Isolation and Characterization of ARA160 as the First Androgen Receptor N-terminal-associated Coactivator in Human Prostate Cells*

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Pei-Wen Hsiao and Chawnnshang Chang‡
From the Departments of Pathology, Urology, and Radiation Oncology, George Whipple Laboratory for Cancer Research, University of Rochester, Rochester, New York 14642 and University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin 53792

The androgen receptor (AR), like other steroid receptors, is a ligand-inducible transcription regulator that can activate or repress its target genes (1). Deletion analysis reveals that the AR molecule has four major functional domains including the DNA-binding domain (DBD), the ligand-binding domain (LBD), the hinge domain, and the N-terminal activation domain (2, 3). There are two activation functions (AFs) identified in AR: the ligand-independent AF-1 in the N-terminal domain, which mediates receptor activity that is dependent only on the presence of the ligand, and AF-2, which mediates transactivation with ligand (5, 6). We have previously shown that the ligand binding domain (LBD) of AR interacts with the coactivator ARA160 (7, 8), and that the ligand binding domain is involved in transactivation of the AR-LBD (9). The LBD of AR also interacts with the coactivator ARA160 (10). In this study, we report the cloning of ARA160, which has the same sequence as TMF (20), and we demonstrate that ARA160 interacts with the AR in a ligand-enhanced manner. Furthermore, co-transfection of ARA160 markedly enhanced the AR-mediated transcriptional activity on both androgen responsive promoters of prostate specific antigen (PSA) gene and mouse mammary tumor virus long terminal repeat (MMTV-LTR), which suggests that ARA160 is an androgen-enhanced N-terminal coactivator for the AR.

ExPERIMENTAL PROCEDURES

Peptide Probe Expression and Library Screening—A human AR (amino acid 38–643) peptide was fused with His-tag and S-tag was expressed in Escherichia coli BL21(DE3)pLysS (Novagen, Madison, WI). The S-tagged AR peptide (S-AR 38–643) was purified by immobilized metal affinity chromatography (Amersham Pharmacia Biotech) and used as a bait in far-Western blotting to screen a human adult testis λ-ZapExpress cDNA library (Stratagene). The expression library was induced by isopropyl-1-thio-D-galactopyranoside according to the manufacturer’s protocol. Eliminating the denaturation and renaturation procedure that was previously used, the membranes were directly blocked with 5% nonfat milk in HBB buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM MgCl2) at 4 °C for at least 6 h. The membranes were incubated with 0.5 μg/ml S-AR 38–643 in blotting buffer (HBB buffer with 1% nonfat milk, 0.02% Nonidet P-40) at 4 °C overnight, and then washed 3 times in washing buffer (HBB buffer with 0.02% Nonidet P-40) at 25 °C for 10 min, followed by incubation with S-protein alkaline phosphatase conjugate in blotting buffer at 25 °C for 30 min. The membranes were extensively washed with washing buffer, and positive clones encoding as S-AR 38–643 associated peptide were further confirmed by far-Western blotting as shown in Fig. 1A.

Far-Western Blot Analysis—The ARA160 cDNA (amino acid 410–888) spanning the overlapping region of the three original ARA160 clones was ligated in frame into pET14b and expressed in E. coli BL21(DE3)pLysS strain. The bacteria total protein extract lysates were resolved by 7.5% Tricine SDS-polyacrylamide gel electrophoresis, and electrotransferred onto Immobilon-P membrane (Millipore). After five washings, the membrane was probed by another truncated S-tag AR N-terminal peptide (S-AR 38–566), and detected essentially as in the library screening procedure described above.

S-protein Affinity Gel Pull-down Assays—Full-length ARA160 cDNA was ligated in frame into pET30b (Novagen) to yield S-tag ARA160 mediates transactivation (4).

Receptor coactivators such as ARA70 (5), ARA55 (6), ARA54 (7), SRC1 (8), TIF1 (9), RIP140 (10), CBP/p300 (11), TIF2 (12), GRIP1 (13), and RAC3/ACTR (14, 15) have been demonstrated to have ligand-dependent association with the LBD of nuclear receptors. Most coactivators have been shown to possess intrinsic activation domains for enhancing receptor transactivity (16, 17). Some coactivators, such as CBP/p300 and SRC1, form a coactivator complex containing histone acetyltransferase activity to modulate chromatin structure, which can influence the accessibility of transcription factors to the chromatin template (18, 19). This suggests a deep impact of steroid hormones on chromatin modulation and transcription regulation.

Here, we report the cloning of ARA160, which has the same sequence as TMF (20), and we demonstrate that ARA160 interacts with the AR in a ligand-enhanced manner. Furthermore, co-transfection of ARA160 markedly enhanced the AR-mediated transcriptional activity on both androgen responsive promoters of prostate specific antigen (PSA) gene and mouse mammary tumor virus long terminal repeat (MMTV-LTR), which suggests that ARA160 is an androgen-enhanced N-terminal coactivator for the AR.

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‡ To whom correspondence should be addressed. Fax: 716-756-4133; E-mail: chang@pathology.rochester.edu.
1 The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; PR, progesterone receptor; ARA160, AR-associated protein 160; TMF, TATA modulatory factor; DBD, DNA binding domain; LBD, ligand binding domain; AF, activation function; GBD, GAL4 DNA binding domain; DHT, 5α-dihydrotestosterone; T, testosterone; E2,17β-estradiol; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; PSA, prostate specific antigen; CMV, cytomegalovirus; Tricine, N-β-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.

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protein in *E. coli* BL21(DE3)pLysS. S-tag ARA160 was purified by immobilized metal affinity chromatography (Amersham Pharmacia Biotech) and analyzed by Western blot. S-protein-agarose beads were saturated with 1 mg/ml bovine serum albumin, and 5 μl of gel for each reaction was loaded with either 500 ng of S-tag ARA160 or 500 μl of bovine serum albumin in 100 μl of binding buffer (20 mM HEPES-KOH, pH 7.5, 200 mM KCl, 10 mM MgCl₂, 20% glycerol, 0.1% Nonidet P-40, 5 μg/ml leupeptin, 5 mM benzamidine, and 0.1 mg/ml bovine serum albumin) at 4 °C with mild agitation for 4 h. After five washes with washing buffer (same as binding buffer without bovine serum albumin), the affinity gels were incubated with 10 μl of various [³⁵S]methionine-labeled AR proteins (in vitro translated by TNT reticulocyte lysate, Promega) in 500 μl of binding buffer at 4 °C with agitation overnight. After five washes with washing buffer, the beads were resuspended in 30 μl of Laemmli sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Relative band intensity was quantified by PhosphorImager (Molecular Dynamics).

**Plasmids**—pSG5-AR, pSG5-ARA70N, pSG5-AR(E708K) mutant, pCMV-AR(T877A) mutant, pCMX-VP16, and pCMX-VP16AR (38–918) were described previously (21, 22). pCMX-GBDARA160 was constructed by ligating full-length ARA160 in frame to pCMX-gal-N (provided by D. Chen, University of Massachusetts). pCMX-ARA160 was constructed by ligating full-length PSA gene promoter (21512 to 112) into pGL3. **Transfection and Reporter Gene Assays**—Human prostate cancer PC-3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected by SuperFect transfection reagent (Qiagen) with 2 μg of DNA mixture as described previously (23). The same amount of parental expression
plasmid was used as a control and indicated as “−” in the figures. Relative luciferase activities were plotted using the activity of DHT-AR in the absence of coactivator as 1. The results were summarized from at least three sets of transfection and presented as mean ± S.E. The results from DU145 cells in Fig. 6, A and B, were transfected by calcium phosphate method as described previously (21).

**RESULTS**

**Cloning of ARA160 as an AR AF-1 Interacting Protein**—The AR N-terminal domain (AF-1) has been demonstrated as an essential element for AR-mediated transactivation. We were interested to know whether AF-1 needs to interact with an AR cofactor for the proper or maximal AR transactivation. To identify the direct AR AF-1 interacting proteins, an S-tag fused AR AF-1 peptide (S-AR 38–643) was used as a probe to screen human testis cDNA library. From 6 × 10⁵ phage plaques, three independent clones corresponding to the same mRNA were isolated from the interaction with the S-AR 38–643. Sequence analysis of these clones revealed a full-length cDNA sequence (3262 base pairs) with an open reading frame of 1093 amino acids (Fig. 1A). We thus named this AR N-terminal-associated protein as ARA160. Sequence comparison in GenBankTM BLAST search showed that ARA160 has the same sequence as TMF. Neither the AR function nor any promoter other than human immunodeficiency virus-LTR has been reported to be regulated by TMF. Recombinant ARA160 expressed in baculovirus or E. coli systems showed the same molecular weight as LNCaP ARA160/TMF (Fig. 1B). Western blot analysis in six human cell lines (LNCaP, DU145, HeLa, H1299, MCF-7, and HepG2) using polyclonal anti-ARA160 antibody that was raised against ARA160 peptide (amino acid 1074–1093) in rabbit, all revealed a ARA160 band (Fig. 1C).

**ARA160 Interacts Directly with AR**—Three different assays (far-Western blotting, co-immunoprecipitation, and affinity gel pull-down) were applied to determine whether ARA160 could interact directly with the AR in vitro. With the far-Western blotting, as shown in Fig. 1D, the E. coli expressed ARA160 peptide (amino acid 410–888, containing the interacting region based on our initial screening assay) could bind directly to another S-tag fused AR N-terminal peptide (S-AR 38–566). Co-immunoprecipitation assay also confirmed the direct interaction between baculovirus expressed AR and ARA160 with anti-ARA160 antibody (data not shown). Finally, the affinity gel pull-down assay showed that S-tag fused ARA160 could pull-down [³⁵S]methionine-labeled in vitro translated AR protein, and this interaction could be significantly enhanced in the presence of 100 nM DHT (Fig. 2A).

Mammalian two-hybrid assays were carried out in human prostate PC-3 cells. When cells were co-transfected with GBD fused ARA160 (GBD-ARA160) and VP16AD fused near full-length AR (amino acid 38–918) (VP16-AR), interaction of these two hybrid proteins formed a functionally transactive complex that activated the transfected GAL4 responsive reporter (pG5E1b-Luc). As shown in Fig. 2B, GBD-ARA160 significantly activated transcription only in the presence of VP16-AR and 10 nM DHT. Together, these mammalian two-hybrid data and the gel pull-down results indicate that androgen enhances AR-ARA160 interaction significantly.

**Influence of ARA160 Expression on the DHT-mediated AR Transactivation**—After demonstrating that AR and ARA160 interaction is enhanced by androgen, we wished to determine whether this interaction could influence the AR-mediated transactivation. As shown in Fig. 3, A and B, co-transfection of ARA160 in increasing amounts enhanced DHT-mediated AR transactivation on MMTV-LTR promoter. When the MMTV-LTR promoter was replaced by PSA promoter, we found that 1 nm DHT could activate AR transactivation 5-fold (using 1.5 kilobase PSA promoter fused with luciferase as reporter). ARA160 could further induce this AR-induced transactivation up to 17-fold (Fig. 3C). This ARA160 enhanced AR transactivation also occurred when we replaced PC-3 cells with Chinese hamster ovary cells (data not shown). Interestingly, unlike our previous C-terminal coactivator ARA70, which shows high specificity for AR, this N-terminal coactivator ARA160 could also enhance GR- and PR-mediated transactivation using the same MMTV-LTR promoter as reporter (Fig. 3, D and E). These data suggest that ARA160 might represent a more general coactivator to the classic steroid receptors, and ARA160 might interact with some conserved region within AR that shares high homology to the other steroid receptors.

To further analyze the ARA160 interaction to AR AF-1 and AF-2, the N-DBD AR (AR 38–643) and the DBD-LBD AR (AR 553–918) peptides were applied in the affinity pull-down assays. As shown in Fig. 4A, not only N-DBD AR peptide can interact with ARA160 (lanes 1–3), the DBD-LBD AR peptide...
can also interact with ARA160 and in a DHT-dependent manner (lanes 4–8). Co-transfection of ARA160 with GBD fused AR AF-1, and AR AF-2 shows that ARA160 enhances the AR AF-1 more than AR AF-2 (Fig. 4B). Notably, the affinity of full-length AR to ARA160 is higher than N-DBD AR or DBD-LBD AR (Fig. 2A versus Fig. 4A), and the ARA160 showed much better enhancement to the full-length AR transactivation, as compared with the AR AF-1 or AR AF-2 transactivation (Fig. 3B versus Fig. 4B). These results indicate that both AF-1 and AF-2 contribute to the AR-ARA160 interaction.

As ARA70 can enhance AR transactivation through the AR LBD (5) and ARA160 can enhance AR transactivation mainly through the AR AF-1 (Fig. 4B), we were interested in determining if any additive or synergistic effects on AR transactivation might occur when both coactivators exist in the same cells. As shown in Fig. 5, whereas ARA160 and ARA70 could enhance AR transactivation up to 6-fold (lane 3 versus 2) and 3-fold (lane 4 versus 2), respectively, co-expression of the AR with ARA160 and ARA70 could further enhance AR transactivation up to 15-fold (lane 5 versus 2). The greater than additive effect suggests that ARA160 and ARA70 can function cooperatively as AR coactivators to enhance AR transactivation.

Influence of ARA160 Expression on the 17β-Estradiol-mediated AR Transactivation—As 17β-estradiol (E2) can also induce androgen target genes in the presence of ARA70 (22), we were interested in knowing if ARA160 can also have similar effects. As shown in Fig. 6A, 10 nM E2 or 1 nM testosterone (T) induced AR-mediated transactivation over 7 and 17-fold, respectively, in the presence of ARA70 in DU145 cells. Under the same conditions, ARA160 had only a marginal enhancing effect on the T- or E2-mediated AR transactivation (Fig. 6B). These contrasting results (ARA70 versus ARA160 in DU145 cells, or ARA160 in PC-3 cells versus DU145 cells) strongly suggest that different coactivators might require different conditions for their maximal or proper enhancing effects. Three ARs, the wild type AR, a lose-of-function mutant AR (AR E708K), and a gain-of-function mutant AR (AR T877A), were applied here to test whether mutations within the AR may influence such contrasting enhancing effects between ARA70 and ARA160 (21, 22). As shown in Fig. 6C, whereas 1 nM T and 10 nM E2...
could modestly induce the wild type AR-mediated transactivation in PC-3 cells co-transfected with ARA160 (lanes 4 and 6 versus lane 2), 10 nM of T, but not 10 nM E2, could induce AR E708K transactivation (Fig. 6D, lanes 4 and 6 versus lane 2). In contrast, both 1 nM T and 10 nM E2 could induce AR T877A transactivation (Fig. 6E, lanes 4 and 6 versus lane 2). These data suggest that the cell environment for each coactivator and the amino acid at the position of 708 and 877 may play very important roles for the E2-mediated AR transactivation, and that ARA160 has much less effect on E2-mediated AR transactivation than ARA70 in DU145 cells.

**DISCUSSION**

Androgens regulate gene transcription through binding to the AR (1, 24). Understanding the transcriptional functions and the regulatory mechanisms of AR is essential for controlling androgen signaling and androgen-regulated prostate cancer growth. Earlier reports suggested that the AR AF-2 may recruit a complex including several AR coactivators, such as ARA70, ARA55, ARA54, and retinal blastoma (5–7, 21), to enhance the AF-2 transactivation function. In contrast, very few coregulators have been reported to selectively bind to the AR AF-1 domain (16), therefore, it will be very important to identify some AF-1 coregulators that can enhance AR AF-1 transactivation.

In this study we were able to isolate and demonstrate that ARA160 can function as a coactivator through the interaction with the AR AF-1. Further data indicate that ARA160 can also interact with DBD-LBD of the AR. Although ARA160 enhances the AR AF-1 more significantly than AR AF-2 (Fig. 4B), these results suggest that ARA160 may physically and functionally serve as a bridge between AR N- and C-terminal activation domain. Moreover, the greater than additive enhancement of ARA160 and ARA70 on AR transactivation as demonstrated in Fig. 5, further supports the hypothesis that the AR can activate transcription through coordinated mechanisms mediated by both the AF-1 and AF-2. Perhaps, androgen-induced AR can recruit the AF-2 coactivator and the AF-1 coactivator to form a higher AR complex, and cooperation between the AF-1 coactivator and the AF-2 coactivator might expand the androgen-AR signal in transactivation.

Nuclear receptor coactivators, such as SRC1, TRAM-1, and p120, have been reported to show significant coactivator activity only when high doses of coactivator expression plasmids are co-transfected with receptor expression plasmids (25, 26). In the case of SRC-1 and TRAM1, the ratio of coactivator plasmid to receptor plasmid reached 20 to 25:1. This higher transfection ratio of coactivator to receptor also occurred with ARA160, as shown in Fig. 3A, more than 10-fold of ARA160 plasmid needs to be co-transfected with AR plasmid for the coactivator enhancing effects. The total ARA160 protein, however, only increases to near 2-fold, even though 30-fold higher ARA160 plasmid than AR plasmid was co-transfected (Fig. 7).

In PC-3 cells, ARA160 becomes a good coactivator to enhance DHT- and T-mediated transactivation in PC-3 cells. These differen-
tial enhancing effects between coactivators support the idea that coactivators require special environments for their maximal or proper function.

Based on the relatively low homology among the N-terminal domain of steroid receptors, we initially expected that ARA160 might function more specifically to AR. However, ARA160 could also enhance GR- and PR-mediated transactivation in transient transfection assays. Possibly, the other portions of the AR, which have relatively higher homology to GR and PR, contribute to their interaction with ARA160. Nevertheless, our data indicated ARA160 enhanced GAL4-SP1 only marginally (1.5-fold) on pG5E1b-Luc (data not shown), suggesting there are still certain preferences for ARA160 to steroid receptors.

Although the apparent molecular mass of ARA160 (160 kDa) is higher than the calculated molecular mass (123 kDa), the running pattern in SDS-polyacrylamide gel electrophoresis is consistent regardless of expression in either eukaryotic or prokaryotic system. These data may imply the post-translational modification of ARA160 to be not very significant. The fact that ARA160 can function as an AR coactivator in multiple cells, including yeast, PC-3, and Chinese hamster ovary, also supports the above suggestion that ARA160 may function well without special modification. Because the baculovirus expressed ARA160 recombinant protein did not show any band shift with PSA-ARE (androgen response element) and core promoter, and the fact that only minimal self-activation occurred when ARA160 was fused to GBD in transfection assays (data not shown), we might be able to conclude that ARA160 will not bind directly to the core promoter sequence of the AR.
target gene and therefore can only function as a coactivator through interaction with the AR in our transfection assays.

In conclusion, we have cloned and identified ARA160 as an AR coactivator through its interaction with the AR N-terminal domain and have shown that ARA160 enhances DHT- and T-mediated AR transactivation in human prostate PC-3 cells. In comparison with ARA70, we found that the cellular environment, ligand, and amino acid within AR are critical for different coactivators to maximally enhance AR transactivation. Further study of the interactions among the AR and all these different AR coactivators will provide us a deeper insight in the regulation of androgen signaling and prostate cancer growth.

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REFERENCES

1. Chang, C., Kokontis, J., and Liao, S. (1988) Science 240, 324–326
2. Evans, R. M. (1988) Science 240, 889–895
3. Beato, M. (1989) Cell 56, 335–344
4. Jenster, G., van der Korput, H. A., Trapman, J., and Brinkmann A. O. (1995) J. Biol. Chem. 270, 7341–7346
5. Yeh, S., and Chang, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5517–5521
6. Fujimoto, N., Yeh, S., Kang, H., Inui, S., Chang, H., Mizokami, A., and Chang, C. (1999) J. Biol. Chem. 274, 8316–8321
7. Kang, H., Yeh, S., Fujimoto, N., and Chang, C. (1999) J. Biol. Chem. 274, 8570–8576
8. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1995) Science 270, 1254–1257
9. LeDouarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Lossos, R. (1995) EMBO J. 14, 2020–2033
10. Cavaillès, V., Dauvois, S., L’Horslet, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) EMBO J. 14, 3741–3751
11. Kamei, Y., Xu, L., Heinzel, T., Torchio, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 403–414
12.Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) EMBO J. 15, 3667–3675
13. Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4948–4952
14. Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479–8484
15. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
16. Onate, S. A., Boonyaratankurat, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O’Malley, B. W. (1998) J. Biol. Chem. 273, 12101–12108
17. Voegel, J. J., Heine, M. J., Tini, M., Yivit, V., Chambon, P., and Gronemeyer, H. (1998) EMBO J. 17, 507–519
18. Ogryzko, V. V., Schiltz, R. L., Rassanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953–969, and references therein
19. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1997) Nature 389, 194–198, and references therein
20. Garcia, J. A., Ou, S. H., Wu, F., Lusis, A. J., Sparkes, R. S., and Gaynor R. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9372–9376
21. Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P.-W., Wang C., Su, C., and Chang C. (1998) Biochem. Biophys. Res. Commun. 248, 361–367
22. Yeh, S., Miyamoto, H., Shima, H., and Chang, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5527–5532
23. Hsiao, P.-W., Lin, D.-L., Nakao, R., and Chang, C. (1999) J. Biol. Chem. 274, 20229–20234
24. Chang, C., Saltzman, A., Yeh, S., Young, W., Keller, E., Lee, H. J., Wang, C., and Mizokami, A. (1996) Crit. Rev. Eukaryotic Gene Expression 5, 97–125
25. Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C.-S., and Chin W. W. (1997) J. Biol. Chem. 272, 27629–27634
26. Monden, T., Wondisford, F. E., and Hollenberg, A. N. (1997) J. Biol. Chem. 272, 29834–29841