

Suppression of Activin-Induced Apoptosis by Novel Antisense Strategy in Human Prostate Cancer Cells

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Apoptosin, a novel gene encoding a mitotic kinase-motif protein, is stimulated by activin, a member of TGF- β family, in human LNCaP prostate cancer cells and in patient tissues. We employed a gene knockout methodology based on the covalent bonding of chemically modified antisense probes to apoptosin mRNAs in LNCaP cells. The mRNA-antisense hybrid duplexes were neither translated nor post-transcriptionally modified, resulting in no protein synthesis. Introducing antisense apoptosin into activin-induced apoptotic LNCaP cells prevented apoptosis, interfered with genomic DNA fragmentation and released cell cycle checkpoint. These findings suggest that the apoptosin, in addition to p53, is important in apoptotic regulation of human prostate cancers. © 1999 Academic Press

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Apoptosin, a human homolog to *Rattus* mitotic checkpoint protein kinase Bub1 (1), was isolated by subtractive hybridization between cancerous and activin-induced apoptotic LNCaP cells (2). In *Saccharomyces cerevisiae*, Bub1 was localized in the nucleus and able to modulate the activation of the spindle assembly checkpoint (3). In *Drosophila*, mutations in Bub1 caused chromosome mis-segregation and apoptotic DNA fragmentation (4). Although the Bub/Mad signaling pathway was recently studied in human mitotic cells, the role of apoptosin in regulating this checkpoint and in apoptosis is still unclear. According to our previous findings (2), apoptosin is highly expressed in activin-induced apoptotic LNCaP cells, in which DNA fragmentation is more significant than cytoplasmic changes. The function of apoptosin and Bub1 appears to be consistent in cell cycle regulation but

different in apoptosis induction. Because the transfection of functional apoptosin is lethal to LNCaP and other tested competent cells, we used an antisense gene knockout method to reveal the function of this novel apoptotic kinase.

Gene knockout technology facilitates the determination of a gene function in living cells. Because traditional probes were nuclease sensitive and mediated through hydrogen-bond affinity (5–7), exceedingly high concentrations were required to obtain biological effects. To increase the specificity and efficiency of antisense targeting, a modified nucleotide probe with covalent binding capability, termed C-probe, was devised to enhance intracellular gene knockout (Fig. 1). The ability to form covalent bonds between two nucleotide sequences has permitted complete elimination of undesired gene homologs through subtractive hybridization procedures (8, 9). To increase the binding force without significant cytotoxicity, a C-probe is preferentially carboxylated on its C-5/C-6 pyrimidines permitting it to form a specific amide-linkage with the C-6/C-2 amino-groups of a targeted mRNA respectively (Fig. 2). C-probes were delivered into activin-induced apoptotic LNCaP cells by liposome mediated transfection (10), to investigate the role of apoptosin gene expression in subsequent DNA laddering and cell growth alterations.

MATERIALS AND METHODS

Cell culture and activin treatment. LNCaP cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum with 100 μ g/ml gentamycin at 37°C under 10% CO₂. For three-day activin induction, LNCaP cells were treated with 200 ng/ml activin per day, while control cells were simply treated with medium. Five days after treatment was initiated, a 56% reduction in growth was observed in the activin-treated cells compared to the control by both microscopy and cell counting as previously reported (11). The two groups of cells were independently trypsinized and mRNAs were purified by poly-(dT) dextran columns (Qiagen, Santa

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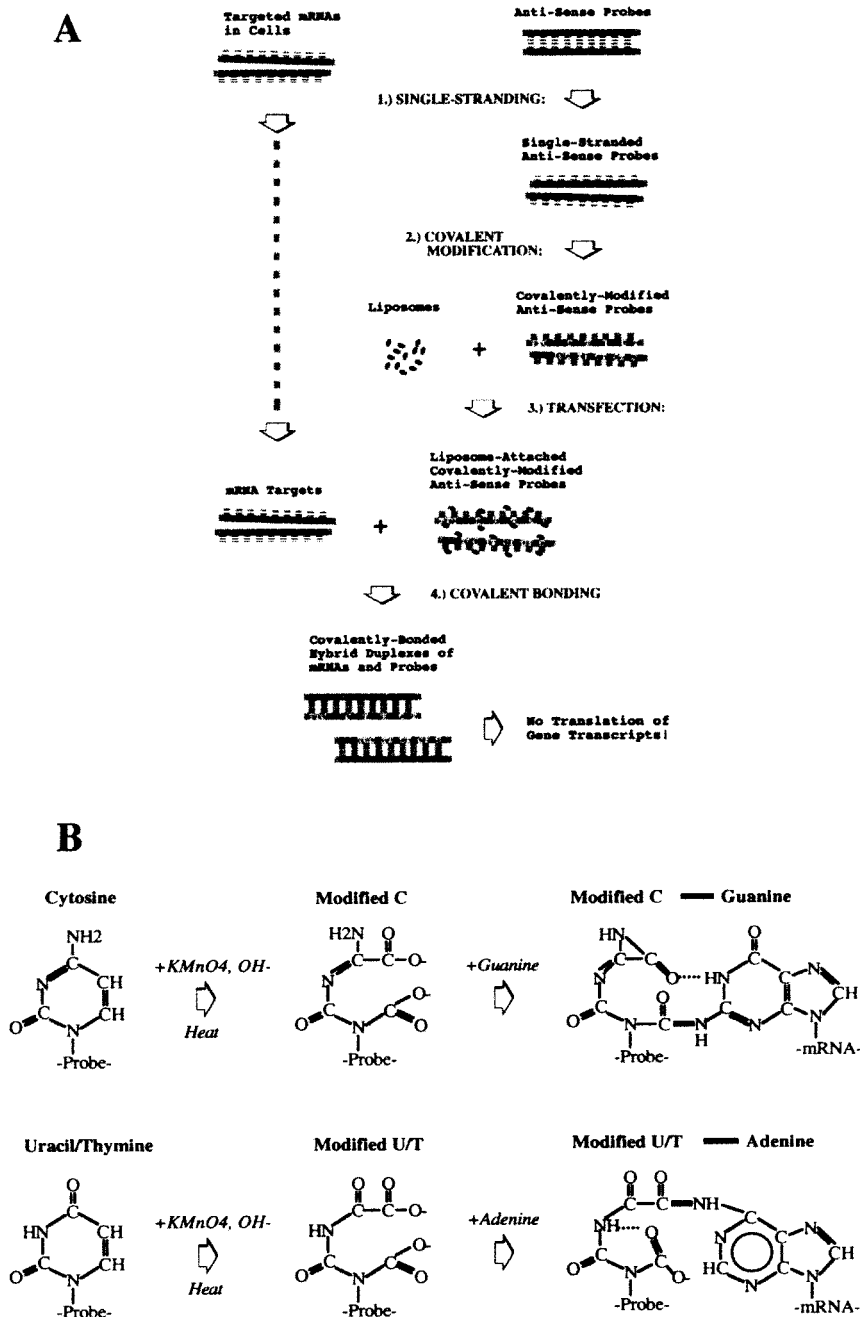


FIG. 1. Schematic protocol. (A) Gene knockout with C-probes, illustrating single-stranding, covalent modification, liposomal transfection, and covalent targeting of an antisense probe. The covalent modification (B) generates carboxyl groups on the C-5/C-6 of modified pyrimidines which can form covalent bonding with the amino groups on the C-6/C-2 of natural purines respectively.

Clarita, CA). The quality of isolated mRNAs (2 μ g) was assessed on 1% formaldehyde-agarose gels.

Preparation of C-probes. Following the single-strand DNA amplification method reported by Medori *et al.* (12), 100 ng of probes were used in a 100 μ l PCR reaction containing 30 pmol of anti-sense primer, 0.3 pmol of sense primer, dNTP mixture (0.2 mM each for dATP, dCTP, dGTP, dTTP and 0.1 mM dUTP), *Taq* DNA polymerase (3.5 U) and 1.5 mM MgCl₂. A thirty cycle PCR amplification was carried out by denaturation at 95°C, annealing at 55°C and extension at 72°C for 1 min in each step. Labeling of probes was achieved

by incorporating [³²P]-dATP (>3000 Ci/mM, Amersham International). The amplified antisense products (up to 30 μ g) were recovered by a microcon-50 filter (Amicon, Beverly, MA), single-stranded by adding 20 μ l alkaline acetyl chloride (3 min, 94°C) and neutralized by 80 μ l Tris buffer (10 mM, pH 7.4). After further purification with a microcon-50 filter in 10 μ l Tris buffer, 20 μ l potassium permanganate reagent (1 mM, pH 10) was added (3 min, 80°C) to generate carboxyl-groups on the C-5/C-6 of pyrimidines in the modified probes. The modified probes can form covalent bonding with amino-groups on the C-6/C-2 of purines from endogenous oligonu-

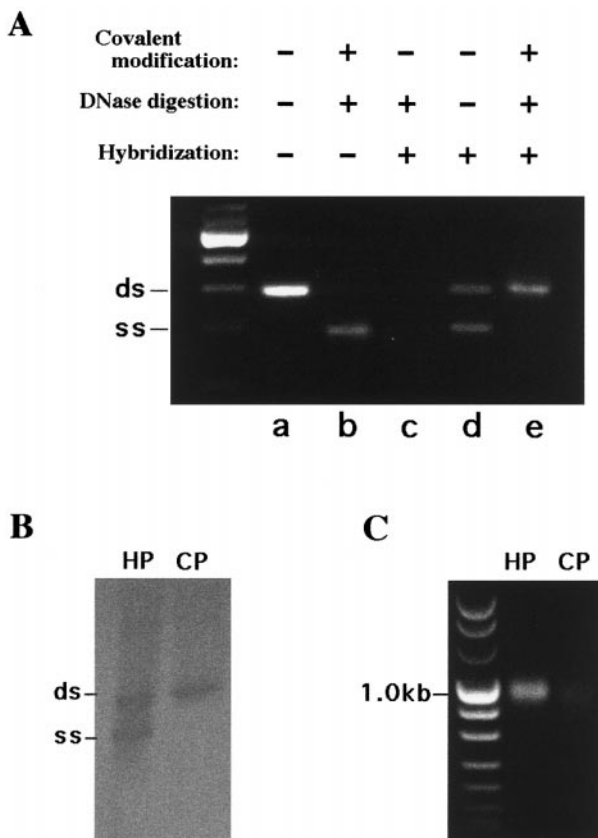


FIG. 2. Comparison between C-probes (CP) and traditional hydrogen-binding probes (HP). (A) *In vitro* analysis of binding efficiency and nuclease resistance by gel electrophoresis of digestions. a, double-stranded (ds) targeted apoptosin DNA fragments (200 ng); b, single-stranded (ss) antisense CP (100 ng); c, hybridization of the targeted DNAs and HP after nuclease digestion; d, same as lane c but without digestion; e, hybridization of CP to the targeted DNAs (100 ng each) after nuclease digestion. (B) Intracellular binding efficiency between apoptosin mRNAs and the two different kinds of antisense probes labeled with ^{32}P . (C) Agarose gel electrophoresis of RT-PCR products amplified from above probe-bound apoptosin mRNAs.

cleotides. The C-probes were finally collected by a microcon-50 filter in pure chloroform containing 3 mM triethylamine and 3 mM ethyl chloroformate at 4°C for 30 min and recollected by another microcon-50 in 10 μl of 20 mM Hepes buffer and prepared for liposome mediated transfection.

Nuclease resistance assay of C-probes. An apoptosin fragment (300 base pairs) served as the target DNA to test the nuclease susceptibility and binding efficiency of an antisense C-probe (300 base oligonucleotide with 70% homology to the apoptosin fragment). Equal amounts of double-stranded fragments and C-probes were mixed for nuclease digestion with or without hybridization. Hybridization was performed at 94°C for 3 min and then 70°C for 16 hours in EEx3 buffer (30 mM EPPS, pH 8.0 at 20°C; 3 mM EDTA). Nuclease digestion was performed with a mixture of DNase I and nuclease S1 (50 U each, Roche) at 25°C for 10 min in 1 \times NS1 buffer (0.2 M NaCl, 50 mM sodium acetate, pH 4.5; 1 mM ZnSO₄, 0.5% glycerol). The results were electrophoresed on a 2% agarose gel as shown in Fig. 2.

Liposomal transfection. Covalently modified probes (1-8 μg) were mixed with 50 μl DOTAP liposome (1 mg/ml; Roche) and applied to a 100 mm culture dish (12 ml) which contained activin-treated

LNCAp cells at 50% confluency. After a 24-hour incubation, the cells took up 60% of the probe-containing liposome. Uptake improved to 100% after two days of incubation. Using liposome-mediated intracellular transfection, C-probes can penetrate at least twenty layers of tumor cells (10). After five days, genomic DNAs were isolated by an apoptotic DNA ladder kit (Roche) and assessed on a 2% agarose gel. The cell growth and morphology were also examined. The mRNAs from the transfected LNCAp cells were isolated by poly-(dT) dextran columns (Qiagen), fractionated on a 1% formaldehyde-agarose gel after a 24-hour incubation period, and transferred onto nylon membranes (Schleicher & Schuell).

Northern blot hybridization. mRNAs were fractionated on 1% formaldehyde-agarose gels and transferred onto nylon membranes (Schleicher & Schuell, Keene, NH). Probes were labeled with the Prime-It II kit (Stratagene, La Jolla, CA) by random primer extension in the presence of [^{32}P]-dATP (>3000 Ci/mM, Amersham International, Arlington Heights, IL), and purified with Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Hybridization was carried out in a mixture of 50% freshly deionized formamide (pH 7.0), 5 \times Denhardt's solution, 0.5% SDS, 4 \times SSPE and 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA (18 h, 42°C). Membranes were sequentially washed twice in 2 \times SSC, 0.1% SDS (15 min, 25°C), and once each in 0.2 \times SSC, 0.1% SDS (15 min, 25°C); and 0.2 \times SSC, 0.1% SDS (30 min, 65°C) before autoradiography.

RESULTS AND DISCUSSION

In order to test the function of apoptosin, we had to block its protein synthesis in cells. For this purpose we tested an improved antisense technology, C-probe. We first desired to show the specificity of the covalent bonds formed by C-probes *in vitro* and *in cell* to prevent unnecessary cytotoxicity and increase targeting specificity.

Covalent Binding Specificity and Nuclease Resistance of Apoptosin C-Probes in Vitro

Although some chemotherapeutic agents can cross-link nucleotide sequences in cells (13, 14), the nonspecific crosslinking feature of these chemicals also causes significant toxicity to normal cells (15). To increase binding specificity without significant cytotoxicity, the carbon 5-6 double bond found in pyrimidines was opened using potassium permanganate followed by oxidation to carboxylic acid (16). The addition of activating agents such as ethyl chloroformate (17) can further activate these carboxyl groups to form an amide-linkage with the amino groups of purines. Such covalent bonding reaction generates low level of carbon dioxide and alcohol which are normally metabolized by cells. Since the covalent bond still maintains the original base pairing specificity, only highly matched homologues can form hybrid duplexes.

The affinity of a C-probe to its homologous gene sequence is greatly enhanced by this covalent interaction. As shown in Fig. 2A, the apoptosin C-probes provide 100% binding efficiency (lane e) compared to 53% in traditional probes (lane d). Also, the inertness between C-probes is rendered by acetylation (18), resulting in high binding efficiency between the C-probe and its target sequences. Moreover, because of their structural modifi-

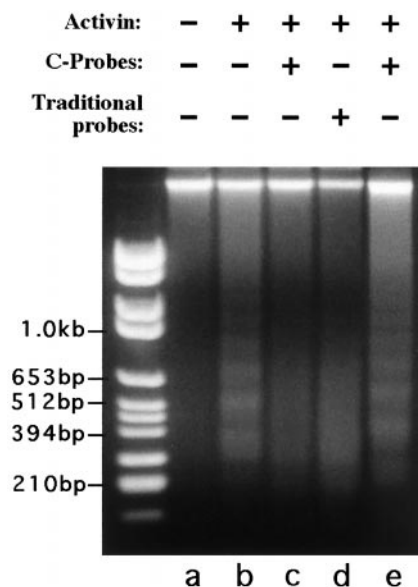


FIG. 3. Analysis of gene knockout effects after transfection of probes into activin-induced apoptotic LNCaP cells. LNCaP genomic DNAs were shown: a, without activin treatment; b, with activin treatment; c, with activin treatment and transfection by anti-apoptosin C-probes; d, with activin treatment and transfection by non-modified anti-apoptosin probes; and e, with activin treatment and transfection by sense-apoptosin C-probes. A better rescue effect results from applying antisense C-probes rather than traditional antisense probes, while the sense probes show no effect on preventing activin-induced apoptosis in LNCaP cells.

cation, they are highly resistant to nuclease digestion (lane b), even after binding with the targeted sequences (lane e). Such selective covalent bonding fully inhibits the functional activity of the targeted gene. Since covalently bound hybrid duplexes cannot be separated in cells, any enzymatic activity requiring single-stranded nucleotide templates will be effectively shut down. It has been shown that even a polymerase chain reaction (PCR) cannot be performed through the covalently bound hybrid duplexes (Fig. 2C).

In-Cell Covalent Targeting of Antisense C-Probes to Apoptosin mRNAs

To compare the efficiency of binding, mRNAs extracted from labeled control and C-probe transfected LNCaP cells were fractionated by electrophoresis. The autoradiogram (Fig. 2B) shows that the C-probes achieved 100% targeting while the traditional hydrogen-binding probes reached only 43% binding efficiency, based on the strength of radioactive emission. Agarose gel electrophoresis of RT-PCR products amplified from the above hybrids indicates that such covalent bonding completely inhibited polymerase extension activity on the targeted gene transcripts whereas the traditional hydrogen-binding did not. We have observed that the C-probes were effectively activated in transfected cells up to three days without significant

degradation or inactivation. The duration of the knock-out effects depends on the expression rate of individual genes in cells.

Genomic and Morphological Alterations of Apoptosin Gene Knockout by C-Probe Transfection

We then tested the ability of suppressing apoptosis with anti-apoptosin C-probes to rescue apoptosis using the activin-induced apoptosis model. The results indicated that the antisense apoptosin C-probes protected activin-treated LNCaP cells from apoptotic DNA fragmentation more efficiently than traditional antisense probes, whereas the sense C-probe of apoptosin had no effect (Fig. 3). The proliferation rate and morphology of activin-treated LNCaP cells were also changed after the C-probe knockout of apoptosin transcripts (Fig. 4).

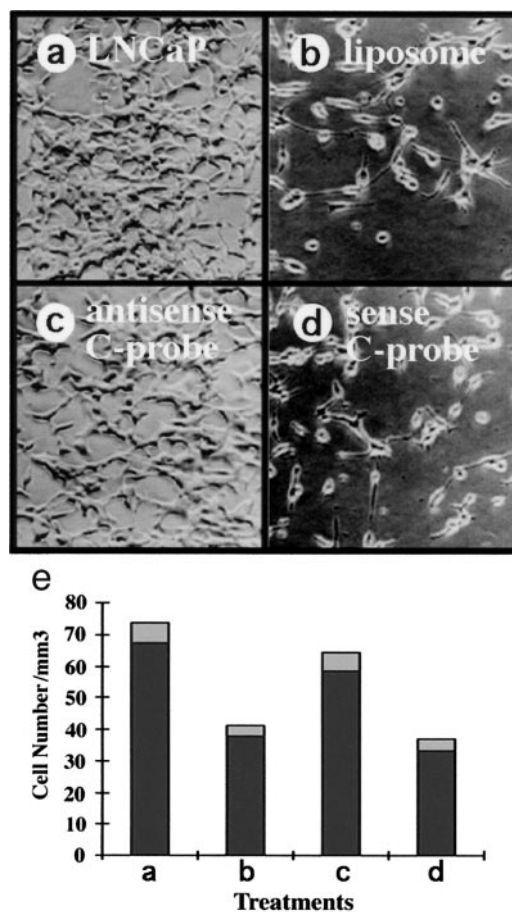


FIG. 4. Gene knockout effects on the cell number and morphology of LNCaP cells. (a) LNCaP cells without treatments. (b to d) Activin-treated cells after different transfections, including liposome only (lane b), anti-apoptosin C-probe (lane c), and sense-apoptosin C-probe (lane d). (e) The cells counted after transfections of lanes a to d demonstrate a 73% rescue effect of anti-apoptosin transfection in the activin-induced cell growth inhibition. The black bar indicates the mean of individual cell numbers counted in each treatment and the gray bar is the variation of each mean ($n = 7$).

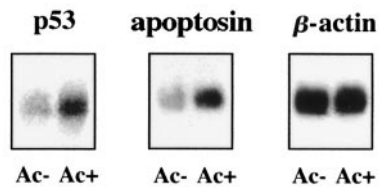
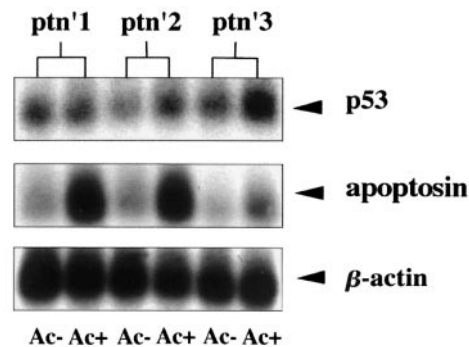
In vitro data from LNCaP cells:**In vivo data from prostatic cancer cells:**

FIG. 5. *In vivo* analysis of p53 and apoptosin expression by Northern blots between activin-positive (Ac+) and activin-negative (Ac-) cells. The patient 1 (ptn'1) represented malignant status, while patients 2 (ptn'2) and 3 (ptn'3) showed intermediate and prostatic intraepithelial neoplasia stage, respectively.

There was a 73% rescue of cell growth after transfecting anti-apoptosin C-probes into activin-treated cells (Fig. 4e). The transfection of either sense-apoptosin C-probes or only liposome carriers had no effect on cell apoptosis and morphology after activin treatments, indicating that the knockout results are highly gene-specific. The function of apoptosin therefore seems related to both cell cycle arrest and apoptosis.

In Vivo Significance of Apoptosin Expression in Activin-Expressing Cancerous Cells

The heterogeneity of progressive cancers usually counteracts the tumor suppression effect of p53. Apoptosin may provide an alternative way to monitor apoptotic regulation following tumor progression. Since the more malignant prostatic cancers contain fewer activin-positive cells, apoptosin appears to play a crucial role in preventing prostatic epithelium from hyperproliferation. Northern blot analysis (Fig. 5) of RNA extracts from either LNCaP cells (*in vitro*) or patients' tissues (*in vivo*) showed higher p53 and apoptosin expression in activin-positive cells. Moreover, based on the *in vivo* data, the p53 expression was more significant in less progressive cancer tissues while apoptosin was significant in more malignant tissues. However, these data only represent possible conditions within three patients as well as LNCaP cells. A larger scale

investigation into the role of apoptosin in cancer research is clearly warranted.

Sequencing data revealed highly conserved kinase domains between apoptosin and Bub1, although the proteins have different functions. While both apoptosin and Bub1 are capable of arresting the cell cycle at the G1 checkpoint, only apoptosin can then trigger DNA fragmentation in apoptotic cells. There are still many questions that need to be answered. For example, what is the target protein for the phosphorylation of apoptosin, how is apoptosin involved in both cell cycle regulation and apoptosis, and which factor activates or represses apoptosin? Current antisense technology successfully facilitates our understanding of loss of apoptosin function in human prostate cancer cells. Because the transduction of this apoptotic kinase is lethal, learning how to exploit other biotechnologies in the further analysis of apoptosin will be a forthcoming challenge.

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