[CANCER RESEARCH 62, 338–340, January 15, 2002]

Advances in Brief

NKX-3.1 Interacts with Prostate-derived Ets Factor and Regulates the Activity of the *PSA* Promoter[1]

Hui Chen, Asit K. Nandi, Xiang Li, and Charles J. Bieberich[2]

Department of Biological Sciences, University of Maryland, Baltimore County, Baltimore, Maryland 21250

Abstract

The *NKX-3.1* homeobox gene maps to human chromosome 8p21, a region that undergoes frequent loss of heterozygosity in prostate tumors. Loss of *Nkx-3.1* function in mice leads to epithelial overgrowth. To further elucidate the molecular basis of NKX-3.1 function, a genetic screen for proteins that interact with NKX-3.1 was performed. Prostate-derived Ets factor (PDEF) was identified as a potential partner of NKX-3.1. Coimmunoprecipitation analyses demonstrated that NKX-3.1 and PDEF are physically associated in prostate epithelial cells. Cotransfection analyses demonstrated that NKX-3.1 can abolish the transcriptional activation function of PDEF on the *prostate-specific antigen* (*PSA*) promoter. These data identify *PSA* as a target gene for NKX-3.1 and provide new insights into the function of this candidate tumor suppressor.

Introduction

Loss of heterozygosity of chromosome 8p occurs in a majority of human prostate tumors (reviewed in Ref. 1). Loss of 8p12–21 appears to be an early event in the oncogenic process and is detected in prostatic intraepithelial neoplasia lesions. Although no bona fide tumor suppressor genes have been mapped to this region, a growing body of evidence points to the *NKX-3.1* gene, which maps to 8p21, as a viable candidate (1). First described in the mouse as an androgen-regulated prostate-restricted gene encoding a homeodomain transcription factor, *NKX-3.1* has come under increasing suspicion as a regulator of prostate cell growth and differentiation (2, 3). Unlike the canonical tumor suppressor p53, in tumors where one *NKX-3.1* allele is lost, the protein-coding region of the remaining allele is not mutated (4). However, a recent immunohistochemical study demonstrated decreased accumulation of NKX-3.1 protein in tumors, and diminished NKX-3.1 correlated with disease progression (5). Although these results are difficult to reconcile with the reported increase in *NKX-3.1* mRNA in prostate tumors, it is possible that post-transcriptional regulation plays an important role in modulating NKX-3.1 levels (6). The strongest evidence that NKX-3.1 regulates prostate growth has emerged from phenotypic analyses of *Nkx-3.1* knockout mice (7–9). Mice heterozygous for a null mutation in *Nkx-3.1* display prostate epithelial hyperplasia and dysplasia, demonstrating that loss of a single allele is sufficient to alter growth control (7). Homozygous null *Nkx-3.1* mutants show more profound epithelial hyperproliferation in addition to defects in branching morphogenesis and secretory protein production (7). To date, no prostate tumors have been reported in *Nkx-3.1* null mutants, suggesting that loss of *Nkx-3.1* alone is not sufficient to induce prostate malignancy.

To further understand the functions of NKX-3.1 in prostate biology, we have performed a screen to identify NKX-3.1-interacting proteins. In this report we demonstrate that NKX-3.1 interacts with PDEF,[3] a prostate-specific transcription factor that positively regulates *PSA* gene expression (10). We further demonstrate that expression of NKX-3.1 abrogates the transcriptional activation function of PDEF on *PSA* regulatory elements. These data provide new insights into the molecular basis of NKX-3.1 function and *PSA* gene regulation in prostate epithelial cells.

Materials and Methods

Yeast Two-Hybrid Screen. A full-length cDNA encoding the R52 allele of human *NKX-3.1* (4) was cloned in frame into pLexA (Clontech Laboratories, Palo Alto, CA), which encodes a full-length LexA protein containing a well-defined DNA binding activity. Expression of the LexA-NKX-3.1 fusion protein was confirmed by Western blot analysis using an anti-LexA polyclonal antibody (Invitrogen, Carlsbad, CA). A human prostate MATCHMAKER cDNA library (Clontech Laboratories), cloned into pB42AD containing a bacterial transcriptional activation domain, was amplified on plates, and plasmid DNA prepared from the library was used to transform a clone of yeast, EGY48[p8op-lacZ], harboring the pLexA/NKX-3.1 bait plasmid. EGY48[p8op-lacZ] also carries a plasmid-based *lacZ* reporter gene and a chromosomal *LEU2* reporter gene, both driven by a multimerized LexA operator site. This transformation yielded 10^7 cfu, and 100,000 independent clones were plated onto −His, −Trp, −Ura, −Leu plates. Eighty-one colonies grew and were patched onto −His, −Trp, −Ura, +X-gal plates. Three colonies that were blue in the presence of X-gal were plated onto +His plates to lose the bait plasmid; DNA prepared from these bait-negative derivatives was used to transform *Escherichia coli* strain DH10B. Sequence analysis of plasmids recovered from one EGY48 transformant revealed a nearly complete open reading frame encoding PDEF (10). The NH_2-terminal coding region of PDEF was completed by PCR.

Cotransfection and Coimmunoprecipitation. A full-length human *PDEF* cDNA was cloned in frame into pcDNA6/Myc-His (Invitrogen), which resulted in the addition of a COOH-terminal MYC epitope. A full-length *NKX-3.1* (R52 allele) cDNA was generated by reverse transcription-PCR using RNA from LNCaP cells. A 10-amino acid HA epitope tag (YPYDVPDYAS) was added to the COOH terminus by PCR, and *NKX-3.1*/HA was cloned into pcDNA3 (Invitrogen). LNCaP cells grown in RPMI 1640 supplemented with 10% FBS were transiently transfected using LipofectAMINE Plus (Invitrogen) according to the manufacturer's recommendations. Transfected cells were harvested after 48 h, and lysates were used for coimmunoprecipitation as described (11). PDEF was precipitated using either a mouse anti-c-*myc* monoclonal antibody (clone 9E10) or a rabbit antimouse PDEF polyclonal antibody (X. Li and C. J. Bieberich, unpublished observations). Immunoprecipitates captured on protein G-Sepharose beads were denatured with SDS-PAGE loading dye, and supernatants were separated on duplicate 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes essentially as described (11). The blots were probed with the anti-c-*myc* monoclonal antibody to detect MYC-tagged PDEF and with a rat anti-HA monoclonal antibody (clone 3F10) to detect the HA-tagged NKX-3.1.

***PSA* Reporter Gene Assays.** The 5.3-kb *PSA* promoter (12) was inserted upstream of the *luciferase* gene in pGL3 (Promega, Madison WI) to generate

Received 9/10/01; accepted 11/30/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1DK54067

[2] To whom requests for reprints should be addressed, at Department of Biological Sciences, University of Maryland, Baltimore County, Baltimore, Maryland 21250. Phone: (410) 455-3125; Fax: (410) 455-3875; E-mail: bieberic@umbc.edu

[3] The abbreviations used are: PDEF, prostate-derived Ets factor; AR, androgen receptor; PSA, prostate-specific antigen; X-gal, 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside

PSA/Luc. PSA/Luc was cotransfected with *PDEF* and *NKX-3.1* together or alone into LNCaP cells using LipofectAMINE Plus. The cells were lysed and the luciferase activity was measured using the Dual Luciferase Reporter assay system (Promega). The pRL-CMV construct, encoding *Renilla* luciferase, was used to normalize the transfection efficiency. Luciferase activity was quantified using a MLX microtiter plate luminometer (Dynex Technologies, Chantilly VA). Luciferase activity was measured in two independent wells of a 12-well plate. The experiment was repeated three times with essentially identical results, and a representative experiment is shown (see Fig. 3).

Northern Analyses. Whole RNA (3 μg) extracted from transfected and untreated LNCaP cells was separated on 1% glyoxal/DMSO/agarose gels (Ambion Inc., Austin, TX) and hybridized to a 523-bp PSA cDNA probe. PSA hybridization signal was quantified using a STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and normalized with respect to the hybridization signal observed when the same blots were probed with a β-actin cDNA probe.

Results and Discussion

The yeast two-hybrid screen for proteins capable of interacting with NKX-3.1 yielded a plasmid encoding *PDEF*. To demonstrate that PDEF was interacting with the NKX-3.1 portion of the LexA/NKX-3.1 fusion protein, the pB42AD/PDEF plasmid was cotransfected into yeast strain EGY48 carrying the parent pLexA vector. These cotransformants were not able to activate the *lacZ* reporter gene, indicating that NKX-3.1 is required for the interaction (Fig. 1). In addition, pLexA/NKX-3.1 together with the empty pB42AD vector also failed to result in activation of the *lacZ* reporter, demonstrating that PDEF is required for the interaction (Fig. 1). These data demonstrate that NKX-3.1 and PDEF are able to physically interact in yeast cells.

To determine whether NKX-3.1 and PDEF were capable of interacting in mammalian cells, LNCaP cells were transiently transfected with expression vectors encoding HA epitope-tagged NKX-3.1 and MYC-tagged PDEF either alone or in combination. Whole cell extracts prepared from LNCaP cells transfected with either NKX-3.1/HA or PDEF/MYC or both were immunoprecipitated with an anti-MYC epitope monoclonal antibody to bring down PDEF/MYC and any associated proteins. Western blot analysis of anti-MYC immunoprecipitates probed with an anti-MYC monoclonal antibody confirmed that PDEF/MYC was captured by the immunoprecipitation (data not shown). Western blot analysis of anti-MYC immunoprecipitates probed with an anti-HA monoclonal antibody revealed the presence of a single component of approximately 38 kDa exclusively in the extracts from cells cotransfected with *PDEF/MYC* and *NKX-3.1/HA* (Fig. 2). A band of identical size was present in lysates of LNCaP cells transfected with *NKX-3.1/HA* (Fig. 2, *Input*), confirming

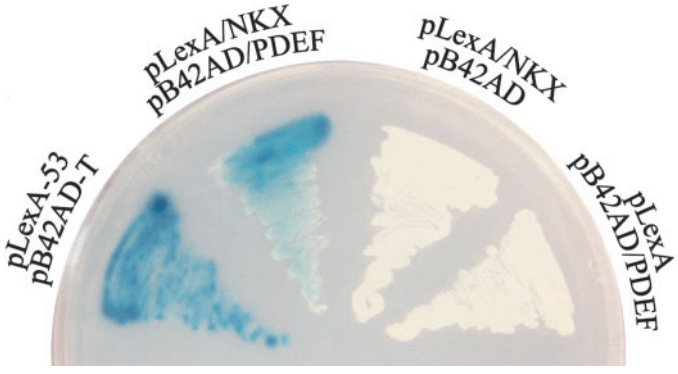

Fig. 1. PDEF interacts with NKX-3.1 in the yeast two-hybrid system. Yeast strain EGY48/[p8op-lacZ] was transformed with the indicated plasmids and selected for cotransformation. To test for interaction, colonies were streaked onto galactose- and raffinose-containing plates (−His−Trp−Ura+X-gal) to assay reporter gene expression. As a positive control, plasmids encoding a LexA/p53 fusion protein (pLexA-53) and an SV40 T antigen (pB42AD-T) were cotransformed into EGY48/[p8op-lacZ].

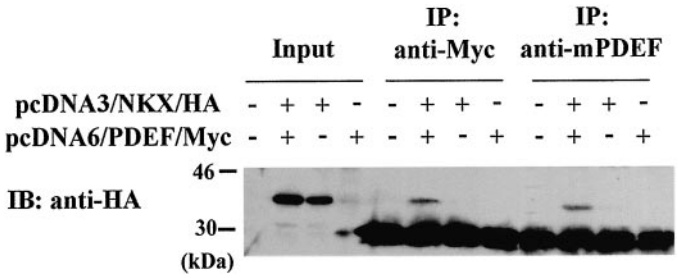

Fig. 2. Coimmunoprecipitation demonstrates interaction between PDEF and NKX-3.1 in LNCaP cells. The table indicates the constructs used to perform the transfection. Constructs pcDNA3/NKX/HA and pcDNA6/PDEF/Myc/were used to express NKX-3.1 and PDEF in cells, respectively. Empty vectors were used to balance the total amount of DNA. Immunoprecipitations of transfected cell lysates were performed using an anti-c-*myc* monoclonal antibody or polyclonal antimouse PDEF antibodies. The blots were probed with an anti-HA monoclonal antibody. The apparent molecular weight of HA-tagged NKX-3.1 was approximately 38,000. The 28-kDa band is immunoglobulin light chain. IP, immunoprecipitation; IB, immunoblotting.

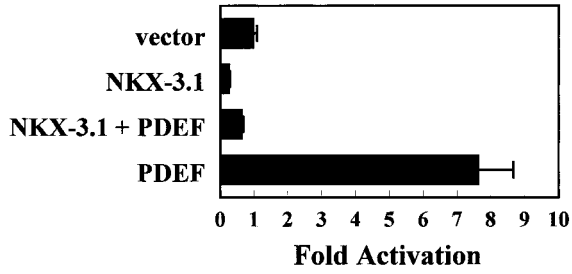

Fig. 3. NKX-3.1 abrogates PDEF-mediated transcriptional activation of the *PSA* promoter. LNCaP cells were cotransfected with the indicated PDEF and/or NKX-3.1 expression vector constructs, and reporter plasmids PSA/Luc and pRL-CMV. pRL-CMV was used to normalize the transfection efficiency. Empty vectors were used to balance the total amount of DNA. Luciferase activity in the lysates was determined 48 h after transfection. IP, immunoprecipitation; IB, immunoblotting.

that this band represents the HA epitope-tagged NKX-3.1 protein. Immunoprecipitation with antimouse PDEF polyclonal antibodies also brought down a complex containing NKX-3.1/HA exclusively in cotransfected cells (Fig. 2). These data demonstrate that NKX-3.1 and PDEF can physically associate in LNCaP cells, in agreement with the yeast two-hybrid analysis.

PDEF encodes a member of the Ets transcription factor family and is expressed predominantly in prostate epithelial cells (10, 13). Although its function in the prostate has not been defined, a recent study has demonstrated that PDEF is capable of synergizing with AR to activate transcription of the *PSA* promoter (10). *In vitro* analyses also showed that PDEF physically interacts with the DNA binding domain of AR.

To determine whether the coexpression of PDEF and NKX-3.1 altered the transcriptional activation function of PDEF on *PSA cis*-regulatory elements, reporter gene assays were performed on LNCaP cells transiently transfected with *NKX-3.1/HA*, *PDEF/MYC*, or both. A 5.3-kb region of the *PSA* promoter driving expression of a luciferase reporter was used to measure PDEF-mediated transcriptional activation. In the absence of transfected NKX3.1, transfected PDEF was able to sustain a nearly 8-fold activation of the *PSA* regulatory region, in good agreement with a previous report (10). Remarkably, cotransfection of *NKX3.1* with *PDEF* resulted in complete abrogation of PDEF-mediated transcriptional activation of the *PSA* regulatory region. In addition, transfection of *NKX-3.1* alone consistently reduced the basal activity of the transfected *PSA* promoter (Fig. 3), most likely by interfering with the function of endogenous PDEF present in LNCaP cells. To determine whether the transfection of *NKX-3.1* also affected the accumulation of endogenous PSA mRNA, RNA extracted

from untreated and *NKX-3.1*-transfected LNCaP cells was analyzed by Northern blot to determine the steady-state level of PSA mRNA. PhosphorImager analyses demonstrated a reproducible decrease of approximately 25% in the level of PSA mRNA in cells transfected with *NKX-3.1* (data not shown). Given the robust transcriptional activity of the *PSA* gene in LNCaP cells, this decrease represents a significant diminution in the level of endogenous PSA mRNA and is consistent with the assays that showed a reduction in the activity of the *PSA* reporter gene in cells transfected with *NKX-3.1*. These data demonstrate that coexpression of NKX-3.1 and PDEF in LNCaP cells has a profound effect on the ability of PDEF to transcriptionally activate the *PSA* promoter, and suggests that their physical interaction may have significant biological consequences.

Our observation that NKX-3.1 can function as a transcriptional repressor of the *PSA* promoter identifies the *PSA* gene as a likely target of NKX-3.1 function in prostate epithelial cells. This observation is consistent with a previous report demonstrating that NKX-3.1 can repress the activity of a basal promoter containing a multimerized NKX-3.1 binding site in TSU-PR1 prostate cells (14). However, mouse Nkx-3.1 has also been reported to act as a transcriptional activator of the smooth muscle γ-actin promoter through physical association with serum response factor. Serum response factor appears to relieve the activity of an inhibitory domain located in the COOH-terminal region of Nkx-3.1, and together they cooperatively activate smooth muscle γ-actin promoter activity (15). These data suggest that the effect of NKX-3.1 on transcription may depend on the nature of interacting partners that are coexpressed at any given time.

The interaction between NKX-3.1 and PDEF also implicates NKX-3.1 as a potential modulator of AR function. PDEF and AR have been shown to cooperatively activate the *PSA* promoter, and PDEF physically interacts with the DNA binding domain of AR *in vitro* (10). NKX-3.1 may compete with AR for binding to PDEF, or it may participate in formation of a complex that includes PDEF and AR, where it could exert its transcriptional repressor function. Given the central role played by AR signaling in prostate growth and disease, it will be of great interest to further dissect the interaction between NKX-3.1 and PDEF and its effect on PDEF-AR interaction.

Acknowledgments

We thank Takehiko Segawa for the gift of the *PSA* promoter plasmid.

References

1. Abate-Shen, C., and Shen, M. M. Molecular genetics of prostate cancer. Genes Dev., *14:* 2410–2434, 2000.
2. Bieberich, C. J., Fujita, K., He, W. W., and Jay, G. Prostate-specific and androgen-dependent expression of a novel homeobox gene. J. Biol. Chem., *271:* 31779–31782, 1996.
3. Sciavolino, P. J., Abrams, E. W., Yang, L., Austenberg, L. P., Shen, M. M., and Abate-Shen, C. Tissue-specific expression of murine Nkx3.1 in the male urogenital system. Dev. Dyn., *209:* 127–138, 1997.
4. Voeller, H. J., Augustus, M., Madike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res., *57:* 4455–4459, 1997.
5. Bowen, C., Bubendorf, L., Voeller, H. J., Slack, R., Willi, N., Sauter, G., Gasser, T. C., Koivisto, P., Lack, E. E., Kononen, J., Kallioniemi, O. P., and Gelmann, E. P. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res., *60:* 6111–6115, 2000.
6. Xu, L. L., Srikantan, V., Sesterhenn, I. A., Augustus, M., Dean, R., Moul, J. W., Carter, K. C., and Srivastava, S. Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. J. Urol., *163:* 972–979, 2000.
7. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. Roles for Nkx3.1 in prostate development and cancer. Genes Dev., *13:* 966–977, 1999.
8. Schneider, A., Brand, T., Zweigerdt, R., and Arnold, H. Targeted disruption of the Nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. Mech. Dev., *95:* 163–174, 2000.
9. Tanaka, M., Komuro, I., Inagaki, H., Jenkins, N. A., Copeland, N. G., and Izumo, S. Nkx3.1, a murine homolog of Ddrosophila bagpipe, regulates epithelial ductal branching and proliferation of the prostate and palatine glands. Dev. Dyn., *219:* 248–260, 2000.
10. Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., Quinn, G., Kas, K., Endress, G., Kunsch, C., and Libermann, T. A. PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. J. Biol. Chem., *275:* 1216–1225, 2000.
11. Houvras, Y., Benezra, M., Zhang, H., Manfredi, J. J., Weber, B. L., and Licht, J. D. BRCA1 physically and functionally interacts with ATF1. J. Biol. Chem., *275:* 36230–36237, 2000.
12. Segawa, T., Takebayashi, H., Kakehi, Y., Yoshida, O., Narumiya, S., and Kakizuka, A. Prostate-specific amplification of expanded polyglutamine expression: a novel approach for cancer gene therapy. Cancer Res., *58:* 2282–2287, 1998.
13. Yamada, N., Tamai, Y., Miyamoto, H., and Nozaki, M. Cloning and expression of the mouse Pse gene encoding a novel Ets family member. Gene, *241:* 267–274, 2000.
14. Steadman, D. J., Giuffrida, D., and Gelmann, E. P. DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1. Nucleic Acids Res, *28:* 2389–2395, 2000.
15. Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3–1, and serum response factor. J. Biol. Chem., *275:* 39061–39072, 2000.

NKX-3.1 Interacts with Prostate-derived Ets Factor and Regulates the Activity of the *PSA* Promoter

Hui Chen, Asit K. Nandi, Xiang Li, et al.

Cancer Res 2002;62:338-340.