The 2′, 5′-Oligoadenylate Synthetase 1b Is a Potent Inhibitor of West Nile Virus Replication Inside Infected Cells*

Anna Kajaste-Rudnitski1,2, Tomoji Mashimo3, Marie-Pascale Frenkiel4, Jean-Louis Guénét5, Marianne Lucas3,4, and Philippe Després1-5

From the 1Interactions Moléculaires Flavivirus-Hôtes and 2Génétique des Mammifères, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris, Cedex 15, France

The 2′, 5′-oligoadenylate synthetase (OAS) proteins associated with endoribonuclease RNase L are components of the interferon-regulated OAS/RNase L system, which is an RNA decay pathway known to play an important role in the innate antiviral immunity. A large body of evidence suggests a critical role for the 1b isoform of the mouse Oas gene (Oas1b) in resistance to West Nile virus (WNV) infection in vivo. WNV is a positive, single-stranded RNA virus responsible for severe encephalitis in a large range of animal species and humans. To investigate the molecular basis for the sensitivity of WNV to the Oas1b antiviral pathway, we established a stable mouse fibroblastic cell clone that up-regulates Oas1b protein expression under the control of the Tet-Off expression system. We showed that murine cells respond to Oas1b expression by efficiently inhibiting WNV replication. The antiviral action of Oas1b was essentially restricted to the early stages in virus life cycle. We found that the inability of WNV to productively infect the Oas1b-expressing cells was attributable to a dramatic reduction in positive-stranded viral RNA level. Thus, Oas1b represents an antiviral pathway that exerts its inhibitory effect on WNV replication by preventing viral RNA accumulation inside infected cells.

The emergence of virulent variant of West Nile virus (WNV) has been associated with a dramatic increase in severity of infection in humans, thus drawing the attention to West Nile illness as a public health concern (1). WNV is an enveloped, plus-stranded RNA virus that belongs to the flavivirus genus (family Flaviviridae) (2). WNV infects the central nervous system and causes viral encephalitis in a large range of animal species (1). The virion is composed of three structural proteins: C (core protein), M (membrane protein), and E (envelope protein). Translation of genomic RNA results in the synthesis of structural proteins C, prM (the intracellular precursor of M) and E, and seven non-structural (NS) proteins NS1 to NS5 (2). In the cytoplasm, the RNA replication begins with the synthesis of negative- and positive-stranded genome-length RNA (2).

The rapid initiation of innate antiviral mechanisms mediated by Type-1 IFNs (IFN-α/β) may be essential for protection against WNV infection (3–5). We reported that IFN-α/β receptor-deficient mice have an increased susceptibility to WNV as compared with immunocompetent mice (6). IFN-α and -β are able to trigger the activation of a specific signal transduction pathway leading to induction of IFN-stimulated genes that are responsible for the establishment of an antiviral state (7). The IFN-induced proteins believed to affect virus replication in single cells are the RNA-specific adenosine deaminase, the proteins of the Mx family, the double-stranded RNA-dependent protein kinase, and the 2′, 5′-oligoadenylate synthetase (OAS) family associated to endoribonuclease RNase L (OAS/RNase L system) (7).

The OAS/RNase L system is an RNA decay pathway known to play an important role in the established endogenous antiviral pathway (7). OAs are a group of double-stranded RNA-dependent enzymes. Binding of enzymatically active OAS to activator viral RNA results in the production of 2′- to 5′-linked oligoadenylates. Latent, monomeric RNase L is enzymatically activated through homodimerization induced by binding to 2′- to 5′-linked oligoadenylate oligomers. Once activated, RNase L degrades single-stranded RNA molecules, including mRNA and viral RNA, by cleaving on the 3′-site of UpXp- sequences (7).

Mouse Oas1 genes are composed of eight (Oas1a to Oas1h) tandemly arranged transcription units (8–10). It has been reported that a nonsense mutation in exon 4 of the Oas1b gene was correlated with the severity of WNV experimental infection of sensitive mice such as BALB/c mice (11, 12). This point mutation might result in a premature stop codon leading to a defective enzyme with amputation of conserved domains that are believed to be critical for its nucleotidyl transferase activity (8, 11–15). So far, all resistant mice to WNV-induced encephalitis such as MBT mice encode a full-length Oas1b protein (11, 12).

A large body of evidence suggests a key role for Oas1b in innate immunity to WNV (3, 11–14, 16). To date, the mechanisms responsible for the antiviral function of Oas1b remain to be elucidated at the cellular level. The promoter region of Oas1b gene exhibits a unique organization capable of triggering gene expression upon viral infection and IFN-α treatment (8). It has been demonstrated that transcription of Oas1b gene is up-regulated by the poly(I-C) in vivo and in vitro (8, 17). In an effort to resolve the molecular basis of the antiviral action of Oas1b, it was important to study its inhibitory effect on WNV replication at the cellular level. An analysis of WNV replication in stable mouse neuroblastoma cell clones transfected with a plasmid expressing Oas1b indicated that full-length protein from resistant MBT mice (Oas1bMBT), but not the mutant form from sensitive BALB/c mice (Oas1bBALB/c), exerted an antiviral effect on WNV growth (6). Because constitutive expression of Oas1bMBT resulted in inhibition of cell growth over passages, we evaluated the anti-WNV mechanisms of Oas1b pathway in...
mouse fibroblastic MEF/3T3 cells using the Tet-Off inducible expression system. In the present study, we demonstrate that the inability of WNV to productively infect MEF/3T3.Tet-Off cells expressing Oas1b is due to the absence of accumulation of positive-stranded viral RNA inside infected cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The MEF/3T3.Tet-Off cell line was purchased from BD Biosciences Clontech. MEF/3T3.Tet-Off cells were maintained in DMEM (Invitrogen), supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, and 100 μg/ml G418 (Invitrogen). The MEF/3T3.Tet-Off/Oas1b cell lines were maintained in DMEM/10% fetal calf serum/4 mM glutamine supplemented with 100 μg/ml G418, 100 μg/ml Hygromycin (BD Biosciences Clontech), and 100 ng/ml tetracycline (Sigma-Aldrich) as a repressor. For Tet withdrawal, cell monolayers are washed three times with non-supplemented DMEM before replacing with DMEM/10% fetal calf serum only supplemented with genotoxic drugs.

**Virus**—Production of low passaged WNV strain IS-98-ST1 (GenBank accession number AF 481684) on mosquito Aedes pseudoscutellaris (AP61) cell monolayers, purification on sucrose gradients, and virus titration by focus immunodetection assay were performed as previously described (10). Virus titers are expressed as focus forming units. For WNV infection, monolayers of cells were adsorbed with highly purified IS-98-ST1 at the indicated multiplicity of infection (m.o.i.) for 1.5 h at 37 °C. After adsorption, cells were washed with DMEM and incubated in DMEM/2% fetal calf serum. To assay its antiviral effect, human IFN-α (BioSource) was directly added to culture medium at 100 IU/ml.

**RT-PCR Amplification of Viral RNA and Southern Blot Analysis**—Viral RNA was extracted from WNV-infected cells using RNA Plus reagent (Qiogene) according to the manufacturer’s recommended procedure. Total RNA (1 μg) was used as template for the production of viral cDNA by RT-PCR using a Superscript One-Step RT-PCR kit (Invitrogen) with primers 5′-WNCa (nucleotides 139–159) and 3′-WNCa (nucleotides 475–495) (Table 1) designated on the basis of the WNV C gene. First-strand cDNA was synthesized using 5′-WNCa primer for 30 min at 50 °C. The PCR amplification was carried out with the following conditions: 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min for 33 cycles. The primers for β-actin mRNA as house-keeping RNA sequence were 5′-actin as forward primer and 3′-actin as reverse primer (Table 1). For analysis of PCR products by Southern blot, plasmid TOPO/WNV.C containing the WNV C gene was radiolabeled with [α-32P]dCTP using the Megaprime kit (Amersham Biosciences) according to the manufacturer’s recommended procedure. The PCR products were electrophoresed using a 2% agarose gel and then analyzed by hybridization using the 32P-labeled plasmid. Real-time RT-PCR analysis of viral RNA accumulation was performed with an ABI Prism 7700 sequence detection system (Applied Biosystems). To prepare intracellular viral RNA, total RNA was extracted from WNV-infected cells using the NucleoSpin RNA II kit (Machery-Nagel) according to the manufacturer’s recommended procedure. Total RNA was first reverse-transcribed into cDNA using the Moloney murine leukemia virus-reverse transcriptase (Invitrogen) with random primer p(ΔN)6 (Roche Applied Science) according to the manufacturer’s instructions. The primers for WNV C gene are 5′-WNCb and 3′-WNCb (see Table 1). 18 S RNA (rRNA) was used as an endogenous sequence control for the normalization of each sample. The primers for rRNA were 5′-rRNA and 3′-rRNA (see Table 1). To ensure the exponential amplification of WNV C sequence, the PCR cycle numbers determined experimentally were 50 cycles. The relative level of viral RNA production in WNV-infected cells was determined using the 2-ΔΔCt method as described in the ABI user guide.

**Establishing the MEF/3T3.Tet-Off/Oas1b Cell Clones**—The plasmids PCR4-TOPO containing the cDNA coding either Oas1bMBT or Oas1balb/c (6, 8) were used as templates for expression of the full-length Oas1b protein (amino acids 1–381) from MTB mice or the truncated form Oas1bAC-term (amino acids 1–254) derived from BALB/c mice. The Oas1b sequences were modified by PCR to be flanked on the 3′ open reading frame end by the c-Myc tag (Fig. 1) followed by a stop-codon and the NotI restriction endonuclease site using primer 3′-Oas1bAC-term or 3′-Oas1bMBT and to be flanked on the upstream end by the BamHI restriction endonuclease site using primer 5′-Oas1b (Table 1). These PCR products were digested with BamHI and NotI and then inserted into the unique BamHI and NotI sites of the pTRE2hyg expression vector (BD Biosciences Clontech) to generate pTRE2/Oas1bAC-term and pTRE2/Oas1b. In this configuration, the Oas1b inserts are under the control of the Tet-Off expression system. The Tet-Off system allows the induction of foreign gene expression by the withdrawal of repressor tetracycline (Tet). MEF/3T3.Tet-Off cells (BD Biosciences Clontech) were transfected in repressing condition with either pTRE2/Oas1bAC-term or pTRE2/Oas1b using transfectant reagent Lipofectamine (Invitrogen) according to the manufacturer’s recommended procedure. The Tet-Off expression system was repressed by adding 100 ng/ml Tet to the culture medium. The transfected cells were selected on growth medium containing inhibitors and then cloned from single cells by limiting dilution. MEF/3T3.Tet-Off/Oas1bAC-term and MEF/3T3.Tet-Off/Oas1b cell clones were first screened for the integration of the Oas1b-coding cDNA by PCR on genomic DNA using 5′-Oas1b as forward primer and 3′-Oas1bAC-term or 3′-Oas1bMBT as reverse primer (Table 1). The amplification products were sequenced to verify the cDNA integrity. To prepare Oas1b mRNA, total RNA was extracted from induced MEF/3T3.Tet-Off/Oas1b cell lines using the RNA Plus reagent followed by a DNase treatment. As a control, total mRNA was extracted from the MEF/3T3.Tet-Off cell clone. Detection of Oas1b mRNA was performed by RT-PCR using the specific primers described above and the primer 3′-c-Myc as reverse primer (Table 1). The level of Oas1b mRNA production in induced cells (+ Tet) relative to that in uninduced cells (+ Tet) was determined by real-time RT-PCR analysis. The primers for Oas1b mRNA were 5′-1b and 3′-1b and for Oas1bAC-term 5′-1bAC-term and 3′-1bAC-term (Table 1). 18 S RNA (rRNA) was used as an endogenous sequence control for the normalization of each sample. To ensure the exponential amplification of Oas1b sequence, the PCR cycle numbers determined experimentally were 50 cycles.

**Establishing the RNase L-deficient Cell Clones**—The BD™ Knock-out Clone & Confirm PCR Kit (BD Biosciences Clontech) was used to design and test the appropriate siRNA constructs for specific RNase L knock-out following the manufacturer’s instructions. The siRNA-82 sequence (Table 1) from nucleotides 1769 to 1787 of the mouse RNase L mRNA coding region (GenBank™ accession number AF281045) was selected from different candidate sequences based on its ability to suppress RNase L mRNA. The pair of complementary oligonucleotides of siRNA-82 was annealed and then inserted into the RNAi-Ready pSiREN-RetroQ vector (BD Biosciences Clontech). The MET/3T3.Tet-Off cell clones were transfected with pSiREN-RetroQ/siRNA-82 using Lipofectamine reagent. The transfected cells were selected on medium containing G418, hygromycin, tetracycline, and puromycin (5 μg/ml, BD Biosciences Clontech) and cloned from single cells by limiting dilution. Stably siRNA-82-transfected cell clones were selected for the inhibition of RNase L mRNA expression by real-time RT-PCR analysis. The
primers for RNase L mRNA were 5′-RL and 3′-RL (Table 1). The 18 S RNA (rRNA) was used as an endogenous sequence control for the normalization of each sample. To ensure the exponential amplification of RNase L sequence, the PCR cycle numbers determined experimentally were 50 cycles. The level of RNase L mRNA production in induced cells was normalized using the BCA protein assay (Pierce). Equal amounts of protein in samples were separated on a 4–12% gradient gel (NuPage Novex Bis-Tris gel) and samples were subjected to a 15% SDS-PAGE.

Western Immunoblot Assay—The down-regulation of RNase L protein expression in siRNA-82-transfected cell clones was verified by Western immunoblot analysis using an goat polyclonal antibody directed against mouse RNase L antibody (clone T16, Santa Cruz Biotechnology). Briefly, cells were lysed with Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid) containing a mixture of protease inhibitors (Roche Applied Science). The concentration of total protein in the cell lysates was quantified using the BCA protein assay (Pierce).

Malonyl-LabTeks (Nalge Nunc International) and infected with WNV strain IS-98-ST1 as described above. WNV-infected cells were fixed with 3% paraformaldehyde in PBS for 20 min, treated with 50 mM NH4Cl in PBS for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 4 min. Viral antigens were stained with WNV hyperimmune ascitic fluid (6) at the dilution of 1:64 in PBS supplemented with blocker solution was used as a secondary antibody. Cell nuclei were stained with Hoechst 33258. The slides were examined with a Leica DMRB fluorescence microscope. The percentage of cells positive for viral antigens is the mean of three independently infected chambers.

Radioimmunoprecipitation Assays—For analysis of viral protein synthesis, WNV-infected cells cultured on 6-well plates were starved with DMEM depleted in methionine (MP Biomedicals) for 60 min and radiolabeled with 200 μCi/ml Tran35S-LabelTM (MP Biomedicals) for 90 min. After three washes with cold PBS, cells were lysed with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, pH 8.0) supplemented with a mixture of protease inhibitors for 10 min at 4 °C. The cell lysates were clarified by centrifugation at 10,000 rpm for 5 min at 4 °C. Radioimmunoprecipitation assay was performed as described previously (18). Viral antigens were analyzed with anti-WNV hyperimmune ascitic fluid. The immunoprecipitated proteins were separated by SDS-PAGE under non-reducing conditions and visualized using the Phosphorlmager (Amersham Biosciences).

For analysis of Oas1b synthesis, MEF/3T3.Tet-off/Oas1b cell lines were seeded in 6-well plates in the presence or absence of Tet for 20 h and then incubated with 2 μM MG132 (Calbiochem) for 5 h. Stable MEF/3T3.Tet-off/WNprM+E cell line7 served as a control. Briefly, cells were pulse-labeled with 500 μCi/ml Tran35S-LabelTM for 30 min and then chased 30 min with DMEM supplemented with 10 mM methionine, 75 μg/ml cycloheximide (Sigma-Aldrich), and 2 μM MG132. Cells were lysed with Nonidet P-40 lysis buffer supplemented with a mixture of protease inhibitors, and protein samples were incubated with anti-c-Myc antibody and Protein–G-Sepharose (Amersham Biosciences). Immune complexes were washed with Nonidet P-40 washing buffers and samples were subjected to a 15% SDS-PAGE.

MTT-based Assays—To assess the cytotoxic effect of MG132 and/or AAV3/Oas1b cell clones, a colorimetric MTT-based assay was performed (Cell Proliferation kit I, Roche Applied Science) according to the manufacturer’s instructions. The cells grown in a 96-well tissue culture plate

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FIGURE 1. The alignment of Oas1b primary amino acid sequences with MBT and BALB/c mouse sequences. The additional amino acids at the C termini of recombinant Oas1b proteins are underlined and in italics. The nine-residue length of the c-Myc epitope tag is underlined and bold.

Oas1b MTB

| BALB/c | MTB |
|--------|-----|
| [1-69] | MEQDLRSIPASKLDKFIEHNLPDTSFCADLRVIDALCALKDRFFRGPRMRASKGKVGKCCALKGR |
| [70-139] | SADLVFLNNTYFEDQNLNQGLTKEIKKOLYEVQHERRFVKEVQSLRPSNRLSFKSLAPDNL |
| [140-209] | KEFKDVLPPAYDLHDHNILKKNQQPYANLISGRTPGLKEGKLLTCEPMGLKRFNLCPRTKLKRILHL |
| [210-279] | VTHWYCLKEKLGDPLPPQYALELLTVAYEYGSRVTKNATQGFRVTLELVTKYKQLRIYWTYVYDFR |
| [280-349] | HQUEVSEYLHQOLKDRPVLDPADTPRNIAGLNPDKWRRLAGEAAMWLYQPCFKYRDSGVPSCWEVPTE |
| [350-381] | VAPVTKYLFCLRIFWLLFWSLFHFFGKTSSGASMQKLISeEDL |

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MTT-based Assays—To assess the cytotoxic effect of MG132 on MEF/3T3 cell clones, a colorimetric MTT-based assay was performed (Cell Proliferation kit I, Roche Applied Science) according to the manufacturer’s instructions. The cells grown in a 96-well tissue culture plate
(10^4 cells/well) were incubated for 20 h with various amounts of MG132. After the incubation period, cells were incubated with MTT labeling reagent for 4 h. After solubilization, the formazan dye is quantified using an enzyme-linked immunosorbent assay reader.

### RESULTS

#### Establishment of MEF/3T3.Tet-Off Cell Clones Expressing Oas1b

To investigate the mechanisms of antiviral action of Oas1b at the cellular level, an inducible MEF/3T3.Tet-Off/Oas1b cell clone expressing...
the Oas1bMBT protein (Oas1b1–381) was established. We reported that mouse cells expressing the Oas1bBALB/c cDNA, which carries a predicted stop codon resulting in a deletion of 30% of the C-terminal sequence were sensitive to WNV infection (6). Thus, we decided to generate the stable MEF/3T3.Tet-Off/Oas1bAC-term cell clone, which contains the Oas1bBALB/c sequence from residues Oas1b-1 to Oas1b-254 as a negative control. The alignment of Oas1b primary amino acid sequences with MBT and BALB/c mouse sequences is shown in Fig. 1.

The MEF/3T3.Tet-Off/Oas1bAC-term and MEF/3T3.Tet-Off/Oas1b cell clones were analyzed for induction of expression of recombinant Oas1b proteins by Tet withdrawal. As determined by RT-PCR assay using specific primers for recombinant Oas1b cDNAs, Oas1b mRNA expression was clearly detected 24 h after induction (Fig. 2A, - Tet). Lower levels of Oas1b mRNA were observed in uninduced cells (Fig. 2A, + Tet). No Oas1b mRNA expression was detected in parental MEF/3T3.Tet-Off cells (data not shown).

Real-time RT-PCR analysis showed that production level of Oas1b mRNA in induced MEF/3T3.Tet-Off/Oas1bAC-term cells was 40 times higher than that found in uninduced cells (Fig. 2B). Stable MEF/3T3.Tet-Off/Oas1b cell clone displayed a lower level of Oas1b mRNA expression compared with that found in MEF/3T3.Tet-Off/Oas1bAC-term cells. A 5-fold increase was observed in induced MEF/3T3.Tet-Off/Oas1b cells compared with uninduced cells (Fig. 2B).

Oas1b Protein Is Metabolically Unstable in Mouse Cells—We assessed the efficiency with which induced MEF/3T3.Tet-Off/Oas1bAC-term and MEF/3T3.Tet-Off/Oas1b cell clones were able to produce recombinant Oas1b proteins. The expression of Oas1b and Oas1bAC-term proteins, which bear a C-terminal c-Myc tag, was first analyzed by immunoblotting of cell lysates with a monoclonal antibody directed against the c-Myc epitope. Expression of recombinant Oas1b proteins could not be detected in induced MEF/3T3.Tet-Off cell clones (data not shown). Also, Oas1b proteins were undetectable in samples from induced MEF/3T3 cell clones by radioimmune precipitation assay (data not shown).

On the basis of these observations, it could be predicted that Oas1b molecules were metabolically unstable. We examined whether Oas1b was subjected to proteolysis by the proteasome (19). The MEF/3T3.Tet-Off/Oas1b and MEF/3T3.Tet-Off/Oas1bAC-term cells were pulse-labeled for 30 min and chased for 30 min in the presence of reversible proteasome inhibitor MG132 at 2 μM. Pulse-chased proteins from cell lysates were immunoprecipitated with anti-c-Myc specific antibody and then analyzed on a 15% SDS-PAGE. The alignment of Oas1b primary amino acid sequences with MBT and BALB/c mouse sequences is shown in Fig. 1.

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ubiquitin/proteasome-dependent proteolytic pathway in the instability of Oas1b molecules in MEF/3T3 cells.

As determined by an MTT-based assay, 20-h treatment with MG132 affected the viability of MEF/3T3 cell clones expressing either Oas1b or Oas1bAC-term in a dose-dependent manner (Fig. 3B). Cell viability was progressively reduced at the doses higher than 50 nM MG132. A 2-fold lower percentage of viable MEF/3T3.Tet-Off/Oas1bAC-term cells upon treatment with up to 500 nM MG132 was observed as compared with MEF/3T3.Tet-Off/Oas1bAC-term cells (Fig. 3B). Thus, Oas1b-expressing MEF/3T3 cells show increased susceptibility to antiproteasome inhibitor as compared with cells expressing the truncated form.

MEF/3T3 Cell Clones Are Permissive to WNV Infection and Sensitive to Antiviral Action of IFN-α—We assessed the susceptibility of uninduced MEF/3T3.Tet-Off cell clones to WNV infection. To test this, MEF/3T3.Tet-Off/Oas1bAC-term and MEF/3T3.Tet-Off/Oas1b cells were infected with WNV strain IS-98-ST1 at 10 m.o.i. At 48 h post-infection, equivalent virus titers were recovered from cell supernatants (Fig. 4A). Extensive necrosis occurred within 72 h (data not shown). Thus, uninduced MEF/3T3.Tet-Off/Oas1bAC-term and MEF/3T3.Tet-Off/Oas1b cell clones show similar susceptibility to WNV infection.

Because IFN-α/β does play a critical role in controlling WNV infection, we investigated the ability of IFN-α to establish an antiviral state in
MEF/3T3.Tet-Off cell clones. The uninduced MEF/3T3.Tet-Off/Oas1b ΔC-term and MEF/3T3.Tet-Off/Oas1b cell clones were treated with 100 IU/ml of IFN-α 5 h prior to WNV exposure. As shown in Fig. 4A, there was a ~2 log reduction in the viral titers recovered from IFN-treated cells, indicating that both MEF/3T3 cell clones display similar susceptibility to IFN-α.

Thus, pretreatment of MEF/3T3.Tet-Off/Oas1b ΔC-term and MEF/3T3.Tet-Off/Oas1b cell clones with IFN-α potently inhibits WNV...
infection. As shown in Fig. 4B, antiviral effect of IFN-α against WNV was essentially preserved within 16 h post-infection. WNV-infected MEF/3T3 cell clones showed resistance to IFN-α when cells were treated past 24 h post-infection. Our data show that IFN-dependent antiviral pathways are functional in MEF/3T3.Tet-Off/Oas1bC-term and MEF/3T3.Tet-Off/Oas1b cell clones, but their antiviral effects are restricted to the early stages of WNV replication.

Ectopic Expression of Oas1b Suppresses WNV Infection—The growth of WNV strain IS-98-ST1 in induced MEF/3T3.Tet-Off/Oas1bC-term was compared with that in uninduced cells (Fig. 5A). To induce Oas1bC-term expression, Tet was removed from the culture medium 24 h prior to virus input. No significant differences were observed either in viral replication (Fig. 5A, left panel) or progeny virus production (Fig. 5A, right panel) between both cell populations regardless of the m.o.i. tested. These results demonstrate the inability of the truncated form of Oas1b to prevent WNV replication inside infected cells.

To investigate the effect of full-length Oas1b protein on infection of mouse fibroblastic cells by WNV, MEF/3T3.Tet-Off/Oas1bC-term cells were exposed to low (0.1 focus forming units/cell) or high input (1–10 focus forming units/cell) of WNV strain IS-98-ST1 (Fig. 5B). In contrast with that found in MEF/3T3.Tet-Off/Oas1bC-term cells, induction of MEF/3T3.Tet-Off/Oas1b cells 24 h prior to virus exposure resulted in dramatic inhibition of WNV replication (Fig. 5B, −Tet). Only few foci of WNV-infected cells were detected by immunofluorescence assay regardless of m.o.i. tested (Fig. 5B, left panel). At low virus input, viral growth was undetectable based on the measurements of viral titers (Fig. 5B, right panel). At 1 m.o.i., there was a >2.5 log reduction in the viral titer recovered from induced MEF/3T3.Tet-Off/Oas1b cells as compared with uninduced cells (Fig. 5B, right panel). The virus progeny was still reduced by at least 90% at the highest m.o.i. tested. Thus, mouse fibroblastic cells respond to Oas1b expression by efficiently inhibiting WNV replication.

We asked whether the apoptotic signaling pathway mediated through the OAS pathway leads to the clearance of WNV-infected cells (7). We found no evidence that the suppression of viral replication was associated with cytopathic effects in infected MEF/3T3.Tet-Off/Oas1b cells as compared with uninduced cells (Fig. 5B, right panel). Thus, the resistance to WNV infection was directly related to the antiviral activity of the Oas1b rather than clearance of virus-infected cells through apoptotic cell death.

Oas1b-dependent Antiviral Activity Is Restricted to the Early Stages of WNV Replication—Kinetic studies were performed to investigate more precisely the antiviral action of Oas1b on WNV replication. MEF/3T3.Tet-Off/Oas1b cells were infected with WNV strain IS-98-ST1 at 10 m.o.i. When the repressor Tet was removed concomitantly to virus input, there was a 1.5 log reduction in the viral titer at 48 h post-infection (Fig. 6A). Induction of Oas1b protein expression at the 24-h time point post-infection reduced the progeny virus production only by −0.5 log. As shown in Fig. 6B, there was a linear correlation between the progressive loss of WNV suppression and the late induction of Oas1b protein expression. We observed that WNV has the ability to escape the Oas1b antiviral action when Tet withdrawal was performed at the time points later than 12 h post-infection. Given that Oas1b-mediated WNV suppression was essentially preserved within the first 10 h of virus life cycle, it is likely that Oas1b acts mainly on the stages of virus replication rather than on viral entry and uncoating of virus particles.

Antiviral Effect of Oas1b Is Associated with a Reduction in Viral Protein Synthesis—Data from the above experiments suggest that a blocking step at early stages of viral infection may be responsible for the inability of WNV to productively infect Oas1b-expressing MEF/3T3 cells. An radioimmune precipitation assay was developed to determine whether Oas1b-mediated WNV inhibition was due to a defect in de novo synthesis of viral proteins. MEF/3T3.Tet-Off/Oas1b and MEF/3T3.Tet-Off/Oas1bC-term cells were infected with WNV strain IS-98-ST1 at 10 m.o.i., and the amounts of viral proteins expressed inside infected cells were assessed for a 90 min-labeling period. Viral proteins were immunoprecipitated from cell lysates with anti-WNV hyperimmune ascitic fluid.

As shown in Fig. 7A, both envelope glycoproteins prM and -E were clearly detected in MEF/3T3.Tet-Off/Oas1bC-term cells. At the 24-h time point post-infection, induction of Oas1bC-term expression increased the viral protein synthesis when compared with that found in uninduced cells (Fig. 7A, compare +Tet and −Tet). The fact that Oas1bC-term-overexpressing MEF/3T3 cells exhibited a great susceptibility to virus replication suggests a pathway through which the truncated form of Oas1b enhances WNV infection inside infected mouse cells.

There was a background level of prM and -E detected in induced MEF/3T3.Tet-Off/Oas1b cells compared with that found in uninduced

![Image](Image.png)
cells (Fig. 7A). Thus, the inability of WNV to productively infect Oas1b-expressing MEF/3T3 cells was attributable to a dramatic reduction in viral protein level.

Kinetic studies showed that induction of Oas1b protein expression after WNV infection was less effective in blocking viral protein synthesis (Fig. 7B). Induction of Oas1b protein expression concomitantly to virus input reduced the de novo synthesis of prM by 85%. There was only a 15% reduction when Oas1b induction started at 20 h post-infection. Thus, the antiviral effect of Oas1b is attenuated once viral replication is well established.

Expression of Oas1b Prevents Viral RNA Accumulation—We determined whether inefficiency of viral protein synthesis was due to a lack of
FIGURE 8. Lack of viral RNA accumulation in cells expressing Oas1b. Induced (+ Tet) and uninduced (- Tet) MEF/3T3.Tet-Off/Oas1b and MEF/3T3.Tet-Off/Oas1b \(^{\mathrm{3C-term}}\) cells were infected with WNV at 10 m.o.i. or mock infected (mock). Tet was removed concomitantly to virus input. A, total RNA was collected from infected cells at different hours post-infection and analyzed by RT-PCR using specific primer couples 5'-WNCa and 3'-WNCa for viral RNA; the \(\beta\)-actin mRNA served as a housekeeping sequence control. The PCR amplification of \(\beta\)-actin mRNA was visualized using a 1% agarose gel. The PCR amplification of WNV C gene was analyzed by Southern blot using \(^{32}\)P-labeled plasmid containing WNV C gene as a probe. B, real-time RT-PCR analysis of viral RNA levels in infected cells 24 h after removal of Tet using primer couples 5'-WNVCb and 3'-WNVCb; the 18 S rRNA served as a sequence control. The relative viral RNA expression levels in respective uninduced cells (- Tet) was assigned a value of 1. Viral RNA expression in induced cell clones relative to the respective value is shown. Error bars indicate deviations from the average. The data are representative of three independent experiments in triplicate. The values were compared statistically according to Student’s \(t\) tests (\(* * *, p < 0.005\); n.s., non significant).
TABLE 1
Oligonucleotide primers used in this study

| Primer | Nucleotide sequence (5’→3’) |
|--------|----------------------------|
| 5′-Actin | ATGGAATGACGATATGCTGC |
| 3′-Actin | GCTGAGAACGGTACAGTGAG |
| 5′-rRNA | GTACCCGGTTGAGACCCTAT |
| 3′-rRNA | CCCATGACATGGTAAAGCG |
| 3′-c-Myc | GTTCTGCGTCCTTCTCTGAGATTC |
| 5′-WNcA | AATATCGTGAAAAAGGGAGATC |
| 3′-WNcA | GTTCTGCTTGGGCTCTTTT |
| 5′-WNcb | GCTTGTGATGTCCTGCA |
| 3′-WNcb | GCTTGTGATGTCCTGCA |
| 5′-Oas1b | ATATAAGCGGCCGCAGT |
| 3′-Oas1b | ATATAAGCGGCCGCAGT |
| 5′-Oas1bAC-term | GCTCAGGAGCGCAAGTCAG |
| 3′-Oas1bMRT | ATATAAGCGGCCGCAGT |
| 5′-1b | ATCTGACGACCCGGCAT |
| 3′-1b | CAAACCAAGAACCAGAAAATACGC |
| 5′-1bAC-term | GCTCAGGAGCGCAAGTCAG |
| 3′-1bAC-term | GCTCAGGAGCGCAAGTCAG |
| 5′-RL | TAAAGGTGGCAGCACAGTC |
| 3′-RL | GGTAGAAGACCAACCAGTCCAC |
| siRNA-82 | CCGCCATTAGAACACTCCGG |

FIGURE 9. RNase L inhibition in siRNA-82-transfected cell clones. A and B, real-time RT-PCR analysis of RNase L mRNA levels in MEF/3T3.Tet-Off/Oas1b-siRNA-82 (A) or MEF/3T3.Tet-Off/Oas1bAC-term/siRNA-82 (B) were analyzed using the primer couple 5′-RL and 3′-RL; the 18S rRNA served as a housekeeping protein control. The 18S rRNA served as a housekeeping protein control.

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accumulation of viral RNA. MEF/3T3 cell clones were infected with WNV strain IS-98-ST1 at 10 m.o.i. (Fig. 8). The repressor Tet was removed concomitantly to WNV input. Total RNA was extracted from MEF/3T3.Tet-Off/Oas1bAC-term/siRNA-82 and MEF/3T3.Tet-Off/Oas1b at the 10-, 16-, and 24-h time points post-infection, and positive-stranded viral RNA was first detected using RT-PCR assay with primers designed on the basis of WNV C gene. The PCR products were analyzed by Southern blot with a specific 32P-labeled probe.

In uninduced MEF/3T3.Tet-Off/Oas1bAC-term cells, the kinetics of the accumulation of positive-stranded viral RNA showed a weak signal at 10 h post-infection followed by a marked increase in production levels past 16 h (Fig. 8A). In response to induction of Oas1bAC-term expression, the accumulation of viral RNA was slightly delayed as compared with uninduced cells (Fig. 8A). This might reflect a possibility that Oas1bAC-term overexpression may have hampered viral RNA replication in the early times of WNV infection. However, there was a comparable level of positive-stranded viral RNA production between uninduced and induced MEF/3T3.Tet-Off/Oas1bAC-term cells at 24 h post-infection (Fig. 8A). At this time point, real-time RT-PCR analysis showed no significant differences in viral RNA accumulation between both cell populations (Fig. 8B, left). Our data show the inability of the truncated form of Oas1b to prevent viral RNA accumulation inside infected MEF/3T3 cells.

In uninduced MEF/3T3.Tet-Off/Oas1b cells, the accumulation of positive-stranded viral RNA could be detected at 24 h post-infection (Fig. 8A). At this time point, there was a background level of intracellular viral RNA in response to Oas1b protein expression. As determined by real-time RT-PCR analysis, there was a 90% reduction in viral RNA
FIGURE 10. Consequence of RNase L deficiency on Oas1b antiviral action. Wild-type and siRNA-82-transfected cells clones were infected 48 h with WNV at 1 m.o.i. Tet was removed concomitantly to virus input. A, MEF/3T3.Tet-Off/Oas1b\textsuperscript{C-term} and MEF/3T3.Tet-Off/Oas1b\textsuperscript{C-term}/siRNA-82 cell clones. B, MEF/3T3.Tet-Off/Oas1b and MEF/3T3.Tet-Off/Oas1b/siRNA-82 cell clones. Infectious particles produced in the supernatants of infected cells were titrated on AP61 cells as described in the legend of Fig. 4. The data are representative of three independent experiments in triplicate. The values were compared statistically according to Student’s t tests (**, p < 0.01; ***, p < 0.001).
production in induced MEF/3T3.Tet-Off/Oas1b cells when compared with that found in uninduced cells (Fig. 8B, right). Thus, the inability of WNV to productively infect MEF/3T3 cells expressing Oas1b can be attributed to a lack of accumulation of positive-stranded viral RNA inside infected cells.

**Antiviral Activity of Oas1b in RNase L-deficient Mouse Cells**—Our data suggest that Oas1b essentially acts on the steps of viral RNA replication. To address the role of RNase L in the Oas1b antiviral pathway, we established small interfering RNA duplexes (siRNA)-mediated down-regulation of RNase L. The sequence of siRNA-82 used in this study is listed in Table 1. The MEF/3T3 cell clones were stably transfected with pSIREN-RetroQ/siRNA-82 to generate the MEF/3T3.Tet-Off/Oas1b/siRNA-82 and MEF/3T3.Tet-Off/Oas1bAC-term/siRNA82 sub-cell clones.

Real-time RT-PCR analysis showed that the siRNA-82 reduced the RNase L mRNA expression level by 60% in MEF/3T3.Tet-Off/Oas1bAC-term/siRNA-82 cells (Fig. 9A) and 90% in MEF/3T3.Tet-Off/Oas1b/siRNA-82 cells as compared with respective wild-type cells (Fig. 9B). The down-regulation of RNase L in stably siRNA-82–transfected cells was verified by an antibody specific to mouse RNase L. A marked reduction in RNase L protein expression was observed in MEF/3T3.Tet-Off/Oas1bAC-term/siRNA-82 (Fig. 9C) and MEF/3T3.Tet-Off/Oas1b/siRNA-82 (Fig. 9D) cells.

We determined the extent to which WNV strain IS-98-ST1 at 1 m.o.i. was capable of replicating in RNase L–deficient cell clones. A moderate reduction in RNase L mRNA expression increased the progeny virus production from infected MEF/3T3.Tet-Off/Oas1bAC-term/siRNA-82 cells by ∼1.5 log when compared with that found in wild-type cells (Fig. 10A). Thus, the down-regulation of RNase L potently enhances WNV replication in mouse fibroblastic cells expressing the truncated form of Oas1b.

Inhibition of RNase L mRNA expression increased the progeny virus production from uninduced MEF/3T3.Tet-Off/Oas1b/siRNA-82 cells only by ∼0.5 log (Fig. 10B). In response to Oas1b protein expression, there was a comparable degree of inhibition in progeny virus production between MEF/3T3.Tet-Off/Oas1b/siRNA-82 and MEF/3T3.Tet-Off/Oas1b cells (Fig. 10B). Thus, siRNA82–mediated inhibition of endogenous RNase L did not rescue WNV replication inside infected MEF/3T3 cells expressing Oas1b. These results suggest that residual levels of RNase L mRNA expression are sufficient to confer antiviral activity in Oas1b-expressing MEF/3T3 cells. We cannot rule out the possibility that Oas1b mediates resistance to WNV replication through an RNase L–independent pathway.

**DISCUSSION**

Recent attention has focused on the role of Oas1b gene in the innate antiviral immunity to WNV in mice (3, 11–14, 16). It was found that expression of the Oas1b sequence in transfected embryo fibroblasts provides some protection against WNV infection (12). In addition, we reported that constitutive expression of Oas1b in stable mouse neuroblastoma cell clones also limited the cell-to-cell spread of WNV (6). These observations are consistent with a model in which WNV is sensitive to the Oas1b antiviral action. The purpose of this study was to investigate the molecular basis for the sensitivity of WNV to the Oas1b antiviral pathway at the cellular level.

Our data suggest that Oas1b is a short-lived molecule in mouse fibroblastic cells that up-regulate the expression of Oas1b sequence under the control of the Tet-Off expression system. An explanation might be that Oas1b is subjected to protein degradation through the N-end rule pathway, which is part of the ubiquitin/proteasome–dependent proteolytic pathway (20). The N-terminal part of Oas1b protein bears a possible degradation signal (N-degron) with the destabilizing N-terminal Gln residue at position 2 of Oas1b followed by two internal lysines at positions 12 and 15, the latter being a potential site of the formation of a multi-ubiquitin chain (Fig. 1) (20, 21). The role of putative N-degron in the metabolic instability of Oas1b requires further analysis. The fact that Oas1b–expressing cells displayed a marked susceptibility to antiprotease–some inhibitor suggests a pathway in which high Oas1b protein level leads to perturbations of cell growth. The precise biochemical mechanism of the growth suppressive properties of OAS pathway still remains elusive (22).

There was a positive correlation between the level of suppression of WNV infection and the inducible expression of Oas1b in mouse fibroblastic cells. Induction of Oas1b protein expression concomitantly to virus input resulted in dramatic inhibition of WNV replication inside infected cells. The Oas proteins are converted from inactive to enzymatically active form by a double-stranded RNA-dependent process (7). The activation of Oas1b protein might be mediated by binding to viral RNA duplexes such as the replicative and intermediate replicative forms produced during WNV replication as well as particular secondary structures within the single-stranded viral RNA (7, 15). Additional studies are undertaken to characterize the viral activator(s) of the Oas1b protein.

The Oas1b–mediated inhibition of viral protein synthesis and the subsequent suppression of viral growth are due to a lack of accumulation of viral RNA inside cells. Our data suggest that expression of Oas1b protein is capable of suppressing viral infection by restricting the ability of WNV to accumulate positive-stranded viral RNA. This is consistent with the previous observation that the levels of single-stranded genomic RNA but not antigenomic RNA, which is detected as viral RNA duplex, are lower in flavivirus infection-resistant as compared with -sensitive mice (13, 23). The antiviral effect of Oas1b against WNV becomes progressively attenuated once virus replication is well established inside infected cells. It is possible that, during the late stages of viral life cycle, viral RNA as well as replication complexes are sequestered in a manner that prevents the antiviral action of Oas1b. Alternatively, the inefficiency of Oas1b could be attributed to specific viral components, which are produced at sufficient levels to preclude the Oas1b pathway (24, 25).

We showed that the IS-98-ST1 strain of WNV, a recent pathogenic isolate used as a viral model for West Nile encephalitis in the Old World (26), has the ability to escape the action of IFN-α inside infected mouse fibroblastic cells. Recent studies suggest that flaviviral IFN antagonists might be involved in the inhibition of IFN-α signaling (25, 27). Flavivirus non-structural proteins such as NS4B and NS5 could be responsible for this effect by blocking the IFN receptor–Janus kinase/signal transducer and activator of transcription signaling pathway inside infected cells (25, 27, 28). Interestingly, hepatitis C virus NS5A protein has been shown to associate with OAS (29). Whether flavivirus–encoded proteins bind to Oas1b and modulate its antiviral activity is a critical issue that will be the subject of further investigation.

The Oas proteins are part of a regulated RNA decay pathway known as the OAS/RNase L antiviral pathway, which has been shown to protect against hepatitis C virus, vaccinia, human immunodeficiency virus, and picornavirus infections (7). To examine the role of RNase L in the antiviral action of Oas1b, we inhibited endogenous RNase L using an siRNA approach and evaluated the ability of WNV to productively infect the RNase L–deficient mouse cells. We found that down-regulation of RNase L mRNA expression enhances WNV replication in MEF/3T3 cells. It is likely that mouse cells respond to WNV infection by triggering the activation of RNase L that affects virus replication inside infected cells. A dramatic reduction in RNase L mRNA expression (90%) failed to
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rescue WNV replication in Oas1b-expressing cells. This might reflect a possibility that small amounts of RNase L enzyme are sufficient to confer antiviral activity to Oas1b. Another possibility might be that Oas1b exhibits a 3’-5’ RNase activity that would be directly responsible for the lack of viral RNA accumulation inside infected mouse cells. Recently, Rogozin et al. (30) reported that human and mouse OAS, predicted to be inactive with nucleotidyltransferase, might have nuclease activity. Sequence analysis identified a putative polymerase β-C domain (PβCD) in the C-terminal part of the Oas1b protein (30). The PβCD domain contains a conserved aspartate residue at position 320 (Fig. 1), which suggests that Oas1b might function as a nuclease. Consistent with this notion, the mutant form of Oas1b, which lacks the C-terminal region encompassing the residue Asp-320, was unable to inhibit WNV replication in infected cells. It has been proposed that truncation mutant of viral protein might be acting as antagonist of endogenous Oas isoenzymes and prevent the RNase L pathway inside infected cells. Consistent with this hypothesis, WNV-infected MEF/3T3 cells displayed a higher level of viral protein synthesis in response to Oas1bΔC-term overexpression.

In conclusion, one of the critical issues to be addressed in the future relates to exact mechanisms by which Oas1b expression results in inhibition of viral RNA production at early stages of WNV infection. Improved knowledge of functional activity of Oas1b will provide new insight into the IFN-stimulated genes that are responsible for establishment of an antiviral state against WNV, opening new perspectives in therapeutic development against flavivirus infection. Experiments are undertaken to validate the antiviral activity of Oas1b against other medically important flaviviruses such as dengue, Japanese encephalitis, and yellow fever viruses.

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