Inflammatory Cytokines Induce Phosphorylation and Ubiquitination of Prostate Suppressor Protein NKX3.1

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Abstract

Inflammation of the prostate is a risk factor for the development of prostate cancer. In the aging prostate, regions of inflammatory atrophy are foci for prostate epithelial cell transformation. Expression of the suppressor protein NKX3.1 is reduced in regions of inflammatory atrophy and in preinvasive prostate cancer. Inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin-1β accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal degradation. The effect of TNF-α is mediated via the COOH-terminal domain of NKX3.1 where phosphorylation of serine 196 is critical for cytokine-induced degradation. Mutation of serine 196 to alanine abrogates phosphorylation at that site and the effect of TNF-α on NKX3.1 ubiquitination and protein loss. This is in contrast to control of steady-state NKX3.1 turnover, which is mediated by serine 185. Mutation of serine 185 to alanine increases NKX3.1 protein stability by inhibiting ubiquitination and doubling the protein half-life. A third COOH-terminal serine at position 195 has a modulating effect on both steady-state protein turnover and on ubiquitination induced by TNF-α. Thus, cellular levels of the NKX3.1 tumor suppressor are affected by inflammatory cytokines that target COOH-terminal serine residues to activate ubiquitination and protein degradation. Our data suggest that strategies to inhibit inflammation or to inhibit effector kinases may be useful approaches to prostate cancer prevention. [Cancer Res 2008;68(17):6896–901]

Introduction

Inflammation has been implicated as a carcinogenic insult in a number of human cancers. For example, transformation of human prostate epithelial cells occurs adjacent to foci of inflammatory atrophy. Inflammation causes the generation of reactive oxygen species that increase the risk of oxidative damage of DNA and generation of mutations (1). Inflammation of the prostate is a risk factor for the development of prostate cancer (2, 3). One of the earliest events in prostate cellular transformation is reduced expression of the haploinsufficient prostate-specific suppressor protein, NKX3.1. The NKX3.1 gene is subject to loss at chromosome 8p21 and/or methylation (4). Intracellular levels of the NKX3.1 protein are reduced in prostate intraepithelial neoplasia, a noninvasive precursor to prostate cancer (4), and in regions of inflammatory atrophy that are precursors to malignant transfor-
that no additional mutations were generated. Twenty four hours before transfection, 750,000 LNCaP and PC-3 cells were plated in FBS-IMEM. Cells were transfected overnight with 500 ng MYC-NKX3.1 expression plasmid or MYC-empty vector, 500 ng green fluorescent protein (GFP) expression plasmid, or 1.5 μg of NKX3.1 expression plasmid or pcDNA3 plasmid using lipofectAMINE Reagent (Invitrogen). The transfection medium was replaced with FBS-IMEM for 24 h before treatment.

**NKX3.1 ubiquitation assay.** To assess the ubiquitination of exogenous NKX3.1, a His Bind Purification kit was used (Novagen). LNCaP cells (2 × 10⁶) were transfected overnight with 2 μg MYC-NKX3.1 or MYC-empty expression plasmid and 3 μg of a histidine-tagged ubiquitin expression plasmid (gift of Dirk Bohmann, University of Rochester, Rochester, NY). Cells were pretreated for 1 h with 100 mmol/L bortezomib followed by a 6-h treatment with 40 ng/mL TNF-α. Cells were harvested in cold PBS and resuspended in 1× Binding Buffer (diluted to volume with 6 mol/L Urea). Samples were sonicated and centrifuged for 30 min at 14,000 × g at 4°C. The supernatant was incubated with 50 μL precharged Ni²⁺ agarose bead slurry for 3 h at room temperature with rotation. Samples were washed with 1× Binding Buffer (6 mol/L Urea) followed by 1× Wash Buffer (6 mol/L Urea). Ubiquitinated proteins were eluded from the Ni²⁺ agarose beads with 4× SDS sample buffer and resolved on a 4% to 20% SDS-polyacrylamide gel. Ubiquitinated NKX3.1 was detected using a mouse monoclonal anti-MYC antibody (1:1,000; Santa Cruz Biotechnologies).

**Immunoprecipitation and immunoblotting.** LNCaP cells transfected with MYC-tagged NKX3.1 with lipofectamine 2000 (Invitrogen) were pretreated with CHX (100 μmol/L) for 15 min and then exposed to 40 ng/mL TNF-α 15 min. An aliquot of cell extract was treated with calf intestinal alkaline phosphatase (CIP; New England Biolabs). Cells were harvested for immunoprecipitation with polyclonal anti-MYC antibody (1:1,000; Santa Cruz Biotechnologies), followed by Western blotting with monoclonal anti-MYC antibody (Santa Cruz) or anti-phosphoserine antibody (Sigma-Aldrich).

**Results**

Cellular NKX3.1 protein levels are critical for maintenance of the prostate epithelial phenotype. We therefore characterized intracellular protein turnover to determine the half-life of NKX3.1. In PC-3 prostate cancer cells, exogenously expressed NKX3.1 has a half-life of ~1 h (Fig. 1A). Members of the natural killer family of homeodomain proteins such as Nkx2.5 and Nkx3.1 have been shown to have increased levels of expression and of protein activity after removal of the peptide domain that lies C terminal to the homeodomain (12, 13). Therefore, we measured the half-life of a C terminal truncated NKX3.1 lacking the 51 amino acids downstream from the homeodomain and observed a prolonged half-life (Fig. 1A). LNCaP prostate cancer cells are one of the few cell lines that express endogenous NKX3.1. In LNCaP, cells exogenous MYC-tagged NKX3.1 has a half-life of ~60 minutes, whereas the COOH-terminal–truncated protein had a half-life of nearly 4 hours (Fig. 1B). We also examined turnover of endogenous NKX3.1 in LNCaP cells by treating cells with bortezomib, a reversible proteasome inhibitor that prolonged half-life of endogenous NKX3.1 but had no effect on the level of exogenous COOH-terminal–truncated protein (Fig. 1C). Bortezomib also blocked turnover of exogenous NKX3.1 expressed in PC-3 prostate cancer cells. Moreover, in the presence of bortezomib, higher molecular weight moieties of NKX3.1 were seen that represented polyubiquitinated NKX3.1 accumulating in PC-3 cells. Under the same conditions, no ubiquitination of NKX3.1(1-183) was seen (Fig. 1D).

We had observed that exposure of LNCaP cells to the inflammatory cytokine TNF-α caused rapid loss of NKX3.1. Similar loss of NKX3.1 was seen when cells were exposed to another

![Figure 1. COOH terminus of NKX3.1 affects protein stability.](https://www.aacrjournals.org/doi/abs/10.1158/0008-5472.CAN-07-4066)
inflammatory cytokine IL-1β (Fig. 2A). In contrast, no effect on NKX3.1 levels was seen in response to the proliferative cytokine IL-6. LNCaP cells are known to express IL-6 receptors and respond to IL-6 (14). A MYC-tagged NKX3.1 fusion protein had a half-life similar to endogenous NKX3.1 in LNCaP cells treated with cycloheximide, indicating that both endogenous and exogenous MYC-tagged NKX3.1 were subjected to similar mechanisms of protein turnover. TNF-α accelerated degradation of full-length MYC-tagged protein. In contrast, truncation of the COOH-terminal domain prolonged protein half-life and conferred resistance to the effect of TNF-α on protein loss (Fig. 2B). The MYC-tagged NKX3.1 fusion protein was ubiquitinated in response to TNF-α, but the COOH-terminal domain truncated protein was resistant to ubiquitination (Fig. 2C). Polyubiquitination most commonly occurs at lysine residues. The COOH-terminal domain of NKX3.1 has lysines at positions 193 and 201. Mutation of either or both lysine residues to arginines had no detectable effect either on steady-state turnover or on TNF-α-induced degradation of NKX3.1 (data not shown). TNF-α causes apoptosis of LNCaP cells, but the effect is not seen until >48 hours after exposure to the cytokine (15). To determine whether caspase activation contributed to NKX3.1 turnover after exposure to TNF-α, we treated cells with the pancaspase inhibitor zVAD-FMK and saw no effect on the degradation of NKX3.1 within 24 hours of exposure to 40 ng/mL TNF-α (data not shown).

To determine what region of COOH-terminal domain influenced NKX3.1 stability, we engineered a series of MYC-tagged deletion constructs whose stability were tested in LNCaP cells. Deletion at amino acids 216, 208, or 200 had no effect on steady-state turnover of NKX3.1. Deletion to amino acid 192 prolonged half-life to a lesser degree than seen with deletion to amino acid 183 (Fig. 3A). The NKX3.1 constructs truncated at amino acids 192 and 183 were also less sensitive to the effect of bortezomib on protein turnover (Fig. 3B). Truncation at amino acid 192 resulted in an increased protein half-life and abrogation of the effect of TNF-α on protein turnover (Fig. 3C, top). MYC-NKX3.1 truncation extended to amino acid 183 caused further increase in protein half-life.

Phosphorylation of a number of proteins, for example IκB and β-catenin, is known to mediate association with ubiquitin ligase and subsequent proteasomal degradation. Computer-based analysis of the COOH-terminal 51 amino acids of NKX3.1 contain three potential phosphorylation sites, all proximal to amino acid 200, at serines 185, 195, and 196 (16). Each of these serines was individually mutated to an alanine residue to abrogate each putative phosphorylation site. In addition, we made compound serine→alanine mutant constructs. The serine mutants were tested both for protein turnover and for sensitivity to TNF-α (Fig. 3C, bottom). Mutation of serine 185 doubled the half-life of NKX3.1 and also increased protein half-life after TNF-α exposure from 25 to 40 minutes. However, NKX3.1(S185A) retained sensitivity to TNF-α, suggesting that serine 185 had a major influence on protein degradation but was not targeted by TNF-α. Mutation of either serine 195 or serine 196 prolonged protein half-life. The serine 195

![Figure 2](http://www.cbs.dtu.dk/services/NetPhos/)

**Figure 2.** TNF-α and IL-1β induce NKX3.1 degradation. A, endogenous NKX3.1 levels in LNCaP cells after exposure to 40 ng/mL TNF-α, IL-1β, or IL-6. Relative levels of NKX3.1 were normalized to β-actin levels and quantitated in the graph. B, TNF-α is shown to accelerate NKX3.1 protein turnover but have no effect on NKX3.1(1-183). MYC-tagged NKX3.1 expression plasmids were transected into LNCaP cells. Cells were treated with 100 μmol/L cycloheximide for 1 h and then exposed to 40 ng/mL TNF-α. C, the effect of TNF-α on ubiquitination of MYC-tagged NKX3.1 and NKX3.1(1-183) in LNCaP is shown. Cells were transfected with MYC-tagged NKX3.1 expression plasmids and with a polyhistidine-tagged ubiquitination expression plasmid. Cells were treated for 1 h with bortezomib and then for 6 h with TNF-α. Cells lysates were exposed to Ni²⁺-charged agarose beads and then subjected to Western blotting with anti-MYC antibody. Input levels of NKX3.1 were determined by Western blot analysis of each total cellular lysate before the addition of Ni²⁺ beads. IB, immunoblotting.
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Figure 3. Determinants of NKX3.1 steady-state and TNF-α-induced turnover. A, COOH-terminal truncation mutants of NKX3.1 were expressed as MYC-tagged constructs and cotransfected with a GFP expression plasmid. Cells were treated with cycloheximide for 1 h and processed for Western blotting with anti-MYC antibody. FL, full-length. B, the effect of bortezomib on levels of full-length and COOH-terminal-truncated MYC-tagged NKX3.1 expression constructs was assayed by Western blot. C: the MYC-tagged NKX3.1 deletion constructs and point mutants were analyzed for half-life after 0, 30, 60, and 120 min of exposure to cycloheximide or to cycloheximide + 40 ng/mL TNF-α. NKX3.1 proteins were detected by Western blotting. Left, maps of the mutant constructs. Right, protein half-lives during turnover or after exposure to TNF-α. At least three separate determinations were done for each value. Mean ± SDs are shown for each half-life.

Mutation attenuated and the serine 196 mutation abrogated the effect of TNF-α on protein degradation because there was no change in the half-life of NKX3.1(S196A) after exposure to TNF-α. Mutation of both serines 195 and 196 enhanced the protein half-life and resistance to TNF-α more than the effect of the serine 196 mutant alone. The compound mutant with altered serine 185 and serine 195 showed a half-life of 110 minutes but retained an effect of TNF-α. In contrast to the serine 185, 196 compound mutant had a prolonged protein half-life and essentially no TNF-α sensitivity. Lastly, simultaneous mutation of serines 185, 195, and 196 resulted in a protein with no sensitivity to TNF-α and a half-life similar to the NKX3.1(1-183) construct. Thus, the effect of COOH-terminal truncation on protein turnover and TNF-α sensitivity was recapitulated by mutation of three serines. To determine whether any of the serine mutations affected nuclear localization of NKX3.1 and thereby affected protein turnover, we expressed each MYC-tagged NKX3.1 construct in LNCaP cells and determined subcellular localization by immunohistochemistry. We observed that all MYC-tagged serine→alanine constructs localized to the nucleus of LNCaP cells (data not shown).
To show directly that the COOH terminus of NKX3.1 was the target for TNF-α-induced phosphorylation, we performed immunodetection of phosphoserine on MYC-tagged NKX3.1. LNCaP cells were transfected with a MYC-NKX3.1 expression vector and pretreated with cycloheximide before 15 minutes of exposure to TNF-α. As shown in the left of Fig. 4A, exposure to TNF-α induced the presence of phosphoserine residues that were sensitive to treatment with CIP (Fig. 4A, right). TNF-α induced serine phosphorylation only on the COOH terminus as shown in Fig. 4B because COOH-terminal truncation abolished immunodetection with anti-phosphoserine antibody. In contrast, deletion of the NH₂-terminal domain upstream from the homeodomain did not affect TNF-α-induced serine phosphorylation. Mutation of serine 196 to alanine specifically abrogated TNF-α-induced serine phosphorylation. The effect was not changed by concurrent mutation of serine 195 to alanine (Fig. 4C). We were also able to show that in the presence of cycloheximide alone, mutation of serine 185 to alanine diminished detection with the anti-phosphoserine antibody, whereas mutation of serines 195 and 196 or COOH-terminal truncation to amino acid 192 had no effect on NKX3.1 serine phosphorylation in the presence of cycloheximide.

Lastly, the COOH-terminal serine mutations were also found to decrease the polyubiquitination of NKX3.1 in the presence of bortezomib and in response to TNF-α (Fig. 5). Thus, the COOH-terminal serines determined both ubiquitination and protein loss after exposure of LNCaP cells to TNF-α.

Discussion

Our data strongly suggest that COOH-terminal phosphorylation was the biochemical signal that initiated ubiquitination and protein degradation both for purposes of NKX3.1 turnover and for degradation in response to TNF-α and perhaps other inflammatory cytokines. Experiments with NKX3.1 mutants showed that serine 185 was targeted for steady-state protein turnover and serine 196 was targeted by TNF-α. The data suggest that serine 195 had a modulating effect on the effects mediated by both serine 185 and serine 196, further augmenting both steady-state turnover and TNF-α-induced degradation. However, phosphorylation at serine 196 seems to be the primary signal for TNF-α-induced degradation of NKX3.1.

Our data showed how inflammation may enhance degradation of a short-lived suppressor protein in prostate epithelial cells. These data provide a mechanism for direct and specific degradation of a suppressor protein by inflammatory cytokines. The data also suggest that anti-inflammatory agents may have activity in prostate cancer prevention. A trial of refecoxib for prostate cancer prevention was closed early when the drug was withdrawn from the market (17). The role of anti-inflammatory agents in prostate cancer prevention remains to be shown. Prostate cancer prevention or treatment might be approached by inhibition of NKX3.1 ubiquitination. Recently, NKX3.1 has been shown to be a target of at least one ubiquitin E3 ligase, TOPORS (18). Interestingly TOPORS is a nuclear protein that associates with PML bodies and also targets p53 for degradation (19, 20). Because we have shown that NKX3.1 ubiquitination is mediated by phosphorylation, it is conceivable that identification of the kinase that targets the NKX3.1 COOH-terminal domain may be a future target for a prostate cancer prevention or treatment strategy. Moreover, the activation of TNF-α receptor is affected by drugs such as etanercept that interfere with ligand availability in the treatment of inflammatory diseases. Thus, there are therapeutic agents available to test the effects of TNF-α inhibition on NKX3.1 expression and prostate epithelial cell transformation.

The control of NKX3.1 levels is complex and is also under control of a kinase that targets NH₂-terminal threonines. In contrast to

Figure 4. TNF-α induces phosphorylation of NKX3.1. A, TNF-α induces NKX3.1 phosphorylation. LNCaP cells, transfected with MYC-tagged NKX3.1, were pretreated with 100 μmol/L cycloheximide for 15 min and then exposed to 40 ng/mL TNF-α for 15 min. Right, blotting was done after one aliquot was treated with CIP. Cells were harvested for immunoprecipitation (IP) with polyclonal anti-MYC antibody followed by Western blotting with either monoclonal anti-MYC or anti-phosphoserine antibody. In B, the effect of COOH-terminal truncation and, in C, S196 and S195 mutations abolish NKX3.1 phosphorylation induced by TNF-α. D, MYC-tagged NKX3.1 constructs as indicated were expressed in LNCaP cells subjected to CHX treatment for 30 min. Immunoprecipitation was with anti-MYC antibody and immunoblotting was done with either anti-phosphoserine antibody or anti-MYC antibody.
Figure 5. Determinants of NKX3.1 ubiquitination in steady-state turnover and after exposure to TNF-α. Ubiquitination of wild-type and mutant MYC-tagged NKX3.1 after cells were treated with bortezomib with or without subsequent exposure to TNF-α was analyzed in LNCaP cell extracts. An expression vector for polyhistidine-tagged ubiquitin was cotransfected into the LNCaP cells. Ubiquitinated proteins were pulled down by Ni²⁺ beads and analyzed by Western blotting using an anti-MYC antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

5. Unpublished data.

The effect of phosphorylation at the COOH-terminal domain, phosphorylation of the NH₂-terminal threonines prolongs NKX3.1 protein half-life (6). Prostate inflammation and inflammatory atrophy can occur without subsequent development of cancer and predispose prostate epithelial cells to malignant transformation. We propose that prostatic inflammation results in reduced intracellular levels of NKX3.1 and thereby predisposes cells to oncogenic transformation. Our unpublished data show that NKX3.1 can mediate proliferative signals via insulin-like growth factor (IGF-I). Thus, reducing NKX3.1 levels may enhance IGF-I–driven cell growth. NKX3.1 also mediates binding of topoisomerase I to DNA (21). In prostate epithelial cells, this interaction influences the efficiency of DNA repair, and therefore, loss of NKX3.1 expression may predispose cells to DNA damage and oncogenic mutations.

We propose that prostate epithelial cell proliferation and transformation are favored by loss of NKX3.1, and therefore, selection of clones with NKX3.1 genetic loss or methylation provide an irreversible growth advantage that frequently represents the first steps in prostate carcinogenesis. In light of this, efforts to prolong NKX3.1 protein half-life are a logical strategy for prostate cancer prevention.
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