

Detection of HBV C Protein Phosphorylation in the Cell

Jaesung Jung^{1,2} and Kyongmin Kim^{1,2*}

Department of Microbiology, Ajou University School of Medicine¹, Department of Biomedical Science, Graduate School of Ajou University², Suwon, South Korea

*For correspondence: kimkm@ajou.ac.kr

[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 ³²P-orthophosphate for 14 h, and then lysed. The ³²Pi-labeled lysates were immunoprecipitated with anti-HBc antibody, and the ³²Pi-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 [³²P_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)

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

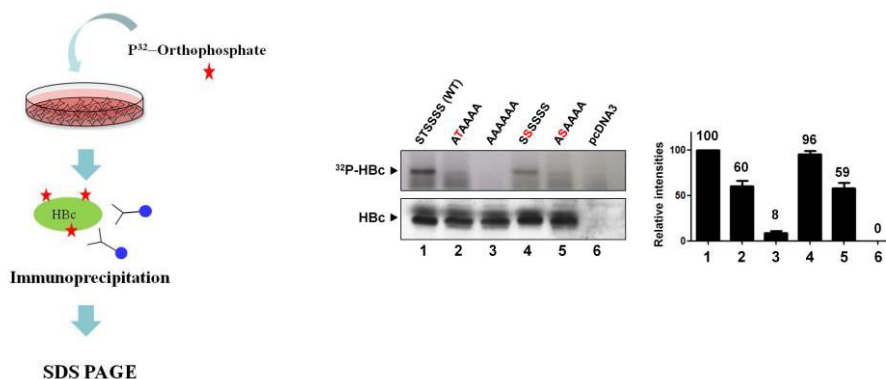
1. Huh7 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified atmosphere at 37 °C in 5% CO₂.
2. Cells were passaged every three days (passage at 80~90% confluency) and 2 x 10⁶ Huh7 cells were seeded 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 μ g of plasmid DNA was mixed with 500 μ l Opti-MEM.
 - b. Add 30 μ l of PEI solution (1 μ g/1 μ l) 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 μ g 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 μ g 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% CO₂ incubator for 6 h in 10 ml DMEM containing 10% dialyzed FBS, then labeled at 37 °C in 5% CO₂ for additional 14 h with 250 μ Ci orthophosphate [³²P_i] per 10⁷ 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.)

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

In vivo phosphorylation assay



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

Recipes

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

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- 25 mM β -glycerophosphate
 - 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

Acknowledgments

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Reference

1. Jung, J., Kim, H. Y., Kim, T., Shin, B. H., Park, G. S., Park, S., Chwae, Y. J., Shin, H. J. and Kim, K. (2012). [C-terminal substitution of HBV core proteins with those from DHBV reveals that arginine-rich 167RRRSQSPRR175 domain is critical for HBV replication](#). *PLoS One* 7(7): e41087.
2. Jung, J., Hwang, S. G., Chwae, Y. J., Park, S., Shin, H. J. and Kim, K. (2014). [Phosphoacceptors threonine 162 and serines 170 and 178 within the carboxyl-terminal RRRS/T motif of the hepatitis B virus core protein make multiple contributions to hepatitis B virus replication](#). *J Virol* 88(16): 8754-8767.