Methods 91 (2015) 13–19

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Antisense-mediated affinity purification of dengue virus ribonucleoprotein complexes from infected cells

Stacia L. Phillips, Mariano A. Garcia-Blanco, Shelton S. Bradrick *

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1055, United States
Department of Microbiology and Molecular Genetics, Center for RNA Biology and Department of Medicine, Duke University, 213 Research Drive, Durham, NC 27710, United States

ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 10 August 2015
Accepted 11 August 2015
Available online 12 August 2015

Keywords:
Dengue virus
Ribonucleoprotein complex
RNA affinity chromatography

ABSTRACT

The identification of RNA-binding proteins that physically associate with viral RNA molecules during infection can provide insight into the molecular mechanisms of RNA virus replication. Until recently, such RNA–protein interactions have been identified predominantly with the use of *in vitro* assays that may not accurately reflect associations that occur in the context of a living cell. Here we describe a method for the specific affinity purification of dengue virus RNA and associated proteins using *in vivo* cross-linking followed by antisense-mediated affinity purification. RNA-binding proteins that specifically co-purify with viral RNA using this method can be identified *en masse* by mass spectrometry. This strategy can potentially be adapted to the purification of any viral RNA species of interest.

1. Introduction

Viral RNAs must navigate a complex cellular environment and be successfully translated, replicated, and/or packaged into progeny virions during the course of a productive infection. Throughout various stages of the viral life cycle, the viral RNA is exposed to, and likely interacts directly with, a number of host cell and viral proteins. Some of these host RNA-binding proteins (RBPs) may bind to viral RNA and impede the establishment of productive infection [1–5]. Alternatively, the virus may co-opt the normal function of cellular RBPs to promote one or more phases of the viral life-cycle. For example, several well-characterized RBPs have been shown to interact with viral RNA and mediate the translation and/or synthesis of viral RNA genomes [6–12]. Therefore, the identification of novel interactions between viral RNA and host RBPs can provide insight into the molecular mechanisms of virus replication.

Until recently, the identification of interactions between viral RNA and host proteins has relied predominantly on *in vitro* systems. One commonly used strategy has been immobilization of *in vitro* transcribed subgenomic regions of viral RNA on solid state resins, followed by incubation with crude or fractionated cell lysates. Proteins that specifically bind to the RNA of interest can be enriched after washing away non-specific interactions and then identified using mass spectrometry [13]. More targeted approaches for the identification of interactions between protein and viral RNA, such as RNA-immunoprecipitation and electrophoretic mobility shift assay, have been used to test the ability of specific RBPs to bind discrete viral RNA regions [3,4,14]. These techniques can be powerful tools for identification and characterization of RNA–protein interactions. However, an important limitation of these strategies is that interactions occurring *in vitro* may not accurately reflect those that occur in the context of virus infection.

One strategy for the characterization of polyadenylated RNP complexes formed *in vivo* involves UV crosslinking followed by purification of RBPs on immobilized oligo(dT) [15–19] A recently described method couples this purification strategy with mass spectrometry for *en masse* identification of interactions between cellular mRNA and protein in living cells. This protocol utilizes an *in vivo* cross-linking approach in which growing cells are exposed to 254 nm UV light, forming covalent cross-links at sites of direct contact between protein and RNA. The cells are then lysed under denaturing conditions and ribonucleoprotein (RNP) complexes are purified from the lysate using immobilized oligo (dT) to specifically purify polyadenylated mRNA with its associated proteins [20,21]. A modification of this strategy was recently used to identify proteins bound to the polyadenylated poliovirus RNA genome in infected cells [22]. Here we report an adaptation of this approach to specifically purify dengue virus (DENV) RNPs from infected cells. The identification of proteins associated with the non-polyadenylated viral genomes during infection, and characterization of their roles in virus replication, will lead to novel insights into the molecular mechanisms of DENV replication.

* Corresponding author at: Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1005, United States.
E-mail address: ssbradri@utmb.edu (S.S. Bradrick).

http://dx.doi.org/10.1016/j.ymeth.2015.08.008
1046-2023/

2. Method overview

Fig. 1 depicts a general overview of the affinity purification method. Infected cells are exposed to 254 nm UV light, inducing covalent cross-links between protein and RNA to which it is directly bound. Mock-infected cells serve as a control for background binding of cellular RNPs. Cells are lysed and viral RNPs are purified by hybridization of antisense biotinylated DNA oligonucleotides to the viral RNA, followed by capture of RNP complexes on streptavidin-coated magnetic beads. After a series of increasingly stringent washes, the bound material is eluted for analysis of RNA and/or protein. RNA recovery is assessed using RT-qPCR. Bound proteins can be liberated from the RNA for targeted or unbiased *en masse* identification.

3. Detailed methods

3.1. Rational design of antisense oligonucleotides by RNase H mapping

RNA molecules can exhibit significant secondary structure and in living cells are associated with RNA-binding proteins. As a result, certain regions of an RNA molecule after UV cross-linking to partner proteins may be unavailable for hybridization to an antisense oligonucleotide. The likelihood that a given antisense oligonucleotide sequence will be able to bind its target RNA and thus be effective in mediating affinity purification may be predicted using theoretical or empirical structural data where available. However, these data are often derived from naked RNA in the absence of protein. One way to determine the accessibility of a given RNA sequence in the context of bound protein is by RNase H mapping of the target RNA (Fig. 2A). This is accomplished by incubation of candidate antisense DNA oligonucleotides with cross-linked cell lysate, followed by treatment with RNase H, which will specifically cleave the RNA in an RNA:DNA hybrid. Cleavage efficiency of the RNA can then be assessed by RT-qPCR using PCR primers that span the predicted cleavage site.

3.2. Materials

1. Cross-linked lysate from DENV-infected Huh7 cells (see Note 1), $\sim 2.5 \times 10^4$ cell equivalents per μL. This corresponds to an approximate protein concentration of 15 mg/ml.
2. Purified RNA from mock-cross-linked cells, heat denatured, 100 ng per reaction.
3. RNase H (NEB).
4. Antisense DNA oligonucleotide (100 μM stock).
5. RQ1 DNase (Promega).
6. 60 mM $CaCl_2$.
7. 60 mM $MgCl_2$.
8. 20 mM EGTA, pH 8.0.
9. RNA purification reagent, e.g. Trizol (Life Technologies).
10. cDNA synthesis and PCR reagents.

3.3. Procedure

1. Assemble 10 μL reactions containing 8.5 μL cell lysate or naked RNA, 1 μL antisense oligonucleotide (100 pmol), and 0.5 μL RNase H (2.5 U).
2. Incubate reactions at 37 °C for 30 min.
3. Add 1 μL RQ1 DNase, 2.5 μL 60 mM $CaCl_2$, and 2.5 μL 60 mM $MgCl_2$.
4. Incubate reactions at 37 °C for 30 min to hydrolyze oligonucleotides.
5. Add 1.5 μL 20 mM EGTA pH 8.0 to stop the DNase reaction.
6. Incubate at 65 °C for 10 min to inactivate DNase.
7. Purify RNA and perform cDNA synthesis and PCR using prevalidated qPCR primers that span the antisense hybridization site.

3.4. Notes

1. RNase H is not compatible with many common detergents used in cell lysis buffers, such as SDS and NP-40 {[23] and data not shown}. Lysis conditions under which RNase H maintains its activity, e.g. hypotonic swelling followed by mechanical lysis, may need to be identified prior to RNase H mapping.

3.5. Results

Fig. 2B shows the results of RNase H mapping using selected antisense oligonucleotides. Oligonucleotides A-H were designed to hybridize to regions distributed across the length of the 10.6 kb DENV type-2 strain New Guinea C (DENV2 NGC) viral genome at approximately 1 kb intervals. The no oligo condition serves as a negative control for background RNA cleavage. Denatured RNA purified from mock-cross-linked cells serves as a positive control. The relative levels of RNase H cleavage under each condition is measured using qPCR primers designed to amplify across the predicted site of cleavage. The amount of RNA remaining is expressed relative to the amount of uncleaved RNA in the no oligo control.

We find that different regions of the viral genome exhibit variable levels of accessibility for hybridization based on the observed differences in cleavage efficiency between naked and cross-linked RNA. RNA cleavage after incubation of the cross-linked viral RNA

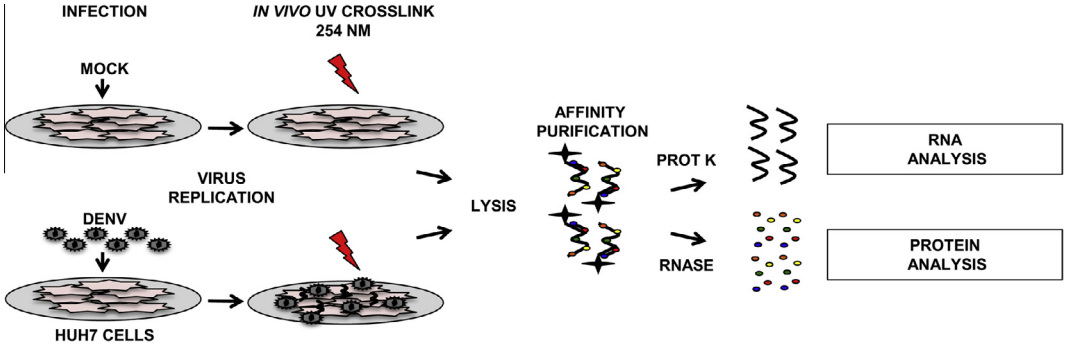

Fig. 1. Schematic of affinity purification technique. Cells are infected with dengue virus at an MOI of 1. Mock infected cells serve as a negative control. RNA–protein cross-links are induced 30 h post-infection by exposing the cells to 254 nm UV. Cells are lysed under denaturing conditions and incubated with biotinylated antisense DNA oligonucleotides. RNA–protein complexes are captured on streptavidin-coated magnetic beads. Protein can be digested with proteinase K for subsequent RNA analysis by RT-qPCR. Alternatively, proteins can be liberated from the RNA by RNase treatment and analyzed by Western blot or *en masse* by mass spectrometry.