Co-immunoprecipitation of Flag-TLR3 or Myc-MSR1 with HCV RNA

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[Abstract] Co-immunoprecipitation assay of TLR3-Flag or Myc-MSR1 with HCV RNA is used to identify direct interaction of viral RNA with host proteins that recognize viral RNA to initiate interferon (IFN) signaling, a crucial antiviral response of the host cells. Both Toll-like receptor 3 (TLR3) and class-A scavenger receptor type 1 (MSR1) proteins recognize viral double-stranded RNA (dsRNA) which may be released into the extracellular milieu or spread from HCV-infected cells to uninfected neighbor cells via cell-to-cell contact, resulting in IFN-β activation that restricts viral propagation. We have found that MSR1 binds extracellular dsRNA, mediating its endocytosis and transport toward the endosome where it is engaged by TLR3, thereby triggering IFN responses in both infected and uninfected cells. We used this assay to demonstrate the pivotal role of MSR1 in mediating TLR3-recognition of the HCV RNA. The assay described in this protocol is based on the conventional protein immunoprecipitation protocol with conditioned buffers that prevent nonspecific RNA degradation by RNase present in the lysate. RNA molecules associated with the Flag-tagged protein were trapped by a specific antibody followed by Protein G capture, extracted and detected quantitatively by RT-PCR assay, followed by agarose-gel electrophoresis for visualization. This method can also be applied to detection of other protein-RNA interactions.

Materials and Reagents

1. Huh-7.5 cells (obtained from Apath, LLC) expressing TLR3-Flag (or other cells stably/transiently expressing Flag/Myc-tagged protein)
2. Culture medium consists of DMEM (Life Technologies, catalog number: 11995065) supplemented with 10% heat-inactivated FBS (Life Technologies, catalog number: 26140079), Penicillin-streptomycin (Life Technologies, catalog number: 15140-148), L-Glutamine (Life Technologies, catalog number: 25030-081) and non-essential amino acids (Life Technologies, catalog number: 11140050)
3. HCV strain HJ3-5 stock prepared in cell culture medium (see Yi *et al.*, 2007)
4. DPBS (Life Technologies, catalog number: 14190-144)
5. RNase inhibitor (RNaseOUT) (Life Technologies, catalog number: 10777-019)
6. Protease inhibitor (Complete Protease Inhibitor Cocktail Tablets) (Roche Diagnostics,

catalog number: 1697498)

7. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
8. Anti-Flag (M2) and anti-Myc (9E10) monoclonal antibodies (Sigma-Aldrich, catalog numbers: F1804 and M4439) and mouse IgG control (Life Technologies, 02-6502)
9. Protein G sepharose (General Electric Company , catalog number: 17-0618-01)
10. TRIzol (Life Technologies, catalog number: 15596-026)
11. Molecular biology grade water (Corning, catalog number: 46-000-CM)
12. Agarose (GenePure LE Agarose) (BioExpress, GeneMate, catalog number: E-3120-500)
13. Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
14. Platinum® *Taq* DNA Polymerase (Life Technologies)
15. Lysis buffer (see Recipes)

Equipment

1. 100 mm plates (Falcon™, catalog number: 353003)
2. Cell scraper (Falcon™, catalog number: 353085)
3. Electrophoresis Gel Box
4. Tube Rotator (Fisher Scientific, catalog number: 05-450-200)
5. Centrifuge (Eppendorf, catalog number: 5415R)
6. Superscript III One-step RT-PCR system (Life Technologies, catalog number: 12574-018)

Procedure

A. Preparation of HCV infected cells

1. Seed the Huh-7.5 cells (1.5 x 10^6 per dish) stably expressing TLR3-Flag onto a 10 cm dish and incubated overnight.
2. Inoculate HCV (strain HJ3-5) at an MOI of 1 (5 ml of HJ3-5 stock at 3 x 10^5 FFU/ml) for 6 h, remove the inoculum, and then replace with 10 ml fresh culture medium.
3. Incubate cells at 37 °C in 5% CO_2 for 72 h.

B. Preparation of the lysates

1. Aspirate culture medium, wash cells twice with DPBS, and scrape the cells into 50 ml tube.
2. Centrifuge at 800 *x g* for 3 min at 4 °C, and remove the supernatant.
3. Resuspend the cell pellet in 1 ml lysis buffer, and rotate the lysate for 5-6 h at 4 °C.
4. Centrifuge the lysate at 15,700 *x g* for 20 min at 4 °C.

5. Transfer the supernatant to 1.5 ml tube and place on ice, and determine the protein content using Protein Assay Kit.

C. Immunoprecipitation

1. Transfer the cell lysate (20 µg of total protein) to a new 1.5 ml tube.
2. Add 1 µg of anti-Flag antibody or mouse IgG control to the lysate and rotate overnight at 4 °C.
3. Add 20 µl Protein G sepharose to the lysate and rotate for 2-3 h at 4 °C.
4. Centrifuge at 800 *x g* for 3 min at 4 °C.
5. Aspirate the supernatant, wash beads with 1 ml lysis buffer and rotate for 10 min at 4 °C.
6. Centrifuge at 800 *x g* for 3 min at 4 °C.
7. Repeat steps C4-5 twice.
8. Suspend the sepharose in 100 µl DPBS and use half for RNA extraction and the remainder for Western blotting to detect the immunoprecipitated protein.

D. RNA extraction and RT-PCR

1. Extract RNA from the sepharose beads by vortexing 15 sec in TRIzol reagent, followed by the standard protocol as indicated in the manufacturer's instruction, and suspend the RNA pellet in 50 µl of nuclease-free water (optional: Add 1 µl of Glycogen before the precipitation of RNA with Isopropanol).
2. Detect HCV RNA bound to TLR3-Flag with SuperScriptIII One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase using an HCV-specific primer pair HCV84FP, 5'-GCCATGGCGTTAGTATGAGTGT-3'; HCV 303RP, 5'-CACCCTATCAGGCAGTACCACAA-3', at an annealing temperature of 55 °C, followed by gel electrophoresis in 1.5% agarose gel. Specific bands (220 bp) can be detected typically with 35-40 PCR cycles.

Recipes

1. Lysis buffer
   1x DPBS
   0.1% Triton X-100
   1x Protease inhibitor cocktail
   RNaseOUT 100 U/ml

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References

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