



## Characterization of retrovirus-based reporter viruses pseudotyped with the precursor membrane and envelope glycoproteins of four serotypes of dengue viruses

Hsien-Ping Hu<sup>a</sup>, Szu-Chia Hsieh<sup>a</sup>, Chwan-Chuen King<sup>c</sup>, Wei-Kung Wang<sup>a,b,\*</sup>

<sup>a</sup> Institute of Microbiology, College of Medicine, National Taiwan University, No 1 Sec1 Jen-Ai Rd, Taipei 100, Taiwan

<sup>b</sup> Department of Internal Medicine, National Taiwan University Hospital, No 7 Chung-Shan S Rd, Taipei 100, Taiwan

<sup>c</sup> Institute of Epidemiology, College of Public Health, National Taiwan University, No 1 Sec1 Jen-Ai Rd, Taipei 100, Taiwan

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### Abstract

In this study, we successfully established retrovirus-based reporter viruses pseudotyped with the precursor membrane and envelope (PrM/E) proteins of each of the four serotypes of dengue viruses, which caused the most important arboviral diseases in this century. Co-sedimentation of the dengue E protein and HIV-1 core proteins by sucrose gradient analysis of the pseudotype reporter virus of dengue virus type 2, D2(HIVluc), and detection of HIV-1 core proteins by immunoprecipitation with anti-E monoclonal antibody suggested that dengue viral proteins were incorporated into the pseudotype viral particles. The infectivity in target cells, as assessed by the luciferase activity, can be inhibited by the lysosomotropic agents, suggesting a pH-dependent mechanism of entry. Amino acid substitutions of the leucine at position 107, a critical residue at the fusion loop of E protein, with lysine resulted in severe impairment in infectivity, suggesting that entry of the pseudotype reporter virus is mediated through the fusogenic properties of E protein. With more and more dengue viral sequences available from different outbreaks worldwide, this sensitive and convenient tool has the potential to facilitate molecular characterization of the PrM/E proteins of dengue field isolates.

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### Introduction

Among the 70 or so arthropod-borne flaviviruses, epidemics of the four serotypes of dengue virus (DENV1, DENV2, DENV3, and DENV4) continue to be a major public health problem in the tropical and subtropical areas (Gubler, 2002; Guzman and Kouri, 2002). It has been estimated that more than 2.5 billion people in over 100 countries are at risk of infection, and approximately 100 million dengue infections occur annually worldwide (Gubler, 2002; Guzman and Kouri, 2002). The clinical presentations of dengue virus infection range from asymptomatic infection, to a relatively mild disease, dengue fever, and severe and potentially life-threatening

diseases, dengue hemorrhagic fever/dengue shock syndrome (Gubler, 2002; Guzman and Kouri, 2002).

Dengue viruses are members of the genus *Flavivirus* in the family *Flaviviridae*. It contains a positive-sense, single-stranded RNA genome of approximately 10,600 bases in length. Flanked by the 5' and 3' non-translated regions, the single open reading frame of the genome encodes a polyprotein precursor, which is subsequently cleaved by cellular and viral protease into three structural proteins, the capsid (C), precursor membrane (PrM), and envelope (E), as well as seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach and Rice, 2001). After the interaction of E protein with the cellular receptor, dengue virus is believed to enter the cell through receptor mediated endocytosis (Guirakhoo et al., 1993; Lindenbach and Rice, 2001; Mukhopadhyay et al., 2005; Randolph and Stollar, 1990). The acidic environment in the endosome triggers a series of conformational changes of E protein, which result in the fusion of viral and endosomal

\* Corresponding author. Institute of Microbiology, National Taiwan University, No 1 Sec1 Jen-Ai Rd, Taipei 100, Taiwan. Fax: +886 2 2391 5293.

E-mail address: [wwang60@yahoo.com](mailto:wwang60@yahoo.com) (W.-K. Wang).

membranes and release of the nucleocapsid into cytoplasm (Lindenbach and Rice, 2001; Modis et al., 2004; Mukhopadhyay et al., 2005). Following translation and genome replication, assembly occurs in the membrane structures derived from ER and the virions are released through the secretory pathway (Lindenbach and Rice, 2001; Mukhopadhyay et al., 2005).

The PrM/E proteins of dengue virus, present as a heterodimer on the surface of the virion, are the major determinants of cellular tropism of dengue virus (Bray et al., 1998; Chen et al., 1995; Gualano et al., 1998; Kawano et al., 1993; Lindenbach and Rice, 2001; Modis et al., 2004; Mukhopadhyay et al., 2005). In addition, PrM/E proteins are the major targets of neutralizing and enhancing antibodies, which are thought to play important roles in the pathogenesis of dengue (Green and Rothman, 2006; Guzman and Kouri, 2002; Halstead, 1988; Lindenbach and Rice, 2001). With the establishment of full-length infectious cDNA clones of dengue viruses and generation of chimeric clones, the functions of PrM/E proteins in the context of viral particles can be investigated (Blaney et al., 2004; Bray and Lai, 1991; Chen et al., 1995; Gualano et al., 1998; Kapoor et al., 1995; Kinney et al., 1997; Lai et al., 1991; Puri et al., 2000; Sriburi et al., 2001; Whitehead et al., 2003a,b). However, the requirement of multiple steps including *in vitro* transcription, RNA transfection and infectivity assay, which is laborious and time-consuming, has restricted its wide use. A convenient system is needed to facilitate molecular characterization of the PrM/E proteins of dengue viruses.

Pseudotype reporter viruses that contain the E protein of one virus and the core and genome with reporter gene of another virus have been shown to be a convenient molecular tool to study the functions of E protein, such as tropism, mechanism of entry, sensitivity to neutralization, enhancement and entry inhibitors (Bartosch et al., 2003; Buonocore et al., 2002; Chan et al., 2001; Cronin et al., 2005; Deng et al., 1997; Fukushi et al., 2005; Giroglou et al., 2004; Hanika et al., 2005; Hsu et al., 2003; Ma et al., 1999; Matsuura et al., 2001; Moore et al., 2004; Nie et al., 2004; Simmons et al., 2004; Wool-Lewis and Bates, 1998). Retrovirus-based reporter viruses pseudotyped with the E proteins of different families of RNA viruses, including simian immunodeficiency virus, hepatitis C virus (HCV), Ebola virus, La Crosse virus, Hantaan virus, and SARS-CoV, have been successfully established (Bartosch et al., 2003; Chan et al., 2001; Deng et al., 1997; Giroglou et al., 2004; Hsu et al., 2003; Ma et al., 1999; Moore et al., 2004; Nie et al., 2004; Simmons et al., 2004; Wool-Lewis and Bates, 1998). In this study, we developed a lentivirus-based pseudotype reporter virus for dengue virus, which contains the core and defective genome of human immunodeficiency virus type 1 (HIV-1), the luciferase reporter gene, and the PrM/E proteins of one of the four serotypes of dengue viruses. We characterized the biochemical properties of the pseudotype reporter viral particles and demonstrated that they enter target cells through a pH-dependent mechanism. Moreover, mutations introduced to a critical residue at the fusion loop of E protein resulted in impairment in infectivity of the pseudotype reporter virus.

## Results

### Generation of pseudotype reporter viruses containing PrM/E protein of DENV2

We first generated a pseudotype reporter virus, D2(HIVluc), by co-transfection of the PrM/E-expressing construct of DENV2, pCB-D2, and the lentivirus-based luciferase reporter construct, pNL4-3.Luc.R-E<sup>-</sup> (Fig. 1A). Since particle formation of lentivirus and dengue virus is known to occur at different subcellular locations, i.e. plasma membrane for lentivirus and ER for dengue virus (Freed and Martin, 2001; Lindenbach and Rice, 2001), we next constructed a chimeric PrM/E-expressing plasmid, pCB-D2VSV, by replacing the TM domain of E protein with the TM and CY domains of VSV G protein, which contained the membrane-targeting signal (Rose and Whitt, 2001), to enhance the surface expression of PrM/E protein and generated the pseudotype reporter virus, D2VSV(HIVluc) (Fig. 1A). Western blot analysis of the cell lysates revealed compatible amounts of PrM and E proteins, indicating that the expression of PrM/E protein was intact in the constructs pCB-D2 and pCB-D2VSV (Fig. 1B). To detect proteins present in the pseudotype reporter viral particles, pellets derived from the

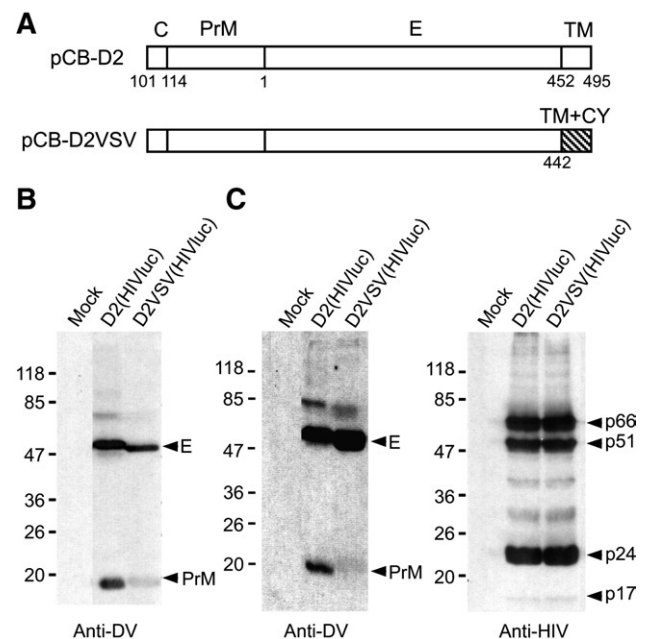


Fig. 1. Generation of pseudotype reporter viruses, D2(HIVluc) and D2VSV (HIVluc). (A) Schematic drawing of constructs expressing the PrM/E proteins of DENV2. PCR product containing the entire *PrM/E* gene and the 3' 42-nucleotides of *C* gene (amino acid residues 101 to 114) of DENV2 was cloned into a pCDNA3-based vector (Chang et al., 2003) to generate pCB-D2. For pCB-D2VSV, the TM domain of the E protein of DENV2 was replaced with the TM and CY domains of VSV-G (hatched bar). The numbers indicate amino acid positions of the C and E proteins. (B, C) Plasmid pCB-D2 or pCB-D2VSV was co-transfected with pNL4-3.Luc.R-E<sup>-</sup> to 293T cells (Connor et al., 1995). 60 h later, cell lysates (B) and pellet lysates (C) derived from sucrose cushion ultracentrifugation were subjected to Western blot analysis by using serum from dengue patient (anti-DV) or HIV-1 patient (anti-HIV). Arrowheads indicate the structure proteins of dengue virus and HIV-1. The size of molecular weight markers is shown in kilodaltons.

supernatants were subjected to Western blot analysis. As shown in Fig. 1C, the PrM and E proteins of dengue virus were detectable by serum from dengue patient. In addition, the matrix (p17), capsid (p24) and reverse transcriptase (p51 and p66) of HIV-1 were detectable by serum from HIV-1 patient (Fig. 1C). These findings suggested that the envelope glycoproteins of dengue virus, PrM and E, and the structural proteins of HIV-1 core were present in the pseudotype reporter viruses, D2 (HIVluc) and D2VSV(HIVluc).

#### *Incorporation of dengue viral proteins into pseudotype reporter viruses*

To further investigate whether the PrM/E proteins were indeed incorporated into the pseudotype reporter viral particles, the pellets derived from supernatants were subjected to sucrose gradient analysis, and the pellet of each fraction to Western blot analysis. Of the fractions derived from D2(HIVluc), while the E protein of DENV2 were detected in fractions 3 to 6 and HIV-1 core proteins in fractions 4 to 6, the majority of E protein and HIV-1 core proteins co-sedimented in fractions 4 and 5, suggesting that they were incorporated into the pseudotype reporter viral particles, D2(HIVluc) (Figs. 2A and B). Moreover, analysis of p24 by ELISA in each fraction revealed that the peak of p24 was in fraction 5, corresponding to a buoyant density of 1.14 g/ml (Fig. 2C). Similarly, analysis of fractions derived from D2VSV(HIVluc) revealed co-sedimentation of the majority of E protein and HIV-1 core proteins with the peak of p24 in fraction 5, which also corresponded to a buoyant density of 1.14 g/ml (Figs. 2E, F, and G). Electron microscopic examination of the pelleted particles of both D2(HIVluc) and D2VSV(HIVluc) revealed spherical particles with homogenous core structure (Figs. 2D and H). Taken together, these findings suggest that the E protein of DENV2 and HIV-1 core proteins are incorporated into the particles of both D2(HIVluc) and D2VSV(HIVluc) with very similar sedimentation properties and morphological features.

Since the buoyant density of D2(HIVluc) and D2VSV(HIVluc) was very similar to that of the HIV-1 gag particle (1.15 g/ml) (Freed and Martin, 2001; Spearman and Ratner, 1996), we carried out an immunoprecipitation experiment, in which pellet D2(HIVluc) derived from culture supernatants of 293T cells co-transfected with pCB-D2 and pNL4-3.Luc.R-E<sup>-</sup>, as well as mixture of pellets each from those transfected with pCB-D2 alone or pNL4-3.Luc.R-E<sup>-</sup> alone, were immunoprecipitated with anti-E MAb and subjected to Western blot analysis. As shown in Fig. 3, HIV-1 core proteins were readily detected in the pellet D2(HIVluc) but not in the mixed pellets of pCB-D2 and pNL4-3.Luc.R-E<sup>-</sup> transfectants, demonstrating that dengue E proteins and HIV-1 gag are present in the same particle.

#### *The pseudotype reporter viruses are infectious*

To investigate whether the pseudotype reporter viruses were infectious, equal amounts of D2(HIVluc), D2VSV(HIVluc) and VSV(HIVluc) reporter viruses, standardized by the levels of

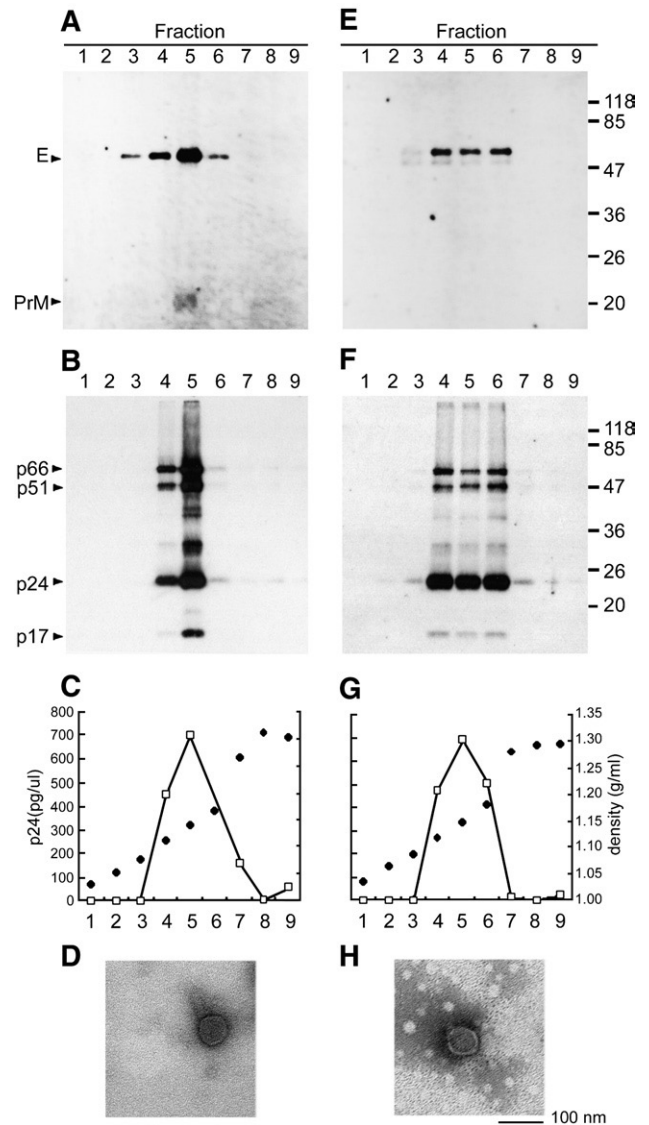


Fig. 2. Sucrose gradient analysis of pseudotype reporter viruses, D2(HIVluc) and D2VSV(HIVluc). Pellets of D2(HIVluc) (A, B) and D2VSV(HIVluc) (E, F) were subjected to sucrose gradient (15% to 80% (wt./vol.)) ultracentrifugation, and pellets derived from each fraction to Western blot analysis by using serum from dengue patient (A, E) or HIV-1 patient (B, F). Arrowheads indicate the structure proteins of dengue virus and HIV-1. The size of molecular weight markers is shown in kilodaltons. Fraction numbers (1 to 9, from top to bottom) are shown above each lane. The amounts of p24 (open square) and buoyant density (closed circle) of each fraction were determined for D2(HIVluc) (C) and D2VSV(HIVluc) (G). Electron micrographs of D2(HIVluc) (D) and D2VSV(HIVluc) (H) were taken after staining with uranyl acetate. Magnification: 100,000 $\times$ . Bar: 100 nm.

p24 (Hsu et al., 2003; Nie et al., 2004), were inoculated into a panel of cell lines that have been reported to support dengue virus replication, including 293T, HeLa, Huh7, HepG2, BHK-21, Vero and K562 (Diamond et al., 2000; Kurane et al., 1990). While VSV(HIVluc), which contained the E protein derived from a virus with broad tropism, infected all the cells tested with the luciferase activity of cell lysates ranging from  $10^5$  to  $10^7$  RLU/s, D2(HIVluc) and D2VSV(HIVluc) infected Huh7 and HepG2 cells with the luciferase activity ranging from  $10^2$  to  $10^3$  RLU/s (data not shown). We therefore used Huh7 or HepG2

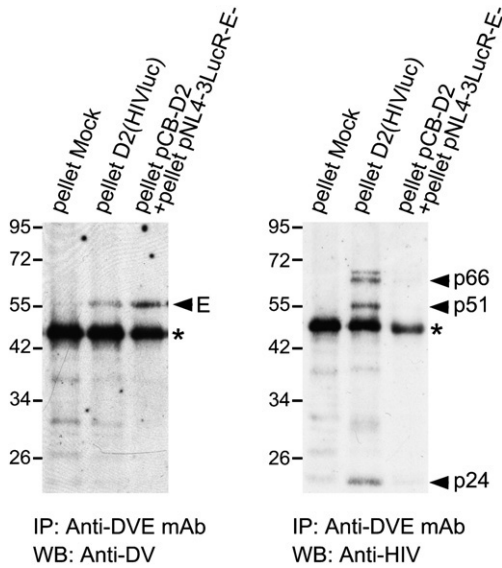


Fig. 3. Immunoprecipitation of the pseudotype reporter virus, D2(HIVluc). Pellet D2(HIVluc) derived from culture supernatants of 293T cells co-transfected with pCB-D2 and pNL4-3.Luc.R-E<sup>-</sup>, as well as mixture of pellets each from those transfected with pCB-D2 alone or pNL4-3.Luc.R-E<sup>-</sup> alone, were immunoprecipitated with anti-E MAb 4G2 (Henchal et al., 1982) and subjected to Western blot analysis by using serum from dengue patient (anti-DV) or HIV-1 patient (anti-HIV). Arrowheads indicate the structure proteins of dengue virus and HIV-1, and asters indicate the mouse heavy chain. The size of molecular weight markers is shown in kilodaltons.

cells as the target cells. To compare the infectivity of D2 (HIVluc) and D2VSV(HIVluc), we titrated down the amounts of p24 in the inoculum and carried out infectivity assay in Huh7 cells. As shown in Fig. 4, a dose-dependent relationship was observed for both D2(HIVluc) and D2VSV(HIVluc). There was no drastic difference in the level of luciferase activity between these two reporter viruses, indicating that replacement of the TM domain of DENV2 with the TM and CY domains of VSV-G dose not result in increase in infectivity of the pseudotype reporter virus. Similar findings were also observed in HepG2 cells (data not shown). We thus focused on the pseudotype reporter viruses that contained the authentic dengue PrM/E

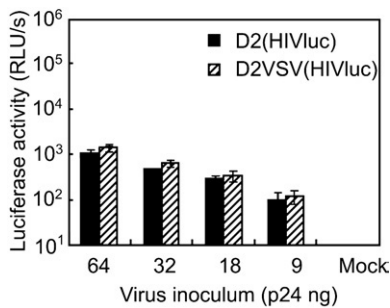


Fig. 4. Relationship between inoculum and luciferase activity of the pseudotype reporter viruses. Increasing amounts of D2(HIVluc) (black bars) and D2VSV (HIVluc) (hatched bars), as standardized by HIV-1 p24, were inoculated into Huh7 cells and the luciferase activity of cell lysates was determined 48 h later. Mock refers to no virus inoculated. The experiments have been repeated more than three times. Data are means of triplicate wells of one representative experiment and the error bars represent the standard errors of the means.

proteins, including D2(HIVluc) and reporter viruses of other serotypes (see below), in our further experiments.

*pH-dependent entry of pseudotype reporter virus*

Like other flaviviruses, dengue virus is believed to enter cells through receptor-mediated endocytosis, a process sensitive to low pH environment in the endosome (Guirakhoo et al., 1993; Lindenbach and Rice, 2001; Mellman et al., 1986; Mukhopadhyay et al., 2005; Randolph and Stollar, 1990). To investigate entry of the pseudotype reporter virus, Huh7 cells were treated with increasing concentrations of two lysosomotropic agents, Bafilomycin A and ammonium chloride, and infected with D2(HIVluc) and VSV(HIVluc), which has been shown to enter cells through a pH-dependent route (Hsu et al., 2003; Nie et al., 2004; Simmons et al., 2004; Wool-Lewis and Bates, 1998). As shown in Fig. 5A, near 100% reduction of the luciferase activities was observed in both D2(HIVluc)-infected and VSV(HIVluc)-infected cells as the concentration of Bafilomycin A increased to 100 nM. Similarly, the luciferase activities of D2(HIVluc)-infected and VSV(HIVluc)-infected cells reduced more than two logs as ammonium chloride increased to 200 mM (Fig. 5B). Of note was that no significant cytotoxicity was found for either Bafilomycin A or ammonium chloride-treated cells at these concentrations, as was reported previously (Hsu et al., 2003). Taken together, these findings suggest that the pseudotype reporter virus, D2(HIVluc), enters cells through a pH-dependent mechanism.

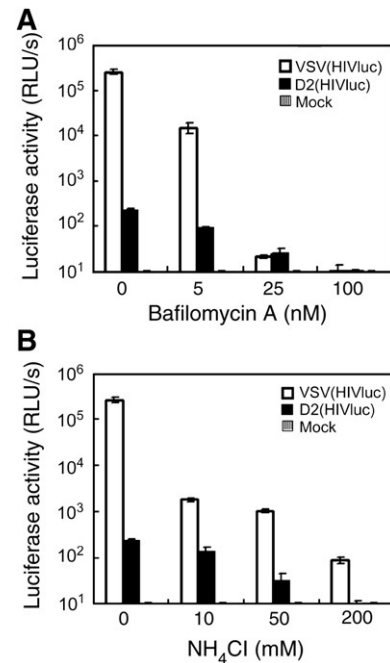


Fig. 5. pH dependency of infectivity of the pseudotype reporter virus, D2 (HIVluc). Huh7 cells were treated with increasing concentration of Bafilomycin A (A) or NH<sub>4</sub>Cl (B) 30 min prior to infection with VSV(HIVluc) (open bars), D2 (HIVluc) (black bars) or mock (hatched bars), and the luciferase activity of cell lysates was determined 48 h later. The experiments have been repeated more than three times. Data are means of triplicate wells of one representative experiment and the error bars represent the standard errors of the means.

### Pseudotype reporter viruses containing PrM/E proteins of other serotypes

To further generate pseudotype reporter viruses of three other dengue serotypes using a similar strategy, we constructed the PrM/E-expressing vectors (pCB-D1, pCB-D3 and pCB-D4) for DENV1, DENV3 and DENV4 (Fig. 6A). Western blot analysis of cell lysates derived from 293T cells co-transfected with pNL4-3.Luc.R-E<sup>-</sup> and pCB-D1, pCB-D3 or pCB-D4 revealed

readily detectable PrM and E proteins, indicating that expression of the PrM/E proteins of these three dengue serotypes was intact (Fig. 6B). Moreover, Western blot analysis of pellets derived from supernatants revealed that HIV-1 core proteins and PrM/E proteins of DENV1, DENV3 and DENV4 were present in the pseudotype reporter viruses, D1(HIVluc), D3(HIVluc) and D4(HIVluc), respectively (Fig. 6C). We then examined the infectivity of these three pseudotype reporter viruses in HepG2 cells. As shown in Fig. 6D, luciferase activities derived from D1(HIVluc)-, D3(HIVluc)-, or D4(HIVluc)-infected cells ranged from 1.6 to  $2.2 \times 10^2$  RLU/s, similar to those from D2(HIVluc)-infected cells.

### Pseudotype reporter viruses containing mutant E proteins

To further demonstrate that entry of the pseudotype reporter virus is mediated through the fusion loop of E protein, mutant pCB-D1 constructs containing amino acid substitutions of the leucine at position 107, which was a critical residue of the fusion loop of E protein (Modis et al., 2004), with a hydrophobic residue (alanine), a negatively charged residue (aspartic acid), or a positively charged residue (lysine) were generated and co-transfected with pNL4-3.Luc.R-E<sup>-</sup> to generate mutant pseudotype reporter viruses (Fig. 7A). As shown in Fig. 7B, PrM/E proteins were readily detectable in cell lysates derived from transfectants of D1-107LA(HIVluc), D1-107LD(HIVluc) and D1-107LK(HIVluc), indicating that the mutations introduced did not affect the expression of PrM/E proteins. Compared with those in the wild type, detectable but less amounts of PrM/E proteins were found in pellets of these mutants, suggesting that incorporation of dengue viral proteins into particles was somewhat affected (Fig. 7C). Of note, the luciferase activity in D1-107LA(HIVluc)-infected cells was similar to that in wild type-infected cells, suggesting that the defect in incorporation of dengue viral proteins into D1-107LA(HIVluc) did not affect its infectivity (Fig. 7D). In contrast, a mild decrease and a drastic decrease in luciferase activity were found in the D1-107LD(HIVluc)-infected and D1-107LK(HIVluc)-infected cells, respectively. Since mutants D1-107LD(HIVluc) and D1-107LK(HIVluc) had the incorporation defect similar to that of D1-107LA(HIVluc), which did not affect infectivity, the decrease in infectivity of these two mutants suggest that amino acid substitutions of the leucine residue 107 with a charged residue, especially a positively charged residue, affect the entry of the pseudotype reporter viruses (Fig. 7D).

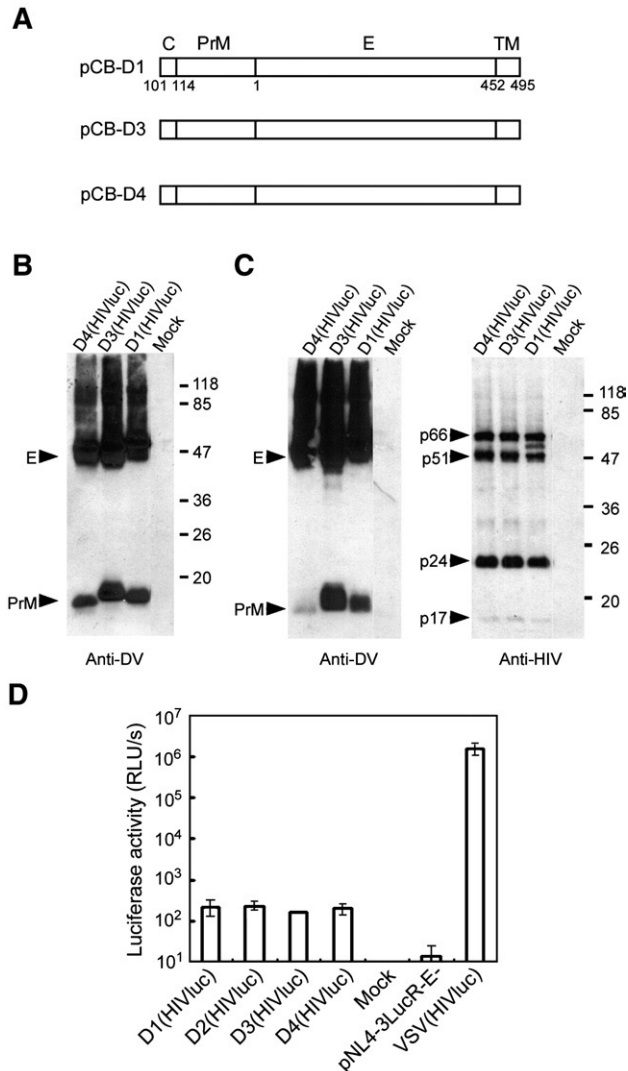


Fig. 6. Generation of pseudotype reporter viruses, D1(HIVluc), D3(HIVluc) and D4(HIVluc). (A) Schematic drawing of constructs expressing PrM/E proteins of DENV1, DENV3 and DENV4, which were generated as described in Fig. 1. (B, C) Plasmid pCB-D1, pCB-D3 or pCB-D4 was co-transfected with pNL4-3.Luc.R-E<sup>-</sup> to 293T cells (Connor et al., 1995). 60 h later, cell lysates (B) and pellet lysates (C) derived from sucrose cushion ultracentrifugation were subjected to Western blot analysis. The data are presented as described for Fig. 1. (D) Equal amounts of the four pseudotype reporter viruses and VSV (HIVluc), as standardized by HIV-1 p24, were inoculated into HepG2 cells and the luciferase activity of cell lysates was determined 48 h later. Mock refers to no virus inoculated, and pNL4-3.Luc.R-E<sup>-</sup> refers to inoculation of pellets derived from pNL4-3.Luc.R-E<sup>-</sup> transfectant. The experiments have been repeated more than three times. Data are means of duplicate wells of one representative experiment and the error bars represent the standard errors of the means.

### Discussion

E protein is the major determinant of cellular tropism, viral entry and neutralization sensitivity of enveloped viruses. With the advancement of molecular technologies, several pseudotype reporter viral systems have been developed for different enveloped viruses and made the investigation of phenotypic properties of E proteins more conveniently and efficiently (Bartosch et al., 2003; Buonocore et al., 2002; Chan et al., 2001; Cronin et al., 2005; Deng et al., 1996, 1997; Fukushi et al., 2005; Giroglou et al., 2004; Hanika et al., 2005; Hsu et al., 2003; Ma et

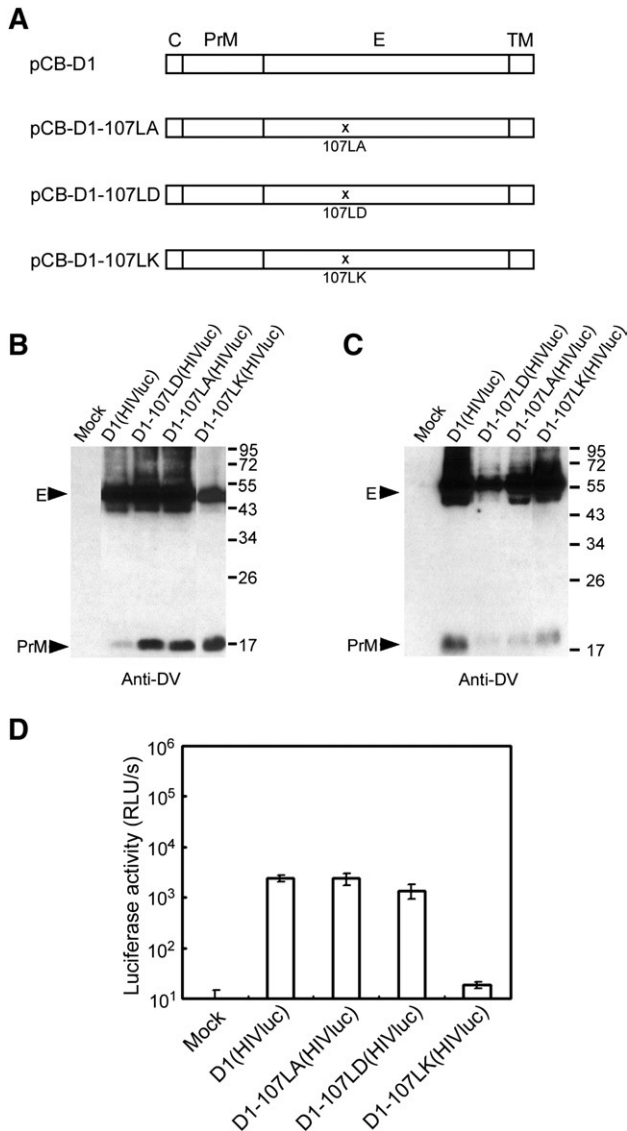


Fig. 7. Pseudotype reporter viruses containing mutant E proteins. (A) Schematic drawing of pCB-D1 constructs expressing mutant E proteins. Plasmid pCB-D1, pCB-D1-107LA, pCB-D1-107LD or pCB-D1-107LK was co-transfected with pNL4-3.Luc.R-E- to 293T cells (Connor et al., 1995). 60 h later, cell lysates (B) and pellet lysates (C) derived from sucrose cushion ultracentrifugation were subjected to Western blot analysis. The data are presented as described for Fig. 1. (D) Equal amounts of the wild type and mutant pseudotype reporter viruses, as standardized by HIV-1 p24, were inoculated into HepG2 cells and the luciferase activity of cell lysates was determined 48 h later. Mock refers to no virus inoculated. The experiments have been repeated more than three times. Data are means of duplicate wells of one representative experiment and the error bars represent the standard errors of the means.

al., 1999; Matsuura et al., 2001; Moore et al., 2004; Nie et al., 2004; Simmons et al., 2004; Wool-Lewis and Bates, 1998). In this study, we have successfully established a retrovirus-based pseudotype reporter virus for four serotypes of dengue viruses. Compared with the traditional infectivity assay for dengue virus, this is a simple, sensitive and convenient tool to study the function of PrM/E proteins in the context of viral particles. To our knowledge, this is the first reporter viral system reported for dengue virus thus far.

Sucrose gradient analysis of the pseudotype reporter viruses revealed co-sedimentation of the majority of dengue E protein and HIV-1 core proteins, suggesting that dengue viral proteins were incorporated into the particles formed by HIV-1 core. The buoyant density of the pseudotype reporter virus, D2(HIVluc), was 1.14 g/cm<sup>3</sup>, which was similar to that of a HIV-1 gag particle (1.15 g/cm<sup>3</sup>) and subviral particles of flaviviruses (1.14 g/cm<sup>3</sup>) but lower than that of a flaviviral particle (range: 1.19 to 1.23 g/cm<sup>3</sup>) (Freed and Martin, 2001; Lindenbach and Rice, 2001; Schalich et al., 1996; Spearman and Ratner, 1996). Therefore, an immunoprecipitation experiment was carried out to further demonstrate that dengue E proteins and HIV-1 gag are indeed present in the same particle (Fig. 3). Of note, since subviral particles of several flaviviruses including dengue virus can be produced by co-expression of PrM and E proteins (Allison et al., 1995; Konishi et al., 1992; Mason et al., 1991; Schalich et al., 1996), these subviral particles may compete with the assembly of our pseudotype reporter viruses and thus account for the relatively low infectivity observed. Re-examination of the electron micrographs revealed some small particles of 30 to 50 nm in diameter, which were smaller than and distinguishable from the dense-core particles of D2(HIVluc) (see Supplementary Fig. 1). The size of D2(HIVluc) was approximately 70 to 80 nm in diameter (Fig. 2D), which was slightly larger than that of a mature flaviviral particle (60 nm) and smaller than that of a HIV-1 gag particle (90 to 100 nm) (Lindenbach and Rice, 2001; Spearman and Ratner, 1996). This suggests that certain change in the arrangement of E proteins might have occurred to accommodate its incorporation into the slightly larger pseudotype reporter particle. However, our observations that the entry of pseudotype reporter virus is mediated through a pH-dependent route (Fig. 5) and affected by the fusion loop mutations (Fig. 7) suggest that the E protein on the pseudotype reporter virus functions similarly to that on the dengue virion.

Because virion assembly and release of dengue virus and HIV-1 occurred at different locations, we sought to generate another pseudotype reporter virus, D2VSV(HIVluc), which contained the membrane targeting domain of VSV-G (Rose and Whitt, 2001), to enhance particle formation at the cell surface. A series of analyses revealed that D2(HIVluc) and D2VSV(HIVluc) had similar biochemical and morphological properties except that the amount of PrM protein relative to that of E protein in the cell and pellet lysates of D2VSV(HIVluc) was less than that of D2(HIVluc) (Figs. 1B and C). Since the TM domain of E protein of tick-borne encephalitis virus, another flavivirus, has been shown to be involved in the interaction with PrM protein (Allison et al., 1999), less amount of PrM protein could be due to the relatively weaker interaction between E and PrM proteins in D2VSV(HIVluc), which lacked the TM domain of DENV2. However, this did not lead to significant difference in infectivity (Fig. 4). Moreover, our findings of no increase in the infectivity of D2VSV(HIVluc), compared with that of D2(HIVluc), would suggest that replacement of the TM domain of DENV2 with the TM and CY domains of VSV-G does not result in increased particle formation at the cell surface and thus increased infectivity. Immunofluorescence study of intracellular distribution of E protein revealed that there was no increase in

the surface expression of E protein in cells producing D2VSV (HIVluc), supporting this interpretation (data not shown). In addition, enzymatic digestion of E proteins in lysates derived from D2(HIVluc) and D2VSV(HIVluc)-producing cells showed predominantly endoglycosidase H-sensitive pattern, suggesting strong ER retention of E proteins for both D2 (HIVluc) and D2VSV(HIVluc) (data not shown). Of note, the stem region (amino acid residues 396 to 452) of DENV2 E protein has been recently reported to contain an ER retention signal (Purdy and Chang, 2005). Thus, our findings that the E protein of D2VSV(HIVluc) retained mainly in ER suggest that the ER retention signal of DENV2 stem region has a stronger effect than the membrane-targeting signal of VSV-G (Rose and Whitt, 2001).

Enveloped viruses enter cells through two main pathways, direct fusion at the plasma membrane and receptor-mediated endocytosis (Lindenbach and Rice, 2001). Our findings that the luciferase activities in D2(HIVluc)-infected cells were completely inhibited by the lysosomotropic agents, Bafilomycin A and ammonium chloride, suggest that the pseudotype reporter virus enters cells through a pH-dependent pathway, a route that was utilized by dengue virus (Guirakhoo et al., 1993; Lindenbach and Rice, 2001; Mukhopadhyay et al., 2005; Randolph and Stollar, 1990). Similarly, the pseudotype reporter virus of HCV, another flavivirus, also utilized the pH-dependent pathway to enter cells (Bartosch et al., 2003; Hsu et al., 2003). Although a variety of cell lines could be productively infected by dengue virus (Diamond et al., 2000; Kurane et al., 1990), only two of them (Huh7 and HepG2 cells, both human cells) were shown to be infected by the dengue pseudotype reporter virus, as revealed by the luciferase activity. To examine whether the pseudotype reporter virus can bind HepG2 cells as well as two common target cells of dengue virus, Vero and BHK-21 cells, equal amounts of D2(HIVluc) and pellets derived from pNL4-3.Luc.R-E<sup>-</sup> transfectant were inoculated to these cells at 4 °C for 1.5 h, and cell lysates containing the bound particles were subjected to Western blot analysis. As shown in Fig. 8, similar amounts of dengue E protein and HIV-1 core proteins were detected in lysates of HepG2, Vero and BHK-21 cells inoculated with D2(HIVluc) but not in those inoculated with pellets of pNL4-3.Luc.R-E<sup>-</sup> transfectant, indicating that D2 (HIVluc) can bind Vero and BHK-21 cells. Similarly, binding to Vero and BHK-21 cells was also observed for D1(HIVluc), D3 (HIVluc) and D4(HIVluc) (data not shown). Thus, the lack of luciferase activity in these two cells could be due to the relatively poor expression of the HIV-based reporter gene in many non-human cells (Besnier et al., 2002; Freed and Martin, 2001). In agreement with this interpretation, VSV(HIVluc), which contained a broad-tropic envelope glycoprotein (VSV-G), had more than two-logs difference ( $10^5$  to  $10^7$  RLU/s) in the luciferase activity in various target cells, with the highest activities in human cell lines, including 293T, Huh7 and HepG2 cells (data not shown). Similarly, another HIV-based  $\beta$ -gal reporter virus pseudotyped with VSV-G had three-log difference in the reporter signal ( $10^4$  to  $10^7$  focus-forming units/ml) in different target cells with the highest signals in human cells (Simmons et al., 2004).

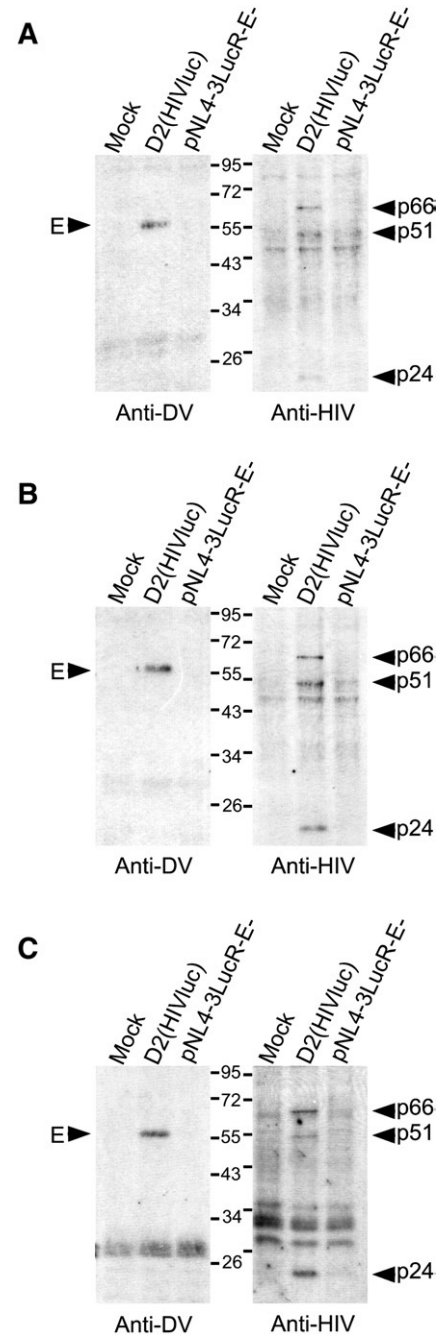


Fig. 8. Binding of the pseudotype reporter virus, D2(HIVluc) to target cells. HepG2 (A), Vero cells (B) and BHK-21 cells (C) were inoculated with equal amounts of D2(HIVluc) and pellets derived from pNL4-3.Luc.R-E<sup>-</sup> transfectant at 4 °C for 1.5 h. After washing, cell lysates were subjected to Western blot analysis. The data are presented as described for Fig. 1.

We have generated pseudotype reporter viruses of other three serotypes and demonstrated that they had similar biochemical properties and infectivity (Fig. 6). Of note, the amount of PrM protein relative to that of the E protein in the cell and pellet lysates of D4(HIVluc) was less than that in other pseudotype reporter viruses. This could be due to the serum used, which was from a confirmed DENV2 patient and recognized the PrM protein of DENV4 less well than that of other serotypes (see Supplementary Fig. 2). During the maturation of flaviviral

particles, PrM protein is cleaved into Pr and M proteins by the *trans*-Golgi resident protein, furin or furin-like protease (Lindenschmidt and Rice, 2001; Stadler et al., 1997). Of note, the cleavage has been shown to be ineffective for dengue virus by several independent studies (Anderson et al., 1997; He et al., 1995; Keelapang et al., 2004; Murray et al., 1993; Randolph et al., 1990; Wang et al., 1999b). We have used mixed sera from dengue patients that contained strong anti-PrM activity to examine the PrM cleavage in our pseudotype reporter viruses, but no discernable M protein was found probably due to ineffective PrM cleavage (data not shown). However, the pH-dependent entry of our pseudotype reporter viruses would suggest incorporation of cleaved M protein, though probably at low level. This may account for the relatively low infectivity observed in this study. Further experiment of treating the pseudotype reporter particles with furin based on the protocol described previously may improve their infectivity (Stadler et al., 1997).

We have also generated pseudotype reporter viruses containing different amino acid substitutions at a critical residue, leucine at position 107, of the fusion loop of E protein. Based on the prediction modeled on crystal structure of E protein and the binding of mutant E proteins to MAbs, mutations introduced to leucine 107 were unlikely to disrupt the overall conformation of E protein (Crill and Chang, 2004; Modis et al., 2004). Our findings that mutants 107LA, 107LD and 107LK affect the incorporation of dengue viral proteins into particles were unexpected (Fig. 7). However, this defect in incorporation does not affect the infectivity of D1-107LA(HIVluc). Since leucine 107 is highly conserved among flaviviruses and mutations introduced to leucine 107 have been shown to impair the fusion activity of E protein in tick-borne encephalitis virus and West Nile virus (Allison et al., 2001; Zhang et al., 2006), the drastic impairment in infectivity of D1-107LK(HIVluc), which had incorporation defect similar to that of D1-107LA(HIVluc), was most likely at the fusion step of entry.

With the current technique of virus isolation and sequencing, more and more dengue viruses from outbreaks in different countries of the world have been isolated, and increasing numbers of E or full-genome sequences are available now (Carrington et al., 2005; Guzman and Kouri, 2002; Zhang et al., 2005). However, an efficient system to characterize the phenotypic properties of different E sequences, which are important to our understanding of the pathogenesis of dengue as well as to the evaluation of candidate vaccines, is lacking. In this regard, our dengue pseudotype reporter viruses have the potential to further molecular characterization of PrM/E proteins of dengue field isolates in the future.

## Materials and methods

### Construction of PrM/E expression plasmids

RNA were extracted from culture supernatants of DENV1 (Hawaii strain), DENV2 (16681 strain), or DENV3 (ID23 strain) infected C6/36 cells, and subjected to RT using random primers as described previously (Lin et al., 2004). Full-length PrM/E genes and the 3' 42-nucleotide region of the C gene

(corresponding to amino acid residues 101 to 114, which encode the signal peptide) of four serotypes of dengue viruses were amplified by using cDNA templates (for DENV1, DENV2 and DENV3) or plasmid p2AXhoI (for DENV4, 814669 strain, kindly provided by Dr. C. J. Lai) (Lai et al., 1991) and the following primer pairs: d1KpnSSA (5'-CTTGGTACCGCCG-CCGCCATGTCTGTGACCATGCTCCTC-3') and d1E495BNotI (5'-CTTCGAGCGGCCGCAATTACGCCTGAACCAT-GACTCCTAG-3') for DENV1; d2KpnSS (5'-CTTGGTACCTC-TAGAGCCGCCGCCATGGCAGGCATGATCATTATG-3') and d2NotI-2402 (5'-TTTCTCGAGCGGCCGCTCAACTAAT-TAGGCCTGCACCATGACTC-3') for DENV2; d3KpnSSA (5'-CTTGGTACCGCCGCCGCCATGACATCGTTCTGTCTCAT-GATG-3') and d3E495BNotI (5'-CTTCGAGCGGCCGCAAT-TAAGCTTGTACCACGGCTCCC-3') for DENV3; d4KpnSSA (5'-CTTGGTACCGCCGCCGCCATGACGATAACATTGCTGT-GCTTG-3') and d4E495BNotI (5'-CTTCGAGCGGCCGCT-CAACTAATTATGCTTGAAGTGTGAAGCCC-3') for DENV4. To generate PrM/E expression plasmids (pCB-D1, pCB-D2, pCB-D3 and pCB-D4), PCR products were digested with *KpnI* and *NotI*, and cloned into respective sites of a pCDNA3-based vector, pCBD2-2J-2-9-1 (kindly provided by Dr. G. J. J. Chang) (Chang et al., 2003). To generate plasmid pCB-D2VSV, primers VSV457-AXcmI (5'-GCCGTTACCAAGTGTGGTGGCAG-TAGTTG GAAAAGCTCTATTG-3') and VSV512BNotI (5'-TTTTCTTTTTCGGCCGCTCAA ACTTACT TTCCAAGTCG-GTTCATC-3') were used to amplify the transmembrane (TM) and cytoplasmic (CY) domains of the glycoprotein of vesicular stomatitis virus (VSV) from plasmid pVSV-G. The PCR product was digested with *XcmI* and *NotI*, and cloned into the respective sites of pCB-D2.

### Generation of pseudotype reporter viruses

Human embryonic kidney cells, 293T cells, were maintained in Dulbecco's modified Eagle medium (DMEM) (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (FBS), 1% antibiotics (penicillin/streptomycin) and 1% HEPES (Gibco Invitrogen, Carlsbad, CA), and seeded in 10-cm<sup>2</sup> dishes 1 day prior to transfection. 6 µg of each of the PrM/E expression plasmids (pCB-D1, pCB-D2, pCB-D2VSV, pCB-D3, pCB-D4) or of the plasmid pVSV-G were co-transfected with 6 µg of a previously described lentivirus-based luciferase reporter construct, pNL4-3.Luc.R-E- (Connor et al., 1995), using the calcium phosphate method (Chen and Okayama, 1987). Briefly, plasmids were mixed with 30 µl of 0.25 M CaCl<sub>2</sub>, 300 µl of 2× BBS, incubated at room temperature for 10 to 20 min, and added dropwise to the cells, which were incubated at 35 °C for 15 h to 24 h and replaced with 15 ml fresh medium. After incubation at 37 °C for 48 h, the cells were washed with 1× PBS once, treated with lysis buffer (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and proteinase inhibitor, Roche, Germany) at 4 °C, followed by centrifugation at 14,000 rpm (851 rotor, Thermo Electron Corporation, Waltham, MA) at 4 °C for 30 min to obtain cell lysates for Western blot analysis (see below). Culture supernatants containing pseudotype reporter virus were clarified by centrifugation at 2500 rpm (A-4-62 rotor, Eppendorf,



Westbury, NY) for 15 min, filtered through 0.22- $\mu$ m pore-sized membranes, layered over a 20% sucrose buffer, and subjected to ultracentrifugation at 19,000 rpm (SW28 rotor, Beckman, Fullerton, CA) at 4 °C for 5 h. The pellets were resuspended in 1 ml medium, filtered through 0.22- $\mu$ m filter, and stored at –80 °C until use (Wang et al., 1999a). The amount of p24 in the pseudotype reporter virus was assessed by an HIV-1 p24 antigen ELISA kit (Murex HIV Antigen Mab, Abbott, IL).

#### *Cells and infectivity of pseudotype viruses*

Huh7 cells and HeLa cells were maintained in DMEM containing 10% FBS and 1% antibiotics (Gibco Invitrogen, Carlsbad, CA). BHK-21 cells and K562 cells were maintained in RPMI-1640 (JRH Buisuebces, Lenexa, KS) containing 5% FBS and 1% antibiotics. HepG2 cells and Vero cells were maintained in minimal essential medium containing 10% FBS and 1% antibiotics. For infectivity assay, cells ( $1 \times 10^5$  cells) were seeded in 24-well plate 1 day earlier, and known amounts (18 to 36 ng of p24) of pseudotype virus were added and incubated at 37 °C for 18 h, followed by replacement with fresh medium. About 48 h later, medium was removed and cells were washed with  $1 \times$  PBS and lysed with 80  $\mu$ l lysis buffer per well (Promega, Madison, WI). 20  $\mu$ l of lysates were tested for luciferase activity using a microplate luminometer (Berthold Detection Systems, Oak Ridge, TN).

#### *Inhibition of entry*

Huh7 cells were pre-incubated with or without different concentration of  $\text{NH}_4\text{Cl}$  or Bafilomycin A (Sigma, St. Louis, MO) at 37 °C for 30 min, infected with known amounts of pseudotype virus (18 ng of p24), and incubated in the presences or absence of inhibitors at 37 °C for 18 h. Fresh medium was replaced and cells were lysed 48 h later to determine luciferase activity as described above.

#### *Cytotoxicity assay*

Huh7 cells were incubated with or without different concentration of inhibitors at 37 °C. After 48 h to 72 h, medium was collected for a cytotoxicity assay (CytoTox 96, Promega, Madison, WI). Briefly, each sample was added with reconstitute substrate mix (50  $\mu$ l) and incubated at room temperature for 30 min. After adding stop solution, the background absorbance (at 490 nm), which was the LDH spontaneously released from cells without inhibitors, was subtracted from the sample absorbance (at 490 nm) to determine the experimental LDH release. The percentage of cytotoxicity was the ratio of the experimental LDH release to the maximum LDH release, which was the LDH released after the freeze–thaw cycle of the cells.

#### *Western blot analysis of cell lysates and particles of pseudotype viruses*

An aliquot (200  $\mu$ l) of the pseudotype reporter virus concentrated by 20% sucrose cushion as described above was

subjected to centrifugation at 14,000 rpm (F-45-30-11 rotor, Eppendorf, Westbury, NY) at 4 °C for 1 h to obtain pellets, which was resuspended in 15  $\mu$ l TNE buffer (50 mM Tris–HCl, pH 8.0, 1 M NaCl, 10 mM EDTA) for Western blot analysis and electron microscopy (see below). Another aliquot (800  $\mu$ l) was subjected to sucrose gradient (15% to 80% (wt./vol.) in TNE buffer) ultracentrifugation at 25,000 rpm (SW41 rotor, Beckman, Fullerton, CA) at 4 °C for 16 h. Each 1 ml fraction was collected from top of the gradient and subjected to ultracentrifugation at 22,000 rpm (SW55 rotor, Beckman, Fullerton, CA) at 4 °C for 1.5 h. Cell lysates, pellets and pellet of each fraction were subjected to 12% SDS–PAGE and Western blot analysis with serum (1:5000) from a dengue patient (Wang et al., 2006) or an HIV-1 patient (Lee et al., 2000) (kindly provided by Dr. C. N. Lee). The density of each fraction was determined by a refractometer (ATAGO, Tokyo, Japan) as described previously (Hsieh et al., 2005).

#### *Electron microscopy*

An aliquot of the pelleted particles was added to a 300-mesh formvar/carbon film, stained with saturated uranyl acetate (Electron Microscopy Sciences, Washington, PA), and examined by electron microscope.

#### *Immunoprecipitation*

15  $\mu$ l of pellets derived from culture supernatants of 293T cells co-transfected with pCB-D2 and pNL4-3.Luc.R-E– as described above, as well as mixture of equal amounts of pellets (15  $\mu$ l) from those transfected with pCB-D2 alone and pNL4-3.Luc.R-E– alone, were incubated with 250  $\mu$ l protein A-Sepharose beads (Amersham Pharmacia Biotech., Piscataway, NJ), and 10  $\mu$ l of a mouse anti-E monoclonal antibody (MAb) 4G2 (1:100) (American Type Culture Collection, Rockville, MD) (Henchal et al., 1982) at 4 °C overnight. After washing four times with 1% NP-40, the immunoprecipitates were mixed with  $2 \times$  sample buffer (500 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.4% bromophenol blue), eluted by heating at 95 °C for 2 min, and subjected to Western blot analysis.

#### *Generation of E mutants of pCB-D1*

Site-directed mutagenesis was performed by using pCB-D1 template and the two-step PCR mutagenesis as described previously to mutate the leucine at position 107 to alanine, aspartic acid or lysine (Liu et al., 2003). In the first PCR, a fragment of 317 bp (fragment A) was amplified by primer pair, d1E2XcmIA (5'-CATGGCCATGCGATGCGTGGGAATAG-3') and d1EL107KB1 (5'-ACCTTTTCCGAATTTCCACAGCCATT-3') for pCB-D1-107LK or by primer pair, d1E2XcmIA and d1EL107A/DB1 (5'-ACCTTTTCCGAAAG/TCCCCACAGCCATT-3') for pCB-D1-107LA and pCB-D1-107LD. Another fragment of 127 bp (fragment B) was amplified by primer pair, d1EL107KA2 (5'-AATGGCTGTGGGAAATTCG-GAAAAGGT-3') and d1E150DraIIIB (5'-ATTTCCCACCTGGTGTGGTC-3') for pCB-D1-107LK or by primer pair,

d1EL107A/DA2 (5'-AATGGCTGTGGGGC/ATTTTCGGAA-AAGGT-3') and d1E150DraIIIB for pCB-D1-107LA and pCB-D1-107LD. In the second PCR, a product of 444 bp was obtained by using mixture of fragments A and B as templates and outer primers (d1E2XcmIA and d1E150DraIIIB), digested with *XcmI* and *DraIII*, and cloned into respective sites of a pCB-D1. All mutants were confirmed by sequencing in the entire 444-bp region to exclude the second site mutation.

#### *Binding of pseudotype reporter virus to target cells*

Huh7, HepG2, BHK-21 or Vero cells ( $1 \times 10^5$ ) were pretreated with cold medium at 4 °C for 30 min and incubated with equal amounts (based on p24) of pseudotype reporter viruses, D1 (HIVluc), D2(HIVluc), D3(HIVluc), D4(HIVluc), and pellets derived from pNL4-3.Luc.R-E- transfectants at 4 °C for 1.5 h. After washing with cold medium twice, cell lysates containing bound particles were subjected to Western blot analysis.

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#### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.virol.2007.06.026](https://doi.org/10.1016/j.virol.2007.06.026).

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