

## HspBP1 Is the Negative Regulator of the Bovine Progesterone Receptor

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**ABSTRACT :** We have investigated whether HspBP1, a Hsp70 binding protein, could have effect on the assembly of the bovine progesterone receptor (bPR) with a chaperone complex consisting of bovine Hsp90 (bHsp90), bovine Hsp70 (bHsp70), Hop, Ydj-1, and p23. The bPR, isolated in its native conformation, loses its function to interact with progesterone hormone in the absence of this protein complex. However, in the presence of bHsp90, bHsp70, Hop, p23 and Ydj-1, its function could be restored *in vitro*. Our findings here indicate that the inclusion of HspBP1 to five-protein system prevented the proper assembly of progesterone receptor-chaperone complex and induce the loss of bPR ability to interact with hormone. Immunoprecipitation assays of bPR with HspBP1 show that the presence of HspBP1 did not have any effect on the assembly of Ydj-1 and bHsp70 with the progesterone receptor. However, further assembly of Hsp90, Hop and p23 was completely prevented and the function of the bPR was lost. *In vitro* competition and protein folding assays indicated that the binding of HspBP1 to bHsp70 prevented the ternary complex formation of bHsp70, bHsp90, and Hop. These results indicate that HspBP1 is a negative regulator of the assembly of Hsp90, Hop and Hsp70, and thus, prevent the proper maturation of unliganded bPR with chaperones assembly system. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 9 : 1261-1267)

**Key Words :** HspBP1, Bovine Progesterone Receptor, Chaperones, Folding

### INTRODUCTION

Progesterone Receptor (PR) is a member of the nuclear receptors, a large and diverse group of proteins that mediate transcriptional activation or repression in the presence of ligand, progesterone (Jensen, 1991; Smith and Toft, 1993). PR, upon binding to progesterone hormone, functions as a transcriptional factor and plays an important role in controlling cell proliferation and differentiation in many organs. It functions as an important physiological factor in ovarian cycle, pregnancy, and various diseases of the female genital tract (Jensen, 1991).

In the absence of progesterone or other activating signals, unliganded PR in cytosol forms heteromeric complexes with several proteins including heat shock proteins, or chaperones, Hsp40, Hsp70 and Hsp90, and their co-regulating proteins (Pratt and Toft, 1997). The heterocomplex of PR can be readily formed under cell free conditions by incubating immunoprecipitated PR with chaperons in rabbit reticulocyte lysate system (Smith et al., 1990). Furthermore, unliganded PR complexes were assembled by incubating purified proteins including Hsp70, Hsp40, Hsp90, Hop and p23 with unliganded PR (Barent et al., 1998; Chen and Smith, 1998; Dittmar et al., 1998; Kosano et al., 1998). Among those proteins, Hsp70, Hsp40 and Hsp90 are three chaperones, which are necessary for maturation of hormone binding ability of PR *in vitro*. Hop and p23 are two additional co-chaperone proteins, which assist the complex formation of three chaperones (Johnson

et al., 1994; Cheung and Smith, 2000). Hop organizes the ternary complex formation of Hsp70 and Hsp90, while p23 binds to Hsp90 and regulates its chaperone function (Smith et al., 1993; Scheuffler et al., 2000; Young and Hartl, 2000). Studies with the complex formation of unliganded PR with chaperoning system using reticulocyte lysate and purified proteins indicated a multistep model pathway for unliganded PR maturation. It has been recently revealed by Toft group that the initial step in the maturation of PR-chaperone complex is the interaction between Hsp40 and PR, followed by the ATP dependent binding of Hsp70 (Hernandez et al., 2002). Hsp90-Hop dimer binds to the ternary complexes of Hsp70-Hsp40-PR. Hsp70 and Hop are dissociated from the complex as p23, a Hsp90 interacting protein, binds to Hsp90 in the presence of ATP (Johnson and Toft, 1995; Hernandez et al., 2002). Thus, the mature progesterone receptor contains Hsp40, Hsp90 and p23 chaperoning system (Cheung and Smith, 2000; Hernandez et al., 2002).

A functional role of chaperones interacting with PR has been that they could maintain the unstable conformation of unliganded hormone receptor until progesterone binds. Another role might be that chaperone complex would repress the binding of the unliganded hormone receptor to DNA site, or induce the release of DNA bound hormone receptor, which would suppress the unnecessary transcriptional activation. Furthermore, chaperone could have effects on the shuttling of hormone receptor between cytoplasm and nucleus through nuclear pore complexes (Pratt and Toft, 1997).

The existence of various regulatory proteins of chaperones involved in PR maturation process implies that

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the unliganded PR-chaperone complex could be regulated in various steps. For example, Hsp70 interacting protein Hip or Hsp46 has been known to be involved in the maturation of PR or GR (glucocorticoid receptor) and influence the transcriptional activation (Cheung and Smith, 2000). In this report, we have analyzed another Hsp70 regulatory protein, HspBP1, which exhibited negative regulatory effect on Hsp70 chaperoning function (Raynes and Guerriero, 1998; Kabani et al., 2002). We showed that HspBP1 prevented the maturation of chaperon complexes by inhibiting the binding of Hop and Hsp90 to Hsp70. Moreover, HspBP1 caused the Hsp90-Hop dissociation from Hsp70 and this process inhibited the ligand binding ability of bovine PR to progesterone.

## MATERIALS AND METHODS

### Plasmids

The human HspBP1 cDNA was amplified by the PCR using cDNA of human liver (BD Bioscience) as template. PCR fragments were subcloned into the *EcoRI*-*XhoI* sites of the pGEX4T1 (Pharmacia-LKB) and pcDNA3.1 expression (Invitrogen) vectors. pcDNA4/his-hPR, the human progesterone receptor (hPR) mammalian cell expression vector, was constructed by PCR amplification using p91023(B)cPR-A as a template (Smith et al., 1995). PCR fragments were subcloned in to the *EcoRI*-*XhoI* sites of the pcDNA4/his expression (Invitrogen) vectors. Bacterial expression system of Ydj-1, p23, and Hop (GenBank™/EBI accession number X56560, M86752, L24804 respectively) were generously supplied by Dr. Toft (Mayo Clinic).

### Preparation of proteins

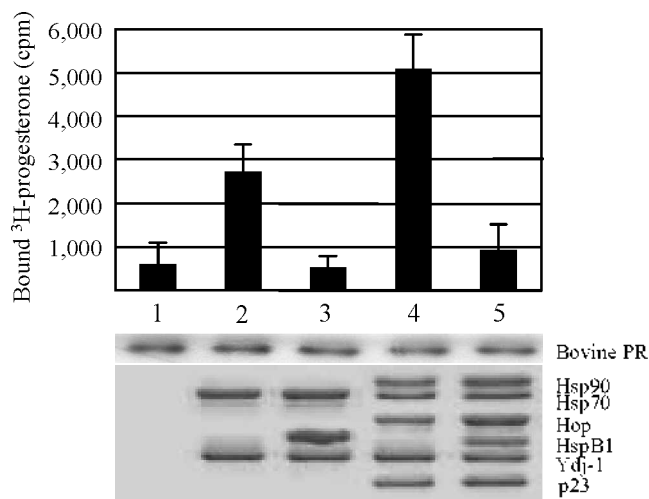
bHsp70 and bHsp90 were prepared from the bovine liver. Tissue homogenates were prepared in 3 volumes of TEN<sub>0.1</sub> (10 mM Tris-HCl (pH 7.2), 0.1 mM NaCl, 0.1 mM EDTA) with protease inhibitors including 0.1 mM leupeptin and 0.1 mM pepstatin. After centrifugation at 13,000 rpm for one hour, the supernatant was purified using DEAE-cellulose chromatography. The purification for bHsp70 was carried out by Resource Q FPLC followed by ATP-agarose affinity column as described previously (Hernandez et al., 2002). bHsp90 was obtained by heparin-Sepharose chromatography. Resource Q FPLC and superdex 200 FPLC as described previously (Hernandez et al., 2002). Ydj-1, p23, and Hop proteins were purified as previously described (Caplan et al., 1992; Johnson et al., 1994; Schumacher et al., 1994). Gst-HspBP1 were purified as follows. An overnight culture of BL21/DE3 transformed with pGEX4T1/HspBP1 was diluted 40-fold in LB medium, cultured for 4 h (OD<sub>600</sub>=0.9) at 30°C and induced with isopropyl β-D-thiogalactopyranoside (Sigma) for 6 h. Crude cell extracts were loaded onto a 100 ml DEAE Sepharose

column (Pharmacia-LKB) and eluted with a 100-400 mM NaCl gradient over five column volumes. The fractions containing Gst-HspBP1 were pooled, re-circulated over 20 mL of glutathione-Sepharose (Pharmacia-LKB), sequentially washed with 1 M NaCl, 0.02 M Tris/HCl (pH 7.2) and 0.1 M NaCl, 0.02 M Tris/HCl (pH 7.2) and eluted with 100 mM glutathione, 0.02 M Tris/HCl (pH 7.2). The fractions containing Gst-HspBP1 were pooled, dialyzed, and concentrated. Protein concentrations were determined using the bicinchoninic acid (Pierce) protein assay relative to standard BSA. Rabbit polyclonal antibody (A1) was made against the purified HspBP1, which were generated from Gst-HspBP1 by thrombin proteolysis.

The bovine progesterone receptor were prepared from bovine oviduct cytosol. Tissue homogenates were prepared in 3 volumes of buffer B (TEN<sub>0.1</sub>, 0.5 mM KCl and 8 mM ATP) with protease inhibitor including 0.1 mM leupeptin and 0.1 mM pepstatin. The inclusion of KCl and ATP removes any receptor-associated proteins (Hernandez et al., 2000). bPR were purified by incubating the cell extracts with anti-bPR antibody (MA1-411, Bioreagents) attached to protein A-Sepharose CL-4B (Amersham Biosciences) in 0.1 M Tris buffer (pH 8.0) and 0.1 M NaCl for 4 h in 4°C with constant rotation, followed by washing with buffer B. bPR bound to the resins were directly used for the complex forming assay.

### Cell biology

The expressions of bPR and HspBP1 in Cos cells were carried out as follows. Cos cells were grown in DMEM supplemented with 10% fetal calf serum (Life Technologies). Transient transfections were performed using Lipofectamine Plus Reagent, according to the manufacturer's guidelines (Life Technologies). Cos cells were transiently transfected with different concentrations of pcDNA3.1/HspBP1 and pcDNA4/his-bPR. Twenty four hours after transfection the cells were washed three times with 1×PBS, and then lysed with a solution containing 1×PBS, 1% Triton, 10 μg ml<sup>-1</sup> pepstatin A, 10 μg ml<sup>-1</sup> leupeptin and 2.5 mM phenyl-methyl-sulfonyl fluoride. The protein concentrations of the lysates were determined by Bio-Rad protein assay (Bio-Rad). Proteins (50 or 100 μg) were resolved using SDS-PAGE gels transferred to nitrocellulose membranes and positively identified with anti-Hsp70 (S5, BioVision), anti-Hsp90 (4A2, MIC), anti-hop (2G6), anti-p23 (BD-612320, Fujisawa Phar.) and anti-his-tag (Amersham) monoclonal antibodies, and anti-HspBP1 (A1) polyclonal antibody. For the precipitation of bPR complexes from cell lysates, anti-his monoclonal antibody was used. Approximately 10 μg of anti-his monoclonal antibody was preincubated with 10 μl of a 50% slurry of protein-G agarose beads by incubating for 1 h at 4°C with constant rotation. Antibody coated beads were



**Figure 1.** HspBP1 inhibits bPR complex formation with progesterone. The hormone binding activities of his-bPR were measured by incubating 30  $\mu$ l of bPR attached to antibody (MA1-411)-protein A complex in the absence (lane 1) or presence of various combinations of proteins including bHsp70 (2  $\mu$ M), Ydj-1 (2  $\mu$ M), bHsp90 (4  $\mu$ M), Hop (2  $\mu$ M), p23 (2  $\mu$ M) and HspBP1 (4  $\mu$ M) (lanes 2-5). Each reaction sample was incubated at 30°C for 30 min for hormone binding activity. Proteins added to the reaction mixtures were shown by Western blot analysis for bPR and SDS-PAGE followed by coomassie brilliant blue staining for other proteins.

then incubated with 20  $\mu$ l of 5% BSA and washed with lysis buffer prior to use in immunoprecipitation reactions. Cell lysate (50  $\mu$ l) in lysis buffer were combined with antibody-beads and rotated for 1 hr at 4°C. Immunoprecipitates were then washed with the lysis buffer 4 times before analysis.

#### Biochemical assay

Folding of guanidine-HCl denatured luciferase (Sigma) *in vitro* was performed as previously described (Minami et al., 1996). Analysis of chaperone and co-chaperone complex formation was carried out as follows. Recombinant purified Hsp70 (0.5-2  $\mu$ M), His-Hop (0.5-2  $\mu$ M) or Gst-HspBP1 (0.5-2  $\mu$ M) were incubated for 15 min to 1 h at 37°C in buffer B (25 mM HEPES pH 7.5, 5 mM MgCl<sub>2</sub>, 50 mM KCl). Glutathione-Sepharose 4B (Amersham-Pharmacia) or Ni<sup>2+</sup>-NTA agarose (Qiagen) was added at a final concentration of 10% (v/v) to reaction mixtures followed by incubation for 30 min at 4°C. Following centrifugation, the pelleted material was washed three times with 100 volumes of buffer C (25 mM HEPES pH 7.5, 5 mM MgCl<sub>2</sub> and 50 mM KCl) with or without 20 mM imidazole. The bound material was eluted with buffer B containing 100 mM glutathione or 200 mM imidazole. The eluted proteins were resolved by 12% SDS-PAGE followed by Coomassie blue staining (Liu, 2003). For

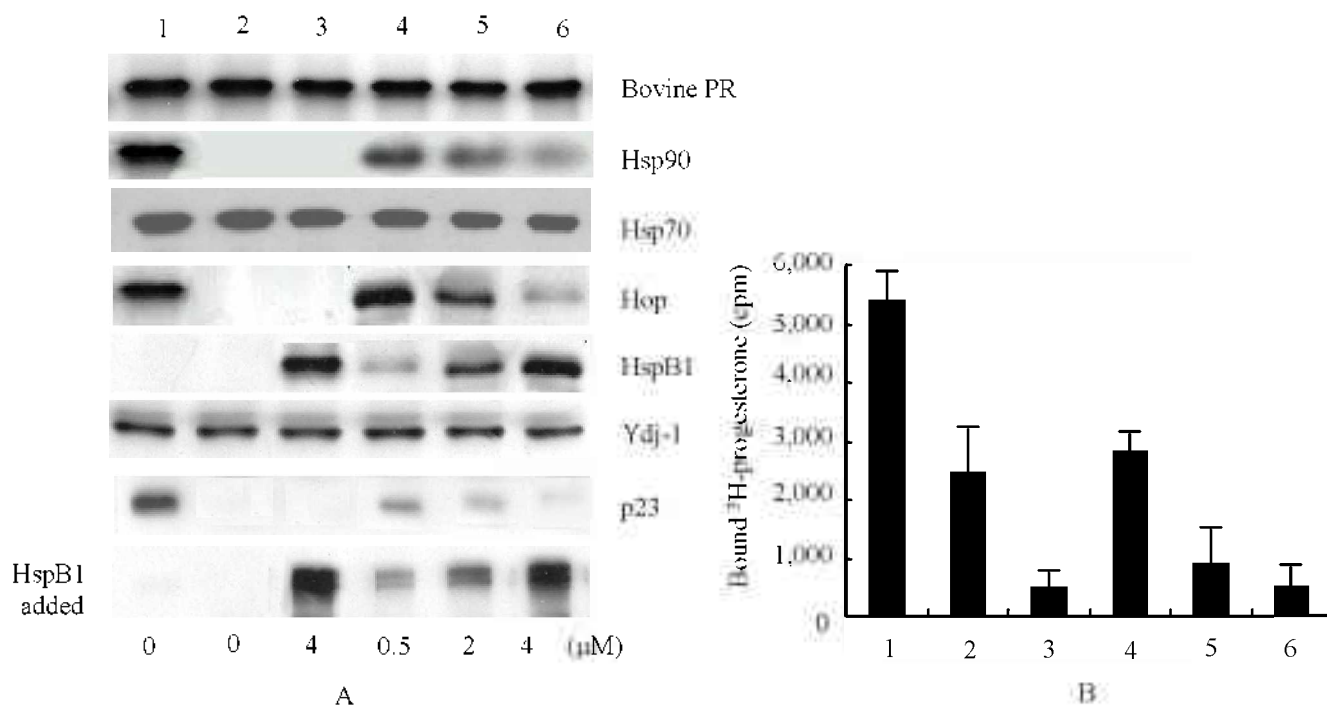
hormone binding assay, samples (30-50  $\mu$ L) were incubated with 20 nM [1, 2-<sup>3</sup>H] progesterone (PerkinElmer Life Science) and 100 nM unlabeled progesterone at 4°C for 2 h. The samples were washed for times with buffer B. The amount of [<sup>3</sup>H] progesterone bound to progesterone receptor was measured using Beckmann scintillation counter (Hernandez et al., 2002).

## RESULTS AND DISCUSSION

### The hormone binding ability of bovine PR can be negatively regulated in the presence of HspBP1.

Bovine his-PR (bPR) can be isolated from bovine oviduct by immunoprecipitation in its native form, which could bind to progesterone hormone. However, the stable structure of bPR is transient, which resulted in the loss of its ability to interact with progesterone. This can be prevented if the bPR is pre-incubated with Hsp70, Hsp40, Hsp90, Hop and p23, which are known as PR complex assembly proteins, prior to incubation with progesterone (Pratt and Toft, 1997; Cheung and Smith, 2000). It has been reported that PR binds initially to Hsp40 followed by Hsp70 (Hernandez, 2002). Hop mediates the subsequent binding of Hsp90 to Hsp70. The binding of the p23 to Hsp90 completes PR complex assembly capable of binding to progesterone (Cheung and Smith, 2000).

The unstable nature of bPR structure in the absence of these assembly proteins is shown in Figure 1. When bPR was incubated without the assembly proteins at 30°C for 30 minutes, it lost most of its ability to bind to the progesterone labeled with <sup>3</sup>H (Figure 1, lane 1). In the presence of bHsp70 and Ydj-1, a yeast homolog of human Hsp40, bPR's ability to bind to the hormone was markedly increased up to 6 times, which indicated that bHsp70 and Ydj-1 could hold the unstable bPR in its native state capable of binding to hormone (Figure 1, lane 2). bPR in the presence of whole bPR assembly proteins, which included bHsp90, bHsp70, Ydj-1, Hop, and p23, showed even higher levels of hormone binding ability up to 12 times compared to bPR in the absence of the assembly proteins (Figure 1, lane 4). These data indicated that for the full function of bPR, five proteins involved in the bPR assembly are required as shown before (Hernandez, 2002). When HspBP1, a negative regulator of Hsp70 chaperone function, was included in the bPR five protein system to identify its effects on the bHsp70 function in the bPR assembly, we observed that it inhibited the hormone binding ability of bPR. It prevented the hormone binding ability of bPR either incubated with bHsp70 and Ydj-1, or bHsp90, bHsp70, Ydj-1, Hop and p23 (Figure 1, lanes 3 and 5). In summary, our results indicate that the HspBP1 has the negative regulatory effect on bPR complex assembly protein, possibly through inhibiting the proper function of Hsp70.



**Figure 2.** HspBP1 inhibits bPR complex formation with bHsp90, Hop and p23, but not with bHsp70 and Ydj-1. A. bPR (30  $\mu$ l) attached to bPR antibody (MA1-411)-Protein A were incubated with bHsp90 (4  $\mu$ M)+bHsp70 (2  $\mu$ M)+Hop (2  $\mu$ M)+Ydj-1 (2  $\mu$ M)+p23 (2  $\mu$ M) (lane 1), bHsp70 (2  $\mu$ M)+Ydj-1 (2  $\mu$ M) (lane 2), bHsp70 (2  $\mu$ M)+Ydj-1 (2  $\mu$ M)+HspBP1 (4  $\mu$ M) (lane 3), bHsp90 (4  $\mu$ M)+bHsp70 (2  $\mu$ M)+Hop (2  $\mu$ M)+Ydj-1 (2  $\mu$ M)+p23 (2  $\mu$ M)+HspBP1 (0.5  $\mu$ M) (lane 4), bHsp90 (4  $\mu$ M)+bHsp70 (2  $\mu$ M)+Hop (2  $\mu$ M)+Ydj-1 (2  $\mu$ M)+p23 (2  $\mu$ M)+HspBP1 (2  $\mu$ M) (lane 5), and bHsp90 (4  $\mu$ M)+bHsp70 (2  $\mu$ M)+Hop (2  $\mu$ M)+Ydj-1 (2  $\mu$ M)+p23 (2  $\mu$ M)+HspBP1 (4  $\mu$ M) (lane 6). Each sample was eluted from bPR antibody bound to Protein A followed by western blot analysis. B. The hormone binding activities of bPR from reaction mixtures of lanes 1 through 6 in Figure 2A were measured as indicated in Figure 1. The hormone binding activity of each bar (1-6) is from the reaction mixtures 1 through 6 of Figure 2A respectively.

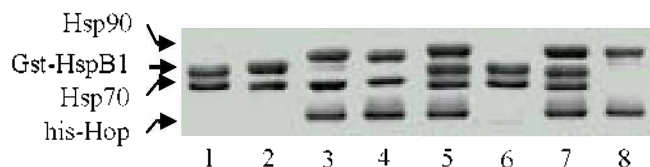
### HspBP1 does not affect the complex formation of bPR/bHsp70/Ydj-1, but inhibits subsequent binding of Hop/Hsp90/p23

To establish whether HspBP1 has any effect on the protein complex assembly with bPR, *in vitro* complex formation assays using immunoprecipitated bPR was carried out. In this assay, bPR bound to its antibody-protein A complexes was incubated with various combinations of assembly proteins, as shown in Figure 1, in the presence of different concentrations of HspBP1. After incubating the proteins at 30°C for 30 min, immunoprecipitated complexes were extensively washed. The precipitated protein complexes were eluted followed by Western blot analysis. In the absence of added HspBP1, the bPR complexes were co-precipitated with either five proteins or bHsp70 and Ydj-1 (Figure 2A, lanes 1 and 2). When HspBP1 were included to the reaction mixtures of bPR, bHsp70 and Ydj-1, we were able to detect the bPR complex containing bHsp70, Ydj-1 and HspBP1 (Figure 2A, lane 3). This indicated that HspBP1 does not interfere with the binding ability of Hsp70 and Ydj-1 to the bPR. Intriguingly, when the increasing amounts of HspBP1 were included in the reaction mixtures

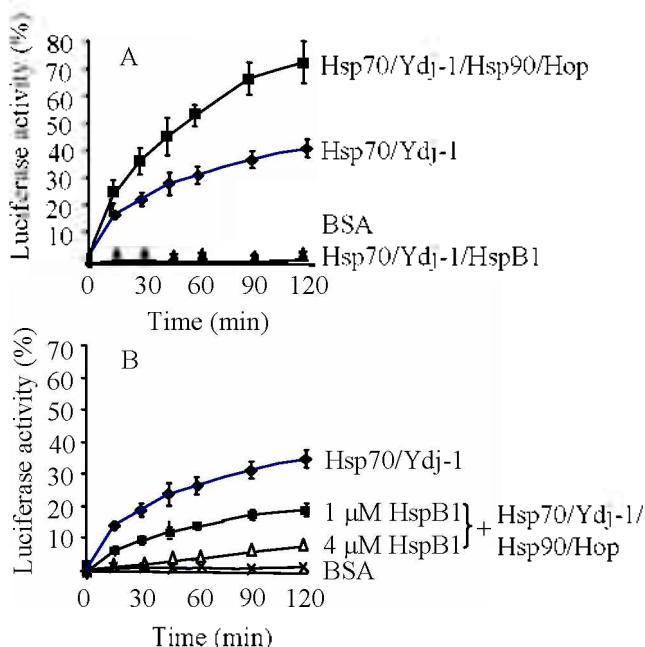
of bPR containing five protein system, the subsequent binding of Hop, bHsp90 and p23 to bPR/bHsp70/Ydj-1 complex was inhibited (Figure 2A, lane 4-6). The inhibitory effect of HspBP1 on bPR complex assembly was concentration dependent, which indicated that HspBP1 might compete with Hop for the interaction with Hsp70, and thus prevent subsequent interaction of bHsp90. The negative regulatory role of HspBP1 on bPR's ability to bind hormone was also observed using the same reaction mixtures of bPR and various combination of proteins used in Figure 2A. The hormone binding abilities of bPR from each lane of Figure 2A are shown in six bars of Figure 2B, respectively. As expected, the hormone binding affinities of bPR with five protein system was decreased reciprocally with the increased levels of HspBP1 (Figure 2B, bars 4-6). The *in vitro* data of complex assembly and hormone binding abilities imply that the binding of HspBP1 prevents the further assembly of Hop, bHsp90 and p23

### The interaction between Hsp70 with HspBP1 prevents the ternary complex of bHsp70/Hop/bHsp90

One possible explanation for the inhibitory effect of



**Figure 3.** HspBP1 prevents the ternary complex formation of Hsp70, Hop and Hsp90. Reaction mixtures containing Gst-HspBP1 (4 μM)+bHsp70 (2 μM) (lane 1), bHsp90 (2 μM)+bHsp70 (2 μM)+his-Hop (2 μM) (lane 3), Gst-HspBP1 (5 μM)+bHsp90 (2 μM)+bHsp70 (2 μM)+his-Hop (2 μM) (lane 5), Gst-HspBP1 (5 μM)+bHsp90 (2 μM)+bHsp70 (2 μM)+his-Hop (2 μM) (lane 7) were incubated and protein complexes were precipitated by glutathione-sepharose (lanes 2, 4, 6) or Ni<sup>2+</sup>-agarose (lane 8). Precipitated proteins were analyzed by SDS-PAGE and visualized with Coomassie blue.



**Figure 4.** The complex formation of Hsp70 and HspBP1 can relieved the Hsp90/Hop mediated enhancement of Hsp70-dependnet folding. A. The folding of chemically denature luciferase (0.5 μM) was measured in the presence of BSA (2 μM) (x), bHsp70 (1 μM)+Ydj-1 (1 μM)+HspBP1 (2 μM) (Δ), bHsp70 (1 μM)+Ydj-1 (1 μM) (◆) and bHsp70 (1 μM)+Ydj-1 (1 μM)+bHsp90 (2 μM)+Hop (2 μM) (■). B. The folding of chemically denature luciferase (0.5 μM) was measured in the presence of BSA (2 μM) (x), bHsp70 (1 μM)+Ydj-1 (1 μM) (◆), bHsp70 (1 μM)+Ydj-1 (1 μM)+bHsp90 (2 μM)+Hop (2 μM)+HspBP1 (1 μM) (■) and bHsp70 (1 μM)+Ydj-1 (1 μM)+bHsp90 (2 μM)+Hop (2 μM)+HspBP1 (4 μM) (Δ).

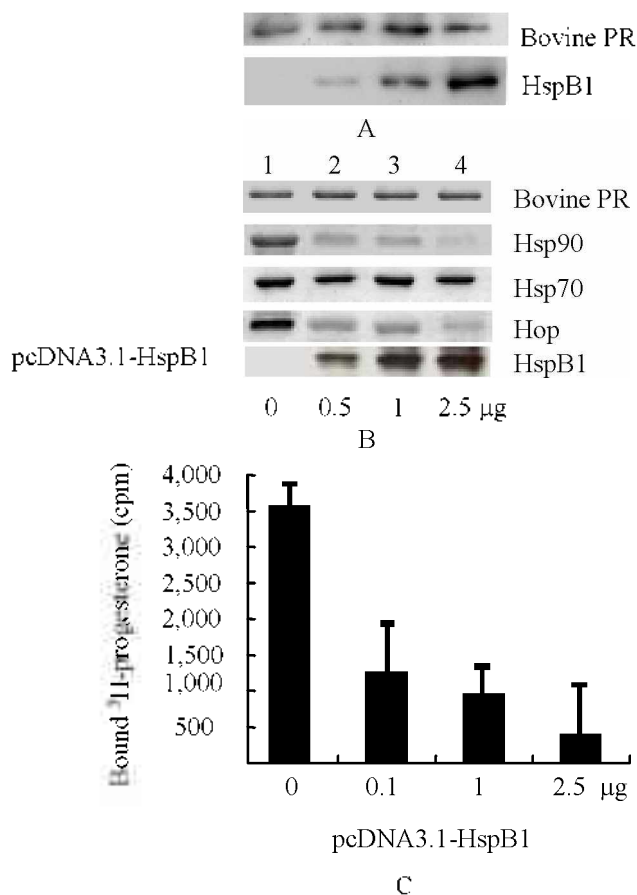
HspBP1 to the binding ability of bPR could be that the binding of HspBP1 to Hsp70 prevents the further protein assembly accomplished by the subsequent binding of Hop and bHsp90. To determine whether the binding of HspBP1 to bHsp70 competes with the Hsp70 and Hop interaction,

complex formation assays were performed using purified proteins. When bHsp70 and Gst-HspBP1 were incubated followed by precipitation using glutathione-Sepharose (Figure 3, lane 1), the complex formation of bHsp70 and HspBP1 were detected (Figure 3, lane 2). We also observed the ternary complex formation between bHsp90, bHsp70 and his-Hop (Figure 3, lanes 3 and 4). Upon addition of Gst-HspBP1 to a reaction mixture containing bHsp90, bHsp70 and his-Hop followed by the precipitation using either glutathione-Sepharose, or Ni<sup>2+</sup>-agarose, we detected that bHsp70 only binds to gst-HspBP1, but not to his-Hop and Hsp90 (Figure 3, lanes 5 to 8). The interaction between HspBP1 and bHsp70 seemed to be dominant over that of bHsp70, bHsp90 and Hop, inasmuch as the addition of HspBP1 almost completely dissociated bHsp70 from Hop and bHsp90 complex. These results were further supported by the inhibitory effect of HspBP1 on the bHsp70-dependent folding of chemically denatured luciferase in the presence of Hop and bHsp90 (Figure 4). Hop and bHsp90 functioned as enhancers of bHsp70 chaperone activity, which increased the folding of denatured luciferase by up to 80%, while HspBP1 had a negative effect on bHsp70-dependent folding (Figure 4A). When HspBP1 was added to reaction mixtures of bHsp70, Hop and bHsp90, the Hsp70-dependent folding of denatured luciferase was inhibited (Figure 4B). The data suggest that HspBP1 has the dominant negative effect over the positive regulatory function of Hop and bHsp90.

In summary our results demonstrated that the negative regulatory effect of HspBP1 on bHsp70-dependent folding is caused by the direct interaction of HspBP1 and bHsp70, which competitively displaced the Hop and bHsp90 complex from bHsp70.

**The expression of HspBP1 prevents the his-bPR complex assembly and decreases the binding ability of his-bPR with hormone in Cos cells**

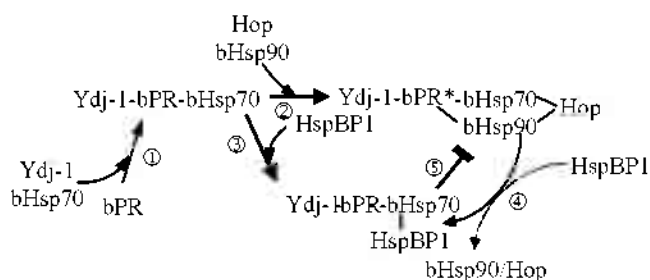
To establish whether HspBP1 also function as a competitive negative regulator of bPR in intact cells, HspBP1 were co-expressed with bPR in Cos cells. As shown in Figure 5A, the various expression levels of HspBP1 in cells were accomplished by transiently transfecting the cells with different concentrations of pcDNA-HspBP1, while the expression levels of his-bPR were kept constant. We next examined the complex formation of his-bPR expressed in cells with assembly proteins including Hsp70, Hsp90 and Hop with or without overexpressed HspBP1. In the absence of HspBP1, his-bPR, which was precipitated using Ni<sup>2+</sup>-agarose, formed complex with Hsp90, Hsp70 and Hop (Figure 5B, lane 1). As the concentrations of overexpressed HspBP1 increased, the dissociating levels of Hop and Hsp90 from bPR was significantly increased. On the other hand, the levels of



**Figure 5.** The expression of HspBP1 prevents the bPR complex assembly and decreases the binding ability of bPR with hormone in Cos cells. A. Cos cells were transiently transfected with pcDNA4/his-bPR and increasing concentrations of pcDNA3.1/HspBP1 (0-2.5 µg). The levels of expressed proteins were detected using Western blot analysis. B. Cos cells from above were immunoprecipitated using anti-his-tag antibody followed by Western blot analysis for each protein. C. The immunoprecipitated bPR complexes were incubated with hormone as indicated in Figure 1.

Hsp70 binding to bPR was kept constant (Figure 5B, lanes 2-4). When the hormone binding ability of each bPR precipitated from Figure 5B were assayed, we were able to detect that the increased levels of HspBP1 displayed higher negative effects on the hormone binding abilities of bPR (Figure 5C). Taken together, the results showed that HspBP1 could competitively inhibit the assembly pathway of bPR with molecular chaperones by displacing Hsp90 and Hop in cells.

Our results as related to the regulatory networks of HspBP1 on bPR are summarized in Figure 6. Initially, bPR forms a ternary complex with Ydj-1 and bHsp70 followed by the complex formation between bHsp70 and Hop, which mediates the binding of bHsp90 to the complex. When HspBP1 is present, it binds to bHsp70, induces dissociation of Hop and bHsp90 from bHsp70 and inhibits the hormone



**Figure 6.** Schematic of the interactions between bPR and chaperone assembly in the presence of HspBP1. bPR forms a ternary complex with Ydj-1 and bHsp70 (①) followed by the additional binding of Hop and bHsp90 (②). In the presence of HspBP1, a bPR/bHsp70/Ydj-1/HspBP1 complex, which is no longer able to bind hormone, is formed (③) and further complex assembly with bHsp90 and Hop is inhibited (⑤). The presence of HspBP1 also dissociates bHsp90/Hop complex from bPR/bHsp70/Hsp40 (④). bPR' represents the fully active form of bPR which can bind to the hormone.

binding ability of bPR.

PR, a sex steroid hormone, is responsible for cell proliferation and differentiation in various organs. However, in the absence of hormone or other activating reagents, PR remains inactive and usually forms heteromeric complexes with chaperones and additional mediators of chaperones. The binding of chaperones maintains PR in functionally stable form, which can be transferred to nucleus, be activated and bind to DNA when progesterone is present. In the absence of chaperon interaction with PR, it fails to achieve its functional state by structural denaturation, which is led to degradation by proteolytic enzymes (Cheung and Smith, 2000). This study shows that with HspBP1, hormone binding ability of bPR is completely prevented, since HspBP1 abolishes the assembly of bPR complex by inhibiting the interaction between Hsp70 and Hop, thus preventing the maturation of PR and chaperone complex. More detailed mechanisms of biological and physiological effects caused by the negative regulation of bPR mediated by HspBP1 remains to be elucidated. One interesting feature of this study is that the function of bPR, a biologically relevant substrate of Hsp70, is affected in the presence of HspBP1, a negative regulator of Hsp70. These indicate that HspBP1 might display various regulatory roles on other Hsp70 substrates by conferring Hsp70 with modulated activities. Our observations also confirm that the maturation of chaperone complex for bPR is an indispensable pathway, which is a necessary step for the full function of bPR.

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