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Novel, anionic, antiviral septapeptides from mosquito cells also protect monkey cells against dengue virus

Chaowanee Laosutthipong [a,b], Nipaporn Kanthong [c], Timothy W. Flegel [a,b,d,*]

[a] Dept. Biotechnology, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[b] Centex Shrimp, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[c] Dept. Biotechnology, Faculty of Science and Technology, Rajamangala University of Technology Tawan-ok, Sriracha, Chonburi 20110, Thailand
[d] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Klong 1, Klong Luang, Pratum Thani 12120, Thailand

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ABSTRACT

We have shown previously that ultrafiltrates (5 kDa cutoff) of cell-free medium from mosquito cell cultures persistently infected with DENV serotype 2 (DENV-2) contained a novel antiviral agent (called viprolaxikine) that could protect pre-treated, naïve mosquito cells from DENV infection. Here, we show that viprolaxikine also reduced DENV-2 titers by almost 4 logs (>99.9%) when compared to Vero cells mock-treated with ultrafiltrates from cultures of uninfected mosquito cells. Protease treatment removed the anti-DENV-2 activity. Pre-incubation for 48-h was required to obtain the maximum, dose-dependent protection against DENV-2, indicating that the antiviral activity was based on the interaction between Vero cells and viprolaxikine rather than direct action of viprolaxikine on DENV-2. Activity was highest against DENV-2, but there was also significant activity against the 3 other DENV serotypes. LC–MS–MS analysis revealed that the active viprolaxikine fraction contained anionic, antiviral peptides, each comprised of 7 amino acids (DDHELQD, DETELQD and DEVMLQD or DEVLMQD) and with a common sequence motif of D-D/E-X-X-X-Q-D. These sequences do not occur in the dengue virus genome, suggesting that the peptides are produced by the host insect cells when persistently infected with DENV-2. These peptides represent a new class of anionic, insect-derived, antiviral peptides with activity against a flavivirus in both mammalian and insect cells.

1. Introduction

Dengue virus (DENV) is divided into four serotypes (DENV-1 to 4) (Westaway, 1997) any of which may cause disease ranging from acute febrile dengue fever (DF) to life-threatening dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). Outbreaks of DF and DHF are still a major public health problem worldwide, especially in tropical and subtropical countries (WHO, 2002).

Persistent, *in vitro* virus infections have been established for many flaviviruses in both mosquito and mammalian cell lines (Chen et al., 1994, 1996; Debnath et al., 1991; Lancaster et al., 1998; Randolph and Hardy, 1988; Schmaljohn and Blair, 1977; Vlaycheva and Chambers, 2002). From high passage cultures of persistently infected insect cells, it has been reported that high numbers of defective interfering particles (DIP) are produced and that they can account for the low infectivity of supernatant solutions for challenged mammalian cells (Huang and Baltimore, 1970; Von Magnus, 1951). An alternate explanation to DIP is that some factor(s) produced by persistently infected insect cells can act as an antiviral agent as previously reported for an antiviral protein (AVP) from mosquito cells persistently infected with Sindbis virus (Luo and Brown, 1993, 1994; Riedel and Brown, 1979).

Similarly, an antiviral preparation from mosquito cells persistently infected with DENV-2 (called viprolaxikine) successfully protected naïve mosquito cells against DENV-2 infection (Kanthong et al., 2010). This suggested that previously reported failure to infect Vero cells with DENV-2 produced from high passage cultures of mosquito cells persistently infected with DENV-2 (Sinarachatanant and Olson, 1973) might be due to viprolaxikine rather than DIP. To test this hypothesis, cell-free medium from C6/36 cells persistently infected with DENV-2 was investigated for the presence of antiviral substances. This revealed that viprolaxikine contained a new class of anionic septapeptides that could protect both C6/36 cells and Vero cells against DENV-2 infection.

2. Materials and methods

2.1. Cell lines, viruses and virus titration

Vero cells (ATCC: CRL-81) were cultured at 37 °C in Minimum Essential Medium (Invitrogen) supplemented with 10%

* Corresponding author at: National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Klong 1, Klong Luang, Pratum Thani 12120, Thailand. Tel.: +662 201 5870; fax: +662 354 7344.
E-mail address: sctwf@mahidol.ac.th (T.W. Flegel).

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heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1.2% antibiotics (Penicillin G and Streptomycin) in a 5% CO_2 incubator. C6/36 cells (*Aedes albopictus*) (ATCC: CRL-1660) were grown in Leibovitz's (L-15) medium containing 10% heat-inactivated FBS, 10% tryptose phosphate broth (TPB), and 1.2% antibiotics (Penicillin G and Streptomycin). Dengue virus serotype-1 (DENV-1) (HAWAII), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (H241) were propagated in C6/36 cells as previously reported (Kanthong et al., 2010) and stored as a stock at −80 °C. Viral infectivity was determined by focus forming units (FFU) and expressed as virus titer of FFU/ml as previously described (Cruz and Shin, 2007) with some modifications (Kanthong et al., 2008).

2.2. Immunofluorescence assays

To determine the proportion of infected cells by flow cytometry, C6/36 cells or Vero cells were washed twice with 1× phosphate buffered saline (PBS) and fixed with 2% formaldehyde in PBS for 1 h. Two additional washings were carried out using 0.1% triton X-100/PBS. Cells were incubated with mouse antibody to DENV at room temperature for 1 h, washed twice with 0.1% triton X-100/PBS and incubated with rabbit anti-mouse IgG conjugated with FITC (F0261, DAKO) for 30 min in the dark. Cells were washed and resuspended followed by FACS analysis (Becton Dickinson). Mock-infected, negative control C6/36 cells were run in parallel.

Immunofluorescence staining of DENV antigen in the cytoplasm of Vero cells was carried out as previously described (Kanthong et al., 2010) using anti-DENV envelope protein antibody labeled with FITC (green fluorescence) while nucleic acids were counterstained with TO-PRO-3 iodide (T-3605, Molecular Probes)(red fluorescence). Results were observed using a confocal laser microscope (FV1000, OLYMPUS).

2.3. Real-time RT-PCR

Viral RNA from culture supernatant was extracted using TRIzol reagent (Invitrogen) and RNA yield was determined using spectrophotometry at 260 nm. Primers for a 150 base pair amplicon of the DENV-2 envelope gene 5′- CAC TGT CAC GAT GGA GTG CT -3′ (forward) and 5′-TGA TCC TTG TGT GTC CGC T-3′ (reverse) were used with a SuperScript One-Step RT-PCR (Invitrogen) kit and Platinum *Taq* DNA polymerase. Real-time RT-PCR was carried out using a Rotor-Gene 6000™ (Corbett Research) machine with the protocol: one cycle of 50 °C for 15 min and 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Relative quantification was achieved by reference to a standard curve prepared using RNA from a DENV-2 stock of known viral titer.

2.4. Persistent dengue virus infections and viprolaxikine preparation

Persistent infections of DENV-2 in C6/36 cells were achieved and viprolaxikine was prepared as previously described (Kanthong et al., 2008, 2010). Before storage at −20 °C, the protein concentration in viprolaxikine preparations was measured using a Bradford protein assay kit (Bio-Rad) and a microplate reader set at OD_{595}.

2.5. Determination of antiviral activity with Vero cells

For time-course activity studies, Vero cell monolayers were incubated with a single addition of persistently infected insect-cell culture filtrate from 0 to 96 h in 96-well plates at 37 °C prior to challenge with serially diluted (10× steps) DENV-2 stock. The filtrate was washed off the cells prior to addition of the viral stock and the exposed cells were assayed for FFU three days later. The negative control consisted of Vero cells pre-incubated in parallel with filtrate from normal insect cell cultures (called naïve cells) followed by washing before challenge with DENV-2.

To compare anti-DENV activity in filtrates, Vero cell monolayers were incubated with a single addition of persistently infected insect-cell culture filtrates from passages 5, 10, 16, and 25 using the optimum filtrate pre-exposure time determined as described above. This yielded the optimum passage number to obtain the highest antiviral activity in the culture supernatant.

To determine the dose dependence of anti-DENV activity, Vero cell monolayers were incubated with a single addition of various quantities (5–200 μl) of persistently infected insect-cell culture filtrates in a total volume of 200 μl for the optimum time determined as described in the previous paragraph. After culture filtrate removal, they were challenged and assayed with DENV-2 as described above. Parallel Vero cell cultures treated with filtrate from uninfected insect cell cultures served as mock-infected cells. The data generated from these experiments were used to define one arbitrary unit of filtrate activity as the volume (μl) or μg protein that resulted in a 50% decrease in viral titer (FFU), and a standard assay was designed where monolayers of naïve Vero cells in a final volume of 100 μl a 96-well microtiter plate (approximately 20,000 cells per well) were prepared and incubated for confluent growth overnight. Then, the old medium was replaced with only 2% FBS-MEM medium or the same mixed with viprolaxikine preparations (final volume 100 μl) and incubated for 48 h before washing and then exposure to DENV-2. The virus titer was determined 3 days later using focus forming assays. Every batch of crude filtrate was calibrated in this way. For convenience, the active filtrate and active fractions of it are referred to here as viprolaxikine.

To determine the antiviral activity of viprolaxikine for other DENV serotypes, Vero cells were treated with filtrates from the 16th passage of C6/36 cells persistently infected with DENV-2 according to the standard assay procedure described above using 50 μl of crude filtrate (1 μg protein) but followed by exposure to each of the 4 DENV serotypes.

2.6. Preliminary purification and mass spectometry of viprolaxikine

Purification of viprolaxikine was based on filtrates from the 16th passage of C6/36 cells persistently infected with DENV-2. Many aliquots (1 ml) of 5 kDa membrane culture filtrates were desalted for removal of low molecular weight contaminants from culture medium using a HiTrap Desalting column (GE Healthcare) and Fast Protein Liquid Chromatography (FPLC) (AKTApurifier). The protein peaks eluted before the salt were collected (2 ml each), pooled and subjected to FPLC using ion exchange chromatography of either the strong cation (HiTrap SP HP) or strong anion (HiTrap Q HP) type eluted using continuous salt gradients ending with 1 M NaCl. Column fractions were tested for antiviral activity by titer with Vero cells. Final purification consisted of reverse phase high performance liquid chromatography (HPLC) (Waters) using a Delta-Pak C_{18} column (2 mm × 150 mm) with buffer A containing 0.1% trifluoroacetic acid (TFA)/H_2O and buffer B containing 0.1% TFA/acetonitrile (ACN). Samples were automatically injected and eluted with a 1–60% ACN gradient over 60 min. Polypeptides were detected by optical density at 256 and 280 nm using a Waters 2487 Dual UV detector, and fractions were dried using a SpeedVac concentrator before re-suspension in culture medium for antiviral testing.

For mass spectrometry, the fraction from the initial HPLC column with highest anti-DENV-2 activity was injected into an Ultimate 3000 Liquid chromatography system (Dionex, USA) that was coupled to an ESI-Ion Trap Mass spectrometry (Bruker, Germany) with electrospray at a flow rate of 300 nl/min to nanocolumn (Onyx, monolithic HDC18, 0.2 mm i.d. × 150 mm). The mobile phase A was 5% ACN in 0.1% formic acid and the mobile