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 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.

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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 fulminating 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 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 mammary 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...

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¶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, N1-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-trimethylammonium methyl sulfate.
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**Experimental Procedures**

**Plasmid Constructions**—Plasmid pcDNA3.1/HDV-2G contains a tandem dimer of the full-length HDV cDNA inserted at the BamHI 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 antigenic 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 the 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 g/l G418 (Promega).

**Recombinant Small Delta Antigen Purification**—To express the small delta antigen, a fragment containing the S-HDAg reading frame 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 A600 reached 0.6, isopropyl-1-thio-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 MgCl2, 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 × 107) 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, equilibration, 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 MgCl2) for 30 min then incubated in 10 ml of kinase buffer containing 50 μM ATP and 20 μCi/ml [γ-32P]ATP at 30 °C for 30 min. After reaction, the gel was soaked in 5% trichloroacetic acid solution to remove nonincorporated [γ-32P]ATP. The gel was dried on a 3MM filter and used for autoradiography.

**Immunoprecipitation**—To immunoprecipitate PKR for in-gel kinase and in vitro kinase assays, protein G-agarose conjugated with 1 μg/ml 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 polyclonal anti-PKR serum (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 aga- rose was washed by 0.5 ml of PKR kinase buffer (15 mM HEPES, 2 mM dithiothreitol, 2 mM MgCl2, and 50 μM ATP) twice. Four micromolar of recombinant S-HDAg and equal volume of [γ-32P]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 sample 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.

**Protein Extraction and Western Blot Analysis**—To detect the S-HDAg expression in S3-HDAg cell, cells (about 1 × 107) were lysed in 1 ml of radiomimmune 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 sample buffer, boiled for 10 min, and
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To deplete the nonspecific antibody in the serum that can interfere with the immunization, culture medium was replaced by fresh 5% fetal calf serum-DMEM for 4 h. 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% acetonitrile in 10 min and subsequent 30–65% acetonitrile 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 MS² scans. The MS scan defined the ion composition in m/z range of 295–1605; the ZOOM scan examined the isotope patterns of the most intense ion in the MS scan; and the MS² 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 identified peptides and their hypothetical phosphopeptides to determine their mass was assigned to all lysine residues that were alkylated in all experiments. The gradient consisted of 5–30% acetonitrile in 10 min and subsequent 30–65% acetonitrile in 50 min.

The phosphorylated peptide, 167FVPNLQGVPEpSPFSRTGE184, with a phosphorylation site at Ser177, 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 for 2 times with 2 mg of peptides, rabbit antiserum against Ser177-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 total HeLa S3 cell lysate was separated in a SDS-polyacrylamide gel containing recombinant S-HDAg (rS-HDAg) and 32P-labeled 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 nuclear 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 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% acetonitrile in 10 min and subsequent 30–65% acetonitrile in 50 min.

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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 mM 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.

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 nuclear 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) and 32P-labeled 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...
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 on 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. 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).

PKR was the S-HDAg kinase identified by in-gel kinase assay. The previously identified S-HDAg kinase activity was detected by previous in-gel kinase assay, we found a 68-kDa protein (the HDAg kinase activity in the same 0.5 M NaCl eluted protein fraction (Fig. 2, upper panel). This kinase was located in the PKR-containing fraction (0.5 M NaCl fraction). 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. 3A, lane 4), whereas such activity vanished in the PKR-depleted cell lysate (Fig. 3A, 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)-agarose (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 PKR784

**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).
lysate harvested from cells with (lanes 2 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). If, 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). 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. 5B, 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. 5C, lane 1). These co-immunoprecipitation experiments further supported an association between S-HDAg and PKR.

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**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).

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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. 5B, 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. 5B, 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. 5C, 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. 6A, 161GAPGGFVPNLQGVPESPFSR was a phosphopeptide. We concluded that a phosphate group was located in serine 177 by observation of derivative s of y4 and b19 fragment ions. Another longer peptide, 161GAPGGFVPNLQGVPESPFSRTGEGGDIRD, could also be doubly phosphorylated. Based on its collision-induced dissociation spectrum, we concluded that serine 180 and threonine 182 were two additional targets (Fig. 6B). 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. 6C, S100). After the nuclear protein extracted in 0.42 M NaCl was removed (Fig. 6C, 0.42 M NaCl), the S-HDAg was extracted when the remainder of the nucleus was treated with 2% Triton X-100 (Fig. 6C; 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. 6C, 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, 161GAPGGFVPNLQGVPESPFSR, was found to be phosphorylated. Its collision-induced dissociation spectrum was identical to the same peptide prepared in vitro (similar to Fig. 6A), which indicated that serine 177 was the dominant phosphorylation site in vivo.

**Suppression of Endogenous PKR by Dominant Negative PKR**—As 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...
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Fig. 5. S-HDAg is associated with PKR and co-localized in the nucleus. 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 nucleus (middle panel), and PKR distributes in cytoplasm and nucleus (left panel). After merging these two pictures, their colocalization in the nucleus 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 complex protein 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.

To detect the phosphorylated S-HDAg, we prepared antibody that specifically recognized Ser\textsuperscript{177}-phosphorylated S-HDAg. The anti-Ser\textsuperscript{177}-phosphorylated S-HDAg serum was raised by immunization of rabbit with \textsuperscript{167}FVPNLQVEPTSPFSRTGE\textsuperscript{184} peptide with the phospho group at serine 177. After serum adsorption to remove nonspecific recognition, this antibody cannot recognize \textit{E. coli}-expressed nonphosphorylated recombinant S-HDAg (Fig. 7A, lane 4). However, it can react with phosphorylated S-HDAg expressed in transfected cells (Fig. 7A, lane 6) or a recombinant S-HDAg variant (serine 177 replaced by aspartic acid) that might simulate the phosphorylated S-HDAg (Fig. 7A, 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. 7B, panel 1, lane 1). The endogenous PKR was also detected by anti-PKR antibody (Fig. 7B, panel 2, lane 1). After co-transfected with wild-type PKR-expressing plasmid, the amount of total PKR increased (Fig. 7B, panel 2, lane 2). However, as PKR suppresses protein translation, the expression of S-HDAg was reduced (Fig. 7B, 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\textsuperscript{177}-phosphorylated HDAg antibody (panel 3). About equal amount of total HDAg was precipitated (Fig. 7B, 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. 7B, 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 \textit{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. 8A, HDV RNA replication was reduced when its cDNA was cotransfected with wild type PKR (Fig. 8A, 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. 8A, 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 COST7 cell line, COS7-S, by ribonucleoprotein transfection. As shown in Fig. 8B, HDV replication was dramatically inhibited by interferon-α in naive COS7 cells (Fig. 8B, 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 r-S-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 \textsuperscript{161}GAPGGGFVPNLLQGVPE\textsubscript{s}PSF\textsubscript{R} 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 \textit{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).
HDAg. The mutant S-HDAg of Ser177 replaced by aspartic acid was also expressed in E. coli blank vector to 293T cells (indicated 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 a-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).

expressed COS7-S cell line (Fig. 8B, lane 3 versus lane 4), despite an increased level of PKR (Fig. 8B, 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 phosphorylate 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 interferon-induced PKR (45), to suppress PKR. In addition, S-HDAg was a substrate for PKR; just as Tat, it could compete against eukaryotic initiation factor-2a and

**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 Ser177 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-serine177-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 a-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.
PKR Phosphorylates Small Delta Antigen

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 poor response of hepatitis D to interferon therapy. In fact, we in vitro phosphorylation pattern and efficiency of S-HDAg in the cells (59).

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 vitro 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 proteins or RNA 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 knockout 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 (PESFP). The (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.

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