The aryl hydrocarbon receptor (AhR) has been shown to interact with an immunophilin-like molecule known as AhR-interacting protein (AIP) and to enhance AhR function. We show here that AIP associates with AhR homologues from mouse and fish, which can bind ligands such as dioxin, but nonligand binding homologues from Caenorhabditis elegans or Drosophila do not bind to AIP. However, a minimal ligand-binding domain of the AhR is incapable of binding AIP. The binding of AIP to AhR in reticulocyte lysate shows several of the characteristics of an hsp90-dependent process, including sensitivity to geldanamycin and temperature and a requirement for ATP or nonhydrolyzable analogues. Purified AIP binds to the C terminus of hsp90, and mutation of a conserved basic residue in the tetra-tricopeptide repeats of AIP (K266A, analogous to K97A in protein phosphatase 5) abolishes binding to hsp90. Mutation of K266A in AIP reduces binding to AhR by 75–80%; the geldanamycin sensitivity of this complex shows that AhR stabilizes the AIP-hsp90-AhR complex. The α-helical C terminus of AIP, which is outside the tetra-tricopeptide repeat domain, is absolutely required for binding to AhR as shown by deletions of the C-terminal 5 amino acids or alanine-scanning mutagenesis, but it is not required for binding of AIP to hsp90. The data support a model where 1) AIP binds to both hsp90 and AhR; 2) hsp90 is required for AhR-AIP binding; and 3) the binding of AhR to AIP stabilizes the AIP-hsp90-AhR complex.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent of a wide range of agonists for the aryl hydrocarbon receptor (AhR) (1). Activation of the AhR by TCDD leads to a wide variety of responses, including acute toxicity, teratogenicity, and cancer (1, 2). The AhR mediates and is required for all the known responses to TCDD (3, 4), and so it is important to understand how the AhR functions. The AhR is constitutively cytoplasmic, bound to hsp90. Upon binding TCDD, it translocates to the nucleus, where hsp90 is removed and a transcriptionally active AhR-arnt complex is formed. The activated, nuclear AhR is subject to controlled degradation (5–7); the mechanisms controlling production of the functional, ligand-binding AhR and its subsequent activation are much less clearly defined.

It has recently been shown that the AhR associates with a novel protein, known as AIP (8), XAP2 (9, 10), or Ara9 (11); this protein was shown to interact with AhR in a yeast two-hybrid assay (8, 11) and also in mammalian cells (9). AIP appears to enhance the sensitivity and magnitude of ligand-induced signaling through the AhR in yeast (11, 12) and in mammalian cells (8, 13). However, it is unclear how AIP enhances AhR function.

AIP possesses similarity to the immunophilins, notably FKBP52. Several of the immunophilins, including FKBP52, are known to bind to hsp90 and are co-chaperones that are associated with nuclear hormone receptors (14). Some of the characteristic features of the hsp90-immunophilin assembly system include dependence on cation (15), temperature and ATP (16) and sensitivity to the specific inhibitor of hsp90, geldanamycin (17–19). Thus one might expect AIP to possess similar properties by analogy to the immunophilins. However, unlike FKBP52, AIP does not bind to FK-506 or rapamycin (11), and there is conflicting evidence about the ability of AIP to bind to hsp90. AIP has been shown to associate with hsp90 in mammalian cells (8) but to bind poorly to hsp90 (12) or not at all (9) in reticulocyte lysates in the absence of the AhR. It is difficult to reconcile these observations, because variation in temperature (16), salt conditions (15), or other conditions used in these papers are critical for the function of hsp90.

We set out to characterize the nature of AIP-AhR binding as a prelude to examining the function of this interaction. We show that, in reticulocyte lysate, AIP-AhR interactions show characteristics of an active process requiring hsp90, we demonstrate a direct interaction between AIP and hsp90, and we show that distinct regions of AIP are required for interaction with hsp90 and AhR, respectively.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The murine AIP plasmids pAIP/GEX4T3 and pAIP/ET28a were described in Ref. 8. For construction of pGEX-AIP1–325, the insert of pAIP/ET28a was amplified with primers 5′-TCAGTGA{TACA}TGGAGGATC{TAC}TGGAAACTGCGAGACAGG-3′, where the sequence in bold type is a BclI site, and 5′-TCAGTGGACTGCAGTCAC{CGGAAAGCAGAGTTT}-3′, where the sequence in bold type identifies an Xhol site and the introduction of a stop codon. The PCR product was cloned into pGEM-T Easy (Promega), excised with BcI and Xhol and subcloned into pGEX-4T3 (Amersham Pharmacia Biotech) digested with BsmI and Xhol. The construct was confirmed by sequencing.
A functional ligand-binding domain of AhR is required but not sufficient for AhR-AIP interaction. A. murine b-1 AhR, C. elegans AhR, Drosophila spinless, F. heteroclitus AhR2, and murine AIP were translated in a reticulocyte lysate system in the presence of [35S]methionine, and the reaction products were incubated together for 1 h at 30 °C. Samples were then immunoprecipitated with an α-T7 tag antibody, run on 10% NuPAGE gel, and phosphorimaged. The vertical bar indicates that tracks 1–3 and 4–7 were run on different gels. The positions of AhR proteins are shown by arrowheads, and AIP is shown by an arrow. M denotes 14C-labeled marker (Amersham Pharmacia Biotech). The AIP construct contains a T7 tag sequence. B. AIP and the AhR ligand-binding domain or the ligand-binding domain by itself were incubated together at the indicated temperature (Temp), followed by immunoprecipitation with α-T7 tag antibody, as described for A. The two right-hand lanes show 100 and 20% of the input AIP and AhR ligand-binding domain proteins, respectively.

Murine AhR b-1 allele cDNAs were previously described (20). The construct pRSET.LBD containing a minimal AhR ligand-binding domain, amino acids L229-P416, was prepared by subcloning the AhR b-1 cDNA into the cloning vector pRSET (Invitrogen). The fragment was amplified by PCR using oligos 5′-GGAGATTACCTCGAGATTTAGGGA-ATTCC-3′, and 5′-GGGTTGATTAGGTAAGGGATCTTTAGGAGGAAA-3′; the BamHI sites are shown in bold type. Mutagenesis was performed with the Promega Gene Editor kit, as described in the manual. Subclones were confirmed by sequencing on both strands. The human hsp90β construct, pET-28a(+), was a kind gift of Professor T. Ratajczak (21).

Protein Expression and Purification—pET and pGEX plasmids were transformed into Escherichia coli BL21 RIL (DE3) and E. coli BL21 (Stratagene), respectively, for expression studies. Bacteria were grown at 37 °C to an A600 nm of 0.6–1.0, and isopropylthigalactoside was added to a final concentration of 1 mM. Induction continued at 37 °C for 2 h, and bacteria were collected by centrifugation. Isolation of histidine-tagged proteins was as described (Invitrogen technical manual), and GST-tagged proteins were isolated as described (Amersham Pharmacia Biotech GST technical manual); samples were dialyzed against 100 mM KCl, 10 mM Tris, pH 7.4, 1 mM DTT, centrifuged to remove particulate matter, and stored at 4 °C. Protein concentration was determined by a Bradford assay (Pierce), using bovine serum albumin as the standard.

Samples were subjected to 10% polyacrylamide gel electrophoresis using the conditions described (Novex technical manual).

PCR of AIP—For transcription and translation of AIP C-terminal mutants, AIP was prepared by PCR using pAIP/ET28a as the template. The 5′ primer, 5′-AGATCTCGATCCGGAAGAATTAATACGACT-3′, included the T7 promoter of the pET28 vector. The 3′ oligonucleotides are named by the amino acid at the site of truncation as follows; 260, 5′-TCATGGGGAAGAATTAATACGACT-3′; 320, 5′-TCATCCGGCAGGTTCGATCCGGAAGAATTAATACGACT-3′; 325, 5′-TCATGGGGAAGAATTAATACGACT-3′; and 325, 5′-TCATGGGGAAGAATTAATACGACT-3′.

The samples were supplemented with either 2 mM ADP, 2 mM ATP plus 10 mM phosphocreatine plus 14 units/ml creatine kinase, or 1 mM ATP; 5 plus 10 mM phosphatase plus 14 units/ml creatine kinase, 2 mM ATP plus 10 mM sodium molydate plus 10 mM phosphocreatine plus 14 units/ml creatine kinase, or no supplement; incubation was for 1 h at 30 °C. Samples were placed on ice, and 0.8 μl of α-T7 tag antibody (Novagen) was added in 200 μl of ice-cold 20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Igepal CA-630, 5 mM EDTA (wash buffer); the samples were incubated with agitation for 1 h at 4 °C. Fifty μl of a 50% slurry of Sepharose CL-4B-protein A conjugate (Amersham Pharmacia Biotech) was added, and incubation continued for a further hour. Each sample was washed four times with 200 μl of ice-cold wash buffer at 4 °C. The aqueous buffer was aspirated, and the protein was eluted into 15–20 μl of 1X reducing sample load buffer (Novex) by heating at 70 °C for 10 min. Samples were then run on a 10% NuPAGE polyacrylamide gel in MES buffer, maintained at 4 °C. The gel was fixed and dried, and samples were analyzed with a Molecular Dynamics PhosphorImager or Storm.

Protein-Protein Interactions—GST, GST-AIP, and hsp90/b530–724 proteins were expressed and purified as described under “Experimental Procedures”; BSA was from Pierce. The indicated amount of protein was incubated in 200 μl of 10 mM KCl, 10 mM Tris, pH 7.4, 1 mM DTT for 1 h at 4 °C, with continuous agitation. 20 μl of a 50% slurry of GST-Sepharose CL-4B/CL-4B beads in the same buffer was added, and incubation continued at 4 °C for a further hour. The samples were washed once with buffer containing 0.2% Igepal CA-630 and three times with buffer. For nickel affinity chromatography, proteins were incubated in a buffer containing 100 mM KCl, 10 mM Tris, 45 mM imidazole, pH 7.4, adsorbed to nickel-nitrioltriacetic acid beads for 1 h and washed as above. Proteins were eluted from the beads with 15–20 μl of reducing load buffer (Novex) by incubation at 70 °C for 10 min, followed by electrophoresis on 10% NuPAGE gel with an MES buffer, at 4 °C. Gels were stained with Coomassie Blue, dried, and scanned.

RESULTS

The AIP construct, pAIP/ET28a, has a N-terminal T7 tag sequence, which was efficiently recognized by an anti-T7 tag monoclonal antibody in an immunoprecipitation assay (Fig. 1). The use of Sepharose beads lacking protein A or an irrelevant monoclonal antibody did not yield any specific immunoprecipitation of the T7-tagged AIP protein (data not shown). AIP and the murine AhR b-1 allele were translated separately in a coupled reticulocyte lysate transcription/translation system in the presence of [35S]methionine. The AhR and AIP translation reactions were mixed and incubated at 30 °C for 1 h, prior to the addition of antibody. Fig. 1A shows that the anti-T7 tag antibody failed to immunoprecipitate the AhR protein by itself. When the T7-tagged AIP was mixed with AhR, both proteins were immunoprecipitated, demonstrating the presence of a complex containing both AhR and AIP. Specific interaction between AIP and AhR was confirmed by showing that AhR bound to a glutathione S-transferase-AIP fusion protein immobilized on glutathione-Sepharose CL-4B, whereas glutathione S-transferase showed minimal interaction with AhR (data not shown).
immunoprecipitated with an a-T7 tag antibody, run on a 10% NuPAGE gel, and phosphorimaged. The two tracks marked input show 100 and 20% of the input AIP and AhR, respectively. The position of AhR proteins is shown by an arrowhead, and AIP is shown by an arrow. B, the data in Fig. 1A were quantified on a PhosphorImager, and plotted with the y axis showing the amount of radioactivity in the AIP band in arbitrary units. The open circles are the total amount of radioactivity in the AhR band in the samples containing both AhR and AIP; the gray circles are the radioactivity in the samples containing AhR alone. The filled circles represent specific binding of AhR to AIP.

FIG. 2. Effect of temperature on AhR-AIP interaction. A, murine b-1 AhR and T7-tagged AIP were translated in a reticulocyte lysate system, and the reaction products were incubated in the indicated combination for 1 h at the indicated temperature (Temp). Samples were then immunoprecipitated with an a-T7 tag antibody, run on a 10% NuPAGE gel, and phosphorimaged. The two tracks marked input show 100 and 20% of the input AIP and AhR, respectively. The position of AhR proteins is shown by an arrowhead, and AIP is shown by an arrow. B, the data in Fig. 1A were quantified on a PhosphorImager, and plotted with the y axis showing the amount of radioactivity in the AIP band in arbitrary units. The open circles are the total amount of radioactivity in the AhR band in the samples containing both AhR and AIP; the gray circles are the radioactivity in the samples containing AhR alone. The filled circles represent specific binding of AhR to AIP.

We examined the effect of nucleotides on AIP-AhR interactions to determine whether AIP-AhR interactions are ATP-dependent processes. After translation of AIP and AhR cDNAs, the reticulocyte lysates were extensively dialyzed by centrifugation using a Centricon column with a molecular mass cut-off of 10 kDa (19). The indicated nucleotide was then added to the mixture, and AhR-AIP interactions were assayed by co-immunoprecipitation with AIP. The process of diafiltration reduced AhR-AIP interactions (Fig. 3A) compared with the nondiafiltered control tracks (compare lanes 1 and 9); the residual binding in diafiltered lysates varied between 5 and 15%, depending on the batch of lysate. This shows that AhR-AIP interactions require a small molecular mass molecule or molecules. ADP provided a small increase in AIP-AHR interactions, but 2 mM ATP restored AIP-AHR interactions (Fig. 3). The nonhydrolyzable ATP analogue, ATPγS, further enhanced the interaction between AIP and AhR, but the combination of ATP and 10 mM sodium molybdate had no further effect over ATP. Thus ATP is necessary for AIP-AHR interactions and can be substituted by nonhydrolyzable analogues.

To characterize the requirement for ATP in AIP-AHR interactions, we used a specific inhibitor of hsp90, geldanamycin, which is a ligand for the hsp90 ATPase domain. Reticulocyte lysates containing AIP or AhR translation products were mixed and incubated in the presence of geldanamycin in Me2SO, or the vehicle, followed by immunoprecipitation. Geldanamycin was a potent inhibitor (Fig. 4A), showing almost total inhibition of AIP-AHR interactions at 1 μg/ml, in good agreement with previous data for the inhibition of hsp90-p23 interactions (19). Me2SO had no significant effect on the co-immunoprecipitation of AhR. AhR is rapidly degraded in cells after addition of geldanamycin (26), but AhR was stable in the presence of geldanamycin in reticulocyte lysates (Fig. 4B), demonstrating that the reduced co-immunoprecipitation of AhR with AIP is not due to degradation of the AhR. The known specificity of geldanamycin as a ligand of the ATP-binding site of hsp90 provides evidence that an ATP-dependent step in AIP-AHR interactions is catalyzed by hsp90 during the process of assembly of an AIP-AHR chaperone complex.

Figs. 2–4 provide evidence for a role of hsp90 in AIP-AHR interactions. To test whether AIP directly binds to hsp90, as opposed to through some intermediary protein in a complex, AIP was expressed as a glutathione S-transferase fusion protein (8) and purified. The C terminus of hsp90 has been shown to bind to several immunophilins containing tetra-tricopeptide repeats (21, 27); the C-terminal hsp90 fragment hsp907530–724 (21) was expressed and purified. As shown in Fig. 5, the hsp90 C-terminal fragment binds to the GST-AIP fusion protein (compare lanes 5 and 9) but does not bind to GST alone.

2 D. R. Bell and A. Poland, unpublished results.
FIG. 3. Effect of nucleotides and analogues on AhR-AIP co-immunoprecipitation. A, immunoprecipitations of T7-tagged AIP with an α-T7 tag antibody, as described for Fig. 1A, except that the co-incubation of AhR (6 μl) and AIP (2 μl) was made up to 40 μl with unprogrammed lysate, and samples contained either 2.5% Me₂SO, or 2.5% dimethyl sulfoxide (DMSO) containing geldanamycin (GA) at final concentrations of 5, 1, 0.2, and 0.04 μg/ml, respectively. B, AhR was translated in a reticulocyte lysate in the presence of [35S]methionine, and aliquots of lysate were run on PAGE, dried, and visualized by phosphorimaging. The track marked GST-AIP was incubated with 15–20 μl of a 50% slurry of GST-agarose/CL-4B beads in the same buffer was added, and incubation continued at 4 °C for a further hour. The samples were washed once with buffer containing 0.2% Igepal CA-630 and three times with buffer. Proteins were eluted from the beads with 15–20 μl of reducing load buffer (Novex), and incubation continued at 70 °C for 10 min followed by electrophoresis on 10% NuPAGE gel with an MES buffer, at 4 °C. The left-hand panel is from a separate gel; 19 and 4.6 μg of GST were incubated alone or with 13 μg of hsp90β530–724. The right-hand panel is from a separate gel; 19 and 4.6 μg of GST were incubated alone or with 13 μg of hsp90β530–724. Solutions containing 10 mM KCl. The positions of the GST, GST-AIP, and hsp90β530–724 proteins were indicated.

FIG. 4. Geldanamycin inhibits the formation of AIP-AhR complexes. A, immunoprecipitations of T7-tagged AIP with an α-T7 tag antibody, as described for Fig. 1A, except that the co-incubation of AhR (6 μl) and AIP (2 μl) was made up to 40 μl with unprogrammed lysate, and samples contained either 2.5% Me₂SO, or 2.5% dimethyl sulfoxide (DMSO) containing geldanamycin (GA) at final concentrations of 5, 1, 0.2, and 0.04 μg/ml, respectively. B, AhR was translated in a reticulocyte lysate in the presence of [35S]methionine, and aliquots of lysate were incubated for 1 h at 4, 30, or 30 °C with the addition of 2.5% dimethyl sulfoxide (DMSO) alone or 2.5% dimethyl sulfoxide containing geldanamycin (GA), at a final concentration of 5 μg/ml. Samples of lysate were run on PAGE, dried, and visualized by phosphorimaging. (compare lanes 1 and 3), demonstrating that the interaction is between AIP and hsp90β530–724. The interaction was specific: bovine serum albumin failed to bind to the GST-AIP protein (lanes 8). The binding between AIP and hsp90β530–724 was equivalent in the presence of NaCl (lane 10) or KCl. Thus AIP binds specifically to the C-terminal region of hsp90β. Neither magnesium ions (2–5 mM) nor 5 mM ATP, CTP, GTP, UTP, or ADP had any effect on the binding of AIP to hsp90β530–724 (data not shown) (28).

To test whether the capacity to bind hsp90 is required for AIP function, we made use of the known structure of the related tetra triopeptide repeat protein, protein phosphatase 5 (29), and the identification of amino acids that are putative contact residues for hsp90 (30). As shown in Fig. 6A, two conserved basic residues in the putative substrate contact groove in protein phosphatase 5 (Lys97 and Arg101), are conserved in FKBP52 and AIP (Lys366 and Lys270, respectively). Lysine 266 in AIP was mutated to alanine and expressed as a GST fusion protein. The purified protein failed to bind the hsp90 C-terminal fragment (Fig. 6, B and C), although wild type AIP bound the hsp90 C-terminal peptide (Fig. 6, B and C).
The Role of hsp90 in AIP-AhR Interactions

AIP was originally shown to bind to AhR in yeast two-hybrid assays and thereby to enhance AhR function in cells (8, 9, 11). However, the detailed mechanisms whereby AIP binds to AhR and enhances its function have remained unresolved. We demonstrate that the formation of an AhR-AIP complex shows several features that are characteristic of an hsp90-dependent process and have defined distinct regions of the AIP protein, which are involved in binding with either hsp90 or the AhR protein.

The murine AhR associates with human, mouse, and simian AIP (8, 9, 11) in a reticulocyte lysate system. We have shown that mouse AIP binds to the AhR2 from F. heteroclitus, which

and Fig. 5). The GST-AIP fusion proteins were tested for copurification with AhR using a glutathione affinity procedure (Fig. 6D). The GST-AIP fusion bound AhR, but glutathione S-transferase by itself failed to interact with AhR. This binding was specific, because it required the presence of the glutathione affinity matrix on the Sepharose CL-4B beads, and no binding was seen in the absence of radiolabeled AhR. Mutation of AIP at K266 consistently reduced the ability of AIP to bind to AhR by 75–80% (Fig. 6D); this binding was hsp90-dependent, because geldanamycin markedly inhibited the binding of AhR to GST-AIP under these conditions (data not shown).

We next sought to define specific regions of AIP that are required for AIP-AhR interactions. C-terminally deleted mutants of AIP were designed to remove the last tetratricopeptide repeat or regions of the C terminus after the repeat sequences (Fig. 7A). The mutants were translated in a reticulocyte lysate system and assayed for their ability to bind to AhR. All of the mutants yielded adequate levels of translation product, but none were able to co-immunoprecipitate AhR (Fig. 7B). An AIP mutant truncated at amino acid 325, lacking only the last 5 amino acids, failed to co-immunoprecipitate AhR, even when present at higher levels than the full-length AIP (Fig. 7C). The last 5 amino acids of AIP appear to be essential for AIP-AhR interactions. To complement the approach of C-terminal deletion, alanine-scanning mutagenesis of the last 5 amino acids (325-GIFS) was undertaken. Replacement of amino acids 327–330 with alanine resulted in almost complete loss of binding to AhR; however, replacement of Gly326 with alanine yielded a mutant that retained partial immunoprecipitation of AhR (Fig. 7D). The C terminus of AIP is required for AIP-AhR interactions, and replacement of any of the last four amino acids ablates AIP-AHR interactions.

Because the full-length AIP protein binds to the hsp90 C terminus, it was of interest to determine whether a C-terminal mutant of AIP, which failed to co-immunoprecipitate AhR in reticulocyte lysate, was also deficient in binding hsp90. The C-terminally truncated AIP, AIP1–325 (Fig. 7C), was expressed as a C-terminal fusion of glutathione S-transferase. Fig. 7E shows that the purified GST-AIP1–325 fusion protein binds to the hsp90(530–724) protein, demonstrating that a failure of AIP C-terminal mutants to bind hsp90 is not responsible for the failure of these mutants to bind to the AhR. The C terminus of AIP is scored as an z-helix by secondary structure prediction programs (data not shown), and a peptide containing the C-terminal 28 amino acids of AIP with an N-terminal cysteine readily assumed an z-helical formation, with 100% z-helical form in 50% methanol, as determined by UV-CD (data not shown). A model summarizing these results and showing the interactions between AIP, AhR, and hsp90 is shown in Fig. 7F.

DISCUSSION

AIP was originally shown to bind to AhR in yeast two-hybrid assays and thereby to enhance AhR function in cells (8, 9, 11). However, the detailed mechanisms whereby AIP binds to AhR and enhances its function have remained unresolved. We demonstrate that the formation of an AhR-AIP complex shows several features that are characteristic of an hsp90-dependent process and have defined distinct regions of the AIP protein, which are involved in binding with either hsp90 or the AhR protein.

The murine AhR associates with human, mouse, and simian AIP (8, 9, 11) in a reticulocyte lysate system. We have shown that mouse AIP binds to the AhR2 from F. heteroclitus, which...
is highly divergent from mammalian AhR genes with 53% amino acid identity (24) but does not bind to AhR homologues from *C. elegans* and *Drosophila*, which do not bind dioxin as a ligand (22) (Fig. 1A). Previous studies had shown that nonoverlapping, N- and C-terminal deletions into the ligand-binding domain of the AhR ablated the ability of AIP to bind to AhR (12, 13), and together, these observations suggest that a functional ligand-binding domain in AhR is required for AIP complex formation. However, a minimal ligand-binding domain of the mouse receptor was unable to interact with the AIP protein (Fig. 1B), demonstrating that additional sequences are necessary for AIP-AhR complex formation. The data are consistent with a model whereby a functional ligand-binding domain is necessary that colocalizes with the site of interaction with hsp90 (25), but additional sequences are also necessary for AhR-AIP complex formation, and these sequences are the site of AIP-AhR interaction. The appearance of dioxin-binding capacity in AhR proteins correlates with the capacity to bind both hsp90 and AIP.

Formation of AIP-AhR complex is temperature-sensitive (Fig. 2), reminiscent of hsp90-mediated chaperone reactions (16, 31), and is suggestive of an ATP-dependent process. AhR and AIP translation products were dialyzed against a buffer that supports hsp90 function (19) but that lacks ATP. They had greatly reduced ability to bind, but this ability was restored by the addition of ATP, ATP plus molybdate, or the nonhydrolyzable ATP analogue, ATP$\gamma$S (Fig. 3A), demonstrating that ATP is required for the formation of the AIP-AhR complex. Because hsp90 hydrolyzes ATP, ATP is required for hsp90 function, and ATP$\gamma$S or ATP plus molybdate can stabilize hsp90 in an active form (17, 32, 33), we hypothesized that hsp90 catalyzed the active step in AIP-AhR complex formation. Geldanamycin is a
specific inhibitor of hsp90, which binds to the ATP-binding pocket (34) and was a potent inhibitor of complex formation between AIP and AhR (Fig. 4A). Geldanamycin did not reduce the amount of AhR in reticulocyte lysates (Fig. 4B), and so the reduced binding of AhR to AIP induced by geldanamycin is not due to degradation of AhR. This observation is in sharp contrast to the rapid degradation of AhR induced by geldanamycin in cultured cells (26). The concordance between temperature sensitivity, requirement for ATP or a nonhydrolyzable analogue, and sensitivity to geldanamycin provides pharmacological evidence that hsp90 is required for binding between AhR and AIP.

However, an obligatory role of hsp90 in AIP function was difficult to reconcile with conflicting literature reports that AIP binds hsp90 poorly in reticulocyte lysates but that it binds to hsp90 in cells (8, 9, 11, 13), not least because of the possibility that AIP may only form complexes hsp90 through the medium of an “adapter” protein in a complex. It was therefore important to resolve whether AIP binds directly or indirectly to hsp90, and we purified the C-terminal domain of hsp90, which is known to bind several immunophilins (21). Fig. 5 shows that purified AIP binds to the C terminus of hsp90, demonstrating a direct interaction between hsp90 and AIP. As a further test, we undertook mutagenesis of AIP to disrupt hsp90 binding, based on the known structure of protein phosphatase 5 (29). Specific residues in protein phosphatase 5 protrude into the solvent in a cleft in the protein, have been identified as essential for hsp90 binding (Lys307 and Arg308) (30), and hence are likely to be hsp90 contact residues. Similarity between protein phosphatase 5, FKBP52, and AIP is highest in the immediate vicinity to the tetratricopeptide repeats (35), and disruption of these structures inhibits binding both to hsp90 and to substrate (36); this α-helical domain adjacent to the tetratricopeptide repeat is known to be critical for the specificity of FKBP52/51 for its substrate progesterone or estrogen receptor (36). Thus, the stabilization of the immunophilin-hsp90 complex by the binding of substrate to the immunophilin α-helix may play a key role in the specificity of immunophilins for their substrates.

In conclusion, we have shown that the binding of AIP to AhR requires hsp90, as evidenced by temperature sensitivity, ATP requirement, and geldanamycin sensitivity; this pharmacological evidence is strongly reinforced by the demonstration that AIP binds directly to the hsp90 C terminus and that mutation of a conserved basic residue, K266A, in the tetratricopeptide repeat domain of AIP abolishes binding to the hsp90 C terminus and substantially reduces binding to AhR. The C-terminal 4 amino acids of AIP are absolutely required for binding to AhR, but not for binding to hsp90, showing that AIP separately makes contact with hsp90 and AhR. The role of AIP in folding the AhR is currently under investigation.

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