Research Article

The Nuclear Factor-κB Pathway Controls the Progression of Prostate Cancer to Androgen-Independent Growth

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Abstract

Typically, the initial response of a prostate cancer patient to androgen ablation therapy is regression of the disease. However, the tumor will progress to an "androgen-independent" stage that results in renewed growth and spread of the cancer. Both nuclear factor-κB (NF-κB) expression and neuroendocrine differentiation predict poor prognosis, but their precise contribution to prostate cancer progression is unknown. This report shows that secretory proteins from neuroendocrine cells will activate the NF-κB pathway in LNCaP cells, resulting in increased levels of active androgen receptor (AR). By blocking NF-κB signaling *in vitro*, AR activation is inhibited. In addition, the continuous activation of NF-κB signaling *in vivo* by the absence of the IκBα inhibitor prevents regression of the prostate after castration by sustaining high levels of nuclear AR and maintaining differentiated function and continued proliferation of the epithelium. Furthermore, the NF-κB pathway was activated in the ARR$_2$PB-*myc*-PAI (Hi-myc) mouse prostate by cross-breeding into a IκBα$^{+/-}$ haploid insufficient line. After castration, the mouse prostate cancer continued to proliferate. These results indicate that activation of NF-κB is sufficient to maintain androgen-independent growth of prostate and prostate cancer by regulating AR action. Thus, the NF-κB pathway may be a potential target for therapy against androgen-independent prostate cancer. [Cancer Res 2008;68(16):6762–9]

Introduction

With the development of the prostate-specific antigen assay for screening, prostate cancer is detected at an earlier stage. However, with 218,890 new cases of prostate cancer, there were still 27,050 deaths in the United States in 2007 due to the disease. If prostate cancer remains localized, therapy such as prostatectomy or radiation therapy can cure the patient. For metastatic prostate cancer, standard treatment uses approaches to block androgen receptor (AR) activity. Androgen ablation therapy (luteinizing hormone–releasing hormone analogues that block the production of testicular androgens and/or antiandrogens that directly block AR activation) in the majority of patients results in initial regression of the disease and a dramatic decrease in serum prostate-specific antigen. Eventually, however, all patients will fail this therapy and the cancer is commonly referred to as "androgen refractory," "androgen-independent," or "castrate-resistant" prostate cancer. At this stage, although there is modest proven benefit with docetaxel treatment, there is no curative treatment. A number of mechanisms have been proposed to explain the acquisition of androgen independence; however, the emerging theme is that the tumor is still dependent on AR signaling (1, 2). The proposed mechanisms that explain continued AR signaling include *AR* gene amplification, resulting in a response to low levels of circulating androgens (3–5), the local synthesis/concentration of androgens (6), *AR* mutations that allow activation by antiandrogens or weak androgens (7), AR activation by growth factors/kinase pathways (4, 8), and/or changes in AR coregulators (9). Because the AR is still a potential target in patients that fail androgen ablation therapy, identifying the pivotal pathway(s) that regulates continued AR signaling can result in new therapeutic approaches to treat the advanced disease.

Neuroendocrine cells are present in the normal and neoplastic prostate (10). Increases in neuroendocrine phenotype of the cancer and neuroendocrine secretory products are closely correlated with tumor progression, androgen independence, and failure of androgen ablation therapy in prostate cancer (11–13). The neuroendocrine phenotype first appears as focal neuroendocrine differentiation of adenocarcinoma in the clinically localized prostate cancers (14, 15). This is not to be confused with neuroendocrine prostate cancer, a rare form of androgen-independent prostate cancer that has the pathologic features of small-cell carcinoma (16). Rather, in neuroendocrine differentiation, the adenocarcinoma phenotype is broadly maintained but the cancer cells begin to also express markers of neuroendocrine cells. For example, expression of chromogranin A, a neuroendocrine protein, is one of the five genes that are reported to serve as outcome predictors for tumor recurrence (17). Although there is no consensus agreement about whether or not neuroendocrine cells are important in advanced prostate cancer (18–20), we have previously published that secreted neuropeptides from neuroendocrine tumor xenografts (NE-10) will support AR activation in adenocarcinoma (LNCaP cells) xenografted at a distant site (4). The virtue of having the neuroendocrine cancer grafted in the same mouse as the otherwise androgen-dependent LNCaP adenocarcinoma line allowed us to follow the progression to androgen

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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independence in castrated mice (4). Therefore, a pathway allowing progression to androgen independence can be activated by neuroendocrine secretions. This conclusion is consistent with a recent report showing that cyclic AMP-dependent protein kinase–differentiated neuroendocrine cells enhance the androgen-independent growth of prostate cancer (21).

Nuclear factor-κB (NF-κB) proteins are an important class of transcriptional regulators. The mammalian NF-κB family contains five members: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), c-Rel, RelB, and RelA (p65; ref. 22). These proteins share a Rel homology domain, which mediates DNA binding, dimerization, and interactions with specific inhibitory factors, the IκBs, which retain NF-κB dimers in the cytoplasm (22, 23). Many stimuli activate NF-κB, mostly through inhibitor of κB (IκB) kinase (IKK)–dependent phosphorylation and subsequent degradation of IκB proteins. The IKK complex activates via its two catalytic subunits, IKKβ and IKKα, the classic and alternative NF-κB signaling pathways, respectively (reviewed in ref. 24). In prostate cancer, the activity of NF-κB is higher in androgen-independent cell lines and androgen-independent xenografts compared with androgen-dependent grafts (25), as well as in metastatic prostate cancer compared with localized disease (26). Further, elevation of NF-κB activity in primary prostate cancer correlates with a poor prognosis (27, 28) and predicts biochemical (prostate-specific antigen) relapse (29, 30). By the analysis of multiple microarray studies, the NF-κB pathway was identified as significantly dysregulated in metastatic prostate cancer (31). However, the precise contribution of NF-κB to prostate cancer progression is unknown.

Thus, both neuroendocrine differentiation and increased expression of nuclear NF-κB in prostate cancer correlate with poor prognosis. This study shows that neuropeptides can activate the NF-κB pathway in LNCaP prostate cancer cells. In addition, activation of NF-κB in the prostate prevents regression after castration of the normal prostate by maintaining both high levels of nuclear AR and continued cell proliferation. Further, activation of NF-κB in the ARR_2PB-myc-PAI transgenic model results in continued growth of the prostatic adenocarcinoma after castration. Therefore, the activation of the NF-κB pathway results in progression of prostate cancer to androgen independence.

Materials and Methods

Cell culture and materials. The human prostate carcinoma cell line LNCaP was obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 (Life Technologies, Inc.) medium containing 5% fetal bovine serum (FBS; Hyclone); 0.1% insulin, transferrin, and selenium (ITS); and 0.1% glutamine (Life Technologies).

Primary culture of NE-10 cells. NE-10 tumor tissue was cut into 1- to 2-mm^3 pieces. The small tissue fragments were placed into 100-mm Primaria tissue culture dishes (Becton Dickinson Labware) and cultured in RPMI 1640 containing 5% FBS, 10% heat-inactivated horse serum (Hyclone), 1% antibiotic-antimycotic (Life Technologies), 50 μg/mL gentamicin (Life Technologies), 1% L-glutamine, 1% sodium pyruvate, and 1 mol/L HEPES at 37°C in a 5% CO_2 incubator. When the explants displayed an initial outgrowth of neuroendocrine cells (usually 1 wk after plating), the culture medium was changed every 2 d. Fibroblast cells that contaminated the cultured neuroendocrine cells were removed by differential trypsinization.

Transient transfection and infection assay. The NGL vector [a NF-κB–responsive reporter vector that has *luciferase* and green fluorescent protein (*GFP*) reporter genes; ref. 32] and ARR_2PB-Luc vector (an AR-responsive reporter vector that does not respond directly to NF-κB; ref. 33), used to measure AR activity, were used in the transfection and infection experiments. LNCaP cells were plated at an initial density of 2.5×10^4 per well in 24-well tissue culture plates. After 24 h, the cells were transfected with Lipofectamine (Invitrogen) for 4 h according to the manufacturer's protocol. After transfection, the cells were treated with conditioned medium (containing neuroendocrine extracts) and neuroendocrine peptides [bombesin (BBS) and gastrin-releasing peptide (GRP), 10^{-8} mol/L each (Sigma)]. To generate the conditioned medium containing neuroendocrine secretions, RPMI 1640 (containing 5% dextran-charcoal-stripped serum, 0.1% ITS and 0.1% glutamine) was added to the neuroendocrine cell culture dish. After 24 h, the medium (containing neuroendocrine secretions) was harvested and transferred to targeted cells (LNCaP cells). RPMI 1640 (containing 5% dextran-charcoal-stripped serum, 0.1% ITS, and 0.1% glutamine) was added to the targeted cells as the control. All experimental groups were tested with a dose-response curve for dihydrotestosterone (10^{-9}–10^{-8} mol/L) with or without bicalutamide (10^{-5} mol/L; Zeneca). The transfection efficiency was determined by cotransfecting pRL-CMV containing the Renilla luciferase reporter gene (Promega). Luciferase activity was determined using the Promega Corp luciferase assay system 24 h after transfection. The values plotted represent the mean of at least three individual samples ± SD. IκBα-DN adenovirus (a mutant avian IκBα with serine to alanine substitutions that prevent phosphorylation and degradation; ref. 34) was used to block NF-κB signaling in the infection experiments and the empty adenovirus was used as control.

Reverse transcription and real-time PCR. Total RNA from LNCaP cells at 48 h after infection with RelA expression vector or empty adenovirus (as control) was extracted using Trizol (Life Technologies), and residual genomic DNA was removed by DNase I (Invitrogen) treatment. The RNA was reverse transcribed using random primers and Superscript II (Life Technologies) according to the manufacturer's protocol. The primers used to amplify *AR* were 5′-ATCAGGGGCGAAGTAGAGCATC-3′ (forward), 5′-AGCCCCACTGAGGGGACAAC C-3′ (reverse). Real-time PCR reactions were carried out in a 20-μL volume using a 96-well plate format and fluorescence was detected using the Bio-Rad I-Cycler IQ Real-time detection system.

Western blot analysis. We extracted cytoplasmic and nuclear proteins from LNCaP cells at 48 h after infection with RelA expression adenovirus vector (35) or empty vector (as control) using a nuclei extraction kit (Pierce) according to the manufacturer's instruction. A 20-μg aliquot of each protein sample was separated on a 4% to 12% Tris-glycine gradient gel (Novex) and then transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 5% skim milk in TBS-1% Tween 20 buffer. The AR antibody (clone N20, Santa Cruz Biotechnology) was added at the optimal concentration (1:1,000) and the blots were incubated for 1 h at room temperature. After washing thrice for 10 min each in TBS-1% Tween 20, incubation was done for 1 h with the secondary horseradish peroxidase–conjugated goat anti-rabbit antibody. The signals were detected using the enhanced chemiluminescence system (Amersham Biosciences).

NE-10 allograft model. All animal studies were conducted in accordance with the principles and procedures outlined by the NIH guide and the Vanderbilt Institutional Animal Care and Use Committee. A small fragment (~50 mg) of neuroendocrine tumor from the NE-10 allograft model (36) was implanted s.c. into the right flank of 6-wk-old male athymic nude mice (BALB/c strain). Mice were separated into two different groups. Eight mice with or without neuroendocrine tumor were subsequently castrated via scrotal approach 2 wk after neuroendocrine tumor implantation. The mice were sacrificed 4 wk after neuroendocrine tumor implantation (2 wk after castration). Prostates were excised and fixed in 10% buffered formalin and paraffin embedded for immunohistochemical analysis.

Prostatic rescue model. The $I\kappa B\alpha^{-/-}$ mice die at 6 to 9 d after birth due to constitutive NF-κB activation (37, 38). Therefore, to examine a mature $I\kappa B\alpha^{-/-}$ prostate requires rescuing the prostate from a newborn $I\kappa B\alpha^{-/-}$ mouse. Prostatic rescue is achieved from $I\kappa B\alpha^{-/-}$ mice that are either embryonic lethal or die shortly after birth by grafting the prostate under the kidney capsule of male athymic nude mice. Prostates from newborn mice ($I\kappa B\alpha^{-/-}$, $I\kappa B\alpha^{+/-}$, and wild type) were grafted under the kidney capsule of male athymic nude mice and allowed to mature for 6 wk in the male host. Then, host mice were castrated for 2 additional weeks. Each experimental group consisted of at least four mice. Prostates

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were excised and fixed in 10% buffered formalin and paraffin embedded for histologic and immunohistochemical analyses.

NF-κB signaling continuously activated prostate cancer mouse model. We developed a constitutively activated NF-κB prostate cancer mouse model (Myc/IκBα$^{+/-}$ mouse) by crossing the IκBα$^{+/-}$ mouse with the ARR_2PB-*myc*-PAI (Hi-Myc) line, which develops invasive adenocarcinoma in the prostate by 6 mo of age (39). Both Myc and Myc/IκBα$^{+/-}$ transgenic mice were castrated at 6 mo and the prostates were harvested at 2 wk after castration for analysis. Each experimental group consisted of at least four mice. Prostates were excised and fixed in 10% buffered formalin and paraffin embedded for histologic and immunohistochemical analyses.

Immunohistochemistry. Paraffin-embedded tissue sections of the prostate were stained immunohistochemically with antibodies against AR (clone N20, Santa Cruz Biotechnology), probasin (M-18, Santa Cruz Biotechnology), and Ki67 (clone TEC-3, DAKO). The primary antibody was incubated at the appropriate concentration (AR, 1:1,000; probasin, 1:1,000; Ki67, 1:1,000) for 1 h at room temperature. The secondary antibody was incubated for 60 min, being either horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse (1:1,000), respectively. Slides were rinsed extensively in tap water, counterstained with Mayer's hematoxylin, and mounted. For quantitation of the prostate proliferation, the cells were counted as positive for Ki67 when nuclear immunoreactivity was observed. The positive cells for Ki67 were counted by monitoring at least 200 luminal epithelial cells from three to five different fields of each sample. Each group had at least four mice. The results are reported as the mean percent ± SE.

Statistical and image analyses. Where appropriate, experimental groups were compared using the Student's two-tailed *t* test, with significance defined as $P < 0.05$. For quantitation of immunoblot data, images were analyzed using Scion Image software, version 1.62 (Scion Corp.).

Results

Neuroendocrine peptides increase the functional activation of NF-κB and AR in LNCaP cells. Many studies have indicated that BBS and GRP are neuroendocrine cell secretory peptides, and the serum BBS levels are significantly elevated in androgen-independent prostate cancer patients (11, 40, 41). In addition, our studies have shown that NE-10 tumors secrete BBS and GRP. To understand how neuroendocrine cells influence prostate/prostate cancer growth and progression, especially after androgen ablation therapy, we investigated the mechanisms by which neuroendocrine-secreted neuropeptides affect AR action using BBS and GRP, the known neuroendocrine-secreted peptides, and neuroendocrine conditioned medium (obtained from primary cultured neuroendocrine cells). To investigate whether the neuroendocrine-secreted peptides affect activation of NF-κB signaling, LNCaP cells were treated with or without BBS and GRP after transient transfection with the NGL vector, a NF-κB responsive luciferase reporter. The neuropeptides (BBS and GRP, 10^{-8} mol/L each) increased the activation of the NGL-luciferase reporter by 3-fold in LNCaP cells in the absence and 2-fold in the presence of androgen (dihydrotestosterone; Fig. 1*A*). These results suggested that neuropeptides increase the functional activation of NF-κB signaling in LNCaP cells both in the presence and absence of androgen.

To further understand how the neuroendocrine-secreted neuropeptides affect AR action, ARR_2PB-Luc, an AR-responsive reporter vector, which does not respond directly to NF-κB, was used to measure AR activity. LNCaP cells were transfected with the ARR_2PB-Luc construct and infected with adenoviral vectors expressing a dominant inhibitor of the NF-κB pathway (IκBα-DN) or empty adenovirus (control; Fig. 1*B*). In the absence of

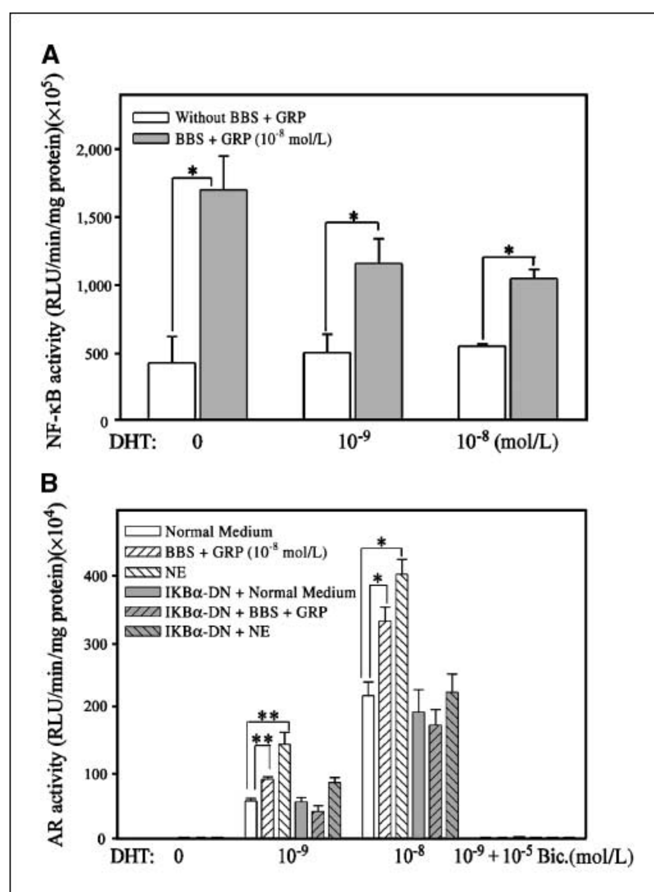

Figure 1. Neuroendocrine peptides increase the functional activation of NF-κB and AR in LNCaP cells. *A,* neuroendocrine peptides (BBS and GRP) increase the activation of NF-κB in LNCaP cells. The activity of NF-κB was determined by luciferase assay of protein extracts following transient transfection of NGL (NF-κB responsive reporter) vector. *Columns,* mean of at least three individual samples; *bars,* SE. *DHT,* dihydrotestosterone; *RLU,* relative light units. *B,* neuroendocrine-secreted factors increase the activity of AR through the NF-κB pathway. The functional activity of AR was determined by luciferase assay of protein extracts following transient transfection of ARR_2PB-Luc (AR-responsive reporter) vector. Adenovirus expressing IκBα-DN was used to block NF-κB signaling. After transfection with ARR_2PB-Luc and infection with IκBα-DN adenovirus (the empty adenovirus was used as control), LNCaP cells were treated with neuroendocrine peptides (BBS and GRP, 10^{-8} mol/L each) and neuroendocrine (*NE*) secretions (conditioned medium containing neuroendocrine extracts). *Bic.,* bicalutamide. *Columns,* mean of at least three individual samples; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

androgen, ARR_2PB promoter activity was not detected in the cells either in the presence or absence of neuroendocrine peptides (BBS and GRP) and neuroendocrine secretions (conditioned medium containing neuroendocrine extracts). In the presence of androgen (10^{-9}–10^{-8} mol/L), the activity of the ARR_2PB promoter, when treated with neuroendocrine peptides (BBS and GRP) or conditioned medium from neuroendocrine cultured cells, was 1.5- to 3-fold higher, respectively, than that when neuroendocrine peptides or neuroendocrine secretions were absent (Fig. 1*B, white columns*). This effect was blocked by bicalutamide, an inhibitor of the LNCaP mutated AR. Statistically significant induction of the reporter occurred even at lower concentrations of androgen (10^{-9} mol/L dihydrotestosterone) plus neuropeptides than with androgens alone. However, the greatest inductions occurred at higher concentrations of androgens (10^{-8} mol/L dihydrotestosterone) plus neuropeptides. The slightly higher activation by neuroendocrine

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secretions relative to BBS plus GRP suggests that other neuropeptides may be present in this conditioned medium. In all cases, the increased AR activity can be blocked by IκBα-DN (i.e., by inhibiting of NF-κB activity; Fig. 1*B, gray columns*). These results indicate that neuroendocrine-secreted factors increase the functional activity of AR through the NF-κB pathway in LNCaP cells.

NF-κB activates transcription and/or stability of the AR in LNCaP cells. To investigate how NF-κB signaling affects activation of the AR, LNCaP cells were infected with adenoviral vectors expressing RelA, the transactivating subunit of NF-κB (an empty adenovirus was used for the control). The data from real-time reverse transcription-PCR (RT-PCR) show that AR mRNA levels are increased in LNCaP cells ~2- to 3-fold after infection with RelA adenovirus independent of the presence of androgen (dihydrotestosterone; $P < 0.05$; Fig. 2*A*). When androgens were absent, Western blot analysis showed that AR protein levels are only increased in the cytoplasm with no change in nuclear protein levels (Fig. 2*B* and *C-a*). However, when androgens and RelA were both present, the AR protein levels showed a small increase in cytoplasm and a large increase in the nuclear compartment (Fig. 2*B* and *C-b*). These affects were

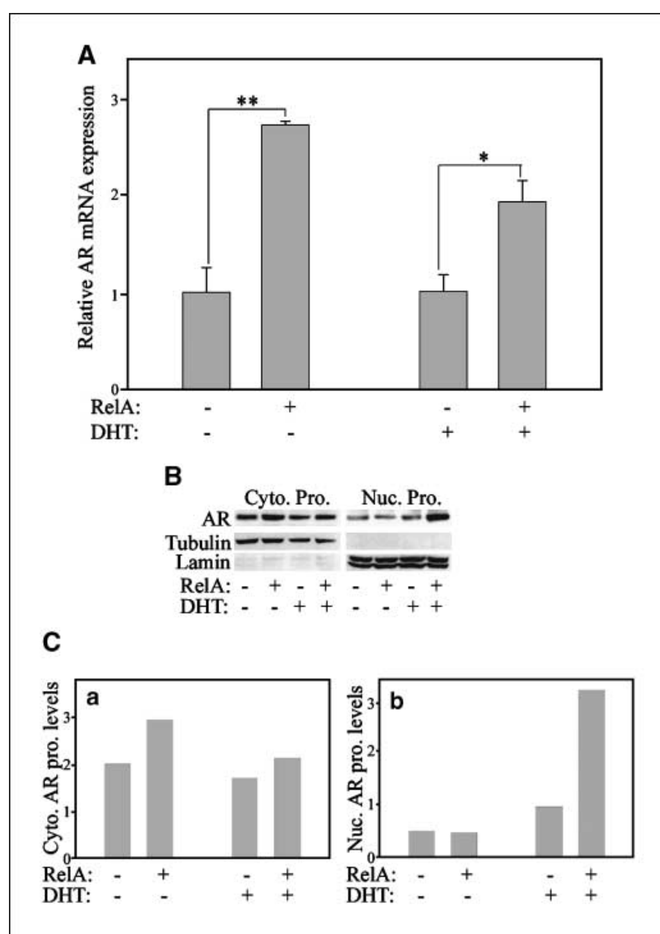

Figure 2. NF-κB activates the transcription and/or stability of the AR in LNCaP cells. *A,* NF-κB (RelA) increases AR mRNA levels in LNCaP cells. The AR mRNA levels of LNCaP cells were quantified by real-time RT-PCR after infection with RelA adenovirus. The amplification of AR was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. *Columns,* mean of at least three individual samples; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). *B,* NF-κB (RelA) increases AR protein levels in LNCaP cells. Cytoplasmic (*Cyto.*) and nuclear (*Nuc.*) protein extracts were harvested from LNCaP cells after infection with RelA adenovirus. Western blot analysis was completed to detect AR protein levels. Lamin and tubulin were used as the control for nuclear and cytoplasmic proteins, respectively. *C,* AR protein expression levels were quantified from the immunoblot by densitometry and the normalized expression of the AR protein (relative to lamin or tubulin) is represented by the histogram adjacent to the immunoblot. *a,* cytoplasmic AR protein; *b,* nuclear AR protein.

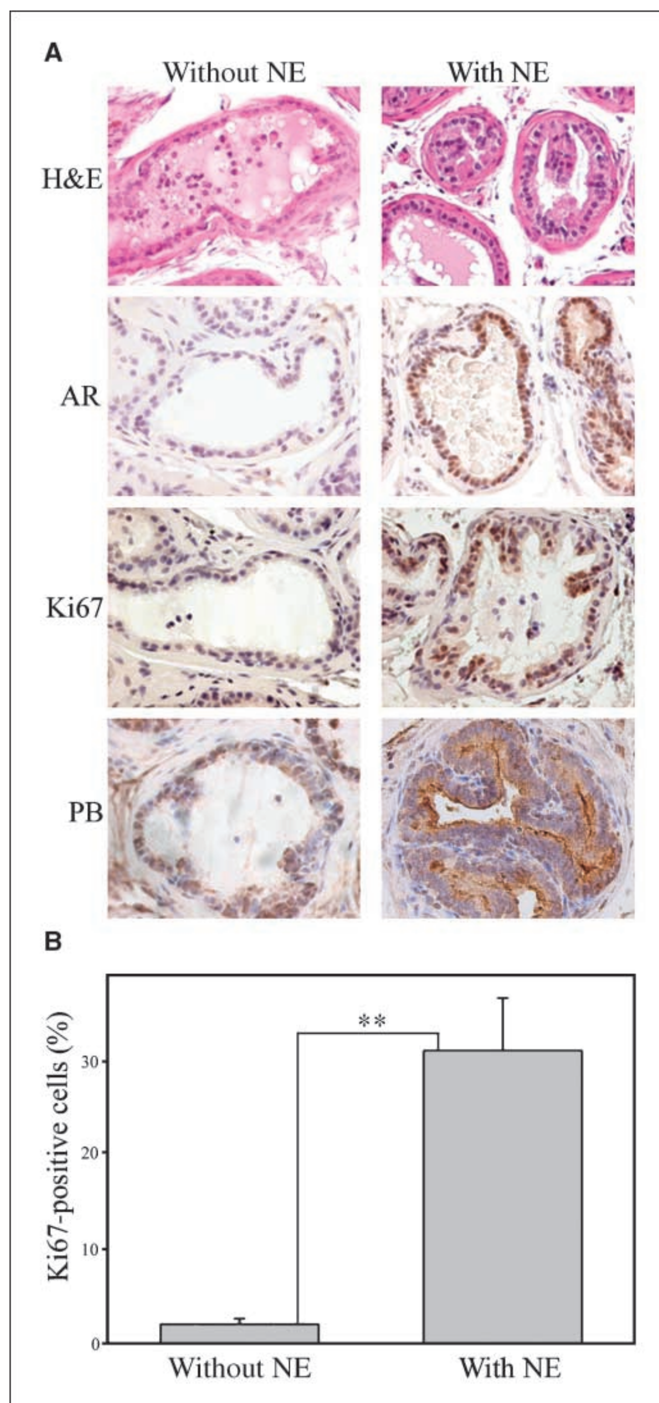

Figure 3. NE-10 neuroendocrine tumor maintains prostate growth and AR expression after castration. *A,* mice with or without neuroendocrine tumor implantation were sacrificed at 2 wk after castration. Immunohistochemical analysis was done to determine AR and probasin (*PB*) expression and proliferation (Ki67) of the prostates. *B,* cells positive for Ki67 were counted by monitoring at least 200 luminal epithelial cells from three to five different fields of each sample and plotted as a percentage of total counted. *Columns,* mean; *bars,* SE. **, $P < 0.01$, Student's *t* test.

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further confirmed by immunocytochemical staining of AR (Supplementary Fig. S1). These results suggest that NF-κB signaling increases the transcription and/or stability of AR in LNCaP cells, resulting in increased AR levels. This datum is consistent with our published *in vivo* observation that neuropeptides increase AR levels 2-fold in LNCaP grafts in castrated mice (4).

NE-10 neuroendocrine tumor maintains prostate growth and AR expression after castration. To investigate the effect of the neuroendocrine cells on NF-κB signaling in the normal prostate, a small fragment (~50 mg) of a neuroendocrine tumor from the NE-10 allograft model (36) was implanted s.c. into the flank of 6-week-old male athymic nude mice. Mice with or without the neuroendocrine tumor were castrated 2 weeks after neuroendocrine tumor implantation. Prostate tissues were harvested 2 weeks after castration for analysis (Fig. 3). The results showed that after castration, AR staining was more intense in nuclei of prostate tissue from mice hosting neuroendocrine allografts, relative to AR in the prostate of mice not carrying the allograft. Castrated allografted mice had proliferative luminal epithelial cells as determined by Ki67 staining, but significantly fewer proliferative luminal epithelial cells were detected in castrated mice without a neuroendocrine tumor graft (Fig. 3*A* and *B*). Further, heterogeneous staining for androgen-regulated probasin occurred in castrated mice bearing the neuroendocrine grafts (Fig. 3*A*), as well as general secretory dorsolateral prostate proteins as detected by the DLP antibody (data not shown; ref. 42). However, some areas in the prostate were negative for these markers of differentiation (data not shown), suggesting that certain populations of cells specifically respond to neuropeptides. These findings indicate that factors secreted from neuroendocrine cells can act systemically to stimulate the continued activation of AR signaling in the absence of testicular androgens, thus maintaining prostatic differentiation and proliferation.

Continuous activation of NF-κB signaling prevents regression of the mouse prostate after castration. To determine if the activation of the NF-κB pathway is sufficient for androgen-independent growth of the prostate, we used a knockout mouse model of IκBα (37), the major inhibitor of NF-κB function (43). IκBα$^{-/-}$ mice die at 6 to 9 days after birth due to constitutive NF-κB activation (37, 38). Therefore, to examine a mature IκBα$^{-/-}$ prostate, we rescued prostates from newborn mice. Prostatic rescue was achieved by grafting the urogenital sinus from 20-day embryonic or newborn mice under the kidney capsule of male athymic nude mice, as previously described (44). Urogenital sinus from wild-type, haploid insufficient (IκBα$^{+/-}$), and IκBα$^{-/-}$ mice were grafted and allowed to mature for 6 weeks in the male athymic nude mouse host. The host mice were then castrated and, after 2 additional weeks, were killed; prostatic grafts were removed from the kidney capsule. As expected, wild-type control prostatic grafts regressed after castration, showing atrophic glands, with limited to undetectable nuclear AR and no Ki67 staining (Fig. 4*A*). However, after castration, IκBα$^{+/-}$ and IκBα$^{-/-}$ prostatic grafts had strong nuclear AR staining (Fig. 4*A*) and significantly greater numbers of luminal Ki67-positive cells than wild-type control prostates (Fig. 4*A* and *B*). This visible difference in AR staining between the wild-type and IκBα knockout (IκBα$^{-/-}$ and IκBα$^{+/-}$)

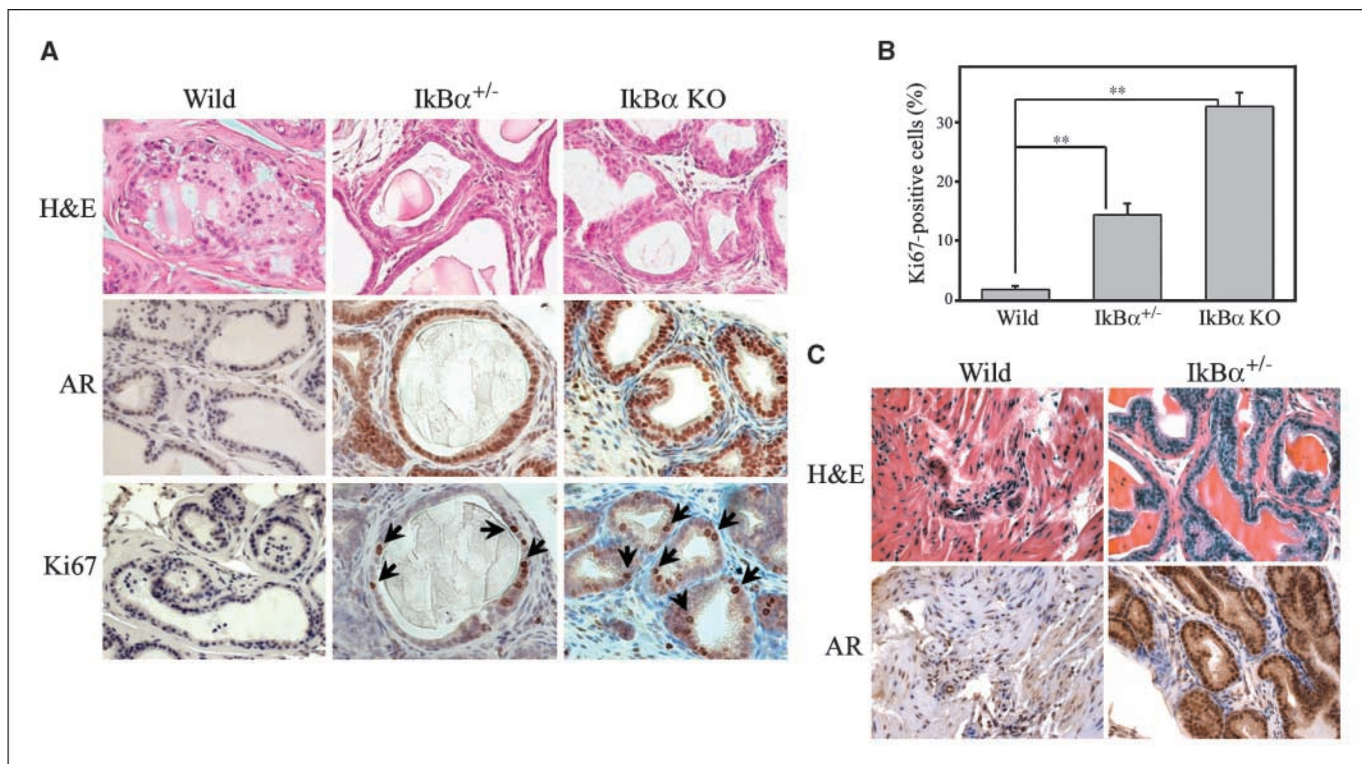

Figure 4. Continuous activation of NF-κB signaling prevents regression of the mouse prostate after castration. *A,* prostates from newborn mice [IκBα$^{-/-}$ (*KO*), IκBα$^{+/-}$, and wild-type] were grafted under the kidney capsule of male athymic nude mice and allowed to mature for 6 wk in the male host. Then, host mice were castrated for 2 additional weeks. Immunohistochemical staining was done to determine AR expression and proliferation (Ki67) of the prostates. *Arrows,* some of the Ki67-positive cells. *B,* cells positive for Ki67 were counted by monitoring at least 200 luminal epithelial cells from three to five different fields of each sample and plotted as a percentage of total counted. *Columns,* mean; *bars,* SE. **, $P < 0.01$, Student's *t* test. *C,* wild-type and IκBα$^{+/-}$ transgenic mice were castrated at 7 to 8 wk of age and the prostates were harvested 6 wk after castration. H&E and immunohistochemical stainings for AR were done (pictures show dorsolateral lobes).

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prostate tissues (Fig. 4*A*) is consistent with our results from real-time RT-PCR (Fig. 2*A*) and Western blot (Fig. 2*B*) *in vitro*.

To further understand the response of NF-κB activation in the prostate to long-term castration, $I\kappa B\alpha^{+/-}$ and wild-type mice were castrated at 7 to 8 weeks of age and the prostates were harvested at 6 weeks after castration. The prostates from wild-type mice showed characteristic features of castration, including involution and fibrosis of the gland. The wild-type prostate had only a few atrophic glands with limited to undetectable nuclear AR staining. The prostates from $I\kappa B\alpha^{+/-}$ mice, however, still maintained more typical glandular structure and strong nuclear AR staining (Fig. 4*C*). These results suggest that constitutive NF-κB signaling prevents the mouse prostate from regressing and maintains prostatic epithelial cell proliferation even up to 6 weeks after castration.

NF-κB signaling controls the progression of prostate cancer to androgen-independent growth. ARR_2PB-*myc*-PAI (Hi-Myc mouse), a transgenic mouse model, was generated using a probasin promoter (ARR_2PB) to target high levels of the human *c-myc* gene to the mouse prostate. These mice develop androgen-dependent invasive adenocarcinoma in the prostate by 6 months of age. With castration, these tumors regressed and had no apparent regrowth (39). To further determine if the activation of the NF-κB pathway is sufficient for androgen-independent growth of prostate cancer, we developed a constitutively NF-κB–activated prostate cancer mouse model (Myc/$I\kappa B\alpha^{+/-}$ mouse). The Myc/$I\kappa B\alpha^{+/-}$ mouse model was developed by crossing the ARR_2PB-*myc*-PAI mouse with the $I\kappa B\alpha^{+/-}$ mouse. Because androgen ablation therapy is the primary clinical treatment for prostate cancer patients with advanced-stage disease, we examined the effect of castration on disease progression in our Myc/$I\kappa B\alpha^{+/-}$ prostate cancer mouse model. Myc and Myc/$I\kappa B\alpha^{+/-}$ mice were castrated at 6 months and the prostates were harvested 2 weeks after castration for analysis. Our results showed that Myc and Myc/$I\kappa B\alpha^{+/-}$ transgenic mice develop invasive adenocarcinoma at 6 months of age (all dorsal and lateral lobes and some anterior and ventral lobes; Fig. 5*A*). The prostates from Myc mice regressed after castration, showing atrophic glands with limited to undetectable nuclear AR staining in all lobes (Fig. 5*A*). The number of luminal Ki67-positive cells significantly decreased ($P < 0.01$) as a result of castration (Fig. 5*B*). In contrast, the prostates from Myc/$I\kappa B\alpha^{+/-}$ mice following castration still had strong nuclear AR staining and luminal Ki67 staining in all lobes (Fig. 5*A*) with no significant decrease ($P = 0.269$) in the number of luminal Ki67-positive cells between intact and castrated mice (Fig. 5*B*). These results indicate that NF-κB signaling controls the progression of prostate cancer to androgen-independent growth in the mouse.

Discussion

Prostate tumors are heterogeneous wherein multiple foci may be genotypically distinct from each other. These multifocal cancers will respond differently to androgen ablation therapy, which leads to selection of androgen-independent cells and/or adaptative changes that result in altered gene expression, giving a subpopulation of cells a selective advantage to survive therapy. For example, focal neuroendocrine differentiation has been observed in the majority of clinically localized prostate cancers (14, 15). During progression to androgen-independent prostate cancer, both nuclear NF-κB localization (31, 45–47) and neuroendocrine differentiation (11–13, 17) occur. Both events predict poor prognosis for the patient, but their precise contribution to prostate cancer progression to androgen independence is unknown.

Neuroendocrine secretions enhance the androgen-independent growth of prostate cancer by increased AR activity (4). In this study, we found that neuroendocrine peptides (BBS and GRP) increased the functional activation of NF-κB and AR. In addition, RelA, a transactivating subunit of NF-κB, increased the transcription and/or stability of AR in prostate cancer cells. These data show that the NF-κB signaling pathway, which can be activated by neuroendocrine secretory factors, is responsible for the androgen-independent growth of prostate cancer. In cell culture, neuroendocrine peptides

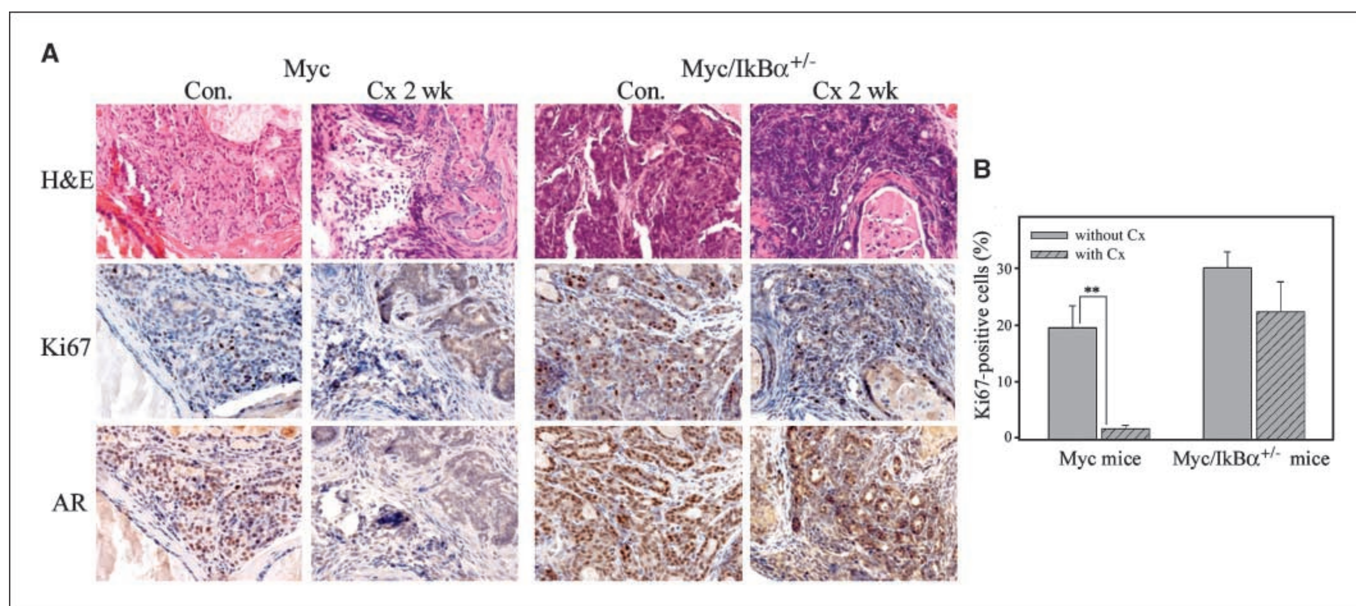

Figure 5. NF-κB signaling controls the progression of prostate cancer to androgen-independent growth. *A,* Myc and Myc/$I\kappa B\alpha^{+/-}$ transgenic mice were castrated at 6 mo of age and the prostates were harvested at 2 wk after castration (*Cx*). Immunohistochemical staining was done to determine AR expression and proliferation (Ki67) of the prostates (pictures show lateral lobes). *B,* cells positive for Ki67 were counted by monitoring at least 200 luminal epithelial cells from three to five different fields of each sample and plotted as a percentage of total counted. *Columns,* mean; *bars,* SE. **, $P < 0.01$, Student's *t* test.

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increased the functional activation of NF-κB, as detected by the NGL reporter, both in the presence and absence of androgen. However, *in vitro*, neuroendocrine peptides increased the functional activation of AR only in the presence of androgen. In addition, although NF-κB (RelA) increased AR expression independent of the presence of androgen, NF-κB increased the functional activation of nuclear AR only in the presence of androgen. These results suggest that neuroendocrine peptides increase the functional activation of AR in the prostate yet still require the presence of androgen, although the androgen levels may be low or there may be weak adrenal androgens such as dehydroepiandrosterone that can now activate the AR. Our *in vivo* experiments showed that prostatic grafts in the kidney capsule from IκBα$^{-/-}$ and IκBα$^{+/-}$ mice continue to function normally in castrated mice. In addition, continuous activation of NF-κB signaling converted androgen-dependent prostate cancer to androgen-independent growth in the ARR$_2$PB-*myc*-PAI transgenic mouse after castration. This suggests that nontesticular androgens are sufficient to activate the AR when NF-κB is constitutively expressed in the castrated mouse. These results are consistent with a previous study that showed that very low levels of testosterone from the adrenal gland and higher levels of weak androgens such as dehydroepiandrosterone would be present in castrated mice (4). Further, neuroendocrine cancers promote LNCaP tumor growth in castrated mice through increased AR expression (4). AR overexpression is associated with increased sensitivity to the growth-stimulating effects at low androgen concentrations in recurrent prostate cancer–derived cell lines (5) and xenografts (48).

In summary, our findings suggest that neuroendocrine secretory proteins will activate the NF-κB pathway in prostate cancer cells, and activation of NF-κB signaling is sufficient to maintain androgen-independent growth of prostate cancer via regulation of AR action. Our report provides the basis to develop a new therapeutic strategy to treat prostate cancer patients after they fail androgen ablation therapy. Thus, the NF-κB pathway may be a potential target for therapy against androgen-independent prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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