MAJOR ARTICLE

Dengue Virus NS5 Inhibits Interferon-α Signaling by Blocking Signal Transducer and Activator of Transcription 2 Phosphorylation

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Type I interferons (interferon [IFN]–α/β) are key mediators of innate antiviral responses. Inhibition of IFN-mediated signal transduction by dengue viruses (DENVs), mosquito-borne flaviviruses of immense global health importance, probably plays a crucial role in determining the outcome of the virus-host interaction. Understanding the molecular basis of IFN antagonism by DENV would therefore provide critical insight into disease pathogenesis and new opportunities for development of antiviral therapies and rationally attenuated vaccines. Here we examine the effects of expression of DENV nonstructural proteins on cellular IFN responses. We show that expression of nonstructural protein 5 (NS5) alone inhibits IFN-α, but not IFN-γ, signaling. Expression of the polymerase domain of NS5 is sufficient to inhibit IFN-α signaling. NS5 binds signal transducer and activator of transcription 2 (STAT2) and inhibits its phosphorylation. NS5 alone did not, however, induce degradation of STAT2, which occurs when all nonstructural proteins are expressed together. We conclude that DENV NS5 is a potent and specific type I IFN antagonist.

Dengue viruses (DENVs) are mosquito-borne flaviviruses that cause a severe febrile illness and sometimes a potentially lethal syndrome called dengue hemorrhagic fever [1]. The global resurgence of dengue is testament to the inadequacy of current control measures despite decades of effort [1–4]. Dengue has become an immense international public health concern; the World Health Organization estimates that there are 50 million dengue infections and 500,000 cases of dengue hemorrhagic fever leading to hospitalization each year [5]. Thus, dengue has a major economic impact in the developing world, through loss of healthy life and exhaustion of limited health resources.

Intensity of DENV replication early in infection is associated with disease severity [6, 7]. During this critical phase, innate antiviral mechanisms mediated by interferon (IFN)–α/β (type I IFN) are potentially the most important pathways of host defense [8]. Cells typically respond to viral infection by secreting IFN-α/β, which binds to cell-surface IFN-α receptors (IFNARs, comprising IFNAR1 and IFNAR2 subunits) on infected and nearby cells [9]. Binding of IFN-α/β to IFNARs leads to activation of the receptor-associated kinases Janus kinase 1 and tyrosine kinase 2 (Tyk2) via tyrosine phosphorylation [10]; in turn, signal transducer and activator of transcription 2 (STAT2) and then STAT1 are phosphorylated and form heterodimers [11], which then associate with the DNA-binding protein p48/IRF-9 to form the transcription factor IFN-stimulated gene (ISG) factor 3 [12]. ISG factor 3 complexes translocate to the nucleus and initiate transcription of ISGs, leading to transcriptional up-regulation of hundreds of cellular genes and induction of an antiviral state [9].

Received 6 April 2009; accepted 22 May 2009; electronically published 15 September 2009.

Potential conflicts of interest: none reported.

Financial support: European Commission (grant FP6-2005-INCO-DEV2-517711), World Health Organization (grant V22/181/158), Peter Samuel Trust.

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The Journal of Infectious Diseases 2009; 200:1261–70
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0022-1899/2009/20008-0013$15.00
DOI: 10.1086/605847

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The ability to counter the human IFN response is probably crucial for viruses to establish infection successfully. Recent studies have demonstrated that DENV has evolved to inhibit IFN-α/β signal transduction [13–17]. Muñoz-Jordan and colleagues observed reduced STAT1 phosphorylation in response to IFN-α/β in cells expressing DENV nonstructural proteins 4B (NS4B), NS4A, and/or NS2A, but no specific mechanism was proposed [13]. IFN-γ signaling, which overlaps with but is distinct from IFN-α/β signaling, was also inhibited. However, subsequent studies have not confirmed this phenotype. We and others have reported that DENV inhibits IFN-α/β but not IFN-γ signaling [15, 16], implying that DENV specifically targets components of the IFN-α/β signal transduction pathway that are not involved in IFN-γ–mediated signaling. Ho et al suggested that DENV blocks Tyk2 phosphorylation in infected cells [16], whereas we found that DENV reduces cellular expression of STAT2 [15], an observation recently corroborated in another study [17]. None of these later studies identified the specific viral components that mediate the observed inhibition of IFN-α/β signal transduction.

Clearer understanding of the molecular basis for DENV antagonism of the IFN response would represent a critical advance; the efficiency with which DENV evades the IFN response in humans is probably an important factor in early viral replication and hence disease pathogenesis. Here we show that DENV nonstructural protein 5 (NS5), a large multifunctional protein that incorporates an N-terminal methyltransferase (MTase) domain [18, 19] and a C-terminal RNA-dependent RNA polymerase (RdRp) domain [20, 21], also specifically blocks IFN-α/β but not IFN-γ signal transduction. NS5 binds STAT2 and inhibits its phosphorylation, hence inhibiting downstream events in the type I IFN response. The RdRp domain of NS5 is sufficient to inhibit the IFN response. NS5 alone did not reduce cellular expression of STAT2, as we reported elsewhere for DENV, implying that additional viral components are necessary for this effect. We conclude that DENV NS5 is a potent and specific type I IFN antagonist.

MATERIALS AND METHODS

Plasmids and lentiviruses. The dual-promoter lentivector pHRSIN-CSGWdINotI_pUb_Em was supplied by a colleague (M. Collins, Dept. of Immunology, University College London) [22]. To increase expression and overcome transcriptional silencing, a leader sequence from murine embrionic stem cell virus [23] was inserted upstream of the transgene. The following DENV (serotype 2, strain New Guinea C) nonstructural genes were amplified using high-fidelity polymerase chain reaction (PCR) (primer sequences available from authors) and cloned into the lentivector: NS1/NS2A, NS2B/NS3, NS4A, NS4B, and NS5 (Figure 1*A*). More lentivectors were constructed encoding the MTase (aa 1–260) and RdRp (aa 223–900) domains separately (NS5-MTase, NS5-RdRP), NS5-A1A2 (residues lysine [Lys] 371/Lys372 and Lys387/Lys388/Lys389 substituted with alanine), and NS5 tagged at the C-terminus with the FLAG epitope (NS5-FLAG). A lentivector encoding only green fluorescent protein (GFP) was employed as a control (empty vector). Lentiviruses were produced as described elsewhere [22, 24].

Cell lines. K562 (human chronic myeloid leukemia) cells were cultured in Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum. K562 cells expressing DENV nonstructural proteins were established by transduction with the lentiviruses just described, at a multiplicity of infection of 4. GFP-positive cells were sorted by flow cytometry (FACSAria; Becton Dickinson). K562 cells stably expressing NS5-A1A2, NS5-MTase, and NS5-RdRp were generated by lentivirus transduction at a multiplicity of infection of 10, without fluorescence-activated cell sorting. K562 cells stably expressing the DENV replicon ΔCprMEPAC2A (Figure 1*A*) were established elsewhere [15].

Immunoblot analysis. Immunoblot analysis was performed according to the method of Jones et al [25]. The following primary antibodies were used: anti-NS1 mouse monoclonal antibody (mAb) 5H5.4 (from Paul Young), polyclonal anti-NS5 raised in rabbit, anti-STAT1 and anti-phosphorylated STAT1 mAbs (Zymed), anti-STAT2 mAb (BD Transduction Laboratories), rabbit polyclonal anti-phosphorylated STAT2 (Upstate Biotechnology), rabbit polyclonal anti-Tyk2 and anti-phosphorylated Tyk2 (Cell Signaling Technology), anti-FLAG M2 mAb (Sigma), and rabbit polyclonal anti-actin (Sigma). Detection was performed using horseradish peroxidase–conjugated goat anti-mouse/rabbit secondary antibodies (Dako) and chemiluminiscence (ECL Plus; Amersham).

Quantitative reverse-transcription PCR. Cells were cultured in RPMI medium containing 2% fetal bovine serum overnight and then treated with IFNα-2a (Roche) or left untreated for 6 h. Total cellular RNA was extracted and reverse transcribed with random hexamer primers according to standard methods. PCR analysis using myxovirus resistance 1 (MX1)– and 2′,5′-oligoadenylate synthetase 1 (OAS1)–specific primers (sequences available from authors) was then performed with QuantiTect SYBR Green (Qiagen) and analyzed on a Rotor-Gene instrument (Corbett Research). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was analyzed in the same samples.

Fluorescence-activated cell sorting analysis. K562 cell lines were cultured in the presence or absence of 100 IU/mL of IFNα-2a for 24 h and analyzed using standard flow cytometry (FACScalibur; Becton Dickinson). The antibodies used were anti–HLA-ABC mAb (Dako), antihuman IFNAR2c mAb (Calbiochem), and allophycocyanin-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch).

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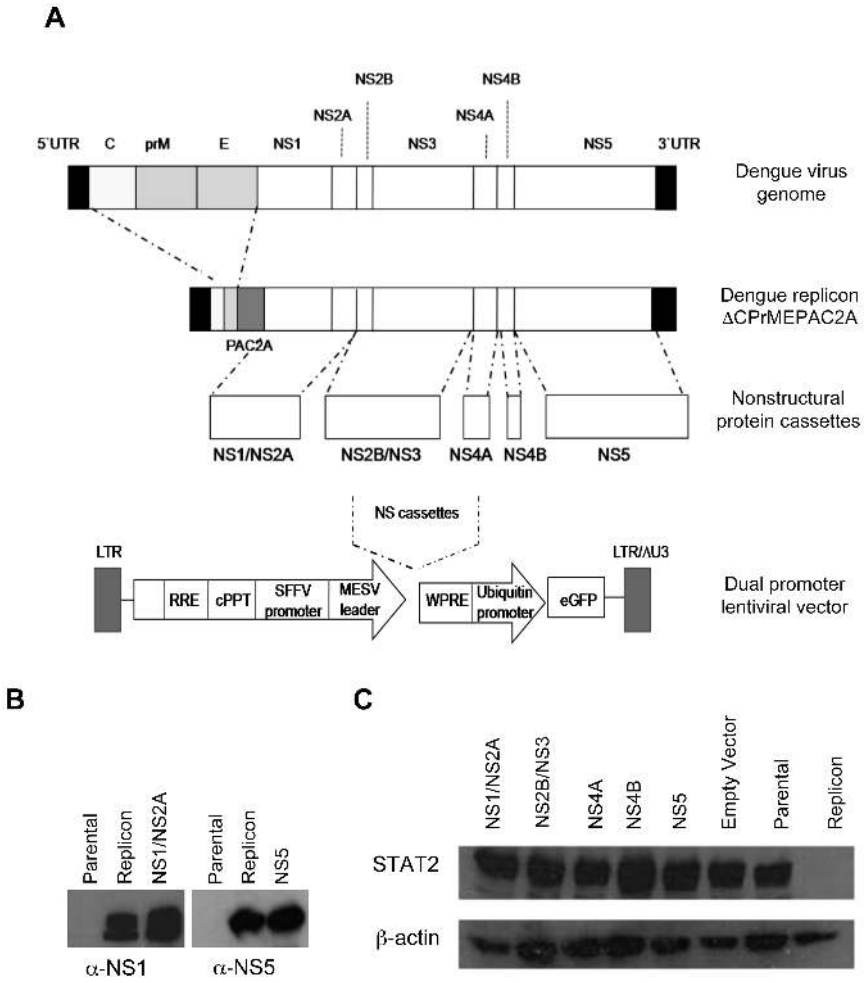

Figure 1. Signal transducer and activator of transcription 2 (STAT2) degraded in the presence of dengue virus (DENV) nonstructural proteins expressed together but not separately. *A,* Schematic representation of DENV genome, DENV replicon ΔCprMEPAC2A, and lentivectors containing DENV nonstructural gene cassettes. The dengue virus genome comprises a single open reading frame encoding 3 structural proteins (C, core; prM, premembrane; E, envelope) and 7 nonstructural genes. Dengue replicon ΔCprMEPAC2A contains a large in-frame deletion within the region encoding the structural genes, replaced with an antibiotic selection cassette encoding puromycin acetyltransferase (PAC). Gene cassettes encoding nonstructural proteins 1/2A (NS1/NS2A), NS2B/NS3, NS4A, NS4B, and NS5 were amplified by polymerase chain reaction (PCR) from the replicon and cloned into the dual promoter lentiviral vector pHRSIN-CSGWdlNotI_pUb_Em. ΔU3 is a 400-nucleotide deletion in a 3′ long terminal repeat (LTR) that abolishes promoter activity. cPPT, central polypurine tract; eGFP, enhanced green fluorescent protein; MESV, murine embryonic stem cell virus; RRE, Rev response element; SFFV, spleen focus-forming virus; UTR, untranslated regions; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. *B,* Levels of NS1 and NS5 proteins in DENV replicon–containing cells and in K562 cells transduced with the corresponding lentivectors. Parental K562 cells were included as a control. Equal numbers of cells were lysed, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). NS1 and NS5 were detected by immunoblot analysis using specific antibodies (α-NS1 and α-NS5). *C,* Comparison of STAT2 levels in K562 cells expressing DENV nonstructural protein cassettes. Equal numbers of cells stably transduced with lentivectors, as indicated, were lysed, and proteins were separated by SDS-PAGE. Cellular levels of STAT2 were analyzed with immunoblotting. Parental K562 cells and K562 cells containing DENV replicons were included as controls.

Coimmunoprecipitation. Immunoprecipitation was performed using the FLAGIPT1 immunoprecipitation kit (Sigma). Briefly, 1×10^7 K562 cells were lysed and then incubated with a resin precoated with anti-FLAG M2 mouse mAb. After extensive washing, the elution step was performed by competition with 3× FLAG peptide. The immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed with immunoblotting for NS5, FLAG, Tyk2, STAT2, and STAT1.

RESULTS

STAT2 degradation in the presence of DENV nonstructural proteins. In other work, we showed that DENV infection or

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replicons, which express all the nonstructural proteins together, reduce cellular STAT2 expression [15]. To investigate further the mechanism underlying decreased STAT2, we first measured STAT2 mRNA levels by quantitative reverse-transcription PCR (RT-PCR) in K562 cells that did or did not contain DENV replicons. We found no difference between these cell types (data not shown), which implies that DENV does not affect STAT2 transcription. We next tested whether a functional proteasome degradation pathway is required for the DENV-mediated reduction in STAT2 levels. K562 cells containing DENV replicons expressed much less STAT2 than parental cells, as expected, but levels of full-length STAT2 were restored by treatment with the proteasome inhibitor bortezomib (Figure 2).

We therefore hypothesized that STAT2 may be targeted by ⩾1 DENV nonstructural protein for proteasome-mediated degradation. To examine the effect of individual nonstructural proteins on STAT2 levels, expression cassettes containing ⩾1 DENV nonstructural genes, collectively spanning the nonstructural region of the DENV genome, were cloned into a dual-promoter lentivector containing GFP (Figure 1*A*). In designing the expression cassettes, we took into account the known subcellular localization and functions of individual nonstructural proteins; NS1 and NS2A, as well as NS3 and its cofactor NS2B, were expressed together [26, 27], and the short 2K sequence between NS4A and NS4B preceded the N-terminus of NS4B [28]. The DENV gene cassettes were transduced into K562 cells, and GFP-positive cells were sorted by flow cytometry. The presence of the transgene RNA transcripts in each transduced cell line was confirmed by RT-PCR (data not shown). Expression of NS1 and NS5 proteins was confirmed by immunoblot analysis, and the levels of expression for both proteins were comparable to those in cells expressing DENV replicons (Figure 1*B*). Antibodies to other DENV nonstructural proteins were not available. We used immunoblot analysis to test whether expression of any of the DENV nonstructural gene cassettes induced STAT2 degradation. As expected, there was a dramatic reduction in STAT2 levels in K562 cells containing DENV replicons (Figure 1*C*), which express all of the nonstructural proteins together. In contrast, none of the nonstructural protein cassettes expressed separately induced a significant reduction in STAT2 levels, suggesting that combinatorial effects of >1 protein may be required.

NS5 inhibition of cellular responses to IFN-α but not IFN-γ. Although separate nonstructural proteins did not reduce cellular STAT2 levels, they might inhibit IFN signaling through a different mechanism. To test this, K562 cells expressing the nonstructural gene cassettes were treated with IFN-α (100 IU/mL) for 6 h, and induction of the ISG MX1 was measured using quantitative RT-PCR. As expected, the presence of DENV replicons dramatically inhibited up-regulation of MX1 transcription in response to IFN-α (Figure 3*A*). MX1

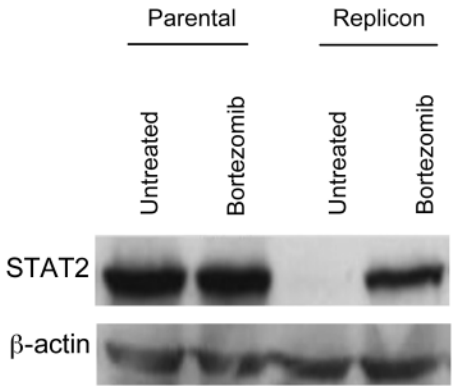

Figure 2. Dengue replicons inducing degradation of signal transducer and activator of transcription 2 (STAT2). Parental K562 cells and replicon-expressing K562 cells were grown for 24 h in the presence or absence of the proteasome inhibitor bortezomib (10 nmol/L) (Janssen-Cilag). Lysates from equal numbers of cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and cellular levels of STAT2 were analyzed with immunoblotting.

transcription was also consistently inhibited in cells expressing NS5 but not in any of the other nonstructural proteins (Figure 3*A*). To confirm this, we measured the dose response of MX1 transcription in response to IFN-α (Figure 3*B*, left panel). As expected, cells transduced with empty lentivirus vector up-regulated MX1 transcription in response to IFN-α in a dose-dependent manner. This was dramatically inhibited in cells expressing NS5.

To exclude an MX1 gene–specific effect, we also measured transcription of a second major antiviral ISG, OAS1, with the same result (Figure 3*B*, right panel). We further tested the ability of NS5 to block the IFN response by testing the whole antiviral pathway in cells that did and cells that did not express NS5, by using a *trans* rescue assay as described elsewhere [15]. Expression of DENV NS5 alone rescued the replication of an IFN-sensitive virus, encephalomyocarditis virus, in the presence of IFN-α (data not shown).

To test whether NS5 specifically inhibits the type I IFN response, we measured up-regulation of the surface antigens HLA-ABC in response to IFN-α (Figure 3*C*, top panel) and IFN-γ (Figure 3*C*, bottom panel). In keeping with our work reported elsewhere, up-regulation of HLA-ABC expression in response to IFN-α, but not IFN-γ, was markedly inhibited in cells containing DENV replicons. We observed the same phenotype in cells expressing NS5. Taken together, these data show that DENV NS5 is a potent and specific type I IFN antagonist.

Mediation of IFN-α antagonism by the polymerase domain of NS5. To determine which region of NS5 is required for IFN antagonism, K562 cells expressing the MTase (aa 1–260) and RdRp (aa 223–900) domains separately were treated with IFN-α, and up-regulation of HLA-ABC at the cell surface was measured by flow cytometry. Expression of the RdRp domain

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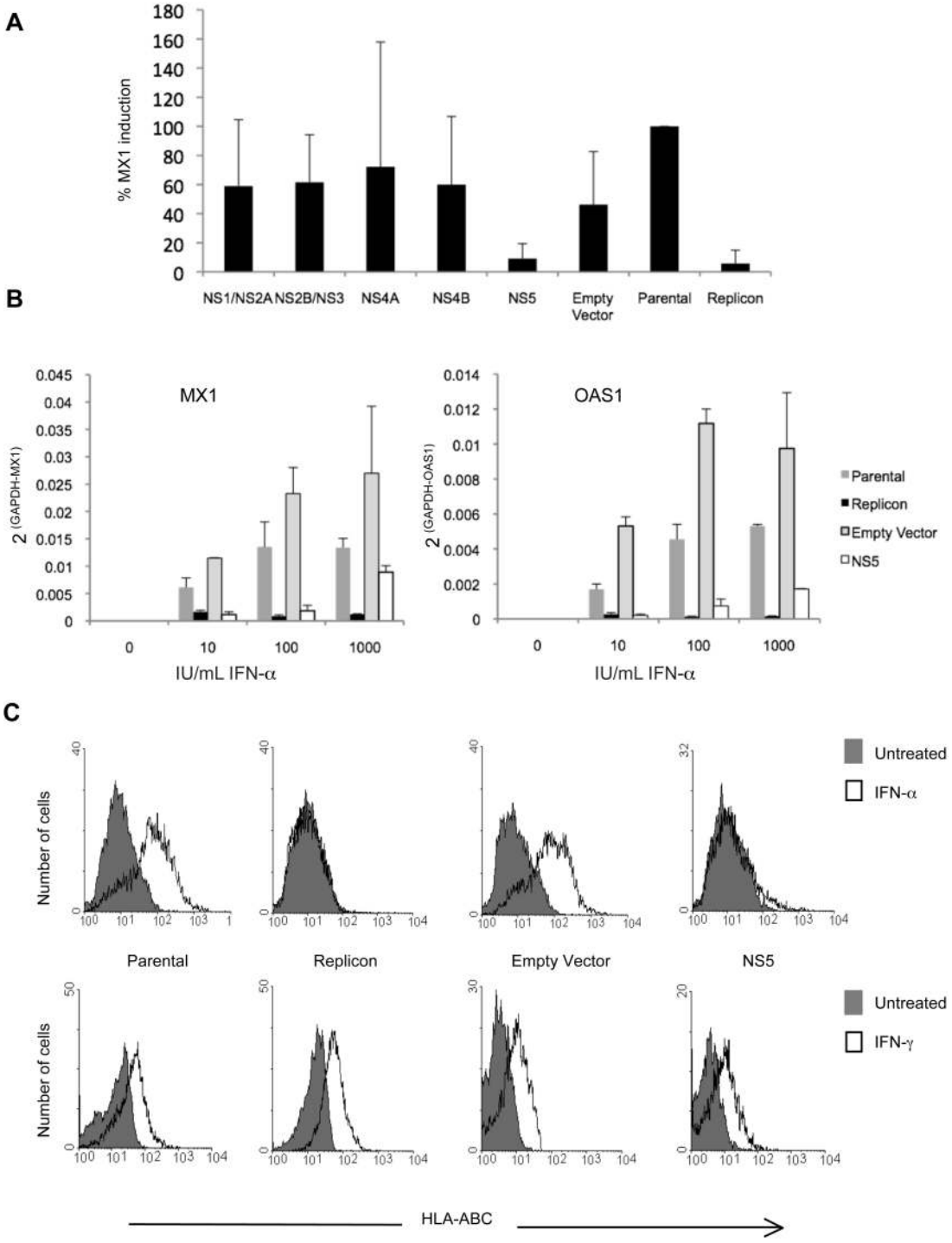

Figure 3. Dengue virus (DENV) nonstructural protein 5 (NS5) inhibiting cellular responses to interferon (IFN)–α but not IFN-γ. *A,* NS5 inhibiting the induction of the IFN-stimulated gene (ISG) myxovirus resistance 1 (MX1). K562 cells expressing DENV nonstructural proteins, as indicated, were treated with 100 IU/mL of IFN-α for 6 h before RNA extraction. MX1 induction was measured by quantitative reverse-transcription polymerase chain reaction (PCR) and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The percentage induction of MX1 was compared with that of parental K562 cells (100%), and the figure shows the mean (+ standard error of the mean) for 3 independent experiments, each performed in duplicate. Replicon-containing cells were included for comparison. *B,* NS5 inhibiting the dose-dependent induction of ISGs. Parental K562 cells, replicon-containing K562 cells, K562 cells transduced with empty lentivirus vector, and K562 cells expressing NS5 were treated for 6 h with different concentrations of IFN-α, as shown. MX1 (*left panel*) and 2′,5′-oligoadenylate synthetase 1 (OAS1) (*right panel*) induction was measured by real-time PCR and normalized to the housekeeping gene GAPDH using the formula $2^{(\text{GAPDH}-\text{GoI})}$ (GoI, gene of interest). *C,* NS5 inhibiting cellular responses to IFN-α but not IFN-γ. NS5-expressing cells and controls, as indicated, were treated for 24 h with 100 IU/mL of either IFN-α (*top panel*) or IFN-γ (*bottom panel*) (*open histograms*) or left untreated (*gray histograms*). Cell surface expression of the IFN-α/γ–inducible antigen HLA-ABC was measured by flow cytometry.

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alone was sufficient to inhibit up-regulation of HLA-ABC in response to IFN-α (albeit less than full-length NS5), but expression of the MTase domain did not inhibit this response (Figure 4*A*). This implies that the RdRp domain of NS5 incorporates a region that is critical for IFN antagonism.

Dengue NS5 is known to shuttle between the cytoplasm and nucleus in infected cells [29, 30], but the role of nuclear localized NS5 remains unclear. To determine whether nuclear localization of NS5 is required for IFN antagonism, the response to IFN-α was also tested in cells expressing a mutant NS5 (NS5-A1A2) incorporating amino acid changes in the nuclear localization signal that prevent translocation to the nucleus [30]. IFN-α–mediated up-regulation of HLA-ABC expression was inhibited efficiently in cells expressing NS5-A1A2, implying that cytoplasmic NS5 blocks the IFN-α response. Together with our finding that NS5 inhibited IFN-α–mediated expression of all ISGs tested, this suggests that DENV NS5 might block an early step in the IFN-α–mediated signal transduction pathway.

NS5 blockage of STAT2 phosphorylation. To determine whether early events in IFN-α signal transduction are inhibited by NS5, we first performed immunoblot analysis of STAT1 phosphorylation in lentivirus-transduced K562 cells, including cells that expressed and cells that did not express NS5. Parental K562 cells and K562 cells containing DENV replicons were included as controls. Steady state levels of STAT1 were similar in all cell lines (Figure 5*A*). As expected, treatment with IFN-α induced STAT1 phosphorylation in K562 cells, and this was significantly inhibited in replicon-containing cells. A similar level of inhibition was observed in cells expressing NS5 alone (Figure 5*A*). In contrast, STAT1 phosphate levels were not reduced in either replicon-containing or NS5-expressing cells in response to IFN-γ (Figure 5*B*), in agreement with the functional data shown in Figure 3*C*. These data imply that early components of the IFN-α signal transduction pathway, but not of the IFN-γ pathway, are targets for inhibition by DENV NS5.

We next performed immunoblot analysis of STAT2 phosphorylation, a key step in IFN-α, but not IFN-γ, signaling. Steady state levels of STAT2 were markedly reduced in DENV replicon–containing cells, as expected, but not in cells expressing NS5 alone. However, STAT2 phosphorylation in response to IFN-α was markedly inhibited in cells expressing NS5 alone, although not to the same degree as in DENV replicon–containing cells (Figure 5*A*). To determine whether inhibition of STAT2 phosphorylation was caused by an effect upstream, we next examined Tyk2 phosphorylation, the preceding step in the IFN-α signal transduction pathway. As shown in Figure 5*A*, the presence of DENV NS5 or replicons affected neither steady state levels of Tyk2 nor Tyk2 phos-

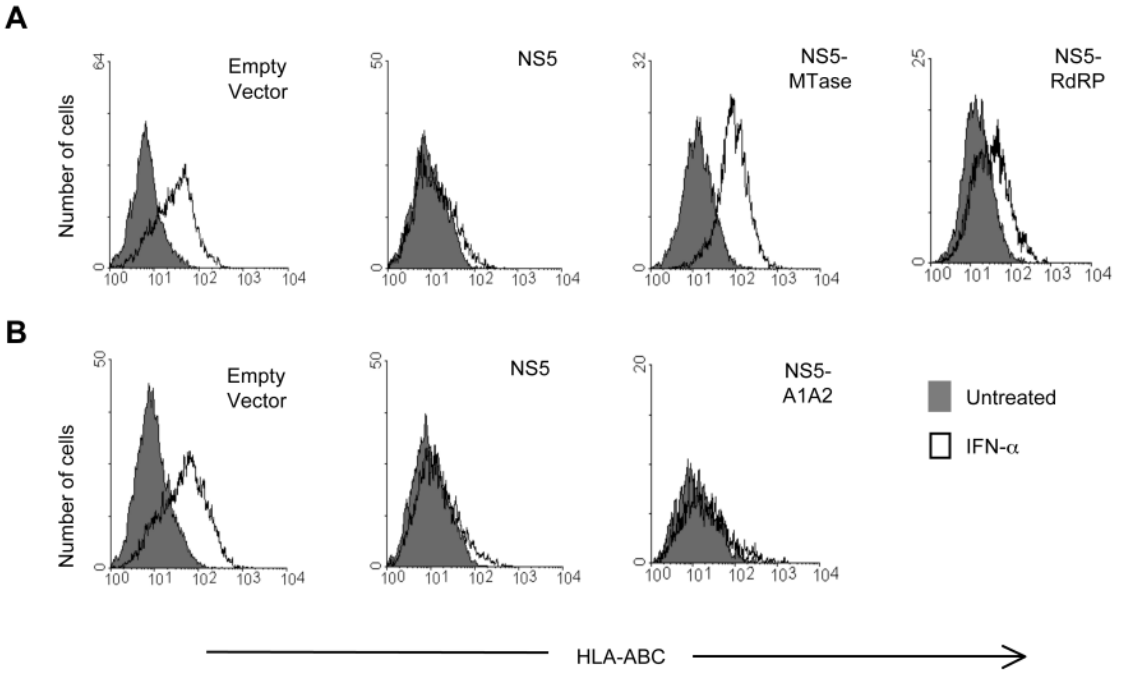

Figure 4. Interferon (IFN)–α antagonism mediated by the polymerase domain of nonstructural protein 5 (NS5) and not requiring NS5 nuclear translocation. K562 cells were transduced with individual functional domains of NS5 (MTase, methyltransferase aa 1–260; RdRp, RNA-dependent RNA polymerase aa 223–900) (*A*) or a mutant of NS5 unable to translocate into the nucleus (NS5-A1A2) (*B*). Cells were treated for 24 h with (*open histograms*) or without (*gray histograms*) 100 IU/mL of IFN-α, and cell surface expression of the IFN-α–inducible antigen HLA-ABC was measured by flow cytometry. Cells transduced with the empty vector and with wild-type NS5 were included as controls.

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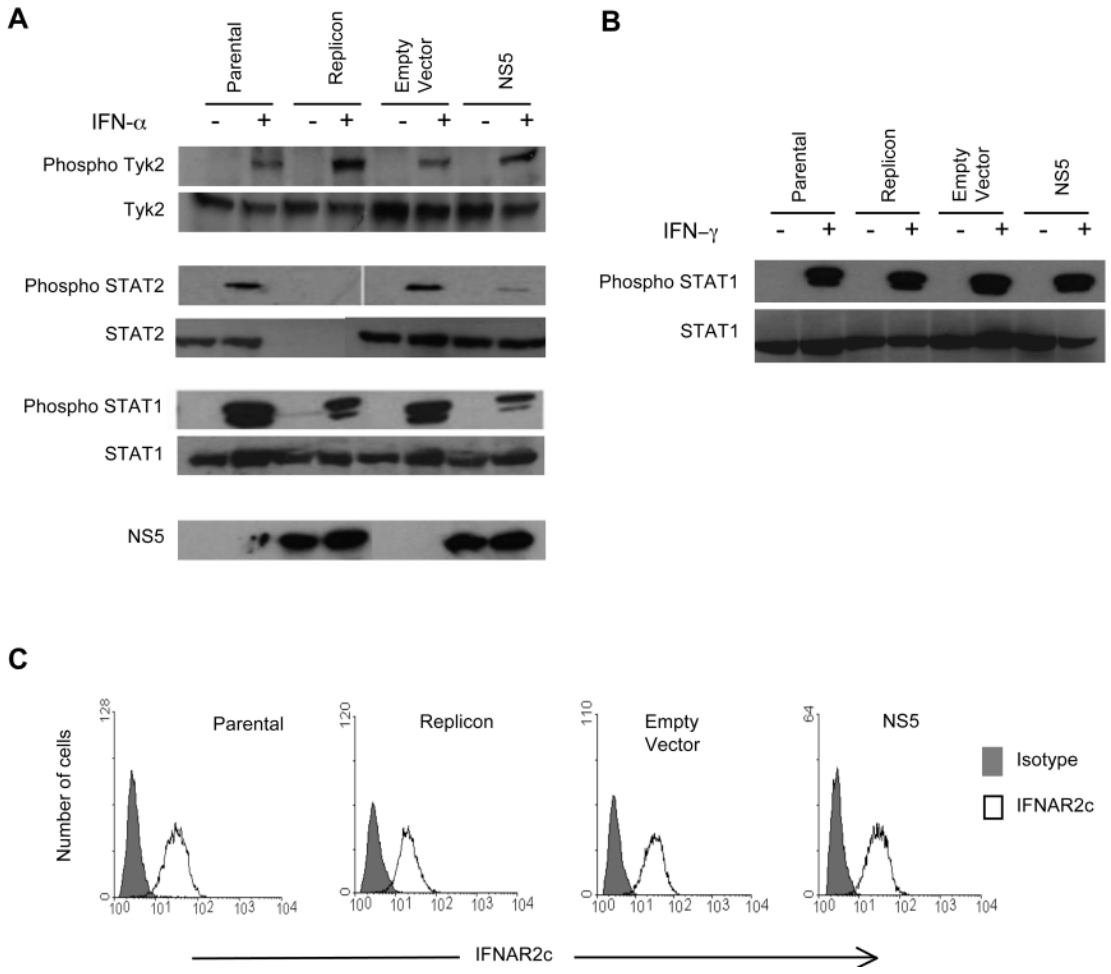

Figure 5. Nonstructural protein 5 (NS5) blocking signal transducer and activator of transcription 2 (STAT2) phosphorylation. *A,* interferon (IFN)–α signaling downstream of tyrosine kinase 2 (Tyk2) activation inhibited in cells expressing NS5. K562 cells expressing NS5 and K562 cells containing dengue virus (DENV) replicons were treated with 100 IU/mL of IFN-α for 15 min or left untreated. Parental K562 cells and K562 cells transduced with empty lentivector were included as controls. Lysates from equal numbers of cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and basal and phosphorylated levels of Tyk2, STAT2, and STAT1 were analyzed with immunoblotting. *B,* NS5 not inhibiting STAT1 phosphorylation in response to IFN-γ. NS5-expressing cells were treated with 100 IU/mL of IFN-γ for 15 min before lysis or left untreated. Proteins were separated by SDS-PAGE, and cellular levels of STAT1 and phosphorylated STAT1 were analyzed with immunoblotting. *C,* NS5 not altering IFN-α receptor levels on the cell surface. NS5-expressing cells and control cells, as indicated, were stained for the surface IFN-α receptor (IFNAR) 2c and analyzed by flow cytometry. Cells stained with the secondary antibody alone (*gray histograms*) were included as negative staining controls.

phorylation. Surface expression of IFNAR2 was also unaffected (Figure 5*C*). Taken together, these data show that DENV NS5 specifically blocks the IFN-α signal transduction pathway by inhibiting STAT2 phosphorylation.

NS5 binding of STAT2. We hypothesized that NS5 could inhibit STAT2 phosphorylation by binding STAT2 and preventing its interaction with activated Tyk2. To test this, K562 cells expressing NS5-FLAG were lysed and NS5-FLAG was immunoprecipitated using an anti-FLAG antibody. Coimmunoprecipitation of relevant components of the IFN-α signal transduction pathway was examined by immunoblot analysis. Figure 6 shows that STAT2, but not Tyk2 or STAT1, coimmunoprecipitates with NS5. Hence NS5, either directly or indirectly, specifically binds STAT2.

DISCUSSION

Type I IFN induces a cellular antiviral state, which plays a major role in limiting the replication and spread of many viruses [8]. Recent studies have suggested that DENV, in common with many (and perhaps all) pathogenic viruses, has evolved mechanisms to counter the human IFN response [13–17]. These mechanisms probably play a crucial role in determining the outcome of the virus-host interaction. Increasing evidence

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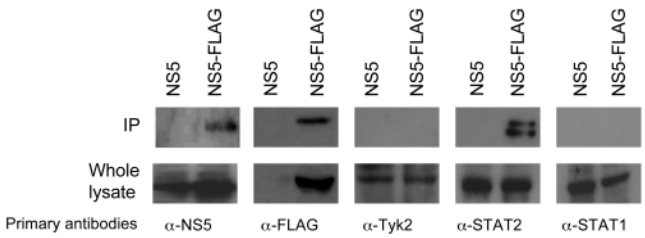

Figure 6. Nonstructural protein 5 (NS5) specifically binding signal transducer and activator of transcription 2 (STAT2). Lysates from K562 cells expressing either NS5 or NS5 tagged at the C-terminus with the FLAG epitope (NS5-FLAG) were immunoprecipitated with anti-FLAG antibodies. Proteins contained in the immunoprecipitates (IP) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed with immunoblotting using specific antibodies against NS5, FLAG, Tyk2, STAT2, and STAT1. Whole-cell lysates were included for comparison. One representative experiment of 4 experiments is shown.

shows that DENV inhibits IFN-mediated signal transduction, which is a broadly effective strategy to counter the IFN response. However, none of the studies to date have both identified a specific DENV component that acts as an IFN antagonist and demonstrated how it inhibits IFN signaling, which was the aim of the current study.

We expressed DENV nonstructural proteins in human cells and showed that NS5 alone specifically inhibits signal transduction in response to IFN-α. NS5 has been implicated as an IFN antagonist for another mosquito-borne flavivirus, Japanese encephalitis virus [31], and for the more distantly related tickborne flaviviruses, Langat virus [32] and tickborne encephalitis virus [33, 34]. The exact mechanism of IFN antagonism by NS5 has not been entirely elucidated for any flavivirus, but all studies have found a block upstream of STAT1 phosphorylation. We found the same in cells expressing DENV NS5 and showed that STAT2 but not Tyk2 phosphorylation was inhibited, which implies that DENV NS5 may block the interaction between Tyk2 phosphate and its substrate STAT2. The demonstration that NS5 can bind STAT2 provides further support for this model, although details of this interaction require further investigation. Our data showing that the RdRp domain of DENV NS5 is sufficient to inhibit IFN-α signaling provide a first step in this direction, suggesting that the residues that mediate IFN antagonism lie within this domain. However, we consistently observed less inhibition in cells expressing the RdRp domain alone compared with full-length NS5. Further experiments are required to determine whether the MTase domain, which does not block IFN signaling when expressed alone, contributes to IFN antagonism, for example, by imparting additional structural stability to NS5.

Our main finding, that DENV NS5 is a specific inhibitor of type I IFN signaling, differs substantially from the finding of an earlier study addressing the DENV components that act as IFN antagonists. Muñoz-Jordan and colleagues reported inhibition of both IFN-α/β– and IFN-γ–mediated STAT1 phosphorylation by DENV NS4B, NS4A, and NS2A but not by NS5 [13]. They suggested that common players involved in both signaling pathways (which do not include STAT2) were likely targets for IFN antagonism by DENV. In view of the importance of counteracting innate immunity, it is plausible that DENV has evolved multiple mechanisms for immune evasion. However, this would not explain why different studies have identified different mechanisms; these differences most likely reflect experimental differences, such as cell type, expression system, and/or DENV strain. We observed a correlation between the level of NS5 expression and the efficiency of IFN-α inhibition (unpublished data), perhaps reflecting stoichiometric antagonism of STAT2. In our lentivirus expression system, NS5 levels in K562 cells were comparable to those in DENV replicon–containing cells, which in turn were similar to levels in DENV-infected cells (unpublished data). It is possible that NS5-mediated inhibition of the IFN response was not detected in other studies because of lower levels of expression [13, 17].

The IFN signaling phenotype of DENV replicon–containing cells, which express all the nonstructural proteins together, is similar to that of cells expressing NS5 alone, except that there is also a marked reduction in STAT2 levels. We show here that this reduction in STAT2 is posttranscriptional and requires a functioning proteasome degradation pathway. Ongoing work is investigating whether STAT2 degradation involves a mechanism independent of NS5 or, more likely, requires the presence of additional nonstructural proteins. By binding STAT2, NS5 may not only block its phosphorylation but also act as a scaffold for other nonstructural proteins and/or cellular components that ultimately target STAT2 for degradation.

An analogous mechanism has been proposed for respiratory syncytial virus (RSV), an unrelated RNA virus that targets STAT2 for proteasome-mediated degradation through a combination of its nonstructural proteins NS1 and NS2 [35]. A consensus sequence (BC box) found in the suppressor of cytokine signaling family of proteins was identified in RSV NS1 and is responsible for recruitment of the ubiquitination complex [35]. Intriguingly, through bioinformatic analysis

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we discovered that the same degenerate BC box sequence (VxxLxxxCxxxA/I/L/V) is highly conserved in DENV serotype 2 NS4B. NS4B alone did not reduce STAT2 levels (Figure 1*C*), and further work is needed to know whether this observation is biologically relevant to the degradation of STAT2 by DENV and, if so, to understand the precise roles of NS5 and NS4B. Whatever the mechanism for targeting STAT2 for degradation, this is likely to represent a highly efficient strategy for blocking the type I IFN response, potentially allowing NS5 to be recycled to bind and inhibit activation of any remaining STAT2.

In conclusion, the results presented in this study are consistent with a model in which DENV NS5 binds STAT2 and thus inhibits its phosphorylation and downstream events in the type I IFN response. During the preparation of this article, Ashour et al [36] reported a parallel study that reached a very similar conclusion. Because they used different experimental techniques, cell lines, and DENV strains, it is highly likely that, taken together, our studies have elucidated a biologically relevant mechanism. Further studies are still important to determine whether NS5-mediated IFN antagonism is conserved among all DENV strains, particularly field isolates, and operates in a range of relevant human cells, including primary cells. Ashour et al demonstrated proteosome-mediated degradation of STAT2 in DENV-infected and replicon-containing cells but not in cells simply expressing full-length NS5, consistent with our own results. In contrast, expression of any construct that generated mature NS5 by N-terminal cleavage (mirroring the processing of NS5 in the DENV polyprotein) did result in reduced STAT2 levels, suggesting that NS5 alone may in fact be sufficient to target STAT2 for degradation when expressed in the appropriate context. Further experiments are needed to understand the role of N-terminal processing of NS5 in targeting STAT2 for degradation.

The emerging data demonstrating that DENV inhibits 2 major arms of the innate immune system, the type I IFN system and natural killer cells [37], emphasize the evolutionary importance of innate immune subversion to these viruses. Differences in IFN antagonism may be an important determinant of differences in pathogenicity observed between dengue strains [38, 39], as has been suggested for another mosquito-borne flavivirus, West Nile virus [40]. Further studies are needed to define the molecular details of the interaction between STAT2 and NS5. Blocking this interaction may provide a novel target for antiviral therapeutics. In addition, precise knowledge of the residues involved in this interaction may enable reverse genetic engineering of DENVs that are disabled in their ability to inhibit IFN-α signaling, as the basis for the design of rationally attenuated vaccine candidates. The results of this study are therefore an important step in defining the molecular pathogenesis of dengue, providing clues that suggest potential new approaches to combat this disease.

Acknowledgments

We thank Dr R. Gitendra Wickremasinghe for sharing his expertise in immunoblot analysis and Angela Strang for expert technical assistance.

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