Cell Stress & Chaperones (1996) 1 (4), 237–250

A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor

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Abstract A variety of regulatory proteins, including different classes of transcription factors and protein kinases, have been identified in complexes with Hsp90. On careful examination of unactivated progesterone receptor complexes, eight different protein participants have been identified, and each can be considered a component of the cytoplasmic molecular chaperone machinery. These proteins are Hsp90, Hsp70, Hip, p60, p23, FKBP51, FKBP52 and Cyp40. Studies in a cell-free assembly system have helped to define a highly ordered, dynamic pathway for assembly of progesterone receptor complexes. In the present study, target proteins other than progesterone receptor were used in this cell-free system to assemble complexes in vitro and to compare the composition of resulting complexes. Targets used were human estrogen receptor, human Fes protein–tyrosine kinase, human heat shock transcription factor Hsf1, and human aryl hydrocarbon receptor. The striking similarity of resulting target complexes with previously characterized progesterone receptor complexes suggest that each of these targets undergoes a common assembly pathway involving multiple chaperone components in addition to Hsp90.

INTRODUCTION

Hsp90 has been identified in association with multiple regulatory proteins, including various transcription factors and protein kinases. Cell-free studies examining the interactions between Hsp90 and the progesterone receptor (PR) have revealed a dynamic, ordered assembly pathway involving multiple components of the cytoplasmic molecular chaperone machinery (Smith 1993; Smith et al 1995). Most of these proteins, including Hsp90, Hsp70, p60, FKBP52 (sometimes termed p59, Hsp56, or FKBP59), and p23 were previously identified as proteins associated with unactivated steroid receptors (reviewed in Smith and Toft 1993). The cyclosporin A-binding protein, Cyp40, first identified in estrogen receptor complexes (Ratajczak et al 1990, 1993), and a novel FK506-binding protein termed either FKBP54 (Smith et al 1993a, 1993b) or FKBP51 (Baughman et al 1995; Yeh et al 1995)[1] are also components of the PR assembly pathway (PRAP). Most recently, the Hsp70-interacting protein Hip (Höhfeld et al 1995; Prapapanich et al 1996) was first

Received 1 May 1996; Revised 17 June 1996; Accepted 18 June 1996

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[1]A cDNA for human FKBP54 has been cloned and sequenced by us (GenBank #U42051) and is about 90% identical to the rat sequences for FKBP51. Since the human sequence also encodes a 51 kDa protein, the term FKBP51 will be adopted by us here and in future studies (Nair SC, Rimerman RA, Toran EJ and Smith DF, manuscript in preparation).

recognized as a 48 kDa transient PRAP component (Smith 1993).

Earlier studies (Smith 1993; Smith et al 1995) on the order and time-course of ATP-dependent PR assembly in rabbit reticulocyte lysate (RL) found that PR rapidly associates with Hsp70 in a process that may indirectly require DnaJ (Caplan et al 1995; Höhfeld et al 1995; Kimura et al 1995). Approximately 2 min after initiating assembly, PR progresses to an intermediate complex containing Hsp70, Hsp90, Hip and p60. By 5–10 min, PR is primarily found in association with Hsp90, p23 and one of the immunophilins. Only at this final assembly stage is PR functionally mature as measured by its ability to bind progesterone with high affinity. Importantly, at the elevated temperature required for efficient PR assembly, Hsp90–PR interactions in the mature complex dissociate with a half-life of approximately 5 min. Following dissociation, unliganded PR, but not ligand-bound PR, rapidly re-enters the PRAP at the initial step of Hsp70 association. Therefore, at maximal conditions for cell-free assembly of functional PR complexes, PR exists in a dynamic, steady state mixture in which most PR exists in the relatively stable mature complex.

Evidence for the existence of a similar dynamic, steady state condition for PR in intact cells was recently provided (Smith et al 1995) using the fungal metabolite geldanamycin (GA). GA binds specifically to Hsp90 (Whitesell et al 1994), but the drug does not block all the actions of Hsp90. The most evident effect of GA-binding is the inhibition of p23 binding to Hsp90 (Johnson and Toft 1995; Smith et al 1995); binding of p60 and immunophilins to Hsp90 is unaffected by GA and formation of intermediate PR complexes containing Hsp90 is unaffected. However, formation of mature PR complexes is blocked in the presence of GA; as a consequence, establishment of PR's hormone binding conformation is prevented (Smith et al 1995). Since GA readily enters cells, intact COS cells expressing a transfected PR cDNA were examined for GA's ability to disrupt progesterone binding and to alter the structure of PR complexes recovered from cell extracts. These results were consistent with those observed from cell-free studies–namely, that GA rapidly depleted progesterone binding and that GA blocked assembly of p23 and immunophilins with PR while enhancing recovery of early and intermediate PR complexes.

Similar effects of GA on glucocorticoid receptor complexes and hormone binding in intact cells have been observed (Whitesell and Cook 1996). Other cellular effects of GA or the closely related compound herbimycin A are:

1. the proteolytic destabilization of v-Src in cells (Whitesell et al 1994)
2. functional disruption of the Ser/Thr kinase Raf (Schulte et al 1995)
3. induction of heat shock proteins (Murakami et al 1991) and activation of heat shock transcription factor (Hsf; Hegde et al 1995).

Since v-Src and some other tyrosine kinases (reviewed by Brugge 1986), Raf (Stancato et al 1993), Hsf (Nadeau et al 1993), and the aryl hydrocarbon receptor (reviewed by Poellinger et al 1992 and Okey et al 1994) have all been reported to interact with Hsp90, the possibility is presented that each of these regulatory proteins traverses the PRAP or a similar pathway of chaperone interactions.

To compare the general nature of chaperone interactions with signaling proteins other than PR, antibodies against various PRAP components were used to examine in vitro assembled complexes of selected target proteins:

1. estrogen receptor (ER)
2. Fes, a cytoplasmic tyrosine kinase
3. the human heat shock transcription factor Hsf1
4. the aryl hydrocarbon receptor.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal IgGs (and respective antigen): AC88 (Hsp90, Sullivan et al 1985); BB70 (Hsp70, Smith et al 1993c); F5 (p60, Smith et al 1993c); FF1 (FKBP51; Smith et al 1993a); JJ3 (p23, Johnson et al 1994); 2G6 (Hip, Prapapanich et al 1996); M17 (ER, Neff et al 1994); M2 (FLAG epitope, Kodak). Mouse monoclonal IgM: 'anti-50' (pp50; Whitelaw et al 1991). Rabbit antisera: UPJ56 (FKBP52, Ruff et al 1992); PA3–022 (Cyp40, Affinity Bioreagents).

Generation of epitope-tagged, recombinant Hsf1

A bacterial expression plasmid-encoding human Hsf1 with an N-terminal epitope tag was generated as follows. First, polymerase chain reaction (PCR) was used to add sequences encoding the FLAG epitope (MDYKDDDDK) at the 5′ end of the open reading frame of a human Hsf1 cDNA (Rabindran et al 1991). The forward oligonucleotide primer (5′-TATCGGCCGCATATGGA-CTACAAGGACGACGATGACAAGGATCTGCCCGTGGGCCCC) contained an NdeI restriction site overlapping an initiation codon, followed by codons for FLAG amino acids and codons 2–7 of Hsf1. The reverse primer (5′-AGTCCCCCTAGAAAGAGAAG-TGGG) was complementary to Hsf1 sequences approximately 700 base pairs downstream from the initiation codon and overlapping a unique BamHI restriction site. Template for the PCR reaction was plasmid Hsf1pBS108 (Rabindran et al 1991).

The 700 bp PCR product was digested with NdeI and BamHI and inserted into the bacterial expression plasmid pET-5A (Novagen). Correct sequence of the PCR product was verified by automated sequencing. The 1.4 kb BamHI/EcoRI digestion fragment from Hsf1pBS108 was subcloned into the engineered pET-5A construct to create a plasmid (pET-5A-Hsf1(2.1))—encoding a full-length Hsf1 with an N-terminal epitope fusion. *Escherichia coli* transformed with pET-5A-Hsf1(2.1) were grown at 23°C to an OD_{600} between 0.6 and 1.0, and FLAG-Hsf1 was induced with 0.4 mM isopropyl–D-galactoside at room temperature for 3 h. Cells were collected by centrifugation and lysed by sonication on ice in 0.1 culture volumes of phosphate buffered saline (PBS) containing 0.3 M NaCl and 1% Tween 20. Disrupted cells were microcentrifuged at 17 000 × *g* for 30 min; the supernatant containing soluble proteins was collected and stored at –80°C.

Baculoviral-mediated expression of ER and Fes constructs in Sf9 cells

The human c-Fes protein tyrosine kinase was expressed as a C-terminal FLAG fusion protein using a baculovirus/Sf9 cell system as described elsewhere (Hjermstad et al 1993). Deletion mutants of Fes lacking the unique N-terminal domain (ΔN), the SH2 domain (ΔSH2), the kinase domain (ΔKin) or the N-terminal and SH2 domains (Kin) were generated by PCR and expressed as C-terminal FLAG fusion proteins in the baculovirus system (Hjermstad et al 1993; Maru et al 1995; Rogers et al 1996). Human estrogen receptor cDNA in a baculoviral vector (Beekman et al 1993) was similarly expressed in Sf9 cells.

Cell extracts were prepared as follows. Sf9 cells were harvested from culture plates and pelleted by centrifugation. Three volumes of lysis buffer (20 mM Hepes, pH 7.8, 400 mM KCl, 20% glycerol, 2 mM dithiothreitol) were added to cell pellets, and the cells were resuspended by pipetting. The suspension was frozen in a dry ice/methanol bath for 5 min and thawed on ice for 30 min. The lysate was mixed and centrifuged to remove debris, and the soluble supernatant was stored at –80°C until needed.

Immunoprecipitations and assembly reactions

For FLAG epitope-tagged antigens, immunoprecipitations were performed with a commercially available affinity resin (M2 resin, Kodak). Since the M2 resin has a high capacity for FLAG-tagged antigens, it was mixed with 9 volumes protein G-Sepharose (Pharmacia) to more readily visualize resin pellets during wash procedures. Other immunoaffinity resins were prepared by preadsorbing the appropriate antibody to protein G-Sepharose. Typically, each SDS-PAGE sample was extracted from a 10 µl resin pellet containing 10 µg antibody.

Immunoprecipitations to isolate target proteins from bacterial or Sf9 cell extracts were performed with gentle rocking at 0–4° for 1 h. Resins were washed 2 times in WB (10 mM Tris, pH 7.4, 50 mM NaCl, 10 mM monothioglycerol, and 1% Tween 20) adjusted to 0.5 M NaCl, then washed 2 times in WB alone.

To examine cell-free assembly of chaperone components with target proteins, the target-resin was added to rabbit RL (1:1 preparation, Green Hectares, Oregon, WI) supplemented with the following, as indicated in the figure legends: an ATP regenerating system (10 mM phosphocreatine plus 50 µg creatine phosphokinase per ml RL); apyrase (10 units per ml RL); or 10 mM EDTA. Assembly reactions were carried out for 30 min at 30° with frequent resin resuspension. Typical proportions in the assembly mixtures were 10 µl resin in 200 µl RL.

In vitro exression and co-precipitation of AhR and Arnt

Plasmids containing cDNA inserts for human AhR (Dolwicki et al 1994) or human Arnt (Hoffman et al 1991) under transcriptional control by SP6 and T7 promoters, respectively, were expressed in a combined in vitro transcription/translation system (TnT lysate, Promega) in the presence of [^{35}S]-methionine according to the manufacturer's recommendations. Aliquots of each synthesis mix were analyzed for levels of incorporated radioactivity by SDS-PAGE and autoradiography. Equivalent amounts of labeled AhR or Arnt mixes were added to RL in the absence or presence of GA (20 µg per ml RL). Specific immunoaffinity resins for p23, p60, Hip, or a negative control were separately added to aliquots of both AhR and Arnt assembly mixes. Samples were incubated for 30 min at 30° with frequent resuspensions. Resin complexes were washed 4 times in WB and analyzed by SDS-PAGE and autoradiography.

Gel electrophoresis and Western immunostaining

Components in immuno-isolated complexes were separated by SDS-PAGE in gels containing 10% acrylamide. Following electrophoresis, total proteins were visualized either by reversible Zn^{2+}-imidazole staining (Fernandez-Patron et al 1992) for subsequent Western immunostaining or by staining with Coomassie brilliant blue R-250, as indicated in the figure legends. Migration positions for individual proteins were marked by small punctures on Zn^{2+}- imidazole-stained gels; the marked gels were then destained in 2% citric acid, equilibrated in transfer buffer and electroblotted to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with PVP buffer (1% polyvinylpyrolidone, 20 mM Tris, pH 7.4, 150 mM

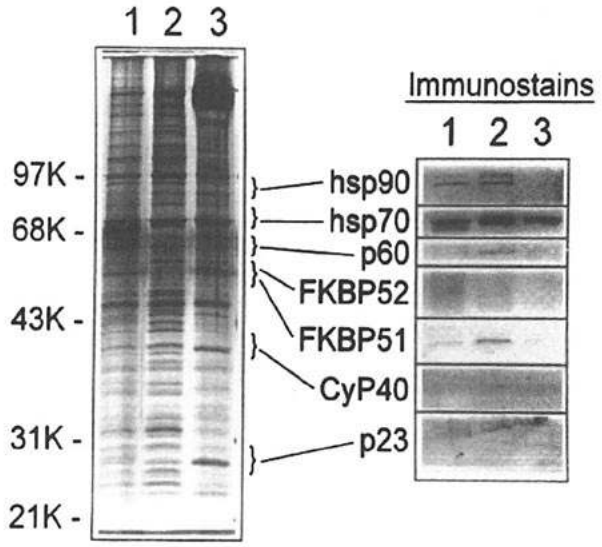
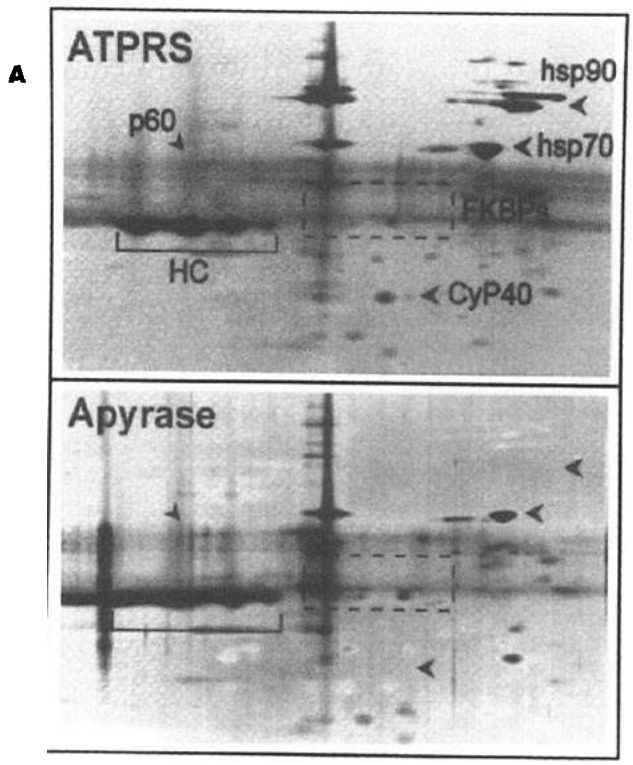


Fig. 1 Binding of chaperone components to control resins. Serum albumin (lane 1), lactalbumin (lane 2), and reduced carboxymethylated lactalbumin (lane 3) were linked to activated Sepharose and treated in rabbit reticulocyte lysate (RL) containing an ATP-regenerating system at 30° for 30 min. Resins were washed and extracted into SDS sample buffer. Proteins bound to each resin were separated by SDS-PAGE and visualized (left-hand panel) and by Western immunostaining (right-hand panel) with antibodies against each of the indicated proteins.

NaCl, 0.1% Tween 20, 0.02% sodium azide), sliced into strips containing individual proteins, and immunostained with specific antibodies. Typical staining reactions consisted of a primary incubation for 2 h at room temperature (RT) in ascites or antiserum diluted 1000- to 2000-fold in PVP buffer. Secondary incubations were for 1 h at RT in a 1:2000 dilution of the appropriate alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG, IgM or rabbit IgG; Southern Biotechnologies Associates, Birmingham. AL). All gel and blot images were digitized by scanning with a laser densitometer (Molecular Dynamics) and edited/annotated with Picture Publisher and Corel Draw software packages.

RESULTS

Relying on in vitro studies to measure chaperone and co-chaperone interactions with a particular target protein, it is important to distinguish chaperone interactions that reflect the presence of non-native, misfolded target protein from interactions occuring with normally folded protein forms. Since each of the following studies relies on resin-immobilized proteins, the binding of chaperone components to different control resins was examined using PR assembly conditions (Fig. 1). Bovine serum albumin (BSA, lane 1), lactalbumin (lane 2), or reduced carboxy-methylated lactalbumin (lane 3) were coupled to CNBr-activated Sepharose. Individual resin samples were incubated at 30° for 30 min in rabbit RL supplemented



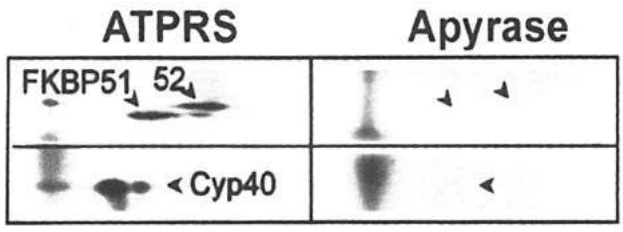


Fig. 2 In vitro assembly of estrogen receptor (ER) complexes. Recombinant human ER was immuno-affinity purified from Sf9 cell extracts and incubated at 30° for 30 min with RL containing and ATP-regenerating system (ATPRS) or treated with an ATPase (Apyrase). ER resin complexes were washed, extracted into urea sample buffer, and separated by two-dimensional PAGE. The basic end of the IEF gel is on the left. (A) Total proteins were visualized by Zn^{2+}-imidazole staining. ER itself does not readily enter the IEF gel, but ER- associated proteins and the anti-ER heavy-chain (HC) are indicated. (B) Immunophilins present in the destained two-dimensional gel were electroblotted to PVDF membrane and detected by Western immunostaining with antibodies specific for FKBP52, FKBP51 or Cyp40.

with an ATP-regenerating system. Resins were washed, extracted with SDS sample buffer, and bound proteins were separated by SDS-PAGE. Proteins were visualized by reversible Zn^{2+}-imidazole staining (left-hand panel) and by Western immunostaining for specific chaperone components (right-hand panel). As is commonly observed in resin-binding assays, Hsp70 and, to a lesser extent, Hsp90 bind non-specifically to each of the resin samples. Note, however, the low non-specific binding observed for p60, the immunophilins and p23. Similarly low levels of non-specific binding were observed with BSA adsorbed to an anti-BSA/protein G-Sepharose resin (not shown) or to various antibody resins lacking an adsorbed antigen (see control lanes in some of the figures below).

Estrogen receptor

Native bovine ER complexes contain Hsp90, Hsp70, FKBP52 (p59), Cyp40 and a protein presumed to be p23 (Ratajczak et al 1993), but in vitro assembly of ER complexes has not been previously examined. In Figure 2, recombinant human ER was immunoisolated from Sf9 cell extracts using protein G-Sepharose preadsorbed with M-17, a mouse monoclonal IgG recognizing an epitope near ER's N-terminus (Neff et al 1994). ER resin was incubated in RL containing an ATP regenerating system (ATPRS) or treated with an ATPase (Apyrase) that blocks assembly of PR complexes (Smith et al 1992). ER resin complexes were washed, and bound proteins were resolved by two-dimensional gel electrophoresis with Zn^{2+}-imidazole staining (Fig. 2A). The presence of immunophilins was verified by Western immunostaining with specific anti-immunophilin antibodies (Fig. 2B). Comparing results in the presence or absence of ATP, the following proteins co-precipitate with ER in an ATP-dependent manner: Hsp90, p60, FKBP52, FKBP51 and CyP40. These results are clearly evident in the gel or blot images shown for each of the proteins except p60. In the upper panel of Figure 2A ('ATPRS'), a faint p60 spot migrates slightly above the keratin bands contaminating the entire width of the gel; though not clear from the reproduced gel image, this spot is absent in the lower panel ('Apyrase'). The three spots seen at about p60's migration coordinates in the lower panel are derived from heavy-chain or other protein that is artifactually streaked in the SDS dimension.

Hsp70 recovery is reduced in the apyrase-treated sample (Fig. 2A); the remaining Hsp70 represents, at least in part, non-specific Hsp70 binding to resin. In addition to the chaperone components shown in Figure 2, p23 also assembled with ER in an ATP-dependent manner (gel region not shown in Fig. 2, but see Fig. 5, lane 5).

Fes kinase

There have been numerous reports that various protein kinases form complexes with Hsp90. Another protein commonly coassociating with Hsp90 in kinase complexes is p50, but this protein has not been observed in steroid receptor complexes. Two of the best characterized kinase–chaperone complexes are those for the Tyr kinase $p60^{v\text{-}src}$ and the Ser/Thr kinase Raf. In in vitro assembly reactions similar to those performed here, Pratt and colleagues have accomplished cell-free assembly of Src (Hutchison et al 1992) and Raf (Stancato et al 1993) with Hsp90 and p50, and they also observed Hsp70 and FKBP52 (Hsp56) in these kinase complexes. To more thoroughly examine a kinase complex for PRAP components, recombinant human Fes, whose viral oncogenic homolog Fps was shown to complex with Hsp90 and p50 (Lipsich et al 1982), was used as a target for in vitro assembly (Fig. 3). Recombinant Fes containing a C-terminal FLAG epitope tag was immunoisolated from Sf9 cell extracts with M2 resin. As shown in Figure 3A, Fes resin (lane 1) was treated in RL containing an ATPRS (lane 2) or containing 10 mM EDTA (lane 3) which chelates Mg^{2+} shown to be needed for PR assembly (Smith et al 1992). As a control, M2 resin lacking Fes was treated in RL plus an ATPRS (Fig. 3, lane 4). Proteins bound to resins were resolved by SDS-PAGE and visualized by reversible Zn^{2+}-imidazole staining (left-hand panel) and Western immunostaining (right-hand panel).

As most readily seen from immunostains, most of the PRAP components, in addition to p50, are recovered with Fes in a Mg^{2+}-dependent manner (compare lanes 2 and 3). Hsp70 recovery was not reduced by EDTA treatment, and p60 recovery was reduced approximately 40% judging from densitometry of the immunostained bands. PRAP components in Fes complexes were similarly sensitive to apyrase treatment (not shown) except that recoveries of Hsp70 and p60 were more greatly reduced than with EDTA treatment. As with other resin controls, little non-specific binding of PRAP components is observed with M2 resin alone (lane 4). Immunostaining of FKBP52 and FKBP51 in samples isolated on M2 resin is complicated somewhat by the co-staining of M2 heavy-chain that leaches from the resin and migrates closely with the FKBPs. M2 heavy-chain staining is most evident in the FKBP51 immunostains where the heavy-chains migrate just above FKBP51.

Hsp90 and p50 interactions have been localized to the kinase domain of v-Src (reviewed in Brugge 1986). To examine whether chaperone interactions with Fes are similarly localized, cell-free assembly reactions were performed with recombinant wild-type and mutant Fes forms containing a C-terminal 9 amino acid (aa) FLAG

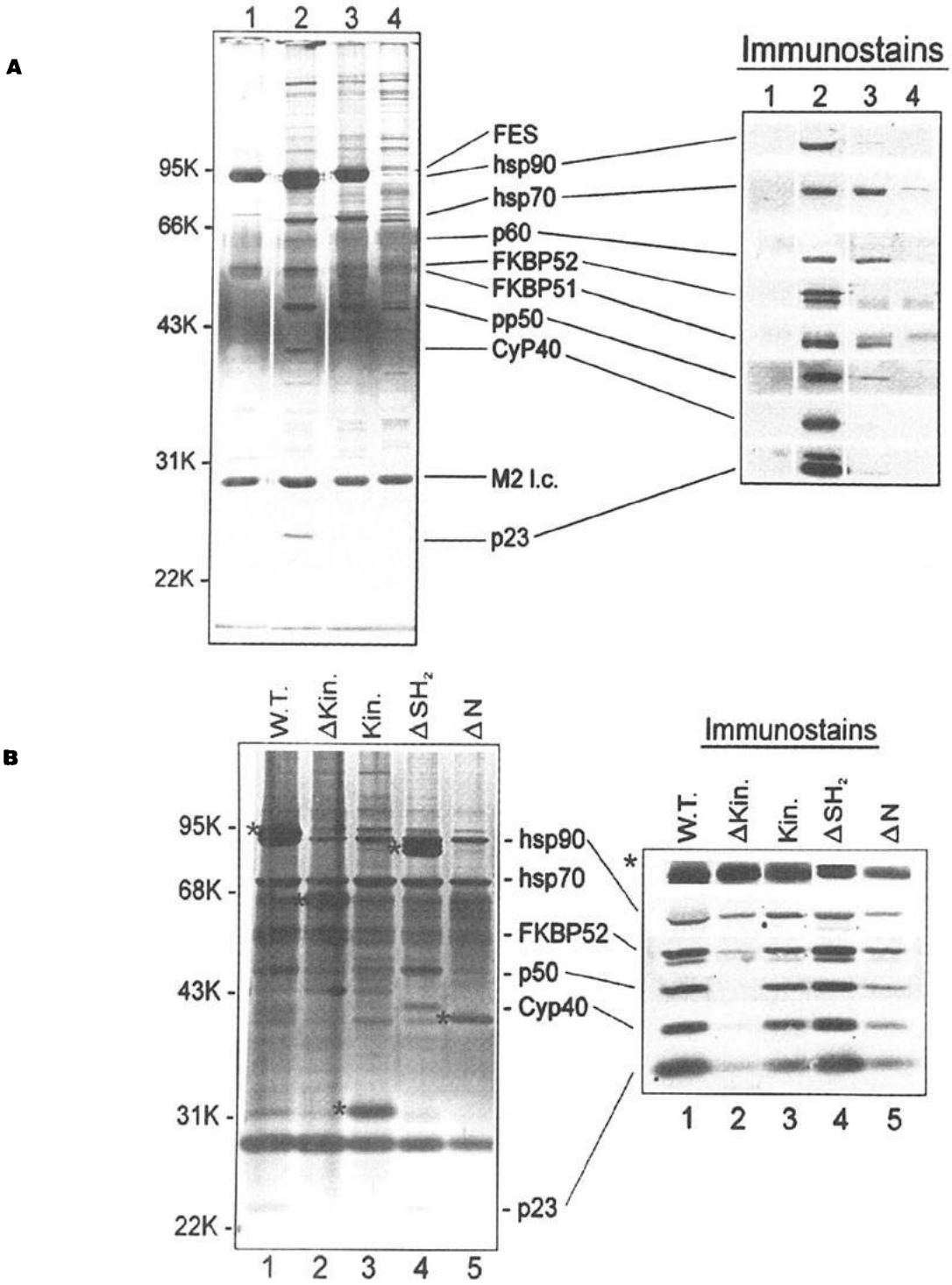


Fig. 3 In vitro assembly of Fes with chaperone components. (A) Recombinant Fes with a C-terminal FLAG epitope tag was immunoaffinity purified from Sf9 cell extract. Fes-resin was untreated (lane 1), or incubated at 30° for 30 min in RL containing either an ATP regenerating system (lane 2) or 10 mM EDTA (lane 3). Antibody resin lacking Fes was similarly incubated in RL plus an ATP regenerating system (lane 4). Resin complexes were washed, extracted into SDS sample buffer, and separated by SDS-PAGE. Total proteins in each sample were visualized by Zn^{2+}-imidazole staining (left-hand panel). The indicated regions of the gel were destained, electroblotted to PVDF membrane and immunostained with antibodies specific for the proteins indicated (right-hand panel). (B) Various mutant forms of Fes were immunoisolated and incubated with RL plus an ATP-regenerating system. Fes forms tested included wild-type Fes (WT, lane 1), a truncation lacking the kinase domain (ΔKin, lane 2), the kinase domain alone (Kin, lane 3), an SH_2 domain deletion (ΔSH_2, lane 4), and an N-terminal truncated product (ΔN, lane 5). Resin complexes were separated by SDS-PAGE and visualized by Zn^{2+}-imidazole staining (left-hand panel) and by Western immunostaining (right-hand panel). Fes forms in the gel and immunostain are indicated with asterisks.

epitope tag (Hjermstad et al 1993; Maru et al 1995; Rogers et al 1996). Fes forms compared were:

1. WT, the 822 aa wild-type protein
2. ΔKin, a truncation mutant (aa 1–540) lacking the C-terminal kinase domain
3. Kin, a truncation mutant (aa 541–822) encompassing the kinase domain
4. ΔSH2, a deletion mutant (aa 451–540 deleted) from which the Src homology 2 domain has been removed
5. ΔN, a truncation mutant (aa 451–822) lacking Fes' unique N-terminal region but containing both the SH_2 and kinase domains. In the Zn^{2+}- imidazole-stained gel (left-hand panel), the relative sizes of Fes forms (*) can be seen; associated proteins were visualized by immunostaining (right-hand panel).

To quantitate differences in protein binding to different Fes forms, the immunostained bands were scanned by laser densitometry. Fes forms were detected by M2 antibody staining of the C-terminal FLAG fusion; since each form contains 1 molar equivalent of FLAG epitope, OD values for associated proteins could be normalized to M2 staining to adjust for the differing molar quantities of Fes protein in each sample. The results are summarized in the Table.

Complexes with ΔKin have no more than 20% WT levels of FKBP52, p50, Cyp40 and p23, but retain about 50% of hsp90 binding. Conversely, the kinase domain alone (Kin) retained 50–90% of WT protein binding levels, indicating that this domain is largely sufficient for the chaperone interactions observed with wild-type Fes. Levels of associated proteins in ΔSH_2 complexes are essentially identical to WT, but levels in ΔN complexes ranged for 60–90% of WT levels. Since ΔN lacks 450 amino acids from the N-terminus of Fes, a partial loss of protein associations due to indirect conformational effects is perhaps not surprising.

Table Recovery of proteins associated with Fes mutant forms

	% of normalized WT level*				
	WT	**ΔKin**	**Kin**	**ΔSH_2**	**ΔN**
Hsp90	100	52	90	90	78
FKBP52	"	21	68	115	87
p50	"	1	71	100	55
Cyp40	"	6	77	100	59
p23	"	18	49	78	67

*Optical density measurements were obtained for the immunostained bands in Figure 3B. Values for Fes-associated proteins were normalized to the level of Fes staining.

Hsf1

Several reports have indicated the presence of Hsp70 (Abravaya et al 1992; Baler et al 1992) in Hsf complexes. Proposals have been made that Hsp70 may serve in an autoregulatory manner to bind and repress Hsf1 when there is limited demand for Hsp70 (reviewed in Morimoto 1993). Whereas this model has appealing features and some experimental support, it has been difficult to demonstrate a direct role for Hsp70 in repressing Hsf1 activity (Rabindran et al 1994; Baler et al 1996).

In vitro interactions between recombinant human Hsf1 containing an N-terminal FLAG epitope tag and PRAP components were examined on a Zn^{2+}-imidazole stained gel (Fig. 4A) and corresponding Western immunostains (Fig. 4B). Hsf1 was immuno-isolated on M2 resin from bacterial cell extract (lane 1) and incubated in RL containing an ATPRS (lane 2) or treated with apyrase (lane 3). M2 resin alone was also incubated with RL plus an ATPRS (lane 4). In the lower panel of Figure 4A, a portion of the gel image was digitally enhanced to highlight the p23 band. Similar to ER and Fes complexes assembled in vitro, all the PRAP components co-precipitate with Hsf1 in an ATP-dependent manner. p50 was not recovered in Hsf1 complexes (immunostain results not shown).

As pointed out with Figure 3, immunostaining for FKBP52 and FKBP51 is complicated by co-staining of M2 heavy-hain. For Figure 4B, the same membrane piece was consecutively stained with anti-FKBP52 then anti-FKBP51. Since the FKBP51 membrane is double-stained, the dominant upper band seen in these lanes is M2 heavy-chain, and FKBP51 is the lower band that is only seen in lane 2.

Since elevated temperature can induce DNA binding by Hsf1 expressed in RL (Sarge et al 1991), temperature effects were examined for changes in the composition of Hsf1 complexes assembled in vitro (Fig. 4C). Hsf1 (left-hand panel) was incubated in RL at 30° (lane 1), as is typical for assembly reactions, and at 37° (lane 2) or 43° (lane 3). In an additional treatment, RL was preheated at 43° for 15 min then returned to 30° for incubation with Hsf1 (lane 4). For comparison, identical conditions were used to assemble PR complexes (right-hand panel). Note that if assembly reactions take place at 43°, both Hsf1 (lane 3) and PR (lane 7) lose association with Hsp90, suggesting that at the highest temperature general assembly with Hsp90 is compromised. However, a distinction between Hsf1 and PR arises when comparing assemblies in preheated RL. Whereas Hsp90 binding to PR is at near normal levels following the 43° pretreatment (lane 8), Hsp90 binding to Hsf1 is minimal (lane 4). This result suggests a heat-dependent alteration of some component in RL that is specifically involved in binding of Hsp90 to Hsf1.

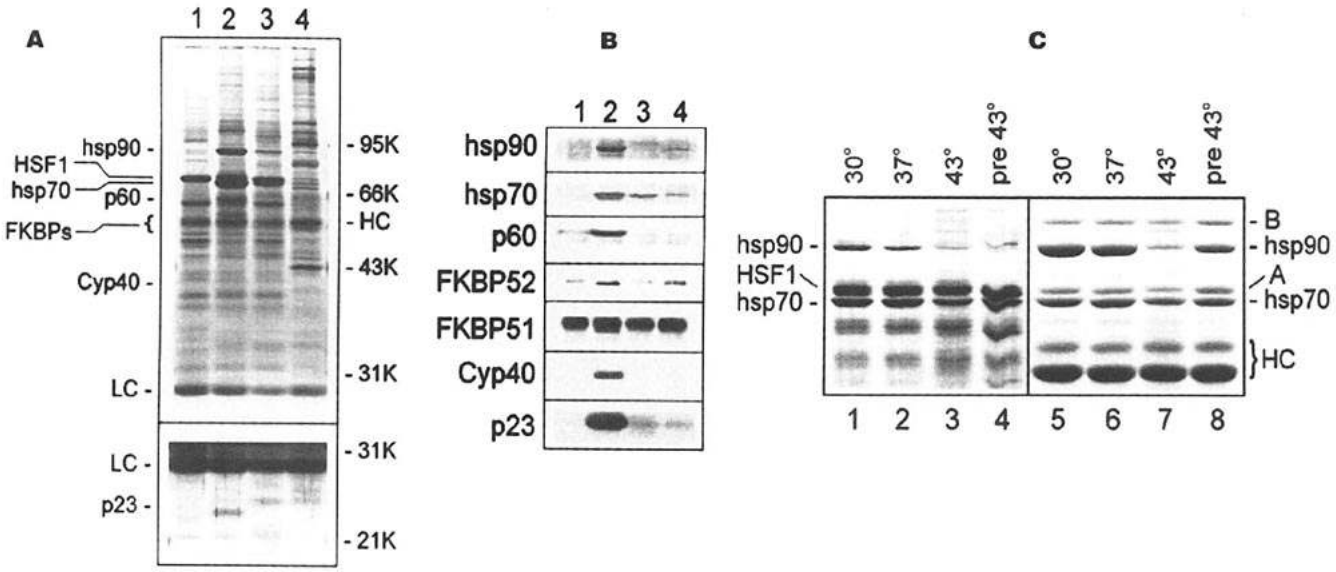


Fig. 4 In vitro assembly of Hsf1 with chaperone components. Recombinant human Hsf1 containing an N-terminal FLAG epitope tag was immunoaffinity purified from Sf9 cell extract. (A) Hsf1–resin was untreated (lane 1) or treated in RL plus an ATP regenerating system (lane 2) or in RL pretreated with apyrase (lane 3). Antibody resin alone was incubated in RL plus an ATP regenerating system (lane 4). Proteins on washed resin complexes were separated by SDS-PAGE and visualized by Zn^{2+}-imidazole staining. In the lower panel, a portion of the digitized gel image was enhanced for visualization of p23. (B) The gel shown in (A) was destained, and proteins were eletroblotted to PVDF membrane. The indicated proteins were visualized by Western immunostaining. (C) Hsf1 (lanes 1–4) and PR (lanes 5–8) complexes were assembled in RL at different temperatures, as indicated above lanes 1–3 and 5–7. For the samples in lanes 4 and 8, RL was preheated at 43° for 15 min and returned to 30° for subsequent incubations. Proteins from washed resin complexes were separated by SDS-PAGE and visualized by Coomassie staining.

Hsp70 synthesis is upregulated in cells treated with either herbimycin A (Murakami et al 1991) or GA (Whitesell et al 1994), two closely related fungal metabolites. Prior speculations on the mechanism for this induction have focused on the apparent ability of these compounds to inhibit some tyrosine kinases. However, the recent discovery (Whitesell et al 1994) that herbimycin A and GA are both Hsp90-binding drugs raises the possibility for an alternate mechanism in their induction of heat shock proteins. In Figure 5, the effect of GA on cell-free assembly of both Hsf1 (lanes 1–3) and ER (lanes 4–6) complexes was examined. Consistent with GA's effects on PR assembly (Smith et al 1995), GA causes

1. a reduction, but not elimination, of target-associated Hsp90
2. complete loss of CyP40 and p23 from both Hsf1 and ER complexes
3. enhanced recovery of p60 in ER complexes.

However, unlike with PR and ER, p60 recovery in Hsf1 complexes appears unaffected by GA treatment (compare lanes 2 and 3). Overall, these results favor the notion that PR, ER and Hsf1 each traverse a similar ordered pathway through the chaperone machinery. Relating specifically to Hsf1, GA's effect on cell-free assembly of Hsf1 complexes and its ability to induce Hsp70 synthesis may be more than coincidental.

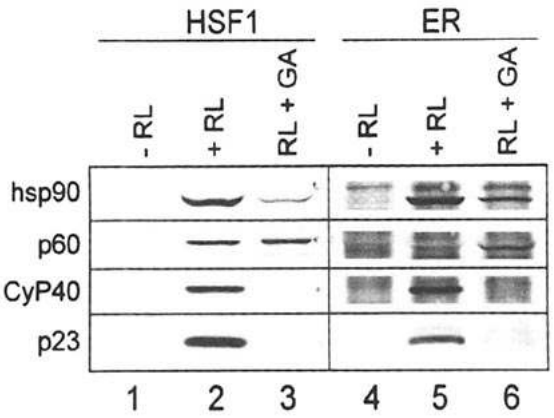


Fig. 5 Geldanamycin (GA) effects on assembly of HSF1 and ER complexes. Immunoisolated Hsf1 and ER were untreated (lanes 1 and 4) or treated in RL plus an ATP regenerating system (lanes 2 and 5) or RL plus 20 mM GA (lanes 3 and 6). SDS-PAGE separations were Western immunostained for the components indicated on the left.

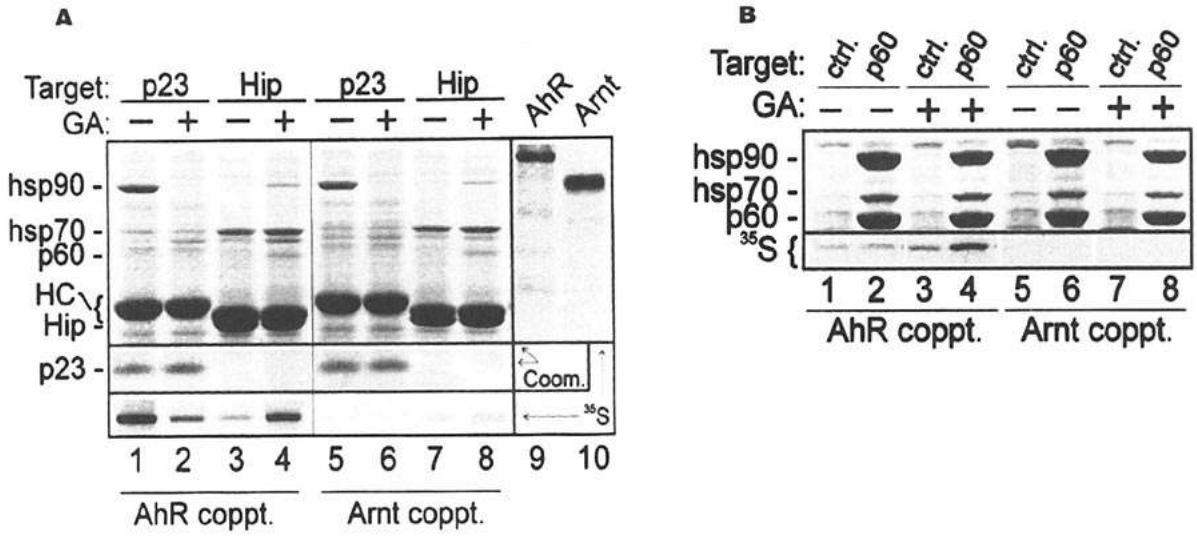


Fig. 6 Aryl hydrocarbon receptor interactions with chaperone components. (A) Plasmids containing a cDNA for either AhR or it partner factor Arnt were expressed in a cell-free transcription/translation system in the presence of [^{35}S]methionine. Aliquots of either synthesis mix was added to RL containing either an ATP regenerating system alone or supplemented with 20 mM GA. During incubations at 30° for 30 min, p23- and Hip-containing complexes were immunoprecipitated from aliquots of each mixture. The precipitates were first examined by SDS-PAGE and Coomassie staining and then by autoradiography, as indicated. Aliquots of the AhR and Arnt synthesis mixtures were also electrophoresed and autoradiographed (lanes 9 and 10). (B) Similar to the results illustrated in (A), RL containing either labeled AhR (lanes 1–4) or Arnt (lanes 5–8) was immunoprecipitated with a control IgG (lanes 1, 3, 5, 7) or anti-p60 antibody (lanes 2, 4, 6, 8). Coomassie-stained gel separations are shown in the upper panel; an autoradiograph from the gel region containing AhR and Arnt is shown in the bottom panel.

Aryl hydrocarbon receptor

A final target for which there is an extensive literature on Hsp90 interactions is the aryl hydrocarbon receptor (AhR). Previous studies have shown that unliganded AhR exists in a complex with Hsp90 (Denis 1988; Perdew 1988), that this association is required for dioxin binding by AhR (Pongratz et al 1992) and that Hsp90 is required for AhR function in vivo (Carver et al 1994; Whitelaw et al 1995). Despite these similarities to steroid receptors, AhR is an unrelated helix–loop–helix class transcription factor. To become an active transcription factor AhR must form a heterodimer with an evolutionarily related protein termed Arnt (reviewed by Hankinson 1994). Interestingly, despite the structural similarities shared by Arnt and AhR, Arnt shows no ligand dependence and displays no interactions with Hsp90.

Lacking sufficient purified protein for studies similar to those presented in earlier figures, an alternate approach was taken to examine whether AhR complexes contain multiple PRAP components. Radioactively labeled AhR was prepared by expressing a human AhR cDNA in a combined cell-free transcription/translation system in the presence of [^{35}S]- methionine. As shown in Figure 6, antibodies recognizing p23, p60 or Hip were tested for their abilities to co-precipitate labeled AhR from RL under various conditions. As a control in these studies, labeled human Arnt was similarly prepared and added to parallel incubations at levels equivalent to AhR.

In Figure 6A, AhR and Arnt-containing samples were immunoprecipitated with either mouse monoclonal antibody JJ3 recognizing p23 or 2G6, a recently developed antibody recognizing Hip (Prapapanich et al 1996). Shown are images of Coomassie-stained gels (pointed out as 'Coom') and autoradiographs of the same gels (bottom panels indicated with '^{35}S') displaying the relative levels of AhR and Arnt in the precipitates. The far right-hand panel (also indicated with '^{35}S') is an autoradiograph of AhR and Arnt synthesis mixes that shows the equivalent levels of AhR or Arnt added to samples and shows the gel mobility of AhR and Arnt relative to other proteins.

The portion of the Coomassie-stained gel image containing p23 is aligned under the larger, cropped gel image. Anti-p23 antibody JJ3 co-precipitates Hsp90 and immunophilins with p23 (Johnson and Toft 1994). As mentioned earlier, GA blocks association of p23 with Hsp90–immunophilin complexes and prevents formation of PR complexes containing p23 and immunophilins. The results of p23 immunoprecipitations in Figure 6A are consistent with earlier findings. Note that GA causes a dissociation of Hsp90 from p23 (lanes 1–2 and 5–6); the FKBPs are not seen since they co-migrate with JJ3 heavy-chain and the Cyp40 gel region has been cropped from the image. As seen in the corresponding

autoradiograph (bottom panel), AhR co-precipitates with p23 in the absence of GA (lane 1) but is greatly reduced in its presence (lane 2). The apparent residual association of AhR with p23 in the presence of GA is somewhat different than PR interactions where the extent of GA-induced disruption of mature PR complexes containing p23 is complete (Smith et al 1995). However, as will be shown in Figure 6B, at least a portion of the residual AhR binding appears to be non-specific. No Arnt co-precipitation with p23 is observed in the presence or absence of GA (lanes 5–6).

Hip interactions with AhR follow the same pattern as observed for PR. First, it has been shown that Hsp90 and p60 can be co-precipitated with Hip/Hsp70 complexes and that the co-precipitation of Hsp90 and p60 are enhanced in the presence of GA (Prapapanich et al 1996). This behavior is again observed in Figure 6A (lanes 3–4 and 7–8); Hip in these lanes is seen migrating just ahead of the 2G6 heavy-chain. AhR co-precipitates with Hip at low levels in the absence of GA (lane 3) but increases in the presence of GA (lane 4). Arnt failed to co-precipitate with Hip under either condition (lanes 7–8) indicating the specificity of Hip's interaction with AhR.

In Figure 6B, co-precipitation of radiolabeled AhR and Arnt from RL with anti-p60 F5 (even numbered lanes) or a control antibody resin (odd numbered lanes) are examined. First, note that a low level of AhR (lanes 1, 3) but not Arnt (lanes 5, 7) appears to bind non-specifically to a control resin. Since AhR is typically bound by Hsp90 while Arnt is not, the greater non-specific binding by AhR may reflect its more disordered structure.

Previous studies have shown that Hsp90 and Hsp70 co-precipitate with p60 (Smith et al 1993c), that each Hsp binds independently to p60 (Chen et al 1996) and that GA has no apparent effect on these interactions (Smith et al 1995; Chen et al 1996). Thus, as expected, a constant pattern of Coomassie-stained bands is observed in all immunoprecipitates (Fig. 6b, upper panel, even numbered lanes). PR in intermediate assembly complexes can be co-precipitated with p60, and this co-precipitation is enhanced by GA (Chen et al 1996). A similar pattern is seen with AhR where co-precipitation in the absence of GA is near background levels (lane 3), but increases substantially in the presence of GA (lane 4).

DISCUSSION

We have hypothesized that distinct Hsp90-associated regulatory proteins may each participate in a highly ordered pathway of chaperone interactions in which Hsp90 is a key component (Smith et al 1995). This pathway is required both in vitro and in vivo in a dynamic manner to establish and maintain PR in a conformation competent for binding hormone. To the extent that direct comparisons have been made, it appears that assembly and maintenance of functional glucocorticoid receptors involves the same chaperone components (reviewed by Pratt 1993) and pathway (Whitesell and Cook 1996), though somewhat different interpretations have been given for chaperone assembly with GR (Pratt 1993; Hutchison et al 1994). In this report, evidence is presented that multiple PRAP components participate in Hsp90 assembly with representatives from various, unrelated classes of regulatory protein. The particular target proteins selected for comparison–ER, Fes, Hsf1 and AhR–are all proteins for which Hsp90 interactions have been previously described and to which Hsp90 association has been ascribed a potential regulatory significance. Using antibodies against individual PRAP components, we demonstrate a strikingly consistent pattern of protein associations in various target protein complexes assembled in vitro.

Similarities of target complexes

In each of the purified target assemblies (ER, Fes, Hsf1), the following associated proteins were identified: Hsp90, Hsp70, p60, FKBP52, FKBP51, CyP40 and p23. Data for the association of p23 with ER and p60 with Fes were obtained but not shown in the figures presented. The evidence for AhR associations was obtained by a different approach and is less comprehensive, but it was shown that antibodies against p23, Hip and p60 co-precipitate AhR in a specific and GA-sensitive manner. Hip interactions with other targets were not examined since suitable antibodies against Hip were only recently developed. In summary, for every PRAP component tested, each can be detected in ER, Fes, Hsf1 and AhR complexes.

As has been observed previously by us and by others, Hsp70 and Hsp90 display non-specific binding to protein-adsorbed resins, so care was taken to distinguish specific and non-specific binding to target resins. Non-specific binding by other chaperone components is usually minimal. In the assays performed here, consideration was given to the possibility that chaperone components detected in association with recombinant proteins may be binding a pool of misfolded protein. However, none of the PRAP components other than Hsp70 displayed significant binding to a commonly studied misfolded substrate, reduced carboxymethylated lactalbumin (Fig. 1, lane 3).

Differences in target complexes

Despite the overall similarity of protein associations, both quantitative and qualitative differences were observed among the target complexes assembled in vitro. The most striking difference is the exclusive presence of p50 in the

Fes kinase complexes that otherwise appear similar to steroid receptor, Hsf1 and AhR complexes. The presence of p50 in Fes complexes is consistent with earlier observations that Hsp90–kinase complexes typically contain p50 while other Hsp90-containing protein complexes do not. As yet, the cloning and sequencing of a p50 cDNA has not been reported. Knowing the amino acid sequence of p50 should provide some clues to its function and perhaps to its preferred interaction with kinases.

A general difference between PR complexes and those that we examined here is the much greater extent to which PR is associated with chaperone components. Although the same assembly pathway appears to be involved with PR and other target proteins, a significant proportion of each target pool appeared to be free from chaperone complexes. In part, this may reflect differences in the stability of target complexes and differences in their tendencies to dissociate during wash procedures. The lower saturation of chaperone binding to targets in the cell-free assembly system may not accurately reflect target distribution in vivo. In the native cellular context, each target protein is probably present at much lower levels relative to chaperone components while the concentration of cytoplasmic constitutents is probably 2- to 3-fold higher than in RL. A further distinction is that RL lacks normal cellular components such as cytoskeleton and membranes that may alter target–chaperone interactions. Thus, we cannot conclude that the target complexes assembled in vitro faithfully represent the composition and distribution of complexes which may exist in vivo. It is noteworthy, though, that in vivo assessments of PR hold up well in comparison with the composition and dynamics of PR complexes assembled in vitro (Smith et al 1995).

The dynamics of in vitro chaperone interactions with ER, Fes, Hsf1 and AhR have not been studied in as much detail as for PR. However, it seems reasonable to propose, given the compositional similarity of various target complexes and the effects of GA on these complexes, that interactions follow the same order as that observed for PR:

1. Hsp70 and perhaps Hip
2. Hsp90–p60–Hsp70 and Hip
3. p23–Hsp90–immunophilins.

However, it does not seem necessary that chaperone interactions with alternate targets proceed with identical dynamics as PR assembly. Based on estimated exchange rates during initial and steady-state assembly of PR complexes, the most stable PR complex is the final one with p23–Hsp90–immunophilin; intermediate complexes containing Hsp70, Hip, p60 and Hsp90 turn over more rapidly. For any particular target in this pathway, the relative stability of complexes at different stages of assembly may diverge from PR dynamics. Little is presently known about the mechanisms that dictate the ordered exchange of chaperone components in this system.

Potential adaptive significance of extended chaperone interactions

An unanswered question from these studies is what function Hsp90 and other assembly components serve in associating with a target protein. As discussed above, it does not seem likely that the multiple chaperone interactions are strictly related to protein folding, since

1. the target proteins do not appear to be generally misfolded
2. commonly studied misfolded substrates such as reduced carboxymethylated lactalbumin do not readily bind the full range of PRAP components.

It is important to recognize, however, that binding of Hsp90 and other PRAP components to PR is not stable at physiological temperatures, but occurs dynamically during steady-state passage of PR through the assembly pathway. Preliminary in vitro studies of Hsf1 and Fes assembly suggest that these complexes are in a similar dynamic state (results not shown). Therefore, as is typical of chaperone interactions, Hsp90 and other assembly components transiently bind to and release from the target protein.

If the chaperone components are behaving normally by displaying transient interactions, then it follows that the 'substrate' protein undergoing continued rounds of chaperone binding must be resistant to changes (folding) that would obviate subsequent recognition by the chaperone machinery. In an earlier report on PR's extended interactions with the chaperone machinery (Smith 1993), it was proposed that PR's structure is specifically adapted for the purpose of remaining in the chaperone machinery for extended periods until hormone binding triggers a conformational change that promotes PR's release from chaperone components. Arguments were presented that extended chaperone interactions, while necessary to maintain the hormone binding ability of PR at elevated temperatures, in fact were originally adapted to repress PR's potential DNA-binding and transcriptional interactions in the absence of hormonal signal.

This proposal bears repeating and generalizing in the context of multiple targets that appear to share a common, repeated set of interactions with chaperone components. The evolutionary rationale for the establishment of PR's extended interaction with the chaperone machinery is based on the presumed passage of a PR precursor through the chaperone machinery in the normal course of nascent chain folding. Pre-existing, but limited, interactions with the chaperone machinery would have afforded a PR precursor the opportunity to extend these

interactions in a regulable manner by retaining a 'misfolded' feature in its ligand-binding domain that is sensitive to hormone binding. Indeed, the binding of all PRAP components is localized to PR's ligand-binding domain.

Generalizing this proposition, any protein passing through the chaperone machinery would be afforded a similar chance to opportunistically co-opt the chaperone machinery for some adaptive purpose. To remain in the chaperone machinery, a protein may simply need to retain a single, perhaps subtle, misfolded feature from its terminal stage of folding. It seems advantageous for extended chaperone interactions to remain transient since this would allow the target access on a regular basis to other cellular components.

A target involved in extended chaperone interactions could be released in any of several ways: a conformational change due to ligand binding, altered phosphorylation state, presence of an interacting macromolecule that effectively competes for chaperone interactions, or others. Some possible functions that would adaptively favor extended chaperone interactions are listed and briefly discussed below.

1. Repression of the target protein's activity. An example would be the proposed repression of PR's DNA-binding ability. The most stable PR complex in the absence of hormone contains an Hsp90 dimer, one immunophilin of 40–50 kDa and p23. The same PR-associated components can be readily identified in a freely existing complex of about 250 kDa. Such a complex bound to a target protein of less than 100 kDa could reasonably be expected to sterically hinder actions by the target.
2. Protection of the target from proteolysis. The most active conformation for a protein may be one that renders the protein susceptible to proteolytic machineries in the cell. Shielding such a protein through chaperone interactions could provide a means for extending the protein's half-life without lessening its activity. Perhaps by coincidence, PR turns over more rapidly in vivo after hormone-dependent activation (Sullivan et al 1988). The increased degradation of PR could contribute to the off-signal following hormone-induced events and thus may be adaptive for progesterone signaling. In yeast cells, Hsp90 is required for proteolytic stabilization of v-Src (Xu and Lindquist 1993) though loss of Hsp90 function did not promote proteolysis of transfected GR (Nathan and Lindquist 1995). In mammalian cells, the Hsp90-binding drug GA has been shown to enhance in vivo degradation of v-Src (Whitesell et al 1994) and glucocorticoid receptor (Whitesell and Cook 1996) correlating with GA's ability to alter chaperone interactions with these targets.
3. Dynamically docking a protein to regulate oligomerization. Each of the target proteins examined here exist in oligomeric complexes in their active state: steroid receptors form homodimers, AhR a heterodimer with Arnt, Hsf-1 a homotrimer, and Fes appears to form a homotetrameric complex (T. E. Smithgall, unpublished observation). Docking of target proteins to Hsp90 has been widely considered, though normally interpreted in the context of a stable Hsp90 interaction with the target. In principal, docking is still a viable concept, but should probably be considered in terms of dynamic, complex interactions with the chaperone machinery.
4. Providing phenotypic diversity in a given target by stabilizing alternate conformational states. As suggested by Susan Lindquist (personal communication), multiple and extended chaperone interactions could be a mechanism to transiently stabilize a target in an otherwise unstable, short-lived conformation that may have functional properties distinct from a more stable conformation. The observation that PR passes through an ordered progression of chaperone complexes, and the suggestion from evidence presented here that other targets do likewise, implies that the target may be conformationally altered in a progressive manner.

Whereas we have not provided mechanistic details for the function of chaperone interactions with any of the target proteins analyzed here, we do feel it is important to recognize that various important regulatory proteins for which Hsp90 interactions have been described, and perhaps other targets that have not been carefully examined may traverse a common pathway of the chaperone machinery. A general adaptive potential for extended interactions in this pathway is the layering of an additional level of control over signaling pathways.

ACKNOWLEDGEMENTS

The following individuals are gratefully acknowledged for providing reagents: David Toft, Mayo Clinic–antibodies AC88, BB70 and JJ3; Gary Perdew, Pennsylvania State University–anti-p50 antibody; Martin Deibel, The Upjohn Company–UPJ56 antiserum; Richard Miksicek–M17 antibody; Robert Kingston, Massachussets General Hospital–Hsf1 cDNA; Christopher Bradfield, Northwestern University–AhR cDNA; Oliver Hankinson, University of California at Los Angeles–Arnt cDNA; and Ming-Jer Tsai, Baylor College of Medicine–ER baculoviral vector. The authors also express their appreciation for technical assistance in these studies provided by Viravan Prapapanich and Vera Bariss. This work was supported by NIH grants DK44923 (DFS), DK48218 (DFS), and CA58667 (TES).

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