行政院國家科學委員會補助專題研究計畫成果報告

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※D型抗原磷酸化的生化分析及在D型肝炎病毒複製的作用及細胞磷酸激酶之尋找(3/3) ※

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計畫類別：■個別型計畫　□整合型計畫

計畫編號：NSC90－2320－B002－170－

執行期間： 90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：陳培哲

計畫參與人員：陳啟武

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：國立台灣大學醫學院

中　華　民　國　90　年　10　月　24　日

一、 中文摘要

D 型肝炎病毒(Hepatitis D virus)的基因為單股的 RNA，於細胞中增殖時中，會產生一種蛋白質，稱為小型 delta 抗原(small delta antigen，S-HDAg，24kDa)，經由 RNA editing 的結果，位於相同的開放閱讀架構(open reading frame)上會產生另外一種更長的蛋白質，稱為大型 delta 抗原(large delta antigen，L-HDAg，27kDa)，所以 L-HDAg 在 C'端較 S-HDAg 多出 19 個胺基酸。兩種蛋白質皆為細胞核內磷酸化蛋白質，以 site-directed mutagenesis 的研究方式，我們已發現：小型 delta 抗原的磷酸會影響 HDV 病毒的增殖。但是其磷酸化的位置以及細胞中控制小型 delta 抗原磷酸的激酶，卻尚未加以定義。

以膠內磷酸(in gel kinase assay)實驗，我們發現有一分子量約 68kDa 的激酶能將小型 delta 抗原磷酸，再經由細胞內蛋白質的純以及文獻的探討，所有實驗數據皆顯示此一 68kDa 激酶是雙股 RNA 活激酶(double-stranded RNA activated kinase，PKR)。此實驗數據總結有：(1)用 PKR 的抗體和 poly(I): (C) agarose 將 PKR 沉澱出來後，能在活體外將小型 delta 抗原磷酸化；(2)將細胞內的 PKR 去除後，此細胞萃取物不再具有磷酸化 S-HDAg 的能力；(3)PKR 活性的減少會減少小型 delta 抗原磷酸；(4)以質譜儀分析更進一步得知：真核細胞中所純的小型 delta 抗原和活體外被 PKR 磷酸化的小型 delta 抗原，其磷酸化位置都位於 serine 177。

D 型肝炎病毒在細胞中的增殖實驗也暗喻著：小型 delta 抗原可能是 PKR 的假性受質(pseudo substrate，decoy substrate)，其磷酸化現象是 D 型肝炎病毒抵禦細胞內免疫系統的一種方式。

在 D 型肝炎病毒的增殖週期中，需要靠 B 型肝炎病毒(Hepatitis B virus)所提供的表面蛋白質(surface antigen)，幫助其組裝(assembly)新的子代病毒顆粒和感染新宿主。此外，因為 HDV 所產生的兩種蛋白質不具有複製酶(replicase)或轉錄酶(transciptase)的活性，所以 D 型肝炎病毒的增殖，或是 delta 抗原的轉錄，必須靠細胞來提供。早期文獻曾用細胞核萃取物來研究 D 型肝炎病毒的增殖與轉錄，顯示它會受 α-amanitin 和 anti-Pol II 抗體(DNA dependent RNA polymerase II)的影響。近來，更以 run-odd assay 證明 D 型炎病毒反基因體(antigenomic sense)向基因體(genomic sense)的複製，是藉由 Pol II 所完成，但是相對的，一直有文獻報

告指出：D 型肝炎病毒兩種基因體的複製，乃至於轉錄，都是由不同的細胞酵素機制所控管。目前仍無活體外的系統來研究基因體向反基因體的複製，所以我們以 run-off assay 來開發此一研究方式。結果發現：HeLa S3 細胞的細胞核萃取物能夠促使基因體的複製，但是它並不會受到 α-amanitin 的影響，而且此活性會因為反應中有二價錳離子而增強，所以初步推論：掌控基因體複製的酵素機制可能不是 Pol II，至於為何種酵素，則可利用本實驗所開發的方式再做更進一步的研究。

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 277, No. 36, Issue of September 6, pp. 33058–33067, 2002
Printed in U.S.A.

The Double-stranded RNA-activated Kinase, PKR, Can Phosphorylate Hepatitis D Virus Small Delta Antigen at Functional Serine and Threonine Residues*

Received for publication, January 22, 2002, and in revised form, May 28, 2002
Published, JBC Papers in Press, June 11, 2002, DOI 10.1074/jbc.M200613200

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Hepatitis D virus (HDV) encodes two proteins, the 24-kDa small delta antigen (S-HDAg) and 27-kDa large delta antigen (L-HDAg) in its single open reading frame. Both of them had been identified as nuclear phosphoproteins. Moreover, the phosphorylated form of S-HDAg was shown to be important for HDV replication. However, the kinase responsible for S-HDAg phosphorylation remains unknown. Therefore, we employed an in-gel kinase assay to search candidate kinases and indeed identified a kinase with a molecular mass of about 68 kDa. Much evidence demonstrated this kinase to be the double-stranded RNA-activated kinase, PKR. The immunoprecipitated endogenous PKR was sufficient to catalyze S-HDAg phosphorylation, and the kinase activity disappeared in the PKR-depleted cell lysate. The S-HDAg and PKR could be co-immunoprecipitated together, and both of them co-located in the nucleolus. The LC/MS/MS analysis revealed that the serine 177, serine 180, and threonine 182 of S-HDAg were phosphorylated by PKR *in vitro*. This result was consistent with previous phosphoamino acid analysis indicating that serine and threonine were phosphorylation targets in S-HDAg. Furthermore, serine 177 was also shown to be the predominant phosphorylation site for S-HDAg purified the from cell line. In dominant negative PKR-transfected cells, the level of phosphorylated S-HDAg was suppressed, but replication of HDV was enhanced. Other than human immunodeficiency virus type 1 trans-activating protein (Tat), S-HDAg is another viral protein phosphorylated by PKR that may regulates HDV replication and viral response to interferon therapy.

Hepatitis delta virus (HDV)[1] is the satellite virus of hepatitis B virus (1, 2), since it requires the hepatitis B virus envelope surface antigen (HBsAg) for viral particle assembly (3–5). Upon superinfection or co-infection with hepatitis B virus, HDV may cause fulminant hepatitis and progressive chronic liver disease (6, 7). The genome of HDV is a circular, single-stranded RNA that resembles the structure of plant viroid (8, 9). HDV contains the ribozyme domains for self-cleavage and self-ligation in both genomic and antigenomic strands of RNA (10, 11). Similar to viroid replication, HDV undergoes a double rolling circle scheme. However, different from viroids, HDV encodes two proteins translated from the same mRNA, small delta antigen (S-HDAg) and large delta antigen (L-HDAg) (12, 13). This viral mRNA is responsible for S-HDAg production. L-HDAg is translated from the same open reading frame through a specific RNA editing process by which the UAG amber termination codon of S-HDAg was converted to UGG tryptophan codon and an additional 19 amino acids were made (14, 15). This adenosine-to-inosine RNA editing is catalyzed by double-stranded RNA adenosine deaminase (15, 16). Although both forms of delta antigens (HDAg) share an identical N-terminal 194 amino acids, their functions are quite different. The S-HDAg is essential for viral replication, whereas L-HDAg inhibits its replication and is required for viral assembly (17–19).

There are several functional domains in HDAg that are responsible for different activities. The N terminus nuclear localization signal and the middle arginine-rich motif mediate HDV RNA transport (20–22). Deletion of the nuclear localization signal or arginine-rich motif leads to the accumulation of HDV RNA in the cytoplasm. The coiled-coil sequence between amino acids 31 and 52 is the delta antigen dimerization signal (23, 24). Furthermore, the nuclear export signal located in the C-terminal domain of L-HDAg is involved in delta antigen exportation to cytoplasm and viral assembly (25). Besides these functional motifs, protein modifications also play important roles in the HDV life cycle. The isoprenylation of L-HDAg has been shown to be required for viral assembly (26). Both forms of HDAg are phosphorylated when they expressed in mammalian cells and infectious hosts (27, 28). Previous phosphoamino acid analysis indicated that L-HDAg was phosphorylated at the serine residue and S-HDAg was phosphorylated at both serine and threonine residues (29, 30). Site-directed mutagenesis in conserved serine and threonine residues of S-HDAg found that substitution of serine 177 by alanine reduced HDV genomic RNA accumulation (31, 32). This result implied the phosphorylation of S-HDAg was probably related to viral replication. To study the underlying mechanism of how delta antigen phosphorylation affects HDV replication, we tried to identify the exact phosphorylation residues and the responsible cellular kinase. By the in-gel kinase assay and subsequent character-

* This work was supported in part by grants from the National Science Council, Executive Yuan, Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HDV, hepatitis D virus; HBsAg, hepatitis B virus envelope surface antigen; HDAg, hepatitis D delta antigen; S-HDAg, hepatitis D small delta antigen; rS-HDAg, recombinant S-HDAg; L-HDAg, hepatitis D large delta antigen; DMEM, Dulbecco's modified Eagle's medium; LC, liquid chromatography; MS, mass spectrometry; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium methyl sulfate.

ization, we found PKR to be the kinase that associated with and subsequently phosphorylated S-HDAg. Furthermore, by the ion trap tandem mass spectrometry, the PKR-phosphorylated residues were identified at serine 177, serine 180, and threonine 182 *in vitro*. Finally, we purified the S-HDAg from a S-HDAg-expressing stable cell line and identified serine 177 as the major phosphorylation residue. *In vivo*, the level of S-HDAg phosphorylation was reduced by overexpressed dominant negative PKR. Besides these biochemical observations, we further found that HDV replication was influenced by PKR activity. This result suggested that PKR participates in the phosphorylation of S-HDAg *in vivo* and influences HDV replication.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Plasmid pcDNA3.1/HDV-2G contains a tandem dimer of the full-length HDV cDNA inserted at the *Xba*I site of the vector pcDNA3.1 (Invitrogen). It transcribes genomic RNA template for replication assay. Plasmid pcDNA3.1/HDV-2AG contains HDV cDNA dimer in the opposite orientation and provides antigenomic RNA for replication. Other HDV-related plasmids used in this experiment were described previously (30, 32). The wild type PKR (PKR-WT) and two dominant negative mutants (PKR-Δ6 and PKR-K296R) (33) were also subcloned into pcDNA3.1 vector.

Cell Lines and Culture Conditions—The N1 cell line was established from HepG2 cell transformed with a trimeric HDV cDNA. HDV RNA replicates constitutively in N1 cells and expresses both small and large delta antigens (34). An S-HDAg-expressing stable cell line, S3-HDAg, was constructed as described previously except that the target cell line is HeLa S3 rather than HuH-7 (35). The expression of S-HDAg in this cell line was confirmed by Western blotting. All of the cell lines used in this report were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. S3-HDAg stable cell line was maintained in DMEM medium containing 1.4 μg/μl G418 (Promega).

Recombinant Small Delta Antigen Purification—To express the small delta antigen, a fragment containing the S-HDAg reading frame was ligated into the *Bam*HI cloning site of pET-15a. The constructed pET-15a-SHDAg was transformed to BL21-CodonPlus (DE3)-RIL competent cells (Stratagene). A single colony was picked and cultured in 10 ml of LB broth containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol overnight. The bacteria were spun down and transferred to 1 liter of LB broth containing 150 μg/ml ampicillin and 34 μg/ml chloramphenicol. When the A_{600} reached 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM and cultured for an additional 3 h. The bacteria were pelleted down and resuspended in 40 ml of lysis buffer (50 mM Tris-Cl, pH 7.5, 10% sucrose, 10 mM $MgCl_2$, 2% Triton X-100, 1 mg/ml lysozyme, and 50 μg/ml DNase). The soluble fraction and inclusion body were separated by centrifugation at 3000 × *g* for 30 min. The recombinant S-HDAg was located in inclusion body. S-HDAg in the inclusion body was further purification by following the procedure described in Ref. 36, except the washing solution containing 2 M urea.

Cell Lysate Preparation and In-gel Kinase Assay—This protocol followed Ref. 37. Briefly, HeLa S3 cells (about 2 × 10^7) were lysed by 0.5 ml of lysis buffer (50 mM HEPES, 100 mM NaCl, 50 mM sodium fluoride, 5 mM glycerophosphate, 2 mM EDTA, 1 mM sodium vanadate, and 1% Triton X-100) and cleared by centrifugation at 14,000 × *g* for 20 min. This lysate was used for the in-gel kinase assay and immunoprecipitation/*in vitro* kinase assay.

For the in-gel kinase assay, purified recombinant S-HDAg was included in the SDS-polyacrylamide gel at a final concentration of 1 mg/ml. After electrophoresis, the gel was sequentially immersed in wash, equilibrium, denaturation, and renaturation buffers. Finally, the gel was equilibrated in 200 ml of kinase assay buffer (15 mM HEPES, 2 mM dithiothreitol, and 2 mM $MgCl_2$) for 30 min then incubated in 10 ml of kinase buffer containing 50 μM ATP and 20 μCi/ml [γ-^{32}P]ATP at 30 °C for 30 min. After reaction, the gel was soaked in 5% trichloacetic acid solution to remove nonincorporated [γ-^{32}P]ATP. The gel was dried on a 3MM filter and used for autoradiography.

Immunoprecipitation and in Vitro Kinase Assay—To immunoprecipitate PKR for in-gel kinase and *in vitro* kinase assays, protein G-agarose conjugated with 1 μg/μl mouse anti-human PKR (Transduction Laboratories) was added to the HeLa S3 cell lysate (500 μg). The same amount of protein G-agarose-conjugated mouse anti-rat PKR serum (Transduction Laboratories) and mouse normal serum (Jackson) were used as negative controls. The PKR also could be precipitated by 20 μl of poly(I:C)-agarose (Amersham Biosciences). For the in-gel kinase assay, the bound PKR was eluted by 20 μl of 8 M urea and then subjected to electrophoresis. For the *in vitro* kinase assay, the PKR-bound agarose was washed by 0.5 ml of PKR kinase buffer (15 mM HEPES, 2 mM dithiothreitol, 2 mM $MgCl_2$, and 50 μM ATP) twice. Four micrograms of recombinant S-HDAg and kinase assay buffer containing 20 μCi/ml [γ-^{32}P]ATP were added to the washed agarose in a final volume of 30 μl and then incubated at 30 °C for 30 min. After reaction, an equal volume of 2× Laemmli sampling buffer was added, and then the mixture was boiled for 10 min. This sample was subjected to 12% SDS-PAGE. The gel was dried on a 3MM filter and used for autoradiography.

Immunofluorescence—An S3-HDAg or N1-stable cell line was cultured in six-well plates. Before immunofluorescence staining, the cells were treated with 1000 units/ml interferon-α (Calbiochem) for 18 h and then fixed with 2.5% paraformaldehyde/phosphate-buffered saline for 30 min at room temperature. After 1% Triton X-100/phosphate-buffered saline soaking, cells were stained with human anti-HDAg serum and mouse anti-human PKR serum (Transduction Laboratories). After further staining by fluorescein isothiocyanate-conjugated anti-human IgG or rhodamine-conjugated anti-mouse IgG (Jackson), the S-HDAg and PKR localization was monitored by a confocal spectral microscope (Leica TCS SP2) according to the manufacturer's protocol.

Purification of S-HDAg Expressed in Eukaryotic Cells—Since the S-HDAg is a nucleoprotein, a procedure modified from Dignam's nuclear extraction protocol was used to remove the cytoplasmic protein to facilitate its purification (38, 39). Briefly, the S3-HDAg cells (about 1 × 10^9) were resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and disrupted by 40 strokes of a Kontes Dounce homogenizer. The homogenate was spun for 10 min at 1000 × *g*, the supernatant was completely removed, and then the nuclei were harvested by further centrifugation at 25,000 × *g* for 20 min. Nuclear pellet was homogenized in 2.5 ml of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) by 10 strokes of the Kontes Dounce homogenizer (B type pestle) and stirred at 4 °C for 30 min. The crude nuclear extract was centrifuged for 30 min at 25,000 × *g*. The remaining nuclear debris was completely lysed by 25 mM phosphate buffer, pH 7.5, 2% Triton X-100. This lysate was further disrupted by a homogenizer until the viscosity disappeared. After 25,000 × *g*, 30-min centrifugation, the S-HDAg was located in the supernatant. This partially purified S-HDAg was further purified by the strong cationic column or anti-HDAg affinity column.

In the strong cationic column purification protocol, the 20HS column (PerSeptive Biosystem; BIOCAD) was pre-equilibrated by five column volumes of equilibrium buffer (35 mM phosphate buffer, pH 7.5, 5 mM NaCl). After loading a 100-mg protein sample, the column was prewashed by 20 column volumes of washing buffer (35 mM phosphate buffer, pH 7.5, 50 mM NaCl). The binding protein was eluted in a stepwise manner with different NaCl concentrations from 0.5 to 2.5 M. The NaCl concentration was increased by 0.25 M in each step. Finally, the column was cleaned by a cleaning solution (35 mM phosphate buffer, pH 7.5, and 3 M NaCl).

For preparing the anti-HDAg affinity column, the anti-HDAg monoclonal antibody (30) was mixed with Poros protein G beads (PerSeptive Biosystem; BIOCAD) at a concentration about 2 mg of antibody/ml of wet beads. After incubation at room temperature for 1 h, the beads were washed with 10 volumes of 0.2 M sodium borate. The washed beads were resuspended in 10 bead volumes of 0.2 M sodium borate and then mixed with methylpimelimidate to bring the final concentration to 20 mM. After coupling at room temperature for 30 min, the reaction was stopped by 0.2 M ethanolamine. The antibody-coupled beads could be packaged into columns by following the manufacturer's protocol. The anti-HDAg affinity column purification protocol is similar to a cationic column except that the elution step was substituted by 20-column volumes of glycine buffer (0.1 M glycine, 0.3 M $MgCl_2$). The eluted S-HDAg-containing fraction was directly analyzed by Western blotting and concentrated by Centricon Plus-20 centrifugal filter device (Millipore Corp.) according to the manufacturer's instructions. The concentrated S-HDAg was subjected to SDS-PAGE. S-HDAg was cut off from the gel after Coomassie Brilliant Blue staining and subjected to in-gel digestion and LC/MS/MS analysis (see blow).

Protein Extraction and Western Blot Analysis—To detect the S-HDAg expression in S3-SHDAg cell, cells (about 1 × 10^7) were lysed in 1 ml of radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktails). For Western blot analysis, about 50 μg of the protein was mixed with an equal volume of 2× Laemmli sampling buffer, boiled for 10 min, and

then subjected to 12% SDS-PAGE. After electrotransfer, the expressed S-HDAg and PKR were monitored by anti-HDAg serum and anti-human PKR serum (Transduction Laboratories) using the ECL Western detection Kit (Amersham Biosciences).

LC/MS/MS Analysis of Phosphorylated Peptides—The preparation of tryptic digest was carried out based on the method described previously (40). Briefly, S-HDAg polypeptide in the gel was reduced by 2% mercaptoethanol, 25 mM NH_4HCO_3, and then modified by 5% 4-vinylpyridine in 25 mM NH_4HCO_3. The pyridylethylated protein was incubated with 1 μg/ml of modified trypsin (Promega) at 37 °C overnight. The tryptic digest was divided into three equal aliquots before storage at −20 °C.

The tryptic digest was analyzed for identification of phosphorylated amino acid residues by LC/MS/MS. All of the LC/MS/MS experiments were performed on an LCQ ion trap mass spectrometer (Thermo Finnigan) coupled on an in-line ABI 1400 high pressure liquid chromatograph (PerkinElmer Life Sciences) equipped with a 150 × 0.5-mm PE Brownlee C18 column (PerkinElmer Life Sciences). The sample was typically loaded in 5% acetonitrile with 0.1% formic acid. The gradient consisted of 5–30% acetronitrile in 10 min and subsequent 30–65% acetronitrile in 50 min.

The first aliquot was analyzed by LC/MS/MS at an automatic mode. The spectra of eluate were collected as successive sets of three different scans: MS, ZOOM, and MS^2 scans. The MS scan defined the ion composition at an *m/z* range of 395–1605; the ZOOM scan examined the isotype patterns of the most intense ion in the MS scan; and the MS^2 scan acquired the mass spectrum of the most intense ion upon collision-induced dissociation. The raw data were subjected to automatic interpretation by Sequest Brower software (Finnigan). The enzyme was not specified during the search, which increased the confidence of identification. The matched peptides had proper cleavage sites. A 105.14-Da mass was assigned to all lysine residues that were alkylated in all experiments. The procedure for identification of phosphopeptides by selected ion chromatogram analysis was described in detail previously (40). Briefly, the selected ion chromatograms were graphed for Sequest-identified peptides and their hypothetical phosphopeptides to determine their retention time. A hypothetical phosphopeptide was considered as putative phosphopeptides only if its retention time was within 5 min of that of the corresponding unmodified peptide. The identities of these putative peptides were verified by LC/MS/MS in a mass-dependent mode. Only the ions with appropriate *m/z* values were selected for ZOOM and MS^2 scan. The acquired collision-induced dissociation spectra were analyzed by Sequest as well as direct inspection.

DNA and Ribonucleoprotein Transfection—In co-transfection experiments, HDV cDNA-expressing plasmid (1 μg) and PKR-expressing plasmid (4 μg) were diluted in 250 μl of OPTI-MEM reduced serum medium (Invitrogen). Ten microliters of LipofectAMINE 2000 (Invitrogen) was diluted in the same medium then mixed with previously prepared plasmid at room temperature for 20 min. Before transfection, 293T or HuH-7 cells (2.4 × 10^5 cells/well) were replaced by 2 ml of serum-free DMEM in the six-well plate and then incubated with the LipofectAMINE 2000-plasmid complex for 6 h. Four days after transfection, total RNA and protein were harvested for HDV RNA and S-HDAg analysis by Northern or Western blot (4).

For ribonucleoprotein transfection, the procedure was modified from a previous report (41). COS7 cells were seeded on a six-well plate (2.4 × 10^5 cell/well) and cultured in a 37 °C incubator overnight. Before transfection, culture medium was replaced by fresh 5% fetal calf serum-DMEM for 4 h. The *in vitro* transcribed HDV dimeric genomic RNA (2 μg) was mixed with recombinant S-HDAg (0.4 μg) in a final volume of 20 μl in 10 mM HEPES buffer at room temperature for 10 min. Ten microliters of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) transfection reagent (Roche Molecular Biochemicals) was diluted in 23 μl of 10 mM HEPES buffer for 15 min and then mixed with the previous RNA-protein complex for another 15 min. The COS7 culture medium was discarded and replaced with 1 ml of 5% fetal calf serum-DMEM and DOTAP-ribonucleoprotein mixture for 16 h. Four days after transfection, total RNA and protein were harvested to detect HDV replication.

Preparation of Antibody against Ser^{177}-phosphorylated S-HDAg—The phosphorylated peptide, ^{167}FVPNLQGVPEpSPFSRTGE^{184}, with a phosphogroup at serine 177 was synthesized (Genemed). To enhance antigenicity, eight multiple antigenic peptides were incorporated (42). The synthesized peptide (2 mg) was dissolved in 0.4 ml of phosphate-buffered saline and 0.6 ml of Freund's complete adjuvant (Invitrogen) buffer. After subcutaneous immunization four times with 2 mg of peptides, rabbit antiserum against Ser^{177}-phosphorylated S-HDAg peptide was acquired. To deplete the nonspecific antibody in the serum that can recognize the nonphosphorylated S-HDAg, the crude rabbit antiserum was adsorbed to nonphosphorylated, recombinant small delta antigen.

The Hybond-C extra (Amersham Biosciences) membrane (about 10 × 10 cm) was immersed in 20 ml of 10 mM Tris-HCl, pH 8.5, 6 M urea buffer containing 20 mg of recombinant S-HDAg at 4 °C. After overnight incubation, the membrane was washed by 40 ml of 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20 buffer three times. About 40 ml of rabbit anti-serine 177-phosphorylated S-HDAg serum was incubated with the membrane saturated by recombinant S-HDAg at 4 °C overnight. The supernatant was harvested and checked for its specificity by Western blotting. The adsorption procedure was repeated until the serum did not recognize the nonphosphorylated recombinant S-HDAg.

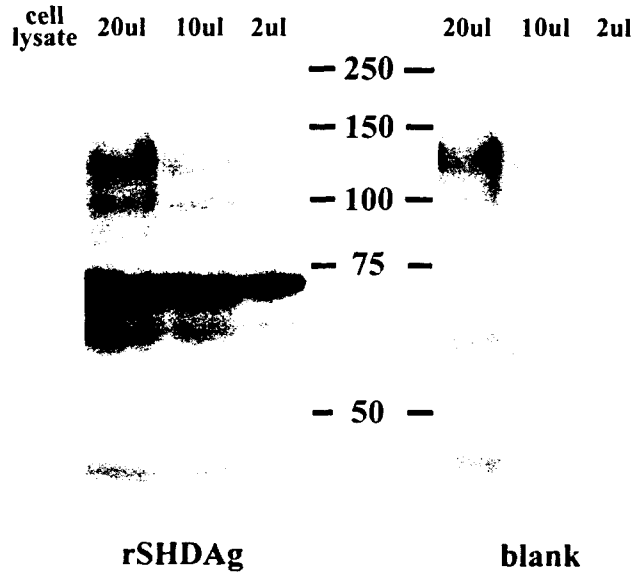

FIG. 1. **Identification of the kinase for HDV S-HDAg by in-gel kinase assay.** In-gel kinase assay with recombinant S-HDAg as the substrate was performed to identify the candidate kinase in HeLa S3 cell lysate. Different amounts of crude cell lysate were separated in rS-HDAg-containing gel (*left panel*) or blank gel (*right panel*). After electrophoresis and the protein renaturation procedure, the gels were soaked in kinase buffer with [γ-^{32}P]ATP. If any kinase could phosphorylate S-HDAg, the gel will exhibit the S-HDAg phosphorylation signal at the corresponding molecular weight of such a S-HDAg kinase. In the *left panel*, a clear band with a molecular mass of about 68 kDa was found. Depending on the amounts of cell lysate loading, the phosphorylation signal gradually diminished (*left panel*; the amounts of loaded lysate decreased from 20 to 2 μl). Other minor bands that appear both in rS-HDAg-containing gel and blank gel are nonspecific signals (compare *left panel* with *right panel*).

RESULTS

S-HDAg was Phosphorylated by a 68-kDa Protein—The *in vivo* orthophosphate labeling experiment revealed that both of the S-HDAg and L-HDAg are phosphorylated proteins (27). To date, the kinase responsible for their phosphorylation has yet to be characterized. The known HDAg-interacting proteins, such as the delta antigen interaction protein A (43) and nucleolar phosphoprotein B23 (44), have no kinase activities. The yeast two-hybrid system and protein fraction method had been tried when searching for HDAg-associated kinase without success in our laboratory. Therefore, we used the in-gel kinase assay system to examine the candidate kinase for S-HDAg phosphorylation.

The total HeLa S3 cell lysate was separated in a SDS-polyacrylamide gel containing recombinant S-HDAg (rS-HDAg). After electrophoresis and protein renaturing, the gel was incubated in a kinase buffer with [γ-^{32}P]ATP. A single major band of ~68 kDa was only specifically found in the rS-HDAg-containing gel (Fig. 1, *left panel*) but not in the control gel without any protein incorporated (Fig. 1, *right panel*). Besides, this ~68-kDa signal was not detected in the gel containing total

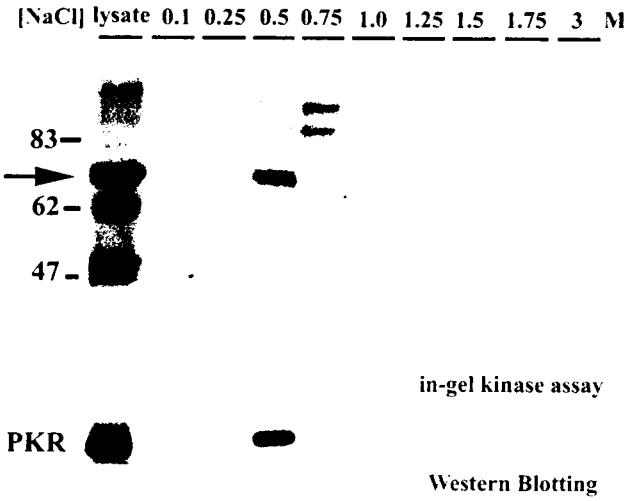

FIG. 2. **PKR and S-HDAg kinase co-locate in the same chromatographic fraction.** The total HeLa S3 protein was fractionated on a cationic exchange column by different NaCl concentration. The concentration of NaCl in each eluted fraction is indicated *above* each *lane*. Every fraction was analyzed in two experiments: the in-gel kinase for detecting S-HDAg kinase (*upper panel*) and Western blotting for monitoring PKR (*lower panel*). The ~68-kDa kinase is indicated by an *arrow*. This kinase was located in the PKR-containing fraction (0.5 M NaCl fraction).

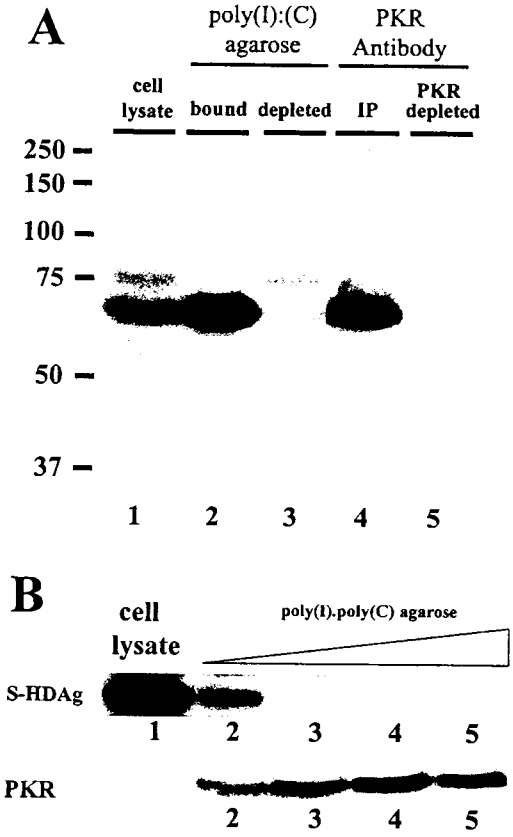

FIG. 3. **PKR is the kinase for S-HDAg.** *A*, PKR precipitation experiments. The ~68-kDa kinase that phosphorylated S-HDAg in the in-gel kinase assay was demonstrated in *lane 1* as a positive control. The cellular endogenous PKR was precipitated by poly(I:C)-agarose (*lane 2*) or anti-human PKR serum (*lane 4*). When this precipitated sample was subjected to an in-gel kinase assay, it could phosphorylate S-HDAg (*lanes 2* and *4*). Furthermore, cell lysates in which PKR was depleted either by poly(I:C)-agarose (*lane 3*) or anti-PKR antibody (*lane 5*) lost kinase activity. *B*, PKR depletion experiments. The *upper panel* shows an in-gel kinase assay that used recombinant S-HDAg as the substrate. In a constant concentration of HeLa S3 cell lysate, gradually depleting the amount of PKR by poly(I:C)-agarose reduced the S-HDAg phosphorylation signal. An increasing amount of precipitated PKR was shown in Western blotting by anti-PKR serum (*lower panel*).

Escherichia coli protein (the *E. coli* was transformed by vector only) as substrate in the in-gel kinase assay (data not shown).

Depending on the amount of loaded HeLa S3 cell lysate (Fig. 1, *left panel*, 20, 10, and 2 μl), the ~68-kDa phosphorylation signal decreased in a dose-dependent manner. Other faint bands around 68 kDa are nonspecific signals. They were also present in the blank gel (*right panel*). These might be other nonspecific kinase from crude total cell lysate.

The 68-kDa S-HDAg Kinase and Double-stranded RNA-activated Kinase Were Co-eluted in Cationic Exchange Chromatography—By previous in-gel kinase assay, we found a 68-kDa protein able to phosphorylate S-HDAg. To purify and identify this kinase, the total cellular proteins of HeLa S3 cell were resolved on a cationic exchange column. Under the NaCl stepwise elution procedure, the crude cell lysate was fractionated, and then each fraction was subjected to in-gel kinase assay. The previously identified S-HDAg kinase activity was detected in the 0.5 M NaCl eluted fraction (Fig. 2, *upper panel*). Besides the ~68-kDa signal, the other phosphorylation signals that appeared in the total lysate were nonspecific signals because they also appeared in the blank gel (data not shown). These signals were probably caused by too much protein in the crude lysate. When the kinase source was fractionated, they did not appear in the gel, such as the signal of ~48- and ~62-kDa protein (Fig. 2, *upper panel*).

Among the known cellular kinase with a molecular mass of ~68 kDa, we were particularly interested in PKR that had been shown to be associated with and activated by HDV RNA (45, 46). Therefore, to determine whether the ~68-kDa S-HDAg kinase was PKR, previous fractionated HeLa S3 cellular protein was analyzed by Western blotting with antibody specific to PKR. Interestingly, the PKR lined up with the S-HDAg kinase activity in the same 0.5 M NaCl eluted protein fraction (Fig. 2, *lower panel*). This strongly suggested that PKR is the S-HDAg kinase identified by in-gel kinase assay.

Immunoprecipitated PKR phosphorylated S-HDAg in the In-gel Kinase Assay—In order to further determine whether the PKR was the S-HDAg kinase, we first tested whether the PKR could catalyze S-HDAg phosphorylation. Thus, PKR was immunoprecipitated from the HeLa S3 total lysate, and then we examined kinase activity for S-HDAg by in-gel kinase assay. As anticipated, we found that the 68-kDa kinase activity was enriched in anti-PKR immunoprecipitate (Fig. 3*A*, *lane 4*), whereas such activity vanished in the PKR-depleted cell lysate (Fig. 3*A*, *lane 5*).

Extensive structural studies of PKR have revealed that its N-terminal end contains a double-stranded RNA-binding motif. If the S-HDAg kinase was PKR, we expected that this 68-kDa S-HDAg kinase would be captured by the double-stranded RNA analog, poly(I:C). After poly(I:C) agarose was incubated with cell lysate, the precipitate was analyzed by in-gel kinase assay and revealed a ~68-kDa band (Fig. 3*A*, *lane 2*). Like the PKR-depleted cell lysate, cell lysate pretreated with poly(I:C)-agarose almost lost S-HDAg phosphorylation activity (Fig. 3*A*, *lane 3*). Furthermore, adding a gradually increasing amount of poly(I:C)-agarose into a constant amount of HeLa S3 cell lysate, we found that more PKR depleted by poly(I:C)-agarose coincided with a weaker S-HDAg phosphorylation signal (Fig. 3*B*, *upper panel*). The poly(I:C)-agarose-precipitated PKR was checked in the Western blotting (Fig. 3*B*, *lower panel*). In comparison with the *upper panel* (S-HDAg in-gel kinase assay) and *lower panel* (Western blotting by anti-PKR serum), it ex-

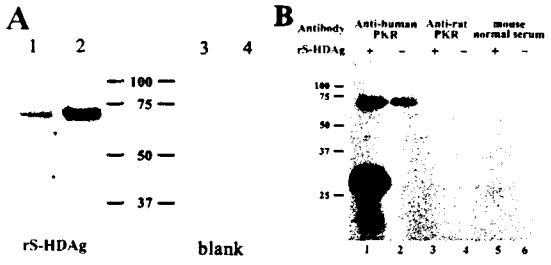

FIG. 4. *A*, interferon-α enhances S-HDAg kinase activity. The total lysate harvested from cells with (*lanes 2* and *4*) or without (*lanes 1* and *3*) interferon treatment. The equal amounts of HeLa S3 cell lysate were subjected to in-gel kinase assay. The S-HDAg phosphorylation signal in interferon-treated lysate (*lane 2*) is more prominent than in lysate not treated with interferon (*lane 1*). Regardless of which lysate was used, the phosphorylation band did not exist in the blank gel (*lanes 3* and *4*). *B*, the *in vitro* kinase assay demonstrated that PKR phosphorylated recombinant S-HDAg. The rS-HDAg was incubated with immunoprecipitated PKR in kinase buffer. The labeled protein was separated in SDS-PAGE. The rS-HDAg was clearly phosphorylated by PKR (*lane 1*, ~27 kDa). It also exhibits the autophosphorylation signal of immunoprecipitated PKR (*lanes 1* and *2*, ~68 kDa). Since the mouse anti-rat PKR serum and mouse normal serum cannot precipitate human PKR, their immunoprecipitated complex cannot phosphorylate rS-HDAg (*lanes 3–6*).

hibited an obvious dose-dependent change. This further supported PKR as an S-HDAg kinase.

Interferon Enhanced S-HDAg Kinase Activity and in Vitro Kinase Assay Demonstrated PKR as S-HDAg Kinase—It has been shown that the PKR activity is up-regulated 3–4-fold upon interferon-α stimulation (47). We wondered whether S-HDAg kinase was also stimulated by the same treatment. The crude cell lysate harvested from the 1000 units/ml interferon-α-treated HeLa S3 cell was subjected to an in-gel kinase assay. The results revealed that the S-HDAg kinase activity indeed increased with PKR activity (Fig. 4*A*, *lane 2*) when compared with that from nontreated HeLa S3 cell (Fig. 4*A*, *lane 1*).

In addition to the in-gel kinase assay, we also developed an *in vitro* kinase assay using immunoprecipitated PKR from HeLa S3 lysate as the kinase source. Without the addition of rS-HDAg, only a ~68-kDa band was phosphorylated (Fig. 4*B*, *lane 2*), which was the autophosphorylated PKR. When the rS-HDAg was added as substrate, a prominent phosphorylated rS-HDAg (of 27 kDa) was detected (Fig. 4*B*, *lane 1*). The specificity was confirmed by control antibodies. The mouse anti-rat PKR serum and mouse normal serum could not precipitate human PKR. When their immunoprecipitated complexes were used as a kinase source, no matter whether the reaction contained rS-HDAg, the rS-HDAg phosphorylation signal was not observed (Fig. 4*B*, *lanes 3–6*). Since rS-HDAg was easily degraded in the purification step, the extra band (Fig. 4*B*, *lane 1*, smaller than 25 kDa) was probably the degraded, phosphorylated rS-HDAg. Since the S-HDAg kinase was only precipitated by anti-human PKR, this clearly showed that S-HDAg was phosphorylated by PKR.

S-HDAg Associated with PKR and Co-localized in the Nucleolus—To verify PKR as the kinase for S-HDAg phosphorylation *in vivo*, we also examined whether PKR and S-HDAg were located in the same subcellular area. The cellular distribution patterns of PKR and S-HDAg were analyzed in S-HDAg-expressing HeLa S3 cells (S3-HDAg) and HDV cDNA-stably transfected HepG2 cells (N1) by confocal microscopy. It has previously been found that PKR localization is heterogeneous in resting cells, but it becomes enriched and concentrated in cytoplasm and nucleolus upon interferon-α treatment (47). Therefore, in S3-HDAg cells treated with interferon-α (*IFN-α*) for 18 h, PKR was detected in cytoplasm and nucleolus (Fig. 5*A*, *left panel*). Meanwhile, S-HDAg was predominantly present in nucleolus (Fig. 5*A*, *middle panel*). The confocal microscopy clearly demonstrated PKR and S-HDAg were co-localized in nucleolus (Fig. 5*A*, *right panel*). This cellular distribution pattern was also observed in HDV replication cells, N1 (data not shown).

Since the potential interaction in nucleolus had been demonstrated, it was intriguing to know whether PKR might interact with S-HDAg *in vivo*. It has been shown that both PKR and S-HDAg are RNA-binding proteins. This led us to consider another possibility, that PKR, S-HDAg, and HDV RNA might form a trimeric complex.

The FLAG-SHDAg was transiently expressed in 293T cells. The endogenous PKR and its associated proteins were immunoprecipitated by anti-PKR serum. When the PKR was precipitated from the lysate, a low amount of S-HDAg was also detected in this anti-PKR immunoprecipitate complex (Fig. 5*B*, *lane 2*). Because the interaction between S-HDAg and PKR seemed very faint, poly(I:C)-agarose was used to capture PKR and its associated proteins. By this method, more S-HDAg was co-precipitated (Fig. 5*B*, *lane 1*). Reciprocal immunoprecipitation using anti-HDAg serum and Western blotting by anti-PKR serum also showed that a significant amount of PKR was co-precipitated with S-HDAg (Fig. 5*C*, *lane 1*). These co-immunoprecipitation experiments further supported an association between S-HDAg and PKR.

S-HDAg Purification and Phosphorylation Site Identification—To identify the PKR phosphorylation residues on S-HDAg, the rS-HDAg was phosphorylated *in vitro* by PKR and subjected to LC/MS/MS analysis. Through analyzing the selected ion chromatograms of S-HDAg peptides, we found a segment of S-HDAg harboring three PKR-phosphorylated residues. As shown in Fig. 6*A*, ^{161}GAPGGGFVPNLQGVPESPFSR^{181} was a phosphopeptide. We concluded that a phosphate group was located in serine 177 by observation of derivatives of y4 and b19 fragment ions. Another longer peptide, ^{161}GAPGGGFVPNLQGVPESPFSRTGEGLDIR^{189}, could also be doubly phosphorylated. Base on its collision-induced dissociation spectrum, we concluded that serine 180 and threonine 182 were two additional targets (Fig. 6*B*). In summary, Ser-177, Ser-180, and Thr-182 constituted a short stretch for PKR phosphorylation.

We also purified S-HDAg from the S3-SHDAg cell line to determine its phosphorylation sites *in vivo*. We first used subcellular fractionation to enrich the S-HDAg. Because the majority of S-HDAg was located in nucleus, it was not detected in the cytosolic fraction (Fig. 6*C*, *S100*). After the nuclear protein extracted in 0.42 M NaCl was removed (Fig 6*C*, 0.42 M NaCl), the S-HDAg was extracted when the remainder of the nucleus was treated with 2% Triton X-100 (Fig. 6*C*; *Triton X-100*). The S-HDAg in this fraction was further purified by ion exchange or anti-HDAg affinity chromatography. The purity of purified S-HDAg was analyzed in an SDS-polyacrylamide gel (Fig. 6*C*, *right panel*), whose identity was verified by Western blotting and LC/MS/MS analysis. The protein was subjected to tryptic digestion and LC/MS/MS analysis. Only one peptide, ^{161}GAPGGGFVPNLQGVPESPFSR^{181}, was found to be phosphorylated. Its collision-induced dissociation spectrum was identical to the same peptide prepared *in vitro* (similar to Fig. 6*A*), which indicated that serine 177 was the dominant phosphorylation site *in vivo*.

Suppression of Endogenous PKR by Dominant Negative PKR Reduced the Phosphorylation of S-HDAg—Although we demonstrated that serine 177 at S-HDAg was the *in vivo* phosphorylation site and phosphorylated by PKR *in vitro*, it remained to be determined whether PKR could influence phosphorylation of S-HDAg in cells. If PKR can phosphorylate S-HDAg in

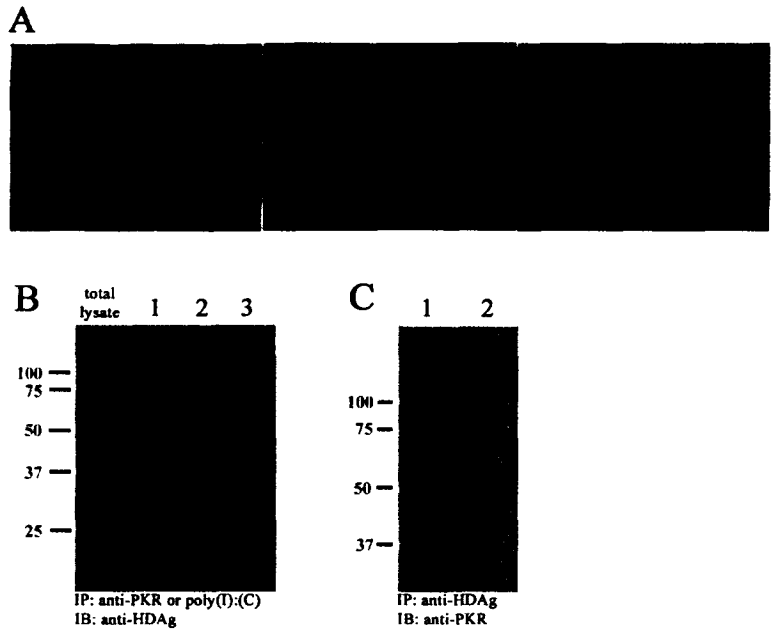

FIG. 5. **S-HDAg is associated with PKR and co-localized in the nucleolus.** *A*, the HeLa S3 cell stably expressing S-HDAg were treated with 1000 units/ml interferon-α for 18 h, and then PKR and S-HDAg localizations were identified by immunofluorescence confocal microscopy. The S-HDAg accumulates in the nucleolus (*middle panel*), and PKR distributes in cytoplasm and nucleolus (*left panel*). After merging these two pictures, their colocalization in the nucleolus is confirmed by confocal microscopy (*right panel*). *B*, S-HDAg was coimmunoprecipitated by anti-PKR serum or poly(I:C)-agarose. The 293T cells were transfected with pFLAG-S-HDAg (*lanes 1* and *2*) or vector only (*lane 3*). The endogenous PKR and its associated protein complex were precipitated by poly(I:C)-agarose (*lane 1*) or anti-PKR antibody (*lane 2*). The expression of FLAG-tagged S-HDAg (indicated by total lysate) was directly analyzed in Western blotting (*IB*). The precipitate complex was also analyzed in Western blotting by anti-HDAg antibody. *C*, the same experiments were performed as in *B* except that the cell lysate was immunoprecipitated by anti-HDAg serum. The co-precipitated endogenous PKR was detected by its specific antibody (*lane 1*). The blank vector control was shown in *lane 2*.

cells, suppression of PKR activity would reduce the phosphorylation of S-HDAg.

To detect the phosphorylated S-HDAg, we prepared antibody that specifically recognized Ser^{177}-phosphorylated S-HDAg. The anti-Ser^{177}-phosphorylated S-HDAg serum was raised by immunization of rabbit with ^{167}FVPNLQGVPEpSPFSRTGE^{184} peptide with the phospho group at serine 177. After serum adsorption to remove nonspecific recognition, this antibody cannot recognize *E. coli*-expressed nonphosphorylated recombinant S-HDAg (Fig. 7*A*, *lane 4*). However, it can react with phosphorylated S-HDAg expressed in transfected cells (Fig. 7*A*, *lane 6*) or a recombinant S-HDAg variant (serine 177 replaced by aspartic acid) that might simulate the phosphorylated S-HDAg (Fig. 7*A*, *lane 5*).

To study the effect of PKR on S-HDAg phosphorylation, we attempted to use dominant negative PKR to suppress the endogenous PKR activity. One dominant negative PKR-expressing plasmid (PKR-K296R) was transfected into 293T cells, together with S-HDAg expression plasmid. Their expressions were identified by anti-PKR or anti-HDAg serum (32). In cells transfected only with S-HDAg-expressing plasmid, the S-HDAg was easily detected (Fig. 7*B*, *panel 1*, *lane 1*). The endogenous PKR was also detected by anti-PKR antibody (Fig. 7*B*, *panel 2*, *lane 1*). After co-transfected with wild-type PKR-expressing plasmid, the amount of total PKR increased (Fig. 7*B*, *panel 2*, *lane 2*). However, as PKR suppresses protein translation, the expression of S-HDAg was reduced (Fig. 7*B*, *panel 1*, *lane 2*). In contrast, in cells receiving dominant negative PKR mutant, the expression of S-HDAg was restored (*panel 1*, *lane 3*), indicating a functional PKR mutant (*panel 2*, *lane 3*).

To evaluate the effect of PKR suppression on S-HDAg phosphorylation, S-HDAg was immunoprecipitated by human anti-HDAg serum first in order to bring down an equal amount of total S-HDAg among various co-transfected cells. The immunoprecipitates were assayed by either rabbit anti-HDAg serum (*panel 4*) or anti-Ser^{177}-phosphorylated HDAg antibody (*panel 3*). About equal amount of total HDAg was precipitated (Fig. 7*B*, *panel 4*). Compared with blank vector or wild type PKR co-transfected cells, the phosphorylated S-HDAg in dominant negative PKR overexpression cells clearly decreased (Fig. 7*B*, *panel 3*, *lane 3*). The data strongly suggested that the PKR might phosphorylate S-HDAg in the cells.

Suppression of PKR Activity Enhances HDV Replication—After showing that PKR probably phosphorylated S-HDAg *in vivo*, we then examined whether modulation of PKR activity could have any effects on HDV replication. To investigate this possibility, the COS7 or HuH7 cells were cotransfected with a replication-competent HDV cDNA clone (HDV-2G) and a plasmid expressing either wild type PKR or either of the two dominant negative PKR mutants (Δ6 or K296R point mutation dominant negative PKR). As shown by the Northern blot analysis of HDV RNA in Fig. 8*A*, HDV RNA replication was reduced when its cDNA was cotransfected with wild type PKR (Fig. 8*A*, *lane 2*), indicating a replication-suppressing effect by wild-type PKR. However, HDV replication increased when it was co-transfected with a dominant negative PKR (Fig. 8*A*, *lanes 3* and *4 versus lane 1*). The results implied that PKR activity could influence HDV replication, and a block of endogenous PKR by dominant negative mutants could enhance HDV RNA replication.

PKR is up-regulated by interferon-α treatment. However, in previous interferon-α treatment experiments, the HDV replication in the S-HDAg-stably expressed cell line was not affected by interferon-α, despite an increased level of PKR (48). To clarify the difference, we performed a similar assay in both COS7 cells and the S-HDAg-stably expressed COS7 cell line, COS7-S, by ribonucleoprotein transfection. As shown in Fig. 8*B*, HDV replication was dramatically inhibited by interferon-α in naive COS7 cells (Fig. 8*B*, *lane 1 versus lane 2*). However, it was not inhibited by interferon-α in the S-HDAg-stably ex-

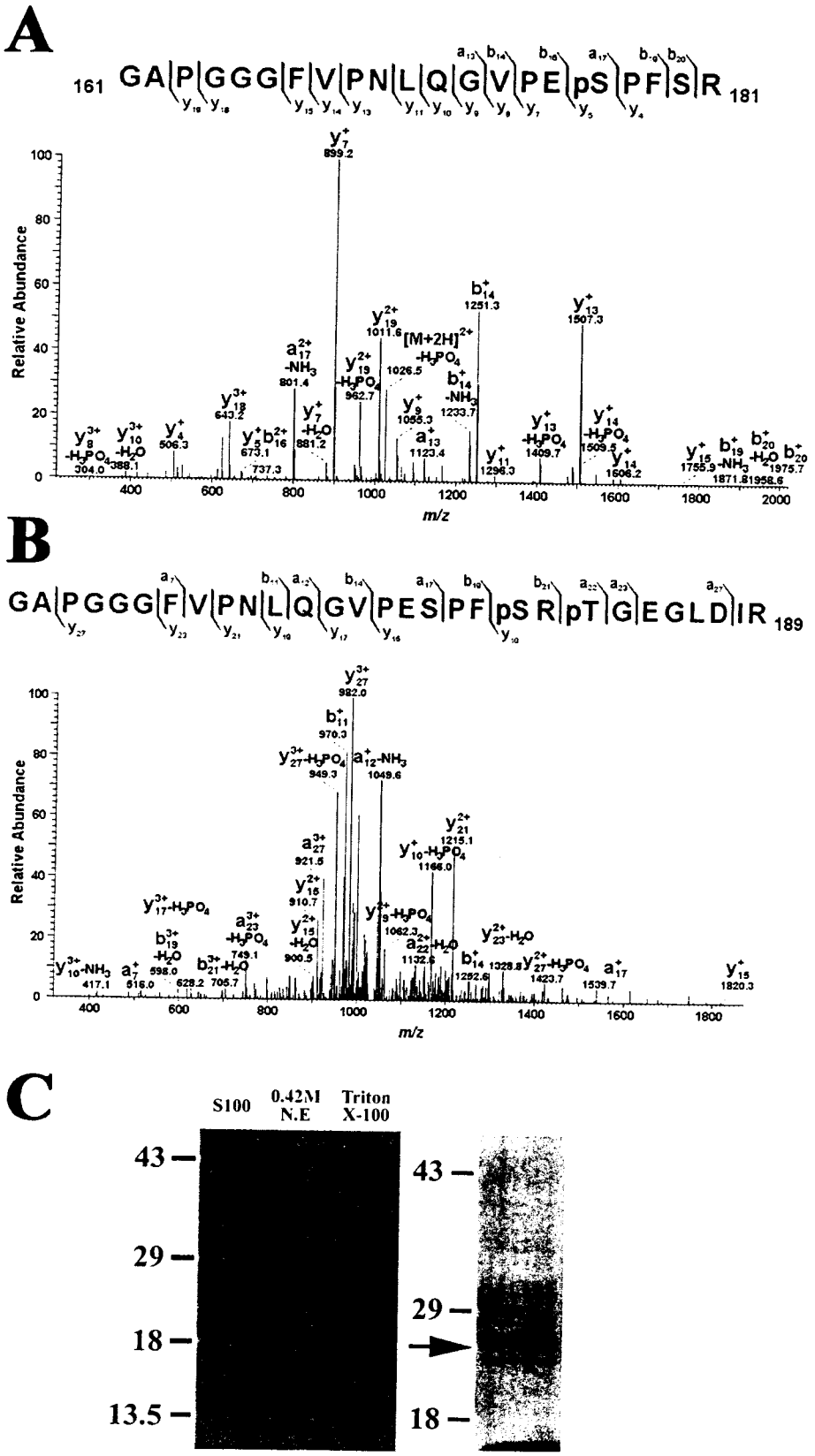

FIG. 6. **Identification of S-HDAg *in vitro* and *in vivo* phosphorylation sites.** The rS-HDAg that was phosphorylated by immunoprecipitated PKR was analyzed by tryptic digestion and mass spectrometry. The LC/MS/MS spectrum shown in *A* and *B* indicated that ^{161}GAPGGGFVPNLQGVPESPFSRTGEGLDIR^{189} was the phosphorylation target. *A*, all of the y ions from y1–5 derived from the phosphopeptide have a mass shift of 80. The y4 and b19 demonstrated that serine 177 is a phosphorylated residue. The *m/z* of the signature fragment is also denoted by the value of 1026.5. *B*, the LC/MS/MS spectrum of serine 180 and threonine 182 doubly phosphorylated peptide is shown in *B*. The identified ion fragment is indicated *above* each *line*. *C*, the related amount of S-HDAg in every subcellular fraction is identified by Western blotting (*left panel*). It indicated that the S-HDAg could only be extracted under Triton X-100 treatment. This portion was further purified by the anti-HDAg affinity column. The purified S-HDAg was analyzed in SDS-PAGE and stained by Coomassie Blue (indicated by an *arrow*).

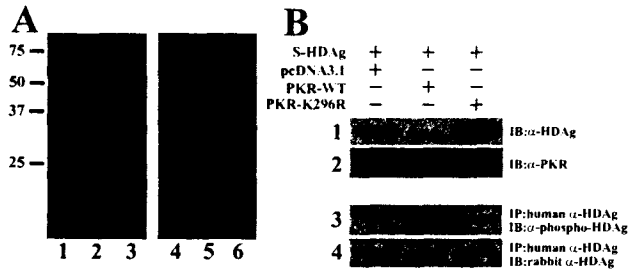

FIG. 7. **S-HDAg phosphorylated status was influenced by PKR.** *A*, specificity of anti-serine 177-phosphorylated S-HDAg serum. *Lanes 1* and *4* show *E. coli* expressed His-tagged recombinant wild type S-HDAg. The mutant S-HDAg of Ser^{177} replaced by aspartic acid was also expressed in *E. coli* (*lanes 2* and *5*). *Lanes 3* and *6* show cellular expressed S-HDAg. These Western blotting data were obtained from rabbit anti-HDAg serum (*left panel*) and adsorbed anti-$serine^{177}$-phosphorylated S-HDAg antibody (*right panel*). *B*, the S-HDAg expression plasmid was cotransfected with wild type, dominant negative PKR, or blank vector to 293T cells (indicated *above* each *lane*). Two days after transfection, the expressed S-HDAg and PKR was directly identified by immunoblotting (*IB*) (*panels 1* and *2*). The S-HDAg was immunoprecipitated (*IP*) by human α-HDAg serum at equal amount (*panel 4, lanes 1–3*). The amount of phosphorylated S-HDAg in the immunoprecipitated S-HDAg was checked by adsorbed anti-serine 177-phosphorylated S-HDAg serum (*panel 3*).

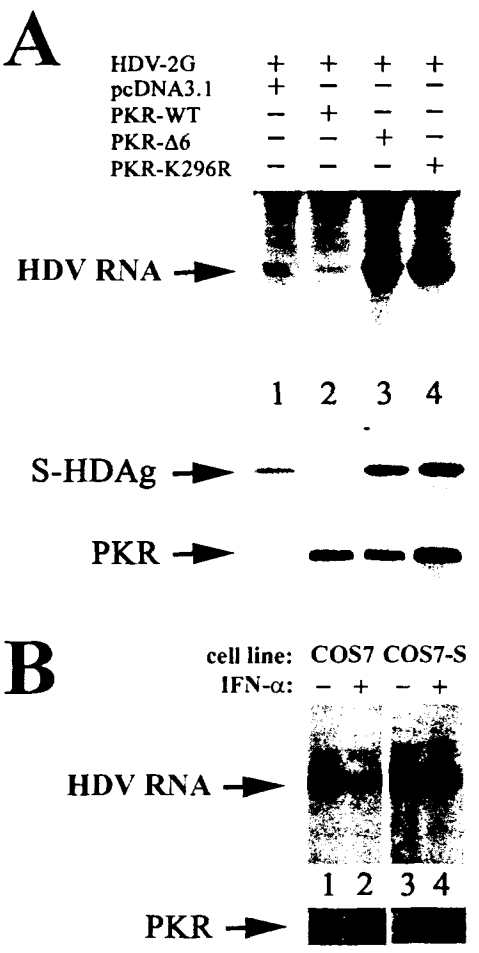

FIG. 8. **HDV replication is interfered by cotransfected PKR.** *A*, all of the cotransfected clones were constructed at the pcDNA3.1 vector. The combinations of plasmids used in this experiment are indicated *above* each *lane*. Four days after transfection, total RNA and protein was harvested. *Upper panel*, Northern blotting data that indicates HDV replication. The S-HDAg and PKR expression levels were identified by Western blotting (*middle* and *lower panels*). *B*, the transfected cell line and interferon-α treatment are indicated *above* each *lane*. For interferon-α treatment, the cells were pretreated with 1000 units/ml interferon-α for 18 h before ribonucleoprotein transfection. Four days after transfection, the HDV RNA was detected by Northern blotting (*upper panel*). The amount of PKR was detected by Western blotting (*lower panel*) using anti-PKR serum.

pressed COS7-S cell line (Fig. 8*B*, *lane 3 versus lane 4*), despite an increased level of PKR (Fig. 8*B*, *lower panel*). The results indicated that a preexisting S-HDAg could antagonize the inhibited effects of PKR.

DISCUSSION

By the in-gel kinase assay, we identified an S-HDAg kinase with an apparent molecular mass of about 68 kDa. This kinase was shown to be the double-stranded RNA-activated kinase, PKR. The protein specifically precipitated by anti-PKR antibodies could phosphorylate recombinant S-HDAg *in vitro*. Furthermore, the kinase activity was eliminated in PKR-depleted cell lysate by anti-PKR antibodies or poly(I:C)-agarose. We also showed the colocalization of S-HDAg and PKR in the nucleolus by confocal microscopy and an association between these two proteins by co-immunoprecipitation. Finally, the residues phosphorylated by PKR *in vitro* shared the conserved serine 177 that was phosphorylated *in vivo*. In addition, the dominant negative PKR also reduced S-HDAg phosphorylation in culture cells. These results suggested PKR as one kinase capable of phosphorylating S-HDAg. Because the PKR was known as an antiviral molecule and could inhibit HDV replication when overexpressed in culture cells, the interaction between S-HDAg and PKR raised an interesting question about the role of PKR in HDV biology. First, this finding might bear biological significance for HDV infections. Since PKR is a downstream effector of interferon, the association between S-HDAg with PKR and being a substrate for PKR might influence the effect of interferon on hepatitis D. Second, we needed to discuss whether PKR was an essential or just a regulatory kinase for S-HDAg and HDV replication.

Possible Implication of PKR Phosphorylation on Interferon Effects for Hepatitis D—In the HDV cDNA-stably transfected cells, HDV replication was not suppressed by interferon treatment, despite an increased level of PKR (48). For hepatitis D patients, interferon has been used for treatment. Unfortunately, the success rate was very low, and post-treatment relapse is common (49, 50). How HDV escapes from interferon activity remains unknown. Since PKR is an important antiviral effector induced by interferon, our finding that PKR could associate with and further phsophorylate S-HDAg may provide some insights into the current failure of interferon therapy for hepatitis D.

Before S-HDAg, the only documented viral protein phosphorylated by PKR is the trans-acting protein (Tat) of human immunodeficiency virus type 1 (51). Tat, however, inhibits the interferon-induced PKR in two ways. In the RNA-dependent manner, Tat cooperated with TAR RNA to block PKR activation. Second, Tat directly interacts with PKR, through a specific region (a hydrophobic motif followed by lysine- and arginine-rich sequences). This region is similar to that in eukaryotic initiation factor-2α, which binds to and is phosphorylated by PKR. In this way, Tat resembles vaccinia virus K3L protein that behaves as a decoy substrate to inhibit PKR activity (52). This may partially account for the failure of interferon therapy for human immunodeficiency virus infection. Our data showed that hepatitis D virus S-HDAg could associate with PKR. Probably, S-HDAg could work together with HDV RNA, a well known inhibitor of PKR (45), to suppress PKR. In addition, S-HDAg was a substrate for PKR; just as Tat, it could compete against eukaryotic initiation factor-2α and

use PKR knock-out cells and determine whether S-HDAg phos-

block PKR activity. The results in this report might explain the poor response of hepatitis D to interferon therapy. In fact, we found that overexpression or preexisting S-HDAg can mitigate the suppressing effects of interferon treatment (Fig. 8*B*).

Phosphorylated Residues and the Nature of Kinases—Besides the serine 177, the phosphorylation of other serine or threonine residues of S-HDAg was also analyzed. We paid special attention to the other conserved residues, serines 2 and 123. The relative abundance of serine 123-containing peptide in LC/MS/MS analysis was adequate. However, no peptides with phosphorylated serine 123 were recovered, no matter whether the S-HDAg was purified from cells or phosphorylated by PKR *in vitro*. This finding was consistent with a previous transfection study that found that substitution of serine 123 by alanine did not have an obvious effect on HDV replication (31). Therefore, serine 123 of S-HDAg is probably not the phosphorylated residue. The third conserved residue, serine 2, was difficult to recover from LC/MS/MS; perhaps the tryptic digested serine 2-containing peptide was too small to identify in the LC/MS/MS spectrum. We could not determine whether serine 2 was a phosphorylated residue by the current method.

Although phosphoamino acid analysis revealed that S-HDAg was phosphorylated at both serine and threonine residues, there are no conserved threonine residues in the S-HDAg sequence among different HDV strains. Among the recovered S-HDAg peptides in LC/MS/MS analysis, all of the threonine residue-containing peptides were involved. Regarding phosphorylated threonine, we have also attempted to verify the other *in vivo* phosphorylated threonine without success. The most feasible explanation is that the protein amount purified from S3-HDAg cells was too low to enable a complete identification of these phosphorylation residues. Probably, a larger amount of S-HDAg was required to resolve the question. Furthermore, the S3-HDAg cells do not contain replicated HDV RNA. Naturally, S-HDAg forms a ribonucleoprotein complex with HDV RNA and associates with many cellular proteins (*e.g.* the B23 or delta antigen interacting protein A). Whether these RNAs or proteins will influence the S-HDAg phosphorylation pattern is unknown. However, for S-HDAg phosphorylated by PKR *in vitro*, the threonines 180 and 182 were consistently found.

The phosphorylated residues of S-HDAg by PKR *in vitro* clustered in a region from amino acid 177 to 182 that includes the conserved serine 177, less conserved serine 180, and threonine 182. In contrast, serine 177 was the only identified *in vivo* phosphorylated residue. If serine 177 could be singly phosphorylated *in vivo*, the results suggested a different phosphorylation pattern and efficiency of S-HDAg in the cells *versus* that by PKR *in vitro*. Nevertheless, PKR phosphorylated S-HDAg at residues adjacent to the critical serine 177. This neighbor effect might affect the phosphorylation of serine 177 and subsequently influence its function on viral replication.

Apart from the phosphorylated residues on S-HDAg, the other critical question was the nature and numbers of kinase for S-HDAg phosphorylation. It was important to know whether PKR was the kinase essential for phosphorylation of S-HDAg or just a regulatory and inessential kinase for S-HDAg phosphorylation. Since the phosphorylated residues differed, although they overlapped, between cellular S-HDAg and PKR *in vitro*, PKR was considered to be a regulatory kinase but not the essential one. The best way to address this question is to use PKR knock-out cells and determine whether S-HDAg phosphorylation was changed. Among all HDV strains, serine 177 was located in a completely conserved motif, Pro-Glu-Ser-Pro-Phe (PESPF). The P*X*(S/T)P motif has been shown to be the phosphorylation site for mitogen-activated protein kinase (extracellular signal-regulated kinase) or Cdc2 kinases (53, 54). However, our preliminary data showed that S-HDAg was not phosphorylated by mitogen-activated protein kinase. We have not examined other candidate kinases yet.

In conclusion, PKR may modulate HDV replication by interacting with the essential viral replication factor, S-HDAg, or by phosphorylating it. Despite these preliminary biochemical and biological observations, determination of the actual mechanism requires further experiments, probably by studying the post-translational modification of S-HDAg and the cellular proteins interacting with S-HDAg or PKR.

Acknowledgment—We thank the laboratory of Dr. S. C. Lee for providing technical assistance in liquid chromatography/tandem mass spectrometry.

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