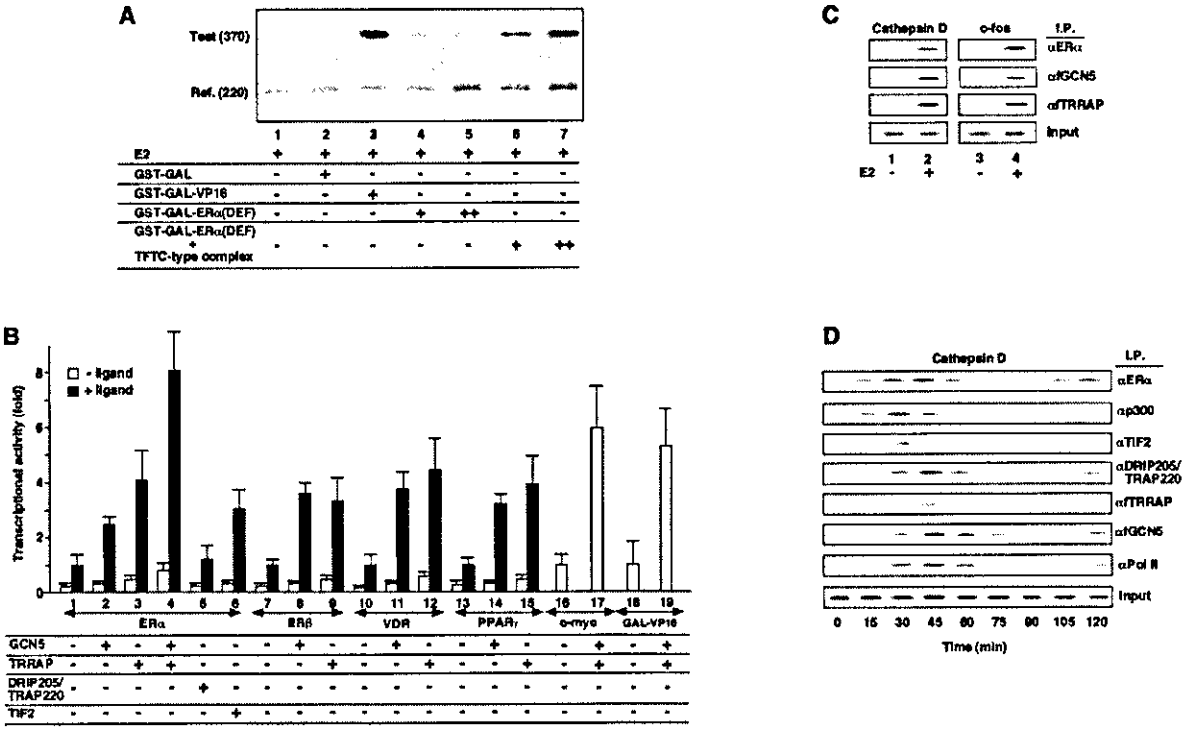

Figure 4. A TFTC-Type TRRAP/GCN5 Complex Acts as an ERα Coactivator

(A) Purified TRRAP/GCN5 complex potentiated the transactivation function of ERα by in vitro transcription. A TFTC-type TRRAP/GCN5 complex was purified according to the schema shown in Figure 2. GST-GAL (25 ng), GST-GAL-VP16 (25 ng), GST-GAL-ERα(DEF) (25[+], 50[+ +] ng) or GST-GAL-ERα(DEF) (25 [+], 50[+ +] ng) along with TRRAP/GCN5 complex was added to a 25 μl reaction mixture containing nuclear extracts in the presence of E_2 (10^{-6} M) and tested for their ability to potentiate the transactivation function of ERα by the in vitro transcription of a reporter plasmid. Reaction mixtures were incubated for 45 min and radiolabeled transcripts visualized on 6% denaturing polyacrylamide gels.

(B) Enhancement of ligand-induced transactivation function of ERα and other NRs by GCN5 and TRRAP in a transient expression assay. COS-1 cells were transfected with the expression vector of ERs (0.5 μg), GAL4DBD-VDR(DEF) (0.5 μg), GAL4DBD-PPAR(DEF) (0.5 μg), pGL-ERE-AdML (1.0 μg), and pML-CMV (10 ng) along with either pcDNA-TRRAP (1.0 μg), pcDNA-GCN5 (1.0 μg), or both in the presence or absence of cognate ligand (10^{-8} M), and the cell extracts used in a luciferase assay. Likewise, the expression vector of c-Myc (0.5 μg) and GAL-VP16 (0.1 μg) along with their reporter plasmids (M4-luc for c-Myc) (Kretzner et al., 1992) were transfected.

(C) Recruitment of ERα and GCN5 to the promoters of estrogen-responsive genes. Soluble chromatin was prepared from MCF-7 cells treated with E_2 (10^{-8} M) for 45 min and immunoprecipitated with antibodies against ERα or FLAG peptide (ChIP assay). Final DNA extractions were amplified using primer pairs that covered the cathepsin D and c-*fos* gene promoters as indicated.

(D) Dynamics of TRRAP/GCN5 complex assembly. Occupancy of the cathepsin D promoter by ERα, different coactivators, and RNA polymerase II (Pol II) at different times as measured by ChIP assay.

clones, not only the TRRAP recruitment, but also that of GCN5, was abolished to the promoter following estrogen treatment, whereas recruitment of the other coactivators was unchanged (Figure 5C). Furthermore, Northern blot analysis showed that the E2-induced expression of the endogenous *c-fos* gene was reduced in the stable cells when compared to the nontransformed MCF-7 cells (Figure 5D), indicating that in the absence of the TFTC-type HAT complex endogenous ERα cannot fully activate endogenous target genes. We next evaluated the growth rate of these stable clones. In the absence of E2, the stable transformants (AS-TRRAP-MCF7) exhibited normal cell growth similarly to the wild-type MCF-7 cells, as previously reported (McMahon et al., 1998) (Figure 5E, left panel). E2 treatments enhanced the growth rate of the wild-type MCF-7 cells (Figure 5E, right panel); however, no significant growth stimulation by E2 was observed in the stable transformants (Figure 5E, right panel). Importantly, reduction in the E2-dependent growth was observed in all of five independent clones (data not shown).

A TFTC-Type Complex Acts as a Third Class of Coactivator Complex for Nuclear Receptors

The GCN5 HAT-containing multiprotein complex isolated in this study appears to belong to the previously described TBP-free TAF_{II}-HAT (TFTC, PCAF, and hSTAGA) complexes (Ogryzko et al., 1998; McMahon et al., 1998; Martinez et al., 1998; Wieczorek et al., 1998; Brand et al., 1999b), since it contains a number of common components. The endogenous GCN5 HAT-containing multiprotein complex isolated from HeLa cells seems to have a molecular mass of about 1.5–2 MDa (Figures 2A–2C, fractions 3 and 4) and contains TRRAP in addition to GCN5. At present, due to the low abundance of this TFTC-type complex, we do not know which are the other TFTC subunits present in this complex. However, when FLAG-GCN5 was exogenously overexpressed in MCF7 cells, we isolated a multiprotein complex that is very similar to the GCN5/PCAF complex that was isolated from HeLa cells by a similar approach (Ogryzko et al., 1998), since our FLAG-GCN5 complex contains TRRAP/PAF400, PAF65β, ADA3, SPT3, and $TAF_{II}30$. Importantly,

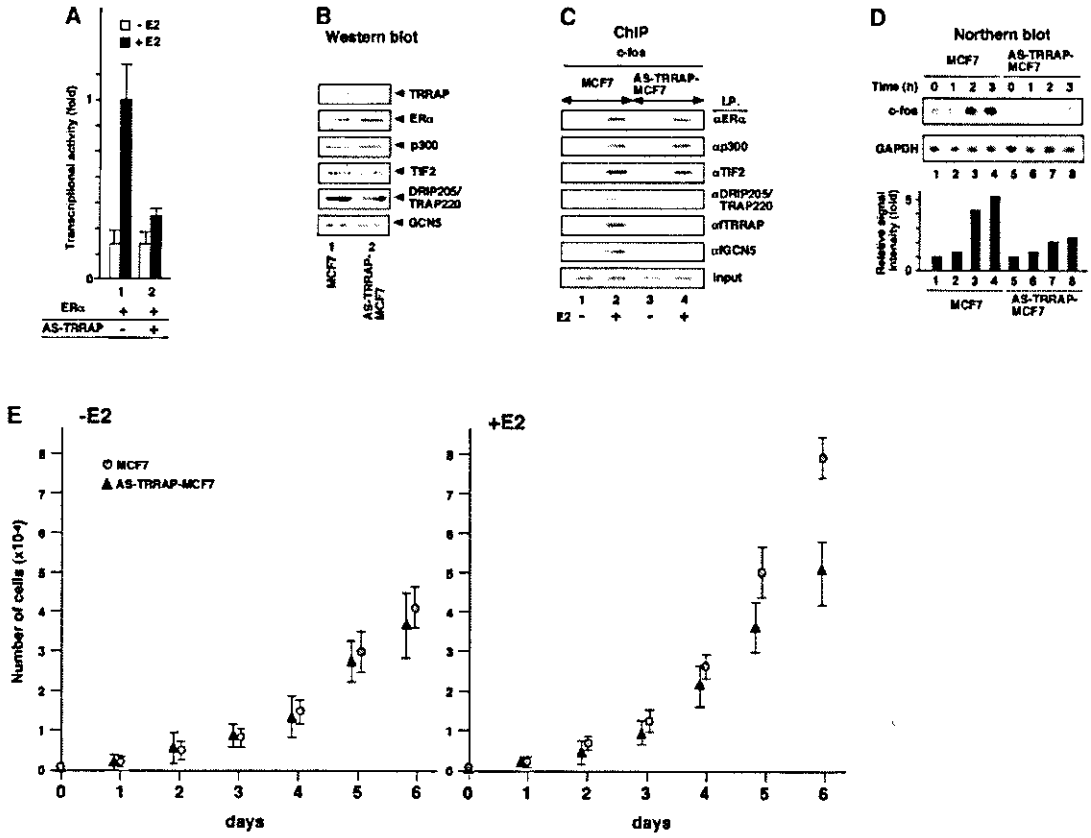

Figure 5. A TFTC-Type TRRAP/GCN5 Complex Is Involved in E_2-Dependent Growth of MCF-7 Cells

(A) Reduced ligand-dependent transactivation function of ERα by expression of TRRAP antisense RNA. COS-1 cells were transfected with ERα (0.5 μg), pGL-ERE-AdML (1.0 μg), and pML-CMV (10 ng), along with pcDNA-AS-TRRAP (1.0 μg) in the presence or absence of E_2 (10^{-8} M), and the cell extracts used in a luciferase assay. pcDNA-AS-TRRAP was transfected into MCF-7 cells and stable transformants selected by G418 (AS-TRRAP MCF7).

(B) Decreased TRRAP protein level in AS-TRRAP-MCF7 cells. Unchanged expression levels of ERα and certain other coactivators were observed by Western blot analysis, whereas TRRAP expresssion level was decreased in the AS-TRRAP-MCF7 cells.

(C) Lack of the TFTC-like complex recruitment to the *c-fos* promoter in the AS-TRRAP-MCF7 cells. No recruitment of the TFTC-like complex components (TRRAP and GCN5) was seen by ChIP assay in MCF7 cells expressing antisense TRRAP RNA. In contrast, under the same conditions, normal recruitment of the p300/TIF2 and the DRIP/TRAP complexes was observed to the *c-fos* promoter by ChIP assay.

(D) Reduced expression of an estrogen target gene in AS-TRRAP-MCF7 cells. The *c-fos* gene expression was examined by Northern blotting with GAPDH expression as an internal control. Densitometric analysis of the *c-fos* gene is shown in the lower panel.

(E) Involvement of TRRAP/GCN5 complexes in E_2-dependent growth of MCF-7 cells. Control MCF-7 cells and AS-TRRAP-MCF7 cells were incubated for the indicated times in the absence (left panel) and presence (right panel) of E_2 (10^{-8} M) and examined by counting cell numbers. Data shown as means ±SD of triplicate cultures of one representative clone used for analyses shown in panels (A)–(D) among five tested clones.

here we demonstrate that the TFTC-type GCN5/TRRAP-containing HAT complexes can function both in vitro and in vivo as coactivator complexes for NRs, and thus, we have defined a third class of coactivator complex important for NR function. The three identified LXXLL motifs in TRRAP protein are responsible for the direct and ligand-dependent interactions with the LBDs of NRs. This finding is in agreement with the suggested accessible location of TRRAP in the TFTC complex as assumed from its structural analysis (Brand et al., 1999a). The ChIP assay suggested that the GCN5 HAT-containing multiprotein complex acts, after recruitment to liganded ERα, about the same time as the DRIP/TRAP complex, and following the p160/CBP complex dissociation from liganded ERα. These data raise two alternative possibilities for the function of these TFTC-type complexes. In the first case, the TFTC-type complex would be functionally identical to that of the DRIP/TRAP complex in potentiating NR function. Note however that differences exist between the ligand-dependent recruitment of the TFTC-type complexes and that of the DRIP/TRAP complex when comparing proteins interacting with liganded LBDs of either ERα (Figure 1B) or VDR (data not shown). Furthermore, the TFTC-type complexes acetylate histone H3 in a nucleosomal context, while the DRIP/TRAP complex does not. Thus, we would prefer a second model in which both of the complexes may be simultaneously (or very quickly each after the other) recruited to ERα, and probably to many other receptors, to allow the formation of an efficient, large transcription initiation complex on chromatin templates, though the molecular mechanism underlying the switch-

ing of the complexes remains elusive. Thus, our findings, together with recent data showing that a TFTC-type TRRAP/GCN5 complex acts as a cofactor complex for c-Myc-mediated transformation (McMahon et al., 1998; Park et al., 2001) and that yeast Tra1 serves as a common target for acidic activators (Brown et al., 2001), raise the interesting possibility that the TFTC-type complex-dependent recruitment of acetyltransferase activity is critical for regulation of gene expression in general.

The lowered expression of endogenous TRRAP by its antisense RNA caused reductions in the E2-dependent cell growth of breast cancer cells (Figure 4F), indicating possible roles of TFTC-type complexes in the estrogen-dependent cell growth of breast cancer cells with enhanced function of c-Myc in cell proliferation. It is of particular interest to clarify the molecular mechanisms of the estrogen-dependent cell growth and the development of acquired E2-antagonist resistance in breast cancer. By identifying components of TFTC-type complexes in breast cancer, it will allow us a better understanding of the tumor development.

Experimental Procedures

Plasmid Construction

TRRAP cDNA (McMahon et al., 1998) was cloned into the pcDNA3 vector (Invitrogen) in an antisense orientation to generate pcDNA-AS-TRRAP. Three consensus estrogen response elements (3x ERE) or eight GAL4 binding elements (8 × 17 M) were inserted into a luciferase reporter plasmid bearing an AdML promoter to give pGL-ERE-AdML and pGL-17M-AdML, respectively. To produce chimeric GST-fusion proteins, cDNA fragments corresponding to LBDs of the NRs, the GAL4-DNA binding domain (1-147 aa) or VP16 transactivation domain were inserted into pGEX 4T-1 vector (Pharmacia Biotech).

Cell Culture

Human MCF-7 breast cancer cells and transformants were routinely maintained in phenol red-free DMEM (Life Technologies, CA) supplemented with 10% FBS (Hyclone, UT). To establish stable transformants, parent MCF-7 cells were transfected with either pcDNA-FLAG-GCN5 or pcDNA-AS-TRRAP by calcium phosphate precipitation (Yanagisawa et al., 1999) and cultured for 2 weeks in the presence of 500 μg/ml G418 for transformant selection (Watanabe et al., 2001). Reduced levels of TRRAP protein were confirmed by Western blotting and the growth rate of cells estimated in the presence or absence of E_2 (10^{-8} M). FLAG-GCN5 transformants were replated into fresh dishes and further cultured for 2 weeks in 500 μg/ml G418. Individual colonies were selected and expanded for further analysis.

Purification and Separation of ERα-Associated Complexes

HeLa nuclear extracts were loaded onto a P11 phosphocellulose column. After extensive washing with washing buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF, and 1 mM DTT), bound proteins were eluted by elution buffer (20 mM Tris-HCl, pH 7.9, 1 M KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF, and 1 mM DTT). Immobilized GST-ERα LBD fusion proteins were preincubated for 1 hr at 4°C in GST binding buffer (20 mM Tris-HCl, pH 7.9, 180 mM KCl, 0.2 mM EDTA, 0.05% NP40, 0.5 mM PMSF, and 1 mM DTT) containing BSA (1 mg/ml) and E_2 (10^{-6} M). Bead-immobilized proteins were then incubated at 4°C for 6–10 hr with P11 column-eluted fractions in the presence of 10^{-6} M E_2. After washing with GST wash buffer (GST binding buffer with 0.1% NP-40) three times, the beads were further washed with a GST wash buffer containing 0.2% *N*-lauroyl sarkosine (Sarkosyl, Sigma, St. Louis, MO). Complexes bound to the E_2-bound-liganded ERα were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris-HCl, pH 8.3, 150 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 5 mM NaF, 0.08% NP-40, 0.5 mg/ml BSA, and 10% glycerol). For purification of the FLAG-GCN5 complex from the MCF-7 stable transformant, elutants from GST-ERα column were further loaded onto 2.5–5 ml of M2 anti-FLAG agarose (Kodak, FL). After washing with binding buffer, the bound proteins were eluted from agarose by incubation for 60 min with 2.5–5 ml of the FLAG peptide (Kodak) in the binding buffer (0.2 mg/ml). For fractionation on glycerol gradient, elutants were layered onto the top of a 4.5 ml linear 100%–40% glycerol gradient in GST binding buffer and centrifuged for 16 hr at 4°C at 40,000 rpm in a SW40 rotor (Beckman, CA). Protein standards used were ovalbumin (44 kDa), β-globulin (158 kDa), and thyroglobulin (667 kDa) (Watanabe et al., 2001).

HAT Assay

The purified fractions were incubated with or without 10 μg calf thymus histones (Type IIA, Sigma) and ^{3}H-labeled acetyl CoA (4.7 Ci/mmol, Amersham, UK) for 30 min at 30°C, spotted onto Whatman P-81 filters, and washed extensively with sodium carbonate buffer (pH 9.1). Radioactivity remaining on the filter was then quantitated by liquid scintillation counting.

GST Pull-Down Assay

GST-fusion proteins were expressed in *Escherichia coli* and bound to glutathione-sepharose 4B beads (Pharmacia Biotech, UK). The in vitro-translated proteins were then incubated with beads in NET-N buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 0.5% NP40) with 1 mM PMSF. Bound proteins were separated by 7.5% SDS-PAGE, lightly stained with Coomasie brilliant blue to verify equal amounts of fusion protein, and then visualized by autoradiography (Endoh et al., 1999).

Immunoprecipitation

After washing MCF7 cells twice with ice-cold phosphate-buffered saline, the collected cells were resuspended in 1 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40), incubated on ice for 30 min, then centrifuged again for 5 min at 500 × g. Sedimented nuclear fractions were resuspended in TNE buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and 1% NP40) and incubated for 30 min on ice. After centrifugation, the supernatants were used as MCF7 whole-cell extracts for immunoprecipitation using anti-ERα antibody (anti-ER Ab-1; NEO MARKERS) following Western blotting using anti-ERα antibody, anti-TRRAP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GCN5 antibody raised against the N-terminal region (Santa Cruz Biotechnology) (Yanagisawa et al., 1999).

In Vitro Transcription

Transcription assay was performed according to previously published methods (Kundu et al., 2000). Briefly, 200 ng DNA was incubated with or without purified activators in 10 μl reaction volumes containing 0.02 mM PMSF and 0.2 mM DTT for 15 min at room temperature; after which, 6 μl rat liver nuclear extract (Conaway et al., 1987) (9 mg/ml protein) was added to each reaction. After incubation for 15 min at room temperature, 9 μl reaction mix containing 5 μl of 5× buffer (50 mM HEPES-KOH, pH 7.6, 15% glycerol, 128 mM KCl, and 30 mM $MgCl_2$, rNTPs), RNase inhibitor, α-^{32}P CTP, and RNase T_1 was added, and incubated for 45 min at 30°C. Reactions were stopped by addition of stop buffer (20 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1% SDS, 5 mM EDTA, and 200 μg/ml tRNA) and treated with proteinase K for 15 min at 37°C. ^{32}P-labeled RNA was extracted by phenol-chloroform, precipitated by ethanol, analyzed on 6% acrylamide 8.3 M urea gels, and visualized by autoradiography.

Transfection and Luciferase Assay

Cells at 40%–50% confluence were transfected with the indicated plasmids using Lipofectamine regent (GIBCO-BRL, CA) in 12-well petri dishes. Total amounts of DNA were adjusted by supplementing with empty vector up to 1.0 μg. Luciferase activity was determined using the Luciferase Assay System (Promega, WI). As a reference plasmid to normalize transfection efficiency, 25 ng of pRL-CMV plasmid (Promega) was cotransfected in all experiments (Takeyama et al., 1999).

ChIP Assay

FLAG-GCN5 transformant cells were cultured for the indicated periods in the presence of E_2 (10^{-8} M) and soluble chromatin prepared using the Acetyl-Histone H4 Immunoprecipitation assay kit (Upstate Biotechnology, CA). Soluble chromatin was Immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designed to amplify Cathepsin D (5′-TCCAGACATCCTCTCTG GAA-3′ and 5′-GGAAGCGGAGGGTCCATTC-3′) (Shang et al., 2000) and c-*fos* (5′-GAAGAGTGGAGAAGGG-3′ and 5′-GAAGCTGTGCT TACGG-3′) from extracted DNA. Optimal PCR conditions to allow semiquantitative measurement were 20 cycles of 30 s at 96°C, 15 s at 58°C, and 1 min at 72°C. PCR products were visualized on 2% agarose/TAE gels.

Northern Blot Analysis

Total cellular RNA was isolated from the indicated cells using ISOGEN reagent (Wako Co., Japan), and 20 μg RNA used for Northern blot analysis with cDNA of c-*fos* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as probes (Takeyama et al., 1997).

Antisense Oligonucleoside Phosphorothioate (AS PS-ODN) Preparation

The sequence of AS-TRRAP PS-ODN was 5′-CTGTGTTGCAA CAAACGCCAT-3′. The control PS-ODN was 5′-CTGAAATGCCG CAAACTTCAT-3′. MCF-7 cells were cultured for 48 hr with PS-ODNs before addition of E_2 (10^{-8} M). After a 24 hr culture with or without E_2, DNA synthesis of these cells was measured using 5-Bromo-2′ deoxy-uridine (BrdU) incorporation method.

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Androgen-Dependent Neurodegeneration by Polyglutamine-Expanded Human Androgen Receptor in *Drosophila*

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Summary

Spinal and bulbar muscular atrophy (SBMA) is an X-linked, adult-onset, neurodegenerative disorder affecting only males and is caused by expanded polyglutamine (polyQ) stretches in the N-terminal A/B domain of human androgen receptor (hAR). Although no overt phenotype was detected in adult fly eye photoreceptor neurons expressing mutant hAR (polyQ 52), ingestion of androgen or its known antagonists caused marked neurodegeneration with nuclear localization and structural alteration of the hAR mutant. Ligand-independent toxicity was detected with a truncated polyQ-expanded A/B domain alone, which was attenuated with cytosolic trapping by coexpression of the unliganded hAR E/F ligand binding domain. Thus, our findings suggest that the full binding of androgen to the polyQ-expanded hAR mutants leads to structural alteration with nuclear translocation that eventually results in the onset of SBMA in male patients.

Introduction

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is a rare degenerative disease of the motor neurons, characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30–50 years of age (Kennedy et al., 1968). Mapping studies and functional analyses of SBMA cases revealed expansions in the number of trinucleotide CAG repeats in the first exon of the *androgen receptor* (*AR*) gene, generating expanded polyQ stretches in the A/B domain of the AR protein (La Spada et al., 1991; Choong and Wilson, 1998; Merry and Fischbeck, 1998). These repeats encode polyglutamine (polyQ) stretches, and it has been found that disease onset occurs when the stretches contain more than 40 glutaminè residues, compared to a range of 8 to 34 polyQ stretches in normal individuals. SBMA patients often suffer mild androgen insensitivity, indicating impaired AR function due to the expanded polyQ stretches (Pinsky et al., 1992). However, it appears unlikely that motor neuronal cell death is caused simply by the loss of AR function, as neurodegeneration is not observed in severe testicular feminization (Tfm) patients that completely lack AR function (Brown et al., 1988; Yong et al., 1994). Like other neurodegenerative diseases involving polyQ stretches, such as Huntington's disease (HD), dentatorubral and pallidoluysian atrophy (DRPLA), and spinocerebellar ataxia atrophy (SCA), formation of mutant protein aggregates is observed in SBMA patients with a loss of selected neuronal populations (Kim and Tanzi, 1998). Abnormal folding of polyglutamine-expanded proteins may cause neural death through a common mechanism, as evidenced by the production of aggregates in these diseases (Ross, 1997). While polyQ-expanded hAR protein expressed in cultured cells has been shown to have reduced transactivation function, ligand binding is indispensable for aggregate formation (Stenoien et al., 1999).

AR is a member of the nuclear receptor superfamily and acts as a ligand-inducible factor to control transcription of a particular set of target genes (Mangelsdorf et al., 1995; Glass and Rosenfeld, 2000). Members of the steroid/thyroid hormone family share common structural features, with distinct functional domains, referred to as domains A to E/F. The highly conserved middle region (C domain) acts as a DNA binding domain (DBD), while the ligand binding domain (LBD) is located in the less well-conserved C-terminal E/F domain. The LBDs of most nuclear receptors, including AR, have been analyzed and are comprised of 12 α helices that form a pocket to capture cognate ligands (Shiau et al., 1998; Poujol et al., 2000). Upon ligand binding, the C-terminal α helix 12 (H12) in the LBD shifts position to create a space, with helices 3 to 5 serving as the key interface following dissociation of corepressor complexes and association of coactivator complexes (Freedman, 1999; Glass and Rosenfeld, 2000; Mckenna and O'Malley, 2002; Yanagisawa et al., 2002). During ligand-induced transactivation, the two N-terminal domains A/B and the steroid receptor LBD act as interacting regions for the coactivator complexes (He et al., 1999; Watanabe et al., 2001; Shang et al., 2002). The autonomous activation function-1 (AF-1) within the A/B domain is ligand independent, while the AF-2 in the LBD is induced upon ligand binding (Kato et al., 1995). Unliganded LBD appears to suppress the function of AF-1, while ligand binding to the LBD is thought to evoke the function of LBD and to restore the A/B domain function through an as yet undescribed intramolecular alteration of the entire steroid receptor structure. As SBMA occurs in men rather than women, we reasoned that a critical step in

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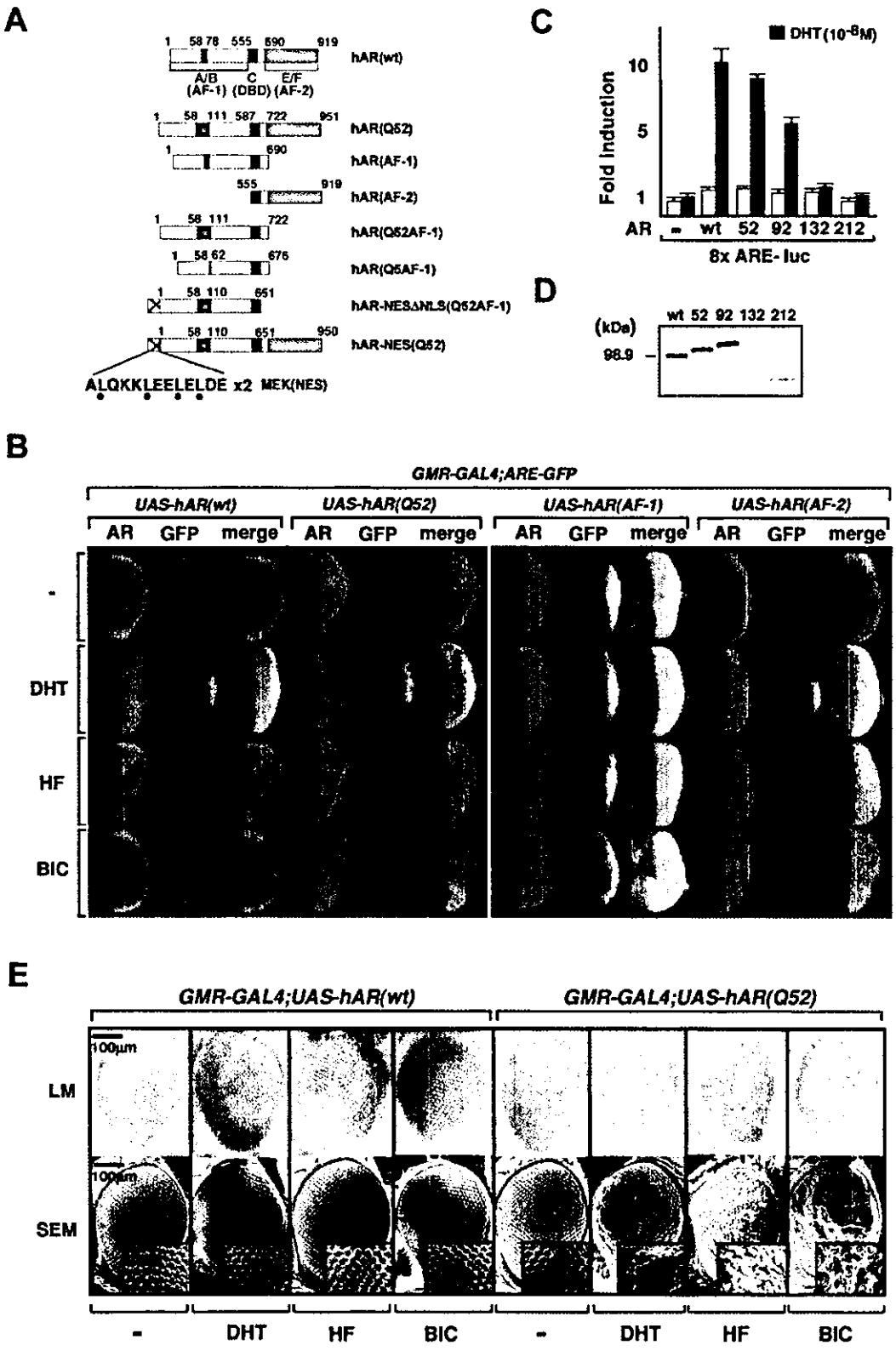

Figure 1. Ligand-Induced Degeneration in Photoreceptor Neurons by hAR Mutants with Expanded PolyQ Stretches

(A) Diagram of the AR constructs. Location of the polyglutamine (polyQ) region (red boxes) in relation to the DNA binding domain (DBD) (black boxes). Transactivation function 1 (AF-1) region is localized within the N-terminal A/B domain, and transactivation function 2 (AF-2) region is

the onset of SBMA could be the structural alteration and nuclear translocation of mutant AR upon binding of significant amounts of androgen.

To test this hypothesis, we investigated the role of hAR mutants with expanded polyQ stretches in neurodegeneration. To this end, we established a *Drosophila* model that ectopically overexpressed a mutated AR in photoreceptor neurons. Although the fly eye has proved to be an effective model in which to observe neuronal degeneration through the expression of other mutant proteins containing polyQ stretches (Jackson et al., 1998; Warrick et al., 1998), no abnormalities were found in eyes expressing a mutant hAR that contained an expanded 52 stretch polyQ (Q52). However, dietary ingestion of dihydroxytestosterone (DHT) induced marked degeneration of the photoreceptor neurons, along with apoptosis, although the mutant hAR still retained reduced transactivation function. Neurodegeneration was induced even in the absence of DHT when only the A/B domain, which harbors the 52 polyQ stretch, was expressed but was abrogated by coexpression of unliganded LBD domain. Surprisingly, known androgen antagonists failed to suppress the DHT-induced neurodegeneration in the Q52 line. Trapping the polyQ-expanded receptor mutants in the cytosol prevented neurodegeneration. Thus, our results suggest that hormone binding and subsequent structural alteration of hAR mutants with nuclear localization appears to be critical for SBMA onset. Furthermore, they reveal that the fly eye model may be useful for the development of novel therapeutic approaches to SBMA.

Results

Targeted Expression of Functional Human Androgen Receptor in *Drosophila*

We investigated the role of hAR mutants that contain expanded polyQ stretches by ectopic expression in the *Drosophila* eye (Figure 1A). The fly eye has proven to be an effective model to observe neuronal degeneration through the expression of other mutant proteins that also contain polyQ stretches (Jackson et al., 1998; Warrick et al., 1998). We first expressed wild-type and mutated hARs (Figure 1A) in photoreceptor neurons and accessory pigment cells in developing eye discs under a *glass multimer reporter* (*GMR*) gene promoter (Moses and Rubin, 1991), using the *Drosophila melanogaster* GAL4-UAS system (Brand and Perrimon, 1993). Using this system, targeted expression of hARs was also achieved in the anterior portion of embryonic segments by a *patched* (*ptc*) gene promoter and to the anterior-posterior boundary area in developing wing discs under a *decapentapregic* (*dpp*) gene promoter (data not shown) (Tanimoto et al., 2000). Northern blot analysis from different tissues of DHT-treated and untreated transgenic flies (data not shown) suggests that ectopic expression of hAR did not affect the expression of endogenous nuclear receptor genes (e.g., *Eip75B*, *ecdysone receptor*) (White et al., 1997). To monitor the ligand-induced transactivation function of hAR, hAR-expressing flies were further crossed to fly lines bearing a *GFP* reporter gene, such that GFP expression could be induced by ligand-bound AR that recognized the consensus androgen response element (*ARE*) in the *GFP* promoter (Yamamoto et al., 2000). Expressed hARs were then detected as red fluorescence in situ using an immunofluorescent antibody. Dietary ingestion of androgen (dihydrotestosterone/DHT) for 5 days from hatching induced remarkable and targeted GFP expression observed as green fluorescence in eye discs of third instar larva by the *GMR* promoter (Figure 1B) and in the other tissues by the *ptc* and *dpp* promoters (data not shown). Two independent transactivation functions (AF-1 in the A/B and AF-2 in the E/F domain of AR) were detected in eye discs (Figure 1B), as observed in cultured mammalian cells (Yamamoto et al., 2000; Ikonen et al., 1997). These observations indicate that ectopic expression of hARs translocated into the nuclei upon DHT binding and activated transcription in the tissues examined.

Ligand-Induced Neurodegeneration in the Fly Line Expressing Polyglutamine-Expanded Human Androgen Receptor

We then characterized the expanded polyQ hAR mutants. The reduction in the hormone-induced transactivation function of hAR mutants in COS-1 cells was dependent on the length of polyQ stretches in the A/B domain (Figures 1A and 1C). An hAR mutant that contained a 52 polyQ stretch [hAR(Q52)] exhibited only a slight reduction in DHT-induced transactivation (Figure 1C) but showed normal translation efficiency as estimated by in vitro translation (Figure 1D), while expres-

localized within the C-terminal E/F domain (gray boxes) containing the ligand binding domains (LBD). Nuclear export signals (NES) derived from MEK (Toyoshima et al., 1998) were tagged at the N terminus.

(B) hAR mutant expression and transactivation function in eye discs. Expression of hAR(wt), hAR(Q52), hAR(AF-1), and hAR(AF-2) in third instar eye imaginal discs driven by *GMR-GAL4* was detected using anti-hAR N-20 and/or C-19 antibodies (left panel). Transactivation function of hAR mutants was assessed using GFP expression (middle panel). A merged image is shown in the right panel.

(C) Reduction in the transactivation function of hAR mutants is dependent on polyQ length. COS-1 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt, Q52, Q92, Q132, or Q212), and 10^{-8} M DHT was added to the medium 6 hr after transfection (black boxes). After 18 hr, firefly luciferase activity (from ARE-tk) was measured to obtain the transfection efficiency, as previously described for Renilla luciferase activity (from pRL-CMV) (Yamamoto et al., 2000).

(D) In vitro translated hARs. Wild-type and mutant hARs were produced by in vitro translation (TNT-coupled in vitro translation system, Promega) in the presence of [^{35}S]methionine. Labeled proteins were separated by SDS-PAGE and analyzed by autoradiography. No stable protein can be produced for hAR(Q132) or (Q212).

(E) Rough-eye phenotype induced in hAR(Q52) lines by DHT or AR antagonists. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult eyes from 5-day-old flies treated as larval with or without 10^{-5} M DHT, HF, or BIC, respectively (closed arrow in Figure 2A). Genotypes are *UAS-hAR(wt)* or *UAS-hAR(Q52) in trans* to *GMR-GAL4*. While no degeneration is detectable after expression of the hAR(wt) protein, severe degeneration was observed in hAR(Q52) lines after treatment with the ligands. Scale bar: whole eye, 100 μm; eye inset, 10 μm.

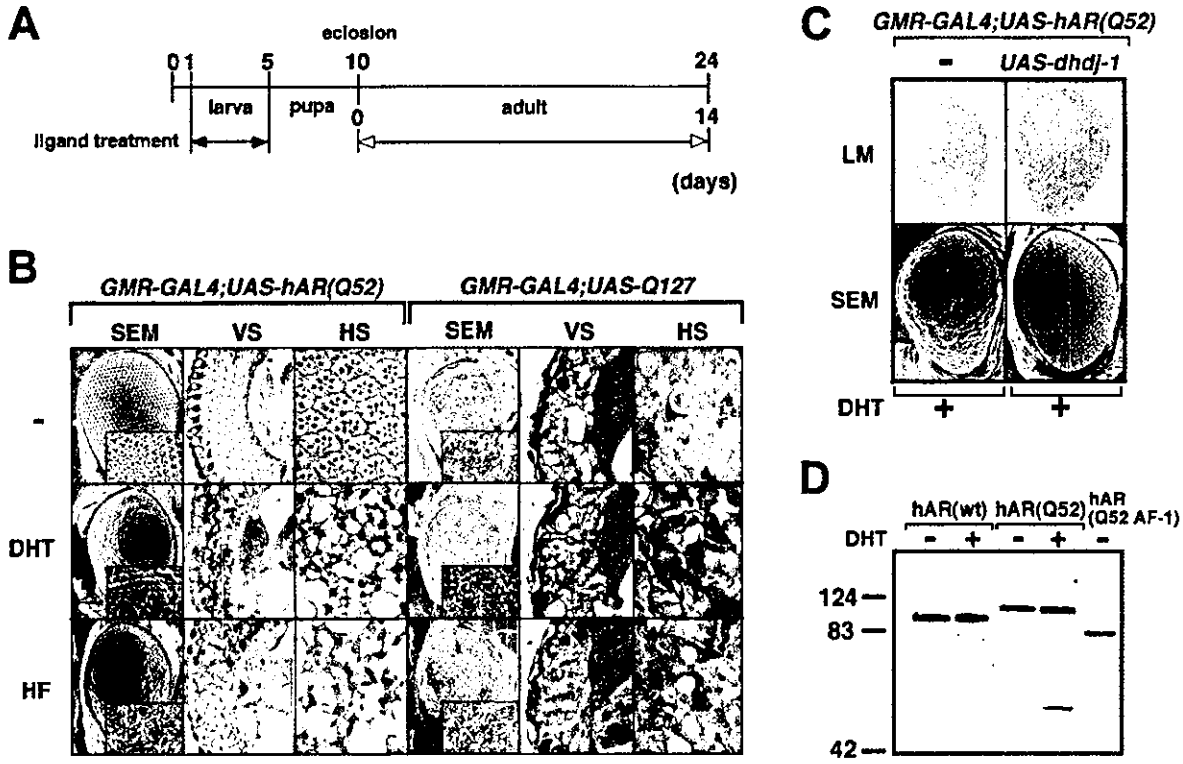

Figure 2. Enhanced Neurodegeneration in Photoreceptor Neurons of hAR(Q52) Lines by Ligand Ingestion

(A) Experimental schedule for ligand treatment. The ligands were given for 5 days after hatching (closed arrow) or for 2 weeks to adult flies after eclosion (opened arrow).

(B) Ligands induced neurodegeneration during adulthood. Genotypes are *UAS-hAR (Q52)* or *UAS-Q127 in trans* to *GMR-GAL4*. Adult transgenic flies were kept for 2 weeks on medium containing vehicle or ligands (10^{-5} M DHT or HF). Scanning electron microscopic images (SEM) of vertical (VS) and horizontal (HS) toluidine blue-stained sections show ligand-dependent degeneration in the hAR(Q52) line but not in the Q127 line. Eyes from six flies were analyzed for each genotype after 2 weeks with or without 10^{-5} M DHT or 10^{-5} M HF from eclosion. Degeneration was also observed in two other independent transgenic lines.

(C) Genetic suppression of ligand-induced neurodegeneration in hAR(Q52) lines by the chaperone component Hsp40. Genotypes are *GMR-GAL4;UAS-hAR(Q52) in trans* to *UAS-dhdj-1(dhsp-40)*. The analysis of fly eyes in the presence of 10^{-5} M DHT by LM and SEM shows a reduction of the pigmentation and rough-eye phenotype by expression of Hsp40 in fly eye.

(D) Expression of the hAR(wt) and hAR mutant proteins in transgenic fly eyes. The intact adult eyes of hAR (wt), hAR(Q52), or hAR(Q52 AF-1), treated with or without DHT for 2 weeks, were dissected and analyzed by Western blotting using an N-terminal-specific anti-hAR antibody (anti-hAR N20). Molecular weights (kDa) are indicated on the left.

sions of hAR(Q132) and hAR(Q212) proteins appeared at very low levels, judging from their translation efficiency (Figure 1D). Transgenic fly lines that expressed hAR(Q52) in the eye under the *GMR* promoter showed nearly normal eye morphology (Figures 1B and 1E) by light microscopy (LM) and scanning electron microscopy (SEM) throughout the life span of the fly, and no other tissues examined appeared to be affected (data not shown). However, we found that ingestion of DHT for 5 days after hatching (Figure 2A) induced marked disruption of the eye, including severely reduced ommatidia numbers and loss of pigmentation with thinned retinas in all lines tested at adult day 0 (see "DHT" treatment in right panels of Figure 1E). Notably, hAR(Q52) expression levels in the eye discs appeared to be unaffected compared to those of hAR(wt), both with and without DHT ingestion (Figure 1B). Despite the marked neurodegeneration in the adult eyes, DHT-induced transactivation function of hAR(Q52) in the eye disc nuclei was still detected (Figure 1B), as seen in COS-1 cells (Figure 1C). We then tested whether androgen antagonists could antagonize hAR(Q52) function and prevent eye disruption. As observed in other tissues, hydroxyflutamide (HF) and bicalutamide (BIC), as expected, depressed hAR(Q52) and hAR(wt) transactivation function but did not affect receptor protein expression levels in the fly eye discs (Figure 1B). Although the antagonists potently blocked DHT-induced hAR transactivation functions (data not shown), surprisingly, both antagonists not only failed to prevent but, indeed, appeared to potentiate eye disruption, leading to even more extreme phenotypes with increased loss of red pigmentation with retinal degeneration as compared to that observed using DHT alone (Figure 1E). The antagonists themselves appeared not to be toxic to the fly eye, as no phenotypic abnormalities were induced in either developing or adult eyes in wild-type hAR lines (Figure 1E) or in any of the tissues examined from normal flies (data not shown).

However, it remained unclear whether the rough-eye phenotype induced by hAR ligand treatment at larval

stages was due to impaired eye formation during development or to neuronal degeneration. To address this issue, AR ligands were given to adult fly lines for 2 weeks following eclosion (Figure 2A). DHT treatment in adults led to severe rough-eye phenotypes in the hAR(Q52) lines in 14 day adults, with loss of photoreceptor neurons, retina, and red pigmentation as observed by SEM and LM with vertical and horizontal sections (VS and HS) (left panels in Figure 2B), clearly indicating that the eye disruption induced by DHT reflects neurodegeneration. The androgen antagonist HF also induced eye disruption in the hAR(Q52) lines (left panels in Figure 2B). The rough-eye phenotype we observed looked similar to that of flies expressing other polyQ mutant proteins (Jackson et al., 1998; Warrick et al., 1998). As expected, neurodegeneration was induced by expressing a fragment with 127 polyQ (right panels in Figure 2B) (Kazemi-Esfarjani and Benzer, 2000). However, no ligand treatments potentiated the rough-eye phenotype (Figure 2B), suggesting that the AR ligands themselves were not enhancing polyQ-induced degeneration. Furthermore, the DHT effect was blocked by coexpression of Hsp40 (dhdj-1), a common suppressor of neurodegeneration by polyQ-expanded proteins (Figure 2C) (Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999). The DHT treatment did not appear to affect the expression levels of hAR(Q52) or hAR(wt) as observed by Western blotting with an antibody against the N-terminal end of hAR (Figure 2D). Most notably, an N-terminal fragment containing the expanded polyQ repeats was generated in a DHT-dependent manner in the adult eyes of the hAR(Q52) line (Figure 2D). No eye disruption was induced in the adult hAR(wt) lines by the hAR ligand treatment (data not shown). Thus, the function of hAR(Q52) in the induction of the rough-eye phenotype, which is induced by ligand binding, cannot distinguish between DHT and androgen antagonists. Again, the features of DHT-dependent neurodegeneration in hAR(Q52) lines appeared to strongly resemble the neural abnormalities in the male SBMA patients, and it would be interesting to compare the effect of anti-androgen treatment in these patients. However, no such clinical data are available.

Nuclear Localization Is Necessary for Expression of the Toxicity by the PolyQ-Expanded hAR A/B Domain

To explore the molecular mechanism of androgen dependency of hAR(Q52)-induced neurodegeneration, we first examined the effect of a truncated polyQ-expanded A/B domain construct, hAR(Q52AF-1) (Figure 1A). A chimeric hAR(Q52AF-1) protein fused only to the nuclear localization signal (NLS) of hAR was sufficient to induce marked toxicity (Figure 3A) with relevant expression levels to those of hAR(Q52) (Figure 2D), along with AF-1 transactivation function, even in the absence of ligand (right panels in Figure 1B). hAR(Q52AF-1) protein was predominantly localized to the nucleus, in agreement with a ligand-independent and constitutive transactivation function (Figure 3B), while nuclear localization of hAR(Q52) was observed only in the presence of a ligand (Figure 3B). The wild-type A/B domain [hAR(AF-1)] alone exhibited slight toxicity due to the wild-type 21 polyQ stretches (Figure 3A), presumably together with more sensitivity of the photoreceptor neurons to polyQ stretch, as expected from previous reports that wild-type disease proteins with normal polyQ stretches could cause neurodegeneration in the fly eyes (Fernandez-Funez et al., 2000). Indeed, a shortening of the polyQ stretches to five repeats in the A/B domain [hAR(Q5AF-1)] resulted in loss of toxicity (Figure 3A). However, we cannot exclude the possibility that the expressed hAR(Q52AF-1) and hAR(AF-1) proteins are in altered structures, which exhibit toxicity more than those of full-length hARs. Notably, the coexpression of unliganded LBD domain [hAR(AF-2)] attenuated the neurodegeneration induced by hAR (Q52AF-1) (Figure 3C), presumably by trapping the hAR(Q52AF-1) in the cytosol. However, DHT treatment aborted this attenuation (Figure 3C). These results indicated that unliganded LBD domain masks the toxic effects of the polyQ stretches as well as their transactivation function in the A/B domains. However, upon ligand binding, the toxic and transactivation functions of the polyQ-expanded A/B domain may be restored, accompanied with translocation into the nuclei.

We further tested whether nuclear localization is necessary for the expression of the toxicity by the expanded polyQ stretches in the hAR A/B domain by hAR mutants with a nuclear export signal (NES) (Toyoshima et al., 1998) (see Figure 1A), which is expected to constitutively retain the cognate protein in the cytosol. The addition of an NES to hAR(AF-1) lacking the D domain harboring the nuclear localization signal (NLS) (Zhou et al., 1994) [hAR-NESΔNLS (Q52 AF-1)] resulted in a predominant localization in the cytosol, and the DHT-induced nuclear localization of hAR(Q52) was prevented by the tagged-NES [hAR-NES(Q52)] (Figure 3B). In agreement with their cytosolic localization and lack of GFP induction by transactivation functions of hARs (Figure 3B), the mutants tagged with the NES exerted no toxicity in the fly eyes (Figure 3D), clearly establishing that nuclear localization is a prerequisite for the onset of neurodegeneration by the hAR mutants.

Ligand Binding Induces Toxicity of the PolyQ-Expanded hAR A/B Domain with Structural Alteration

We then directly analyzed androgen-induced alterations in hAR structure using a GST pull-down assay. While androgen-dependent interactions between the A/B and E/F domains were observed for hAR(wt) (Figure 4A, lane 3), hAR(Q52) exhibited ligand-dependent dissociation (Figure 4A, lane 5). This indicated that structural alterations took place upon ligand binding for both wild-type hAR and hAR(Q52), irrespective of the distinct structures of unliganded hAR(Q52) compared to hAR(wt). The ligand-induced alterations in receptor structure were further visualized using a trypsin digestion assay. Again, it was evident from the digestion patterns that structural alterations induced by DHT binding for hAR(wt) and hAR(Q52) were not identical (Figure 4B), in agreement with the observations in the adult eyes of hAR(Q52) lines that DHT treatment induced the generation of a fragment containing the polyQ repeats (Figure 2D). Thus, these results suggest that structural alterations of hAR mutants by ligand binding rendered the polyQ-expanded A/B domain more accessible to proteolysis, resulting in

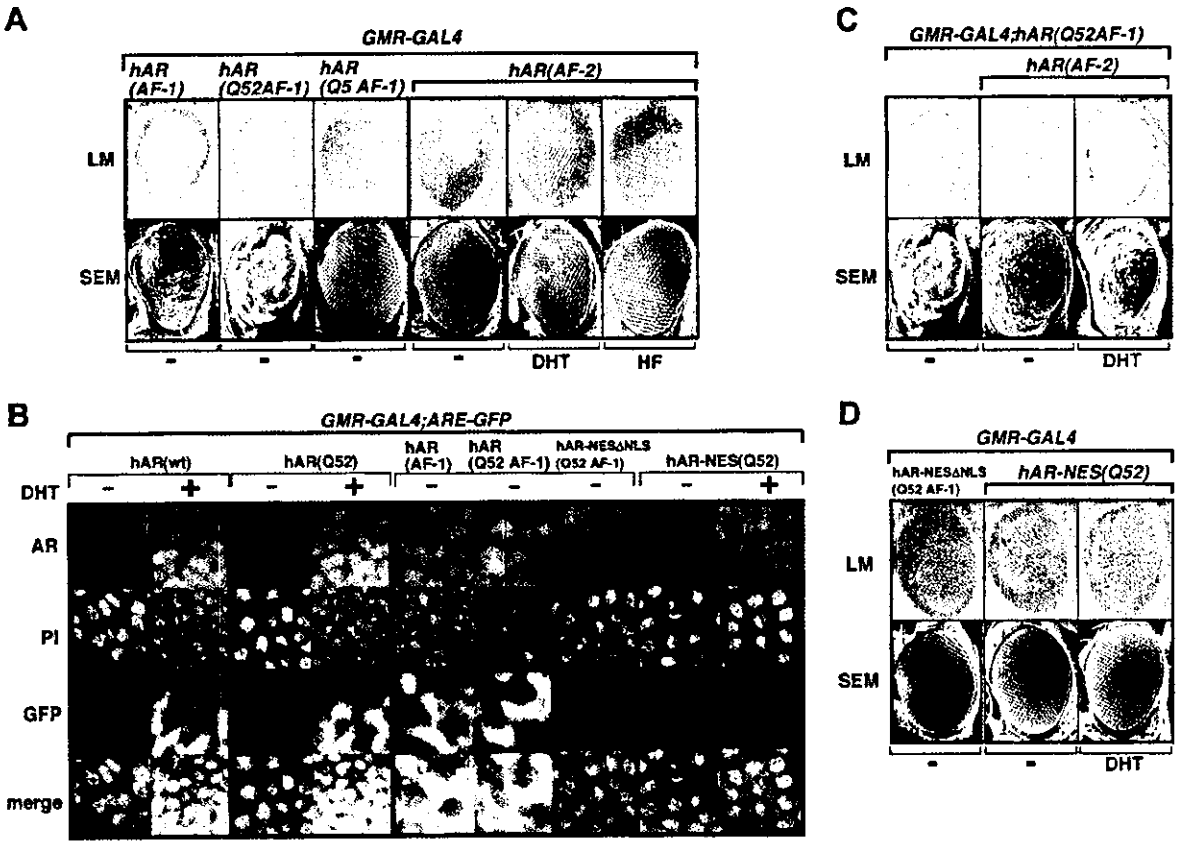

Figure 3. PolyQ-Expanded hAR AF-1 Alone Is Sufficient to Cause Neurodegeneration

(A) Ligand-independent neurodegeneration by hAR (Q52 AF-1). Genotypes are *UAS-hAR (AF-1)*, *UAS-hAR (Q52 AF-1)*, *UAS-hAR (Q5 AF-1)*, or *UAS-hAR (AF-2) in trans* to *GMR -GAL4*. Transgenic flies were kept on medium containing vehicle or ligands (10^{-5} M DHT or HF). While hAR(AF-1) and hAR(Q52 AF-1) induced degeneration even without ligand treatment, expression of hAR(Q5 AF-1) did not induce detectable degeneration, even after the treatment of ligands.

(B) Localization of hAR mutants in the third instar eye imaginal discs. hAR(AF-1) and (Q52 AF-1) predominantly localized to nuclei irrespective of DHT ingestion, but the nuclear localization of hAR(wt) and hAR(Q52) required DHT. Expression of hAR(wt), hAR(Q52), hAR(AF-1), hAR(Q52 AF-1), hAR-NESΔNLS(Q52 AF-1), and hAR-NES(Q52) in third instar eye imaginal discs driven by *GMR -GAL4* were detected using anti-hAR N-20 (left panel), and chromosomal DNA was stained with propidium iodide (PI), transactivation function of hAR mutants assessed using GFP expression (GFP), and merged image are shown in the right panel.

(C) Attenuation of hAR(Q52 AF-1)-induced neurodegeneration by unliganded hAR(AF-2). Genotypes are *GMR-GAL4;UAS-hAR(Q52 AF-1) in trans* to *UAS-hAR(AF-2)*. Without DHT, the expression of hAR(AF-2) suppressed the degeneration induced by hAR(Q52 AF-1). Treatment with DHT (10^{-5} M) abolished this effect.

(D) No toxicity of hAR mutants in the cytosol. Genotypes are *GMR-GAL4;UAS-hAR NES*ΔNLS*(Q52 AF-1)* or *UAS-hAR NES(Q52)*. Transgenic flies were kept on medium containing vehicle or ligands (DHT 10^{-5} M). The addition of a nuclear export signal to hAR(Q52 AF-1) or (Q52) abolished the toxic effect.

the generation of fragments that could potentially be toxic.

Discussion

The Transactivation Function of hAR Expressed in *Drosophila* Is Maintained without Affecting the Endogenous Nuclear Receptor System

Drosophila melanogaster possesses a number of endogenous nuclear receptors that are functionally homologous to members of the vertebrate nuclear receptor superfamily (White et al., 1997). Of the fly nuclear receptors, the physiological role of the ecdysone receptor (EcR) has been well-documented (Bender et al., 1997). Like vertebrate steroid hormone receptors, the transactivation function of EcR is completely dependent upon ligand binding. Specific DNA elements that bind EcR and other fly nuclear receptor molecules are thought to be composed of a directly repeated 5′-AGGTCA-3′ core motif (DRs), whereas vertebrate steroid hormone receptor homodimers bind a pair of core motifs arranged as inverted core motifs (IRs). The opposite orientation between DRs and IRs is thought to render ectopic expression of vertebrate steroid receptors in *Drosophila* unable to compete with endogenous fly receptors in DNA binding. However, as there are functional similarities in gene regulation between vertebrate and insect nuclear receptors, it is speculated that there may be a common coregulatory system that supports the transactivation function of nuclear receptors. Indeed, two

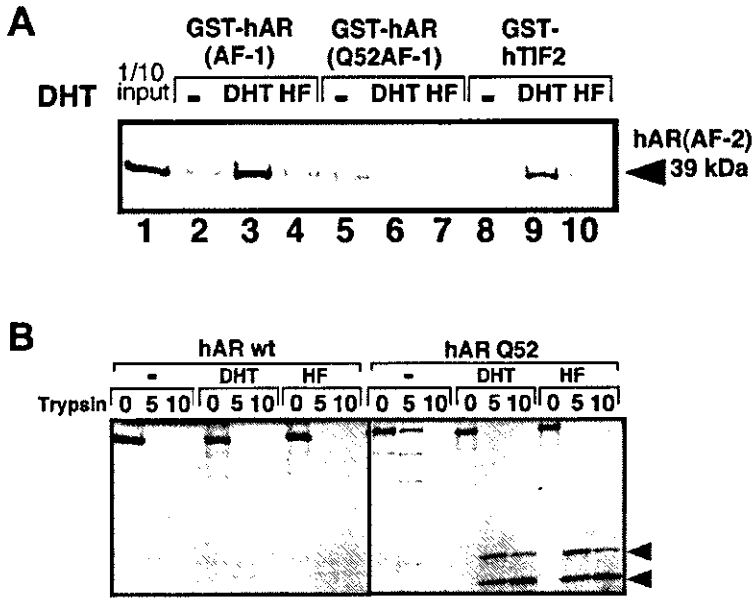

Figure 4. Structural Alteration Induced by Ligand Binding Differs between Wild-Type and Mutant hARs

(A) Ligand-induced dissociation of hAR(AF-2) and hAR(Q52 AF-1) in vitro. Interaction was assessed by incubating a GST fusion protein with either hAR(AF-1) [GST-AR(AF-1)], mutant hAR(AF-1) with Q52 [GST-AR(Q52 AF-1)], or GST-hTIF2 as a positive control, with in vitro translated [^{35}S]methionine-labeled hAR LBD by pcDNA3-hAR 560-919. A ligand-induced interaction between hAR LBD [hAR(AF-2)] and hAR A/B domain [hAR(AF-1)] was observed, while a ligand-depedent dissociation is seen for the hAR A/B domain mutant hAR(Q52 AF-1).

(B) Different structural alterations of hAR(wt) and hAR(Q52) induced by ligand binding. In vitro translated hAR(wt) and hAR(Q52) were incubated with or without DHT or HF (10^{-5} M) and were digested with 0, 5, or 10 ng of trypsin for 10 min at 32°C with (right panel) or without (left panel) recombinant hAR LBD expressed in *E. coli*. Two hAR (Q52)-specific fragments (arrow bands) can be detected by autoradiography after ligand treatment and digestion.

homologs to mammalian nuclear receptor coactivators CBP and AIB1 have been recently identified in *Drosophila* (Akimaru et al., 1997; Bai et al., 2000).

Ectopic expression of hAR targeted to particular tissues by specific promoters appeared functional in ligand-induced transactivation in all tissues tested, including the eye. Both unliganded and liganded hAR(wt) were nontoxic in the transgenic flies under all conditions studied, such that no phenotypic abnormalities were observed in any of the tissues. Also, the activities of ingested AR ligands agreed well with results obtained using mammalian cultured cells and intact mammals. Thus, it is clear that the fly system will be a useful tool in helping to dissect the function of vertebrate steroid hormone nuclear receptors and for the genetic screening of coregulators and chromatin remodeling factors that are essential for the ligand-induced transactivation function of steroid receptors.

Polyglutamine-Expanded Human Androgen Receptor Induces Degeneration of Photoreceptor Neurons

Ligand binding induces the transactivation function of AR, including the structural alteration required to activate the transcription of target genes via direct binding to specific DNA promoter elements (Freedman, 1999; Poujol, et al., 2000). However, it is unlikely that the ligand-induced neurodegeneration caused by polyQ-expanded AR mutants is directly related to the binding of mutant AR to the promoters of genes involved in neuronal cell death. First, the androgen antagonists did not induce transactivation function of the human AR mutants (Figure 1B) but induced neurodegeneration (Figure 2B). Second, the features of late-onset neurodegeneration in the eyes of Q52 transgenic fly lines appeared indistinguishable from transgenic flies expressing other polyQ-expanded disease proteins (Warrick et al., 1998; Jackson et al., 1998). Third, previous studies that extensively analyzed common events in those transgenic fly lines suggested that aggregate formation may cause neurodegeneration, which also appears to occur in Q52 lines. These neurodegenerate events are thought to be mediated through factors associated with the expanded polyQ stretches in the aggregates and not through any innate function of the disease proteins. This hypothesis is further supported by the fact that no phenotypic abnormalities in the fly eye expressing wild-type hAR were observed, even in the presence of ligand. Thus, ligand-induced neurodegeneration caused by AR mutants is most likely directly due to the expanded polyQ stretches in the hAR A/B domain and not to indirect alterations in hAR function.

Onset of Neurodegeneration by hAR Mutants Is Hormone Dependent

The androgen receptor is one of several neurodegenerative disease proteins which harbors an expanded polyQ stretch. However, in sharp contrast to other neurodegenerative disease-associated mutant proteins, SBMA develops only in men (Kennedy et al., 1968; Choong and Wilson, 1998). In the present study, we clearly show in an intact animal model that the onset of neurodegeneration is completely dependent on androgen binding to mutant hAR and nuclear translocation. Moreover, the mutated hAR A/B domain (Q52 AF-1) alone, when transported into nuclei, was sufficient to promote androgen-independent toxicity as well as transactivation, whereas coexpression of unliganded LBD abrogated the neurodegeneration induced by the A/B domain mutant, presumably by trapping the A/B domain in the cytosol. Although the structures of unliganded and liganded hAR mutants are likely to differ from androgen-bound wild-type hAR, polyQ-expanded hAR A/B domains appeared to be functionally exposed, like the wild-type hAR A/B domain, only upon androgen binding (Yamamoto et al., 2000; Ross, 1997; Watanabe et al., 2001). These findings

strongly indicate that the polyQ-expanded AR A/B domains in the SBMA patients are functionally and physically masked by the unliganded ligand binding domain in the cytosol. Androgen binding to mutant hARs induced structural alterations and translocation into the nuclei that resulted in toxicity. Thus, together with the fact that serum androgen levels in adult men are 10 to 20 times higher than in women, the androgen-dependent onset of the neurodegeneration in the fly eye may explain why only men suffer SBMA.

Nuclear Localization of PolyQ-Expanded hAR Mutants Depends on Ligand Binding

The nuclear localization of hAR mutants, like huntingtin and spinocerebellar ataxia type 1 (SCA1) mutants (Klement et al., 1998; Saudou et al., 1998), appears to be critical for the onset of neurodegeneration, since the toxicity of the hAR mutants was abolished when the polyQ-expanded hAR mutants were trapped in the cytosol by tagged-NES, even in the presence of ligand (Figures 3C and 3D). Moreover, the polyQ-expanded A/B domain mutant [hAR(Q52AF-1)], which constitutively localizes in the nuclei (Figure 3B) with the autonomous transactivation function (Figure 1B), caused ligand-independent neurodegeneration (Figure 3A). Nevertheless, the neurodegeneration induced by hAR(Q52AF-1) was attenuated by coexpression of the unliganded LBD E/F domain [hAR(AF-2)] (Figure 3C), which appears to trap the A/B domain mutant in the cytosol. More interestingly, known androgen antagonists clinically applied in androgen-dependent prostate cancer (Ruijter et al., 1999) failed to attenuate the DHT-induced neurodegeneration in the hAR(Q52) line. Although these antagonists were effective in blocking transactivation function of hAR mutants in the fly eyes (Figure 1B), nuclear translocation of the polyQ-expanded hAR mutants was unlikely to be inhibited, as antagonist-induced nuclear translocation of hAR has been previously reported (Tomura et al., 2001). These findings of the nuclear events are further supported by the recent report that neurodegeneration induced by polyQ repeats in huntingtin in the adult fly eyes required a transcriptional cofactor, CBP (Steffan et al., 2001).

Ligand Binding Causes Structural Alteration of hAR Mutants to Expose the PolyQ-Expanded A/B Domain

Ligand binding to nuclear receptors induces structural alterations, dissociation of corepressor complexes, and association of coactivator complexes for ligand-dependent transactivation (Freedman, 1999; Glass and Rosenfeld, 2000; Mckenna and O'Malley, 2002; and Yanagisawa et al., 2002). Crystallographic analyses of the structural changes in LBDs of many nuclear receptors, including AR, revealed that H12 is drastically shifted, while other helices are also repositioned upon ligand binding (Poujol et al., 2000). The angle of H12 movement is ligand-type dependent and determines the agonistic/antagonistic action of the ligand. Improper H12 shifting and impaired pocket formation of the other helices by antagonist binding may result in the lack of recruitment of coactivator complexes to the ligand-bound LBD (Shiau et al., 1998). These findings suggest a general molecular basis by which structural alterations caused by agonist or antagonist binding modulate AF-2. In contrast, due to technical limitations of structurally analyzing the whole nuclear receptor, little is known of the structural basis of A/B domain structural alteration upon ligand binding and subsequent AF-1 induction. However, it is evident that an intramolecular structural alteration involving the entire receptor molecule takes place after ligand binding that exposes the A/B domain and allows AF-1 activation (Kato et al., 1995; Watanabe et al., 2001; Kitagawa, et al., 2002). The A/B domain may be exposed upon ligand binding, such that coactivator complexes are recruited after dissociating from the ligand-bound LBD domain. Alternatively, it is also possible that coactivator complexes recruited to the LBD upon ligand binding form a bridge to the A/B domain by releasing an inhibitory factor that suppresses AF-1.

While mutated hAR A/B domains appear to be functionally exposed upon ligand binding, the resultant structural alterations in hAR mutants are likely to differ from those of ligand-bound wild-type hAR. While known androgen antagonists are capable of inactivating the transactivation function of mutant hAR structures, the structurally altered mutant hAR still appears to be in a position to exhibit the toxicity. Judging from expression levels in fly eyes treated with and without DHT, it is unlikely that the half-life of the hAR(Q52) protein is significantly affected by ligand binding. It has been previously demonstrated that some mutant polyQ proteins are alternatively cleaved by proteases (Merry et al., 1998; Stenoien et al., 1999). It has been suggested that these truncated proteins are toxic (Ross, 1997; Kim and Tanzi, 1998). We find that ligand binding of the mutant hAR receptor results in the generation of an N-terminal fragment (see Figure 2D). It is therefore possible that ligand binding results in a conformational change of the hAR, making it more accessible to proteolysis, which allows for the generation of a potentially toxic polyQ-containing fragment.

A Clue to Rescue SBMA/Kennedy Disease

All together, these findings suggest that nuclear localization with the ligand-dependent structural alteration is critical for the onset of neurodegeneration by hAR. Although cellular formation of aggregates by polyQ-expanded hAR mutants in cultured cells (Stenoien et al., 1999; Simeoni et al., 2000) and subsequent inhibition of aggregate formation in the cytosol by antagonists (Becker et al., 2000) have been previously reported, it appears likely that the nuclear events caused by the polyQ-expanded hAR mutants are required for SBMA pathogenesis. If the mutated receptors could be trapped in the cytosol by a novel ligand, the toxicity of mutant hAR might be prevented or at least reduced. For this reason, androgen antagonists that still permit nuclear localization would not be useful therapeutically for the treatment of SBMA. Thus, the hAR(Q52) *Drosophila* line is a useful SBMA model for drug development and for genetic screening for factors involved in androgen-induced neurodegeneration. In conclusion, we propose that SBMA may be treated by giving patients novel hAR ligands that prevent nuclear translocaton of hAR.

Experimental Procedures

Transactivation Assay

COS-1 cells were maintained in Dulbecco's modified Eagle's medium without phenol red, supplemented with 5% fetal-calf serum stripped with dextran-coated charcoal (Kato et al., 1995). COS-1 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt, Q52, Q92, Q112, or Q212). Cells were incubated for 18 hr in the absence or presence of 10^{-8} M DHT and then assayed for luciferase activity as previously described.

In Vitro Translation System

Wild-type and mutant AR proteins were produced by in vitro translation of respective cDNAs in pSG5 in the presence of [^{35}S]methionine (Promega) (Takeyama et al., 1997).

Drosophila Stocks and Generation of Transgenic Flies

All general fly stocks and the *ptc-GAL4* line were obtained from the Bloomington *Drosophila* Stock Center. Transgenic constructs together with pπ 25.7 wc transposase were microinjected into 5–30 min old w^{1118} embryos reared at 18°C, using a micromanipulator (Leica). Several transgenic lines were generated (Tsuneizumi et al., 1997). The AR mutant cDNAs in *pCaSpeR3* (see Figure 1A) and an *ARE-GFP* reporter construct (*GFP-TT* in *pCaSpeR3* with a consensus ARE in its promoter) were constructed specifically for microinjection into *Drosophila*. Plasmid rescue and sequencing were performed to confirm the presence of AR mutants in the transgenic lines. Target chromosomes were separated from those carrying the *GAL4*-driver by crossing with flies harboring second and third balancer chromosomes CyO and TM3. *GAL4*-driver lines used were as follows: *GMR-GAL4* line, expressing GAL4 in the retina driven by the glass multimer reporter (Moses and Rubin, 1991); *dpp-GAL4* line, in the anterior-posterior boundary area in developing wing disc; and *ptc-GAL4* line, in the anterior portion of embryonic segments. The *UAS-dhdj-1* and *UAS-Q127* lines were the generous gift of Dr. Kazemi-Esfajani (Kazemi-Esfarjani and Benzer, 2000).

Histology

Tissues were dissected and fixed for 20 min in 4% formaldehyde (Tanimoto et al., 2000) and incubated with a primary antibody, hAR (N-20), to recognized the N-terminal A/B domain of AR (Santa Cruz Biotechnology, Inc). Cy5-conjugated AffinityPure donkey anti-rabbit IgG (Jackson Immunoresearch) was used as the secondary antibody for immunofluorescence staining. Toluidine blue stainings of adult eyes was performed on 1 μm thick vertical or horizontal serial sections (VS or HS) (Kanuka et al., 1999). Chromosomal DNA was stained with propidium iodide (PI). Confocal microscopy was carried out on a Zeiss confocal laser scanning system 510, and results were assessed with Adobe Photoshop 5.0 (Adobe). For scanning electron microscopy (SEM) images, whole flies were dehydrated in ethanol, critical-point dried, and analyzed with a JEOL JSM 6100 microscope.

Western Blot Analysis

To detect hAR proteins and fragments containing the polyQ-expanded N-terminal regions, cell lysates of intact adult eyes with or without ligand were separated by 7.5% SDS-PAGE and detected with hAR (N-20) antibody (Santa Cruz Biotechnology, Inc).

GST Pull-Down Assay

Human AR A/B domain (AF-1) and its Q52 mutant (Q52 AF-1) were expressed as GST fusion proteins [GST-AR(AF-1) and GST-AR(Q52 AF-1), respectively] in *E. coli*, as previously described, and bound to glutathione-Sepharose 4B beads (Pharmacia Biotech). The ^{35}S-labeled AR deletion mutant together with DNA and ligand binding domains CDE/F were incubated with beads bound with either GST-AR(AF-1) or GST-AR(Q52 AF-1) in the absence or presence of 10^{-6} M DHT or HF in NET-N buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA) with 1 mM PMSF. Bound proteins were separated by 7.5% SDS-PAGE and lightly stained with Coomasie brilliant blue to verify equal protein loading and then visualized by autoradiography.

Trypsin Digestion Assay

Labeled translation mixtures were incubated at 25°C with or without 10^{-6} M DHT or HF for 30 min. Limited trypsinization was performed by addition of trypsin solutions (5 μg/ml or 10 μg/ml trypsin) at 32°C and stopped with equivalent amounts of 2× SDS sample buffer.

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Late onset of obesity in male androgen receptor-deficient (AR KO) mice

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Abstract

An androgen receptor (AR) null mutant mice line was generated by means of a Cre-lox P system. The male ($AR^{L-/Y}$) (KO) mice exhibited typical features of testicular feminization mutant (Tfm) disease in external reproductive organs with growth retardation. The growth curve of the male AR KO mice was similar to that of the wild-type female littermates until the 10th week of age, but thereafter a drastic increase in the growth was observed with development of obesity. Clear increase in the wet weights of white adipose tissues, but not of brown adipose tissue, was found in the 30-week-old male AR KO mice. However, no significant alteration in serum lipid parameters and food intake was observed. Thus, the present results suggest that AR may serve as a negative regulator of adipose development in adult males.

Keywords: Androgen; Androgen receptor; AR KO mice; Tfm disease; Obesity; Adipocyte; Lipid accumulation

Androgens exert a wide variety of actions in target tissues like male reproductive organs, brain for expression of sexual behaviors, and skeletal tissues as anabolic actions [1,2]. Most of such androgenic actions are believed to be mediated by tissue-specific transcriptional controls of a particular set of target genes through nuclear androgen receptor (AR) [3,4]. AR is a member of the nuclear receptor gene superfamily and acts as a ligand-inducible transcription factor [5,6]. Upon ligand binding, AR, like the other nuclear receptors, recruits distinct classes of co-regulators and co-regulator complexes for ligand-dependent transcriptional control [6,7].

Such androgen actions are well documented especially in male animals, however, little is clear about the molecular basis of androgen actions in the target tissues, due to the lack of a null mutant animal line deficient in AR (AR KO). As the mammalian AR gene locates on the X chromosome and is a single copy gene in males, loss of AR function by genetic mutations leads to androgen insensitivity and infertility in males with female-like phenotypes known as testicular feminization syndrome (Tfm) [8,9]. Such infertility in male AR mutant has prevented the generation of AR KO ($AR^{-/Y}$) mice line by a conventional gene disruption method.

To define physiological functions of AR in male and female animals, we applied a Cre-lox P system to establish AR KO mice line [10]. Male AR KO ($AR^{L-/Y}$) mice exhibited typical Tfm abnormalities, which have been well documented in the Tfm rodents and patients [8,9]. Most notably, unlike the reported Tfm mice, neither AR transcript nor AR protein was detectable in AR KO ($AR^{L-/Y}$) mice to date [10], indicating that our AR KO mice have advantage over naturally mutated Tfm animals as an AR null mutant to evaluate the physiological function of AR.

During analyses of the male AR KO mice [10], we became aware that the AR KO mice develop obesity, but its onset appeared only after growth maturation. Although the growth curve of the male AR KO mice was similar to that of the wild-type female littermates until 10 weeks of age, thereafter it caught up in a following couple of weeks and came over that of the wild-type male littermates. Remarkable increases in white adipose tissues were seen, but the levels of serum lipid

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markers and the food intake appeared unaffected by AR inactivation. Such a clear accumulation of lipids in adipocytes was not found in female $AR^{(L-/L-)}$ mice. Thus, these results suggest that AR serves as a negative regulator of adipocyte development in adult males.

Materials and methods

Animal conditions and food intake. AR KO mice were generated by targeting disruption of AR gene by means of a Cre-lox P system, as previously described [10,11]. All mice were given a standard laboratory chow diet (4.4% w/w fat) and water ad libitum. The growth rates of male $AR^{L-/Y}$ and $AR^{+/Y}$ were monitored from the birth. Food intakes of $AR^{L-/Y}$ and $AR^{+/Y}$ mice were monitored for 12 weeks from the 12th week. For hormone treatments, a 60-day time-release pellet (Innovative Research of America) containing either DHT (10 mg/pellet), E2 (0.25 mg/pellet) or placebo (PLA) was implanted subcutaneously in 10-week-old $AR^{L-/Y}$ and $AR^{+/Y}$ mice 2 weeks after gonadectomy under avertin anesthesia. The body fat contents were evaluated by a dual-energy X-ray absorptiometry (DXA, PIXImus2; LUNAR) according to the methods by Sjogren et al. [12].

Histological Analysis. Subcutaneous, infrarenal, intraperitorial, and gonadal fat pads were taken from mice and the wet weights were measured. The tissues were fixed with 4% paraformaldehyde and frozen in tissue-Tek OCT compound. Ten-micrometer cryosections were stained with hematoxylin and eosin, and examined by light microscopy [13].

Serum measurements. Blood was collected by cardiac puncture from anesthetized mice after 20 h fasting. Serum levels of cholesterol, triglycerides, and free fatty acids were determined by an enzymatic colorimetric method (ACS-ACOD; Eiken Chemicals) [13].

Statistical analysis. The data were evaluated by Student's *t* test and a one-way analysis of variance (ANOVA) followed by post hoc comparison using Fisher's protected least significant difference (Fisher's PLSD) test.

Results

Late onset of obesity of male AR KO mice

The AR floxed male ($AR^{L3/Y}$) mice grew up normally with no overt abnormality in behaviors and metabolisms, and appeared completely normal in reproduction. Male AR floxed mice were then crossed with CMV-Cre transgenic mice [11] to generate female heterozygotes ($AR^{+/L-}$) for further production of male AR $KO^{(L-/Y)}$ mice [10].

The male AR ($AR^{L-/Y}$) KO mice exhibited growth retardation and the growth curve was indistinguishable from that of the wild-type female littermates up to the 10th week (Fig. 1A). However, thereafter, the rapid increase in the growth of male AR KO mice was seen, and until the 12th week the body weights of the male AR KO mice exceeded over those of the wild-type male littermates. The late onset of drastic increase in the growth curve in the male AR KO mice was understandable as development of clear obesity (Fig. 1B, upper panel).

Increased adipocytes in male AR KO mice

Reflecting obesity of male AR KO mice, significant increases of wet tissue weights in subcutaneous, infrarenal, and intraperitorial white adipose tissues (WATs) were observed at the 30th week of age (Fig. 1B lower panel, and Fig. 2A) and a clear lipid accumulation was seen in the subcutaneous WAT (Fig. 2B) and infrarenal and intraperitorial WATs (data not shown). In contrast, the sizes of gonadal adipocyte and brown adipocyte tissue (BAT) appeared unchanged by AR inactivation. Such a clear increase was not detected in the WATs of the 8-week-old male AR KO mice, consistent with the total body fat contents (Fig. 2C). To get an insight into the rapid increases in WATs of male AR KO mice after the 8th week of age, we first monitored the food intake for 12 weeks from the 12th to 24th week. Inconsistent with the drastic lipid accumulation in the male AR KO mice, no increase in food intake was seen (Fig. 3A). Likewise, no significant alteration in serum markers related to lipid metabolism was detected in the male AR KO mice at the 8th week (data not shown) and at the 10th week of age (Fig. 3B).

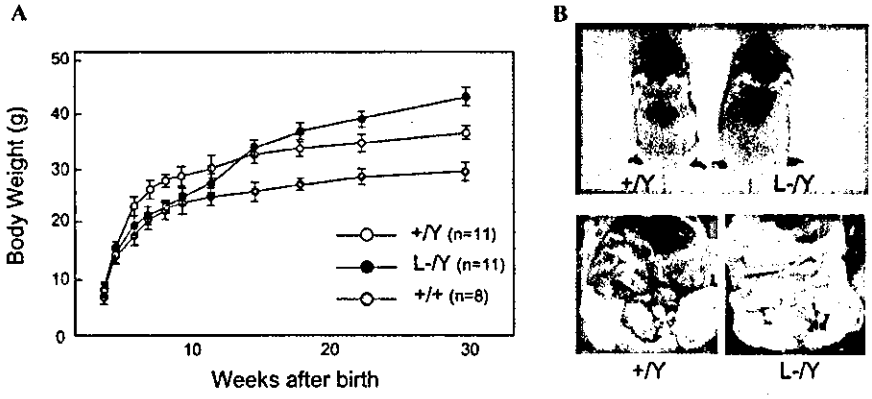

Fig. 1. Late onset of obesity in male AR KO mice. (A) Growth curves of the 30-week-old $AR^{+/Y}$ and $AR^{L-/Y}$ male mice and female $AR^{+/+}$ mice. Circles represent means ± SE. (B) External and intraabdominal appearance of 30-week-old $AR^{+/Y}$ and $AR^{L-/Y}$ mice.

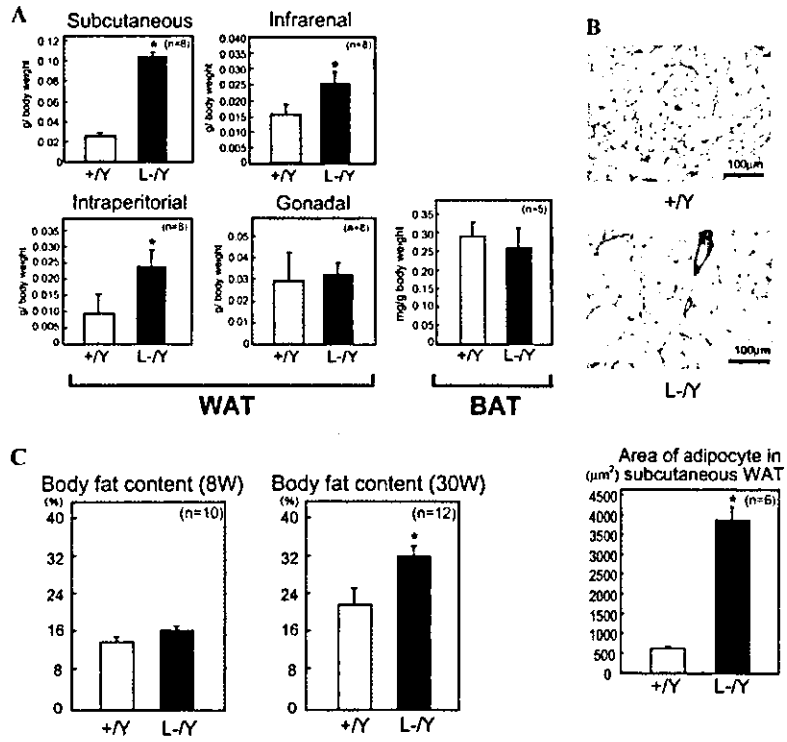

Fig. 2. Increased wet weights in white adipose tissues in male AR KO mice. (A) Wet weights of WATs and BAT from 30-week-old $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars) mice. Bars represent means ± SE (* $P < 0.005$). (B) Subcutaneous WATs from 30-week-old $AR^{+/Y}$ and $AR^{L-/Y}$ were fixed and sectioned and stained with hematoxylin and eosin (scale bars: 50 μm). Area of adipocyte in subcutaneous WAT from 30-week-old $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars) is displayed. (C) Fat proportion of 8- and 30-week-old $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars). Bars represent means ± SE (* $P < 0.005$).

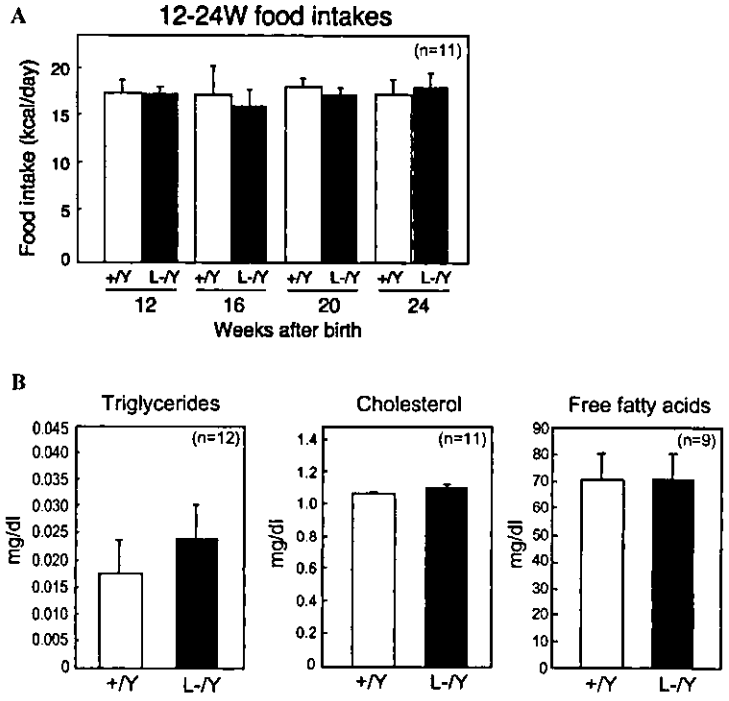

Fig. 3. No alteration in serum lipid markers and food intake in male AR KO mice. (A) Serum contents of triglycerides, total cholesterol, and free fatty acids in 30-week-old $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars) mice. Bars represent means ± SE. (B) Daily food intakes of 12–24-week-old $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars) mice. Bars represent means ± SE.

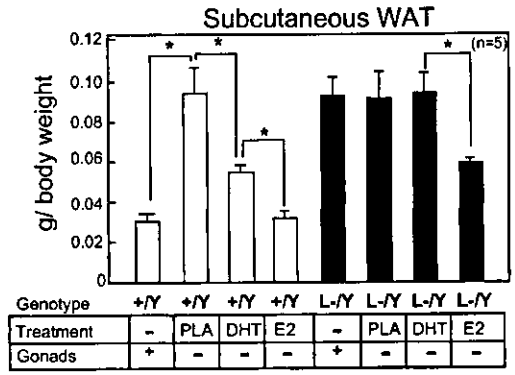

Fig. 4. Suppressive actions of sex steroid hormones in male adipose development. Wet weights of subcutaneous WAT from intact and hormone (DHT, E2, or placebo) treated $AR^{+/Y}$ (empty bars) and $AR^{L-/Y}$ (filled bars) mice. Bars represent means ± SE (*$P < 0.005$).

Treatment of estrogen attenuated the late onset of lipid accumulation of the male AR KO mice

As the estrogen signaling mediated through estrogen receptor α (ERα) is shown to suppress the development of WATs by the observations of male mice deficient in either ERα or aromatase [14,15], we wondered if such estrogenic action is intact or not in the male AR KO mice. As expected, a treatment with dihydrotestosterone (DHT) for 12 weeks to the 10-week-old male AR KO mice had no suppressive action, but 17β-estradiol (E2) was potent enough to attenuate the increase of wet weights in subcutaneous WAT (Fig. 4). In contrast, both of DHT and E2 were effective in preventing the subcutaneous WAT development in the wild-type male littermates. Thus, these findings suggest that the androgen-AR system has a negatively regulatory role in adipocyte development in male adult animals.

Discussion

We have succeeded in generating a null mutant mouse line by means of a Cre-lox P system [10], and the male AR KO ($AR^{L-/Y}$) mice exhibited the typical features of Tfm syndrome [8,9] including female-like outlook of external reproduction organs, degenerated testes, no ovary, and blunted vagina [10]. Male AR KO mice showed growth retardation, but the growth curve was similar to that of the wild-type female littermates up to the 8th week. However, thereafter obesity developed in the male AR KO mice with remarkable increases in WATs. Moreover, a DHT treatment with wild-type male mice attenuated adipogenesis, in agreement with previous studies in vitro [16–18]. Thus, these results suggest that the androgen-AR signaling system is a negative factor for adipocyte development in the adult male animals.

There are a number of factors involved in adipogenesis [19]. Among them, an estrogen-ER system has been recently shown to play a negative role in adipocyte development like the androgen-AR signaling presented here. Interestingly, the suppressive actions of these sex hormones were seen in WATs, but not in BAT [14,15]. However, there was a clear sex difference between two sex hormone actions in adipogenesis. Impaired estrogen signaling induced either by ERα or aromatase inactivation in mice resulted in increases in WATs in both sexes [14,15]; indeed an estrogen treatment was suppressive for adipocyte development in the male AR KO mice. However, obesity did not develop in the female AR KO ($AR^{L-/L-}$) mice at 30 weeks old with no significant difference in body weights when compared to the wild-type female mice (data not shown). Moreover, the onset of the obesity in male AR KO mice was only after growth maturation, while increases in WATs were detected in the ERα KO mice at the 8th week of age [14]. It is also notable that no wet weight increase in gonadal WAT was detected in the male AR KO mice even at 30 weeks of age, unlike male ERα KO mice. Thus, it is likely that the suppressive action of androgen in adipocyte differentiation is male-specific and late-onset.

Irrespective of clear increases in WATs in the adult AR KO male mice, food intake appeared unaffected with no alterations in serum lipid parameters. But glucose tolerance in the adult male AR KO may be impaired with insulin resistance. These observations point out a possibility that energy expenditure may decrease by AR inactivation, leading to the accumulation of lipid in WATs. As the ERα KO mice are the case [14], it is of great interest to evaluate the energy expenditure in the male AR KO mice. Alternative possibility is that the adipocyte development in the adults may be impaired by AR inactivation through an alteration in the expression of critical factors in adipogenesis.

The molecular basis of male-specific androgen actions in preventing adipogenesis in adult males is unknown at the present time. However, it is possible that the expressions of adipogenic factors are under the control of androgen-AR signaling. These transcriptional controls are expected to include positive and negative regulations, since like the other members of the nuclear receptor gene superfamily, AR may serve as a dual transcriptional regulatory factor upon the target gene promoters [3–7].

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