La protein is required for efficient translation driven by encephalomyocarditis virus internal ribosomal entry site

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Translation of internal ribosomal entry site (IRES)-dependent mRNAs is mediated by RNA-binding proteins as well as canonical translation factors. In order to elucidate the roles of RNA-binding proteins in IRES-dependent translation, the role of polypyrimidine tract-binding protein (PTB) and La protein in encephalomyocarditis virus (EMCV) IRES-dependent translation was investigated. PTB was required for efficient EMCV IRES-driven translation but, intriguingly, an excess of PTB suppressed it. Such a translational suppression by surplus PTB was relieved by addition of La protein. A possible role for La protein in IRES-dependent translation is discussed.

Introduction

Translation via internal ribosomal entry sites (IRES) of eukaryotic mRNAs was first discovered for picornaviral mRNAs (Jang et al., 1988, 1989; Pelletier & Sonenberg, 1988). Ever since, the list of mRNAs containing IRES elements has been rapidly expanding and includes viral mRNAs and cellular mRNAs (Gan & Rhoads, 1996; Bernstein et al., 1997; Macejak & Sarnow, 1991; Oh et al., 1992; Teerink et al., 1995; Tsukiyama-Kohara et al., 1992; Vagner et al., 1995). However, little is known about the detailed mechanism of IRES-dependent translation except that certain primary sequences and secondary/tertiary structures in the IRES elements are important for efficient translation.

The IRES-dependent translation requires most of the initiation factors used in cap-dependent translation (Pestova et al., 1996a, b; Scheper et al., 1992). However, it is generally believed that some RNA-binding proteins that specifically interact with IRES elements are also required for IRES-dependent translation. One such protein is a cytoplasmic RNA-binding protein of 57 kDa known as polypyrimidine tract-binding protein (PTB) or heterogeneous nuclear ribonucleoprotein (hnRNP) I. It has been suggested that PTB regulates pre-mRNA splicing in the nucleus (Gil et al., 1991; Patton et al., 1991) and enhances IRES-dependent translation of encephalomyocarditis virus (EMCV) mRNA in the cytoplasm (Borovjagin et al., 1994; Jang & Wimmer, 1990; Witherell et al., 1995).

Another cellular protein, human La autoantigen (also known as p52 and SS-B) has also been shown to stimulate IRES-dependent translation of poliovirus mRNA and hepatitis C virus (HCV) mRNA. Originally, La protein was identified as an autoantigen recognized by sera from patients with systemic lupus erythematosus and Sjögren’s syndrome (Tan, 1989). The La protein belongs to a group of RNA-binding proteins containing the RNA recognition motif (Kenan et al., 1991). La protein is involved in regulation of initiation and termination of transcription by RNA polymerase III (Gottlieb & Steitz, 1989a, b; Maraia et al., 1994; Maraia, 1996). In addition, La protein is associated with polymerase II RNA transcripts such as U1 RNA (Madore et al., 1984) as well as viral RNAs including Epstein–Barr virus-encoded RNAs (Toczyński & Steitz, 1991; Lee & Deng, 1992), adenovirus VA RNAs (Francoeur & Mathews, 1982), vesicular stomatitis virus leader RNAs (Kurilla & Keene, 1983), the 5’ NTR of picornaviruses (Meerovitch et al., 1993; Svitkin et al., 1994a), and the HIV TAR sequence (Chang et al., 1994). In the case of poliovirus and HCV, addition of purified La to rabbit reticulocyte lysates (RRL) stimulates virus IRES-dependent translation (Meerovitch et al., 1993; Svitkin et al., 1994a). The binding of La protein to the HIV TAR sequence alleviates the translational repression exerted by the TAR sequence on a downstream reporter gene (Svitkin et al., 1994b). All these observations support the conclusion that the La antigen has a role in the translational regulation of some mRNAs.

In this report, we confirm the translational enhancing effect of PTB on certain EMCV mRNAs by depleting the translation mixture of PTB and then re-adding purified PTB to it. Interestingly, surplus PTB reduces translation driven by EMCV
IRES. The inhibitory effect of surplus PTB can be alleviated by the addition of purified La protein. PTB outcompetes La protein in the binding to the same site(s) on the EMCV IRES. Possible roles of PTB and La protein in EMCV IRES-dependent translation are discussed.

Methods

- **Plasmid construction.** Enzymes used for cloning and modification of DNA were purchased from New England Biols and Boehringer Mannheim. Plasmid pSK(+)/CATA-ECAT was constructed by replacing the Bip IRES sequence of pSK(+) /CATA-BCAT (Y. K. Kim, B. Hahn & S. K. Jang, unpublished data) with the EMCV 5’ NTR sequence from pBS-ECAT (Jang & Wimmer, 1990). Upon in vitro transcription by T7 RNA polymerase, plasmid pSK(+)/CATA-ECAT produces a dicistronic mRNA containing a CAT gene with a truncated C terminus followed by the EMCV IRES and a full-length CAT gene.

- **In vitro transcription.** Plasmid DNAs were purified by the polyethylene glycol precipitation method (Sambrook et al., 1989) and then linearized with appropriate restriction enzymes. The linearized DNAs were extracted with phenol–chloroform and ethanol-precipitated. Transcription was performed by incubation with T7 RNA polymerase (Boehringer Mannheim) for 90 min at 37°C as described by the manufacturer. To yield capped mRNA, 1 mM m7GpppG (Pharmacia Biotech) was included in the transcription reaction mixture. The concentration of the RNA transcripts was determined by UV spectrophotometry. BamH I-digested pSK(+)/CATA-ECAT DNA was used to produce the dicistronic mRNA (CATA-EMCV IRES-CAT). 32P-Labelled EMCV IRES (nt 260–488) or a full-length EMCV IRES probe was produced by in vitro transcription of pBS-ECAT digested with HindIII or Boll in the presence of [α-32P]UTP (NEN).

- **Depletion of endogenous PTB from RRL and HeLa lysate.** Partial depletion of endogenous PTB from the micrococcal nuclease-treated RRL (Promega) was performed as described by Niepmann (1996). RRL (1 ml) was adjusted with 250 mM potassium acetate and incubated with 75 µl poly(U)-Sepharose (Pharmacia Biotech) for 30 min at 4°C with gentle agitation. The resin was then removed by centrifugation. This step was repeated twice. Cytoplasmic S-10 extracts of HeLa S3 cells were prepared as described by Oh et al. (1998). The endogenous PTB from HeLa S3 extracts was removed by poly(U)-Sepharose as a step in the depletion of the RRL.

- **In vitro translation.** In vitro translation in the RRL was performed in 20 µl reaction mixtures including control or depleted RRL plus mRNA at a final concentration of 6 nM. Translation reactions of EMCV mRNAs in the HeLa cytoplasmic extracts were performed in 12.5 µl translation mixture containing 40 nM mRNA as described by Rose et al. (1978). Translation reactions were carried out for 1 h at 30°C in the presence of [35S]methionine (NEN). Translation products were analysed by SDS–PAGE. The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. The gel was dried and exposed to Kodak XAR-5 or Agfa Curix RP1 for 12–18 h. Efficiency of the translation was measured with a densitometer (Bioimage 50S Series; B. I. System) or phosphorimager.

- **Protein purification.** Human La cDNA from HeLa total mRNA was cloned in pGEX-KG using the RT–PCR method. La protein was produced in E. coli cells and the cell pastes were harvested and resuspended in lysis buffer (20 mM sodium phosphate (pH 7.2), 10 mM EDTA, 10 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 300 mM NaCl). After lysis, pre-swollen glutathione Sepharose beads (Pharmacia Biotech) were incubated with the cell extracts for 2 h at 4°C. After centrifugation, the pelleted beads were washed three times with lysis buffer. To cleave the La protein from GST–La, the resuspended pellet beads were incubated with thrombin in cleavage buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2.5 mM CaCl2, 0.1% DTT] for 3 h at room temperature. Glutathione Sepharose beads were removed by centrifugation, and then the supernatant containing the cleavage product of GST–La was loaded onto a glutathione Sepharose 4B column equilibrated with lysis buffer. The flowthrough from the glutathione Sepharose 4B column containing most of the La was applied directly to a poly(U)-Sepharose column (Pharmacia Biotech). Bound La was eluted with a linear gradient of 0–1–2 M NaCl. Fractions were analysed on a gel and those containing La were pooled and dialysed against LD buffer [10 μM HEPES–KOH (pH 7.5), 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 10% glycerol]. The purification steps for recombinant PTB have been described elsewhere (Oh et al., 1998).

- **UV cross-linking of labelled RNAs and proteins.** UV cross-linking reactions were performed essentially as described by Meerovitch et al. (1989) with slight modifications. RNA probes (2 x 105 to 4 x 105 c.p.m.) purified with push columns (Stratagene) were incubated at 30°C for 30 min with 75 µg RRL or 40 µg HeLa extract in 30 µl reaction mixture. After RNA binding, the reaction mixtures were irradiated with UV light on ice for 30 min using a UV-Stratalinker (Stratagene). Unbound RNA was removed by digestion with 20 µg RNase A, 200 U RNase T1 and 1 U RNase V1 (cobra venom nuclease; Pharmacia Biotech) at 37°C for 20 min. The RNA–protein complexes were analysed by 12% or 15% SDS–PAGE followed by autoradiography.

- **Western blot analysis.** HeLa extracts or RRL were resolved by 12% SDS–PAGE and then transferred to a nitrocellulose paper (Amersham). The membrane was blocked overnight with 5% skimmed milk in TBS buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Tween 20) and then incubated with anti-La MAb (3B9) for 2 h. The antibody was kindly provided by M. Bachmann, Institut für Physiologische Chemie, Johannes-Gutenberg Universität, Germany. Horse-radish peroxidase-conjugated anti-mouse IgG was used as secondary antibody. To visualize bands, the membrane was developed with the ECL method following the supplier's instructions (Amersham).

Results

PTB protein enhances EMCV IRES-dependent translation

EMCV mRNA is efficiently translated in RRL containing a lot of endogenous PTB (Jang & Wimmer, 1990; Luz & Beck, 1990). The requirement of PTB in the translation driven by EMCV IRES has been well documented by depletion and re-addition experiments (Kaminski et al., 1995). We repeated the depletion and re-addition experiment to confirm the requirement of PTB in the EMCV IRES-dependent translation. Poly(U)–Sepharose was used for depletion of endogenous PTB in the RRL. The level of the depletion of endogenous PTB was monitored by a UV cross-linking assay with 32P-labelled full-length EMCV IRES (Fig. 1a, lanes 1 and 2). PTB remained below the detection level in the poly(U)-depleted RRL (Fig. 1a, lane 2). The translational activity of the PTB-depleted RRL was...
ECMV IRES-dependent translation requires La protein.

**Fig. 1.** Effects of PTB and La on EMCV IRES-dependent translation in depleted RRL. Schematic diagram of the dicistronic mRNA (CATΔ-ECAT) is shown. CAT and CATΔ represent the full-length CAT gene and the C terminus-truncated CAT gene, respectively. The positions of the 7-methyl guanosine cap structure (m7GpppG) and the EMCV IRES are indicated. (a) Effect of PTB depletion on EMCV IRES-dependent translation. RRL was treated with poly(U)-Sepharose resin. The presence of PTB was investigated by UV cross-linking to 32P-labelled RNA corresponding to full-length EMCV IRES. Labelled proteins from 75 µg control RRL (lane 1) or poly(U)-depleted RRL (lane 2) were analysed by 12% SDS–PAGE. Translational efficiencies of CATΔ-ECAT mRNA (final concentration 6 nM) in control and poly(U)-depleted RRL are shown in lanes 3 and 4, respectively. (b) Effects of purified PTB and La on EMCV IRES-dependent translation. The capped dicistronic mRNAs (CATΔ-ECAT) shown in the upper panel in (a) were expressed either in the control (lane 1) or the PTB-depleted RRL (lanes 2–16). The amounts of PTB added to the translation reaction mixtures are indicated at the top of the panel. La or BSA (1 µg) was added to the translation reaction mixtures with PTB (lanes 7–10, 12–15, respectively) or without PTB (lanes 11 and 16, respectively). No protein was added to the reactions in lanes 1 and 2. CAT and CATΔ gene products are indicated by arrows. The intensities of the bands were measured using a phosphorimager. The relative translational efficiencies were normalized, taking the relative ratio of CAT/CATΔ product in the control RRL as 100%.

**Fig. 2.** Effect of La on EMCV IRES-dependent translation in poly(U)-depleted RRL. The effect of La on translation was investigated (a) without, (b) with 4 ng or (c) with 250 ng PTB. A capped dicistronic mRNA (CATΔ-ECAT) was used in the control (lane 1 in each panel) or poly(U)-depleted RRL (other lanes in each panel). The amounts of exogenously added PTB, La, and BSA are indicated at the top of each panel. CAT and CATΔ gene products are indicated by arrows. Relative translational efficiencies were normalized (see legend to Fig. 1).
Y. K. Kim and S. K. Jang tested using capped dicistronic mRNA (CAT-ECAT). This mRNA contains a CAT gene with a truncation at the C-terminus (CATΔ) translated by ribosome scanning, and a full-length CAT that is translationally directed by the EMCV IRES (Fig. 1a, upper panel). In the depleted RRL, the translational efficiency of CAT driven by the EMCV IRES was drastically reduced (6–4-fold), whereas CATΔ expression was only slightly reduced (1–4-fold) (Fig. 1a, compare lanes 3 and 4).

**Surplus PTB reduces translation driven by EMCV IRES in poly(U)-depleted RRL**

The effect of PTB on translation driven by EMCV IRES was also investigated by addition of purified PTB in poly(U)-depleted RRL. Addition of up to 100 ng purified PTB to the depleted RRL gradually increased translational activity driven by EMCV IRES (Fig. 1b, lanes 2–5). Full activity for internal initiation was restored by adding 100 ng purified recombinant PTB, which is close to physiological concentration (about 5 µg/ml; Kaminski et al., 1995). To our surprise, further addition of PTB to the depleted RRL reduced translation driven by EMCV IRES drastically (Fig. 1b, lane 6). Translation of cap-dependent mRNA (CATΔ) was not affected by the addition of PTB (Fig. 1b, lanes 1–6).

**La protein alleviates the inhibitory activity of excess PTB**

It has been suggested that La protein binds to poliovirus and EMCV IRES and plays an important role in poliovirus-dependent translation (Meerovitch et al., 1993; Svitkin et al., 1994a; Witherell & Wimmer, 1994). We investigated the effect of La protein on translation driven by EMCV IRES in dicistronic configuration [Fig. 1a, upper panel (CATΔ-ECAT)]. The identity of purified La was confirmed by Western blot analysis using a MAb against human La (3B9; data not shown). We also confirmed that La protein bound strongly to HCV 5’ NTR, enhancing internal initiation via HCV IRES (data not shown; Ali & Siddiqui, 1997). The addition of 1 µg purified recombinant La to the poly(U)-depleted RRL alleviated the inhibitory effect of PTB on EMCV IRES-dependent translation (Fig. 1b, compare lanes 6 and 10). BSA, a negative control protein, did not alleviate this inhibitory effect (Fig. 1b, lane 15). La protein by itself did not enhance the translation driven by EMCV IRES in poly(U)-depleted RRL (Fig. 2a) or in the presence of 4 ng PTB (Fig. 2b). Translational activation by La protein in the presence of excess PTB (250 ng) was dosage-dependent (Fig. 2c, compare lanes 3–7). The more specific depletion of endogenous PTB in the RRL was performed by using biotinylated RNA corresponding to EMCV IRES (nt 260–488) which strongly binds to PTB; the same interplay between PTB and La protein was also observed (data not shown).

Intriguingly, addition of PTB to undepleted RRL did not affect the translation driven by EMCV IRES (Fig. 3a). This may suggest the presence of a cellular factor(s), which alleviates the inhibitory activity of PTB in the undepleted RRL, and it excludes the possibility of the existence of a translational inhibitor in the purified PTB solution. The addition of purified La protein to undepleted RRL did not affect either cap-dependent translation or IRES-dependent translation (Fig. 3b). These data indicate that La protein, or a putative factor

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**Fig. 3. Effects of purified PTB and La on EMCV IRES-dependent translation in undepleted RRL.** (a) Effect of PTB on cap- and EMCV IRES-dependent translation in control RRL. A capped CATΔ-ECAT mRNA was used in the translation reaction in the control RRL. The amounts of PTB added to the translation reaction mixtures are indicated on the top of the panel. (b) Effect of La on cap- and EMCV IRES-dependent translation in control RRL. Capped CATΔ-ECAT mRNA at 1 nM (lanes 1–4) or 6 nM (lanes 5–8) concentration was used in the translation reactions. The amounts of La added to the translation reaction mixtures are indicated on the top of the panel. No exogenous La protein was added in lanes 1 and 5. The CAT and CATΔ gene products are indicated by arrows.
related to La protein activity, exists in undepleted RRL in amounts sufficient to prevent inhibition by surplus PTB.

The effect of PTB and La protein on translation driven by EMCV IRES was also investigated using HeLa cell extract. The level of depletion of endogenous PTB in the HeLa extract by poly(U)-Sepharose was monitored by the UV cross-linking method with \(^{32}\)P-labelled full-length EMCV IRES or EMCV IRES spanning nts 260–488 (Jang et al., 1989). Less than 1% of PTB remained in the poly(U)-depleted HeLa cell extract (Fig. 4, compare lanes 1 and 3 with lanes 2 and 4). The efficiency of CAT translation via EMCV IRES was drastically reduced (6- to 6-fold), whereas the expression of CATΔ, which is directed by scanning, was only slightly reduced (1- to 3-fold) after poly(U) depletion (Fig. 4, compare lanes 5 and 7 with lanes 6 and 8). Translation driven by EMCV IRES remained the same regardless of the mRNA concentration in poly(U)-depleted HeLa extract (Fig. 4, lanes 6 and 8). On the other hand, translation driven by EMCV IRES exhibited the mRNA concentration-dependency in the poly(U)-untreated extract. Moreover, addition of purified PTB to the poly(U)-depleted HeLa cell extract partially restored the translational efficiency of CAT driven by the EMCV IRES (Fig. 5). Interestingly, translation driven by EMCV IRES was not reduced by excess of PTB even though the HeLa extract had been depleted by poly(U)-resin treatment (Fig. 5, lanes 6 and 7).

In order to understand the reason for the discrepancy between the RRL and HeLa cell extract, we compared the La protein level in RRL and HeLa cell extract using Western blot analysis with an anti-La MAb (3B9) (Fig. 6). At least 20-fold or higher levels of La protein were detected in the HeLa cell extract compared to levels in the RRL (Fig. 6, compare lanes 5–8 with lanes 9–11). The level of La protein in RRL was slightly reduced by poly(U) depletion (Fig. 6, compare lanes 1 and 3 with lanes 2 and 4). Much more La protein existed in the HeLa cell extract than in the RRL, regardless of poly(U) treatment, which is consistent with previous observations (Meerovitch et al., 1989, 1993; Svitkin et al., 1994a). Thus, the high level of La protein in HeLa cells may have prevented the reduction of EMCV mRNA translation by excess PTB in the poly(U)-depleted HeLa extract (Fig. 5).

**PTB inhibits binding of La protein to the EMCV IRES**

The binding patterns of PTB and La protein to the EMCV IRES were investigated using the UV cross-linking technique...
Fig. 6. Levels of La protein in RRL and HeLa extracts before and after poly(U) depletion. Western blot analyses were performed with MAb against human La (3B9). RRL (lanes 1–4 and 9–11) or HeLa extracts (lanes 5–8) were used as protein sources with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5, 7 and 9–11) poly(U) depletion. Amounts of loaded proteins are indicated at top of the panel. The La protein is indicated by an arrow.

Fig. 7. PTB competition with La protein for binding to EMCV IRES. The IRES binding efficiency of purified PTB and La was monitored by UV cross-linking assay. 32P-Labelled full-length EMCV IRES was used as probe. The amounts of protein used in the reactions are indicated at the top of the panel. Molecular mass size markers are indicated on the left side of the panel. The positions of PTB and La are indicated by arrows.

Discussion

It has been shown that more than 400 nt are required for full activity of EMCV IRES (Jang & Wimmer, 1990). This is by far enough for the binding of several proteins. In fact, several cellular proteins such as PTB have been reported to bind to EMCV IRES (Borovjagin et al., 1994; Jang et al., 1988, 1989; Jang & Wimmer, 1990; Kolupaeva et al., 1996; Witherell & Wimmer, 1994). However, the roles of these proteins in translation remain obscure. Here, we report on the effects of PTB and La protein on translation driven by EMCV IRES, which provides some clues towards the understanding of the functions of these proteins in translation. PTB was required for efficient translation driven by EMCV IRES. Surplus PTB, on the other hand, reduced translation driven by EMCV IRES in poly(U)-depleted RRL. The translational inhibition by excess PTB could be alleviated by addition of La protein (Fig. 1b). Restoration of EMCV mRNA translation by La protein seems to differ from non-specific translational activation of cap-dependent mRNA by La protein as shown by Svitkin et al. (1996). These authors have shown activation of cap-dependent mRNA by general RNA-binding proteins. However, La protein and PTB, which do possess RNA-binding activity, had little influence on cap-dependent translation under the reaction conditions used in this study. Moreover, unlike La protein, hnRNP E2 (an RNA-binding protein) failed to restore EMCV IRES-dependent translation in the presence of surplus PTB (data not shown). These data therefore indicate that La protein is involved in the translation driven by EMCV IRES in a specific manner.
PTB may enhance EMCV IRES-dependent translation by facilitating the RNA to assume a proper conformation of the IRES. In this case, PTB may function as a scaffolding protein holding RNA segments in a certain conformation. Several biochemical properties support this hypothesis. Firstly, deletion mutants of PTB that bound to the EMCV IRES with wild-type affinity and specificity did not enhance EMCV translation (Kaminski et al., 1995). Secondly, PTB binds to several sites on the EMCV IRES (Jang & Wimmer, 1990; Kolupaeva et al., 1996; Witherell et al., 1993, 1995; Witherell & Wimmer, 1994). Lastly, PTB can form oligomers in solution and it contains several RNA-binding domains (Oh et al., 1998; Perez et al., 1997).

Based on the biochemical properties of PTB and La protein, we can speculate how excess PTB may inhibit translation of EMCV IRES-dependent translation and how La protein could alleviate the inhibitory effect. Surplus PTB could be deleterious to IRES function, since non-specific binding of the excess PTB to the IRES element could inhibit IRES function by blocking the binding of other essential cellular factor(s). One of the possible essential factors is La protein, which was shown to play an important role in picornavirus IRES-dependent translation (Svitkin et al., 1994a). Purified La protein bound well to the EMCV IRES (Fig. 7, lane 6), and PTB inhibited La protein binding to the EMCV IRES (Fig. 7, lanes 2 and 3). In such a scenario, inhibition and restoration of translation by PTB and La protein could be explained as follows. Residual La protein in the poly(U)-depleted RRL may not be enough to compete with the excess PTB for RNA-binding. Addition of excess La protein to the translation mixture lets translation resume because of adequate competition for RNA binding with the surplus PTB. In this case, La protein plays an active role in EMCV IRES-dependent translation. Alternatively, La protein may play a passive role in EMCV IRES-dependent translation by preventing abnormal binding of PTB to the EMCV IRES with its RNA helicase activity (Bachmann et al., 1990; Huhn et al., 1997; Xiao et al., 1994). Prevention of abnormal binding of PTB to the EMCV IRES may thus result in the alleviation of the inhibitory effect of PTB.

Rather large quantities of purified La protein were required for the restoration of the EMCV IRES-dependent translation (Fig. 2c). This may have been due to the specific activity of the La protein expressed in E. coli. The purified protein may not have possessed full activity possibly because of a lack of modification or for other reasons. Alternatively, the limiting factor removed in the poly(U)-depleted RRL may not be the La protein at all, but a factor related to the activity of La protein. This putative factor may enhance La protein activity by facilitating RNA binding of La protein to a specific site. The detailed activational mechanism of PTB and La protein in translation remains to be elucidated.

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