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Dengue virus NS1 protein interacts with the ribosomal protein RPL18: This interaction is required for viral translation and replication in Huh-7 cells

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ABSTRACT

Given dengue virus (DENV) genome austerity, it uses cellular molecules and structures for virion entry, translation and replication of the genome. NS1 is a multifunctional protein key to viral replication and pathogenesis. Identification of cellular proteins that interact with NS1 may help in further understanding the functions of NS1. In this paper we isolated a total of 64 proteins from DENV infected human hepatic cells (Huh-7) that interact with NS1 by affinity chromatography and immunoprecipitation assays. The subcellular location and expression levels during infection of the ribosomal proteins RPS3a, RPL7, RPL18, RPL18a plus GAPDH were determined. None of these proteins changed their expression levels during infection; however, RPL-18 was redistributed to the perinuclear region after 48 hpi. Silencing of the RPL-18 does not affect cell translation efficiency or viability, but it reduces significantly viral translation, replication and viral yield, suggesting that the RPL-18 is required during DENV replicative cycle.

Introduction

The persisting epidemics of dengue disease in the world as well as the increasing number of cases, especially of the severe forms of the infection, along with the growing number of countries where dengue outbreaks are reported, make this disease a severe public health problem (Gubler and Meltzer, 1999). Despite multiple studies conducted by several research groups to produce a vaccine or an effective antiviral treatment, so far neither is available (Chokephaibulkit and Perng, 2013). Dengue virus (DENV), a member of the *Flaviviridae* family and *Flavivirus* genus is a single-stranded RNA virus with positive polarity, which encodes for ten viral proteins, three structural, capsid (C), membrane (M) and envelope (E) proteins and seven non-structural NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

Because of the austerity of the viral genomes, viruses require during their replicative cycle the participation of different molecules and organelles to enter into the host cell, to translate and replicate their genome and to exit from the cell. The use of various strategies to identify molecules required during DENV infection has allowed to identify proteins involved in viral particle or E protein binding to the cell surface (Chen et al., 1997; Jindadamrongwech et al., 2004; Navarro-Sanchez et al., 2003; Reyes-Del Valle et al., 2005; Sakoonwatanyoo et al., 2006; Tassaneetrithep et al., 2003; Vega-Almeida et al., 2013), proteins that bind to the 5′ and 3′ untranslated regions of the viral genome (De Nova-Ocampo et al., 2002; Yocupicio-Monroy et al., 2007; Yocupicio-Monroy et al., 2003), as well as molecules that modify their location or expression levels during infection. Interestingly, although DENV replicative cycle occurs within the cytoplasm and specifically in the endoplasmic reticulum (ER), several of the proteins that bind to the viral RNA are located in the nucleus (Agis-Juarez et al., 2009; Blaney et al., 2005; De Nova-Ocampo et al., 2002). Thus, it is clear that there are major changes in the cellular physiology during infection. One of the tools used to identify and characterize molecules involved in viral replicative cycles is to identify cellular proteins which interact with viral proteins. One of the less known DENV viral protein is NS1. This is a protein of approximately 46–50 kDa with two glycosylation sites (N130 and N207) (Smith and Wright, 1985) and one of the most conserved proteins among the four DENV serotypes and even among flaviviruses (Deubel et al., 1988; Mackow et al., 1987). The precise function of NS1 in DENV replication is unclear, but NS1 is known to co-localizes with double-stranded RNA in infected cells

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and to be a necessary cofactor for viral RNA replication (Lindenbach and Rice, 1997, 1999; Mackenzie et al., 1996). In addition to the cytoplasmic localization of NS1, this protein has the particularity to be associated with the outer face of the plasma membrane, via a GPI link, and to be secreted during infection (Gutsche et al., 2011; Winkler et al., 1988). In its membrane-associated form, NS1 behaves as a dimer, whereas in its soluble form it behaves as a hexamer (Gutsche et al., 2011; Winkler et al., 1988). In patients, NS1 circulates at high levels in serum during the first 5–7 days after infection (de la Cruz-Hernandez et al., 2013). High levels of NS1 in patients' sera correlate with disease severity and with the risk of developing Dengue Hemorrhagic fever (DHF) (Avirutnan et al., 2006; de la Cruz-Hernandez et al., 2013). Thus, it has been postulated that NS1 protein is directly involved in the pathogenesis of DHF. Finally, it has been reported that anti-NS1 antibodies protected mice from infection with DENV (Falgout et al., 1990; Henchal et al., 1988).

It is likely that NS1 interacts with cellular proteins that contribute to its multifunctionality. The identification of host cell proteins that interact with NS1 may help to better understand the function of this protein during DENV replicative cycle. Thus, in this paper we used two different strategies to isolate cellular proteins that interact with NS1. First, a His-tag recombinant NS1 protein produced in bacteria was used as bait to separate cellular proteins with affinity to NS1. Second, a polyclonal anti-NS1 antibody was used in immunoprecipitation assays with DENV infected cell extracts to isolate cellular proteins with affinity to NS1. The cell proteins bound to NS1 were eluted with salt and identified by MALDI ToF, MS/MS. A total of 64 proteins were identified that belong to different functional groups such as ribosomal and histone proteins as well as cytoskeleton proteins. The subcellular distribution of some of the proteins identified such as GAPDH and RPL18 was altered in Huh-7 infected cells. Since RPL18 was redistributed to the perinuclear region during DENV infection, its participation in DENV replicative cycle was analyzed by siRNA techniques. RPL18 silencing reduces significantly viral translation and replication as well as viral yield suggesting that the RPL18 is required during DENV replicative cycle.

Results

Purification of recombinant NS1

To identify cellular proteins that interact with the nonstructural protein NS1 from DENV, the first step was to express and purify recombinant DENV2 NS1 protein (rNS1) in *E. coli*. The expression of the rNS1 was induced by IPTG and the recombinant protein was purified by metal affinity resin as described in the Materials and methods section. The full sequence of the cloned NS1 protein and the Western blot carried out to corroborate the nature of the expressed protein are shown in Fig. 1 of Supplemental material. After washing with different concentrations of imidazole, the rNS1 protein, with a molecular weight of approximately 55 kDa, was coupled to the resin (lane R, Fig. 2A, Supplemental material). The presence and purity of the rNS1 in the resin was confirmed by Western blot assay using an anti-NS1 polyclonal antibody (lane R, Fig. 2A and B, Supplemental material). Moreover, the rNS1 migrates as a single band of 55 kDa in a native gel, suggesting that the monomer is the isoform present in the preparation (lanes 1 and 2 Fig. 2C Supplemental material). No specific bands were observed after the analysis of lysates from induced bacteria transformed with the same plasmid without the NS1 sequence used as controls (Fig. 3A and B, Supplemental material).

Table 1
Functional classification of the NS1-interacting proteins identified of mass spectrometry. To investigate the biological process and signaling pathway, we used DAVID Bio-informatics Resources (http://david.abcc.ncifcrf.gov/) and the proteins were also searched through REACTOME pathway database (http://www.reactome.org).

Biological process	Proteins
Microtubule based process	8
Translation	33
Nucleosome assembly	4
Glycolysis	4
Stress response	3
Transport	2
Other process	12

Cellular proteins that interact with DENV NS1 in infected human liver cells

To isolate cellular proteins that interact with NS1 two different strategies were used: affinity chromatography using the rNS1 protein as bait and immunoprecipitation assays using polyclonal anti-NS1 antibodies.

For the affinity chromatography assay, the rNS1 protein, coupled to the cobalt column, and the control resin were incubated with cell extracts from uninfected Huh-7 cells, and after six washes with different concentrations of NaCl, (lanes 1–6, Fig. 3, Supplemental material), two elutions, at 1000 and 1500 mM of NaCl were performed. The majority of the proteins were eluted at 1000 mM NaCl (Fig. 4A Supplemental material). Most of the proteins obtained in this faction interact specifically with rNS1 because none of them were observed in the elution fraction of the negative controls (Fig. 4B, Supplementary material). Mass spectrometry (Maldi ToF) analysis of the eluted proteins allowed the identification of 80, 70 and 72 proteins in each of the three independent experiments from Huh-7 cells. These proteins were able to interact directly or indirectly with rNS1 and were absent in the proteins identified in the control resin.

The immunoprecipitation assays were carried out with anti-NS1 polyclonal antibody or rabbit IgG used as negative control, cross linked to protein-G agarose and infected Huh-7 cells cytoplasmic extracts. The immunoprecipitated proteins were washed five times (lanes 1–5, Fig. 5A and B, Supplemental material) and eluted as described in the Material and methods section. The proteins obtained in the elution fraction interact specifically with NS1 because none of them were immunoprecipitated with the anti-rabbit IgG used as negative control (lanes E, Fig. 5A and B, Supplemental material). Mass spectrometry (Maldi ToF) analysis of the immunoprecipitated proteins allowed to identify 70, 86 and 85 proteins from 3 independent experiments. A total of 64 proteins that were identified by the 2 methods and in the 3 independent experiments were considered as true NS1-interacting proteins (Table 2).

Functional characterization of NS1 interacting proteins

In an attempt to further characterize the cell proteins identified, the functional categorization and subcellular localization was determined based on a Swiss-Prot TrEMBL database search. The 64 proteins identified are involved in six different biological processes: 33 out of the 64 proteins identified are involved in translation; 8 out of 64 are involved in microtubule based process; 4 out of the 64 are involved in nucleosome assembly and glycolysis, 3 and out of the 64 are involved in stress response and 2 in transport (Table 1). Most proteins identified are cytoplasmic proteins (23.2%) and are ribosomal proteins (48.2%). Interestingly, 8.9% of the cell proteins identified are nuclear proteins, while only 1.7% of the proteins identified are from mitochondrion, 3.5% from membrane and 1.7% are secreted (Fig. 1).