directly inhibiting the transcription of Wnt target genes or, rather, acts largely through the induction of KCNIP4. The physiological relevance of the modest inhibitor effect of Nurr1 on Wnt-1 signaling remains to be studied, but for cell culture our preliminary microarray data revealed that Wnt-1-regulated genes were, on average, 22% less responsive in 293F cells overexpressing Nurr1 than in parental controls (mean of two replicates with different subclones [n = 135 RNAs]; $P < 0.001$) (unpublished observations). It will be interesting to determine if Nurr1$^+$ neuronal precursors respond differentially to Wnt signaling compared to Nurr1$^-$ cells in vivo.

In addition to the well-studied role for Wnt signaling during CNS development of the VM dopaminergic system, aberrant Wnt signaling has been implicated in several psychiatric and neurological disorders, such as bipolar disorder (19) and schizophrenia (16). Given the importance of the dopaminergic system in these diseases, it is conceivable that Wnt signaling could, in part, affect the activity of Nurr1. Interestingly, both LiCl and valproic acid, drugs useful in a number of mental disorders, inhibit GSK3β, and recent data suggest that certain antipsychotics modulate this system as well (1, 2, 30). We show here that naturally occurring Nurr1 mutants have a reduced ability to inhibit β-catenin transcription from a TCF/LEF element and appear to differentially regulate β-catenin accumulation and KCNIP4 promoter activation. Future studies that determine if the functional interplay between β-catenin and Nurr1 varies with disease state and is modulated by genetic factors will be of interest.

ACKNOWLEDGMENTS

We thank T. Iwatsubo for the kind gift of KCNIP4 antibodies, Ung-il Chung for technical support with handling retrovirus, and J. Yanagisawa for critical reading of the manuscript. We also thank H. Higuchi and Y. Nagasawa for manuscript preparation.

This work was partially supported by the 19th Research Fellowship from the Naito Memorial Foundation (2003) and by a research fellowship from the Uehara Memorial Foundation (2004).

REFERENCES

1. **Alimohamad, H., N. Rajakumar, Y. H. Seah, and W. Rushlow.** 2005. Antipsychotics alter the protein expression levels of beta-catenin and GSK-3 in the rat medial prefrontal cortex and striatum. Biol. Psych. **57:**533–542.
2. **Alimohamad, H., L. Sutton, J. Mouyal, N. Rajakumar, and W. J. Rushlow.** 2005. The effects of antipsychotics on beta-catenin, glycogen synthase kinase-3 and dishevelled in the ventral midbrain of rats. J. Neurochem. **95:**513–525.
3. **Armogida, M., A. Petit, B. Vincent, S. Scarzello, C. A. da Costa, and F. Checler.** 2001. Endogenous beta-amyloid production in presenilin-deficient embryonic mouse fibroblasts. Nat. Cell Biol **3:**1030–1033.
4. **Cadigan, K. M., and Y. I. Liu.** 2006. Wnt signaling: complexity at the surface. J. Cell Sci. **119:**395–402.
5. **Castelo-Branco, G., and E. Arenas.** 2006. Function of Wnts in dopaminergic neuron development. Neurodegener. Dis. **3:**5–11.
6. **Castelo-Branco, G., K. M. Sousa, V. Bryja, L. Pinto, J. Wagner, and E. Arenas.** 2006. Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. Mol. Cell. Neurosci. **31:**251–262.
7. **Castelo-Branco, G., J. Wagner, F. J. Rodriguez, J. Kele, K. Sousa, N. Rawal, H. A. Pasolli, E. Fuchs, J. Kitajewski, and E. Arenas.** 2003. Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc. Natl. Acad. Sci. USA **100:**12747–12752.
8. **Castro, D. S., M. Arvidsson, M. Bondesson Bolin, and T. Perlmann.** 1999. Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2. J. Biol. Chem. **274:**37483–37490.
9. **Chan, H. M., and N. B. La Thangue.** 2001. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J. Cell Sci. **114:**2363–2373.
10. **Chu, Y., K. Kompoliti, E. J. Cochran, E. J. Mufson, and J. H. Kordower.** 2002. Age-related decreases in Nurr1 immunoreactivity in the human substantia nigra. J. Comp. Neurol. **450:**203–214.
11. **Chu, Y., W. Le, K. Kompoliti, J. Jankovic, E. J. Mufson, and J. H. Kordower.** 2006. Nurr1 in Parkinson's disease and related disorders. J. Comp. Neurol. **494:**495–514.
12. **Codina, A., G. Benoit, J. T. Gooch, D. Neuhaus, T. Perlmann, and J. W. Schwabe.** 2004. Identification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting. J. Biol. Chem. **279:**53338–53345.
13. **Danielian, P. S., and A. P. McMahon.** 1996. Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. Nature **383:**332–334.
14. **Daniels, D. L., and W. I. Weis.** 2005. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat. Struct. Mol. Biol. **12:**364–371.
15. **Darragh, J., A. Soloaga, V. A. Beardmore, A. D. Wingate, G. R. Wiggin, M. Peggie, and J. S. Arthur.** 2005. MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling. Biochem. J. **390:**749–759.
16. **Dean, B.** 2002. Understanding the pathology of schizophrenia: recent advances from the study of the molecular architecture of postmortem CNS tissue. Postgrad. Med. J. **78:**142–148.
17. **Forman, B. M., K. Umesono, J. Chen, and R. M. Evans.** 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell **81:**541–550.
18. **Galleguillos, D., A. Vecchiola, J. A. Fuentealba, V. Ojeda, K. Alvarez, A. Gomez, and M. E. Andres.** 2004. PIASgamma represses the transcriptional activation induced by the nuclear receptor Nurr1. J. Biol. Chem. **279:**2005–2011.
19. **Gould, T. D., and H. K. Manji.** 2002. The Wnt signaling pathway in bipolar disorder. Neuroscientist **8:**497–511.
20. **Hermanson, E., B. Joseph, D. Castro, E. Lindqvist, P. Aarnisalo, A. Wallen, G. Benoit, B. Hengerer, L. Olson, and T. Perlmann.** 2003. Nurr1 regulates dopamine synthesis and storage in MN9D dopamine cells. Exp. Cell Res. **288:**324–334.
21. **Holla, V. R., J. R. Mann, Q. Shi, and R. N. DuBois.** 2006. Prostaglandin E2 regulates the nuclear receptor NR4A2 in colorectal cancer. J. Biol. Chem. **281:**2676–2682.
22. **Jiang, C., X. Wan, Y. He, T. Pan, J. Jankovic, and W. Le.** 2005. Age-dependent dopaminergic dysfunction in Nurr1 knockout mice. Exp. Neurol. **191:**154–162.
23. **Joseph, B., A. Wallen-Mackenzie, G. Benoit, T. Murata, E. Joodmardi, S. Okret, and T. Perlmann.** 2003. p57(Kip2) cooperates with Nurr1 in developing dopamine cells. Proc. Natl. Acad. Sci. USA **100:**15619–15624.
24. **Kang, D. E., S. Soriano, X. Xia, C. G. Eberhart, B. De Strooper, H. Zheng, and E. H. Koo.** 2002. Presenilin couples the paired phosphorylation of beta-catenin independent of axin: implications for beta-catenin activation in tumorigenesis. Cell **110:**751–762.
25. **Killick, R., C. C. Pollard, A. A. Asuni, A. K. Mudher, J. C. Richardson, H. T. Rupniak, P. W. Sheppard, I. M. Varndell, J. P. Brion, A. I. Levey, O. A. Levy, M. Vestling, R. Cowburn, S. Lovestone, and B. H. Anderton.** 2001. Presenilin 1 independently regulates beta-catenin stability and transcriptional activity. J. Biol. Chem. **276:**48554–48561.
26. **Kim, K. S., C. H. Kim, D. Y. Hwang, H. Seo, S. Chung, S. J. Hong, J. K. Lim, T. Anderson, and O. Isacson.** 2003. Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. J. Neurochem. **85:**622–634.
27. **Kim, S. Y., K. C. Choi, M. S. Chang, M. H. Kim, Y. S. Na, J. E. Lee, B. K. Jin, B. H. Lee, and J. H. Baik.** 2006. The dopamine D2 receptor regulates the development of dopaminergic neurons via extracellular signal-regulated kinase and Nurr1 activation. J. Neurosci. **26:**4567–4576.
28. **Kitagawa, H., R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, and S. Kato.** 2003. The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. Cell **113:**905–917.
29. **Kouzmenko, A. P., K. Takeyama, S. Ito, T. Furutani, S. Sawatsubashi, A. Maki, E. Suzuki, Y. Kawasaki, T. Akiyama, T. Tabata, and S. Kato.** 2004. Wnt/beta-catenin and estrogen signaling converge in vivo. J. Biol. Chem. **279:**40255–40258.
30. **Kozlovsky, N., S. Amar, R. H. Belmaker, and G. Agam.** 2006. Psychotropic drugs affect Ser9-phosphorylated GSK-3 beta protein levels in rodent frontal cortex. Int. J. Neuropsychopharmacol. **9:**337–342.
31. **Le, W. D., P. Xu, J. Jankovic, H. Jiang, S. H. Appel, R. G. Smith, and D. K. Vassilatis.** 2003. Mutations in NR4A2 associated with familial Parkinson disease. Nat. Genet. **33:**85–89.
32. **Liu, J., H. Wang, Y. Zuo, and S. R. Farmer.** 2006. Functional interaction between peroxisome proliferator-activated receptor gamma and beta-catenin. Mol. Cell. Biol. **26:**5827–5837.
33. **Maira, M., C. Martens, E. Batsche, Y. Gauthier, and J. Drouin.** 2003. Dimer-specific potentiation of NGFI-B (Nur77) transcriptional activity by the protein kinase A pathway and AF-1-dependent coactivator recruitment. Mol. Cell. Biol. **23:**763–776.
34. **Morohashi, Y., N. Hatano, S. Ohya, R. Takikawa, T. Watabiki, N. Takasugi,**

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

Y. Imaizumi, T. Tomita, and T. Iwatsubo. 2002. Molecular cloning and characterization of CALP/KChIP4, a novel EF-hand protein interacting with presenilin 2 and voltage-gated potassium channel subunit Kv4. J. Biol. Chem. **277:**14965–14975.

35. **Mulholland, D. J., S. Dedhar, G. A. Coetzee, and C. C. Nelson.** 2005. Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? Endocr. Rev. **26:**898–915.
36. **Murphy, E. P., and O. M. Conneely.** 1997. Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. Mol. Endocrinol. **11:**39–47.
37. **Nantermet, P. V., J. Xu, Y. Yu, P. Hodor, D. Holder, S. Adamski, M. A. Gentile, D. B. Kimmel, S. Harada, D. Gerhold, L. P. Freedman, and W. J. Ray.** 2004. Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland. J. Biol. Chem. **279:**1310–1322.
38. **Nordzell, M., P. Aarnisalo, G. Benoit, D. S. Castro, and T. Perlmann.** 2004. Defining an N-terminal activation domain of the orphan nuclear receptor Nurr1. Biochem. Biophys. Res. Commun. **313:**205–211.
39. **Palacino, J. J., M. P. Murphy, O. Murayama, K. Iwasaki, M. Fujiwara, A. Takashima, T. E. Golde, and B. Wolozin.** 2001. Presenilin 1 regulates beta-catenin-mediated transcription in a glycogen synthase kinase-3-independent fashion. J. Biol. Chem. **276:**38563–38569.
40. **Perissi, V., and M. G. Rosenfeld.** 2005. Controlling nuclear receptors: the circular logic of cofactor cycles. Nat. Rev. Mol. Cell. Biol. **6:**542–554.
41. **Porfiri, E., B. Rubinfeld, I. Albert, K. Hovanes, M. Waterman, and P. Polakis.** 1997. Induction of a beta-catenin-LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. Oncogene **15:**2833–2839.
42. **Powzaniuk, M., S. McElwee-Witmer, R. L. Vogel, T. Hayami, S. J. Rutledge, F. Chen, S. Harada, A. Schmidt, G. A. Rodan, L. P. Freedman, and C. Bai.** 2004. The LATS2/KPM tumor suppressor is a negative regulator of the androgen receptor. Mol. Endocrinol. **18:**2011–2023.
43. **Sacchetti, P., R. Carpentier, P. Segard, C. Olive-Cren, and P. Lefebvre.** 2006. Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1. Nucleic Acids Res. **34:**5515–5527.
44. **Sacchetti, P., T. R. Mitchell, J. G. Granneman, and M. J. Bannon.** 2001. Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. J. Neurochem. **76:**1565–1572.
45. **Sakurada, K., M. Ohshima-Sakurada, T. D. Palmer, and F. H. Gage.** 1999. Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development **126:**4017–4026.
46. **Salthun-Lassalle, B., E. C. Hirsch, J. Wolfart, M. Ruberg, and P. P. Michel.** 2004. Rescue of mesencephalic dopaminergic neurons in culture by low-level stimulation of voltage-gated sodium channels. J. Neurosci. **24:**5922–5930.
47. **Satoh, J., and Y. Kuroda.** 2002. The constitutive and inducible expression of Nurr1, a key regulator of dopaminergic neuronal differentiation, in human neural and non-neural cell lines. Neuropathology **22:**219–232.
48. **Saucedo-Cardenas, O., J. D. Quintana-Hau, W. D. Le, M. P. Smidt, J. J. Cox, F. De Mayo, J. P. Burbach, and O. M. Conneely.** 1998. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc. Natl. Acad. Sci. USA **95:**4013–4018.
49. **Sierra, J., T. Yoshida, C. A. Joazeiro, and K. A. Jones.** 2006. The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev. **20:**586–600.
50. **Singh, R., J. N. Artaza, W. E. Taylor, M. Braga, X. Yuan, N. F. Gonzalez-Cadavid, and S. Bhasin.** 2006. Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with beta-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. Endocrinology **147:**141–154.
51. **Soriano, S., D. E. Kang, M. Fu, R. Pestell, N. Chevallier, H. Zheng, and E. H. Koo.** 2001. Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer factor-1 signaling independently of beta-amyloid precursor protein and notch processing. J. Cell Biol. **152:**785–794.
52. **Takayama, S., I. Rogatsky, L. E. Schwarcz, and B. D. Darimont.** 2006. The glucocorticoid receptor represses cyclin D1 by targeting the Tcf-beta-catenin complex. J. Biol. Chem. **281:**17856–17863.
53. **Volakakis, N., M. Malewicz, B. Kadkhodai, T. Perlmann, and G. Benoit.** 2006. Characterization of the Nurr1 ligand-binding domain co-activator interaction surface. J. Mol. Endocrinol. **37:**317–326.
54. **Volpicelli, F., C. Perrone-Capano, P. Da Pozzo, L. Colucci-D'Amato, and U. di Porzio.** 2004. Modulation of nurr1 gene expression in mesencephalic dopaminergic neurones. J. Neurochem. **88:**1283–1294.
55. **Wang, Z., G. Benoit, J. Liu, S. Prasad, P. Aarnisalo, X. Liu, H. Xu, N. P. Walker, and T. Perlmann.** 2003. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature **423:**555–560.
56. **Wansa, K. D., J. M. Harris, and G. E. Muscat.** 2002. The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment. J. Biol. Chem. **277:**33001–33011.
57. **Xia, X., S. Qian, S. Soriano, Y. Wu, A. M. Fletcher, X. J. Wang, E. H. Koo, X. Wu, and H. Zheng.** 2001. Loss of presenilin 1 is associated with enhanced beta-catenin signaling and skin tumorigenesis. Proc. Natl. Acad. Sci. USA **98:**10863–10868.
58. **Yanagisawa, J., H. Kitagawa, M. Yanagida, O. Wada, S. Ogawa, M. Nakagomi, H. Oishi, Y. Yamamoto, H. Nagasawa, S. B. McMahon, M. D. Cole, L. Tora, N. Takahashi, and S. Kato.** 2002. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol. Cell **9:**553–562.
59. **Zetterstrom, R. H., L. Solomin, L. Jansson, B. J. Hoffer, L. Olson, and T. Perlmann.** 1997. Dopamine neuron agenesis in Nurr1-deficient mice. Science **276:**248–250.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

Molecular and Cellular Biology, July 2007, p. 4807–4814 Vol. 27, No. 13
0270-7306/07/$08.00+0 doi:10.1128/MCB.02039-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

The Pituitary Function of Androgen Receptor Constitutes a Glucocorticoid Production Circuit[▽]

Junko Miyamoto,[1]† Takahiro Matsumoto,[1,2]† Hiroko Shiina,[1] Kazuki Inoue,[1] Ichiro Takada,[1] Saya Ito,[1] Johbu Itoh,[3] Takeo Minematsu,[4] Takashi Sato,[1] Toshihiko Yanase,[5] Hajime Nawata,[5] Yoshiyuki R. Osamura,[4] and Shigeaki Kato[1,2]*

Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan[1]; ERATO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchisi, Saitama 332-0012, Japan[2]; Teaching and Research Support Center[3] and Department of Pathology,[4] Tokai University School of Medicine, Boseidai, Isehara, Kanagawa 259-1193, Japan; and Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan[5]

Received 1 November 2006/Returned for modification 18 December 2006/Accepted 16 April 2007

Androgen receptor (AR) mediates diverse androgen actions, particularly reproductive processes in males and females. AR-mediated androgen signaling is considered to also control metabolic processes; however, the molecular basis remains elusive. In the present study, we explored the molecular mechanism of late-onset obesity in male AR null mutant (ARKO) mice. We determined that the obesity was caused by a hypercorticoid state. The negative feedback system regulating glucocorticoid production was impaired in ARKO mice. Male and female ARKO mice exhibited hypertrophic adrenal glands and glucocorticoid overproduction, presumably due to high levels of adrenal corticotropic hormone. The pituitary glands of the ARKO males had increased expression of proopiomelanocortin and decreased expression of the glucocorticoid receptor (GR). There were no overt structural abnormalities and no alteration in the distribution of cell types in the pituitaries of male ARKO mice. Additionally, there was normal production of the other hormones within the glucocorticoid feedback system in both the pituitary and hypothalamus. In a cell line derived from pituitary glands, GR expression was under the positive control of the activated AR. Thus, this study suggests that the activated AR supports the negative feedback regulation of glucocorticoid production via up-regulation of GR expression in the pituitary gland.

Sex steroid hormones exert a wide variety of biological actions. They are also involved in pathological events, such as the development of hormone-dependent cancers in reproductive organs (5, 37). In vertebrates, sex hormones play a pivotal role in male reproductive function and metabolic control. Most sex steroid actions are mediated through transcriptional control of target genes by nuclear receptors (NRs). NRs form a gene superfamily and act as transcriptional factors (9, 20). Sex hormone receptors have been shown to transactivate particular sets of target genes in a hormone-dependent manner through direct DNA binding to specific elements in target gene promoters. Hormone receptors activated by hormone binding recruit a number of coregulator-coregulator complexes for transactivation (28). These complexes then affect transcription through chromatin remodeling (12, 17, 22) and histone modification (1, 7). Hormone binding to the receptors may also transrepress target genes. The mechanisms of hormone-dependent transrepression of steroid receptors likely involve protein-protein interactions and are thus more diverse than that of transactivation (8, 10, 13, 21).

The molecular mechanisms behind the regulation of gene transcription by hormones and their NRs are complicated. Gene disruption studies have clarified the role of various NRs in steroid hormone action. By combining a Cre-loxP system with a canonical gene disruption approach, we succeeded in disrupting the androgen receptor (AR) on the X chromosome in mice in a manner that did not result in male infertility (14). Male AR null mutant (ARKO) mice exhibit abnormalities typical of testicular feminization mutants, including female external genitalia with atrophic testis and impaired sex behavior (29). Growth of the male ARKO mice is partially retarded, with impaired bone growth coupled with high bone turnover (16). The male mice also develop late-onset obesity (30). In contrast, no clear phenotypic abnormalities are present in female ARKO mice. However, normal folliculogenesis does require the AR, which suggests that androgen/AR signaling is also physiologically important in females (32).

To study how and why obesity develops in ARKO males, we began by examining the adrenal glands, which were hypertrophic in both males and females. In the present study, we explored the molecular basis of this observation. Dissection of the gland revealed that the layers of the zona fasciculata were thicker and coupled to the remaining layers of the X-zone (fetal zone). The hypertrophy resulted from a hypercorticoid state. Adrenal corticotropic hormone (ACTH) overproduction was driven by impaired negative feedback through the hypothalamus-pituitary-adrenal (HPA) axis. No clear alteration in the numbers of hormone-producing cells in the pituitary glands and hypothalamus was detected, but there were increased proopiomelanocortin (POMC) and decreased glucocorticoid

* Corresponding author. Mailing address: Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Phone: 81-8-5841-8478. Fax: 81-3-5841-8477. E-mail: uskato@mail.ecc.u-tokyo.ac.jp.
† J.M. and T.M. contributed equally to this work.
▽ Published ahead of print on 30 April 2007.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

receptor (GR) expression levels of transcripts in the ARKO pituitary glands. Androgen-induced GR gene activation was further confirmed in a pituitary gland-derived cell line (AtT-20 cells). These findings suggest that androgen/AR signaling in the pituitary gland supports the normal feedback system of glucocorticoid production through the HPA axis.

MATERIALS AND METHODS

Animals. ARKO mice were generated by targeted disruption of the AR gene by means of a Cre-loxP system (19) and maintained as described previously (16, 29, 30, 32). Experiments were performed with 2- to 25-week-old male mice. All mice protocols were approved by the Animal Care and Use Committee of the University of Tokyo (31, 40).

Cell culture. Adherent AtT-20 cells, a murine corticotropic tumor cell line, were cultured in a 5% CO_2 atmosphere at 37°C with Dulbecco's modified Eagle's medium-Ham's F12 at 1:1 containing 15% fetal calf serum (FCS) and penicillin-streptomycin. 3T3-L1 cells, a murine preadipocyte cell line, were cultured with Dulbecco's modified Eagle's medium containing 10% FCS. FCS in the culture media was replaced with charcoal-treated FCS for 1 week prior to the administration of 5α-dihydrotestosterone (DHT). For Northern and Western blot analyses, the cultured AtT-20 cells were subcultured in six-well plates. After incubation for 24 h, DHT (10^{-7} M) was added to the medium.

Histology and immunohistochemistry. Adrenal glands and pituitary glands were fixed by immersion with 4% paraformaldehyde for 24 h at 4°C. They were then embedded in paraffin, sliced into 4-μm sections by standard methods, and mounted onto silane-coated slides. After perfusion by 0.9% saline followed by 4% paraformaldehyde, brains were postfixed in the same fixative for 2 h at 4°C and soaked in phosphate-buffered saline containing 20% sucrose. Frontal sections were cut at 30-μm thickness using a cryostat. Serial sections were divided into four groups and used for single-labeling immunohistochemistry for the GR, corticotropin-releasing hormone (CRH), α-melanocyte-stimulating hormone (α-MSH), or thionin to allow determination of the areas to be measured.

Immunostaining was carried out using antibodies as described below (34). The primary antibodies included rabbit polyclonal anti-human AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-GR (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human ACTH (DAKO, Carpinteria, CA), mouse anti-human luteinizing hormone β (Immunotech, Marseille, France), mouse anti-human follicle-stimulating hormone β (DAKO, Carpinteria, CA), mouse anti-human thyroid-stimulating hormone β (Advanced Immunochemical Inc., CA), rabbit anti-rat glycoprotein hormone (kindly supplied by A. F. Parlow, the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, MD), rabbit anti-human growth hormone (DAKO, Carpinteria, CA), and rabbit anti-rat prolactin (kindly supplied by A. F. Parlow, NIDDK).

After treatment with 0.5% H_2O_2 (30 min) and 5% normal serum (1 h), the sections were incubated for 24 h at 4°C with specific primary antibodies. The sections were then incubated with secondary antibodies and an avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories). The signals were visualized with diaminobenzidine and the nuclei were counterstained with hematoxylin.

For dual labeling of ACTH and the AR or GR, a single staining of the AR or GR was first performed as described above. After the primary antibodies were removed by treatment with 0.1 M glycine, sections were incubated with anti-ACTH antibodies followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G (DAKO, Carpinteria, CA). The signals were visualized with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

Detection of proliferation and apoptosis of adrenal gland cells. Eight-week-old mice were injected intraperitoneally (i.p.) with the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU) (30 mg/g body weight [BW]) every 12 h five times (25). Mice were fully anesthetized and their adrenal glands removed 12 h after the last injection. Incorporated BrdU was detected immunohistochemically using a mouse monoclonal anti-BrdU antibody. The proliferative index was defined as the number of BrdU-positive cells per microscopic field. Five fields per mouse were counted for each of three wild-type (WT) and three ARKO mice.

Cells undergoing apoptosis were identified by digoxigenin labeling of the free 3′OH ends of fragmented DNA by use of terminal deoxynucleotidyltransferase (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] assay). Assays were performed on sections from the same tissue blocks used for BrdU immunohistochemistry. Sections were counterstained with hematoxylin to facilitate cell counting. The fraction of apoptotic cells was defined as the fraction of diaminobenzidine-positive cells per total number of cells. Five fields per mouse were counted for each of three WT and three ARKO mice.

Serum endocrine parameters. A circadian rhythm experiment and dexamethasone suppression tests were performed on 8-week-old male mice as previously described (2). For the circadian rhythm experiment, blood was collected at 08:00 or 18:00 h. For the dexamethasone suppression tests, mice were injected i.p. with different doses of dexamethasone (0, 2, or 5 μg/20 g BW) in 0.3 ml of 0.9% saline. Injections were performed between 08:00 and 08:30 h and blood was collected 6 h later. Mice were fully anesthetized and blood was collected by cardiac puncture. Plasma ACTH and serum corticosterone were measured using radioimmunoassay kits (IRMA; Mitsubishi, Tokyo, Japan) at SRL (Tokyo, Japan), according to the manufacturers' instructions. Measurements were independently duplicated, and interassay variability and buffer dilution were corrected for by using internal correction factors.

RNA extraction and mRNA quantitation. Total mRNA was extracted from pituitary glands with TRIzol (Invitrogen) for reverse transcription-PCR (RT-PCR) and Northern blotting (35). To remove any possible DNA contamination prior to semiquantitative RT-PCR, the DNA was digested with RNase-free DNase. The digested total mRNA (2 μg) was subjected to RT using SuperScript reverse transcriptase (Invitrogen) primed by oligo(dT) primers. After first-strand cDNA synthesis, 1 ml from a 5% reaction mixture was diluted serially (2- to 128-fold). Amplification was performed with *rTaq*DNA polymerase (Takara) using primer pairs for GAPDH as an internal control to allow for concentration estimation (38). Expression levels of transcripts were measured using the standardized cDNA and specific primer pairs. The validity of the PCR products was confirmed by direct sequencing.

Western blot analysis. The lysates of mouse tissue and AtT-20 cells were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (15, 39). Membranes were probed with rabbit polyclonal anti-GR antibody (M-20; Santa Cruz Biotechnology) and goat polyclonal anti-β-actin antibody (I-19; Santa Cruz Biotechnology) as an internal control. The blots were visualized using peroxidase-conjugated anti-rabbit antibody and anti-goat antibody, together with an ECL detection kit (Amersham Biosciences). The small interfering RNA analysis used AR and control small interfering RNA (Ambion), and transfection was accomplished with the Lipofectamine 2000 system (Invitrogen).

Luciferase reporter assay. GR promoter regions (upstream regions of exons 1B, 1C, and 2) were cloned by PCR and subcloned into a luciferase reporter gene driven by a tk promoter (tk-luc). PCR primers were as follows: for 1B Fw, 5′-GGCATAGTTAGGCCACTAAAGAGA-3′; for 1B Rv, 5′-GGGAGAAGT TGCAAAGCAGA-3′; for 1C Fw, 5′-CTGGAGCAGCAAATGTCAAG-3′; for 1C Rv, 5′-AGCTCGCAAAATGGAGGAG-3′; for 2 Fw, 5′-GGATCTGGCGT CCTTTTC-3′; and for 2 Rv, 5′-CCACATTATCTCTGATCCGATT-3′.

For the luciferase reporter assay, cultured cells were transfected with the indicated plasmids using the Lipofectamine Plus reagent (Invitrogen) into 24-well plates at 40 to 50% confluence. The total amount of DNA was adjusted by supplementing with empty vector up to 1.0 μg/well. Luciferase activity was determined using a dual luciferase assay system (Promega). As a reference plasmid to normalize the transfection efficiency, 1.5 ng/well of pRL-CMV plasmid (Promega) was cotransfected in all experiments.

Statistical analysis. Values are given as the means ± standard deviations. Comparisons between two groups were made by Student's *t* test. *P* values of <0.05 were accepted as statistically significant.

RESULTS

High serum levels of ACTH and corticosterone in male ARKO mice. The male ARKO ($AR^{L-/Y}$) mice exhibited growth retardation in comparison to WT male mice until 10 weeks of age but then showed catch-up growth over the next few weeks. Thereafter, the male ARKO mice weighed more than the WT mice and developed severe obesity (Fig. 1A) as previously reported (6, 30). Obesity to this extent was not seen for female ARKO ($AR^{L-/L-}$) mice. To identify causes for the late-onset obesity in male ARKO mice, serum endocrine parameters were measured. We found that serum corticosterone levels in male ARKO mice were elevated at 8 weeks of age and became significantly higher at 13 and 20 weeks ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 1B).

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

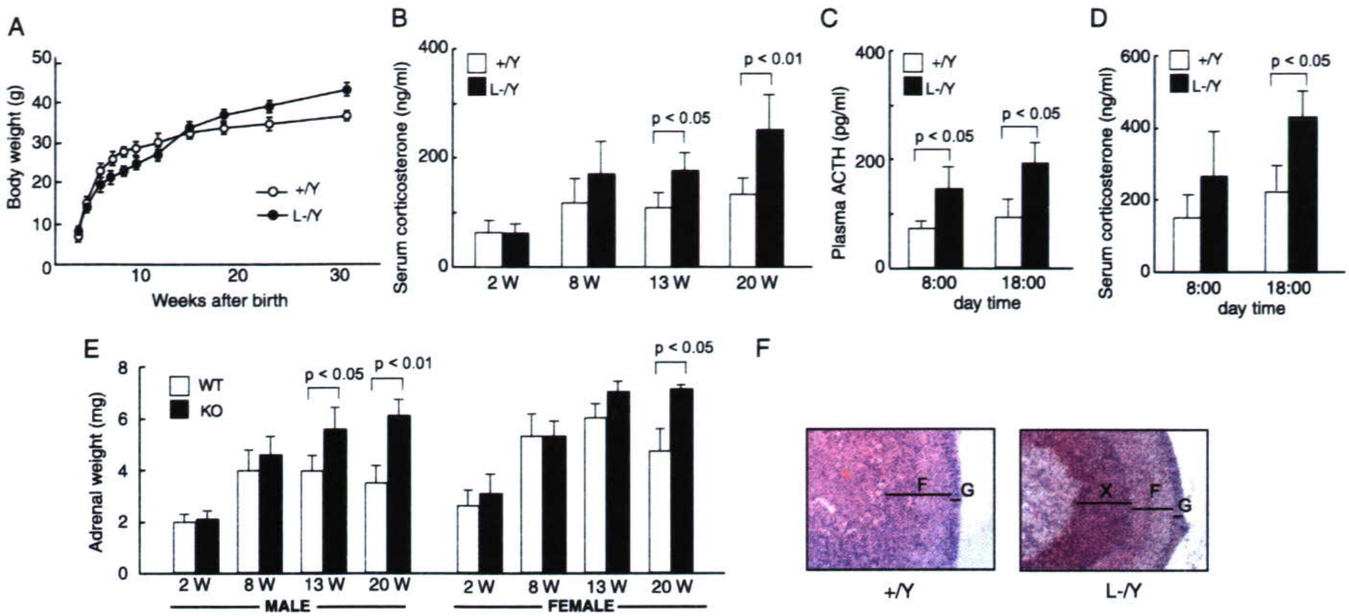

FIG. 1. Hypertrophic adrenal glands with high serum levels of ACTH and corticosterone in ARKO mice. (A) Growth curves of ARKO and WT littermate mice. The floxed AR mice (female, $AR^{L+/L+}$; male, $AR^{L+/Y}$) were crossed with Cre-CMV transgenic mice to generate ARKO male ($AR^{L-/Y}$) and female ($AR^{L-/L-}$) mice (16, 30). (B) Serum corticosterone levels of ARKO and WT mice at 2, 8, 13, and 20 weeks (W) of age. (C) Plasma ACTH levels of ARKO and WT mice measured in the morning (8:00) and evening (18:00). (D) Serum corticosterone levels of ARKO and WT mice in the morning (8:00) and evening (18:00). (E) Adrenal gland weights of male and female ARKO and WT mice at 2, 8, 13, and 20 weeks of age. (F) Histology of ARKO and WT adrenal glands. All sections were stained with hematoxylin and eosin. F, zona fasciculata; G, zona glomerulosa; X, X-zone.

To more carefully examine the hypercorticoid state in male ARKO mice, we measured the serum levels of corticosterone and its upstream hormone ACTH. Measurements were taken both in the morning and in the evening, as these hormones exhibit a circadian rhythm. As expected, the 8-week-old WT and ARKO mice had low levels of both hormones in the morning and higher levels in the evening (Fig. 1C and D). Overall, the ARKO males tended to have high levels of ACTH and corticosterone at any time compared to WT mice. They had significantly high levels of plasma ACTH at both 8:00 and 18:00 and high levels of corticosterone at 18:00 compared to WT mice ($P < 0.05$) (Fig. 1C and D). However, for female ARKO mice, though serum levels of these hormones tended to be higher than in WT littermates, the differences were not statistically significant (data not shown). The obesity seen for these mice was likely the result of their hypercorticoid state, as centripetal obesity is a typical symptom of Cushing's syndrome. In the following experiments, we explored the etiology of the hypercorticoid state in the ARKO mice.

Hypertrophic adrenal glands in ARKO mice. To investigate the hypercorticoid state in the ARKO mice, we first examined the adrenal glands. The adrenal glands in the ARKO males clearly weighed more than the glands of WT mice at 13 weeks of age (Fig. 1E). This coincided with the onset of obesity and the hypercorticoid state. Likewise, in ARKO females, the adrenal glands also increased in size in comparison to what was seen for WT littermate females; however, the growth was not as pronounced as that in ARKO males (Fig. 1E). The adrenal glands of male ARKO mice were then used for subsequent experiments.

The adrenal cortex forms the major part of the gland and is divided into three layers in mammals: the zona glomerulosa, immediately beneath the capsule, followed by the zona fasciculata and the zona reticularis. The zona reticularis is replaced in rodents by the X-zone, which develops prenatally and begins to degenerate at pubertal maturity in males. In mice, corticosterone, the major glucocorticoid in rodents, is produced in the zona fasciculata, while aldosterone, the most potent mineralocorticoid, is formed in the zona glomerulosa. Hematoxylin and eosin staining of adrenal glands in 13-week-old mice revealed that the enlargement of the adrenal glands in ARKO males was caused by cellular hypertrophy of the zona fasciculata as well as by a failure of X-zone (fetal zone) regression (Fig. 1F). Since glucocorticoids are produced in the zona fasciculata, it is likely that the overproduction of corticosterone is the result of the hypertrophy in this area.

Increased proliferation and decreased apoptosis in the adrenal cortex of ARKO males. The failed regression of the X-zone in 13-week-old ARKO males raised the possibility of impaired cell death or decreased apoptosis in the adrenal cortex. Indeed, the percentage of apoptotic cells in the zona fasciculata, detected by TUNEL assay in the ARKO males, was clearly less (19.5%) than that for WT mice (33.1%) (Fig. 2A). When actively proliferating cells of the adrenal glands were counted by BrdU labeling in WT and ARKO males, 2.5 times more BrdU-labeled cells/section were found in ARKO mice. This suggests increased proliferation in the adrenal cortex of ARKO mice (Fig. 2B). Thus, the hypercorticoid state likely results from the overproduction of glucocorticoid by the hypertrophic zona fasciculata. This hypertrophy is caused by chronic exposure to high levels of ACTH.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

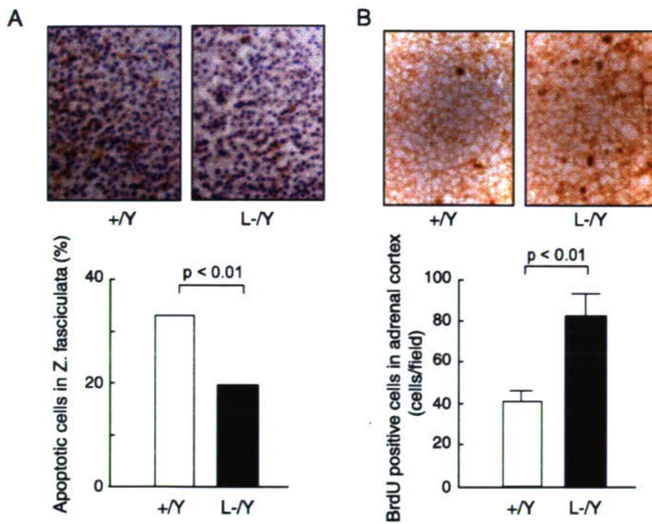

FIG. 2. Increased proliferation and decreased apoptosis in ARKO adrenal glands. (A) Decreased apoptosis in the ARKO adrenal glands. Histogram showing the number of TUNEL-positive cells in the zona fasciculata (Z. fasciculata). (B) Increased proliferation in the ARKO adrenal cortex. Histogram showing the number of BrdU-positive cells.

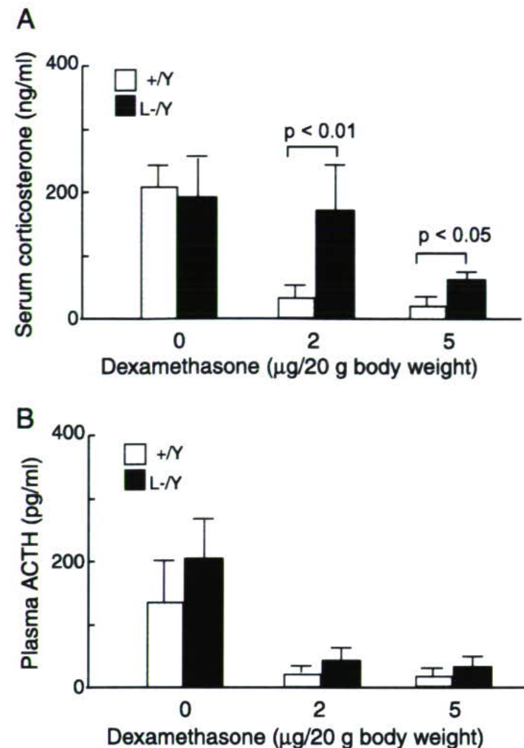

FIG. 3. Impairment of the HPA negative feedback system of glucocorticoid production in ARKO mice. (A) Serum corticosterone levels of ARKO and WT mice in the dexamethasone suppression test. Trunk blood was collected from ARKO and WT mice 6 hours after injection with increasing doses of dexamethasone. (B) Plasma ACTH levels of ARKO and WT mice in the dexamethasone suppression test.

The HPA negative feedback system for glucocorticoid production is impaired in ARKO males. Glucocorticoid synthesis is regulated by a negative feedback loop via the HPA axis involving CRH and ACTH, produced by the hypothalamus and pituitary glands, respectively. We assessed whether the axis was intact and functioning normally in the ARKO males with a dexamethasone suppression test. As expected, serum corticosterone levels were down-regulated in 8-week-old WT mice 6 hours after i.p. injection of either 2 µg/20 g BW or 5 µg/20 g BW of dexamethasone (Fig. 3A). In ARKO mice, injection with 2 µg/20 g BW of dexamethasone did not suppress the serum levels of corticosterone. However, a dose of 5 µg/20 g BW was effective in lowering the serum levels of corticosterone in ARKO mice (Fig. 3A). This is similar to the high-dose dexamethasone suppression seen for patients with central Cushing's syndrome. Plasma ACTH levels in both the ARKO and WT males were decreased 6 h after dexamethasone injection at both the low and high doses; however, suppression was less sensitive in the ARKO males than in the WT males (Fig. 3B). No statistical difference in ACTH levels was detected between ARKO and WT males in this suppression test.

No overt abnormalities were present in the hypothalami or pituitary glands of ARKO males. The results of the suppression tests suggested that the adrenal hypertrophy of the ARKO males resulted from the hyperfunction of the hypothalami and/or pituitary glands. To address this issue, the hypothalami and pituitary glands of 8-week-old ARKO males were histologically examined. No overt abnormalities were detected in sections of the ARKO mice stained with hematoxylin and eosin (Fig. 4A). Immunohistochemical staining of the pituitary glands demonstrated similar numbers of cells expressing pituitary hormones in WT and ARKO mice (Fig. 4B). AR protein expression was detectable in several types of hormone-producing cells in the WT males but was absent in the ARKO mice (data not shown).

Increased POMC expression and decreased GR expression in the pituitary glands is caused by AR deficiency. To address if hormone production was intact following AR inactivation, we examined the mRNA expression levels of pituitary hormones by RT-PCR. As shown in Fig. 5A, the expression of luteinizing hormone β, follicle-stimulating hormone β, and thyroid-stimulating hormone β, as well as that of the orphan NRs (Nur77 and Nurr1), appeared unaltered by AR deficiency. The POMC mRNA levels, however, were clearly up-regulated in males (Fig. 5A) but not in females (Fig. 5B). The up-regulation of POMC mRNA was confirmed by Northern blot analysis (Fig. 5C). This finding is consistent with the high ACTH levels observed for the ARKO mice. In contrast, pituitary GR expression was decreased at both the mRNA and protein levels (Fig. 5A and D). Decreased GR gene expression in ARKO males was also seen for the spleen but not for the other tested tissues (Fig. 5E), suggesting tissue-specific regulation of GR expression by the AR. Interestingly, a clear decrease in the GR mRNA levels was not detected in the total brain RNA of ARKO males (Fig. 5E). Additionally, there was no alteration in the numbers of GR and CRH immunoreactive cells in the hypothalamic paraventricular nucleus in the male ARKO brain (Fig. 4D). These results suggest that the androgen/AR signaling system affects the negative feedback regulation of glucocorticoid production via pituitary GR expression. This view is further supported by the observation that colocalization of ACTH with the AR and/or GR in the pituitary

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

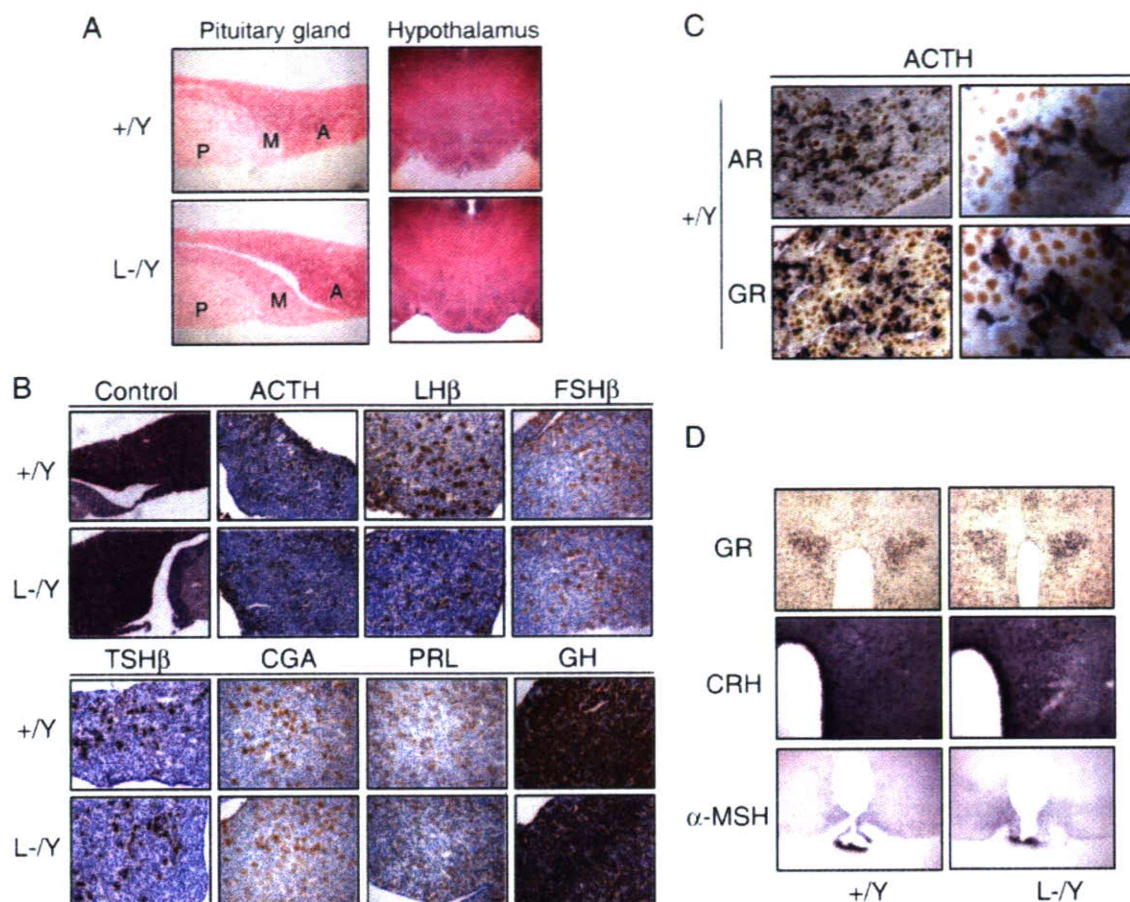

FIG. 4. Histological appearance of the hypothalamus and pituitary gland in ARKO mice. (A) No clear alteration in morphology of the hypothalami or pituitary glands of ARKO mice. Sections of pituitary glands and hypothalami were stained with hematoxylin and eosin. A, anterior lobe; M, intermediate lobe; P, posterior lobe. (B) No overt abnormality in the distribution of cells expressing pituitary hormones in ARKO mice by immunohistochemical staining. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β; CGA, glycoprotein hormone; PRL, prolactin; GH, growth hormone. (C) Pituitary ACTH (black/gray) colocalized with AR (brown) or GR (brown) (left) and its higher magnification (right) in WT mice as detected by immunostaining with specific antibodies. (D) No clear alterations in the GR and CRH (in the paraventricular nucleus) and α-MSH (in the arcuate nucleus) immunoreactive neurons in the hypothalami of ARKO mice.

glands of WT mice was detected by double immunostaining with specific antibodies (Fig. 4C).

Induction of the GR gene by DHT in a pituitary cell line. Finally, to confirm that GR expression levels in the pituitary glands of ARKO mice were reduced, we tested whether DHT induced GR gene expression in cultured cells. DHT treatment of the pituitary cell line AtT-20 for 4 h induced expression of the GR gene (Fig. 6A) and protein (Fig. 6B). Unexpectedly, the expression levels of POMC mRNA (Fig. 6A) and protein (Fig. 6B) were reduced. The AR effect was confirmed following treatment with an AR antagonist (Flutamide) (Fig. 6A and B) and RNA interference (Fig. 6C). Reflecting tissue-specific regulation of GR expression by the AR in intact animals, no response to DHT in the expression levels of either GR or POMC was seen in 3T3-L1 preadipocytes (Fig. 6A to C, lower panels). No consensus androgen response elements or closely related sequences are present up to −3 kb in the GR promoter. However, the intron between exons 1A and 1B of the GR gene (33) was found to counter androgen responsiveness in a transient expression assay in AtT-20 cells (Fig. 6D). This suggests that this element is responsible for androgen-induced GR expression in the pituitary.

DISCUSSION

Hypertrophic and hyperplastic adrenal glands are associated with a hypercorticoid state in male mice deficient for the AR. A hypercorticoid state was observed in sexually mature male mice deficient for the AR. It likely resulted from glucocorticoid overproduction by the hypertrophic and hyperplastic zona fasciculata of the adrenal gland. The ARKO mice demonstrated hyperplasia of the X-zone, which normally regresses by the time sexual maturity is attained (11). TUNEL assays and BrdU labeling confirmed that the hypertrophy and hyperplasia of adrenal glands resulted from decreased apoptosis and increased cell proliferation. Chronic ACTH stimulation causes zona fasciculata cell hypertrophy and hyperplasia, and ACTH is a potent inhibitor of apoptosis in the adrenal cortex (36). Thus, the findings for the ARKO males are consistent with exposure to high levels of ACTH.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

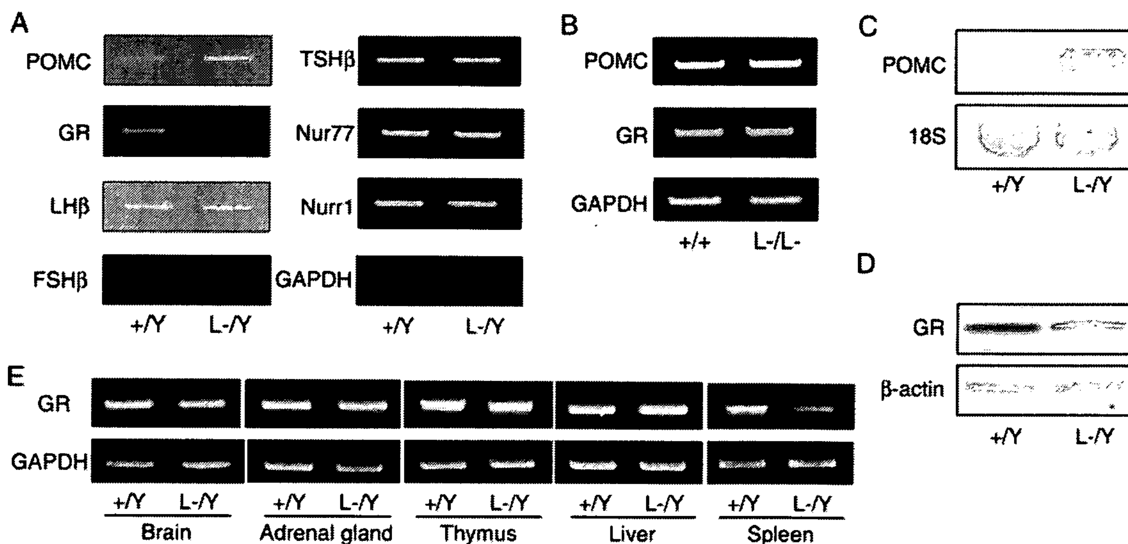

FIG. 5. Altered expression levels of gene transcripts involved in the HPA axis. (A) Increased POMC and decreased GR expression levels of transcripts in ARKO pituitary by semiquantitative RT-PCR. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β. (B) No significant alterations of POMC and GR mRNA levels in the pituitary glands of female ARKO ($AR^{L-/L-}$) mice. (C and D) Northern blot analyses showing clear up-regulation of POMC mRNA levels and down-regulation of GR mRNA levels in the ARKO pituitary. (E) Tissue-specific reduction of GR transcripts in ARKO mice. GR expression levels are down-regulated only in the spleen and pituitary in male ARKO mice.

The hypertrophic and hyperplastic adrenal glands in the ARKO mice probably resulted from high levels of serum ACTH, derived from high POMC transcript levels in the pituitaries of ARKO mice. Studies with transgenic mice expressing antisense RNA against the GR in the brain and anterior pituitary demonstrate that the GR mediates the negative feedback regulation of glucocorticoid production through HPA axis activity (26, 36). Consistent with this observation, the male ARKO mice had low pituitary GR mRNA levels but no difference in the distribution of pituitary hormone-producing cells compared to WT animals. Thus, our findings suggest that the activated AR in the pituitary gland is needed to express pituitary GR at a sufficiently high level to participate in the negative feedback regulation of glucocorticoid production. The X-zone, which is considered a fetal zone, regresses during sexual maturation and reappears after gonadectomy (11). The molecular basis underlying X-zone regression during sexual maturation remains to be investigated. However, our results raise the possibility that the activated AR in adrenal glands induces X-zone repression by the induction of apoptosis. Consequently, the identification of AR target genes expressed in the X-zone is another interesting direction to pursue.

Liganded AR augments GR gene expression in the pituitary gland. We found that GR gene expression was impaired in the pituitary glands of ARKO males. We presumed that the reduced GR levels led to increased expression of the POMC gene, with subsequent high levels of serum ACTH. This idea was supported by the observation that the suppression of ACTH production by exogenous glucocorticoids was partially impaired in the ARKO mice. Moreover, the DHT-activated AR enhanced the GR mRNA levels in a pituitary cell line but not in 3T3-L1 preadipocytes. The effect of DHT was most likely mediated by a response element in an upstream region of the GR promoter exon 1B (33). Thus, the activated AR directly induces the pituitary GR in a cell-specific manner. How this is accomplished on a molecular level remains to be elucidated.

Do androgen/AR signaling disorders link with an ACTH-dependent hypercorticoid state? A hypercorticoid state in humans is well known to cause Cushing's syndrome, in which patients suffer from a number of disorders such as centripetal obesity, facial rounding, glucose intolerance, hyperinsulinemia, and impaired lipid and bone metabolism (23). Most of these lesions are a reflection of glucocorticoid-driven gluconeogenesis. The hypercorticoid state may result from either endogenous disorders or chronic treatment with exogenous glucocorticoid. Endogenous causes of Cushing's syndrome are further classified as ACTH dependent or independent (18). The ACTH-dependent syndrome is characterized by up-regulated levels of ACTH; however, the molecular basis underlying the ACTH overproduction remains to be investigated. It is possible that sex steroids are involved, but this has not yet been fully addressed.

The male ARKO mice exhibited abnormalities similar to those seen for ACTH-dependent Cushing's syndrome patients. Since we detected up-regulation of the pituitary POMC transcript, other POMC-derived peptides might have contributed to the onset of obesity in male ARKO mice. For example, α-MSH in the neurons of the hypothalamus plays a central role in appetite control and energy homeostasis (3, 4). Although we detected no clear alteration in α-MSH immunoreactivity in the arcuate nuclei of the hypothalami of male ARKO mice, it will be of interest in future experiments to examine the melanocortin receptor system in ARKO brain. In contrast to the male ARKO mice, ARKO females did not display some of the abnormalities, such as obesity. It is possible that the lack of obesity in female ARKO mice may result from activation of estrogen receptors (ERs). ERs activated by high physiological

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

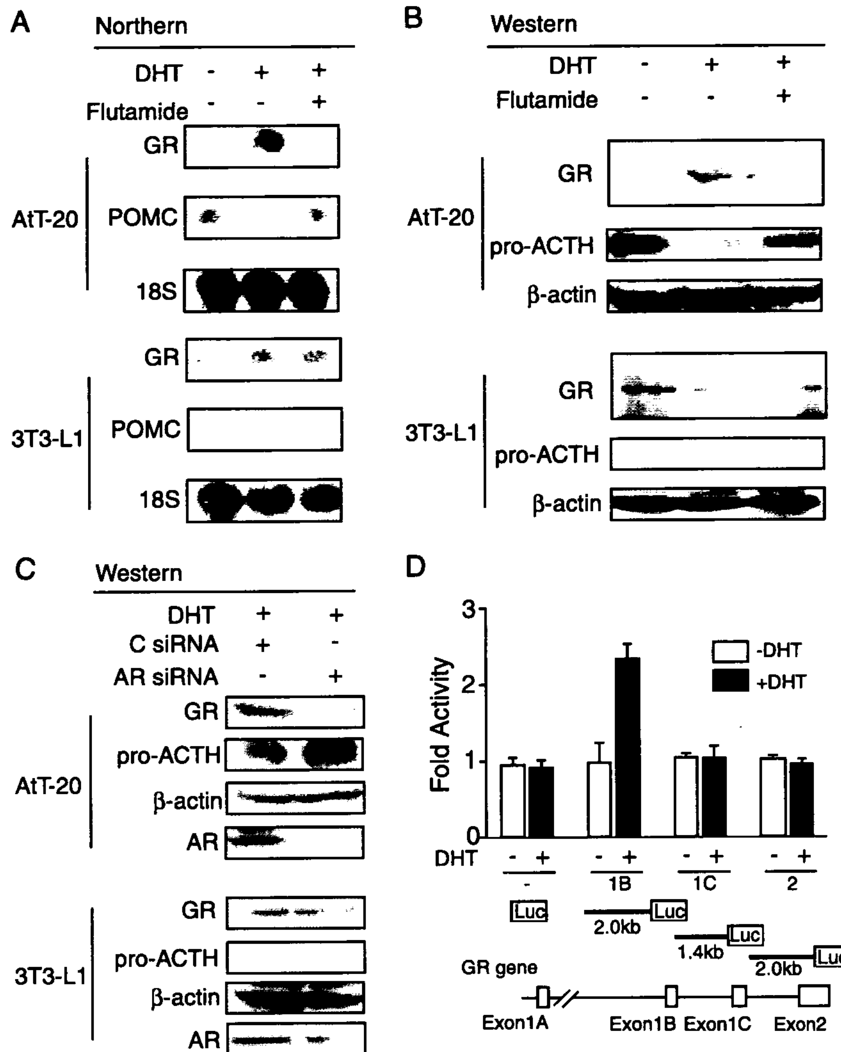

FIG. 6. Cell-type-specific regulation of the GR by activated AR. (A) Regulation of GR and POMC gene expression by treatment with either DHT or an AR antagonist (Flutamide) in the cultured cells as analyzed by Northern blot analysis. (B) Expression of the GR and pro-ACTH proteins was analyzed by Western blot analysis. (C) The significance of AR in the GR gene regulation was tested by AR RNA interference (with small interfering RNA [siRNA]) in the cultured cells. C siRNA, control siRNA. (D) Luciferase assay was performed with a series of the GR promoter regions in AtT-20 cells. After transfection with each of the promoter tk-luciferase vectors, the transfected cells were incubated with or without 10^{-7} M DHT.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

levels of endogenous estrogens are effective in maintaining the proper levels of pituitary GR mRNAs needed to control POMC gene expression. This idea is indeed supported by the finding of unaltered levels of GR and POMC transcripts in the pituitary glands of the female ARKO mice. Moreover, estrogen treatment in female rats is shown to suppress serum levels of ACTH (27, 41). The common but gender-specific putative functions of the AR and ER in the brain have already been described in the context of mouse sexual behavior (24, 29). Though the possible ER functions remain to be studied for female ERKO mice, the present study suggests that the activated AR potentiates the negative HPA feedback regulation of glucocorticoid production through up-regulation of GR expression levels. Our study implies that the AR may be a potential therapeutic target for ACTH-dependent Cushing's syndrome.

In conclusion, the present study suggests that the androgen/AR signaling system is a negative pathway for glucocorticoid secretion in adult male mice. ARKO mice showed decreased GR expression in the pituitary glands and increased circulating ACTH and glucocorticoid. Androgens may increase the sensitivity of the HPA negative feedback loop to glucocorticoids by increasing GR expression in the pituitary gland, leading to suppression of adrenal cortical function. Thus, we presume that activated AR in the pituitary gland is a component of the negative feedback system for glucocorticoid production.

ACKNOWLEDGMENTS

We thank members of the KO project team for experimental support, A. F. Parlow (NIDDK, National Hormone and Peptide Program, Torrance, CA) for kindly providing antisera, and H. Higuchi 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 S.K.).

REFERENCES

1. **Bannister, A. J., and T. Kouzarides.** 2005. Reversing histone methylation. Nature **436**:1103–1106.
2. **Barden, N., I. S. Stec, A. Montkowski, F. Holsboer, and J. M. Reul.** 1997. Endocrine profile and neuroendocrine challenge tests in transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuroendocrinology **66**:212–220.
3. **Coll, A. P., I. S. Farooqi, B. G. Challis, G. S. Yeo, and S. O'Rahilly.** 2004. Proopiomelanocortin and energy balance: insights from human and murine genetics. J. Clin. Endocrinol. Metab. **89**:2557–2562.
4. **Cone, R. D.** 2005. Anatomy and regulation of the central melanocortin system. Nat. Neurosci. **8**:571–578.
5. **Couse, J. F., and K. S. Korach.** 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. **20**:358–417.
6. **Fan, W., T. Yanase, M. Nomura, T. Okabe, K. Goto, T. Sato, H. Kawano, S. Kato, and H. Nawata.** 2005. Androgen receptor null male mice develop late-onset obesity caused by decreased energy expenditure and lipolytic activity but show normal insulin sensitivity with high adiponectin secretion. Diabetes **54**:1000–1008.
7. **Fischle, W., Y. Wang, and C. D. Allis.** 2003. Histone and chromatin cross-talk. Curr. Opin. Cell Biol. **15**:172–183.
8. **Fujiki, R., M. S. Kim, Y. Sasaki, K. Yoshimura, H. Kitagawa, and S. Kato.** 2005. Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. EMBO J. **24**:3881–3894.
9. **Glass, C. K., and M. G. Rosenfeld.** 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. **14**:121–141.
10. **Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387**:43–48.
11. **Howard, J. M., J. M. Olney, J. P. Frawley, R. E. Peterson, and S. Guerra.** 1955. Adrenal function in the combat casualty. AMA Arch. Surg. **71**:47–58.
12. **Ito, T., M. Bulger, M. J. Pazin, R. Kobayashi, and J. T. Kadonaga.** 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell **90**:145–155.
13. **Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell **85**:403–414.
14. **Kato, S.** 2002. Androgen receptor structure and function from knock-out mouse. Clin. Pediatr. Endocrinol. **11**:1–7.
15. **Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, et al.** 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science **270**:1491–1494.
16. **Kawano, H., T. Sato, T. Yamada, T. Matsumoto, K. Sekine, T. Watanabe, T. Nakamura, T. Fukuda, K. Yoshimura, T. Yoshizawa, K. Aihara, Y. Yamamoto, Y. Nakamichi, D. Metzger, P. Chambon, K. Nakamura, H. Kawaguchi, and S. Kato.** 2003. Suppressive function of androgen receptor in bone resorption. Proc. Natl. Acad. Sci. USA **100**:9416–9421.
17. **Kitagawa, H., R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, and S. Kato.** 2003. The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. Cell **113**:905–917.
18. **Lacroix, A., N. Ndiaye, J. Tremblay, and P. Hamet.** 2001. Ectopic and abnormal hormone receptors in adrenal Cushing's syndrome. Endocr. Rev. **22**:75–110.
19. **Li, M., A. K. Indra, X. Warot, J. Brocard, N. Messaddeq, S. Kato, D. Metzger, and P. Chambon.** 2000. Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis. Nature **407**:633–636.
20. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, et al.** 1995. The nuclear receptor superfamily: the second decade. Cell **83**:835–839.
21. **Murayama, A., M. S. Kim, J. Yanagisawa, K. Takeyama, and S. Kato.** 2004. Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. EMBO J. **23**:1598–1608.
22. **Narlikar, G. J., H. Y. Fan, and R. E. Kingston.** 2002. Cooperation between complexes that regulate chromatin structure and transcription. Cell **108**:475–487.
23. **Newell-Price, J., X. Bertagna, A. B. Grossman, and L. K. Nieman.** 2006. Cushing's syndrome. Lancet **367**:1605–1617.
24. **Ogawa, S., A. E. Chester, S. C. Hewitt, V. R. Walker, J. A. Gustafsson, O. Smithies, K. S. Korach, and D. W. Pfaff.** 2000. Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). Proc. Natl. Acad. Sci. USA **97**:14737–14741.
25. **Ohtake, F., K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama, and S. Kato.** 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature **423**:545–550.
26. **Pepin, M. C., F. Pothier, and N. Barden.** 1992. Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. Nature **355**:725–728.
27. **Redei, E., L. Li, I. Halasz, R. F. McGivern, and F. Aird.** 1994. Fast glucocorticoid feedback inhibition of ACTH secretion in the ovariectomized rat: effect of chronic estrogen and progesterone. Neuroendocrinology **60**:113–123.
28. **Rosenfeld, M. G., V. V. Lunyak, and C. K. Glass.** 2006. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev. **20**:1405–1428.
29. **Sato, T., T. Matsumoto, H. Kawano, T. Watanabe, Y. Uematsu, K. Sekine, T. Fukuda, K. Aihara, A. Krust, T. Yamada, Y. Nakamichi, Y. Yamamoto, T. Nakamura, K. Yoshimura, T. Yoshizawa, D. Metzger, P. Chambon, and S. Kato.** 2004. Brain masculinization requires androgen receptor function. Proc. Natl. Acad. Sci. USA **101**:1673–1678.
30. **Sato, T., T. Matsumoto, T. Yamada, T. Watanabe, H. Kawano, and S. Kato.** 2003. Late onset of obesity in male androgen receptor-deficient (AR KO) mice. Biochem. Biophys. Res. Commun. **300**:167–171.
31. **Sekine, K., H. Ohuchi, M. Fujiwara, M. Yamasaki, T. Yoshizawa, T. Sato, N. Yagishita, D. Matsui, Y. Koga, N. Itoh, and S. Kato.** 1999. Fgf10 is essential for limb and lung formation. Nat. Genet. **21**:138–141.
32. **Shiina, H., T. Matsumoto, T. Sato, K. Igarashi, J. Miyamoto, S. Takemasa, M. Sakari, I. Takada, T. Nakamura, D. Metzger, P. Chambon, J. Kanno, H. Yoshikawa, and S. Kato.** 2006. Premature ovarian failure in androgen receptor-deficient mice. Proc. Natl. Acad. Sci. USA **103**:224–229.
33. **Strahle, U., A. Schmidt, G. Kelsey, A. F. Stewart, T. J. Cole, W. Schmid, and G. Schutz.** 1992. At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc. Natl. Acad. Sci. USA **89**:6731–6735.
34. **Suzawa, M., I. Takada, J. Yanagisawa, F. Ohtake, S. Ogawa, T. Yamauchi, T. Kadowaki, Y. Takeuchi, H. Shibuya, Y. Gotoh, K. Matsumoto, and S. Kato.** 2003. Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat. Cell Biol. **5**:224–230.
35. **Takeyama, K., S. Kitanaka, T. Sato, M. Kobori, J. Yanagisawa, and S. Kato.** 1997. 25-Hydroxyvitamin D3 1alpha-hydroxylase and vitamin D synthesis. Science **277**:1827–1830.
36. **Thomas, M., M. Keramidas, E. Monchaux, and J. J. Feige.** 2004. Dual hormonal regulation of endocrine tissue mass and vasculature by adrenocorticotropin in the adrenal cortex. Endocrinology **145**:4320–4329.
37. **Wilson, J. D.** 1999. The role of androgens in male gender role behavior. Endocr. Rev. **20**:726–737.
38. **Yanagisawa, J., H. Kitagawa, M. Yanagida, O. Wada, S. Ogawa, M. Nakagomi, H. Oishi, Y. Yamamoto, H. Nagasawa, S. B. McMahon, M. D. Cole, L. Tora, N. Takahashi, and S. Kato.** 2002. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol. Cell **9**:553–562.
39. **Yanagisawa, J., Y. Yanagi, Y. Masuhiro, M. Suzawa, M. Watanabe, K. Kashiwagi, T. Toriyabe, M. Kawabata, K. Miyazono, and S. Kato.** 1999. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. Science **283**:1317–1321.
40. **Yoshizawa, T., Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto, and S. Kato.** 1997. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat. Genet. **16**:391–396.
41. **Young, E. A., M. Altemus, V. Parkison, and S. Shastry.** 2001. Effects of estrogen antagonists and agonists on the ACTH response to restraint stress in female rats. Neuropsychopharmacology **25**:881–891.

Downloaded from mcb.asm.org at University of Tokyo on January 20, 2008

ELSEVIER

Molecular and Cellular Endocrinology 265–266 (2007) 168–173

MCE
Molecular and Cellular Endocrinology
www.elsevier.com/locate/mce

Review

1α,25$(OH)_2D_3$-induced DNA methylation suppresses the human CYP27B1 gene

Mi-Sun Kim[a], Ryoji Fujiki[a], Hirochika Kitagawa[a], Shigeaki Kato[a,b,*]

[a] *The Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[b] *ERATO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan*

Abstract

CYP27B1 is a critical enzyme of Vitamin D biosynthesis that hydroxylates 25$(OH)D_3$ at the final step of the biosynthetic pathway. The CYP27B1 gene is expressed primarily in kidney and negatively controlled by Vitamin D receptor. We have characterized the negative vitamin D response element and its binding protein, a bHLH transcription factor. This factor directly binds to the 1αnVDRE and activates transcription, but its transcriptional activity is suppressed by the ligand-activated Vitamin D receptor through recruitment of histone deacetylase. We have shown that histone deacetylation is a critical step for chromatin structure remodeling in suppression of the CYP27B1 gene. We have further demonstrated that, in addition to histone acetylation, this transrepression by VDR requires DNA methylation in the CYP27B1 gene promoter. Thus, transcriptional regulation of the CYP27B1 gene appears to be mediated by dual epigenetic modifications.
© 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: CYP27B1; Transrepression; VDIR; VDR; WSTF

Contents

1. Introduction ... 168
2. Vitamin D negatively regulates expression of the 25$(OH)D_3$ 1α-hydroxylase gene ... 169
3. WSTF potentiates the ligand-induced transrepression by VDR of the human CYP27B1 gene promoter ... 169
4. WSTF promotes recruitment of unliganded VDR to the CYP27B1 gene promoter ... 169
5. DNA methylation followed histone deacetylation in the VDR-meditaed transrepression of the CYP27B1 gene ... 171
6. Discussion ... 171
   6.1. Transrepression of the CYP27B1 by liganded VDR requires two epigenetic modifications ... 171
   6.2. WINAC supports ligand-induced transrepression by VDR on the human CYP27B1 gene promoter region ... 171
   Acknowledgements ... 172
   References ... 172

1. Introduction

Vitamin D_3 1α-hydroxylase (CYP27B1) is a critical enzyme of Vitamin D biosynthesis that hydroxylates 25$(OH)D_3$ at the final step of the biosynthetic pathway (Kato et al., 1998; Takeyama et al., 1997). Lipophilic ligands, such as Vitamin D, as well as thyroid/steroid hormones, are thought to exert their physiological effects through transcriptional control of target genes via cognate nuclear receptors (NRs) (Mangelsdorf et al., 1995). The Vitamin D receptor (VDR, NR1I1) is a member of this NR superfamily that is activated by its ligand Vitamin D_3. A number of co-regulator complexes that support ligand-dependent transcriptional control have been identified, and these complexes can be classified into three categories according to the function (Glass and Rosenfeld, 2000). Members of the first co-regulator complex class regulate transcription directly, through a physical interaction with general transcription factors and RNA polymerase II (Gu et al., 1999; Rachez et al., 1998). Members of the second co-regulator complex class covalently modify his-

* Corresponding author at: The Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Tel.: +81 3 5841 8478; fax: +81 3 5841 8477.
E-mail address: uskato@mail.ecc.u-tokyo.ac.jp (S. Kato).

0303-7207/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2006.12.014

tone tails, for example by acetylation, in promoter nucleosomal arrays (Heinzel et al., 1997; Kamei et al., 1996; Onate et al., 1995; Yanagisawa et al., 2002). The major function of the third class of complexes is chromatin remodeling, which involves the ATP-dependent dynamic remodeling of chromatin structure (Ito et al., 1997; Kitagawa et al., 2003; Narlikar et al., 2002). Chromatin remodeling complexes utilize energy from ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. As chromosomal DNA is generally packed as nucleosomal arrays, chromatin-remodeling complexes are thought to render specific promoter regions accessible to other co-regulator complex classes and sequence-specific regulators.

Recently, we identified a novel multifunctional ATP-dependent chromatin remodeling complex, designated WINAC, which consists of 13 subunits (Kitagawa et al., 2003). It contains SWI/SNF chromatin remodeling complex components and DNA replication-related factors. VDR interacts with WINAC in a ligand-independent manner through the Williams syndrome transcription factor (WSTF). WSTF contains a bromodomain that is adjacent to a zinc-finger motif common for the (BAZ) protein family. Members of this family harbor both a PHD finger and bromodomain in their C-terminal domain (Jones et al., 2000). As bromodomains have been recently shown to bind acetylated histones, it is possible that WSTF serves as an adaptor protein for acetylated histones (Hassan et al., 2002; Margueron et al., 2005; Martin and Zhang, 2005), facilitating the association between WINAC and chromatin.

2. Vitamin D negatively regulates expression of the 25(OH)D_3 1α-hydroxylase gene

Expression of CYP27B1 is negatively regulated by Vitamin D[1α,25$(OH)_2D_3$] (Murayama et al., 2004; Murayama et al., 1998; Takeyama et al., 1997). We recently reported that a bHLH-type activator, VDR-interacting repressor (VDIR), directly binds to the negative Vitamin D response element (1αnVDRE) in the human CYP27B1 gene promoter, thus activating its transcription (Fig. 1) (Murayama et al., 2004). However, ligand-induced association between VDR and VDIR results in ligand-induced repression (transrepression) of the CYP27B1 gene expression. This transrepression is associated with VDIR switching from a co-activator complex containing histone acetyltransferase (HAT) to a co-repressor complex containing histone deacetylase (HDAC) (Murayama et al., 2004).

3. WSTF potentiates the ligand-induced transrepression by VDR of the human CYP27B1 gene promoter

We have previously shown that WINAC supports ligand-induced transactivation through chromatin remodeling (Kitagawa et al., 2003). However, it remained unclear if ligand-induced transrepression by VDR requires WINAC. To address this question, the role of WSTF in ligand-induced VDR/VDIR transrepression was studied in MCF7 cells, which express the endogenous CYP27B1 gene. Transrepression at the CYP27B1 promoter was monitored in transfection assays using a luciferase reporter plasmid under the control of a human CYP27B1 gene promoter segment (~700 bp upstream region of the transcription start site) containing two consensus 1αnVDRE sequences recognized by VDIR. Using gene-specific RNAi, we found that endogenous VDR, VDIR and WSTF are involved in the ligand-induced suppression of the CYP27B1 gene promoter (Fig. 2A) that is consistent with previously shown physical association of these three factors (Kitagawa et al., 2003; Murayama et al., 2004).

4. WSTF promotes recruitment of unliganded VDR to the CYP27B1 gene promoter

To test whether WSTF was recruited to VDIR via liganded VDR in the nuclei of living cells, we performed a ChIP assay on the endogenous CYP27B1 gene promoter. In agreement with

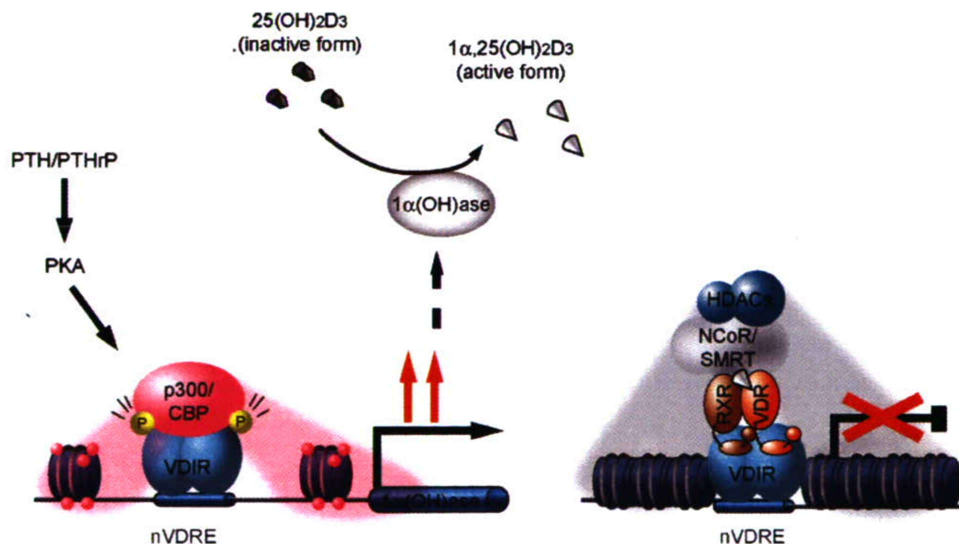

Fig. 1. Schematic view of ligand-induced transrepression by VDR in the human CYP27B1 gene promoter. A bHLH-type activator, VDIR, binds to the 1αnVDRE, and activates transcription. PKA, signaling downstream of PTH/PTHrP, phosphorylates VDIR leading to the association of VDIR with HAT co-activator p300/CBP. Upon binding 1α,25$(OH)_2D_3$, the VDR associates with VDIR promoting dissociation of the HAT co-activator and recruitment of HDAC co-repressor.

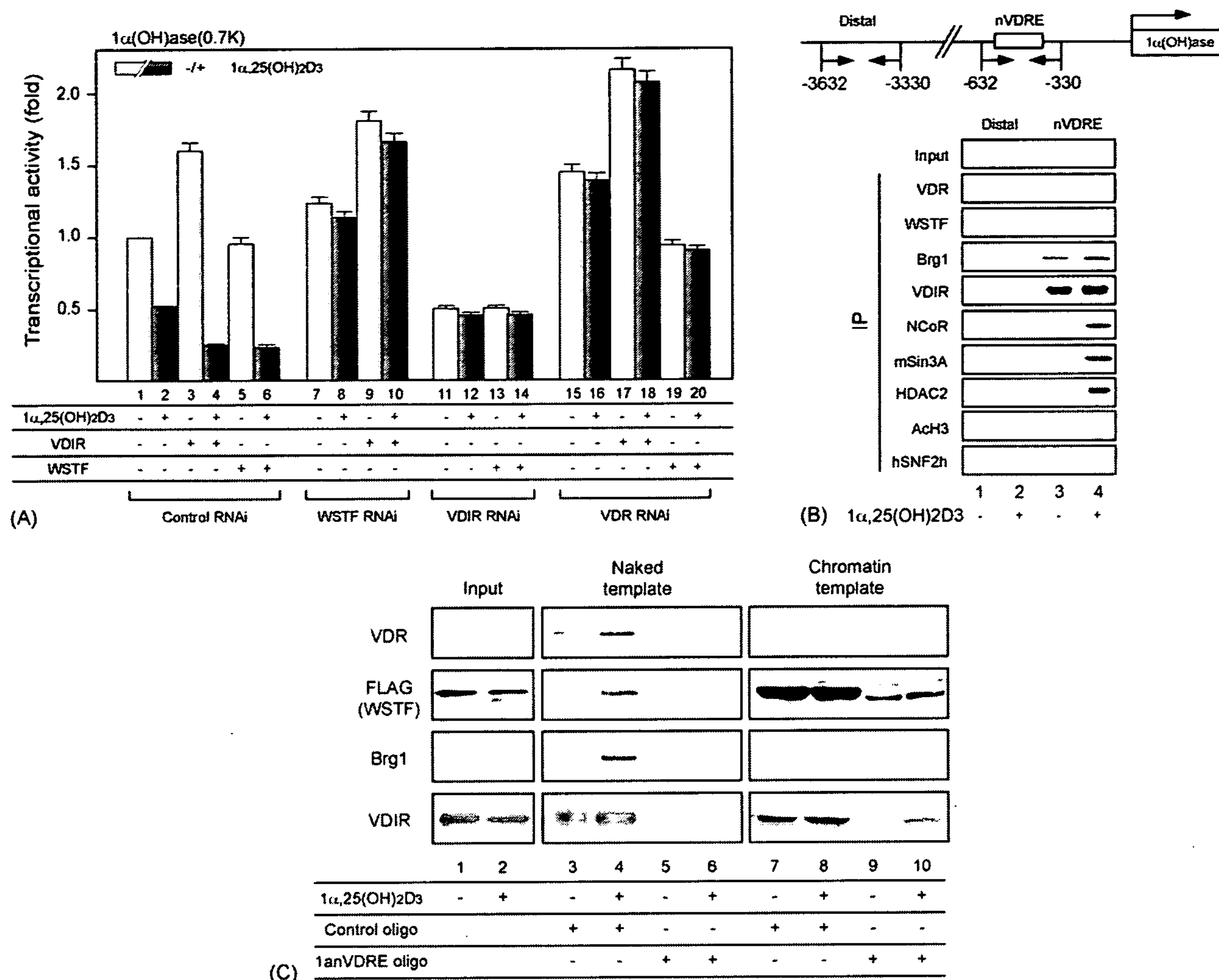

Fig. 2. WSTF enhances VDIR-mediated transrepression of CYP27B1 gene expression through chromatin remodeling. (A) The effect of gene-specific knockdown of endogenous WSTF, VDIR or VDR on CYP27B1 promoter expression. Gene-specific knockdown by factor-targeted RNAi was confirmed by Western blots using the respective antibodies (data not shown). MCF7 cells were transfected with 0.3 μg of the indicated siRNAs; 48 h later, a luciferase reporter gene containing a CYP27B1 promoter segment harboring the nVDRE was transfected into the cells. Luciferase activity was assessed after 12 h culture in the presence or absence of 1α,25(OH)$_2$D$_3$ (10^{-8} M). (B) Recruitment of VDR, WSTF, VDIR, and other co-regulators to the endogenous CYP27B1 gene promoter. For ChIP analysis, soluble chromatin was prepared from MCF7 cells treated with 1α,25(OH)$_2$D$_3$ (10^{-8} M) for 45 min and immunoprecipitated with the indicated antibodies. Extracted DNA samples were amplified using primer pairs that covered the CYP27B1 negative VDRE region (1αnVDRE) (Kato et al., 2003; Murayama et al., 2004). As a control, DNA samples were amplified with primer pairs covering a region 3 kb upstream from 1αnVDRE. (C) Stabilization of the ligand-free VDR/WSTF complex on the CYP27B1 promoter required chromatin-structured DNA *in vitro*. Whole cell extracts from MCF7 cells stably expressing FLAG-WSTF treated with or without 1α,25(OH)$_2$D$_3$ (10^{-8} M) were mixed with immobilized templates. The template beads were then concentrated using a magnet and analyzed by Western blotting using the indicated antibodies. The data of panels A–C are basically similar to those in our previous report (Fujiki et al., 2005).

previous reports (Kitagawa et al., 2003; Murayama et al., 2004), VDIR was constitutively bound to the 1αnVDRE (Fig. 2B). As WSTF RNAi remarkably attenuated the promoter occupancy by VDR in the absence of ligand, WSTF appeared to facilitate the binding of unliganded VDR at the 1αnVDRE (Fig. 2B).

To examine the mechanism by which WSTF targets unliganded VDR to the promoter *in vitro*, we determined that factors are indispensable for the promoter targeting of unliganded VDR by employing an immobilized DNA/chromatin template recruitment assay. DNA fragments containing either 1αnVDRE (−60 to −615) or the CYP27B1 distal region (−3632 to −3032) were end-biotinylated to allow their immobilization onto streptavidin beads. Whole cell extracts from MCF-7 cells that stably expressed FLAG-tagged WSTF and treated with or without 1α,25(OH)$_2$D$_3$ were incubated with either naked or chromatin fragments of the promoter DNA. Proteins bound to the promoter DNA were then analyzed by immunoblotting (Fig. 2C). WSTF and VDR bound to naked DNA templates only in the presence of ligand, while VDIR stably associated with naked DNA templates even in the absence of ligand (Fig. 2C). In contrast, for the chromatin templates with HeLa histone octamers, recruitment of WSTF and VDR was ligand-independent (Fig. 2C), indicating a role for DNA-bound VDIR in the stable association of VDR/WSTF with chromatin. Thus, considering all of these results, we conclude that WINAC facilitates VDR-mediated transrepression of the CYP27B1 gene through a physical inter-

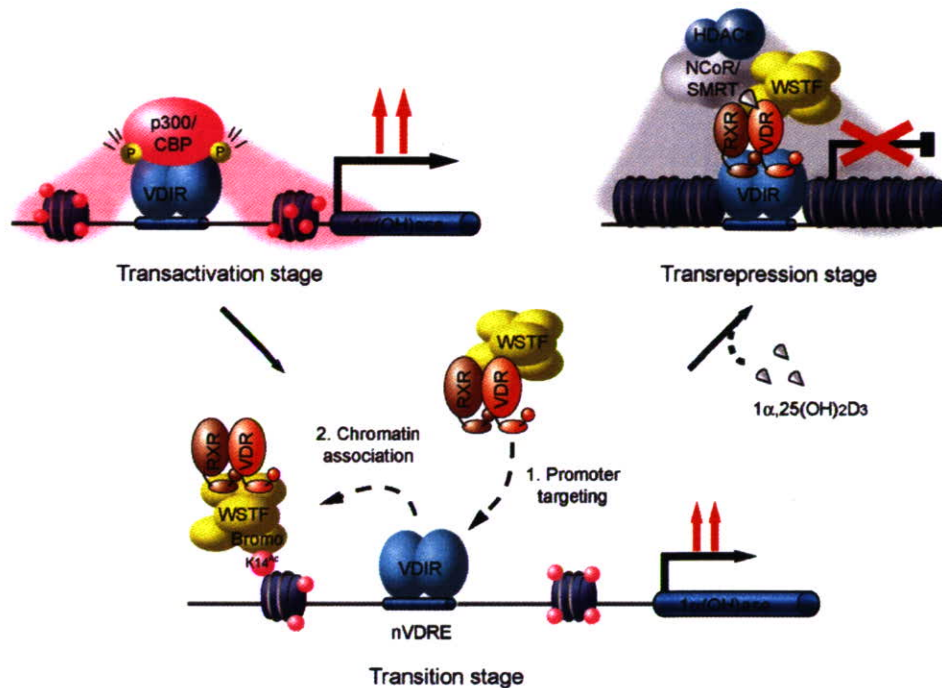

Fig. 3. Model demonstrating the role of WINAC in the ligand-induced transrepression function of VDR at the CYP27B1 gene promoter. p300 is recruited to VDIR, which was phosphorylated via PKA signaling, and acetylates the nucleosomes at the VDRI in the CYP27B1 gene promoter (Transactivation stage). WINAC, along with VDR, sequentially targets VDIR through interaction between unliganded VDR and VDIR, and is retained on the acetylated promoter via the WSTF bromodomain (Transition stage). Upon $1\alpha,25(OH)_2D_3$ binding, HDAC co-repressor complexes are recruited to the ligand-bound VDR/VDIR complex and deacetylate the nucleosomes. WINAC then exerts its ATP-dependent chromatin remodeling activity (Transrepression stage) (see also the figure in Fujiki et al., 2005).

action between the WSTF bromodomain and an acetylated nucleosomal array (Fujiki et al., 2005) (Fig. 3).

5. DNA methylation followed histone deacetylation in the VDR-meditaed transrepression of the CYP27B1 gene

Histone deacetylation by recruited HDACs to VDR/VDIR at the 1αnVDRE is a critical step for remodeling chromatin structure at the CYP27B1 gene promoter in VDR-mediated transrepression (Murayama et al., 2004). However, TSA (a HDAC inhibitor) could not fully abrogate $1\alpha,25\ (OH)_2D_3$-induced transrepression (Fig. 4A), suggesting that an additional mechanism may contribute to this transcriptional repression. To explore this unknown mechanism, we purified a $1\alpha,25(OH)_2D_3$-dependent VDIR-VDR co-repressor complex. By biochemical purification and consequent mass spectrometric sequence analyses, we identified a DNA methyltrasferase as a part of this co-repressor complex. The DNA methyltrasferase interacted with both VDR and HDAC2 in a ligand-dependent manner (Fig. 4B). Indeed, we found that treatment with $1\alpha,25(OH)_2D_3$ induced DNA methylation at CpG sites in the promoter and exon regions of the CYP27B1 gene (data not shown). All together, these findings suggest that ligand-induced DNA methylation in the promoter may contribute, at least in part, to continuous transcriptional repression by VDR, and provide a novel mechanism of a ligand-dependent transrepression by NR that links repressive histone modification with epigenetic repression through DNA methylation (Kim et al., unpublished results).

6. Discussion

6.1. Transrepression of the CYP27B1 by liganded VDR requires two epigenetic modifications

We have shown that the CYP27B1 gene nVDRE is composed of two E-box like motifs (Murayama et al., 2004). This E-box like motif(s), serving as a VDIR binding site, also appears to confer a negative response to $1\alpha,25(OH)_2D_3$ at other VDR target gene promoters (Kim et al., in press). Thus, it appears that this E-box type motif acts as a nVDRE, and that DNA-bound VDIR is directly targeted by the VDR. In the present study, we have provided evidence that, in addition to hitsone deacetylation by VDR/VDIR-recruited HDAC, transrepression by VDR/VDIR also requires DNA methylation. It is conceivable that both these steps of epigenetic modifications may required for the transrepression of other VDR negative target genes by VDR/VDIR. Therefore, characterization of nVDREs in other VDR negative target genes together with identification of their binding factors would provide a better understanding of the action of Vitamin D.

6.2. WINAC supports ligand-induced transrepression by VDR on the human CYP27B1 gene promoter region

A large number of co-regulator complexes appear to support transcription control by NRs at multiple but sequential steps (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Pascual et al., 2005). ATP-dependent chromatin remodeling complexes are considered to facilitate the promoter-specific

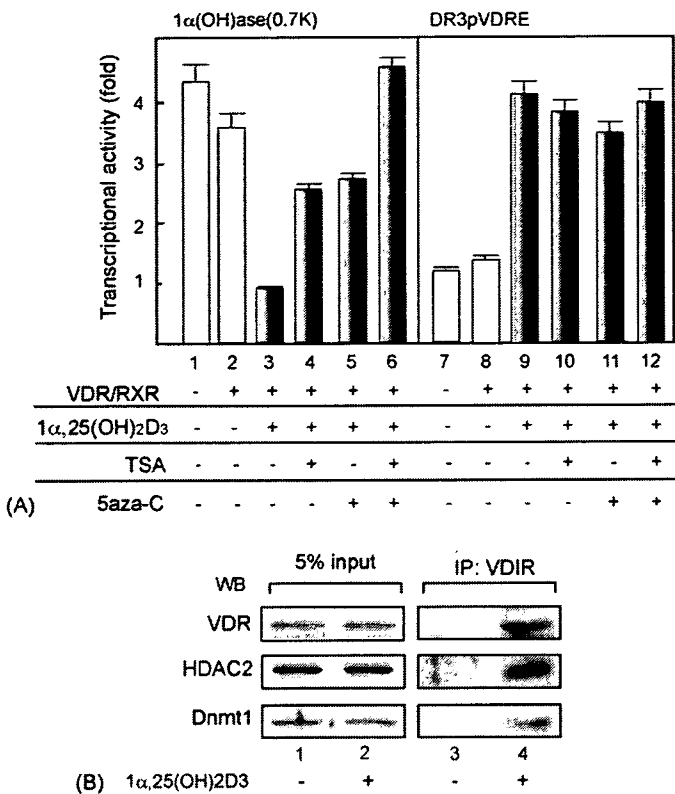

Fig. 4. Ligand-induced transrepression of the CYP27B1 gene by VDR mediates DNA methylation. (A) The effects of TSA (HDAC inhibitor) or 5aza-C (DNA methylation inhibitor) on transrepression of a luciferase reporter under control by a human CYP27B1 gene promoter segment containing 1αnVDRE. 293F cells (human embryonic kidney) were treated with or without TSA (10^{-6} M), 5aza-C (10^{-5} M) or both. After 36 h, the luciferase reporter plasmid and VDR/RXR expression vectors were transfected into the cells. Luciferase activity was assessed as described in Fig. 2. (B) 1α25$(OH)_2D_3$-dependent association between endogenous Dnmt1 (DNA methyltransferase) and VDIR *in vivo*. Whole cell extracts from 293F cells treated with or without 1α,25$(OH)_2D_3$ were immunoprecipitated with anti-VDIR antibodies, followed by Western blot analysis using anti-VDR, anti-Dnmt1 or anti-HDAC2 antibodies.

recruitment of other co-regulator complexes (Emerson, 2002; Narlikar et al., 2002). We have previously reported that WINAC dysfunction resulted in a failure of proper transcriptional regulation by VDR, possibly because of impairment of co-regulator recruitment to VDR-target gene promoters (Kato et al., 2003). These findings strongly suggested that ATP-dependent chromatin remodeling activity is indispensable for subsequent co-regulator recruitment in response to ligand binding (Kitagawa et al., 2003).

It has been suggested that ligand-unbound VDR/RXR on the VDRE mainly associates with a HDAC complex to repress target genes (Murayama et al., 2004). According to this model of a positive VDRE, ligand binding leads to co-repressor dissociation from VDR. In contrast, the WINAC assists recruitment of HDAC co-repressor complex in VDIR-mediated transrepression on a negative VDRE (Fujiki et al., 2005). These imply that the set of factors/complexes associated with unliganded VDR on negative VDREs differ from those associated with positive VDREs in the VDR target gene promoters. Indeed, ligand binding significantly increased the interaction of VDR/WINAC with a HDAC complex (Fujiki et al., 2005) Hence, in addition to ligand-induced transactivation by VDR, WINAC also has an important role in mechanisms of VDR-mediated transrepression. The proposed mechanism of the ligand-induced transrepression by VDR in the present study appears to be dependent on the promoter-content, since it is unlikely that all of the VDR target gene promoters for Vitamin D-induced transrepression harbor VDIR binding sites. Another mechanism of ligand-induced transrepression may be found for other promoters negatively controlled by VDR or some other NRs, like recently reported data on the transrespressive function of PPARγ (Pascual et al., 2005).

Acknowledgements

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 S.K.).

References

Emerson, B.M., 2002. Specificity of gene regulation. Cell 109, 267–270.

Fujiki, R., Kim, M.S., 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.

Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121–141.

Gu, W., Malik, S., Ito, M., Yuan, C.X., Fondell, J.D., Zhang, X., Martinez, E., Qin, J., Roeder, R.G., 1999. A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Mol. Cell. 3, 97–108.

Hassan, A.H., Prochasson, P., Neely, K.E., Galasinski, S.C., Chandy, M., Carrozza, M.J., Workman, J.L., 2002. Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell 111, 369–379.

Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K., Rosenfeld. M.G., 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature 387, 43–48.

Ito, T., Bulger, M., Pazin, M.J., Kobayashi, R., Kadonaga, J.T., 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell 90, 145–155.

Jones, M.H., Hamana, N., Nezu, J., Shimane. M., 2000. A novel family of bromodomain genes. Genomics 63, 40–45.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K., Rosenfeld, M.G., 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85, 403–414.

Kato, S., Suzawa, M., Takada, I., Takeyama, K., Yanagizawa, J., Fujiki, R., Kitagawa, H., 2003. The function of nuclear receptors in bone tissues. J Bone Miner. Metab. 21, 323–336.

Kato, S., Yanagisawa, J., Murayama, A., Kitanaka, S., Takeyama, K., 1998. The importance of 25-hydroxyvitamin D_3 1 alpha-hydroxylase gene in vitamin D-dependent rickets. Curr. Opin. Nephrol. Hypertens. 7, 377–383.

Kim, M.S., Fujiki, R., Murayama, A., Kitagawa, H., Yamamoto, K., Yamamoto, Y., Mihara, M., Takeyama, K., Kato, S., in press. 1alpha,25(OH)2D_3-induced transrepression by vitamin D receptor through E-box-type elements in the human parathyroid hormone gene promoter. Mol. Endocrinol., 2006 November 9 [Epub ahead of print].

Kitagawa, H., Fujiki, R., Yoshimura, K., Mezaki, Y., Uematsu, Y., Matsui, D., Ogawa, S., Unno, K., Okubo, M., Tokita, A., Nakagawa, T., Ito, T., Ishimi, Y., Nagasawa, H., Matsumoto, T., Yanagisawa, J., Kato, S., 2003. The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. Cell 113, 905–917.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.

Margueron, R., Trojer, P., Reinberg, D., 2005. The key to development: interpreting the histone code? Curr. Opin. Genet. Dev. 15, 163–176.

Martin, C., Zhang, Y., 2005. The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell. Biol. 6, 838–849.

McKenna, N.J., O'Malley, B.W., 2002. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108, 465–474.

Murayama, A., Kim, M.S., Yanagisawa, J., Takeyama, K.I., Kato, S., 2004. Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. EMBO J. 23, 1598–1608.

Murayama, A., Takeyama, K., Kitanaka, S., Kodera, Y., Hosoya, T., Kato, S., 1998. The promoter of the human 25-hydroxyvitamin D_3 1 alpha-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1 alpha,25(OH)2D_3. Biochem. Biophys. Res. Commun. 249, 11–16.

Narlikar, G.J., Fan, H.Y., Kingston, R.E., 2002. Cooperation between complexes that regulate chromatin structure and transcription. Cell 108, 475–487.

Onate, S.A., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270, 1354–1357.

Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., Li, A.C., Perissi, V., Rose, D.W., Willson, T.M., Rosenfeld, M.G., Glass, C.K., 2005. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature 437, 759–763.

Rachez, C., Suldan, Z., Ward, J., Chang, C.P., Burakov, D., Erdjument-Bromage, H., Tempst, P., Freedman, L.P., 1998. A novel protein complex that interacts with the vitamin D_3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. Genes Dev. 12, 1787–1800.

Takeyama, K., Kitanaka, S., Sato, T., Kobori, M., Yanagisawa, J., Kato, S., 1997. 25-Hydroxyvitamin D_3 1alpha-hydroxylase and vitamin D synthesis. Science 277, 1827–1830.

Yanagisawa, J., Kitagawa, H., Yanagida, M., Wada, O., Ogawa, S., Nakagomi, M., Oishi, H., Yamamoto, Y., Nagasawa, H., McMahon, S.B., Cole, M.D., Tora, L., Takahashi, N., Kato, S., 2002. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol. Cell. 9, 553–562.

A reduction state potentiates the glucocorticoid response through receptor protein stabilization

Hirochika Kitagawa[1], Ikuko Yamaoka[1,2], Chihiro Akimoto[1], Ikuko Kase[1], Yoshihiro Mezaki[1], Takafumi Shimizu[1] and Shigeaki Kato[1,2,*]

[1]*The Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032, Japan*
[2]*ERATO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan*

The intracellular redox state regulates all biological processes including gene expression. The glucocorticoid receptor (GR), a hormone-dependent transcription factor, is affected by the redox state. GR translocation from the cytoplasm to the nucleus is regulated by oxidative stress. The molecular mechanism of how the redox state affects GR transcriptional regulation, however, has not been clarified. We identified a deoxidizing agent, cobalt chloride ($CoCl_2$), that potentiates the GR transcriptional effects by stabilizing endogenously expressed GR protein as well as exogenously over-expressed one without affecting GR mRNA level. Consequent GR protein stabilization enhanced co-factor recruitments on the target gene promoters. These results support the existence of a novel redox-dependent mechanism of GR transcriptional regulation mediated by receptor protein stabilization.

Introduction

The intracellular environment results from the coordination of numerous signaling pathways. These pathways control the most basic biological events, including gene transcription. One component of the intracellular environment is the redox state, which is a reflection of the intracellular concentrations of reactive oxygen species. The intracellular redox state plays a key role in all cellular events including the modulation of gene expression. How the redox state affects gene expression is poorly understood (Kim *et al.* 2002; Rahman *et al.* 2004, 2006; Pouyssegur & Mechta-Grigoriou 2006).

Transcription regulatory factors mediate chromatin reorganization and histone modification, and are involved in the key steps of gene expression. These factors require several classes of co-regulator/co-regulator complexes (Kishimoto *et al.* 2006; Rosenfeld *et al.* 2006). Protein modification of the transcription factors themselves also affects transcription (Kumar *et al.* 2004). The glucocorticoid receptor (GR), a member of the nuclear receptor gene superfamily, is one transcription factor regulated in this manner (Rhen & Cidlowski 2005; Qiu *et al.* 2006).

Communicated by: Kohei Miyazono
**Correspondence*: E-mail: uskato@mail.ecc.u-tokyo.ac.jp

The GR engages in both positive and negative regulation of transcription. Its roles are diverse and include participation in glucose homeostasis and the suppression of inflammation (Smoak & Cidlowski 2004). Hormone binding induces translocation of the GR from the cytosol into the nucleus. In hormone-dependent transactivation, the GR, activated by hormone binding, binds as a homodimer to the consensus glucocorticoid responsive element (GRE) in the target gene promoters. Hormone-induced transrepression of inflammatory genes by the GR is mediated mainly by indirect GR association with the binding sites for AP-1 and NF-κB (Jonat *et al.* 1990; Heck *et al.* 1994; Ogawa *et al.* 2005). Thus, the intracellular translocation of the GR is a critical step to initiate gene regulation and appears to be modulated through the other intracellular signals (Saklatvala 2002; Rogatsky & Ivashkiv 2006).

In the present study, we tested how redox conditions modulate the function of the hormone-bound GR in hormone-induced gene regulation. H_2O_2-induced oxidative stress retained hormone-bound GR in the cytosol. In contrast, a reduced condition induced by cobalt chloride ($CoCl_2$) potentiates the function of the hormone-bound GR in transactivation through protein stabilization, but not in transrepression. Thus, the present findings indicate that a reduction state potentiates the response to glucocorticoids through GR protein stabilization.

DOI: 10.1111/j.1365-2443.2007.01131.x
© 2007 The Authors
Journal compilation © 2007 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd.

Results

To test if the redox state regulated GR functions, the effects of oxidative stress induced by H_2O_2 and reduction with $CoCl_2$ were examined with a luciferase reporter assay. H_2O_2 is a strong oxidative agent commonly used for the induction of cellular oxidative stress. $CoCl_2$ is a hypoxia mimetic reagent which reduces the oxidative environment in living cells (Zhang *et al.* 2002). $CoCl_2$ changes cellular conditions such as the NAD^+ : NADH ratio and modulates the structure of several proteins. Three types of reporters [Glucocorticoid responsive element (GRE), AP-1 responsive element (AP1-RE) and NF-κB responsive element (NFκB-RE)] were utilized with the reporter plasmids. A GR point mutant (C481S), known to remain in the nucleus after oxidative stress, was also used (Okamoto *et al.* 1999; Tanaka *et al.* 2000).

As expected, dexamethasone (Dex), a synthetic GR agonist stimulated GR-mediated transactivation through the GRE. It potently repressed transcription through the AP1-RE and NFκB-RE in the luciferase assay. H_2O_2 treatment attenuated GR function, while $CoCl_2$ potentiates it. Similar effects were seen on Dex-induced transrepression by the GR, but the effects were not significant (Fig. 1A). However, the Dex-induced function of the GR mutant (C481S) was sensitive to the treatment of $CoCl_2$, but not H_2O_2. This indicated that H_2O_2-induced oxidative stress caused the retention of Dex-bound GR in the cytosol (Fig. 1A). Redox state-mediated transcriptional regulation was also confirmed with endogenously expressed GR in A549 cells using the same luciferase assays without GR transfection (Fig. 1B; Wang *et al.* 2004). Another deoxidizing agent *N*-acetyl-L-cysteine (NAC) had the same effect as $CoCl_2$ in all assays, confirming the effect of reduction on the GR function (data not shown). Reflecting the GR protein stabilization by $CoCl_2$, the expression of the endogenous GR target genes were up-regulated, although the mRNA level of GR was unaltered (Fig. 1C).

Next, we confirmed the cellular localization of the GR in a reduction state by expressing a chimeric human GR protein fused to GFP, in 293F cells. In the absence of ligands, the GR was located mainly in the cytosol and H_2O_2 treatment induced clear cytosolic localization (Fig. 2A, top panel). Dex treatment induced the nuclear localization of the GR, but H_2O_2 inhibited GR translocation as previously reported (Okamoto *et al.* 1999). However, H_2O_2 treatment failed to block the nuclear translocation of the C481S mutant (Fig. 2A, left panels). These results indicated that the inhibitory action of H_2O_2 on the transcriptional activity of the GR was correlated with the retention of the GR in the cytosol. In contrast, $CoCl_2$ treatment did not alter the locations of Dex-bound and unbound GR (Fig. 2, right panel). These results suggested that another regulatory mechanism potentiates the function of Dex-bound GR.

To explore the molecular mechanism underlying potentiation of the GR by $CoCl_2$, we measured the expression levels of the GR over time. Measurements were made upon Dex addition since several steroid hormone receptors like ERs or AR are destabilized by ligand binding (Dennis & O'Malley 2005; Garside *et al.* 2006; Ohtake *et al.* 2007). In the 293F GR expressing stable transformant, the expression level of the GR was down-regulated in a time-dependent manner after Dex treatment (Fig. 3B). Furthermore, Dex-induced down-regulation of the GR was also seen with endogenous GR protein in A549 cells (Fig 3A,C). In the presence of $CoCl_2$, Dex-induced down-regulation of GR protein levels was not detected. This suggested that $CoCl_2$ interfered with Dex-induced destabilization of the GR protein. As a proteasome inhibitor MG132 was also effective in attenuating Dex-induced destabilization, Dex-induced destabilization of the GR protein appeared to be mediated by a ubiquitin-proteasome cascade that was presumably blocked by $CoCl_2$ (Fig. 3B,C).

To address if stabilization of the GR protein by $CoCl_2$ reflected the transcriptional potentiation of Dex-bound GR at the promoter level, GR recruitment to its target gene promoters was tested by a ChIP assay. An ATPase chromatin remodeling factor, Brg-1, was utilized as a representative co-factor for the GR as previously reported (Nagaich *et al.* 2004). The presence of $CoCl_2$ enhanced the recruitment of the GR as well as Brg-1 to three different gene promoters which contained either GRE, AP1-RE or NFκB-RE (Fig. 3D). Since the protein stability of Brg-1 was unaltered by either Dex or $CoCl_2$ (data not shown), the potentiation of GR function by $CoCl_2$ most likely resulted from increased co-factor recruitment by the stabilized GR protein in the target gene promoters.

Discussion

During the decade, the regulation of nuclear receptor transcription was extensively analyzed with the development of biochemical approach and the improvement of protein identification methods using mass spectrometry (Yanagisawa *et al.* 2002; Kitagawa *et al.* 2003; Takezawa *et al.* 2007). Various types of transcriptional co-factors have been already identified, most of which are related to chromatin reorganization (Perissi & Rosenfeld 2005; Rosenfeld *et al.* 2006). Accumulating knowledge suggests

© 2007 The Authors
Journal compilation © 2007 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd.

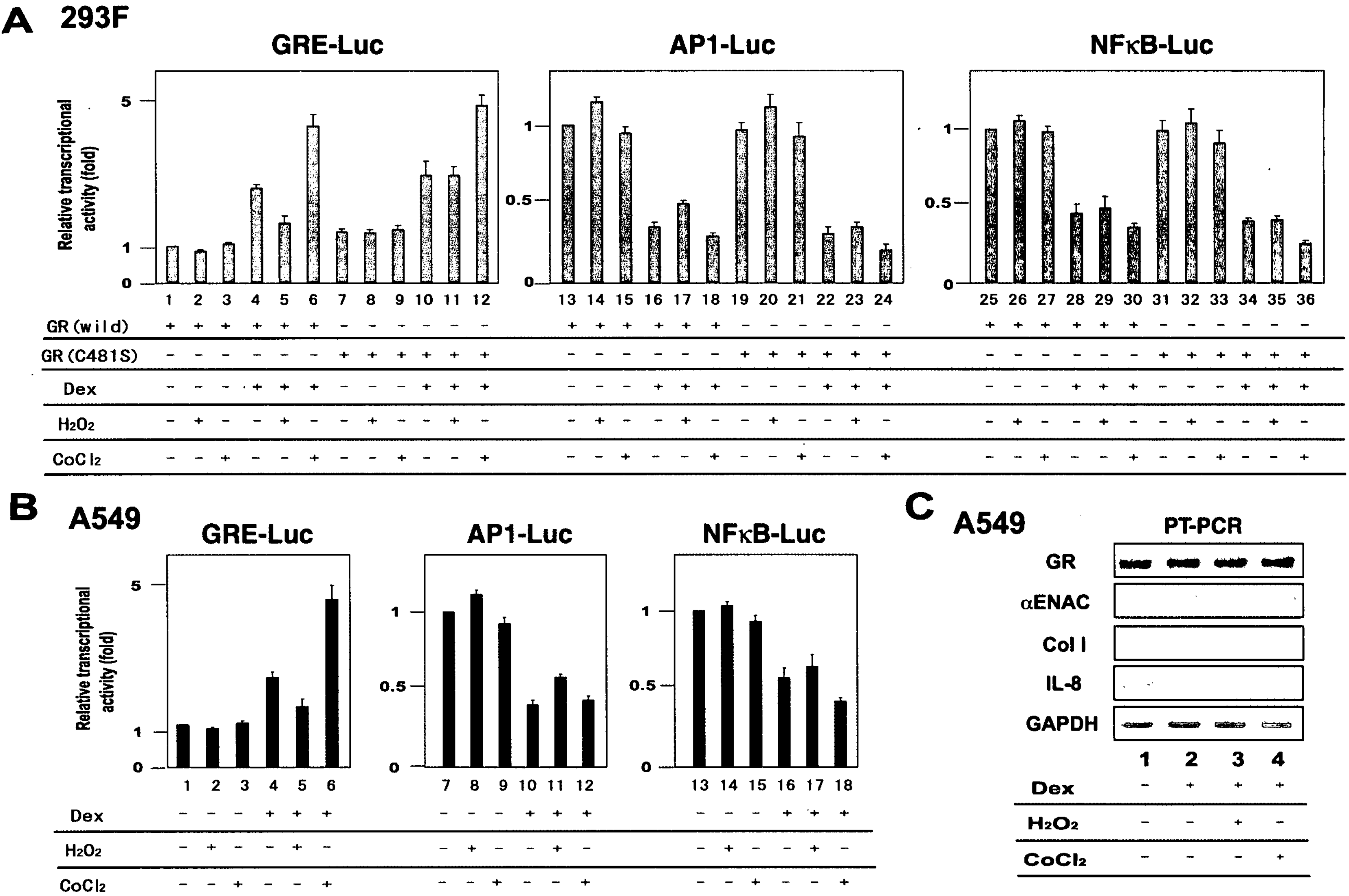

Figure 1 Ligand-dependent transcriptional regulation of the glucocorticoid receptor (GR) is redox state-dependent. (A) Luciferase assays were performed in 293F cells transfected with each of the indicated reporter plasmids (300 ng) and human GR or GR mutant (C481S) expression vectors (50 ng). For AP1-Luc, expression vectors were for c-Jun and c-Fos, and for NFκB-Luc, expression vectors were for p50 and p65. Vectors were simultaneously transfected (50 ng each). Dexamethasone (10^{-7} M), H_2O_2 (0.1 mM) and $CoCl_2$ (0.2 mM) were added 3 h after transfection. (B) Luciferase assays were performed in A549 cells as explained in (A) without transfection of the human GR or GR mutant (C481S). (C) GR target genes were really regulated by redox state in A549 cells. Total RNA was extracted 16 h after indicated stimulations. RT-PCR of GR and its target genes were performed as described in Experimental procedures.

© 2007 The Authors
Journal compilation © 2007 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd.

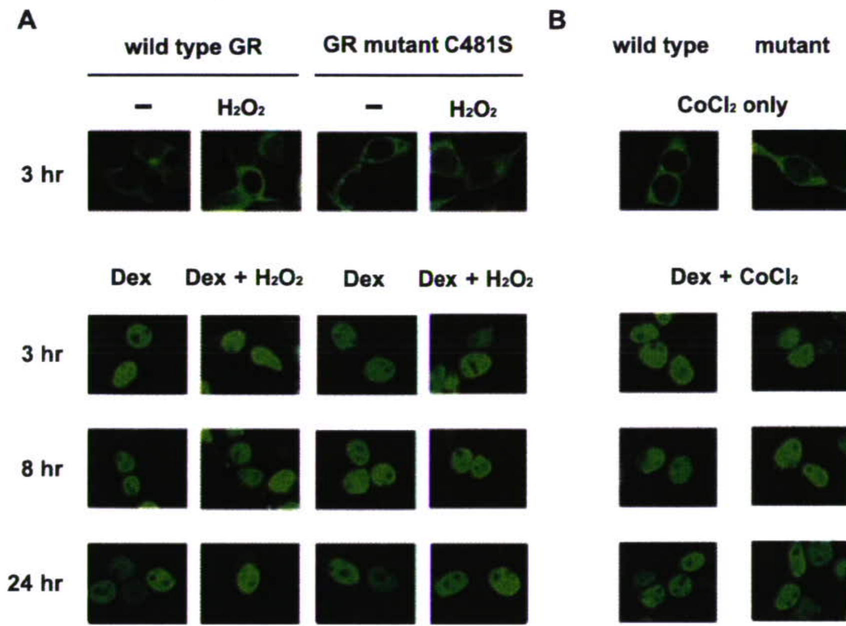

Figure 2 The ligand induced change in cellular localization of the glucocorticoid receptor (GR) is redox state-dependent. (A) Oxidative stress affects the cellular localization of the GR. 293F cells were transfected with expression vectors of GFP-GR or GFP GR mutant (C481S). Dex (10^{-7} M) and H_2O_2 (0.1 mM) were added at the indicated times before fixing. (B) Cobalt chloride ($CoCl_2$) did not affect GR localization. Dex (10^{-7} M) and $CoCl_2$ (0.2 mM) were added at the indicated times before fixing. After 24 h of transfection, the cells were scanned using a Zeiss confocal laser scanning system 510, and results were assessed with Adobe Photoshop 5.0 (Adobe) (Miyamoto *et al.* 2007).

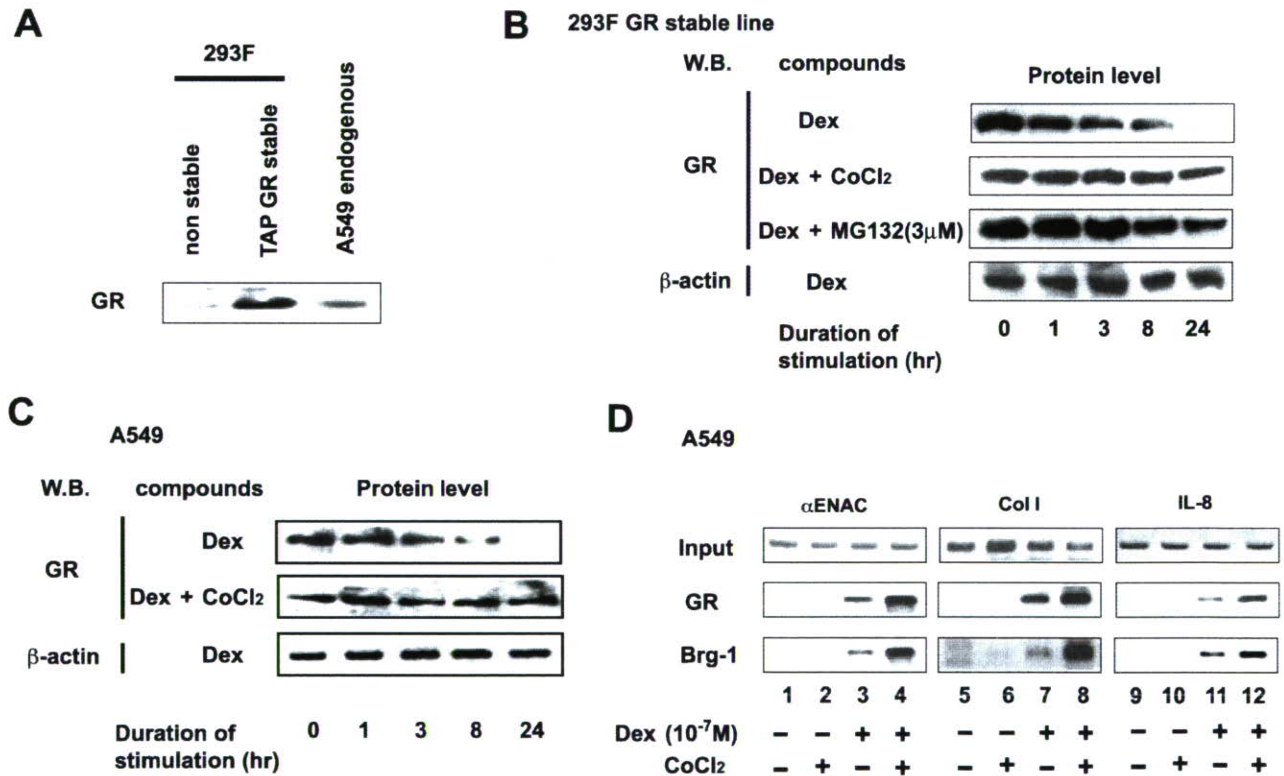

Figure 3 $CoCl_2$ stabilizes the GR protein and co-factor recruitment. (A) Expression of the glucocorticoid receptor (GR) in indicated cells. Cell extracts were immunoprecipitated with GR antibody and subjected to Western blotting. (B) $CoCl_2$ stabilized the GR protein. Whole cell extract from 293F cells with stable expression of the GR were immunoprecipitated at an indicated time upon stimulation with Dex or Dex + $CoCl_2$ and Western blotted as shown in (A). MG132 (3 μM) was also used for cell stimulation. (C) Endogenous GR protein was also stabilized by $CoCl_2$ in A549 cells. A549 cells were treated and processed in the same way as (B). (D) Co-factor recruitment was also stabilized by $CoCl_2$. A ChIP assay was performed as described in the Experimental procedures.

© 2007 The Authors
Journal compilation © 2007 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd.