Polypyrimidine tract-binding protein is relocated to the cytoplasm and is required during dengue virus infection in Vero cells

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The 3′ untranslated region (3′UTR) of the dengue virus (DENV) genome contain several sequences required for translation, replication and cyclization processes. This region also binds cellular proteins such as La, polypyrimidine tract-binding protein (PTB), Y box-binding protein 1, poly(A)-binding protein and the translation initiation factor eEF-1α. PTB is a cellular protein that interacts with the regulatory sequences of positive-strand RNA viruses such as several picornaviruses and hepatitis C virus. In the present report, it was demonstrated that PTB translocates from the nucleus to the cytoplasm during DENV infection. At 48 h post-infection, PTB, as well as the DENV proteins NS1 and NS3, were found to co-localize with the endoplasmic reticulum marker calnexin. Silencing of PTB expression inhibited virus translation and replication, whilst overexpression of PTB augmented these processes. Thus, these results provide evidence that, during infection, PTB moves from the nucleus to the cytoplasm and plays an important role in the DENV replicative cycle.

INTRODUCTION

Dengue virus (DENV), a member of the family Flaviviridae, is the causative agent of dengue fever, dengue haemorrhagic fever and dengue shock syndrome. The single-stranded, positive-polarity RNA genome of approximately 11 kb contains a type I cap at the 5′ end and lacks a poly(A) tail at the 3′ end. The single open reading frame (ORF) encodes a polyprotein that generates three structural proteins – envelope (E), membrane and capsid (C) – and seven non-structural proteins – NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Flanking the ORF, the viral RNA contains two untranslated regions (UTRs) involved in various functions such as initiation and regulation of virus translation, replication and assembly (Lindenbach & Rice, 2001; Proutski et al., 1999). The 3′UTR of DENV and other mosquito-borne flaviviruses contains a conserved stem–loop structure within the last ~96 nt (Brinton & Dispoto, 1988; Brinton et al., 1986; Grange et al., 1985; Mohan & Padmanabhan, 1991). Additionally, there are two pairs of conserved sequences (5′CS1, 3′CS1, 5′UAR and 3′UAR) that together induce DENV cyclization (Alvarez et al., 2005a, b; Hahn et al., 1987). These motifs are essential for negative-strand RNA synthesis of DENV (Ackermann & Padmanabhan, 2001; Alvarez et al., 2005a, b; Villordo & Gamarnik, 2009; You & Padmanabhan, 1999) and other flaviviruses (Bredenbeek et al., 2003; Corver et al., 2003; Jones et al., 2005; Khromykh et al., 2001; Lo et al., 2003; Nomaguchi et al., 2004). On the other hand, sequences present within a large stem–loop structure located at the 5′ end as well as a conserved oligo(U) track function as the promoter for viral polymerase activity (Lodeiro et al., 2009). Moreover, an RNA secondary structure present in the coding region of DENV type 2 (DENV-2) directs translation, start-codon selection and replication of the viral genome (Clyde & Harris, 2006; Clyde et al., 2008). Although it has been shown that the cyclization process does not require the presence of cellular or viral proteins, it is likely that viral and cellular proteins are actually required as trans-acting factors to facilitate or regulate viral replication.

Translation and replication of flaviviruses occurs in close association with cellular membranes (Mackenzie et al.,
et al., 2009; Westaway et al., 1997, 1999, 2003). The viral proteins associated with the replication complex are the two hydrophobic viral proteins, NS2A and NS4A, the abundant luminal protein NS1, and the NS5 and NS3 proteins. NS5 has methyltransferase and RNA-dependent RNA polymerase activities, whilst NS3 is a serine protease, NTPase/RNA helicase and 5′ RNA triphosphatase (for a review, see Lindenbach & Rice, 2001; Luo et al., 2008). Consistent with their putative role in viral replication, both NS3 and NS5 exist as a complex in DENV-infected cells (Kapoor et al., 1995) and interact with the 3′- and 5′UTR of Japanese encephalitis virus and DENV RNA, respectively (Chen et al., 1997; Filomatori et al., 2006; Lodeiro et al., 2009; Luo et al., 2008). Additionally, some sequences present in the 3′- and 5′UTR are able to interact with cellular proteins such as La, polypyrimidine tract-binding protein (PTB), translation elongation factor 1α, Y box-binding protein 1, heterogeneous nuclear ribonucleoproteins (hnRNPs) A1, A2/B2 and Q, protein disulfide isomerase, poly(A)-binding protein (PABP) and calreticulin (De Nova-Ocampo et al., 2002; García-Montalvo et al., 2004; Paranjape & Harris, 2007; Polacek et al., 2009; Yocupicio-Monroy et al., 2003, 2007).

Although the specific functions of all of these cellular proteins is unclear, in other viruses it has been described in detail that they participate in RNA stability, as well as in translation and replication of the viral genome (Bushel & Sarnow, 2002; Shi & Lai, 2005). PTB, a 57 kDa protein (also called hnRNPI), is a member of the hnRNP family and contains four RNA-recognition motifs. This protein functions in a large number of cellular processes including pre-mRNA splicing and mRNA localization and stabilization (Sarnow, 2002; Shi & Lai, 2005). PTB, a 57 kDa protein (also called hnRNPI), is a member of the hnRNP family and contains four RNA-recognition motifs. This protein functions in a large number of cellular processes including pre-mRNA splicing and mRNA localization and stabilization (Sarnow, 2002; Shi & Lai, 2005). PTB has been implicated in the translation and replication of different viruses, including other flaviviruses such as hepatitis C virus (HCV) (Aizaki et al., 2006; Ali & Siddiqui, 1995; Gutiérrez et al., 1997; Sawicka et al., 2008). Moreover, a phosphorylated form of PTB was found associated with the membrane-bound HCV replication complex (Chang & Luo, 2006). The protein achieves all of these functions alone or in the presence of other viral or cellular proteins. PTB, a multifunctional protein, binds to the 3′ stem–loop region of DENV, which is known to play an important role during virus translation and replication. In the present report, we have demonstrated that PTB translocates from the nucleus to the cytoplasm during DENV infection in Vero cells. This translocation was only observed in Vero cells; in Huh7 cells, as described previously, only a partial translocation of PTB from the nucleus to the cytoplasm was detected (Anwar et al., 2009; Jiang et al., 2009), suggesting that some cell-type-specific factors present in Vero cells might be conditioning the massive translocation of PTB. At 48 h post-infection (p.i.), PTB and DENV NS1 and NS3 proteins were found to co-localize with the endoplasmic reticulum (ER) marker calnexin. Silencing of PTB inhibited DENV translation and replication, whilst its overexpression augmented translation and replication of the virus. In summary, our results provide evidence that, during infection, PTB moves from the nucleus to the cytoplasm and plays an important role in the DENV replicative cycle.

**METHODS**

**Cells and viruses.** Vero and Huh7 cells were grown in Advanced Medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) at 37 °C in a 5 % CO2 humidified atmosphere. DENV-2 was propagated in suckling mice brains, as described previously (Gould & Clegg, 1991).

**Immunofluorescence.** To evaluate intracellular PTB localization, cells infected with DENV-2 were processed for immunofluorescence at 24 or 48 h p.i. Cells were washed with PBS, fixed with 4 % paraformaldehyde for 30 min, washed again and permeabilized with acetone for 3 min on ice. Cells were washed and incubated with blocking solution (PBS containing 10% FCS, 3% BSA and 10 mM glycerol) for 1 h at 37 °C. After this treatment, cells were washed and incubated overnight at 4 °C with mouse anti-PTB antibody (Santa Cruz Biotechnology) diluted 1:200 in PBS. Finally, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG as secondary antibody, according to the manufacturer’s protocol. For co-localization experiments, goat anti-calnexin (Santa Cruz Biotechnology) and mouse anti-NS1 (a gift from Dr Ada Alves, Instituto Oswaldo Cruz, Brazil) were used as primary antibodies. Finally, cells were incubated with anti-goat IgG conjugated to Alexa Fluor 555 or anti-mouse IgG conjugated to Alexa Fluor 647. Following additional washes, slides were mounted in Vectashield containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and examined for specific fluorescence with a confocal microscope (TCS SP2; Leica).

**Western blotting.** To study PTB localization, cells were infected with DENV2 at an m.o.i. of 10. At the appropriate times p.i., cells were collected and fractionated into nuclear and cytoplasmic components as described previously (Hu et al., 1998). Briefly, cells were washed three times with ice-cold PBS, scraped and pelleted by low-speed centrifugation. The cells were resuspended in 500 μl buffer C [10 mM Tris/HCl (pH 7–8), 5 mM MgCl2, 10 mM KCl, 0.3 mM EGTA, 0.5 mM dithiothreitol, 3 M sucrose, 10 mM β-glycerophosphate, 2 mM ZnCl2] and incubated on ice for 20 min. NP-40 was added to a final concentration of 0.5%. The cellular membrane fraction and nuclei were harvested following centrifugation at 7200 g for 20 min at 4 °C. The cytoplasmic fractions (supernatants) were removed and the pellets (cellular membranes and intact nuclei) were washed three times with buffer C and resuspended in 125 μl buffer D [20 mM Tris/HCl (pH 7–8), 5 mM MgCl2, 320 mM KCl, 0.2 mM EGTA, 0.5 mM dithiothreitol, 2 mM ZnCl2]. After a 15 min incubation on ice and sonication, nuclear extracts (supernatants) were separated from membrane fractions by low-speed centrifugation.

To monitor PTB levels, total cell lysates were obtained at 24, 48 and 72 h post-transfection (p.t.) from cells transfected with either small interfering RNA (siRNA) or pCMV-SPORT-PTB1 (a plasmid promoting transient but high-level expression of PTB through a cytomegalovirus promoter; donated by Dr Mariano García-Blanco, Duke-NUS Graduate Medical School Singapore) and analysed by Western blotting. Briefly, the growth medium was removed and the transfected Vero cells were washed once with PBS. Cell pellets were resuspended in 50 μl lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg leupeptin ml−1, 1 mM PMSF and protease cocktail. **Downloaded from www.microbiologyresearch.org by...**
inhibitor (Mini Complete, Roche Applied Science), incubated on ice for 10 min and centrifuged at 15000 g for 15 min. Supernatants were collected, run on a 10 % SDS-PAGE gel and transferred to a nitrocellulose membrane (Trans Blot Transfer; Bio-Rad) at 16 V for 30 min. The membranes were probed and developed using SuperSignal West Dura Extended Duration Substrate (Pierce). Band intensities from the Western blots were measured using ID Image Analysis Software (Kodak). PTB was detected with a polyclonal anti-PTB antibody (Santa Cruz Biotechnologies). The monoclonal anti-actin antibody, used as a loading control, was a gift from Dr Manuel Hernández (CINVESTAV, Mexico).

**Preparation of DENV replicon DNA for in vitro transcription.**

Thirty micrograms of a DENV replicon DNA with a green fluorescent protein (GFP) reporter gene was linearized for *in vitro* transcription by restriction digestion with *Eco*RI (Promega) in a reaction volume of 100 μl. The DNA was extracted twice with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) followed by chloroform extraction once and ethanol precipitation with 1/10 vol. 3 M sodium acetate (pH 5.2) and 2.5 vols 100 % ethanol, followed by a wash in 2 vols 70 % ethanol after centrifugation of the pellet. The DNA was resuspended in 10 μl nuclease-free water and quantified by gel electrophoresis (0.5 % agarose gel).

**In vitro transcription of DENV replicon RNA.** An AmpliCap SP6 High Yield Messenger Maker kit (Epicentre Technologies) was used for *in vitro* transcription, as recommended by the manufacturer. Briefly, 2 μg linear replicon DNA was combined with 5–8 μl 2 × CapPremix, 2 μl 10 × AmpliCap SP6 RNA polymerase buffer, 2 μM 100 mM GTP, 2 μl 100 mM dithiothreitol, 2 μl SP6 RNA polymerase and 40 U Prime RNase Inhibitor (Eppendorf) in a 20 μl reaction. The reaction was incubated at 40 °C for 2 h. After transcription, DNA was removed by the addition of 1 U DNase I followed by incubation at 37 °C for 30 min.

**Transfection of DENV replicon RNA.** RNA transfections were done by electroporation using a Gene Pulser (Bio-Rad). For each well of a six-well or 12-well plate, cells resuspended in 0.4 ml Opti-MEM (Invitrogen) were mixed with approximately 2 μg replicon RNA in 0.4 cm cuvettes. The cells were pulsed at 850 V and 25 μF with resistance set to ∞. Following electroporation, the cells were diluted with complete medium, transferred to a six-well plate and incubated at 37 °C in a 5 % CO₂ humidified atmosphere. Expression of GFP was evaluated at 2 h after transfection for translation and at 96 h after replication. A time-course experiment was also carried out after transfection to monitor GFP expression.

**Overexpression of PTB.** Vero cells were transfected by electroporation using a Gene Pulser (Bio-Rad). Cells grown in six-well plates (2 × 10⁶ cells per well) were resuspended in Opti-MEM and mixed with 10 μg plasmid pCMV-SPORT-PTB1 in 0.4 cm cuvettes or with the empty vector as a control. The cells were pulsed at 850 V and 25 μF with resistance set to ∞. Following electroporation, the cells were diluted in complete medium, transferred to six-well plates and incubated at 37 °C in a 5 % CO₂ humidified atmosphere. Forty-eight hours after transfection, the cells were transfected with the DENV replicon RNA and the expression of GFP was evaluated after 2 and 96 h.

**Silencing of PTB by siRNA.** PTB siRNA (Santa Cruz Biotechnology) was used to silence the expression of PTB. siRNA transfections were carried out according to the protocol recommended by the manufacturer. As a negative control, an irrelevant siRNA was used. In brief, cells were plated to 60 % confluency in a 12-well plate using 1 ml antibiotic-free, normal growth medium. The following day, 1 μg siRNA was mixed with 100 μl Opti-MEM. In a separate Eppendorf tube, 3 μl Lipofectamine 2000 (Invitrogen) was diluted in 100 μl Opti-MEM and allowed to incubate at room temperature for 10 min. The two mixtures were combined, allowed to incubate at room temperature for 30 min and then diluted to 1 ml with 800 μl Opti-MEM. A 200 μl aliquot of the mixture was added directly to the cells, and transfection with the siRNAs was carried out at 37 °C for 7 h, followed by the addition of 1 ml normal growth medium and additional incubation for 48 h. On day 4, cells were retransfected using 3 μg siRNA mixed with 3 μl Lipofectamine 2000. After the second transfection, cells were electroporated with DENV replicon RNA and reseeded into a culture dish containing glass coverslips. The expression of GFP was evaluated after 2 and 96 h.

**RESULTS**

**Co-localization of PTB with DENV non-structural proteins**

DENV translation and replication occur in membranous compartments of the ER (Welsch et al., 2009). As PTB is located mainly in the nucleus in association with hnRNA, then, if PTB is required during the DENV replicative cycle, it should be present in the cytoplasm and specifically in the membranous compartment in which DENV is translated and replicated. The redistribution of PTB from the nucleus to the cytoplasm has been described previously in other systems in which PTB plays a role (Aizaki et al., 2006; Chang & Luo, 2006; Grover et al., 2008; Gutiérrez et al., 1997).

To determine the location of PTB during DENV infection, Vero cells were infected with DENV at an m.o.i. of 10 and the location of PTB was analysed by confocal microscopy and Western blot analysis at 24 and 48 h p.i. A mouse polyclonal anti-PTB antibody was used as the primary antibody in both assays. The results clearly showed that, in uninfected cells, PTB was located in the nucleus (Fig. 1a, i and ii). In contrast, in infected cells, PTB was located throughout the cytoplasm at 24 h p.i. and very little or no fluorescence was observed in the nucleus (Fig. 1a, iii). Furthermore, at 48 h p.i., PTB was located mainly in the perinuclear region of the cell (Fig. 1a, iv).

To corroborate the redistribution of PTB from the nucleus to the cytoplasm, cell extracts obtained from nuclei and cytoplasm were prepared from DENV-infected and uninfected cells and the location of PTB was evaluated by Western blotting. PTB was detected mainly in nuclear extracts and to a lesser extent in cytoplasmic extracts in uninfected cells (Fig. 1b), whilst in infected cells, PTB was detected mostly in the cytoplasmic fraction and to a lesser extent in the nuclear fraction (Fig. 1b). The amount of PTB detected in the cytoplasm of infected cells was almost three times the amount detected in the cytoplasm of uninfected cells (Fig. 1b). Similarly, the amount of PTB detected in the nucleus of infected cells was reduced to less than half compared with the amount of PTB observed in the nucleus of uninfected cells (Fig. 1b). Thus, Western blot analysis confirmed that PTB moves from the nucleus to the cytoplasm of DENV-infected Vero cells.
The massive relocation of PTB protein in Vero cells contrasts with the discrete movement reported for this protein in DENV-infected Huh7 cells (Anwar et al., 2009; Jiang et al., 2009). To confirm this difference, Huh7 cells were infected with DENV at an m.o.i. of 10, as in Vero cells, and at 48 h p.i., the location of PTB was analysed by confocal microscopy. PTB was located mainly in the nucleus of uninfected and infected Huh7 cells (Fig. 2). However, at 48 h p.i., a discrete amount of PTB protein could be observed in the cytoplasm of infected Huh7 cells (Fig. 2), confirming previous observations (Anwar et al., 2009; Jiang et al., 2009). Furthermore, PTB present in the nucleus as well as the discrete amount of PTB present in the cytoplasm co-localized with DENV replicase, the non-structural protein NS5 (Fig. 2, Merge).

In an attempt to determine whether the PTB present in the cytoplasm in Vero cells was present in the ER and specifically in the sites in which DENV is translated and replicated, co-localization assays using confocal microscopy were performed. Initially, a co-localization assay between PTB and the ER marker calnexin was performed using anti-PTB and anti-calnexin antibodies. Extensive co-localization of PTB with calnexin was observed (Fig. 3), indicating that PTB in infected cells moves from the nucleus to the ER. Translation and replication of DENV occurred in membranes of this organelle, as indicated by co-localization of NS1 and NS3, the two components of viral replicative complexes, with calnexin (Fig. 3). To confirm that PTB protein was present in the same compartment as the DENV replicative complexes, co-localization assays were performed using anti-NS3 antibodies. PTB protein co-localized with NS3 in the ER (Fig. 3). The co-localization of PTB and NS5 was less evident because most NS5 protein is present in the nucleus of infected cells, as has been demonstrated previously (Kapoor et al., 1995; Rawlinson et al., 2009). These results, taken together, suggest that PTB protein is translocated to the cytoplasm and co-localizes with markers of the ER, as well as with NS1 and NS3 proteins, which are components of the DENV replicative complex, supporting the idea that PTB is present in the same compartments where part of the replicative cycle of DENV occurs.

Overexpression of PTB increases DENV translation and replication. To analyse the possible role of PTB in DENV replication, Vero cells were transfected with a DENV-2 replicon (DENV2rep). This replicon contained the complete sequence of DENV-2 strain New Guinea C under the control of the SP6 promoter. The structural genes, with the exception of specific nucleotides of the N-terminal...
sequence of the C protein and the C-terminal region of the E protein, were replaced by the GFP gene. An internal ribosome entry site (IRES) region from encephalomyocarditis virus (EMCV) was inserted between the GFP and E genes to allow constitutive expression of the non-structural proteins (T. Daikoku and others, unpublished data). It has been demonstrated previously that the expression mediated by EMCV IRES is not altered by either the absence or overexpression of PTB (Chang & Luo, 2006).

To study the time course of GFP expression in mammalian cells, DENV2rep was transcribed and 5’ capped in vitro and the RNA was transfected into Vero cells by electroporation. The transient expression of DENV2rep in transfected cells was monitored by measuring GFP activity at different times p.t. The first peak of GFP occurred at 2 h p.t. and then declined. At 24 h, the GFP signal started to accumulate until it reached a maximum at 96 h p.t. The first rise in GFP corresponded with translation, whilst the second represented replication of the viral RNA (Fig. 4).

To study the role of PTB in DENV replication, the protein was overexpressed by transfecting the plasmid pCMV-SPORT-PTB1. This plasmid promotes a transient but high-level expression of the PTB through a cytomegalovirus promoter. Vero cells were transfected with this plasmid or with an empty vector as the control, and the expression of PTB was monitored by Western blotting. An increased amount (15 %) of PTB could be observed in PTB-transfected cells compared with control cells by 24 h p.t., which became considerably higher compared with control cells at 48 h (68 %) and 72 h (92 %) p.t. (Fig. 5a). The high expression level of PTB was maintained in transfected cells for more than 96 h (data not shown).

Under the conditions described above, at 2 h p.t. with DENV2rep, the amount of GFP protein, measured in arbitrary units, produced by the cells that overexpressed PTB was significantly higher than the amount produced in control cells (14.669 vs 3.472, $P < 0.005$), supporting the idea that PTB plays a role in DENV translation (Fig. 5b, c).

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**Fig. 3.** Co-localization of PTB with DENV non-structural proteins NS1, NS3 and NS5 and with calnexin. Vero cells were infected with DENV-2 at an m.o.i. of 10; at 48 h p.i., the cells were fixed and processed for immunofluorescence. The upper three rows show cells that were processed for the co-localization of PTB (green) and calnexin, NS3 and NS5, respectively (red). The lower two rows show cells that were processed for the co-localization of DENV NS1 and NS3 proteins (green), respectively, with calnexin (red). Merged images showed extensive co-localization of PTB, NS1 and NS3 with the ER marker calnexin (yellow). Nuclei stained with DAPI in the lower panels are shown in blue. The experiments were repeated three times with identical results.

**Fig. 4.** Kinetics of GFP production in Vero cells transfected with the DENV replicon. Vero cells were transfected with the DENV replicon and at different times after transfection, cells were fixed and processed for confocal microscopy to visualize GFP expression. No green signal was observed in cells not transfected with the replicon. The amount of GFP expressed was measured by confocal microscopy from 30 cells selected randomly and is expressed in arbitrary units.
Furthermore, a significantly higher amount of GFP was also detected in cells overexpressing PTB compared with control cells at 96 h p.t. (27.607 vs 12.598, \( P<0.005 \)) (Fig. 5b, c). This result suggested that the increase in RNA replication could be due to the increase in translation.

**Silencing of PTB inhibits DENV translation and replication**

To confirm further the role of PTB in DENV replication, the expression of PTB was knocked down using a specific siRNA. Silencing of PTB protein in Vero cells transfected with specific and non-specific siRNAs was monitored by Western blotting. At 24 h p.t., the expression of PTB protein was reduced dramatically compared with cells transfected with the control siRNA. This silencing was maintained for at least 72 h (Fig. 6a). To study the role of PTB in DENV replication, PTB-silenced and control cells were retransfected with DENV2rep and the amount of GFP was determined at 2 and 96 h p.t. At both time points, the amount of GFP present in silenced cells was only up to 25% of the amount of GFP observed in cells transfected with control siRNA (Fig. 6b, c), indicating that silencing of PTB induced a 75% reduction in processes such as translation and replication of DENV. These results are in full agreement with the results obtained after the overexpression of PTB and confirmed our previous conclusion that PTB plays a role in DENV translation and replication.

**DISCUSSION**

The reduced number of genes encoded in viral genomes makes viruses dependent on host proteins and the cellular machinery. Consequently, it is likely that, during the replicative cycle, viral RNA remains associated with several cellular and viral proteins. This fact correlates with multiple reports regarding the participation of host factors in the replicative cycle of positive-strand RNA viruses (Chang & Lou, 2006; Meerovitch et al., 1993; Paranjape & Harris, 2007; Polack et al., 2009; Raha et al., 2004). For DENV, one of the proteins that interacts with the 3'UTR is PTB (De Nova-Ocampo et al., 2002). As PTB is a nuclear factor, the first aspect to analyse was its location in DENV-infected cells. After 24 h of DENV infection, PTB was translocated noticeably from the nucleus to the cytoplasm. Moreover, after 48 h of infection, PTB was located mainly in the perinuclear region of the cells. In other systems in which PTB participates in virus translation and/or replication, this protein has also been shown to move from the nucleus to the cytoplasm (Aizaki et al., 2006; Grover et al., 2008). However, in recent reports, Anwar et al. (2009) and Jiang et al. (2009) observed only partial translocation of PTB from the nucleus to the cytoplasm of DENV-infected Huh7 cells. To verify the difference between the two cell types, an analysis of PTB location in Huh7 cells was performed. Our results confirmed that this significant translocation of the protein to the cytoplasm occurred only in Vero cells. The differences observed in the pattern of PTB translocation to the cytoplasm in these two cell lines could be related to intrinsic differences in the cell types. Among these is the property that Vero cells do not express interferon-stimulated genes.
It is well known that translocation of PTB to the cytoplasm occurs under cellular stress induced by virus infection or apoptosis (Aizaki et al., 2006; Back et al., 2002; Domitrovich et al., 2005). The nuclear export signal (NES) of PTB is required for efficient nucleocytoplasmic transport of this protein. Although the phosphorylation of a serine residue present in the NES results in accumulation of PTB in the cytoplasm (Xie et al., 2003), changes in the subcellular localization of PTB can take place without detectable changes in phosphorylation (Knoch et al., 2006). Consequently, it would be interesting to evaluate the phosphorylation level of PTB in DENV-infected cells. In agreement with Anwar et al. (2009) and Jiang et al. (2009), we also could not detect differences in PTB migration by SDS-PAGE, indicating that post-translational modification or processing of the NES or any other proteolytic processing of the PTB does not occur during DENV infection. As other proteins such as La also translocate from the nucleus to the cytoplasm of infected cells (Yocupicio-Monroy et al., 2007), it is likely that a group of nuclear proteins is translocated to the cytoplasm in response to DENV infection. This phenomenon has been observed with other viruses such as poliovirus (Park et al., 2008). It will be interesting to dissect the mechanisms that induce translocation of these nuclear proteins.

In this study, we showed that PTB protein co-localizes in the perinuclear region with ER markers such as calnexin, as well as with viral NS1, NS3 and to a lesser extent NS5, all of which are components of the viral replicative complex. Anwar et al. (2009) and Jiang et al. (2009) observed translocation of PTB and its co-localization with the viral protein NS4A in the ER of Huh7 cells. In our study, the co-localization of PTB with NS4A was not evaluated; however, it has been shown that NS4A interacts with NS1 (Lindenbach & Rice, 1999) and thus it would be expected that PTB would also co-localize with NS4A in Vero cells.

Our results also indicated that PTB participates in DENV replication. This statement is supported by the fact that overexpression of PTB increased virus translation and replication, whilst silencing of PTB induced the opposite effect. Translation and replication of positive-strand RNA viruses are closely related processes. It is clear from our results that silencing or overexpression of PTB altered the amount of GFP expression due to translation or replication of the viral RNA from replicon-transfected cells. At present, we cannot distinguish between the possibilities that the increase in GFP expression in the presence of PTB overexpression is due to an increase in virus replication or an increase in GFP half-life. In contrast with our results, Anwar et al. (2009) and Jiang et al. (2009) reported that PTB does not participate in DENV translation in Huh7 cells. Intrinsic differences between these two cell types may explain the differential effects observed in the two studies.

Translation of DENV RNA is not completely understood. It has been reported that, when cap-dependent translation is inhibited, DENV RNA translation can still proceed by switching from cap-dependent translation initiation to a non-IRES-mediated form of translation initiation that requires the presence of the 5' and 3'UTRs (Edgil et al., 2006). As PTB interacts with the 3'UTR and this region is required for non-canonical virus translation, it is likely that this protein plays a role in both modes of translation. On the other hand, the overexpression or silencing of PTB also alters virus replication. In HCV, another member of the family Flaviviridae, PTB also plays an important role in
both IRES-dependent translation and virus replication (Aizaki et al., 2006).

In summary, using both overexpression and silencing of PTB, we obtained evidence indicating that this protein is required for DENV translation and replication. This result correlates with the massive translocation of PTB from the nucleus to the cytoplasm of Vero cells. Although the exact mechanism of action of the PTB protein remains unknown, this work provides a framework for several lines of follow-up studies to understand the role of PTB in the DENV life cycle.

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PTB protein modulates dengue virus replication


