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Highly efficient production of a dengue pseudoinfectious virus

Xiaowu Pang^{a,b,*}, Yinhan Guo^c, Yanfei Zhou^{b,1}, Wenchuan Fu^c, and Xinbin Gu^a

^aDepartments of Oral Pathology, College of Dentistry, Howard University, Washington, District of Columbia ^bTegen Biomedical Co., Rockville, Maryland ^cOriental Tengen Technology Development Co., Ltd. Biomedicals, Beijing, China

Abstract

Dengue is a major infectious disease that affects people living in tropical and subtropical regions around the world. The causative agents are dengue virus serotype 1, 2, 3, and 4 (DENV1, 2, 3, and 4). Developing a vaccine for dengue is a high priority for public health, but traditional methods have faced numerous obstacles due to the unique immunopathogenesis of dengue virus infection. Here, we report a novel dengue vaccine candidate based on dengue pseudoinfectious virus (PIV) produced by the incorporation of a dengue subgenomic replicon into viral particles in highly efficient packaging cells. The subgenomic replicon was constructed by deleting the capsid protein (C) gene from the dengue viral genome and optimizing the signal peptide sequence of pre-membrane protein (prM) to facilitate the formation of viral particles. Packaging cells were developed for inducible expression of a bi-protein Cpr, where the protein pr is the “pr” segment of viral protein prM that holds the protein C on the endoplasmic reticulum (ER). When the replicon was introduced into the packaging cells, protein C was released from the bi-protein Cpr by a replicon-encoded viral protease. Coordinate expression of viral structural proteins by the replicon and packaging cells led to the incorporation of the replicon into viral particle to produce PIVs. Animal tests showed that the dengue PIV vaccine was highly immunogenic and the immune response protected mice challenged with a hundred-fold LD₅₀ inoculation of dengue virus. The method described here has the potential to be applied to vaccine development for other flaviviruses.

Keywords

Dengue vaccine; pseudoinfectious virus; replicon; packaging cell; immunogenicity

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*Address correspondence to Xiaowu Pang, xpang@howard.edu, 1-(202) 806-0345.

¹Current work at Viral Diseases Branch, Walter Reed Amy Institute of Research, Silver Spring, Maryland.

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1. Introduction

Dengue virus (DENV) infection is the most widespread vector-borne human viral infection in the world, with an estimated incidence of up to 390 million cases annually [1]. The predominant disease caused by DENV is dengue fever, a self-limited febrile illness often accompanied by minor hemorrhagic manifestations [2]. Every year approximately 250,000–500,000 cases of DENV infection develop into a more serious form called dengue hemorrhagic fever/shock syndrome (DHF/DSS), which has fatality rates as high as 20% [3]. The four dengue serotypes (DENV1, 2, 3, and 4) co-circulate in affected areas and any one is able to cause the full spectrum of dengue disease, from mild fever to DHF/DSS. While DENV infection appears to provide life-long immunity against re-infection by the same serotype, it only induces limited cross-serotype protective immunity, and people exposed to secondary DENV infections often have residual cross-reactive antibodies and T-cells from the initial infection that can actually exacerbate the disease [4, 5]. A high frequency of severe dengue disease has also been observed following primary infections of infants born to dengue-immune mothers when the infant's maternal antibodies are reduced to sub-neutralizing levels [6]. Due to antibody-dependent enhancement (ADE) of dengue infections, an effective dengue vaccine must induce and maintain high levels of virus-neutralizing antibodies to ensure long-term protection against all four serotypes. Since the 1920s, tremendous effort has been spent on DENV vaccine development using a diverse array of potential immunogens, including inactive (killed) virus, attenuated live virus, chimeric virus, recombinant poxvirus, recombinant subunits, and the DNA sequence encoding a putative antigen. However, these approaches have faced challenges in producing long-term protective immunity against all four DENV serotypes with acceptable safety, stability, and universality [7, 8, 9]. Therefore, there are numerous efforts underway to develop and test new candidate vaccines [10,11].

The use of pseudoinfectious viruses (PIV) is a new direction for the development of a dengue vaccine. PIV has been demonstrated to induce high immunogenicity in a safe manner [12]. Furthermore, the unique characteristics of PIV single-round infections minimize the effect of viral growth interference among serotypes, since a dominant serotype in a tetravalent dengue live viral vaccine is associated with its higher replication potential and predominance of viraemia [13, 14]. As a result, dengue PIV vaccines can potentially induce a balanced immune response to all four DENV serotypes. The major challenge for the application of PIV in dengue vaccine development is the production of high titers of dengue PIV by stable cell lines.

The goal of the present study was to create a highly efficient DENV PIV production system based on stably transfected cells. High efficiency of PIV formation was achieved by coordinated expression of viral structural proteins by DENV replicon and packaging cells to incorporate the replicon into viral particles to form PIVs. Animal test results show that DENV PIV vaccine is highly immunogenic and protects mice challenged with a hundred-fold LD50 inoculation of dengue virus. The implication of these findings for dengue and other flavivirus vaccines is discussed.

2. Materials and Methods

2.1. Creation of DENV2 replicons

DENV2 replicon cDNA plasmids were derived from full-length cDNA clone plasmids of DENV2 NGC [15]. The construct is based on plasmid pRS424, which is a yeast-bacterium shuttle vector used for the assembly and mutagenesis of full-length infectious DENV cDNAs in yeast cells [16, 17]. The “infectious” cDNA copy of DENV2 in the construct was used as a basis for creating the replicon in this study. The primers used to create a PCR fragment containing a deletion of the viral capsid protein (C) coding sequence with or without an optimized signal peptide of membrane precursor protein (prM) are shown in Figure 1. The PCR products generated with primer pairs D2/dc(5′u) and D2/dc(3′A), D2/dc(5′u) and D2/dc(3′B), or D2/dc(5′u) and D2/dc(3′C) contained corresponding deletions as shown in Figure 1. For construction of a replicon with an optimized signal peptide sequence for protein prM, the first PCR product was generated with primers D2/dc(5′u) and L4(3′) with D2/ C(A) replicon cDNA as a template. The second PCR product was generated with primers PQAQA and D2/1431R with DENV2 cDNA plasmid as the template. A fused PCR product was created with primers D2/dc(5′u) and D2/1431R and the first and second PCR products as templates. Mutations (Fig. 1) were introduced into the pRS424/D2 by homologous recombination between a PCR fragment bearing the mutations and a linearized plasmid DNA in yeast cells as described previously [18]. Positive clones were confirmed by DNA sequencing. The replicon RNAs were produced by *in vitro* transcription, and the replicon RNA was introduced into BHK21 cells or BHK21-based packaging cells by electroporation [15]. To verify the replication of the DENV replicon in normal cells, re-suspended cells were seeded onto 3 chamber slides (BD Falcon, Bedford, MA) and DENV antigen-positive cells were identified by immunofluorescence analysis [15] at 48 hours, 96 hours or 1 week post transfection. For the packaging cells, one tenth of the replicon-transfected cells were seeded onto chamber slides and rest were plated onto one well of a six-well plate. The cells in the six-well plate were examined daily for cytopathic effect (CPE) and the supernatant was collected for testing PIV production.

2.2. Development of packaging cell lines

The packaging cells were created to provide viral protein Cpr for replicon encapsidation. The plasmid pCpr was developed based on the commercial pLVX-Tight-Puro vector (Clontech) using standard methods. Briefly, the DENV2 Cpr cDNA was synthesized with codons optimized for high expression in mammalian cells. An internal ribosome entry site (IRES) DNA fragment and an enhanced green fluorescent protein (EGFP) DNA fragment were created by PCR using pLVX-tet-off advanced vector plasmid DNA and pEGFP/C1 as templates, respectively. The three DNA fragments (C-pr, IRES, and EGFP) were fused together by PCR to form the expression cassette Cpr-IRES-EGFP. The cassette was clone into a pLVX-Tight-Puro vector to yield plasmid pCpr as shown in Figure 2. The plasmid pCpr clones were further confirmed by DNA sequencing.

Lentiviral particles containing pLVX-tet-off advanced vectors, pLVX-Tight-Puro-Luc Control Vectors or pCpr vectors were produced by using the Lentiphos HT Packaging System (Clontech) following the manufacturer’s instructions. To create inducible expression

cells, 5×10^5 BHK21 cells were seeded into six-well plates in fresh growth medium and incubated overnight at 37°C in a humidified incubator under 5% CO_2 . The following day the cells were transduced with pLVX-tet-off lentiviral particles. Two days following transduction, the antibiotic Geneticin (G418) (Sigma) was added at a concentration of 400 $\mu\text{g}/\text{ml}$ for selection of G418-resistant cell clones. These clones were then analyzed for inducibility of expression by transduction with pLVX-Tight-Puro-Luc lentiviral vectors, and 24 hours following transduction cells were grown in medium in the presence (1 $\mu\text{g}/\text{ml}$) or absence of doxycycline. Luciferase activity was measured after 48 hours of induction using bioluminescence imaging with a Xenogen IVIS instrument (Caliper Life Sciences, Hopkinton, MA) based on the manufacturer's protocol. The BHK-Tet-Off cell clone displaying the highest fold of induction was used to establish a stable BHK cell line expressing the DENV2 structural gene cassette Cpr by transduction with pCpr lentiviral particles as described above. At 48 hours following the second transduction, cells were subjected to limited dilution into two 96-well plates in fresh growth medium containing 400 $\mu\text{g}/\text{ml}$ of G4185, 1 $\mu\text{g}/\text{ml}$ of doxycycline, and 5 $\mu\text{g}/\text{ml}$ of puromycin. When cell clones in the 96-well plate were visible, cell culture medium was replaced with doxycycline-free medium with 10% tetracycline-free FBS. After 48 hours of induction, several high-expression clones were selected based on levels of green fluorescence under a microscope. To select the most efficient packaging cell line, the high-expression cell clones were electroporated with DENV2 replicon RNA and cultured without doxycycline to determine whether they were able to produce PIV. The titers of PIV (in infectious units [IU] per milliliter) present in harvested culture fluids (CFs) were determined by flow cytometry [19]. The expression of Cpr mRNA in the packaging cells was confirmed by sequencing after reverse transcription-PCR amplification of total RNA isolated from the cells.

2.3. DENV PIVs growth curve

The PIVs growth curve in packaging cell line BHK/Cpr was analyzed in doxycycline-free growth medium supplemented with 10% tetracycline-free FBS. Packaging cells were seeded at a concentration of 1×10^6 per 100-mm dish. After a 24 h incubation at 37°C , cells were infected with DENV PIV at an MOI of 0.01 IU/cell, and then incubated in doxycycline-free growth medium. For comparison, BHK-21 cells were infected by wild-type parent DENV2 under the same conditions. Aliquots of the culture fluid were removed daily and stored at -80°C . The titer of DENV PIVs in each sample was determined as described above.

2.4. Animal tests

For animal studies, DENV PIVs were purified and concentrated by ultracentrifugation as described previously [20]. DENV PIV safety as a vaccine candidate was investigated by intracranial (i.c.) inoculation of virus or PIV into 3- to 4-day-old AG129 mice (parent mice were kindly provided by Herbert W. Virgin IV, Washington University School of Medicine). Groups of 10 2- to 3-day-old AG129 suckling mice were inoculated intracranially with 1×10^5 IU of dengue PIVs or with 5 PFU of parent DENV2 as a positive control. Mice were monitored for 14 days for signs of disease and death. Animals that were moribund and appeared to be unable to survive until the next day were humanely euthanized and scored as "dead" the following day.

For DENV PIV potency and efficacy studies, eight-week-old AG129 mice were used to test the ability of a single intraperitoneal (i.p.) inoculation of DENV PIV to prevent lethal DENV2 infection. Mice were assigned to one of 5 groups of 10 animals. The first group received 100 μ l of diluent (phosphate buffered saline, PBS). The second, third, fourth, and fifth groups received 1×10^3 IU, 1×10^4 IU, 1×10^5 IU, and 1×10^6 IU of DEN PIV in 100 μ l of PBS, respectively. On day 27 post vaccination, blood samples were collected from mouse tail veins and pooled by group. A plaque reduction neutralization (PRNT) assay was used to measure titers of DENV2-specific neutralizing antibodies in mouse sera as described previously [17]. To assess the protective efficacy of the vaccine, all mice were challenged at 28 days post-vaccination with $100 \times 50\%$ lethal doses (LD_{50}) of DENV2. All animals were weighed daily from 3 days prior to challenge until 28 days post-challenge and examined for signs of infection. Animals that appeared to be unable to survive until the next day (based on lethargy, weight loss, and paralysis) were humanely euthanized and scored as “dead” the following day. All surviving animals were euthanized at 28 days post-challenge. Guidelines for the humane treatment of animals were followed as approved by the Howard University Animal Care and Use Committee.

3. Results

3.1 DENV subgenomic replicon development

We previously constructed several DENV replicons [21, 22]. To investigate the influence of protein C deletion on the efficiency of viral protease cleavage of the truncated substrate, three replicons with different sized deletions in protein C were created (Fig. 1). DENV protein C belongs to the α -fold class with four helices ($\alpha 1$ - $\alpha 4$) [23]. Amino acid residues 1–20 have a labile conformation. The first three replicons were created to test how the α -helix domains influence viral protease cleavage. The main features of these constructs are as follows: D2/ C (A) contains no α -helix domain; D2/ C (B) contains the $\alpha 4$ domain; and D2/ C (C) contains the $\alpha 2$, $\alpha 3$, and $\alpha 4$ domains. In general, the smaller the deletion the higher the efficiency of PIV production. To overcome the dependence of VLP formation on inefficient viral protein cleavage, the replicon D2/ C (A) was further modified by introducing amino acid substitutions with PQAQA at the COOH terminus and inserting amino acids LLLL in the hydrophobic region of the prM signal peptide sequence (Fig. 1). The changes in signal peptide sequence enhanced signal peptidase-mediated cleavage, therefore uncoupling signal peptidase cleavage of the prM protein from its dependence on prior cleavage in the cytoplasm by the viral NS2B/NS3 protease. Once introduced into BHK-21 by electroporation, the D2/ C(L4) replicon RNA was able to amplify itself and express viral proteins, as measured by RT-PCR and indirect-immunofluorescence assays, without production of infectious viral particles (Figure 3 and data not shown).

3.2. Packaging cell lines development

Of the three structural proteins needed for replicon packaging, proteins prM and E can be provided by the replicon. However, stable cell lines produced by DNA transfection failed to produce high enough expression levels of protein C for sufficient production of PIV. Therefore, in order to increase viral protein C concentration in the viral packaging site on the ER membrane, we developed a DNA plasmid expressing protein C in the form of Cpr.

Consequently, the majority of protein Cpr remains on the membrane of endoplasmic reticulum (ER) until protein C is freed by viral protease NS2b/NS3. Stable cell lines derived from Cpr plasmid-transfected BHK21 cells were capable of producing a high titer of PIV, but long term passage of the cells resulted in a gradual loss of PIV production. To develop a stable packaging cell line for large scale PIV production, long-term stability of production cells is necessary. Since we have found that cells transduced by lentiviral vectors usually demonstrate higher stability than cells transfected by plasmid DNA, we utilized the “Lenti-X™ Tet-Off® Advanced Inducible Expression System” (Clontech), which has low base line expression and high inducibility. To facilitate selection of high expression clones, a bicistronic expression cassette of Cpr and green fluorescent protein (GFP) was introduced into pLVX-tight-puro vector to produce the pCpr vector (Fig. 2). BHK/Tet-off cells were developed by transducing BHK-21 cells with high titer of pTet-off lentiviral vectors. Twenty clones were analyzed for inducible expression. Clone 8 showed the highest inducibility of luciferase expression and was used to establish a stable BHK cell line to express DENV protein Cpr. The cells were transduced with pCpr lentiviral vectors and subjected to puromycin selection (see methods). To select an efficient packaging cell line, fifteen cell clones were electroporated with replicon D2/ C(L4) RNA and cultured in medium without doxycycline. The preliminary screening for PIV production cell clones was conducted by indirect immunofluorescent staining as shown in Figure 3. Ten of the 15 clones were capable of producing PIVs. The most efficient cell clone, #1b (referred to as BHK/Cpr), produced 2×10^6 IU/ml of PIVs and was used in all further studies.

3.3 Biological characterization of PIV

We sought to optimize PIV production by mimicking wild-type DENV replication. In order to compare PIVs and wild-type DENVs growth in packaging cells, cells were transfected with the same amount of *in vitro* transcribed replicon RNA or DENV RNA. Samples of the media were taken daily, and the titers of virus were subsequently determined. PIV and DENV2 grew at similar rates on respective cells (Fig. 4). However, the titers of PIV were slightly lower than DENV2, suggesting that the production system could further be optimized.

3.4. Determination of dengue PIV vaccine immunogenicity and safety in AG129 mice

The AG129 mouse model is one of the best small animal models for early stage dengue vaccine testing [24]. AG129 mice lack alpha/beta IFN and gamma IFN receptor genes, which permits infection by all four serotypes of DENVs regardless of age. The high safety level of the PIV vaccine was verified by intracranial injection of suckling mice. In this study, all 10 mice injected with 5 PFU of wild-type DENV2 developed paralysis of the hind legs at 4 days postinoculation and had to be sacrificed. In contrast, all PIV-injected mice remained healthy and demonstrated normal development for the duration of the experiment (21 days) (Fig. 5a). These results indicate the absence of infectious DENVs in PIV preparations and also demonstrated the high safety level of PIV as a vaccine candidate.

To determine PIV immunogenicity, mice were inoculated by the i.p. route with four different doses of PIV (see Materials and Methods). The induction of dengue virus-specific neutralizing antibodies was taken as an indicator of immunogenicity. Sera from same group

animal were pooled and analyzed for DENV2-specific neutralizing antibodies. All immunized animals developed a neutralizing antibody response by 27 days after injection, even at the lowest dose (Table 1). Significantly, antibody titers in each group were clearly dose-dependent (Table 1). Twenty-eight days post-inoculation, the animals were challenged by i.p. inoculation with 100 LD₅₀ (Fig. 5b). This dose produced 100% mortality in control animals by day 15. In the low dose-vaccinated animal groups, vaccination with any of the doses of PIV evaluated in this experiment produced significant delays in the onset of both illness and death in these animals (Fig. 5b). The group with 1x 10⁴ IU of PIV injection induced partial protection against a lethal DENV2 challenge. Four of the 10 mice in this group survived the experimental period. Over the 28-day course of observation, there was not a single animal that died in groups inoculated with 1x 10⁵ IU and 1x10⁶ IU of the PIVs. The results from this experiment show a clear correlation of protection from morbidity and mortality with vaccine dose (Fig. 5b).

Discussion

DENVs are members of the flavivirus genus of the flaviviridae family [25]. Like all flaviviruses, translation of the DENV genome produces a large polyprotein that is cleaved co- and post-translationally into at least 10 proteins [26]. The N-terminal quarter of the polyprotein encodes structural proteins (C-prM-E). Host signal peptidase is responsible for cleavage between C-prM, prM-E, and E-NS1 [27, 28, 29]. Interestingly, the cleavage of C-prM by signal peptidase is delayed until protein C is removed by viral protease NS2B/NS3 [30, 31]. This seems to be due to the combination of a fairly short (14 amino acids) signal sequence and suboptimal residues at the signalase cleavage site [32]. One explanation for this unusual processing strategy could be to delay structural protein processing and, consequently, virion formation until late in the infection when viral genome RNA levels are high [33, 34]. To satisfy the viral protein processing strategy in PIV virion formation, a major challenge is that the viral structural proteins are translated from separated messenger RNAs (mRNAs): protein C from cellular mRNA and protein prM and E from replicon RNA. For the DENV replicon, the deletion of protein C gene reduces the viral protease NS2B/NS3 activity at the C-prM site, which, in turn, reduces cleavage of C-prM by signalase [35, unpublished data]. In order to overcome the dependence of signalase cleavage on the release of signal sequence from protein C, we optimized the signal peptide sequence by elongating the signal sequence with the insertion of four leucine amino acids between methionine and leucine and replacing PTVMA with PQAQA (see Methods). The changes to the signal peptide sequence did not influence the replicon replication and expression in cells, but greatly increased VLP production. In our sequential transfection study, viral protein C expressed by second plasmid was sufficient to support PIV formation. However, in stably transfected cells, protein C is continually synthesized and a high proportion of protein C is located in the cell nucleus [36, 37, 38]. As a result, a very high level expression in transfected cells was needed to provide enough protein C at the viral packaging site to support PIV formation. To enhance the concentration of protein C at the viral packaging site, protein C was expressed in the form of bi-protein Cpr, where “pr” is the pr segment of prM. Protein pr is translocated into the endoplasmic reticulum (ER) by the hydrophobic signal peptide at the C-terminal of protein C. Consequently, the majority of protein Cpr

remains on the ER membrane until protein C is freed by the viral protease NS2b/NS3, similar to the natural DENV structural polyprotein processing that involves temporal and spatial coordination of virion formation by regulated cleavage of an ER membrane-spanning C-prM intermediate. This study demonstrates that this approach creates a highly efficient PIV production system with a viral titer similar to the parent virus replicated on the same type of cells (Fig. 4).

Infection with wild-type DENV induces a high titer of neutralizing antibody and recovery from infection results in life-long immunity to the homotypic DENV [5], which suggests that a vaccine for dengue is very feasible. However, the development of a DENV vaccine has been complicated by a requirement to protect simultaneously against the four serotypes of DENV to mitigate the potential to induce an immune response that would exacerbate the disease [39]. Historically, live virus vaccine induces long-term immunity, but it has proven difficult to achieve a balanced immune response to all four serotypes with live tetravalent DENV vaccines due to growth interference among serotypes in the host. On the other hand, inactivated viral tetravalent DENV vaccines are able to produce a balanced immune response but are usually not able to produce long-term immunity. In contrast with these traditional approaches, the unique biological characteristics of dengue PIV may provide the combined advantages of both live and inactivated viral vaccines. Specifically, PIV can infect host cells and express viral antigens *in vivo*, thus stimulating both arms of immune response similar to a live virus vaccine. Meanwhile, genetic deletion of encoding region for the structural protein prevents the virus from proceeding to a second round infection, reducing virus growth interference among the four serotypes of DENV and potentially inducing balanced immunity to all four serotypes of DENVs. Such an approach would mitigate concerns regarding ADE. Indeed, the results from animal tests revealed dose-dependent DENV2-specific responses in the PIV-inoculated mice and a clear correlation of protection from morbidity and mortality with vaccine dose (Fig. 5 and Table 1). Taking into account the dose-dependent response to the PIV vaccine, future studies will determine whether a DENV PIV vaccine is able to induce a balanced immune response to all four serotypes of DENVs if the tetravalent vaccine is composed of an adequate dose of each serotype.

In conclusion, we have developed a DENV PIV production system based on mimicking natural DENV replication. Coordinate expression of viral structural proteins by the replicon and packaging cells led to the incorporation of the DENV subgenomic replicon into virus particles. Animal tests demonstrated that a single dose of dengue PIV vaccine was able to elicit neutralizing antibodies in mice and provide protection from lethal challenge with DENV2. The experiments with intracranial injection of suckling mice suggest that the PIV vaccine has an excellent safety level. Due to the high degree of similarity in flavivirus genomic organization, the method described here has the potential to be applied to vaccine development for other flaviviruses.

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Highlights

- We explore the DENV replication mechanism to develop a high efficient production system for dengue PIV.
- We investigate the immunogenicity of dengue PIV with AG129 mice model.
- We show that PIV-based vaccines are inherent high safe level.
- We prove that dengue PIV can potential induce balance immune response against all four serotypes of DENV.
- The approach described in this study can be used for other flavivirus vaccines development.

Fig. 1. Diagram of the creation of DENV2 replicons. (A) The PCR primers used for the creation of viral protein C gene deletions are shown. The insertions and substitutions are in shown in bold. (B) The replicons D2/ C(A), D2/ C(B), and D2/ C(C) were made by deleting the coding region for protein C from amino acids (AA) 21 through 95, 21 through 76, and 21 through 63, respectively. Replicon D2/ C(L4) has the same deletion as D2/ C(A) plus a modified signal sequence of prM with an insertion of LLLL and replacement of PTVMA with PQAQA (see Materials and Methods).

Fig. 2.

Generation and characterization of the stable packaging cell line BHK/Cpr. (A) Schematic representation of the lentiviral vectors used for the generation of the stable packaging cell line BHK/Cpr (see Materials and Methods). (B) Inducible expression of EGFP in packaging cell BHK/Cpr. tTA-Adv, a Tet-controlled transactivator; IRES, EMCV internal ribosome entry site; Puro^r, puromycin N-acetyltransferase; Neo, neomycin resistance gene; P_{tight}, modified Tet-responsive promoter.

Fig. 3. Amplification and spread of PIV in packaging cell line BHK/Cpr. BHK-21 and BHK/Cpr cells were transfected with D2/C(L4) replicon RNA, and one tenth of transfected cells were inoculated into a chamber slide. Indirect immunofluorescence analysis with 4G2 monoclonal antibody was performed at 2 and 3 days after transfection.

Fig. 4.

Growth characteristics of DENV2 and PIVs in packaging cells. The cells were transfected by electroporation with 2 μ g of the indicated RNA (see Materials and Methods). The results demonstrate the influence of signalase cleavage on PIV production.

Fig. 5. Characterization of PIV as a dengue vaccine candidate in AG129 mice. (A) Kaplan-Meier curve showing onset of death following I.C. inoculation of wild type DENV2 or 1×10^5 IU of PIV in suckling mice. (B) Kaplan-Meier curve showing onset of death following challenge with 100× LD50 of DENV2 NGC (see Materials and Methods).

TABLE 1

Neutralizing antibody induced in mice immunized with PIV

PIV Dose, IU	PRNT ₅₀
0 (PBS)	<10
1×10^3	80
1×10^4	160
1×10^5	320
1×10^6	590