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Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production

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ABSTRACT

Dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), caused by dengue virus (DENV) infection, are important public health problems in the tropical and subtropical regions. Abnormal hemostasis and plasma leakage are the main patho-physiological changes in DHF/DSS. A remarkably increased production of cytokines, the so called 'cytokine storm', is observed in the patients with DHF/DSS. A complex interaction between DENV proteins and the host immune response contributes to cytokine production. However, the molecular mechanism(s) by which DENV nonstructural protein 5 (NS5) mediates these responses has not been fully elucidated. In the present study, yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx) was identified. The *in vivo* relevance of this interaction was suggested by co-immunoprecipitation and nuclear co-localization of these two proteins in HEK293 cells expressing DENV NS5. HEK293 cells expressing DENV NS5-K/A, which were mutated at the nuclear localization sequences (NLS), were created to assess its functional roles in nuclear translocation, Daxx interaction, and cytokine production. In the absence of NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase the DHF-associated cytokine, RANTES (CCL5) production. This work demonstrates the interaction between DENV NS5 and Daxx and the role of the interaction on the modulation of RANTES production.

1. Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* family, which contain a single positive-stranded RNA genome encoding a single polypeptide precursor. Host and viral proteases cleave this polypeptide into three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), respectively. Clinical symptoms of DENV infection range from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which typically occurs in cases of reinfection with a different serotype of DENV. Patients with DHF present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration [1]. In addition, increased levels of cytokines – so called 'cytokine storm', which relate to the pathogenesis of severe DENV infection, are observed in the patients with DHF/DSS [2].

A complex interaction between DENV proteins and the host immune response contributes to DHF/DSS [3–9]. Both inhibition of the antiviral response and stimulation of cytokine production by DENV proteins have been reported. For example, DENV NS2B3 complex, DENV NS4B and DENV NS5 contribute to the inhibition of type I IFN response [3,5,7]. DENV NS4B and DENV NS5 also enhance the production of DHF-associated cytokines [8,9].

DENV NS5 is a bi-functional enzyme containing three domains [10–12]. The N-terminus from residues 1 to 368 contains a 2′-*O*-methyltransferase while the C-terminus from residues 405 to 900 contains an RNA-dependent RNA polymerase. The interdomain region contains nuclear localization sequences (NLS), which are divided into aNLS and bNLS, respectively. Mutations in both aNLS and bNLS result in the accumulation of DENV NS5 in the cytoplasm [13].

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In the present study, a yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx) which serves as transcription repressor was identified. The roles of DENV NS5 in nuclear translocation, Daxx interaction, and cytokine production were tested and DENV NS5 was demonstrated to interact with human Daxx to increase RANTES production.

2. Materials and methods

2.1. Yeast two-hybrid screening

Two-hybrid screening was performed by the interaction mating method described by Finley and Brent [14]. DENV NS5 was PCR amplified by *Pfu* DNA polymerase from a cDNA clone of DENV serotype 2 strain 16681 [15] using nucleotide primers, 5′TTG ACT GTA TCG CCG GGA ACT GGC AAC ATA3′ and 5′CCG GAA TTA GCT TGG CTG CAG CCA CAG AAC TCC TCG3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 94 °C for 5 min and followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, extension at 72 °C for 3 min, and one cycle of final extension at 72 °C for 10 min. Subsequently, the amplified DNA was cloned by yeast recombination [16] into the yeast expression vector pEG-NRT [17], which was derived from pEG202 [18], and contains a *HIS3* selectable marker. The constitutive ADH promoter is used to express DENV NS5 with the DNA binding domain protein LexA. Nuclear localization sequences, 5′recombination tag (5′RT), multiple cloning site (MCS) and 3′recombination tag (3′RT) were inserted between LexA and ADH terminator. The resulting bait plasmid, pEG-NS5, was verified by DNA sequencing and transformed into *Saccharomyces cerevisiae* strain RFY 206 (MATa *his3A200 leu2-3 lys2A201 ura3-52 trp1A::hisG*) containing a Lexop-*lac*Z reporter plasmid, pSH18–34 [14]. A galactose inducible HeLa cell cDNA prey library was constructed in plasmid pJZ 4–5 containing a *TRP1* selectable marker and transformed into strain RFY 231 (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) [14]. The bait strain then was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil and leucine (gal/raf -u, -h, -w, -l) to select for diploids. The production of a DENV NS5 binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU*2 reporter. Putative positive clones were patched to four indicator plates: (glu/-u, -h, -w, -l), (gal/raf -u, -h, -w, -l), (glu/X-Gal-u, -h, -w), and (gal/raf/ X-Gal-u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ $lacZ^+$ phenotype by transformation into a Trp^- *Escherichia coli* strain KC8 [14]. Putative positive library plasmids were purified and subjected to DNA sequencing and BLAST analysis.

2.2. Generation of HEK293 cells expressing DENV NS5, or DENV NS5-K/A

DENV NS5 with a C-terminal FLAG-tag was PCR amplified by *Pfx* DNA polymerase (Invitrogen) from plasmid pET-DENV-2-NS5 using nucleotide primers, 5′ACA GGA TCC ACC ATG GGA ACT GGC AAC ATA GGA GAG ACG3′ and 5′TGT CTC GAG TTA CTT GTC ATC GTC ATC CTT GTA ATC CCA CAG AAC TCC TGC TTC TTC C3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94 °C for 5 min and followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 3 min, and one cycle of final extension at 68 °C for 10 min. The PCR product was sub-cloned into plasmid pcDNA3.1/Hygro (Invitrogen) and the fidelity of the insert in the resulting plasmid, pcDNA3.1/DENV NS5, was verified by DNA sequencing.

Site-directed mutagenesis was employed to generate plasmid pcDNA3.1/DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A. Firstly, DENV NS5 (K387A, K388A, K389A) was amplified by PCR using plasmid pcDNA3.1/DENV NS5 as a template and nucleotide primers, 5′GGA AAG AAT TAG GGG CGG CAG CGA CAC CCA GGA TGT G3′ and 5′CAC ATC CTG GGT GTC GCT GCC GCC CCT AAT TCT TTC C3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94 °C for 2 min and followed by 18 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 68 °C for 18 min. The PCR product was digested with *Dpn*I to eliminate methylated parental DNA template and transformed into competent *E.coli* strain DH5. The correct mutant clone with K387A, K388A, K389A was verified by digestion with *Fnu4*HI and DNA sequencing. Secondly, DENV NS5 (K371A, K372A, K387A, K388A, K389A), was amplified by PCR using plasmid pcDNA3.1/DENV NS5(K387A, K388A, K389A) as a template and nucleotide primers, 5′CCG AAA GAA GGC ACG GCG GCA CTA ATG AAA ATA AC3′ and 5′GTT ATT TTC ATT AGT GCC GCC GTG CCT TCT TTC GG3′. The PCR reaction and transformation is similar to those described previously. The correct mutant clone with K371A, K372A, K387A, K388A, K389A, namely DENV NS5-K/A, was verified by digestion with *Fnu4*HI and DNA sequencing.

HEK 293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1/DENV NS5 or pcDNA3.1/DENV NS5-K/A by Lipofectamine® 2000 transfection (Invitrogen). Two days after transfection, the cells were collected, fixed and permeabilized with 0.1% Triton X-100 in PBS. Re-suspended cells were blocked with DMEM containing 1% BSA before incubation with mouse anti-FLAG (Sigma) antibody for 1 h. After incubation, the cells were washed twice with chilled DMEM containing 1% BSA. Then, goat anti-mouse antibody conjugated with FITC (Molecular Probes) was used as secondary antibody for 30 min. The cells were washed again and analyzed by using FACSort™ flow cytometer (Becton–Dickinson). HEK293 cells expressing either DENV NS5, or DENV NS5-K/A were also tested for the presence of DENV NS5 by Western blot analysis using anti-FLAG (Sigma).

2.3. Co-immunoprecipitation

Forty-eight h post-transfection, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were lysed in RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA, and protease inhibitor cocktail. Five micrograms of goat anti-Daxx antibody (Santa Cruz Biotechnology) or 5 μg of mouse anti-FLAG antibody were added to lysates. The mixture was incubated with gentle rotation at 4 °C for 6 h. The incubation was continued 24 h after addition of Protein G Sepharose (Amersham Pharmacia Biosciences). Subsequently, Protein G Sepharose was collected by centrifugation at 13,000*g* for 5 min and washed twice with 0.1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA. The bound proteins were eluted by boiling in SDS–PAGE loading buffer, separated by SDS–PAGE, and transferred to nitrocellulose membrane. After blocking with 5% skim milk, the membranes were incubated with either a mouse anti-FLAG antibody or a rabbit anti-Daxx antibody followed by probing with either a rabbit anti-mouse antibody conjugated-horseradish peroxidase (HRP) or a swine anti-rabbit antibody conjugated-HRP (DakoCytomation), respectively. Chemiluminescent signals generated by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were detected using a G:BOX chemiluminescence imaging system (Syngene).

2.4. Co-localization

HEK293 cells were grown on coverslips and then transfected with plasmid expressing either DENV NS5 or DENV NS5-K/A for 48 h. The transfected cells were fixed with 0.1% formaldehyde in

PBS, rinsed, permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% BSA. The cells were stained at RT for 1 h with both a mouse anti-FLAG and a rabbit anti-Daxx primary antibodies, washed and incubated at RT for 1 h with both an Alexa 488-conjugated rabbit anti-mouse Ig antibody (Molecular Probes) and a Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch Laboratories) as secondary antibodies. Fluorescent images were captured with a confocal microscope (model LSM 510, Carl Zeiss).

2.5. Real-time RT-PCR

Total RNA from HEK293 cells expressing either DENV NS5, or DENV NS5-K/A and cultured in the presence of 50 ng/ml TNF-α (Santa Cruz biotechnology) was isolated using Trizol reagent (Invitrogen). Cytokines were quantified by real-time RT-PCR using the following primer pairs: IL-8: 5′-TCC TGC AGA GGA TCA AGA CA-3′ and 5′-GAG CAC TTG CCA CTG GTG TA-3′, CXCL9: 5′-CAG ATT CAG CAG ATG TGA AGG A-3′ and 5′-GAA ATT CAA CTG GTG GGT GGT-3′, RANTES: 5′-CAA GGA AAA CTG GGT GCA GA-3′and 5′-TCT CCC GTG CAA TAT CTA GGA A-3′, respectively. The assay was performed using LightCycler® 480 SYBR Green I Master Mix (Roche) and a LightCycler® 480 Instrument equipped with a 96-well thermal cycler (Roche). Briefly, RNA samples were reverse-transcribed using the SuperScript^{III} First-Strand Synthesis System (Invitrogen). Then cDNA templates were subjected to a 10-min initial denaturation at 95 °C prior to 50 cycles of PCR (95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s) in the presence of *Taq* DNA polymerase and the gene-specific primer pairs. The mRNA level was normalized with human beta-actin mRNA using the ΔΔCt method [19].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Production of RANTES in HEK293 cells expressing either DENV NS5 or DENV NS5-K/A in the presence of TNF-α, was measured by Instant ELISA® (eBioscience) according to the manufacturer's instruction.

3. Results and discussion

3.1. Identification of DENV NS5 interacting proteins by a yeast two-hybrid screen

To identify human proteins that interacted with DENV NS5, we screened over 10^7 clones from a HeLa cDNA library using DENV NS5 as bait. Thirty putative positive clones were obtained. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lacZ* plasmid and bait plasmid and again tested on the indicator plates. Twelve library plasmids were purified and subjected to DNA sequencing. BLAST analysis revealed that the twelve clones corresponded to the proteins Daxx, Fas-associated factor 1 (FAF1), Calpain 2, Protein phosphatase 1 (PP-1), Splicing factor (SF3a), and Double-strand break repair protein (Mre11A).

The specificity of the interaction between DENV NS5 and Daxx, is shown in Fig. 1A wherein cells containing the DENV NS5 and Daxx, exhibited galactose-dependent leucine prototrophy and *lacZ* expression. Daxx has a strong transcriptional repression activity and can bind to several transcription factors in the nucleus [20,21]. In addition, the majority of Daxx is present in the nucleus similar to that of DENV NS5 [22,23]. Daxx is a 740 amino acid protein that contains amino-terminal amphipathic helices (PAH1, PAH2), a coiled–coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). Among them, the S/P/T domain can mediate the interaction of Daxx with numerous proteins. The region of Daxx that interacted with DEN NS5 was verified in the prey plasmid by DNA sequencing and BLAST analysis and the 211 carboxyl-terminal residues, which cover the S/P/T domain than can bind to multiple proteins including DENV capsid protein in the nucleus of DENV-infected cells, were identified [24] (Fig. 1B).

3.2. Interaction of DENV NS5 and Daxx in the nucleus of HEK293 cells expressing DENV NS5

The interaction of DENV NS5 and Daxx was confirmed in mammalian HEK293 cells. Co-immunoprecipitation of DENV NS5 and

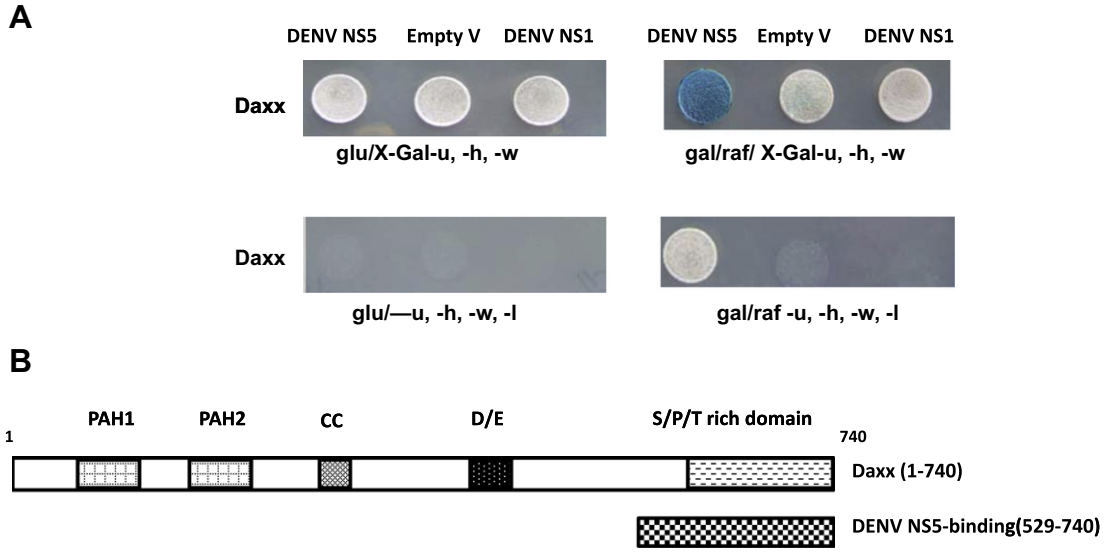

Fig. 1. DENV NS5-human Daxx interaction in a yeast two-hybrid system. (A) Yeast strain RFY231 was co-transformed with a bait plasmid, a Daxx prey plasmid and *lacZ* reporter plasmid. The bait plasmids used were pEG-NS5, expressing the lexA-DEN NS5 fusion protein, the empty bait plasmid pEG202, or an unrelated bait plasmid pEG-NS1 (DENV NS1). A specific interaction was indicated by galactose-dependent β-galactosidase expression, as evidenced by blue colonies on the galactose containing X-Gal plate and white colonies on the glucose containing X-Gal plate, and by galactose-dependent growth on the leucine deficient plate. (B) The region of Daxx that interacted with DEN NS5 was the 211 carboxyl-terminal residues, a region which binds multiple cellular proteins.

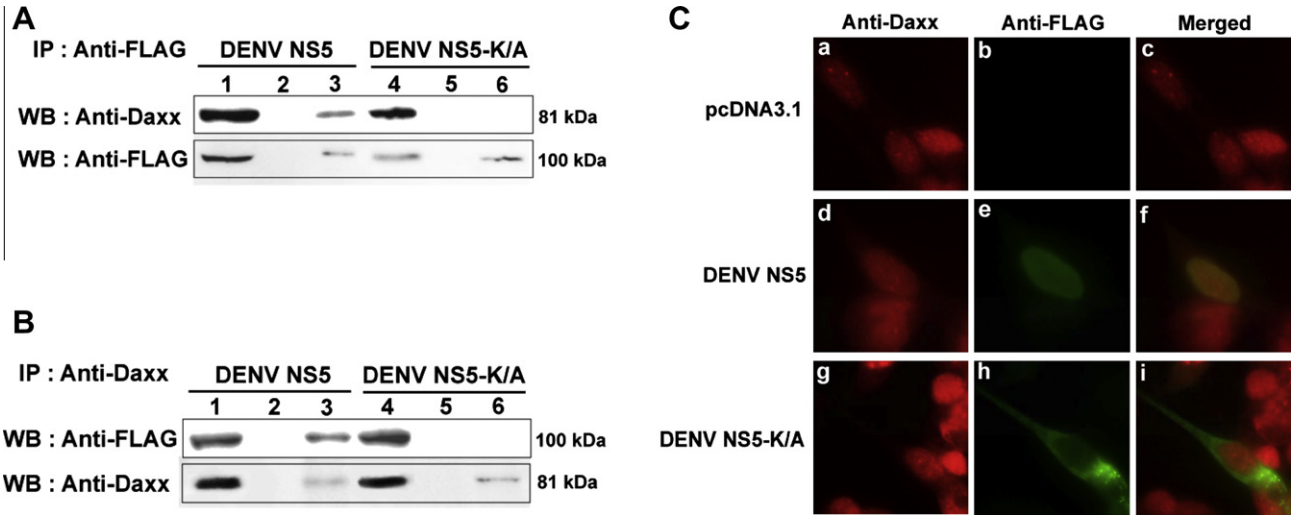

Fig. 2. Interaction between DENV NS5 and human Daxx. (A) Lysates of HEK293 cells expressing DENV NS5 or DENV NS5-K/A were immunoprecipitated with a mouse anti-FLAG antibody. Immune complexes were detected by Western blot analysis using either a mouse anti-FLAG or a rabbit anti-Daxx antibodies. Lane 1, and 4: input; Lane 2, and 5: IP with no antibody; Lane 3 and 6: IP with anti-FLAG antibody. (B) Lysates of HEK293 cells expressing DENV NS5 or DENV NS5-K/A were immunoprecipitated with a rabbit anti-Daxx antibody. Immune complexes were detected by Western blot analysis using either a mouse anti-FLAG antibody or a rabbit anti-Daxx antibody. Lane 1, and 4: input; Lane 2, and 5: IP with no antibody; Lane 3 and 6: IP with anti-Daxx antibody. (C) HEK293 cells expressing DENV NS5 or DENV NS5-K/A were fixed and immunostained with rabbit anti-Daxx antibody (panel a, d, and g) and mouse anti-FLAG antibody (panel b, e, and h). The merged image (panel c, f, and i) demonstrated co-localization between DENV NS5 and Daxx.

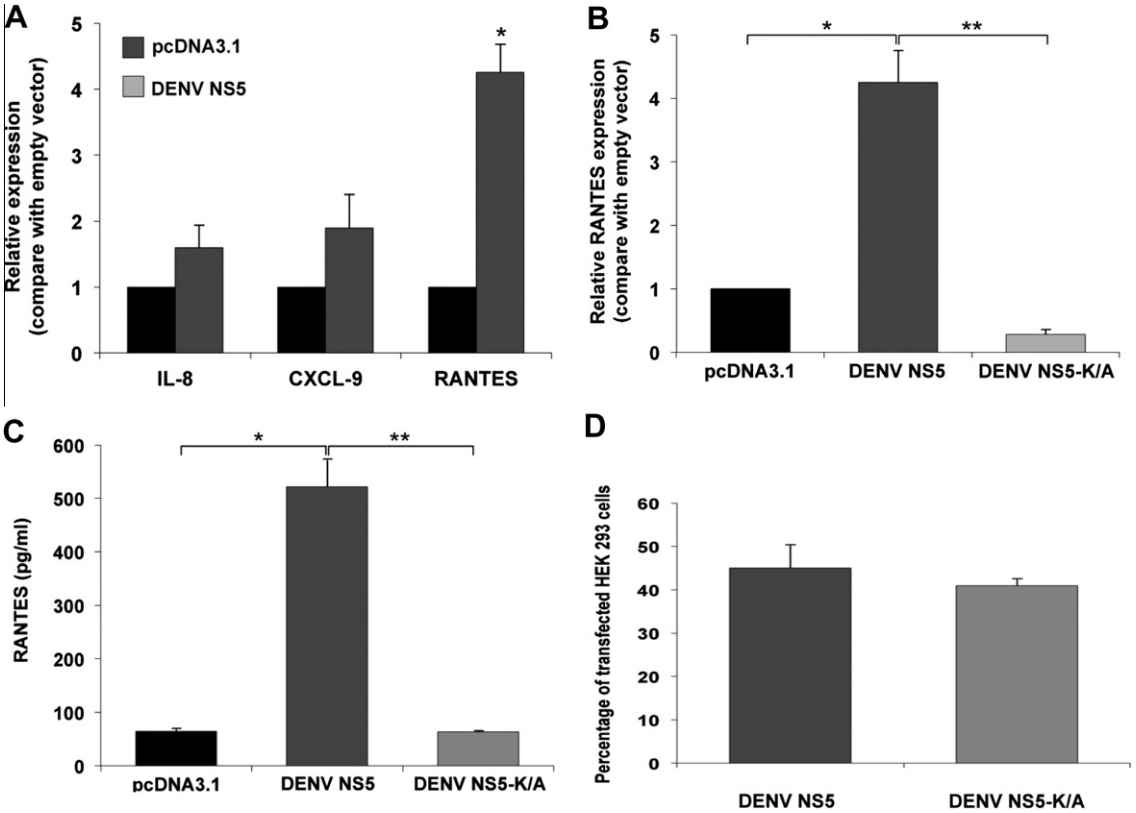

Fig. 3. Interaction between DENV NS5 and human Daxx modulates RANTES production. (A) The amount of mRNA of DHF-associated cytokines was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. Relative gene expression (fold change) was determined by real-time PCR using primers specific to IL-8, CXCL-9 and RANTES. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and empty vector ($^{*}p < 0.05$). (B) The mRNA expression of RANTES was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. Relative gene expression (fold change) of RANTES was determined by real-time PCR. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and DENV NS5-K/A ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) The production of RANTES was measured by ELISA in HEK293 cells expressing DENV NS5 in the presence of TNF-α. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and DENV NS5-K/A ($^{*}p < 0.05$, $^{**}p < 0.01$). (D) The expression of DENV NS5 or DENV NS5-K/A was determined by flow cytometry using a mouse anti-FLAG antibody.

Daxx was observed in HEK293 cells expressing DENV NS5. Anti-flag antibody co-immunoprecipitated Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 (Fig. 2A and B). Co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 (Fig. 2C). These data supported the proposed interaction of DENV NS5 and Daxx and further suggested that association occurs mainly in the nucleus of HEK293 cells expressing DENV NS5.

3.3. Interaction DENV NS5 and Daxx modulates RANTES production

DENV NS5 was previously shown to enhance the production of IL-8 [9]. In addition, enhancement of TNF-α-stimulated NF-κB activation by DENV NS5 was reported [25]. In the present study, the level of DHF-associated cytokines including IL-8, CXCL-9 and RANTES was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. The mRNA expression of RANTES was significantly up-regulated in HEK293 cells expressing DENV NS5 compared to empty vector-transfected HEK293 cells (Fig. 3A). RANTES is a member of the C–C chemokine family and its expression was increased both in DENV-infected cell lines and DENV-infected patients [26] and activation of RANTES production by other viral infections was reported to be regulated by NF-κB [27,28]. Therefore, enhancement of TNF-α-stimulated NF-κB activation of RANTES production may modulated by DENV NS5.

Interaction between DENV NS5 and Daxx, which normally interacts with NF-κβ [29], may modulate RANTES production. Therefore, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were created to assess in the role of NS5 nuclear translocation in Daxx interaction and RANTES production. Site-directed mutagenesis was employed to generate mutant DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A, Whilst DENV NS5 was present in the nuclei of HEK293 cells expressing DENV NS5, HEK293 cells expressing DENV NS5-K/A had no detectable DENV NS5 protein in the nucleus (Fig. 2C). Secondly, co-immunoprecipitation and co-localization of DENV NS5-K/A and Daxx was examined. Co-immunoprecipitation of DENV NS5 and Daxx was observed only in HEK293 cells expressing DENV NS5, but not in HEK293 cells expressing DENV NS5-K/A. Anti-flag antibody co-immunoprecipitates Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 in HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 2A and B). Furthermore, co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 2C). Finally, the RANTES production in either HEK293 cells expressing DENV NS5 or DENV NS5-K/A. was measured by real-time RT-PCR and ELISA, respectively. As expected, both mRNA expression and RANTES production were significantly higher in HEK293 cells expressing DENV NS5 than those in HEK293 cells expressing DENV NS5-K/A (Fig. 3B and C). This difference was not due to differences in transfection efficiency or protein expression since DENV NS5 and DENV NS5-K/A were expressed at similar levels as determined by immunofluorescence staining (Fig. 3D). Thus, in the absence of a functional NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase RANTES secretion.

As TNF-α signaling activates NF-κB and RANTES production during DENV infection [2,30] and NF-κB is known to activate the RANTES promoter [28], DENV NS5 may increase the amount of NF-κB available to activate RANTES expression through its interaction with Daxx, which normally interacts with NF-κB [29] (Fig. 4). The molecular mechanisms that control the competitive binding of DENV NS5, Daxx, and NF-κB require further investigation. However, this work is the first to demonstrate the *in vivo* interaction between DENV NS5 and Daxx and its role in modulating RANTES production.

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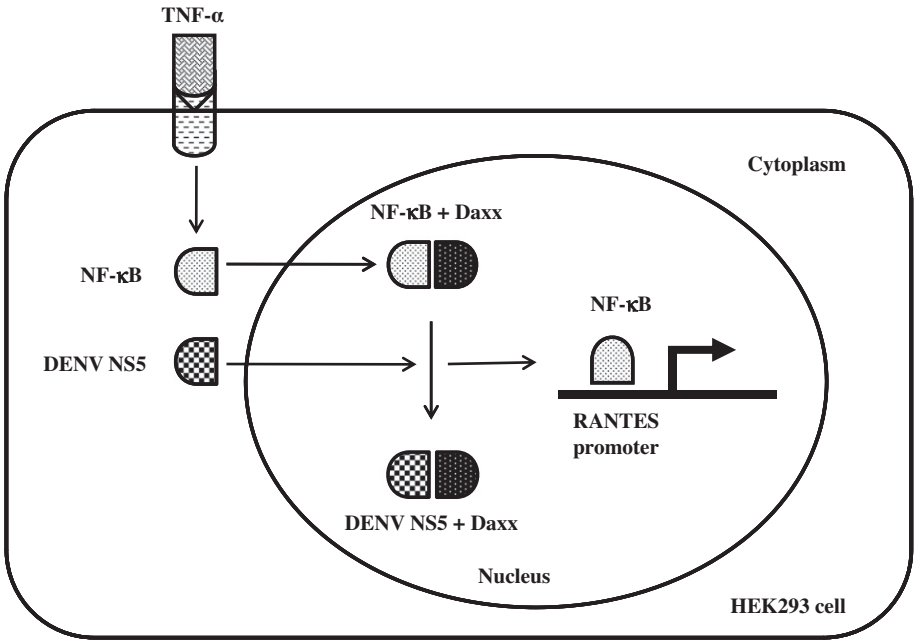

Fig. 4. Proposed model for modulation of Rantes production by DENV NS5. TNF-α signaling activates NF-κB and the competitive binding between DENV NS5 and Daxx limits the interaction between Daxx and NF-κB thereby releasing NF-κB to activate RANTES production.

reagents of yeast two-hybrid system, and Dr. Nopporn Sitthisombut, Chiang Mai University, for pBluescript II KS containing cDNA of DENV serotype 2 strain 16681.

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