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Review

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Molecular Genetics of Vitamin D-Dependent Hereditary Rickets

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Key Words

Hereditary rickets · Vitamin D · Vitamin D receptor · Vitamin D_1 alpha-hydroxylase · Vitamin D receptor KO mice

Abstract

Vitamin D exerts a wide variety of biological actions. The active form of vitamin D, 1α,25$(OH)_2D_3$, is biosynthesized from cholesterol. The final, critical step in this biosynthesis is conversion from 25-hydroxyvitamin D_3 to 1α,25$(OH)_2D_3$ by the enzyme 25-hydroxyvitamin D_3 1α-hydroxylase(CYP27B1)[1α(OH)ase]. 1α,25$(OH)_2D_3$ transcriptionally controls the expression of a particular set of target genes mediated through nuclear vitamin D receptor(VDR) acting as a ligand-inducible factor. Two types of vitamin D-dependent hereditary rickets (VDDR) are known to be caused by mutations in the 1α(OH)ase and VDR genes. The 1α(OH)ase gene is responsible for VDDR type I, and VDR for type II. Both of the diseases display an autosomal-recessive trait, but clinical features and response to administrated 1α,25$(OH)_2D_3$ are distinct. The phenotypes of the gene KO mice deficient of 1α(OH)ase and VDR exhibited the clinical abnormalities observed in the VDDR patients.

Molecular Mechanism of Vitamin D Actions

Biosynthensis of Vitamin D Synthesis

The precursor of vitamin D, 7-dehydrocholesterol, is biosynthesized from cholesterol, then converted into vitamin D_3 by UV light in the skin. Vitamin D is also ingested from the diet, as vitamin D_2 (ergocalciferol) mainly from plants, and vitamin D_3 (cholecalciferol) from animals [1, 2]. A hormonal form of vitamin D, 1α,25$(OH)_2D_3$, is metabolically formed through two steps of hydroxylation at the final stage (fig. 1) [see refs in 3, 4]. First, vitamin D is hydroxylated in the liver to 25-dihydroxyvitamin D_3 [25(OH]D_3), which is subsequently hydroxylated in the kidney to 1α,25$(OH)_2D_3$. For metabolic inactivation of 25(OH)D_3, or 1α,25$(OH)_2D_3$, the 24-hydroxylation to form 24,25$(OH)_2D_3$ or 1α,24,25$(OH)_3D_3$, is the first step in degradation of vitamin D. The serum level of 1α,25$(OH)_2D_3$ is kept constant in the normal state, and is strictly regulated in response to factors controlling calcium homeostasis. The regulation of 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ production by these factors is conducted by altering the activities of the enzymes that hydroxylate vitamin D derivatives. Vitamin D_3-25-hydroxylase (CYP27) catalyzes hepatic 25-hydroxylation, and renal 1α-hydroxylation is catalyzed by 25-hydroxyvitamin D_3 1α-hydroxylase[1α(OH)ase, CYP27B1]. The first step of metabolic inactivation of vitamin D metabolites by 24-hydroxylation is catalyzed by 25(OH)D_3–24-hydroxylase-

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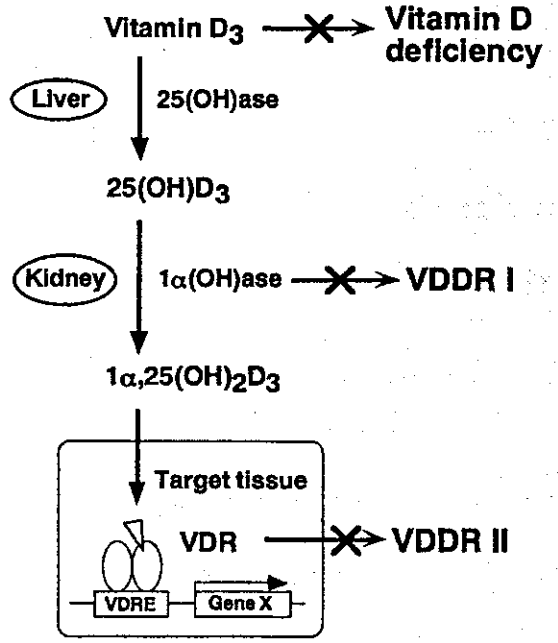


Fig. 1. Rickets related with vitamin D. The biosynthesis pathway of 1α,25(OH)$_2D_3$ and the mode of 1 α,25(OH)$_2D_3$ action are illustrated. The defects in these processes cause rickets. Nutritional vitamin D deficiency, and the the defect of the renal 1 α(OH)ase activity by genetic mutations(VDDRI patients) result in short supply of vitamin D. The mutated VDR in the VDDRII patients is unable to respond to 1α,25(OH)$_2D_3$, resulting in the rickets. A hormonal form of vitamin D acting as a ligand specific for VDR, 1α,25(OH)$_2D_3$, is metabolically formed through two steps of hydroxylations at the final stage. First, vitamin D is hydroxylated in the liver to 25-dihydroxyvitamin D_3 [25(OH]D_3) by the vitamin D_3–25-hydroxylase(CYP27). Subsequently, 25-hydroxyvitamin D_3 1α-hydroxylase[1α(OH)ase] in kidney mainly undergoes conversion into 1α,25(OH)$_2D_3$.

(CYP24) [4]. The gene expression of the CYP24 gene is under positive control by liganded VDR, while the CYP27B1 gene expression is negatively regulated by liganded VDR (fig. 2).

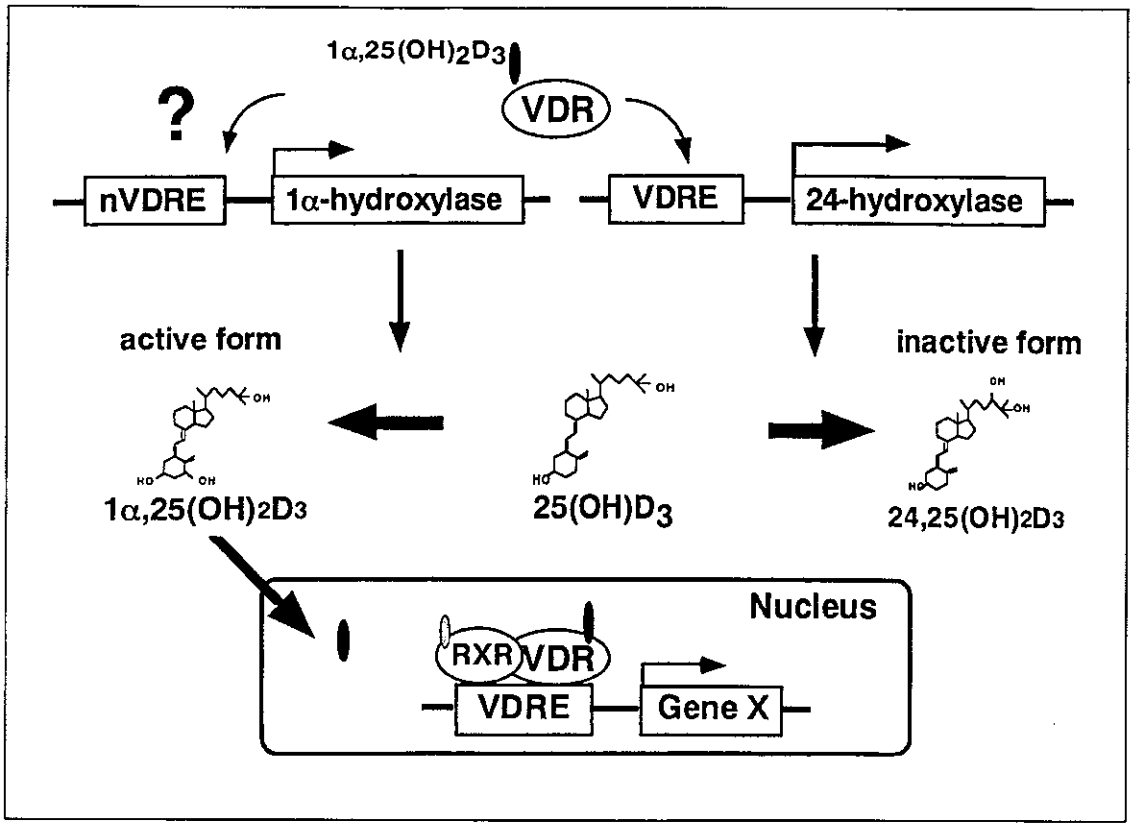
Molecular Mechanism of Transcriptional Control by Vitamin D Receptor

Hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25(OH]$_2D_3$), acts as a ligand for the vitamin D receptor(VDR), and the liganded VDR activates the target gene expression at the transcriptional level (fig. 3) [5, 6]. VDR forms homodimer or heterodimer with one of three retinoid X receptors (RXRα, RXRβ, RXRγ). The VDR homodimer or VDR-RXR heterodimer binds to specific enhancer elements referred to as vitamin D response elements (VDREs) for the 1α,25(OH)$_2D_3$-induced transactivation [7]. For the ligand-induced transactivation by VDR, coactivators interacting with VDR in a ligand-dependent way have recently been shown to be essential for formation of the initial transcription complex with RNA polymerase II (fig. 2, 3) [8]. They include the SRC-1/TIF2 160-kD protein family, CBP/p300 protein family, SRA (a RNA coactivator), and then other [see refs in 8–11]. Most interestingly, these coactivators themselves are histone acetylases (HATs) to modulate chromatine structure for activating gene expression [12, 13]. These coactivators are speculated to form a complex. More recently, another coactivator complex has been identified as the DRIP/TRAP complex, which has no HAT activity [8, 14, 15]. In contrast to coactivators, the corepressors, SMRT and NCoR were found to associate with ligand-unbound thyroid receptor (TR) and all-trans retinoic acid receptor (RAR) to repress their ligand-induced transactivation functions [16, 17]. However, these corepressors appear not to show interaction with ligand-unbound VDR [5, 18].

Vitamin D-Dependent Hereditary Rickets

Phenotypic Manifestations of VDDR I and VDDR II Patients

Vitamin D-dependent rickets type I (VDDR I), also known as pseudovitamin D-deficient rickets (PDDR): VDDR I, is an autosomal-recessive disorder, and the clinical course is similar to that of nutritional rickets due to simple vitamin D deficiency. The patients give birth normally, but develop typical features of rickets within the first to second years of life. Muscle weakness, growth retardation, and bone deformity hypocalcemia, along with secondary hyperparathyroidism and aminoaciduria are seen, and some of them eventually suffer convulsions or tetany as the initial event. Particular features of the VDDR I patients include normal levels of 25(OH)D, but reduced levels of 1α,25(OH)$_2D_3$ [19]. Massive doses of either vitamin D or 25(OH)D_3 are effective to cure rickets, and a normal physiological dose of 1α,25(OH)$_2D_3$ are more potent for therapeutic treatments [20]. From these observations of the VDDR I patients, it had been suggested for a long time that VDDR I patients are genetically defective in 1α(OH)ase.



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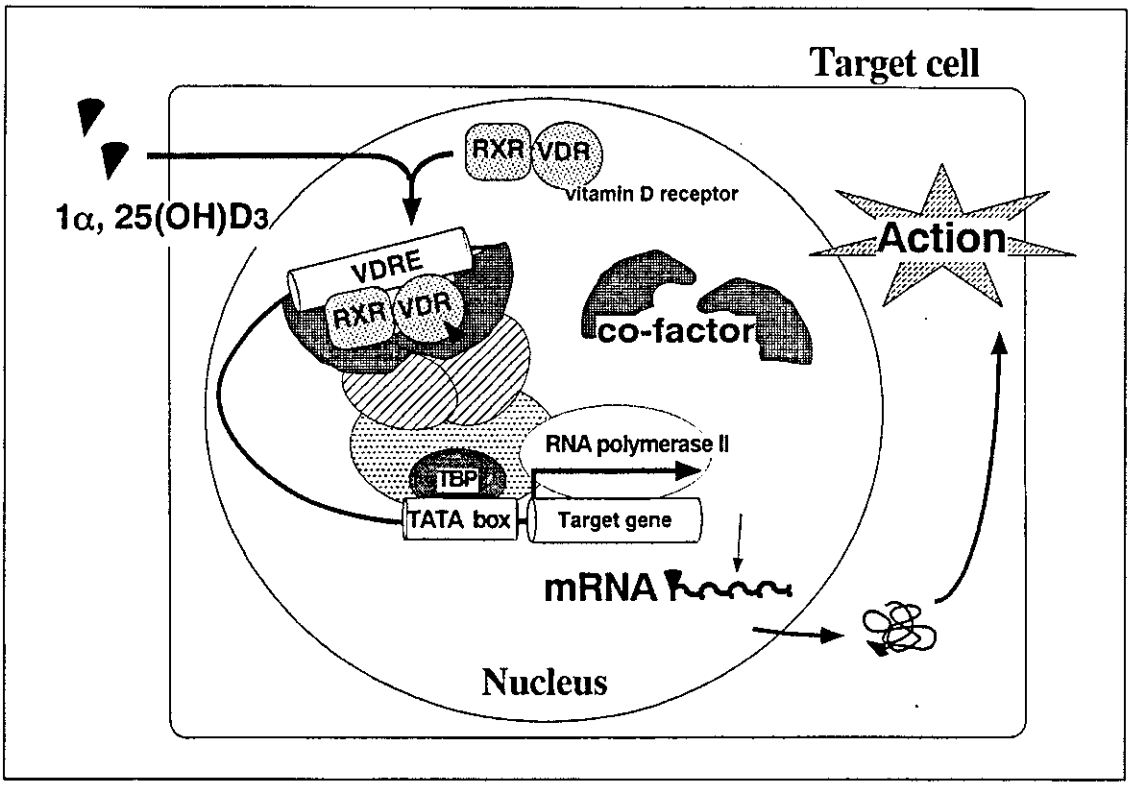

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Fig. 2. Regulations of two hydroxylases by VDR in vitamin D biosynthesis.
Fig. 3. Schema of molecular mechanism of vitamin D actions through VDR-mediated gene expression.

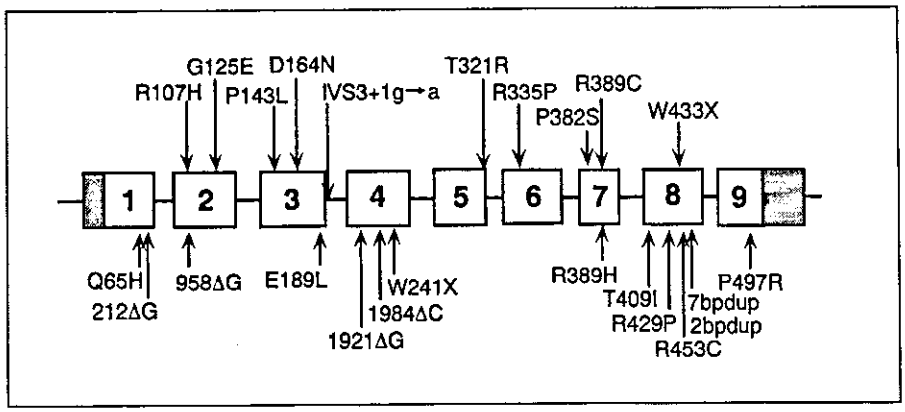

Fig. 4. Genetic mutation in the CYP27B1(25-hydroxyvitamin D_3 1α-hydroxylase gene found in VDDR I patients.

Vitamin D-dependent rickets type II (VDDR II), also known as hypocalcemic vitamin D-resistant rickets (HVDRR). VDDR II is also inherited as an autosomal recessive trait, and exhibits the almost same clinical features as the VDDR I patients, but unlike VDDR I, serum levels of $1\alpha,25(OH)_2D_3$ are high in the VDDR II patients. The physiological doses of $1\alpha,25(OH)_2D_3$ are unable to rescue the rachitic abnormality [19], suggesting that the VDDR II patients lack physiological response to [21]. Alopecia is seen only in some VDDR II patients, and supposed to be a best symptom to distinguish from the other rickets.

The clinical presentation and therapeutic response in VDDR II show marked heterogeneity. The onset of their symptoms are usually before 2 years of age; however, late onset (in their teens to adults) was reported in several sporadic cases [22]. Alopecia varies from sparse hair to total alopecia without eye lashes, and this seems to be a marker of a more severe form of the disease. Patients with normal hair usually retain the response to pharmacological doses of vitamin D or $1\alpha,25(OH)_2D_3$; however, the patients with severe alopecia lack calcemic response even to higher doses of calcitriol, and are required to take long-term intravenous calcium infusions [23]. Life-long therapy is usually required, although sporadic cases of remissions maintained off therapy have been described [24]. Even if rachitic abnormalities such as osteomalacia and the lowered levels of serum minerals in the VDDR II patients are improved in response to the treatments, no rescue of hair loss is found.

Mutations in the 1α-Hydroxylase Gene Cause Vitamin D-Dependent Rickets Type I

Genetic defects in the enzymes responsible for biosynthesis of $1\alpha,25(OH)_2D_3$ is supposed to cause insufficient production of $1\alpha,25(OH)_2D_3$, leading to rickets (fig. 1). As 1α(OH)ase is a final and rate-limiting enzyme in $1\alpha,25(OH)_2D_3$ biosynthesis [4, 25], genetic mutations in the 1α(OH)ase to lose its enzymatic activity is supposed to cause a hereditary rickets. A group of hereditary rickets patients exhibiting low serum levels of $1\alpha,25(OH)_2D_3$, referred to as vitamin D dependency type I(VDDR I), had been considered to be caused by mutation in the 1α(OH)ase gene. Indeed, inactivating mutations in the 1α(OH)ase gene have been identified in the VDDR I patients by us [26] and others [27]. It was thus established at the molecular level that the 1α(OH)ase gene is responsible for VDDR I. To date, various mutations in this gene have been identified spreading over all exons (fig. 4) [28, 29]. The recent reports of the 1α(OH)ase(CYP27B1)$^{-/-}$ KO mice confirmed these observations by describing almost all of the rachtic abnormality found in the VDDR I patients. Most remarkably, the KO mice showed no alopecia event though severe rickets have been developed (table 1) [30].

Mutations in the VDR Gene Cause Hereditary Type II Rickets

In contrast to the VDDR I patients, VDDR II patients genetically lack response to physiological doses of $1\alpha,25(OH)_2D_3$. The mutation searches of the VDDR II patients led to the identification of mutations in the human VDR gene impairing the VDR function as a ligand-inducible transcriptional factor [5, 6, 18, 31, 32] (fig. 1–3).

Several missense mutations in the DNA binding domain (DBD) of the VDR are reported and these VDR mutant proteins expressed in vitro have been shown to lose DNA-binding activities, though the ligand bindings to the mutants were normal. Therefore, such VDR

Table 1. Summary of phenotypes of VDDR patients and mice disrupted in the responsible genes

	Serum $1\alpha,25(OH)_2D_3$	Response to $1\alpha,25(OH)_2D_3$	Alopecia	Cause	KO mice
Nutritional VD deficiency	↘	+	–	VD	
Type I rickets	↘	+	–	1α(OH)ase	No alopecia Dardenne et al. [30]
Type II rickets	↗	–	+	VDR	Alopecia Yoshizawa et al. [35]

mutants are shown transcriptionally inactive in gene regulations through VDRE. Alternatively, mutations in the ligand binding domain (LBD) cause inability to bind the ligands, leading no physiological response to $1\alpha,25(OH)_2D_3$ [31–33]. However, some missense mutants still hold transactivation function in response to the increased concentration of $1\alpha,25(OH)_2D_3$. A missense mutation in the hormone binding domain resulting in lack of heterodimerization with RXR have been also reported [34].

It appears that complete loss of VDR function by DBD mutations or premature stop mutations generally cause alopecia or hair loss, whereas patients with mild impairments of the VDR function such as LBD mutations do not appear to develop alopecia. Thus, the type of mutation in the VDR gene seems to reflect the severity of the rickets.

VDR KO mice as the animal model of the VDDR II patients have been established [35, 36]. A null mutation of the VDR gene in mice, indeed, caused rickets with typical features such as growth retardation, impaired bone formation, hypocalcemia and alopecia [35, 36], which are seen in VDDR II patients [31–33]. The VDR in skin appears to heterodimerize with RXRα for its function for normal development of hair follicule [37]. Even though the most of rachitic abnormalities were rescued by dietary supplements of minerals to normalize serum minerals [38, 39], alopecia is not improved at all, in complete agreement with the VDDR II patients (table 1) [31–33].

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Androgen Receptor Structure and Function from Knock-out Mouse

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Abstract Most androgen actions are considered to be mediated by the androgen receptor (AR) of the target genes. The AR is a fairly large molecule because of the long A/B domains of its N-terminal, but the independent roles of the AR as well as those of the estrogen receptors have largely remained unknown mainly due to lack of the AR knockout (ARKO) mice line. The authors have succeeded in generating the ARKO mouse by means of a conditional targeting with the Cre/loxP system. ARKO males grew healthily although they showed signs of the typical feature of testicular feminization mutation (Tfm), and hormonal assay revealed significantly lower serum androgen and higher LH levels than in wild type (WT) males. The serum estrogen levels were, however, comparable in ARKO and WT. Another hallmark of the ARKO males was a state of high bone turnover osteopenia, in which the acceleration in bone resorption clearly exceeded bone formation. Aiming at a quick differentiation of an androgen dependent polyQ disease such as Kennedy's disease, the authors also developed the Drosophila fly-eye model in which the wild type and the polyQ expanded human AR (hAR) was induced in the eyes of Drosophila. When androgen was administered to the flies induced with the polyQ expanded hAR, their optical nerves were devastated.

Key words: androgen receptor (AR), androgen receptor knockout mouse (ARKO), Kennedy's disease, prostatic cancer, testicular feminization mutation (Tfm)

Androgen Receptor

The androgen receptor (AR), a member of the steroid hormone receptor superfamily, is composed of a fairly large protein in comparison with thyroid hormone receptors (TR), vitamin D receptors (VDR), retinoid receptors (RXR) and estrogen receptors (1–3). This is because the A/B domains of the N-terminal of the AR that include a polyQ repeat are much longer than those of other receptors (4, 5). Androgen controls the expression of genes via the AR, in which the AR positively or negatively regulates the expression of the target genes acting as androgen dependent transcription factors, under the existence of co-activators (5, 6). When the AR acts on the DNA of the genes, the complex of co-activators interact as a trigger with the basal transcription factor and the AR to start the transcription.

Recent studies of 2 subtypes of estrogen receptors, ERα and ERβ and found that,

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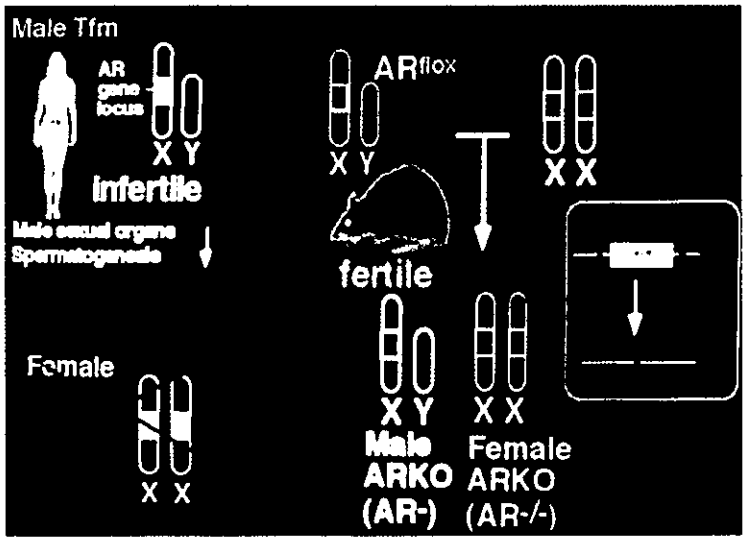

Fig. 1 Strategy for generating ARKO mice line. When the male ARflox mouse with a partially modified AR gene locus integrated by lox P sites and the female transgenic mouse (Cre Tg+) generated by applying recombinase Cre were mated, all the AR genes were disrupted during embryogenesis and an ARKO mice line was obtained.

especially in the knockout mouse, clear phenotypes such as osteoporosis were not manifested perhaps because the plasma level of androgen had been extremely high (7). This may be explained by the fact that androgen is the precursor of estrogen in the female mouse. It has been also reported that in the aromatase knockout female mouse, the circulating testosterone levels are very high (8). Such being the case, there was a demand for developing the androgen receptor knockout (ARKO) mouse to investigate the actions of sexual steroid hormones individually. Androgen is required for the genital organs as well as for sexual behavior not only in males but also in females. And in the clinical aspect, it is well known that some prostatic cancer can be androgen-dependently aggravated (9). A clarification of these issues was also expected with the development of the ARKO mouse.

Androgen Knockout Mouse

There were basic and technical difficulties in generating an ARKO mouse. When the AR gene is mutated in the male mouse, the mouse turns out to be a phenotypic female without either normal female or male genitalia and is infertile (10, 11). Moreover, as the AR gene is located only on the X chromosome, there is no male heterozygote of the AR gene - disrupted animals to transfer the mutated AR gene. It is therefore impossible to obtain a female homozygote by either naturally occurring genetic mutations or the conventional targeted gene disruption method, so that animals which have a recessive genotypic change in the AR gene cannot be generated by means of the usual methods.

Such being the case, we planned to introduce the recombinase Cre/Lox-P base sequence (Cre-lox P system) into the mouse AR gene locus to generate an ARKO mouce line. Our strategy is summarized in Fig. 1. To begin with, we

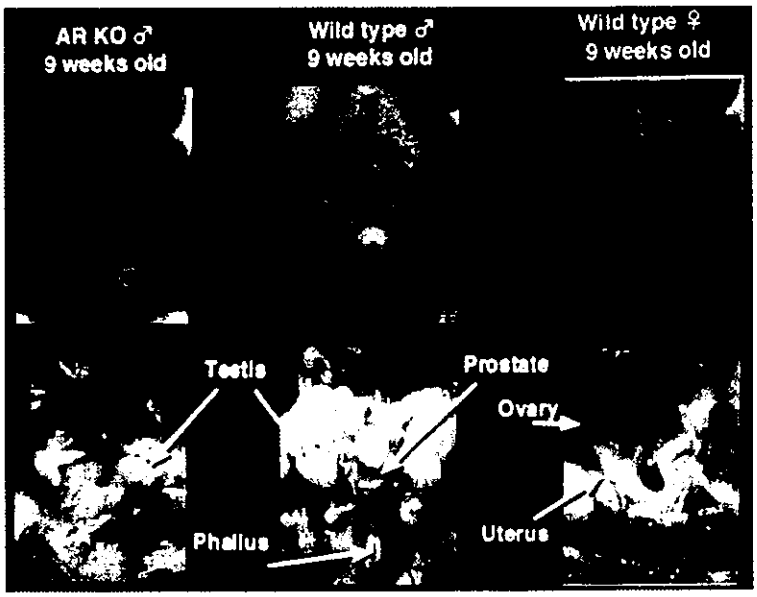

Fig. 2 Appearance and anatomy of ARKO and Wild Type mice. The male ARKO looked like a complete female but had the small testes and a cecum-like vagina but had no uterus or ovaries.

generated a potential AR knockout mouse (floxed AR) by introducing the lox P sites, into the AR gene by homologous recombination in ES cells. Three lox P sites were successively introduced into the first intron of the mouse AR gene as shown in Fig. 1. The male floxed AR mice are completely fertile/normal so far, and have normal expression and function of the AR, but under the partially modified AR gene. On the other hand, a female transgenic mouse was generated by applying the recombinase Cre, a capsid of a DNA breaking enzyme, which induces a recombination at the site between the 2 lox P sequences in the same direction. In this way, in the Cre transgenic female mouse (CreTg+), one of the two AR genes has been disrupted, as shown in Fig. 1, to generate female CreTg+ mice with heterozygous disruption of the AR gene. When the male floxed AR mice and these female CreTg+ mice were mated, the AR gene was disrupted by expressed Cre under the CMV strong promoter during the embryogenesis. We have obtained one homozygous female ARKO out of 16 newborn, and one male ARKO out of 8.

The appearance of the male and female ARKOs and the female wild type (WT) are shown in Fig. 2. The male ARKO which looks like a complete female had small testes and a cecum-like vagina but no uterus or ovaries; and was similar to the clinical Tfm. The histological findings such as the hypertrophic Leydig cells suggested impaired spermatogenesis. The growth curves for 56 days after the birth of the female ARKO mouse were very similar to those of the WT female, but those of the male ARKO were clearly retarded in comparison with those of the WT male and were rather similar to those of the female.

Estimation of plasma hormone levels in the male ARKO revealed noticeably lower androgen as well as a luteinizing hormone, but the estradiol level was almost the same as that of the wild type (Fig. 3). These findings suggest that we can investigate the effect of androgens

independently by using the ARKO mouse in which only the AR is disrupted while the estrogen receptors remain intact.

The bone densitometry showed noticeable osteopenia, and the 3D-CT indicated that both the trabecular bone and cortical bone volumes were greatly reduced in the ARKO male mouse in comparison with that of the WT littermate male mouse at 616 weeks of age. Since the bone volumes result from bone remodeling which is the coupling of the formation/resorption of the bone, we compared bone formation and resorption of the proximal tibia in the ARKO and WT male by means of a histomorphometric analysis. Unexpectedly, the bone formation in the ARKO male exceeded that of the WT male by 15~20% (Fig. 4). On the other hand, the bone resorption in the ARKO male was more remarkable and exceeded that of the WT male by 40~50%. In view of these results, we concluded that the reduction in the bone found in the ARKO male was due to the high bone turnover osteopenia.

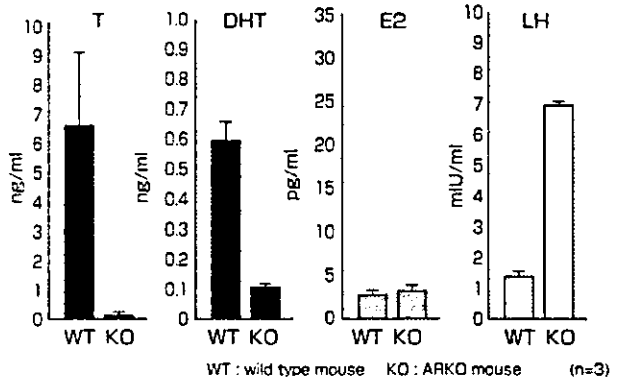

Fig. 3 Plasma hormone levels in WT and ARKO mice. Lower androgen levels and a higher LH level were observed in the ARKO mouse in comparison with those of the wild type mouse, whereas estrogen levels were comparable. WT: wild type mouse, KO: ARKO mouse.

A characteristic change was seen in the body fat composition. More than 10 weeks after birth the ARKO male became fat and its weight exceeded the normal growth curve; and the accumulation of white fat which was almost double that in the WT male was recognized under

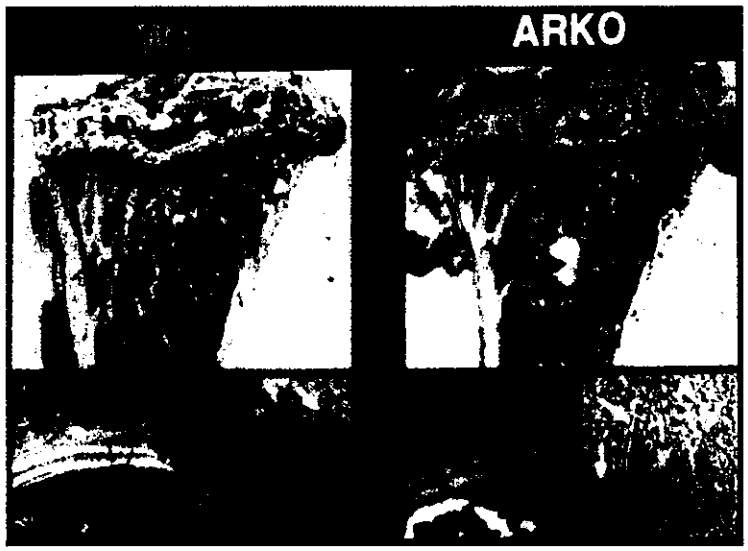

Fig. 4 Histomorphometric analysis of the proximal tibiae. Cartilaginous ossification was rather accelerated in the ARKO male.

celiotomy. Since there were no clear differences in serum lipids, especially in total cholesterol and free fatty acid, the AR might have suppressed the differentiation of the adipose cells. On the other hand, the sexual behavior of the ARKO mouse either as male or female was found not to be normal; nevertheless normal gonadal differentiation was found in the ARKO female. It was therefore considered that abnormal sexual behavior resulted in a smaller number of offspring about half of that of the WT female.

An important disease group other than the testicular feminization mutation (Tfm) and androgen insensitivity syndrome (AIS) that is related to the mutation of the AR gene is the triplet repeat disease, or so-called polyQ expansion, in which the poly Q repetitions of the A/B domain of the N-terminal are expanded (4, 5). SBMA (spinobulbar muscular atrophy) is one of the polyQ diseases and also called Kennedy's disease. Other polyQ diseases such as Huntington's disease, spino-cerebellar ataxis (SCA1) and Machado-Joseph disease are seen both in males and females (12, 13), whereas manifestation of SBMA cannot be seen in the female, even if she is a carrier. Since the AF-1 functions of the A/B domain are androgen-dependent, the reason that the disease occurs only in the male was considered to be due to the concentration of androgen.

Drosophila Fly-Eye Model

Aiming at proving this theory, we tried to use the Drosophila fly-eye model. As the lifespan of the fly is short, we thought we could quickly obtain assay results.

The fly possesses nuclear receptors (14). For example, it has the receptors for ecdysone, metamorphotic hormone, and its partner gene, the ultrabithorax gene. The latter is identical to the human retinoid receptor (RXR). Since the ecdysone receptor of the fly functions as a heterodimer, its DNA binding site is considered to be a direct repeat sequence; on the other hand, the DNA binding site of the human steroid hormone receptor that functions as the homodimer is of a palindrome sequence. Such being the case, we expressed the human AR (hAR) in the fly-eye, the tissue/stage specifically, by using GAL4 UAS, a conditional gene expression system (15), expectating that this AR expression would not impair the functions of the intrinsic receptors in the fly. Then, the reporter gene, a DNA sequence, which can bind to the marker GFP (green fluorescent protein), was bound to the GFP (Fig. 5). In such a fly-eye model, the AR expression can be detected as red by staining it with the antibody; and the transcription function can be recognized as green fluorescent.

Naturally the human AR has about 20 polyQ repetitions but when we induce too many repeats into the AR, the transcription ability is reduced and also the *in vitro* protein biosynthesis becomes suppressed. Consequently, we judged that about 52 repetitions would be optimal for monitoring the transcription activity and the nerval death. When androgen is fed to the fly that has expressed a wild type AR (ARwt), a green fluorescence is seen in the eye without any abnormal changes. But when the polyQ repeat AR is expressed, the optical nerves (photo-receptor neurons) of the fly are devastated unless the androgen feeding is discontinued; which means that the nervous system disorders are androgen dependent. When cartinostatic agents for prostatic cancer such as hydroxy flutamide and bicalutamide were administered concomitantly, the nerve disorders of the fly were rather worsened. The results justify the development of a new-type anti-androgen for the treatment of prostatic cancer. As the AR is expressed in the nucleus and disrupts the optical nerves while keeping the transactivations, it was clarified that the disorder is based on an intranuclear event; and we recognized that an androgen-dependent apoptosis was concurrently taking place.

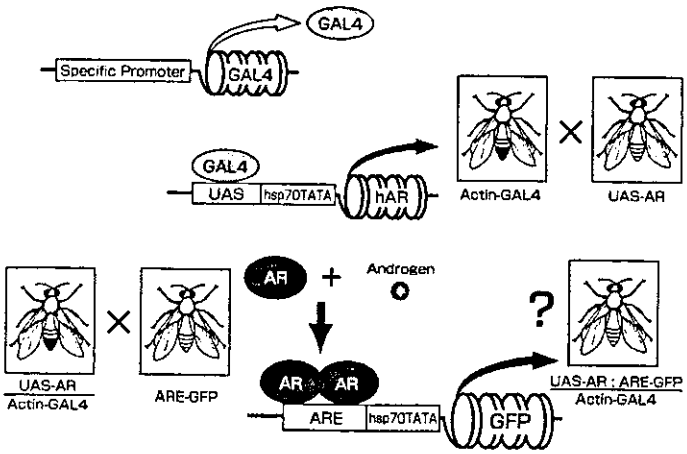

Fig. 5 Inducing hARs in the Drosophila eye. Human ARs, wild type and polyQ expanded, were induced in Drosophila eyes with GAL4 UAS, then the GFP reporter genes were induced to monitor LAR function.

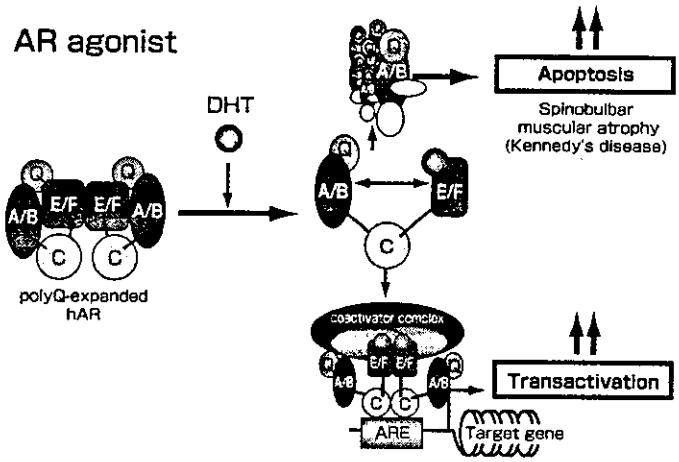

Fig. 6 Androgen-dependent structural alteration caused by the polyQ expanded hAR. It is considered that the polyQ expanded AR is inactive in the transactivation without the agonists (androgens); but in the presence of the agonists, it alters the molecular structure and also recruits the coactivators, and the polyQ repeat induces apoptosis by aggregation.

Fig. 6 illustrates a speculation on the ligand-dependent structural changes in the polyQ-expanded hAR. The hAR that is inactive in the transactivitiy without the ligand (androgen) gains transactivities in the presence of androgen due to its structural changes and also by recruiting coactivators (9, 16); whereas the polyQ repeat induces apoptosis due to its

aggregating property. Since the level of plasma testosterone is much lower in female patients (1/20~1/30), than in male patients, the polyQ aggregation may be unlikely to occur. On the other hand, most androgen antagonists inhibit the transactivity of the AR by inhibiting recruitment of the coactivators; but they may not induce a structural change in the AR that eliminates aggregation by polyQ repeat. Most polyQ diseases including Kennedy's disease are of late onset; and the disorders in the gonadal function and skeletal muscles appear after middle age. And on the other hand, the sensitivity of the fly-eye in expressing the polyQ repeat AR slightly changes depending on the stage.

In view of these results, we consider that for the management of Kennedy's disease, an antiandrogen treatment, such as an orchidectomy or the development of a new ligand that induces a structural change in polyQ-expansion, may be required.

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Nuclear Receptor Function Requires a TFTC-Type Histone Acetyl Transferase Complex

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Summary

Nuclear receptors (NRs) regulate transcription in a ligand-dependent way through two types of coactivator complexes: the p160/CBP histone acetyl transferase (HAT) complex and the DRIP/TRAP/SMCC complex without HAT activity. Here we identified a large human (h) coactivator complex necessary for the estrogen receptor α (ERα) transactivation. This complex contains the GCN5 HAT, the c-Myc interacting protein TRRAP/PAF400, $TAF_{II}30$, and other subunits. Similarly to known TFTC (TBP-free TAF_{II}-containing)-type HAT complexes (hTFTC, hPCAF, and hSTAGA), TRRP directly interacted with liganded ERα, or other NRs. ERα transactivation was enhanced by the purified complex in vitro. Antisense TRRAP RNA inhibited estrogen-dependent cell growth of breast cancer cells. Thus, the isolated TFTC-type HAT complex acts as a third class of coactivator complex for NR function.

Introduction

Liphophilic ligands such as steroid/thyroid hormones and fat-soluble vitamins are thought to exert their actions through transcriptional controls of target genes by their cognate nuclear receptors (NRs). NRs form a steroid/thyroid hormone superfamily and act as a ligand-inducible transcription factor (Beato et al., 1995; Mangelsdorf et al., 1995; Chambon., 1996). From their functional and structural similarities, NR proteins are divided into six (or five) functional domains designated as A–F. The most-conserved DNA binding domain is located in the C domain, and the less-conserved C-terminal E/F domain is mapped as the ligand binding domain (LBD). The N-terminal A/B and the LBD are responsible for ligand-dependent transactivation function of NRs (Tora et al., 1989). The N-terminal activation function (AF-1) in the A/B domain is constitutively active on its own, while the AF-2 function in the LBD is induced upon ligand binding. However, the activities of both of AFs are dependent on cell type and promoter content (Tora et al., 1989; Beato et al., 1995; Watanabe et al., 2001).

NRs require coactivator complexes along with basic transcription machinery to activate transcription (Freedman, 1999; Glass and Rosenfeld, 2000), like the other activators. Two distinct classes of NR coactivator complexes have been identified to date to directly associate with and activate AF-2 of NRs. One class (designated as p160/CBP complex hereafter) contains CBP/p300 (Kamei et al., 1996; Ogryzko et al., 1996) and p160 (SRC-1/TIF2/AIB1) family proteins (Onate et al., 1995; Voegel et al., 1996; Anzick et al., 1997; Torchia et al., 1997) and the others (Yanagisawa et al., 1999; DiRenzo et al., 2000; Watanabe et al., 2001), which are supposed to modulate chromatin structure in terms of their intrinsic histone acetyltransferase (HAT) activities and the action of the associated chromatin-remodeling proteins. The other class is a non-HAT coactivator complex, DRIP/TRAP/SMCC (Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Rachez et al., 1999). Both p160/CBP and DRIP/TRAP complexes look equally potent with regard to activating the function of most liganded NRs in vitro, while their functional difference is suggested by the findings that the two complexes are sequentially recruited from the p160/CBP complex to the DRIP/TRAP complex to liganded NRs bound upon the target gene promoters (Shang et al., 2000). Moreover, in mice, lack of ligand response in target tissues was observed in only limited, but not in all, members of NRs by gene disruption of major components in these complexes (Xu et al., 1998; Ito et al., 2000). Considered together with the cell type- and promoter-content-specific properties of the AFs, a possibility is raised that the other unknown coactivator complexes and/or novel factors in the known complexes support transactivation function of NRs.

To address this issue, we purified HAT complexes associated with liganded estrogen receptor α (ERα) LBD from the HeLa cell nuclear extract. One of purified, large multiprotein complexes contained GCN5 HAT (Wang et al., 1997), the c-Myc-interacting protein TRRAP/PAF400 (McMahon et al., 1998; Ogryzko et al., 1998; Vassilev et al., 1998), and TAFII30 (Jacq et al., 1994), which are common factors shared with hTFTC, hPCAF, and hSTAGA HAT coactivator complexes (Martinez et al., 1998; Ogryzko et al., 1998; Wieczorek et al., 1998; Brand

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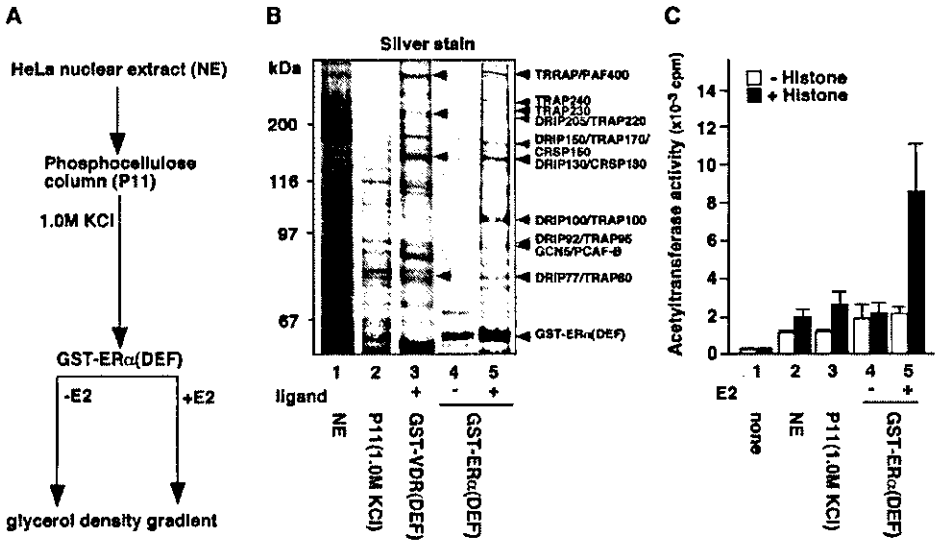

Figure 1. Purification and Identification of Proteins Interacting with E_2-Bound ERα

(A) Purification schema for E_2-bound ERα-interacting proteins. Nuclear extracts prepared from HeLa S3 cells were applied to a P11 phosphocellulose column. After extensive washing with wash buffer containing 0.15 M KCl, bound proteins were eluted with wash buffer containing 1.0 M KCl. Eluted fractions were then incubated with immobilized GST-ERα(DEF) in the presence or absence of E_2 (10^{-6} M). ERα-interacting proteins were eluted from the GST-ERα(DEF) column by N-lauroyl sarkosine.

(B) Identification of ligand-dependent ERα(DEF)-interacting proteins. A panel of fractions, as indicated, was subjected to SDS-PAGE followed by silver staining. Total HeLa S3 nuclear extract (lane 1), fraction eluted from P11 column [P11(elute)] (lane 2), the eluted fractions from liganded-GST-VDR(DEF) column [GST-VDR(DEF) + 1,25$(OH)_2D_3$] (lane3), and unliganded- and liganded-GST-ERα(DEF) columns [GST-ERα(DEF) − E2; GST-ERα(DEF) + E2] (lanes 4 and 5) are shown. Proteins eluted from the GST-ERα(DEF) column (lane 4) were examined by mass spectrometry and identified proteins indicated at the right side of the panel.

(C) Histone acetyltransferase (HAT) activity in purified fractions. The indicated fractions were incubated with either free histones (closed bars) or BSA (open bars), together with ^{3}H-labeled acetyl-CoA, and assayed for acetyltransferase activity (HAT) in a filter binding assay (Rachez et al., 1998). HAT activity is quantitated as radioactivity (cpm) of ^{3}H-labeled acetyled histones.

et al., 1999a, 1999b). Three LXXLL motifs mapped in the middle of TRRAP served as a direct and ligand-dependent surface for ERα and other NRs. Coexpression of TRRAP and GCN5 cooperatively enhanced the ligand-induced transactivation function in vivo, and the ERα transactivation function was potentiated by the purified complex in vitro. A chromatin immunoprecipitation (ChIP) analysis revealed that the TRRAP/GCN5 complex is recruited in an estrogen-dependent manner to the target gene promoters for ERs. Thus, the present study suggests that the purified TFTC-type HAT complex functions as a third class of NR coactivator complexes.

Results and Discussion

Purification of a Multiprotein Complex Associated with Liganded ERα AF-2 Domain

To identify coactivator complexes for ERα, a HeLa cell nuclear extract-derived fraction, which was prepurified on a phosphocellulose column (P11) (see Experimental Procedures), was incubated with a glutathione-S-transferase (GST)-fused LBD of ERα in the presence or absence of 17β-estrogen (E2), and found that the 1.0 M KCl elute fraction from the P11 column contains abundant amounts of putative complexes (Figure 1A). Proteins that interacted with ERα LBD were separated by SDS-PAGE and silver stained (Figure 1B). A number of proteins bound the ERα LBD in a ligand-dependent way (Figure 1B, compare lanes 4 and 5), and interestingly, some of them were not detected by purification on the liganded LBD of the vitamin D receptor (VDR) using the same purification procedure (Figure 1B, lane 3). As HAT activities of p160/CBP and other coactivator complexes are considered to acetylate nucleosomes to facilitate transcription initiation (Kuo and Allis, 1998), we measured whether proteins binding to ERα LBD had HAT activity (Rachez et al., 1998) (Figure 1C). ERα-interacting proteins dissociated from the liganded GST-ERα LBD exhibited significantly higher HAT activity than those eluted either from the nonliganded ERα LBD or the phosphocellulose column (Figure 1C, compare lanes 3 and 4 to lane 5). To identify the proteins that bound to the liganded ERα LBD, we performed the peptide mass fingerprinting of polypeptides specifically bound to the liganded ERα LBD. The obtained masses and the apparent molecular weight of the different polypeptides revealed that the fraction eluted from the liganded ERα LBD contained the GCN5 HAT (Wang et al., 1997) together with the c-Myc interacting protein TRRAP/PAF400 (McMahon et al., 1998; Ogryzko et al., 1998; Vassilev et al., 1998), suggesting that protein complexes containing these factors bind specifically to the liganded ERα LBD. Moreover, in agreement with previous results (Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Rachez et al., 1999), several components of the DRIP/TRAP/SMCC complex were also identified from this fraction and the liganded VDR LBD-bound fraction (Figure 1B, lanes 3 and 5). In contrast, the p160 family of

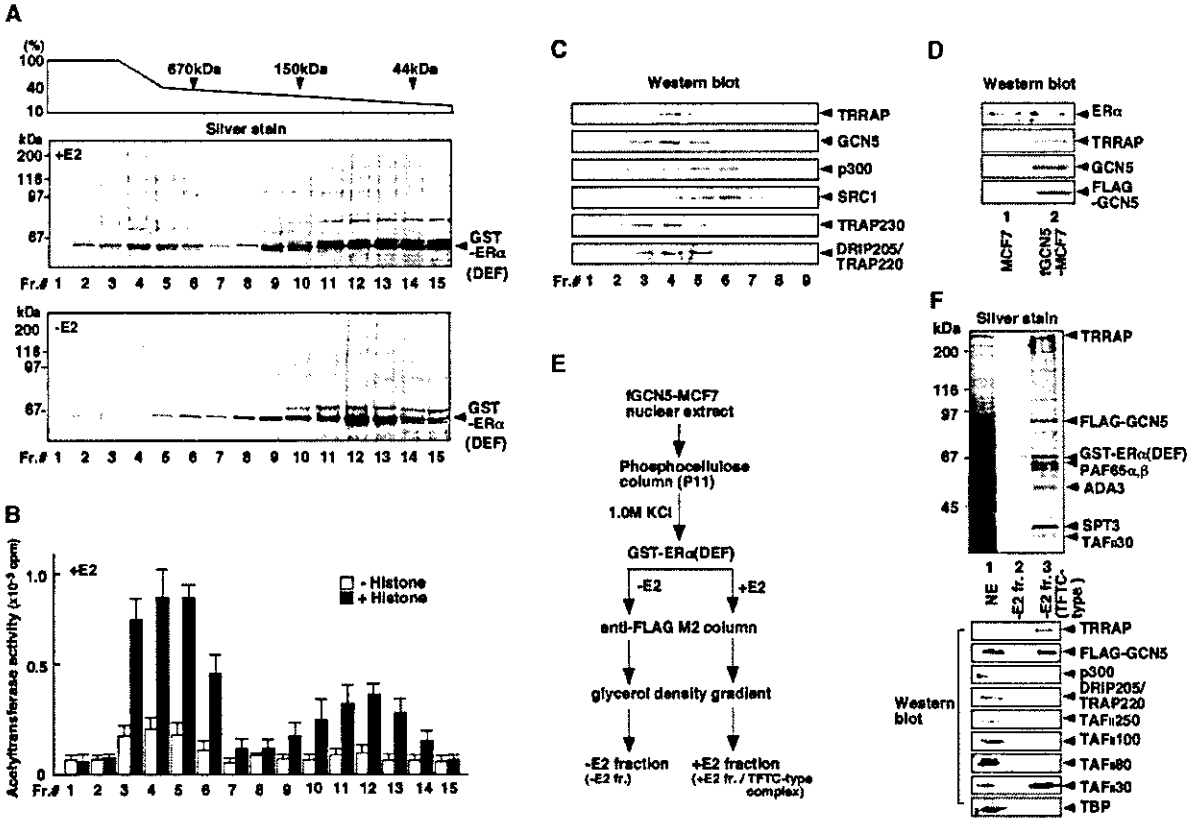

Figure 2. A TRRAP/GCN5 Complex Interacts with ERα(DEF) in a Ligand-Dependent Manner

(A) Glycerol gradient analysis. Eluted fractions from a P11 column were passed over an immobilized GST-ERα(DEF) column in the absence or presence of 10^{-6} M E_2. Protein complexes associated with GST-ERα(DEF) were then dissociated from the reduced glutathione and applied to 10%–40% glycerol gradients. Upper panel, SDS-PAGE analysis of glycerol gradient fractions of GST-ERα(DEF)-interacting proteins in the presence of E_2 (10^{-6} M). Lower panel, fractions of GST-ERα(DEF)-interacting proteins in the absence of E_2. The positions of marker proteins of known molecular masses are shown above the panels.

(B) HAT activity in glycerol gradient fractions. HAT activity in each gradient fraction was quantitated in the presence (closed bars) or absence (open bars) of histones.

(C) Western blot analysis of glycerol gradient fractions. To identify proteins contained in each gradient fraction, Western blot analysis was performed using specific antibodies against TRRAP, GCN5, p300, SRC-1, TRAP230, and DRIP205/TRAP220.

(D) Ectopic expression of FLAG-tagged GCN5 in MCF7 stable transformants. Expression of exogenous FLAG-GCN5 and endogenous TRRAP and ERα proteins in wild-type MCF7 (lane 1) or MCF7 cell line constitutively expressing FLAG-GCN5 (fGCN5-MCF7) (lane 2) were tested by Western blot analysis using antibodies against FLAG, TRRAP, and ERα.

(E) Purification schema of GCN5 complexes from MCF7 stable transformants. ERα-interacting complexes were purified from nuclear extracts of MCF7 stable transformants ectopically expressing FLAG-GCN5 using phosphocellulose P11 and GST-ERα(DEF) columns. Protein complexes containing GST-ERα(DEF) were eluted from glutathione beads with reduced glutathione and then applied to anti-FLAG affinity columns. Protein complexes containing FLAG-GCN5 were then eluted from anti-FLAG affinity resin by FLAG peptide for further analyses.

(F) Ligand-dependent interaction of TRRAP/GCN5 complexes with ERα. Fractions eluted from anti-FLAG affinity resin in the presence of E_2 ($+E_2$ fraction) and absence of E_2 ($-E_2$ fraction) were subjected to SDS-PAGE followed by silver staining. Separated proteins were further identified by both mass spectrometric (upper panel) and Western blot (lower panel) analyses using antibodies as indicated at the right side of the panel.

proteins, CBP/p300 or p68/p72 (Endoh et al., 1999; Watanabe et al., 2001) was not identified from this liganded ERα LBD-bound fraction by mass spectrometry, presumably due to their low abundance in the prepurified fraction.

To test whether a GCN5-type HAT complex or other HAT complexes contribute to the measured HAT activity in the ERα-bound fraction, the ERα-interacting proteins were further fractionated according to their molecular masses by a glycerol gradient (Figure 2A). The HAT activity in each fraction was then measured (Figure 2B). In parallel, proteins in each fraction were separated by SDS-PAGE and silver stained (Figure 2A), or analyzed by Western blot using specific antibodies against TRRAP, GCN5, components of the p160/CBP complex (p300, SRC-1), and components of the DRIP/TRAP complex (DRIP205/TRAP220, TRAP230) (Figure 2C). In glycerol gradient fractions 3 to 6, where liganded ERα LBD-bound proteins were separated, protein complexes with a molecular mass larger than 670 kDa were clearly visible (Figure 2A, upper panel), whereas these complexes were absent in the control fractions where proteins were eluted from the nonliganded ERα LBD (Figure 2A, lower panel). HAT assays revealed that fractions containing

multiprotein complexes with more than 670 kDa possess high HAT activities (Figure 2B). Western blot analysis revealed the presence of GCN5 HAT together with TRRAP, DRIP205/TRAP220 in fractions 3 and 4 (Figure 2C), while other HATs, such as p300 and SRC-1, were detected in fractions 5 and 6 (Figure 2C). Thus, our results indicate that an endogenous human GCN5/TRRAP HAT-containing complex binds liganded ERα, together with previously identified coactivators (such as the DRIP/TRAP/SMCC complex, p300 and SRC1).

Direct and Ligand-Dependent Interaction of ERα AF-2 Domain with a TFTC-Type HAT Complex through Three LXXLL Motifs in TRRAP

To better characterize and identify other components of the GCN5/TRRAP complex that bind to the liganded ERα LBD, we generated a stable MCF-7 human breast cancer cell line expressing FLAG-tagged GCN5, since GCN5 and TRRAP are common components shared with hTFTC (McMahon et al., 1998; Wieczorek et al., 1998; Brand et al., 1999a, 1999b), hPCAF (Ogryzko et al., 1998), and hSTAGA (Martinez et al., 1998) complexes to form a class of coactivator complexes with slightly different subunit compositions. An expression of FLAG-GCN5 in the selected clone (fGCN5-MCF7) was detected by Western blot analysis using an antibody against the FLAG epitope (Figure 2D). The ERα-bound complexes containing FLAG-GCN5 were then purified according to the same scheme as before (Figure 2E). In this complex, FLAG-GCN5 was copurified together with TRRAP and a 30 kDa TAF_{II} ($hTAF_{II}30$) (Jacq et al., 1994), whereas p300, DRIP205/TRAP220, other $hTAF_{II}$s ($TAF_{II}250$, $TAF_{II}100$, and $TAF_{II}80$), and hTBP were undetectable by Western blot analysis (Figure 2F, lower panel). Protein identification of the purified proteins by mass-spectrometric analysis identified TRRAP, GCN5, PAF65α, β, hADA3, hSPT3, and $hTAF_{II}30$ (Figure 2F, upper panel), which are all common components of TFTC and PCAF complexes (Ogryzko et al., 1998; Wieczorek et al., 1998; Brand et al., 1999b). These findings suggest that the complex purified from MCF7 cells belongs to a class of the known GCN5 HAT-containing TFTC-type complexes and that these complexes may have a coactivator activity for NRs.

Ligand-dependent interactions of NRs with their coactivators are known to be mediated through direct contacts between LXXLL motif(s) in the coactivators and the most C-terminal α-helix (helix12) of the NR LBD (Heery et al., 1997; Voegel et al., 1998; Glass and Rosenfeld, 2000). To identify directly interacting components for ERα in this TFTC-like complex, we searched the amino acid sequences in all of the known components of TFTC, hSTAGA, and PCAF/GCN5 and found that TRRAP contains ten LXXLL motifs in its entire sequence. The fact that the yeast homolog of TRRAP, Tra1, participates in direct activator interactions critical for transcription activation (Brown et al., 2001), together with the finding that TRRAP was abundant in our purification process, further suggested that TRRAP is the factor that recruits the purified TFTC-like complex to ERα. Thus, we analyzed the direct and ligand-dependent interaction of TRRAP with ERα by a GST pull-down assay. The full length of TRRAP exhibited direct and ligand-dependent interaction with ERα, and the region responsible for this interaction was mapped in between amino acids 984–1214 of TRRAP, harboring three LXXLL motifs (Figure 3A). A series of mutations in each of the three motifs that introduce amino acid substitution (Figure 3B) demonstrated that all of the three motifs contribute the efficient interaction with E2-bound ERα (Figure 3B). Importantly, ligand-dependent interactions were observed also with other NRs, such as in ERβ, VDR, and PPARγ (Figure 3C), as expected from the property of the LXXLL motif (Heery et al., 1997; Voegel et al., 1998). We then tested by immunoprecipitation whether the direct and ligand-dependent interaction between ERα and TRRAP takes place also in the cells. Endogenous ERα was immunoprecipitated from MCF-7 cells grown in the absence and presence of 17β-estradiol and tested for the presence of TRRAP and other subunits (Figure 3D). Western blot analysis showed that both endogenous TRRAP and GCN5 are coprecipitated with ERα in an E2-dependent manner (Figure 3D). Thus, the endogenous ERα is able to stably associate with the endogenous GCN5/TRRAP HAT complex. To further study the association of the GCN5/TRRAP HAT complex and ERα, the purified FLAG-GCN5-containing complex was disrupted by sonification and then incubated with GST- ERα LBD in the absence or presence of E2. A significant retention of TRRAP by the liganded ERα was found by Western blotting and mass-spectrometric analysis (Figure 3E), indicating again that TRRAP serves as a direct surface in the TRRAP/GCN5 complex for ligand-dependent interactions with ERα and the other NRs. This is in agreement with the recent finding that Tra1, the yeast homolog of TRRAP, serves as a common and direct target for acidic activators (Brown et al., 2001).

Potentiation of Nuclear Receptor Transactivation Function by the TFTC-Type Complex

To investigate whether the TRRAP/GCN5 complex acts indeed as a coactivator complex of ERα, a cell-free in vitro transcription assay was performed with the purified FLAG-GCN5 complex as prepared for mass-spectromic analysis (Figure 2E). The chimeric protein of the ERα AF-2 LBD domain fused to the GAL4 DNA binding domain was expressed as a GST-fusion protein, purified, and applied to this assay with a G-less cassette reporter plasmid bearing five GAL4-DNA binding sites (17 × 5 m). The addition of the purified GCN5/TRRAP HAT complex to the rat liver nuclear extract increased the activation of the liganded ERα 3- to 4-fold when compared with the activation obtained with the liganded ERα alone (Figure 4A, compare lanes 4 and 5 to 6 and 7). Moreover, in a transient expression assay using a luciferase reporter driven by an estrogen response element (ERE)-containing promoter, coexpression of either TRRAP or GCN5 enhanced the E2-induced transactivation of ERα (Figure 4B, lanes 2 and 3). We obtained a comparable enhancement of activation with the previously described cofactor TIF2 (Voegel et al., 1996) (Figure 4B, lane 6), while DRIP205/TRAP220 was not very potent in this assay (lane 5). Ligand-dependent transactivation of other NRs (such as ERβ, VDR, and PPARγ) was also enhanced by expression of either TRRAP or GCN5 (Figure 4B, lanes 7–15), in agreement with the previous report that hTRβ transactivation function is enhanced by

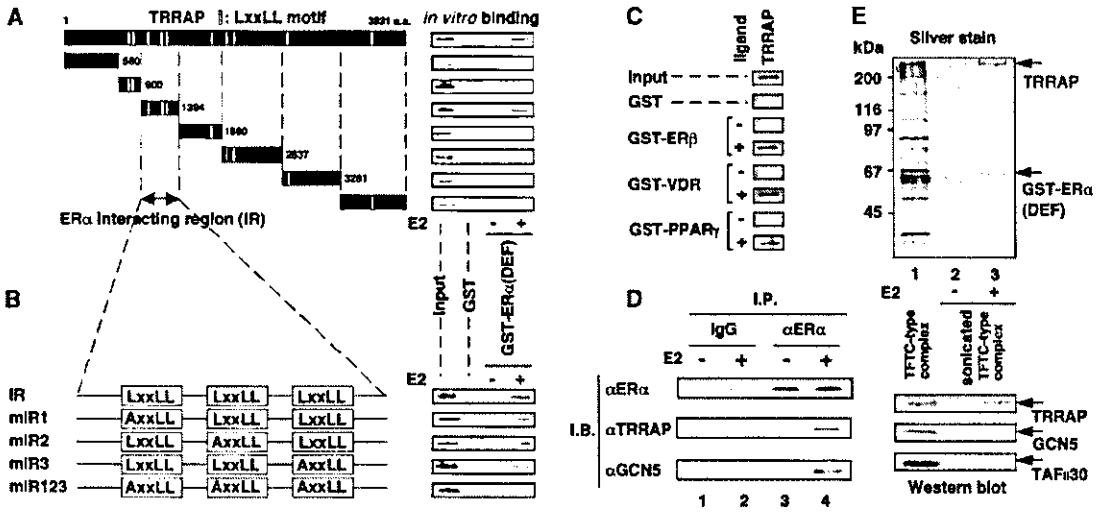

Figure 3. Ligand-Dependent Interaction of a TFTC-Type TRRAP/GCN5 Complex with ERα via LXXLL Motifs in TRRAP

(A) Direct, ligand-dependent interaction of TRRAP with ERα in vitro. To map the ERα-interacting region of TRRAP protein, deletion mutants of TRRAP were translated in vitro and incubated with either GST or GST-ERα(DEF) immobilized on glutathione-Sepharose beads in the presence or absence of 10^{-6} M E_2. Bound proteins were subjected to SDS-PAGE followed by autoradiography.

(B) The three LXXLL motifs in the ERα-interacting region of TRRAP are essential for E_2-dependent interaction. A series of mutants with amino acid replacements in the LXXLL motifs were examined for ligand-dependent interaction with ERα.

(C) Ligand-dependent interaction of TRRAP with other NRs. In vitro-translated full-length TRRAP protein was incubated with GST-fused LBD of the indicated NRs in the presence or absence of cognate ligand (10^{-6} M). Bound proteins were subjected to SDS-PAGE followed by autoradiography.

(D) E_2-dependent interaction between ERα and TRRAP/GCN5 complexes in vivo. MCF-7 cells were lysed and subjected to immunoprecipitation using mouse anti-ERα or normal mouse immunoglobulin as a control in the presence or absence of E_2 (10^{-6} M). Precipitates were Western blotted with antibodies to ERα, GCN5, or TRRAP as indicated.

(E) Direct, E_2-dependent interaction between ERα and TRRAP. Purified TRRAP/GCN5 complex was disrupted by sonication and incubated with immobilized GST-ERα(DEF) resin in the presence or absence of E_2 (10^{-6} M). Bound proteins were separated by SDS-PAGE and identified by mass spectrometry (upper panel) and Western blot (lower panel).

GCN5 (Anafi et al., 2000). Coexpression of TRRAP together with GCN5 additively enhanced the ligand-induced transactivation of ERα (Figure 4B, lane 4), further supporting the idea that the identified TFTC-type complex serves as a coactivator complex during the ERα activation process. Furthermore, transactivation functions of c-Myc (Kretzner et al., 1992) and VP16 were potentiated by expressions of TRRAP and GCN5 (Figure 4B, lanes 17 and 18), in agreement with the previous findings (McMahon et al., 1998; Brown et al., 2001).

To investigate whether the TFTC-type complex is indeed recruited to liganded ERα similarly to the other known coactivator complexes in the nuclei of living cells, we performed a ChIP assay with the promoters of estrogen-responsive endogenous target genes. In agreement with the idea that a TFTC-type complex plays a coactivator role, after estrogen treatment, ERα was recruited together with GCN5 and TRRAP to the promoter regions of the cathepsin D and c-*fos* genes, harboring EREs, within 30 min after E2 stimulation (Figures 4C and 4D). Interestingly, GCN5 and TRRAP were recruited to the cathepsin D promoter slightly after the recruitment of p300 and TIF2 by the liganded ERα but appeared synchronous with that of the DRIP205/TRAP220 complex (Figure 4D). These results suggest that the TFTC-type HAT complex is recruited to the ERα-stimulated promoters after the p300 HAT complex and about the same time as the DRIP/TRAP complex. Thus, it seems that the first cofactor that acts upon ERα binding to modify the chromatin is p300/CBP (Figure 4D) and that the DRIP/TRAP and the TFTC-type complex are recruited subsequently, at about the same time, to the ERα to further modify the chromatin and to facilitate transcription initiation.

Finally, in order to test whether the direct interaction observed between TRRAP and ERα plays a biological role in the estrogen actions, the function of TRRAP was examined in the estrogen-dependent cell growth of MCF-7 breast cancer cell lines by reducing its endogenous expression level, since both of TRRAP and GCN5 are major components in a complex required for c-Myc-mediated transformation (McMahon et al., 1998; Park et al., 2001). To this end, an antisense expression vector was generated by inserting the TRRAP cDNA into the CMV expression vector in the antisense orientation (AS-TRRAP). In agreement with our above described results, the ligand-dependent transactivation function of ERα was reduced by the transient expression assay when cotransfected with AS-TRRAP (Figure 5A, lane 2). We then established several stable cell lines constitutively expressing antisense RNA for TRRAP in MCF7 cells (AS-TRRAP-MCF7). In AS-TRRAP-MCF7 cells, decreased level of the TRRAP protein was observed, whereas expression levels of the other coactivators and ERα were unchanged when compared with the parental MCF-7 cells (Figure 5B). In agreement with the above results showing that GCN5 and TRRAP are both subunits of a TFTC-type coactivator complex in the AS-TRRAP-MCF7