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The large form of human 2′,5′-Oligoadenylate Synthetase (OAS3) exerts antiviral effect against Chikungunya virus

Anne-Claire Bréhin [a], Isabelle Casadémont [b,c,d], Marie-Pascale Frenkiel [a], Cécile Julier [b,c,1], Anavaj Sakuntabhai [b,c,d,*], Philippe Desprès [a,*]

[a] Unité Interactions Moléculaires Flavivirus-Hôtes and Centre National de Référence des Arbovirus, Institut Pasteur, 75724 Paris, France
[b] Unité de Génétique des Maladies Infectieuses et Autoimmunes, Institut Pasteur, 75724 Paris, France
[c] Inserm U730, Institut Pasteur, 75724 Paris, France
[d] Laboratoire de Génétique de la Réponse aux Infections chez l'Homme, Institut Pasteur, 75724 Paris, France

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ABSTRACT

Chikungunya virus (CHIKV) becomes one of the most important mosquito-borne alphavirus in the medical field. CHIKV is highly sensitive to antiviral activity of Type-I interferons (IFN-α/β). Here, we investigated the role of IFN-induced 2′,5′-Oligoadenylate Synthetase (OAS) family in innate immunity to CHIKV. We established inducible human epithelial HeLa cell lines expressing either the large form of human OAS, OAS3, or the genetic variant OAS3-R844X which is predicted to lack about 20% of the OAS3 protein from the carboxy terminus. HeLa cells respond to ectopic OAS3 expression by efficiently inhibiting CHIKV growth. The characteristic of the antiviral effect was a blockade in early stages of virus replication. Thus, OAS3 pathway may represent a novel antialphaviral mechanism by which IFN-α/β controls CHIKV growth. HeLa cells expressing the truncated form of OAS3 were less resistant to CHIKV infection, raising the question on the involvement of OAS3 genetic polymorphism in human susceptibility to alphavirus infection.

Introduction

Chikungunya fever is an arbovirosis of major impact in public health in Asia and Africa. Chikungunya virus (CHIKV) has been responsible for an unprecedented magnitude of outbreaks in the Indian Ocean (Schuffenecker et al., 2006) and in India where hundreds of thousands of people have been infected since 2005 (Epstein, 2007). Humans infected with CHIKV typically experience acute illness with incapacitating polyarthralgia, severe muscle pain, and stiffness in the joints. CHIKV infection in muscle tissue could explain some features of clinical manifestations (Ozden et al., 2007; Couderc et al., 2008).

CHIKV, Sindbis virus (SINV), and Semliki Forest virus (SFV) are members of the *Alphavirus* genus of the Togaviridae family. Alphaviruses are lipid enveloped, positive-sense, single-stranded (ss) RNA viruses that replicate in the cytoplasm of the infected cells (for review Strauss and Strauss, 1994; Griffin, 2007). The alphavirion contains an 11.5 kb, single-stranded RNA genome of positive polarity. The genomic RNA is directly translated into the non-structural proteins 1 to 4 (nsP-1 to -4), which are encoded by the 5′ two-thirds of the genome. The structural proteins are encoded within the 3′ one-third of the genomic RNA and are translated from the subgenomic 26S RNA. The three major structural proteins C (capsid), E2 and E1 (the both envelope glycoproteins) together with genomic RNA form the alphavirion. Alphavirus infection results in rapid inhibition of cellular machinery, which favours the production of viral RNA and proteins inside the host cells (for review Strauss and Strauss, 1994).

Alphaviruses such as CHIKV are highly sensitive to the antiviral activity of Type-I interferons (IFN-α/β) (Desprès et al., 1995; Ryman et al., 2005; Lenschow et al., 2007; Shabman et al., 2007; Sourisseau et al., 2007; Zhang et al., 2007; Couderc et al., 2008; Tesfay et al., 2008).

Innate antiviral mechanisms mediated by IFN-α/β are potentially the most important pathways of host cell defence limiting viral replication (for review, Samuel, 2001). Recent reports have shown that IFN-α/β is able to trigger the activation of a specific signal transduction pathway leading to induction of IFN-stimulated genes (ISGs) that are responsible for the establishment of an antialphaviral state (Lenschow et al., 2007; Zhang et al., 2007). To date, the ISGs thought to affect alphavirus replication are ISG15, ISG20, P56, ZAP, and

* Corresponding authors. A. Sakuntabhai is to be contacted at Génétique de la Réponse aux Infections Chez l'Homme, Institut Pasteur 28, rue du Dr Roux, 75724 Paris, Cedex 15, France. Fax: +33 0145688929. P. Desprès, Interactions Moléculaires Flavivirus-Hôtes and Centre National de Référence des Arbovirus Institut Pasteur 25, rue du Dr Roux, 75724 Paris, Cedex 15, France. Fax: +33 0140613774.
E-mail addresses: anavaj@pasteur.fr (A. Sakuntabhai), philippe.despres@pasteur.fr (P. Desprès).
[1] Present address: Inserm U730, Centre National de Génotypage, CP5721, 91057 Evry, France.

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Table 1
Amino acid differences between available OAS3 sequence (GenBank access NM-006178) and OAS3 cDNAs used in this study

AA position	NM-006178	OAS3	OAS3-R844X
18	Arg	Lys	Lys
844	Arg	Arg	*Opal*
865	Trp	Ser	–

Viperin (Lenschow et al., 2007; MacDonald et al., 2007; Zhang et al., 2007).

It is currently unknown whether any members of IFN-induced 2′,5′-Oligoadenylate Synthetase (OAS) family may play a role in the establishment of an antialphaviral state. The OAS/RNase L is a RNA decay pathway known to play an important role in the established endogenous antiviral pathway (Justesen et al., 2000; Sarkar et al., 1999; for review, Samuel, 2001; Ryman et al., 2005; Silverman, 2007; Randall and Goodbourn, 2008). Human OAS is a family of enzymes encoded by three closely linked genes on chromosome 12q24.2, with the following order: small (OAS1, p40/p46), medium (OAS2, p69/71), and large (OAS3, p100) OAS isoforms. The OAS proteins contain one (p40/p46), two (p69/71), or three (p100) repeats of the basic OAS module. Very little information is available on the biological properties of OAS3 (for review, Rebouillat and Hovanessian, 1999). The IFN-dependent OAS proteins are a group of double-strand (ds) RNA-dependent enzymes (for review Rebouillat and Hovanessian, 1999; Rebouillat et al., 2000). Binding of enzymatically active OAS to activator viral RNA results in the production of 2′- to 5′-linked oligoadenylates (2-5A). Latent, monomeric RNase L is enzymatically activated through homo-dimerization induced by binding to 2-5A oligomers. Once activated, RNase L degrades single-stranded RNA molecules including mRNA and viral RNA (for reviews, Samuel, 2001; Silverman, 2007).

We and others demonstrated that 1b isoform of mouse OAS gene (Oas1b) is a critical component of innate immunity to West Nile virus *in vivo* and *in vitro* (Mashimo et al., 2002; Perelygin et al., 2002; Kajaste-Rudnitski et al., 2006). We also reported that Oas1b is capable of suppressing flavivirus infection in RNase L-deficient mouse cells (Kajaste-Rudnitski et al., 2006). In the case of alphaviruses, a body of evidence exists to suggest that IFN-mediated inhibition of virus growth does not require RNase L (Ryman et al., 2005; for review, Silverman, 2007). Because the large form of human OAS is presumably not involved in RNase L activation (for review, Rebouillat and Hovanessian, 1999), we investigated whether OAS3 exerts an antiviral effect on CHIKV infection in human epithelial cells.

Results and discussion

Susceptibility of HeLa.Tet-Off cells to CHIKV

Our preliminary data showed that a high level of OAS3 expression after transfection with non-inducible DNA expression vectors resulted in great instability of OAS3 in transfected human cell lines (data not shown). To assess the antiviral activity of the large form of human OAS, we decided to establish a tetracycline-inducible human epithelial cell line with a stably integrated human OAS3 cDNA (Table 1) that allows induction of OAS3 expression by the removal of tetracycline (Tet) repressor.

To our knowledge, no stable cell lines expressing the large form of human OAS have been reported to date. Our previous attempts to generate a stable human cell line which allows inducible expression of OAS3 showed that HeLa.Tet-Off cell line was available for production of OAS3. To validate the susceptibility of HeLa.Tet-Off cells to CHIKV infection, these cells were exposed to an increasing input of La Réunion CHIKV strain 06-49 (Schuffenecker et al., 2006; Bréhin et al., 2008) (Fig. 1A). An analysis of CHIKV replication in HeLa.Tet-Off cells infected at 1 multiplicity of infection (MOI) showed that production of progeny virus reached ~7 log FFU.mL^{-1} at 18 h p.i. At this time point of infection, CHIKV infection resulted in detection of ~50% cells that were positive in flow cytometry analysis with anti-CHIK.E2 MAb 3E4 (Bréhin et al., 2008) (data not shown). Thus, HeLa.Tet-Off cells show susceptibility to CHIKV growth.

Recent attention has focused on the role of IFN-α/β in the antiviral innate immune responses against CHIKV in human cells (Sourisseau et al., 2007; Couderc et al., 2008). Thus, we examine the ability of human IFN-α to establish an antiviral state in HeLa.Tet-Off cells. Treatment of HeLa.Tet-Off cells with 1000 IU.mL^{-1} human IFN-α 5 h prior CHIKV exposure (1 MOI) resulted in ~1.5 log reduction in virus progeny production at 18 h p.i. (Fig. 1B). Thus, IFN-dependent antiviral pathways are functional in HeLa.Tet-Off cells and provide protection against CHIKV at cellular level. However, CHIKV-infected HeLa.Tet-Off cells showed complete resistance to IFN-α at 5 h p.i. once virus replication is well established (Fig. 1C).

Establishment of OAS3-expressing HeLa cell lines

To investigate the antiviral effect of OAS3 against CHIKV, we established a HeLa.Tet-Off/OAS3 cell clone that up-regulates OAS3 protein expression under the control of the Tet-Off expression system. The recombinant OAS3 protein is composed of three adjacent OAS units (domains I, II, and III) including three potential active catalytic

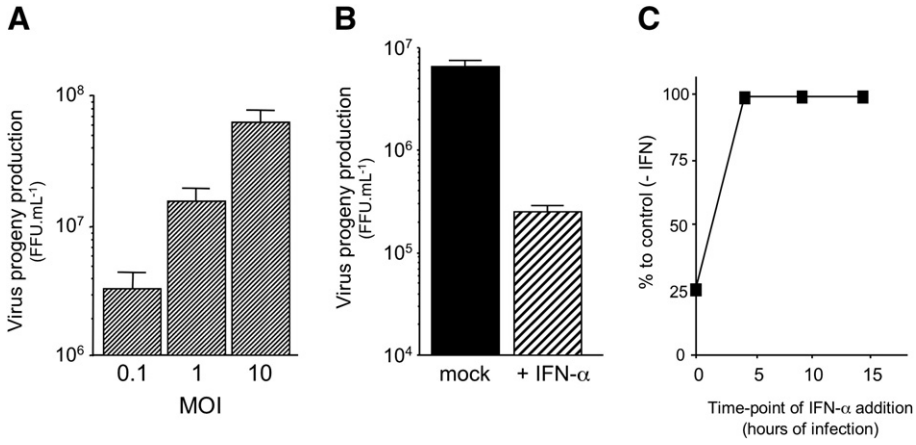

Fig. 1. Susceptibility of HeLa cells to CHIKV. In (A), HeLa.Tet-Off cells were infected with CHIKV at different MOI. At 20 h p.i., virus particles produced in the supernatants were titered on AP61 cells. In (B), HeLa.Tet-Off cells were treated with 1000 IU.mL^{-1} human IFN-α or mock-treated (control) 5 h prior to CHIKV 06-49 input at 1 MOI. Virus progeny productions were determined at 18 h p.i as described above. In (C), antiviral action of IFN-α on CHIKV growth. HeLa.Tet-Off cells were infected with CHIKV at 1 MOI. Cells were treated with 1000 IU.mL^{-1} human IFN-α at various time points p.i. Percentage of CHIKV progeny production in IFN-treated cells relative to that in mock-treated cells (% to control) at 20 h p.i.

sites (for review, Rebouillat and Hovanessian, 1999) (Fig. 2A). A number of HeLa.Tet-Off/OAS3 cell clones were capable of OAS3 production and the most efficient designated HeLa.Tet-Off/OAS3#3C4 was used in all further studies. Expression of recombinant OAS3 in these cells was assessed by immunoblotting assay with a polyclonal immune serum directed against the C-terminal region of human OAS3 protein (Fig. 2B). As a positive control, the production of endogenous OAS3 molecules was clearly detected in IFN-treated HeLa.Tet-Off cells (Fig. 1D, control, +*IFN*). whereas no basal expression of OAS3 was observed in resting cells (Fig. 1D, control, *mock*). Immunoblot analysis detected a basal level of OAS3 molecules in uninduced HeLa.Tet-Off/OAS3 cells indicating that shut-off was incomplete (Fig. 1D, *OAS3*, +*tet*). When the repressor Tet was removed 24 h from the culture medium, there was an increase in production level of recombinant OAS3 protein (Fig. 1D, *OAS3*, −*tet*).

By sequencing the 16 exons, flanking introns and 2 kb region 5′ to the *OAS3* gene in forty-eight healthy Caucasians individuals, we identified a single-nucleotide polymorphism (SNP) at the first position of codon CGA-844 where the substitution T for C resulted in a non-sense mutation (OAS3.R844X) (Table 1). We further genotyped this SNP in 180 healthy Caucasian individuals and identified two heterozygous individuals, resulting in an overall estimate of allele frequency of 0.5%. OAS3.R844X is expected to result in a truncated form of OAS3 protein, lacking about 20% from the carboxy-terminus (Fig. 1C). Consequently, we constructed a stable HeLa.Tet-off/OAS3$^{\Delta C\text{-term.}}$# 1C cell clone expressing the OAS-1 to OAS3–843 sequence. The OAS3$^{\Delta C\text{-term.}}$ lacks the D^{889} residue which composes the triad defining the active catalytic site of the third domain of OAS3 (Sarkar et al., 1999; Rebouillat et al., 2000). Immunoblot analysis using immune serum directed against the N-terminal region of human OAS3 protein detected the truncated form of OAS3 protein only in induced HeLa.Tet-off/OAS3$^{\Delta C\text{-term.}}$ cells (Fig. 2C).

Antiviral effects of OAS3 against CHIKV

To determine the extent by which CHIKV was capable of replicating in OAS3-expressing HeLa cells, HeLa.Tet-Off/OAS3 cells were exposed to CHIKV at 1 MOI (Fig. 2A). Flow cytometry analysis of CHIKV-infected HeLa.Tet-Off cells with Cy^3-conjugated MAb 3E4 detected nearly 40% of E2-positive cells (Fig. 3A, control) at 18 h p.i. whereas pre-treatment of these cells with IFN-α resulted in ~5% cells that were positive for viral antigen (Fig. 3A, +*IFN*-α). In contrast with that found in parental cells, only 9 to 11% of HeLa.Tet-Off/OAS3 cells were detected positive for CHIKV infection. There was a comparable degree of inhibition in CHIKV replication between uninduced (Fig. 3A, +*tet*) and induced HeLa.Tet-Off/OAS3 cells (Fig. 3A, −*Tet*). Thus, human epithelial cells respond to OAS3 expression by efficiently inhibiting CHIKV infection.

To further assess the efficiency with which OAS3 inhibits CHIKV growth, virus progeny production in HeLa.Tet-Off/OAS3 cells was compared with that in parental HeLa.Tet-Off cells (Fig. 3B). Studies were performed without Tet to avoid any inhibitory effect of antibiotic on infectivity of released CHIKV. The culture fluids were harvested at 18 h p.i. and examined by infectivity assay on mosquito AP61 cells. We found that expression of recombinant OAS3 protein reduced the progeny virus production by 1.5 to 2.0 log regardless of the MOI tested. At 24 h p.i., there was ~3.0 log reduction in the viral titer at the lower MOI tested. However, viral growth was reduced only by ~0.5 log when CHIKV infection was performed at high virus input (10 MOI). This relative inefficiency of OAS3 could be attributed to viral components which are produced at sufficient levels to attenuate its antiviral activity.

Measurements of LDH activity released from CHIKV-infected HeLa.Tet-Off/OAS3 cells showed no significant loss of cell viability within the first 24 h of infection as compared to infected parental cells (data not shown). Thus, the resistance to CHIKV infection was directly related to the antiviral activity of the OAS3 rather than elimination of virus-infected HeLa cells through cytopathic effects.

Kinetic studies showed that OAS3-mediated inhibition of CHIKV was effective during virus life cycle (Fig. 3C). At 12 h p.i., there was a 2.0 log reduction in the viral titer recovered from HeLa.Tet-Off/OAS3 cells as compared with HeLa.Tet-Off cells. Induction of OAS3 protein expression was still able to reduce the progeny virus production by at least 1.5 log at 24 h p.i. Given that OAS3-mediated inhibition of CHIKV was essentially preserved within the 24 h p.i., it is likely that CHIKV was unable to counteract the antiviral action of OAS3 to its own advantage.

We assessed the ability of mutant OAS3.R844X to inhibit CHIKV growth. Induced HeLa.Tet-Off/OAS3 and HeLa.Tet-off/OAS3$^{\Delta C\text{-term.}}$ cells were infected with CHIKV 06-49 at 1 MOI. Analysis of viral growths showed that ectopic expression of OAS3$^{\Delta C\text{-term.}}$ resulted in a lower efficiency of CHIKV inhibition as compared to full-length OAS3 protein (Fig. 3D). This result suggests that SNP at codon CGA-844 may have an impact on the antiviral activity of OAS3. The finding that

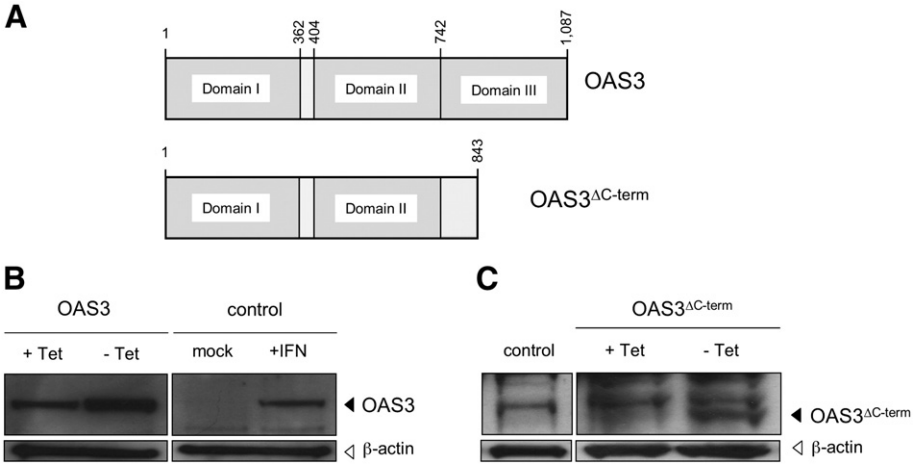

Fig. 2. Detection of OAS3 in inducible HeLa.Tet-Off/OAS3 cell lines. In (A), the graph depicts the structures of full-length OAS3 protein and the truncated form OAS3$^{\Delta C\text{-term.}}$. In (B), immunoblot analysis of OAS3 expression in HeLa.Tet-Off cells incubated with IFN-α (control, +IFN) for 24 h or mock-treated (control, mock) and uninduced (OAS3, +Tet) or induced (OAS3, −Tet) HeLa.Tet-Off/OAS3 cells using anti-OAS3 C-term. antibodies. To ensure an optimal production of recombinant OAS3 protein, tetracycline was removed 24 h from the culture medium. The β-actin served as a house-keeping protein control. In (C), detection of the truncated form of OAS3 in HeLa.Tet-Off/OAS3$^{\Delta C\text{-term.}}$ cells. HeLa.Tet-Off cells (control) and uninduced (+Tet) or induced (−Tet) HeLa.Tet-Off/OAS3$^{\Delta C\text{-term.}}$ cell lysates were analyzed by immunoblotting with anti-OAS3 N-term antibodies. The β-actin served as a house-keeping protein control.

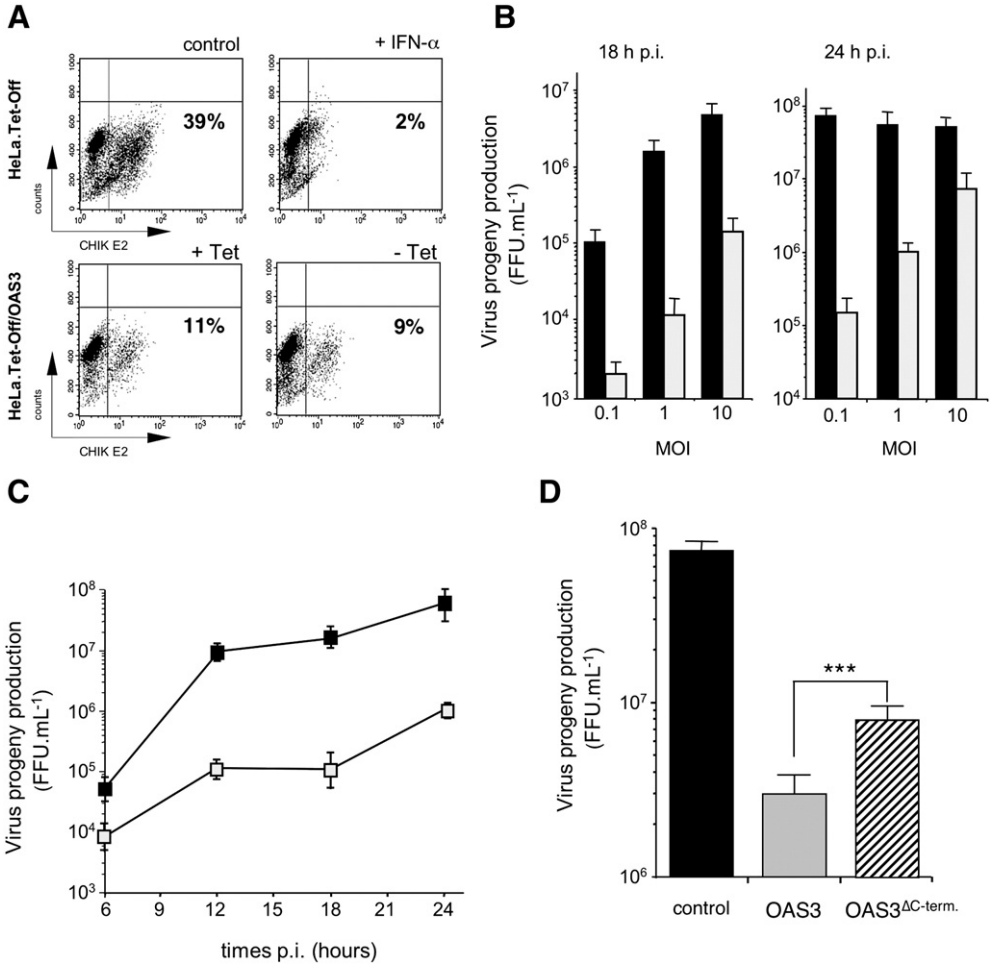

Fig. 3. Inhibition of CHIKV growth in OAS3-expressing HeLa cells. In (A), cells were infected 18 h with CHIKV 06-49 at 1 MOI and analyzed by flow cytometry with Cy^3-conjugated MAb 3E4 directed against CHIKV E2 glycoprotein. Percentage of infected HeLa.Tet-Off cells incubated with 1000 $IU.mL^{-1}$ IFN-α (+IFN-α) 24 h prior virus input or mock-treated (control), and infected HeLa.Tet-Off/OAS3 cells in the presence (+*tet*) or in absence (−*tet*) of Tet. The background of mock-infected cells was <0.5%. In (B), HeLa.Tet-Off (close box) and induced HeLa.Tet-Off/OAS3 (grey box) cells were infected with CHIKV at different MOI and virus progeny productions were determined at 18 and 24 h p.i. In (C), induced HeLa.Tet-Off/OAS3 cells were infected with CHIKV at 1 MOI and viral growth was monitored at various times p.i. In (D), induced HeLa.Tet-Off/OAS3 (OAS3) and HeLa.Tet-Off/$OAS3^{\Delta C\text{-term.}}$ ($OAS3^{\Delta C\text{-term.}}$) cells were infected with CHIKV at 1 MOI and virus progeny productions were determined at 18 h p.i. The values were compared statistically according to Student's *t* tests (***: $P<0.001$).

$OAS3^{\Delta C\text{-term.}}$-expressing cells displayed resistance to CHIKV infection indicates that OAS-1 to OAS3-843 sequence is still able to provide significant protection.

Antiviral action of OAS3 against alphaviruses

We examined whether the large form of human OAS is able to inhibit growth of other alphaviruses such as SINV and SFV in human epithelial cells (Fig. 4A). HeLa.Tet-Off and induced HeLa.Tet-Off/OAS3 cells were infected with alphaviruses at 1 MOI. In response to OAS3 protein expression, there was a comparable degree of inhibition (~2 log) in progeny virus production between SINV and SFV at 18 h p.i. Thus, different alphaviruses can be greatly suppressed by the antiviral activity of OAS3.

We examined whether OAS3 displays antiviral activity against other RNA viruses of medical interest such as flavivirus (positive-sense ssRNA) and morbillivirus (negative-sense ssRNA). Because human epithelial cells are permissive to Schwarz (Schw.) vaccine strain of measles virus (MV) and live-attenuated 17D vaccine strain of yellow fever virus (YFV), parental Hela.Tet-Off and HeLa.Tet-Off/OAS3 cells were exposed to $MV_{Schw.}$ or YFV 17D. There were no obvious differences in $MV_{Schw.}$ replication between both cell populations (data not shown). Also, there was no significant inhibition of YFV 17D growth in OAS3-expressing HeLa cells (Fig. 4A). Since IFN-α was able to establish an antiviral state against YFV strain 17D in infected HeLa.Tet-Off cells (Fig. 4B), we can rule out the possibility that over-expression of OAS3 resulted in Type-I IFN induction that might account for any antiviral effects observed in human epithelial cells. Taken together, these results show that alphaviruses are particularly sensitive to OAS3 expression in human cells.

Mechanisms of OAS3-mediated CHIKV inhibition

In an effort to resolve the molecular basis of the antiviral action of OAS3, we first determined whether inhibition of CHIKV growth was due to a lack of accumulation of structural virus proteins. Total proteins were extracted from Hela.Tet-Off and HeLa.Tet-Off/OAS3 cells at the 18 h p.i. and C, pE2 (precursor of E2), E2, and E1 were detected by immunoblot using mouse anti-CHIKV polyclonal antibodies or anti-CHIKV E2 MAb 3E4 (Fig. 5A). There was a background level of structural virus proteins in response to OAS3 protein expression. Thus, the inability of CHIKV to productively infect OAS3-expressing HeLa cells is associated with a severe reduction in viral protein synthesis.

We determined whether the inefficiency of viral protein synthesis was due to a lack of accumulation of viral RNA. Total RNA was extracted

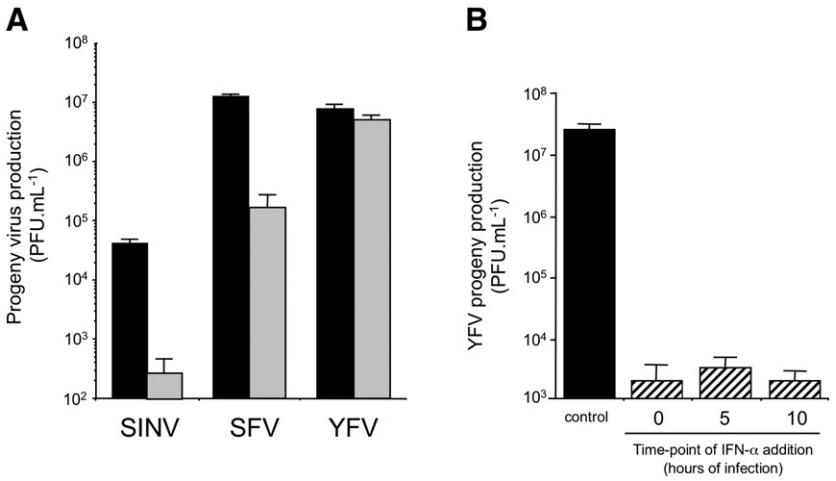

Fig. 4. Antialphaviral activity of OAS3. In (A), HeLa.Tet-Off (close box) and induced HeLa.Tet-Off/OAS3 (grey box) cells were infected at 1 (SINV and SFV) or 10 (YFV) MOI and virus progeny productions were determined at 18 h (SINV and SFV) or 72 h (YFV) p.i. In (B), HeLa.Tet-Off cells were infected with YFV 17D at 1 MOI and treated with 1000 IU.mL^{-1} human IFN-α at various time points p.i. or mock-treated (control). Virus progeny productions were determined at 72 h p.i.

from Hela.Tet-Off and HeLa.Tet-Off/OAS3 cells at the 8 h time point post-infection and production of genomic and subgenomic viral RNAs was analyzed using RT-PCR assay with primers designed on the basis of CHIKV E2 gene. As determined by real-time RT-PCR analysis, there was a 2 log reduction in viral RNA level recovered from OAS3-expressing cells when compared to that found in parental cells (Fig. 5B). Thus, OAS3 expression prevents viral RNA accumulation inside infected cells. To address the role of RNase L in the antiviral action of OAS3, we inhibited endogenous RNase L using a small interfering RNA duplex (siRNA) approach. Specific targeting siRNA 1890 (Table 2) was able to reduce RNase L expression level after transfection of HeLa.Tet-Off/OAS3 cells (Fig. 5C). Since siRNA-mediated inhibition of RNase L was ineffective to promote CHIKV growth in OAS3-expressing HeLa cells (Fig. 5C), it seems unlikely that OAS3 affects CHIKV replication through a RNase L-dependent pathway.

Concluding remarks

Our data suggest a role for the large form of human OAS in the established endogenous antiviral pathway against CHIKV infection.

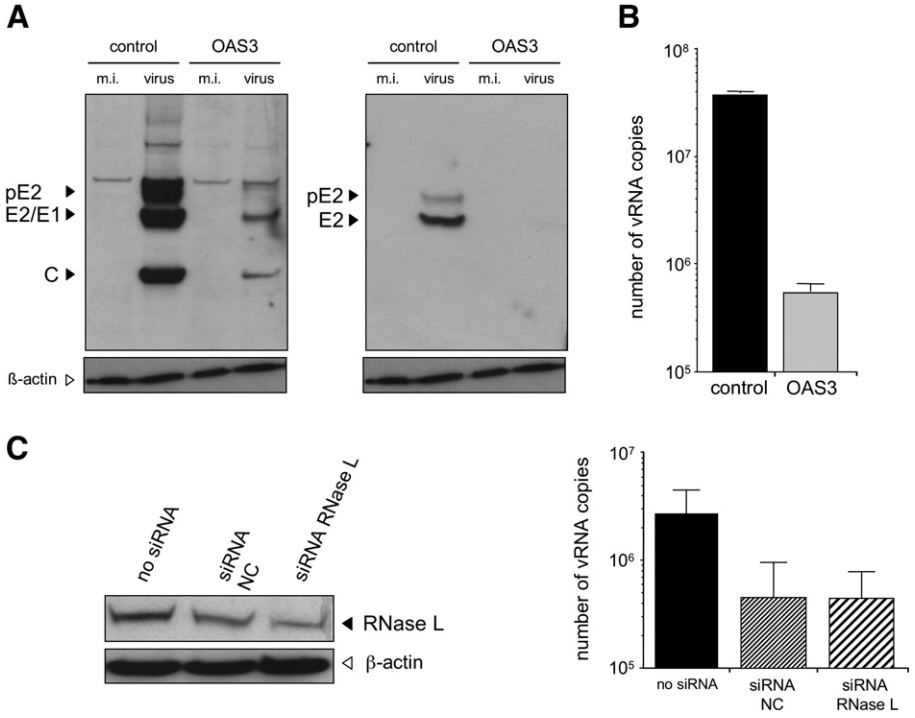

Fig. 5. Consequences of OAS3 expression for CHIKV replication. In (A), immunoblot assay was performed on cell extracts from HeLa.Tet-Off and HeLa.Tet-Off/OAS3 cells infected with CHIKV (virus) or mock-infected (m.i.) using anti-CHIK HMAF (left) or anti-CHIKV E2 MAb 3E4 (right). The β-actin served as protein control. In (B), real-time RT-PCR analysis of viral RNA production in HeLa.Tet-Off cells (control) and induced HeLa.Tet-Off/OAS3 cells (OAS3) infected with CHIKV at 1 MOI was performed at 8 h p.i. The quantification of viral RNA copies was performed as described in the Materials and Methods. In (C), a consequence of down-regulation of RNase L on OAS3 inhibition of CHIKV. Left, immunoblot analysis of RNase L inhibition in HeLa.Tet-Off/OAS3 cells transfected 20 h with nontargeting negative control siRNA (siRNA NC), specific targeting siRNA 1890 (siRNA RNase L) or mock-transfected (no siRNA). The design and the production of siRNA 1890 were made by GeneCust Co. (Luxembourg). Right, induced HeLa.Tet-Off/OAS3 cells transfected 20 h or mock-transfected were infected with CHIKV at 1 MOI. Real-time RT-PCR analysis of viral RNA production was assessed at 8 h p.i. as described above. Error bars indicate deviations from the average.

Table 2
Sequences of primers used in this study

Primer	Nucleotide sequence (5′→3′)
OAS3.R844X-F	GTGGTGTTCCTCAGCTGCTT
OAS3.R844X-R	CATCGTCTGGGATGTCAGTG
pTet-OAS3-univ1	ATTAATGCGGCCGCAACGAAACCAGAAATCCGAAGGCC
pTet-OAS3-rev2	AACTAACTATAG**TCA**AAGGTCCTCCTTGGAGATGAGCTTCTGTTCCACAGCAGCCTTCACTGGCCATGGCTGGATGGG
pTet-OAS3-univ-1bis	ATTAATGCGGCCGCATGGACTTGTACAGCACCCCGGCCGCTGCG
pTet-OAS3-rev-3bis	AACTAACTATAG**TCA**AAGGTCCTCCTTGGAGATGAGCTTCTGTTCGATCTCGGAGATGATCTCGGCCCGCCGCTTGTTGCC
OAS3ecto-For	CGGACGGTGCTGGGGCTCGTG
OAS3ecto-Rev	CTTGTCTTCGAGTAGAGGCTTCTC
Chik/E2/9018/+	CACCGCCGCAACTACCG
Chik/E2/9235/−	GATTGGTGACCGCGGCA
GAPDH-For	GGGAGCCAAAAGGG
GAPDH-Rev	GGGGACACGGAAGG
hRNaseL-For	GCGTGAAGCTGCTGAAACTT
hRNaseL-Rev	ACTTGGGTTTGGTGCAGAGG
siRNA 1660	GCUCAGUUCUGGCACCAAATT
	UUUGGUGCCAGAACUGAGCTG
siRNA 1890	AGUGGACGACUAAGAUUAATT
	UUAAUCUUAGUCGUCCACUTG

Enzyme recognition site are underlined. Sequence complementary to a stop codon are shown in bold.

We showed that OAS3 acts on the stages of alphavirus replication inside infected cells. However, we cannot rule out the possibility that OAS3-mediated inhibition of CHIKV was also due to a block early in virus life cycle, e.g., viral entry and uncoating of virus particles.

We consider that the ability of OAS3 to inhibit alphavirus growth may be important for the development of antiviral molecules against CHIKV. One of the critical issues to be addressed in the future relates to a precise biochemical mechanism of OAS3-dependent antialphaviral activity. Experiments will be undertaken to determine whether antiviral effect of OAS3 may be mediated by binding to viral RNA duplexes such as particular secondary structures within the genomic or antigenomic RNA as well as replicative forms produced during alphavirus replication (for review, Griffin, 2007).

There was a background level of intracellular viral RNA in response to OAS3 protein expression. Using a siRNA approach, we showed that reduction in RNase L expression did not rescue the inability of CHIKV to productively infect human cells expressing OAS3. This might reflect a possibility that OAS3 mediates resistance to alphavirus infection by a RNase L-independent mechanism. We showed that expression of OAS3 was effective in blocking viral protein synthesis inside infected human cells. Recently, it was reported that IFN-α/β could control alphavirus replication in inhibiting cap-dependent translation of viral RNA (Zhang et al., 2007; Tesfay et al., 2008). On the basis of this observation, it is tempting to speculate that OAS3 possesses inhibitory translation activity that might compromise viral protein production. Whether OAS3 potently inhibits alphaviral mRNA translation is a critical issue that will be the subject of further investigation.

A siRNA approach was developed to determine whether reduction in OAS3 expression was effective to promote CHIKV replication in HeLa cells exposed to IFN. Although specific targeting siRNA 1660 (Table 2) was able to reduce OAS3 mRNA expression after transfection of IFN-treated cells, no rescue of CHIKV growth was observed in these cells (data not shown). This might reflect the possibility that redundant antiviral activities of the different ISGs prevent the detection of individual effects of OAS3 against CHIKV.

Very little information is available on the human genetic susceptibility to alphavirus infection. Screening the *OAS3* gene for polymorphism identified the genetic variant OAS3.R844X in Caucasian individuals. *In vitro*, CHIKV was less sensitive to the antiviral activity of OAS3 protein lacking the C-terminal Domain III. The notion that genetic polymorphism of OAS3 could control its antialphaviral activity opens a new avenue for elucidating the role of human OAS genes in the pathogenesis of alphavirus-related diseases such as Chikungunya fever.

Materials and methods

Cell cultures

The HeLa.Tet-Off cell line was purchased from BD Biosciences Clontech. HeLa.Tet-Off cells are maintained in DMEM (Invitrogen), supplemented with 10% heat-inactivated foetal calf serum (FCS), 4 mM glutamine and 200 μg mL^{-1} G418 (Invitrogen). The HeLa.Tet-Off/OAS3 cell line is maintained in DMEM/10% FCS/4 mM glutamine supplemented with 200 μg mL^{-1} G418, 100 μg mL^{-1} Hygromycin (BD Biosciences Clontech), and 2 μg mL^{-1} Tetracyclin (Sigma-Aldrich).

Viruses

Production of clinical isolate CHIKV strain 06-49 on mosquito *Aedes pseudoscutellaris* (AP61) cell monolayers and virus titration were performed as previously described (Bréhin et al., 2008). Infectivity titers were expressed as focus forming units (FFU) on AP61 cells. SINV strain AR339, SFV strain SF64, and YFV strain 17D (STAMARIL, Aventis-Pasteur) were propagated on African green monkey kidney (VERO) cell line and infectivity titers were expressed as plaque forming units (PFU) on VERO cells. In order to assay its antiviral effect, human IFN-α (BioSource) was directly added to culture medium.

Genotyping

The SNP OAS3.R844X was genotyped by PCR-RFLP assay using the couple of primers OAS3.R844X-F and OAS3.R844X-R (Table 2). The 183 bp-long PCR product was subjected to digestion with Bgl II and the detection of two fragments of 115 and 68 bp indicated the presence of OAS3.R844X.

Establishing HeLa.Tet-Off cell lines expressing OAS3 proteins

The plasmids PCR4-TOPO (Invitrogen) containing the cDNA coding for two overlapping regions of the large form of human OAS were used as templates for expression of the full-length OAS3 protein (aa 1 to 1087) or the truncated form $OAS3^{\Delta C\text{-term.}}$ (aa 1 to 843) (Table 1). The OAS3 sequences were modified by PCR to be flanked on the 3′ open reading frame end by an additional sequence (EQKLISKEDL) followed by a stop-codon with the couple of primers pTet-OAS3-univ1 and pTet-OAS3-rev2 for OAS3 and the couple of primers pTet-OAS3-univ1bis and pTet-OAS3-rev2bis for $OAS3^{[1-843]}$ (Table 2). The OAS3 sequences are flanked by the NotI and EcoRV restriction

enzymatic sites at the downstream and upstream ends, respectively. The PCR products were digested with NotI and EcoRV and then inserted into the unique sites NotI and EcoRV of pTRE2hyg expression vector (BD Biosciences Clontech) to generate pTRE2/OAS3 and pTRE2/OAS3$^{[1-843]}$. HeLa.Tet-Off cells were transfected with pTRE2/OAS3 or pTRE2/OAS3$^{[1-843]}$, selected on growth medium containing inhibitors and then cloned from single cells by limiting dilution in the presence of 10 μg mL^{-1} repressor Tetracycline (Tet). For Tet withdrawal, cell monolayers were trypsined and cells were washed at least five times with non-supplemented DMEM before replacing with DMEM/10% FBS supplemented only with genotoxic drugs. The level of recombinant OAS3 mRNA production in induced cells (−Tet) relative to that in uninduced cells (+Tet) was determined by RT-PCR analysis using the couple of primers OAS3-For and OAS3-Rev (Table 2). Based on these experiments, the inducible HeLa.Tet-Off/OAS3#3C4 and HeLa.Tet-Off/OAS3$^{\Delta C\text{-term.}}$#1C cell clones were selected. Both HeLa.Tet-Off/OAS3 cell lines were maintained under repressing condition in the presence of 2 μg mL^{-1} Tet.

Real-time RT-PCR

Analysis of viral RNA accumulation was performed with an ABI Prism 7700 sequence detection using the SYBR® Green PCR essentially as described previously (Kajaste-Rudnitski et al., 2006). The primers for endogenous OAS3 mRNA are OAS3endo-For and OAS3endo-Rev (Table 2). The primers for CHIKV E2 gene are Chik/E2/9018/+ and Chik/E2/9235/− (Table 2). GAPDH mRNA was used as an endogenous sequence control for the normalization of each sample. The primers GAPDH-For and GAPDH-Rev are listed in Table 2. For the quantification of viral RNA copies, an *in vitro* CHIK RNA transcript encoding the N-terminal region of E2 was performed to build the standard curve as previously described (Vazeille et al., 2007).

Immunoblot assays

Cellular proteins were subjected to immunoblot analysis as previously described (Bréhin et al., 2008). Viral protein expression was detected using anti-CHIKV hyperimmune mouse ascitic fluids (HMAF) or anti-CHIK.E2 MAb 3E4 (Bréhin et al., 2008). OAS3 protein expression was detected using anti-OAS3 N-term (Santa-Cruz Biotechnology) or OAS3 C-term (Abgent) antibodies. RNase L protein expression was detected using a mouse monoclonal anti-RNase L antibody (clone 2E9, Zymed Laboratories). β-actin expression was detected using a mouse monoclonal β-actin antibody (Sigma-Aldrich).

Flow cytometry analysis

Cells were detached and then fixed with 3.2% paraformaldehyde in PBS. Fixed cells were permeabilized, stained with Cy^3-conjugated MAb 3E4 and analyzed by flow cytometry as described previously (Bréhin et al., 2008).

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