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HBV Polymerase Interacts Independently with N-Terminal and C-Terminal Fragments of Hsp90β

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Hsp90 is an abundant chaperone protein that assists the folding of specific proteins, such as steroid receptors, protein kinases, and so on, for their proper function. TP and RT domains of HBV polymerase have been also shown to be associated with Hsp90. Therefore, the identification of the binding sites within Hsp90, responsible for forming Hsp90/HBV Pol complex, is important for the understanding of HBV replication. In this study, cotransfection and immunoprecipitation experiments were performed to localize the binding sites of HBV pol to Hsp90. Our data show that HBV pol interact independently with both N-terminal and C-terminal fragments of Hsp90. Further analysis showed that N-terminal fragment (1–302) of Hsp90 interacts with both TP and RT domains of HBV pol, whereas C-terminal fragment (438–723) interacts with only RT domain. In conclusion, we showed that HBV pol independently interacts with N-terminal and C-terminal fragments, but not the middle fragment (327–438) of Hsp90.

Hsp90 is known to play a role in folding of signal-transducing proteins such as steroid hormone receptors, protein kinases and so on (1, 2). Members of the Hsp90 gene family are well conserved from bacteria to humans and can be located in the cytosol, the endoplasmic reticulum and chloroplasts. Hsp90 forms several discrete subcomplexes, each containing a distinct group of co-chaperones that function in protein folding pathway; Hsp70, p23, Hop, and immunophilin (1–4). But it is not required for the maturation or maintenance of most proteins *in vivo.*

Recently, the crystal structure of N-terminal fragment of Hsp90 was elucidated in complex with ATP (5, 6). X-ray crystallography also revealed that geldanamycin (GA), a novel antitumor drug that prevents Hsp90 function, binds specifically to the ATP binding site of Hsp90 (6, 7). Two chaperone sites exist in Hsp90 that differ in their substrate specificity. The C-terminal fragment recognizes folded substrates whereas the N-terminal fragment binds preferentially unfolded (poly) peptides (8, 9).

Defining the sites involved in the interaction of Hsp90 with its substrate proteins is important to understand the molecular mechanism of this chaperone and its substrate proteins. At least two sites located in the N-terminal and central (for glucocorticosteroid receptor, 206–291 and 446–581) or in the central and C-terminal (for progesterone receptor, 381–441 and 601–677) parts of Hsp90 play a role in the interaction of the steroid receptor (10, 11). The interaction sites of Hsp90 with a number of protein kinases, p53, telomerase, nitric oxide synthase and HBV polymerase are not yet precisely determined (12–15).

Hepatitis B viruses (HBV) are a group of double–stranded DNA viruses that replicate through the reverse transcription pathway (16–18). HBV replication is not initiated by the tRNA-priming reaction but by the protein priming reaction using terminal protein as a primer (protein-priming reaction) (19–23). Compared to HIV-1 RT (reverse transcription), HBV Pol contains an additional domain which is the terminal protein (TP) domain located at the N-terminal region of RT domain (20).

Recently, HBV Pol that suffers many conformational changes for replication was reported to be associated with Hsp90 (15, 24, 25). The formation of the RNP complex between HBV Pol and ϵ RNA depends on the Hsp90 protein (15). RNP formation requires ATP hydrolysis, the function of HSP70 and chaperone partner for Hsp90 (p23) (24). In addition, the HBV Pol/chaperon complex is also packaged into a nucleocapside (24). In this study, to take a step toward an understanding of the complicate HBV replication mechanism involved in Hsp90, we defined the binding

Abbreviations used: HBV, hepatitis B virus; MBP, maltose binding protein; pol, polymerase; TP, terminal protein; RT, reverse transcriptase; RH, RNase H; Hsp90, heat shock protein 90.

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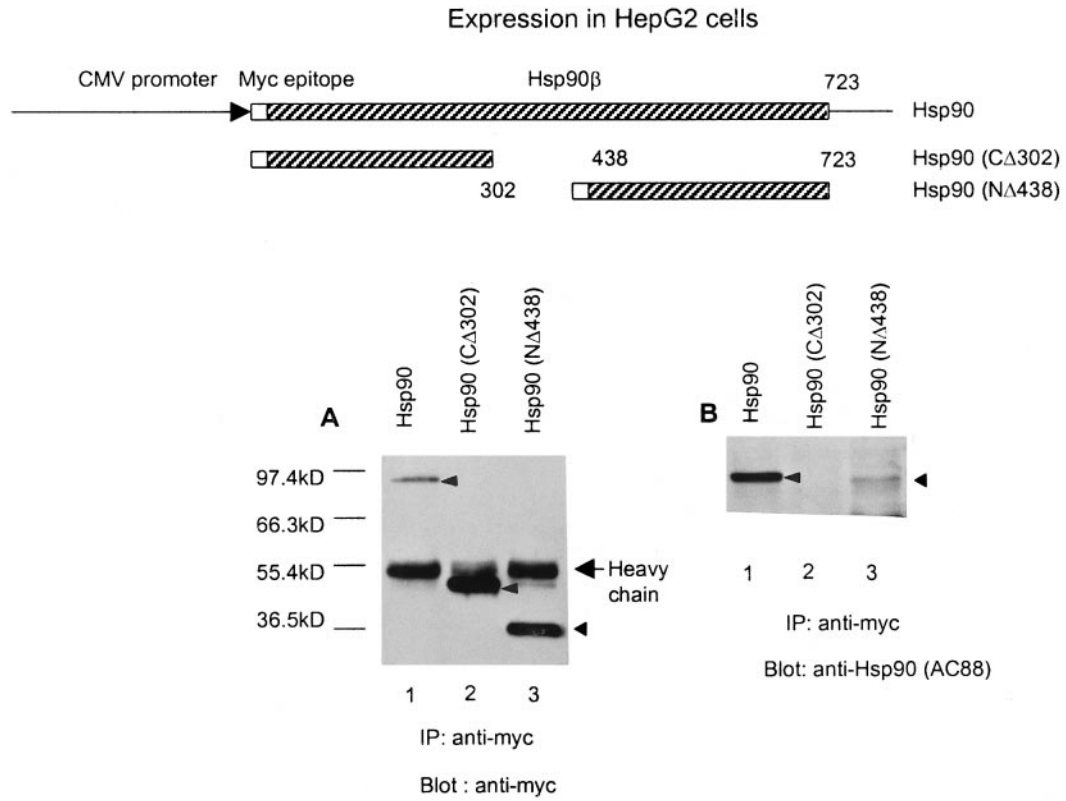

FIG. 1. Expression and purification of myc/Hsp90 protein in HepG2 cells. Immunoblot using anti-myc antibody (A) and anti-Hsp90 antibody (B) of the immunopurified myc/Hsp90 derivatives by using anti-myc antibody (9E10). Lanes 1–3, myc/Hsp90, myc/Hsp90 (CΔ302) and myc/Hsp90 (NΔ438), respectively. The immunoreactive band detected by AC88 in lane 3 is endogenous Hsp90 that is copurified with myc/Hsp90 (NΔ438). An arrowhead represents the specific band detected by anti-myc and anti-HSP90 antibody. An arrow indicates the heavy chain of immunoglobulin.

domains within Hsp90 responsible for the formation of Hsp90–HBV Pol complex.

MATERIALS AND METHODS

Plasmid. For the expression of HBV pol proteins in HepG2 cells, pCMV/MBPOL construct expressing HBV pol proteins in fusion with MBP (maltose binding protein) was used (25). To express the TP (CΔ199) and RT + RH (NΔ336) domains of HBV pol, the coding region of the mutants generated by PCR was replaced with a wild type coding region of pCMV/MBPOL.

To isolate the coding fragment of Hsp90 β, the DNA fragment was amplified by RT-PCR using primers harboring the NotI restriction enzyme site (forward primer: 5′-AAA GCG GCC GCA TGC CTG AGG AAG TGC AC-3′, Reverse primer: 5′-GCG GCC GCT CTA GAG GTA CCC TCG AGG AGC TCG GCC-3′). The cDNA template of HepG2 cell was used as the template. The coding region of Hsp90 β was inserted into the *Not*I site of the Rc/CMV containing the myc epitope (pCMV/mycHSP90). As a result of subcloning, the coding region of Hsp90 β was fused to the myc-epitope at N-terminal region. pCMV/mycHSP90 (CΔ302), coding residues 1–302, was constructed by the filling-in of *Eco*RI on the pCMV/mycHSP90. The pCMV/mycHSP90 (NΔ438), coding residues 438–724, was generated by using the HindIII site as a result of deletion (amino acid residue 1–438) in pCMV/mycHSP90.

To express and purify Hsp90 β in *E. coli*, 6× His-tagged Hsp90 coding region was subcloned by RT-PCR into *Sal*I–*Not*I of pET28b (Novagen, Inc.) (pET/HSP90) (25). The pET/HSP90 (CΔ302) mutant was constructed by the filling-in of *Eco*RI on the pET/HSP90. The C-terminal portion of Hsp90, encoding 444–724 amino acid residues, was amplified by PCR using 5′-primer and 3′-primer, harboring *Sal*I and *Not*I restriction enzyme sites, respectively. The fragment was ligated into pET28b.

For the expression of GST/Hsp90 mutants, DNA fragments, coding N-terminal fragment (CΔ302), middle fragment (327–438), large C-terminal fragment (NΔ438) and small C-terminal fragment (643–724) from pCMV/mycHSP90 were subcloned into *Eco*RI, *Bam*HI/*Hin*dIII, *Hin*dIII and *Nco*I/*Hin*dIII sites of pGEX-KG (26), respectively.

For TP and RT domains of HBV Pol expression in the insect cell, FLAG-tagged TP and RT coding region were cloned by the PCR into the baculovirus transfer vector pFASTBAC HTb (Life Technologies Inc.) (unpublished data).

Cell culture, transfection. The human hepatoblastoma HepG2 cells, used for the transfection of the pol expression constructs, were cultured on a 100-mm dish in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Life Technologies Inc.). DNA transfection was performed by a liposome method using FuGENE 6 (Roche Molecular Biochemicals) with a total of 20 μg DNA, according to the protocol supplied by the manufacturer. HepG2 cells were plated on a 100-mm dish a day before the transfection. The cells were 60% confluent on the day of the transfection. 200 μl of serum-free MEM was mixed with 20 μl of FuGENE6. After 5 min, this solution was added to tube containing DNA and it was gently tapped. After the incubation for 15 min at room temperature, FuGENE6: DNA solution was added to the cells. The next 2 days, the

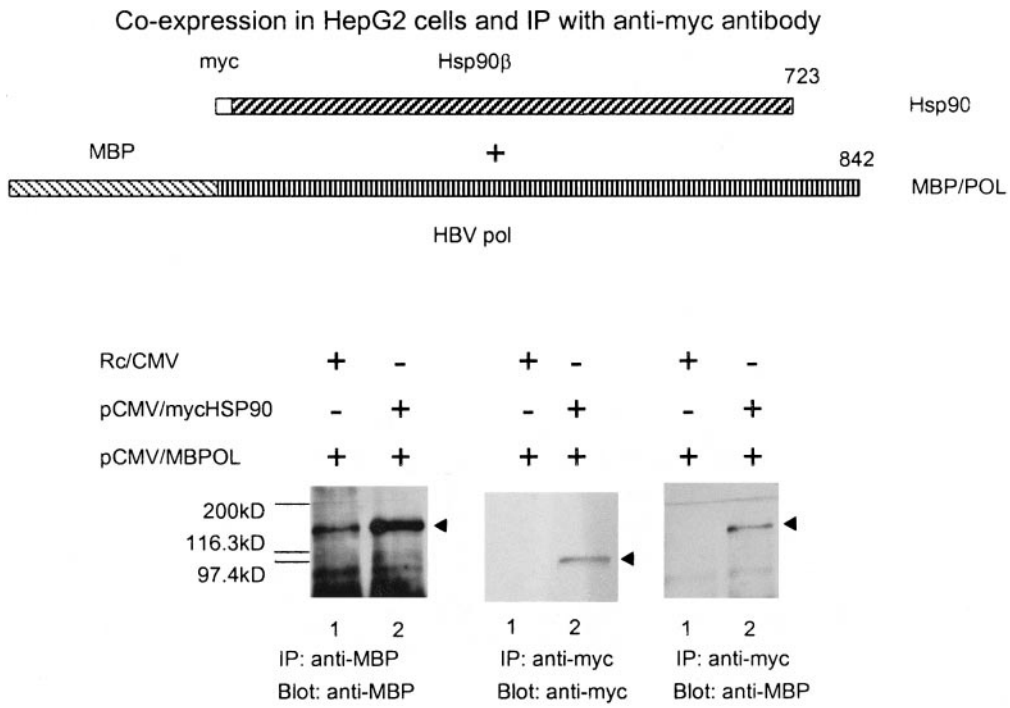

FIG. 2. HBV pol protein was coimmunoprecipitated with Hsp90 from the cotransfected HepG2 cells with pCMV/MBPOL (10 μg) and pCMV/mycHSP90 (10 μg). The cotransfected HepG2 cells (one 100-mm dish) were immunoprecipitated using anti-MBP antibody (HAM-19) and anti-myc antibody (9E10) as described under Materials and Methods. Immunoblot analyses were performed using anti-MBP polyclonal antibody (25) and anti-myc antibody (9E10). Left and middle panels of the immunoblot show the expression of MBP/POL and myc/HSP90. Right panel shows the copurified MBP/POL protein with myc/Hsp90. Plasmids used for transfection are indicated on each panel. The plasmid, Rc/CMV, was used to compensate the DNA quantity for the same transfection efficiency. An arrowhead indicates the full-length protein detected by anti-MBP antibody and anti-myc antibody.

transfected cells were used for the purification and immunoblot analysis. In the case of cotransfection, the DNA quantities of pCMV/mycHSP90 derivatives and pCMV/MBPOL derivatives are the same: 10 μg for each construct. The plasmid, Rc/CMV was used to compensate the DNA quantity per each transfection for the same transfection efficiency.

Expression and purification of FLAF/TP and FLAG/RT mutants. 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 the recombinant baculoviruses were as previously described (27) except that the viruses were generated by the Bac to Bac system (Life Technologies Inc.), in which transposition in bacteria creates the recombinant baculovirus genome rather than homologous recombination in insect cells (28). For the expression of recombinant FLAG/TP and FLAG/RT, the Sf9 cells were grown in TNMFH supplemented with 5% fetal bovine serum 48 h after the infection of recombinant virus. FLAG/TP and FLAG/RT fusion proteins were immunoaffinity-purified by the agarose-cross-linked anti-FLAG monoclonal antibody (M2) according to the supplier's instruction (Sigma Chemical Co.).

Immunoprecipitation and immunoblotting. The transfected HepG2 cells (100-mm dish) were washed with ice-cold PBS, and were lysed in 2 ml of immunoprecipitation (IP) buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each leupeptin, aprotinin, and bestatin). The lysate was centrifuged at 21,000*g* for 15 min. Samples from the supernatant were precleared with protein A–Sepharose (PAS). Each 1ml of supernatant was incubated for 2 h at 4°C with 2 μg of anti-MBP antibody (HAM-19) (29) and 2 μg of anti-myc antibody (9E10; Sigma Chemical Co.). MBP/POL or myc/Hsp90 was immunoprecipitated with the addition of PAS beads. The PAS beads were washed five times with 1 ml of ice-cold IP buffer and the bound proteins were solubilized by boiling in the sample buffer for 5 min. After SDS–PAGE, the proteins were transferred to PVDF membranes, and the membranes were probed with anti-MBP polyclonal antibody (25), anti-myc antibody (9E10) and anti-Hsp90 antibody (AC88). Anti-Hsp90 antibody (AC88) was purchased from StressGen Biotechnologies Corp.). Immunoreactive proteins were visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham Pharmacia Biochemical Inc.).

Expression and purification of Hsp90 mutant in E. coli. For the expression of recombinant Hsp90 β protein, bacterial cells harboring the clone (pGEX/HSP90, pET/HSP90) were pelleted from overnight culture (100 ml) by centrifugation. After resuspension in 1 liter of fresh LB medium containing antibiotics (100 μg/ml ampicillin for pGEX/HSP90 derivatives, 50 μg/ml kanamycin for pET/HSP90 derivatives), the bacteria were incubated for 1 h at 37°C. Protein expression was induced with 1 mM IPTG overnight at 18°C. The bacteria were harvested by centrifugation.

In the case of GST fusion protein, the bacterial pellet was resuspended in 10 ml PBS buffer containing 0.5% Triton X-100. GST/Hsp90 fusion mutant proteins were isolated by agarose-conjugated glutathione bead (Amersham Pharmacia Biochemical Inc.) as described in the manual provided by supplier. The anti-GST antibody used in immunoblot was purchased from Sigma Chemical Co.

In the case of His-tagged Hsp90 proteins, the bacterial pellet was resuspended in 10 ml binding buffer (20 mM Hepes [pH 7.9], 500 mM NaCl, 5 mM imidazole, 0.5% NP-40). The freeze–thawed bacteria were sonicated on ice. Lysate was recovered by centrifugation. His-tagged Hsp90 proteins were purified from crude extract by chromatography on Ni-NTA-agarose (QIAGEN). Unbound proteins were

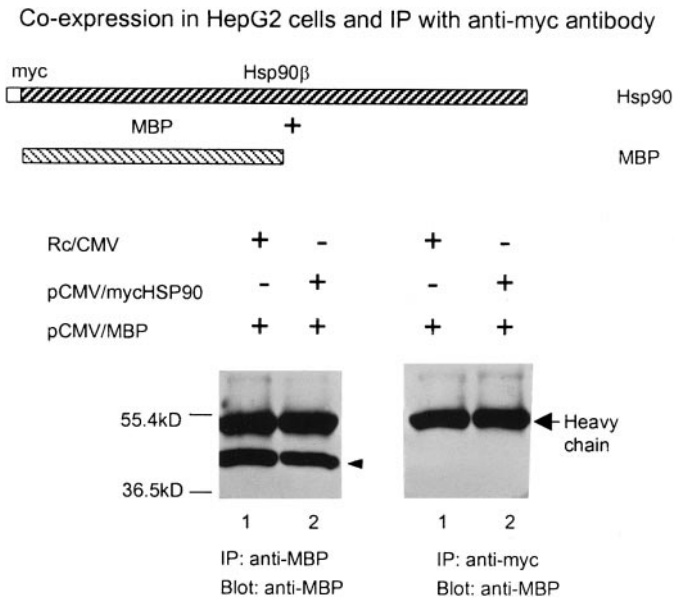

FIG. 3. None of MBP was coimmunoprecipitated with Hsp90 from the cotransfected HepG2 cells with pCMV/MBP (10 μg) and pCMV/mycHSP90 (10 μg). Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 2. Plasmid DNAs used for transfection and antibodies used for immunoprecipitation and immunoblotting are indicated on each panel. An arrowhead points out the full-length protein detected by anti-MBP antibody and anti-myc antibody. An arrow indicates the heavy chain of immunoglobulin.

washed twice with the binding buffer, twice with washing buffer I (20 mM Hepes [pH 7.9], 500 mM NaCl, 50 mM imidazole) and twice with washing buffer II (20 mM Hepes [pH 7.5], 20 mM NaCl), successively. The absorbed protein was recovered by the elution with Affi-Gel binding buffer (0.1 M Hepes [pH 7.5], 80 mM $CaCl_2$) containing 200 mM imidazole. To remove imidazole in the eluates, they were dialyzed in the Affi-Gel binding buffer for 8 h at 4°C. The recombinant Hsp90 mutant proteins (25 μg) were incubated with 100 μl Affi-Gel 10 bead (Bio-Rad Laboratories) in the Affi-Gel binding buffer for 4 h. Subsequently, the unbound protein was recovered by centrifugation in order to check the cross-linking efficiency. The efficiency was approximately 90%. The bead was incubated in 0.1 M ethanolamine [pH 8.0] to block the activated Affi-Gel 10 bead. Until usage, Hsp90-crosslinked bead was stored at 4°C.

In vitro protein binding studies. To analyze the association of HBV pol with GST/Hsp90 fusion proteins, the purified FLAG/TP and FLAG/RT proteins (1 μg) from an insect cell were incubated with the agarose-conjugated GST and GST/Hsp90 mutants (2 μg per each mutant) in a PBS buffer containing 10% glycerol, separately. After rotating the samples for 4 h at 4°C, the beads were sedimented by a brief centrifugation and washed five times with 1 ml of ice-cold PBS. The bound proteins were eluted with 30 μl of freshly prepared 10 mM reduced glutathione (pH 8.0), and the eluate was boiled in a sample buffer. The presence of FLAG/TP and FLAG/RT in the samples was analyzed by immunoblot using anti-FLAG antibody (M2).

To assay the interaction of His/Hsp90 with TP and RT domains, the purified FLAG/TP and FLAG/RT (1 μg) were added to the Affi-Gel 10 bead charged with the immobilized Hsp90 mutant (2.5 μg per each mutant) in a PBS buffer containing 10% glycerol. After vigorous washing with the PBS buffer, the bead was analyzed by an immunoblot as described above.

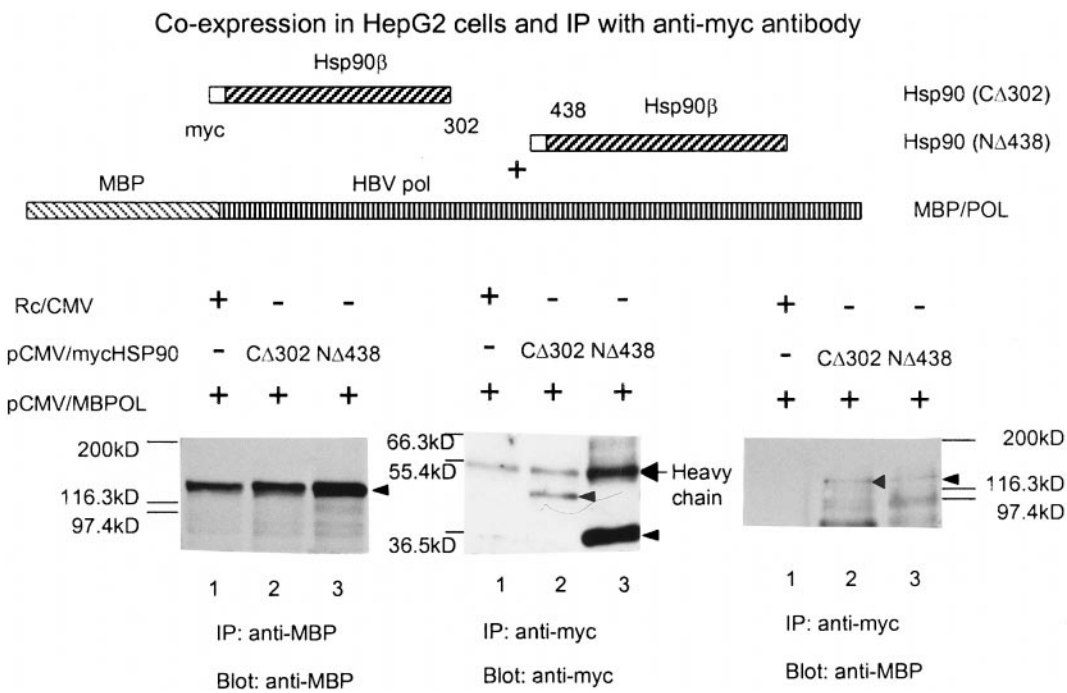

FIG. 4. N-terminal and C-terminal fragments of Hsp90 were coimmunoprecipitated with MBP/POL. After cotransfection of HepG2 cells with pCMV/MBPOL (10 μg) and pCMV/mycHSP90 mutants (10 μg per each mutant), one 100-mm dish of the cotransfected HepG2 cells were immunoprecipitated using anti-MBP antibody (HAM-19) and anti-myc antibody (9E10). Immunoblot analyses were performed using anti-MBP polyclonal antibody (25) and anti-myc antibody (9E10). Plasmid DNAs and antibodies used are indicated on each panel. Left and middle panels of the immunoblot show the expression of MBP/POL and myc/HSP90 mutants. Right panel shows the copurified MBP/POL protein with myc/Hsp90 mutants. An arrowhead indicates the full-length proteins detected by anti-MBP antibody and anti-myc antibody. An arrow indicates the heavy chain of immunoglobulin. Data are representative of similar results obtained in at least two experiments.

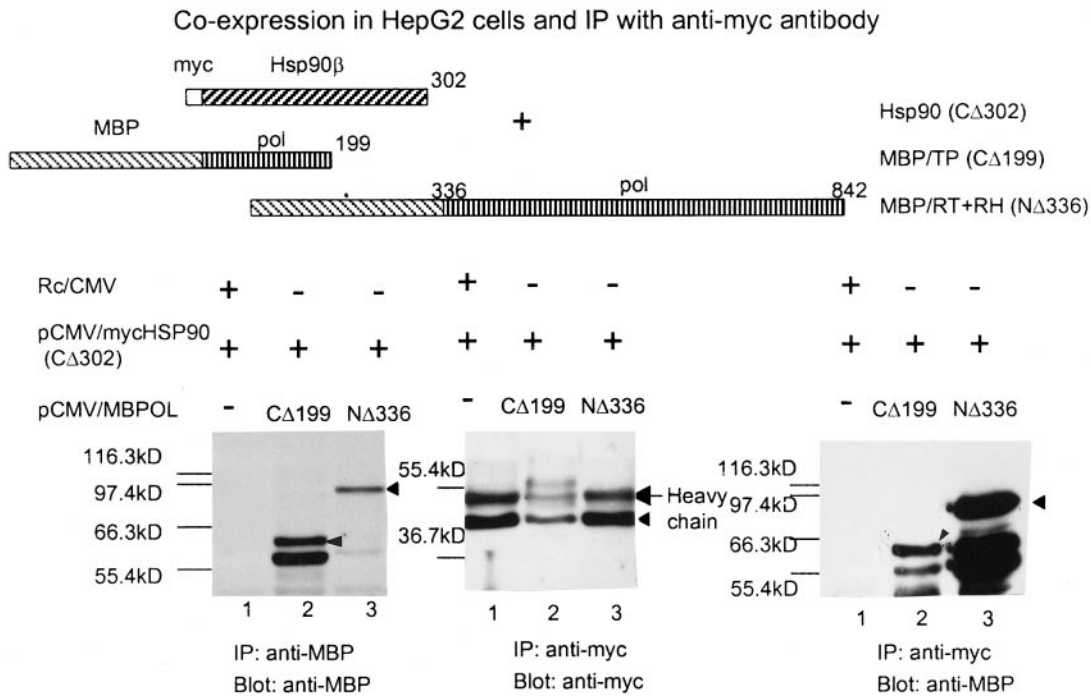

FIG. 5. N-terminal fragment of Hsp90 was coimmunoprecipitated with TP and RT domain of HBV pol. Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 4. Plasmid DNAs and antibodies used are indicated on each panel. An arrowhead indicates the full-length protein detected by anti-MBP antibody and anti-myc antibody. The purified MBP/POL mutants from the transfected HepG2 cells with pCMV/MBPOL derivatives comprise the degradation products detected by anti-MBP antibody (lanes 2 and 3). Left and middle panels of the immunoblot show the expression of MBP/POL derivatives and myc/HSP90 derivatives. Right panel shows the copurified MBP/POL derivatives with myc/Hsp90 derivatives. An arrow indicates the heavy chain of immunoglobulin. Data are representative of similar results obtained from at least three experiments.

RESULTS AND DISCUSSION

Hsp90β Interacts with HBV pol

In the previous study (25), it was demonstrated that the immunopurified HBV pol protein complex from HepG2 cells, which were transfected with HBV pol gene (pCMV/MBPOL), contained the endogenous Hsp90 proteins. As a way to further investigate the interaction between HSP 90 and HBV pol, we expressed myc-tagged human Hsp90β gene (pCMV/mycHsp90) in HepG2 cells. Myc-tagged human Hsp90β (myc/HSP90) was immunopurified by anti-myc antibody (9E10) and was detected at about 90 kDa on the immunoblot by anti-myc antibody (9E10) and anti-Hsp90 antibody (AC88) (Fig. 1).

To confirm whether Hsp90 β interacts with HBV pol in HepG2 cells, we cotransfected pCMV/MBPOL (25) and pCMV/mycHSP90 into HepG2 cells. The eluates from immunoprecipitation by anti-MBP antibody (HAM-19) or anti-myc antibody (9E10) were analyzed by an immunoblot using anti-MBP polyclonal antibody (25) or anti-myc antibody (9E10), confirming the expression of MBP/POL and myc/HSP90 proteins in the cotransfected cells (Fig. 2, left and middle panel). The immunopurified myc/HSP90 complex contained MBP/POL protein as shown in Fig. 2 (lane 2 in the right panel), suggesting that Hsp90β interacts with HBV pol protein. This result, however, cannot exclude the possible association of MBP fusion protein with Hsp90β, not HBV pol protein itself. To eliminate this possibility, we cotransfected pCMV/MBP which encodes only MBP without HBV pol, with pCMV/mycHSP90 and examined its possible interaction with myc/HSP90 by immunoprecipitation followed by immunoblotting. Figure 3 shows MBP is not coimmunoprecipitated with myc/HSP90 when it is expressed alone. Taken together, our data show that Hsp90β interacts with HBV pol.

Both N-Terminal and C-Terminal Fragments of Hsp90β Interact with HBV pol

In the previous studies (8, 9), it was shown that Hsp90 possesses two independent chaperone sites with differential specificity; N-terminal and C-terminal fragments. C-terminal fragment binds to partially folded proteins and N-terminal fragment preferentially binds to unfolded peptides. To define the recognition site for HBV pol on Hsp90, C-terminal or N-terminal deletion constructs of Hsp90β was cotransfected with HBV pol gene; pCMV/mycHSP90 (CΔ302) and pCMV/mycHSP90 (NΔ438) code N-terminal fragment and

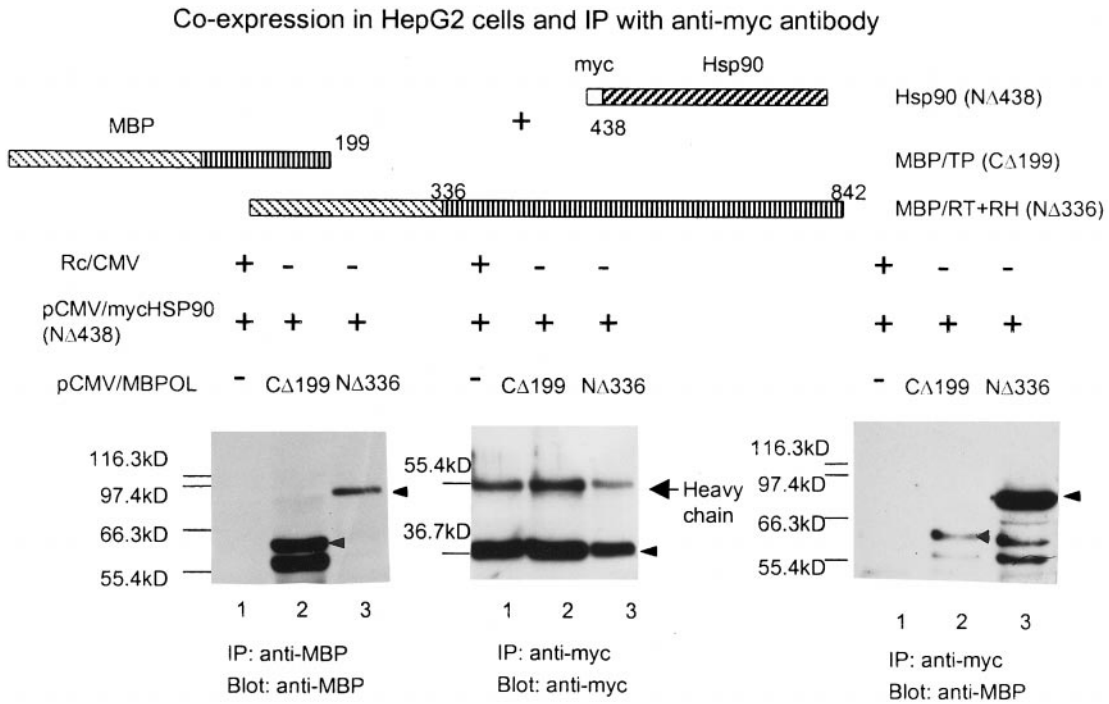

FIG. 6. C-terminal fragment of Hsp90 was coimmunoprecipitated with TP and RT domains of HBV pol. Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 5. The used DNA for transfection and the used antibodies are described at each panel. Left and middle panels of the immunoblot show the expression of MBP/POL derivatives and myc/HSP90 derivatives. Right panel shows the copurified MBP/POL derivatives with myc/Hsp90 derivatives. An arrowhead indicates the full-length protein detected by anti-MBP antibody and anti-myc antibody. An arrow indicates the heavy chain of immunoglobulin. Data are representative of similar results obtained from at least three experiments.

C-terminal fragment of Hsp90 β, respectively. The recombinant Hsp90 deletion mutants were immunopurified by anti-myc antibody (9E10) and detected at 51 and 37 kDa on the immunoblot (lanes 2–3 of Fig. 1A and lanes 2–3 of Fig. 4, middle panel). The size (51 kDa) of N-terminal fragment on SDS–PAGE was different from the estimated size (37 kDa). This difference has been observed in the previous reports (30, 31). The immunoblot result from cotransfection with pCMV/MBPOL and pCMV/mycHSP90 derivatives showed that MBP/POL proteins were coimmunoprecipitated with both C-terminal and N-terminal Hsp90 mutant proteins (Fig. 4, lanes 2–3 of right panel). These facts indicate that N-terminal and C-terminal fragments of Hsp90 β can interact with HBV pol protein in HepG2 cells.

Interactions of N-Terminal Fragment with TP and RT Domains, and C-Terminal Fragment with RT Domain

We have shown that TP and RT domains of HBV pol associate with Hsp90 independently in the previous study (25). In this study, to localize the binding sites for TP and RT domains on Hsp90, cotransfection and immunoprecipitation were performed as following. For the transient expression of TP and RT domains, pCMV/MBPOL (CΔ199) and pCMV/MBPOL (NΔ336) were used. First, in co-transfection with pCMV/mycHSP90 (CΔ302) and pCMV/MBPOL derivatives, the immunoprecipitate using anti-myc antibody (9E10) contained MBP/TP (CΔ199) and MBP/RT + RH (NΔ336) proteins (Fig. 5, lanes 2–3 of right panel). Second, in cotransfection with pCMV/mycHSP90 (NΔ438) and pCMV/MBPOL derivatives, MBP/TP (CΔ199) and MBP/RT + RH (NΔ336) proteins were present in the immunoprecipitate using anti-myc antibody (9E10) (Fig. 6, lanes 2–3 of right panel). The immunoblot result of immunoprecipitate indicates that both N-terminal and C-terminal fragments of Hsp90 interact with TP and RT + RH domains (Figs. 5 and 6).

In the previous results (32–34), it was demonstrated that Hsp90 is a dimer and the dimerization domain lies in the C-terminal fragment of Hsp90. The C-terminal fragment of Hsp90 expressed in HepG2 cells can form a heterodimer with an endogenous Hsp90. The presence of endogenous Hsp90 in the immunopurified C-terminal fragment (NΔ438) elucidates this fact (Fig. 1B, lane 3). Accordingly, it can be hypothesized that the binding of TP and RT domains to C-terminal fragment of Hsp90 may result from the interaction of TP and RT domains with an endogenous Hsp90, not from the interaction with C-terminal fragment itself. We tested this hypothesis by GST pull-down assay using

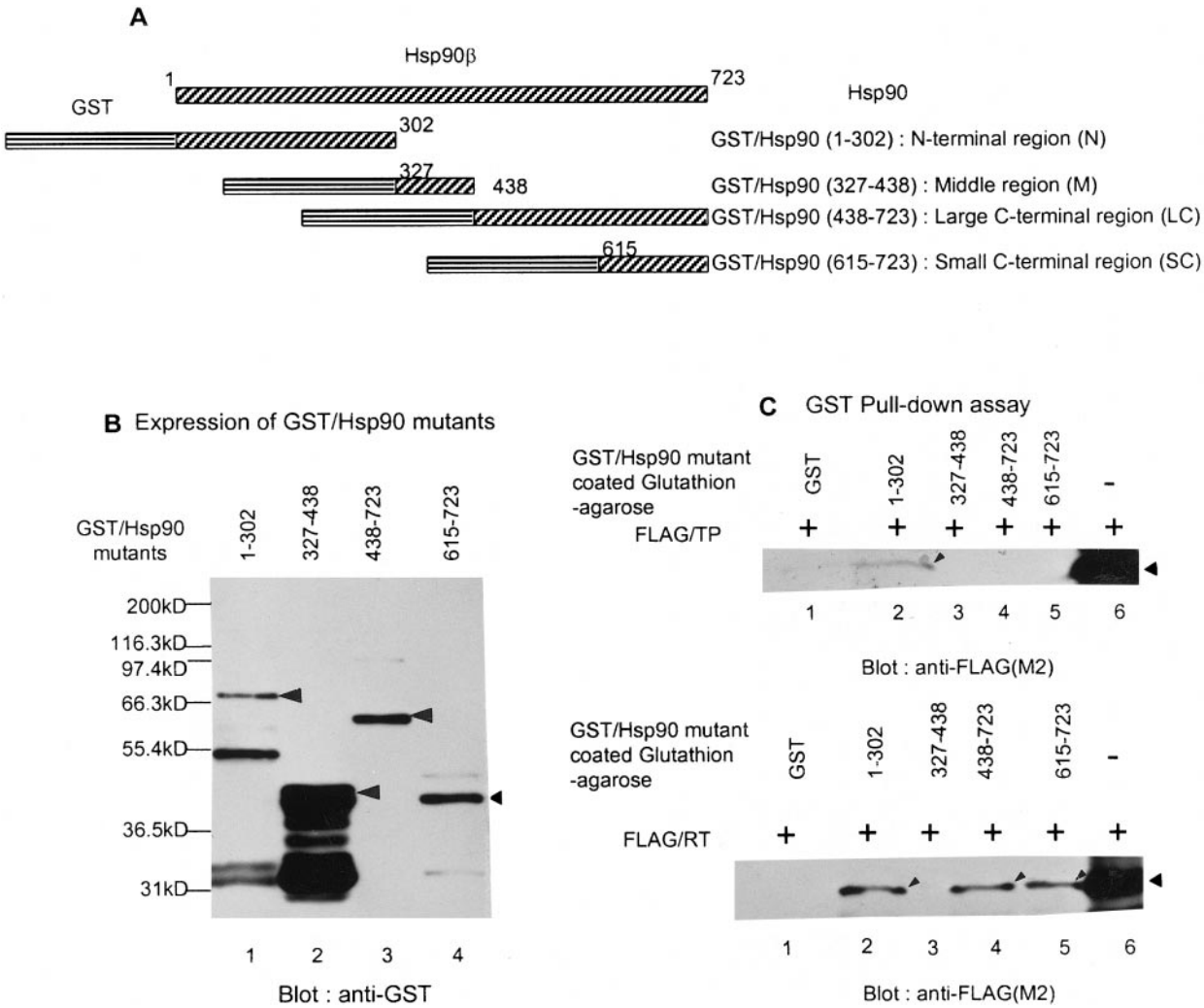

FIG. 7. Interaction of TP and RT domains with GST/Hsp90 deletion mutant proteins. (A) Schematic diagram of the GST/Hsp90 mutants. (B) Expression of GST/Hsp90 mutants in *E. coli*. The purified proteins were analyzed on SDS–PAGE and immunoblotting using anti-GST antibody. An arrow indicates the full-length protein detected by anti-GST antibody. Lanes 1–4, the purified GST/Hsp90 mutants, 1–302, 327–438, 438–723, and 615–723, respectively. (C) GST fusion proteins and GST (each 2 μg) bound to glutathione affinity matrix were incubated with the purified FLAG/TP (upper panel) or FLAG/RT (lower panel) from Sf9 insect cells. After washing, the proteins retained on the matrix were detected by immunoblot analysis using anti-FLAG antibodies. Lanes 1–5, the GST/Hsp90 mutants bound to glutathione agarose, GST (negative control), 1–302, 327–438, 438–723, and 615–723. Lane 6 is the purified FLAG/TP or FLAG/RT protein (200 ng) as positive control. Results are representative of three experiments with similar results. An arrow indicates the specific band detected by anti-FLAG antibody (M2).

bacterially expressed Hsp90 mutants (C-terminal and N-terminal fragments). This assay was used to remove the heterodimer formation between C-terminal fragment and wild-type Hsp90 because of absence of Hsp90 in *E. coli*. After the purification of GST/Hsp90 mutant proteins (Fig. 7), the beads bound with GST/Hsp90 mutant proteins were incubated with the purified FLAG/TP and FLAG/RT from insect cell. The evidence shown in Fig. 7 indicates that 1) TP domain interacts with N-terminal fragment of Hsp90 (1–302), 2) RT domain interacts with N-terminal fragment (1–302) and C-terminal fragment (615–724) and 3) none of TP and RT domains interact with the middle region of Hsp90.

To confirm the above result, we performed another experiment using His-tagged Hsp90 mutant proteins expressed in *E. coli*. Two purified His/Hsp90 mutant proteins (C-terminal and N-terminal fragments) were cross-linked with Affi-Gel 10 as described under Materials and Methods. Affi-Gel 10 cross-linked with both His/Hsp90 (CΔ302) or His/Hsp90 (NΔ444) were incubated with the purified FLAG/TP and FLAG/RT. The immunoblot of the eluate show that N-terminal fragment binds to TP and RT domains and C-terminal fragment binds to RT domain only (Fig. 8). These results are consistent with the results of GST pull-down assay.

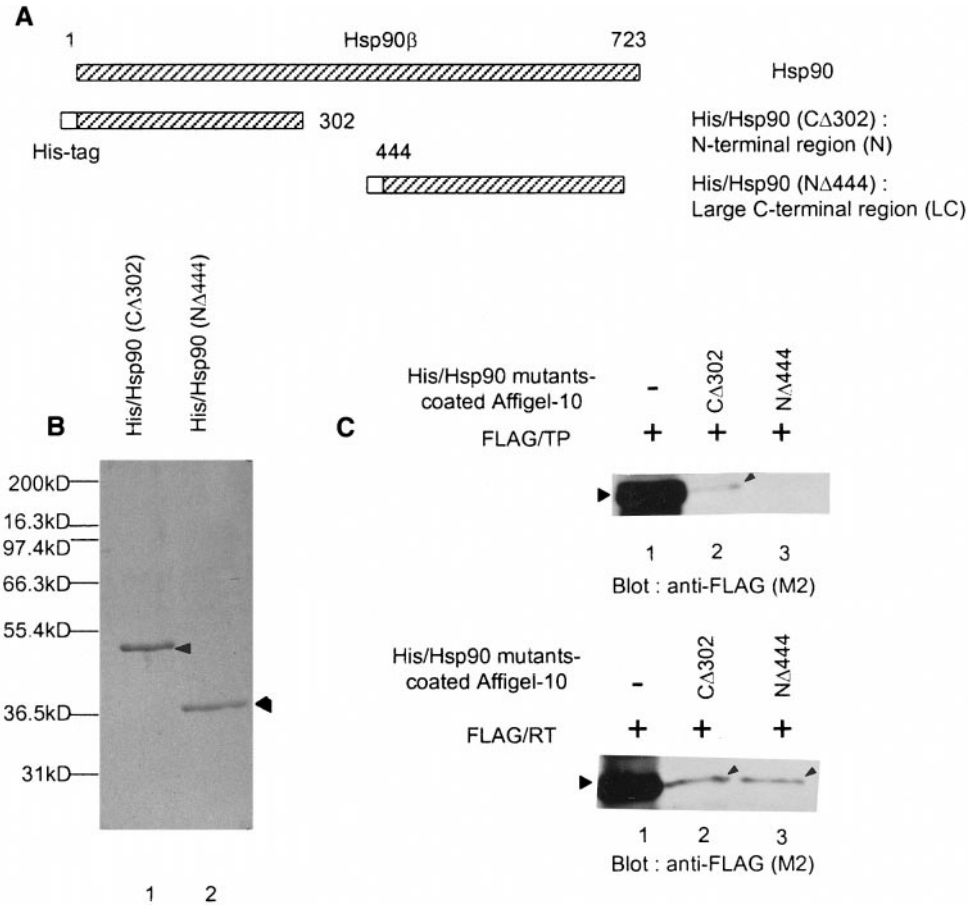

FIG. 8. Interaction of TP and RT domains with His/Hsp90 deletion mutant proteins. (A) Schematic diagram of the His/Hsp90 mutants. (B) Expression of His/Hsp90 mutants in *E. coli*. The purified proteins were analyzed on SDS–PAGE and stained by Coomassie brilliant blue. An arrow indicates the purified His/Hsp90 proteins. Lanes 1–2, the purified His/Hsp90 mutants, 1–302 and 444–723, respectively (C) His/Hsp90 fusion proteins (2.5 μg) cross-linked to Affi-Gel 10 were incubated with the purified FLAG/TP (upper panel) or FLAG/RT (lower panel) from Sf9 insect cells. After washing, the proteins retained on the matrix were detected by Western blot analysis using anti-FLAG antibody. An arrow indicates the specific band detected by anti-FLAG antibody (M2). Lane 1 is the purified FLAG/TP or FLAG/RT protein (200 ng) as positive control. Lanes 2–3, the His/Hsp90 mutants cross-linked to bead, 1–302 and 444–724. Results are representative of three experiments with similar results.

Our data did not rule out the possibility that the association of GST/Hsp90 mutants (C-terminal fragment) with FLAG/TP and FLAG/RT may have resulted from the binding of the GST/Hsp90 mutants to insect Hsp90 proteins which can be copurified with FLAG/TP and FLAG/RT. But, C-terminal fragment of Hsp90 mutant expressed in *E. coli* did not interact with endogenous Hsp90 from insect cells (data not shown). If there is the interaction of C-terminal fragment with insect Hsp90 protein, the result of GST pull-down assay must be similar to that of the coimmunoprecipitation (Figs. 4–6). However, C-terminal fragment binds to RT domain, but not TP domain, in protein binding assay using the bacterially expressed Hsp90 mutants. We think that the interaction of C-terminal fragment with TP in HepG2 cells resulted from the interaction of C-terminal fragment with endogenous Hsp90 protein.

Consequently, we demonstrated that HBV pol binds to N-terminal and C-terminal chaperon fragments, with each having a different substrate preference (8, 9), but not the middle region of Hsp90. This implies that HBV pol may utilize both the two independent chaperon sites of Hsp90 for viral genome replication. These sites are different from the subregions of the Hsp90 molecule that allow interaction with the progesterone receptor and the glucocorticosteroid receptor (10, 11). Although the significance of this difference has yet to be further studied, these results will be useful for the understanding of molecular mechanism of HBV pol in replication.

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