Available online at www.sciencedirect.com
ScienceDirect
Virology 356 (2006) 35–44
VIROLOGY
www.elsevier.com/locate/yviro

Binding of the polypyrimidine tract-binding protein-associated splicing factor (PSF) to the hepatitis *delta* virus RNA

Valerie S. Greco-Stewart, Catherine St-Laurent Thibault, Martin Pelchat *

Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Room 4223A, Ottawa, ON, Canada K1H 8M5

Received 6 March 2006; returned to author for revision 4 April 2006; accepted 9 June 2006
Available online 30 August 2006

Abstract

The hepatitis *delta* virus (HDV) has a very limited protein coding capacity and must rely on host proteins for its replication. A ribonucleoprotein complex was detected following UV cross-linking between HeLa nuclear proteins and an RNA corresponding to the right terminal stem–loop domain of HDV genomic RNA. Mass spectrometric analysis of the complex revealed the polypyrimidine tract-binding protein-associated splicing factor (PSF) as a novel HDV RNA-interacting protein. Co-immunoprecipitation demonstrated the interaction between HDV RNA and PSF both *in vitro* in HeLa nuclear extract and *in vivo* within HeLa cells containing both polarities of the HDV genome. Analysis of the binding of various HDV-derived RNAs to purified, recombinant PSF further confirmed the specificity of the interaction and revealed that PSF directly binds to the terminal stem–loop domains of both polarities of HDV RNA. Our findings provide evidence of the involvement of a host mRNA processing protein in the HDV life cycle.

Keywords: Hepatitis *delta* virus; RNA binding protein; RNA replication; Splicing factor; Polypyrimidine tract-binding protein; Mass spectrometry; UV cross-linking; Co-immunoprecipitation; PSF; RNA virus

Introduction

There is increasing evidence that cellular factors are substantially involved in viral RNA replication and transcription (Lai, 1998, 2005; Taylor, 2006). These cellular proteins are often associated with RNA-processing pathways or translation machinery, suggesting that RNA viruses might actively subvert normal cellular RNA processing or translation proteins for viral RNA synthesis. This is particularly crucial for the hepatitis *delta* virus (HDV) which has an extremely small genome and possesses a very limited protein coding capacity (Taylor, 2006). HDV must thus rely heavily on host components for most steps of its replication and transcription.

HDV has a small (~1700 nucleotides, nt) single-stranded, circular RNA genome and requires the hepatitis B virus (HBV) envelope proteins for encapsidation and dissemination (Chen et al., 1986; Rizzetto et al., 1977; Wang et al., 1986). Its genome folds into an unbranched, rod-like structure including two self-cleaving motifs (*delta* motifs; Kuo et al., 1988b) and a single open reading frame (ORF) encoding two viral proteins (HDAgs; Wang et al., 1986; Weiner et al., 1987). These two proteins are identical in sequence, except that the large HDAg (HDAg-L) contains 19 additional amino acids at its C-terminus resulting from RNA editing of the termination codon of the small HDAg (HDAg-S) gene (Luo et al., 1990; Wong and Lazinski, 2002). Although they are identical over most of their lengths, each protein has a distinct function. HDAg-S (195 amino acids) is essential for HDV replication (Kuo et al., 1989), while the HDAg-L (214 amino acids) is necessary for virion assembly and is reported to be a dominant negative inhibitor of replication (Chang et al., 1991).

In the currently accepted model, HDV replicates in mammalian cells by a symmetrical, rolling circle mechanism (Chen et al., 1986; Taylor, 2006). Replication of the infectious circular monomer (which is assigned genomic polarity by convention and accumulates at a greater intracellular abundance than the antigenomic species) produces linear, multimeric

* Corresponding author. Fax: +1 613 562 5452.
E-mail address: mpelchat@uottawa.ca (M. Pelchat).

0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.06.040

strands which are subsequently self-cleaved and ligated, yielding antigenomic polarity circular monomers. Using the latter RNAs as templates, the same three steps are repeated to generate the genomic progeny. Nuclear RNA polymerase II (RNAP II) is considered to be involved in the replication and transcription of HDV.

HDAg mRNA has a distinct 5′-end and is post-transcriptionally processed with a 5′-cap and a 3′-poly(A) tail (Gudima et al., 2000), which are typical features of transcripts generated by RNAP II. Low doses of α-amanitin, a mycotoxin that specifically inhibits DNA-dependent RNA transcription by RNAP II, inhibit the accumulation of HDV mRNA and processed unit-length genomic RNA species in infected cells (Fu and Taylor, 1993; Macnaughton et al., 1991). Additionally, in *in vitro* transcription assays using nuclear extract (NE) from HeLa cells and RNA derived from the left terminal stem–loop domain of HDV antigenomic RNA, synthesis of the complementary strand was possible (Filipovska and Konarska, 2000). Accumulation of this RNA product was highly sensitive to α-amanitin. This sensitivity was partially abrogated in experiments conducted in NE from cells containing an α-amanitin-resistant allele of the largest subunit of RNAP II, suggesting the involvement of RNAP II in this reaction. However, the transcription did not proceed by *de novo* initiation, but rather by cleavage of the RNA template followed by extension of the new 3′ end, generating a chimeric template/transcript product. Conversely, because the accumulation of the antigenomic species is resistant to higher doses of α-amanitin, it is suggested that the synthesis of the antigenome could be carried out by a polymerase other than RNAP II (Macnaughton and Lai, 2002; Macnaughton et al., 2002; Modahl et al., 2000).

To date, few proteins are reported to be associated with HDV. HDAg associates with HDV genomic RNA to form a ribonucleoprotein (RNP) complex in the HDV-containing HBV virion and in transfected cells (Chang et al., 1988; Lin et al., 1990; Ryu et al., 1993). In addition, HDAg can function as an RNA chaperone and modulate the ribozyme activity of HDV RNA (Huang and Wu, 1998). Host proteins that were identified to be associated with HDV biology can be divided in two categories: HDAg- or HDV RNA-interacting proteins. The host proteins reported to interact with HDAg are the cellular double-stranded RNA-dependent protein kinase (PKR), nucleolin, and a cellular homolog of HDAg named *delta*-interacting protein A (DIPA; Brazas and Ganem, 1996; Chen et al., 2002; Lee et al., 1998). The host proteins observed to interact directly with HDV RNA are PKR, the negative elongation factor (NELF), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the small form of adenosine deaminase acting on RNA (ADAR-1; Circle et al., 1997; Robertson et al., 1996; Wong and Lazinski, 2002; Yamaguchi et al., 2002). ADAR-1 is reported to carry out the post-transcriptional RNA editing of the HDV genome and GAPDH is believe to be involved in the regulation of HDV ribozyme activity (Lin et al., 2000). However, the physiological significance of the interaction of most of these proteins remains enigmatic and the identification of additional host factors involved in viral replication is required to gain a better understanding of HDV replication.

To identify additional host proteins involved in HDV replication, we analyzed the protein content of a ribonucleoprotein complex produced by UV cross-linking an HDV-derived RNA previously reported to act as an RNA promoter *in vitro* (Beard et al., 1996) to proteins included in NE from HeLa cells. Mass spectrometry of the complex led to the identification of the polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) as an HDV-binding protein. Using co-immunoprecipitation with HeLa NE proteins and electrophoretic mobility shift assays (EMSAs) of a series of HDV-derived RNAs with purified, recombinant hexahistidine-tagged PSF, we have found that PSF binds directly to both terminal stem–loop domains of both polarities of HDV RNA. In addition, the interaction between HDV RNA and PSF was demonstrated *in vivo* using HeLa cells containing both polarities of HDV RNA. Together, these results suggest a role for host mRNA processing proteins in HDV replication and/or propagation and the possible involvement of PSF in the life cycle of HDV.

Results

Formation of a specific complex between the right terminal stem–loop domain of genomic HDV RNA and HeLa NE proteins

In order to identify host proteins that interact with HDV RNA, we used a 208 nt RNA fragment containing 199 nt derived from the right terminal stem–loop domain of the genomic polarity of HDV (R199G; nucleotides 1541 to 61) as bait. We selected this fragment because it includes the reported initiation site for HDAg mRNA transcription (i.e. position 1630; Gudima et al., 2000) and a similar HDV-derived RNA fragment was previously shown to be able to initiate transcription *in vitro* using HeLa NE proteins (Beard et al., 1996). In addition, the stem–loop structure conformation of this domain has been confirmed *in vitro* under approximately physiological conditions (Beard et al., 1996). Fig. 1 shows the location, the sequence, and the putative secondary structure of the radiolabelled synthetic RNA used as bait in our study.

We performed UV cross-linking assays of internally radiolabelled R199G with proteins contained in HeLa NE. To obtain a better cross-linking yield, photoreactive 4-thio-uridine was randomly incorporated into R199G during its *in vitro* synthesis. In addition, an excess amount of total yeast tRNA was added to each sample to ensure the specificity of the interaction. Following UV irradiation and treatment with RNase A to remove excess probe, the mixtures were fractionated by SDS-PAGE. UV cross-linking of NE proteins to the radiolabelled bait generated a single major band migrating as a species of approximately 250 kDa (Fig. 2). This complex was only detected in the presence of NE, indicating that formation of the radiolabelled complex was dependent on the presence of the NE proteins. This complex was also detected in the presence of heparin, poly(A) RNA, and P11.60 RNA as non-specific competitors. P11.60 is a small RNA fragment derived from the terminal stem–loop domain of the peach latent mosaic viroid genome which has been demonstrated to have promoter activity