

Dynamic Nucleotide-dependent Interactions of Cysteine- and Histidine-rich Domain (CHORD)-containing Hsp90 Cochaperones Chp-1 and Melusin with Cochaperones PP5 and Sgt1*

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Tae-Joon Hong^{†1}, Sangkyu Kim^{†1}, Ah Ram Wi^{†1}, Peter Lee[‡], Miae Kang[§], Jae-Hoon Jeong[§], and Ji-Sook Hahn^{†2}

From the [†]School of Chemical and Biological Engineering, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-744 and the [§]Research Center for Radiotherapy, Korea Institute of Radiological and Medical Sciences, 75 Nowon-gil, Nowon-gu, Seoul 139-706, Republic of Korea

Background: Hsp90 cochaperones are regulating interactors of Hsp90, but interactions between themselves are not well known.

Results: CHORD-containing Hsp90 cochaperones interact with cochaperones PP5 and Sgt1 in the presence of ATP.

Conclusion: Conformational changes induced by ATP binding play an important role in the regulation of interactions between cochaperones.

Significance: Interactions between cochaperones might have Hsp90-independent roles that are regulated by cellular ATP concentration.

Mammals have two cysteine- and histidine-rich domain (CHORD)-containing Hsp90 cochaperones, Chp-1 and melusin, which are homologs of plant Rar1. It has been shown previously that Rar1 CHORD directly interacts with ADP bound to the nucleotide pocket of Hsp90. Here, we report that ADP and ATP can bind to Hsp90 cochaperones Chp-1 and PP5, inducing their conformational changes. Furthermore, we demonstrate that Chp-1 and melusin can interact with cochaperones PP5 and Sgt1 and with each other in an ATP-dependent manner. Based on the known structure of the Rar1-Hsp90 complex, His-186 has been identified as an important residue of Chp-1 for ADP/ATP binding. His-186 is necessary for the nucleotide-dependent interaction of Chp-1 not only with Hsp90 but also with Sgt1. In addition, Ca²⁺, which is known to bind to melusin, enhances the interactions of melusin with Hsp90 and Sgt1. Furthermore, melusin acquires the ADP preference for Hsp90 binding in the presence of Ca²⁺. Our newly discovered nucleotide-dependent interactions between cochaperones might provide additional complexity to the dynamics of the Hsp90 chaperone system, also suggesting potential Hsp90-independent roles for these cochaperones.

Hsp90 (heat shock protein of 90 kDa) is a conserved, ubiquitous, and abundant molecular chaperone involved in a broad spectrum of key cellular functions. The Hsp90 homodimer works as a “molecular clamp” undergoing open and closed cyclic conformational changes driven by binding and hydrolysis of ATP (1). The activity of the Hsp90 chaperone machinery is

regulated by dynamic association of various Hsp90 cochaperones during this chaperone cycle. Hsp90 cochaperones are known to modulate the ATPase activity of Hsp90, to stabilize certain conformations of Hsp90 dimer, to recruit particular classes of client proteins to Hsp90, and to exert post-transcriptional modifications on some client proteins or the Hsp90 chaperone complex (2, 3). Some cochaperones have their own chaperone activities, suggesting that cochaperones may also have Hsp90-independent cellular functions (4–8).

The Hsp90 cochaperones Sgt1 and Rar1 are involved in plant immunity by regulating the immune sensors called resistance proteins (R proteins)³ (9–11). Sgt1 consists of the tetratricopeptide repeat (TPR), CS (CHORD-containing proteins and Sgt1), and SGS (Sgt1-specific) domains. Sgt1 acts as a client adaptor linking R proteins to Hsp90 by binding to R proteins through its SGS domain while interacting with Hsp90 through the CS domain (12, 13). It also bridges Skp1 and Hsp90 in yeast (14), revealing its function as a multidomain adaptor. Rar1, which contains two cysteine- and histidine-rich domains (CHORDs), has been suggested to facilitate the recruitment of R proteins to Hsp90 through interacting simultaneously with the Sgt1 CS domain and the Hsp90 N-terminal domain, supporting the adaptor function of Sgt1 (15).

In mammals, Sgt1 also plays an essential role in innate immune responses mediated by Nod-like receptor proteins such as Nod1 and Nalp3, the mammalian homologs of R proteins (16, 17). However, the roles for Chp-1 and melusin, the two mammalian CHORD-containing homologs of Rar1, in innate immunity have not yet been clarified. Chp-1 and melusin contain the additional C-terminal CS domain as well as the two conserved CHORDs (18). Both Chp-1 and melusin interact with Hsp90, suggesting their potential roles as Hsp90 cochap-

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[†] These authors contributed equally to this work.

[‡] To whom correspondence should be addressed. Tel.: 82-2-880-9228; Fax: 82-2-888-1604; E-mail: hahnjs@snu.ac.kr.

³ The abbreviations used are: R protein, resistance protein; TPR, tetratricopeptide repeat; CHORD, cysteine- and histidine-rich domain.

Nucleotide-dependent Interactions of Hsp90 Cochaperones

erones (5, 19). In addition, Chp-1 and melusin have been shown to interact with cochaperones PP5 (protein phosphatase 5) and Sgt1, respectively (5, 20). Melusin is a muscle-specific interactor of β -integrin (21) involved in the signaling pathway that senses and responds to mechanical stress in heart. Upon mechanical stress, melusin induces cardiac hypertrophy, while protecting heart from dilation and failure (22). On the other hand, Chp-1, also known as morgana, has been shown to inhibit Rho kinase II, preventing centrosome amplification (23). Like Chp-1, Sgt1 in *Drosophila* participates in centrosome maturation through stabilizing Polo, the upstream kinase of Rho kinase (24, 25). In addition, the Hsp90-Sgt1 chaperone complex is engaged in kinetochore formation in both yeast and mammals (26, 27). Therefore, both Chp-1 and Sgt1 seem to be involved in mitosis, although their genetic or physical connections *in vivo* remain elusive. In addition, it is not known whether melusin and Chp-1 carry out these functions as Hsp90 cochaperones or as Hsp90-independent regulators. Both Chp-1 and melusin have their own intrinsic chaperone activities (5, 8), but it has not been demonstrated whether they have Hsp90-independent functions related to this stand-alone chaperone activity.

In our previous study (20), we identified the interaction between Chp-1 and PP5 *in vivo*. PP5 is a Ser/Thr phosphatase involved in multiple cellular functions such as the MAPK signaling pathway, cell cycle progression, DNA damage repair, and regulation of transcription factors (28). The enzymatic activity of PP5 is autoinhibited by its TPR domain, which can be relieved by binding of the TPR domain to the Hsp90 C-terminal MEEVD sequence or fatty acids such as arachidonic acid (29, 30). Ppt1, the yeast homolog of PP5, dephosphorylates Hsp90 (31), whereas the known substrates of PP5 include the glucocorticoid receptor (32), Raf-1 (33), and tau (34, 35). In addition, both Ppt1 and PP5 dephosphorylate another Hsp90 cochaperone, Cdc37 (36).

In this study, we identified Chp-1 and PP5 as nucleotide-binding proteins. In addition to the previously known interaction between Chp-1 and PP5 (20), we newly identified the interaction between Chp-1 and Sgt1. Interestingly, these interactions were dramatically enhanced in the presence of ATP. Moreover, these nucleotide-dependent interactions were also conserved in melusin, revealing the similarities of the two CHORD-containing proteins. Furthermore, we show a regulatory role for Ca^{2+} in the melusin/Hsp90 and melusin/Sgt1 interactions. These results suggest that Hsp90 cochaperones retain the property of nucleotide-dependent interaction not only with Hsp90 but also with themselves, implying that each component of the Hsp90 chaperone machinery may participate in the dynamic assembly of multiple complexes depending on the cellular environment.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Plasmids containing cDNAs from mouse Hsp90 α , mouse Chp-1, and human PP5 were described previously (20). Human Sgt1a was amplified from an I.M.A.G.E. expressed sequence tag clone (2985858) by PCR. To generate N-terminally His-tagged proteins, Hsp90 α , PP5, and Sgt1 ORFs were cloned into pET28b vector. Mouse melusin, Chp-1, and Chp-1(H186A) ORFs were cloned into the

pET15b vector. For the production of GST-tagged proteins, Hsp90, Sgt1, and melusin ORFs were cloned into the pGEX-4T-1 vector (GE Healthcare), and PP5 and Chp-1 ORFs were cloned into pGEX-3X.

Protein Expression and Purification—N-terminally GST-tagged proteins and N-terminally His-tagged proteins were expressed in *Escherichia coli* strain Rosetta gami2(DE3)pLysS. The bacteria were grown in LB medium at 37 °C and induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside. Subsequently, the proteins were affinity-purified using glutathione-agarose resin (Novagen) for GST-tagged proteins and nickel-nitrilotriacetic acid affinity chromatography (GE Healthcare) for His-tagged proteins. His-tagged proteins were further purified using a Superdex 200 prep grade gel filtration column (GE Healthcare). Purified proteins were dialyzed against 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl and stored at -70 °C.

ADP- and ATP-Agarose Binding Assay—The purified proteins were incubated with N^6 -ADP (C_8 -ADP)- or N^6 -ATP-agarose resin (Sigma) at 4 °C in buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 50 μ M ZnCl₂, 1 mM DTT, and 5 mM MgCl₂) supplemented with 0.1% Nonidet P-40 and protease inhibitor mixture (Calbiochem). The incubated resin was washed with buffer B (20 mM Tris-HCl (pH 8.0) and 150 mM NaCl), and the bound proteins were eluted with 10 mM ADP or by boiling in SDS-PAGE sample buffer. The pulldown fractions were analyzed by Western blotting with anti-GST and anti-His antibodies (Santa Cruz Biotechnology).

Circular Dichroism Spectroscopy—The secondary structures of the proteins were analyzed using a JASCO J815 spectropolarimeter in the far-UV spectral region at room temperature. The spectra were recorded in buffer A with or without 1 mM ADP or ATP.

GST Pulldown Assay—The purified GST-tagged proteins were prebound to the resin by incubating the proteins with glutathione-agarose resin for 2 h at 4 °C in buffer A with 0.1% Nonidet P-40 and protease inhibitor mixture. The prebound resin was washed twice with buffer B. His-tagged proteins were then added to the prebound resin and incubated for 2 h at 4 °C in the presence or absence of 5 mM nucleotide. After washing the resin three times with buffer B, samples were analyzed by Western blotting with anti-GST and anti-His antibodies. To observe the effect of Ca^{2+} on the protein interactions, various concentrations of CaCl₂ was added to buffer A, and 1 mM CaCl₂ was added to buffer B used to wash the resin.

RESULTS

Hsp90 Cochaperones Chp-1 and PP5, but Not Sgt1, Bind to ADP and ATP—The crystal structure of the complex of the plant Hsp90 N-terminal, Sgt1 CS, and Rar1 CHORD-II domains shows that Rar1 CHORD-II directly interacts with the ADP bound to the nucleotide pocket of the Hsp90 N-terminal domain (15). In a recent proteomic analysis, Chp-1 was also shown to exhibit ADP-dependent interaction with Hsp90 (37). These results raised the possibility of the direct interaction of Chp-1 with nucleotides, independent of Hsp90. To test this, we examined the binding of Chp-1 and two other cochaperones, PP5 and Sgt1 (Fig. 1A), to ADP or ATP linked to agarose through the N^6 -position of adenine. As shown in Fig. 1B, Chp-1

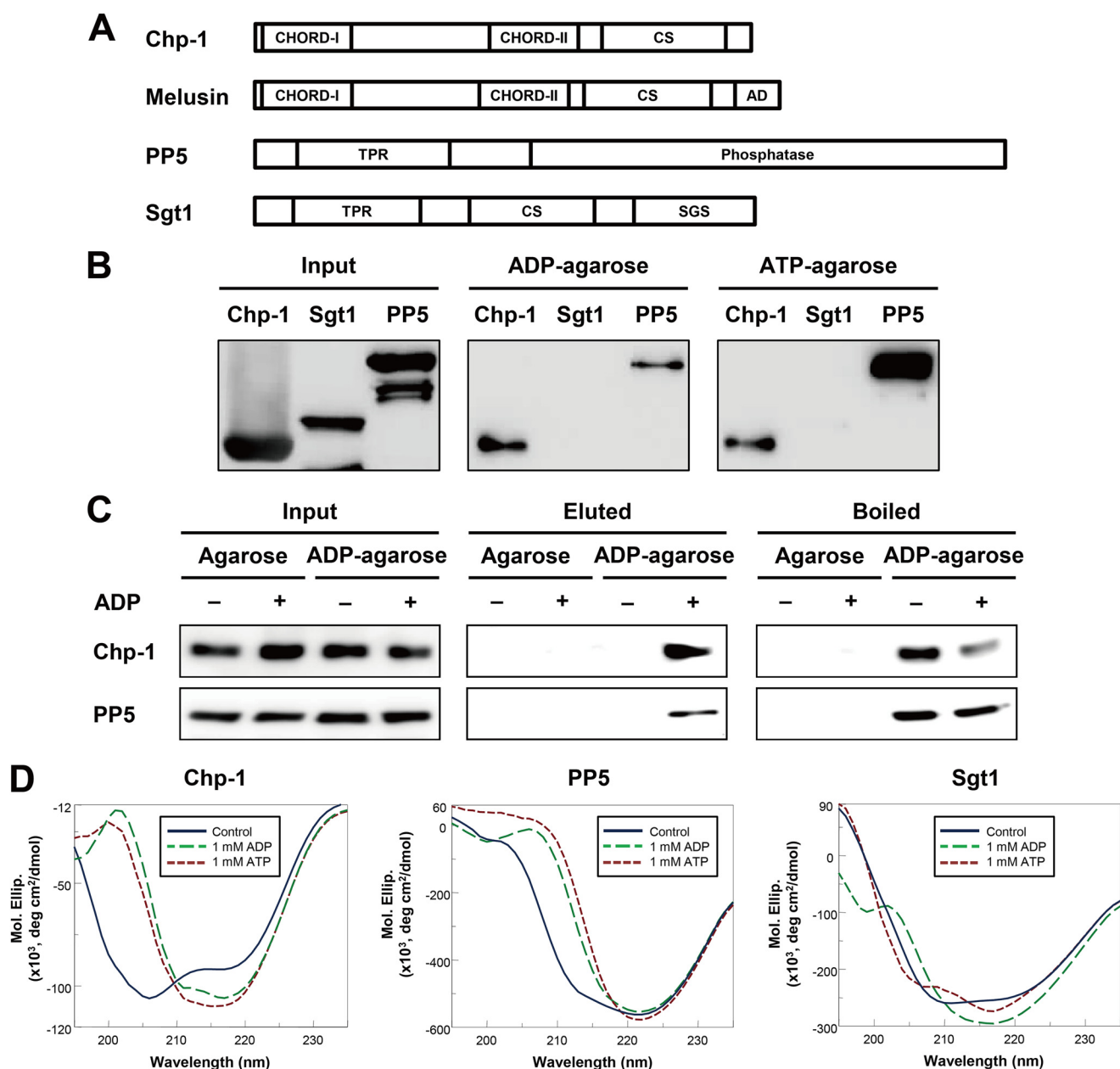


FIGURE 1. Nucleotide binding property of Hsp90 cochaperones Chp-1 and PP5. *A*, schematic diagram of the domain structures of Hsp90 cochaperones related to this work. *AD*, acidic domain. *B*, His-tagged mouse Chp-1, human PP5, and human Sgt1 proteins were incubated with N^6 -ADP-agarose and N^6 -ATP-agarose, and the bound proteins were eluted by boiling and detected by Western blotting. *C*, ADP elution of Chp-1 and PP5. Chp-1 or PP5 was incubated with control agarose or ADP-agarose and then eluted with 10 mM ADP to confirm specific binding. The remaining proteins bound to the agarose beads were detected after boiling. *D*, far-UV CD spectra of Chp-1, PP5, and Sgt1 in the absence or presence of 1 mM ADP or ATP. *Mol. Ellip.*, molar ellipticity; *deg*, degrees.

and PP5, but not Sgt1, bound to ADP- and ATP-agarose. Chp-1 and PP5 did not bind to the control agarose beads, and the proteins bound to ADP-agarose were eluted with ADP, confirming their specific binding to ADP (Fig. 1C). Furthermore, the changes in the CD spectra of Chp-1 and PP5 reflected the conformational changes in the proteins in the presence of ADP or ATP (Fig. 1D). In contrast, the CD spectra of Sgt1 showed little difference upon the addition of nucleotides (Fig. 1D), which is consistent with the previous experiment showing no interaction of Sgt1 with ADP- or ATP-agarose. These results suggest that not only Hsp90 but also Hsp90 cochaperones Chp-1 and PP5 possess the properties of nucleotide binding and subsequent conformational change. This might be a common

regulatory mechanism shared by Hsp90 and its cochaperones Chp-1 and PP5, although these cochaperones have no ATPase activity.

ADP and ATP Enhance Interactions between Cochaperones—A previous study demonstrated the interaction between Chp-1 and the PP5 TPR domain *in vivo* (20), but its biological significance remains elusive. Other CHORD-containing proteins, plant Rar1 and mammalian melusin, have been shown to interact with Sgt1 (5, 11). Because ADP and ATP bind to Chp-1 and PP5, inducing their conformational changes, we asked whether ADP and ATP could affect the interactions between the cochaperones. Chp-1, PP5, and Sgt1 were purified as GST- and His-tagged proteins, and the interactions between the proteins

Nucleotide-dependent Interactions of Hsp90 Cochaperones

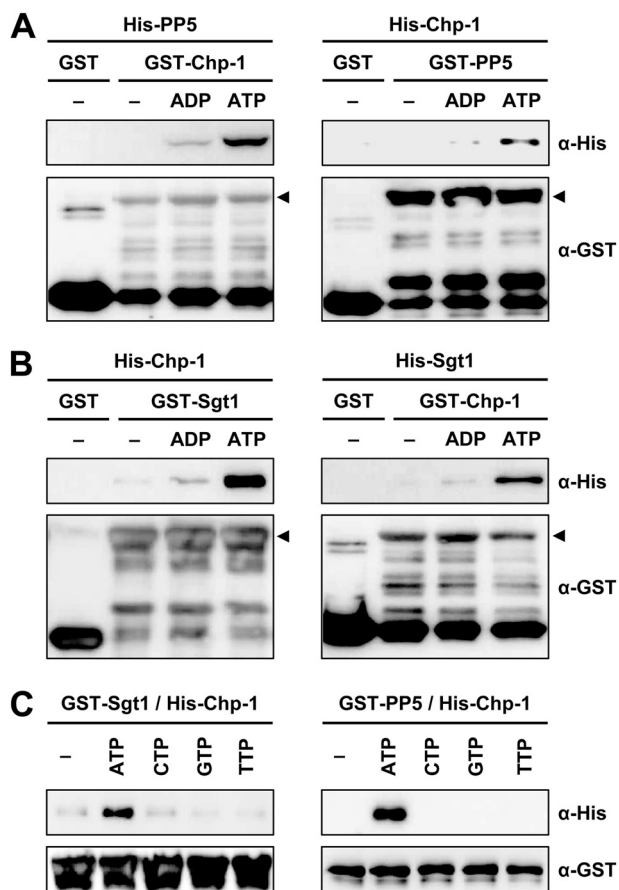


FIGURE 2. Nucleotide-dependent interactions between Hsp90 cochaperones. PP5/Chp-1 (A) and Chp-1/Sgt1 (B) interactions were examined by reciprocal GST pull-down experiments. Each GST-tagged cochaperone protein was incubated with another His-tagged cochaperone with or without 5 mM ADP or ATP. The full-length protein bands are indicated as arrowheads. Smaller bands are degradation products. C, the Chp-1/Sgt1 (left) and PP5/Chp-1 (right) interactions were analyzed by GST pull-down assays in the absence or presence of 5 mM ATP, CTP, GTP, or TTP. The pull-down fractions were analyzed by Western blotting.

were observed *in vitro* in the presence or absence of the nucleotides. Interestingly, the interaction between Chp-1 and PP5 increased in the presence of ADP and ATP in the reciprocal GST pull-down experiments (Fig. 2A). ATP enhanced the interaction more effectively compared with ADP. Furthermore, we newly identified the interaction between Chp-1 and Sgt1, which was also enhanced by ATP (Fig. 2B). Although ADP and ATP induced comparable levels of conformational changes in Chp-1 and PP5 in the CD spectra (Fig. 1, B and C), the interactions between the cochaperones showed preferential enhancement in the presence of ATP compared with ADP. Therefore, the γ -phosphate moiety of ATP might contribute to the enhancement of interactions between cochaperones. To further verify the nucleotide specificity in the interaction enhancement between the cochaperones, we tested the effects of all four NTPs in GST pull-down assays. Among the four NTPs, only ATP exclusively enhanced the interaction of Chp-1 with Sgt1 or PP5, suggesting the specific binding of the adenine base to Chp-1 (Fig. 2C).

Melusin Shows Nucleotide-dependent Interaction with PP5 and Sgt1—Melusin, a CHORD-containing Chp-1 homolog, has two N-terminal CHORDs, a central CS domain, and a C-termi-

nal acidic domain (see Fig. 5A). The similarity of its structure to Chp-1 gave rise to the possibility that melusin might also have the property of nucleotide-dependent interactions with other cochaperones. Like GST-Chp-1, GST-melusin strongly interacted with PP5 and Sgt1 in the presence of ATP (Fig. 3). ADP also exerted a minor effect on enhancement of the interactions. Therefore, the ATP-dependent interactions with PP5 and Sgt1 are the conserved properties shared by Chp-1 and melusin.

Chp-1 and Melusin Can Form Homo- and Heterocomplexes in a Nucleotide-dependent Manner—Next, we tested the possibility of interaction between the CHORD-containing proteins. GST pull-down experiments showed interaction of His-Chp-1 with GST-melusin as well as GST-Chp-1 in the presence of ADP or ATP (Fig. 4A). As in the other interactions between cochaperones, ATP exerted a higher enhancement of the interaction compared with ADP. Moreover, His-melusin also exhibited a nucleotide-dependent interaction with GST-melusin (Fig. 4B). These results suggest that Chp-1 and melusin can form homo- or heterocomplexes in a nucleotide-dependent manner.

His-186 of Chp-1 Is Involved in the Specific Binding of ADP or ATP to Chp-1—The crystal structure of the complex of the Rar1 CHORD-II, Sgt1 CS, and Hsp90 N-terminal domains shows that the imidazole ring of His-188 of Rar1 CHORD-II interacts directly with the β -phosphate of ADP that is bound to the Hsp90 N-terminal domain (15). Because this residue is conserved in Chp-1 as His-186 (Fig. 5A), we tested whether the H186A mutation could affect the nucleotide binding to Chp-1. Chp-1(H186A) had diminished affinity for ADP-agarose compared with wild-type Chp-1 (Fig. 5B). This result indicates that, like His-188 of Rar1, His-186 of Chp-1 contributes to the nucleotide binding property of Chp-1. Accordingly, the Chp-1(H186A) mutant showed a weaker nucleotide-dependent interaction with Hsp90 compared with wild-type Chp-1 (Fig. 5C). Unlike the interactions of Chp-1 with PP5 and Sgt1, which were stimulated mainly by ATP, the Chp-1/Hsp90 interaction was enhanced more by ADP than by ATP. Such ADP-dependent interactions of Hsp90 with Rar1 and Chp-1 have been demonstrated previously (15, 37).

The reduction in the ADP-agarose binding affinity of Chp-1(H186A) also suggests that His-186 is engaged in the binding to free ADP or ATP even in the absence of Hsp90. Therefore, we investigated whether the mutation of His-186 could also affect the nucleotide-dependent binding of Chp-1 to Sgt1. As shown in Fig. 5D, the nucleotide-dependent interaction with Sgt1 was greatly reduced in Chp-1(H186A) compared with wild-type Chp-1. These results suggest that conformational changes induced by ATP binding to His-186 might enhance the interaction of Chp-1 with Sgt1.

Ca²⁺ Further Enhances Nucleotide-dependent Melusin/Hsp90 and Melusin/Sgt1 Interactions—It is known that Ca²⁺ can bind to the C-terminal acidic domain of melusin, negatively regulating its interaction with the cytoplasmic domain of β -integrin *in vitro* (21). Therefore, we examined the effect of Ca²⁺ on the nucleotide-dependent interactions of melusin with Hsp90, Sgt1, and PP5. Unlike the interaction between Chp-1 and Hsp90, ADP and ATP exerted similar levels of binding enhancement between melusin and Hsp90. The addition of

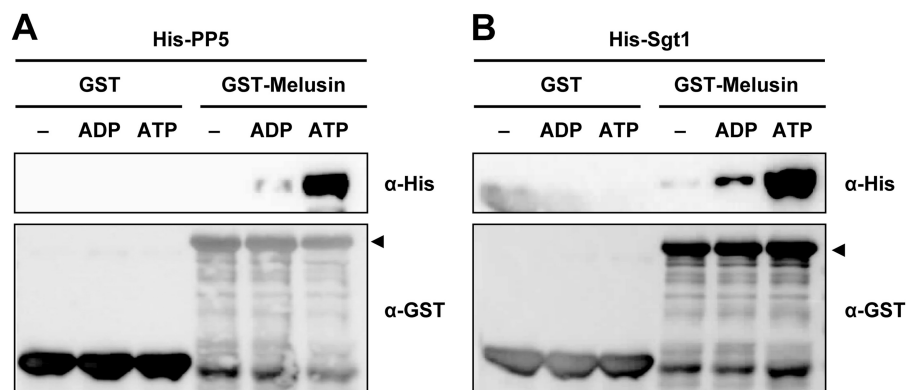


FIGURE 3. Nucleotide-dependent interactions between melusin and other Hsp90 cochaperones. GST-melusin was incubated with the His-tagged Hsp90 cochaperones PP5 (A) and Sgt1 (B) with or without 5 mM ADP or ATP. The pull-down fractions were analyzed by Western blotting.

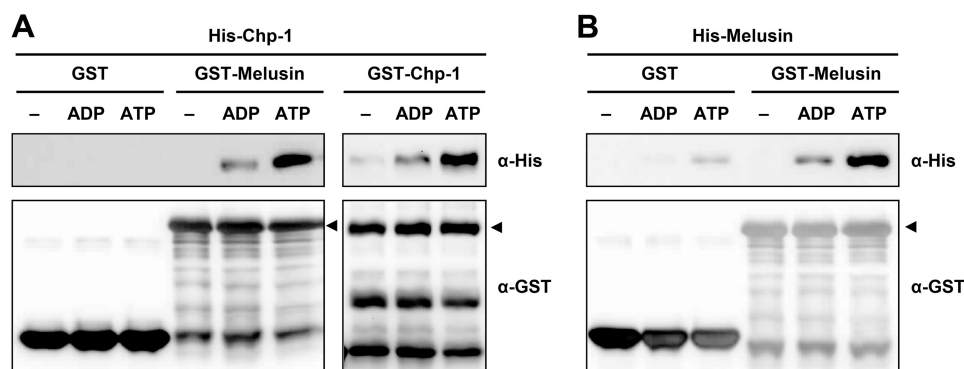


FIGURE 4. Nucleotide-dependent homo- and heterocomplex formation by Chp-1 and melusin. A, His-Chp-1 was incubated with GST, GST-melusin, or GST-Chp-1 with or without 5 mM ADP or ATP. B, His-melusin was incubated with GST or GST-melusin with or without 5 mM ADP or ATP. The pull-down fractions were analyzed by Western blotting.

Ca²⁺ further enhanced the nucleotide-dependent interaction between melusin and Hsp90, especially in the presence of ADP, resulting in a nucleotide preference similar to that of the Chp-1/Hsp90 interaction (Fig. 6A). This enhancement of the interaction was dependent on the concentration of Ca²⁺ to some extent (Fig. 6D). In contrast, Chp-1, which does not contain the C-terminal Ca²⁺-binding acidic domain, showed no enhancement of the interaction upon the addition of increasing amounts of Ca²⁺ in the presence of ADP (Fig. 6D).

In addition, the ATP-dependent interaction between melusin and Sgt1 was further enhanced in the presence of Ca²⁺ in a concentration-dependent manner (Fig. 6, B and D). However, the addition of Ca²⁺ did not affect the interaction between melusin and PP5 (Fig. 6C). These results imply that melusin undergoes additional conformational change upon Ca²⁺ binding, which makes melusin more capable of interacting with Hsp90 and Sgt1.

DISCUSSION

Hsp90 cochaperones work as modulators of the Hsp90 chaperone machinery through dynamic association with the Hsp90 homodimer during its ATPase cycle. Various Hsp90 cochaperones are involved in multiple cellular functions, some of which are suggested to be Hsp90-independent (7).

In this study, we have demonstrated novel nucleotide binding properties of Hsp90 cochaperones Chp-1 and PP5. Interestingly, interactions of Chp-1 with other cochaperones such as PP5 and Sgt1 were greatly enhanced by ATP and, to a lesser

extent, by ADP. Such nucleotide-dependent interactions with PP5 and Sgt1 were also conserved for melusin, another CHORD-containing protein in mammals. Furthermore, Chp-1 and melusin could form homo- and heterocomplexes by interacting in the same nucleotide-dependent manner. Therefore, conformational changes induced by ATP binding might play important roles in regulating the interactions among these Hsp90 cochaperones.

The structural determination of the complex between plant Rar1 CHORD-II and the Hsp90 N-terminal domain has revealed that Rar1 directly binds to ADP bound in the nucleotide pocket of Hsp90 (15). CHORD-I is also predicted to bind to Hsp90 through the same pattern of interaction, which allows binding of a single Rar1 molecule to both N-terminal binding sites of an Hsp90 dimer (15). His-188 in CHORD-II of Rar1, engaged in binding to the β -phosphate of ADP, is also conserved in Chp-1 and melusin (Fig. 5A). We demonstrated that His-186 of Chp-1, equivalent to His-188 of Rar1, plays an important role in the nucleotide-dependent binding of Chp-1 to Hsp90. For enhancement of the Chp-1/Hsp90 interaction, ADP played a more prominent role than ATP, consistent with a previous report (37). Such a nucleotide preference could possibly be related to the differences in the ADP- and ATP-bound conformations of Hsp90. A steric overlap between CHORD and the closed conformation of ATP-bound Hsp90 is observed when the complex of Rar1 CHORD-II and the Hsp90 N-terminal domain is superimposed on the structure of ATP-bound

Nucleotide-dependent Interactions of Hsp90 Cochaperones

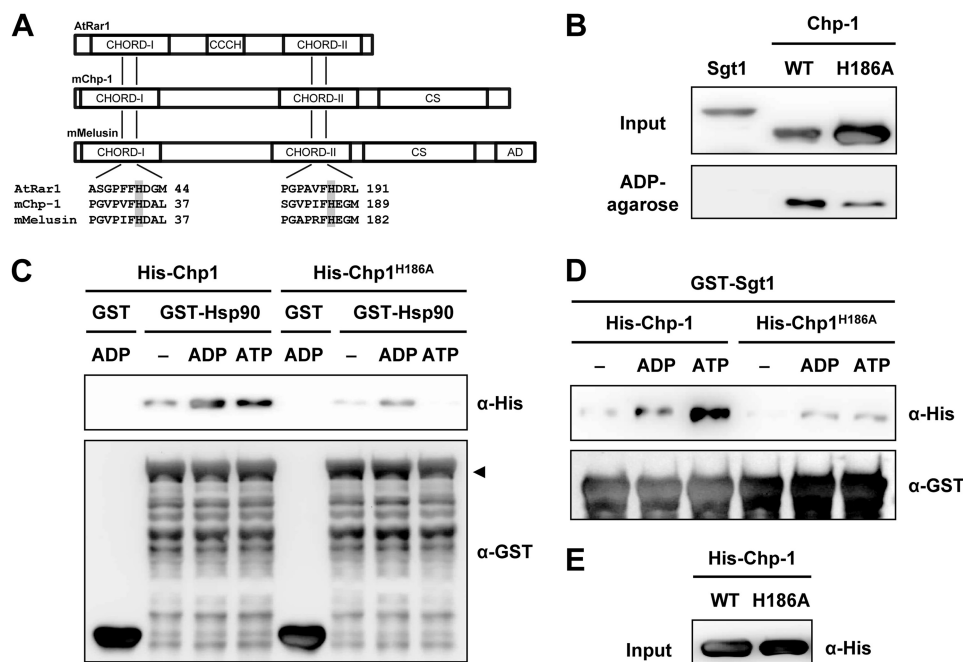


FIGURE 5. Contribution of nucleotide binding property of Chp-1 to Chp-1/Hsp90 and Chp-1/Sgt1 interactions. *A*, schematic diagram of the structures of the CHORD-containing proteins *Arabidopsis thaliana* (*At*) Rar1, mouse (*m*) Chp-1, and mouse melusin. The conserved His residues involved in nucleotide binding in *A. thaliana* Rar1 are shaded. AD, acidic domain. *B*, His-Sgt1, His-Chp-1, and His-Chp-1(H186A) were incubated with N^6 -ADP-agarose, and the bound proteins were eluted and detected by Western blotting. His-Chp-1 and His-Chp-1(H186A) were incubated with GST-Hsp90 (C) or GST-Sgt1 (D) with or without 5 mM ADP or ATP. The pull-down fractions were analyzed by Western blotting. *E*, the amounts of His-Chp-1 and His-Chp-1(H186A) used for the GST pull-down experiments shown in C and D were analyzed by Western blotting.

full-length Hsp90 (15). Different preferences for different Hsp90 conformations are also known for other Hsp90 cochaperones, leading to sequential exchange of the cochaperones in the Hsp90 chaperone complex during progression of the Hsp90 cycle.

Remarkably, Chp-1(H186A) showed a reduced nucleotide-dependent interaction not only with Hsp90 but also with Sgt1. In the plant Hsp90-Rar1-Sgt1 ternary complex, the opposite faces of Rar1 CHORD-II bind simultaneously to Hsp90 and the Sgt1 CS domain (15). However, the residues involved in the CHORD-II/CS domain interaction are not conserved in Chp-1, melusin, and mammalian Sgt1. Therefore, it is not clear yet how Chp-1 or melusin interacts with Sgt1. However, if Chp-1 does not interact with both Hsp90 and Sgt1 through the same interacting surface where ADP or ATP acts as a bridge, the conformational changes in Chp-1 induced by nucleotide binding to His-186 might be responsible for the nucleotide-dependent interaction between Chp-1 and Sgt1. The fact that Chp-1(H186A) showed reduced binding affinity for ADP-agarose also supports the notion that His-186 is one of the residues involved in direct nucleotide binding even in the absence of Hsp90. Considering the results together, although the same nucleotide-binding sites in Chp-1 are involved in the regulation of Chp-1/Hsp90 and Chp-1/Sgt1 interactions, the mode of regulations might be quite different for these two interactions.

We have shown that Chp-1 and melusin share similar nucleotide dependence and preference for interactions with other cochaperones. On the other hand, unlike Chp-1, melusin did not show a clear preference of ADP in binding to Hsp90. Interestingly, we found that Ca^{2+} not only enhanced the melusin/Hsp90 interaction but also allowed melusin to acquire the ADP

preference for Hsp90 binding. Ca^{2+} also enhanced the nucleotide-dependent melusin/Sgt1 interaction. This is opposite the interaction of melusin with β -integrin, which is diminished by Ca^{2+} (21). These results imply that nucleotides and Ca^{2+} act cooperatively in the regulation of melusin to be released from β -integrin and to bind Hsp90 or other cochaperones.

Several interactions between Hsp90 cochaperones have been shown to be crucial for the regulation of cellular functions. In yeast, the dimerization of Sgt1 has been demonstrated to be important for Sgt1/Skp1 binding and kinetochore assembly (38). In addition, the yeast Pih1/Tah1 cochaperone heterodimer plays a role in the recruitment of client proteins such as core ribonucleoproteins to Hsp90 (39). The interaction of plant Rar1 and Sgt1 in the Hsp90 chaperone complex is well known to be involved in plant resistance (9–11). The newly identified interactions between cochaperones might be part of the regulatory mechanisms to assemble the Hsp90 chaperone machinery, as in the case of the plant Rar1/Sgt1 interaction. The Chp-1/Sgt1 interaction can be expected to be a mammalian equivalent of the Rar1/Sgt1 interaction, although the role for Chp-1 in innate immunity needs to be clarified (16). The three CHORD-containing proteins Rar1, Chp-1, and melusin seem to interact with Sgt1, but, except for the Rar1/Sgt1 interaction, the physiological functions of these interactions are still elusive. In the case of PP5, some of its functions are mediated by its interaction with Hsp90 (32, 33, 36, 40), but it has not yet been clarified whether PP5 dephosphorylates all of its substrates as an Hsp90 cochaperone or as an Hsp90-independent phosphatase. Although the Hsp90-independent roles of the cochaperone/cochaperone interactions are largely unknown, we cannot rule out the possibility that these cochaperones could have their

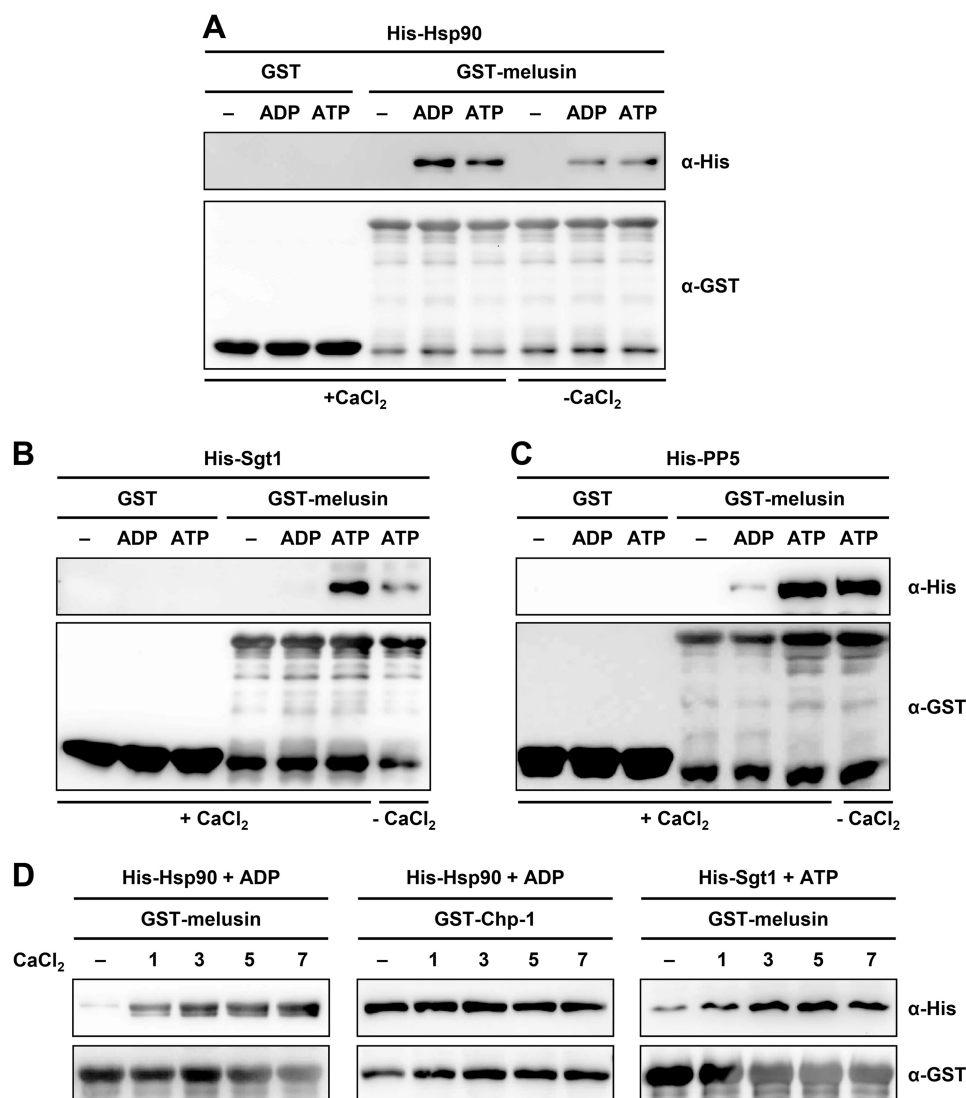


FIGURE 6. Effect of Ca²⁺ on interaction of CHORD proteins with Hsp90 or Hsp90 cochaperones. A, GST pull-down assay was performed with GST-melusin and His-Hsp90 with or without 1 mM Ca²⁺ in the presence of 5 mM ADP or ATP. GST-melusin was incubated with His-Sgt1 (B) or His-PP5 (C) with or without 3 mM Ca²⁺ in the presence of 5 mM ADP or ATP. The GST pull-down fractions were analyzed by Western blotting. D, GST-tagged melusin (left) or Chp-1 (middle) was incubated with His-tagged Hsp90 in the presence of 5 mM ADP, and GST-melusin was incubated with His-Sgt1 in the presence of 5 mM ATP (right) with increasing concentrations of Ca²⁺. The GST pull-down fractions were analyzed by Western blotting.

own Hsp90-independent roles, which can be regulated by nucleotide-dependent interactions with other cochaperones demonstrated in our work. In fact, both Chp-1 and melusin possess their own chaperone activities (5, 8), implying their potential Hsp90-independent functions *in vivo*.

In this study, we have demonstrated interactions among cochaperones that are dependent mainly on ATP. Therefore, cellular ATP concentration, which can fluctuate depending on cellular energy status and the cell cycle (41), physiological conditions affecting mitochondrial function (42), and stresses such as metabolic or oxidative stress (43), could possibly influence the cochaperone/cochaperone interactions and the biological processes regulated by the cochaperones. In addition, cellular Ca²⁺ levels might play important roles in the regulation of melusin. Although the biological functions regulated by these interactions remain to be elucidated, this study will serve as a first step to investigate novel regulatory mechanisms for dynamic assembly of the Hsp90 chaperone machinery and to

explore potential Hsp90-independent functions of these cochaperones.

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