J. Biochem. 126, 1025-1032 (1999)

Identification of Heat Shock Protein 90-Associated 84-kDa Phosphoprotein[1]

Hong Ro Kim, Kwang Il Kang, Ho Sung Kang,[2] and Han Do Kim

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea

Received July 6, 1999; accepted September 20, 1999

In eukaryotic cells, HSP90 is associated with several protein kinases and regulates their activities. HSP90 was also reported to possess an autophosphorylase activity. In this study, we examined *in vitro* autophosphorylation of HSP90, which was purified from chick muscle. We show that HSP90 was not phosphorylated *in vitro*, but an 84-kDa protein (p84) was highly phosphorylated. P84 was neither HSP90 nor its degradative product, as it was not detected by an antibody (BF4) specific to HSP90 in denaturing immunoprecipitation and Western blot analysis. Phosphorylation of a protein similar to p84 was also detected with purified human brain and HeLa HSP90, indicating that p84 is present in many different types of cells. P84 appeared to exist as large complexes, as determined by HPLC and native gel electrophoresis. Native immunoprecipitation using anti-HSP90 (BF4)-conjugated Affi-gel revealed that this phosphoprotein is specifically associated with HSP90. The interaction of p84 and HSP90 was not affected by p84 phosphorylation. In addition, p84 phosphorylation was prevented by the presence of divalent cations such as Mg^{2+} and Mn^{2+}. In contrast, p84 phosphorylation was significantly activated by addition of exogenous Ca^{2+} between 100 and 500 μM, although it was blocked by higher concentrations (>1 mM) of Ca^{2+}. HSP90, but not p84, could be phosphorylated by casein kinase II. Finally, p84 phosphorylation was specifically prevented by hemin, but not by other kinase inhibitors, indicating that p84 phosphorylation may be regulated by heme-regulated protein kinase.

Key words: HRI, HSP90, p84, phosphorylation.

Heat shock protein 90 kDa (HSP90) is ubiquitously present in prokaryotic and eukaryotic cells. HSP90 is an abundant cytosolic protein in normal cells and further synthesized in stressful conditions such as exposure to elevated temperatures. HSP90 has been suggested to play an important role(s) in both stressed and normal cells. In normal cells, HSP90 is found in association with a number of regulatory proteins including steroid receptors (*1*), retinoid receptor (*2*), helix-loop-helix transcriptional factor such as MyoD (*3*), HSF (*4*), p53 (*5*), cytoskeletal proteins (*6, 7*), calmodulin (*8*), and $\beta\gamma$ subunit of G proteins (*9*). HSP90 also forms complexes with several protein kinases, including casein kinase II (*10*), double-stranded DNA-activated protein kinase (*11*), heme-regulated eIF-2α kinase (HRI) (*12*), retroviral transforming viral protein pp60$^{v\text{-}src}$ (*13*), CDK4 (*14*), and Wee1 (*15*). HSP90 has been suggested to play a role(s) in the regulation of activities of these target proteins to stabilize and maintain the proteins in an inactive, partially unfolded, or unassembled state. Despite this information, the exact function of HSP90 is incompletely understood.

Of HSP90-associated kinases, HRI was originally found in reticulocytes and has been subsequently identified in other types of cells such as murine erythroleukemia (MEL) cell, K562 and HeLa cell (*16, 17*). HRI is a serine/threonine kinase and is implicated in the translational regulation in eukaryotic cells by phosphorylating α subunit of eIF-2 at serine-51 (*18, 19*). Phosphorylation of eIF-2α by HRI results in the accumulation of inactive eIF-2·GDP complex, thus inhibiting the formation of the eIF-2α GTP·Met-tRNA$_f$ ternary complex and arresting initiation of protein synthesis (*18, 19*). HRI can be phosphorylated and activated by casein kinase II (*18, 19*). In addition, HRI phosphorylation and activation is regulated by hemin, sulfhydryl oxidant, and a wide variety of stressful stimuli, including exposure to heat shock, oxidative stress, and glucose starvation (*12, 18, 19*). The enzymatic activity of HRI is also regulated by HSP90 (*20, 21*).

In cells, HSP90 exists as both phosphorylated and nonphosphorylated forms. HSP90 can be phosphorylated by casein kinase II that is associated with HSP90 (*10*).

[1] This work was supported by the Basic Science Research Institute Program (BSRI-97-4409), Ministry of Education, and a Grant (94-0401-10-01-3) from the Korea Science and Engineering Foundation, Korea.

[2] To whom correspondence should be addressed: Tel: +82-51-510-2275, Fax: +82-51-513-9258, E-mail: hspkang@hyowon.cc.pusan.ac.kr

Abbreviations: HSP90, heat shock protein 90 kDa; PBS, phosphate-buffered saline; LSB, Laemmli sample buffer; HRI, heme-regulated eIF-2α kinase; DBR, 5,6-dichlorobenzimidazole riboside; chelerythrine chloride, 1,2-dimethoxy-*N*-methyl[1,3]benzodioxolo[5,6-*c*]-phenanthridinium chloride; H-7, 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine; Genistein, 4′,5,7-trihydroxyisoflavone; tyrphostin A1, [4-methoxybenzylidene]malononitrile; tyrphostin A25, [3,4,5-trihydroxybenzylidene]malononitrile; tyrphostin A63, (4-hydroxybenzyl)malononitrile.

Recently, it was demonstrated that HSP90 possesses an ATP-binding site and ATPase activity, like such heat shock proteins as HSP70 and HSP60 (*4, 22-24*). In addition, HSP90 is known to possess an autophosphorylase activity (*23*). HSP90 undergoes changes in secondary and tertiary structures in the presence of ATP (*25-27*). The conformational change of HSP90 may be important in the regulation of functions of its associated proteins (*22-29*).

In this study, to examine the autophosphorylase activity of HSP90, we have purified HSP90 from chick muscle by conventional column chromatography (DEAE, hydroxylapatite, and Sephacryl S-300) and heparin-affinity chromatography (*30, 31*). Here we show that HSP90 is not autophosphorylated, but an 84-kDa protein (p84) that is specifically associated with HSP90 is highly phosphorylated. Phosphorylation of a protein similar to p84 was also detected with purified human brain and HeLa HSP90. We further show that the phosphorylation of p84 is regulated by divalent cations such as by Ca^{2+}, Mg^{2+}, and Mn^{2+}. Finally, p84 phosphorylation was specifically prevented by hemin, but not by other kinase inhibitors, indicating that p84 phosphorylation may be regulated by heme-regulated protein kinase.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (>185 TBq/mmol) and Western blotting detection system were purchased from Amersham. DEAE-Sepharose CL-6B and Sephacryl S-300 HR were from Pharmacia LKB Biotechnology (Sweden). Bio-Gel HT hydroxylapatite resin, Affi-Gel Hz immunoaffinity kit and all other chromatography media and apparatus were obtained from Bio-Rad. Protein Pak 300SW column and HPLC system were from Millipore. Sepharose CL-4B, heparin-agarose (type I), chelerythrine chloride, ovalbumin, BSA, DBR, hemin, H-7, tyrphostin A1, and tyrphostin A63 were purchased from Sigma. Geldanamycin, calphostin C, staurosporine, genistein, herbimycin A, tyrphostin A25, and wortmannin were obtained purchased from Calbiochem. Purified human brain and HeLa HSP90 were from StressGen. Monoclonal anti-HSP90 antibody (BF4) and purified casein kinase II were kindly provided by Dr. Etienne-Emile Baulieu (Lab. of Hormones, 94276 Le Kremlin-Bicetre Cedex, France) and Dr. Young-Seuk Bae (Kyungpook National University, Korea), respectively.

Purification of HSP90—HSP90 was purified as described previously by Welch *et al.* (1982) and Yonezawa *et al.* (1988). Chicken skeletal muscle was excised, and about 200 g of muscle was homogenized on ice with a blender in 5 volumes of buffer BT (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 0.5% Triton X-100) containing 0.1 M KCl, 1 μM aprotinin, 1 μM leupeptin, 1.5 μM pepstatin A, and 1 mM PMSF. The homogenate was passed through a layer of cheesecloth. The filtered mixture was centrifuged at 10,000×g for 20 min, and the supernatant was applied to a DEAE Sepharose CL-6B (2.5×10 cm) that had been preequilibrated in buffer BT. After washing the column with buffer BT, the adsorbed proteins were eluted by a linear gradient of 20 to 300 mM NaCl in Buffer BT. Fractions containing HSP90 were applied to a hydroxylapatite column (2.5×10 cm) equilibrated with buffer CT (20 mM K_2HPO_4, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, 3 mM β-mercaptoethanol, 0.1% Triton X-100). After washing the column with buffer CT, the proteins were eluted by a linear gradient of 20 to 300 mM K_2HPO_4 in Buffer CT. Fractions containing HSP90 were pooled and concentrated to 5 ml with Amicon YM30. The concentrated sample was applied to gel filtration through a Sephacryl S-300 column (1×100 cm) equilibrated with buffer B, then fractions containing HSP90 were pooled. To eliminate the activity of casein kinase II, the pooled sample was incubated with heparin-agarose gel (type I) equilibrated with buffer B containing 90 mM KCl at 4°C for 2 h. After spinning down, the supernatant was stored at −70°C.

HPLC Analysis—HPLC analysis was performed by using Protein-Pak 300SW column (0.75×30 cm, Millipore) equilibrated with buffer M (50 mM Mes-NaOH, pH 7.0, 5 mM β-mercaptoethanol). The flow rate was 1 ml/min and 0.25-ml fractions was collected. The column was calibrated with the following proteins (Sigma): carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa). Excluded volume (V_0) and total volume (V_t) values were determined by the elution peak of blue dextran (Sigma). Standard curves were plotted according to the relation of the logarithm of molecular weight to elution volume (V_e) of the protein being studied or the logarithm of molecular weight to the distribution coefficient K_D, which is defined as the ratio $(V_e - V_0)/(V_t - V_0)$.

In Vitro Phosphorylation of Purified HSP90 Preparation—*In vitro* phosphorylation of HSP90 preparation was carried out at 37°C for 1 h in 40 μl of buffer M containing 4 μg HSP90 and 2 μM [γ-^{32}P]ATP (>185 TBq/mmol, >5,000 Ci/mmol) (*23, 32*). In some experiments, divalent cations and protein kinase inhibitors were added in the reaction mixture. The reaction was stopped by boiling for 10 min in 1×LSB (40 mM Tris, pH 6.8, 7.5% glycerol, 1% SDS, 2.5% β-mercaptoethanol, and 0.006% bromophenol blue), and the proteins were analyzed by SDS-PAGE and autoradiography (*33*). Quantitation of phosphorylated bands was carried out using a Molecular Dynamics Phosphorimager (Molecular Dynamics) and Imagequant software.

SDS-PAGE and Two-Dimensional Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% polyacrylamide gel as described by Laemmli (*33*). To prepare samples for SDS-PAGE, the proteins in 1×LSB were heated at 100°C for 10 min and quantified by the Bradford method (Bio-Rad). After electrophoresis, the gels were stained with Coomassie Brilliant Blue. Molecular weight calibration of protein markers (Sigma) was performed using SigmaPlot (Jandel).

For two-dimensional gel electrophoresis, the proteins were solubilized in native electrophoresis sample buffer (40 mM Tris, pH 6.8, 7.5% glycerol, 2.5% β-mercaptoethanol, and 0.006% bromophenol blue) and applied 7.5% native slab gels without SDS, then electrophoresed in the first dimension at 4°C. The native gel was treated with LSB, laid onto SDS-polyacrylamide gel, and electrophoresed. Gels were then stained with Coomassie Brilliant Blue and analyzed by autoradiography.

Preparation of Anti-HSP90 Coupled Affi-Gel—Prior to

coupling to Affi-Gel Hz gel, antibody solution was desalted by using Econo-Pac 10DG column (Bio-Rad). Anti-HSP90 antibody (BF4) was oxidized with $NaIO_4$ in the dark. The mixture of 4 ml of BF4 (1-5 mg/ml) and 400 μl of 20 mg/ml $NaIO_4$ was incubated at room temperature for 1 h, then $NaIO_4$ was completely removed by using a desalting column. Antibody-coupled gel slurry was washed with PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.0, 100 mM NaCl) containing 500 mM NaCl, then adjusted to buffer M. The antibody-coupled gels were stored at 4°C until immunoprecipitation.

Immunoprecipitation—For native immunoprecipitation, purified HSP90 preparation (4 μg) was precleared in 30 μl of Sepharose CL-4B [25% (w/v)] in buffer M at 4°C for 1 h. The precleared supernatants were mixed with either rat preimmune serum-coupled protein G or anti-HSP90 Affi-Gel (40 μl) in buffer M and incubated at 4°C for 2 h. Immunocomplexes and supernatants were then separated by centrifugation, and the immunocomplexes were washed five times with 400 μl of buffer M. The immunocomplexes and supernatants were phosphorylated with [γ-^{32}P]ATP at 37°C for 1 h as described above. Adsorbed HSP90 and associated proteins were eluted by boiling in 1×LSB at 100°C for 10 min, then analyzed by SDS-PAGE and autoradiography.

In some cases, purified HSP90 preparation was first *in vitro* phosphorylated, then native and denaturing immunoprecipitation were carried out with either protein G, rat preimmune serum-coupled protein G, or anti-HSP90 (BF4) conjugated Affi-Gel as described above. In denaturing immunoprecipitation, phosphorylated HSP90 preparation was boiled in 2×LSB at 100°C for 10 min, adjusted to 0.1% SDS, then immunoprecipitated. The immunoprecipitates were washed five times with either buffer M. The resulting pellets were analyzed by SDS-PAGE and fluorography.

Western Blot Analysis—Immunoblotting was carried by transferring proteins from SDS-acrylamide gels onto nitrocellulose filter paper. The sheet was incubated with 3% bovine serum albumin (BSA) in PBS at 37°C for 1 h. After washing three times with PBS, the sheet was incubated with anti-HSP90 antibody (BF4) at room temperature for 2 h. The sheet was washed and incubated at room temperature for 2 h in the same buffer containing peroxidase-conjugated rabbit anti-rat IgG antibody (Sigma). After washing, the protein bands were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham).

RESULTS AND DISCUSSION

An 84-kDa Protein (p84), but Not HSP90, Is Highly Phosphorylated In Vitro—Both HSP90 and GRP94, an ER-resident HSP90 homologue, were reported to possess an autophosphorylating activity (*23, 32*). To examine *in vitro* autophosphorylation of HSP90, HSP90 was purified from chick muscle by sequential conventional chromatography including DEAE-Sepharose CL-6B ion exchange, hydroxylapatite, and Sephacryl S-300 gel filtration as described previously (*30, 31*). Recently, it was reported that HSP90 could be copurified with and phosphorylated by casein kinase II (*10, 34, 35*). Thus, to eliminate the trace activity of casein kinase II from HSP90 preparation, we further purified HSP90 by heparin column as described previously (*34*). As shown in Fig. 1, the purified HSP90 was seen as a single band in Coomassie-stained gels, and the overall purity of this preparation was over 95% (panel A, lane 1). *In vitro* phosphorylation of purified HSP90 from chick muscle was carried out in 40 μl of buffer M containing 2 μM [γ-^{32}P]ATP at 37°C for 1 h. Under these conditions, HSP90 was not phosphorylated, but a polypeptide (p84) that has an apparent molecular weight of 84 kDa was intensively phosphorylated (panel A, lane 2). This 84-kDa protein appeared to be neither HSP90 itself nor its breakdown fragment, since it migrated faster than HSP90 on SDS-polyacrylamide gels (*i.e.*, HSP90 was marked with radioactive ink, and p84 was positioned below the radioactive mark) and was not detected by anti-HSP90 antibody (BF4) in Western blot analysis (data not shown). The phosphorylation of p84 appeared to increase continuously until 3 h in a time-dependent manner by a saturatable enzymatic reaction (data not shown). In addition, *in vitro* phosphorylation with HSP90 from HeLa cells purchased from StressGen also showed the phosphorylation of a similar protein to p84 (panel B). A similar observation was made with human brain HSP90 purchased from StressGen (data not shown). Thus, p84 is likely to be ubiquitously present in various types of cells. Although this p84 was highly phosphorylated, it was co-purified with HSP90 in a very small amount and thus could be detected by silver staining (Fig. 1C).

To test whether HSP90 could be *in vitro* phosphorylated, full-length cDNA of chicken HSP90α, cloned into the pBlueBacHisC transfer vector (Invitrogen), was cotransfected with linearized baculovirus DNA into Sf9 insect cells, and HSP90 was purified by BF4-conjugated affinity chromatography. The purified HSP90 was not phosphorylated and the phosphorylation of p84 was not detected (data not shown). These results suggest that HSP90 itself may be not phosphorylated.

P84 Is Specifically Associated with HSP90—To determine whether p84 could be separated from HSP90 preparation, HPLC was carried out using a Protein-Pak 300SW column, and the HPLC fractions were analyzed for *in vitro* phosphorylation (Fig. 2). However, p84 could not be separated from HSP90 preparation by HPLC. As shown in Fig. 2C, the peak of p84 phosphorylation overlapped the elution profile of HSP90. Both HSP90 and p84 were eluted as a major peak at about 780 kDa, and two minor peaks of approximately 320-340 kDa and 100 kDa in size, respectively. The second peak migrating at about 320-340 kDa is presumed to be close to the predicted molecular weight for such a trimer or tetramer. The third peak eluted with an apparent molecular mass of 100 kDa may be monomeric form of either HSP90 or p84. Our estimated native size of HSP90 is different from that of HSP90 reported to exist predominantly as a dimeric form with rod-like geometry (*31*). This discrepancy may be due to the exclusion of nonionic detergents such as Triton X-100 in the buffer for HPLC. Analysis of the densitometric data in Fig. 2C revealed that HSP90 and p84 exist as oligomeric (including trimeric/tetrameric) and monomeric forms in the ratio of 12:1 and 3.3:1, respectively. Thus, most of HSP90 (>92%) appeared to exist as large complexes with other proteins. A significant amount (75-80%) of p84 is likely to be present in complexes with other proteins, although the monomeric

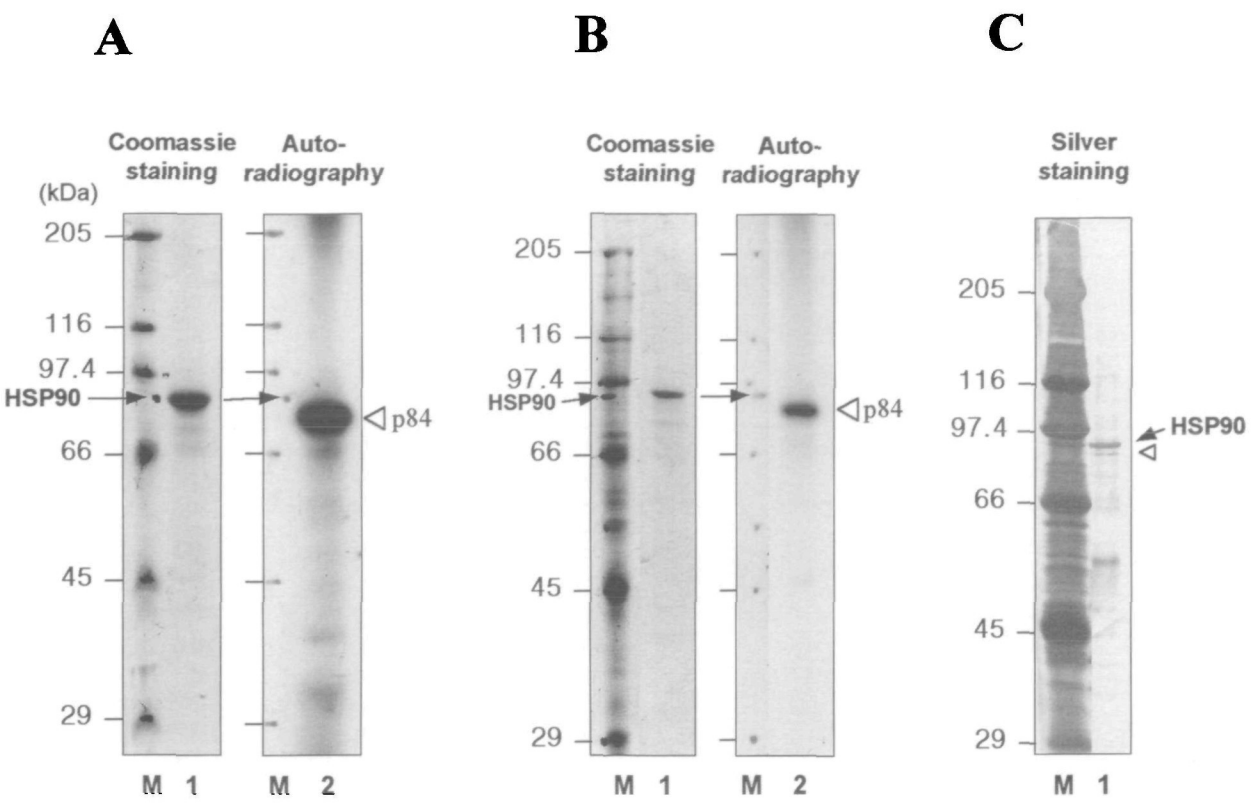

Fig. 1. ***In vitro* phosphorylation of purified chick and human HeLa HSP90.** (A and B) Purified chick and human HeLa HSP90 (4 μg) were phosphorylated *in vitro* in 40 μl of buffer M containing 2 μM [γ-^{32}P]ATP at 37°C for 1 h. After reaction, the proteins were analyzed with 10% SDS-PAGE gel/Coomassie Brilliant Blue staining (lane 1) and autoradiography (lane 2). A, chick HSP90; B, human HeLa HSP90. (C) Purified chick HSP90 was analyzed by SDS-PAGE and silver staining. Size markers are shown in lane M (carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; BSA, 66 kDa; phosphorylase *b*, 97.4 kDa; β-galactosidase, 116 kDa; rabbit myosin, 205 kDa). Note that the position of HSP90 is marked with radioactive ink and that of p84 is below the radioactive mark.

p84 was also detected. These results suggest that p84 may be physically associated with HSP90, although it is not feasible to determine the exact stoichiometry of the interaction between HSP90 and p84 since we could detect only the phosphorylated p84, not p84 itself. It is noteworthy that the monomeric p84 could be phosphorylated *in vitro* similar to the oligomeric p84.

To examine whether p84 exists as large complexes with HSP90, native gel electrophoresis was carried out (Fig. 3). The purified HSP90 was *in vitro* phosphorylated, then separated in the first dimension by native gel electrophoresis in the absence of SDS. The native electrophoresed gels were treated with 1×LSB and laid onto 10% SDS-polyacrylamide gel, and the gel was electrophoresed. HSP90 complex was separated into three distinct bands with apparent molecular masses of 700, 300, and 100 kDa (Fig. 3, arrowheads). The native size of p84 was similar to that of HSP90. These results are in consistent with our HPLC data, confirming that p84 exists as large complexes with HSP90 or other proteins. Many other reports have revealed that HSP90 interacts with other proteins including several steroid receptors, kinases, cytoskeleton and other families of heat shock proteins *in vivo* and *in vitro* (*1-15*). Since p84 was copurified with HSP90, p84 might be physically associated with HSP90.

To examine the possible specific interaction of HSP90 with p84, HSP90 was immunoprecipitated using anti-HSP90 antibody under native conditions and the resulting immunoprecipitates and supernatants were *in vitro* phosphorylated (Fig. 4A). In the immunoprecipitation with BF4, the phosphorylated p84 was detected along with HSP90 in the immunoprecipitates (panel A, lane 6), but not in the supernatants or the first wash-out (lanes 5 and 7). In the immunoprecipitation experiment using pre-adsorbed beads with rat preimmune serum, the phosphorylation of p84 was detected in the supernatant and the first wash-out (lanes 2 and 4), but not in the immunoprecipitates (lane 3). These results suggest that the phosphorylated p84 may be specifically associated with HSP90. It is noteworthy that in the immunoprecipitation using preimmune serum, the phosphorylation of p84 was observed to a similar extent in the first wash-out as well as in the supernatants, while most of HSP90 was found in the supernatants. This may be due to a difference in non-specific binding affinity to beads between free p84 and complexes of HSP90 and other proteins including p84.

As shown in Fig. 2, p84, which exists as a monomeric form, could be phosphorylated in the same way as HSP90-associated p84. However, we could not detect the phosphorylation of p84 as a monomeric form in the supernatants or in the first wash-out of native immunoprecipitation with BF4 (Fig. 4A, lanes 5 and 7). These results suggest that p84 may be phosphorylated only in association with HSP90 or in the presence of HSP90.

Interaction of p84 and HSP90 Is not Altered by p84 Phosphorylation—The phosphorylation reaction plays a

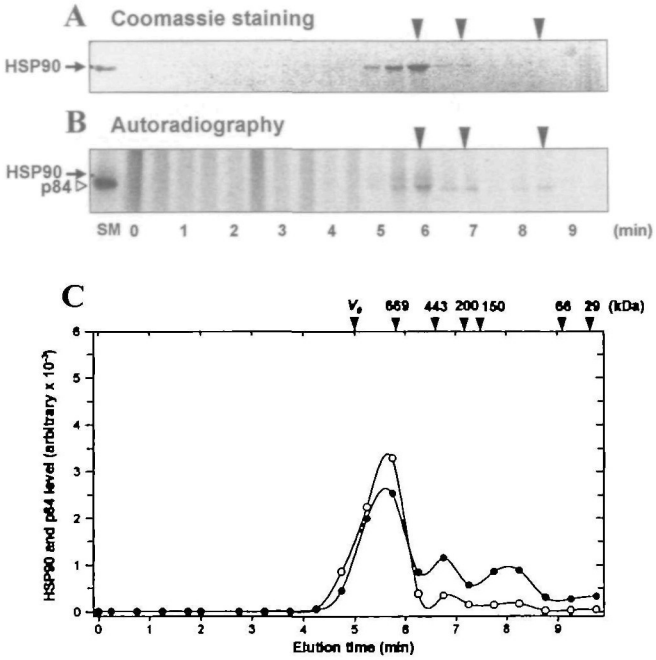

Fig. 2. **High-performance size-exclusion chromatography (HPLC) of HSP90 and p84.** HSP90 was purified on a Protein-Pak SW-300 column (Millipore) equilibrated with degassed buffer M. The flow rate was 1 ml/min and proteins were detected at 280 nm. Every second fraction was phosphorylated, as described in Fig. 1. The phosphorylated proteins were analyzed by SDS-PAGE/Coomassie Brilliant Blue staining (A) and autoradiography (B). Lane SM, the starting material for HPLC. Protein amounts of HSP90 (○) and radioactivities of the phosphorylated p84 (●) in each fraction were plotted as a function of elution time (C). The elution time of HPLC and HPLC size markers are shown at the down and top of the plot, respectively. Molecular masses used are as follows: carbonic anhydrase, 29 kDa; BSA, 66 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 669 kDa. The void volume (V_0) for the column was 4.86 ml, determined by the exclusion of the initial peak of blue dextran (Sigma).

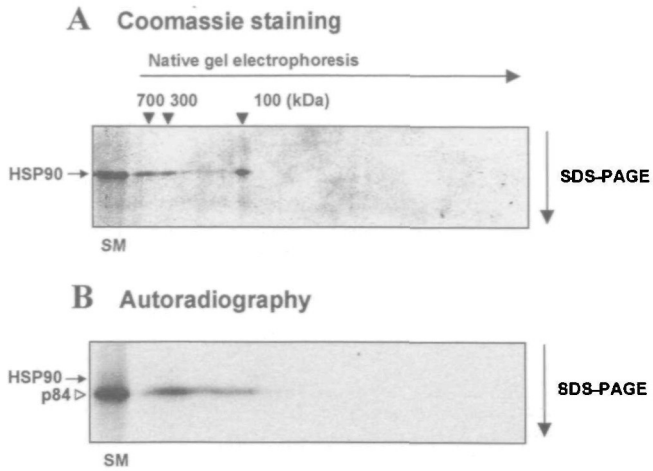

Fig. 3. **Two dimensional PAGE of HSP90 and p84.** HSP90 was *in vitro* phosphorylated with 2 μM [γ-^{32}P]ATP at 37°C for 1 h as described in Fig. 1. The phosphorylated sample was separated in the first dimension under non-denaturing conditions, then the gel was loaded on SDS-10% polyacrylamide gel and electrophoresed. The gel was stained with Coomassie Brilliant Blue (A), then subjected to autoradiography (B). SM, the starting material.

key role(s) in the regulation of enzymatic activity by inducing the conformational change of proteins. To explore whether p84 phosphorylation could affect the p84/HSP90 complex, HSP90 preparation was *in vitro* phosphorylated, then immunoprecipitated under both native and denaturing conditions using no antibody, rat preimmune serum, or anti-HSP90 antibody coupled Affi-gel (Fig. 4B). Immunoprecipitation using either no antibody (lanes 2 and 5) or preimmune serum (lanes 3 and 6) did not show the precipitation of either HSP90 or p84. HSP90, but not p84, was precipitated in denaturing immunoprecipitation using BF4 (lane 7), while the phosphorylated p84 was detected in the supernatants of denaturing immunoprecipitation (data not shown). These results confirmed that p84 is different from HSP90 and that BF4 is highly specific to HSP90. Native immunoprecipitation using anti-HSP90 coupled Affi-gel showed the coprecipitation of the phosphorylated p84 and HSP90 (lane 4). These results suggest that the interaction of HSP90 and p84 may be not altered by the phosphorylation of p84.

HSP90 possesses an ATP-binding site and binds to ATP-agarose (*22-24*). Recently, geldanamycin, a benzoquinone ansamycin that specifically binds to ATP-binding site of HSP90, has been shown to induce the conformational change of HSP90, resulting in the release of associated proteins from HSP90 and degradation of target proteins (*25, 28, 29*). Thus, geldanamycin has been used as a specific inhibitor of HSP90 and prevents the activity of associated kinase (*25, 27*). To test whether geldanamycin could affect the phosphorylation of p84, we performed *in vitro* phosphorylation in the presence of geldanamycin (100 μg/ml) at 37°C for 1 h (Fig. 5). The treatment with geldanamycin did not exert any inhibitory effect on the p84 phosphorylation. Therefore, although p84 is specifically associated with HSP90, p84 phosphorylation is not likely to be linked to the interaction of p84 and HSP90 (panel B of Figs. 4 and 5).

Characterization of the Kinase Responsible for p84 Phosphorylation—To determine which kinase activity is responsible for the phosphorylation of p84, the effects of protein kinase inhibitors on the phosphorylation of p84 were examined (Fig. 6A): 5 mM DBR, 1 μM calphostin C, 10 μM chelerythrine chloride, 100 μM H-7, 10 nM staurosporine, 100 nM genistein, 0.2 μg/ml herbimycin, 250 μM tyrphostin A25, 100 μM tyrphostin A1, 500 μM tyrphostin A63, 200 μM hemin, and 100 nM wortmannin were used to inhibit the activity of casein kinase II, protein kinase C, protein tyrosine kinase, HRI, and PI3-kinase. The phosphorylation of p84 was significantly inhibited only by hemin, and not by other kinase inhibitors. In addition, heparin, an inhibitor of casein kinase II (*10*), did not affect the phosphorylation of p84 (data not shown). These results suggest that heme-regulated protein kinase may be responsible for the p84 phosphorylation.

Typical heme-regulated protein kinase is HRI, which is mostly present in reticulocytes (*12, 16, 17*). P84 may be HRI, as the molecular weight of p84 is similar to that of HRI (*34-36*). Since both HRI and HSP90 have been reported to be specific substrates of casein kinase II, we examined whether HSP90 and p84 are phosphorylated *in vitro* by casein kinase II (Fig. 6B). The phosphorylation of a protein by casein kinase II was detected. The position of the

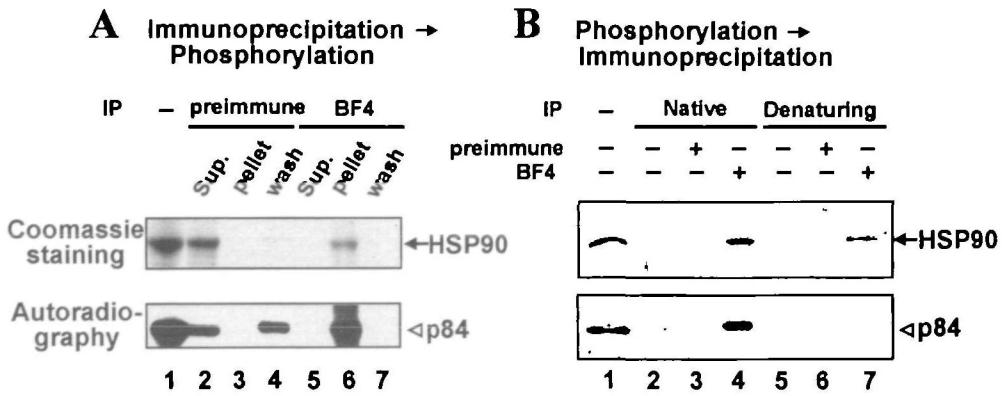

Fig. 4. **Specific interaction of p84 with HSP90.** (A) Native immunoprecipitation was carried out with rat preimmune serum (lanes 2-4) or anti-HSP-90 (BF4) conjugated Affi-Gel (lanes 5-7) at 4°C for 2 h in buffer M condition. The immunoprecipitates were washed 5 times with buffer M, then *in vitro* phosphorylated with [γ-^{32}P]ATP as described in Fig. 1. After reaction, the proteins were analyzed by 10% SDS-PAGE/Coomassie Brilliant Blue staining and autoradiography. Lane 1, the starting material; lanes 2 and 5, the supernatant; lanes 3 and 6, the immunoprecipitate; lanes 4 and 7, the first wash-out. (B) HSP90 preparation was *in vitro* phosphorylated, then native (lanes 2-4) and denaturing (lanes 5-7) immunoprecipitation was carried out with no antibody (lanes 2 and 5), rat preimmune serum (lanes 3 and 6), or anti-HSP90 (BF4) (lanes 4 and 7) conjugated Affi-Gel at 4°C for 2 h. The precipitates were washed five times with buffer M. The phosphorylated proteins were analyzed by 10% SDS-PAGE and autoradiography. Lane 1, the starting material for immunoprecipitation.

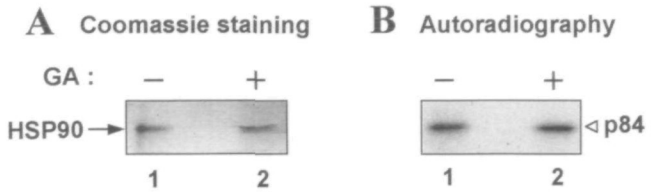

Fig. 5. **Effect of geldanamycin, a HSP90 specific inhibitor, on the p84 phosphorylation.** *In vitro* phosphorylation was carried out with HSP90 (4 μg) preparation in the absence (lane 1) or presence (lane 2) of 100 μg/ml geldanamycin (GA) at 37°C for 1 h. The phosphorylated proteins were analyzed by SDS-PAGE/Coomassie Brilliant Blue staining (panel A) and autoradiography (panel B).

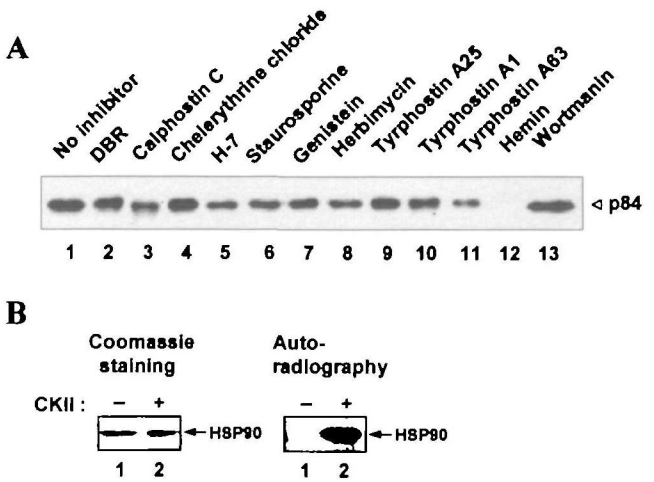

Fig. 6. **Effects of protein kinase inhibitors and exogenous casein kinase II on the p84 phosphorylation.** (A) *In vitro* phosphorylation of p84 was performed in the absence (lane 1) or presence of 5 mM DBR (lane 2), 1 μM calphostin C (lane 3), 10 μM chelerythrine chloride (lane 4), 100 μM H-7 (lane 5), 10 nM staurosporine (lane 6), 100 nM genistein (lane 7), 0.2 μg/ml herbimycin (lane 8), 250 μM tyrphostin A25 (lane 9), 100 μM tyrphostin A1 (lane 10), 500 μM tyrphostin A63 (lane 11), 200 μM hemin (lane 12), or 100 nM wortmannin (lane 13) in 40 μl of reaction mixture at 37°C for 1 h. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. (B) *In vitro* phosphorylation of HSP90 was performed without (lane 1) or with (lane 2) purified casein kinase II (CKII) in the reaction mixture (20 mM Tris-HCl, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 5 mM EGTA) at 37°C for 1 h, and the phosphorylated proteins were immunoprecipitated with BF4 under denaturing conditions, then analyzed by SDS-PAGE/Coomassie staining and autoradiography.

phosphoprotein was slightly higher than that of 84 and the same as that of HSP90. The phosphoprotein was identified as HSP90 by immunoprecipitation of the samples with BF4 under denaturing conditions after the phosphorylation (panel B, lane 2). Thus, p84 is not likely to be a substrate of casein kinase II. In addition, the phosphorylation of p84 was not affected by treatment with 1 mM *N*-ethylmaleimide, a known activator of HRI (data not shown). Taken together, these results suggest that p84 may be phosphorylated by an unknown heme-regulated kinase different from HRI.

Effects of Divalent Cations on 84 Phosphorylation—Next, we examined whether p84 phosphorylation is regulated by divalent cations. As shown in Fig. 7, p84 phosphorylation was significantly activated by addition of exogenous Ca^{2+} at 100-500 μM, but it was blocked by higher concentrations (>1 mM) of Ca^{2+} (panel A). In addition, treatment with EGTA inhibited the p84 phosphorylation, suggesting that a small amount of divalent cations such as Ca^{2+} is present in purified HSP90 preparation (panel C). In contrast, p84 phosphorylation was prevented by the presence of divalent cations such as Mg^{2+} (panel B) and Mn^{2+} (panel C) in a dose-dependent manner.

Csermely and Kahn demonstrated that HSP90 could be *in vitro* autophosphorylated, especially in the presence of higher concentration of Ca^{2+} (10 mM) (*23*). However, we could not detect the HSP90 phosphorylation in the presence of 10 mM Ca^{2+}. The reason for this discrepancy is not clear at present. Since we could not detect the phosphorylation of chicken HSP90 which was overexpressed in Sf9 insect cells and purified by BF4-affinity chromatography, it is not likely to be due to a difference between the tissues from which HSP90 was purified. It is possible that the phosphorylated protein that Csermely and Kahn identified as HSP90 may be p84, since p84 exhibits a very similar mobility to HSP90 in SDS-PAGE and thus could be mistaken for HSP90. In this regard, it is interesting that p84 and Csermely and Kahn's protein (HSP90) share some properties: their

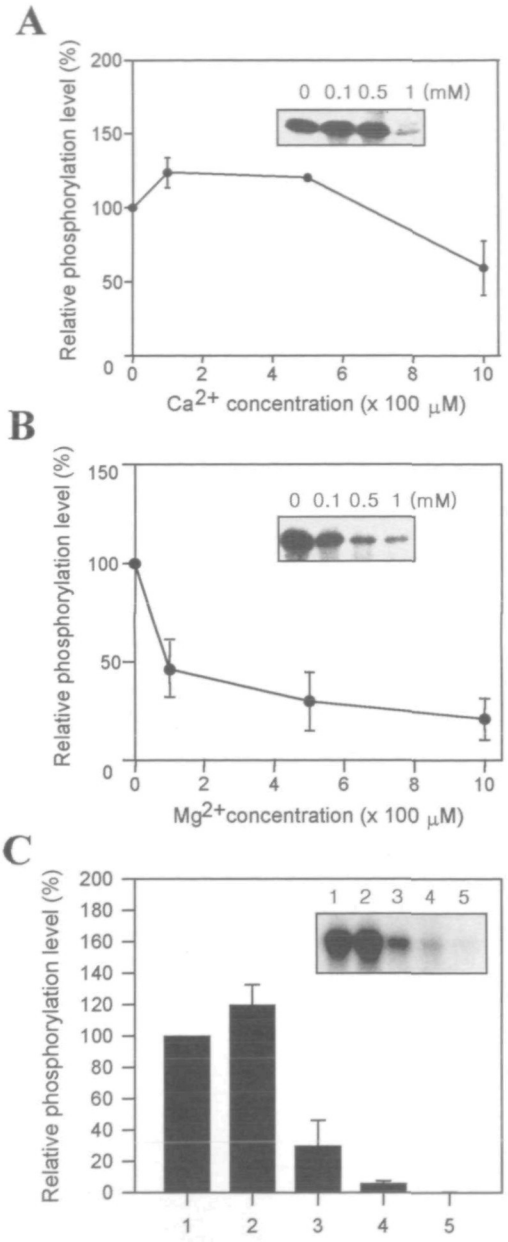

Fig. 7. **Effects of divalent cations on the p84 phosphorylation.** (A and B) *In vitro* phosphorylation of p84 was performed with various concentrations of Ca^{2+} (A) and Mg^{2+} (B). The phosphorylated proteins were analyzed by SDS-PAGE/autoradiography and densitometric scanning. (C) *In vitro* phosphorylation was also performed with p84 in the absence (lane 1) or presence of 100 μM $CaCl_2$ (lane 2), 500 μM $MgCl_2$ (lane 3), 100 μM $MnCl_2$ (lane 4), or 500 μM EGTA (lane 5). The phosphorylated proteins were analyzed by SDS-PAGE/autoradiography and densitometric scanning.

phosphorylation is inhibited by Mg^{2+} and activated by Ca^{2+} (although Ca^{2+} concentration for maximal phosphorylation is different as mentioned above), and both have the ability to bind to ATP-agarose (data for p84 not shown).

In summary, our data indicate that HSP90 does not possess autophosphorylase activity. We further identified an HSP90-associated 84-kDa phosphoprotein that can be phosphorylated by the activity of heme-regulated protein kinase. The phosphorylation of p84 was detected in the HSP90 preparation purified from chick, human brain, and HeLa cells, indicating that p84 is present in various types of cells. The phosphorylation of p84 was controlled by divalent cation such as Ca^{2+}, Mg^{2+}, and Mn^{2+}. Further studies on the role(s) of HSP90 in the phosphorylation of p84 may help us to understand the function(s) of HSP90.

REFERENCES

1. Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E.E., Feramisco, J.R., and Welch, W.J. (1985) The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. *EMBO J.* **4**, 3131-3135
2. Holley, S.J. and Yamamoto, K.R. (1995) A role for hsp90 in retinoid receptor signal transduction. *Mol. Biol. Cell* **6**, 1833-1842
3. Shaknovich, R., Shue, G., and Kohtz, D.S. (1992) Conformational activation of a basic helix-loop-helix protein (MyoD1) by the C-terminal region of murine hsp90 (hsp84). *Mol. Cell. Biol.* **12**, 5059-5068
4. Nadeau, K., Das, A., and Walsh, C.T. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J. Biol. Chem.* **268**, 1479-1487
5. Sepehenia, B., Paz, I.B., Dasgupta, G., and Momand, J. (1996) Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. *J. Biol. Chem.* **271**, 15084-15090
6. Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H., and Yahara, I. (1986) Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. *Proc. Natl. Acad. Sci. USA* **83**, 8054-8058
7. Sanchez, E.R., Redmond, T., Scherrer, L.C., Bresnick, E.H., Welch, M.J., and Pratt, W.B. (1988) Evidence that the 90-kilodalton heat shock protein is associated with tubulin containing complexes in L cell cytosol and in intact PrK. *Cell. Mol. Endocrinol.* **2**, 756-760
8. Minami, Y., Kawasaki, H., Suzuki, K., and Yahara, I. (1993) The calmodulin-binding domain of mouse 90-kDa heat shock protein. *J. Biol. Chem.* **268**, 9604-9610
9. Inanobe A., Takahashi, K., and Katada, T. (1994) Association of the $\beta\gamma$ subunits of trimeric GTP-binding proteins with 90-kDa heat shock protein, hsp90. *J. Biochem.* **115**, 486-492
10. Miyata, Y. and Yahara, I. (1995) Interaction between casein kinase II and the 90-kDa stress protein, HSP90. *Biochemistry* **34**, 8123-8129
11. Lees-Miller, S.P. and Anderson, C.W. (1989) The human double-stranded DNA-activated protein kinase phosphorylates the 90-kDa heat shock protein, hsp90α at two NH_2-terminal threonine residues. *J. Biol. Chem.* **264**, 17275-17280
12. Matts, R.L. and Hurst, R. (1989) Evidence for the association of the heme-regulated eIF-2α kinase with the 90-kDa heat shock protein in rabbit reticulocyte lysate in situ. *J. Biol. Chem.* **264**, 15542-15547
13. Brugge, J.S., Erikson, E., and Erikson, R.L. (1981) The specific interaction of the Rous sarcoma virus transforming protein, $pp60^{src}$, with two cellular proteins. *Cell* **25**, 363-372
14. Stepanova, L., Leng, X., Parker, S.B., and Haeper, J.W. (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of hsp90 that binds and stabilizes Cdk4. *Genes Dev.* **10**, 1491-1502
15. Aligue, R., Akhavan-Niak, H.M., and Russell, P. (1994) A role for hsp90 in cell-cycle control: Wee1 tyrosine kinase activity requires interaction with hsp90. *EMBO J.* **13**, 6099-6106
16. Oimsted, E.A., O'Brien, L., Henshaw, E.C., and Panniers, R. (1993) Purification and characterization of eukaryotic initiation factor (eIF)-2α kinases from Ehrlich ascites tumor cells. *J. Biol. Chem.* **268**, 12552-12559
17. Pal, J.K., Chen, J-J., and London, I.M. (1991) Tissue distribution and immunoreactivity of heme-regulated eIF-2α kinase determined by monoclonal antibodies. *Biochemistry* **30**, 2555-2562
18. Chen, J-J. and London, I.M. (1995) Regulation of protein synthesis by heme-regulated eIF-2α kinase (Review). *TIBS* **20**,

105-108
19. Haro, C., Mendez, R., and Santoyo, J. (1996) The eIF-2α kinases and the control of protein synthesis (Review). *FASEB* **10**, 1378-1387
20. Xu, Z., Pal, J.K., Thulasiraman, V., Hahn, H.P., Chen, J-J., and Matts, R.L. (1997) The role of the 90-kDa heat shock protein and its associated cohorts in stabilizing the heme-regulated eIF-2α kinase in reticulocyte lysates during heat stress. *Eur. J. Biochem.* **246**, 461-470
21. Uma, S., Hartson, S.D., Chen, J-J., and Matts, R.L. (1997) Hsp90 is obligatory for the heme-regulated eIF-2α kinase to acquire and maintain an activable conformation. *J. Biol. Chem.* **272**, 11648-11656
22. Prodromou, C., Roe, C.M., O'Brien, R., Ladbury, J.E., Piper, W.P., and Pearl, L.H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the hsp90 molecular chaperone. *Cell* **90**, 65-75
23. Csermely, P. and Kahn, C.R. (1991) The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. *J. Biol. Chem.* **266**, 4943-4950
24. Jakob, U., Scheibei, T., Bose, S., Reinstein, J., and Buchner, J. (1996) Assessment of the ATP binding properties of hsp90. *J. Biol. Chem.* **271**, 10035-10041
25. Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U., and Pavletich, N.P. (1997) Crystal structure of an hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* **89**, 239-250
26. Csermely, P., Kajtar, J., Hollosi, M., Jalsovszky, G., Holly, S., Kahn, C.R., Gergely, P. Jr., Soti, C., Mihaly, K., and Somogyi, J. (1993) ATP induces a conformational change of the 90-kDa heat shock protein (hsp90). *J. Biol. Chem.* **268**, 1901-1907
27. Grenert, J.P., Sullivan, W.P., Fadden, P., Haystead, T.A.J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H-J., Schulte, T.W., Sausville, E., Neckers, L.M., and Toft, D.O. (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J. Biol. Chem.* **272**, 23843-23850
28. Smith, D.F., Whitesell, L., Nair, S.C., Chen, S.Y., Prapapanich, V., and Rimerman, R.A. (1995) Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Cell Biol.* **15**, 6804-6812
29. Whitesell, L. and Cook, P. (1996) Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. *Mol. Endocrinol.* **10**, 705-712
30. Welch, W.J. and Feramisco, J.R. (1982) Purification and the major mammalian heat shock proteins. *J. Biol. Chem.* **257**, 14949-14959
31. Yonezawa, N., Nishida, E., Sakai, H., Koyasu, S., Matsuzaki, F., Iida, K., and Yahara, I. (1988) Purification and characterization of the 90-kDa heat shock protein from mammalian tissues. *Eur. J. Biochem.* **177**, 1-7
32. Csermely, P., Miyata, Y., Schnaider, T., and Yahara, I. (1995) Autophosphorylation of grp94 (Endoplasmin). *J. Biol. Chem.* **270**, 6381-6388
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
34. Lees-Miller, S.P. and Anderson, C.W. (1989) Two human 90-kDa heat shock proteins are phosphorylated in vivo at conserved serines that are phosphorylated in vitro by casein kinase II. *J. Biol. Chem.* **264**, 2431-2437
35. Miyata, Y. and Yahara, I. (1992) The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. *J. Biol. Chem.* **267**, 7042-7047
36. Mendez, R. and Haro, C. (1994) Casein kinase II is implicated in the regulation of heme-controlled translational inhibitor of reticulocyte lysates. *J. Biol. Chem.* **269**, 6170-6176