INTRODUCTION

Influenza virus infection efficiently shuts off the expression of the host cell genes (Skehel, 1972), while maintaining an efficient translation of viral proteins. During influenza virus infection, the virus evades the inhibition of protein synthesis through the inhibition of the double-stranded RNA-activated kinase (Lee et al., 1992; Lu et al., 1995; Polyak et al., 1996). Cellular protein synthesis shutoff may be the result of several alterations induced by the virus during infection. These include: (i) cap-snatching of cellular pre-mRNAs (Krug et al., 1986); (ii) inhibition of cleavage and polyadenylation of cellular pre-mRNAs (Chen & Krug, 1999; Nemeroff et al., 1998); (iii) nuclear retention of poly(A)-containing cellular mRNAs (Fortes et al., 1994); (iv) degradation of cytoplasmic cellular mRNAs (Beloso et al., 1992; Inglis, 1982; Zürcher et al., 2000); and (v) preferential utilization of the translation machinery by the viral-specific mRNAs (Katze et al., 1986).

Influenza virus mRNAs have a capped 5' end followed by a 10–12 nt long untranslated region of cellular, heterogeneous sequences generated by cap-snatching, which precede a viral-encoded, highly conserved sequence that is common to all influenza virus genes. The 3' end of the viral mRNAs is polyadenylated by a reiterative copy of a U5–7 track present near the 5' end of the viral RNA (Luo et al., 1991; Poon et al., 1998, 1999; Robertson et al., 1981). Although viral mRNAs are formally equivalent to cellular ones, influenza virus infection specifically enhances viral mRNA translation, with the conserved sequences contained within the 5'-untranslated region (5'UTR) playing a critical role (Garfinkel & Katze, 1993). Recently, a specific interaction of the cellular RNA-binding protein GRSF-1 with the 5'UTR of the viral nucleoprotein mRNA has been described. This protein specifically stimulates nucleoprotein mRNA translation in HeLa cell extracts (Park et al., 1999). In addition, viral mRNAs are preserved from the generalized degradation of cytoplasmic mRNAs that takes place in the course of infection (Beloso et al., 1992; Inglis, 1982).

NS1 protein is the only non-structural protein of influenza virus (Barret et al., 1979; Lamb & Choppin, 1979). It accumulates in the nucleus of the infected cell at early times but is also present in the cytoplasm later in the infection, where it is associated with polysomes (de la Luna et al., 1995; Falcón et al., 1999; Krug & Etkind, 1973). In view of its interaction with several viral and cellular factors, NS1 has been

PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes

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It has previously been shown that influenza virus NS1 protein enhances the translation of viral but not cellular mRNAs. This enhancement occurs by increasing the rate of translation initiation and requires the 5'UTR sequence, common to all viral mRNAs. In agreement with these findings, we show here that viral mRNAs, but not cellular mRNAs, are associated with NS1 during virus infection. We have previously reported that NS1 interacts with the translation initiation factor eIF4GI, next to its poly(A)-binding protein 1 (PABP1)-interacting domain and that NS1 and eIF4GI are associated in influenza virus-infected cells. Here we show that NS1, although capable of binding poly(A), does not compete with PABP1 for association with eIF4GI and, furthermore, that NS1 and PABP1 interact both in vivo and in vitro in an RNA-independent manner. The interaction maps between residues 365 and 535 in PABP1 and between residues 1 and 81 in NS1. These mapping studies, together with those previously reported for NS1–eIF4GI and PABP1–eIF4GI interactions, imply that the binding of all three proteins would be compatible. Collectively, these and previously published data suggest that NS1 interactions with eIF4GI and PABP1, as well as with viral mRNAs, could promote the specific recruitment of 43S complexes to the viral mRNAs.
implicated in many of the alterations indicated above that occur during influenza virus infection. It recognizes double-stranded RNA, U6 snRNA, poly(A), viral mRNA and viral mRNA (Fortes et al., 1994; Hatada & Fukuda, 1992; Hatada et al., 1997; Maríon et al., 1997b; Qiu & Krug, 1994, 1995), as well as the 30 kDa subunit of the CPSF complex, PABPII and NS1-BP, a nuclear protein that might be involved in splicing (Chen & Krug, 1999; Nemeroff et al., 1998; Wolff et al., 1998). NS1 has anti-interferon properties. The role of its double-stranded RNA-binding domain in counteracting the antiviral pathways has been pointed out (Talon et al., 2000; Wang et al., 2000b). NS1 binding to the 30 kDa subunit of the CPSF complex has also been implicated as being responsible for its anti-interferon properties (Noah et al., 2003).

NS1 is also involved in the stimulation of viral mRNA translation (de la Luna et al., 1995; Enami et al., 1994; Katze et al., 1986; Maríon et al., 1997a; Park & Katze, 1995), a function in which its interaction with the 5′-terminal conserved sequences of viral mRNAs is important (de la Luna et al., 1995; Park & Katze, 1995). We have identified two cellular targets of NS1 that may be relevant for this function – the human Staufen protein (Falcón et al., 1999; Maríon et al., 1999) and the eIF4GI subunit of the eIF4F translation initiation factor (Arango´ n et al., 2000). Mapping of the eIF4GI-binding domain in the NS1 protein has indicated that the first 113 N-terminal amino acids of the protein were sufficient to bind eIF4GI, but not the first 81 residues. The first mutant has previously been shown to be a translational enhancer, while the second is defective in this activity (Maríon et al., 1997a). Since NS1 is able to bind poly(A) RNA (Qiu & Krug, 1994) and its binding to eIF4GI protein takes place in a region located close to the poly(A)-binding protein (PABP1)-interacting domain (Arango´ n et al., 2000), we considered the possibility that NS1 could replace PABP1 at the 3′ poly(A) tail of viral mRNAs and, correspondingly, that an NS1–eIF4GI interaction could impair PABP1–eIF4GI association. In this report we show that NS1 associates specifically with viral mRNAs and does not compete with the eIF4GI–PABP1 interaction in infected cells. On the contrary, NS1 is also able to interact with PABP1 in vivo and in vitro. Our data support a model for the preferential translation of viral mRNAs in which NS1 would promote their association to polysomes by interaction with eIF4GI and PABP1 proteins, as well as with the 5′-terminal conserved sequences of viral mRNAs.

METHODS

Biological materials. The COS-1 cell line was obtained from Y. Gluzman (Gluzman, 1981). Cell cultures were grown in Dulbecco’s modified Eagle’s medium containing 5% foetal bovine serum. The influenza virus A/Victoria/3/75 strain was grown in Madin–Darby canine kidney cells as reported previously (Ortí ´n et al., 1998). Plasmid pGEX-2T containing the cDNA of the human poly(A)-binding protein 1 (pGST-PABP1) was kindly provided by T. Preiss. Plasmids expressing the NS1 protein or its deleted versions have been previously described (Aragón et al., 2000). The preparation of antisera specific for NS1 protein has been reported previously (Maríon et al., 1997b; Nieto et al., 1992). Rabbit antisera specific for the eIF4GI protein were prepared as reported (Aragón et al., 2000). Rabbit antisera specific for PABP1 were obtained by immunizing animals with purified glutathione S-transferase (GST)–PABP1 fusion protein, expressed and purified from bacteria.

Mutant construction. The plasmid pGEX-2T-PABP1 expressed a GST–PABP1 fusion protein lacking the first nine amino acids of human PABP1. To obtain carboxy-deletion mutants of the GST–PABP1 protein, pGEX-2T-PABP1 plasmid was digested with HindIII or SpeI endonucleases, blunt-ended and self-ligated. The resulting plasmids pGEX-2T-PABP1 234 and pGEX-2T-PABP1 319 expressed a GST–PABP1 fusion protein containing the N-terminal 234 or 319 amino acids of PABP1, lacking the first 9 amino acids, fused to GST. To obtain amino-deletion mutants, pGEX-2T-PABP1 was digested with Bsal and BamHI, with BplI and BamHI or with NcoI and BsrGI. In all cases the digestion mixture was blunt-ended and the fragments of interest were isolated and self-ligated. The resulting plasmids pGEX-2T-PABP1 Δ1–307, pGEX-2T-PABP1 Δ1–365 and pGEX-2T-PABP1 Δ1–535 expressed GST–PABP1 fusion proteins lacking 307, 365 or 535 amino acids at the N terminus, respectively.

Protein expression and purification. The His–NS1 and His–eIF4GI 157–550 proteins were purified as previously described (Arango´ n et al., 2000). GST protein, GST–PABP1 and its mutant derivatives were expressed in E. coli DH5 cells harbouring plasmids pGEX-2T, pGEX-2T-PABP1, pGEX-2T-PABP1 Δ1–307, pGEX-2T-PABP1 319, pGEX-2T-PABP1 Δ1–365, pGEX-2T-PABP1 234 and pGEX-2T-PABP1 Δ1–535. The proteins were purified on glutathione–Sepharose according to the manufacturer’s instructions (Pharmacia Biotechnology). Briefly, expression was induced with 1 mM IPTG for 2 h at 37 °C. The cells were resuspended in buffer containing 5 mM sodium phosphate, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, pH 7-4 (supplemented before use with 1 mM PMSF, 1 mM TPCK, 1 mM TLCK and 0-1% 2-mercaptoethanol) and sonicated. After removal of cell debris by centrifugation, the supernatant was incubated with glutathione–Sepharose 4B resin equilibrated in the same buffer by rocking for 30 min at 4 °C. After extensive washes with the same buffer, the proteins were eluted with 10 mM glutathione in 50 mM Tris/HCl at pH 8·0.

In vitro transcription/translation. Plasmids encoding NS1 protein or mutants thereof were used for in vitro transcription/translation using the Promega Tnt coupled system. In all cases the genes were expressed under the T7 promoter and a 35S-labelled methionine/ cysteine mixture (1400 µCi ml−1) was added to the cell-free protein synthesis system. After 2 h of incubation at 30 °C, the mixture was centrifuged at 10 000 g for 10 min at 4 °C and the supernatants centrifuged again at 250 000 g for 2 h at 4 °C. The post-ribosomal supernatants were then used as a source of recombinant protein for in vitro binding studies.

Western blotting. This was done as described previously (Sanz-Ezquierdo et al., 1995). The following primary antibodies were used: for eIF4GI, a mixture of rabbit antibodies against N-terminal or C-terminal peptides of eIF4GI (1:2000 dilution each) (Aragón et al., 2000); for PABP1 protein, a rabbit antiserum raised against GST–PABP1 fusion protein (dilution 1:1000); for NS1 protein, a rabbit anti-NS1 serum prepared by hyperimmunization with His–NS1 protein (Maríon et al., 1997a) (1:100 dilution) or a rat anti-NS1 serum against His–NS1 protein (1:400); for His-tagged proteins, a rabbit anti-His peroxidase-conjugated serum (Santa Cruz Biotechnology) (1:10 000 dilution) and for GST-tagged proteins, a rabbit anti-GST-serum (Sigma) (1:10 000 dilution).
m.o.i. of 10. After the incubation time, the cells were washed with ice-cold PBS, scraped off the plates and lysed in a buffer containing 150 mM NaCl, 1 mM MgCl₂, 10 mM Tris/HCl, pH 8·5 and 0·5 % Igepal (extraction buffer). The extracts were clarified by centrifugation at 10 000 g for 15 min and used for communoprecipitation assays. The extracts were incubated with the corresponding antibody for 2 h at 4 °C and applied to protein A-Sepharose. When indicated, the extracts were treated with micrococcal nuclease for 37 °C before the addition of the antibody. For Western blot assays, the immunoprecipitates were washed four times with extraction buffer, boiled in Laemmli sample buffer and analysed by SDS-PAGE.

To analyse the RNA associated with NS1 protein, the immunoprecipitates from either mock-infected or influenza virus-infected cells were washed seven times with extraction buffer and twice with RIPA buffer (10 mM Tris/HCl, pH 7·5, 150 mM NaCl, 1 % sodium deoxycholate, 0·1 % SDS and 1 % Igepal) and the resulting protein A-Sepharose–IgG complexes were used to isolate the associated RNA as previously described (Marion et al., 1997b).

Pull-down experiments. For pull-down experiments with GST fusion proteins, GST, GST–PABP1 or mutant GST–PABP1 proteins were purified as described above and bound to Sepharose 4B–glutathione resins. Purified His–NS1 or His–elf4GI 157–550, in vitro–translated NS1 or its deletion mutants were added and incubated for 1 h at room temperature in a buffer containing 150 mM NaCl, 10 mM Tris/HCl, pH 8·5, 1·5 mM MgCl₂ and 0·5 % Igepal. After incubation, the resin was washed three times with 10 volumes of the same buffer and the bound proteins were analysed by Western blot assays.

RNA analysis. To identify the RNA associated with NS1 in vivo, the immunoprecipitates were extensively washed as described above. These immunoprecipitates or total cellular extracts prepared in 150 mM NaCl, 10 mM Tris/HCl, pH 8·5, 1·5 mM MgCl₂ and 1 % Igepal were DNase treated as described (Ortínez et al., 1980; Perales & Ortín, 1997), incubated with 50 µg proteinase K ml⁻¹ for 30 min at 37 °C and phenol extracted. The RNAs were heated for 15 min at 55 °C in 7·5 % formaldehyde in 10× SSC and applied to nylon filters. Duplicate filters were hybridized with negative-polarity riboprobes specific for the NP, M, vimentin or β-tubulin genes.

RESULTS

Characterization of RNAs associated with NS1 in influenza virus-infected cells

Influenza virus infection causes a switch from cellular to viral protein synthesis and a decrease in cellular RNAs present in the cytoplasm of the infected cell. The amount of cytoplasmic RNA gradually decreases with progression of the infection (Beloso et al., 1992; Inglis, 1982; Zürcher et al., 2000). Thus, at 5 h post-infection (p.i.), the accumulation levels of abundant endogenous mRNAs such as β-tubulin or vimentin are around 50–60 %, but at 8 h p.i. the steady-state levels of endogenous mRNAs are only around 10–15 % relative to uninfected cells (Beloso et al., 1992; Zürcher et al., 2000). These degradation data are compatible with the induction of a ribonuclease that uses cellular mRNAs as targets or with a preferential translation of viral mRNAs, which would account for the degradation of non-translated cellular RNAs.

The association of NS1 with elf4GI (Aragon et al., 2000) could be the basis of the efficient translation of influenza virus mRNAs during infection. In line with this possibility, NS1 protein has been found to be associated with viral mRNAs (Marion et al., 1997a). To ascertain the specificity of this association, we analysed the presence of viral and cellular mRNAs in NS1 immunoprecipitates at various times after infection with influenza virus. Cytosolic extracts from mock-infected or virus-infected cells were used to determine the accumulation of viral and cellular mRNAs by dot-blot hybridization. In addition, the extracts were used for immunoprecipitation with NS1-specific or control antibodies, and the presence in the immunoprecipitates of different cellular and viral mRNAs was studied. Owing to the degradation of cellular RNAs that occurs during influenza virus infection, dot-blot hybridization was used instead of Northern assays to evaluate the total amount of RNAs present in the preparations, since otherwise some hybridization signals could be lost. The results are presented in Fig. 1. The total amount of influenza NP or M mRNA increased with progression of the infection (Fig. 1, Total). The presence of NP and M mRNA associated with NS1 was clearly visible in the NS1-specific immunoprecipitates (Fig. 1, Ipp Ab-NS1). In contrast, the mRNAs encoding β-tubulin and vimentin were not present in these immunoprecipitates. Neither NP nor M, β-tubulin or vimentin mRNAs were present in the control immunoprecipitates (Fig. 1, Ipp Control). Quantitative determination of the hybridization signals from at least five independent experiments indicated that the ratio of mRNA present in the anti-NS1 versus control immunoprecipitates was around ten times higher for influenza mRNA than for cellular mRNA. These data indicate that during infection NS1 protein associates specifically with influenza virus mRNA.

PABP1 remains associated with viral mRNAs during influenza virus infection

NS1 protein is a translational activator (de la Luna et al., 1995; Egorov et al., 1998; Enami et al., 1994; Salvatore et al., 2002). This activation is modulated by the 5′ extracistronic common sequences present in influenza virus mRNAs (de la Luna et al., 1995; Enami et al., 1994). Thus, the 5′UTR sequence of the M segment stimulates NS1-mediated translation of M1 protein in transfected cells (Enami et al., 1994) and these 5′ extracistronic sequences can in part account for the enhancement of the translation initiation rate of viral-like mRNAs mediated by NS1 in transfected cells (de la Luna et al., 1995). In agreement with the contribution of these 5′UTR sequences to NS1 activity, gel-shift and UV cross-linking assays have shown in vitro interaction of NS1 with these sequences (Park & Katze, 1995). On the other hand, influenza mRNAs have a poly(A) tail at their 3′ ends and it has been shown that NS1 binds poly(A) both in vivo and in vitro (Qiu & Krug, 1994). In view of the NS1-specific association with viral mRNAs that we observed in infected cells (Fig. 1) and the ability of NS1 to bind poly(A), we asked whether the 3′ end of viral mRNAs could be associated with NS1 instead of the usual cytosolic PABP1 present on cellular mRNAs. Moreover, as PABP1 associates with elf4GI mRNAs, whether the 3′ end of viral mRNAs could be associated with NS1 instead of the usual cytosolic PABP1 present on cellular mRNAs. Moreover, as PABP1 associates with elf4GI mRNAs.
(Imataka et al., 1998; Tarun & Sachs, 1996), as does NS1 protein (Aragón et al., 2000), allowing a circularization of cellular mRNAs, we explored the possibility that NS1 could behave as a virus substitute for PABP1 protein. To test this possibility, we carried out coimmunoprecipitation assays using eIF4GI-specific antibodies in extracts of mock- and virus-infected cells. The results are presented in Fig. 2. Total amounts of eIF4GI and PABP1 did not change, while NS1 protein progressively accumulated with time of infection (Fig. 2, Total). NS1 protein appeared to be associated with translation initiation factor eIF4GI as previously described and PABP1 remained bound to eIF4GI during infection (Fig. 2, Ipp α-eIF4GI, 5 h and 8 h). Since at these times after infection most of the mRNAs being translated are viral, these results indicate that NS1 does not displace the association between PABP1 and eIF4GI.

**Characterization of translational proteins associated with NS1 in influenza virus-infected cells**

Mapping studies in vitro have indicated that NS1 binds at the N-terminal end of eIF4GI, in a region close to the PABP1-interacting domain (Aragón et al., 2000). Since PABP1 is associated with eIF4GI in influenza virus-infected cells, these results suggest that NS1 and PABP1 could interact in viral mRNA translation initiation complexes. To explore this possibility, we carried out coimmunoprecipitation assays using anti-NS1 serum, and the presence of NS1

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**Fig. 1.** Association of viral mRNAs with NS1 in influenza virus-infected cells. Cultures of COS-1 cells were mock-infected or infected with influenza virus A/Victoria/3/75. Cytoplasmic extracts were prepared at 5 or 8 h p.i. and immunoprecipitated with either pre-immune (Ipp Control) or immune (Ipp Ab-NS1) anti-NS1 sera. Samples of the immunoprecipitates (Ipp) or the extracts prior to immunoprecipitation (Total) were used to isolate total RNAs, which were blotted on to nylon filters and probed with riboprobes specific for NP, M, β-tubulin or vimentin mRNAs. The results show data from a representative experiment.

**Fig. 2.** Association of eIF4GI and PABP1 with influenza virus infection. Cultures of COS-1 cells were mock-infected or infected with influenza virus A/victoria/3/75. Cytoplasmic extracts were prepared at different times p.i. and immunoprecipitated with either immune (I) or pre-immune (C) anti-eIF4GI sera. Samples of the immunoprecipitates (Ipp) or the extracts prior to immunoprecipitation (Total extracts) were separated by SDS-PAGE. The gels were blotted on to Immobilon and the filters were analysed by Western blotting using anti-eIF4GI, anti-PABP1 or anti-NS1 sera.
Influenza virus NS1 interacts with PABP1 protein

and associated proteins in total extracts and immunoprecipitates was evaluated by Western blotting. The results are presented in Fig. 3(A). NS1 accumulation in total cell extracts increased with time of infection, whereas accumulation of PABP1 and eIF4GI did not change (Fig. 3A, left). eIF4GI protein was effectively coimmunoprecipitated with NS1 (Fig. 3A, α-NS1), as previously described (Aragón et al., 2000). When the content of PABP1 protein in NS1 immunocomplexes was analysed, we found that PABP1 was indeed present in the complexes (Fig. 3A, α-NS1). The amount of PABP1 coimmunoprecipitated with NS1 was higher than that expected from its association with eIF4GI. This is apparent from the ratios of PABP1/eIF4GI in the NS1-specific and eIF4GI-specific immunoprecipitates (Fig. 3A, right). These results suggested that the presence of PABP1 in the NS1-specific immunoprecipitates was due not only to the coimmunoprecipitation of eIF4GI protein but also to an additional interaction between NS1 and eIF4GI.

Fig. 3. In vivo association of NS1 with PABP1. Cultures of COS-1 cells were mock-infected or infected with influenza virus A/Victoria/3/75 at an m.o.i. of 10. (A) Total cytoplasmic extracts were prepared at various hours p.i., separated by SDS-PAGE, blotted on to Immobilon and analysed by Western blotting. Cytosolic extracts were immunoprecipitated with anti-eIF4GI (α-eIF4GI), anti-NS1 (α-NS1) or with pre-immune serum (Control) and the immunoprecipitates separated by SDS-PAGE. The filters were analysed by Western blotting using the antibodies described in Fig. 2. (B) Total cytoplasmic extracts (Total extracts) were prepared and processed as in (A). Cytosolic extracts were immunoprecipitated with anti-PABP1 (α-PABP) or with pre-immune serum (C) and the immunoprecipitates separated by SDS-PAGE, blotted to Immobilon and analysed by Western blotting as in Fig. 2. When indicated (+), the cytosolic extracts were treated with micrococcal nuclease (RNase) before the immunoprecipitation.
PABP1. To characterize this *in vivo* association further, we carried out coimmunoprecipitation assays to evaluate the presence of NS1 in PABP1 immunocomplexes derived from influenza virus-infected cells. The results are presented in Fig. 3(B). The presence of NS1 in the PABP1 immunocomplexes was evident (Fig. 3B, α-PABP). Since both, PABP1 and NS1 are poly(A)-binding proteins, we re-evaluated their *in vivo* association after micrococcal nuclease treatment to analyse the contribution of RNA in the interaction. Fig. 3(B, right) shows that these two proteins remained associated after RNase treatment (Fig. 3B, α-PABP, +). Taken together, these data suggest that NS1 interacts with PABP1 *in vivo*, in addition to its interaction with the translation initiation factor eIF4GI.

**NS1 and PABP1 interact directly**

Since both NS1 and PABP1 are RNA-binding proteins and both interact with eIF4GI, we set out to determine whether the association detected *in vivo* reflects a direct or an indirect interaction. PABP1 was expressed and purified from bacteria as a GST fusion protein and bound to glutathione–Sepharose as described in Methods. As a negative control, GST protein was expressed and purified in parallel. The Coomassie staining of purified GST and GST–PABP1 is shown in Fig. 4(A). NS1 protein was expressed and purified from bacteria as a His-tagged protein (Aragón et al., 2000) and used for pull-down experiments with GST- or GST–PABP1-containing resins. As an additional specificity control, we used His-tagged eIF4GI 157–550 protein, a fragment of eIF4GI that interacts with NS1 (Aragón et al., 2000) and does not contain the PABP1-binding domain (Imataka et al., 1998). The results are presented in Fig. 4(B). NS1 protein was retained by GST–PABP1-containing resin but not by GST-containing resin. On the other hand, GST–PABP1 resin was unable to retain His-tagged eIF4GI 157–550 protein. These results indicate that NS1–PABP1 binding does not require the presence of any other proteins. Additionally, the pull-down experiments were repeated with purified His–NS1 and GST–PABP1 extensively treated with RNase to eliminate non-specifically bound bacterial RNA. The results obtained clearly show that such treatment did not affect the NS1–PABP1 interaction and indicate that these two proteins are able to interact directly *in vitro* without the participation of other factors.

**Mapping the PABP1-binding domain in the NS1 protein**

NS1 interacts directly with PABP1 (see above) and eIF4GI factor (Aragón et al., 2000), the latter being recognized by NS1 sequences located between residues 81 and 113. Therefore, it was important to determine the PABP1-binding domain in the NS1 protein and to test whether both interactions were compatible or not. Thus, we carried out pull-down experiments with GST–PABP1–Sepharose and *in vitro*-labelled NS1 protein or mutants thereof. The results are shown in Fig. 5. An NS1 mutant containing the first 81 amino acids of the protein was positive for PABP1 interaction. Conversely, an NS1 mutant lacking the first 81 N-terminal amino acids had completely lost its PABP1-binding capacity. NS1 deletion mutants containing the 152 or 171 N-terminal amino acids were also positive in this assay. Although the binding activity of NS1 protein containing the first 81 amino acids was weaker than that obtained with mutants 172 or 152, the binding was reproducible. The mapping results of NS1–PABP1 binding described above indicate that distinct domains in the protein are responsible for the association with PABP1 and eIF4GI.
Mapping the NS1-binding domain in the PABP1 protein

Sequence analysis of the gene encoding PABP1 from several organisms has revealed that it contains four conserved RNA recognition motifs (RRMs I–IV). Each RRM consists of two short stretches of conserved amino acids within a unit length of 90–100 residues. Downstream of the tandem array of RRMs, PABP1 contains a less well-conserved proline-rich C-terminal portion of variable length (Adam et al., 1986; Grange et al., 1987; Sachs et al., 1986; Zelus et al., 1989).

Studies in several species have revealed that RRMs I and II bind poly(A) sequences with high affinity (Burd et al., 1991; Kühn & Pieler, 1996). Furthermore, the region comprised by RRM I and II supports interaction with eIF4GI and its homologue in humans, eIF4GII (Imataka et al., 1998), while this binding activity is carried out by RRM II in Saccharomyces cerevisiae (Kessler & Sachs, 1998; Sachs et al., 1986). On the other hand, RRM III and IV also possess RNA-binding activity but their affinity for poly(A) sequences is lower than that observed for RRM I and II (Burd et al., 1991; Kühn & Pieler, 1996). The C-terminal part of PABP1 forms specific homophilic protein–protein interactions (Kühn & Pieler, 1996) and contains the cleavage site for coxsackievirus and enterovirus 2A proteases, which may be involved in the shutoff of cellular protein synthesis (Joachims et al., 1999; Kerekatte et al., 1999). A scheme showing the organization of the human PABP1 is shown in Fig. 6(A).

In order to analyse the region of PABP1 involved in NS1-binding activity, a set of GST–PABP1 deletion mutants was used to evaluate their ability to bind purified His–NS1 protein (Fig. 6). GST protein and GST–PABP1 mutants purified from bacteria were bound to glutathione-containing resins and used for pull-down experiments with purified His–NS1 (Fig. 6B). The left panel shows the accumulation of the different mutants used in the assay and the panel on the right shows the NS1 retention in the different mutant-containing resins. PABP1 mutants lacking the first 307 or 365 N-terminal amino acids were positive for NS1 interaction. In contrast, mutants containing the first 234 or 319 amino acids were either negative (GST–PABP1 234) or weakly positive (GST–PABP1 319). Furthermore, a deletion mutant containing the last 98 amino acids was also unable to interact. These results indicate that the NS1-interacting domain resides between amino acids 365 and 535 of PABP1, a region that does not contain any RNA-binding activity.

These data, together with the NS1–PABP1 interaction observed after RNase treatment in both in vivo (Fig. 3) and in vitro (Fig. 4) assays, indicate that an RNA bridge is not responsible for NS1–PABP1 binding, although RNA could modulate their interaction.

DISCUSSION

Virus–cell interactions affecting translational factors

Viruses have developed different strategies to positively regulate the translation of their mRNAs using translation factors as targets. Picornavirus infection induces the cleavage of eIF4GI protein and leads to a drastic inhibition of translation of cellular capped mRNAs, whereas translation of picornavirus uncapped mRNAs is unaffected (Etchison et al., 1982). eIF4GI cleavage generates a C-terminal fragment able to carry out picornavirus mRNA internal initiation. This process involves the direct entry of the 43S initiation complex to the initiation codon, mediated by a cis-acting element known as the internal ribosome entry site (IRES) (Jackson & Kaminski, 1995). The initiation process depends on IRES binding to translation factors. Thus, the IRES from pestiviruses and hepatitis C viruses interacts directly with eIF3 factor (Buratti et al., 1998; Pestova et al., 1998). In contrast, encephalomyocarditis virus requires initiation factors eIF4A, eIF4B, eIF4GI, eIF3 and eIF2 to mediate IRES-dependent initiation (reviewed in Pestova & Hellen, 1999). The IRES element of foot-and-mouth disease
virus binds to eIF4B and eIF4GI translation proteins (Lopez de Quinto & Martinez-Salas, 2000; Meyer et al., 1995). Recently, it has been reported that point substitutions in the IRES that decrease its interaction with eIF4GI lead to severe reduction of IRES activity in vivo (Lopez de Quinto & Martinez-Salas, 2000), indicating that eIF4GI acts as a linker to recruit the translational machinery in IRES-dependent initiation. PABP1 also plays a role in the shutoff of cellular protein synthesis during picornavirus infection.

A significant body of evidence indicates that eIF4GI cleavage is only partially responsible for the shutoff of host protein synthesis. Thus, different treatments can prevent the host translation shutoff but not eIF4GI cleavage (Bonneau & Sonenberg, 1987; Irurzun et al., 1995), and complete cleavage of eIF4GI results in a reduction but not elimination of cellular protein synthesis (Keiper & Rhoads, 1997; Lamphear & Rhoads, 1996). During coxsackievirus infection, PABP1 is proteolytically cleaved at a specific site.

![Fig. 6. Mapping of the NS1-interacting domain in the PABP1 protein. (A) Scheme of the PABP1 functional domains and the GST–PABP1 deletion mutants used in (B). (B) The C-terminal domain of PABP1 interacts with NS1. Left panel: Coomassie staining of the GST–PABP1 proteins expressed and purified from bacteria. Right panel: purified His–NS1 was incubated with matrices containing GST or GST–PABP1 deletion mutants. After incubation and extensive washing, the retained protein was analysed by SDS-PAGE and blotted on to Immobilon. The filters were analysed by Western blotting with anti-His peroxidase-conjugated serum (Ab-HisPx).]
separating the N-terminal RRMAs from the C-terminal homodimerization domain (Kerekatte et al., 1999). This cleavage correlates better with the timing of host translational shutoff than does the cleavage of eIF4GI (Kerekatte et al., 1999). PABP1 is also degraded during poliovirus infection and this degradation induces a concurrent loss of translational activity in vitro (Joachims et al., 1999).

Rotaviruses have a segmented genome consisting of 11 molecules of double-stranded RNA. Their mRNAs are capped but non-polyadenylated (Estes & Cohen, 1989). The viral mRNAs have 5′- and 3′UTRs of variable length that are flanked by two different sequences common to all rotavirus genes. NSP3 is a non-structural protein that plays a critical role in translation regulation of rotavirus mRNAs. The protein can be cross-linked to the consensus sequence located at the 3′ end and is communoprecipitated with eIF4GI factor (Piron et al., 1998). NSP3 interacts with the same region of eIF4GI that interacts with PABP1 (Piron et al., 1998). As a consequence, during rotavirus infection, PABP1 dissociates from eIF4GI, probably impairing the translation of polyadenylated mRNA and leading to the cellular shutoff. The enhancement of viral mRNA translation by NSP3 has also been observed in vitro, and the phenotype of NSP3 mutants has shown that both its RNA- and eIF4GI-binding domains are required to enhance the translation of viral mRNAs (Vende et al., 2000), indicating that interaction with these molecules is required to obtain a fully translational activation.

Picornaviruses and rotaviruses have both developed mechanisms to ensure efficient viral protein synthesis, concomitant with an impairment of endogenous protein translation. In both cases, an appropriate virus translation correlates with an efficient eIF4GI–viral element interaction. These regulatory mechanisms involve changes in the normal composition of the translation machinery, cleavage of eIF4GI and PABP1 proteins during picornavirus infection or the release of the eIF4GI-bound PABP1 protein during rotavirus infection. These data suggest that modulation of these two factors or a coordinate regulation of both proteins could be sufficient to regulate translation. These changes finally give rise to a drastic reduction in the translation of cellular mRNAs that are 5′ capped and 3′ polyadenylated and require fully active translation initiation complexes.

NS1 associates with translation initiation factors

Influenza virus mRNAs are formally equivalent to the endogenous mRNAs and a different strategy has been selected by the virus to enhance specifically the translation of viral mRNAs. Previous work has demonstrated that NS1 protein interacts directly and specifically with eIF4GI factor in vitro and that they associate in vivo during influenza virus infection in what appears to be a feature common to other viruses to improve virus translation (Aragón et al., 2000). The results presented in this report indicate that NS1 and PABP1 also interact directly in vitro and are associated in the infection (Figs 3 and 4). Mapping studies have shown that the interactions among NS1, eIF4GI and PABP1 are compatible. Thus, the eIF4GI- and PABP1-interacting domains map to positions 81–113 and 1–81 of the NS1 protein, respectively. The PABP1-interacting domain in eIF4GI maps to position 132–160 (Imataka et al., 1998), whereas residues 157–550 are involved in NS1 interaction (Aragón et al., 2000). Finally, eIF4GI-binding domain is located in the region 1–175 of PABP1 (Imataka et al., 1998), while residues 365–535 are required for NS1 interaction. Although NS1 also interacts with a different poly(A)-binding protein, PABII, amino acids 223–237 of NS1 are required for this interaction. PABII is a nuclear protein required for the processive elongation of poly(A) chains catalysed by the poly(A) polymerase, which does not share sequence homology with the cytoplasmic PABP1 (Chen & Krug, 1999; Nemeroff et al., 1998; Li et al., 2001). Sequence comparison has shown that PABP1 is very conserved among different species, from yeast to humans (Burd et al., 1991). The highest homology is found in the RRMs and the last C-terminal 75 amino acids (more than 94 % identity). Several proteins interact at the C-terminal conserved region of PABP1. Among them, Paip1 stimulates translation (Craig et al., 1998) and Paip2 competes with Paip1 for binding to PABP1 and represses translation (Khaleghpour et al., 2001). The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) (Hoshino et al., 1999) and the polymerase of zucchini yellow mosaic potyvirus (Wang et al., 2000a) also bind to PABP1. Interestingly, NS1 binds to PABP1 in a non-conserved region different from that used by the other PABP1-interacting proteins (Roy et al., 2002). The possibility exists that the NS1–PABP1 interaction is restricted to species to which influenza virus has become adapted during evolution.

The interaction between eIF4GI and PABP1, which facilitates translation initiation of polyadenylated mRNAs in yeast and stimulates translation in Xenopus oocytes and mammalian cells (Gray et al., 2000; Imataka et al., 1998; Sachs et al., 1986; Tarun & Sachs, 1996; Tarun et al., 1997; Wakiyama et al., 2000), may be reinforced by the cross-interaction of both factors with NS1 protein in influenza virus-infected cells. Furthermore, the communoprecipitation of viral mRNAs, but not cellular mRNAs, with anti-NS1 antibodies (Fig. 1) strongly supports previous data indicating a specific interaction of NS1 with viral mRNA (Katze et al., 1986; Marión et al., 1997b; Park & Katze, 1995) and suggests an NS1-mediated enhancement of virus translation. Virus-specific mRNAs would be efficiently associated with translation initiation complexes by the concurrent interaction of NS1 protein with their 5′ end as well as with eIF4GI and PABP1. These complexes would effectively recruit ribosomes to the 5′ end of viral mRNAs at the expense of cellular ones.

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