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型肝炎之病毒及免疫反應-D型肝炎病毒RNA　※
※　複製及組合之研究）　　　　　　　　　　　　　　※
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計畫類別：☐個別型計畫　☑整合型計畫

計畫編號：NSC 89－2315－B002－013－MH

執行期間：88 年 08 月 01 日至 89 年 07 月 31 日

計畫主持人： 陳培哲

共同主持人：

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☐赴國外出差或研習心得報告一份

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執行單位：國立台灣大學醫學院臨床醫學研究所

中 華 民 國 89 年 10 月 26 日

行政院國家科學委員會專題研究計畫成果報告
Preparation of NSC Project Reports

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一、中文摘要:

D 型肝炎病毒(Hepatitis Delta Virus，HDV) 僅轉譯一種蛋白質-delta 抗原。delta 抗原有兩種型式，分別為大型及小型 delta 抗原，兩種抗原參與 D 型肝炎病毒生活史中不同的階段。大型 delta 抗原是一個磷酸化蛋白質，絲胺酸為其磷酸化殘基，而小型 delta 抗原的磷酸化現象仍有待釐清。

為研究 delta 抗原的磷酸化，本篇建立一種特殊的雙向電泳系統分離磷酸化與非磷酸化的 delta 抗原，名為非平衡 pH 梯度電泳 (NEPHGE) 。結果顯示小型 delta 抗原有多種磷酸同型体 (phosphorylated isoform)，而大型 delta 抗原只有一種。此外，磷酸基酸分析(phosphoamino acid analysis,PAA) 確定絲酸(serine)及酥酸(threonine)皆為小型 delta 抗原的的磷酸化殘基.因此,兩種 delta 抗原均為磷酸化蛋白質,但具不同的磷酸化型態。在病毒的顆粒中只有非磷酸同型体的小型 delta 抗原存在，且小型 delta 抗原的磷酸同型体數目會隨著細胞轉染的天數而增加，顯示細胞內的磷酸化過程與小型 delta 抗原的生物功能有關。運用定位點突變 (site-directed mutagenesis) 法置換小型 delta 抗原內絲胺酸及酥胺酸以探討此一關連性。得知小型 delta 抗原 95 殘基上酥胺酸突變成丙胺酸(alanine)，破壞了小型 delta 抗原幫助 D 型肝炎病毒雙向複製的能力，而 177 及 182 殘基上絲胺酸及酥胺酸的突變，只破壞了 D 型肝炎病毒單一方向的複製(antigenomic polarity)。同時，177 及 182 突變株的磷酸化程度低於野生型小型 delta 抗原。因此，絲胺酸 177、酥胺酸 95 及 182 可能為小型 delta 抗原上的磷酸化殘基，但仍待進一步的生化鑑定。磷酸化似乎在 D 型肝炎病毒複製過程中掌控了 D 型肝炎病毒 RNA 模板的選擇。此外， 其他發現都指向絲胺酸 177 殘基可能是小型 delta 抗原上的磷酸化位置之一， 例如(1)當 177 殘基以絲胺酸存在時，TPA 可增小型 delta 抗原磷酸化程度達三倍。(2)在雙向電泳膠中，177 突變株呈現不同的磷酸化形態。所以推小型 delta 抗原的磷酸化在 D 型肝炎病毒複製的機制中扮演重要調控角色。

英文摘要:

Hepatitis B surface antigens(L-, M-, and S-HBsAgs) compose the envelopes of three kinds of viral particles, including the infectious hepatitis B virus (HBV) particle, the HBsAg Subviral particle, and the hepatitis D virus (HDV) paticle. It is interesting to understand the morphogenic mechanism of these viral or subviral particles, of which HDV particle was especially focuced. In this thesis, the studies were divided into three chapters on the HbsAg glycosylation, the molecular chaperones, and the HbsAg minimun region involed in HDV morphogenesis:<I>.HbsAg to be glycosylated is required for efficient HDV assembly; <II>.Five molecular chaperones, especially the calnexin, an endoplasmic reticulum(ER) membrane-bound chaperone usually involved in promoting the correct folding and oligomerization of many glycoproteins and providing unique control, are not involved in the assembly by HBsAg or HDV particle; <III>.HbsAg C-terminal region, especially the last 15 amino acid (a.a.) residues could be divided into two

functional subdomains, playing roles in HBsAg secretion and HDV morphogenesis, respectively.

The study was focused on whether HBsAg glycosylaton is involved in the process of HDV assembly. HDV is a defective virus requiring the HBV to provide HBsAgs as the envelope protein. The HBsAgs are posttranslationally modified by N-linked glycosylation. After the N-linked glycosylation of HBsAg was blocked by tunicamycin treatment, the packaging of HDV in the culture system was suppressed to a level as low as 5-10% of the untreated. The extent of inhibition correlated with the increased concentration of tunicamycin. In contrast, the loss of HBsAg glycosylation did not affect the efficiency of assembly of HBV particles. When the N-linked glycosylation site of HBsAg at a.a. 146 was mutated from Asn to Gln, the mutant HBsAg packaged a modest amount of HDV particles only very late after transfection. Therefore, it was due to the loss of glycosylation of HBsAg, rather than the loss of glycosylation of ther cellular proteins, that suppressed HDV assembly. The quantity and kinetics of production of HDV particles in culture system were significantly reduced by the depletion of HBsAg glycosylation. Therefore, HDV, similar to influenza and vesicular stomatitis viruses, depends on glycosylation of envelope protein as a signal for envelope maturation and for virion formation. Results in chapter I showed that HBsAg glycosylation is involved but not essential in the process of HDV assembly.

Secondly, five molecular chaperones, including the ER membrane-bound calnexin, were examined if they are involved in the HBsAg folding or in the HDV assembly. HBsAg deglycosylation selectively inhibited the kinetics of HDV assembly and the production of HDV particle but not the formation of HBsAg or HBV particles as shown in chapter I. To address the reason that glycosylation of envelope protein was important for HDV assembly, several hypotheses were raised: <1>. HBsAg glycosylation could enhance the interaction with the components of HDV virions and facilitate the virion formation; <2>.HBsAg glycosylation may influence the matutation or the trafficking of HBsAg in the cells, thus affectiong its capability or rate in interacting with HDV components prior to assembly; <3>. A molecular chaperone is involved in the interaction with the glycan of HBsAg and helps its folding; and <4>. HBsAg glycosylation may produce an appropriate confirmation for the HBsAg to interact effectively with the HDV viral components.

As virogenesis of many viruses requires the participation of different molecular chaperones residing in the host cells,therefore, in chapter II, the study was focused on several molecular chaperones if being involved in folding and maturation of the HBsAg and the HDV particles. Of the molecular chaperones, calnexin was discovered recently and reported to interact with the glycan of many glycoproteins

newly synthesized. Besides calnexin, several other molecular chaperones, including BiP, Hsp70, Hsc70, and Grp94, all reported to be important for protein folding and maturation, were also studied. Results in chapter II showed that these five molecular chaperones are not involved in the HBsAg folding or in the HDV assembly.

Finally, the correlation of the structure and the functions the HBsAg C-terminus in the HDV assembly was studied. HDV can be experimentally packaged by the S-HBsAg, a polypeptide of 226 a.a. residues encoded by the HBV genome. S-HBsAg is able to interact with HDV-encoded L-HDAg, thereby, resulting in HDV morphogenesis. By using deletion and point mutation analyses, the S-HBsAg was minimized to its C-terminal 15-residue region shown as two functional subdomains responsible for HBsAg particle secretion and HDV formation, respectively. As one mainly in the carboxyl-end of HBsAg (a.a.212- a.a. 226) plays a role in the interaction with L-HDAg and in the HDV assembly. These two functional subdomains may overlap due to the correlated structural elements in the HBsAg C-terminus. The downstream 6-residue region also contributed to HDV packaging possibly through an secondary structure and/or the hydrophobicity. The upstream 9-residue region spanning a.a. 212- a.a. 220 of S-HBsAg overlapped a lately predicted α -helical structure. This structure interacts directly with the L-HDAg or mediates an allosteric effect on other regions of the HBsAg molecule. The interaction between HBsAg and L-HDAg is through a hydrophobic force that is discussed in the thesis. In conclusion, the results suggested that HBsAg interacts with L-HDAg through the C-terminal helical structure comprised of hydrophobic residues in an appropriate topology. The study also agreed in large with the predicted secondary structure model of HBsAg that in the C-terminal region beyond a.a. 170 at least two transmembrane helices exist.

JOURNAL OF VIROLOGY, Dec. 1999, p. 10540–10545 Vol. 73, No. 12
0022-538X/99/$04.00+0

Characterization of the Phosphorylated Forms and the Phosphorylated Residues of Hepatitis Delta Virus Delta Antigens

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Received 26 May 1999/Accepted 23 August 1999

Hepatitis delta virus (HDV) replication requires both the cellular RNA polymerase and one virus-encoded protein, small delta antigen (S-HDAg). S-HDAg has been shown to be a phosphoprotein, but its phosphorylation status is not yet clear. In this study, we employed three methods to address this question. A special two-dimensional gel electrophoresis, namely, nonequilibrium pH gradient electrophoresis, was used to separate the very basic S-HDAg. By carefully adjusting the pH of solubilization solution, the ampholyte composition, and the appropriate electrophoresis time periods, we were able to clearly resolve S-HDAg into two phosphorylated isoforms and one unphosphorylated form. In contrast, the viral large delta antigen (L-HDAg) can only be separated into one phosphorylated and one unphosphorylated form. By metabolic ^{32}P labeling, both immunoprecipitated S-HDAg and L-HDAg were found to incorporate radioactive phosphate. The extent of S-HDAg phosphorylation was increased upon 12-*O*-tetradecanoylphorbol-13-acetate treatment, while that of L-HDAg was not affected. Finally, phosphoamino acid analysis identified serine and threonine as the phospho residues in the labeled S-HDAg and only serine in the L-HDAg. Therefore, HDV S- and L-HDAgs differ in their phosphorylation patterns, which may account for their distinct biological functions.

Hepatitis delta virus (HDV) is the smallest member of negative-strand RNA viruses and possesses a circular genome of 1.7 kb (19, 21, 29). Replication of HDV is entirely through RNA intermediates and proposed to proceed by a double rolling-circle mechanism (19, 29). Hepatitis delta antigen (HDAg) is the only known protein encoded by HDV and exists as two major forms: small HDAg (S-HDAg; 195 amino acids) and large HDAg (L-HDAg; 214 amino acids) (29, 30, 31). Both forms of HDAg are translated from the same initiation codon of one open reading frame (ORF), but L-HDAg contains an additional 19 amino acids at the C terminus of S-HDAg (31) because of RNA editing of the original termination codon during replication. Hence, they have different biological functions: S-HDAg is essential for viral replication (18); however, L-HDAg exerts a dominant-negative role in HDV replication and is involved in viral assembly (5, 10).

L-HDAg was first shown as a nuclear phosphoprotein when expressed in mammalian cells, and a previous result indicated that L-HDAg is phosphorylated only at the serine residue(s) (6). In a subsequent study, both L- and S-HDAg were found to be phosphorylated in insect cells (16). S-HDAg was further demonstrated to be a phosphoprotein in mammalian cells (35), and the phosphorylation level of S-HDAg is reported to be downregulated by casein kinase (CK II)- and protein kinase C (PKC)-specific inhibitors, whereas that of L-HDAg is downregulated only by CK II inhibitor (35). A recent study (2) tried to distinguish the phosphorylated from the unphosphorylated delta antigens by using an NEPHGE (nonequilibrium pH gradient electrophoresis)-sodium dodecyl sulfate (SDS) system instead of the conventional isoelectric focusing (IEF)-SDS system. The results of that study revealed ca. 20 to 40% of L-HDAg to be phosphorylated. However, in contrast to previous studies, hardly any phosphorylated S-HDAg was detected (2).

The different results concerning the phosphorylation status of S-HDAg may be reconciled by at least two factors. First, because of its nonequilibrium nature, separation of the isoforms of basic protein by NEPHGE has been empirical. Careful optimization of running conditions is required to achieve the best resolution. Second, protein phosphorylation is subjected to modulation by many signals; thus, different cells and culture conditions may influence the extent of S-HDAg phosphorylation. In this study, we tried to address the question by optimizing the conditions for NEPHGE by carefully adjusting many parameters in order to separate the phosphorylated forms of both HDAgs from the unphosphorylated forms.

Separation of HDAg isoforms of different pI by fine-tuning the NEPHGE. Because the IEF allows a clear separation of proteins and their isoforms of different pIs, it is usually used in separating the unphosphorylated from the phosphorylated forms of a protein (32, 34). However, it has been noted that the standard IEF would not give satisfactory resolution in separating very basic or acidic proteins (23, 33). According to the predicted pI values of S-HDAg and L-HDAg (10.2 and 9.9, respectively), both HDAgs seemed to be very basic, and it was difficult to reach pH equilibrium in conventional IEF. Therefore, we adapted an alternative approach, namely, NEPHGE, which was developed to resolve very basic protein by using a nonequilibrium condition rather than true focusing (1, 23, 33). To investigate S- and L-HDAgs in the NEPHGE system, two plasmids, pCDAg-S and pCDAg-L, which express S- and L-HDAg, respectively, were cotransfected into HuH-7 cells. pCDAg-S contained the S-HDAg ORF of HDV (nucleotides 46 to 781) under the control of human cytomegalovirus immediate-early promoter. To construct pCDAg-L, the site-directed mutagenesis (Transformer Site-Directed Mutagenesis Kit;

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Clontech Laboratories, Inc.) was employed to change the stop codon (UAG) in the S-HDAg ORF of pCDAg-S to UAA (encoding Trp). On day 3 posttransfection, cells were washed twice with cold TBS (150 mM NaCl, 20 mM Tris; pH 7.5) and lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 0.2% NP-40; 1% Triton X-100; 0.1% SDS). The cell lysates were centrifuged at 12,000 rpm for 20 min to remove debris. For preclearing, the supernatant was incubated with a 1/10 volume of normal mouse serum (50 mg/ml; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 4°C and then with 25 μl of protein G-agarose beads (Boehringer Mannheim) for another 1 h at 4°C. After centrifugation at 12,000 rpm for 1 min, precleared supernatant was reacted for 1 h at 4°C with a mouse monoclonal anti-HDAg antibody (5 μg/ml), D9-3, followed by another 1 h at 4°C with 25 μl of protein G-agarose. D9-3 was raised in BALB/c mice by inoculation of S-HDAg expressed in *Escherichia coli*. After centrifugation at 12,000 rpm for 1 min, the pellet was washed with 1 ml of RIPA buffer once, 1 ml of high-salt buffer (25 mM HEPES, pH 7.5; 1% Triton X-100; 1% deoxycholate; 0.1% SDS; 500 mM NaCl; 5 mM EDTA) twice, and then low-salt buffer (25 mM HEPES, pH 7.5; 0.2% Triton X-100; 1 mM EDTA) once.

The immune complex was resuspended in 50 μl of urea–NP-40 solubilizer (9 M urea, 4% NP-40; 1.6% Pharmalyte [pH 3.5 to 10; Amersham Pharmacia Biotech AB], 0.4% Servalyte [pH 9 to 11; Serva FeinBiochemica GmbH and Co.], and 1% dithiothreitol [adjusted to pH 3.0]) and held at room temperature for 10 min. After dissolution, the supernatant was loaded onto the well of one cylindrical gel (12 cm [length] by 4 mm [diameter]) and covered with 100 μl of 4 M urea overlay solution. The gel was made with 3.3% acrylamide (30% acrylamide and 1.8% bisacrylamide) containing 9 M urea, 2% NP-40, 1.6% Pharmalyte (pH 3.5 to 10) and 0.4% Servalyte (pH 9 to 11). Finally, the gels were installed vertically in electrophoresis apparatus (Desaga GmbH, D-6900 Heidelberg, Germany) with 20 mM NaOH in the lower chamber and 10 mM H_3PO_4 in the upper chamber. Lysozyme and trypsinogen (Sigma, St. Louis, Mo.) were used as the reference for pI markers and run in parallel cylindrical gels.

In NEPHGE, proteins migrate in nonequilibrium pH gradient; therefore, it is important to find the critical window (defined by the running volt-hour) at which the proteins are best focused (23, 33). Proteins did not enter gels completely if the electrophoresis time period was insufficient. On the other hand, prolonged period of electrophoresis made proteins in acidic ends migrate backwards off the gel and caused the collapse of the pH gradient in the basic ends. Therefore, outside the critical window, proteins will clump together, spread into a broader pattern, or intermingle with others, and this results in poor separation (23). In order to fine-tune the NEPHGE, gels were electrophoresed at four different volt-hour levels: 600, 800, 1,300, and 1,600 V · h, respectively. Gels were electrophoresed at 400 V for 1 h and then run at 800 V at different periods of time with the current reversed. After electrophoresis, the cylindrical gels were extruded from the glass tubes by syringes, and each one was soaked in 10 ml of equilibration buffer (10% glycerol, 4.9 mM dithiothreitol, 2% SDS, 0.125 M Tris; adjusted to pH 6.8) for 10 min. Then, the tube gel was laid on top of the second-dimension gel. The second dimension was the standard discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) employing a 20-by-20-by-0.15-cm gel (12% separation gel and 5% stacking gel) with a beveled plate to assemble the glass-plate sandwich (BRL Vertical Gel Electrophoresis System, model V16-2). This plate provided a wider space to accommodate the thick cylindrical gel from the NEPHGE. After equilibration, the extruded tube gel was laid on top of the stacking gel and sealed with 0.5% agarose. Electrophoresis was carried out overnight (70 V, 16 to 18 h), and gels were electrotransferred onto nitrocellulose membrane (Hybond-c Super; Amersham Pharmacia Biotech). Both HDAgs were detected by Western blot probing with human polyclonal anti-HDAg antibody.

Based upon the migration of the molecular weight marker and recognition of HDAgs by specific antibody in a subsequent Western blot, we identified both HDAgs in the NEPHGE-SDS gel (Fig. 1). In the shorter electrophoresis periods (600 and 800 V · h), both the S- and L-HDAgs migrated as a large, clumped spot without separation, probably due to insufficient time for electrophoresis. Because of a higher pI, the S-HDAg is noted moving farther right than the L-HDAg (Fig. 1B). Notably, in the appropriate volt-hour (in the case of 1,300 V · h), the L-HDAg separates into two spots (Fig. 1C), and the S-HDAg resolves into three spots. However, with a longer electrophoresis period, the L-HDAg is still separated into two spots (Fig. 1D), but the S-HDAg now becomes broader again. This clumped signal may result from the S-HDAg isoforms residing in the collapsed pH gradient at the basic end of gel after prolonged electrophoresis. The results suggested that the electrophoresis period needs to be experimentally titrated, so different pI isoforms of one basic protein can be resolved.

Effect of protein dephosphorylation on pI isoforms of both HDAgs. If the observed pI isoforms of both HDAgs were generated by differential phosphorylation, then the stepwise decrease in pI value should be caused by the incorporation of phosphates. The incorporation with one phosphate into the protein is noted to decrease its pI, which varies from 0.04 to 0.46 pI units (11, 12, 32, 34). Phosphorylation on more residues will further reduce its pI and generate more isoforms. However, treatment with phosphatase will remove the phosphates, and then the dephosphorylated protein should become a single species with the same pI. To test this, immunoprecipitated HDAgs were treated with alkaline phosphatase before the NEPHGE–SDS-PAGE and detected by Western blot as described above. The immunoprecipitated HDAgs were resuspended in 90 μl 1× dephosphorylation buffer (50 mM Tris, 0.1 mM EDTA; pH 8.8) for 10 min at 30°C. Then, 10 μl of calf intestinal alkaline phosphatase (Boehringer Mannheim) was added into reaction buffer (100 U/ml) for 1 h at 37°C, followed by another 1 h at 42°C. The sample was washed with 1 ml of high-salt buffer once and 1 ml of low-salt buffer once and then dissolved in the solubilizer for NEPHGE–SDS-PAGE. In the untreated samples, the L-HDAgs are separated into two spots and the S-HDAgs are separated into three spots (Fig. 2A). After alkaline phosphatase treatment, both the L- and S-HDAgs were reduced to only one spot (Fig. 2B). This spot migrated to the same distance as the isoform with the highest pI in untreated HDAgs (Fig. 2A, indicated by open triangles). It supported that the isoform with the highest pI value is the unphosphorylated HDAg. Those isoforms with lower pI values were phosphorylated S- or L-HDAgs, which could be dephosphorylated by alkaline phosphatase treatment (Fig. 2A, indicated by solid triangles).

These data demonstrated that the L-HDAg contains two isoforms, one unphosphorylated and the other perhaps monophosphorylated. For S-HDAgs, other than the unphosphorylated one, there exist two obvious phosphorylated isoforms (probably mono- and diphosphorylated). However, there could be small amounts of S-HDAg with higher phosphorylation, since minor spots with even lower pIs sometimes were seen (data not shown). Although there was another posttranslational modification for L-HDAg farnesylation (13, 14, 24),