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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