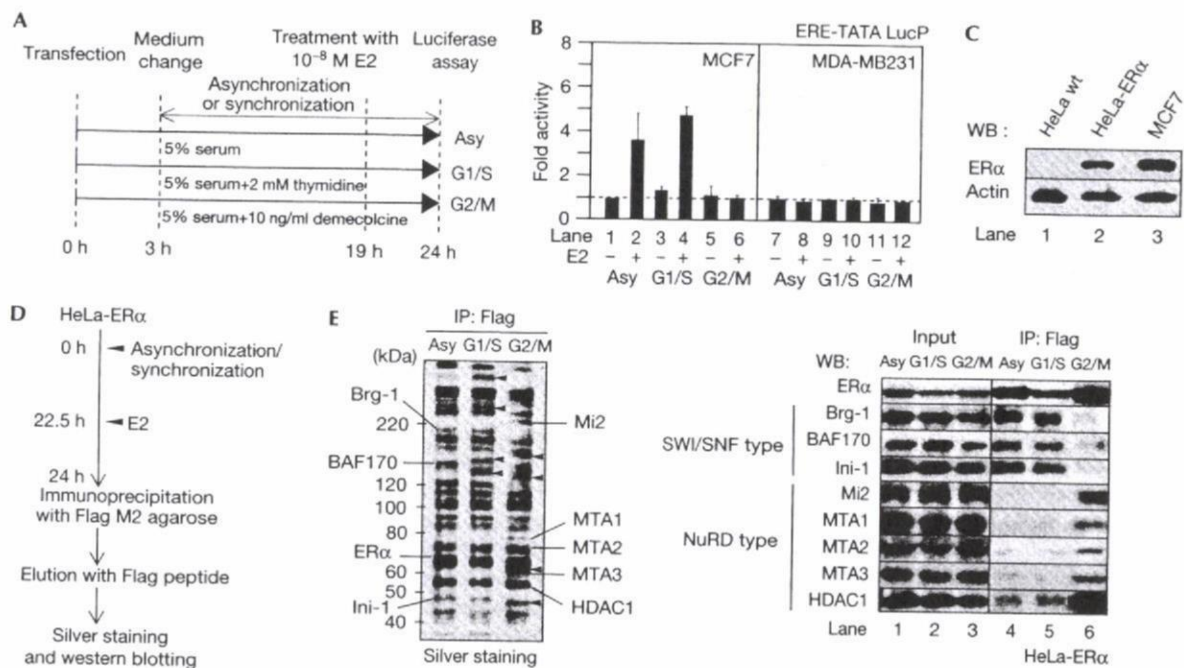

Fig 1 | Behaviour of oestrogen receptor-α over the cell cycle. (A) Schematic for cell-cycle synchronization. (B) The E2-induced transactivation functions of ERα in asynchronous (Asy) cells or synchronized cells at G1/S or G2/M phase. Luciferase assays were performed in MCF7 or MDA-MB132 cells transfected with an ERE-TATA luc reporter plasmid (ERE-TATA LucP). (C) Establishment of a HeLa S3 stable transformant expressing Flag-HA-ERα (HeLa-ERα). A pcDNA3 expression vector of ERα, tagged with Flag and HA, was introduced into HeLaS3 cells. Expression levels of ERα in HeLa-ERα cells were compared with those in the MCF7 cell line. A 10 μg portion of whole extracts was subjected to SDS–polyacrylamide gel electrophoresis for western blotting (WB). (D) Purification schematic of the cell-cycle-dependent interactants of liganded ERα. (E) The interactants of Flag-ERα were separated by SDS–polyacrylamide gel electrophoresis and visualized by silver staining (left panel) and western blotting (WB; right panel). The arrowheads indicate G1/S- or G2/M-phase-specific ERα interactants. E2, 17β-oestradiol; ER, oestrogen receptor; HA, haemagglutinin; HDAC1, histone deacetylase 1; IP, immunoprecipitation; wt, wild type.

specific cell-cycle stages are an area of considerable interest for further research. Here, the cell-cycle-dependent switching of chromatin-remodelling complexes is described.

RESULTS

Transactivation function of ERα was lowered at G2/M

To investigate whether the transactivation function of activated ERα varies across the cell cycle, we tested the transactivation function of endogenous ERα activated by 17β-oestradiol (E2) in synchronized MCF7 (ERα-positive) or MDA-MB231 cells (ERα-negative breast carcinoma cell line; Fig 1A; supplementary Fig S1 online). The synchronization of each phase was confirmed by DNA content analysis using flow cytometry, as reported previously (Takezawa *et al*, 2007). In a luciferase assay with an ERE-TATA reporter (a PEST sequence was tagged to luciferase for rapid protein degradation (Hill *et al*, 2001)), the transcriptional activity of ERα seemed to be unaltered at G1/S (Fig 1B, compare lane 4 with lane 2 in asynchronous cells), whereas at G2/M, its activity was significantly lower in MCF7 cells (Fig 1B, compare lane 2 with lane 6). As expected, the ERα activity was undetectable in MDA-MB231 cells.

To explore the molecular basis of cell-cycle-dependent alteration of ERα activity, we generated a HeLa S3 stable transformant cell line (HeLa-ERα) expressing human ERα tagged with Flag and haemagglutinin epitopes at the amino termini (Fig 1C). The cell-cycle-dependent transcriptional activity of ERα was also confirmed in these cells (supplementary Fig S2A online).

Given that cell-cycle-related proteins such as cyclins are regulated by both their cellular localization and their relative abundance over the cell cycle (Moore *et al*, 2003), we examined the expression levels and localization of ERα in synchronized HeLa cells. The expression levels of ERα were slightly reduced at G1/S and were higher at G2/M (supplementary Fig S2B online, left panel). Immunofluorescence using an ERα antibody showed that ERα was localized mainly in the nucleus and seemed to associate with chromosomes at G2/M, as well as G1/S, regardless of stimulation with E2 (supplementary Fig S2B online, right panel). Certain classes of sequence-specific regulators, however, dissociated from mitotic chromosomes (Martinez-Balbas *et al*, 1995; Nuthall *et al*, 2002; Prasanth *et al*, 2003).

As ERα is localized in chromosomal areas even at G2/M, we suggested that ERα-interacting proteins define the transactivation function of ERα in each cell-cycle phase. To test this, we biochemically purified ERα interactants from synchronized cells in the presence of E2 (Fig 1D). Nuclear extracts of asynchronous or synchronized HeLa stable transformants were subjected to sequential affinity column purification by using an anti-Flag M2 affinity resin column. The interactants were separated by

©2008 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

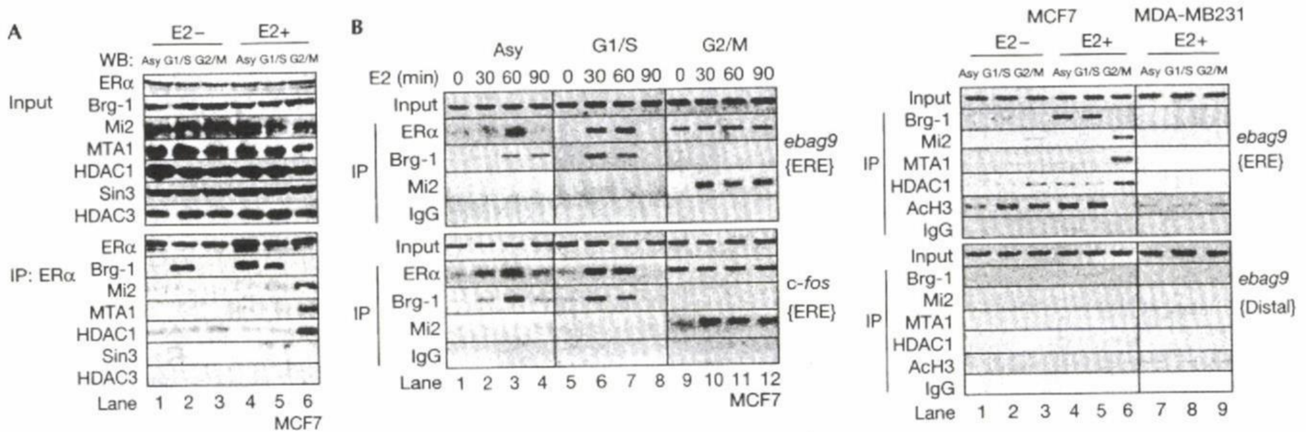

Fig 2 | Cell-cycle-dependent exchange of chromatin-remodelling complexes. **(A)** G2/M-phase-specific exchange of chromatin-remodelling complexes associating with ERα. The nuclear extracts were prepared from synchronized MCF7 cells treated with E2 for 1.5 h. ERα interactants were co-immunoprecipitated (IP) with an ERα antibody from the nuclear extracts of MCF7 and then subjected to western blotting (WB). **(B)** Cell-cycle-dependent recruitment of components of the chromatin-remodelling complex on the *ebag9* and *c-fos* promoters. By using asynchronous (Asy) or synchronized MCF7 cells or MDA-MB231 cells treated with E2 for the indicated time (left panel), the chromatin immunoprecipitation assay was performed with specific antibodies for the tested factors. For PCR, primers were designed to the *ebag9* and *c-fos* promoter regions including the ERα response element site. The right panel shows the results of the chromatin immunoprecipitation assay with the target promoter regions ({ERE} and {Distal}) 60 min after E2 treatment. The antibodies against the indicated proteins were used in MCF7 and MDA-MB231 cells (used as a negative control). E2, 17β-oestradiol; ER, oestrogen receptor; HDAC, histone deacetylase.

SDS–polyacrylamide gel electrophoresis and silver stained. By comparing the interactants of the cells at G1/S versus G2/M, we found that the association of ERα with several interactants was cell-cycle stage dependent (Fig 1E, left panel, arrowheads). As ERα activity was lowered at G2/M, we focused on cell-cycle-stage-specific interactants and identified them by using mass fingerprinting. One of them, Mi2, was a G2/M-phase-specific interactant; Mi2 is a core ATPase component of the NuRD-type chromatin-remodelling complex (Sif, 2004; Denslow & Wade, 2007). Western blotting verified known components of the NuRD complex in the interactants only at G2/M, and detected components of the SWI/SNF complex in asynchronous and G1/S-phase cells (Fig 1E, right panel).

Switch of ERα-bound chromatin-remodelling complexes

From those purification results, we hypothesized that, at G2/M, association of ATP-dependent chromatin-remodelling complexes with ERα leads to suppression of the transactivation function of ERα in the ERα target gene promoters. To test this hypothesis, the association of ERα with the components of the NuRD-type complex was examined by immunoprecipitation of ERα from the nuclear extracts of asynchronous or synchronized MCF7 cells (Fig 2A) and HeLa-ERα cells (Fig 1E, right panel), treated with or without E2. The core components of the NuRD-type complex were detected in the immunoprecipitants of endogenous ERα from the E2-treated MCF7 cells only at G2/M (Fig 2A, lane 6). The core component of the SWI/SNF-type chromatin-remodelling complex, Brg-1, was co-immunoprecipitated with activated ERα in asynchronous cells as reported previously (Belandia *et al*, 2002) and in synchronized MCF7 cells at G1/S in a ligand-independent manner (Fig 2A). However, the association of Brg-1 with ERα disappeared at G2/M (Fig 2A). Thus, there is a cell-cycle-dependent switching of the ATP-dependent chromatin-remodelling complexes associated with ERα. Next we examined the recruitment of components of the complex and ERα to oestrogen response elements in the endogenous oestrogen receptor binding site *ebag9* (Tsuchiya *et al*, 2001) and *c-fos* gene (Ohtake *et al*, 2003) promoters by chromatin immunoprecipitation analysis of synchronized MCF7, MDA-MB231 and HeLa-ERα cells (Fig 2B; supplementary Fig S2C online). Consistent with co-immunoprecipitation data with ERα from synchronized MCF7 cells at G2/M, the components of the NuRD-type complex were recruited to both gene promoters in an E2-dependent manner (Fig 2B, right panel, lanes 3,6). E2-dependent recruitment of Brg-1 was seen in both an asynchronous and G1/S-phase-specific manner (Fig 2B, left panel). Unexpectedly, at G2/M, ERα anchored to the promoter regions in an E2-independent manner (Fig 2B, left panel).

NuRD complex inhibits ERα transcriptional activity at G2/M

To investigate whether a NuRD-type chromatin-remodelling complex co-represses the function of ERα in cells at G2/M, we examined the cellular colocalizations of Mi2 with E2-bound/unbound ERα by using immunofluorescence with antibodies against Brg-1, Mi2 and ERα (Fig 3A). At G1/S, Brg-1 and Mi2 were distributed primarily in the nucleus and ERα was partly colocalized with Brg-1 but not Mi2 (Fig 3A, left panel). At G2/M, however, Mi2 was detected on the mitotic chromosomes and partly colocalized with ERα only in the presence of E2 (Fig 3A, right panel). Brg-1 was localized in the cytosol apart from the mitotic chromosomes, as reported previously (Muchardt *et al*, 1996). Thus, ERα seemed to associate with a NuRD-type chromatin-remodelling complex on mitotic chromosomes at G2/M in the presence of E2.

©2008 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

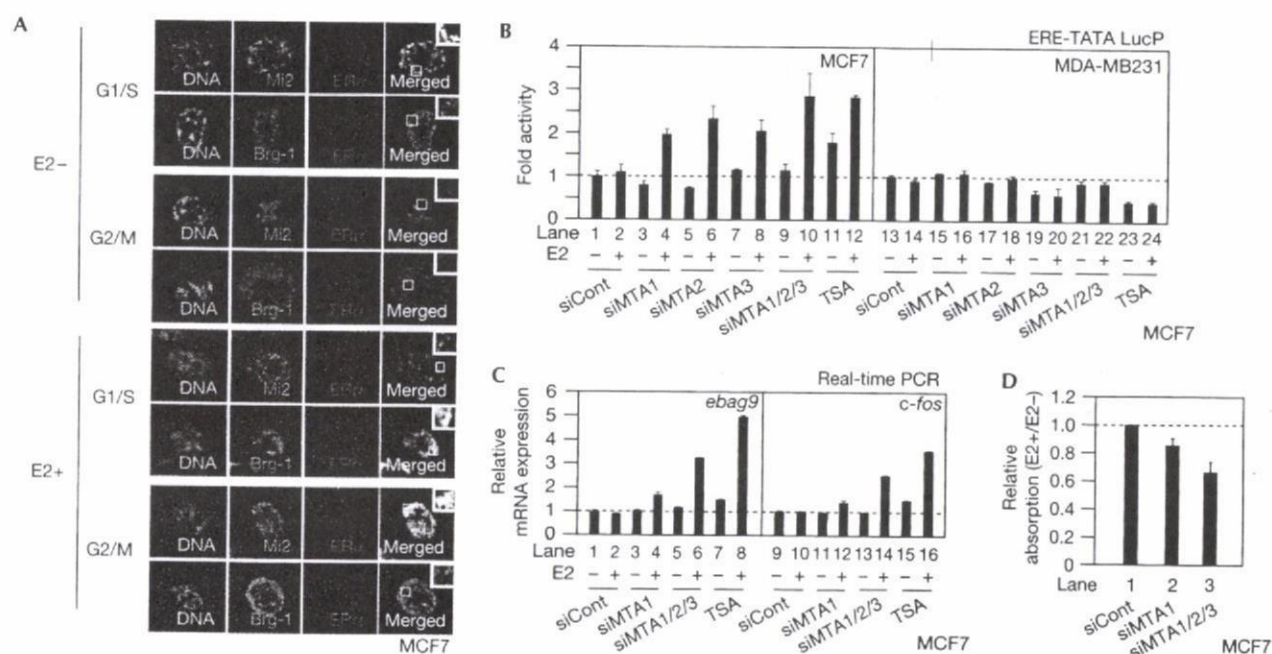

Fig 3 | Co-repressive functions of components of the NuRD-type complex for oestrogen receptor-α at G2/M. (A) Colocalization of liganded ERα with chromatin-remodelling factors at G1/S or G2/M. MCF7 cells were synchronized at G1/S or G2/M and then treated with or without E2 (for 30 min). Brg-1 or Mi2 (green) colocalized with ERα (red) at G1/S or G2/M, respectively. DNA was stained with To-PRO (blue). (B) Abrogation of the G2/M-phase-specific suppression of transactivation function by TSA or siMTA1/2/3. Synchronization at G2/M and transfection were performed as shown in Fig 1A. Short interfering RNA (siRNA) for MTA1/2/3 was transfected into MCF7 or MDA-MB231 cells 36 h before synchronization. TSA treatment was carried out at the same time as E2 treatment. (C) Repression of ebag9 or c-fos expression at G2/M by components of the NuRD-type complex. The expression levels of the indicated genes were measured by quantitative real-time PCR with total RNA extracted from synchronized MCF7 cells at G2/M. E2 and/or TSA treatments were for 6 h. (D) Lowered E2-dependent cell proliferation of MCF7 cells by MTA knockdown. MCF7 cells transfected with siRNAs were cultured with or without E2 for 4 days. A cell proliferation assay was performed by using the Cell-Counting Kit-8 (Dojindo, Kumamoto, Japan). The data are shown as the ratio of E2-dependent proliferation in siMTA cells to siControl cells (siCont). E2, 17β-oestradiol; ER, oestrogen receptor; TSA, trichostatin A.

Finally, the putative co-repressive function of Mi2 for ERα was tested in MCF7 cells synchronized at G2/M (Fig 3B). The E2-dependent transcriptional activity of ERα was lower at G2/M. Either knockdown of the known components (MTA1/2/3) of the NuRD-type complex (supplementary Fig S2D online) or treatment with an HDAC inhibitor, trichostatin A (TSA), abrogated the G2/M-phase-specific suppression of the transactivation function of ERα (Fig 3B). The MTA family member proteins (MTA1/2/3) seemed to associate physically with ERα in a glutathione S-transferase pull-down assay (supplementary Fig S2E online). This was consistent with the function of a NuRD-type complex at least partly mediating G2/M-phase-specific suppression (Fig 3B). Furthermore, either RNA interference of MTAs or TSA treatment restored the endogenous expression of *ebag9* or *c-fos* at G2/M phase (Fig 3C). Knockdown of MTAs resulted in a decrease in E2-dependent proliferation of MCF7 cells (Yanagisawa *et al*, 2002; Fig 3D). These findings further support the hypothesis that a NuRD-type complex at G2/M lowers the E2-dependent transcriptional activity of ERα and mediates oestrogen-induced cell proliferation (Mazumdar *et al*, 2001; Manavathi *et al*, 2007).

DISCUSSION

Consistent with a previous report (Belandia *et al*, 2002), ERα associated with components of the SWI/SNF-type chromatin-remodelling complex, and these complexes were recruited to ERα target gene promoters in asynchronous cells. Similarly, at G1/S, the components were also recruited (Fig 2B). Thus, the SWI/SNF-type complex seems to support the ligand-induced transactivation function of liganded ERα in a cell-cycle-stage-specific manner (Fig 4, left panel).

In this study, we biochemically identified Mi2 as an ERα interactant in the cells at G2/M; associations with the other components of the NuRD-type chromatin-remodelling complex were also detected. Furthermore, recruitment of the core components of the NuRD-type complex to the ERα target gene promoters was seen only at G2/M and not at the other stages (Fig 2). Given that the NuRD-type complex contains HDAC1 (Denslow & Wade, 2007), which, in this study, interacted with ERα at G2/M, the NuRD-type complex might suppress the transcriptional function of ERα at G2/M (Fig 4, right panel). As knockdown of MTAs lowered E2-dependent cell proliferation, this association seems to be significant for the actions of E2. Although we cannot exclude the possibility that activated ERα induces expression of co-repressor genes responsible for cell-cycle regulation during G2/M, it is more likely that the expression of ERα target genes is suppressed through association with the NuRD-type complex. ERα might be transcriptionally silent at G2/M, but it might still contribute to cell-cycle progression by reorganizing

©2008 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

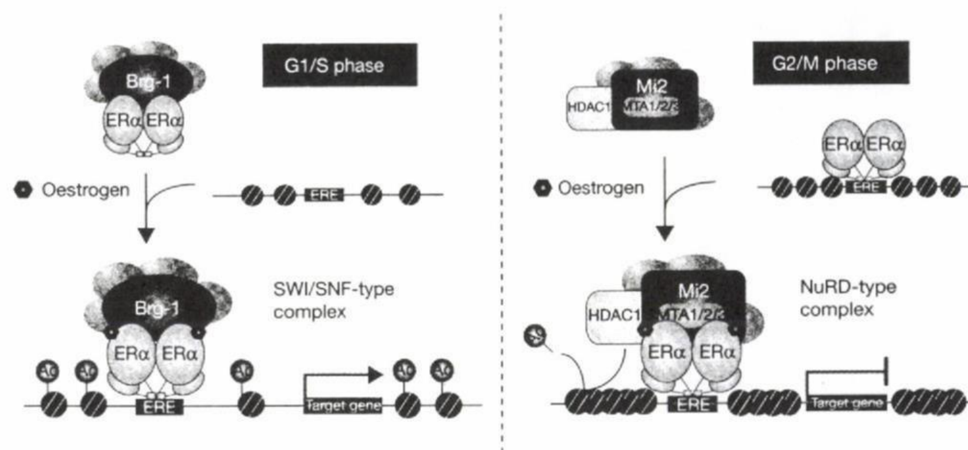

Fig 4 | Schematic representation of the cell-cycle-dependent exchange of chromatin-remodelling complexes. ERα exerts cell-cycle-dependent transcriptional activity by switching of components of the associated ATP-dependent chromatin-remodelling complex. The transcriptional activity of liganded ERα is co-activated by components of the SWI/SNF-type complex at G1/S, whereas it is co-repressed by components of the NuRD-type complex at G2/M. ER, oestrogen receptor; ERE, oestrogen response element; HDAC1, histone deacetylase 1.

chromatin structure through its association with the NuRD-type complex. At present, the molecular basis of cell-cycle-dependent switching of the two ATP-dependent chromatin-remodelling complexes remains to be uncovered. A switching factor seems to associate, at least transiently, with these complexes and is presumably functional at specific cell-cycle stages. The appearance of such a factor might be under cell-cycle regulation, similar to a recently characterized cell-cycle-dependent co-repressor (Ret-CoR) that represses the constitutively transrepressive-type nuclear receptor (Takezawa *et al*, 2007).

METHODS

Cell-cycle analysis. Cells were synchronized 24 h after spreading (for suspension culture, at a cell density of 2×10^6/ml) by treatment with 2.5 mM thymidine (Wako, Osaka, Japan) at G1/S phase or 10 ng/ml demecolcine (Wako) at G2/M phase under phenol red-free DMEM containing 5% serum. Each treatment was for 24 h except the luciferase assay. Cell synchronization was determined by using flow cytometry, as reported previously (Takezawa *et al*, 2007).

Transfection. Transfection with Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's protocol, before treatment with synchronization reagents. E2 and/or TSA treatment was 5 h before cell collection.

Purification of the ERα interactants. Nuclear extracts were prepared as described previously (Yanagisawa *et al*, 2002; Kitagawa *et al*, 2003; Ohtake *et al*, 2007; Takezawa *et al*, 2007) from synchronized HeLa-ERα cells treated with E2 for 1.5 h. Extracts ($\sim$400 mg) were loaded onto an anti-Flag M2 affinity resin column (approximately 200 μl bed volume) and were washed extensively with washing buffer containing 300 mM KCl. Bound proteins were eluted from the column by incubation with 5 × volume of Flag peptide (100 μg/ml; Sigma, St Louis, MO, USA) three times for 5 min. Each sample was applied onto a 2–15% gradient polyacrylamide gel for mass fingerprinting (Ohtake *et al*, 2007; Takezawa *et al*, 2007).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS
We thank F. Ohtake, S. Sawatsubashi, R. Fujiki, M.-s. Kim and A. Yokoyama for helpful discussion and plasmids; S. Fujiyama for technical assistance; H. Higuch, K. Motoi and H. Yamazaki for manuscript preparation; and H. Ito and A. Miyajima for flow cytometric analysis. This work was supported in part by a grant-in-aid for priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to H.K. and S.K.).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES
Bannister AJ, Kouzarides T (2005) Reversing histone methylation. *Nature* **436:** 1103–1106
Baumann C, Korner R, Hofmann K, Nigg EA (2007) PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. *Cell* **128:** 101–114
Belandia B, Parker MG (2003) Nuclear receptors: a rendezvous for chromatin remodeling factors. *Cell* **114:** 277–280
Belandia B, Orford RL, Hurst HC, Parker MG (2002) Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *EMBO J* **21:** 4094–4103
Berger SL (2007) The complex language of chromatin regulation during transcription. *Nature* **447:** 407–412
de la Serna IL, Ohkawa Y, Imbalzano AN (2006) Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. *Nat Rev Genet* **7:** 461–473
Denslow SA, Wade PA (2007) The human Mi-2/NuRD complex and gene regulation. *Oncogene* **26:** 5433–5438
Deroo BJ, Korach KS (2006) Estrogen receptors and human disease. *J Clin Invest* **116:** 561–570
Fujiki R, Kim MS, Sasaki Y, Yoshimura K, Kitagawa H, Kato S (2005) Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. *EMBO J* **24:** 3881–3894
Groth A, Rocha W, Verreault A, Almouzni G (2007) Chromatin challenges during DNA replication and repair. *Cell* **128:** 721–733
Hill SJ, Baker JG, Rees S (2001) Reporter-gene systems for the study of G-protein-coupled receptors. *Curr Opin Pharmacol* **1:** 526–532
Kitagawa H *et al* (2003) The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113:** 905–917
Klose RJ, Zhang Y (2007) Regulation of histone methylation by demethylimination and demethylation. *Nat Rev Mol Cell Biol* **8:** 307–318

©2008 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128:** 693–705

Lee KK, Workman JL (2007) Histone acetyltransferase complexes: one size doesn't fit all. *Nat Rev Mol Cell Biol* **8:** 284–295

Li B, Carey M, Workman JL (2007) The role of chromatin during transcription. *Cell* **128:** 707–719

Manavathi M, Singh K, Kumar R (2007) MTA family of coregulators in nuclear receptor biology and pathology. *Nucl Recept Signal* **5:** 1–8

Mangelsdorf DJ *et al* (1995) The nuclear receptor superfamily: the second decade. *Cell* **83:** 835–839

Martinez ED, Danielsen M (2002) Loss of androgen receptor transcriptional activity at the G(1)/S transition. *J Biol Chem* **277:** 29719–29729

Martinez-Balbas MA, Dey A, Rabindran SK, Ozato K, Wu C (1995) Displacement of sequence-specific transcription factors from mitotic chromatin. *Cell* **83:** 29–38

Mazumdar A, Wang RA, Mishra SK, Adam L, Bagheri-Yarmand R, Mandal M, Vadlamudi RK, Kumar R (2001) Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. *Nat Cell Biol* **3:** 30–37

Mellor J (2005) The dynamics of chromatin remodeling at promoters. *Mol Cell* **19:** 147–157

Moore JD, Kirk JA, Hunt T (2003) Unmasking the S-phase-promoting potential of cyclin B1. *Science* **300:** 987–990

Muchardt C, Reyes JC, Bourachot B, Leguoy E, Yaniv M (1996) The hbrm and BRG-1 proteins, components of the human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis. *EMBO J* **15:** 3394–3402

Narayanan R, Edwards DP, Weigel NL (2005) Human progesterone receptor displays cell cycle-dependent changes in transcriptional activity. *Mol Cell Biol* **25:** 2885–2898

Nuthall HN, Joachim K, Palaparti A, Stifani S (2002) A role for cell cycle-regulated phosphorylation in Groucho-mediated transcriptional repression. *J Biol Chem* **277:** 51049–51057

Ohtake F *et al* (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* **423:** 545–550

Ohtake F *et al* (2007) Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* **446:** 562–566

Prasanth KV, Sacco-Bubulya PA, Prasanth SG, Spector DL (2003) Sequential entry of components of the gene expression machinery into daughter nuclei. *Mol Biol Cell* **14:** 1043–1057

Rosenfeld MG, Lunyak VV, Glass CK (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* **20:** 1405–1428

Sif S (2004) ATP-dependent nucleosome remodeling complexes: enzymes tailored to deal with chromatin. *J Cell Biochem* **91:** 1087–1098

Swedlow JR, Hirano T (2003) The making of the mitotic chromosome: modern insights into classical questions. *Mol Cell* **11:** 557–569

Takezawa S *et al* (2007) A cell cycle-dependent co-repressor mediates photoreceptor cell-specific nuclear receptor function. *EMBO J* **26:** 764–774

Tsuchiya F, Ikeda K, Tsutsumi O, Hiroi H, Momoeda M, Taketani Y, Muramatsu M, Inoue S (2001) Molecular cloning and characterization of mouse EBAG9, homolog of a human cancer associated surface antigen: expression and regulation by estrogen. *Biochem Biophys Res Commun* **284:** 2–10

Workman JL (2006) Nucleosome displacement in transcription. *Genes Dev* **20:** 2009–2017

Yanagisawa J *et al* (2002) Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. *Mol Cell* **9:** 553–562

©2008 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Biochemical and Biophysical Research Communications 370 (2008) 541–546

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings

Fumiaki Ohtake [a,b], Atsushi Baba [a], Yoshiaki Fujii-Kuriyama [c,d], Shigeaki Kato [a,b,*]

[a] Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[b] ERATO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchisi, Saitama 332-0012, Japan
[c] TARA Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan
[d] SORST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchisi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 4 March 2008
Available online 19 March 2008

Keywords:
Dioxin receptor
AhR
Sex hormone receptor
Estrogen
Cross-talk
Transcription
Ubiquitin ligase
Proteasomal degradation

ABSTRACT

The arylhydrocarbon receptor (AhR) mediates sex steroid hormone-related actions in both normal physiology and in dioxin toxicity. In addition to regulation of direct target genes, the ligand-activated AhR associates with estrogen or androgen receptors (ERα or AR) to regulate transcription as a functional unit. Given that endogenous and exogenous AhR-ligands are structurally diverse, it is unclear whether cross-talk regulation of ERα/AR by the activated AhR is an intrinsic function of the AhR or the result of ligand-type-selective differences. To ensure uniform activity of the AhR irrespective of ligand-type-specific differences, we employed CA-AhR, which lacks the ligand-binding domain and has a constitutive activity. We found that CA-AhR, in the absence of a ligand, acted as a transcriptional co-regulator for the unliganded ERα/AR as well as for mutants of ERα/AR lacking a ligand-binding domain. CA-AhR was recruited to estrogen-/androgen-responsive promoters with endogenous ERα/AR. Moreover, CA-AhR had an E3 ubiquitin ligase activity and promoted proteasomal degradation of ERα/AR. Thus, these findings indicate that the cross-talk function of the AhR with sex hormone receptors is an intrinsic function of the AhR.
© 2008 Elsevier Inc. All rights reserved.

The arylhydrocarbon receptor (AhR) is a member of the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors. The AhR mediates the toxic effects of dioxins such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) [1–3], in part by modulating estrogen and androgen signaling [4,5]. AhR-deficient mice exhibit both abnormal estrous cycles and defective ovarian follicle maturation in females [6], and developmental defects in the prostate of males [7]. This implies a sex hormone-related innate function of the AhR.

The transcriptional activity of the AhR is primarily regulated by ligand-dependent translocation to the nucleus [3]. Unliganded AhR is sequestered in the cytosol by interacting with the Hsp90-XAP2 (also called AIP or ARA9) chaperon complex through the PAS-B region [1–3]. Ligand binding to the PAS-B region of the AhR induces dissociation of the Hsp90-XAP2 and subsequent translocation of the AhR to the nucleus[1–3]. The AhR then dimerizes with Arnt, recognizes the xenobiotic responsive element (XRE), and recruits co-activators [3]. The AhR induces expression of direct target genes such as the drug metabolizing enzymes CYP1A1 and CYP1A2 [1–3]. Disruption of inhibitory PAS-B function by ligand binding is therefore expected to be sufficient to induce transcriptional activity of the AhR [8]. In fact, a mutant AhR that lacks a PAS-B region (CA-AhR) is constitutively active and exhibits transcriptional activity irrespective of lack of ligand-binding capacity [9].

AhR exhibits other regulatory functions by modulating the function of other transcription factors, including Rb/E2F [10], NF-κB [11], and the estrogen (ERα and ERβ) [12–16] and androgen (AR) [16] receptors. These cross-talk pathways are important components that mediate the functions of endogenous and exogenous AhR-ligands. As for the estrogen-related adverse effects of dioxins, ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within the ERs [12–16]. By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα and represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [12]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [13] or repress [14] AhR-mediated transcription. Reflecting this functional cross-talk, Arnt also acts as a co-regulator for ERα [17]. In addition, it has been recently shown that the liganded AhR promotes ubiquitination and proteasomal degradation of ERα and the AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [16]. Thus, complexes of the AhR with ERs or

* Corresponding author. Address: Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Fax: +81 3 5841 8477.
E-mail address: uskato@mail.ecc.u-tokyo.ac.jp (S. Kato).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.03.054

AR appear to regulate transcription as functional units by multiple mechanisms.

Several diverse types of AhR-ligands have been reported to date, including endogenous [tryptamine, indole-3-acetic acid, and indirubin], exogeneous [TCDD, and benzo(a)pyrene], and synthetic [3-methylcholanthrene (3MC), and β-naphthoflavone (βNF)] ligands [18]. Since these compounds are structurally diverse, and the physiological ligand(s) for the AhR has not been defined, it is unclear whether modulation of ERα and AR function by the liganded AhR may be attributed to an intrinsic function of the AhR.

To ensure uniform activity of the AhR in our experiments, irrespective of ligand-type-specific differences, we employed CA-AhR which lacks binding capacities for the Hsp90-XAP2 as well as for ligands [8]. CA-AhR has constitutive transcriptional activity in the absence of ligands [8], and CA-AhR transgenic mice exhibit dioxin-exposure-related phenotypes in various tissue such as the liver and stomach [9]. These characteristics render this mutant particularly suitable for studying the intrinsic function of the AhR irrespective of ligand-type-specific differences. In this study, we demonstrated that CA-AhR acts as a transcriptional co-regulator for the unliganded ERα/AR, even in the absence of AhR-ligands. Moreover, CA-AhR promoted proteasomal degradation of both ERα and the AR. Therefore, these findings indicate that cross-talk with the sex hormone receptors is an intrinsic function of the AhR that is not subject to ligand-type-selective differences.

Materials and methods

Plasmids. Expression plasmids for the AhR, Arnt, constitutively active AhR, ERα, ERαΔD/E/F, AR, and ARΔE/F were previously described [12]. Luciferase reporter plasmids [estrogen-responsive element (ERE)-Luciferase, androgen-responsive element (ARE)-Luciferase, and xenobiotics-responsive element (XRE)-Luciferase] were described previously [12].

Cells, transfection, and luciferase assay. Human breast cancer-derived MCF-7 cells, human endometrial cancer-derived Ishikawa cells, human prostate cancer-derived LNCaP cells, and human renal cancer-derived 293T cells were cultured as previously described [16]. Cells were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS and transfected with the receptor expression vectors and the luciferase reporter plasmids [16]. Cells were then treated with E_2 (10 nM), DHT (10 nM), 3-methylcholanthrene (3MC) (1 μM), β-naphthoflavone (β-NF) (1 μM), or MG132 (10 μM), for 24 h (Luciferase assays) or for 3 h (Western blotting).

For the Luciferase assays [19], cells at 40–50% confluence were transfected with the indicated plasmids [0.25 μg reporter plasmids, 0.025 μg ERα, 0.1 μg ERαΔD/E/F, AhR/Arnt (+, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), 0.1 μg AR, 0.1 μg ARΔE/F] using Lipofectamine reagent (Gibco–BRL). Luciferase activity was determined with the Luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 2.5 ng pRL-CMV plasmid (Promega) was co-transfected. All values represent averages ±SD of at least three independent experiments.

In vitro ubiquitination assay. The *in vitro* ubiquitination assay was performed as previously described, with several modifications [16]. The CA-AhR immunocomplex was purified using anti-FLAG antibody from MCF-7 cells transfected with FLAG-HA-CA-AhR together with HA-DDB1 and myc-TBL3. The immunocomplex was incubated with recombinant ubiquitin and reaction buffer [50 mM Tris–HCl (pH 7.4), 5 mM MgCl, 2 mM NaF, 2 mM ATP and ATP-regenerating system, 0.6 mM DTT, and 12 μg ubiquitin (Calbiochem), 60 ng E1(Calbiochem), 0.3 μg E2 mixture set (Calbiochem)]. The self-ubiquitination of CA-AhR was detected by Western blotting using an anti-HA antibody.

ChIP assays. ChIP assays were performed essentially as previously described, with several modifications [12]. MCF-7 or LNCaP cells were transfected with 5 μg FLAG-CA-AhR in a 100 mm dish. After 36 h, the cells were treated with a transcription inhibitor α-amanitin (2.5 μM) for 2 h, released by a washing twice with PBS for 10 min and medium change. After the medium change, the cells were cross-linked at the indicated time (min).

More detailed methods are supplied as Supplementary information.

Results and discussion

CA-AhR activates the transcriptional function of unliganded ERα and AR

First, we tested if CA-AhR was indeed constitutively active in a xenobiotic-responsive element (XRE)-driven reporter assay in endometrial tumor-derived Ishikawa cells. CA-AhR activated XRE-mediated transcription in the absence of AhR-ligand as efficiently as 3MC-bound wild-type AhR (Supplementary Fig. 1).

Under this experimental condition, the effects of CA-AhR on ERα- and AR-mediated transcription were examined with the reporter assays. A reporter plasmid containing either a consensus estrogen-responsive element (ERE) or a consensus androgen-responsive element (ARE) was co-transfected with different amounts of either wild-type AhR or CA-AhR into Ishikawa cells (for ERE assays) or kidney-derived 293T cells (for ARE assays). When the wild-type AhR was transfected, transfection-dosage-dependent activation of the ERE- or ARE-Luciferase activity in the presence of, but not in the absence of, AhR-ligands [3MC and βNF], was observed (Fig. 1A and B). In that reporter assay, we found that CA-AhR significantly activated the ERE- or ARE-Luciferase activity in a transfection dosage-dependent manner in the absence of AhR-ligands (Fig. 1A and B). The activation function of CA-AhR for ERα-/AR-mediated transcription was comparable with that of the ligand-bound wild-type AhR, as expression levels of these AhR derivatives were not significantly different (Fig. 1C). We obtained similar results on the observed CA-AhR modulation of ERα function in mammary tumor-derived MCF-7 cells (data not shown).

CA-AhR activates ERα and AR mutants which lack C-terminal ligand-binding domains

To further demonstrate direct modulation of ERα-/AR-mediated transcription by the AhR, we employed ERα and AR mutants which lack C-terminal ligand-binding domains (LBDs; also known as E/F regions) [12]. Nuclear receptors exhibit two activation functions (AFs), AF-1 and AF-2 [20]. The AF-1 transactivation function in the A/B domain is ligand-independent, while the AF-2 function in the LBD is induced in a ligand-dependent manner through conformational change [20]. The LBD-deficient ERα and AR derivatives [ERαΔD/E/F and ARΔE/F] have DNA-binding activity but retain only AF-1 function [12].

As shown in lane 2 of Fig. 2A and B, transfected ERαΔD/E/F and AR ΔE/F mutants exhibited AF-1 activity, which was unaffected by the presence of E_2 or DHT, respectively (Fig. 2A and B, lane 12). Nonetheless, CA-AhR activated ERαΔD/E/F- and AR ΔE/F-mediated transcription in a transfection dosage-dependent manner in the absence of ERαAR ligands (lanes 9–11). The wild-type AhR, however, activated ERαΔD/E/F- and AR ΔE/F-mediated transcription only in the presence of 3MC (lanes 6–8). Importantly, since neither CA-AhR nor the LBD mutant of ERαAR has a functional ligand-binding domain, it is highly unlikely that the modulation of ERαΔD/E/F and AR ΔE/F by CA-AhR can be attributed to overlapping ligand-responsibility.

CA-AhR is recruited to estrogen- and androgen-responsive promoters in the absence of ligands

To explore the function of CA-AhR in the endogenous chromatin context, we tested whether CA-AhR was functional in endogenous estrogen-/androgen-responsive promoters by a chromatin immunoprecipitation (ChIP) assay. For this purpose, we used MCF-7 cells and LNCaP cells. Endogenous ERα and AR functionally bind to estrogen-responsive *c-fos* promoters in MCF-7 cells and androgen-responsive *PSA* promoters in LNCaP cells, respectively [12,21]. The wild-type AhR, upon ligand treatment, is recruited to the XRE-containing promoters at 30–60 min, and dissociates from the promoters after 60 min. The ligand-activated AhR is recruited to and dissociates from estrogen-responsive promoters together with ERα on a similar time course [12]. In order to synchronize the transcriptional cycle of CA-AhR in the absence of ligands, we

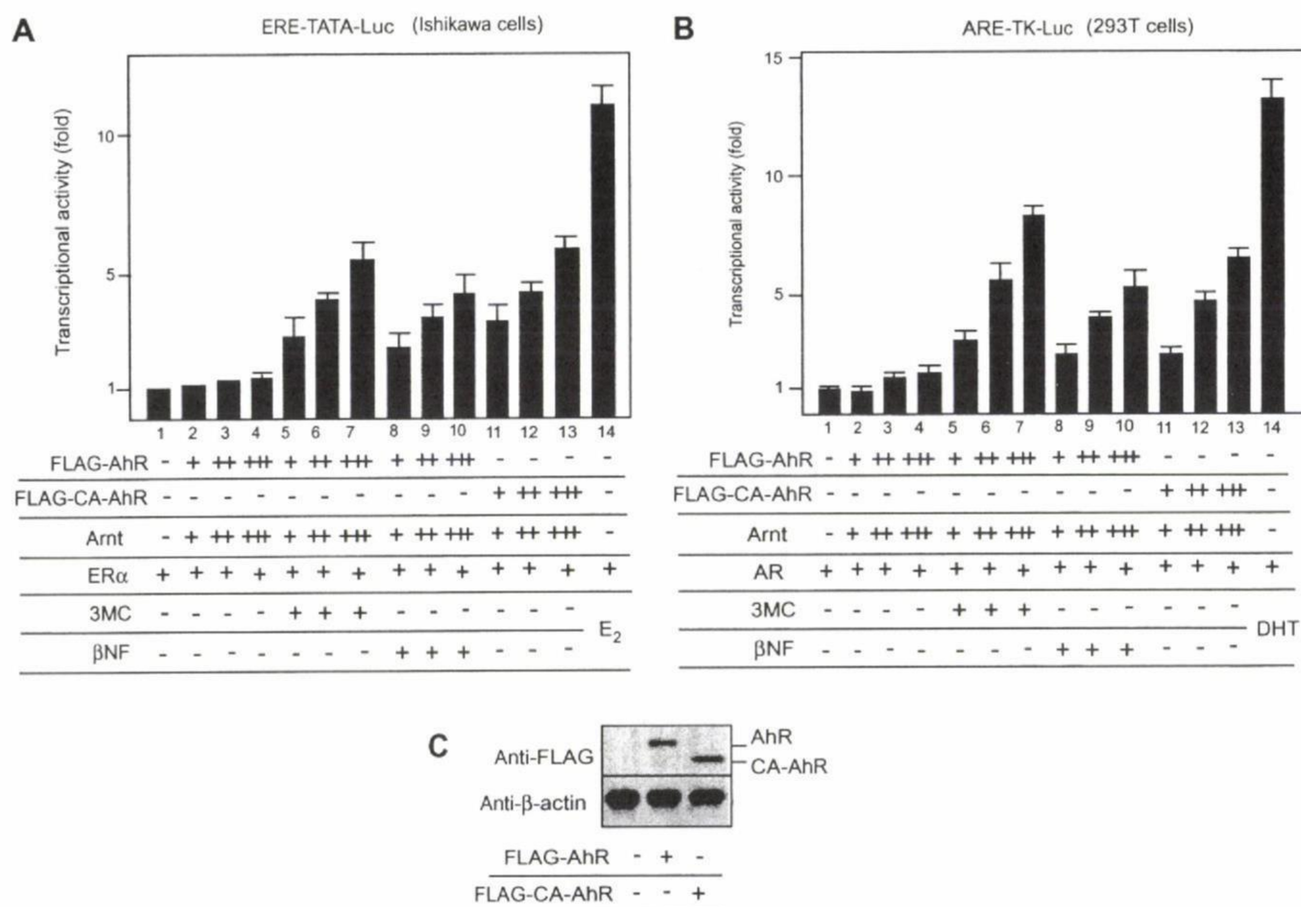

Fig. 1. The constitutively active AhR activates transcription through the unliganded ERα and AR in the absence of AhR-ligands. Ishikawa cells (A) or 293T cells (B) were transfected with the reporter plasmid bearing ERE (A) or ARE (B) together with the indicated expression plasmids (AhR/Arnt; +, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), in the presence or absence of the indicated ligands (3MC, 1 μM; βNF, 1 μM), and a Luciferase assay performed. Data are means ± SD of three independent experiments. (C) 293T cells were transfected with the indicated expression vectors (0.5 μg for FLAG-AhR and FLAG-CA-AhR), and the lysates subjected to Western blotting.

used the α-amanitin-release method, which is widely used in similar time-course ChIP experiments [22]. The MCF-7 cells and LNCaP cells transfected with CA-AhR were treated with α-amanitin, a transcription inhibitor, for two hours, and were then released by washing and a medium change.

Upon release from α-amanitin, CA-AhR was recruited to the *c-fos* promoter at 30–60 min in MCF-7 cells. Interestingly, endogenous ERα was co-recruited with CA-AhR to the *c-fos* promoter following a similar time-course in CA-AhR-transfected cells. No significant recruitment of ERα was observed 60 min after α-amanitin release in non-transfected cells (Fig. 3A). Similarly, CA-AhR was recruited to the *PSA* promoter at 60 min in LNCaP cells, and transfection of CA-AhR induced co-recruitment of endogenous AR on the *PSA* promoter on a similar time-course as CA-AhR (Fig. 3B). These results suggest that CA-AhR associates with endogenous ERα or AR on estrogen- or androgen-responsive promoters, respectively, as a transcriptional co-regulator.

CA-AhR has ubiquitin ligase activity and promotes proteasomal degradation of ERα in the absence of ligands

Finally, we tested if CA-AhR acted as an E3 ubiquitin ligase in the absence of a ligand. The ligand-activated AhR assembles a $CUL4B^{AhR}$ complex consisting of CUL4B/DDB1/Rbx1/TBL3/AhR/Arnt [16]. It recognizes ERα and the AR and promotes their ubiquitin–proteasome-mediated degradation [16]. Therefore, in this study the ubiquitin ligase activity of CA-AhR was verified. The CA-AhR immunocomplex, which included the components of $CUL4B^{AhR}$, was prepared, and the *in vitro* ubiquitination assay performed. The CA-AhR complex has an E3 ubiquitin ligase activity that is E1/E2-enzyme-dependent *in vitro*, as revealed by its self-ubiquitination activity (Fig. 4A).

The effects of CA-AhR on the degradation of ERα and the AR were then examined. ERα was co-transfected with either the wild-type AhR or CA-AhR into MCF-7 cells. After incubation with ligands and/or a proteasomal inhibitor MG132 for 6 h, cell lysates were prepared and the protein levels of ERα measured with Western blotting. To avoid detection of endogenous ERα protein in the non-transfected cells, we transfected FLAG-tagged ERα and detected ERα with a FLAG antibody. As shown in Fig. 4B, CA-AhR promoted degradation of ERα in the absence of the AhR-ligand in a transfection dosage-dependent manner (Fig. 4B, lanes 4 and 5). In contrast, wild-type AhR promoted degradation of ERα only in the presence of the AhR-ligand (lanes 2 and 3). A proteasomal inhibitor MG132 abolished the promotion of degradation, confirming a ubiquitin–proteasome pathway mediated mechanism (lane 6). Proteasomal degradation of the wild-type AhR itself was promoted by the AhR-ligand (lanes 2 and 3), consistent with previous reports [1–3].

Consistently, when the AR was co-transfected with either the wild-type AhR or CA-AhR into 293T cells, we found that CA-AhR promoted proteasomal degradation of the AR in the absence of AhR-ligand (Fig. 4C). The wild-type AhR promoted degradation of the AR only in the presence of a ligand (data not shown). These re-

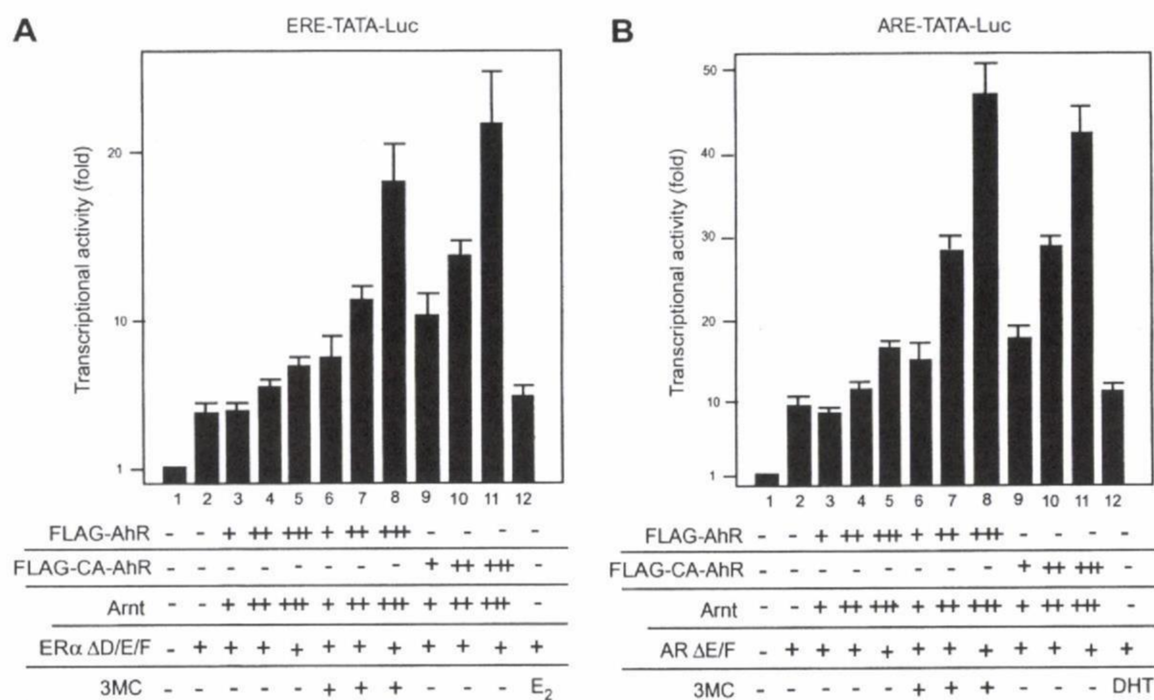

Fig. 2. CA-AhR activates mutants of ERα and AR which lack ligand-binding domains. Ishikawa cells (A) or 293T cells (B) were transfected with the indicated plasmids, and the Luciferase assay was performed as in Fig. 1. Data are means ± SD of three independent experiments. The data show that CA-AhR modulates the transcriptional activity of ERαΔD/E/F and AR ΔE/F in the absence of ligands.

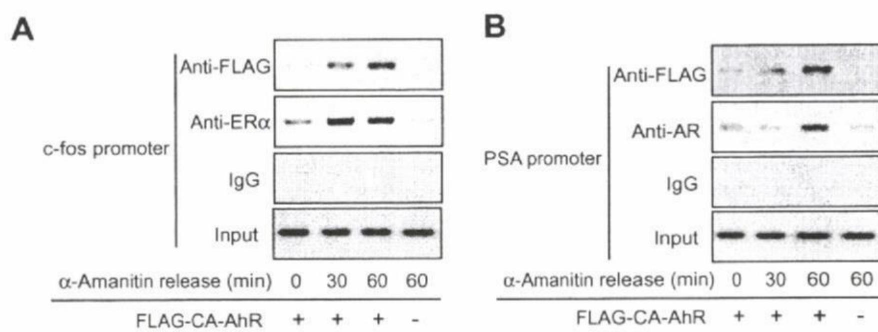

Fig. 3. Promoter occupancy of CA-AhR together with ERα or the AR in the absence of AhR-ligands. MCF-7 cells (A) or LNCaP cells (B), in which endogenous ERα (A) or the AR (B) was functional, were transfected with FLAG-tagged AhR or CA-AhR as indicated. The cells were treated with 10 μM α-amanitin for 2 h, and then subjected to the ChIP assay at the indicated time (min) after release from α-amanitin by a medium change. Samples were immunoprecipitated with the indicated antibodies, and promoter DNAs were amplified by PCR as indicated.

sults suggest that the activated AhR directly modulates stability of ERα and AR proteins by its ubiquitin ligase activity, irrespective of the ligand.

AhR as a transcriptional co-regulator for ERα/AR

Accumulating evidence suggests some of the actions of the AhR are mediated through cross-talk pathways with other transcription factors, including Rb [10], NF-κB [11,23], and nuclear receptors [5,12–16]. We and other groups have previously reported that the ligand-activated AhR directly associates with ERα and the AR to regulate transcription [12–16]. Moreover, AhR-dependent degradation of ERα has been independently reported [15,16]. To provide additional evidence for these AhR-ERα/AR cross-talk pathways, we have shown that CA-AhR, which lacks the ligand-binding domain and has a constitutive activity [8,9], modulates the functions of ERα and the AR in the absence of AhR-ligands. This suggests that activation of the AhR, either by ligand binding or by deletion of an inhibitory domain, directly induces a regulatory AhR-ERα/AR cross-talk pathway. Moreover, we have shown that the ubiquitin ligase activity of CA-AhR is also intact in the absence of a ligand. These results suggest that modulation of ERα and the AR is an intrinsic function of the AhR.

Importantly, we have shown that the mutants of ERα and AR which lack the C-terminal ligand-binding domains are also functionally regulated by CA-AhR. This directly excludes the possibility of cross-binding of AhR-ligands to ERα and the AR. Consistent with this, it was recently demonstrated that the AhR-ligand 3MC does not directly bind to ERα [24]. Together, these results indicate that the active form of the AhR, but not the ligand itself, is required for AhR-ERα/AR cross-talk. Thus, the present data support the existence of a 'direct cross-talk' pathway in which the AhR directly associates with ERα/AR and regulates their function as a transcriptional co-regulator. In the nuclear receptor superfamily of tran-

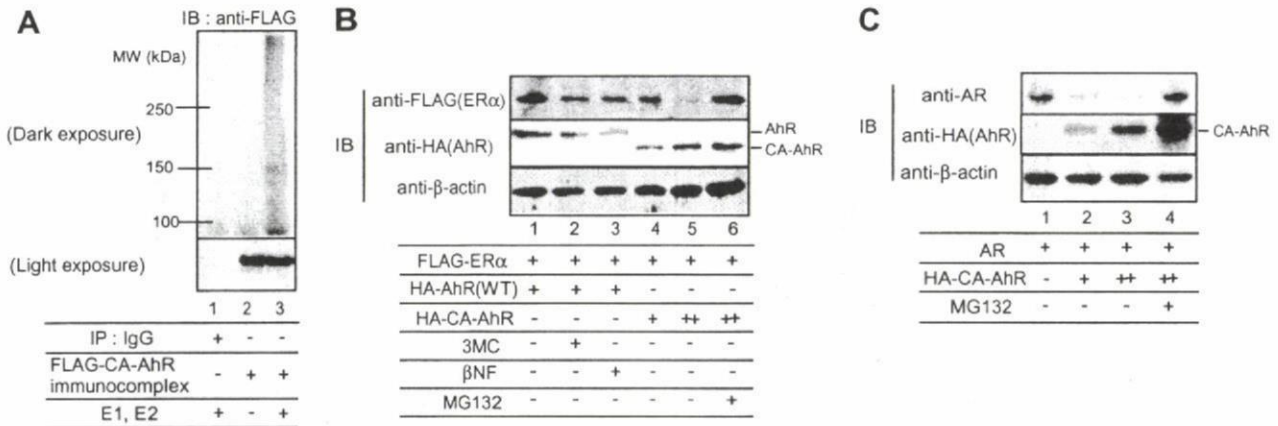

Fig. 4. CA-AhR promotes proteasomal degradation of ERα and the AR. (A) A FLAG-CA-AhR immunocomplex and IgG immunocomplex, prepared from MCF-7 cells, were subjected to the *in vitro* ubiquitination assay. The self-ubiquitination of CA-AhR was detected by Western blotting as indicated. (B,C) MCF-7 cells (B) or 293T cells (C) were transfected with the indicated plasmids [0.25 μg FLAG-ERα, 0.25 μg AR, 0.5 μg HA-AhR, HA-CA-AhR (+, 0.5 μg; ++, 1.0 μg) in 6-well dish]. Twenty-four hours after transfection, the cells were incubated with the indicated ligands or vehicle, then lysed and subjected to Western blotting with the indicated antibodies.

scription factors, ligand-type-selective differences in receptor conformational change affect the interaction with specific co-regulators [21,25]. Given that AhR-ligands are structurally diverse, ligand-specific differences in the modulation of AhR conformation may result in differential interaction with ERα/AR, CUL4B, and co-activators such as p300. This may lead to differential regulation of cross-talk pathways.

In the cross-talk pathways, AhR, like other co-regulators [26], both positively and negatively regulates other transcription factors. For instance, AhR associates with Rb/E2F1 and cooperatively regulates transcription, both positively [27,28] and negatively [10]. Similarly, AhR and NF-κB pathways converge in either a cooperative [11] or inhibitory [23] manner, depending on the cellular conditions. Our presented data indicate that the AhR assembles both a transcriptional co-regulator complex and a ubiquitin ligase complex. These complexes may explain the bi-phasic functions of the AhR-ligand in the regulation of other transcription factors, and are likely related to the physiological function of the AhR.

Acknowledgments

We thank Dr. J. Mimura for valuable comments, K. Motoi and M. Yamaki for manuscript preparation. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to Y.F.-K. and S.K.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.054.

References

[1] Y.Z. Gu, J.B. Hogenesch, C.A. Bradfield, The PAS superfamily: sensors of environmental and developmental signals, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 519–561.
[2] L. Poellinger, Mechanistic aspects–the dioxin (aryl hydrocarbon) receptor, Food Addit. Contam. 17 (2000) 261–266.
[3] J. Mimura, Y. Fujii-Kuriyama, Functional role of AhR in the expression of toxic effects by TCDD, Biochim. Biophys. Acta 1619 (2003) 263–268.
[4] D.B. Carlson, G.H. Perdew, A dynamic role for the Ah receptor in cell signaling? Insights from a diverse group of Ah receptor interacting proteins, J. Biochem. Mol. Toxicol. 16 (2002) 317–325.
[5] D.R. Boverhof, J.C. Kwekel, D.G. Humes, L.D. Burgoon, T.R. Zacharewski, Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus, Mol. Pharmacol. 69 (2006) 1599–1606.
[6] T. Baba, J. Mimura, N. Nakamura, N. Harada, M. Yamamoto, K. Morohashi, Y. Fujii-Kuriyama, Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction, Mol. Cell Biol. 25 (2005) 10040–10051.
[7] T.M. Lin, K. Ko, R.W. Moore, U. Simanainen, T.D. Oberley, R.E. Peterson, Effects of aryl hydrocarbon receptor null mutation and in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on prostate and seminal vesicle development in C57BL/6 mice, Toxicol. Sci. 68 (2002) 479–487.
[8] J. McGuire, K. Okamoto, M.L. Whitelaw, H. Tanaka, L. Poellinger, Definition of a dioxin receptor mutant that is a constitutive activator of transcription: delineation of overlapping repression and ligand binding functions within the PAS domain, J. Biol. Chem. 276 (2001) 41841–41849.
[9] P. Andersson, J. McGuire, C. Rubio, K. Gradin, M.L. Whitelaw, S. Pettersson, A. Hanberg, L. Poellinger, A constitutively active dioxin/aryl hydrocarbon receptor induces stomach tumors, Proc. Natl Acad. Sci. USA 99 (2002) 9990–9995.
[10] A. Puga, S.J. Barnes, T.P. Dalton, C. Chang, E.S. Knudsen, M.A. Maier, Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest, J. Biol. Chem. 275 (2000) 2943–2950.
[11] C.F. Vogel, E. Sciullo, W. Li, P. Wong, G. Lazennec, F. Matsumura, RelB, a new partner of aryl hydrocarbon receptor-mediated transcription, Mol. Endocrinol. 21 (2007) 2941–2955.
[12] F. Ohtake, K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama, S. Kato, Modulation of oestrogen receptor signalling by association with the activated dioxin receptor, Nature 423 (2003) 545–550.
[13] J. Matthews, B. Wihlen, J. Thomsen, J.A. Gustafsson, Aryl hydrocarbon receptor-mediated transcription: ligand-dependent recruitment of estrogen receptor alpha to 2,3,7,8-tetrachlorodibenzo-p-dioxin-responsive promoters, Mol. Cell Biol. 25 (2005) 5317–5328.
[14] T.V. Beischlag, G.H. Perdew, ER alpha-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription, J. Biol. Chem. 280 (2005) 21607–21611.
[15] M. Wormke, M. Stoner, B. Saville, K. Walker, M. Abdelrahim, R. Burghardt, S. Safe, The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes, Mol. Cell Biol. 23 (2003) 1843–1855.
[16] F. Ohtake, A. Baba, I. Takada, M. Okada, K. Iwasaki, H. Miki, S. Takahashi, A. Kouzmenko, K. Nohara, T. Chiba, Y. Fujii-Kuriyama, S. Kato, Dioxin receptor is a ligand-dependent E3 ubiquitin ligase, Nature 446 (2007) 562–566.
[17] S. Brunnberg, K. Pettersson, E. Rydin, J. Matthews, A. Hanberg, I. Pongratz, The basic helix-loop-helix-PAS protein ARNT functions as a potent coactivator of estrogen receptor-dependent transcription, Proc. Natl. Acad. Sci. USA 100 (2003) 6517–6522.
[18] J. Mimura, K. Yamashita, K. Nakamura, M. Morita, T.N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, Y. Fujii-Kuriyama, Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor, Genes Cells 2 (1997) 645–654.
[19] I. Takada, M. Mihara, M. Suzawa, F. Ohtake, S. Kobayashi, M. Igarashi, M.Y. Youn, K. Takeyama, T. Nakamura, Y. Mezaki, S. Takezawa, Y. Yogiashi, H. Kitagawa, G. Yamada, S. Takada, Y. Minami, H. Shibuya, K. Matsumoto, S. Kato, A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation, Nat. Cell Biol. 9 (2007) 1273–1285.
[20] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, et al., The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.

[21] Y. Shang, X. Hu, J. DiRenzo, M.A. Lazar, M. Brown, Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription, Cell 103 (2000) 843–852.
[22] R. Metivier, G. Penot, M.R. Hubner, G. Reid, H. Brand, M. Kos, F. Gannon, Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter, Cell 115 (2003) 751–763.
[23] C.E. Ruby, M. Leid, N.I. Kerkvliet, 2,3,7,8-Tetrachlorodibenzo-p-dioxin suppresses tumor necrosis factor-alpha and anti-CD40-induced activation of NF-kappaB/Rel in dendritic cells: p50 homodimer activation is not affected, Mol. Pharmacol. 62 (2002) 722–728.
[24] E. Swedenborg, J. Ruegg, A. Hillenweck, S. Rehnmark, M. Hedengran Faulds, D. Zalko, I. Pongratz, K. Pettersson, 3-Methyl cholanthrene displays dual effects on ER{alpha} and ER{beta} signaling in a cell-type specific fashion, Mol. Pharmacol. 22 (2007) 304–316.
[25] K. Takeyama, Y. Masuhiro, H. Fuse, H. Endoh, A. Murayama, S. Kitanaka, M. Suzawa, J. Yanagisawa, S. Kato, Selective interaction of vitamin D receptor with transcriptional coactivators by a vitamin D analog, Mol. Cell Biol. 19 (1999) 1049–1055.
[26] M.G. Rosenfeld, V.V. Lunyak, C.K. Glass, Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response, Genes Dev. 20 (2006) 1405–1428.
[27] S. Shimba, K. Komiyama, I. Moro, M. Tezuka, Overexpression of the aryl hydrocarbon receptor (AhR) accelerates the cell proliferation of A549 cells, J. Biochem. 132 (2002) 795–802.
[28] C.J. Elferink, N.L. Ge, A. Levine, Maximal aryl hydrocarbon receptor activity depends on an interaction with the retinoblastoma protein, Mol. Pharmacol. 59 (2001) 664–673.

doi: 10.1507/endocrj.K08E-177

ORIGINAL

Nuclear Receptor DAX1 in Human Prostate Cancer: A Novel Independent Biological Modulator

Yasuhiro NAKAMURA[1], Takashi SUZUKI[1], Yoichi ARAI[2] and Hironobu SASANO[1]

[1] *Department of Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan*
[2] *Department of Urology, Tohoku University Graduate School of Medicine, Sendai, Japan*

Received July 4, 2008; Accepted September 16, 2008; Released online October 1, 2008

Correspondence to: Hironobu Sasano, M.D., Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980- 8575, Japan

Abstract. The orphan nuclear receptor DAX1 (dosage-sensitive sex reversal-AHC critical region on the X chromosome gene 1; *NR0B1*) has been known for its various roles in human development, specifically sex determination and steroidogenesis. Its expression has been reported in endocrine and sex steroid-dependent neoplasms such as human adrenocortical, pituitary, endometrial, and ovarian tumors. Prostate cancer is also sex steroid-dependent tumor in which androgens play important roles in the pathogenesis and development via androgen receptor (AR). DAX1 is also reported to repress AR activity in human prostate cancer cell line (LNCaP) but its biological roles have remained unclear in the human prostate cancer. The aim of this study is to examine the expression of DAX1 in human prostate cancer using immunohistochemistry in order to evaluate its possible biological and/or clinical significance. In this study, we examined the DAX1 immunoreactivity in human prostate cancer obtained from surgery (n=40), and correlated the findings with clinicopathological features of the patients. Twenty-one cases were defined as positive cases for DAX1 immunoreactivity (53%). Immunoreactivity for DAX1 was inversely and significantly correlated with Gleason score ($P<0.05$). However, DAX1 immunoreactivity was not significantly correlated with the status of sex steroid receptors we examined. DAX1 immunoreactivity is considered a new biological modulator of human prostate cancer, but independent to the status of sex steroid receptors in human prostate cancer tissues.

Key words: DAX1, prostate, cancer, immunohistochemistry

PROSTATE cancer is known as sex steroid-dependent tumor in which androgens play important roles in the pathogenesis and development via androgen receptor (AR) (1, 2). In addition, estrogen receptor beta (ERβ) and progesterone receptor (PR) is also expressed in prostate cancer and is known to modify its biological significance (3-5). Therefore, it becomes interesting to examine the regulatory mechanisms of expression of these steroid receptors in human prostate cancer.

The orphan nuclear receptor DAX1 (dosage-sensitive sex reversal-AHC critical region on the X chromosome gene 1; *NR0B1*) is a recently characterized member of the orphan nuclear receptor family (6, 7). DAX1 is widely distributed in the reproductive and endocrine systems (8, 9). In addition, DAX1 is known to act as a negative regulator of steroid production (10-13). DAX1 has also been demonstrated to repress Ad4BP/SF-1-mediated transactivation of other steroidogenic genes, and to act as a corepressor for AR, PR, and ERβ (14-20). These interactions could play significant roles by influencing sex-steroid signaling pathways. However, the expression of DAX1 has not been examined in detail in human prostate cancer. Therefore, in this study, we examined the status and relative abundance of DAX1 in human prostate cancer, and correlated the

findings with the status of AR, ERβ, PR, and other clinicopathological findings in order to examine the possible biological significance of this unique transcription factor.

Materials and Methods

Patients and tissues

Forty surgical pathology specimens of prostate carcinoma were obtained from the patients who underwent prostatectomy from 1998–2003 at the Department of Urology, Tohoku University Hospital (Sendai, Japan). The specimens were retrieved from surgical pathology files of Tohoku University Hospital. The mean age of the patients was 65.9 y (range: 54-77 y). All the patients examined in this study did not receive radiation, chemotherapy, or hormone therapy prior to surgery. Clinical data, including patient age, serum prostate specific antigen (PSA) concentration, lymph node status, and clinical stage according to the International Union Against Cancer TNM classification (1987), and Gleason score were retrieved from the patient charts describing individual patient histories. The histological grades of each tumor were evaluated by two of the authors (Y.N. and T.S.). All the specimens were fixed with 10% formalin and embedded in paraffin wax at the Department of Pathology, Tohoku University Hospital. The Ethic's Committee at Tohoku University School of Medicine approved the research protocol for this study (2003-146).

Antibodies

Rabbit polyclonal antibody for DAX1 was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Antibodies against AR and Ki-67 were purchased from DAKO Corporation (Carpinteria, CA) and Immunotech (Marseilles, France), respectively. Antibodies for ERβ and PR were also commercially obtained from Gene Tex, Inc., (San Antonio, TX) and NeoMarkers Co. Ltd. (Fremont, CA), respectively.

Immunohistochemistry

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan) and has been previously described in detail (2, 4). For immunostaining, the slides were heated in an autoclave at 120 °C for 5 min in citric acid buffer (2 mmol/l citric acid and 9 mmol/l trisodium citrate dehydrate, pH 6.0) after deparaffinization for antigen retrieval. The dilutions of primary antibodies used in our study were as follows: DAX1, 1: 500; AR, 1: 100; ERβ, 1: 1,500; PR, 1: 200; and Ki-67, 1: 50. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution [1 mmol/l 3,3'-DAB, 50 mmol/l Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Tissue sections of the normal adrenal gland were used as positive controls for DAX1, an invasive ductal carcinoma of the breast were used as positive controls for ERβ and PR, and normal prostate tissue was used as a positive control for AR. As for negative controls, immunohistochemical preabsorption tests were performed for DAX1, and normal rabbit IgG was also used instead of the primary antibody. No specific immunoreactivity was detected in these tissue sections (data not shown).

Scoring of immunoreactivity

Evaluation of DAX1, AR, ERβ, PR, and Ki-67 immunoreactivity was performed in high-power fields (×400) using a standard light microscope. These immunohistochemical expression levels were independently reviewed by two of the authors (Y.N. and T.S.). In all cases examined, a total of more than 500 tumor cells from three different representative fields were counted independently by the two aforementioned authors, and the percentage

immunoreactivity (i.e. labeling index [LI]), was determined. After completely reviewing the immunostained sections of each lesion, all cases were divided into the following two groups: + (more than 10% positive cells); – (fewer than 10% positive cells) for DAX1 immunoreactivity.

Statistical analysis

Values for patient age, serum PSA levels, and LI for AR, ERβ, PR, and Ki-67 were presented as the mean±95% confidence interval (95% CI), and associations between DAX1 immunoreactivity described above were evaluated using the unpaired *t*-test. Statistical differences between immunoreactivity for DAX1 and stage, lymph node status, histological grade, and Gleason score were evaluated in a cross-table using the χ^2-test. $P<0.05$ was considered significant.

Results

DAX1 immunoreactive protein was detected in the nuclei of prostate carcinoma cells, but not in the normal prostate (Figure 1). Twenty-one cases were defined as positive cases for DAX1 immunoreactivity (53%). There was a significant inverse or negative correlation between DAX1 immunoreactivity and Gleason score of carcinoma (Table 1) ($P<0.05$). Immunoreactivity for DAX1 was not significantly correlated with other clinicopathological parameters including patients' age, concentration of serum PSA levels, pT stage, lymph node status, or LI of Ki-67 (Table 1). There were no significant correlations between DAX1 immunoreactivity and AR, ERβ, or PR, immunoreactivity (Table 2).

Discussion

In this study, we first demonstrated that immunoreactivity for DAX1 was detected in human prostate cancer in approximately 50% of the cases we examined. In addition, immunoreactivity for DAX1 was inversely and significantly correlated with Gleason score.

To the best of our knowledge, this study is the first report to examine the relative abundance of DAX1 protein in human prostate cancer tissues. DAX1 was previously reported in human testis, ovarian follicle, corpus luteum, and adrenal, and has been in general postulated to be associated with development and steroidogenesis of these tissues (21-25). The presence of DAX1 was also reported in adrenal, endometrial, and ovarian tumor (19, 26-28). DAX1 mRNA was absent in human normal prostate, while it was expressed in androgen-dependent prostate cancer cell lines, i.e. LNCaP cells (20, 29). Therefore, DAX1 may play an important role in development of human prostate cancer but not in normal prostate.

DAX1 protein has been reported to be localized mainly in the nuclei of various cells and to be postulated to work as a nuclear orphan receptor in the human normal and neoplastic tissues (19, 21-28). Results of recent studies suggest that DAX1 was also detected in the cytoplasm, functioning as a potent corepressor for steroid formone receptors in mammalian cells (30-32). However, in our present study, DAX1 immunoreactivity was clearly detectable in the nuclei of prostate carcinoma cells but not so in their cytoplasm, which is compatible with results of most previously reported studies (19, 21-28). Therefore, these results all suggest that DAX1 protein is predominantly present in the nuclei of prostate carcinoma cells and may play an important role as a nuclear orphan receptor in human prostate cancer tissues. However, it requires further investigations to clarify the significance of DAX expression in both the nucleus and cytoplasm in human prostate carcinoma cells.

Gleason score is the most important and established predictors of biologic behavior of human prostate carcinoma (33). In this grading system, the five basic grade patterns are used to generate a histologic score, which ranges from 2 to 10, by adding the primary grade pattern and the secondary grade pattern. Gleason score 2–4 carcinoma is commonly regarded as well-differentiated, Gleason score 5–7 as moderately differentiated, and Gleason score 8–10 as poorly differentiated (33). However, Gleason score 7 carcinoma is known to harbor an element of high-grade pattern carcinoma, and is intermediate in clinical aggressiveness between patterns 5–6 and 8–10, and is postulated not to be included in a moderately differentiated category (33-36). Therefore, the 2–6 *vs* 7 *vs* 8–10 lump is considered most appropriate for low patient numbers in a research setting, as used in this study (33).

In our present study, the status of DAX1 immunoreactivity was inversely and significantly correlated with Gleason score of the cases. Saito *et al.* previously reported that there is a significant inverse correlation between DAX1 immunoreactivity and histological grade in endometrial carcinoma, suggesting that DAX1 may inhibit cell proliferation and the progression of endometrial carcinoma (19). DAX1 expression level is postulated to be associated with the differentiation of mammary epithelial cells (37). In contrast, Abd-Elaziz *et al.* reported that DAX1 immunoreactivity is considered to be an independent marker of poor prognosis or adverse clinical outcome in patients with epithelial ovarian carcinoma (28). DAX1 was, however, reported not to be associated with any clinicopathological factors including histological grades in human breast cancer (31). In our study, DAX1 immunoreactivity was detected in approximately 50% of prostate cancer tissues. The loss of expression or decreased expression of DAX1 in endometrial carcinoma was reported to result in active or increased intratumoral steroids metabolism or production in endometrial carcinoma, which subsequently result in the estrogen-dependent proliferation of carcinoma cells (19). Similarly, the loss of expression or decreased expression of DAX1 in prostate cancer may play an important role in intratumoral steroid metabolism or production, which subsequently influences the proliferation of prostate carcinoma cells. However, further *in vitro* investigations are required to clarify the precise roles of DAX1 in regulating cell growth and development in human prostate carcinoma cells.

DAX1 has been reported by Zhang *et al*. to play important roles in the regulation of ER transactivation (30). DAX1 has been also reported to inhibit the transcriptional activity of liganded ER by a sequential mechanism, possibly involving the recruitment of corepressors (16). Saito *et al.* also reported that there was a statistically significant positive correlation between DAX1 and ERα and β expression levels, suggesting that this nuclear factor may inhibit the proliferation and progression of endometrial carcinoma through inhibition of estrogenic actions, possibly by interacting with ER present in carcinoma cells (19). However, the status of DAX1 immunoreactivity was not significantly correlated with that of AR, ERβ, or PR in all the cases of prostate carcinoma cases which we examined. These findings suggest that DAX1 does not regulate the expression levels of these steroid hormone receptors in human prostate carcinoma cells compared to the cases of human ovarian and endometrial cancer tissues. However, DAX1 was also reported to suppress agonist-dependent activity of AR in human prostate carcinoma cell lines and PR in human breast cancer cells (20). In our study, 21 cases were defined as positive for DAX1 immunoreactivity (53%). Among these DAX-1 positive cases, the case with relatively high expression levels (LI more than 10%) was detected in 20 cases for AR, 20 cases for ERβ, and 8 cases for PR (data not shown), respectively. These findings also suggest the co-localization of DAX1 and these steroid hormone receptors resulting in their interaction and possible regulation by DAX-1 in the function of these steroid hormone receptors in

human prostate cancer tissues. It awaits further examinations to clarify the correlation between DAX1 and these steroid hormone receptors in human prostate cancer tissues.

In summary, we demonstrated that DAX1 protein was detected in approximately 50% of prostate cancer tissues, and significant inverse or negative correlation was present between DAX1 immunoreactivity and Gleason score of carcinoma. These findings indicate that this nuclear orphan receptor is considered to be a new biological modulator of human prostate cancer.

Acknowledgements

This work was partly supported by the grants from the Japanese Ministry of Health, Labour and Welfare for Researches on Intractable Diseases and Risk Analysis Research on Food and Pharmaceuticals. This work was also partly supported by Grant-in-Aid for Scientific Research (18390109) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Public Trust Haraguchi Memorial Cancer Research Fund, and Kurozumi Medical Foundation.

References

1. López-Otín C, Diamandis EP (1998) Breast and prostate cancer: an analysis of common epidemiological, genetic and biochemical features. Endocr Rev 19:365-396.
2. Nakamura Y, Suzuki T, Nakabayashi M, Endoh M, Sakamoto K, Mikami Y, Moriya T, Ito A, Takahashi S, Yamada S, Arai Y, Sasano H (2005) In situ androgen producing enzymes in human prostate cancer. Endocr Relat Cancer 12:101-107.
3. Härkönen PL, Mäkelä SI (2004) Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol 92:297-305.
4. Nakamura Y, Suzuki T, Fukuda T, Ito A, Endo M, Moriya T, Arai Y, Sasano H (2006) Steroid sulfatase and estrogen sulfotransferase in human prostate cancer. Prostate 66:1005-1012.
5. Hiramatsu M, Maehara I, Orikasa S, Sasano H (1996) Immunolocalization of oestrogen and progesterone receptors in prostatic hyperplasia and carcinoma. Histopathology 28:163-168.
6. Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Moser C, Walker AP, McCabe ER, Meitinger T, Monaco AP, Sassone-Corsi P, Camerino G. (1994) An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 372:635-645.
7. Swain A, Zanaria E, Hacker A, Lovell-Badge R, Camerino G (1996) Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. Nat Genet 12:404-409.
8. Tamai KT, Monaco L, Alastalo TP, Lalli E, Prvinen M, Sassone-Corsi P (1996) Hormonal and developmental regulation of DAX1 expression in Sertoli cells. Mol Endocrinol 10:1561-1569.
9. Ikeda Y, Swain A, Weber TJ, Hentges KE, Zanaria E, Lalli E, Tamai KT, Sassone-Corsi P, Lovell-Badge R, Camerino G, Parker KL (1996) Steroidogenic factor 1 and DAX1 colocalization in multiple cell lineages: potential kinks in endocrine development. Mol Endocrinol 10:1261-1272.
10. Lalli E, Melner MH, Stocco DM, Sassone-Corsi P (1998) DAX1 blocks steroid production at multiple levels. Endocrinology 139:4237-4243.
11. Zazopoulos E, Lalli E, Stocco DM, Sassone-Corsi P (1997) DNA binding and transcriptional repression by DAX1 blocks steroidogenesis. Nature 390:311-315.
12. Sugawara T, Lin D, Holt JA, Martin KO, Javitt NB, Miller WL, Strauss JF 3rd (1995) Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR

doi: 10.1507/endocrj.K08E-177

stimulates mitochondrial cholesterol 27 hydroxylase activity. Biochemistry 34: 12506-12512.
13. Stocco DM, Clark BJ (1996) Regulation of acute production of steroid in steroidogenic tissue. Endocr Rev 17:221-244.
14. Parker KL, Schimmer BP (1997) Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 18:361-377.
15. Takayama K, Sasano H, Fukaya T, Morohashi K, Suzuki T, Tamura M, Costa MJ, Yajima A (1995) Immunohistochemical localization of Ad4-binding protein with correlation to steroidogenic enzyme expression in cycling human ovaries and sex cord stromal tumors. J Clin Endocrinol Metab 80:2815-2821.
16. Crawford PA, Dorn C, Sadovsky Y, Milbrandt J (1998) Nuclear receptor DAX1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. Mol Cell Biol 18: 2949-2956.
17. Hanley NA, Rainey WE, Wilson DI, Ball SG, Parker KL (2001) Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15:57-68.
18. Wang ZJ, Jeffs B, Ito M, Achermann JC, Yu RN, Hales DB, Jameson JL (2001) Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1. Proc Natl Acad Sci USA 98: 7988-7993.
19. Saito S, Ito K, Suzuki T, Utsunomiya H, Akahira J, Sugihashi Y, Niikura H, Okamura K, Yaegashi N, Sasano H (2005) Orphan nuclear receptor DAX1 in human endometrium and its disorders. Cancer Sci. 96:645-652.
20. Agoulnik IU, Krause WC, Bingman WE 3rd, Rahman HT, Amrikachi M, Ayala GE, Weigel NL (2003) Repressors of androgen and progesterone receptor action. J Biol Chem 278:31136-31148
21. Guo W, Burris TP, McCabe ER (1995) Expression of DAX-1, the gene responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism, in the hypothalamic-pituitary-adrenal/gonadal axis. Biochem Mol Med 56:8-13.
22. Sato Y, Suzuki T, Hidaka K, Sato H, Ito K, Ito S, Sasano H (2003) Immunolocalization of nuclear transcription factors, DAX-1 and COUP-TF II, in the normal human ovary: correlation with adrenal 4 binding protein/steroidogenic factor-1 immunolocalization during the menstrual cycle. J Clin Endocrinol Metab 88:3415-3420.
23. Ozisik G, Achermann JC, Meeks JJ, Jameson JL (2003) SF1 in the development of the adrenal gland and gonads. Horm Res 59 Suppl 1:94-98.
24. Beuschlein F, Keegan CE, Bavers DL, Mutch C, Hutz JE, Shah S, Ulrich-Lai YM, Engeland WC, Jeffs B, Jameson JL, Hammer GD (2002) SF-1, DAX-1, and acd: molecular determinants of adrenocortical growth and steroidogenesis. Endocr Res 28:597-607.
25. Hanley NA, Rainey WE, Wilson DI, Ball SG, Parker KL (2001) Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15:57-68.
26. Shibata H, Kobayashi S, Kurihara I, Saito I, Saruta T (2003) Nuclear receptors and co-regulators in adrenal tumors. Horm Res 59 Suppl 1:85-93.
27. Abd-Elaziz M, Moriya T, Akahira J, Nakamura Y, Suzuki T, Sasano H (2005) Immunolocalization of nuclear transcription factors, DAX-1 and Ad4BP/SF-1, in human common epithelial ovarian tumors: correlations with StAR and steroidogenic enzymes in epithelial ovarian carcinoma. Int J Gynecol Pathol 24:153-163.
28. Abd-Elaziz M, Akahira J, Moriya T, Suzuki T, Yaegashi N, Sasano H (2003) Nuclear receptor DAX-1 in human common epithelial ovarian carcinoma: an independent prognostic factor of clinical outcome. Cancer Sci 94:980-985.

29. Ho J, Zhang YH, Huang BL, McCabe ER (2004) NR0B1A: an alternatively spliced form of NR0B1. Mol Genet Metab 83:330-336.
30. Zhang H, Thomsen JS, Johansson L, Gustafsson JA, Treuter E (2000) DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J Biol Chem 275:39855-39859.
31. Conde I, Alfaro JM, Fraile B, Ruíz A, Paniagua R, Arenas MI (2004) DAX-1 expression in human breast cancer: comparison with estrogen receptors ER-alpha, ER-beta and androgen receptor status. Breast Cancer Res 6:R140-148.
32. Holter E, Kotaja N, Mäkela S, Strauss L, Kietz S, Jänne OA, Gustafsson JA, Palvimo J, Treuter E (2002) Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. Mol Endocrinol 16:515-528.
33. Humphrey PA (2004) Gleason grading and prognostic factors in carcinoma of the prostate. Mod Pathol 17:292-306.
34. Epstein JI, Partin AW, Sauvageot J, Walsh PC (1996) Prediction of progression following radical prostatectomy: a multivariate analysis of 721 men with long-term follow-up. Am J Surg Pathol 20:286–292.
35. Albertsen PC, Hanley JA, Gleason DF, Barry MJ (1998) Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. JAMA 280:975–980.
36. Barry MJ, Albertsen PC, Bagshaw MA, Blute ML, Cox R, Middleton RG, Gleason DF, Zincke H, Bergstralh EJ, Jacobsen SJ (2001) Outcomes for men with clinically nonmetastatic prostate carcinoma managed with radical prostatectomy, external beam radiotherapy, or expectant management. Cancer 91:2302–2314.
37. Helguero LA, Hedengran Faulds M, Förster C, Gustafsson JA, Haldosén LA (2006) DAX-1 expression is regulated during mammary epithelial cell differentiation. Endocrinology 147:3249-3259.

doi: 10.1507/endocrj.K08E-177

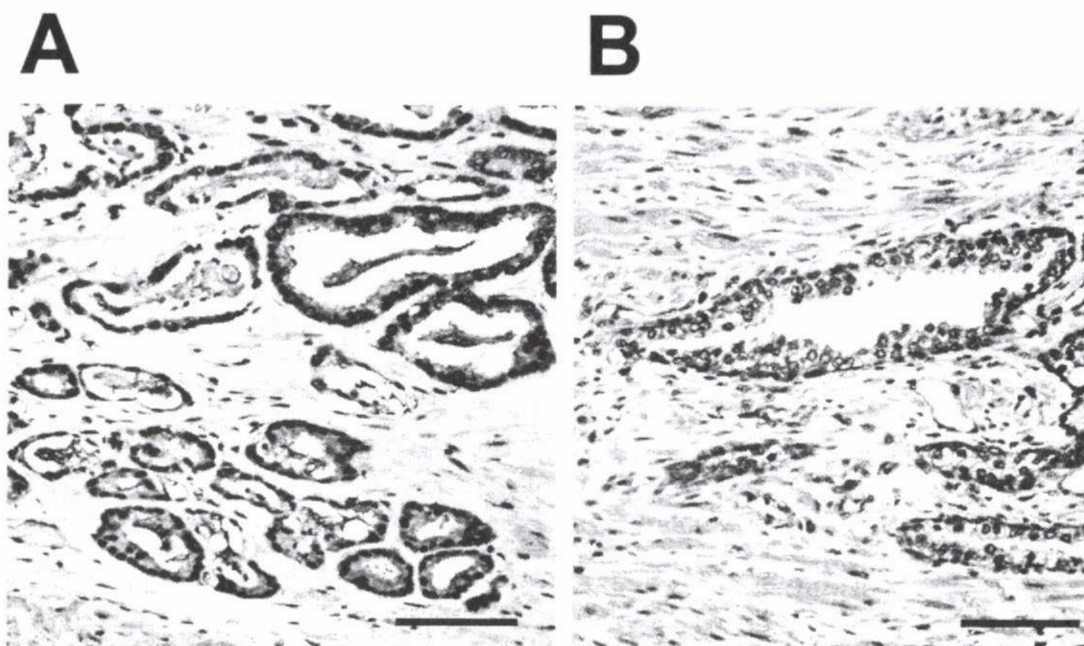

Fig. 1. Immunoreactivity of DAX1 in human prostate cancer tissues. DAX1 immunoreactive protein was detected in the nuclei of prostate carcinoma cells (A), but it was negligible in the epithelial cells of non-neoplastic prostate glands (B). Bar = 10 μm.

doi: 10.1507/endocrj.K08E-177

Table 1. Correlation between DAX1 immunoreactivity and clinicopathological parameters in human prostate cancer tissues (* $P<0.05$)

	Positive (n=21)	Negative (n=19)	*P* value
Age (years)	66.1±1.1	65.7±1.2	0.802
PSA (ng/ML)	12.0±2.3	16.7±3.6	0.270
Gleason score			
2–6	7 (17.5%)	1 (2.5%)	
7	10 (25.0%)	6 (15.0%)	
8–10	4 (10.0%)	12 (30%)	0.009*
Stage			
pT2	11 (27.5%)	7 (17.5%)	
pT3	10 (25.0%)	12 (30.0%)	0.324
Lymph node status			
Positive	2 (5.0%)	0 (0%)	
Negative	19 (47.5%)	19 (47.5%)	0.168
Ki-67 LI (%)	7.6±1.1	7.8±1.5	0.903

Table 2. Correlation between DAX1 immunoreactivity and the expression level of sex steroid receptors in human prostate cancer tissues

	Positive (n=21)	Negative (n=19)	*P* value
AR LI (%)	76.3±5.2	72.0±4.4	0.531
ERβ LI (%)	44.5±6.4	39.9±6.6	0.624
PR LI (%)	10.1±2.6	5.7±2.2	0.214