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AWARD NUMBER: DAMD17-02-1-0142

TITLE: Vitamin D Treatment of Prostate Cancer: The Inhibitory Role of IGFBP-3

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REPORT DATE: January 2006

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2006	Final Addendum	15 Dec 2004 – 14 Dec 2005

4. TITLE AND SUBTITLE

Vitamin D Treatment of Prostate Cancer: The Inhibitory Role of IGFBP-3

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0142

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Stanford University
Stanford, California 94305-5401

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Calcitriol plays a critical role in maintaining mineral homeostasis but also exhibits antiproliferative activity in many cancers. We have shown that the antiproliferative actions of calcitriol in LNCaP human prostate cancer cells are mediated mainly by induction of insulin-like growth factor binding protein–3 (IGFBP-3). We also found that androgens increase expression of IGFBP-3 and cause a major enhancement of IGFBP-3 stimulation by calcitriol. The purpose of this study was to determine the molecular mechanisms involved in calcitriol and androgen regulation of IGFBP-3. We cloned 6 kb of the IGFBP-3 promoter and demonstrated its responsiveness to calcitriol and androgen in transactivation assays. Computer analysis identified a putative vitamin D response element (VDRE) and a potential androgen response element (ARE) in the IGFBP-3 promoter. We proved each to be inducible by calcitriol or androgen. Mutations created in the VDRE or ARE resulted in a loss of IGFBP-3 induction confirming the critical response element sequences. Chromatin immunoprecipitation assays demonstrated that calcitriol recruited VDR/RXR heterodimers to the VDRE site and androgen recruited the AR/AR homodimer to the ARE site. In conclusion, we have identified a functional VDRE and ARE in the human IGFBP-3 promoter that directly mediates the action of calcitriol and androgen to regulate IGFBP-3 expression.

15. SUBJECT TERMS
vitamin D, calcitriol, prostate cancer, vitamin D receptor, VDRE, IGFBP-3, antiproliferation, androgens, androgen receptor, ARE

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	19	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

We have previously shown that calcitriol (1,25-dihydroxyvitamin D_3) the active hormonal form of vitamin D, is antiproliferative in several prostate cancer (PCa) models including human prostate cancer cell lines (1-3). This has led us to hypothesize that calcitriol may be a useful therapy for PCa (4). In studies of the LNCaP human PCa cell line, we further demonstrated that calcitriol induction of insulin-like growth factor binding protein–3 (IGFBP-3) was necessary for the antiproliferative activity (5). The IGFBP-3 gene appears to mediate the antiproliferative activity of calcitriol by inducing a subsequent gene, p21, an inhibitor of the cell cycle. Prevention of IGFBP-3 expression, using antibodies to immunoneutralize IGFBP-3 or antisense to prevent IGFBP-3 mRNA transcription, abrogated the antiproliferative activity of calcitriol and the induction of p21. We therefore concluded that induction of IGFBP-3 is the critical step in calcitriol's antiproliferative activity in the LNCaP cells (5).

IGFBP-3 is the major binding protein for circulating IGF-1, a potent mitogen (6). IGFBP-3 has activities that are antimitogenic because it sequesters IGF-1. However, it has recently become clear that IGFBP-3 also has IGF-independent actions that are antiproliferative and proapoptotic (7). These activities make IGFBP-3 a potentially important factor in halting cancer cell growth (5, 8, 9, 10). The circulating blood level of IGFBP-3 has also been studied as a potential marker of PCa risk (11).

The goal of the current project is to further investigate the role of IGFBP-3 in the antiproliferative actions of vitamin D in PCa. Calcitriol regulates target genes by binding to the vitamin D receptor (VDR) (12). The calcitriol-bound VDR dimerizes with retinoid X receptor (RXR) and this complex then binds to a vitamin D regulatory element (VDRE) in the promoter region of target genes (13). Therefore, to study the vitamin D regulation of IGFBP-3, we set out to determine whether a VDRE was present in the promoter region of the IGFBP-3 gene.

In addition, we hoped to resolve another controversy about the nature of calcitriol regulation of the critical gene, p21. This gene is a cyclin-dependent kinase (Cdk) inhibitor and an important regulator of the cell cycle and cell proliferation. In our studies, calcitriol appeared to regulate IGFBP-3 that then regulated p21. In contrast, the studies of Liu et al (14) concluded that calcitriol directly regulated p21. The differences could be resolved, at least partially, by demonstrating a VDRE in the IGFBP-3 promoter region and demonstrating direct regulation of IGFBP-3 by calcitriol.

BODY

Calcitriol Regulation of IGFBP-3

In our Statement of Work, our major goal was to investigate the regulation of IGFBP-3 by calcitriol in PCa cells. We have clearly demonstrated that calcitriol’s antiproliferative actions in some PCa cells are mediated by induction of IGFBP-3 (5).

The second goal of this project was to determine the molecular mechanism of calcitriol’s action on IGFBP-3. We have successfully identified a functional VDRE in the distal region of the IGFBP-3 promoter (15). The induction of IGFBP-3 by calcitriol is directly mediated via VDR interaction with this VDRE.

Identification of an ARE in the IGFBP-3 Promoter

IGFBP-3 the most abundant circulating IGF binding protein, inhibits cell growth and induces apoptosis by both IGF-I dependent and independent pathways. The ability of IGFBP-3 to inhibit tumor growth has been demonstrated in many cancers including PCa. High concentrations of androgens, that inhibit the growth of the LNCaP human PCa cell line, have been shown to have both positive and negative effects on IGFBP-3 expression by different labs. To further explore the relationship between IGFBP-3 and androgens, we examined IGFBP-3 expression in LNCaP cells (16). We demonstrate that IGFBP-3 expression can be induced by 10 nM of the synthetic androgen R1881 or DHT. Transactivation assays show that the 6 kb IGFBP-3 promoter sequence directly responds to androgen treatment. *In silico* analysis identified a putative ARE at -2879/-2865 in the IGFBP-3 promoter. A single point mutation in this ARE disrupted transactivation by R1881. Combining the data obtained from EMSA, chromatin immunoprecipitation and mutational analysis, we conclude that a novel functional ARE is present in the IGFBP-3 promoter that directly mediates androgen induction of IGFBP-3 expression. Furthermore, we found that the combination of androgens and calcitriol significantly potentiated the IGFBP-3 promoter activity, suggesting that enhanced induction of the expression of the endogenous IGFBP-3 gene may contribute to the greater inhibition of LNCaP cell growth by combined calcitriol and androgens (16). Since androgens are well known to stimulate PCa growth, and androgen deprivation therapy causes PCa to regress, the stimulation by androgens of this anti-proliferative and pro-apoptotic protein is paradoxical and raises interesting questions about the role of androgen-stimulated IGFBP-3 in PCa.

Calcitriol-Androgen Interaction, Inhibition of LNCaP Cell Proliferation by Androgens

In the androgen-dependent LNCaP human prostate cancer (PCa) cell line calcitriol exerts its anti-proliferative action predominantly through inducing cell cycle arrest. We have previously shown that growth arrest by calcitriol is primarily mediated by induction of IGFBP-3, which subsequently increases the expression of the cell cycle inhibitor p21. We also have identified functional response elements for androgens and calcitriol in the IGFBP-3 promoter. In this study we show that low doses of the synthetic androgen R1881 that stimulate LNCaP cell proliferation, do not induce IGFBP-3 expression, while high doses of R1881 that inhibit cell growth, significantly increase expression of IGFBP-3 (17). Importantly, we demonstrate that calcitriol and androgens in combination synergistically up-regulate IGFBP-3 expression through VDR and AR response elements within the IGFBP-3 promoter. The hormone combination inhibits cell growth better than either hormone alone. siRNA blockade of IGFBP-3 expresssion abrogates the growth inhibition by calcitriol as well as by androgens. Furthermore, we demonstrate that the growth inhibitory dose of R1881 leads to an increase in the cyclin dependent kinase inhibitors, p21 and p27 and G1 arrest. These changes can be blocked or partially reversed by IGFBP-3 siRNA. Addition of exogenous IGFBP-3 protein causes growth inhibition. Our data suggest that IGFBP-3 mediates the anti-proliferative effect of high doses of androgen partly through p21 and p27 pathways and that IGFBP-3 plays an important role in the androgen-dependent biphasic growth regulation of LNCaP cells (17).

Other IGFBP-3 Regulators

We have also studied the interaction of other important regulators of IGFBP-3 including 9-cis retinoic acid [(9 cis-RA), the RXR ligand], rosiglitazone [a peroxisome proliferator-activated receptor (PPAR)γ ligand], and tricostatin A [TSA, a histone deacetylase (HDAC) inhibitor] using Northern blot analysis as well as our established IGFBP-3 promoter-reporter transactivation system. We found that all of these regulators significantly enhanced calcitriol's up-regulation of IGFBP-3 expression. These findings suggest that these agents given in combination with calcitriol would increase calcitriol's antiproliferative activity. It is possible that combination therapy would allow the use of lower doses of calcitriol to achieve a therapeutic antiproliferative effect and thereby diminish the hypercalcemic side-effects of calcitriol in prostate cancer therapy.

Calcitriol Analogs

Another goal of the project was to evaluate IGFBP-3 regulation by several calcitriol analogs that are less hypercalcemic than calcitriol. In other words, we wanted to know whether ability to induce IGFBP-3 could be used to explore the antiproliferative activity of the calcitriol analogs on PCa cells. To establish this assay, we measured both the effect of the analogs on PCa cell proliferation as well as real-time PCR to assess the analog's ability to induce IGFBP-3. We used LNCaP cells for both the growth studies and the IGFBP-3 induction. Analogs EB1089, Ro24-5531, KH1060 and Ro27-0574, each at 10 nM, resulted in over 10-fold increase in IGFBP-3 mRNA compared to the vehicle treatment. The analogs inhibited cell proliferation by over 65%. In comparison, 10 nM calcitriol induced a 2-3 fold of induction of IGFBP-3 mRNA and about 55% inhibition of cell growth. The studies indicate that these analogs have a higher potency than calcitriol at both IGFBP-3 induction and antiproliferative activity.

Differential Regulation of Polymorphic Variants of IGFBP-3

Recently Deal et al. (18) found a novel polymorphism that varies between an "A" or a "C" nucleotide at –202 from its transcription start site in the IGFBP-3 promoter. The "A" allele is more active than the "C" allele that is consistent with the relationship between genotype and circulating IGFBP-3 levels in the serum (AA>AC>CC). The potency of calcitriol analogs as antiproliferative agents may be predicted by their ability to induce IGFBP-3 in an allele-specific manner. On the other hand, the VDR gene also exists in several polymorphic forms. Especially important is the FokI polymorphism at the translation start site that alters the translated protein. The FokI polymorphism results in two VDR variants, the "f" variant (427 amino acids) and "F" variants (3 amino acids shorter) (19, 20). Epidemiologic studies have reported that the FokI polymorphism correlates with the risk of osteoporosis and PCa. Thus we hypothesized that the FokI polymorphism in the VDR gene contributes to the efficacy of calcitriol action on the level of induction of target genes. Since calcitriol's antiproliferative activity in LNCaP cells is dependent on stimulation of IGFBP-3, the interaction of VDR and IGFBP-3 polymorphisms may determine the response of the IGFBP-3 gene to calcitriol therapy.

To assess whether the VDR FokI polymorphism exhibits differential activity to activate the IGFBP-3 promoter variants, the two VDR variants, "f" and "F", were tested for transactivation potency on the IGFBP-3 A and C variants. Plasmids expressing the VDR variants were co-transfected with the different IGFBP-3 promoters. Both VDR variants were tested on each IGFBP-3 promoter. We found that the combination of VDR-f with IGFBP-3-C did not respond to calcitriol in LNCaP cells. The other three combinations (VDR-F/IGFBP-3-A, VDR-F/IGFBP-3-C, or VDR-f/IGFBP-3-A) induced gene expression.

To explore the mechanism by which the IGFBP-3 polymorphic forms differentially regulate the level of gene expression, we performed gel shifts assays. The experiments used nucleic extracts isolated from LNCaP cells using the oligonucleotide probe containing the A or C allele at -202 of the IGFBP-3 promoter. Both alleles produced similar binding complexes but showed different binding activity with the A allele probe stronger than the C allele. This result is consistent with the transcriptional activity of the IGFBP-3 promoter with the A allele being stronger than the C allele. Point mutations in promoter DNA near –202 identified the critical nucleotides involved in the DNA-protein complex. Supershifts further characterized N-Myc in this complex that might contribute to the differential regulation of IGFBP-3 expression.

Nuclear Actions of IGFBP-3

The last part of this project is to study the nuclear mechanisms of IGFBP-3 interaction with RXR and VDR to inhibit PCa cell growth. It has been postulated that IGFBP-3 translocates to the nucleus and binds to RXR altering nuclear receptor function (21, 22). Using GST pull-down assays, we found that IGFBP-3 specifically binds to RXR and not VDR. In transactivation assays, we failed

to see that over-expressed IGFBP-3 changes the 24-hydroxylase promoter's response to calcitriol. We have not completed this area of study and can not yet reach any conclusions about interactions of IGFBP-3 and either RXR or VDR that have been postulated by Cohen and associates (22, 23).

Analysis of Calcitriol-Regulated Prostate Cancer Cell Gene Expression Profiles

Although not part of the SOW, this grant helped to support our studies of all of the genes regulated by calcitriol in the LNCaP human PCa cell. These studies used cDNA microarrays to evaluate the entire profile of genes up- or down-regulated by calcitriol (24). It is of interest that IGFBP-3 had the greatest fold-induction of all genes in the LNCaP cell by calcitriol. At 6 hours, IGFBP-3 achieved a statistically significant up-regulation (2.4-fold) supporting the idea of direct regulation. At 24 hours IGFBP-3 was 33.2-fold stimulated over control cells, the highest level by far of any gene in LNCaP cells. These results confirm the importance of the IGFBP-3 response to calcitriol in LNCaP cells.

Studies of Prostate cancer Cells with Mutant Androgen Receptor

The grant also helped to support studies of mutant androgen receptors (ARs) that resulted in "promiscuious" binding of glucocorticoids to the androgen binding site. Although not part of the SOW these studies are closely related to our major goal of improving therapy for prostate cancer patients. This paper demonstrated that the synthetic glucocorticoid triamcinolone did not bind to the mutant AR whereas many glucocorticoids such as cortisol did. These findings were part of the preliminary data that allowed us to submit a new DAMD grant proposal to the prostate cancer program to study triamcinolone therapy of androgen-independent prostate cancer (PC030122).

KEY RESEARCH ACCOMPLISHMENTS

- Identified the presence of both VDRE and ARE sites in the IGFBP-3 promoter.
- Proved that both calcitriol and androgen directly regulate IGFBP-3.
- Found that the combination of calcitriol and androgen greatly enhances expression of IGFBP-3 and cell growth inhibition.
- Found that androgen has a biphasic regulatory effect on expression of IGFBP-3. 10^{-11} M of R1881 does not affect IGFBP-3 till $5x10^{-11}$ M. This may explain the biphasic actions of androgens on PCa cell growth.
- Demonstrated that calcitriol indirectly regulates p21/p27 and the effect is mediated through IGFBP-3 induction.
- Demonstrated that the growth inhibitory effect of high concentrations of androgens are due, at least in part, to the increased expression of IGFBP-3 and its regulation of p21 and p27.
- Characterized important VDRE and ARE with unique characteristics in an important calcitriol target gene.
- Provided information on the promoter region of the IGFBP-3 gene.
- Established an advanced biotechnique, the ChIP assay, to study DNA-protein interactions in our lab that will be very useful to investigate additional hormone receptor-DNA interactions.
- Demonstrated the induction of IGFBP-3 via action on its promoter by other important regulators including retinoids, PPAR ligands and HDAC inhibitors.
- Provided data on possible approaches to combination therapy that would improve the therapeutic potency of calcitriol in PCa therapy.

REPORTABLE OUTCOMES

All of the above mentioned research accomplishments are reportable. We had two publications, one in Molecular Endocrinology and one in Endocrinolgy describing the identification of functional VDRE and ARE in the IGFBP-3 promoter (15, 16 and also see Appendix). We wrote another manuscript describing the role of IGFBP-3 in mediating the biphasic actions of androgens on the growth of LNCaP cells and it is submitted for publication (17). We have provided our IGFBP-3 promoter-reporter constructs (15) to five investigators including D. Edward P. Gelmann (Georgetown University), Dr. Hiroshi Nakagawa (University of Pennsylvania), Dr. Jianming Xu (Baylor College of Medicine), Dr. Yu-Chung Yang (Case Western Reserve University), and Dr. Shuyuan Yeh (University of Rochester). This saves the other labs time and labor and provides them with important reagents making their research efforts possible so that it is very beneficial to our research community.

PUBLICATIONS

Krishnan A V, Zhao XY, Swami S, Brive L, Peehl DM, Ely KR, and Feldman D, 2002 A glucocorticoid-responsive mutant androgen receptor (AR^{ccr}) exhibits unique ligand specificity: Therapeutic implications for androgen-independent prostate cancer. Endocrinology, 143, 1889-1900

Peng L, Malloy PJ, Feldman D 2004 Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18:1109-19

Krishnan AV, Shinghal R, Raghavachari N, Brooks JD, Peehl DM, Feldman D 2004 Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. Prostate 59:243-51

Peng L, Malloy PJ, Wang J, Feldman D 2006 Growth Inhibitory Concentrations of Androgens Up-Regulate Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) Expression *via* an Androgen Response Element in LNCaP Human Prostate Cancer Cells. Endocrinology (in press)

Peng L, Wang J, Malloy PJ, Feldman D 2006 The Role of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) in Mediating the Biphasic Actions of Androgens on the Growth of LNCaP Human Prostate Cancer Cells. (submitted for publication)

CONCLUSIONS

Our research has elucidated the presence of a VDRE and an ARE in the distal promoter region of the IGFBP-3 gene. These findings establish the fact that calcitriol or androgen directly regulates IGFBP-3 via a classical hormone response element. Since only a modest number of VDREs or AREs have been characterized, these findings add to the series of known VDREs or AREs and provide useful data for further elucidation of additional response elements. The research also demonstrates how this critical gene is regulated and provides DNA sequence data for the IGFBP-3 promoter region to support further analysis of additional regulators or modulators of IGFBP-3 gene expression. Most importantly, the work clarifies how calcitriol or androgen induces this most important regulator of PCa cell growth.

Our study has also demonstrated enhanced calcitriol action to induce IGFBP-3 by other important regulators including retinoids, rosiglitazone (a diabetes drug that binds to PPARs) and HDAC inhibitor and has evaluated the potency of several calcitriol analogs with less hypercalcemic activity. The results have provided new insights into improving calcitriol's therapeutic efficacy in PCa therapy.

REFERENCES

1. Skowronski RJ, Peehl DM, Feldman D 1993 Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. Endocrinology 132:1952-60
2. Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D 1994 Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer Res 54:805-10
3. Skowronski RJ, Peehl DM, Feldman D 1995 Actions of vitamin D3, analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D3. Endocrinology 136:20-6
4. Zhao XY, Feldman D 2001 The role of vitamin D in prostate cancer. Steroids 66:293-300.
5. Boyle BJ, Zhao XY, Cohen P, Feldman D 2001 Insulin-like growth factor binding protein-3 mediates 1 alpha,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. J Urol 165:1319-24
6. Pollak M 2001 Insulin-like growth factors and prostate cancer. Epidemiol. Rev. 23:59-66
7. Rajah R, Valentinis B, Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181-8
8. Pollak M, Beamer W, Zhang JC 1998 Insulin-like growth factors and prostate cancer. Cancer Metastasis Rev 17:383-90.
9. Grimberg A, Cohen P 2000 Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. J Cell Physiol 183:1-9.
10. Ali O, Cohen P, Lee KW 2003 Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. Horm Metab Res 35:726-33
11. Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, Giovannucci E 2002 Insulin-like growth factor -I (IGF-I) and IGF binding protein -3 as predictors of advanced-stage prostate cancer. J. Natl. Cancer Inst. 94:1099-1106
12. Feldman D, Malloy PJ, Gross C 2001 Vitamin D: biology, action and clinical implications. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press, San Diego, vol 1:257-303

13. Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13:325-49
14. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP 1996 Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142-53
15. Peng L, Malloy PJ, Feldman D 2004 Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18:1109-19
16. Peng L, Malloy PJ, Wang J, Feldman D 2006 Growth Inhibitory Concentrations of Androgens Up-Regulate Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) Expression *via* an Androgen Response Element in LNCaP Human Prostate Cancer Cells. Endocrinology (in press)
17. Peng L, Wang J, Malloy PJ, Feldman D 2006 The Role of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) in Mediating the Biphasic Actions of Androgens on the Growth of LNCaP Human Prostate Cancer Cells. (submitted for publication)
18. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, Hudson T, Stampfer M, Pollak M 2001 Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. J Clin Endocrinol metab 86:1274-80.
19. Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R, Feldman D 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. J Bone Miner Res 11:1850-5
20. Feldman D 1997 Androgen and vitamin D receptor gene polymorphisms: the long and short of prostate cancer risk [editorial; comment]. J Natl Cancer Inst 89:109-11
21. Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM, Cohen P 2000 Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor- regulate transcriptional signaling and apoptosis. J Biol Chem 275:33607-13
22. Lee KW, Cohen P 2002 Nuclear effects: unexpected intracellular actions of insulin-like growth factor binding protein-3. J Endocrinol 175:33-40
23. Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP, Cohen P 2004 Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. J Biol Chem 279:469-76.
24. Krishnan AV, Shinghal R, Raghavachari N, Brooks JD, Peehl DM, Feldman D 2004 Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. Prostate 59:243-51.

0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(10):4599–4607

doi: 10.1210/en.2006-0560

Growth Inhibitory Concentrations of Androgens Up-Regulate Insulin-Like Growth Factor Binding Protein-3 Expression via an Androgen Response Element in LNCaP Human Prostate Cancer Cells

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IGF binding protein-3 (IGFBP-3), the most abundant circulating IGF binding protein, inhibits cell growth and induces apoptosis by both IGF-I-dependent and -independent pathways. The ability of IGFBP-3 to inhibit tumor growth has been demonstrated in many cancers including prostate cancer (PCa). High concentrations of androgens, which inhibit the growth of the LNCaP human PCa cell line, have been shown to have both positive and negative effects on IGFBP-3 expression by different laboratories. To further explore the relationship between IGFBP-3 and androgens, we examined IGFBP-3 expression in LNCaP cells. We demonstrate that IGFBP-3 expression can be induced by 10 nM of the synthetic androgen R1881 or dihydrotestosterone. Transactivation assays show that the 6-kb IGFBP-3 promoter sequence directly responds to androgen treatment. *In silico* analysis identified a putative androgen response element (ARE) at −2879/−2865 in the IGFBP-3 promoter. A single point mutation in this ARE disrupted transactivation by R1881. Combining the data obtained from EMSA, chromatin immunoprecipitation and mutational analysis, we conclude that a novel functional ARE is present in the IGFBP-3 promoter that directly mediates androgen induction of IGFBP-3 expression. Furthermore, we found that the combination of androgens and calcitriol significantly potentiated the IGFBP-3 promoter activity, suggesting that enhanced induction of the expression of the endogenous IGFBP-3 gene may contribute to the greater inhibition of LNCaP cell growth by combined calcitriol and androgens. Because androgens are well known to stimulate PCa growth and androgen deprivation therapy causes PCa to regress, the stimulation by androgens of this antiproliferative and proapoptotic protein is paradoxical and raises interesting questions about the role of androgen-stimulated IGFBP-3 in PCa. (*Endocrinology* 147: 4599–4607, 2006)

IGF BINDING PROTEIN (IGFBP)-3 is a member of the IGFBP family that bind the potent mitogen IGF-I with high affinity and specificity (1–3). It is thought that IGFBPs serve to extend the half-life of IGF-I as well as transport and modulate the biological actions of IGFs on target cells. IGFBP-3 is the most abundant circulating carrier protein binding more than 75% of serum IGF-I (1–3). In addition to sequestering IGF-I to diminish the growth stimulating action of IGF-I in an IGF-dependent manner, IGFBP-3 also exhibits multiple IGF-independent actions, including inhibition of cell growth and induction of apoptosis (1–7). Other steps in the mechanism of IGFBP-3 action include binding to TGFβ type V receptor (8), translocation to the nucleus via the importin β-subunit (9), and interacting with the nuclear retinoid X receptor-α (10).

Epidemiological studies have suggested that high plasma levels of IGF-I and low concentrations of IGFBP-3 are associated with increased risk of prostate cancer (PCa) (11–13), suggesting that high IGF-I and low IGFBP-3 may be potential risk markers of PCa. IGFBP-3 has been shown to promote apoptosis and inhibit cellular proliferation in a variety of cell lines including PCa cells in an IGF-independent manner (1–7, 14–16). Recently Liu *et al.* (17) reported that the combination of IGFBP-3 with retinoid X receptor ligands resulted in a synergistic effect to suppress the growth of PCa xenografts by inducing apoptosis. Using a transgenic mouse model, Silha *et al.* (18) demonstrated that at the early stages of the development of prostate cancer, overexpression of IGFBP-3 attenuates tumor growth via IGF-dependent actions, and as the cancer advances, IGFBP-3 suppresses tumor growth entirely through IGF-independent mechanisms (3, 18). Both animal and preclinical studies provide evidence that IGFBP-3 is an important antitumor agent (1–7, 17–19).

Previously we found that calcitriol induction of IGFBP-3 importantly contributed to its antiproliferative actions (20). We identified a functional vitamin D response element (VDRE) in the IGFBP-3 promoter that directly mediates calcitriol actions to induce IGFBP-3 expression (21). p53 has also been shown to regulate IGFBP-3 synthesis via a direct protein/gene interaction (22). In LNCaP cells either positive or negative regulation of IGFBP-3 expression by androgens has been reported by several groups (23–27). Goossens *et al.* (23), Arnold *et al.* (25), and Kojima *et al.* (27) showed an inhibitory effect of androgens on expression of IGFBP-3. On the other hand, Martin and Pattison (24) observed a stimulatory effect

First Published Online July 6, 2006

Abbreviations: AR, Androgen receptor; ARE, androgen response element; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation assay; FACS, flow cytometric analysis; FBS, fetal bovine serum; GR, glucocorticoid receptor; IGFBP, IGF binding protein; LUC, luciferase; PCa, prostate cancer; qRT-PCR, quantitative real-time RT-PCR; TK, thymidine kinase; VDRE, vitamin D response element.

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of androgens on IGFBP-3. The reason for these conflicting results remains unclear.

Androgens are essential for the development of the normal prostate and the maintenance of prostate function (28). In addition to the well-known ability of androgens to stimulate prostate growth and androgen deprivation therapy to cause prostate cancer regression (29–31), a number of studies have demonstrated that androgens can exert antiproliferative and prodifferentiative actions on PCa cells (32–37). Other studies in LNCaP cells demonstrate that low doses of androgen stimulate cell growth, whereas high concentrations result in inhibition of cell growth (33, 38–40). The mechanism for these divergent or biphasic actions remains unknown. Androgen actions are mediated by the androgen receptor (AR), a member of the steroid, thyroid, retinoid receptor superfamily of nuclear transcription factors (28). AR binds as a homodimer to androgen response elements (AREs) containing an inverted repeat of the core sequence (5′-TGTTCT-3′) separated by a three-nucleotide spacer (41).

In the present study, we used the androgen-responsive LNCaP cells as a model to study the regulation of IGFBP-3 by growth inhibitory concentrations of androgens and determine the molecular mechanism involved. We provide the first evidence that the induction of IGFBP-3 by androgens is mediated through a novel functional ARE site in the distal region of the IGFBP-3 promoter and is possibly involved in the growth-inhibitory action of androgens. Furthermore, we demonstrate that the interaction of calcitriol and androgens leads to a substantial increase in IGFBP-3 promoter activity that is mediated through their respective response elements in the IGFBP-3 promoter.

Materials and Methods

Cell culture

The LNCaP cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium containing 5% fetal bovine serum (FBS) at 37 C in a humidified atmosphere with 5% CO_2. During all treatments, the serum concentration was reduced to 2% FBS.

Cell growth assay

LNCaP cells were plated at low density (5×10^4) in six-well plates and grown in 5% FBS-RPMI 1640 medium. After 24 h incubation, the cells were treated with vehicle or androgens in triplicate for 6 d. Fresh medium with androgen was replenished 72 h later in all experiments. At d 6, the cells were processed for DNA quantitation using the Burton reagent (42).

Flow cytometric analysis (FACS)

LNCaP cells were plated at 3×10^5 per well in six-well plates in 5% FBS-RPMI 1640 medium. On the next day, the cells were treated with ethanol or R1881 (at 0.1 or 10 nM). After 2 d of growth, the cells were trypsinized and harvested. The pellets were then washed in PBS buffer containing 5 mM EDTA and fixed in ice-cold 0% ethanol for at least 1 h at 4 C (43). RNA in the fixed cells was digested for 30 min at room temperature with the addition of 100 μg/ml RNase A. Then cells were stained with 50 μ/ml propidium iodide (Sigma, St. Louis, MO). The cell cycle distribution was determined by FACS and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Preparation of RNA and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated using RNeasy minikit (QIAGEN, Valencia, CA). cDNA was synthesized from 2.5 μg of total RNA with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR analysis was performed using DNA Engine Opticon fluorescence detection system (MJ Research, Foster City, CA). The PCR was set up in 20 μl with 0.25 μM of each primer; 2 μl of 1:3 diluted cDNA template; and 10 μl of 2× Master Mix containing modified DNA polymerase, PCR buffer, SYBR Green I, 5 mM $MgCl_2$, and 4 mM deoxynucleotide triphosphate mix including deoxyuridine 5-triphosphate supplied from the DyNAmo SYBR Green qRT-PCR kit (MJ Research). The cycling was performed using the following program: 95 C 5 min, 40 cycles of 94 C 30 sec, 58 C 30 sec, and 72 C 30 sec. Comparative cycle threshold method ($2^{-\Delta\Delta CT}$) was used to semiquantitate the target signal change in treatment *vs.* control (44). The TATA-binding protein gene was used as an internal control to normalize the amount of cDNA added to the PCR. The following primers were used: IGFBP-3, 5′-AAGTTCCACCCCCTCCATTC-3′ (sense), 5′-TCTTCCATTTCTCTACGGCAG-3′ (antisense); TATA-binding protein, 5′-TGCTGAGAAGAGTGTGCTGGAG-3′ (sense), 5′-TCTGAATAGGCTGTGGGGTC-3′ (antisense). AQ: A

Determination of IGFBP-3 secretion by LNCaP cells

IGFBP-3 levels in conditioned medium secreted by LNCaP cells were measured using an ELISA kit according to the manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX).

Plasmid constructions

The IGFBP-3 promoter reporter constructs containing −5992/+55, −3590/+55, and −1901/+55 fragment in pGL3-Basic (Promega, Madison, WI) were previously reported (21). A 1.85-kb fragment from −3590 to −1753 was cloned into the heterologous thymidine kinase (TK) promoter-driven luciferase reporter (LUC) (45). Synthetic oligonucleotides for IGFBP-3 ARE (−2879/−2865) were annealed and cloned into *Mlu*I/*Xho*I sites in TK-LUC. Annealed BP3-VDRE oligonucleotides (21) were ligated to the BP3-ARE/TK construct to generate the BP3-ARE/VDRE construct. A single-point mutation (G to T) in the IGFBP-3 ARE site was introduced into the −3590/+55/pGL3-LUC reporter construct using the GeneEditor *in vitro* site-directed mutagenesis system (Promega) following the manufacturer's instructions. Oligonucleotides containing point mutations in the BP3-ARE or BP3-VDRE sequence with *Mlu*I/*Xho*I site overhangs were synthesized and directly cloned into the TK-LUC vector. Positive clones were confirmed by sequencing.

Transfection analysis

LNCaP cells were plated at a density of 3–5 $\times$ 10^5 cells in six-well plates the day before transfection. At approximately 75% confluence, the cells were transfected with 1 μg DNA using 2 μl of TransIT-Insecta transfection reagent per well (Mirus, Madison, WI). Ten nanograms of the *Renilla* luciferase plasmid pRL-null (Promega) were included in each transfection to control for the transfection efficiency. After 20 h the cells were treated with ethanol or 10 nM R1881, a synthetic androgen, in the absence or presence of calcitriol and incubated for an additional 18 h. The cells were lysed using passive lysis buffer (Promega). Luciferase activity was determined using the dual-luciferase assay system (Promega) that was normalized to the *Renilla* luciferase activity. Activity of each construct was expressed as fold induction over control.

Isolation of nuclear extracts and EMSA

Nuclear extracts from LNCaP cells were isolated using the 1-h minipreparation technique (46). EMSAs were performed with modifications as described previously (21, 47). Annealed BP3-ARE oligonucleotides (5′-CCAAAGGCTCTTTCAGTTCTAGGA-3′) were radiolabeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. DNA binding reactions were carried out in a total volume of 20 μl containing 4 mM HEPES (pH 7.9), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 2 μg poly(dI:dC), and 5 μg BSA. Nuclear extracts (10 μg) were added along with 0.1–0.4 ng radiolabeled probes (50,000 cpm/reaction) and incubated for 20 min at room temperature. For competition assays, a 100-fold molar excess of unlabeled oligonucleotides was added to the binding reaction mixture 15 min before the addition of the labeled probe.

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The DNA-protein complexes were separated on 4% nondenaturing polyacrylamide gels (29:1) and processed as described previously (21).

Chromatin immunoprecipitation assay (ChIP)

Confluent LNCaP cells were treated with ethanol or 10 nM R1881 for 20 min and subjected to ChIP assay using AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (21). The purified DNA fragments were subjected to PCR using primers (sense: 5′-AGATTTCCTGTCTCCACCAATACG-3′; antisense: 5′-TGCTTTTCTTCTGCTCAGCCC-3′), which amplify a 259-bp fragment spanning the BP3-ARE in the IGFBP-3 promoter. The PCR was carried out at 95 C for 5 min and then 30 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec with a final extension for 8 min at 72 C.

Western blot analysis

LNCaP cells were treated with vehicle or R1881 for 40 h. Extracts were prepared in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA] containing a protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN). Supernatants were subjected to SDS-PAGE and Western blot analysis using p21, p27, and β-actin mouse monoclonal antibodies (Santa Cruz Biotechnology) as described previously (45).

Results

Induction of growth arrest and stimulation of cyclin-dependent kinase (CDK) inhibitors by androgens in LNCaP cells

Previous studies from different groups have demonstrated that LNCaP cells exhibit a biphasic growth pattern in response to androgens with low concentrations stimulating cell proliferation and high concentrations inducing growth arrest (33, 38–40, 48). In our hands, LNCaP cells have always shown this biphasic effect (40). As shown in Fig. 1A, 0.1 nM R1881 almost doubled the amount of DNA, whereas 1 and 10 nM R1881 caused an approximate 50% reduction in DNA content, compared with the vehicle control, reflecting a typical biphasic growth response of LNCaP cells to androgens, either R1881 or dihydrotestosterone. In concurrent experiments, we examined the effect of the growth-inhibitory dose of R1881 on the level of the CDK inhibitors p21 and p27 by Western blot, proteins associated with cell cycle arrest. Consistent with previous reports (49, 50), addition of 10 nM R1881 caused an increase in CDK inhibitors p21 and p27 (Fig. 1B). This increase resulted in about 11% more cells accumulating in the G_1 phase of the cell cycle accompanied by a decrease in the $S/G_2/M$ fraction as shown in Table 1. In contrast, 0.1 nM R1881 with growth-stimulatory effect resulted in a small but significant decrease in the proportion of cells in G_1 with an increase in $G_2/M/S$ phase. Our observations confirm previous reports that G_1 arrest contributes to growth inhibition induced by high concentrations of androgens (48).

TABLE 1. Effecs of R1881 treatment on cell cycle in LNCaP cells

Treatment	G_1 (%)	G_2/M/S (%)
Ethanol	70.4 ± 0.9	21.9 ± 0.8
R1881, 0.1 nM	65.6 ± 0.7[a]	26.3 ± 0.6[a]
R1881, 10 nM	81.3 ± 1.4[b]	10.6 ± 1.1[b]

LNCaP cells were treated with different doses of R1881 for 48 h. Cell cycle was analyzed by flow cytometry as described in *Materials and Methods*. Results showing the percentage of the cells in each phase are the mean ± SE of triplicate sample. The data represent one of two independent experiments.

[a] $P \leq 0.05$, compared with ethanol.

[b] $P \leq 0.01$, compared with ethanol.

A growth-inhibitory dose of androgen up-regulates IGFBP-3

To determine whether high concentrations of androgens affect the expression of IGFBP-3, an important growth regulator, we treated LNCaP cells with 10 nM of androgens and analyzed IGFBP-3 gene expression over time. As shown in Fig. 2A, R1881 treatment, relative to control, elicited a significant 2.5-fold increase in IGFBP-3 gene transcription after 6 h. The IGFBP-3 mRNA levels continued to increase with longer incubation times. IGFBP-3 protein levels were unchanged at 6 h but increased after 12 h and remained elevated after 24 and 48 h (Fig. 2B). Addition of cycloheximide, a protein synthesis inhibitor, failed to block IGFBP-3 induction by androgens (data not shown). These results suggest that R1881 may directly regulate the expression of IGFBP-3 in LNCaP cells. Similar findings were obtained with dihydrotestosterone (data not shown).

We next determined whether the increase in IGFBP-3 by R1881 was AR dependent using the androgen antagonist casodex. Addition of 10 μM casodex blocked IGFBP-3 induction by R1881 demonstrating that IGFBP-3 induction is mediated by the AR (Fig. 2C).

Transactivation of the IGFBP-3 promoter by androgens

To determine whether androgens directly regulate the transcription of IGFBP-3, we transfected LNCaP cells with IGFBP-3 promoter reporter constructs previously described by our laboratory (21). As shown in Fig. 3, the −1901/+55

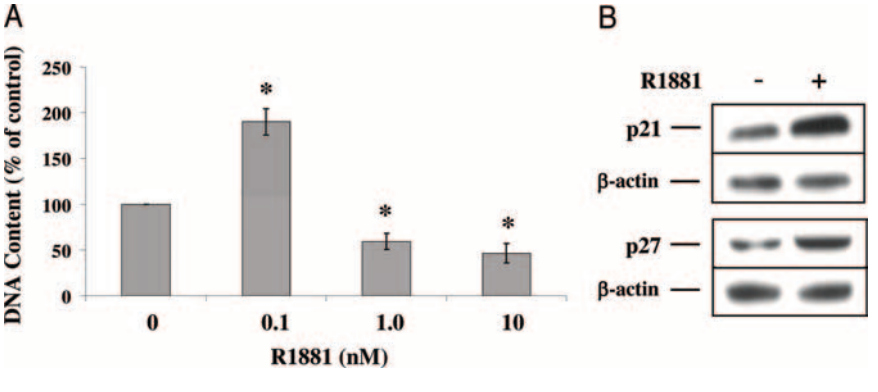

FIG. 1. A growth-inhibitory dose of R1881 induces the levels of p21 and p27 in LNCaP cells. A, LNCaP cells were treated with increasing concentrations of R1881 (0–10 nM). Media were changed every 3 d and fresh hormones added. On d 6, the cells were harvested for DNA assay. The DNA content of each treatment was expressed as percentage of control, the mean ± SE of three experiments. *, $P < 0.05$, compared with the control group. B, LNCaP cells were treated with vehicle or 10 nM R1881 for 48 h. The cell pellets were subjected to protein extraction and then Western blot analysis of p21, p27, and β-actin.

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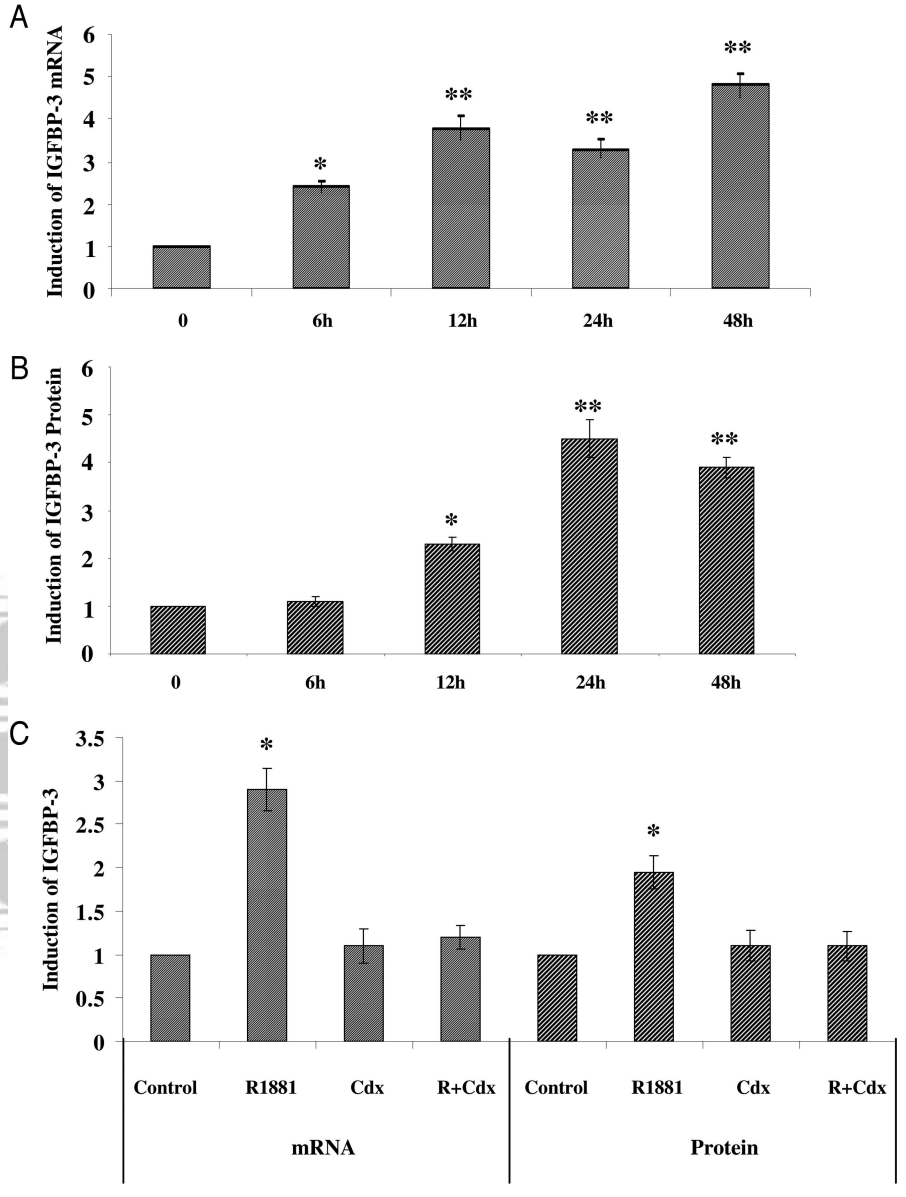

FIG. 2. Growth-inhibitory doses of androgens increase IGFBP-3 mRNA and protein expression in LNCaP cells. Confluent LNCaP cells were treated with vehicle or 10 nM R1881 and samples taken over time (A and B). A, RNA was prepared for qRT-PCR analysis. B, The conditioned medium was collected for determining IGFBP-3 levels using an ELISA. C, LNCaP cells were treated with 10 nM R1881 in the absence or presence of 10 μM casodex (Cdx) for 16 h and then processed for IGFBP-3 mRNA and protein. Values are expressed as fold change over control. The data represent three separate experiments (mean ± SE). *, $P < 0.05$; **, $P < 0.01$, compared with the vehicle-treated group.

construct was unresponsive to R1881 treatment, whereas the −3595/+55 construct exhibited approximately 1.9-fold induction by R1881. No further increase in transactivation was observed when fragments up to −5992 were tested. The results show that the region from −3595 to −1901 in the IGFBP-3 promoter contains a potential ARE.

Identification of a functional ARE in the IGFBP-3 promoter

To determine whether the sequence between −3590 and −1901 can act as an enhancer element, we cloned a 1.85-kb fragment from −3590 to −1753 into a luciferase reporter driven by the TK-LUC promoter (45). As shown in Fig. 4A, in transactivation assays in LNCaP cells, the 1.85 kb sequence exhibited approximately 2-fold increase in luciferase activity after R1881 treatment. Addition of casodex blocked the induction by R1881 (Fig. 4A). The empty TK-LUC vector was unresponsive to R1881 (data not shown).

In silico analysis of this 1.85-kb fragment identified a potential ARE located between −2879 and −2865. As shown in Fig. 4B, the putative BP3-ARE (5′-**GGCTCT** TTC **TGTTCT**-3′) contains two partial direct repeats separated by a three-nucleotide spacer. (The *bold letters* indicate the consensus sequence.) The BP3-ARE is 92% identical with the ARE of the secretory component promoter (SC ARE1.2) and 87% identical with the ARE consensus sequence (41, 51, 52). The SC ARE1.2 has been characterized as a high-affinity AR-specific ARE (51).

To determine whether the BP3-ARE sequence functions as an ARE, we subcloned the BP3-ARE sequence directly into the TK-LUC vector (BP3-ARE/TK). LNCaP cells were transfected with the BP3-ARE/TK heterologous reporter. Treatment with R1881 resulted in an approximately 2-fold induction of luciferase activity (Fig. 4C). To further confirm the BP3-ARE acts as an enhancer element, we created a G to T mutation (TGTTCT to TtTTCT) (Fig. 5A) in the BP3-ARE sequence in the −3590/+55 native promoter reporter con-

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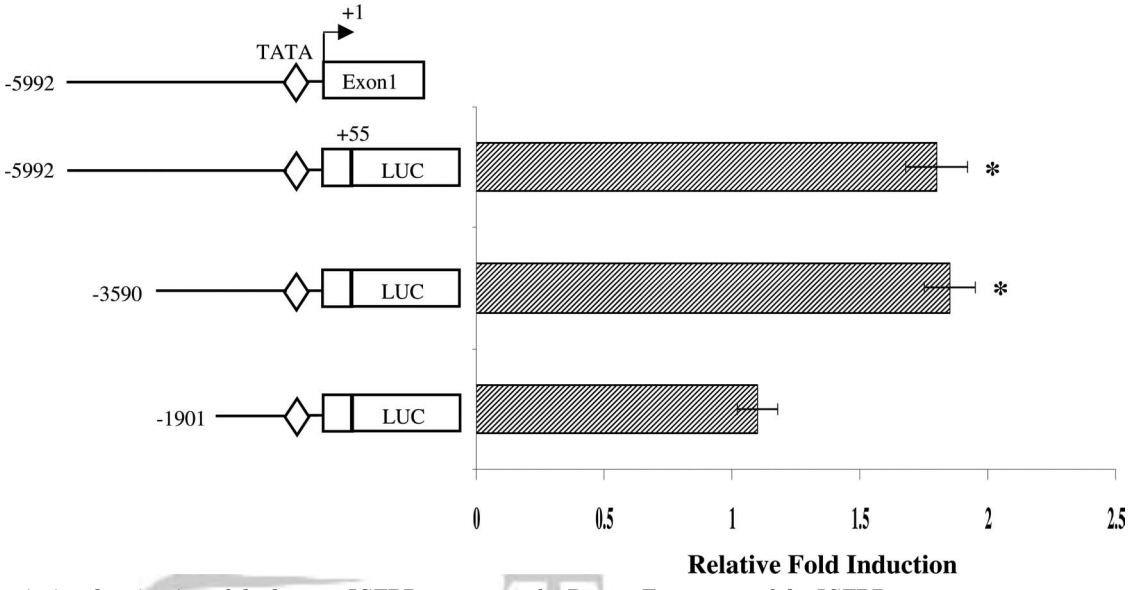

FIG. 3. Transcriptional activation of the human IGFBP-3 promoter by R1881. Fragments of the IGFBP-3 promoter sequence were cloned into the pGL3-basic reporter vector as previously described (21). Transfected LNCaP cells were treated with vehicle or 10 nM R1881 for 18 h. A *Renilla* luciferase expression vector was used to control for transfection efficiency. The activity of each construct is expressed as treatment *vs.* control that is set at 1. Each value represents the mean ± SE of three independent transfections, each performed in triplicate. *, $P < 0.05$, compared with the control group.

struct as well as the BP3-ARE/TK heterologous construct. As shown in Fig. 5B, the mutation resulted in a loss of androgen inducibility for both constructs.

Confirmation of a novel ARE in the IGFBP-3 promoter

EMSAs were used to determine whether AR binds to the putative IGFBP-3 ARE sequence. [^{32}P]-labeled BP3-AREs were incubated with LNCaP nuclear extracts in the presence or absence of R1881. A strong specific DNA-protein complex was observed with the androgen-treated cell extract (Fig. 6A, lane 1). When a 100-fold molar excess of unlabeled BP3-ARE or SC ARE1.2 oligonucleotide was added, the shifted band signal was diminished (Fig. 6A, lanes 2–3). In contrast, the DNA binding was not competed when unlabeled mutant BP3-AREmt oligonucleotides were added (Fig. 6A, lane 4).

ChIP assays were performed to determine whether R1881 recruits AR to the chromatinized BP3-ARE *in vivo*. LNCaP cells were treated with or without 10 nM R1881 and then subjected to ChIP assay. PCR was performed with the purified immunoprecipitated DNA using primers designed to amplify the IGFBP-3 promoter sequence spanning the BP3-ARE. As shown in Fig. 6B, in the AR antibody (lanes 5–6) immunoprecipitation reactions, a 259-bp DNA fragment was amplified only in the androgen-treated cells (lane 6), indicating that the AR interacts with the BP3-ARE *in vivo* in a ligand-dependent manner. The results further confirm the presence of a functional ARE in the natural chromatin structure in intact cells.

Taken together, our data strongly demonstrate the presence of a functional BP3-ARE in the human IGFBP-3 promoter that is activated by the AR in response to the androgens.

Androgens and calcitriol synergistically activate the IGFBP-3 promoter through their respective response elements

We previously demonstrated that calcitriol up-regulates IGFBP-3 through a VDRE in the IGFBP-3 promoter (21). The VDRE site is approximately 400 bp upstream of the ARE site in the IGFBP-3 promoter. The physical position of each response element in the native promoter is shown in Fig. 7A. To examine the combined effects of androgens and calcitriol on the IGFBP-3 promoter, a DNA fragment containing BP3-ARE and BP3-VDRE sequences in the IGFBP-3 promoter was cloned into the TK-LUC vector. Mutant constructs containing either a mutated ARE site or mutated VDRE site or both mutated sites were also examined. As shown in Fig. 7B, the intact sequence responded to each hormone individually and exhibited a greater activation in the presence of both ligands. Mutations in either the ARE or VDRE sites resulted in the loss of response to the corresponding hormone. Mutation of both response elements eliminated the response to both ligands.

Discussion

In this study we first verified that our LNCaP cell system displayed a biphasic growth response to androgens (Fig. 1A) as previously reported (33, 38–40). A growth-inhibitory dose of R1881 induced the levels of the CDK inhibitors p21 and p27 (Fig. 1B), thus causing accumulation of cells in G_1 (Table 1). In contrast, the growth-stimulatory dose of R1881 triggered an increase in cells in the $S/G_2/M$ phase (Table 1). In our LNCaP cells, we found that growth-inhibitory doses of androgens up-regulated IGFBP-3 gene expression by a direct interaction of the AR with an ARE in the IGFBP-3 promoter. Based on the ability of casodex to block the induction, the androgen action on IGFBP-3 is ligand and AR dependent (Figs. 2C and 4A). The presence of a functional ARE motif is

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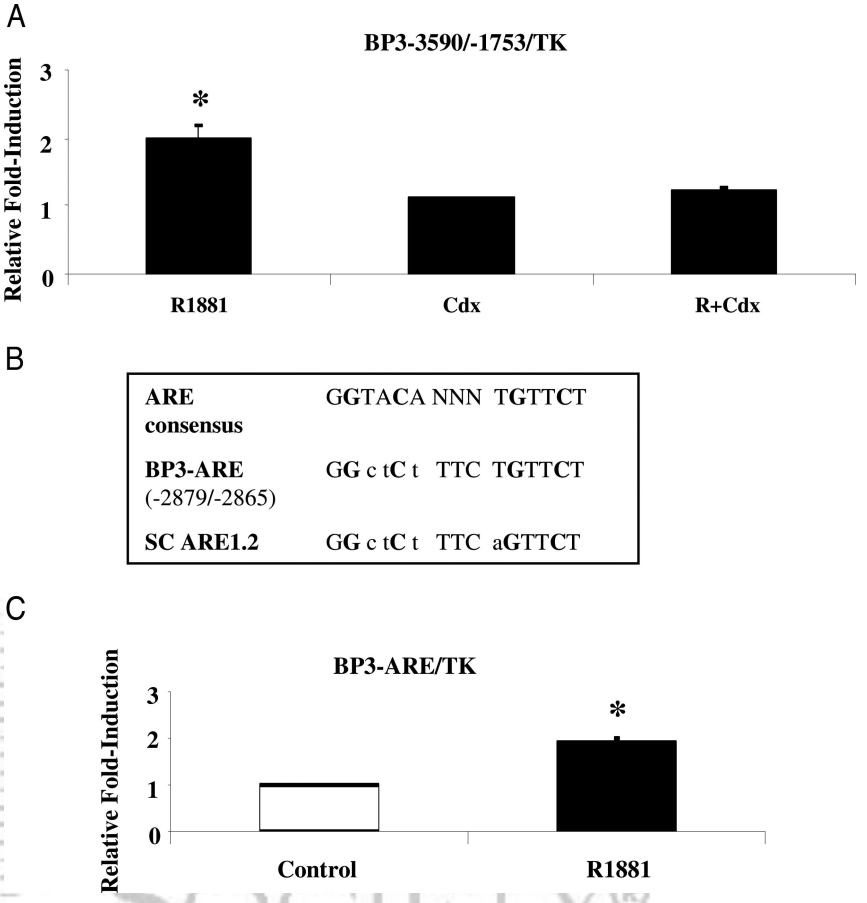

FIG. 4. Characterization of an ARE in the IGFBP-3 promoter. A, The TK-LUC heterologous construct containing the 1.85-kb fragment from −3590 to −1753 of the IGFBP-3 promoter was transfected into LNCaP cells and then treated with 10 nM R1881 or 10 μM casodex (Cdx) or 10 nM R1881 plus 10 μM casodex (Cdx+R). The activity of the construct was expressed as fold increase over control. B, Comparison of a potential ARE site at −2879/−2865 in the IGFBP-3 promoter (BP3-ARE) to the ARE consensus and the well-characterized ARE in the secretory component promoter (SC ARE1.2). The consensus nucleotides for AR binding are illustrated in *bold*, and *lower-case letters* represent variations form the consensus. C, The potential ARE was cloned into the TK-LUC vector. The construct was transfected into LNCaP cells and treated with vehicle or 10 nM R1881. The activity of the construct is displayed as fold induction by R1881 over the control. All transfections were carried out in triplicate, and the data represent the mean ± SE of three experiments. *, $P <$ 0.05, compared with the vehicle-treated group.

strongly supported by the fact that the BP3-ARE sequence confers androgen responsiveness in both its natural promoter setting and a heterologous promoter (Figs. 3 and 4).

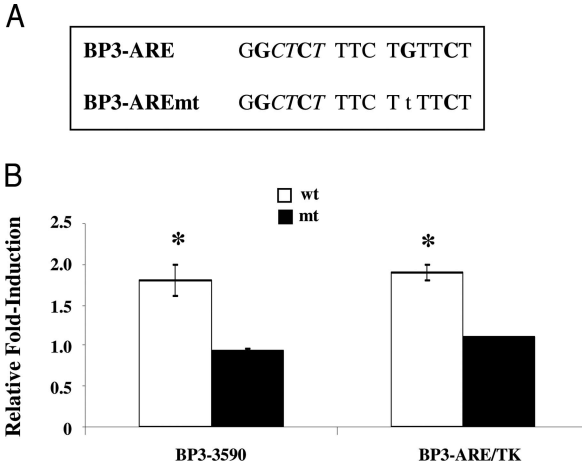

FIG. 5. The BP3-ARE is a functional ARE. A, Sequences of wild-type BP3-ARE or mutated BP3-ARE (BP3-AREmt). The *lower-case letter* represents the mutated base. B, The BP3-AREmt mutation (A) was introduced into the BP3–3590/+55 promoter construct and the BP3-ARE heterologous construct. These constructs were transfected into LNCaP cells and treated with vehicle or 10 nM R1881 for 18 h. The activity of each construct is expressed as fold induction over control. Data represent three independent experiments (mean ± SE), each performed in triplicate. wt, Wild type; mt, BP3-AREmt. *, $P < 0.05$, compared with the vehicle-treated group.

Mutations in the BP3-ARE eliminate androgen induction by either heterologous or natural promoters (Fig. 5). EMSAs and ChIP assays demonstrate specific recognition and binding of AR to the ARE sequence *in vitro* and *in vivo* (Fig. 6). Furthermore, we find that the combination of calcitriol and androgens results in a greater induction in IGFBP-3 promoter activity than is individually mediated through the interaction of their receptors with their respective hormone response elements (Fig. 7).

The BP3-ARE sequence exhibits a high degree of similarity with the SC ARE1.2 (5′-**GGCTCT** TTC **AGTTCT**-3′) located in the secretory component (sc) gene and the PB-ARE-2 (5′-**GGTTCT** TGG **AGTACT**-3′) found in the probasin promoter. Each of these AREs has been demonstrated to be specifically recognized by the AR with high binding affinity (51, 53). There are high homologies among steroid nuclear receptors, the glucocorticoid receptor (GR), progesterone receptor, and AR so that they sometimes are capable of overlapping into the same response element. The ability of some response elements to be highly selective for the AR probably acts in concert with other mechanisms to generate androgen-specific regulation of transcription *in vivo* (41). AQ: D

It is known that within the steroid nuclear receptor core binding site (5′-TGTTCT-3′) guanine and cytosine at position 2 and 5, respectively, are essential for high-affinity binding of this family including GR and progesterone receptor as well as AR (28, 54). On the other hand, the residues at position 3 and 4 determine specific binding of the family members.

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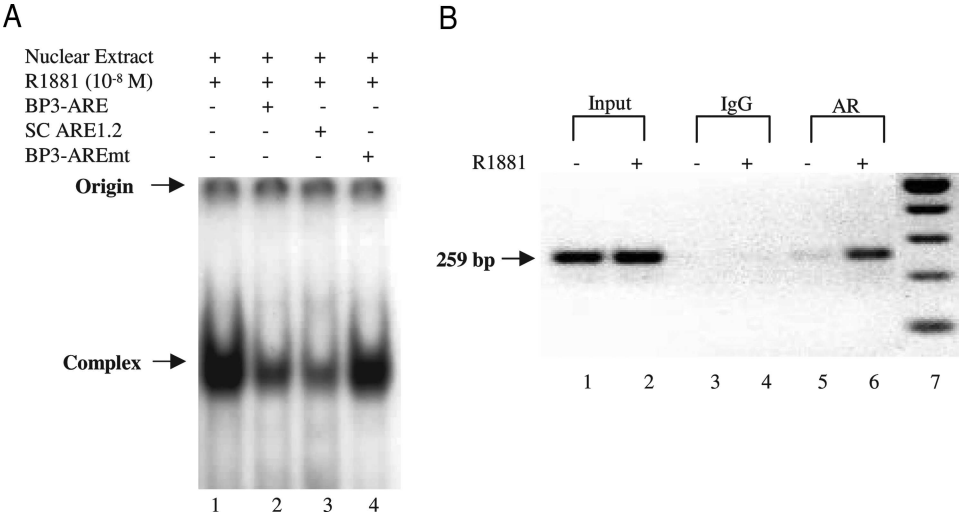

FIG. 6. The AR binds to the BP3-ARE *in vitro* and *in vivo*. A, EMSA showing specific binding of the BP-3 ARE with LNCaP nuclear extracts. LNCaP cells were treated with vehicle or 10 nM R1881 for 2 h and nuclear extracts prepared. The nuclear extracts were incubated with radiolabeled BP3-ARE in the absence (lane 1) or presence of 100-fold molar excess of cold competitor oligonucleotides as indicated. B, ChIP assay. LNCaP cells were treated with vehicle or 10 nM R1881 for 20 min. The cells were collected and subjected to ChIP assay using an AR antibody. The 259-bp PCR product encompasses the BP3-ARE sequence in the IGFBP-3 promoter. Lanes 1–2, Input DNA; lanes 3–4, IgG, mouse IgG; lanes 5–6, AR, anti-AR antibody; lane 7, 100-bp DNA ladder.

Verrijdt *et al.* (51) demonstrated that in the SC ARE1.2 (5′-**GGC*T*CT** TTC **AGTTCT**-3′), the thymidine residue at position 4 in the 5′ half-site is responsible for specific AR binding as opposed to GR binding. Therefore, the striking resemblance of the BP3-ARE and SC ARE1.2 and the presence of a thymidine at position 4 in the 5′ half-site of the BP3-ARE (5′-**GGC*T*CT** TTC **TGTTCT**-3′) suggest that the BP3-ARE may be an AR-selective element.

IGFBP-3 expression has been shown to be regulated by a variety of specific growth stimulators and inhibitors including TGFβ (55, 56), retinoic acid (56), calcitriol (20, 21, 57), TNFα (58), the histone deacetylase inhibitors trichostatin A and sodium butyrate (59), and androgens (23–27) as well as p53 (22, 56–58). Calcitriol exerts its antiproliferative actions by promoting G_0/G_1 cell cycle arrest, inducing IGFBP-3 production and stimulating apoptosis (60). In the androgen-dependent LNCaP cell line, calcitriol up-regulates the expression of the IGFBP-3 gene through a VDRE located at −3296/−3282 in the distal region of the IGFBP-3 promoter (21). Calcitriol-induced IGFBP-3 expression leads to increased expression of p21, causing cell cycle arrest (20). Evidence has shown that up-regulation of IGFBP-3 expression plays an important role in the *in vivo* growth-inhibitory effects initiated by calcitriol and its analogs (60, 61). On the other hand, the effect of androgens on the regulation of the IGFBP-3 gene remains controversial (23–27). Here we show a direct stimulatory effect of androgens on IGFBP-3 expression via AR-dependent action on the IGFBP-3 promoter. This stimulatory effect is similar to data from Martin and Pattison (24). On the contrary, Goossens *et al.* (23), Arnold *et al.* (25),

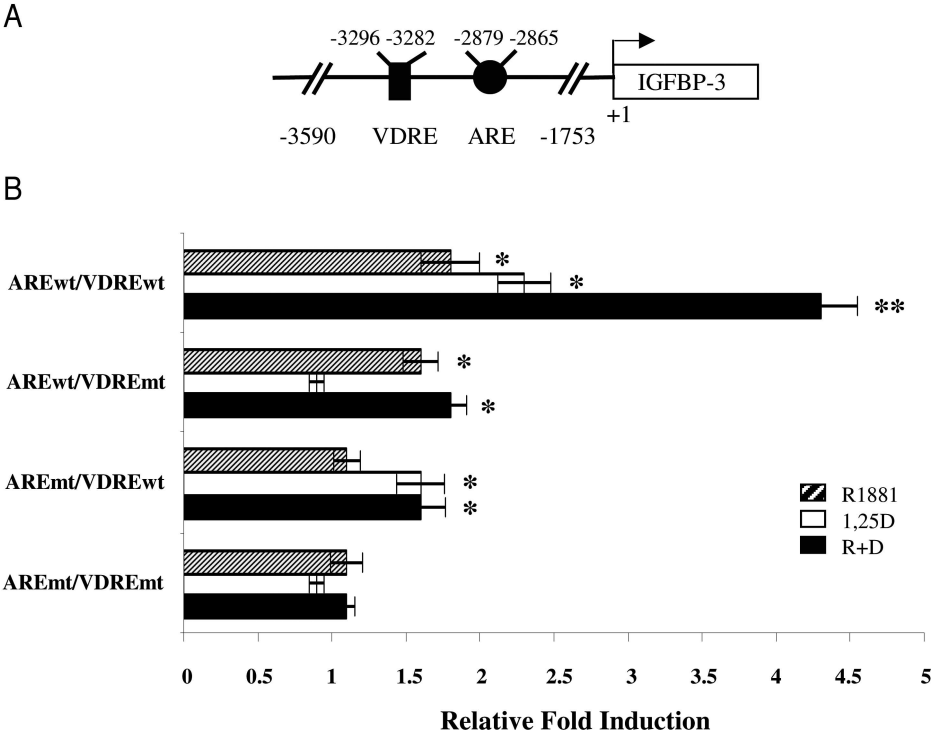

FIG. 7. IGFBP-3 regulation by R1881 and calcitriol is directly mediated through interaction with ARE and VDRE in the IGFBP-3 promoter. A, Schematic structure of the IGFBP-3 promoter showing positions of hormone response elements VDRE −3296/−3282 and ARE −879/−2865 relative to the transcription start site (+1). B, Single copies of the wild-type (wt) or mutant (mt) ARE and wt or mt VDRE oligonucleotide were cloned into the TK-LUC vector. The heterologous constructs were transfected into LNCaP cells and treated with vehicle, 10 nM R1881 (R), 10 nM calcitriol (1,25D), or 10 nM R1881 plus 10 nM calcitriol (R+D) for 18 h. The transactivation activity of each construct is expressed as treatment *vs.* control, which is set as 1. Each value represents the mean ± SE of three transfections, each performed in triplicate. *, $P < 0.05$; **, $P < 0.01$, when compared with the vehicle-treated group.

and Kojima *et al.* (27) reported a down-regulation of IGFBP-3 by androgens in LNCaP cells. The different observations between these groups may be a result of cell passage or differences in cell culture conditions or cell densities that can alter steroid responsiveness, even within the same cell line (62). However, our studies convincingly demonstrate that high concentrations of androgens directly up-regulate the expression of the IGFBP-3 gene through a functional ARE site in the IGFBP-3 promoter. These findings also reveal a cross-talk between the two cell growth regulators, IGFBP-3 and androgens, at the molecular level. The findings also raise an interesting possibility that induction of IGFBP-3 expression may be involved in the androgen mediated antiproliferative actions that have been reported in LNCaP cells (33–35, 40).

In addition, our observation of enhanced IGFBP-3 promoter activity by the interaction of androgens and calcitriol in transactivation assays suggests enhanced induction of the expression of the endogenous IGFBP-3 gene by these two hormone classes. The two regulatory elements, VDRE and ARE, are separated by approximately 400 bp and appear to interact or cooperate to regulate the level of IGFBP-3 expression. In a separate study we found that the two hormones in combination resulted in a more than 40-fold increase in IGFBP-3 mRNA and protein levels. Further detailed studies of the cross-talk between androgens and calcitriol on IGFBP-3 expression and the role of IGFBP-3 in regulating cell growth are described in that paper (Peng, L., J. Wang, P. J. Malloy, and D. Feldman, submitted for publication). This action may reflect the enhanced inhibition of LNCaP cell growth by combined calcitriol and androgens (63). These studies provide new insights into potential interactions between calcitriol and androgens, two regulators of IGFBP-3 expression.

The paradox raised by these studies is that androgens are well-known stimulators of prostate cancer growth (29, 64), yet they directly induce expression of IGFBP-3, an antiproliferative and proapoptotic protein (1–3). We speculate that some of the growth-inhibitory actions of androgens noted in some conditions (33–35, 37) may be related in part to IGFBP-3 induction. In a recent study, Hendriksen *et al.* (65) defined the AR pathway by selecting 200 genes from PCa xenografts, cell lines, and patient-derived tissues. Their conclusions were that in the well-differentiated state of early PCa, AR activity was involved in stimulating genes involved in growth promotion as well as growth inhibition and cell differentiation, resulting in a low growth rate. In high-grade PCa, there is a selective down-regulation of some AR target genes that inhibit proliferation or stimulate apoptosis or differentiation. Although the authors did not find IGFBP-3 in their arrays, their conclusions provide an attractive hypothesis to explain how androgens can induce a growth inhibiting prodifferentiating gene.

Acknowledgments

We thank Dr. Larisa Nonn (Stanford University Medical Center, Stanford, CA) for her advice on setting up FACS experiments. We greatly appreciate Gu Zhang (Genentech, South San Francisco, CA) for her help on FACS data analysis.

Received April 27, 2006. Accepted June 28, 2006.

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This work was supported by Department of the Army Grant DAMD17-02-1-0142 and National Institutes of Health Grant DK 42482.

Disclosure summary: the authors have nothing to disclose.

References

1. **Hwa V, Oh Y, Rosenfeld RG** 1999 The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20:761–787
2. **Firth SM, Baxter RC** 2002 Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824–854
3. **Cohen P** 2006 Insulin-like growth factor binding protein-3: insulin-like growth factor independence comes of age. Endocrinology 147:2109–2111
4. **Rajah R, Valentinis B, Cohen P** 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-β1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181–12188
5. **Rajah R, Khare A, Lee PD, Cohen P** 1999 Insulin-like growth factor-binding protein-3 is partially responsible for high-serum-induced apoptosis in PC-3 prostate cancer cells. J Endocrinol 163:487–494
6. **Kim HS, Ingermann AR, Tsubaki J, Twigg SM, Walker GE, Oh Y** 2004 Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. Cancer Res 64:2229–2237
7. **Santer FR, Bacher N, Moser B, Morandell, D, Ressler S, Firth SM, Spoden GA, Sergi C, Baxter RC, Jansen-Durr P, Zwerschke W** 2006 Nuclear insulin-like growth factor binding protein-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis. Cancer Res 66:3024–3033
8. **Leal SM, Liu Q, Huang SS, Huang JS** 1997 The type V transforming growth factor β receptor is the putative insulin-like growth factor-binding protein 3 receptor. J Biol Chem 272:20572–20576
9. **Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA, Baxter RC** 2000 Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin β subunit. J Biol Chem 275:23462–23470
10. **Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM, Cohen P** 2000 Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-α regulate transcriptional signaling and apoptosis. J Biol Chem 275:33607–33613
11. **Chokkalingam AP, Pollak M, Fillmore CM, Gao YT, Stanczyk FZ, Deng J, Sesterhenn IA, Mostofi FK, Fears TR, Madigan MP, Ziegler RG, Fraumeni JF, Hsing AW** 2001 Insulin-like growth factors and prostate cancer: a population-based case-control study in China. Cancer Epidemiol Biomarkers Prev 10:421–427
12. **Shi R, Berkel HJ, Yu H** 2001 Insulin-like growth factor-I and prostate cancer: a meta-analysis. Br J Cancer 85:991–996
13. **Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, Giovannucci E** 2002 Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. J Natl Cancer Inst 94:1099–1106
14. **Oh Y, Gucev Z, Ng L, Muller HL, Rosenfeld RG** 1995 Antiproliferative actions of insulin-like growth factor binding protein (IGFBP)-3 in human breast cancer cells. Prog Growth Factor Res 6:503–512
15. **Valentinis B, Bhala A, DeAngelis T, Baserga R, Cohen P** 1995 The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol Endocrinol 9:361–367
16. **Hong J, Zhang G, Dong F, Rechler MM** 2002 Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. J Biol Chem 277:10489–10497
17. **Liu B, Lee KW, Li H, Ma l, Lin GL, Chandraratna RA, Cohen P** 2005 Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands synergize on prostate cancer cell apoptosis *in vitro* and *in vivo*. Clin Cancer Res 11:4851–4856
18. **Silha JV, Sheppard PC, Mishra S, Gui Y, Schwartz J, Dodd JG, Murphy LJ** 2006 Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. Endocrinology 147:2112–2121
19. **Ali O, Cohen P, Lee KW** 2003 Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. Horm Metab Res 35:726–733
20. **Boyle BJ, Zhao XY, Cohen P, Feldman D** 2001 Insulin-like growth factor binding protein-3 mediates 1α,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. J Urol 165:1319–1324
21. **Peng L, Malloy PJ, Feldman D** 2004 Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18:1109–1119
22. **Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, Kley N** 1995 Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 377:646–649

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23. **Goossens K, Esquenet M, Swinnen JV, Manin M, Rombauts W, Verhoeven G** 1999 Androgens decrease and retinoids increase the expression of insulin-like growth factor-binding protein-3 in LNcaP prostatic adenocarcinoma cells. Mol Cell Endocrinol 155:9–18
24. **Martin JL, Pattison SL** 2000 Insulin-like growth factor binding protein-3 is regulated by dihydrotestosterone and stimulates deoxyribonucleic acid synthesis and cell proliferation in LNCaP prostate carcinoma cells. Endocrinology 141:2401–2409
25. **Arnold JT, Le H, McFann KK, Blackman MR** 2005 Comparative effects of DHEA *vs.* testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. Am J Physiol Endocrinol Metab 288:E573–E584
26. **Murthy S, Agoulnik IU, Weigel NL** 2005 Androgen receptor signaling and vitamin D receptor action in prostate cancer cells. Prostate 64:362–372
27. **Kojima S, Mulholland DJ, Ettinger S, Fazli L, Nelson CC, Gleave ME** 2006 Differential regulation of IGFBP-3 by the androgen receptor in the lineage-related androgen-dependent LNCaP and androgen-independent C4–2 prostate cancer models. Prostate 66:971–986
28. **Gelmann EP** 2002 Molecular biology of the androgen receptor. J Clin Oncol 20:3001–3015
29. **Hellerstedt BA, Pienta KJ** 2002 The current state of hormonal therapy for prostate cancer. CA Cancer J Clin 52:154–179
30. **Nelson WG, De Marzo AM, Isaacs WB** 2003 Prostate cancer. N Engl J Med 349:366–381
31. **Culig Z, Steiner H, Bartsch G, Hobisch A** 2005 Mechanisms of endocrine therapy-responsive and unresponsive prostate tumours. Endocr Relat Cancer 12:229–244
32. **Bruchovsky N, Lesser B, Van Doorn E, Craven S** 1975 Hormonal effects on cell proliferation in rat prostate. Vitam Horm 33:61–102
33. **Sonnenschein C, Olea N, Pasanen ME, Soto AM** 1989 Negative controls of cell proliferation: human prostate cancer cells and androgens. Cancer Res 49:3474–3481
34. **Olea N, Sakabe K, Soto AM, Sonnenschein C** 1990 The proliferative effect of "anti-androgens" on the androgen-sensitive human prostate tumor cell line LNCaP. Endocrinology 126:1457–1463
35. **Umekita Y, Hiipakka RA, Kokontis JM, Liao S** 1996 Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. Proc Natl Acad Sci USA 93:11802–11807
36. **Joly-Pharaboz M, Ruffion A, Roch A, Michel-Calemard L, Andre J, Chantepie J, Nicolas B, Panaye G** 2000 Inhibition of growth and induction of apoptosis by androgens of a variant of LNCaP cell line. J Steroid Biochem Mol Biol 73:237–249
37. **Chuu CP, Hiipakka RA, Fukuchi J, Kokontis JM, Liao S** 2005 Androgen causes growth suppression and reversion of androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in athymic mice. Cancer Res 65:2082–2084
38. **Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim Z, Amsel I, Shaw N, Prins GS, Kozlowski JM** 1995 Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. Endocrinology 136:796–803
39. **Esquenet M, Swinnen JV, Heyns W, Verhoeven G** 1996 Control of LNCaP proliferation and differentiation: actions and interactions of androgens, 1α,25-dihydroxycholecalciferol, all-trans retinoic acid, 9-cis retinoic acid, and phenylacetate. Prostate 28:182–194
40. **Zhao XY, Ly LH, Peehl DM, Feldman D** 1997 1α,25-dihydroxyvitamin D3 actions in LNCaP human prostate cancer cells are androgen-dependent. Endocrinology 138:3290–3298
41. **Verrijdt G, Haelens A, Claessens F** 2003 Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol Genet Metab 78:175–185
42. **Burton K** 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323
43. **Husbeck B, Nonn L, Peehl DM, Knox SJ** 2006 Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells. Prostate 66:218–225
44. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2[-$\Delta\Delta$C(T)] method. Methods 25:402–408
45. **Peng L, Payne AH** 2002 AP-2γ and the homeodomain protein distal-less 3 are required for placental-specific expression of the murine 3β-hydroxysteroid dehydrogenase VI gene, Hsd3b6. J Biol Chem 277:7945–7954
46. **Deryckere F, Gannon F** 1994 A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. Biotechniques 16:405
47. **Ikezoe T, Chen SS, Yang Y, Heber D, Taguchi H, Koeffler HP** 2003 PC-SPES: Molecular mechanism to induce apoptosis and down-regulate expression of PSA in LNCaP human prostate cancer cells. Int J Oncol 23:1461–1470
48. **de Launoit Y, Veilleux R, Dufour M, Simard J, Labrie F** 1991 Characteristics of the biphasic action of androgens and of the potent antiproliferative effects of the new pure antiestrogen EM-139 on cell cycle kinetic parameters in LNCaP human prostatic cancer cells. Cancer Res 51:5165–5170
49. **Lu S, Liu M, Epner DE, Tsai SY, Tsai MJ** 1999 Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. Mol Endocrinol 13:376–384
50. **Hofman K, Swinnen JV, Verhoeven G, Heyns W** 2001 E2F activity is biphasically regulated by androgens in LNCaP cells. Biochem Biophys Res Commun 283:97–101
51. **Verrijdt G, Schoenmakers E, Alen P, Haelens A, Peeters B, Rombauts W, Claessens F** 1999 Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element. Mol Endocrinol 13:1558–1570
52. **Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombautws W** 2001 Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol 7:23–30
53. **Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, Snoek R, Hamel A, Bock ME, MacDonald BS, Nickel BE, Chang C, Liao S, Cattini PA, Matusik RJ** 1993 Characterization of two *cis*-acting DNA elements involved in the androgen regulation of the probasin gene. Mol Endocrinol 7:23–36
54. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
55. **Oh Y, Muller HL, Ng L, Rosenfeld RG** 1995 Transforming growth factor-β-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. J Biol Chem 270:13589–13592
56. **Gucev ZS, Oh Y, Kelley KM, Rosenfeld RG** 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor β2-induced growth inhibition in human breast cancer cells. Cancer Res 56:1545–1550
57. **Colston KW, Perks CM, Xie SP, Holly JM** 1998 Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3. J Mol Endocrinol 20:157–162
58. **Rozen F, Zhang J, Pollak M** 1998 Antiproliferative action of tumor necrosis factor-α on MCF-7 breast cancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. Int J Oncol 13:865–869
59. **Tsubaki J, Choi WK, Ingermann AR, Twigg SM, Kim HS, Rosenfeld RG, Oh Y** 2001 Effects of sodium butyrate on expression of members of the IGF-binding protein superfamily in human mammary epithelial cells. J Endocrinol 169:97–110
60. **Krishnan AV, Peehl DM, Feldman D** 2005 Vitamin D and prostate cancer. In: Feldman D, Pike JW, Glorieux FH, ed. Vitamin D. San Diego: Elsevier Academic Press; 1679–1707
61. **Stewart LV, Weigel NL** 2005 Role of insulin-like growth factor binding proteins in 1α,25-dihydroxyvitamin D(3)-induced growth inhibition of human prostate cancer cells. Prostate 64:9–19
62. **Moffatt KA, Johannes WU, Hedlund TE, Miller GJ** 2001 Growth inhibitory effects of 1α, 25-dihydroxyvitamin D(3) are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. Cancer Res 61:7122–7129
63. **Zhao XY, Peehl DM, Navone NM, Feldman D** 2000 1α,25-Dihydroxyvitamin D3 inhibits prostate cancer cell growth by androgen-dependent and androgen-independent mechanisms. Endocrinology 141:2548–2556
64. **DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI** 2003 Pathological and molecular aspects of prostate cancer. Lancet 361:955–964
65. **Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, Jenster G** 2006 Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020

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