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Differential action of pateamine A on translation of genomic and subgenomic mRNAs from Sindbis virus

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ABSTRACT

Pateamine A (Pat A) is a natural marine product that interacts specifically with the translation initiation factor eIF4A leading to the disruption of the eIF4F complex. In the present study, we have examined the activity of Pat A on the translation of Sindbis virus (SINV) mRNAs. Translation of genomic mRNA is strongly suppressed by Pat A, as shown by the reduction of nsP1 or nsP2 synthesis. Notably, protein synthesis directed by subgenomic mRNA is resistant to Pat A inhibition when the compound is added at late times following infection; however, subgenomic mRNA is sensitive to Pat A in transfected cells or in cell free systems, indicating that this viral mRNA exhibits a dual mechanism of translation. A detailed kinetic analysis of Pat A inhibition in SINV-infected cells demonstrates that a switch occurs approximately 4 h after infection, rendering subgenomic mRNA translation more resistant to Pat A inhibition.

Introduction

Translation of cellular and viral mRNAs can take place by a number of mechanisms depending on the mRNA and the context of its translation. The vast majority of cellular mRNAs contain a cap structure at their 5′ end and are translated following the canonical mechanism that involves the recognition of the cap structure by the heterotrimeric factor eIF4F followed by the interaction of the preinitiation 43S complex with the mRNA (Sonenberg and Hinnebusch, 2009). The eIF4F complex is composed of the cap-binding factor eIF4E, the helicase and ATPase enzyme eIF4A and the scaffolding protein eIF4G (Gingras et al., 1999). Unwinding of the secondary structure present in the mRNA leader sequence is accomplished by eIF4AI or eIF4AII, which are functionally interchangeable isoforms with 90% similarity (Parsyan et al., 2011). After RNA unwinding, the 40S ribosomal subunit containing several initiation factors linearly scans the leader sequence until an AUG codon is encountered in a good sequence context (Kozak, 1991). Initiation of translation can also occur by a mechanism which is independent of the cap structure whereby initiation takes place at an internal sequence located at the 5′ untranslated region (5′-UTR) of the mRNA, known as the Internal Ribosome Entry Site (IRES) (Au and Jan, 2014; Komar et al., 2012; Niepmann, 2009). This element promotes the direct interaction of preinitiation complexes, or even 40S ribosomal subunits, to an internal region of the mRNA leader sequence that can be followed by scanning until the initiation codon is reached (Au and Jan, 2014; Chamond et al., 2014). The number of eIFs that participate in this initiation mechanism, as well as the molecular events that occur to build up the 80S initiation complex, depends on the particular IRES analyzed. Yet another mechanism of translation has been observed with Sindbis virus (SINV) subgenomic mRNA (sgmRNA), which contains a cap structure and is translated by a scanning mechanism of its leader sequence, where cap recognition and linear scanning are accomplished without the participation of crucial eIFs, such as eIF2 or eIF4A (Garcia-Moreno et al., 2014). SINV belongs to the alphavirus genus in the *Togaviridae* family and contains a positive-stranded RNA as genome, which is delivered to the cytoplasm after virus entry (Brown and Hernandez, 2012; Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994). This genomic mRNA (gmRNA) directs the synthesis of early nonstructural proteins (nsP1-4), which are involved in RNA replication and transcription. In contrast, the sgmRNA is transcribed and translated in the late phase of the virus life cycle and gives rise to the production of structural proteins concomitant with the inhibition of cellular mRNA translation (Sanz et al., 2014). Interestingly, SINV sgmRNA exhibits a dual mechanism of translation depending on the context in which it is translated. Thus, translation of this mRNA

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does not require eIF2, eIF4G nor eIF4A in infected cells (Castelló et al., 2006; Garcia-Moreno et al., 2013; Sanz et al., 2009; Ventoso et al., 2006). In contrast, these factors are necessary to initiate protein synthesis on sgmRNA in cell free systems or in transfected cells.

Inhibitors of cellular functions are very valuable as therapeutic agents, but they also represent important tools to help unravel the molecular events involved in a given cellular or viral process. This is the case for translation inhibitors, which have been widely employed to explore the processes of mRNA translation (Lindqvist and Pelletier, 2009; Vázquez, 1979). More recently, high throughput screening methods have led to the discovery of a number of new translation inhibitors with promising applications in molecular biology (Cencic et al., 2011, 2012). One such molecule is pateamine A (Pat A), a natural marine compound synthesized by the sponge *Mycale* sp. (Hood et al., 2001; Low et al., 2007). Pat A targets eIF4A and enhancing its helicase and ATPase activities disrupts its interaction with eIF4G while promoting the formation of a stable complex between eIF4A and eIF4B (Bordeleau et al., 2005, 2006; Low et al., 2005). This disruption may lead to an inhibition of the interaction of the preinitiation complexes with mRNA (Bordeleau et al., 2006), or to the stalling of initiation complexes at the leader region of mRNA *in vitro* (Low et al., 2005). Thus, translation of capped mRNAs that require the eIF4F complex is blocked. In contrast, hepatitis C virus (HCV) mRNA is not inhibited by Pat A, although other mRNAs bearing picornavirus IRES elements are blocked by this compound (Bordeleau et al., 2006; Low et al., 2005). Additionally, Pat A induces the formation of stress granules (SG) by a pathway independent of eIF2α phosphorylation (Dang et al., 2006). In the present work, we have tested the activity of Pat A on the translation of SINV gmRNA and sgmRNA, both of which contain a cap-structure at the 5′ end. Our results show that protein synthesis directed by sgmRNA is resistant to Pat A inhibition, whereas gmRNA translation is blocked. Moreover, resistance of sgmRNA to Pat A is only observed in SINV-infected cells, but not when this mRNA is translated out of the infection context. This represents the first example of a capped mRNA that is resistant to Pat A.

Results

Early translation of SINV gmRNA. Inhibition of nsP synthesis by Pat A

The first step in the SINV replication cycle after virus entry is the translation of the input gmRNA that has been delivered to the cytoplasm (Hernandez et al., 2014). The schematic representation of gmRNA, sgmRNA and the different constructs used in this work are shown in Fig. 1a. To analyze the action of Pat A on translation, BHK cells were initially infected with SINV for 1 h to allow virus entry. Then, increasing amounts of the inhibitor were added and cells were incubated for one additional hour. Synthesis of nsP1and nsP2 was analyzed by immunoblotting using specific polyclonal antibodies. Used at a concentration of 100 nM, Pat A markedly inhibited the synthesis of nsP1 and nsP2 (Fig. 1b and c). Next, translation of gmRNA was assayed by transfection of a non-replicative RNA lacking most of the coding region of nsP4 and bearing the luciferase gene embedded within the nsP3 sequence (see SV-Luc ΔnsP4 scheme in Fig. 1a). Synthesis of luciferase directed by this mRNA was strongly inhibited by Pat A in transfected BHK cells (Fig. 1d). The extent of inhibition was similar to that observed with a control cap-Luc mRNA, whereas synthesis of luciferase directed by CrPV IGR-Luc mRNA was moderately stimulated by Pat A. The cap-Luc contains the cellular leader sequence of luciferase mRNA, while CrPV IGR IRES has the intergenic region (IGR) from cricket paralysis virus (CrPV) genome that confers translatability in the absence of any eIFs (Jan and Sarnow, 2002). This finding indicated that Pat A has no effect on the elongation or termination steps of translation and is consistent with the idea that Pat A is a selective inhibitor of eIF4A. Therefore, SINV gmRNA requires this initiation factor for its translation early during infection.

To further analyze the synthesis of nsPs and to test the formation of SG by Pat A, BHK cells were treated with Pat A or sodium arsenite, an inducer of oxidative stress, and immunocytochemistry was used to analyze SG formation. Treatment of control uninfected BHK cells with Pat A (400 nM) resulted in TIA-1 release from the nucleus to the cytoplasm and stimulated formation of SGs at a level similar to that observed with sodium arsenite (Fig. 2). As expected, the synthesis of nsP2 was diminished by Pat A in SINV infected cells, as assessed by reduced staining with an antibody against nsP2 (Fig. 2). The amount of nsP2 observed in presence of 200 μM sodium arsenite may correspond to partial inhibition by this compound. Formation of SG was abrogated in SINV-infected cells at 3 h post infection (hpi), perhaps due to the production of nsP3 before treatment with the inhibitors (Panas et al., 2012).

Previous observations indicated that Pat A blocks eIF4A in an irreversible manner (Bordeleau et al., 2005; Low et al., 2005). Thus, we tested the potential irreversibility of Pat A inhibition directly on protein synthesis in SINV infected cells in order to assess the blockade of other steps of SINV replication, such as the synthesis of late viral proteins. To this end, BHK cells were infected with SINV (10 pfu/cell) and cells were treated from 2 to 3 hpi with 200 nM Pat A. Subsequently, the inhibitor was extensively washed out and cells were replenished with fresh medium and protein synthesis monitored for several hours after washing. As shown in Fig. 3, the application of Pat A in uninfected cells for only 1 h potently blocked cellular mRNA translation even several hours after washing off the inhibitor. On the other hand, Pat A strongly blocked the remaining cellular mRNA translation, and also late viral proteins in SINV-infected BHK cells treated from 2–3 hpi. This blockade extended over the ensuing hours even in the absence of Pat A, demonstrating that this compound exerts an irreversible inhibition of translation.

Translation of SINV sgmRNA to produce late viral proteins. Action of Pat A

Viral RNA replication gives rise to the negative-stranded RNA, which contains two promoters: one located at the 3′-end and one located internally. Viral transcription using this internal promoter on negative RNA generates sgmRNA. Translation of this messenger gives rise to the structural viral proteins, which are synthesized as a large precursor that is proteolytically cleaved to render the mature viral proteins. The initiation of translation of sgmRNA at late stages of infection is carried out by a mechanism that does not require certain eIFs (Garcia-Moreno et al., 2013, 2014; Sanz et al., 2009, 2013). The inhibition of the synthesis of SINV structural proteins was examined by radioactive labeling from 5 to 6 hpi using different concentrations of Pat A. Translation of cellular mRNAs was blocked by 32.5% with 100 nM Pat A and this inhibition increased to 70% with 200 nM Pat A (Fig. 4a and b). At these concentrations, the translation of SINV sgmRNA was only marginally affected and a concentration of 400 nM Pat A was required to provoke a reduction of viral protein synthesis of 50%. However, this inhibition may not have been due solely to the blockade of eIF4A activity, but perhaps also to side-effects of the inhibitor on other cellular functions. Nevertheless, it can be concluded that a concentration of Pat A that reduced cellular protein synthesis by approximately 70% inhibited SINV sgmRNA translation by only ~20%, suggesting that initiation of translation

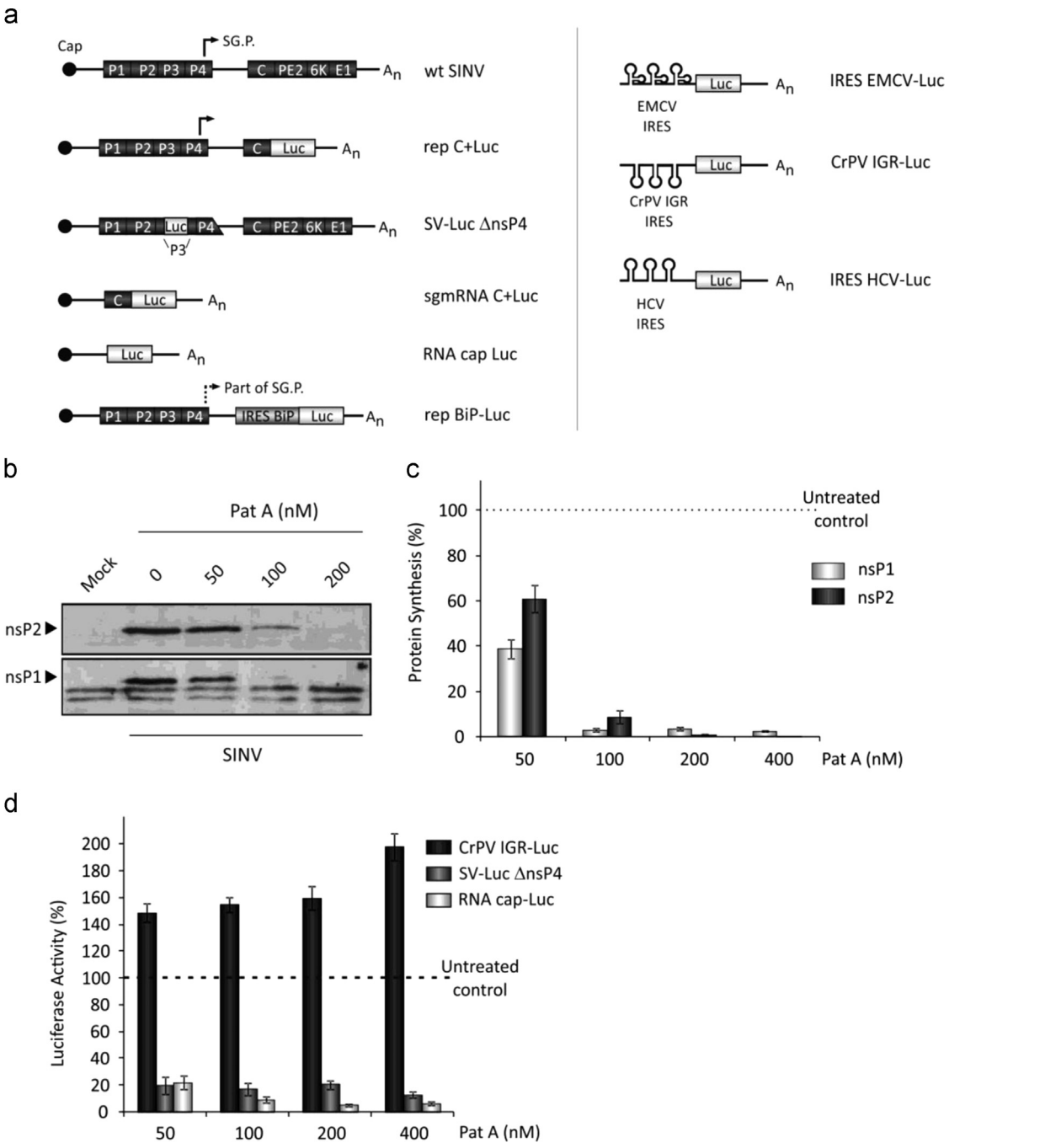

Fig. 1. Translation of SINV gmRNA. Effect of Pat A. **(a)** Schematic representation of the RNAs employed in this work. **(b)** BHK cells were mock-infected or infected with SINV at a multiplicity of infection (MOI) of 10 pfu/cell. At 1 hpi, cells were treated with vehicle or Pat A for 1 h at the indicated concentrations. SINV proteins nsP2 (upper panel) and nsP1 (lower panel) were analyzed by western blot. The results shown are representative of three independent experiments. **(c)** Densitometric values of western blots of the viral proteins nsP1 and nsP2 are expressed as the percentage of untreated samples. The results represent the mean ± SD of three independent experiments. **(d)** *In vitro* synthesized RNAs gmRNA SV-Luc ΔnsP4, RNA cap-Luc and CrPV IGR-Luc were transfected into BHK cells with Lipofectamine 2000. Different concentrations of Pat A (50, 100, 200 and 400 nM) or cycloheximide (100 mg ml^{-1}) were added at 1 hpt and cells were incubated for 1 h before analysis of luciferase activity. Values obtained from cycloheximide-treated cells were used to subtract the amount of luciferase synthesized prior to Pat A addition. Luciferase activity values of Pat A-treated cells are expressed as percentage of untreated samples. The results represent the mean ± SD of three independent experiments.

of this viral messenger does not require eIF4A or intact eIF4F complex at later times of the viral life cycle. Of note, the inhibition of endogenous translation was less efficient when compared with transfected cap-Luc mRNA (Fig. 1c). This result is consistent with the idea that disruption of the eIF4F complex has a greater impact on *de novo* translation of mRNAs as compared to protein synthesis directed by preexisting mRNAs already engaged in the polysome (Novoa and Carrasco, 1999).

As with most alphaviruses, SINV is an arthropod borne virus (arbovirus) that has two natural hosts for its transmission. Thus, aside from vertebrate cells, SINV also infects insect cells, giving rise to a productive infection without apparent inhibition of host protein synthesis. Therefore, we next explored the action of Pat A on translation in mosquito C6/36 cells infected of SINV (10 pfu/cell) and treated with different concentrations of the compound. As a control, the activity of Pat A was also examined in uninfected

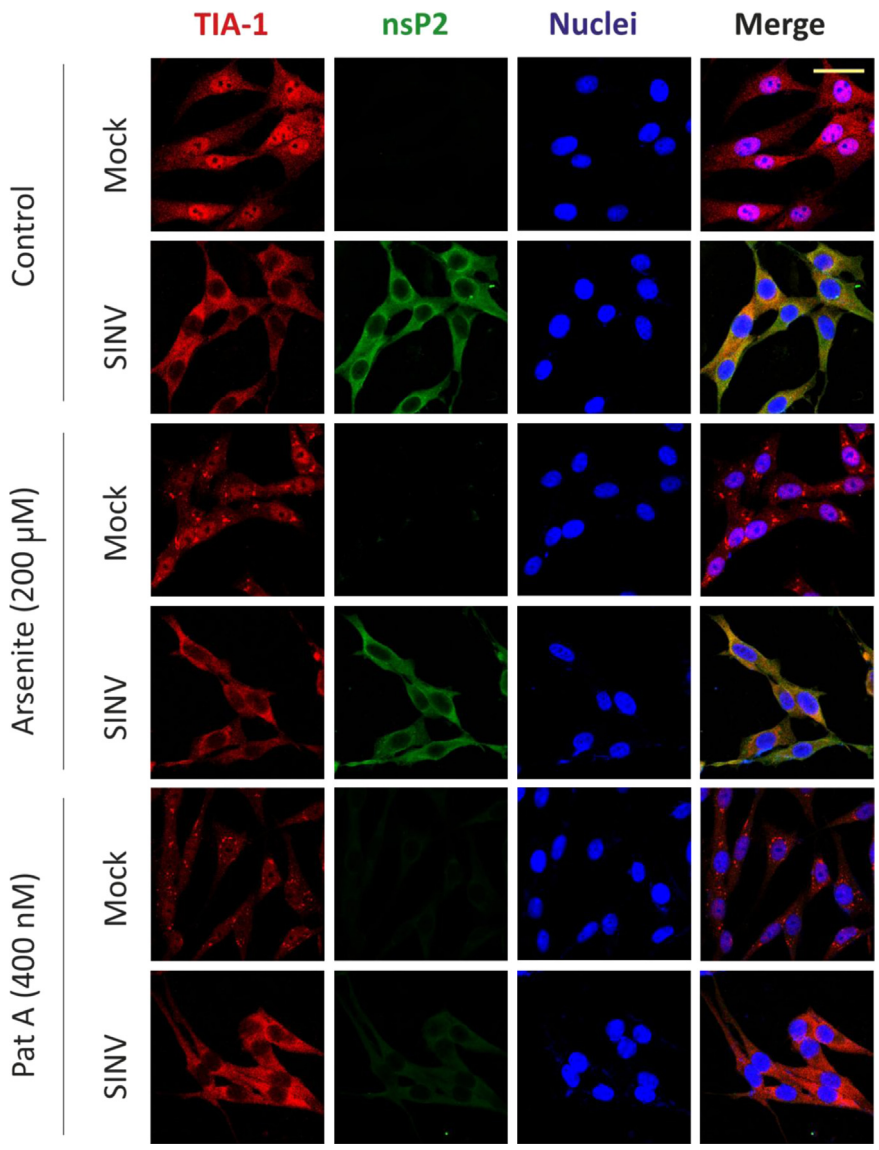

Fig. 2. Pat A-induced formation of stress granules. Blockade by SINV infection. BHK cells seeded on glass coverslips were mock-infected or infected with SINV (MOI of 10 pfu/cell). At 2 hpi, cells were treated or not with Pat A (400 nM) or sodium arsenite (200 μM) for 1 h. At 3 hpi, cells were fixed, permeabilized and processed for immunofluorescence using anti-TIA-1 (red), anti-nsP2 (green) and DAPI (blue). Images were acquired with a confocal microscope and subsequently processed with Zeiss Zen 2010B sp1 and Zen 2008 software (Zeiss). Merged images show the simultaneous visualization of TIA-1, nsP2 and nucleic acids. Scale bar represents 30 μm. The results shown are representative of three independent experiments.

mosquito cells. Analogous to vertebrate cells, protein synthesis was also inhibited by Pat A in insect cells (Fig. S1). In contrast, sgmRNA translation at 7 hpi was more resistant to inhibition by Pat A than cellular protein synthesis, even though the shut-off of host translation does not occur in mosquito cells. Therefore, the translation of sgmRNA appears to be more eIF4A/eIF4F-dependent in insect cells than in vertebrate settings. This result indicates that the resistance to Pat A in infected cells is specific for sgmRNA translation and does not occur with other mRNAs that are translated in the same cell.

It should be possible that Pat A resistance of SINV sgmRNA was due to the fact that it is synthesized in large amounts from SINV replicons and that these newly-synthesized mRNAs are located in specific foci in close proximity to components of the protein synthesizing machinery (Sanz et al., 2009). To test this possibility, the sequence of luciferase gene preceded by a cellular IRES element was cloned in place of SINV sgmRNA (see rep BiP-Luc scheme Fig. 1a). The sgmRNA that is rendered after transfection of rep BiP-Luc bears the IRES from the cellular mRNA that encodes for the chaperone BiP (binding immunoglobulin protein). Translation of this mRNA is independent of eIF2α phosphorylation (Fernandez et al., 2002) and therefore can be translated in BHK cells that replicate SINV RNA. Interestingly, Pat A strongly blocked luciferase synthesis directed by this mRNA, indicating that it requires eIF4A for translation under these conditions (Fig. 5). As a control SINV rep C+luc was tested. In this case, the inhibition of sgmRNA translation by Pat A was lower as compared to rep BiP-Luc. Luciferase synthesis was assayed by measuring its activity (Fig. 5). In conclusion, only protein synthesis directed by SINV sgmRNA was resistant to Pat A, whereas under the same conditions translation driven by a cellular IRES was sensitive to this inhibitor.

Pat A inhibits sgmRNA translation out of the viral infection context

We next questioned whether the resistance of sgmRNA translation to Pat A was an intrinsic property of the RNA structure or whether such a resistance was due to conditions existing in cells

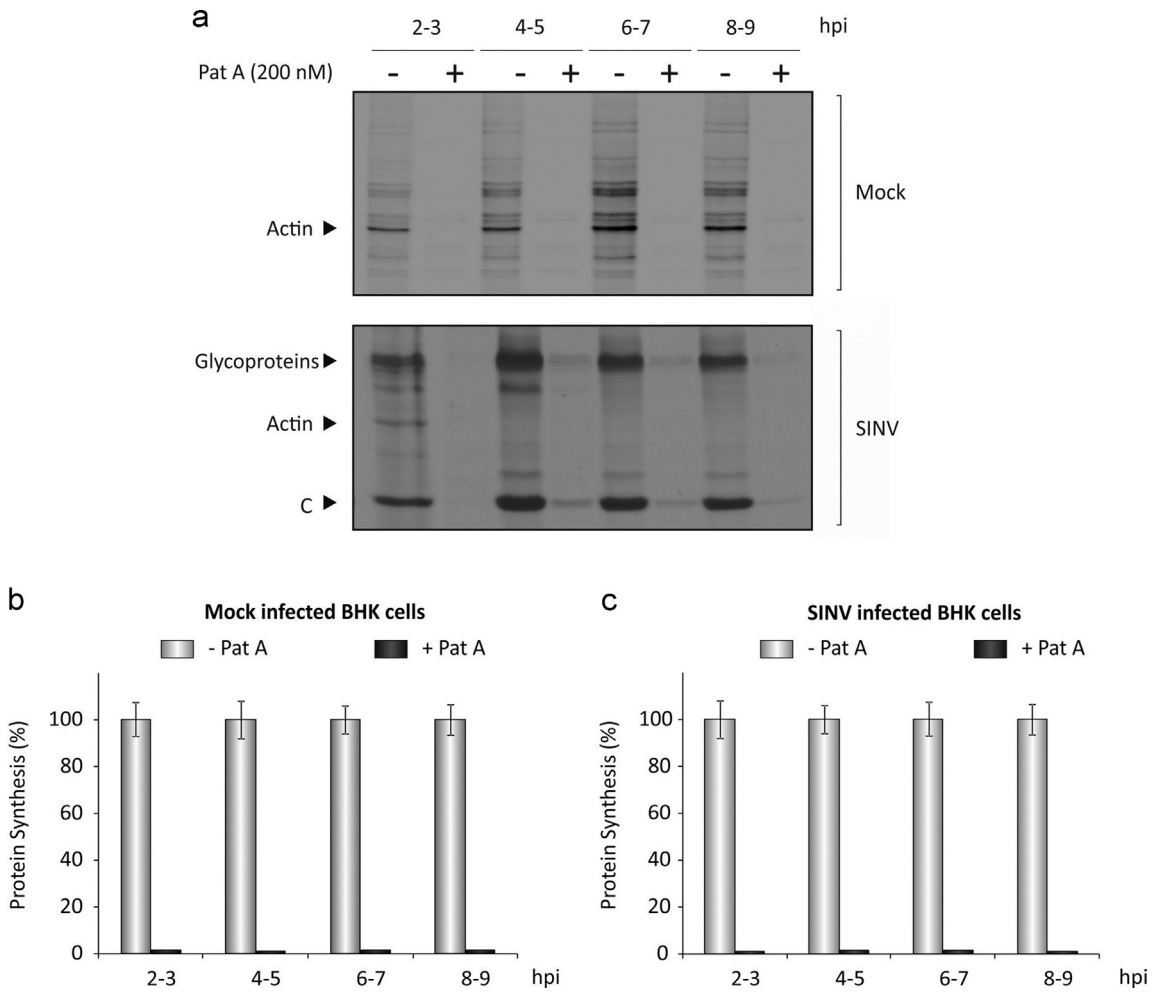

Fig. 3. Pat A blocks protein synthesis in an irreversible manner. **(a)** BHK cells were infected with SINV at a MOI of 10 pfu/cell for 1 h. Cells were then treated or not with Pat A (200 nM) from 1 to 2 hpi. Subsequently, Pat A was washed out and replaced by fresh medium. At the indicated times, cells were labeled with [^{35}S]Met−Cys for 1 h. Radiolabeled proteins were separated by SDS-PAGE, followed by autoradiography. The results shown are representative of three independent experiments. **(b)** The percentage of cellular (actin) and **(c)** viral C protein synthesis in cells treated or not with Pat A (200 nM) were calculated from values obtained by densitometric scanning of the corresponding bands. The results are mean ± SD of three independent experiments.

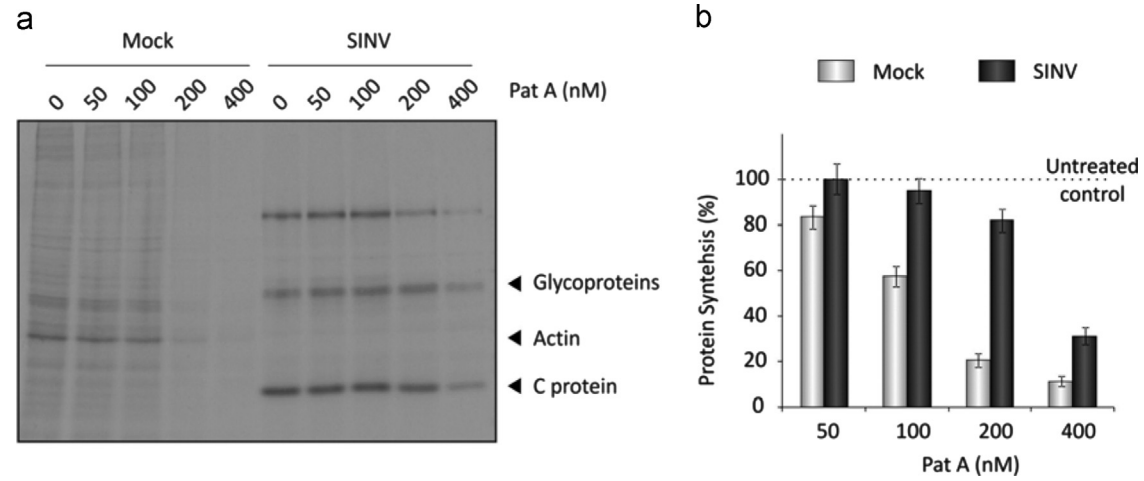

Fig. 4. Action of Pat A on the translation of SINV sgmRNA. **(a)** BHK cells were either mock-infected or infected with SINV (MOI of 10 pfu/cell). From 5 to 6 hpi, cells were treated or not with the indicated concentrations of Pat A while they were labeled with [^{35}S]Met−Cys. Radiolabeled proteins were separated by SDS-PAGE, followed by autoradiography. The results shown are representative of three independent experiments. **(b)** Values of cellular and viral protein synthesis were obtained by densitometric scanning of the radioactive signal and are expressed as the percentage of untreated samples. The results represent the mean ± SD of three independent experiments.

replicating SINV RNA. Initially, different mRNAs, including sgmRNA, were transfected into BHK cells and luciferase synthesis was measured after addition of increasing concentrations of Pat A. Cycloheximide was added at the same time and served to establish the amount of luciferase synthesized before the addition of Pat A, and this was subtracted from these samples. As positive controls of

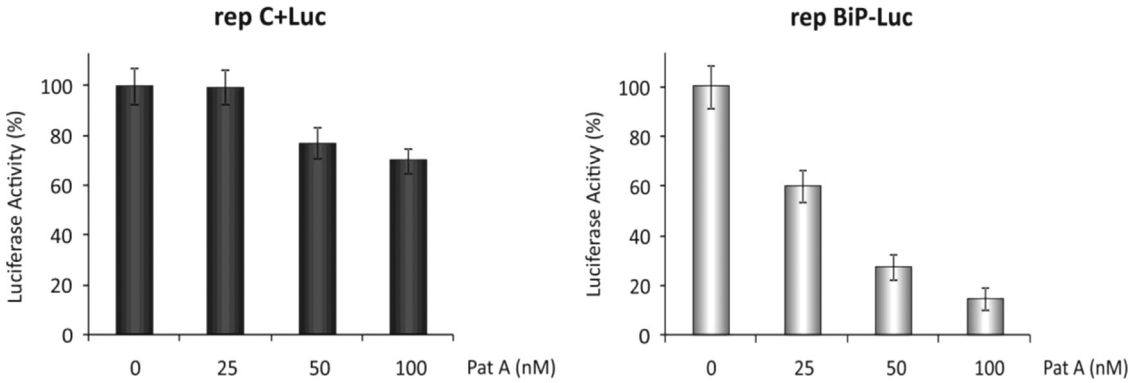

Fig. 5. Luciferase synthesis from rep C+Luc and rep BiP-Luc. Action of Pat A. BHK cells were transfected with *in vitro* synthesized RNAs rep C+Luc and rep BiP-Luc with Lipofectamine 2000. Different concentrations of Pat A (25, 50 and 100 nM) or cycloheximide (100 μg ml^{-1}) were added at 5 hpt and cells were incubated for 2 h before analysis of luciferase activity. Values obtained from cycloheximide-treated cells were used to subtract the amount of luciferase synthesized prior to Pat A addition. Luciferase activity values of Pat A-treated cells are expressed as percentage of untreated samples. The RLU values obtained once the luciferase obtained in presence of cycloheximide was subtracted were rep C+Luc: 4864404 ± 359965.9; rep BiP+Luc: 1591818 ± 135304.5. These luciferase values were taken as 100% of control. The results represent the mean ± SD of three independent experiments.

mRNAs that utilize eIF4A, we employed the eIF4A-dependent cap-Luc and IRES EMC-Luc mRNAs. CrPV IGR-Luc and IRES HCV-Luc mRNAs served as negative controls as these are not dependent on eIF4A for initiation. As shown in Fig. 6a, SINV sgmRNA was blocked by Pat A in transfected BHK cells to an extent similar to that observed with cap-Luc mRNA, while CrPV IGR-Luc and IRES HCV-Luc mRNAs were in fact stimulated by Pat A. This stimulation was presumably due to the fact that the inhibition of cellular mRNA translation avoids the competition with translation driven by CrPV or HCV IRES. In contrast, IRES EMC-Luc mRNA was partially inhibited by Pat A, in agreement with *in vitro* results (Bordeleau et al., 2005; Low et al., 2005). Further evidence that Pat A could inhibit sgmRNA translation out of the context of active infection was obtained through *in vitro* translation assays using rabbit reticulocyte lysates (RRL). Increasing concentrations of Pat A inhibited *in vitro* translation directed by sgmRNA C+Luc as well as by cap-Luc or IRES EMC-Luc mRNAs (Fig. 6b). Interestingly, the optimal translation of sgmRNA C+Luc required high concentrations of KCl (Fig. 6c), as occurs with a variety of viral mRNAs which are translated late during infection (Koch et al., 1980). Moreover, the inhibition of sgmRNA C+Luc translation by Pat A was higher at the optimal concentration of KCl (Fig. 6c). Collectively, these findings indicate that the structure of sgmRNA is not responsible for its resistance to inhibition by Pat A in infected cells; instead, this viral messenger requires eIF4A for initiation of protein synthesis in uninfected cells.

Determination of the stage during SINV infection when translation becomes resistant to eIF4A

To further assess the activity of Pat A on sgmRNA translation in BHK cells that replicate SINV RNA, we made use of rep C+Luc (see scheme, Fig. 1a). This SINV replicon was firstly synthesized by *in vitro* transcription from the corresponding plasmid. After transfection of rep C+Luc, C protein was analyzed at two distinct time points in order to assess viral C production early during the late phase or at later times. The activity of Pat A on the production of C protein from 3 to 5 or from 6 to 8 h post transfection (hpt) was estimated by western blotting using specific rabbit polyclonal antibodies (Fig. S2). As a control, we measured in parallel the amount of protein C synthesized prior to Pat A treatment by adding cycloheximide. Surprisingly, 100 nM Pat A was strongly inhibitory (91%) for C production at early times of the late phase, whereas this inhibition was lower (20%) as replication progressed (Fig. S2). These findings suggested that sgmRNA translation requires eIF4A at early times and becomes less dependent on this factor at later infection times.

Additionally, the above results indicated that gmRNA translation was sensitive to inhibition by Pat A very early during SINV infection (Fig. 1), whereas at late times sgmRNA translation was more resistant to the inhibition of eIF4A (Fig. 4). This behavior of sgmRNA to Pat A only occurs in SINV-infected cells or in cells replicating SINV RNA (Fig. S2), suggesting that when viral infection progresses there is a switch from a mechanism of initiation of protein synthesis dependent on eIF4A to a mode of translation that is less dependent of this factor. To determine more accurately when this switch takes place, SINV-infected cells were treated with 200 nM Pat A at different hpi and protein synthesis was analyzed by radioactive labeling followed by SDS PAGE and fluorography. During the initial hours of infection, cellular translation was potently blocked by Pat A (Fig. 7a and b). Conversely, the synthesis of viral C protein was observed from 2 hpi and was concomitant with the increased shut-off of cellular protein synthesis. Notably, from 2–3 and 3–4 hpi, the synthesis of C protein was drastically reduced by Pat A, whereas from 4 hpi sgmRNA translation became more resistant to the inhibitor (Fig. 7a–c). Therefore, at early periods during the late phase sgmRNA translation was sensitive to Pat A, suggesting that a change occurs after that time which confers less dependency on eIF4A for the initiation of translation of sgmRNA in infected cells (Fig. 7c). Thus, Pat A constitutes a good tool for future studies to investigate the molecular nature of this switch. Further support to the concept that sgmRNA translation is more dependent on eIF4A at early times of the late phase was obtained by hippuristanol, the other selective inhibitor of eIF4A (Garcia-Moreno et al., 2013). Indeed, analysis of the inhibition of sgmRNA translation at different times p.i. by hippuristanol clearly indicates that this inhibition varies and becomes more resistant as infection progresses (Fig. S3).

Discussion

From the perspective of therapeutic agents and molecular tools, marine organisms are providing a very interesting number of natural compounds for investigation (Singh and Pelaez, 2008; Stonik and Fedorov, 2014; Vera and Joullié, 2002). Since the discovery of didemnins, the first marine natural products administered to humans (Lee et al., 2012), the number of new inhibitors of cellular functions from marine sources continues to rise (Skropeta and Wei, 2014). This is the case for hippuristanol and Pat A, two natural compounds produced by invertebrate marine organisms (Lindqvist and Pelletier, 2009). These

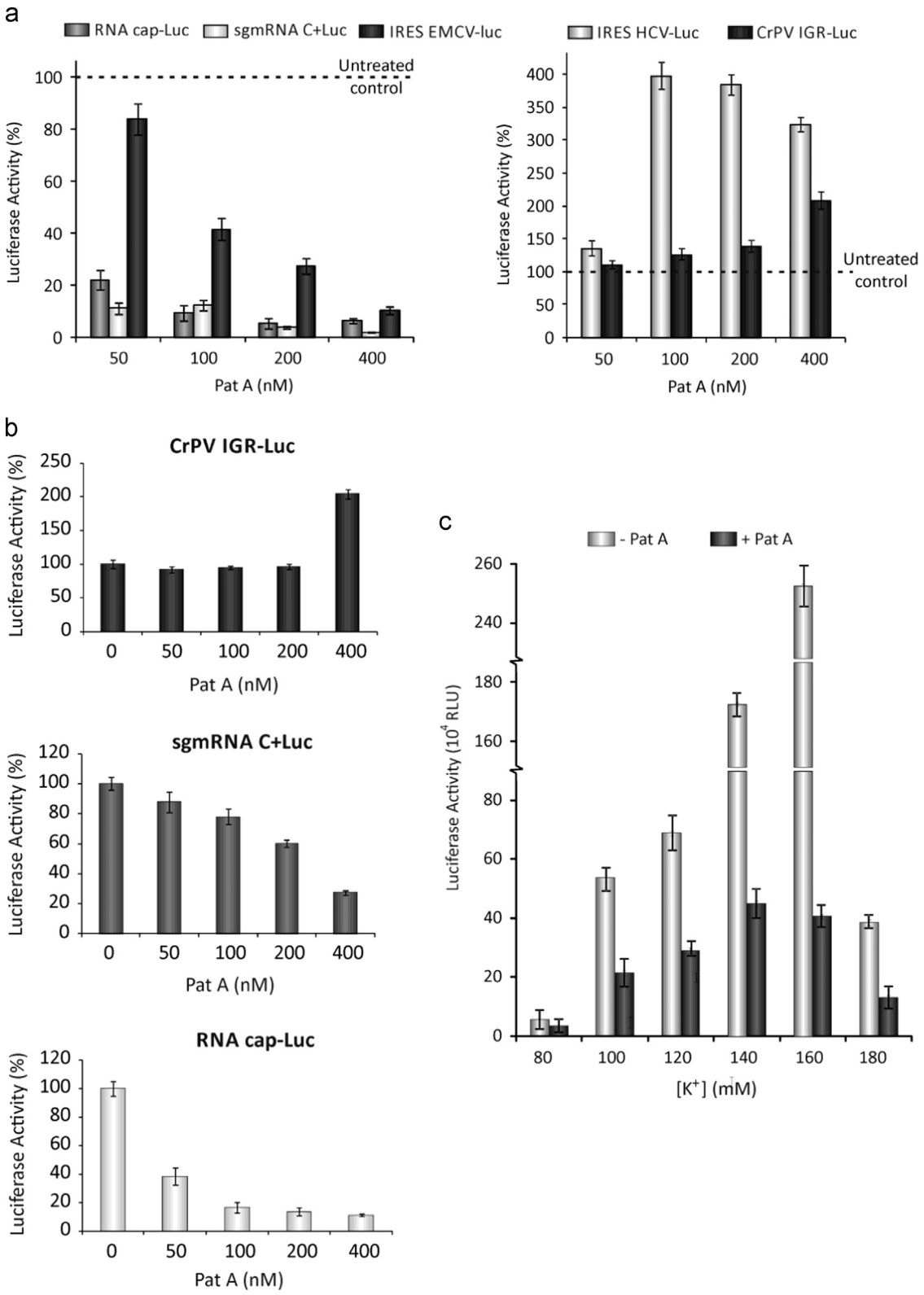

Fig. 6. Translation of sgmRNA outside the viral infection context. Activity of Pat A. **(a)** *In vitro* synthesized sgmRNA C+Luc and control RNAs eIF4A-dependent (RNA cap-Luc and IRES EMCV-Luc (left panel)) and eIF4A-independent (IRES HCV-Luc and CrPV IGR-Luc (right panel)) were transfected into BHK cells with Lipofectamine 2000. Cells were then incubated with different concentrations of Pat A (50, 100, 200 and 400 nM) or cycloheximide (100 μg ml^{-1}) for 1 h before analysis of luciferase activity. Values obtained from cycloheximide-treated cells were used to subtract the amount of luciferase synthesized prior to Pat A addition. Luciferase activity values of Pat A-treated cells are expressed as percentage of untreated samples. The results are mean ± SD of three independent experiments. **(b)** CrPV IGR-Luc (upper panel), sgmRNA C+Luc (medium panel) and RNA cap-Luc (lower panel) were generated by *in vitro* transcription using T7 RNA polymerase and then, *in vitro* translated using nuclease-treated rabbit reticulocyte lysate (RRL), programmed with 200 ng of the different mRNAs. Luciferase activity values of Pat A-treated cells are expressed as percentage of untreated samples. The results are mean ± SD of three independent experiments. **(c)** Effect of potassium [K^+] on *in vitro* translation of sgmRNA C+Luc and its inhibition by Pat A. *In vitro* transcribed sgmRNA C+Luc (200 ng) was translated in RRL at different concentrations of [K^+] (80, 100, 120, 140, 160 and 180 mM) and treated or not with 200 nM of Pat A. Luciferase activity results are mean ± SD of three independent experiments. The percentage values of Pat A-treated cells relative to their respective untreated cells are indicated in the figure.

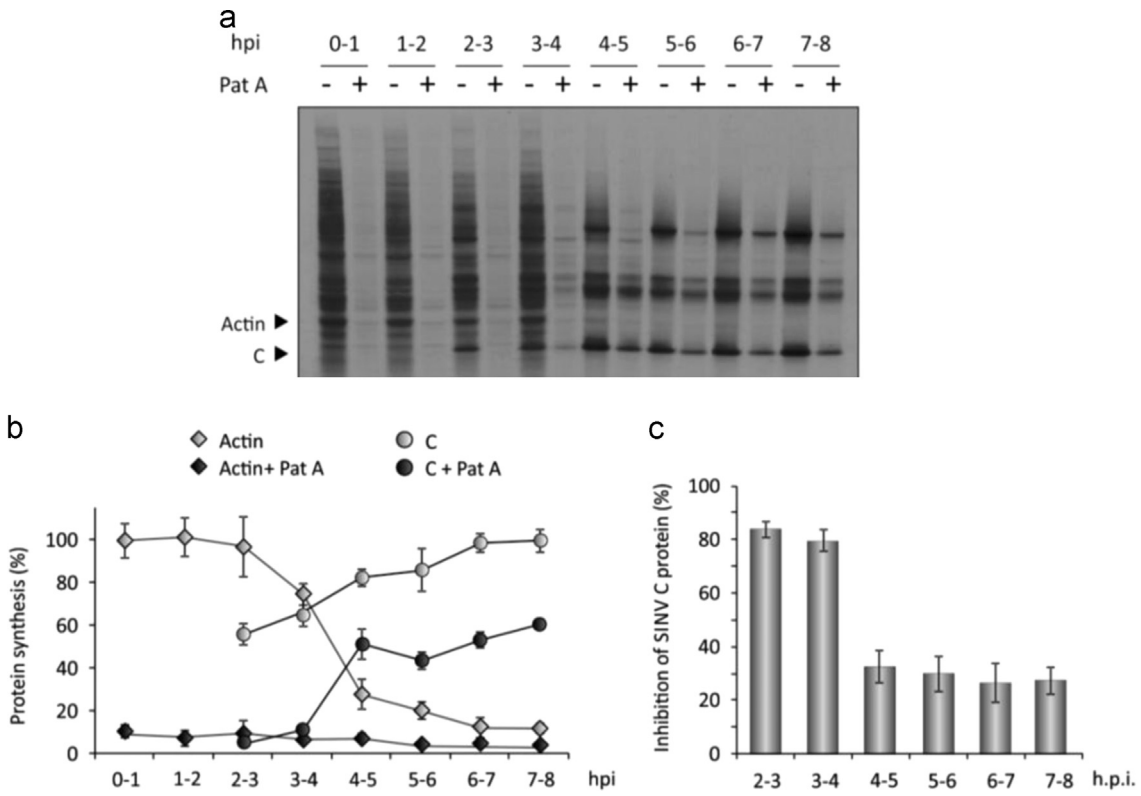

Fig. 7. Time-course of Pat A resistance of sgmRNA translation. **(a)** BHK cells were infected with SINV (MOI of 10 pfu/cell). At the indicated times post infection, cells were treated with vehicle or Pat A for 1 h at a concentration of 200 nM while they were labeled with [^{35}S]Met – Cys. Radiolabeled proteins were separated by SDS-PAGE, followed by autoradiography. The results shown are representative of three independent experiments. **(b)** The percentage of cellular (actin) and viral C protein synthesis in cells treated or not with Pat A (200 nM) were calculated from values obtained by densitometric scanning of the corresponding bands. The results are mean ± SD of three independent experiments. **(c)** Inhibition of Pat A on viral C protein is represented as the percentage of corresponding untreated samples. These results represent the mean ± SD of three independent experiments.

agents selectively inhibit the initiation of protein synthesis by targeting eIF4A, thus blocking the activity of the eIF4F complex. These inhibitors can be useful to ascertain the participation of eIF4A in the translation of some cellular and viral mRNAs. As reported in this work, Pat A strongly blocks the synthesis of SINV nsPs when used at early times post infection. This inhibition of viral translation is irreversible such that treatment for only 1 h is sufficient to block the synthesis of viral proteins at late times, as well as the inhibition of cellular translation. The arrest of late viral protein synthesis is most likely due to the inhibition of viral RNA replication and transcription that is accomplished by nsPs. Therefore, we can conclude that SINV gmRNA requires eIF4A for translation and the blockade of nsP synthesis abrogates the production of sgmRNA. The possibility that Pat A may affect other steps different to initiation is not supported by the finding that translation driven by the CrPV IRES is not only resistant, but is actually stimulated by Pat A, indicating that the elongation or termination processes of mRNA translation are not affected by this compound. The stimulation of CrPV IRES translation by Pat A may be a consequence of the inhibition of cellular protein synthesis and the concomitant release from mRNA competition. However, it is formally possible that Pat A affects other cellular functions, such as the redistribution of nuclear proteins, and that these reactions may affect sgmRNA translation at high concentrations of Pat A. The potential repercussions of these alterations for protein synthesis remains to be investigated, but we believe that the most important activity of Pat A on mRNA translation is its selective interaction with eIF4A, leading to the disruption of the eIF4F complex (Bordeleau et al., 2005; Low et al., 2005).

The results obtained with Pat A on the translation of SINV sgmRNA reinforce the view that eIF4A and the eIF4F complex are dispensable in infected cells (Castelló et al., 2006; Garcia-Moreno et al., 2013; Sanz et al., 2009). Thus, at concentrations of 200 nM Pat A there is a profound inhibition of cellular translation, while sgmRNA is only slightly blocked. Curiously, as observed with other translation inhibitors, protein synthesis directed by sgmRNA is negatively affected out of the replication complex (Garcia-Moreno et al., 2013). Therefore, our present results with Pat A are consistent with the concept that this messenger exhibits a dual mechanism for its translation and consequently the structure of sgmRNA does not confer independence for several eIFs (Sanz et al., 2009). This has been also clearly established for the requirement of eIF2. In this case, there is a stem-loop structure downstream of the AUG initiation codon (DLP) that confers eIF2 independence, but only in infected cells (Garcia-Moreno et al., 2013, 2014; McInerney et al., 2005; Ventoso et al., 2006). However, this DLP structure is not involved in providing independence for eIF4A (Garcia-Moreno et al., 2013). The structural requirements necessary for eIF4A-independent translation of sgmRNA remain to be investigated.

SINV infected cells undergo a drastic modification during infection, from a Pat A-sensitive status at early times to a more resistant status during the late phase of the viral cycle. Our kinetic analyses indicate that this change occurs at about 4 hpi. From this time onwards, translation of sgmRNA becomes independent of several eIFs, including the eIF4F complex. Future studies will be needed to determine the precise modifications that take place in SINV-infected cells to alter the mechanism of initiation of translation.

Methods

Cell lines and viruses

The cell lines used in this work were Baby hamster kidney (BHK-21) cells and *Aedes albopictus* C6/36 cells, both obtained from

ATCC. BHK-21 cells were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). C6/36 cells were cultured at 28 °C without CO_2 in M3 medium supplemented with 10% FCS. SINV derived from the pT7SVwt infective clone was used (Sanz and Carrasco, 2001). Viral infections of BHK-21 cells were carried out in DMEM without serum for 1 h at 37 °C, whereas infections of C6/36 cells were performed at 28 °C. Subsequently, medium was removed and infection was continued in DMEM with 5% FCS or M3 medium with 10% FCS, respectively, at the temperatures indicated. Infections with SINV were carried out at a multiplicity of 10 pfu per cell.

Plasmids and recombinant DNA procedures

The plasmids employed in this work are listed and described in Table S1. Plasmids were used as DNA templates for *in vitro* transcription with T7 or Sp6 RNA polymerases. pT7 SVwt (Sanz and Carrasco, 2001) was used as the parental plasmid for all of the constructs. The luciferase gene was derived from the plasmid pKS-Luc (Sanz et al., 2007).

Plasmid rep Bip-Luc was prepared with a product obtained after two consecutive PCRs between HpaI and SphI sites in rep C+luc. In the first PCR, oligonucleotides 5′ HpaI (5′-GCTATGGCGTTAACCGGTCTG-3′) and 3′ Nexo SV-Bip (5′-GGCCGGCGTCGACCTGCTGACTATTTAGG-3′) were used plus rep C+luc as DNA template. The other PCR product was obtained using 5′ Nexo SV-Bip (5′-CCTAAATAGTCAGCAGGTC-GACGCCGGCC-3′) and 3′ Luc SphI (5′-CCCGGGGCATGCGAGAATCT-GACGCAG-3′) oligonucleotides plus pBS-BIP-IRES-FFL-pA as DNA template, kindly provided from Dr. M. Hentze (EMBL Heidelberg, Germany). Oligonucleotides 5′ HpaI and 3′ Luc SphI with a mixture of the above products as DNA template were employed in the next PCR.

Antibodies

Rabbit polyclonal antibodies raised against SINV C protein and rat polyclonal antibodies raised against bacterially produced nsP1 were produced in our laboratory. Rabbit polyclonal anti-nsP2 was a kind gift from Dr. Richard W Hardy (Indiana University, USA). Goat polyclonal anti-TIA-1 was purchased from Invitrogen and Santa Cruz Biotechnology. Anti-rabbit and anti-rat immunoglobulin G antibodies coupled to peroxidase were purchased from Amersham. Specific antibodies conjugated to Alexa 488 or Alexa 555 (A-21202 and A-21432 respectively) were obtained from Invitrogen.

Inhibitors

The following chemical inhibitors were used: pateamine A was purified as previously described (Bordeleau et al., 2005), hippuristanol (Bordeleau et al., 2006), sodium arsenite (Riedel-de Haën) and cycloheximide (Sigma).

In vitro RNA transcription and translation

Linearized plasmids were used as templates for *in vitro* RNA transcription using T7 or SP6 RNA polymerases (New England Biolabs), as previously described (Garcia-Moreno et al., 2013). *In vitro* translation was carried out in nuclease-treated rabbit reticulocyte lysate (RRL) (Promega). One hundred nanograms of *in vitro* transcribed mRNAs were added to the translation mixture. Protein synthesis was estimated by measuring luciferase activity.

RNA transfection

In vitro transcribed RNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the supplier's recommendations.

Measurement of luciferase activity

Luciferase activity was determined as described by Sanz et al. (2014).

Analysis of protein synthesis and western blotting

Protein synthesis was analyzed at the indicated times by replacing growth media for 1 h with 0.2 ml of methionine/cysteine-free DMEM supplemented with 1 μl of EasyTagTM EXPRESS ^{35}S Protein Labeling, [^{35}S]Met−Cys (11 mCi ml^{-1}, Perkin Elmer) per well of an L-24 plate. Labeling medium also included inhibitors when the action of these compounds was assayed. Radioactive proteins and samples for western blotting were analyzed as described (Garcia-Moreno et al., 2013). Protein synthesis was quantified by densitometry using a GS-800 Calibrated Densitometer (Bio-Rad).

Immunofluorescence assays

Fixation, permeabilization and confocal microscopy were performed as described (Madan et al., 2008) using a confocal laser scanning and multiphoton microscope LSM 710 coupled to an inverted microscope (Axio Observer, Zeiss). Primary antibodies used were: rabbit polyclonal anti SINV nsP2, and goat polyclonal anti-TIA-1 at a 1:500 dilution. Specific antibodies conjugated to Alexa 488 and Alexa 555 were employed as secondary antibodies at a 1:1000 dilution. DAPI (4′-6-diamidino-2-phenylindole) was used to stain the nuclei.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.05.002.

References

Au, H.H., Jan, E., 2014. Novel viral translation strategies. Wiley Interdiscip. Rev. RNA 5, 779–801.
Bordeleau, M.-E., Matthews, J., Wojnar, J.M., Lindqvist, L., Novac, O., Jankowsky, E., Sonenberg, N., Northcote, P., Teesdale-Spittle, P., Pelletier, J., 2005. Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation. Proc. Natl. Acad. Sci. USA 102, 10460–10465.
Bordeleau, M.-E., Cencic, R., Lindqvist, L., Oberer, M., Northcote, P., Wagner, G., Pelletier, J., 2006. RNA-mediated sequestration of the RNA helicase eIF4A by Pateamine A inhibits translation initiation. Chem. Biol. 13, 1287–1295.
Brown, D.T., Hernandez, R., 2012. Infection of cells by alphaviruses. Adv. Exp. Med. Biol. 726, 181–199.
Castelló, A., Sanz, M.A., Molina, S., Carrasco, L., 2006. Translation of Sindbis virus 26S mRNA does not require intact eukariotic initiation factor 4G. J. Mol. Biol. 355, 942–956.
Cencic, R., Hall, D.R., Robert, F., Du, Y., Min, J., Li, L., Qui, M., Lewis, I., Kurtkaya, S., Dingledine, R., et al., 2011. Reversing chemoresistance by small molecule inhibition of the translation initiation complex eIF4F. Proc. Natl. Acad. Sci. USA 108, 1046–1051.

Cencic, R., Galicia-Vázquez, G., Pelletier, J., 2012. Chapter twenty – inhibitors of translation targeting eukaryotic translation initiation factor 4A. In: Eckhard Jankowsky (Ed.), Methods in EnzymologyAcademic Press, pp. 437–461.
Chamond, N., Deforges, J., Ulryck, N., Sargueil, B., 2014. 40S recruitment in the absence of eIF4G/4A by EMCV IRES refines the model for translation initiation on the archetype of Type II IRESs. Nucleic Acids Res. 42, 10373–10384.
Dang, Y., Kedersha, N., Low, W.-K., Romo, D., Gorospe, M., Kaufman, R., Anderson, P., Liu, J.O., 2006. Eukaryotic initiation factor 2alpha-independent pathway of stress granule induction by the natural product pateamine A. J. Biol. Chem. 281, 32870–32878.
Fernandez, J., Yaman, I., Merrick, W.C., Koromilas, A., Wek, R.C., Sood, R., Hensold, J., Hatzoglou, M., 2002. Regulation of internal ribosome entry site-mediated translation by eukaryotic initiation factor-2alpha phosphorylation and translation of a small upstream open reading frame. J. Biol. Chem. 277, 2050–2058.
Garcia-Moreno, M., Sanz, M.A., Pelletier, J., Carrasco, L., 2013. Requirements for eIF4A and eIF2 during translation of Sindbis virus subgenomic mRNA in vertebrate and invertebrate host cells. Cell. Microbiol. 15, 823–840.
Garcia-Moreno, M., Sanz, M.A., Carrasco, L., 2014. Initiation codon selection is accomplished by a scanning mechanism without crucial initiation factors in Sindbis virus subgenomic mRNA. RNA 21, 93–112.
Gingras, A.C., Raught, B., Sonenberg, N., 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68, 913–963.
Hernandez, R., Brown, D.T., Paredes, A., 2014. Structural differences observed in arboviruses of the alphavirus and flavivirus genera. Adv. Virol. *2014*, 259382.
Hood, K.A., West, L.M., Northcote, P.T., Berridge, M.V., Miller, J.H., 2001. Induction of apoptosis by the marine sponge (Mycale) metabolites, mycalamide A and pateamine. Apoptosis Int. J. Program. Cell Death 6, 207–219.
Jan, E., Sarnow, P., 2002. Factorless ribosome assembly on the internal ribosome entry site of cricket paralysis virus. J. Mol. Biol. 324, 889–902.
Koch, G., Bilello, J.A., Kruppa, J., Koch, F., Oppermann, H., 1980. Amplification of translational control by membrane-mediated events: a pleiotropic effect on cellular and viral gene expression. Annu. N. Y. Acad. Sci. 339, 280–306.
Komar, A.A., Mazumder, B., Merrick, W.C., 2012. A new framework for understanding IRES-mediated translation. Gene 502, 75–86.
Kozak, M., 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266, 19867–19870.
Lee, J., Currano, J.N., Carroll, P.J., Joullié, M.M., 2012. Didemnins, tamandarins and related natural products. Nat. Prod. Rep. 29, 404–424.
Lindqvist, L., Pelletier, J., 2009. Inhibitors of translation initiation as cancer therapeutics. Future Med. Chem. 1, 1709–1722.
Low, W.-K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N.S., Merrick, W.C., Romo, D., Liu, J.O., 2005. Inhibition of eukaryotic translation initiation by the marine natural product pateamine A. Mol. Cell 20, 709–722.
Low, W.-K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N.S., Rzasa, R.M., Shea, H.A., Li, S., Park, K., Ma, G., et al., 2007. Isolation and identification of eukaryotic initiation factor 4A as a molecular target for the marine natural product Pateamine A. Methods Enzymol. 431, 303–324.
Madan, V., Castelló, A., Carrasco, L., 2008. Viroporins from RNA viruses induce caspase-dependent apoptosis. Cell. Microbiol. 10, 437–451.
McInerney, G.M., Kedersha, N.L., Kaufman, R.J., Anderson, P., Liljeström, P., 2005. Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. Mol. Biol. Cell 16, 3753–3763.
Niepmann, M., 2009. Internal translation initiation of picornaviruses and hepatitis C virus. Biochim. Biophys. Acta 1789, 529–541.
Novoa, I., Carrasco, L., 1999. Cleavage of eukaryotic translation initiation factor 4G by exogenously added hybrid proteins containing poliovirus 2Apro in HeLa cells: effects on gene expression. Mol. Cell. Biol. 19, 2445–2454.
Panas, M.D., Varjak, M., Lulla, A., Eng, K.E., Merits, A., Karlsson Hedestam, G.B., McInerney, G.M., 2012. Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. Mol. Biol. Cell 23, 4701–4712.
Parsyan, A., Svitkin, Y., Shahbazian, D., Gkogkas, C., Lasko, P., Merrick, W.C., Sonenberg, N., 2011. mRNA helicases: the tacticians of translational control. Nat. Rev. Mol. Cell Biol. 12, 235–245.
Sanz, M.A., Carrasco, L., 2001. Sindbis virus variant with a deletion in the 6K gene shows defects in glycoprotein processing and trafficking: lack of complementation by a wild-type 6K gene in *trans*. J. Virol. 75, 7778–7784.
Sanz, M.A., Castelló, A., Carrasco, L., 2007. Viral translation is coupled to transcription in Sindbis virus-infected cells. J. Virol. 81, 7061–7068.
Sanz, M.A., Castelló, A., Ventoso, I., Berlanga, J.J., Carrasco, L., 2009. Dual mechanism for the translation of subgenomic mRNA from Sindbis virus in infected and uninfected cells. PloS One 4, e4772.
Sanz, M.A., Redondo, N., García-Moreno, M., Carrasco, L., 2013. Phosphorylation of eIF2α is responsible for the failure of the picornavirus internal ribosome entry site to direct translation from Sindbis virus replicons. J. Gen. Virol. 94, 796–806.
Sanz, M.A., García-Moreno, M., and Carrasco, L. (2014). Inhibition of host protein synthesis by Sindbis virus: correlation with viral RNA replication and release of nuclear proteins to the cytoplasm. Cell. Microbiol.
Schlesinger, M.J., Schlesinger, S., 1996. Togaviridae and their replication. Field's Virol. Ed BN Fields Al pp, 825–843.
Singh, S.B., Pelaez, F., 2008. Biodiversity, chemical diversity and drug discovery. Prog. Drug Res. Fortschritte Arzneimittelforschung Prog. Rech. Pharm 65 (141), 143–174.
Skropeta, D., Wei, L., 2014. Recent advances in deep-sea natural products. Nat. Prod. Rep. 31, 999–1025.
Sonenberg, N., Hinnebusch, A.G., 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136, 731–745.
Stonik, V.A., Fedorov, S.N., 2014. Marine low molecular weight natural products as potential cancer preventive compounds. Mar. Drugs 12, 636–671.
Strauss, J.H., Strauss, E.G., 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. 58, 491–562.
Vázquez D., 1979. Inhibitors of protein biosynthesis (Molecular Biology Biochemistry and Biophysics).
Ventoso, I., Sanz, M.A., Molina, S., Berlanga, J.J., Carrasco, L., Esteban, M., 2006. Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. Genes Dev. 20, 87–100.
Vera, M.D., Joullié, M.M., 2002. Natural products as probes of cell biology: 20 years of didemnin research. Med. Res. Rev. 22, 102–145.