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

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

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

Abstract

The NKK-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 NKK-3.1 function, a genetic screen for proteins that interact with NKK-3.1 was performed. Prostate-derived Ets factor (PDEF) was identified as a potential partner of NKK-3.1. Coimmunoprecipitation analyses demonstrated that NKK-3.1 and PDEF are physically associated in prostate epithelial cells. Cotransfection analyses demonstrated that NKK-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 NKK-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 NKK-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, NKK-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 NKK-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 NKK-3.1 protein in tumors, and diminished NKK-3.1 correlated with disease progression (5). Although these results are difficult to reconcile with the reported increase in NKK-3.1 mRNA in prostate tumors, it is possible that post-transcriptional regulation plays an important role in modulating NKK-3.1 levels (6). The strongest evidence that NKK-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 null mutants, suggesting that loss of Nkx-3.1 alone is not sufficient to induce prostate malignancy.

To further understand the functions of NKK-3.1 in prostate biology, we have performed a screen to identify NKK-3.1-interacting proteins. In this report we demonstrate that NKK-3.1 interacts with PDEF, a prostate-specific transcription factor that positively regulates PSA gene expression (10). We further demonstrate that expression of NKK-3.1 abrogates the transcriptional activation function of PDEF on PSA regulatory elements. These data provide new insights into the molecular basis of NKK-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 NKK-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-NKK-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/NKK-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 NH2-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 NKK-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 the 5.3-kb Promotor 1 PSA reporter gene and a luciferase gene in pGL3 (Promega, Madison WI) to generate a reporter plasmid. Following transfection, 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 denaturated 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 NKK-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

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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 luminoimeter (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/DSMO/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 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 that this band represents the HA epitope-tagged NKX-3.1 protein. Immunoprecipitation with antimouse PDEF polyclonal antibodies also brought down a complex containing HA epitope monoclonal antibody to bring down PDEF/MYC and any associated proteins. 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 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.

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**References**

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