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Nemo-Like Kinase Induces Apoptosis and Inhibits Androgen Receptor Signaling in Prostate Cancer Cells

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Abstract

BACKGROUND—The mitogen-activated protein kinases (MAPKs) regulate cell growth, differentiation, and stress responses, and many critical signaling pathways are subject to cross-regulation by MAPK signaling. Previous studies have yielded evidence of cross-talk between the MAPK pathways and androgen receptor (AR) signaling, which plays a critical role in growth control of both normal prostate and prostate cancer (PCa). Objective of this study was to evaluate the expression of MAPK-like protein nemo-like kinase (NLK) in PCa and its effects on AR-mediated transcription.

METHODS—Real-time PCR and IHC were used to evaluate levels of NLK in prostatic samples. Effects of over-expression of NLK on apoptosis and proliferation were determined using Western blot and flow cytometry. Effects on AR signaling were evaluated using over-expression and knockdown of NLK in PCa cells in combination with PCR, Western blotting and reporter assays.

RESULTS—Our results show that the expression of NLK is decreased in PCa metastases in comparison to normal prostate epithelium and primary PCa. Our results also show that over-expression of NLK resulted in induction of apoptosis, which was more pronounced in AR-expressing LNCaP versus AR-negative PC-3 cells. Higher levels of NLK decreased levels of AR mRNA and protein as well as inhibited AR-mediated transcription.

CONCLUSIONS—NLK expression is altered during PCa progression and it is involved in regulation of AR signaling in these cells. A deeper understanding of the roles of NLK in regulation of AR-mediated transcription and control of PCa progression may point the way to new modes of therapeutic intervention in this disease. *Prostate* 69: 1481–1492, 2009.

Keywords

prostate cancer; nemo-like kinase; androgen receptor; apoptosis

INTRODUCTION

As we strive to increase our understanding of prostate cancer (PCa) progression, it is important to delineate mechanisms underlying regulation of AR signaling and alterations in AR signaling pathway associated with PCa progression.

The androgen receptor (AR), a key modulator of growth and development of the prostate, is a critical factor involved in progression of PCa [1,2]. Association of androgens with AR promotes its nuclear localization and subsequent binding to androgen-response elements (ARE) in the promoters of androgen-regulated genes. AR-directed transcription is tightly controlled and the growing list of co-regulators of AR reflects the complexity of this signaling network [3]. Recently, attention has focused on the hypothesis that aberrant activation of AR through various signal transduction pathways plays an important role in tumor progression [4–6]. Achieving a better understanding of the molecular interactions and cross-talk between these signaling pathways may have a dramatic positive impact on therapeutic strategies for prevention and cure of PCa [1,2,4,6,7].

The MAPK pathways regulate cell growth, differentiation, and stress responses. Previous studies have yielded evidence of cross-talk between the MAPK pathways and AR signaling (reviewed in Refs. [8,9]), for example, AR is involved in androgen-mediated induction of *Xenopus* oocyte maturation via the MAPK-signaling cascade [10–12]. In PCa, constitutive activation of the Ras/MAPK signaling pathway has been shown to reduce the androgen requirement of PCa cells for growth and tumorigenicity [13], and recent reports indicate that MAP kinase kinase 1 (MEKK1) inhibits AR signaling and causes apoptosis in PCa cells [14,15].

Nemo-like kinase (NLK), a MAPK-like kinase, belongs to the proline-directed serine/threonine protein kinase superfamily, which consists of MAPKs and cyclin-dependent kinases (CDKs) [16,17]. Initial genetic studies in *C. elegans* indicated a role for NLK in regulation of the wnt signaling pathway [18,19]. In *C. elegans* NLK is present in complex with T-cell factor 4 (TCF-4) [18,20,21], and suppresses the activity of the TCF/lymphoid enhancer factor (LEF) family of transcription factors [22,23]. In zebrafish, however, NLK is an essential co-activator of wnt signaling, but over-expression of NLK can also negatively regulate the suppressive activity of TCF [24]. NLK has been shown to alter dramatically the activity of other transcription factors, including NF- κ B, Smads, AP1, and p53 [25,26].

We report here our investigation of expression of NLK and its effects on AR signaling in PCa cells. We have found that NLK expression is decreased in PCa metastases versus normal prostate and primary PCa, and that NLK induces apoptosis in PCa cells. We have also shown that inhibition of expression of endogenous NLK leads to increases in AR-mediated transcriptional activity, while over-expression of NLK dramatically inhibits this activity. Increased NLK expression correlated with decreased levels of AR and NLK was present in complex with AR in PCa cells. The observed involvement of NLK in AR-dependent signaling is consistent with a major role for this MAPK-like protein in control of PCa growth and progression, indicating that NLK signal transduction might be potential area for development of new therapies.

MATERIALS AND METHODS

Tissue Samples

Tissue samples were obtained from consented patients at the University of Washington Medical Center between 1999 and 2004. All studies were done following approval by the local Internal Review Board. Normal prostatic and PCa tissues were obtained as excess tissue from patients undergoing radical prostatectomy. For RT-PCR, samples of normal prostate and areas containing at least 80% cancer were micro-dissected from the same sample to produce matched pairs. Metastases were obtained through a rapid autopsy program [27]. For immunohistochemistry, the samples were fixed in 10% buffered formalin and embedded in paraffin. Bone metastases were decalcified in 5% formic acid before paraffin embedding.

Real-Time RT-PCR Analysis

STAT-60 (Tel-Test, Friendswood, TX) was used for RNA extractions from samples according to the manufacturer's instructions. A bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to assess preservation of 28S:18S ribosomal RNA. First-strand cDNA was transcribed from 1.0 µg RNA using an Advantage RT-PCR kit according to manufacturer's instructions (Clontech, Palo Alto, CA). Real-time PCR was carried out as we described previously [28]. The primers used were: NLK: CTTTGAGCCTGTCACCAATCCAA and CTTCCACCATCACTCCCACACCA; Prostate specific antigen (PSA): CCCCAGAATCACCCGAGCAG and ACCAGAGGAGTTCTTGACCCAAAA; AR: GGAAGTTGTGCATGCGGTACTCA and CCTGGCTTCCGCAACTTACAC; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): TGCACCACCAACTGCTTAGC and GGCATGGACTGTGGTCATGAG; epithelial glycoprotein (EGP): GCTGGAATTGTTGTGCTGGTTATTTTC and TGTGTCCATTGCTATTTCCCTTCTTC. Standard curves for each amplicon were generated from four-fold dilutions of LNCaP cDNA. Reactions were carried out in duplicate and expression levels were calculated from standard curves. Relative expression of NLK, AR and PSA were normalized to EGP (patient's samples) or GAPDH (cells).

Immunohistochemistry (IHC)

Five-µm sections were used for IHC, which was performed by standard procedure that we have described elsewhere [29]. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6, 10 min) and sections were incubated 1 hr at room temperature with a rabbit polyclonal anti-NLK antibody (4 µg/ml; Abcam, Cambridge, MA). Control slides were incubated with rabbit IgG instead of the primary antibody. Specific immunostaining, defined as optically dense reaction product within nuclei was assessed on a three-point scale: intense, faint, or absent reaction product. A quasi-continuous "NLK score" was created by multiplying each intensity level (0 for no stain, 1 for faint stain, 2 for intense) by the corresponding percentage of cells at that intensity in the slide staining, and then summing the results.

Cell Culture

Tissue culture media and supplements were purchased from Invitrogen, Inc. (Grand Island, NY). LNCaP, an AR-expressing, androgen-dependent PCa cell line; PC-3, an AR-negative, androgen-independent PCa cell line, and CHO cells, Chinese hamster ovary cells, were purchased from ATCC (Manassas, VA). C4-2, a subline of LNCaP cells, was obtained from Urocor, Inc. (Oklahoma City, OK). C4-2 cells express AR and are androgen-independent [30,31]. LAPC-4 cells, which express wild-type AR and are androgen-dependent were obtained from Dr. Sawyers (University of California, Los Angeles, CA) [32]. For AR mRNA degradation studies, LNCaP cells were transfected with NLK or mock transfected. After 40 hr, media were replaced with serum-free media for 2 hr, after which 10 mg/ml DRB (Sigma, Inc., Minneapolis MN) was added. Cells were harvested after 2, 4, and 8 hr of treatment.

Plasmids

NLK and kinase-negative NLK (NLK(K155M)) expression plasmids were gifts from Dr. Matsumoto (Nagoya University, Japan). The ARE-luciferase reporter construct and wild-type AR-pcDNA were provided by Dr. Plymate (University of Washington, Seattle, WA), and the 5.8-kb PSA promoter-luc plasmid was provided by Dr. Kim (Fred Hutchinson Cancer Research Center, Seattle, WA).

Transient Transfection and Reporter Assay

The Amaxa Nucleofector was used for transfection of PCa cells using solution V and program T-27, and CHO cells using solution T and program U-23 according to the manufacturer's recommendations (Amaxa Biosystems, Inc., Gaithersburg, MD). Cells (1×10^6 , LNCaP, C4-2, LAPC-4, PC-3 or CHO) were transfected with ARE reporter plasmid or PSA-promoter reporter plasmid (1 μ g) and, if specified, with the effector plasmids—AR, NLK, and NLK(K155M) (1 μ g or as specified), or 1 μ M siRNA NLK (SMARTpool siRNAs and four different siRNA to NLK, Dharmacon, Lafayette, CO) or siCONTROL, a non-targeting siRNA (Dharmacon). The TK renilla luciferase plasmid (2 ng) was transfected under the same conditions to enable normalization of transfection efficiencies. Cells were then incubated for 48 hr, lysed and subjected to a dual-luciferase assay according to manufacturer's recommendations (Promega, Madison, WI). For the reporter assays the amounts of plasmids transfected were normalized with pcDNA3.1. For examination of apoptosis, proliferation, IF and Western blot analysis control cells were mock transfected.

Apoptosis and Cell-Cycle Distribution Analyses

Effects on cell cycle distribution and apoptosis were examined in mock-transfected and NLK-transfected cells by flow cytometry 48 hr post-transfection. For cell cycle analysis, cells were resuspended in a 10 μ g/ml solution of 4,6-diamidino-2-phenylindole (DAPI) and 0.1% NP-40 in a Tris-buffered saline solution (pH 7.0) and were analyzed using an Influx cytometer (Cytospea, Seattle, WA). Analyses were performed with MultiCycle software (Phoenix Flow Systems, San Diego, CA). The Annexin V-FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA) was used according to the instructions of the manufacturer to detect apoptosis. Apoptosis was also examined by Western blot by detection of cleaved PARP using anti-PARP antibody (Cell Signaling Technologies, Beverly, MA).

Immunofluorescence

After transfection LNCaP cells were seeded onto Lab-Tek Chamber Slides (Nalge Nunc Naperville, IL). After 48 hr cells were fixed and incubated with a rabbit polyclonal anti-NLK antibody (5 μ g/ml, Abcam) or a mouse monoclonal anti-human AR antibody (1:60, BioGenex, San Ramon, CA) for 1 hr, and then with an goat-anti-mouse Alexa Fluor 546 or a goat anti-rabbit Alexa Fluor 488 at dilution 1:400 for 1 hr, followed by mounting with ProlongGold anti-fade reagent w/DAPI ((Invitrogen Corp.)

Western Blotting

Western blots were performed using total cell lysates as we have described previously [33]. Total cell lysates were prepared from mock-transfected and NLK-transfected cells 48 hr after transfection. Total protein levels were determined using the Bio-Rad DC Protein Assay kit (BioRad, Hercules, CA). Western blots were performed with a rabbit polyclonal anti-NLK antibody (Abcam); a mouse monoclonal anti-human AR antibody (BioGenex); and a rabbit polyclonal anti-human actin antibody (Sigma). Immunoreactivity was detected using an ECL detection kit (GE Healthcare, Piscataway, NJ).

Immunoprecipitation (IP)

Three hundred to 500 μ g of total proteins from mock-transfected and NLK-transfected cells were incubated with 2 μ g of a rabbit polyclonal anti-human AR antibody (C-19, Santa Cruz Biotechnologies, Inc.) pre-bound to Immunopure immobilized Protein A-agarose beads (Pierce, Rockford, IL) for 1 hr at room temperature. To ensure specificity of the detected associations, control IPs with rabbit IgG instead of the primary antibodies were performed under the same conditions. Immune complexes were used for Western blots as described above.

PSA Measurement

Levels of PSA in media were measured using the IMx Total PSA Assay (Abbott Laboratories, Abbott Park, IL) 48 hr post-transfection.

RESULTS

NLK Expression in Prostatic Tissues

We first examined expression of NLK in matched pairs of normal prostate and PCa samples from the same patient obtained from radical prostatectomy ($n = 12$). Serial dilutions of LNCaP cDNA were used to create a calibration curve to calculate relative expression of NLK. EGP mRNA levels were used to normalize for the epithelial cell content of the samples, since multiple cell types can be present in these samples. The analysis of real-time RT-PCR results revealed that levels of NLK mRNA were lower in PCa versus NP in all pairs examined ($51.27 \pm 7.78\%$, $P = 0.0007$), with decreases of more than 50% in 7/12 samples (Fig. 1A). We have also detected NLK mRNA in prostate cancer cell lines LNCaP, C4-2, PC-3, and LAPC-4 (data not shown).

Expression of NLK protein in PCa cell lines and prostatic samples was evaluated using Western blot and IHC, respectively. Western blot with antibody against NLK residues 500–515 which does not show any significant homology to other MAPKs, detected two bands in all PCa cells; a dominant band of expected molecular weight (~65 kDa) and a weaker band of ~50 kDa (Fig. 1B). More differentiated cells LNCaP, C4-2 and LAPC-4 showed higher levels of NLK than undifferentiated PC-3 cells.

IHC was used to examine levels of NLK in samples from patients with PCa. Our results show strong NLK immunoreactivity in nuclei of the epithelium of normal prostate and PCa cells (Fig. 1C). There was considerable heterogeneity in the nuclear localization of NLK in both types of tissue. In this small sample set, the analysis of the quasi-continuous NLK score did not reveal significant differences in the NLK score between normal prostate and PCa, (NP ($n = 5$): 148 ± 96 , PCa ($n = 7$): 174 ± 83). When PCa metastases were evaluated, we again observed significant heterogeneity in NLK nuclear immunoreactivity in all types of metastases (i.e., lymph nodes, bone, liver; 28 samples from 13 patients, Fig. 1C). Despite the heterogeneity of the staining, the NLK score of metastases was significantly lower (72 ± 54) than that of NP ($P = 0.001$) and PCa ($P = 0.0003$). Some cytoplasmic staining was detected in normal epithelium, primary PCa, and in metastases. Negative-control staining was performed with rabbit IgG instead of the anti-NLK antibody at the same concentration, and no immunoreactivity was detected on control slides (data not shown).

NLK Induces Apoptosis in PCa Cells

To examine whether NLK plays a role in control of PCa cells proliferation, LNCaP and PC-3 cells were transfected with NLK and viable cells were counted using Trypan Blue assay 2 days post-transfection. NLK over-expression decreased numbers of LNCaP cells to $58.85 \pm 7.80\%$ of the control mock-transfected cells ($P = 0.0004$, Fig. 2A), and smaller decreases were detected in PC-3 cells ($88.35 \pm 7.71\%$, $P = 0.041$). Because of the decreased cell number, we next investigated whether NLK causes apoptosis and/or cell-cycle arrest in these cells. To elucidate whether NLK expression induces apoptosis in PCa cells, we examined the levels of the 86-kDa fragment of poly (ADP-ribose) polymerase (PARP) in NLK-transfected PCa cells. NLK expression increased levels of the 86-kDa fragment of PARP in LNCaP and PC-3 cells suggesting apoptosis via activation of caspases (Fig. 2B). Levels of cleaved PARP were higher in LNCaP cells versus PC-3 cells which is in concordance with larger decreases in cell numbers of LNCaP cells. To ensure that the observed differences in PARP cleavage were not related to different levels of NLK in

LNCaP cells and PC-3 cells, we determined that similar levels of NLK were expressed in both cell types (Fig. 2B). Since we observed larger effects of NLK on LNCaP cells we used these cells for our additional investigations. Our data showed that the effects of NLK on PARP cleavage and LNCaP cell number were concentration dependent, and the increased levels of cleaved PARP were concordant with decreased cell numbers (Fig. 2C). FACS analysis confirmed the induction of apoptosis (~2-fold induction) by NLK in LNCaP cells (Fig. 2D, representative results are shown). Our analysis also showed that cell-cycle distribution was not altered in LNCaP cells by NLK expression (Fig. 2E).

NLK Inhibits AR-Directed Transcription in PCa Cells

Since inhibition of AR signaling induces apoptosis of PCa cells, and NLK expression induced apoptosis in AR-positive LNCaP cells to a higher extent than in AR-negative PC-3 cells, we focused on investigation whether NLK is involved in regulation of AR-mediated transcription. First, we investigated whether AR-mediated transcription is affected by endogenous NLK. Our results show that pooled siRNA directed to NLK decreased levels of NLK mRNA (Fig. 3A), and that this led to a 1.5-fold enhancement of AR-mediated signaling in comparison to LNCaP cells transfected with siCONTROL non-targeting siRNA using reporter assays with ARE-luc construct (Fig. 3B). To demonstrate the specificity of the effects detected with the pooled siRNA, we then used four different siRNAs against NLK. All of the NLK siRNAs decreased levels of NLK mRNA (Fig. 3A) and also caused increases in AR-mediated signaling (Fig. 3B). These results suggest a biological role of NLK in regulation of AR-mediated transcription in PCa cells. To further investigate the role of NLK in regulating AR-mediated transcription, we over-expressed NLK in LNCaP, C4-2, and LAPC-4 cells. Transfection with NLK resulted in large increases in NLK mRNA levels (Fig. 3C) and in inhibition of AR-mediated transcriptional activity in all three PCa cell lines tested (Fig. 3D). To determine whether kinase activity is involved in NLK effects on AR-mediated transcription, we employed a kinase-negative mutant NLK(K155M), in which Lys-155 has been converted to Met [18]. After transfection approximately equivalent expression levels of NLK and NLK(K155M) were detected in LNCaP cells by Western blot. In contrast to wild-type NLK, kinase-negative NLK(K155M) increased AR-directed transcription, possibly in a dominant-negative fashion (Fig. 3E).

AR is a transcription factor whose main activity is exerted in the nucleus, and therefore we investigated localization of NLK in LNCaP cells. Immunofluorescent staining clearly showed that both endogenous and over-expressed NLK is present in nuclei of LNCaP cells and co-localize with AR (Fig. 3F).

Since ARE-luc is an artificial promoter, we extended our studies to further substantiate the concept that NLK functions as a negative regulator of AR-mediated transcription by using a 5.8-kb prostate-specific antigen (PSA) promoter-luc construct. Over-expression of NLK suppressed activation of the PSA promoter in LNCaP cells to a similar extent seen with the ARE-luc construct (Fig. 4A). The biological relevance of this observation was confirmed by decreased levels of endogenous PSA mRNA and secreted PSA protein in conditioned media of NLK-transfected LNCaP cells (Fig. 4A). In addition, knockdown of endogenous NLK expression using multiple siRNAs to NLK resulted in significant increases in activation of the PSA promoter, again supporting the biological role of NLK in PCa cells (Fig. 4B).

To confirm the inhibitory effects of NLK on AR-mediated transcription in cells of a different phenotype we transfected AR-negative PC-3 and CHO cells with AR and NLK. AR-mediated transcription in AR-transfected PC-3 cells (Fig. 5A) and CHO cells (data not shown) was also inhibited by NLK. Because NLK inhibited AR-mediated transcription in multiple models, we wanted to investigate whether this inhibition is specific or may be due to general inhibition of transcription. For these experiments we used pGL3 parental plasmid.

Our results showed that basal pGL3-luc activity was not affected by NLK expression (Fig. 5B) suggesting that the inhibition of AR-mediated transcription by NLK was not due to general inhibition of transcription.

NLK Decreases Levels of AR mRNA and AR Protein

To begin to unravel the mechanism of NLK inhibition of AR-mediated transcription we examined whether NLK is involved in regulation of the AR expression. Real-time PCR showed that levels of AR mRNA were decreased in NLK-transfected LNCaP cells versus mock-transfected LNCaP cells (Fig. 5C), while abolishment of NLK using siRNA resulted in increased levels of AR mRNA in these cells (Fig. 5D). Lower levels of mRNA can be the result of multiple mechanisms including alteration of the mRNA stability and/or transcription. Therefore, next we investigated whether NLK expression alters AR mRNA stability. No differences in AR mRNA degradation were detected between NLK-transfected and mock-transfected LNCaP cells after treatment with DRB up to 8 hr (data not shown). To further examine whether NLK regulated AR transcription, we evaluated effects of NLK on AR mRNA levels in AR-transfected CHO cells, in which the expression of AR is driven by the CMV promoter. We did not observe any significant down-regulation of AR mRNA by NLK in these cells (Fig. 5C). These results suggest that NLK is involved in regulation of AR transcription. Western blot results were concordant with the PCR data; lower levels of AR protein were detected in NLK-transfected LNCaP cells versus mock-transfected LNCaP cells, while no differences in AR levels were observed between NLK-transfected and mock-transfected AR-over-expressing CHO cells (Fig. 5D).

Our results using AR-transfected PC-3 and CHO cells showed that NLK, in addition to decreasing AR levels, also inhibits AR-mediated transcription. Since MAPKs were shown to regulate AR activity in our preliminary experiments, we set out to determine whether NLK associates with AR as a potential mode of regulation of AR-mediated transcription. IP results showed that NLK is present in a complex with AR in NLK transfected LNCaP cells. These results points to additional mechanisms of NLK regulation of AR-mediated transcription, potentially similar to NLK's regulation of TCF4, Smad and NF- κ B activities. We did not detect any interactions in parental LNCaP cells, which might be due to a limited sensitivity of this assay.

DISCUSSION

To date no reports have been published on expression or roles of NLK in normal prostate or PCa. To investigate the possible involvement of NLK in progression of PCa and establish relevance to this disease, we first examined NLK expression by RT-PCR, Western blot, and IHC. Expression of NLK in normal prostatic epithelium and in PCa suggests that this kinase may be involved in regulation of growth of prostatic epithelium. Our RT-PCR results showed that NLK expression is decreased in concert with the development of cancer. In contrast to the mRNA, we have not seen significant differences in NLK protein when normal prostatic tissues and primary PCa were evaluated by IHC. This might be due to small sample set evaluated and/or lower sensitivity of IHC in comparison to PCR methodology. Therefore to conclusively determine whether NLK may be a potential biomarker for PCa and have a prognostic value will require analyses of a larger cohort of patients. However, IHC analysis of PCa metastases revealed significant decreases in nuclear localization of NLK in PCa metastases versus NP and primary PCa. These differences may be biologically significant, because our results showed that NLK co-localizes with AR in nuclei, and the principal action of AR as a transcription factor takes place in nuclei. These results demonstrate deregulation of the NLK signaling cascade in progression of PCa. Further studies with larger cohorts of patients are needed to investigate associations of NLK

expression with clinical parameters of this disease and determine whether NLK has promise as a prognostic marker.

The current first-line therapy for recurrent PCa is androgen ablation, which suppresses AR-mediated transcription and stimulates apoptosis of PCa cells. Our results show that over-expression of NLK recapitulates both aspects of this treatment in the LNCaP model. Our hypothesis is that a portion of the pro-apoptotic activity of NLK is related to inhibition of AR-mediated transcription. In AR-positive LNCaP cells, NLK expression resulted in much larger decreases in cell number and larger increases in levels of cleaved PARP when compared to AR-negative PC-3 cells, suggesting that induction of apoptosis in PCa cells is partially dependent on expression of AR; although other mechanisms are also involved. Induction of apoptosis via over-expression of NLK has been also reported in human colon cancer cells, which do not express AR [25,26]. Interestingly, there is a literature report that MEKK1, a member of the MAPKK family, induced apoptosis in PCa cells but increased the rate of AR-mediated transcription [14]. These observations together suggest that NLK activity depends on the cell type, and that the pro-apoptotic effects of the MAPK and MAPKK enzymes are exerted through a variety of mechanisms.

Our results show that NLK is expressed in PCa cells lines, is present in protein complexes with AR, and is involved in regulation of the AR signaling cascade in these cells. Importantly, using multiple PCa models our results showed that NLK inhibition of AR-mediated signaling is not limited to the mutated form of AR found in LNCaP and C4-2 cells, since NLK also inhibited AR-mediated transcription LAPC-4 cells, which express wild-type AR as well as wild-type AR transfected PC-3 cells.

In AR-positive PCa cells, NLK expression reduced levels of AR mRNA by inhibiting transcription of the AR gene and also inhibited AR-mediated transcription. These observations contrast with results in the TCF-4 and NF- κ B systems, where NLK inhibits signaling of these transcription factors, but their levels are not decreased [26,34]. To our knowledge this is the first observation of suppression of the level of a transcription factor by NLK. We hypothesize that the inhibitory effects of NLK on the wnt and NF- κ B pathways, which were described in other models may be involved in the down-regulation of AR-mediated transcription, since both of these NLK-regulated pathways are involved in AR regulation [35–38]. Reduction in levels of AR may prove to be a valuable means of achieving more complete suppression of AR signaling, especially in view of the fact that androgen ablation by chemical or surgical castration, even combined with AR antagonists, has not led to complete blockade of AR-mediated transcription.

CONCLUSIONS

Our results show that NLK nuclear localization is decreased in advanced PCa versus normal prostatic epithelium and primary PCa, and that NLK induces apoptosis and inhibits AR signaling in PCa cells. Mechanisms involved in reduced AR-mediated transcription include decreased levels of AR mRNA, as well as inhibition of AR-transcriptional activity. This suggests the possibility that exploitation of NLK signaling may lead to new means of blocking AR signaling as a different treatment option for advanced PCa. Further studies will be required to delineate the precise mechanisms of NLK action in this system and elucidate the value of this pathway as a therapeutic target in PCa.

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Fig. 1.

NLK mRNA and protein are expressed in prostatic samples and cell lines. **A:** Real-time PCR was used to quantify levels of NLK mRNA in matched pairs of normal prostatic tissues and PCa. Expression of NLK was normalized to the content of epithelial cells based on EGP mRNA levels. Expression of NLK messages is significantly decreased in PCa versus normal prostate epithelium. **B:** Western blot analysis shows presence of NLK protein in PCa cell lines. Strong 65 kDa band corresponds to expected molecular weight of NLK. **C:** IHC was used to investigate immunoreactivity of NLK in prostatic tissues. Representative examples are shown for: (1) normal prostate; (2 and 3) prostate cancer; (4 and 5) lung metastases of PCa; (6) lymph node metastasis of PCa; (7) ommental metastasis of PCa; and (8 and 9) bone metastases of PCa. NLK exhibit strong nuclear reactivity in NP and PCa, while in

metastases weaker nuclear staining was observed with increased cytoplasmic immunoreactivity.PCa.* $P < 0.05$.

Fig. 2.

NLK induces apoptosis in PCa cells. LNCaP and PC-3 cells were transfected with NLK plasmid or mock-transfected (control cells) as described in Materials and Methods Section. Cells were harvested 48 hr after transfection. **A:** Over-expression of NLK decreased the LNCaP cell numbers ~50%, while smaller decreases were detected in AR-negative PC-3 cells. **B:** NLK expression in LNCaP cells resulted in cleavage of PARP. Smaller increases in PARP cleavage were detected in PC-3 cells. **C:** Increases in PARP cleavage in LNCaP cells were concentration dependent and concordant with decreases in cell numbers. NLK expression was confirmed by Western blot. **D:** Flow cytometry confirmed the induction of apoptosis in LNCaP cells, over-expression of NLK caused a ~2.25-fold increase in apoptosis. **E:** Over-expression of NLK did not alter the cell-cycle distribution of LNCaP cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3.

NLK inhibits AR-mediated transcriptional activity. **A:** Transfection of NLK siRNA significantly decreased NLK mRNA in LNCaP cells. Control cells were transfected with siCONTROL RNA as described in method section. **B:** Knockdown of endogenous NLK augments AR-mediated transcription in LNCaP cells. ARE promoter activity was measured in the presence or absence of siRNAs against human NLK or siCONTROL in LNCaP cells. **C:** Transient transfection of NLK into LNCaP, C4-2, and LAPC-4 cells resulted in large increases in NLK mRNA in these cells. **D:** Over-expression of NLK inhibited AR-mediated transcription in PCa cells. The ARE reporter construct was transfected alone or co-transfected with NLK into LNCaP, C4-2, and LAPC-4 cells. The amount of plasmid was

normalized using pcDNA 3.1. **E:** Using NLK(K155M), which lacks kinase activity, we showed that NLK-kinase activity is critical for the inhibition of AR-mediated transcription by NLK. **F:** Immunofluorescent analysis showed that NLK co-localizes with AR in nuclei of LNCaP cells. Results are plotted as means $\pm$ standard error. * $P < 0.05$.

Fig. 4.

NLK inhibit expression of PSA in LNCaP cells. **A:** The 5.8 kb PSA promoter-luc construct was transfected alone or co-transfected with NLK into LNCaP cells. NLK almost completely inhibits PSA promoter activation. **B:** NLK-transfected and mock-transfected LNCaP cells were harvested 48 hr after transfection for RT-PCR analysis of PSA mRNA levels. NLK expression reduced levels of PSA mRNA and protein in LNCaP cells. **C:** Total PSA was determined in conditioned media of LNCaP cells 48 hr after transfection using the IMxTotal PSA Assay. NLK decreased levels of PSA secreted in condition media. **D:** Knockdown of NLK using siRNA resulted in increases in activation of PSA promoter as determined by reporter assay. All experiments were run in duplicate and repeated two to four times. Results are plotted as means $\pm$ standard error. * $P < 0.05$.

Fig. 5.

A: To confirm the NLK effects on AR-mediated transcription PC-3 cells were transfected with ARE-luc reporter and AR with and without NLK. The amount of transfected plasmid was matched using pcDNA3.1. AR transcriptional activity was suppressed by NLK in AR-expressing PC-3 cells. **B:** To demonstrate specificity of NLK effects on ARE-mediated transcription ARE-luc reporter construct or pGL-3 plasmid was transfected alone or co-transfected with NLK into LNCaP cells and luciferase activity was measured after 48 hr. Basal pGL3-luc activity was not affected by NLK in LNCaP cells suggesting that the inhibition of AR-mediated signaling by NLK was not due to general inhibition of transcription. **C:** Real-time PCR showed that NLK transfection decreased levels of AR mRNA in LNCaP cells, while levels of AR mRNA in CHO cells transfected to express AR under a CMV promoter were not altered by NLK. Control LNCaP and CHO cells were mock-transfected. These results suggest that the regulation of AR mRNA occurs at the level of transcription. **D:** Knockdown of endogenous NLK in LNCaP cells resulted in increases in AR mRNA levels, again supporting our assumption that NLK is involved in regulation of AR transcription. **E:** NLK expression decreased levels of AR in LNCaP cells but not in CHO cells expressing AR under a CMV promoter. **F:** Over-expressed NLK associates with AR in LNCaP cells. Total protein isolated from LNCaP cells transfected to over-express NLK and mock-transfected LNCaP cells were subjected to immunoprecipitation with a rabbit anti-human AR polyclonal antibody as described in method section. To ensure specificity control reactions with rabbit IgG were also performed. Results are plotted as means $\pm$ standard error. * $P < 0.05$.