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Localization of HSP90 Binding Sites in the Human Hepatitis B Virus Polymerase

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The fact that HSP90 proteins and their chaperonin partners play an important role in ϵ RNA binding of duck HBV Pol protein during duck HBV replication has been reported. To elucidate the molecular basis of HBV Pol/HSP90 interaction, we have characterized the HSP90 interaction to HBV Pol. We found that human HBV Pol protein upon synthesis in rabbit reticulocyte lysate formed a complex with HSP90 *in vitro* as duck HBV Pol did. In addition, HSP90 protein was copurified with MBP/POL protein expressed in HepG2 cells, suggesting that human HBV Pol protein is associated with HSP90 *in vivo*. To localize the HSP90 interaction site region, several deletion mutants of HBV Pol translated *in vitro* were immunoprecipitated with anti-HSP90 antibody. The result indicates that C-terminal regions of the TP and RT domains interact with HSP90 independently.

Hepatitis B viruses (HBV) are a group of double-stranded DNA virus that replicates through a reverse transcription pathway (1–3). HBV employ a different replication mechanism from HIV-1 which also use reverse transcription; HBV replication is not initiated by tRNA-priming reaction but by protein priming reaction using terminal protein as primer (protein-priming reaction) (4–8). Compared to HIV-1 RT, HBV Pol contains an additional domain, the terminal protein domain that exists at the N-terminal region of RT domain (5).

HBV replication mechanism has been determined in detail by several experiments. After the formation of a ribonucleoprotein (RNP) complex by binding of Pol protein to a short RNA sequence located at the 5′ end of pregenomic RNA, termed ϵ (9–13), protein-priming reaction in which a nucleotide becomes covalently linked to a tyrosine residue within the TP domain of Pol occurs (5–8). The RNP complex containing a pregenomic RNA and Pol is packaged into core particles and subsequent replication steps proceed in their particles (10–12). In the protein priming reaction, the sequence in a bulge in the 5′ copy of ϵ serves as the template for the added nucleotides (14–16). As a result of protein priming reaction, the terminal protein is considered to be released from the active site of the RT domain. Following this priming reaction, the primer/Pol complex translocates to another *cis* element in the 3′ copy of DR1 (Direct Repeat 1), where the synthesis of minus strand DNA continues (17–22). After termination of minus-strand DNA synthesis at the 5′ end of pregenomic RNA, Pol primes plus strand with the help of an RNA oligomer derived from the 5′ end of pregenomic RNA which has been excluded from the degradation of RNA template by RNaseH and their RNA oligomer translocates to a homologous DR2 on minus-strand DNA (23–26). Once plus-strand DNA synthesis has reached the 3′ end of minus strand DNA, a successive strand translocation to the 5′ end of minus-strand DNA occurs, resulting in a noncovalently closed, partially double stranded, circular genome DNA. These replication steps are so complex that these would not occur via single peptide Pol protein. Instead, several cellular factors will be required for these replication steps.

HSP90 is a molecular chaperone associated with the folding of signal-transducing proteins such as steroid hormone receptors and protein kinases (27, 28). It is not required for the maturation or maintenance of most proteins *in vivo*. HSP90 forms several discrete subcomplexes, each containing distinct group of co-chaperones that function in protein folding pathway. The association between the HSP90 complex and its substrate is not static but dynamic. HSP90 acts in concert with other chaperones to help refold denatured protein and aid in folding some proteins into a competent state.

Recently, the fact that HBV Pol that suffers many conformational changes for replication is associated with HSP90 was reported (29, 30). The formation of the RNP complex between duck HBV Pol and ϵ RNA de-

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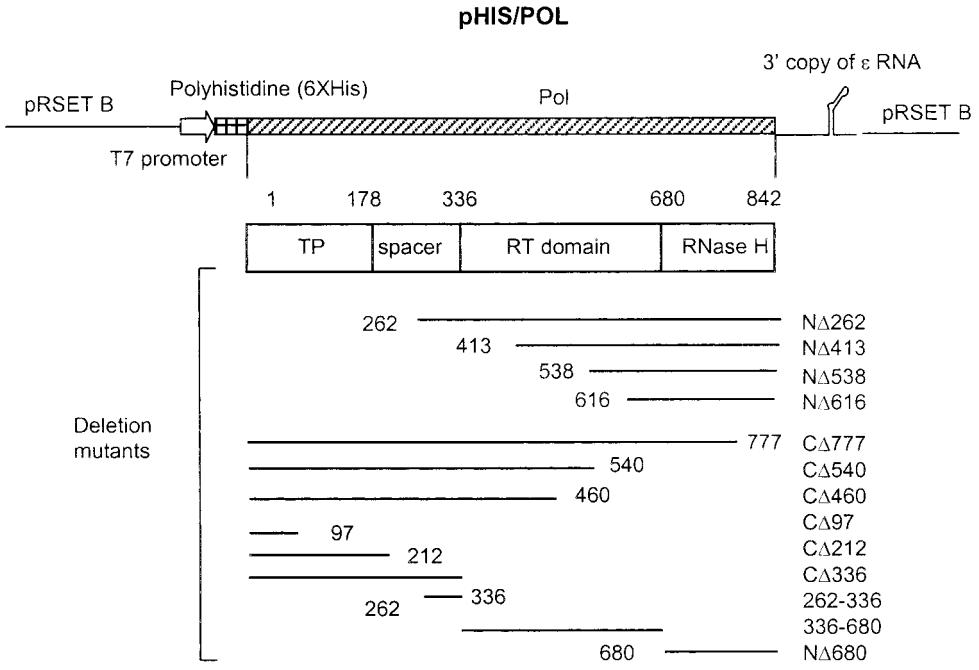

FIG. 1. Schematic representation of the expression cassettes, pHIS/POL and the domain region of Pol protein. The position of the *pol* gene deletion mutant is indicated. These mutants were cloned into pCMV/MBPOL through the substitution of wild-type pol.

pend on the HSP90 protein (29). The RNP formation requires ATP hydrolysis and function of HSP70 and chaperone partner for HSP90 (p23) (30). In addition, the HBV Pol/chaperon complex is also packaged into nucleocapside (30). This association was proved only in duck HBV Pol but not in human HBV Pol (29). Although human HBV Pol shares a high similarity to duck HBV Pol, they are not identical in their sequences. Duck HBV Pol expressed by *in vitro* translation possessed accurate protein-primed activity (6); however, human HBV Pol synthesized by *in vitro* translation did not have it. These reasons make us investigate whether human HBV Pol can interact with HSP90, which facilitate the RNP complex formation. As a step toward an understanding of HBV replication involved in HSP90, we mapped the sequence within the human HBV Pol responsible for forming HSP90-HBV Pol complex.

MATERIALS AND METHODS

Plasmid construction. We introduced a *Pst*I restriction site at the beginning of the coding region of MBP/POL protein derived from pMPH (31), by using the site-directed mutagenesis (32). The pCMV/MBPOL construct was made by ligating the 4.2-kb *Pst*I fragment containing MBP/POL into downstream of cytomegalovirus and T7 RNA polymerase promoter, derived from Rc/CMV (33).

For *in vitro* synthesis of HBV Pol, 6XHis tagged Pol protein expression construct was generated (Fig. 1). The Pol coding region and 3′ copy of epsilon from pMPH was cloned into *Sac*I/*Hin*dIII sites in pRSETb (Invitrogen Inc.). The result plasmid, pHIS/POL contains T7 RNA polymerase promoter, 6XHis tagged to Pol region and 3′ copy of epsilon. In additional deletion mutants, the coding region of all mutants derived from plasmids, pMPH (31), including truncated mutant of Pol was replaced with wild type coding region of pHIS/POL. The mutants including 1–97, 262–336, and 1–336 were generated by the result of frame-shift mutation through fill-in of restriction enzyme sites (*Apa*I, *Xho*I) in constructs from pHIS/POL (CΔ212, NΔ262 and wild type, respectively).

To express and purify HSP90 β in *E. coli*, 6XHis tagged HSP90 coding region was subcloned by RT-PCR into *Sal*I–*Not*I of pET28b (Novagen, Inc.). All plasmids were verified by DNA sequencing.

For HBV Pol expression in the insect cell, FLAG-tagged Pol coding region was cloned by PCR into the baculovirus transfer vector pFASTBAC HTb (Gibco-BRL) (unpublished data).

Cell culture and transfection. The Sf9 cell line of *Spodoptera frugiperda* was grown in TNMFH supplemented with 5% fetal bovine serum. The methods for growth, isolation, and assay of recombinant baculoviruses were as previously described (34) except that the viruses were generated by the Bac to Bac system (Gibco BRL, Gaithersburg, MD), in which transposition in bacteria creates the recombinant baculovirus genome rather than homologous recombination in insect cells (35). For the expression of recombinant FLAG/POL, the sf9 cell was grown in TNMFH supplemented with 5% fetal bovine serum 48 h after the infection of recombinant virus.

The human hepatoblastoma HepG2 cells, used for transfection of the HBV genome and Pol expression constructs, were cultivated in a 10-cm dish in minimum essential medium supplemented with 10% fetal bovine serum (Life Technologies Inc.). DNA transfection was performed with liposome method using FuGENE 6 (Roche Molecular Biochemicals) with total 15 μg DNA, according to the protocol supplied by manufacturer. For cotransfection, 15 μg of polymerase expression constructs was mixed and transfected. Three days later cells were harvested and isolation of MBP/POL from transfected tissue culture cells.

Antibody and Western blotting analysis. For immunoprecipitation, the anti-MBP monoclonal antibodies (HAM-19) (36) were supplied by Dr. Kil-Lyong Kim of KIST (Korea Institute of Science and Technology) and the monoclonal antibody against the HSP90 anti-

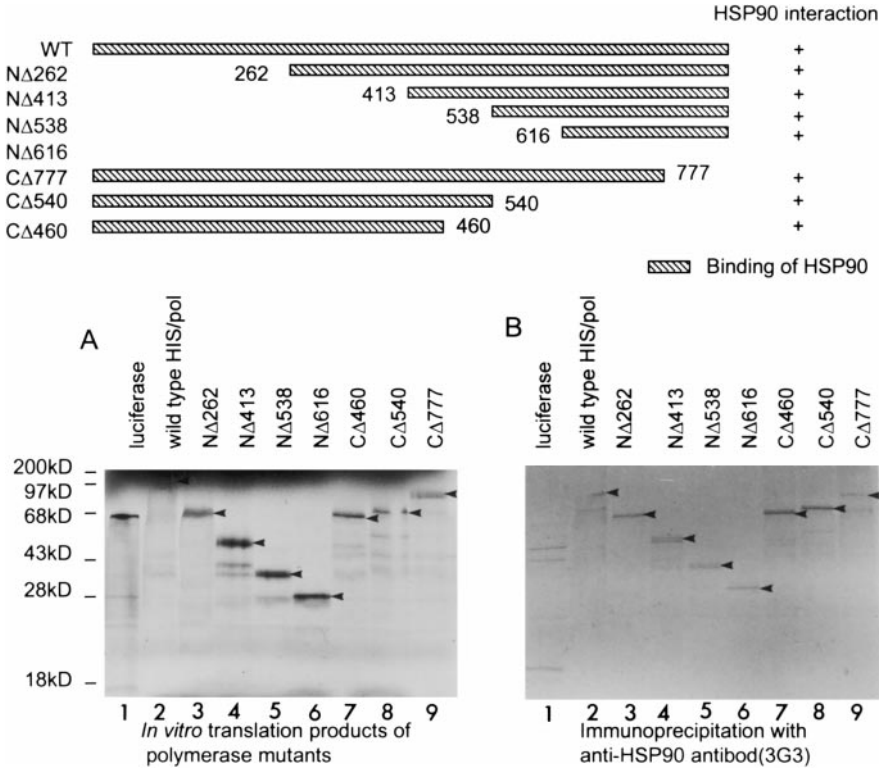

FIG. 2. Association of human hepatitis B virus Pol protein to HSP90 *in vitro*. (A) *In vitro* translation products of HBV Pol wild type and mutants. (B) Immunoprecipitation using anti-HSP90 antibody (3G3) from *in vitro* translation products. Arrowheads indicate each translation product. The detail experimental method is described under Material and Methods. Repeated experiments gave similar results. Diagram displays the result of immunoprecipitation experiment.

gen (3G3) was purchase from Affinity BioReagents Inc. The goat anti-mouse IgM (IgG fraction) was obtained from Sigma Chemical Co. For Western blotting analysis, we used the anti-MBP polyclonal antibody (1:20,000 dilution in PBS containing 1% BSA and 0.05% Tween 20), which was made by the third injection of MBP antigen to purify through amylose resin in rabbit, anti-HSP90 monoclonal antibody (AC88) obtained from Stressgene, anti-polyhistidine monoclonal antibody purchased from Roche Molecular Biochemicals and anti-FLAG monoclonal antibody (M2) purchased from Sigma Chemical Co. The anti-rabbit and mouse polyclonal antibody conjugated horseradish peroxidase, purchased from Sigma Chemical Co. was used as a secondary antibody. The band was detected by using of ECL Western blotting system (Amersham Pharmacia Biotech Inc.).

In vitro translation. The His/POL proteins were translated by TNT coupled reticulocyte lysate in the presence of L-[^{35}S]methionine (Amersham Pharmacia Biotech Inc.), for 90 min at 30°C according to the protocol provided by the supplier (Promega Corp.). Reaction volume of translation was 50 μl. Translation was stopped by addition of cyclohexamide (final concentration of 20 μg/ml).

Immunoprecipitation. To purify the MBP/POL protein, the transfected cells were harvested and resuspended in RIPA buffer (25 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% DOC and 0.1% SDS) containing protease inhibitor cocktail (Roche Molecular Biochemicals). After centrifugation, supernatant was mixed with 2 μg of anti-MBP monoclonal antibody (HAM19) and protein A–Sepharose L-4B (Amersham Pharmacia Biotech Inc.) for 2 h at 4°C. Thereafter, the immunobeads were washed four times with RIPA buffer. Their beads were used for Western blotting analysis.

To immunoprecipitate HSP90 complex and its interacted proteins, the translation products (48 μl) were mixed with 1 ml TNEG buffer (10 mM Tris–HCl [pH 7.6], 50 mM NaCl, 4 mM EDTA, 10% glycerol) and 2.5 μg of anti-HSP90 antibody (3G3). Almost all of endogenous HSP90 in 50 μl of *in vitro* translation product was sufficient to be immunopurified by 2.5 μg of anti-HSP90 antibody. After 1 h incubation of their mixture on ice, the goat anti-mouse IgM (IgG fraction) and protein A–Sepharose (CL4B) were added to the mixture and further incubation. After washing with TNEG buffer containing 200 mM NaCl, the immunoprecipitates or the translation products (2 μl) were separated on SDS–PAGE. The gel was dried and autoradiographed.

Protein purification of His/HSP90 protein and Ni–NTA pull-down assay. His/HSP90 fusion proteins were produced and purified by the Ni–NTA bead (Novagen, Inc) according to the protocol provided by the supplier (Novagen, Inc). The FLAG/POL fusion proteins were immunoaffinity-purified by the agarose-cross-linked anti-FLAG monoclonal antibody M2 according to the supplier's instruction (Sigma Chemical Co). For the purpose of the studying *in vitro* association, Ni–NTA-bound His/HSP90 proteins (1 μg) were directly incubated with the purified FLAG/POL protein (5 μg) in the binding buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, 10% glycerol) for 2 h at 4°C. The bound complex was subsequently washed three times, and eluted with 400 mM imidazole-containing binding buffer. After SDS–PAGE, they were immunoblotted with anti-FLAG antibody.

RESULTS AND DISCUSSION

To investigate whether human HBV Pol is associated with HSP90, we performed the immunoprecipitation experiment using anti-HSP90 monoclonal antibody (3G3) with *in vitro* translation products. HSP90 is

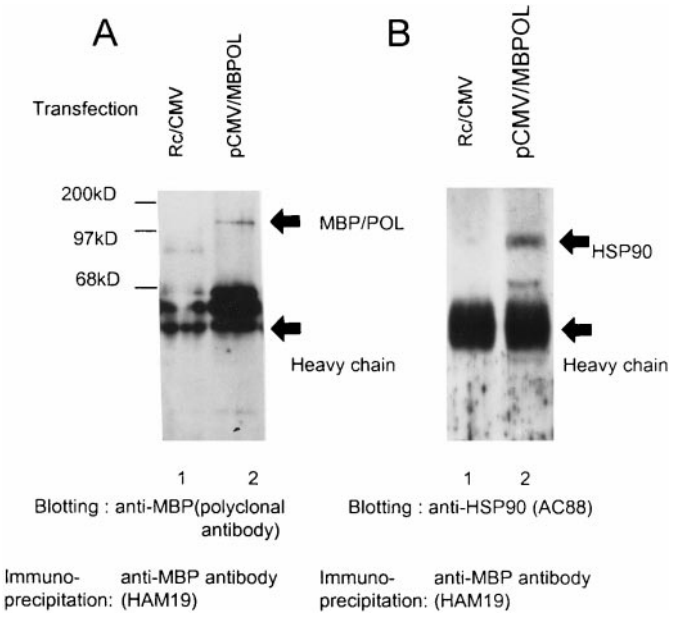

FIG. 3. Association of MBP/POL and HSP90 *in vivo.* After transfection of pCMV/MBPOL, cells were collected and extracted with RIPA buffer. Lysate from pCMV/MBPOL transfected cells were immunoprecipitated with monoclonal antibody against MBP (IgG), immunoprecipitates were immunoblotted for MBP (A) and HSP90(B). Endogenous HSP90 proteins were detected with anti-HSP90 antibody (AC88).

a very abundant protein in rabbit reticulocyte lysate. The HBV Pol product if interact with HSP90 will be coimmunoprecipitated with HSP90 bound to the anti-HSP90 antibody. After *in vitro* translation of wild type HBV Pol by rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine, the endogenous HSP90 was immunoadsorbed from the translation products of [^{35}S]methionine-labeled HBV Pol with the anti-HSP90 monoclonal antibody (3G3) that could immunoprecipitate the complexed HSP90. As expected, [^{35}S]methionine-labeled Pol was coimmunoprecipitated with anti-HSP90 monoclonal antibody (3G3) (Fig. 2). But, luciferase as negative control was not immunoprecipitated with 3G3 antibody (Fig. 2). This result indicates that human HBV Pol is associated with HSP90.

The intact HBV Pol in hepatocyte might differ from the Pol expressed in rabbit reticulocyte lysate. Therefore, we examined the possibility to form complex between HSP90 and HBV Pol expressed in HepG2, hepatoblastoma. Because of the difficulty of expression, HBV Pol was expressed as MBP (maltose binding protein) fusion protein to enhance the expression level and to purify it easily. After the transfection of MBP/POL expression construct (pCMV/MBPOL) and pRC/CMV as negative control, cells were lysed with RIPA buffer and MBP/POL protein was immunoaffinity-purified by anti-MBP antibody as described under Materials and Methods. Their immunoprecipitates were analyzed on SDS–PAGE and immunoblotted for the presence of MBP/POL or endogenous HSP90 with either anti-MBP antibody or anti-HSP90 antibody, respectively. Western blotting analysis using anti-MBP polyclonal anti-

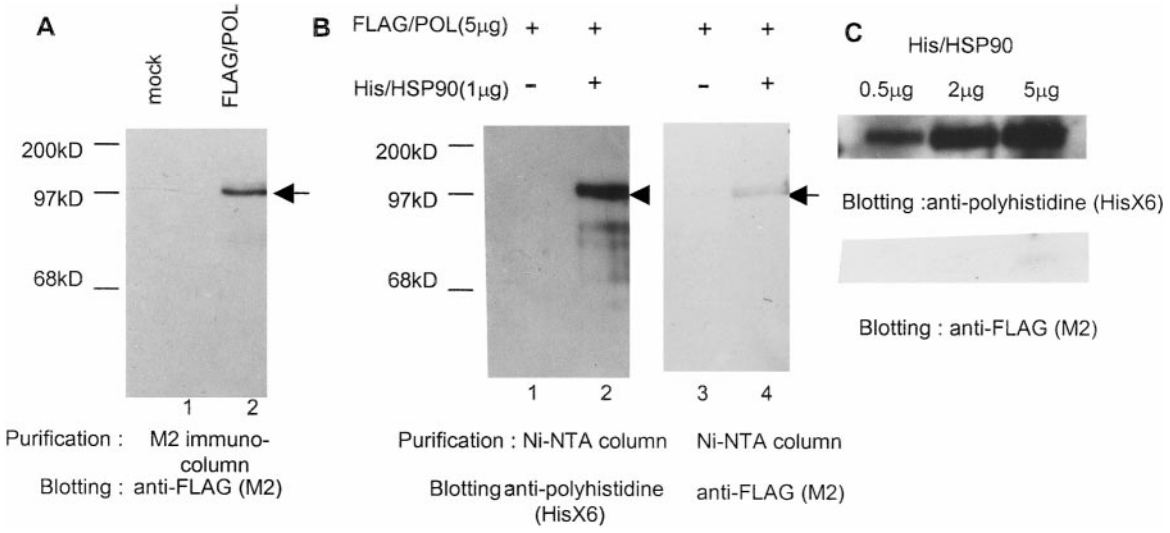

FIG. 4. The recombinant His/HSP90 proteins were assayed *in vitro* for binding to the purified FLAG/POL as indicated the Ni–NTA pull-down assay and Western blotting with the indicated antibody as described under Materials and Methods. (A) The FLAG/POL protein purified with M2 column and immunoblotted with anti-FLAG antibody. Lane 1: The purified protein from non-infected insect cell lysate. Lane 2: The protein from FLAG/POL expression virus infected sf 9 cell lysate. An arrow indicates the full-length FLAG/POL protein. (B) His/HSP90 (1 μg-bound Ni–NTA bead (lanes 2 and 4) or nonbound Ni–NTA bead (lanes 1 and 3) were mixed with purified 5 μg of FLAG/POL. After binding for 2 h on ice, and washing with the binding buffer, the purified complex proteins were immunoblotted with anti-HIS antibody (lanes 1 and 2) or anti-FLAG antibody (lanes 3 and 4). An arrowhead and arrow indicate His/HSP90 and FLAG/POL, respectively. (C) Cross-reactivity between His/HSP90 and anti-FLAG antibody. 0.5–5 μg His/POL protein is analyzed on SDS–PAGE and immunoblotted with anti-HIS antibody (upper panel) and anti-FLAG antibody (lower panel).

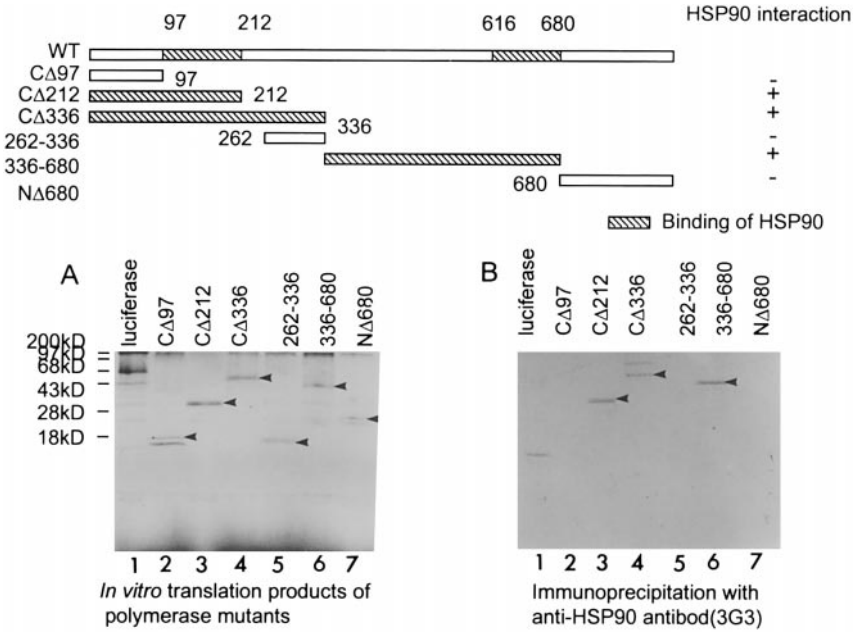

FIG. 5. Two binding regions of HSP90 within HBV Pol. (A) *In vitro* translation products of HBV Pol mutants. (B) Immunoprecipitates obtained by using anti-HSP90 antibody (3G3). Diagram displays the result of immunoprecipitation experiment. In this immunoprecipitation experiment, more fine deletion mutants were used. Arrowheads indicate each translation product. We think that an additional upper band except CΔ336 product shown at lane 4 of B might be the artifact (Pol mutant) derived from internal translation initiation of pHIS/POL (CΔ336), because pHIS/POL (CΔ336) was generated from minor modification of pHIS/POL (wild type) and contained all the coding region except the presence of internal stop codon of Pol ORF (open reading frame). This product was not shown at lane 4 of A. That is because of low translation efficiency.

body showed the presence of full-length and degradation products of MBP/POL on blot (Fig. 3A). Only, the endogenous HSP90-specific band was detected on the blot of immunoprecipitates of pCMV/MBPOL-transfected cell (Fig. 3B), indicating that the recombinant MBP/POL protein is associated with endogenous HSP90 protein *in vivo*.

We could not rule out the possibility that an association of HSP90 with MBP/POL expressed in HepG2 cell resulted from the binding of HSP90 with MBP region of MBP/POL protein. However, the purified MBP/POL protein pool had many MBP/POL degradation products that contained MBP region and short polymerase region; the purified protein contained lower quantity of Pol than that of MBP. Moreover, the quantity of co-purified HSP90 is very low (Fig. 3B). These two facts imply that HSP90 does not interact with MBP but Pol. If MBP is associated with HSP90, much more HSP90 protein will exist in the MBP/POL protein pool purified by the anti-MBP antibody. In previous report, MBP did not affect on hormone binding function and HSP90 interaction in MBP/hMR (human mineral corticoid receptor) fusion protein (37). Therefore, we think that MBP does not impede the interaction of Pol with HSP90. Taken together, human HBV Pol interacts with HSP90 *in vivo*.

There is the possibility of non-specific complex formation between HBV Pol and HSP90. Because of this possibility, we tested the association between His/HSP90 produced from *E. coli* and the FLAG/POL produced in the insect cell. The Ni–NTA-bound His/HSP90 (1 μg) was allowed to associate with FLAG/POL purified by immuno-affinity chromatography (anti-FLAG, M2). As shown at Fig. 4, FLAG/POL was found to interact with recombinant HSP90 *in vitro*. There is a possibility of non-specific interaction between the anti-FLAG antibody and the His/HSP90 protein. To investigate whether there is cross-reactivity between the anti-FLAG and the His/HSP90 protein, 0.5–5 μg of His/HSP90 proteins were immunoblotted with anti-FLAG antibody. In case of 5 μg His/HSP90 protein, a very weak reactivity of anti-FLAG antibody (M2) against His/HSP90 appeared. Because the His/HSP90 (1 μg) protein were used in the Ni–NTA pull-down assay, it was thought that the indicated band in lane 4 of Fig. 4B is not His/HSP90 but FLAG/POL protein.

Recently, the known HSP90 target proteins are so diverse in structure and function that the structural conformational features that define HSP90 target remain elusive (27, 28). To localize the interaction site between HSP90 and HBV Pol, Pol mutants expressed by using the rabbit reticulocyte lysate were immunoprecipitated with anti-HSP90 monoclonal antibody (3G3). In various C-terminal and N-terminal deletion mutants of HBV Pol, all the derivatives were copurified with anti-HSP90 antibody (Fig. 2). This result indi-

cates that two regions of Pol interact with HSP90 independently. To identify the exact interaction sites on HBV Pol that are responsible for HSP90, we generated deletion mutants of each domain and performed the immunoprecipitation experiments as described above. As shown in Fig. 5, TP domain (CΔ212) and RT domain (336–680) were immunoprecipitated with anti-HSP90 monoclonal antibody (3G3) but RNaseH (NΔ680) domain was not. This fact suggests that TP and RT domain is associated with HSP90, independently. The absence of CΔ97 and 262–336 in immunoprecipitates with 3G3 imply that C-terminal region of TP domain is important for HSP90 interaction. In previous result (Fig. 2), the fact that CΔ616 could be immunoabsorbed by antibody (3G3) has been shown. The immunoprecipitation results of CΔ616 and RNaseH (NΔ680) implicate that thumb region of RT domain, 616–680 is involved in HSP90 interaction. Conclusively, C-terminal region of TP domain and RT domain mediate HBV Pol/HSP90 interaction independently.

The fact that the RNaseH domain does not interact with HSP90 might be related to ϵ RNA binding activity of HBV Pol that requires HSP90. That is because RNase H domain is not essential for ϵ RNA binding activity (38). This is consistent with the report by Hu and Seeger (29) that the interaction of HSP90 with HBV Pol influences the RNP formation between Pol and ϵ RNA

In this report, we have described the determination of HSP90-interaction site in HBV Pol. These results showed that C-terminal region of TP and RT domain are important for HSP90 binding. This report should be useful in further exploration of interaction site between Pol and HSP90 and in delineating of HBV replication.

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