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The knockdown of eIF4AI interferes with the respiratory syncytial virus replication cycle

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Summary. – The respiratory syncytial virus (RSV) is one of the main etiological agents in acute respiratory infections. To date, the replicative cycle of this virus is not completely known, and the events as well as the role of cellular and viral proteins that participate in the infectious cycle of RSV are still a matter of intense research. An important protein that is a control point for many viruses is the helicase eIF4AI, which participates at the beginning of the cap-dependent translation of eukaryotes and cap-independent translation of certain viral mRNAs. Recently, eIF4AI has been considered as a potential viral therapeutic target. In order to understand the role of eIF4AI during the infectious cycle of RSV, we evaluated the effect of eIF4AI knockdown on the amount of positive-strand viral RNA and viral progeny of this virus. Our results showed a decrease for both parameters, suggesting a possible involvement of eIF4AI during replicative cycle of RSV. In addition, using confocal microscopy, it was observed that eIF4AI colocalized with RSV viral protein, supporting the possible participation of eIF4AI during the replicative cycle of RSV.

Keywords: eIF4AI; RSV; translation; antiviral

Introduction

Viruses are obligate intracellular parasites that require the cellular machinery to be able to replicate themselves and synthesize the proteins that form them. A process involved in the viral replicative cycle is the initiation of the cellular translation, a limiting event in the protein synthesis (Montero *et al*., 2015). More than a dozen proteins called eukaryotic Initiation Factors (eIFs) and regulatory proteins participate in the initiation (Pestova *et al*., 2001). Together, the eIFs intervene in the binding of the ribosomal subunit 40S and in the scanning to the start codon to allow the incorporation of the ribosomal subunit 60S and proceed to the elongation phase of translation (Gingras *et al*., 1999; Pestova *et al*., 2001).

The binding of the ribosomal subunits 40S, eIF1, eIF1A, eIF3 and the ternary complex (eIF2, GTP, and tRNA-methionine-initiator) to the messenger RNA (mRNA) is a determining step in the speed of the translation start (Gingras *et al*., 1999)</style> 1999. This binding is regulated by a complex of eIFs known as eIF4F, which is formed by the proteins eIF4E, eIF4AI, and eIF4G (Preiss *et al*., 2003). These proteins are often the target of regulation by viruses (Montero *et al*., 2015, 2019).

The protein eIF4AI is the prototype member of the DEAD-box family of RNA helicases (Rogers *et al*., 1999; Andreou *et al*., 2013). There are three isoforms of eIF4A: eIF4AI, eIF4AII, and eIF4AIII. All three isoforms have been

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Abbreviations: DMEM = Dulbecco's Minimum Eagle Medium; eIF = eukaryotic Initiation Factors; hpi = hours post-infection; hpt = hours post-transfection; HRP = horseradish peroxidase; IBAGs = granules associated to inclusion bodies; MOI = multiplicity of infection; RSV = respiratory syncytial virus; SGs = stress granules; siRNA = small interference RNA

reported to exhibit functional differences (Li *et al*., 1999; Galicia-Vazquez *et al*., 2012). The most studied is eIF4AI, whose function has been mainly implicated in translation (Montero *et al*., 2019). Within eIF4F complex, eIF4AI has an important role in unfolding of the secondary structure in the 5'-untranslated region (5'-UTR) of mRNA (Pestova *et al*., 1996; Gingras *et al*., 1999). Moreover, in the stress cellular response, cytoplasmic aggregates are generated where the translation is arrested. These cytoplasmic aggregates are known as stress granules (SGs) containing eIF4AI (Mazroui *et al*., 2006).

The importance of eIF4AI has not only been observed in the cellular translation process. Certain pathologies have been associated with the alteration of the expression of this factor, such as Alzheimer disease, some types of cancer, and viral infections (Eberle *et al*., 1997; Bottley *et al*., 2010; Tsumuraya *et al*., 2011; Jin *et al*., 2013). Some natural compounds such as silvestrol, hippuristanol, and panteamine A, inhibit the activity of eIF4AI (Bordeleau *et al*., 2005; Low *et al*., 2005; Bordeleau *et al*., 2006; Cencic *et al*., 2009), and these are being tested as possible antivirals with promising results in cases where the virus replicative cycle is dependent on eIF4AI, like Ebola, Zika and Hepatitis E (Bordeleau *et al*., 2006; Elgner *et al*., 2018; Todt *et al*., 2018; Montero *et al*., 2019).

The respiratory syncytial virus (RSV) is one of the main etiological agents responsible for acute respiratory infections in both upper and lower tract (Hall, 2001). Similarly to other viruses, RSV requires certain eIFs to synthesize its own proteins and complete its replicative cycle (Montero *et al*., 2015; Montero *et al*., 2019). Given the record of the participation of eIF4AI in different viral life cycles and the fact that it has been considered as a potential viral therapeutic target, it is important to evaluate its role during RSV infection. In this study, through RNA interference, the effect of eIF4AI knockdown on the viral progeny and positive-strand RNA synthesis of RSV was evaluated, and the intracellular localization of eIF4AI throughout the replicative cycle of RSV was determined by implementing immunodetection and epifluorescence and confocal microscopy.

Materials and Methods

Cells, virus, and treatment. The cellular line HEp-2 was cultured in Dulbecco's Minimum Eagle Medium (DMEM) with FBS at 10%. The lysate of RSV utilized was provided by Dr. Beatriz Gomez Garcia and Evelyn Rivera Toledo from the School of Medicine of the Universidad Nacional Autónoma de México (UNAM). The virus was propagated in the Hep-2 cells using DMEM without FBS as previously described (Perez-Gil *et al*., 2015).

eIF4AI knockdown. The eIF4AI knockdown was performed by means of RNA interference using siRNAs. Validated siRNA was acquired from Santa Cruz Biotechnology (SC-40554) and it was used according to the manufacturer's instructions. Rhinovirus siRNA (WD01357486; Sigma-Aldrich) was used as an irrelevant control. The siRNA transfection was performed in HEp-2 monolayers with a confluence between 90–100% per manufacturer's protocol. The transfection mix containing siRNA was added to the cells at a concentration of 150 pmol/well of a 48-well plate and was incubated for 36 hours (h) at 37°C with 5% CO_2. After the incubation, cells were infected with RSV at MOI of 3 and lysed at 8 or 32 hours post infection (hpi).

Microtiter. A 96-well plate with HEp-2 cells at a confluence of 100% was infected in duplicate with RSV 2-fold serial dilutions using DMEM as diluting agent. The infected cells were incubated for two hours at 37°C with 5% CO_2 under agitation. The next step was the removal of the non-absorbed virus and cells were incubated with DMEM for 20 h, after which the cells were fixed with 80% acetone diluted in PBS for 40 min at room temperature. The cells were then incubated with mouse anti-RSV fusion protein antibody (SC-57999; Santa Cruz Biotechnology) for two hours at room temperature and later incubated with protein A peroxidase (HRP) (Sigma-Aldrich; 101023) for one hour. The HRP substrate was prepared in 8 ml of acetate buffer 0.05 M pH 5, 10 µl of hydrogen peroxide and 1.5 ml of carbazole 4 mg/ml and it was incubated for 30 min in the dark. The reaction was stopped with running water. The number of foci-forming units (FFU) was calculated as follows: FFU/ml: (20)(5.5)(number of foci)(dilution).

Western blot. HEp-2 cells cultivated in 48-well plates were transfected with the corresponding siRNAs and infected with RSV at a multiplicity of infection (MOI) of 3 with two hours of absorption. The cells were incubated for 32 h at 37°C with 5% CO_2 and then lysed, as described previously (Montero *et al*., 2006). The samples were separated by SDS-PAGE at 10% and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with the primary rabbit anti-eIF4A antibody (PA5-30216; Invitrogen) or rabbit anti-vimentin antibody and subsequently with secondary HRP-conjugated anti-rabbit IgG. Finally, a chemiluminescent reagent was added (Perkin Elmer) to obtain the signal. The load adjustment of the proteins was performed with Coomassie Brilliant blue staining.

Real-time PCR. After the siRNA transfection and RSV infection as described above, the RNA was extracted with TRIzol Reagent® (15596026; Invitrogen) according to the manufacturer's instructions. The reverse transcription was performed using the SuperScript® III First-Strand Synthesis System for RT-PCR (18080051; Invitrogen). The relative quantification and employment of oligonucleotides capable of detecting the positive-strand RNA of RSV was performed according to the specifications of real-time PCR methodologies for the quantification of viral RNA of RSV already described (Bannister *et al*., 2010;

Boukhvalova *et al*., 2010). As internal control, β-globin was used with a PCR product of 140 base pairs and with following primers 5'-GGGCTGTCATCACTTAGACCTCAC-3' (forward primer) and 5'-CCGCTGTCAGAAGCAAATGTAAGCAATAG-3' (reverse primer). The mRNA expression of β-globin was measured in each sample for normalization purposes.

Immunofluorescence. The HEp-2 cells were cultured on round coverslips in a 48-well plate and were infected with 100 µl of RSV at MOI 3. Indirect immunofluorescence was performed as was already described (Montero *et al*., 2006) using the following antibodies: mouse anti-eIF4AI, which also can detect eIF4AII (SC-377315; Santa Cruz Biotechnology), and polyclonal goat anti-RSV (AB1128; Chemicon). The secondary antibodies used were Alexa 568 anti-mouse (A10037; Invitrogen) and Alexa 488 anti-goat (A-11078; Invitrogen). The images were taken with an epifluorescence microscope Eclipse 80i and the software NIS-Elements BR 3.2 with 64 bits (both made by Nikon, Tokyo, Japan) and a confocal microscope (Leica Microsystems, Software Leica TCS SPE LASX version 3.7).

Results

eIF4A knockdown reduces the positive-strand RNA synthesis and the number of infectious viral particles

It is known that certain viruses that synthesize their proteins via cap-dependent mechanism require eIF4AI during their life cycle (Montero *et al*., 2019). To evaluate whether the eIF4AI knockdown affects, directly or indirectly, the transcription or the replication of RSV, the relative amount of positive-strand RNA produced in cells transfected with siRNA against eIF4AI (siRNAeIF4AI) was determined by real-time PCR. The analysis of the RSV positive-strand RNA at different times post-infection (pi) showed a strong and significant reduction by 93% at 8 hpi and 86.5% at 32 hpi in comparison with the control that corresponds to cells transfected with irrelevant siRNA (siRNAirre) (Fig. 1a).

To evaluate the impact of eIF4AI knockdown on the generation of viral progeny, the cells transfected with siRNAeIF4AI or with siRNAirre were infected at 36 h post-transfection (hpt) and lysed by cryofracture at 32 hpi. The samples were used to quantify the viral progeny using microtiter of infectious foci assay. The amount of virus was normalized with respect to the siRNAirre, considering it as 100%. The results showed a significant reduction of the viral progeny by 32.33±9.06% in cells transfected with siRNAeIF4AI compared to the control (siRNAeIF4AI vs. siRNAirre; p = 0.0001 determined by a paired Student's t-test) (Fig. 1b). The eIF4AI knockdown was corroborated by Western blot and the vimentin detection was used as loading control (Fig. 1c).

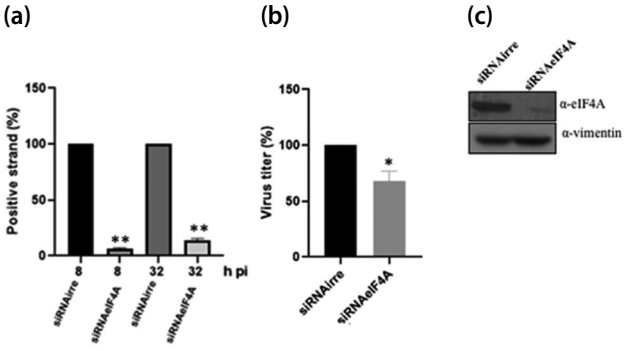

Fig. 1

The knockdown of eIF4A reduces both viral positive-strand RNA level and the viral progeny

(a) HEp-2 cells transfected with siRNAeIF4A or siRNAirre were infected by RSV at 36 h pt and lysed at 8 or 32 h pi. The RNA was extracted with TRIzol and then quantified to perform the reverse transcription. The level of RSV positive-strand RNA for each siRNA was determined by real-time PCR. The results are expressed in percentage of positive-strand RNA as the arithmetic mean ± standard deviation of two independent experiments performed in triplicates. siRNAirre was set to 100%. hpi: hours post-infection; hpt: hours post-transfection; siRNAirre; irrelevant siRNA. **p >0.001 was determined by a paired Student's t-test. **(b)** HEp-2 cells were transfected with the siRNAeIF4A or siRNAirre. After 36 h, the cells were infected with RSV at MOI 3 and lysed 32h. Amount of infectious RSV particles was normalized to the virus obtained with siRNAirre, the virus amount is shown as percentage. Data is shown as an arithmetic mean ± standard deviation of five independent experiments. siRNAeIF4A vs. siRNAirre; *p >0.05, determined by the paired Student's t-test. **(c)** Western Blot analysis of eIF4A. The proteins were transferred and the membrane was incubated with the indicated antibodies, as has been described in Materials and Methods. The vimentin detection was used as loading control. Paired Student's *t*-test was used for statistical analysis. siRNAirre: irrelevant siRNA.

eIF4AI colocalizes with RSV protein

It has been found that the inhibition of translation initiation leads to the formation of SGs by different mechanisms, including alteration of eIF4AI function (Mazroui *et al*., 2006) and a study on the RSV replication cycle showed that SGs start to form at 12 hpi (Lindquist *et al*., 2010). For that reason, we were interested in identifying the intracellular localization of eIF4AI in RSV-infected cells. In this study, we detected eIF4AI at different times post infection and found that a portion of eIF4AI starts to accumulate in the cellular cytoplasm at 4 hpi. The accumulation continues as the viral cycle evolves, apparently until 20 hpi. The distribution analysis of eIF4AI during infection with RSV by epifluorescence microscopy showed a probable colocalization between viral proteins and eIF4AI in some cells at the analyzed times from 4 to 40 hpi (Fig. 2a). To corroborate this colocalization, representative times were chosen at 10, 24 and 36 hpi and a confocal microscopy analysis was performed using an

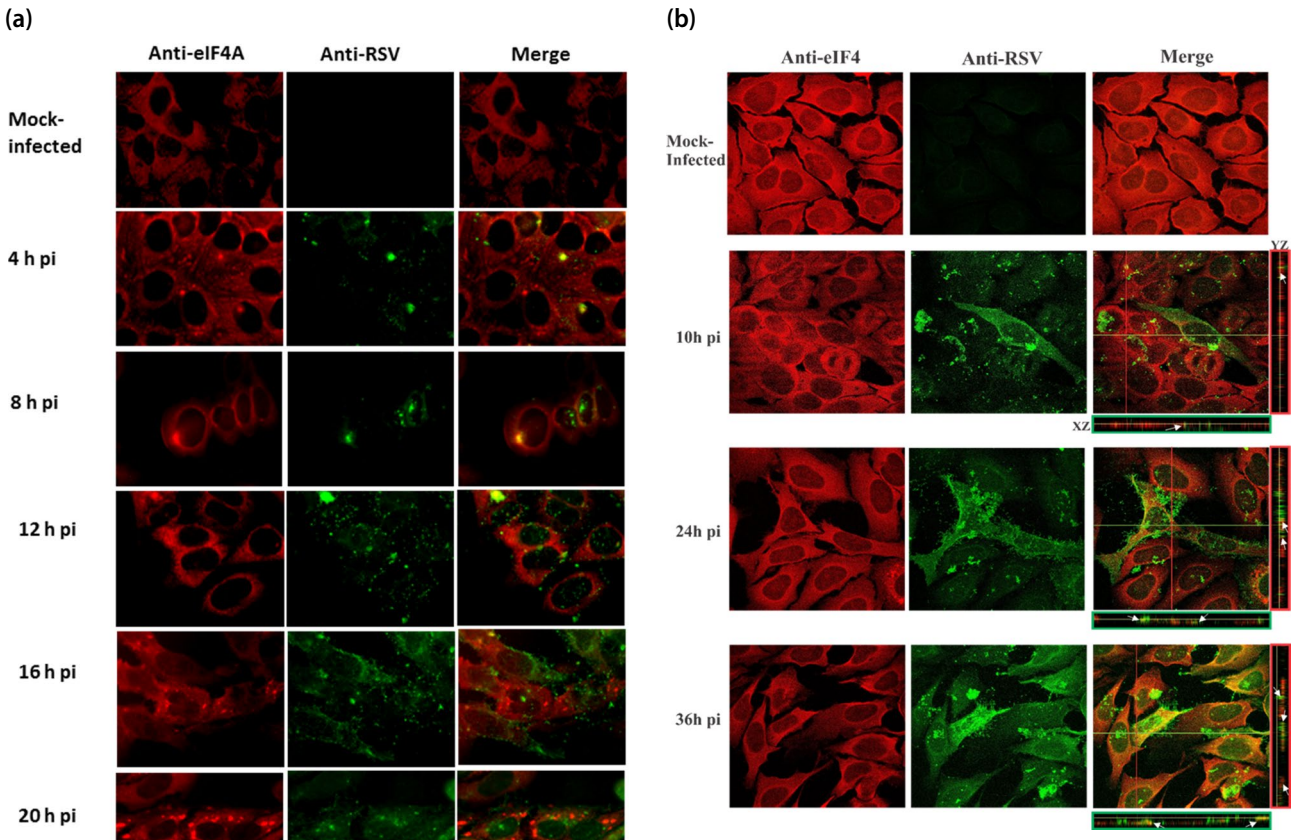

Fig. 2

eIF4A modifies its distribution pattern during RSV infection and colocalizes with viral proteins

HEp-2 cells were cultured on coverslips, infected with RSV and harvested at different times pi. Then the cells were fixed and co-stained with antibodies directed against eIF4A and RSV proteins. Representative images of three independent experiments are shown. **(a)** Kinetics of the infection by RSV by epifluorescence microscopy at the indicated times. **(b)** Confocal microscopy images of Z section projections of eIF4 and RSV staining and their colocalization (merge). The orthogonal projections of Z series at the digital sections, indicated with the horizontal green line and vertical red line and shown as YZ (red box) of XZ (green box) images, confirm the colocalization of eIF4 and RSV staining (white arrows) at the different times pi. h pi: hours post-infection.

antibody against eIF4AI and a polyclonal antibody for the detection of the RSV proteins. The colocalization was identified as viral protein accumulates that covered little accumulations of eIF4AI in some cells, at times 10, 24 and 36 hpi (Fig. 2b). The colocalization analysis of the obtained z sections by confocal microscopy was performed and the Manders coefficient (used to quantify the degree of colocalization) was determined. The Manders coefficient has values ranging from 0 to 1, where 1 is the value with the highest colocalization (Dunn *et al.*, 2011). Here, the Manders coefficient was determined to reach the values of more than 0.9 at the analyzed times.

Discussion

The genome of viruses does not code for all the necessary proteins for their replication. For that reason, they need to infect a cell to complete their replicative cycle. Some viruses can control the cells to their benefit within few hours. A cellular process that is a common regulation target for many viruses is the cellular translation, which is complex and not entirely understood (Toribio *et al*., 2010; Au *et al*., 2014; Montero *et al*., 2015, 2019). Helicase eIF4AI is one of the translation factors, in which viruses converge in their regulation (Montero *et al*., 2019) and for that reason the function of this factor in the context of RSV infection was the objective of our study.

It has been described that the function of eIF4AI is primarily in the processes of cellular translation. However, for some viruses, the direct or indirect effect of eIF4AI inhibition or knockdown is observed in other steps within the replicative cycle, such as the replication and the transcription (Elgner *et al*., 2018; Muller *et al*., 2018; Todt *et al*., 2018). Ebola virus, hantavirus, Dengue, SARS-CoV, and hepatitis C encode for viral proteins with helicase activity (Adedeji *et al*., 2012; Swarbrick *et al*., 2017; Montero *et al*., 2019; Shu *et al*., 2019). On the other hand, when cells infected with these viruses are treated with drugs that interfere with eIF4AI activity, a negative impact in virus replicative cycle is observed (Biedenkopf *et al*., 2017; Muller *et al*., 2018). This may indicate either an important role of eIF4AI in these viral cycles, or it can be an indirect effect. The specific function of eIF4AI in the context of virus infection is not yet known and should be studied in more detail. In this study, the synthesis of eIF4AI was reduced by RNA interference and, as a result, a strong reduction in the positive-strand RNA and a less profound but significant decrease of the viral progeny was observed, indicating that eIF4AI might be important for RSV replicative cycle. The amount of viral genomes does not directly correspond to the number of infectious viral particles. A similar effect has been observed when silvestrol was used to inhibit the function of eIF4A (Elgner *et al*., 2018). We speculate that even though there was a low number of viral mRNAs, the viral translation and/or viral morphogenesis was slightly affected by the eIF4AI knockdown. Whether the synthesis of viral proteins has also been affected by the eIF4AI knockdown is under investigation. In this context, previous studies suggested that the translation of messenger RNA of RSV is independent of the eIF4F complex due to dephosphorylation of 4EBP1, which is a protein that sequesters eIF4E and affects the cap-dependent translation (Perez-Gil *et al*., 2015). However, some viruses interrupt the formation of eIF4F but depend on eIF4AI or eIF4G to synthesize their proteins (Montero *et al*., 2019). This shows the need of more detailed studies to corroborate whether the RSV mRNA requires eIF4AI for its translation.

By immunodetection and epifluorescence microscopy, eIF4AI is observed in the cytoplasm in a homogeneous pattern under basal conditions. However, it is known that if the eIF4AI function is altered, the formation of SGs is triggered (Mazroui *et al*., 2006). The infection by some viruses leads to the formation of SGs without interfering with their replicative cycles (Reineke *et al*., 2013). In the case of RSV the formation of SGs during the infection was observed (Lindquist *et al*., 2010). In addition to the SGs formation, granules associated to inclusion bodies (IBAGs) have also been observed. IBAGs are considered as dynamic structures and sites for recruiting the newly synthesized mRNA and the viral transcription anti-terminator M2-1 (Rincheval *et al*., 2017). In this work, the staining of eIF4AI in RSV-infected cells showed a clear change from a homogeneous cytoplasmic staining to aggregates that remain throughout the curse of infection. Importantly, it was noted that the RSV viral proteins colocalized in some of these aggregates formed by eIF4AI but not in the entirety of them. This observation might mean that a portion of the eIF4AI could be forming IBAGs and another portion forming SGs; however, we did not use any marker for SGs or IBAGs, thus we still do not know the nature of these aggregates formed by eIF4AI. It would not be surprising if the presence of eIF4AI in viral aggregates is associated with a possible function in RSV transcription-replication processes as it has been described for other viruses (Elgner *et al*., 2018; Muller *et al*., 2018; Todt *et al*., 2018), either as a helicase or other function for the viral replication. Clearly, additional research is needed to understand the nature of aggregates formed by eIF4AI and its colocalization with RSV viral proteins, which is fundamental for a better understanding of the role of eIF4AI during the replicative cycle of RSV.

Finally, since the eIF4AI knockdown affects the RSV replicative cycle in a negative way, drugs that impair the function of eIF4AI, like silvestrol, hippuristanol, and pateamine A (Bordeleau *et al*., 2005, 2006; Low *et al*., 2005; Cencic *et al*., 2009), could be employed as potential treatment against RSV infection, in a similar way as it has been observed against other viruses like Ebola, Epstein-Barr virus and some corona- and picornaviruses (Patton *et al*., 2015; Biedenkopf *et al*., 2017; Grünweller and Hartmann 2017). This possibility is of great importance, as so far, there is no effective antiviral treatment for RSV infection and it is a virus with high mortality rate worldwide (Shi *et al*., 2017). To our knowledge, this is the first report that suggests a possible involvement of eIF4AI in the replicative cycle of RSV. Whether drugs that disrupt the function of eIF4AI could also affect the replicative cycle of RSV needs to be examined.

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