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Influenza virus polymerase confers independence of the cellular cap-binding factor eIF4E for viral mRNA translation

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ABSTRACT

The influenza virus mRNAs are structurally similar to cellular mRNAs nevertheless; the virus promotes selective translation of viral mRNAs despite the inhibition of host cell protein synthesis. The infection proceeds normally upon functional impairment of eIF4E cap-binding protein, but requires functional eIF4A helicase and eIF4G factor. Here, we have studied whether the presence of *cis* elements in viral mRNAs or the action of viral proteins is responsible for this eIF4E-independence. The eIF4E protein is required for viral mRNA translation *in vitro*, indicating that *cis*-acting RNA sequences are not involved in this process. We also show that PB2 viral polymerase subunit interacts with the eIF4G protein. In addition, a chimeric mRNA containing viral UTR sequences transcribed by the viral polymerase out of the infection is successfully translated independently of an impaired eIF4E factor. These data support that the viral polymerase is responsible for the eIF4E independence of influenza virus mRNA translation.

Introduction

Influenza virus uses an unusual transcription mechanism in which capped and polyadenylated viral mRNAs are synthesized by the viral polymerase, a heterotrimer composed of three subunits named PA, PB1 and PB2 (Elton et al., 2005). Viral mRNA synthesis is primed by short-capped oligonucleotides of around 10 to 12 nucleotides that are generated from host cell nuclear mRNAs by a viral endonuclease activity (Plotch et al., 1981). The cap recognition and binding is achieved by the PB2 subunit (Blaas et al., 1982; Ulmanen et al., 1981), while the PA subunit seems to be required for the cleavage of the cap-oligonucleotides (Dias et al., 2009; Yuan et al., 2009). In addition, the 3′-end of viral mRNAs is polyadenylated by the reiterative copy of a U_{5-7} track present near the 5′ end of the genomic negative sense viral RNA (Luo et al., 1991). Consequently, although synthesized by different pathways, cellular and viral mRNAs are both structurally similar.

Influenza virus efficiently shuts off host cell protein synthesis (Garfinkel and Katze, 1993). Moreover, upon infection, viral mRNAs are selectively translated (Garfinkel and Katze, 1993; Park and Katze, 1995), while the initiation and elongation of cellular mRNA translation are inhibited (Katze et al., 1986). The translation initiation eIF4F complex plays a pivotal role in the translation of capped-mRNAs. It is a heterotrimer formed by eIF4E, the cap-binding factor that is required for cap-dependent translation, the eIF4A helicase and the scaffolding eIF4G factor. The eIF4G protein binds to eIF3, which in turn, mediates the recruitment of the 40S ribosomal subunit triggering the translation initiation of the mRNAs bound to the eIF4F complex (see, for a review, Gingras et al., 1999). Since viral and cellular mRNAs are formally equivalent, influenza virus must have developed sophisticated strategies to discriminate and favor translation of its own mRNAs. Among the key factors that have been related with viral translation regulation, NS1 protein plays an important role contributing to the selective translation of viral mRNAs in the infected cell, especially for those produced later in the virus life cycle. This activation is mediated by its functional interaction with the 5′ UTR of the viral mRNAs that are conserved in every viral mRNA (de la Luna et al., 1995; Enami et al., 1994; Park and Katze, 1995). In addition, the interaction of NS1 with the eIF4GI factor (Aragón et al., 2000) and with the polyA binding protein I (Burgui et al., 2003) appears to be essential for this process. However, viral mRNAs are selectively translated in a mutant virus lacking NS1 protein, suggesting that other viral factors might be involved in the preferential translation of viral mRNAs that takes place within the infected cells (Salvatore et al., 2002).

Regarding the eIF4F complex, influenza virus infection alters the phosphorylation state of eIF4E and eIF4G, and these changes have been related with the inhibition of host-cell protein synthesis and the selective translation of viral mRNAs (Feigenblum and Schneider, 1993). In agreement with these data, we have previously shown that the translation of influenza virus mRNAs and the viral infection proceed efficiently when the eIF4E cap-binding protein is functionally

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impaired, even when a recombinant influenza virus lacking NS1 protein is used (Burgui et al., 2007). In addition, we have recently characterized that the other two components of the eIF4F complex, eIF4A and eIF4G, are essential for viral translation both in *in vivo* and *in vitro* analyses and, hence, should not be related with selective translation in the infected cell (Yángüez et al., 2011).

Among the possible trans-acting proteins that could be involved in viral protein synthesis, we also described that the influenza virus polymerase binds to translation preinitiation complexes and that the infection increases the binding of the eIF4GI factor to cap-structures under conditions of eIF4E–eIF4GI disassociation triggered by overexpression of a non-phosphorylatable 4E-BP1 protein (Burgui et al., 2007). These data suggest a role for the viral polymerase in overriding the dependence of viral mRNA translation on the eIF4E factor, as it could behave as the cap-binding factor that mediates eIF4G recruitment to the viral mRNA. Here, we have assessed whether the viral polymerase or the presence of *cis* structural elements in viral mRNAs is the responsible for the eIF4E independence. This process could be part of the mechanism underlying the selective translation of viral mRNAs that takes place in the infected cell.

Results

Study of the presence of structural cis elements in influenza virus mRNAs

As mentioned, influenza virus infection proceeds normally in the absence of functional eIF4E factor. Thus, rapamycin treatment, eIF4E gene silencing and overexpression of constitutively hypophosphorylated 4E-BP1, which provokes eIF4E–eIF4G dissociation, do not impair viral mRNA translation in the infected cells (Burgui et al., 2007). Common structural determinants within influenza virus mRNAs could mediate their independence for the cap-binding factor as, for instance, internal ribosome entry sites (IRES) that are capable of directly recruiting the translation machinery (Kieft, 2008; Martinez-Salas et al., 2008). Therefore, we carried out *in vitro* experiments to examine whether influenza virus mRNAs contain sequences that would confer eIF4E independence.

Requirements for eIF4E in vitro

We compared the translation efficiency of a dicistronic cap-CAT-EMCV:IRES-luciferase RNA containing the encephalomyocarditis disease virus IRES, with isolated RNAs from influenza virus infected cells in conditions of limited eIF4E availability. Accordingly, *in vitro* translation preparations were depleted of eIF4E by adding purified 4E-BP1 protein followed by incubation with a cap-Sepharose resin. After removal of the bound complexes by centrifugation, the eIF4E-depleted lysates were used to translate *in vitro* transcribed cap-CAT:EMCV-IRES-Luc RNA or purified cytoplasmic RNA from infected cells (Fig. 1A). The depletion of eIF4E produced a clear decrease in cap-dependent CAT protein synthesis, while IRES-driven luciferase synthesis remained unaffected. Similarly, the synthesis of viral proteins diminished under these conditions. Further addition of purified recombinant His-eIF4E protein partially recovered the translation of CAT and viral proteins in the eIF4E-depleted preparations.

We also used a different approach to analyze the possible presence of specific structures in the viral mRNAs that might be involved in the low dependence on eIF4E. Accordingly, both *in vitro* transcribed dicistronic cap-CAT:EMCV-IRES-Luc RNA and cytoplasmic RNA from infected cells were then assayed in the presence or absence of increasing concentrations of purified 4E-BP1 to inhibit the interaction of eIF4E with eIF4G. The proteins synthesized were metabolically labeled and analyzed by SDS-polyacrylamide gels (SDS-PAGE) (Fig. 1B). Translation of the IRES-driven luciferase was not affected by the addition of 4E-BP1 even at the highest doses. By contrast, the translation of the CAT gene, which occurs in a cap-dependent

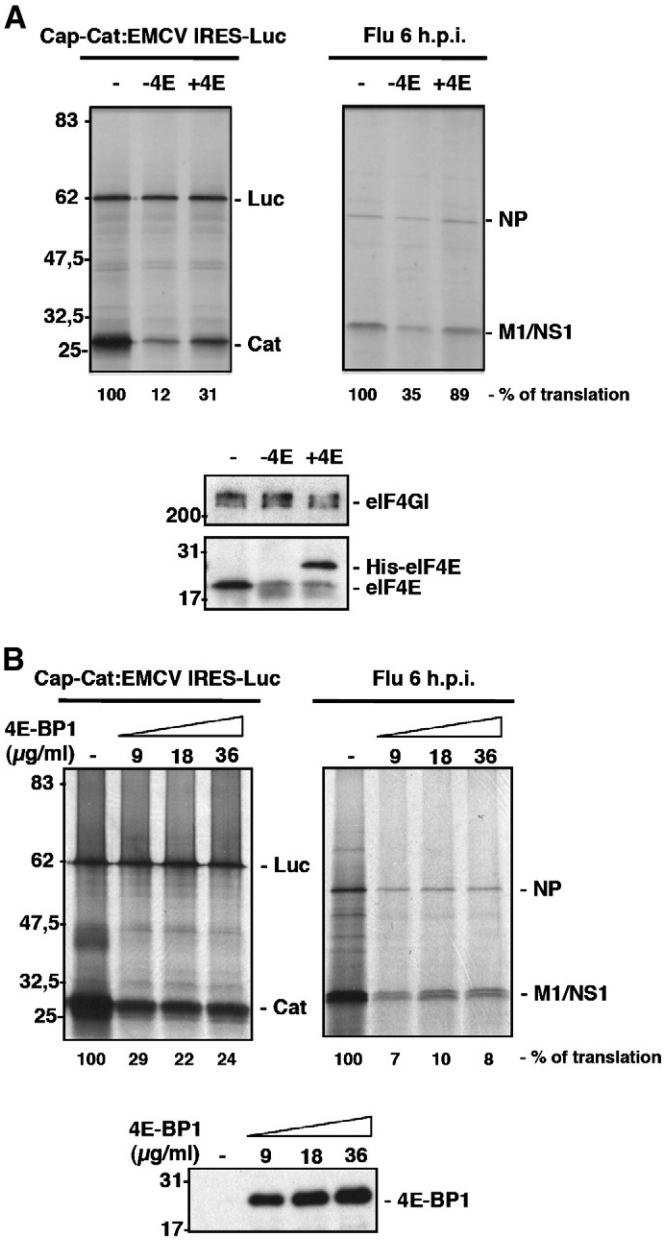

Fig. 1. Functional impairment of eIF4E inhibits the *in vitro* synthesis of influenza virus proteins from isolated viral RNAs. (A) Rabbit reticulocyte extracts were depleted of eIF4E by the addition of purified 4E-BP1 protein and incubation with a 7mGTP resin. After removal of the bound complexes, the eIF4E-depleted lysates were used to assess the translation of *in vitro* transcribed cap-CAT:EMCV-IRES-Luc RNA or purified cytoplasmic RNA from infected cells (−4E lanes). Subsequently, purified His-eIF4E recombinant protein was added (+4E lanes). The bottom panel shows the amounts of the indicated proteins in the eIF4E-depleted preparations and after the addition of recombinant His-eIF4E protein. The synthesized proteins were metabolically labeled and analyzed by SDS-PAGE. (B) Cytoplasmic RNA from HEK293T infected cells isolated after 6 hpi and dicistronic cap-CAT:EMCV IRES-Luc RNA obtained by *in vitro* transcription were used for *in vitro* translation in reticulocyte lysates, with or without increasing concentrations of purified 4EBP1. The bottom panel shows the amounts of 4EBP1 added to the reactions. The synthesized proteins were processed as described in part (A).

manner, clearly diminished in the presence of 4E-BP1. Similarly, the addition of purified 4E-BP1 significantly reduced the translation of viral mRNAs. These results indicate that isolated influenza virus mRNAs behave like standard capped-mRNAs in terms of eIF4E dependence *in vitro*. Therefore, the low requirement for functional eIF4E observed *in vivo* is not due to an inherent property of the viral mRNA (*e.g.* the presence of *cis* elements), but it is more likely the consequence of specific factors acting in *trans*.