Development of a rapid and quantitative method for the analysis of viral entry and release using a NanoLuc luciferase complementation assay

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Abstract

Subviral particles (SVPs) self-assemble and are released from cells transfected with expression plasmids encoding flavivirus structural proteins. Flavivirus-like particles (VLPs), consisting of flavivirus structural proteins and a subgenomic replicon, can enter cells and cause single-round infections. Neither SVPs or VLPs possess complete viral RNA genomes, therefore are replication-incompetent systems; however, they retain the capacity to fuse and bud from target cells and follow the same maturation process as whole virions. SVPs and VLPs have been previously employed in studies analyzing entry and release steps of viral life cycles. In this study, we have developed quantitative methods for the detection of cellular entry and release of SVPs and VLPs by applying a luciferase complementation assay based on the high affinity interaction between the split NanoLuc luciferase protein, LgBiT and the small peptide, HiBiT. We introduced HiBiT into the structural protein of West Nile virus and generated SVPs and VLPs harboring HiBiT (SVP-HiBiT and VLP-HiBiT, respectively). As SVP-HiBiT emitted strong luminescence upon exposure to LgBiT and its substrate, the nascently budded SVP-HiBiT in the supernatant was readily quantified by luminometry. Similarly, the cellular entry of VLP-HiBiT generated luminescence when VLP-HiBiT was infected into LgBiT-expressing cells. These methods utilizing SVP-HiBiT and VLP-HiBiT will facilitate research into life cycles of flaviviruses, including WNV.

Keywords

Flavivirus; Virus entry; Virus release; Luciferase complementation assay

1. Introduction

The genus *Flavivirus* includes important human pathogens e.g. yellow fever virus, dengue virus, Zika virus and West Nile virus (WNV). Flavivirus virions are enveloped particles formed by three structural proteins: envelope (E), membrane (M) and core (C). Capsidless subviral particles (SVPs) self-assemble and are released from cell lines expressing E and the glycoprotein precursor of M (prM) of flaviviruses (Blazevic et al., 2016; Hanna et al., 2005; Inagaki et al., 2016; Makino et al., 2014; Ohtaki et al., 2010; Op De Beeck et al., 2003; Qiao et al., 2004; Yoshii et al., 2012). Flavivirus-like particles (VLPs) consist of E, M, C proteins and a subgenomic replicon (Pierson et al., 2006). Both SVPs and VLPs are replication-incompetent virus particles, however, they undergo the same maturation process as whole virions and are thus capable of fusing to target cells (Pierson and Diamond, 2013; Schalich et al., 1996). Therefore, SVPs and VLPs have been previously used as antigens in serosurveys and for immunization (Inagaki et al., 2016; Ohtaki et al., 2010; Qiao et al., 2004), and have also been applied to the establishment of model systems for the investigation of cellular entry and extracellular release of virions (Blazevic et al., 2016; Hanna et al., 2005; Kobayashi et al., 2016; Makino et al., 2014; Op De Beeck et al., 2003; Phongphaew et al., 2017; Pierson et al., 2006; Yoshii et al., 2012).

The split luciferase complementation assays are a well-characterized approach to study protein-protein interactions. Recently, Dixon *et al*. developed a novel luciferase complementation assay by splitting the NanoLuc (Nluc) luciferase protein into two subunits and optimizing them (Dixon et al., 2016); which are referred to as smBiT and LgBiT by the manufacturer (Promega, Madison, WI). SmBiT is an 11 amino acid peptide with low binding affinity for LgBiT and is therefore suitable for protein interaction assays by fusion to proteins of interest (Dixon et al., 2016). Through the screen of smBiT, “peptide 86” was

identified which consists of 11 amino acids with high binding affinity for LgBiT and shows spontaneous complementation. Peptide 86 is referred to as HiBiT (Promega).

The purpose of the present study was the establishment of a simple and sensitive method for the analysis of viral entry and release using SVPs and VLPs with an Nluc luciferase complementation assay. We have generated SVPs and VLPs harboring HiBiT in the viral particles, which we term SVP-HiBiT and VLP-HiBiT, respectively. To validate the experimental utility of SVP-HiBiT and VLP-HiBiT, we have examined these properties in cellular entry and release experiments.

2. Materials and Methods

2.1. Cell culture

293T cells were propagated in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. A549 cells (RIKEN BRC, Tsukuba, Japan) and Vero cells (JCRB Cell Bank, Osaka, Japan) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

2.2. Plasmids

Plasmids pCMV-SVP expressing prM and E, and pCXSN-C expressing C of WNV 6-LP strain were constructed as described previously (Phongphaew et al., 2017). Plasmid pCMV-SVP-HiBiT carrying HiBiT flanked by GS linker GSSGGSSGVSGWRLFKKISGSSGGSSG (the HiBiT sequence is underlined) was constructed by inverse PCR using pCMV-SVP as a template. Plasmid pCXSN-C-HiBiT was constructed by adding a GS linker and HiBiT, GSSGGSSGVSGWRLFKKIS, at the C terminus of C protein by inverse PCR using pCXSN-C. The WNV replicon plasmid, pWNIIrep-GFP, was kindly provided by Dr. Doms (Pierson et al., 2006). The self-inactivating lentiviral vector expressing constructs (CSII-CMV-MCS-IRES2-Bsd, pCAG-HIVgp, pCMV-VSV-G-RSV-Rev) were kindly provided by Dr. Miyoshi (RIKEN BRC). CSII-CMV-LgBiT-IRES2-Bsd was constructed by sub-cloning LgBiT fragment which was amplified from pBiT1.1-C[TK/LgBiT] (Promega, Madison, WI) by PCR.

2.3. Preparation of SVPs and VLPs

For generation of SVPs, expression plasmids pCMV-SVP or pCMV-SVP-HiBiT were transfected into 293T cells on collagen type 1 coated dish with polyethylenimine MAX

(Polysciences, Warrington, PA) according to the manufacture's protocol. For generation of VLPs, mixture of pCMV-SVP, pWNIIrep-GFP and pCXSN-C or pCXSN-C-HiBiT were co-transfected into 293T cells as described above. After 36 h post-transfection, the culture supernatants were harvested, filtrated through a 0.45 μm syringe filter and then pelleted by ultracentrifugation using a SW32 rotor (Beckman Coulter, Brea, CA) for 3 h at 153,720 *g*. The pellets were resuspended in PBS (−) and used for analyses.

2.4. Protein blotting

293T cells were transfected with the SVP expression plasmids using polyethylenimine MAX and cultured for 36 h. Cells were lysed in lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA] supplemented with cOmplete ULTRA protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were separated by SDS-PAGE and transferred onto Amersham Protran Supported nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) for HiBiT blotting and Immobilon-P PVDF membrane (Merck Millipore, Darmstadt, Germany) for immunoblotting. For HiBiT detection, protein-transferred membranes were incubated with LgBiT protein (Promega) in TBS plus 0.05% Tween-20. After addition of Nano-Glo HiBiT Blotting Substrate (Promega), luminescence was detected by VersaDoc MP 5000 imaging system (Bio-Rad Laboratories, Hercules, CA). For immunoblotting, protein-transferred membranes were blocked with 5% skimmed milk and stained with anti-WNV E protein monoclonal antibody (3.91D, Merck Millipore) or anti-WNV M protein polyclonal antibody (NB100-56743, Novus Biologicals, Littleton, CO), following by staining with anti-mouse or rabbit IgG secondary antibody conjugated with horseradish peroxidase (BioSource International Camarillo, CA). Anti-β-actin polyclonal antibody conjugated with horseradish peroxidase

(PM053-7, MBL, Nagoya, Japan) was used as a loading control antibody. Immune complexes were detected with Immobilon western chemiluminescent HRP substrate (Merck Millipore) and VersaDoc MP 5000 imaging system.

2.5. Quantification of the amount of SVPs

SVPs were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using anti-flavivirus E protein monoclonal antibody clone 402 (kindly provided by Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan) as described previously (Makino et al., 2014; Takahashi et al., 2009). For quantification of HiBiT-tagged SVPs, the culture supernatants or fractions containing SVPs were harvested and mixed with LgBiT protein, Nano-Glo luciferase assay buffer and Nano-Glo luciferase assay substrate (Promega) on a 96 well black plate for 10 min. Luminescence was detected on a luminometer (GloMax-Multi detection system, Promega).

2.6. Sucrose gradient sedimentation analysis

After incubation with or without 1% Triton X-100 for 30 min at 4°C, SVPs were overlaid onto 10-50% (w/v) discontinuous sucrose gradient in PBS (−) and ultracentrifuged using a SW41 rotor (Beckman Coulter) at 4°C for 14 h at 247,606 *g*. Fractions (500 µl each) were carefully collected from the upper phase and SVPs in each fraction were quantified as described above.

2.7. Gene knockdown

To achieve efficient gene knockdown of Rab11, two different pairwise combinations of Silencer Select siRNAs targeting *Rab11a* and *Rab11b* (s16702 and s17648, or s16703 and

s17649, Ambion; Thermo Fisher Scientific, Waltham, MA) were examined. Silencer Select negative control no. 1 siRNA was used as non-targeting control siRNA. A549 cells were transfected with siRNAs at a final concentration of 20 nM each by reverse transfection protocol with Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific). 48 h post-transfection, cells were transfected with pCMV-SVP-HiBiT and cultured for 24 h. HiBiT-tagged SVPs in the supernatants were quantified as described above. The knockdown efficiency was confirmed by western blotting with anti-Rab11 monoclonal antibody (610656, BD Biosciences, San Jose, CA).

2.8. Infection of VLPs and VLP-HiBiT

Vero cells seeded on 96 well plates were infected with VLPs and VLP-HiBiT. At 48 h post-infection, cellular nuclei were stained with Hoechst 33342 (Molecular Probes; Thermo Fisher Scientific). For quantitative evaluation of infectivity, images were captured with an automated microscope IN Cell Analyzer 2000 (GE Healthcare), and GFP positivity was calculated by counting the number of nuclei and GFP-expressing cells in each well with IN Cell Investigator software (GE Healthcare). To obtain LgBiT stably expressing Vero cells (Vero-LgBiT), a lentiviral vector was prepared by co-transfection with CSII-CMV-LgBiT-IRES2-Bsd, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev to 293T cells. Vero-LgBiT was established by infecting Vero cells with the lentiviral vector and maintained in medium containing 10 μg/ml of blasticidin S HCl (Gibco; Thermo Fisher Scientific). LgBiT expression was confirmed by western blotting with anti-Nluc polyclonal antibody (provided by Promega). VLP-HiBiT was infected to Vero-LgBiT on 96 well black clear bottom plates (353219, Falcon; Corning, Corning, NY) with Nano-Glo Live Cell Reagent (Promega). Luminescence was detected on GloMax-Multi detection system.

2.9. Assessing the effects of chemical inhibitors on cellular entry of VLP-HiBiT

To neutralize the infectivity of VLP-HiBiT, VLP-HiBiT was preincubated with either 10 μg/ml of dextran sulfate (Wako Pure Chemical Industries, Osaka, Japan) or 20 μg/ml of anti-WNV E monoclonal antibody (3.91D, Merck Millipore) for 30 min, and then infected onto Vero-LgBiT with Nano-Glo Live Cell Reagent. Mouse IgG3 B10 (Beckman Coulter) was used as isotype control antibody. To inhibit the endocytosis, Vero-LgBiT cells were preincubated with either 100 μM of Dynasore (Abcam, Cambridge, UK) or 30 μM of Pitstop 2 (Abcam) for 30 min. The mixture of VLP-HiBiT and Nano-Glo Live Cell Reagent was then added to cells in the presence of chemical inhibitors. After incubation for 30 min at 37°C, luminescence was detected on GloMax-Multi detection system. The cytotoxicity of chemical inhibitors was measured using CellTiter-Glo 2.0 Assay (Promega).

2.10. Statistical analyses

One-way analysis of variance (ANOVA) with Dunnett's test was used to determine statistical significance.

3. Results

3.1. Generation and detection of WNV SVPs tagged with HiBiT

The flavivirus E protein consists of a large ectodomain and two small transmembrane domains (Pierson and Diamond, 2013; Zhang et al., 2003). We introduced the HiBiT amino acid sequence, VSGWRLFKKIS, flanked with GS linker between two transmembrane domains of WNV E protein and obtained the plasmid, pCMV-SVP-HiBiT (Fig. 1A). Therefore, HiBiT is predicted to be localized in the interior of the viral envelope. To generate WNV SVPs tagged with HiBiT (SVP-HiBiT), 293T cells were transfected with pCMV-SVP-HiBiT. The expression of E and prM protein was confirmed on 293T cells by immunoblotting with anti-WNV E and M antibodies, respectively (Fig. 1B). E protein fused with HiBiT immobilized on the blotting membrane was also found to bind to LgBiT protein and exhibited luminescence following treatment with LgBiT protein and luciferase substrate (Fig. 1B). The luminescence was specifically observed from the supernatant of cells transfected with pCMV-SVP-HiBiT, increasing in a time-dependent manner, when the supernatant was incubated with LgBiT protein and luciferase substrate (Fig. 1C), indicating the extracellular secretion of E protein with HiBiT.

To assess the physiological properties of the secreted E protein tagged with HiBiT isolated from the supernatant, the supernatant was concentrated and subjected to sucrose gradient sedimentation analysis. E protein tagged with HiBiT, as well as non-tagged E protein, peaked in the 40% sucrose fraction (Figs. 2A and 2B). Disruption of membrane integrity by treatment with Triton X-100 resulted in a shift of the peak to low-density fractions. These sedimentation patterns were similar to those of flavivirus SVPs (Inagaki et al., 2016; Makino et al., 2014). Taken together, these results indicate that cells expressing prM and E protein fused to HiBiT secrete SVPs harboring HiBiT with similar physiological

property to non-tagged SVPs.

3.2. Application of SVP-HiBiT to quantitative measurement of extracellular release of WNV SVPs

Antigen-capture sandwich ELISA are commonly used for the quantification of viral antigens, including SVPs (Konishi and Fujii, 2002; Takahashi et al., 2009). We compared antigen-capture sandwich ELISAs with the SVP-HiBiT luciferase assay. SVP-HiBiT was two-fold serially diluted and subjected to sandwich ELISA with anti-E protein monoclonal antibody (Takahashi et al., 2009). Although ELISA optical density (O.D.) values were correlated with the dilution of SVP-HiBiT, the quantitative range of the assay was limited (Fig. 3A). The serially diluted SVP-HiBiT was also subjected to the luciferase assay by mixing with LgBiT protein and substrate. Luminescence correlated with the dilution of SVP-HiBiT over a broad dynamic range (Fig 3B). These results demonstrate that the luciferase assay of SVP-HiBiT could represent a more quantitative measurement of SVPs than traditional antigen-capture ELISA approaches.

We have previously shown that the small GTPase Rab11 is involved in the trafficking and release of WNV virions (Kobayashi et al., 2016). To examine whether the budding of SVP-HiBiT follows the same pathway of infectious WNV virions, we measured the extracellular release of SVP-HiBiT on Rab11-depleted cells. The expression of Rab11 was markedly decreased by co-transfection with two different combinations of siRNAs targeting *Rab11a* and *Rab11b* (Rab11a+b KD1 and KD2) (Fig. 3C). Control and Rab11-depleted cells were transfected with pCMV-SVP-HiBiT and the culture supernatants were subjected to the luciferase assay. The luciferase signals from the supernatant of Rab11-depleted cells were significantly lower than those of mock-transfected cells (Fig.

3D). This result suggests that the extracellular release of SVPs was inhibited by the depletion of Rab11, corresponding to the previous study using infectious whole virus (Kobayashi et al., 2016).

3.3. Generation and application of VLPs tagged with HiBiT to quantitative measurement of cellular entry of WNV VLPs

We next generated HiBiT-tagged VLPs (VLP-HiBiT) by fusing HiBiT with WNV C protein. The VLPs consist of E, M, C proteins and WNV replicon RNA incorporating a GFP-expression cassette. Vero cells infected with VLP-HiBiT expressed GFP, while the expression rate was slightly lower than that of cells infected with the original VLP without HiBiT (Fig. 4A and 4B). This indicated that VLP-HiBiT can cause infectious entry into target cells. We hypothesized that VLP-HiBiT shows luminescence in LgBiT-expressing cells when VLP-HiBiT releases the HiBiT-tagged C protein into the cytoplasm at the membrane fusion step. The cell permeable substrate is available and can be added to culture medium for quantitative measurement in living cells as detailed in the Materials and Methods section. LgBiT-stably expressing Vero cells (Vero-LgBiT) were established by the infection with a lentiviral vector carrying an LgBiT expression cassette. The expression of LgBiT was confirmed by immunoblotting with an antibody against Nluc that cross-reacts with LgBiT (Fig. 4C). The inoculation of VLP-HiBiT with substrate showed a gradual increase in luminescence and the signal peaked at 30 min, while the luminescence signals did not change following addition of substrate only (Fig. 4D). The luminescence signal derived from VLP-HiBiT also increased in a dose-dependent manner (Fig. 4E). We next examined whether the detected luminescence corresponds to the cellular entry of VLP-HiBiT. It has been previously reported that the monoclonal antibody clone 3.91D

targeting the WNV E protein neutralizes WNV infection (Frost et al., 2012). Indeed, pretreatment of VLP-HiBiT with the monoclonal antibody 3.91D, but not an isotype control antibody, significantly reduced luminescence signal in the Vero-LgBiT (Fig. 4F). Flaviviruses attach to cell surfaces and then enter into cells *via* clathrin-mediated endocytosis (Chu and Ng, 2004; Pierson and Diamond, 2013). Dextran sulfate, a soluble glycosaminoglycan, inhibits the attachment and subsequent entry of flaviviruses (Su et al., 2001; Talarico et al., 2005). Dynasore is a dynamin inhibitor and blocks endocytosis, including the infectious entry of flaviviruses (Kalia et al., 2013; Piccini et al., 2015). Pitstop 2 is a selective clathrin inhibitor and has been used to block viral entries *via* clathrin-mediated endocytosis (Garrison et al., 2013; von Kleist et al., 2011). Treatment with all three inhibitors significantly decreased the luminescence in Vero-LgBiT cells infected with VLP-HiBiT (Fig. 4G), and no cytotoxicity was observed under the assay conditions (Fig. 4H). These results indicate that inoculation of VLP-HiBiT to LgBiT-expressing cells emits luminescence, which is dependent upon the cellular attachment and subsequent entry of VLP-HiBiT.

4. Discussion

Reporter genes such as β-galactosidase, fluorescent proteins and luciferases provide ways to detect and quantify the amount of proteins easily by fusing with the targets of interest. We have introduced the HiBiT oligopeptide sequence to the WNV structural proteins and obtained SVP-HiBiT and VLP-HiBiT. E protein tagged with HiBiT and SVP-HiBiT were easily detected by an Nluc complementation assay with the substrate and LgBiT protein. In a proof-of-concept study, we then applied the luciferase assay with SVP-HiBiT and VLP-HiBiT in experiments quantifying cellular entry and release of SVPs and VLPs.

We used this experimental system to examine the quantitative properties of SVP-HiBiT release from transfected cells and found that the luciferase assay with SVP-HiBiT was a more sensitive, quantitative method with a broader dynamic range compared to antigen-capture ELISA (Figs. 2A and 2B). Moreover, the development of luminescence signals was rapid and accomplished within 10 min (data not shown). The luminescent signal from the supernatant containing SVP-HiBiT was also significantly decreased by the depletion of Rab11 (Fig. 3D), which is required for the efficient extracellular release of WNV (Kobayashi et al., 2016). As various flavivirus SVPs have been used in previous studies (Blazevic et al., 2016; Hanna et al., 2005; Op De Beeck et al., 2003; Yoshii et al., 2012), the SVP-HiBiT represents a useful tool for the analysis of virion release.

Other studies have employed virus-like particles (VLPs) harboring a subgenomic replicon with a reporter gene expression cassette to estimate viral entry by quantifying reporter gene expression. As this assay method requires stable transcription and translation of the reporter gene from the endocytosed VLPs, in some cases, it has been difficult to evaluate the signal decrease due to the inhibition of VLP entry or gene expression steps

(Phongphaew et al., 2017). It is also laborious and prone to error to count the number of fluorescent-labeling VLPs in cells under microscopy. In Fig. 4, we developed a VLP-HiBiT entry assay using LgBiT-expressing cells. Theoretically, the luminescence is emitted by direct interaction between the LgBiT protein and HiBiT-tagged C protein after the membrane fusion of VLP-HiBiT. Indeed, the luminescence was specifically observed in LgBiT-expressing cells infected with VLP-HiBiT under live-cell conditions and the signal was dependent on the endocytic entry of VLP-HiBiT. This assay can therefore also be used as a model system for the analysis of viral entry steps.

Previous studies have generated VLPs and infectious viral particles fused with whole firefly luciferase for the quantification of retroviral entry and release steps (Kolokoltsov and Davey, 2004; Saeed et al., 2006; Sakuragi et al., 2006). Unlike the larger whole luciferase proteins, such as firefly luciferase and NLuc (550 and 171 amino acids, respectively), HiBiT consists of only 11 amino acids and would be predicted to be less likely to interfere with the maturation and activity of viral proteins. Although WNV is a biosafety level-3 agent and requires a containment laboratory to handle; SVPs and VLPs cause non-productive infection and can therefore be used in a conventional biosafety level-2 laboratory. Taken together and considering these simple, time-saving and quantitative properties of the assay system using SVP-HiBiT and VLP-HiBiT, it will be directly amenable to high-throughput screening to identify host and pathogen-derived factors implicated in viral entry and release steps.

Competing financial interests

The authors have no competing interests to declare.

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Figure Legends

Fig. 1. Detection of HiBiT-tagged WNV E protein

(A) Schematic representation of the plasmids expressing WNV SVPs. Arrowheads and hatched boxes indicate putative cleavage sites and transmembrane domains, respectively. (B) 293T cells were transfected with SVP expressing plasmids shown in (A). HiBiT-fused WNV E protein was detected by protein blotting with anti-WNV E antibody and LgBiT protein. WNV prM protein was detected with anti-WNV M antibody. β-actin was used as a loading control. (C) 293T cells were transfected with SVP-expressing plasmids and the culture supernatants were harvested at the indicated time points. HiBiT-fused WNV E protein in the supernatants was detected by luciferase assay with LgBiT protein. Data are means ± S.D. of triplicates from a representative experiment. RLU, relative light units.

Fig. 2. Sucrose gradient sedimentation analysis of SVPs

SVPs (A) and SVP-HiBiT (B) were treated with or without 1% Triton X-100 (open squares or closed circles, respectively), and then fractionated by 10-50% sucrose gradient ultracentrifugation. Each fraction of SVPs and SVP-HiBiT was subjected to ELISA (A) and luciferase assay with LgBiT protein (B), respectively. O.D., optical density.

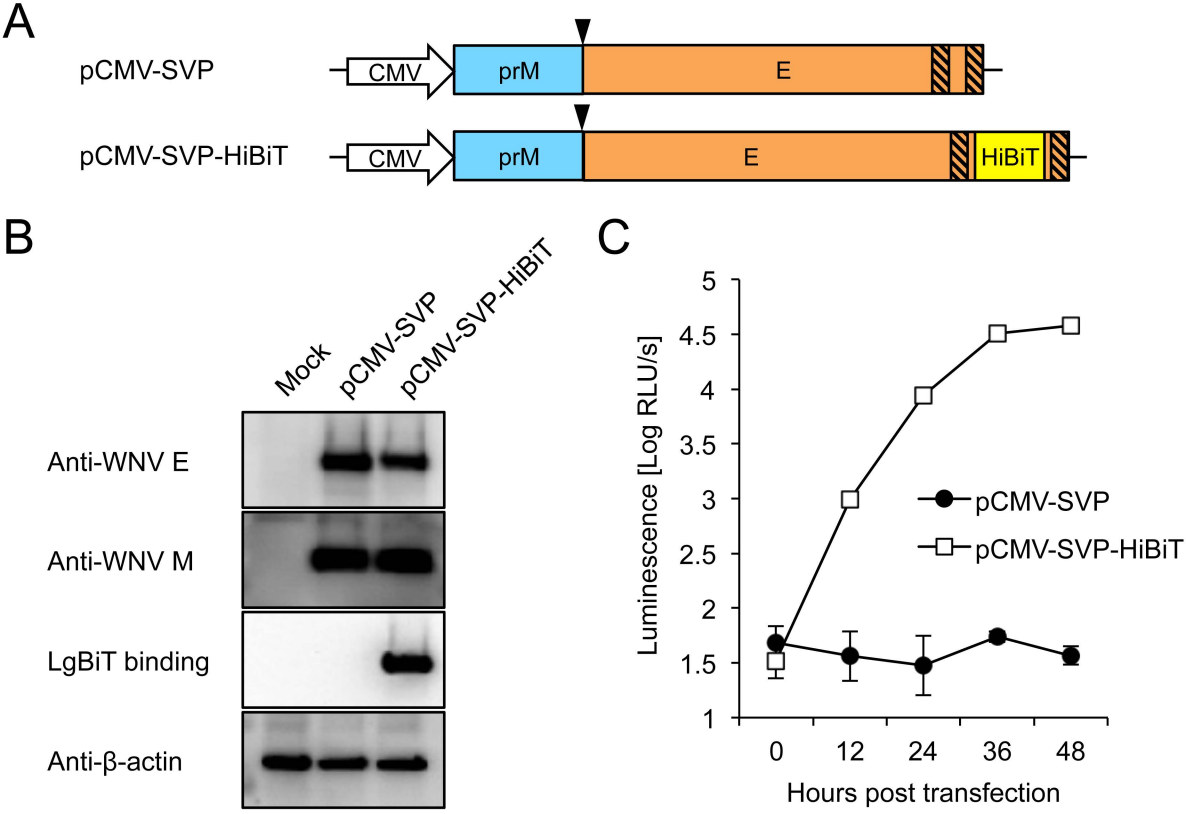
Fig. 3. Quantitative analysis of extracellular release of SVP-HiBiT

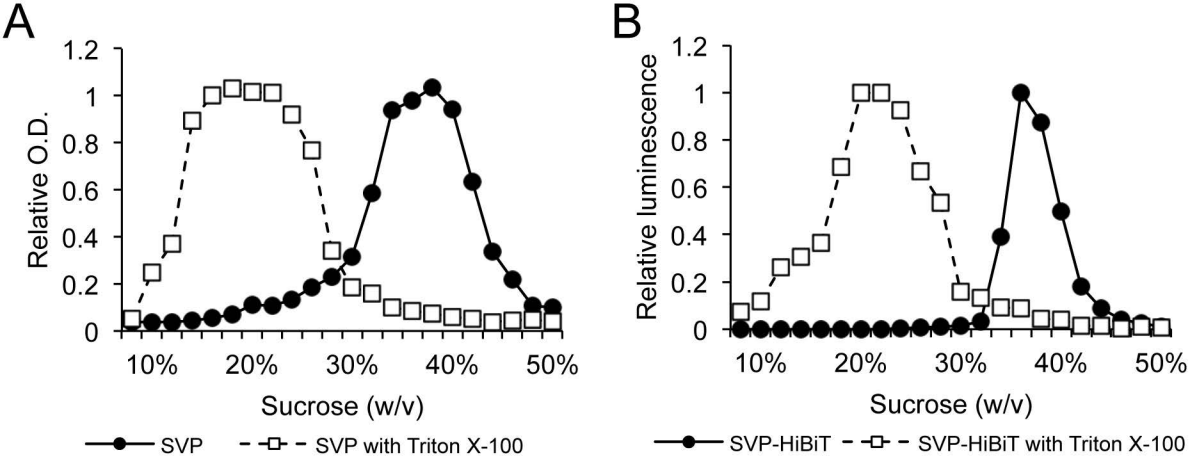
(A and B) Serially diluted SVP-HiBiT was subjected to an antigen-capture sandwich ELISA (A) or luciferase assay with LgBiT protein. O.D., optical density. RLU, relative light units. (C) A549 cells were transfected with non-targeting control siRNA or pairwise combinations of siRNAs targeting *Rab11a* and *Rab11b* (Rab11a+b KD1 and KD2). The expression level of Rab11 was examined by immunoblotting. (D) Control or

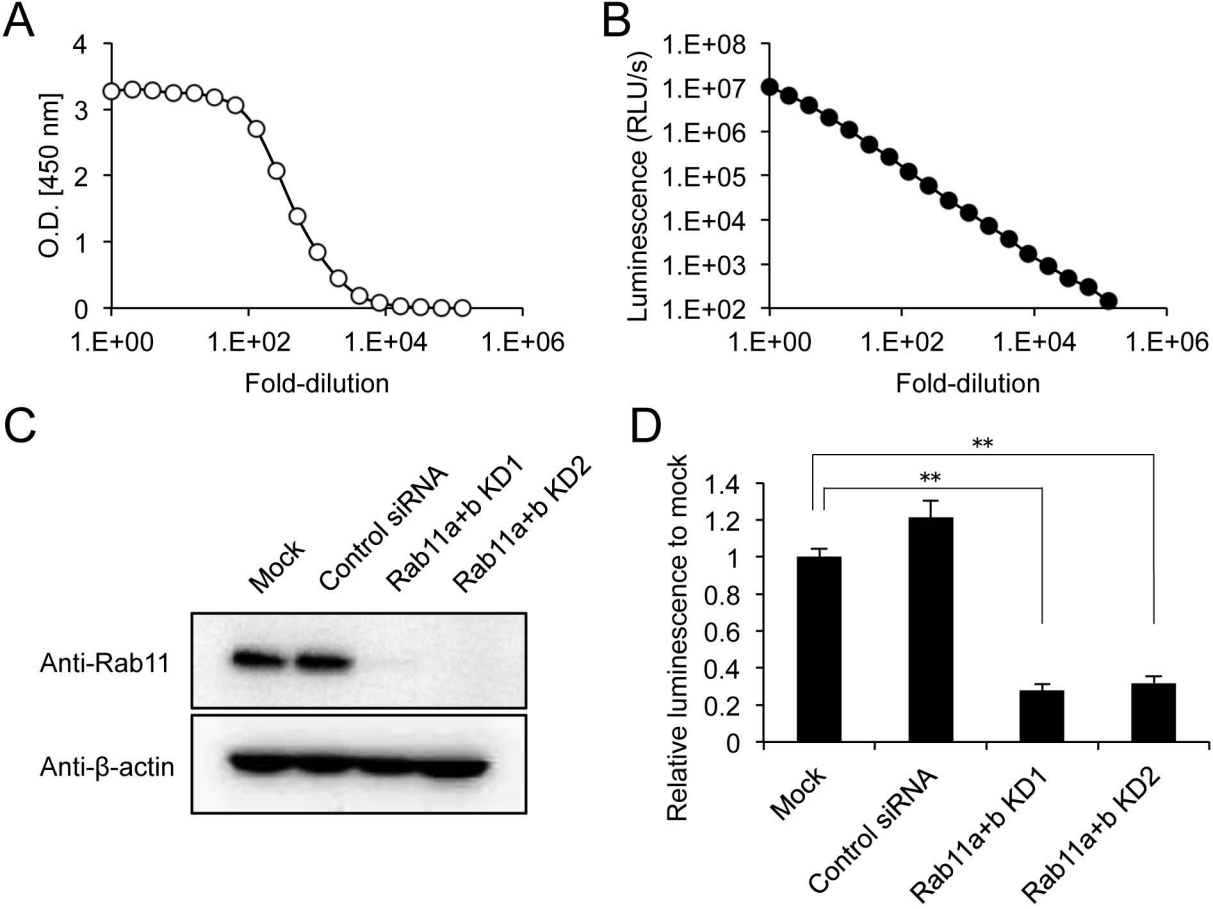
Rab11-knockdown cells were transfected with pCMV-SVP-HiBiT. The amount of SVP-HiBiT in the supernatant was quantified by luciferase assay with LgBiT protein. Data are means ± S.D. of triplicates from a representative experiment. ** $p < 0.001$ by statistical analyses using one-way ANOVA with Dunnett's test.

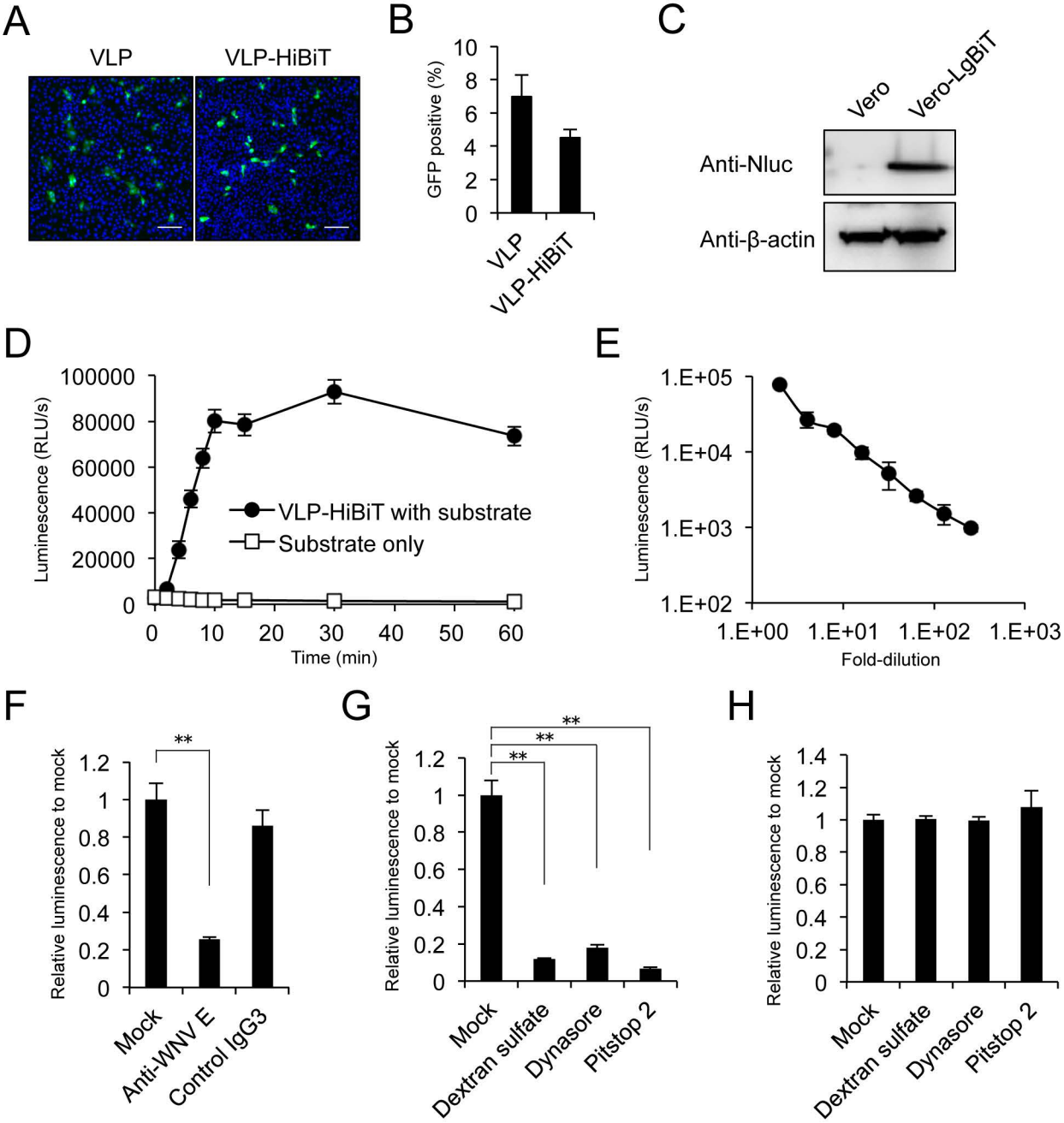
Fig. 4. Quantitative analysis of cellular entry of VLP-HiBiT

(A and B) Vero cells were infected with either VLPs or VLP-HiBiT. After 48 h, GFP expression (green) and nuclei stained by Hoechst 33342 (blue) were examined by fluorescence microscopy (A). Scale bars, 100 μm. GFP positive rates were calculated by counting the number of nuclei and GFP-expressing cells (B). (C) Vero cells infected with a lentiviral vector carrying LgBiT expression cassette. The expression of LgBiT was examined by immunoblotting using anti-Nluc antibody. (D) Vero-LgBiT cells were infected with VLP-HiBiT with substrate (closed circles) or treated with substrate only (open squares). Luminescence was measured at the indicated time points. RLU, relative light units. (E) Vero-LgBiT cells were infected with serially diluted VLP-HiBiT with substrate. Luminescence was measured 30 min post-infection. RLU, relative light units. (F) VLP-HiBiT preincubated with 20 μg/ml of monoclonal antibodies was infected onto Vero-LgBiT cells with substrate. Luminescence was measured 30 min post-inoculation. (G) Vero-LgBiT cells were infected with VLP-HiBiT plus substrate in the presence of 10 μg/ml of dextran sulfate, 100 μM of Dynasore or 30 μM of Pitstop 2. Luminescence was measured 30 min post-inoculation. (H) After incubation with chemical inhibitors for 1 h, cytotoxicity was measured using the CellTiter-Glo 2.0 Assay. All data are means ± S.D. of triplicates from a representative experiment. ** $p < 0.001$ by statistical analyses using one-way ANOVA with Dunnett's test.


A
pCMV-SVP
CMV
prM
E
pCMV-SVP-HiBiT
CMV
prM
E
HiBiT
B
Mock
pCMV-SVP
pCMV-SVP-HiBiT
Anti-WNV E
Anti-WNV M
LgBiT binding
Anti-β-actin
C
Luminescence [Log RLU/s]
5
4.5
4
3.5
3
2.5
2
1.5
1
0
12
24
36
48
Hours post transfection
pCMV-SVP
pCMV-SVP-HiBiT


A
Relative O.D.
1.2
1
0.8
0.6
0.4
0.2
0
10%
20%
30%
40%
50%
Sucrose (w/v)
SVP
SVP with Triton X-100
B
Relative luminescence
1.2
1
0.8
0.6
0.4
0.2
0
10%
20%
30%
40%
50%
Sucrose (w/v)
SVP-HiBiT
SVP-HiBiT with Triton X-100


A
O.D. [450 nm]
4
3
2
1
0
1.E+00
1.E+02
1.E+04
1.E+06
Fold-dilution
B
Luminescence (RLU/s)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+00
1.E+02
1.E+04
1.E+06
Fold-dilution
C
Mock
Control siRNA
Rab11a+b KD1
Rab11a+b KD2
Anti-Rab11
Anti-β-actin
D
Relative luminescence to mock
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Mock
Control siRNA
Rab11a+b KD1
Rab11a+b KD2


A
VLP
VLP-HiBiT
B
GFP positive (%)
10
8
6
4
2
0
VLP
VLP-HiBiT
C
Vero
Vero-LgBiT
Anti-Nluc
Anti-β-actin
D
Luminescence (RLU/s)
100000
80000
60000
40000
20000
0
VLP-HiBiT with substrate
Substrate only
0
10
20
30
40
50
60
Time (min)
E
Luminescence (RLU/s)
1.E+05
1.E+04
1.E+03
1.E+02
1.E+00
1.E+01
1.E+02
1.E+03
Fold-dilution
F
Relative luminescence to mock
**
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Anti-WNV E
Control IgG3
G
Relative luminescence to mock
**
**
**
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Dextran sulfate
Dynasore
Pitstop 2
H
Relative luminescence to mock
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Dextran sulfate
Dynasore
Pitstop 2