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

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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。

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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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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-

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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.

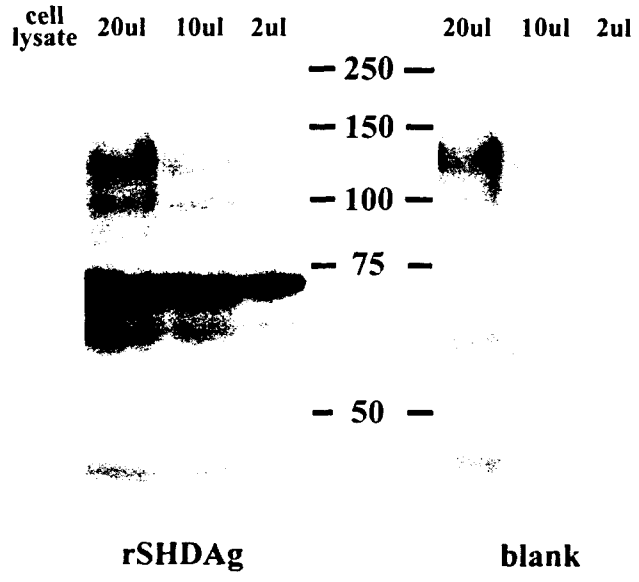

FIG. 1. **Identification of the kinase for HDV S-HDAg by in-gel kinase assay.** In-gel kinase assay with recombinant S-HDAg as the substrate was performed to identify the candidate kinase in HeLa S3 cell lysate. Different amounts of crude cell lysate were separated in rS-HDAg-containing gel (*left panel*) 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