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Detection of HBV C Protein Phosphorylation in the Cell

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[Abstract] Among the seven serines and one threonine in the carboxyl-terminus of HBV C protein, all but one (serine 183) appear in the context of RxxS/T consensus phosphoacceptor motifs and also overlap with other consensus motifs, such as S/TP, RS, SPRRR, RRRS/T, or RRxS/T, suggesting that various cellular kinases phosphorylate these residues. To determine whether threonine and/or serine (serines 157, 164, 170, 172, 178, and 180, and threonine 162, adw subtype) of HBV C protein are indeed phosphoacceptor residues in cells, Huh7 were transfected with a series of C-protein-expressing mutants, labeled with ^{32}P-orthophosphate for 14 h, and then lysed. The ^{32}Pi-labeled lysates were immunoprecipitated with anti-HBc antibody, and the ^{32}Pi-labeled immunoprecipitated C proteins were detected by autoradiography.

Materials and Reagents

1. Huh7 hepatoma cells (Japanese Collection of Research Bioresources Cell Bank, catalog number: JCRB0403)
2. Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gibco®, catalog number: 12800-017)
3. Fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 16000-044)
4. Penicillin/streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)
5. Plasmid; HBV WT C protein (STSSSS) in plasmid HBV-P-def of pcDNA3 backbone, phosphoacceptor-site mutants (ATAAAA, AAAAAA, SSSSSS, and ASAAAA) in plasmid HBV-P-def in pcDNA3 backbone, pcDNA3-GFP
6. Polyethylenimine (Polysciences, catalog number: 23966)
7. Opti-MEM (Life Technologies, Gibco®, catalog number: 31985-062)
8. OptiMEM (Life Technologies, Gibco®, catalog number: 31985-070)
9. Dialyzed FBS (Life Technologies, Gibco®, catalog number: 26400044)
10. 1 mCi orthophosphate [$^{32}P_i$] (PerkinElmer Inc., catalog number: NEX053)
11. Polyclonal rabbit anti-HBc antibody (home-made, Jung *et al.*, 2012)
12. Protein A/G Plus agarose beads (Calbiochem®, catalog number: IP05)
13. Tris-HCl (pH 8.5) (Sigma-Aldrich, catalog number: T6066)
14. EDTA (Sigma-Aldrich, catalog number: E5134)
15. Nonidet P-40 (Sigma-Aldrich, catalog number: CA630)
16. NaF (Sigma-Aldrich, catalog number: 201154)

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17. β-glycerophosphate (USB, catalog number: 155-56-2)
18. Sodium orthovanadate (Sigma-Aldrich, catalog number: s6508)
19. Protease inhibitors (Calbiochem®, catalog number: 535142)
20. Tris-HCl (pH 6.8) (Sigma-Aldrich, catalog number: T6066)
21. SDS (Sigma-Aldrich, catalog number: L-3771)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
23. Bromophenol blue (Sigma-Aldrich, catalog number: 114391)
24. Glycerol (USB, catalog number: 16374)
25. Lysis buffer (see Recipes)
26. 2x sample buffer (see Recipes)

Equipment

1. 10-cm dishes (Corning Incorporated, catalog number: 430167)
2. PVDF membranes (Bio-Rad Laboratories, AbD Serotec®, catalog number: 162-0177)

Procedure

1. Huh7 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified atmosphere at 37 °C in 5% CO_2.
2. Cells were passaged every three days (passage at 80~90% confluency) and 2 x 10^6 Huh7 cells were seeded in 10-cm dishes one day before the transfection.
3. Next day, Huh7 cells were transfected using polyethylenimine (PEI).
   PEI transfection method:
   a. In a sterile tube, total 10 μg of plasmid DNA was mixed with 500 μl Opti-MEM.
   b. Add 30 μl of PEI solution (1 μg/1 μl) to DNA-Opti-MEM solution and then vortex immediately.
   c. Incubate 15 min at room temperature.
   d. Then add PEI/DNA-Opti-MEM mixture to cells.
   For transfections, 10 μg of plasmid encoding HBV STSSSS (WT) or phosphoacceptor-site mutant C proteins in a P-deficient mutant backbone were used. As a transfection control, 1 μg of green fluorescent protein (GFP)-expressing plasmid was included in the transfection mixture.
4. Transfection experiments were repeated at least three times.
5. Forty-eight hours after transfection, Huh7 cells were incubated at 37 °C in 5% CO_2 incubator for 6 h in 10 ml DMEM containing 10% dialyzed FBS, then labeled at 37 °C in 5% CO_2 for additional 14 h with 250 μCi orthophosphate [$^{32}P_i$] per 10^7 cells, rinsed with ice-cold phosphate-buffered saline, and lysed with 1 ml of lysis buffer. (Isotope was added directly onto the cells containing DMEM-dFBS.)

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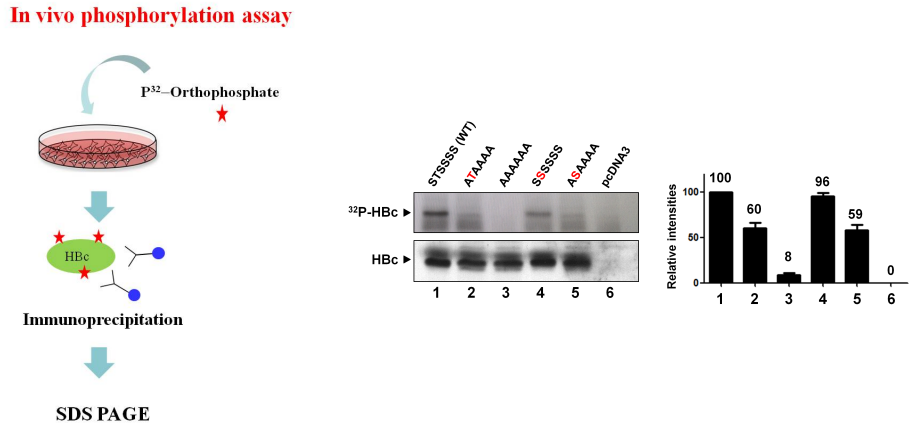
6. Lysates were transferred to 1.5 ml tube, incubated on ice for 10 min for further lysis, spin down at 13,000 rpm at 4 °C for 2 min, and supernatant was transferred to fresh 1.5 ml tube.
7. The supernatant lysates were incubated for 2 h at 4 °C with 1 µl of polyclonal rabbit anti-HBc antibody on the rotator.
8. Next, 15 µl of protein A/G Plus agarose beads was added, and the mixture was incubated at 4 °C for 1 h and centrifuged at 1,500 rpm for 10 sec. (Protein A/G Plus agarose bead preparation: Take 15 µl of bead/sample to fresh 1.5 ml tube, add 1 ml lysis buffer, vortex briefly, spin down at 1,500 rpm for 10 sec, and discard supernatant. Repeat above washing procedures 2 more times.)
9. The pellets in 1.5 ml tube (containing beads bound to immune complexes of radiolabeled WT or mutant C proteins and anti-HBc antibodies) were washed twice with 1 ml lysis buffer, eluted by boiling (100 °C for 5 min) in 15 µl of 2x sample buffer, and separated by SDS-PAGE on 13.5% gels. Running time was 3 h at 80 voltage.
10. Separated proteins were transferred to PVDF membranes (250 mA 1 h 30 min) and subjected to autoradiography.

Representative data



Compared to AAAAAA mutant of which the phosphoacceptor sites were all abolished to alanine, STSSSS (WT) and other mutants were all ^{32}Pi-labeled.

Recipes

1. Lysis buffer
   50 mM Tris-HCl (pH 8.5)
   2 mM EDTA
   0.5% Nonidet P-40
   50 mM NaF
   25 mM β-glycerophosphate

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2 mM sodium orthovanadate, and protease inhibitors

2. 2x sample buffer
   100 mM Tris-HCl (pH 6.8)
   4% SDS
   200 mM β-mercaptoethanol
   0.2% bromophenol blue
   20% glycerol

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