A Novel Genetic System for Analysis of Co-activators for the N-Terminal Transactivation Function Domain of the Human Androgen Receptor

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Androgen receptor (hAR) regulates transcription of target genes in a ligand-dependent manner and recruits a number of co-activators for the ligand-induced transactivation via the N-terminal, activation function-1 (AF-1), and C-terminal, AF-2, transactivation domains. But the co-regulator functions on each of AR domains have not yet been fully understood. We have established a Drosophila transgenic system in which hAR and its deletion mutants are ectopically expressed in fly tissues together with an AR response element (ARE)-GFP reporter gene, and have confirmed that hAR was functional in ARE transactivation without affecting the expression of endogenous genes. We found that transcriptional activity of the hAR AF-1 domain was markedly reduced in Drosophila deficiency mutants of homologs for known mammalian co-activators of the AR ligand-dependent AF-2 domain. This suggests that hAR AF-1 recruits co-activators previously known only to interact with the AF-2 domain. Therefore, Drosophila with the hAR AF-1 transgene provides a relevant genetic system in which to uncover novel functions of vertebrate steroid hormone receptors and to screen for novel AF-1 co-regulators.

Key words: androgen receptor; transactivation; coactivator; histone acetylation; transcriptional mediator

The members of the steroid/thyroid nuclear hormone receptor superfamily act as ligand-inducible transcription factors that regulate transcription of particular sets of target genes involved in diverse physiological processes.1) Based on structural and functional similarities, the nuclear receptors are divided into functional domains designated A to E(F). The DNA binding domain is a highly conserved middle region (C domain), while the ligand binding domain (LBD) is located in the less conserved C-terminal E/F domain and is comprised of twelve α-helixes forming a pocket to capture cognate ligands.2,3) The N-terminal A/B domain and the C-terminal domain are required for the ligand-induced transactivation function of nuclear receptors. The autonomous activation function-1 (AF-1) in the A/B domain is the least conserved and constitutively active on its own, while AF-2 activation of the LBD E/F domain is dependent on ligand binding. The transactivation function of nuclear receptors requires a variety of common co-activator complexes that in many cases show ligand-dependent interactions (direct or indirect) with the AF-2 domain. A large number of regulatory complexes implicated in AF-2 transcriptional regulation have been identified.4–6) Among them, two HAT co-activator complexes have been characterized, one composed of factors belonging to the p160 (SRC-1/TIF2/AIB-1) family and another of p300/CBP, both of which function to modify histones through intrinsic histone acetyltransferase (HAT) activities.7,8) The other HAT co-activator complex has been recently identified by us.9) Besides the HAT complexes, a non-HAT co-activator complex was biochemically identified and designated the thyroid hormone/vitamin D receptor-associated protein (TRAP/DRIP)/Mediator complex, and represents a different type of non-HAT co-activator complex.10,11) This complex was further shown to be essential for TR function in an in vitro transcription system.12,13) Although the TRAP220 subunit in this complex interacted directly with TR and subsequent analysis with TRAP220/C0 fibroblasts confirmed a

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Abbreviations: AR, androgen receptor; ARE, AR response element; AF-1, activation function-1; DHT, dehydrotestosterone; HAT, histone acetyltransferase; AIB-1, Amplified in Breast Cancer; CBP, CREB-binding protein; TRAP, thyroid hormone receptor-associated protein

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receptor-selective function for TRAP220, the interaction of other subunits, such as TRAP80 and 100, their functional roles are not fully understood. Thus, their functions to the AF-2 have been addressed to some extent, but those for AF-1 are largely unknown.

*Drosophila* not only possesses homolog of most mammalian signaling proteins, transcriptional co-regulators, and basal transcription factors, but also expresses nuclear receptors like the ecdysone receptor (EcR), which is structurally and functionally homologous with the members of the vertebrate nuclear hormone receptor superfamily. The fact that *Drosophila* homologs for mammalian HAT co-activators (CBP and AIB1) and TRAP/Mediator have been identified recently, and the findings of their co-activator functions on glucocorticoid receptor (GR) in cultured *Drosophila* embryonic Schneider’s cell line 2, indicate that *Drosophila* and mammalian transcription factors are functionally interchangeable.

In the present study, to identify and characterize co-activators for AF-1 of the hAR, we established a transgenic fly model system by ectopically expressing hAR wild type (wt) and its deletion mutants in *Drosophila* tissues. When the hAR expression was induced by tissue-specific GAL4-divers, ligand-dependent hAR transactivation was observed in a given tissue. No abnormality was detected, however, in the expression of either EcR or its downstream genes. The transactivation function of hAR(AF-1) was significantly reduced in all of the fly deletion mutants of homologs for known mammalian AF-2 interacting co-activator complex components, TRAP80, 100, p160, and CBP, showing that the fly co-activators for the AF-2 function also act to enhance the AF-1 function. Taken together, our data provide evidence that transgenic *Drosophila* expressing human ARs represents a potent and functionally relevant system in which to evaluate AR synthetic ligands and androgen-like compounds and to identify and characterize novel nuclear receptor co-regulators.

**Materials and Methods**

**Fly stocks and genetics.** All general fly stocks were obtained from the Bloomington *Drosophila* Stock Center. The AR mutant cDNAs in pCaSpeR3 and an ARE-GFP reporter construct (GFP-TT in pCaSpeR3 with a consensus ARE in its promoter) were used to generate several transgenic lines, as previously described. Target chromosomese were separated from those carrying the *Gal4*-driver by crossing with flies harboring second and third balancer chromosomes CyO and TM3. The *Gal4*-driver lines used were as follows: the *gmr-Gal4* line, expressing GAL4 in the retina driven by the Glass Multimer Reporter; the *dpp-Gal4* line, expressing GAL4 in the anterior-posterior boundary area in developing wing discs driven by the *dpp* (blk) promoter; and the *ptc-Gal4* line, expressing GAL4 in the anterior portion of embryonic segments driven by a patched (ptc) gene promoter. The TRAP80 delution line, l(3)s2956, was obtained from Szeged while the TRAP100 delution line, BL-01670, the AIB-1 delution line, l(2)01351, and the AIB-1 dominant overexpression line, BL-6378 (UAS-Ta), were obtained from BDGP. The CBP deletion line, *nej*, was obtained from S. Ishii.

**Immunofluorescence and Histology.** Tissues were dissected and fixed for 20 min in 4% formaldehyde and incubated with a primary antibody, either hAR (N-20) or (C-19) (Santa Cruz Biotechnology, Inc.), then Cy5-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch) was used as a secondary antibody for immunofluorescence staining. Conforcal microscopy was performed on the Zeiss conforal laser scanning system 510, and images were assessed using the Adobe Photoshop 5.0 (Adobe) software program.

**Cell Culture and Transactivation assay.** *Drosophila* embryonic Schneider’s cell line 2 were maintained in Schneider’s *Drosophila* medium, supplemented with 5% fetal calf serum. S2 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt or AF-1). Cells were incubated for 18h in the absence or presence of 10^{-8} M DHT, hydroxyflutamide (HF), and bicalutamide (BIC) and then assayed for luciferase activity as previously described.

**RNAi experiments.** The dsRNAs were transfected after they were annealed. The dsRNAi constructs of TRAP80-RNAi, TRAP100-RNAi, and CBP(nejire)-RNAi were from Open Biosystems Co. Original dsRNAs of the AIB-1(taiman)-RNAi are constructed from the 5'-region (1 to 700 bases) in AIB-1 ORF cDNA.

**Results and Discussion**

Just as we previously showed that the selective and ectopic expression of hAR mutants with expanded polyglutamine in fly eyes caused neurodegeneration, the hARs were expressed in selected tissues using the GAL4-UAS system. The cDNA for wild-type hAR [hAR(wt)] or AR(AF-1) was inserted into an expression vector under the control of the hsp70 promoter containing UAS. After establishment of UAS transgenic lines, the flies were crossed with *Drosophila* from GAL4-driver lines that expressed GAL4 under a tissue-specific promoter. To monitor the ligand-induced transactivation
function of hAR, these flies were further crossed with flies bearing a GFP reporter gene with consensus sequences for androgen response element (ARE) in their promoter. Using this system, tissue-specific expression of hAR(wt) was induced in the developing eye disc under a Glass Multimer Reporter (gmr) gene promoter (left panel in Fig. 1A), in the middle area in the developing wing disc under a decapentaplegic (dpp) gene promoter (middle panel), and in the anterior portion of embryonic segments under a patched (ptc) gene promoter (right panel). No phenotypic abnormalities were observed in flies with any of the transgene constructs. 

Expressed hARs were detected in situ using an immunofluorescent antibody, and ingestion of DHT induced GFP expression, observed as green fluorescence, only in tissues that ectopically expressed hAR (Fig. 1A). Significantly, since no green fluorescence was detected in flies cultured on medium without androgens (Fig. 1A), it appears that Drosophila does not produce endogenous hAR ligands. Well-known androgen antagonists, such as hydroxyflutamide (HF) and bicalutamide (BIC), did not induce GFP expression in the presence of DHT (Fig. 1A). Antagonized effects of AR (AF-2) transactivation were also detected (Fig. 1B, lanes 8–10). Both unliganded and liganded hAR (wt, AF-1 and AF-2) were non-toxic in the transgenic flies under all conditions studied as no phenotypic abnormalities were detected in any of the tissues. The biological activity in Drosophila of ingested AR ligands appeared to be identical to ligand activity in cultured mammalian cells or intact mammals.

Targeted expression of hAR(wt) in several tissues at different developmental stages caused no overt abnormalities in the transgenic fly, even after 5 d of dietary ingestion of DHT and/or antagonists. Nonetheless, it is difficult to exclude the possibility that the function of endogenous coactivators, essential for endogenous nuclear receptor action, was affected by the overexpressed hAR, resulting in altered endogenous nuclear receptor function. The Drosophila nuclear receptor Eip75B is known to be under the transcriptional control of EcR/USP heterodimers as a response element for the ecdysone receptor has been identified in its gene promoter. In our experimental system, DHT ingestion did not appear to affect the expression of endogenous Eip75B or EcR genes even in tissues that expressed high levels of hAR (Fig. 2). Thus it seems unlikely that hAR expression in the fly significantly interferes with endogenous regulatory processes mediated by Drosophila nuclear receptors.

The properties of hAR (AF-1) are distinct from those of AF-2 in mammalian cells and it is not clear whether cofactors interact specifically with one domain or the other. In this study, we tested the transactivation function of the AF-1 domain in Drosophila deficiency mutants of homologs of mammalian co-activators for nuclear receptors known to interact with...
their AF-2 domain. hAR (AF-1) expression targeted by GMR-Gal4 was observed in the third instar larva eye disc (Fig. 3). Ligand-independent ARE-GFP transactivation by hAR (AF-1) was clearly evident in these flies, while no enhancement of ligand-dependent transactivation in AR (AF-1) was detected (data not shown). We then crossed these flies expressing hAR (AF-1) with Droso-

Fig. 2. Transgenic Flies Do Not Display Aberrant Expression of Endogenous Ecdysone Receptor (EcR) and Eip75B Genes.

Expression of EcR and Eip75B was measured in third instar larvae of UAS-hAR (lanes 1 and 8), GAL4-driver (lanes 2, 4, 6, 9, 11, and 13), and GAL4-driver: UAS-AR (3, 5, 7, 10, 12, and 14) lines in the absence or presence of DHT (1 × 10⁻⁵ M). Polyadenylated RNA (poly(A)⁺-RNA) was purified by oligo(dT) affinity chromatography and 10 µg poly(A)⁺-RNA was separated by electrophoresis on 1% agarose-1.1 M formaldehyde gels, and transferred to nitrocellulose membranes. Northern blots were probed with cDNA for the Ecdysone receptor (EcR), Eip75B, and β-actin.

Fig. 3. AR (AF-1) Transactivation in Drosophila Is Induced by Its Endogenous Co-activators.

A. AR (AF-1) transactivation in the eye disc is reduced in mutants deficient in homologs of mammalian co-activators. Genotypes are GMR-GAL4, UAS-hAR(AF-1); ARE-GFP, in trans to TRAP80 mutant [l(3)s2956], TRAP100 mutant (BG01670), AIB-1 mutant (tai01351), overexpressed AIB-1 (UAS-tai), or CBP mutant (nej). GMR-GAL4 expressed AR (AF-1) was detected with anti-hAR N-20 antibody in the third instar eye imaginal discs (upper panel). The transactivation function of AR (AF-1) was evaluated by levels of GFP expression and green fluorescence (middle panel) and merged images are shown in the lower panel. The relative abundance of GFP activities is corrected by anti-AR antibody-stained AR protein levels using NIH images and indicated as mean ± for at least three samples from different eye discs. B. AR transactivates via endogenous co-activators in the Drosophila Schneider’s cell line 2. S2 cells were transfected with the expression vectors of an ARE-luciferase reporter plasmid, 0.1 µg of AR (wt) expression plasmid and 0.2 µg of dsRNAi constructs [Trasp80-RNAi, Trap100-RNAi, AIB-1 (tai) RNAi, and CBP (nej) RNAi] were transfected as indicated in the images in the absence or presence of DHT (1 × 10⁻⁸ M). The data presented are from a representative experiment out of the five independent experiments performed. Luciferase reporter activity of AR transactivation in the absence of DHT is presented as fold induction. Values are mean ± SD.
Tai (p160), and nej(CBP), whose mammalian counterparts are known to be recruited by the nuclear receptor AF-2 domain but their interaction with the AF-1 domain has not been demonstrated. The transactivation by AR (AF-1) in the eye discs of resulting hybrids was significantly reduced, while hAR (AF-1) expression levels estimated by anti-hAR antibody were unaffected. On the contrary, an overexpression of tai (p160) driven by GMR-Gal4 markedly enhanced the AR (AF-1) transcriptional activity in the eye disc. Finally, we attempted to determine by transient expression assay in S2 cells whether the AR (wt) transactivation function reduces with dsRNAi constructs which repress endogenous expression of the co-activators Trap80, Trap100, AIB-1, and CBP. The DHT-induced transactivation function of AR (wt) was assessed 72 h after as RNAi transfection and was severely attenuated nearly to basal transcription levels (Fig. 3B, compare lane 2 with lanes 4, 6, 8, and 10). A similar effect on AR (AF-1) transactivation was observed both in mammalian cells and S2 cells (data not shown). Thus, by employing the well characterized and defined Drosophila genetic mutant system, we were able to demonstrate that the hAR (AF-1) domain functionally associates with co-activators previously known only to interact with the AF-2 domain of nuclear receptors.

The genetic approach in animals is a powerful technique that offers the advantage of being able to detect endogenous transcription co-regulators associated with a given transcription factor in vivo under different physiological conditions or different genetic backgrounds. In this study, we established a Drosophila model system by ectopically expressing functional hAR and its mutants. The application of genetic screening using these fly lines may help us to identify novel hAR (AF-1) co-regulators and determine fine mechanisms and processes through which hAR (AF-1) regulates gene expression.

Although the findings here indicate that both the p160/CBP HAT co-activator and the TRAP/Mediator are important in hAR-mediated gene expression, supporting the previous observations in vitro, several recent studies suggest a requirement for chromatin remodeling complexes in gene regulations by nuclear receptors in addition to their association with co-activators. For example, the ATP-dependent ISWI chromatin remodeling complex is required for retinoic acid receptor/RXR binding to chromatin templates. Furthermore, the SWI/SNF chromatin remodeling complexes, including WINAC, have been shown to play a critical role in vitamin D receptor/RXR and several nuclear receptor-mediated transactivation processes. Drosophila has been also been shown to possess chromatin remodeling factors that appear to be functionally conserved from yeast to mammals, and transcriptional controls by hAR in flies are supposed to require such chromatin remodeling complexes. While studies in yeast implicate a temporal role for the SWI/SNF complex in gene regulation preceding the recruitment of HAT coactivators, experiments with nuclear receptors and other activators in mammalians suggest that the SWI/SNF complexes function only after the recruitment of HAT co-activator complexes. Future genetic studies are required to reveal a stage or temporal role for each of the co-activator complexes in the context of recruitment of chromatin remodeling factors on nuclear receptor target promoters, and further to identify a novel molecule bridging between a co-activator complex and a chromatin remodeling complex.

In addition to genetic studies, functional screening for ligands with desired biological activities is also applicable by means of ligand-dependency in AR transactivation. Indeed, we showed that HF and BIC, major androgen antagonists, did not induce AR mediated transactivation in vivo and in S2 cells, suggesting that the structural alteration of the ligand binding domain in hAR with androgen antagonists causes an association with endogenous co-repressors. Recently, it has been reported that an EsR co-regulator, SMRT, a Drosophila homolog of the mammalian co-repressor SMRT, associates with endogenous Sin3A similar to its vertebrate counterpart. These findings perhaps indicate that the basic mechanism of transrepression by NRs is conserved between vertebrates and insects. Since mammalian HDAC co-repressor factors including SMRT are recruited to an antagonist-bound AR on the mammalian androgen-responsive gene promoters, it is conceivable that antagonist-bound hAR in flies also recruits SMRT for transrepression. To test this hypothesis and to determine the molecular basis of antagonist-induced transrepression by AR, identification of the antagonist-bound hAR-SMRTER or other functionally similar complexes is of interest.

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