained a high affinity for SDC2. However, we cannot exclude the possibility that a small proportion of DEN2 quasispecies, which had SDC2 affinity, was circulating in the patient’s blood and was selectively enhanced during the passages. If SDC2-sensitive quasispecies are circulating in a patient’s blood, it may have an influence on the pathogenicity of DENV and thus it will be crucial to confirm and characterize such possible quasispecies in patients’ blood in future work.

Finally, these studies will be useful as a model for further evaluating HS sensitivity of DENV strains and other HS-sensitive flaviviruses. Also, these findings will help to clarify

**Fig. 6.** Gene expression of sdc2 varies among cloned K562 cell lines. Gene expression of sdc2 was compared among K562 clones, HEK293 and HUVEC cell lines by RT-PCR, with gapdh expression as the internal control.

**Fig. 7.** DENV susceptibility of sdc2 knock-in K562-rcb0027 cells. DENV-infected K562-rcb0027/puro and K562-rcb0027/SDC2-WT cells after infection with 19 DENV strains derived from patients in the Philippines, Thailand, Bangladesh and Vietnam, and four tissue culture-adapted DENV strains (DEN1 Hawaii, DEN2 NGB, DEN2 16681 and DEN4 no. 17) were used for this assay. Cells were collected at day 4 p.i. and DENV-infected cells were measured by flow cytometry. *Infectivity was below the detectable level.
the detailed mechanism of HS-mediated DENV entry and pathogenesis.

**METHODS**

Cells and viruses. The C6/36 Aedes albopictus mosquito cell line (Igarashi, 1979) was cultured at 28 °C in minimum essential medium (MEM). Vero cells (African green monkey kidney-derived cell line), BHK cells (baby hamster kidney cell line) and HEK293 cells (human embryonic kidney cell line), all from ATCC, were cultured at 37 °C in MEM. K562-rchb0027 cells, a human lymphoid cell line (RIKEN BioResource Center), and K562 clone cells (described in this article) were cultured at 37 °C in RPMI 1640 medium (Gibco). HUVEC cells, a human umbilical vein endothelial cell line (Lonza), were cultured at 37 °C in EBM-2 medium supplemented with EGM-2 BulletKit (endothelial cell growth factors; TaKaRa) and 0.1 U penicillin ml⁻¹/0.1 mg streptomycin ml⁻¹. All media except EBM-2 were supplemented with 10% FBS and 0.1 U penicillin ml⁻¹/0.1 mg streptomycin ml⁻¹. K562 cells were cloned by limiting dilution and five clones were obtained. DENV infectivity and binding in these clones were checked by flow cytometry as described below. Vero and BHK cells were grown in 12- and 96-well plates for virus titration using plaque and focus-formations assays, respectively. A DENV (DEN2 16681 strain; GenBank accession no. U87411) infectious clone (Kinney et al., 1997) was used for this study. Tissue culture-adapted DENV, i.e. DEN1 Hawaii, DEN2 NGB and DEN4 no. 17, were used for this study. Patient-derived DENVs, i.e. BDH16-09 (GenBank accession no. JN036372), BDH83-06 (JN036378) and BDH127-07 (JN036380) from Bangladesh; 00st-12A (GU772287), 99st-12A, 99st-310, 99st-338, 99st-628, SLMC50 and SLMC318 from the Philippines; THN07/93 (AF002434), TG-235, S.M.-2H2, BT.M.-93, THNHA-37, THNHO-33 and ThNHA-43 from Thailand; and VH18-C from Vietnam were also used in this study. Several DENV isolates were not completely sequenced, but their serotype was identified by using a conventional RT-PCR method. DENVs were propagated in C6/36 cells and the viruses were titrated and used for various assays.

**Plasmid construction.** Total mRNA was extracted from the lysates of K562-cloned3 cells by using a FastPure RNA kit (TaKaRa) and cDNA was synthesized by reverse transcription (SuperScript III; Invitrogen). The sdc2-wt gene was amplified from this cDNA using the following primers: F: 5′-GTTAGAACCGTCCGGTCCAGGTTATC-3′ and R: 5′-GGCTGTCGATGACTCCTTG-3′. The 12D11/7E8 anti-flavivirus antibody was used for detecting DEN2 E glycoprotein by flow cytometry as described previously (Kinoshita et al., 2009). This 12D11/7E8 anti-flavivirus antibody was used for titrating DENV. Goat anti-human SDC2 (sc-9492; Santa Cruz Biotechnology) and donkey anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology) antibodies were used for flow cytometry. Mouse anti-β-actin (sc-69789), rabbit anti-human SDC2 (sc-15348), goat anti-mouse IgG-HRP (sc-2005; all from Santa Cruz Biotechnology) and anti-rabbit IgG-HRP (A102PT; American Qualex Antibodies) were used for immunoblotting. Heparin sodium (081-00136; Wako) was used for the heparin inhibition assay. Heparinase I and III (50-0120-012; IBEX Technology Inc.) were used for the heparinase assay.

**Microarray.** In total, 1 × 10⁶ K562-rchb0027 and K562-cloned3 cells were harvested. Total mRNA was extracted by using a FastPure RNA kit and the mRNA concentration was adjusted to 100 ng µl⁻¹. mRNA expression of these cells was measured by a commercial microarray service (Bio Matrix Research). After evaluating the RNA quality, double-stranded cDNA was synthesized from 200 ng RNA by following the protocol of the Quick Amp Labelling kit (Amersham). Then, hybridization was performed using a Whole Human Genome Oligo Microarray (Agilent). At this time, cDNAs of K562-cloned3 and K562-rchb0027 cells were labelled with Cy3 and Cy5, respectively, or switched to Cy5 and Cy3, respectively. After hybridization, plates were washed and fluorescence was read using an Agilent Microarray Scanner. Individual fluorescence was calculated by using Feature Extraction software (Agilent). Genes of K562-cloned3-upregulated plasma-membrane proteins were listed. These genes were analysed further by RNA interference (RNAi) silencing.

**RNa silencing.** The siRNAs were designed and synthesized by Invitrogen Stealth Select RNA Services. K562-cloned3 cells and HUVEC cells (1.0 × 10⁶) were transfected with 40 nM siRNA in six-well plates using Lipofectamine RNAiMAX reagent (Invitrogen). For analysis of DEN2 binding, cells were collected 2 days after siRNA treatment; for DEN2 infectivity, cells were collected 1, 2 and 3 days after siRNA treatment.

**Immunoblotting.** In total, 5 × 10⁶ cells of blood and bone-marrow cell lines and 1 × 10⁶ HUVEC cells were harvested. Cells were washed with PBS buffer and lysed by 8 M urea, 2% CHAPS, 20 mM DTT (50 µl) for 30 min on ice. After collecting the supernatants, 50 µl × 2 sample buffer (62.5 mM Tris/HCl, pH 6.8, 4% SDS, 10% mercaptoethanol and 0.1% bromophenol blue) was added to the samples, and the samples were heated for 3 min at 95 °C. Ten microlitres of sample was loaded onto a 10–20% polyacrylamide gradient gel (e-PAGEI; ATTO), and samples were separated by SDS-PAGE. Western blot was performed following a protocol described previously (Yu et al., 2007). For the primary antibody reaction, PVDF membranes were treated with mouse anti-β-actin (1:1000 dilution) or rabbit anti-SDC2 (1:200 dilution) polyclonal antibodies. For the
secondary antibody reaction, PVDF membranes were treated with the corresponding anti-mouse IgG–HRP (1:1000 dilution) or anti-rabbit IgG–HRP (1:1000) antibodies.

RT-PCR. For RT-PCR, 1 × 10^6 cells were harvested. Total mRNA was extracted by using a FastPure RNA kit. Then, cDNAs were synthesized by reverse transcription (SuperScript III). Partial sequence of sd2 genes was amplified from the cDNAs using these primers: F4 (5′-GTAGAGATGATGACCACTTGTTGCGT-3′) and R4 (5′-GGATGGTGGTTGCGT-GTCTCC-3′). Partial sequences of gapdh genes were amplified as an internal control from the same cDNAs using these primers: F5 (5′-TCTCATGACAGAATCCTTGTTGCGT-3′) and R5 (5′-CTCTTCC-TCTTGTGCCTTGTTGCGT-3′).

Heparin and heparinase treatment. Virus samples (5.0 × 10^5 p.f.u.) were treated or not with 50 U heparin sodium ml⁻¹ for 1 h at 37 °C. Then, each virus sample was added to 1 × 10^5 cells and incubated for 1 h on ice. K562-clones and Vero cells (1.0 × 10^5 cells) were treated with 0, 0.1, 0.5, 1.0, and 5.0 μM heparinase I and III for 1 h on ice. Then, virus samples were added to the cells and incubated for 1 h on ice. Detection of DEN2-binding cells was done by following the protocol described below.

Preparation of flow-cytometry samples. To detect cell-surface SDC2, 1 × 10^5 PBS-washed cells were treated with goat anti-human SDC2 (1:20 dilution) or normal goat IgG control (1:40 dilution) for 30 min. After cells were washed twice with 1 ml PBS, cells were treated with anti-goat IgG–PE (1:20 dilution) for 30 min on ice. Then, cells were washed with 1 ml PBS twice, and resuspended in 500 μl PBS for measurement by flow cytometry.

Flow cytometry was performed by using a FACScan (Beckman Coulter) with a 488 nm laser for detecting PE fluorescence. Ten thousand cells were used for data analysis.

Virus-binding and -infectivity assays. For the virus-binding assay, 1 × 10^5 cells were washed with PBS, and then virus samples were added to cell pellets on ice. After 1 h incubation on ice, the cells were washed twice with 1 ml Hanks’ balanced salt solution (HBSS; Gibco) supplied with 0.5 % BSA and 0.05 % NaN₃. Twenty microlites of Alexa Fluor 488-labelled 12D11/7E8 anti-flavivirus antibody was next added to the cells on ice. After 30 min incubation on ice, cells were washed twice with 1 ml HBSS. Finally, cells were resuspended in 500 μl PBS buffer.

For the virus-infectivity assay, 5 × 10^5 blood and bone-marrow cells were infected with the virus at an m.o.i. of 1, and 1 × 10^5 HUVEC cells were infected with the virus at an m.o.i. of 5. After the indicated no. of days p.i., cells were harvested and washed twice with 1 ml HBSS. Then, 100 μl fixation buffer (4.0 % paraformaldehyde in PBS) was added for 20 min incubation at room temperature. After washing the cells with 1 ml HBSS, 100 μl permeabilization buffer (1 % Nonidet P-40 in PBS) was added for 5 min incubation at room temperature. Cells were washed with 1 ml HBSS, after which 20 μl Alexa Fluor 488-labelled 12D11/7E8 anti-flavivirus antibody (40 μg ml⁻¹) was added to the cells. After 30 min incubation on ice, cells were washed twice with 1 ml HBSS, then resuspended in 500 μl PBS buffer.

Flow cytometry was performed by using a FACScan with a 488 nm laser for detecting Alexa Fluor 488 fluorescence. Blood and bone-marrow cells (1 × 10^5) and HUVEC cells (1 × 10^5) were used for data analysis.

ACKNOWLEDGEMENTS

We acknowledge Lyre Anni E. Murao and Corazon C. Buerano from the Department of Virology, Institute of Tropical Medicine, Nagasaki University and Global COE Program, Nagasaki, Japan, for English proofreading and helpful advice, and Richard M. Kinney at the Centers for Disease Control and Prevention, Division of Vector-borne Diseases, Fort Collins, CO, USA, for kindly donating the DEN2 16681 strain infectious clone. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (no. 1865913), a Grant-in-Aid for JSPS Fellows (Japan Society for the Promotion of Science), MEXT, Japan, the Global COE program, MEXT, Japan, the Japan Initiative for Global Network on Infectious Diseases (J-GRID), MEXT, Japan, and the Research and Japan Student Services Organization, Japan.

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