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Biochemical Pharmacology 65 (2003) 941–948
Biochemical Pharmacology
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p23 enhances the formation of the aryl hydrocarbon receptor–DNA complex

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Received 11 April 2002; accepted 13 August 2002

Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that requires heterodimerization with its partner, the Ah receptor nuclear translocator (Arnt), for activation of transcription. The heterodimer specifically recognizes the dioxin response element (DRE), which contains a core sequence (5′-TNGCGTG-3′). This AhR/Arnt/DRE complex has been well characterized and can be observed readily by the gel shift assay. Human AhR and Arnt with a C-terminal histidine tag have been expressed functionally using a baculovirus expression system. However, after purification of these proteins using the metal resin, they are not able to bind the response element in a ligand-dependent manner unless crude extracts, such as the rabbit reticulocyte lysate (RRL), are reconstituted with these proteins. Proteins in the RRL are responsible for this restoration of the gel shift complex because the activity is sensitive to both heat and proteolytic treatments. We have examined whether hsp90 and p23 are among the protein factors in the RRL that are responsible for this activity. By performing fractionation studies using filtration devices and immunodepletion studies, we have selectively fractionated these proteins. Among all the fractions, the centricon-10 retentate, which contains 100% of p23 but no hsp90, possessed the most enriched activity. Purified bacterial-expressed p23 restored the gel shift complex; the mechanism was mediated at the heterodimerization step and was hsp90-dependent.

Keywords: Ah receptor; Arnt; DRE; p23; hsp90; Protein factors; Gel shift assay

1. Introduction

The function of hsp90 has been studied for a variety of biological events such as protein folding, assembly of functional steroid receptors and basic helix-loop-helix containing transcription factors, nuclear transport, DNA binding, interaction with cytoskeleton and protein kinases, regulation of the cell cycle, and proteolysis (for a review, see [1,2]). Among these events, one that has been well documented is that hsp90 and its associated proteins play an important role in conforming the ligand binding domain of the glucocorticoid receptor [3], the mineralocorticoid receptor [4], the progesterone receptor [5], the androgen receptor [6], and the AhR [4,7,8]. In particular, we are interested in the roles of these chaperone proteins in AhR signaling. For example, hsp90 has been shown to be important for masking the DNA binding domain of the AhR while maintaining the receptor in a ligand-ready state in the cytoplasm [4]. The ligand-dependent dissociation of hsp90 from the AhR requires the presence of p23, suggesting that p23 is important for the ligand responsiveness of the AhR [9]. In addition, binding of p23 with the AhR/hsp90 complex is essential for the ligand-dependent nuclear import of the AhR [10].

Both human AhR and Arnt have been overexpressed in a baculovirus system in an effort to generate reagents to delineate the signaling mechanism of the AhR [11]. The recombinant AhR and Arnt contained a six-histidine tag at their C-termini so that they could be affinity purified using

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Abbreviations: hsp90, heat shock protein 90; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; RRL, rabbit reticulocyte lysate; C100R, C100 retentate; C100WR, C100 washed retentate; C100F, C100 filtrate; C10R, C10 retentate; MWCO, molecular weight cut off; TBE, Tris–borate–ethylenediaminetetraacetic acid eletrophoresis buffer; IPTG, isopropylthio-β-D-galactoside; βNF, β-napthoflavone; 6xHis, six histidine tagged; ThioHisCΔ553, thioredoxin-CΔ553 fusion protein; PCR, polymerase chain reaction; NBT, nitrotetrazolium blue; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

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doi:10.1016/S0006-2952(02)01650-7

a metal resin such as the TALON resin. After purification, the AhR and Arnt were unable to form the ligand-dependent AhR/Arnt/DRE complex in a gel shift assay. Interestingly, upon reconstitution with a variety of crude protein extracts, the gel shift complex was restored. Since the reconstitution using a wheat germ lysate was sensitive to heat treatment, protein factors were initially suggested to be required for the AhR/Arnt/DRE complex formation [11]. It has been well documented that hsp90 associates with the cytoplasmic AhR. In addition, hsp90 appears to translocate along with the ligand-bound AhR into the nucleus, followed by the dissociation of hsp90 caused by the binding of Arnt to the liganded AhR [9,12,13]. Since p23 is known to associate with hsp90, it is conceivable that it may translocate along with the liganded AhR/hsp90 complex into the nucleus. Here, we examined the possible involvement of hsp90 and p23 in the formation of the AhR/Arnt/DRE complex by employing a screening gel shift assay using TALON-purified AhR and Arnt.

2. Materials and methods

2.1. Materials and reagents

RRL was purchased from Green Hectares. The AC88 monoclonal IgG against hsp90 was purchased from StressGen, and the JJ3 monoclonal IgG against human p23 from Affinity Bioreagents. Immobilized protein-A was purchased from Pierce, geldanamycin from CalBiochem, and apyrase from the Sigma Chemical Co. TALON-purified baculovirus-expressed human AhR and Arnt were prepared according to previously published protocols [11]. ThioHisCΔ553, a thioredoxin fusion protein of the human AhR deletion construct CΔ553 which lacks 553 amino acids from the C-terminus, was expressed in *Escherichia coli* and then purified using the TALON resin (manuscript in preparation). The anti-AhR polyclonal antibody (R5690 4B) was raised against the human AhR construct (amino acid 1-295, CΔ553 with a His tag at the N-terminus) in rabbits (Alpha Diagnostic). Centricons® YM10 and YM100 Centrifugal Filter Devices were purchased from Millipore. All other unspecified reagents and chemicals were purchased from either Fisher or Sigma.

2.2. Gel shift assay

Due to different preparations of baculovirus-expressed AhR and Arnt used in this paper, the amount of TALON-purified AhR (0.5 to 2 μL) and Arnt (0.1 to 0.2 μL) were optimized for each gel shift assay. AhR and Arnt were incubated/activated (30°, 10 min) in HEDG (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) in the presence or absence of other proteins (such as RRL, RRL fractions, BSA, casein, or non-fat dry milk), followed by the addition of poly(dI-dC) in the presence of 100 mM KCl (10 min, room temperature) and then 100,000 cpm of the [^{32}P]DRE (10 min, room temperature), which was synthesized according to a previously published protocol [11]. In samples where ligand activation of the AhR was required, the agonist βNF (7 μM final concentration) was added prior to the activation step. In some cases, polyclonal antibody (R5690 4B) or preimmune serum (PIS) was included after the addition of [^{32}P]DRE. Samples were loaded onto a 4% nondenaturing polyacrylamide gel that had been pre-equilibrated overnight at 4° with 0.5× TBE and were subjected to electrophoresis at a constant voltage of 185 V for approximately 1.5 hr at 4°. Migration of DNA-bound complexes were analyzed after autoradiography of the dried gels.

2.3. Western blotting

Western blots using different antibodies were performed according to published protocols [11]. In brief, after the transfer, the nitrocellulose membrane was incubated with the primary antibodies overnight at 4°, followed by incubation with the corresponding secondary antibodies coupled with alkaline phosphatase for 1 hr at room temperature. A colorimetric assay was performed using NBT/BCIP.

2.4. Fractionation studies

We modified the RRL fractionation protocols described in the literature [3]. One milliliter of RRL was centrifuged at 1000 *g* in a Centricon YM-100 filter unit at 4°, until approximately 70% of the lysate had been filtered. C100R was washed copiously by centrifugation (1000 *g*, 4°) with about 20 mL of 0.1 M potassium phosphate buffer (pH 7.4). The resultant filtrate (C100WF) was concentrated 25-fold using a Centricon YM-10 filter unit (2500 *g* at 4°) to give C10R. All fractions were stored at −80°.

2.5. Immunodepletion studies

Protein-A trisacryl beads (25% in HEDG) were precoated with 5 μL of the anti-p23 monoclonal antibody JJ3 by incubation for 1 hr at 4°. After that, 200 μL (3.6 mg) of C10R was added to the precoated beads, followed by incubation for 5 hr at 4°. The immunodepleted supernatants were subjected to western blotting and gel shift assay.

2.6. Bacterial expression of human p23

The human p23 cDNA was generated by PCR using Ambion FirstChoice PCR-Ready human liver cDNA as the template. A second PCR reaction was performed to generate *Bam*HI and *Sac*I sites at the 5′ and 3′ ends, respectively, of the p23 cDNA, using the purified PCR product from the first reaction. After subcloning into the Promega pGEM-T vector, the full-length p23 cDNA was cloned into the *Bam*HI and *Sac*I sites of the Qiagen pQE80L vector.

This cloned vector was used to express a 6xHis tag at the N-terminus of p23 in JM109, and the full-length p23 protein was subsequently purified using the TALON resin purchased from BD Biosciences Clontech, according to protocols described previously [11].

3. Results

3.1. RRL proteins responsible for restoration of the ligand-dependent AhR/Arnt/DRE complex

The uncharged RRL, when reconstituted with the AhR and Arnt in the gel shift assay, clearly restored the ligand-dependent gel shift, and this activity was not a general protein effect since BSA alone did not restore the complex (Fig. 1, lanes 1–3, 11). In addition, the AhR/Arnt/DRE gel shift complex formed using the baculovirus-expressed AhR and Arnt showed a stronger intensity than the gel shift band generated using the same amount of the RRL-expressed

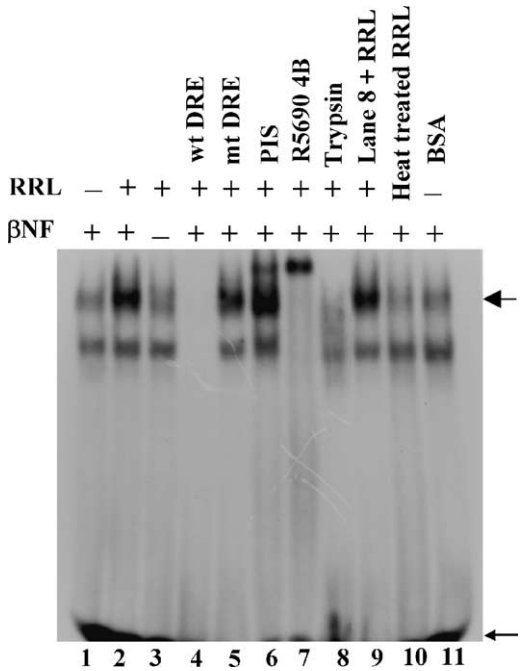

Fig. 1. Protein factor-dependent formation of the AhR/Arnt/DRE gel shift complex using RRL. TALON-purified baculovirus-expressed human AhR and Arnt were included in all lanes. Lanes 1 and 11 are the negative controls plus βNF with no protein factors and BSA, respectively. Lanes 2 and 3 are positive controls with RRL plus 7 μM βNF and RRL plus DMSO (vehicle), respectively. Lanes 4 to 9 are the same as lane 2 except that: lane 4, 100-fold excess of unlabeled wild-type (wt) DRE was included; lane 5, 100-fold excess of unlabeled mutated (mt) DRE was added; lane 6, rabbit preimmune serum (PIS, 3 μL) was included; lane 7, anti-AhR rabbit polyclonal R5690 4B (3 μL) was included; lane 8, RRL was treated with trypsin prior to reconstitution; and lane 9, same as lane 8 except that another dose of RRL was included. Lane 10 was reconstituted with RRL preheated at 56° for 3 hr. The amount of protein factors added to all lanes (except for lane 1 which had no protein factors and lane 9 which had two doses of RRL) was normalized to 10 μg. The upper arrow indicates the Ahr/Arnt/DRE complex, whereas the lower arrow indicates the free probe. This gel shift assay was repeated twice to confirm our findings.

AhR and Arnt, suggesting that more of the properly folded AhR and Arnt were expressed in the S*f*9 cells than in the RRL system (data not shown). The very weak gel shift bands observed in lanes 1, 3, 10, and 11 corresponded to the ligand-independent AhR gel shift (or "background") that was routinely observed when the baculovirus-expressed AhR and Arnt were used. The identity of the AhR/Arnt/DRE complex was confirmed using an unlabeled excess of either wild-type DRE or mutated DRE and anti-AhR polyclonal antibody R5690 4B (Fig. 1, lanes 4–7). This restoration was abolished when the RRL was treated previously with heat for 3 hr at 56° (Fig. 1, lane 10). Furthermore, tryptic digestion studies were performed, and the results showed that the activity was sensitive to proteolysis. A small amount of trypsin (2.5 μg/mL final concentration) was added to the RRL for 10 min at 30°. After treatment, the protease inhibitor leupeptin (2 μg/mL) was added to the trypsin-treated RRL to inactivate the trypsin so that no further proteolysis could occur during the subsequent gel shift assay. As the control, another dose of RRL was added to the trypsin-treated sample right before the gel shift assay (Fig. 1, lanes 8 and 9). The RRL activity was sensitive to both heat and proteolysis, suggesting that protein factors present in the RRL were responsible for the AhR/Arnt/DRE complex formation.

3.2. Enriched protein factor activity in the C10R fraction

Since our data suggested that there were proteins present in the RRL that were essential to restore the AhR/Arnt/DRE gel shift complex, we next examined if these proteins could be enriched. Using Centricon YM100 (100 kDa MWCO) and YM10 (10 kDa MWCO) filter devices, we obtained C100R, C100WR, C100F, and C10R fractions. The C10R fraction, which contained most, if not all, of the p23 content (Fig. 2B), had enriched protein factor activity (Fig. 2A). In contrast, the C100R and C100WR fractions, which contained the hsp90 in RRL, did not show any appreciative enrichment of the gel shift activity (Fig. 2A, top, lanes 1–6, and below). The AhR/Arnt/DRE complex caused by C10R was AhR- and DRE-specific since the anti-AhR antibodies, but not the preimmune serum, abolished the gel shift complex and an excess amount of unlabeled wild-type DRE, but not mutated DRE, abolished the band as well (Fig. 2A, top, lanes 7–10). In addition, the C10R fraction that lacked p23 restored only 40% of the C10R gel shift intensity (quantified using UN-SCAN-IT® software), suggesting that the activity in the C10R was partially caused by p23 (Fig. 2C).

3.3. p23-dependent restoration of the AhR/Arnt/DRE complex

Since the gel shift activity correlated well with the p23 content, we examined whether p23 could be one of the

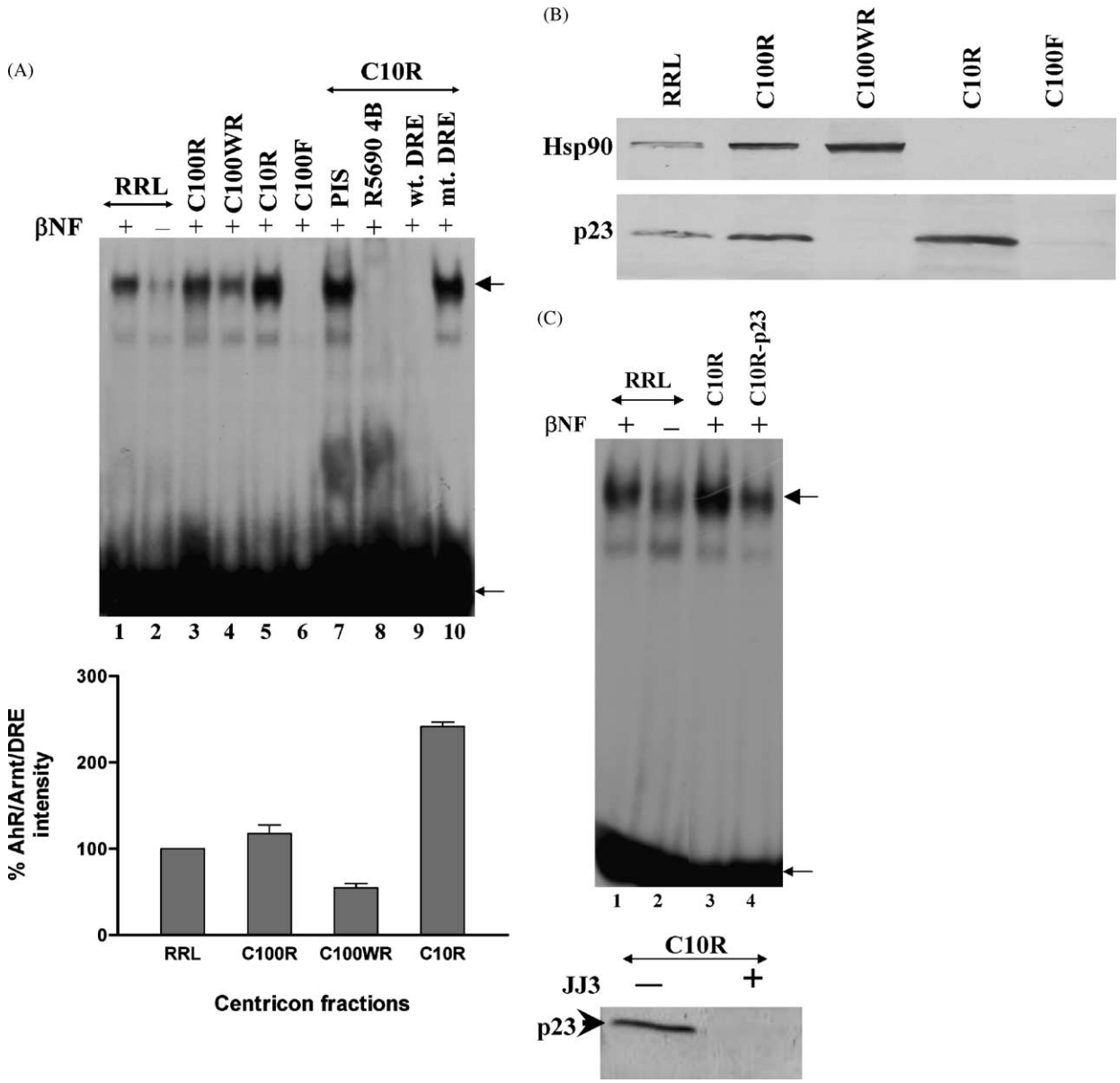

Fig. 2. Activity of protein factors in different Centricon fractions (A, top panel) gel shift assay showing activity of different Centricon fractions. All lanes contained TALON-purified baculovirus-expressed human AhR and Arnt and 10 μg of Centricon fractions. Lanes 1 and 2 are the positive controls with RRL ± 7 μM βNF. Lanes 3–6 contain different Centricon fractions as follows: lane 3, C100R; lane 4, C100WR; lane 5, C10R; and lane 6, C100F. Lanes 7–10 are the same as lane 5 except that lane 7 contains 1 μL of rabbit preimmune serum (PIS); lane 8, 1 μL of the anti-AhR rabbit polyclonal R5690 4B; lane 9, 100-fold excess of wild-type (wt) DRE; and lane 10, 100-fold excess of mutated (mt) DRE. All lanes except lane 2 contained 7 μM βNF. The upper arrow indicates the Ahr/Arnt/DRE complex, whereas the lower arrow indicates the free probe (A, bottom panel) graph showing the quantification of the AhR/Arnt/DRE gel shift complex. The gel shift assay of the different Centricon fractions as shown in the top portion of panel A was repeated five times, and the intensity of the gel shift bands was measured by UN-SCAN-IT software. The gel shift band produced by RRL was arbitrarily set as 100%. The error bars define the standard error of measurement. (B) Western blots of different Centricon fractions. Fifty micrograms of RRL and Centricon fractions were loaded to detect hsp90 (AC88, 1:1000 dilution), whereas 30 μg of each were used to detect p23 (JJ3, 1:1000 dilution) (C, top panel) Gel shift assay using the p23-immunodepleted C10R fraction. All lanes contained TALON-purified baculovirus-expressed human AhR and Arnt and 10 μg protein fractions ± 7 μM βNF. Lane 1, RRL plus βNF; lane 2, RRL minus βNF; lane 3, C10R plus βNF; and lane 4, p23-immunodepleted C10R plus βNF. The upper arrow indicates the AhR/Arnt/DRE complex, whereas the lower arrow indicates the free probe (C, bottom panel) Western blot of C10R (10 μg) with or without p23 immunodepletion using JJ3. The arrow indicates p23. The gel shift assay (A) and immunoprecipitation (B) were repeated twice and once, respectively, to confirm our findings. For panel C, the gel shift assay (above) was repeated three times, and the western blot (below) was repeated twice.

protein factors required for AhR/Arnt/DRE complex formation by using the gel shift sample with purified p23. Full-length human p23 was cloned and expressed as a histidine-tagged protein in *E. coli*. An abundant amount of p23 was expressed after IPTG induction, according to Coomassie blue staining results (Fig. 3A, top panel). The identity of p23 was determined by western blotting (Fig. 3A, bottom panel). The crude *E. coli* extracts containing p23 clearly restored the AhR/Arnt/DRE complex, whereas the same extracts containing the empty vector did not (Fig. 3B, lanes

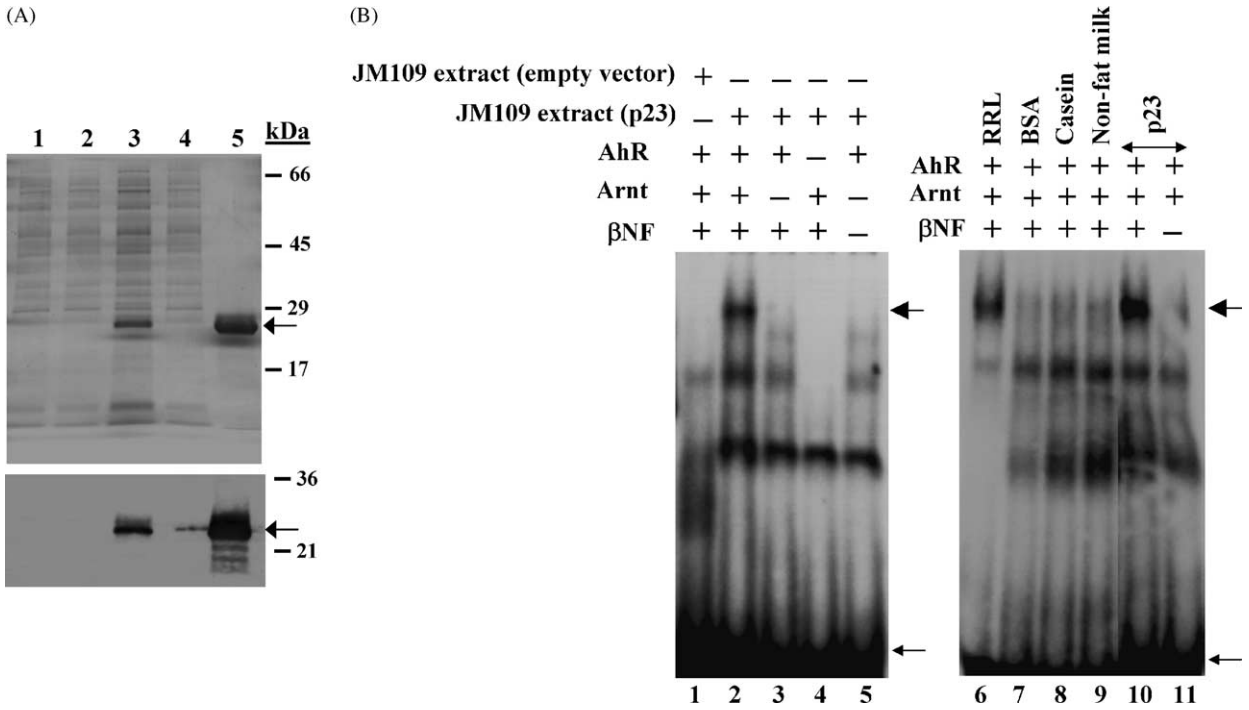

Fig. 3. Effect of p23 on the AhR/Arnt/DRE complex. (A) Coomassie blue staining (top panel) and western blots (bottom panel) of bacterial-expressed p23. The numbers on the right y-axis represent the protein size (kDa). Top panel: lane 1, IPTG-induced crude JM109 extract containing the empty pQE80 vector; lane 2, crude JM109 extract containing the empty pQE80 vector; lane 3, IPTG-induced crude JM109 extract containing the pQE80-p23 vector; lane 4, crude JM109 extract containing the pQE80-p23 vector; lane 5, TALON-purified p23. Bottom panel: Same lanes as above using JJ3 to detect p23. All lanes contained 2 μg of protein. The arrow indicates p23. (B) Gel shift assay showing the p23-dependent formation of the AhR/Arnt/DRE complex. TALON-purified baculovirus-expressed human AhR and Arnt were used in all lanes. Each lane was reconstituted with: lane 1, IPTG-induced crude JM109 extract containing the empty pQE80 vector; lane 2, IPTG-induced crude JM109 extract containing the pQE80-p23 vector; lane 3, same as lane 2 except without Arnt; lane 4, same as lane 2 except without AhR; lane 5, same as lane 3 except without βNF; lane 6, RRL; lane 7, BSA; lane 8, casein; lane 9, Carnation non-fat dry milk; lanes 10 and 11, TALON-purified p23. All lanes, except for lanes 5 and 11, contained 7 μM βNF. Lanes 1–5 and 6–11 were reconstituted with 20 and 10 μg of protein, respectively. The upper arrow indicates the AhR/Arnt/DRE complex, whereas the lower arrow indicates the free probe. This gel shift assay was repeated twice to confirm our findings.

1 and 2). This gel shift complex contained both the AhR and Arnt since the removal of either AhR or Arnt abolished the gel shift complex (Fig. 3B, lanes 3–5). Purified p23 restored the AhR/Arnt/DRE complex in a ligand-dependent manner (Fig. 3B, lanes 10 and 11), and this restoration was not caused by a general protein effect since BSA, casein, and non-fat dry milk alone did not restore the gel shift complex (Fig. 3B, lanes 7–9). In addition, the gel shift restoration required the reconstitution of p23 at the dimerization step (Fig. 4A, lanes 1–4). Incubation of the p23-treated gel shift sample with either geldanamycin (5 μg/mL) or apyrase (3.2 U/mL) significantly suppressed the AhR/Arnt/DRE complex formation (Fig. 4A, lanes 6–8). On the contrary, neither geldanamycin nor apyrase had an effect on the C10R-dependent formation of the gel shift complex (Fig. 4A, lanes 9–11). In addition, when CΔ553 was used instead of the full-length AhR, p23 failed to restore the gel shift complex (Fig. 4B).

4. Discussion

Since our TALON-purified baculovirus AhR and Arnt are unable to form the ligand-dependent AhR/Arnt/DRE complex without reconstitution with crude protein extracts in the gel shift assay, this assay thus provides us with a unique opportunity to search for proteins that restore the formation of the AhR/Arnt/DRE complex. Interestingly, another group has reported that the purified AhR/Arnt complex from rat livers is fully capable of binding to the DRE [14]. That AhR/Arnt complex, which was purified using the DRE affinity column, represented the "final" form of the AhR/Arnt/DRE complex. We believe that essential protein–protein interactions precede final complex formation and that these interactions are protein factor-dependent. Here, we examined whether hsp90 and p23 are involved in this complex formation. Fractionation of the RRL using different Centricon filter devices was effective in enriching the contents of the protein factors essential for the restoration of the AhR/Arnt/DRE gel shift complex. Our western blot results on various Centricon fractions showed that the C100 membrane retained hsp90 and its associated protein p23 very well after 70% of the RRL content was filtered through the C100 device (Fig. 2B, RRL and C100R). We found that p23 was eluted completely in the filtrate after copious washing of the C100R with phosphate buffer, consistent with published data [15]. The resulting C100WR gave only weak gel shift activity—this

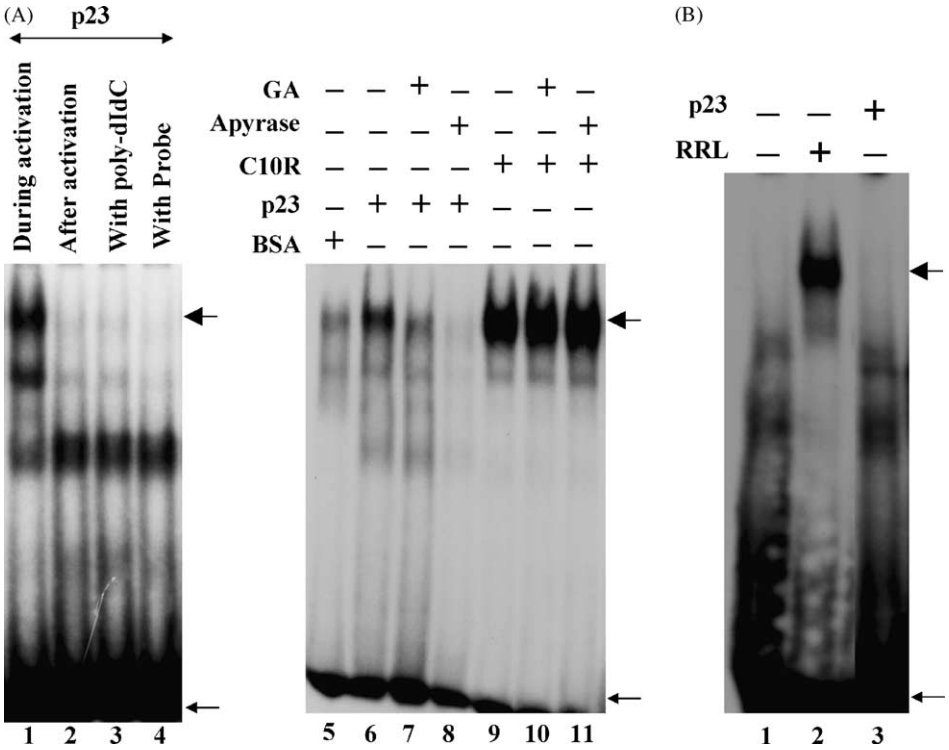

Fig. 4. Mechanism of the p23-dependent formation of the AhR/Arnt/DRE complex. (A) All lanes contained the TALON-purified baculovirus-expressed human AhR and Arnt, 7 μM βNF, and 10 μg of reconstituted protein. Conditions of each lane were as follows: lane 1, purified p23 added at the activation step; lane 2, purified p23 added after the activation step but before the poly(dI-dC) addition; lane 3, purified p23 added with poly(dI-dC); lane 4, purified p23 added with the [^{32}P]DRE; lane 5, BSA negative control plus DMSO vehicle; lane 6, repeat of lane 1 plus DMSO vehicle; lane 7, same as lane 6 plus 5 μg/mL of geldanamycin (GA) in DMSO; lane 8, same as lane 1 plus 3.2 U/mL of apyrase; lane 9, C10R at the activation step plus DMSO vehicle; lane 10, same as lane 9 plus 5 μg/mL of GA in DMSO; and lane 11, same as lane 9 but no DMSO, plus 3.2 U/mL of apyrase. The upper arrow indicates the AhR/Arnt/DRE complex, whereas the lower arrow indicates the free probe. (B) All lanes contained TALON-purified bacterial-expressed ThioHisCΔ553 and baculovirus Arnt and 10 μg of RRL or purified p23. Conditions of each lane were as follows: lane 1, no protein factors; lane 2, RRL; and lane 3, purified p23. The upper arrow indicates the CΔ553/Arnt/DRE complex, whereas the lower arrow indicates the free probe. These gel shift assays were repeated twice to confirm our findings.

was the first evidence that led us to suspect that p23 might be one of the protein factors. The concentrated C100WF fraction, obtained by centrifugation through a Centricon filter unit with a 10 kDa MWCO, which we also called C10R, contained p23, but not hsp90 (Fig. 2B, C10R). Importantly, this filtrate fraction showed enriched gel shift activity. Thus, our data supported the hypothesis that hsp90 is unlikely to be one of the protein factors in the RRL. This finding is in line with our expectation for three reasons. First, hsp90 has been reported to repress the DNA binding activity of the AhR [4]. Thus, one would expect that hsp90 probably does not promote the binding of the AhR–Arnt heterodimer to the DRE. Second, the wheat germ lysate, which does not contain hsp90, is fully capable of restoring the AhR/Arnt/DRE complex [11]. Third, the TALON-purified AhR fraction was by no means purified. Many Sf9 proteins were co-eluted with the 6xHis AhR during affinity purification. We suspected that many associated proteins of the AhR were co-purified with the receptor, and one of the obvious proteins would be the insect hsp90. But yet this AhR fraction does not form the gel shift complex, suggesting that proteins other than hsp90 are probably involved.

To investigate the role of p23 in the formation of the AhR/Arnt/DRE complex, we performed immunodepletion studies to deplete the p23 content in C10R prior to gel shift reconstitution. The results clearly showed that p23 was essential for the activity since C10R minus p23 had significantly less ability to restore the AhR gel shift complex (Fig. 2C). However, we cannot exclude the possibilities that (i) protein factors other than p23 were co-immunoprecipitated, (ii) protein factors other than p23 were non-specifically precipitated in the presence of protein A, and (iii) the immunodepletion protocol itself inhibited protein factor activity. In fact, it is likely that protein factors other than p23 are present in C10R since the p23-depleted C10R restored the AhR gel shift to a similar extent as the RRL (Fig. 2C, lanes 1 and 4). In addition, the same amount of C10R as the purified p23 gave a much more pronounced gel shift complex (Fig. 4A, lanes 6 and 9). Interestingly, unlike the p23-dependent gel shift complex, the C10R-dependent AhR complex was not altered in the presence of geldanamycin or apyrase, suggesting that there are multiple mechanisms involved in restoring the AhR/Arnt/DRE complex (Fig. 4A, lanes 6–11). Nonetheless, bacterial-expressed p23 unambiguously showed that p23 by itself restored the ligand-dependent AhR gel shift complex (Figs. 3B and 4A).

Next, we examined the p23-mediated mechanism involved in restoring the AhR/DNA complex. It has been

reported that p23 is essential in conferring the ligand-dependent formation of the AhR/Arnt/DRE complex in Hepa-1 cells [9] and in allowing the pendulin-mediated nuclear translocation of the AhR [10]. In addition, formation of the stable complex of hsp90 and p23 is essential for the XAP2 (also called Ara9 or AIP) mediated cytoplasmic retention of the AhR [16]. These events describe the roles of p23 while the AhR is in the cytoplasm. Here, we proposed an additional function of p23 after the AhR undergoes nuclear translocation. Our data suggest that p23 assists the heterodimerization of the AhR and Arnt (which is known to occur after nuclear translocation of the AhR complex) because in order for p23 to allow the AhR/Arnt/DRE complex formation in the gel shift assay, p23 had to be reconstituted at the activation (dimerization) step (Fig. 4A, lanes 1–4). Unfortunately, it is difficult to design an *in vivo* or a cell line study to differentiate the function of p23 after nuclear translocation from the known p23 functions (i.e. in AhR nuclear import and ligand-dependent dissociation of hsp90 from the AhR). These gel shift studies using semi-purified preparations provided an alternative means to elucidate the possible function of p23 after AhR nuclear translocation.

In addition, this p23 effect appeared to be hsp90-dependent based on the following three lines of evidence. First, p23 could not restore the AhR gel shift complex in the presence of geldanamycin (Fig. 4A, lanes 6 and 7). It is known that geldanamycin binds to the ATP binding site of hsp90 and, in turn, prohibits the association of p23 with hsp90 [10]. Inhibition of this p23 activity by geldanamycin suggested that hsp90 is required for p23 to elicit this effect. Second, ATP appeared to be important for the generation of the p23-dependent AhR/Arnt/DRE complex because when the gel shift sample was treated with an ATPase apyrase, p23 did not restore the AhR gel shift complex (Fig. 4A, lanes 6 and 8). It has been reported that association of p23 and hsp90 is an ATP-dependent event [5]. Without ATP, p23 could not interact with hsp90 and, hence, no gel shift restoration was observed. However, when exogenous ATP was included in the gel shift samples, no enhancement of any gel shift complex was observed, suggesting that ATP present in the TALON-purified AhR or Arnt was adequate for the association of p23 with hsp90 (data not shown). This hsp90-dependent p23 effect is consistent with our assumption that insect hsp90 is present in the TALON-purified AhR fraction. This assumption was supported by the observation that a protein, similar in size to human hsp90, in the TALON-purified AhR fraction was detected using western analysis that was immunoreactive with an anti-human hsp90 monoclonal antibody (data not shown). Another group has shown that the 9S fraction of the Hepa-1 C4 cytosol, which did not contain p23, formed the AhR/Arnt/DRE complex in their gel shift assay [9]. Since the other group used the RRL-expressed Arnt which contained all of the protein factors required to form the gel shift complex, significant gel shift activity would be expected and the p23 effect on the AhR/DNA complex could not be examined directly. Third, when a constitutively active construct of the AhR (CΔ553, 553 amino acids at the C-terminus have been deleted) was used in the gel shift assay instead of the full-length AhR, p23 was unable to restore the gel shift complex (Fig. 4B). We know that CΔ553 dimerizes with Arnt and then the heterodimer binds to the DRE in a ligand-independent manner because the domain required for hsp90 interaction on the AhR is deleted in the CΔ553 construct such that hsp90 is no longer necessary for the ligand responsiveness of the receptor. We know that this CΔ553 also requires protein factors for the formation of the AhR/Arnt/DRE complex as well (Fig. 4B, lanes 1 and 2). However, p23 could not restore the CΔ553/Arnt/DRE complex, suggesting that the hsp90 interaction with the AhR is necessary for this p23 activity. It is important to note that p23 may act as a chaperone protein along with hsp90 to facilitate the formation of the AhR–Arnt heterodimer. Even though this chaperone mechanism of hsp90 and p23 for AhR function has been well established, there are no data suggesting that this mechanism is necessary after nuclear translocation of the AhR. Here, we suggested that the chaperone property of these proteins may be important for the heterodimerization to occur.

In summary, we have provided evidence that there are protein factors in the RRL necessary to form the ligand-dependent AhR/Arnt/DRE complex. This restoration activity can be concentrated using Centricon filter devices, and p23 is one of the protein factors involved in restoring this AhR gel shift complex. Mechanistically, p23 appears to promote AhR/Arnt/DRE complex formation in an hsp90-dependent manner by assisting with the heterodimerization of the AhR and Arnt. However, current literature shows that p23 does not interact directly with either the AhR or Arnt [9,10]. At present, our data are consistent with our hypothesis that the association of p23 with hsp90 favors the displacement of hsp90 from the liganded AhR by Arnt. In addition, phosphorylation may very well be involved here since we know that tyrosine phosphorylation has been implicated, but poorly understood, for binding of the AhR–Arnt heterodimer to the DRE [17]. The detailed mechanism for p23-mediated restoration of the AhR gel shift complex remains to be investigated.

Acknowledgments

This work is supported by a grant from the National Institute of Environmental Health Sciences (ES09794).

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