THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 41, Issue of October 13, pp. 31682–31688, 2000

Printed in U.S.A.

Heat Shock Protein 90 Mediates Protein-protein Interactions between Human Aminoacyl-tRNA Synthetases*

Received for publication, December 12, 2000, and in revised form, July 24, 2000
Published, JBC Papers in Press, July 25, 2000, DOI 10.1074/jbc.M909965199

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Heat shock protein 90 (hsp90) is a molecular chaperone responsible for protein folding and maturation *in vivo*. Interaction of hsp90 with human glutamyl-prolyl-tRNA synthetase (EPRS) was found by genetic screening, co-immunoprecipitation, and *in vitro* binding experiments. This interaction was sensitive to the hsp90 inhibitor, geldanamycin, and also ATP, suggesting that the chaperone activity of hsp90 is required for interaction with EPRS. Interaction of EPRS with hsp90 was targeted to the region of three tandem repeats linking the two catalytic domains of EPRS that is also responsible for the interaction with isoleucyl-tRNA synthetase (IRS). Interaction of EPRS and IRS also depended on the activity of hsp90, implying that their association was mediated by hsp90. EPRS and IRS form a macromolecular protein complex with at least six other tRNA synthetases and three cofactors. hsp90 preferentially binds to most of the complex-forming enzymes rather than those that are not found in the complex. In addition, inactivation of hsp90 interfered with the *in vivo* incorporation of the nascent aminoacyl-tRNA synthetases into the multi-ARS complex. Thus, hsp90 appears to mediate protein-protein interactions of mammalian tRNA synthetases.

Mammalian aminoacyl-tRNA synthetases (ARSs)[1] are unique in their formation of a macromolecular complex. This complex consists of at least eight different ARS polypeptides (1–3) and three auxiliary protein factors (4–6). Although the existence of this complex has been known for more than two decades, its assembly process and dynamic status are not well understood. The structure of the multi-ARS complex must accommodate their reaction substrates without steric hindrance, since the component enzymes carry out aminoacylation reactions simultaneously. Thus, it is intriguing how the components are assembled and maintained in the complex.

The multi-ARS complex contains three auxiliary proteins, p18, p38, and p43. Since p38 interacts with most of the ARS components, it was proposed to be a scaffold for the assembly of the multi-ARS complex (5). The proposed role of p38 suggests that the formation of the multi-ARS complex is assisted by nonsynthetase factors, although ARSs themselves also interact with each other (7). Since assembly of many functional complexes in the cell is facilitated by their specific chaperones, we suspected that chaperones might be involved in the biogenesis of the multi-ARS complex (8–11).

This possibility was first suggested by the results showing the interaction between heat shock protein 90 (hsp90) and human glutamyl-prolyl-tRNA synthetase (EPRS), a component of the multi-ARS complex. Although hsp90 is known to protect cellular proteins from heat denaturation or other proteotoxic stresses (12, 13), it also plays a role under normal physiological conditions. hsp90 is present as two highly homologous isoforms, α and β (14, 15), and consists of two functional domains linked by a flexible and charged hinge region. The N-terminal domain interacts with ATP (16) or its inhibitor, geldanamycin (GA), and is also responsible for target binding (17). The C-terminal domain is involved in dimerization, which is essential for both function and target interaction (18). Although the structural details of hsp90 are well studied, its working mechanism and target selection remain elusive. Here, we report that hsp90 associates with the complex-forming ARSs and mediates their protein-protein interactions.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—To identify proteins interacting with the three repeats of EPRS, the cDNA encoding the peptide from Val^{573} to Lys^{889} of EPRS (EPRS-L) was subcloned into pLex202 vector using *Eco*RI and *Sal*I sites and used as bait to screen a human HeLa cDNA library expressing B42 fusion proteins (19). The DNA encoding full-length hsp90 was cleaved from pGEX2T-hsp90α (20) (kindly provided by T. Takagi, Tohoku University, Japan) with *Bam*HI and *Sma*I and ligated to pGEX4T-1. The insert was then cleaved with *Bam*HI and *Xho*I and religated into pLexA202. The resulting plasmid was cleaved again with *Eco*RI and *Xho*I to release the insert, which was then ligated to pJG4–5 (B42 fusion vector). Positive interactions were confirmed by both cell growth on leucine-depleted yeast synthetic medium and blue colony formation on the 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal, 5 mM)-containing medium.

LexA-ARS Construction—All of the tested genes encoding the full-length ARSs were isolated from the original clones by polymerase chain reaction using their specific primers and ligated into the appropriate LexA fusion vectors. Detailed information for each construct is available upon request. The original ARS plasmids, except for those of human valyl-tRNA synthetase and human tyrosyl-tRNA synthetase, were kindly provided from Dr. K. Shiba (Cancer Institute, Japan). pG7a-1 for valyl-tRNA synthetase and pHYTS3-WT for tyrosyl-tRNA synthetase were obtained from Dr. R. D. Campbell (University of Oxford) (21) and Dr. E. A. First (Lousiana State University Medical Center) (22), respectively. Polymerase chain reaction products used for

* This work was supported in part by a grant from the National Creative Research Initiatives of the Ministry of Science and Technology of Korea (to S. K.) and by a grant from the Korea Science and Engineering Foundation (H. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ARS, aminoacyl-tRNA synthetase; XRS, ARS for amino acid(s) X; GA, geldanamycin; TSA, trichostatin A; RC, radicicol.

construction were confirmed by DNA sequencing, and expression of the LexA-ARS hybrid proteins was determined by immunoblotting with anti-LexA antibody (data not shown).

Co-immunoprecipitation—HeLa cells were harvested, and proteins were extracted by ultrasonication. Endogenous EPRS was incubated with anti-EPRS rabbit antiserum and precipitated with protein A-agarose. The precipitated proteins were resolved by SDS gel electrophoresis, transferred onto Immobilon P membrane (Millipore Corp.), and immunoblotted with anti-hsp90 antibody (Transduction Laboratories) and the ECL system following the manufacturer's instruction (Amersham Pharmacia Biotech). Precipitation of EPRS and isoleucyl-tRNA synthetase (IRS) was confirmed by immunoblotting with their respective antibodies.

Preparation of Antibodies—Polyclonal rabbit antibodies were raised against EPRS, IRS, LRS, MRS, QRS, RRS, p43, and p38. After expression as a His tag protein, the full-length MRS, p43, and p38 (denatured), EPRS (the native peptide of three repeats from Val^{573} to Lys^{889}), LRS (denatured peptide of C-terminal 236 amino acids), QRS (native N-terminal 236 amino acids), and RRS (native N-terminal 72 amino acids) were purified using nickel affinity chromatography (Invitrogen) and used for antibody preparation (details will be available upon request). Antibodies were prepared as described previously (23). The purified anti-IRS rabbit antibody was obtained from Dr. K. Shiba.

In Vitro Pull-down Assay—hsp90α fused to GST or GST alone was expressed from pGEX2T-hsp90α or pGEX2T, respectively, in *E. coli* BL21 (DE3). Cells were lysed by ultrasonication, and protein extracts were prepared. EPRS-L was synthesized by *in vitro* translation in the presence of [^{35}S]methionine using pcDNA3-EPRS-L. The *in vitro* translated EPRS-L was mixed with the *Escherichia coli* protein extract containing GST-hsp90 or GST in binding buffer composed of 40 mM HEPES, pH 7.6, 20% glycerol, 1 mM DTT, 0.3 μM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (pepstatin A, leupeptin, antipain, and chymostatin; 5 μg/ml each). GA (30 μM) or ATP (5 mM) was added as indicated to the binding mixture to assess their effect on the interaction between hsp90 and EPRS. Glutathione-Sepharose was added to the binding mixture and incubated overnight at 4 °C with rotation. The reaction mixture was then washed three times with binding buffer containing 0.1% Triton X-100, and GST or GST-hsp90 was eluted with 15 mM reduced glutathione. The eluted proteins were separated by SDS gel electrophoresis, and the presence of EPRS-L was determined by autoradiography.

Reporter Assay—Induction of the reporter genes, β-galactosidase, and *leu2* in the two hybrid assay was determined as described below. For the β-galactosidase assay, yeast (EGY48 strain) expressing the two proteins tested was grown overnight in a yeast synthetic medium (Ura^-, His^-, Trp^-, 2% glucose). After checking absorbance at 600 nm, the cells were transferred in a yeast medium (Ura^-, His^-, Trp^-, 2% galactose, 0.2% Me_2SO). Equivalent numbers of cells were lysed in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0) containing one drop of 0.1% SDS and two drops of chloroform for 15 min at 30 °C, and then 200 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside was added to determine the β-galactosidase activity. When a yellow color appeared, the reaction was stopped with 500 μl of 1 M Na_2CO_3. The samples were centrifuged briefly, and the absorbance was measured at 420 and 550 nm. The β-galactosidase activity was calculated using the formula units = $(1000 \times (A_{420} - 1.75 \times A_{550}))/(\text{time} \times \text{volume} \times A_{600})$. To determine the induction of the *leu2* gene, the EGY48 strain expressing LexA-IRS-C (from Glu^{966} to Phe^{1266}) and B42-EPRS-L was cultivated in 5 ml of yeast synthetic medium (Ura^-, His^-, Trp^-, 2% glucose) at 30 °C overnight with shaking. Cells (1.5 ml) were harvested and resuspended in 1.5 ml of yeast synthetic medium (Ura^-, His^-, Trp^-, Leu^-, 2% galactose, and 0.5% raffinose) and then spun down again and resuspended in 1.5 ml of the same broth. 0.2 ml of cells were then transferred to the same broth containing 0.001% SDS and each of the antibiotics, trichostatin A (TSA) (24), GA, and radicicol (RC) (25). These cells were cultivated at 30 °C overnight with shaking, and the number of the cells was determined by hemocytometer after the same time interval. The growth of the EGY48 strain alone or expressing LexA-IRS-C and B42 in the presence of these antibiotics was also determined using the same methods, except that the cells were cultivated in yeast synthetic medium containing leucine.

Partial Purification of Multi-ARS Complex from Bovine Liver—Fresh bovine liver (100 g) was immersed in buffer A (25 mM KPO_4, pH 7.5, 10% glycerol, 0.1 mM EDTA, and 10 mM β-mercaptoethanol) containing protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin (each 5 μg/ml) and 0.1 mM phenylmethylsulfonyl fluoride) to remove blood, and this process was repeated five times. The liver was then homogenized (PowerGen 700), and the lysate was centrifuged at

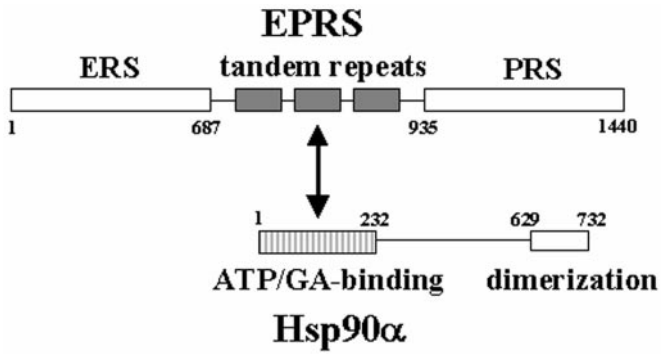

FIG. 1. **Structure and interaction of human EPRS and hsp90.** Human EPRS contains 1440 amino acids. It consists of two catalytic domains (the N-terminal glutamyl- and the C-terminal prolyl-tRNA synthetase (*ERS* and *PRS*, respectively)) linked by three tandem repeats of 57 amino acids (40, 41). Human hsp90α contains 724 amino acids (42), consisting of the N- and C-terminal domains responsible for ATP or GA binding and dimerization, respectively (16, 17). The peptide-spanning tandem repeat domain of EPRS interacts with the N-terminal domain of hsp90.

26,000 $\times$ *g* for 45 min. After removing the tissue debris, the supernatant was recentrifuged at 100,000 $\times$ *g* for 1 h to obtain the postribosomal supernatant. Polyethylene glycol 6000 (Fluka) was added to a final concentration of 2% (w/v) to the supernatant with stirring and mixed for 30 min. The solution was then centrifuged at 12,000 $\times$ *g* for 20 min, the supernatant was removed, and then PEG 6000 was added to a final concentration of 15% (w/v). This solution was then centrifuged, and the pellet was dissolved in buffer A and filtered through a 0.4-μm filter disc. After the filtrate was dialyzed in buffer A, the solution was loaded onto gel filtration column (Bio-Gel A-5m; Bio-Rad). The eluted fractions containing the multi-ARS complex were determined by the aminoacylation activity of IRS. The fractions were then collected and loaded onto a Blue Sepharose (Bio-Rad) column pre-equilibrated with buffer A. After washing the column, the bound proteins were eluted using a gradient of 0–1.3 M KCl. Fractions showing the IRS activity were collected and dialyzed in buffer A and loaded onto a hydroxyapatite column (Bio-Rad). After washing, the proteins in the column were eluted using a phosphate gradient from 100 to 400 mM. The IRS-positive fractions were collected, concentrated, and finally loaded onto a Superdex 200 HR (Amersham Pharmacia Biotech) column. The eluted proteins in each fraction were monitored by absorbance at 280 nm and immunoblotting with the mixture of anti-ARS antibodies as indicated.

Assay of Multi-ARS Complex Assembly—Assembly of the complex-forming ARSs was determined by co-immunoprecipitation of the components with anti-EPRS antibody. HeLa cells were treated with different concentrations of GA (from 5 to 20 μM) for 24 h or treated with 20 μM GA for the indicated time (from 6 to 24 h). Cells were then washed twice with PBS and methionine-free minimal essential medium (Sigma) containing the indicated concentration of GA and incubated for 30 min. Then [^{35}S]methionine (100 μCi/ml) in 10% serum was added to the cells for 1 h. Cells were then washed twice with phosphate-buffered saline, and complete medium (Dulbecco's modified Eagle's medium) containing 1 mM cold methionine was added to the medium for 1 h. The cells were then harvested and lysed with the lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Nonidet P-40, 12 mM β-glycerophosphate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and aprotinin 5 μg/ml). Equal amounts of the proteins extracted from each treatment condition were then mixed with anti-EPRS antibody (5 μg) and immunoprecipitated. ARSs co-precipitated with EPRS were resolved by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with a mixture of their specific antibodies (EPRS, IRS, LRS, MRS, and QRS). The nascent ARSs in the whole cell extracts or in the immunoprecipitates were determined by autoradiography. Autoradiography was quantified using a phosphor image analyzer (BAS-3000; Fuji).

RESULTS

hsp90 Identified as an EPRS-interacting Protein—Human EPRS contains three tandem repeats linking the catalytic domains of the N-terminal glutamyl- and the C-terminal prolyl-tRNA synthetase (Fig. 1). It has been previously shown that this peptide region is responsible for the interaction with the

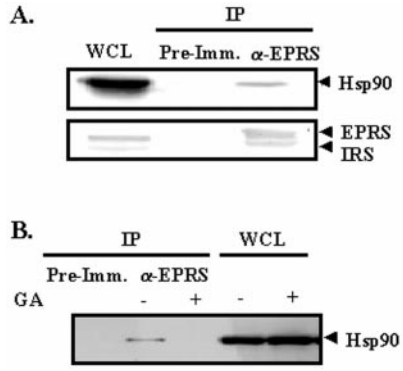

FIG. 2. **Co-immunoprecipitation (*IP*) of EPRS and hsp90.** *A*, the endogenous EPRS in HeLa cells was immunoprecipitated with anti-EPRS antiserum, and co-precipitation of hsp90 and IRS was determined using their corresponding antibodies. The cellular expression of hsp90 was determined in whole cell lysate (*WCL*). Preimmune rabbit serum was used as a control. IRS associated with EPRS in the multi-ARS complex was also co-precipitated with EPRS. *B*, co-immunoprecipitation of EPRS and hsp90 was also determined from the HeLa cells cultivated with or without GA.

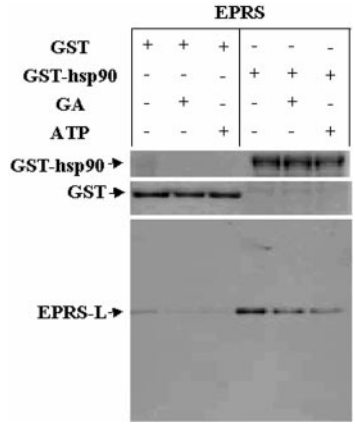

FIG. 3. ***In vitro* interaction of EPRS and hsp90.** A peptide spanning the tandem repeats of EPRS (from Val^{573} to Lys^{889}) was used for *in vitro* pull-down assay. This peptide was synthesized by *in vitro* translation in the presence of [^{35}S]methionine, while hsp90 was expressed as a GST fusion protein. Overexpressed GST alone or GST-hsp90 was mixed with the *in vitro* translation mixture of the EPRS peptide and then pulled down using glutathione-Sepharose. Co-precipitation of the radioactively labeled EPRS was determined by SDS-polyacrylamide gel electrophoresis and autoradiography. The hsp90 inhibitor GA (30 μM) or ATP (5 mM) was added to the indicated binding reaction to assess its effects on the interaction between hsp90 and EPRS.

C-terminal tandem repeats of human IRS (26) as well as nucleic acids (27). We screened a human HeLa cDNA library to test whether additional proteins could associate with the repeated region of EPRS. From this screening, human hsp90α was selected to interact with the tandem repeats of EPRS via its N-terminal ATP binding domain (Fig. 1).

Co-immunoprecipitation of hsp90 with EPRS—hsp90's interaction with EPRS was further confirmed by co-immunoprecipitation. HeLa cells were lysed, and endogenous EPRS was immunoprecipitated with antibody raised against the EPRS peptide. Co-precipitation of hsp90 with EPRS was then determined with anti-hsp90 antibody (Fig. 2*A*). IRS was previously shown to interact with EPRS (26, 27) and was also detected in the immunoprecipitate of EPRS.

To test whether this interaction depends on the activity of hsp90, HeLa cells were cultivated in the absence and presence of GA**,** a specific inhibitor of hsp90 (17, 28), and the effect of GA on the interaction of EPRS and hsp90 was determined by immunoprecipitation. While the same level of hsp90 was detected in the whole cell lysates of the HeLa cells cultured with and without GA, hsp90 was not co-immunoprecipitated with EPRS in the presence of GA (Fig. 2*B*). This indicates that the interaction of EPRS and hsp90 depends on the chaperone activity of hsp90.

In Vitro Interaction of EPRS and hsp90—The interaction of hsp90 with EPRS was further analyzed by *in vitro* pull-down experiments. The EPRS peptide was synthesized by *in vitro* translation in the presence of [^{35}S]methionine, and hsp90 was prepared as GST fusion protein. Either GST alone or GST-hsp90 mixed with the EPRS peptide was affinity-purified using glutathione-Sepharose, and co-purification of the EPRS peptide was determined by autoradiography. The EPRS peptide was specifically eluted as a complex with GST-hsp90 but not with GST alone (Fig. 3).

The binding sites for GA and ATP are both located in the N-terminal domain of hsp90 that is also responsible for the interaction with EPRS (Fig. 1). Since our co-immunoprecipitation results indicated that the interaction of hsp90 and EPRS was sensitive to the presence of GA, we also tested the effect of these compounds on the interaction of hsp90 and EPRS using an *in vitro* pull-down assay. EPRS binding to hsp90 was partially inhibited by the addition of GA or ATP (Fig. 3). The GA- or ATP-induced dissociation of hsp90 from the bound peptide was previously reported (29).

Effect of Geldanamycin on the Interaction between EPRS and IRS—Chaperones such as hsp 90 play roles in protein folding, trafficking, degradation, and assembly. EPRS is one of the components for the multi-ARS complex**,** and previously we have shown that it interacts with IRS. We hypothesized that hsp90 might facilitate the interaction between EPRS and IRS; thus, the effect of hsp90 inhibitors such as GA and RC was tested on the interaction of these two peptides using the yeast two-hybrid system. Since heat shock proteins are highly conserved among different organisms and yeast hsp90 homologue is also sensitive to GA (30, 31), we treated yeast cells with GA or RC to determine whether these antibiotics affect the interaction between EPRS and IRS. TSA, which affects transcription by inhibiting histone deacetylase (24), was also employed as a control. Yeast EGY48 cells were cultivated in the presence of each of these drugs individually for 48 h to assess whether these drugs affected cell growth. Cell growth was very slightly affected by treatment with these drugs, suggesting that they were not toxic (Fig. 4, *right*). This result is also consistent with previous reports (31). The same host cells expressing both LexA-IRS-C and B42-EPRS-L were grown in yeast synthetic medium without leucine in the presence of the indicated drug. Cell growth in the leucine-depleted medium should be affected by the expression level of *leu2* (the reporter gene for the interaction between EPRS and IRS). The growth of cells treated with GA or RC was inhibited by about 80% compared with those without drug or treated with TSA (Fig. 4, *left*). As a control, the same cells expressing LexA-IRS-C and B42 were treated with these drugs and cultivated in the yeast medium with leucine. The growth of these cells was only slightly affected by the treatment with these drugs (Fig. 4, middle). These results suggest that the interaction of EPRS and IRS is specifically affected by the inhibition of hsp90, further supporting that this interaction is mediated by hsp90. Similar results were obtained when another reporter gene, β-galactosidase, was used for these experiments (data not shown).

Co-elution of hsp90 with Sub-ARS Complex—Although hsp90 was co-immunoprecipitated with EPRS (Fig. 2), we do not know whether hsp90 is associated with EPRS in the multi-ARS complex. The presence of hsp90 has not been previously reported in the purified multi-ARS complex, implying that it

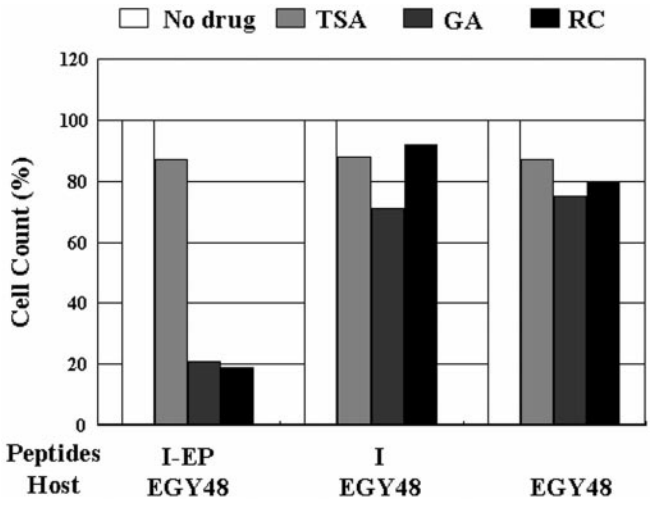

FIG. 4. **Effect of hsp90 inhibitors on interaction between EPRS and IRS.** The yeast tester stain, EGY48, expressing the LexA-IRS-C and B42-EPRS-L was cultivated in yeast synthetic medium lacking leucine for 48 h with no drug (*white bar*), TSA (10 μg/ml; *light gray bar*), GA (5.6 μg/ml; *dark gray bar*), and RC (10 μg/ml; *black bar*), and the cell number was counted using a hemocytometer. At the same time, EGY48 itself or the cells expressing LexA-IRS-C and B42 were cultivated in yeast synthetic medium with leucine as described above to determine whether these antibiotics had any nonspecific effects on the cells.

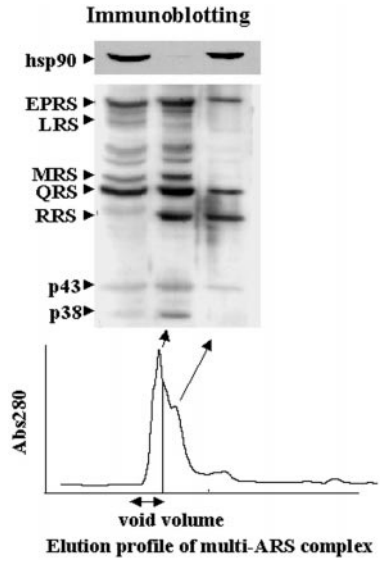

FIG. 5. **Association of hsp90 with the sub-ARS complex.** The multi-ARS complex was partially purified from bovine liver as described under "Experimental Procedures." The sample containing the multi-ARS complex was loaded onto a gel filtration column, and the proteins were fractionated. The eluted proteins were resolved using SDS gel electrophoresis, and some of the components for the multi-ARS complex (EPRS, LRS, MRS, QRS, p43, and p38) were detected with their corresponding antibodies (*upper panel*). The *leftmost lane* shows the components present in the multi-ARS complex before gel filtration chromatography. The *middle* and *rightmost lanes* show the components detected in the major peak fraction eluted in void volume and the accompanying peak fraction, respectively. The corresponding elution profile is shown in the *lower panel*.

either may not be present in or may be weakly associated with this complex. We thus investigated if hsp90 is present in the purified multi-ARS complex. To determine whether hsp90 is present in either the partially purified multi-ARS complex or in its intermediate subcomplexes, we purified the multi-ARS complex and evaluated hsp90 association. The complex was enriched by differential precipitation and by chromatography using several different columns. Although a majority of hsp90 was removed during purification, a slight amount of hsp90 co-eluted with the fractions containing the multi-ARS complex (data not shown). Finally, the proteins in the partially purified complex were separated using a sizing column, and the elution profile was monitored. A major protein peak was observed in the void volume indicative of a macromolecular complex, which was also accompanied by a minor peak (Fig. 5). The fractions found in these peaks were subjected to SDS gel electrophoresis, and several components of the complex (EPRS, LRS, MRS, QRS, RRS, p43, and p38) were identified by immunoblotting with a mixture of their corresponding antibodies. While the major peak contained all of these components, the minor peak only showed the signals for EPRS, QRS, RRS, and p43. The presence of these four components is consistent with the interaction map determined by genetic and cross-linking analyses (7, 23, 32). hsp90 was not found in the major peak containing more components of the multi-ARS complex but was present in the fraction containing the sub-ARS complex. Based on the molecular weight of hsp90, the free form of hsp90 is not expected to be present in this fraction. Thus, hsp90 appears to be associated with the sub-ARS complex but not with the complete multi-ARS complex.

Preferential Interactions of hsp90 with Complex-forming ARSs—The results above suggest that hsp90 would be more generally involved in the molecular interactions of the complex-forming ARSs. We thus investigated whether there is a distinction between which ARSs interact with hsp90. Interactions of hsp90 with different ARSs were tested by yeast two-hybrid assay. A total of 15 full-length human ARSs, and the nonsynthetase components, p18, p38, and p43, were fused to LexA and tested for the interaction with B42-hsp90. Among the ARSs tested, LRS, MRS, QRS, KRS, DRS, and RRS form a complex with nonsynthetase protein factors, p18, p38, and p43, while the ARSs, CRS, GRS, HRS, SRS, TRS, VRS, WRS, and YRS have not been found in this complex (1–3). Among the complex-forming ARSs, hsp90 showed strong interactions with MRS, QRS, DRS, and p38. In contrast, no significant interactions were observed with the non-complex-forming ARSs, although weak interactions were observed with GRS, HRS, and WRS (Fig. 6). These results suggest that hsp90 interacts preferentially with some of the complex-forming ARSs.

Assembly of Nascent Complex-forming ARSs Is Disrupted by hsp90 Inhibitors—The experiments above showed that the interaction between EPRS and IRS was severely affected by the addition of hsp90 inhibitors (Fig. 4). In addition, hsp90 is bound to the sub-ARS complex but not to the complete multi-ARS complex (Fig. 5) and also preferentially associates with some of the complex-forming ARSs (Fig. 6). All of these results imply that hsp90 may be involved in the associations of other complex-forming ARSs, in addition to the pair of EPRS and IRS. If this were the case, inhibition of hsp90 would be predicted to either disrupt or retard the assembly of the complex-forming ARSs.

To address this possibility, we decided to monitor whether the incorporation of the newly synthesized ARSs to the multi-ARS complex is affected by the activity of hsp90. In these experiments, HeLa cells were treated for different times or with different concentrations of GA, and the nascent polypeptides were labeled *in vivo* with [^{35}S]methionine. The cells were then harvested, and proteins were extracted from the cells and immunoprecipitated using anti-EPRS antibody. The ARS components (IRS, LRS, MRS, QRS, RRS, and KRS) co-precipitated with EPRS were then separated by SDS-gel electrophoresis and quantified by autoradiography. Cellular protein synthesis was determined by autoradiography of the protein extracts.

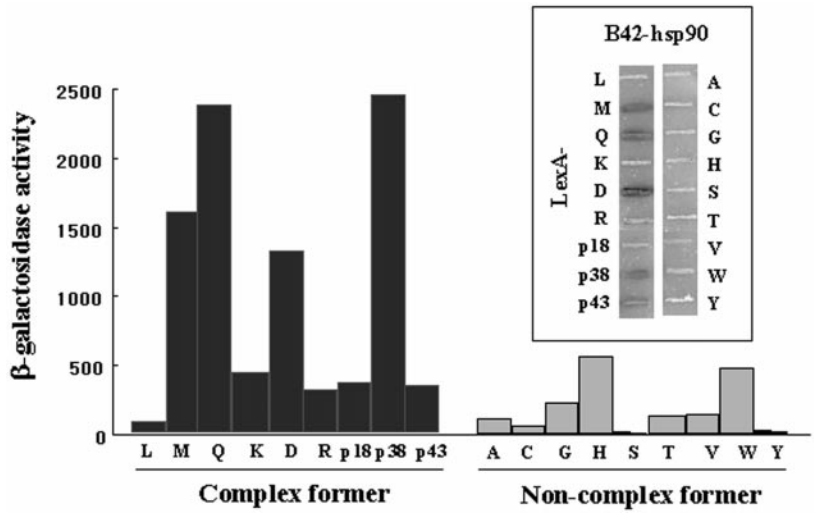

FIG. 6. **Interactions of hsp90 with complex-forming ARSs.** A total of nine complex-forming proteins (six ARSs and two auxiliary factors, p38 and p43) and nine non-complex-forming ARSs were tested for interaction with hsp90 by yeast two-hybrid assay. Positive interactions were determined by the induction of β-galactosidase on a yeast synthetic plate containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (5 mM) (*inset*) or in liquid medium using o-nitrophenyl β-D-galactopyranoside as a substrate (*bar graph*). Similar results were obtained from three independent experiments.

The total amounts of the co-precipitated ARSs were compared by the immunoblotting of the ARS components using their respective antibodies.

First, the cells were treated with 20 μM GA and harvested at different time intervals. The harvested cells were lysed, and the multi-ARS complex was precipitated using anti-EPRS antibody from the extracted proteins. Immunoblotting with the corresponding ARS antibodies showed that the similar amounts of the ARS components were precipitated at each time point (Fig. 7*A*, *middle panel*). The precipitated ARS complex was then subjected to autoradiography. The amount of EPRS precipitated with anti-EPRS antibody was decreased to about 75% of the control by the treatment of GA for 24 h. The incorporation of other co-precipitated ARSs was decreased from 0 to 30% under the same conditions (Fig. 7*A*, *left panel* and *line plot*). Autoradiography of the total cellular protein showed that protein synthesis was not affected by treatment with GA (Fig. 7*A*, *right panel*). This suggests that inactivation of hsp90 interferes with assembly of the nascent ARSs.

To further confirm this result, cells were then treated with different concentrations of GA, and its effects on the incorporation of the ARS components to the complex were monitored. Cells treated under each concentration were harvested, and the multi-ARS complex were extracted and precipitated with anti-EPRS antibody as described above. The incorporation of the nascent ARS components was also determined by autoradiography. The assembly of the nascent ARS components in the absence of GA was determined by the immunoprecipitation of the ARS complex with anti-EPRS antibody and used as a control (Fig. 7*B*, *left panel*, *leftmost lane*). Then the effect of different concentrations of GA on the assembly of the ARS components was determined by the same method. The incorporation of the nascent ARSs to the complex was impaired by GA treatment in a dose-dependent manner (Fig. 7*B*, *left panel* and *line plot*). The cellular protein synthesis was not affected by the GA treatment as determined by the autoradiography of the whole cell extract (Fig. 7*B*, *right panel*). The stability of the preexisting multi-ARS complex remained the same without GA treatment during the cultivation of the cells (data not shown). These results indicate that the incorporation of the nascent ARS components to the multi-ARS complex was sensitive to the activity of hsp90.

DISCUSSION

Although hsp90 is one of the most abundant cellular proteins, it is highly restrictive in target selection under normal physiological conditions. Identified targets of hsp90 include steroid hormone receptors, kinases, regulatory factors, and enzymes (13, 33). However, the cellular concentration of hsp90 is much higher than its known target molecules, suggesting that more hsp90 substrates remain to be identified. In addition, the role of hsp90 in the formation of macromolecular protein complexes is not well understood. Herein, we suggest that hsp90 associates with a subset of mammalian ARSs to facilitate their protein-protein interactions.

The N-terminal domain of hsp90α responsible for the binding to GA and ATP (16, 17) associates with the EPRS peptide that also interacts with other complex-forming ARSs (26, 27). The association of hsp90 with EPRS was sensitive to hsp90 inhibitor, GA, and ATP (Figs. 2 and 3), suggesting a specific interaction. In addition, the ATP-induced dissociation of hsp90 from EPRS is consistent with a previous report that millimolar concentrations of ATP induce the dissociation of hsp90 from actin filaments (34). Taken together, these results indicate that the chaperone activity of hsp90 is required for its interaction with EPRS.

While hsp90 preferentially associated with the sub-ARS complex, it was not found to be present in the multi-ARS complex, suggesting that it may be involved in the assembly process but not the maintenance of the multi-ARS complex (Fig. 5). This is also reminiscent of a recent report on the role of a mitochondrial protein, Bcs1p, in the assembly of the cytochrome bc1 complex (8). This protein also binds to a subcomplex to facilitate the assembly pathway of the cytochrome complex. A possible role of hsp90 in the macromolecular assembly of ARSs was further supported by the inhibitory effect of GA or RC on the interaction between EPRS and IRS (Fig. 4) and, more directly, on the assembly of the nascent complex-forming ARSs *in vivo* (Fig. 7). Although the cellular protein synthesis was not affected by the GA treatment, the amount of the nascent ARSs co-precipitated with anti-EPRS was decreased by the inactivation of hsp90. This result implies that the stability of ARSs is sensitive to the activity of hsp90. In this regard, it is worth noting that CFTR is destabilized by interfering with its interaction with hsp90 (35). Thus, ARSs not incorporated to the complex may be either degraded or aggregated. However, the stability of the preexisting ARS complex did not seem to be severely affected by GA treatment because the similar amounts of the multi-ARS complex were precipitated from both treated and untreated cells with anti-EPRS antibody (Fig. 7, *A* and *B*, *middle panel*). Similarly, hsp90 is important for the membrane association of newly synthesized Src-kinase $p56^{lck}$, but not its maintenance (36).

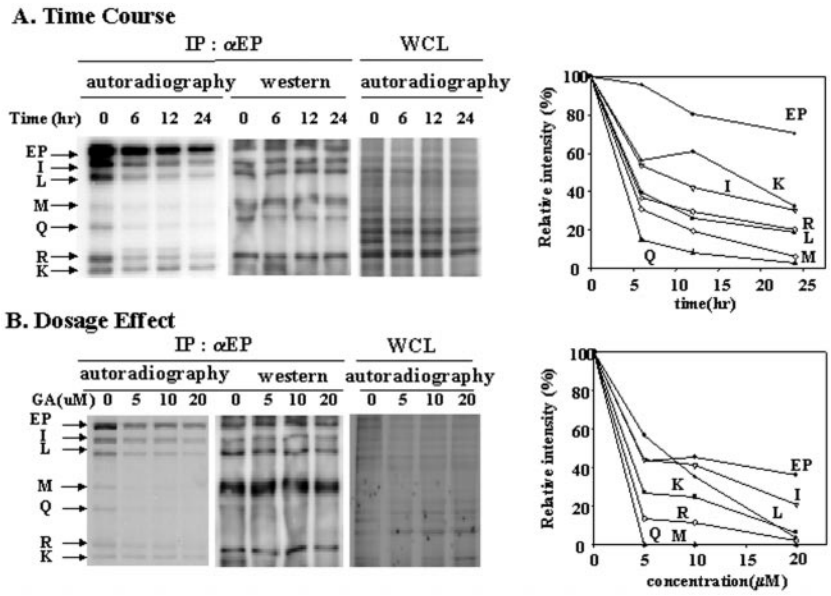

FIG. 7. **Effect of GA on the incorporation of nascent ARSs to the multi-ARS complex.** Newly synthesized cellular proteins were labeled with [^{35}S]methionine. Then the multi-ARS complex was immunoprecipitated with anti-EPRS antibody, and the incorporation of the nascent ARSs to the multi-ARS complex was monitored by autoradiography (*leftmost panel*). The amounts of the immunoprecipitated ARSs were determined by Western blotting with the mixture of their respective antibodies (*middle panel*). The effect of GA on the cellular protein synthesis was determined by autoradiography of the whole cell lysate (*rightmost panel*). The relative intensities of the ARS components found in the multi-ARS complex were quantified by scanning each band in the autoradiograph and plotted. HeLa cells were treated with GA (20 μM) and harvested at the indicated times (*A*), or the cells were cultivated for 24 h at the indicated concentration of GA (*B*).

The interaction of hsp90 with ARSs appears to be dynamic based on the induction level of the reporter gene *leu2* in two-hybrid analysis (Fig. 6). Under physiological conditions, ATP is present at millimolar levels; thus, most of hsp90 would be expected to be associated with ATP *in vivo* (29). Further, since ATP stimulated the dissociation of hsp90 from EPRS (Fig. 3), it would be predicted that the cellular interactions of hsp90 with ARSs would be dynamic. Nonetheless, the potential of ARSs for the interaction with hsp90 appears to be well correlated with their propensity for the complex formation (Fig. 6).

The known substrates for hsp90 do not share sequence or structural characteristics. Furthermore, ARSs interacting with hsp90 do not show any common sequence or structural features. Instead, the two classes of ARS that interact and do not interact with hsp90 may differ in conformational stability or folding characteristics. Perhaps, the complex-forming ARSs may be unstable in isolation and need to be associated with chaperones like hsp90 until they are associated with the neighboring ARSs. Among the non-complex-forming ARSs, some members including GRS, HRS, and WRS showed slightly higher affinity to hsp90 than others. Interestingly, these ARSs also contain motifs homologous to the EPRS repeats in their N-terminal extensions (37–39). Further, the potential for the association of GRS with the complex-forming IRS has been previously suggested, although it is classified as a non-complex former (26). Thus, hsp90 may be also involved in protein-protein interactions of some non-complex-forming ARSs.

Various cellular machineries exist as macromolecular complexes. Thus, it is important to understand the biogenesis and dynamics of these complexes. The involvement of chaperones in macromolecular assembly has been reported in many cases. The formation of ribulose bisphosphate carboxylase-oxygenase is assisted by the plant homologue of *E. coli* groEL (9, 10), and the assembly of bacteriophage capsids also involves chaperones (11). Here, we suggest that hsp90 may play a role in mediating protein-protein interactions between the complex-forming ARSs.

Acknowledgments—We thank Drs. K. Shiba, E. D. First, R. D. Campbell, and T. Takagi for kindly providing materials and Dr. W. Seol for critical reading and advice.

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