Advances in Brief

Prostatic Nerve Growth Factor Inducible A Gene Binds a Novel Element in the Retinoblastoma Gene Promoter

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Abstract

We are investigating the role of the early response transcription factor, nerve growth factor inducible A gene (NGFI-A), as a modulator of retinoblastoma (RB) gene transcription in prostate cells. Examination of the RB promoter reveals a novel element GCGGGGGGAG located at nucleotides 152–144 upstream of the methionine initiation codon. This sequence shares strong homology with the consensus NGFI-A binding element GC-GGGGGGCG varying by a single nucleotide. In DNA binding assays, an NGFI-A fusion protein and the native protein product of the NGFI-A gene purified from prostate cancer cells bound specifically to an oligonucleotide containing the RB promoter element. Gene expression studies in rat ventral prostate demonstrated a 1.9-fold increase in RB mRNA following castration that parallels a 2.7-fold induction of NGFI-A mRNA. In summary, the in vitro DNA binding data and the transient coregulation of rat NGFI-A and RB following castration suggests that the RB gene may be transcriptionally regulated by NGFI-A in prostate cells.

Introduction

A variety of extracellular stimuli are essential in signaling normal cellular differentiation and mitogenesis. The nuclear arm of this signal transduction process is conveyed through the actions of transcriptional regulatory proteins that regulate the expression of genes required for the various phenotypic changes in the cell. Conversely, if this mechanism is altered or insufficient, the fate of the cell may be that of uncontrolled, neoplastic growth.

Allelic loss at the RB locus has been extended beyond retinoblastoma to include a variety of cancers (1–4). The association of RB mutations with prostate cancer has also been demonstrated (5); however, the significance of RB inactivation in the molecular etiology of prostate cancer has yet to be determined.

We have described the expression of the early response transcription factor NGFI-A in prostate and demonstrated its close association with apoptosis of prostate cancer cells; however, the identity of target genes and the specific function of NGFI-A in prostate remains unknown. Results from this study indicate that RB gene transcription in prostate may be regulated in part by NGFI-A.

Materials and Methods

DNA Binding Assays. A fusion protein consisting of the bacterial trpE protein and NGFI-A was produced using the pATH 1 expression vector (6). The construct was made by ligating a 1677-nucleotide BglII/XhoI (nucleotides 313–1990 of the NGFI-A cDNA) containing the entire coding region of the NGFI-A gene into the BamHI and XbaI sites of pATH 1. The plasmid was transformed into Escherichia coli DH5α cells, and the fusion protein was induced and purified as previously described (7). The synthetic oligonucleotide, GCGGGGGGCGGGGGGGGG (NO1A), consists of a dimer of the previously described NGFI-A binding site (8). The RB binding site was also synthesized as a dimer of the sequence GCGGGGGGCGGGGGGAGG (RB1A). Fifty ng of the NGFI-A/trpE fusion protein were incubated in separate reactions with [32P]-labeled NO1A and RB1A for 15 min at room temperature in GS buffer (50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [pH 7.5], 100 mM NaCl, 1 mM EDTA, 5 mM spermidine, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) containing 100-fold excess of a nonspecific oligonucleotide. Protein-DNA complexes were then resolved by electrophoresis through a 8% nondenaturing acrylamide gel and subjected to autoradiography.

The human prostate cancer cell line LNCaP (9) was propagated in Dulbecco’s Eagle’s medium:F-12 medium (1:1) supplemented with 10% fetal calf serum and 2 mM glutamine (GBHC, Grand Island, NY). The cells were kept at 37°C in a humidified atmosphere of 5% CO₂. TPA-treated (10 nM) and untreated LNCaP cells were lysed in a denaturing immunoprecipitation buffer, and NGFI-A immunoprecipitation was carried out as previously described (7). The resulting immune complex was collected on protein A sepharose beads (Sigma Chemical Co., St. Louis, MO), electrophoresed though an 8% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto nitrocellulose. The protein blot was prehybridized at room temperature in renaturation buffer [100 mM KCl-25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.8)-10 μM ZnCl₂-1 μM dithiotreitol-20% glycerol] containing 5% nonfat dry milk and salmon sperm DNA (1 μg/ml). [32P]-labeled RB1A was added to a final concentration of 10⁶ cpm/ml, and binding was allowed to proceed at 25°C for 30 min. The protein blot was washed in renaturation buffer and subjected to autoradiography.

Gene Expression Studies in Castrates. Sprague-Dawley rats (250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed in the standard environment 1 week before use. Castration was performed through scrotal incision under ketamine, xylazine, and acepromazine anesthesia. Rats were killed by decapitation at the appropriate time points following castration, and the ventral prostate was excised, trimmed of adipose tissue, and immediately frozen in liquid nitrogen until use.

Total RNA was extracted from frozen prostate tissue, and mRNA was isolated using the PolyATtract mRNA isolation system (Promega Corp., Madison, WI). Northern blot analysis was performed on these mRNA samples. Restriction fragments from the NGFI-A, human RB, and β-actin cDNAs were labeled with [α-32P]dCTP using the Random Primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). After hybridization, filters were washed and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL) with intensifying screens at ~70°C. The resulting autoradiogram was subjected to image analysis using a CCD-72 camera (DAGE-MTI, Michigan City, IN), and grey scale values were quantitated using a Matrox MVP image processing board.

Results

We have examined a novel regulatory mechanism of RB gene transcription in prostate. It was previously reported that a 103-nucleotide deletion in the promoter of the RB gene was discovered in a case of prostate cancer in which RB expression was absent (5). Upon examination of the deleted sequence, we identified the 9-nucleotide element GCGGGGGAG located at nucleotides 152–144 upstream of the methionine initiation codon.
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Prostate cells destined to undergo apoptosis. Three of these genes, as demonstrated by inhibition of binding with unlabeled RB1A prior to tracts were unable to bind the oligonucleotide. Specificity was demonstrated by inhibition of binding with unlabeled NO1A and RB1A oligonucleotides.

In the androgen-sensitive prostate cancer cell line LNCaP, NGFI-A gene induction exhibits early response kinetics when induced with the phorbol ester TPA. Maximum expression of the NGFI-A gene in LNCaP cells is achieved within 1 h of TPA addition. Subsequently, synthesis of the protein peaks within 90 min following induction of the gene. To determine if native NGFI-A expressed in prostate cells could bind \(^{32}P\)-labeled RB1A, NGFI-A was immunoprecipitated from LNCaP cells treated with 10 nM TPA for 90 min using an NGFI-A-specific antisem (7), and the immune complex was used in a Southwestern DNA binding assay (10) (Fig. 2B). Immunopurified NGFI-A and control NGFI-A fusion protein bind the RB1A oligonucleotide, whereas immunoprecipitates from uninduced LNCaP extracts were unable to bind the oligonucleotide. Specificity was demonstrated by inhibition of binding with unlabeled RB1A prior to incubation with \(^{32}P\)-labeled RB1A (data not shown).

Ablation of androgen in the rat induces the expression of genes in prostate cells destined to undergo apoptosis. Three of these genes, c-fos, c-myc (11), and NGFI-A, are transcriptional regulatory genes. Our DNA binding data suggest that NGFI-A may have a regulatory role in RB expression in the prostate. If RB is a biological target gene of NGFI-A in prostate cells, then coregulation of NGFI-A and RB might be expected in regressing prostate following castration. We compared mRNA levels of NGFI-A and RB in rat ventral prostate following castration to determine if reciprocal or parallel patterns of expression are observed. mRNA was extracted from the ventral prostates of male rats at various time points following castration, and the expression of NGFI-A and RB was examined (Fig. 3). Single transcripts of 4.7 and 3.2 kilobases for RB and NGFI-A, respectively, were constitutively expressed in uncastrated control animals. To confirm equal loading of the mRNA samples, the filter was washed and reprobed with rat \(\beta\)-actin cDNA. The autoradiogram was quantitated using an image analyzer, and the data was plotted to show the relative expression of NGFI-A and RB normalized to \(\beta\)-actin transcripts. The 3.2-kilobase NGFI-A transcript increased 2.7-fold 48 h following castration and then decreased steadily over the next 5 days. There was a 1.9-fold increase in the 4.7-kilobase RB transcript that paralleled the induction of NGFI-A. A duplicate experiment demonstrated an identical induction profile of NGFI-A and RB expression relative to \(\beta\)-actin (data not shown).

Discussion

Attention has begun to focus on transcription factors as agents of malignant transformation. Overexpression or loss of transcriptional regulatory genes is often associated with neoplasia. Many of these genes have the ability to directly alter patterns of gene expression, resulting in epigenetic changes that convert a normal cell to a neoplastic state (12).

The zinc finger DNA-binding domain has been identified in a number of transcription factors exhibiting diverse biological roles (13, 14). One of the most intensely studied groups of zinc finger transcription factors is the GSG family whose members share a common "zinc finger" DNA-binding domain which transcriptionally regulates genes containing the 9-nucleotide element CCGGGGGCG (15). The prototype, NGFI-A, was cloned from differentiating PC12 cells and displays rapid and transient expression in response to various extracellular signals (16); however, only the cardiac \(\alpha\)-myosin heavy chain gene has been identified as a potential NGFI-A target gene (17).

Loss of RB gene expression reported in two types of cancer appears to result from a loss of gene transcription. In hereditary retinoblastoma...
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toma, single-nucleotide mutations have been identified in potential SP1 and ATF elements within the RB promoter (18). Such mutations could potentially compromise normal transcription of the RB gene. Additionally, a study of testicular germ-cell malignancies showed that 100% of the tumors investigated exhibited diminished RB mRNA in spite of a normal gene, again suggesting alterations in RB gene transcription (19). RB function has been the subject of intense study resulting in greater knowledge of its biological and pathogenic significance; however, elucidation of regulatory mechanisms that govern the transcriptional state of the RB gene have not been thoroughly investigated.

This study demonstrates a potential mechanism of RB transcriptional regulation that may be altered during tumorigenesis of the prostate. We have determined that a novel 9-nucleotide element contained in the RB promoter is specifically recognized by the early response transcription factor NGFI-A. This element is lost as part of a larger deletion mutation in a case of prostate cancer in which RB expression is absent (5). The in vitro DNA binding studies coupled with the temporal pattern of RB and NGFI-A expression in the ventral prostate of castrated rats suggests that NGFI-A can recognize and bind to a novel element in the promoter of the RB gene, possibly regulating the transcription of RB in prostate cells.

References

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