

# Curcumin sensitizes prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L by inhibiting nuclear factor- $\kappa$ B through suppression of $\text{I}\kappa\text{B}\alpha$ phosphorylation

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## Abstract

Epidemiologic studies suggest that diet rich in plant-derived foods plays an important role in the prevention of prostate cancer. Curcumin, the yellow pigment in the spice turmeric, has been shown to exhibit chemopreventive and growth inhibitory activities against multiple tumor cell lines. We have shown previously that curcumin and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L interact to induce cytotoxicity in the LNCaP prostate cancer cell line. In this study, we investigated the mechanism by which curcumin augments TRAIL-induced cytotoxicity in LNCaP cells. Subtoxic concentrations of the curcumin-TRAIL combination induced strong apoptotic response in LNCaP cells as demonstrated by the binding of Annexin V-FITC and cleavage of procaspase-3. Furthermore, LNCaP cells express constitutively active nuclear factor- $\kappa$ B (NF- $\kappa$ B), which is inhibited by curcumin. Because NF- $\kappa$ B has been shown to mediate resistance to TRAIL-induced apoptosis in tumor cells, we investigated whether there is a relationship between NF- $\kappa$ B activation and resistance to TRAIL in LNCaP prostate cancer cells. Pretreatment with curcumin inhibited the activation of NF- $\kappa$ B and sensitized LNCaP cells to TRAIL. A similar increase in the sensitivity of LNCaP cells to TRAIL-induced apoptosis was observed following inhibition of NF- $\kappa$ B by dominant negative mutant  $\text{I}\kappa\text{B}\alpha$ , an inhibitor of NF- $\kappa$ B. Finally, curcumin was

found to inhibit NF- $\kappa$ B by blocking phosphorylation of  $\text{I}\kappa\text{B}\alpha$ . We conclude that NF- $\kappa$ B mediates resistance of LNCaP cells to TRAIL and that curcumin enhances the sensitivity of these tumor cells to TRAIL by inhibiting NF- $\kappa$ B activation by blocking phosphorylation of  $\text{I}\kappa\text{B}\alpha$  and its degradation. [Mol Cancer Ther 2004;3(7):803–12]

## Introduction

Prostate cancer is the second leading cause of cancer-related deaths in the United States (1, 2). Androgen reduction therapy is largely effective against hormone-sensitive tumors, but hormone-refractory clones often emerge after hormone therapy (3). Because apoptosis is the underlying mechanism by which most anticancer therapies including chemotherapy, radiation, and antihormonal therapy kill tumor cells, novel agents that may sensitize drug-resistant tumor cells for induction of apoptosis by conventional therapies could lead to the regression and improved prognosis of the refractory disease (4, 5). Indeed, chemotherapeutic agents have been shown to sensitize tumor cells to killing by death ligands such as tumor necrosis factor- $\alpha$  and Fas ligand (Apo1L or CD95L; refs. 6, 7). However, the use of these apoptosis-inducing antineoplastic agents is limited by unacceptable systemic toxicity. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L, a recently discovered member of the tumor necrosis factor superfamily, is a potent inducer of apoptosis in various cancer cell lines including prostate, colon, breast, skin, kidney, lung, brain, and hematologic tumor cell lines, with little cytotoxicity to the normal cells (8, 9). TRAIL is also effective in inhibiting the tumor growth *in vivo* (10, 11), and unlike the severe inflammatory response syndrome induced by tumor necrosis factor- and the hepatotoxicity of Fas ligand (12, 13), treatment of mice and nonhuman primates with TRAIL is without systemic toxicity (14). Recently, however, recombinant human TRAIL was shown to induce massive apoptosis in normal human liver hepatocytes but not in hepatocytes isolated from the livers of mice, rat, or nonhuman primates (15). The hepatotoxicity of soluble recombinant human TRAIL used in this study, as well as its toxicity toward normal keratinocytes shown in another report (16), was shown to result from the modifications introduced to the TRAIL molecule to stabilize and enhance trimerization of the three subunits of TRAIL. It is clear now that only the polyhistidine tagged or leucine zipper motif containing versions of recombinant human TRAIL with low zinc contents are toxic to the normal cells (17, 18).

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Although the exact cause of prostate cancer is not known, epidemiologic studies suggest that, in addition to race and age, diet is a prominent risk factor for prostate cancer (19). While a high-fat diet has been implicated in the occurrence of human cancers, including prostate cancer, several polyphenolic compounds found in common plant-derived foods have been recognized for their anticarcinogenic properties (20). Curcumin (diferuloyl-methane), the yellow pigment found in the spice turmeric extracted from the rhizome of the plant *Curcuma longa*, has been shown to have strong anti-inflammatory, antioxidant, and anti-lipoxygenase/cyclooxygenase activities (21, 22). Because of its ability to scavenge free radicals and inhibit inflammation, curcumin has been investigated for chemoprevention of cancers. Curcumin inhibited the induction of cancers of skin, forestomach, duodenum, tongue, colon, and mammary glands in models of chemical carcinogenesis in mice and rats (23-25). Exposure of tumor cell lines to curcumin *in vitro* inhibited cell proliferation and induced apoptosis (26-30). It has also shown therapeutic efficacy against human prostate cancer xenografts in nude mice (31). Together, these studies demonstrate that curcumin could potentially boost the antitumor activity of conventional cancer therapies and immunologic agents such as TRAIL. Indeed, we have reported previously that combined treatment with curcumin and TRAIL induces strong cytotoxic response in LNCaP cells characterized by the activation of effector caspase-3 and caspase-9 and the release of cytochrome *c* from mitochondria (32).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) regulates the transcription of genes involved in immune and inflammatory responses (33) and acts as a survival factor by protecting tumor cells from apoptosis-inducing tumor necrosis factor- $\alpha$ , TRAIL and radiochemotherapy (34-38). Because curcumin has been shown to inhibit NF- $\kappa$ B (29, 39), we hypothesized that curcumin might sensitize prostate cancer cells to TRAIL by inhibiting NF- $\kappa$ B. In this report, we show that there is a strong correlation between the constitutively active NF- $\kappa$ B and the resistance of LNCaP cells to induction of apoptosis by TRAIL. Curcumin sensitized LNCaP cells to TRAIL-induced apoptosis by inhibiting NF- $\kappa$ B activation by blocking the phosphorylation of I $\kappa$ B $\alpha$ , an inhibitor of NF- $\kappa$ B.

## Materials and Methods

### Reagents and Antibodies

Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-cleaved caspase-3 (ASP175) and anti-phospho-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-NF- $\kappa$ B antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TransAm NF- $\kappa$ B kit was from Active Motif (Carlsbad, CA). Texas red-conjugated anti-rabbit IgG antibody was from Vector Laboratories (Burlingame, CA). TRAIL and anti-TRAIL receptor (TRAIL-R) antibodies were purchased from R&D Systems (Minneapolis, MN). A 100 mmol/L solution of curcumin was prepared in DMSO, and all test concentrations were prepared by diluting the stock solution in tissue culture medium.

### Cell Lines

LNCaP cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Hyclone, Logan, UT), 100 units/mL penicillin G, 100  $\mu$ g/mL streptomycin sulfate, 1  $\mu$ g/mL hydrocortisone, and 100 nmol/L testosterone as described previously (40). Cells were cultured at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air and were maintained by subculturing cells twice a week.

PZ-HPV-7 cells, a human prostatic epithelial cell line obtained by transfecting normal prostatic epithelial cells with human papilloma virus type 18 DNA (41), were obtained from American Type Culture Collection and cultured in keratinocyte serum-free medium supplemented with recombinant human epidermal growth factor (5 ng/mL) and bovine pituitary extract (0.05 mg/mL). PZ-HPV-7 cells show transformed morphology but are nontumorigenic in nude mice.

### Measurement of Cell Viability [3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium, Inner Salt Assay]

Cells ( $2 \times 10^4$ ) were seeded into each well of a 96-well plate in 100  $\mu$ L tissue culture medium. After 24-hour incubation to allow cells to adhere, cells were treated with either curcumin, TRAIL, or a combination of the two agents. Cultures were incubated for an additional 48 hours. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using CellTiter 96 Aqueous One Solution Proliferation Assay System (Promega). This assay measures the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron coupling reagent phenazine methosulfate. MTS and phenazine methosulfate were added to the culture wells, and the mixture was incubated for 3 hours at 37°C. Absorbance was measured at 490 nm using a microplate reader and is directly proportional to the number of viable cells in the cultures. Percentage cytotoxicity was calculated from the loss of cell viability in cultures.

### Staining with Annexin V-FITC

Induction of apoptosis was assessed by the binding of Annexin V to phosphatidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, LNCaP cells untreated or treated with curcumin, TRAIL, or a combination of the two agents for 48 hours were resuspended in the binding buffer provided in the Annexin V-FITC Detection Kit II (BD Biosciences Pharmingen, San Diego, CA). Cells were mixed with 2  $\mu$ L Annexin V-FITC reagent and incubated for 30 minutes at room temperature in the dark. Stained cells were analyzed by fluorescent-activated cell sorting on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

### Measurement of TRAIL-R

To measure the expression of TRAIL-R, 10<sup>6</sup> LNCaP cells were treated with curcumin (10  $\mu$ mol/L) for 48 hours. After incubation, cells were washed, blocked with Fc block

(anti-CD16/CD32), and reacted with primary goat anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3, or anti-TRAIL-R4 antibodies (5  $\mu\text{g}/\text{mL}$ ) for 45 minutes on ice. Cells were washed once with PBS and incubated with phycoerythrin-conjugated donkey anti-goat secondary antibody (1:50 dilution) for 45 minutes at 4°C. Cells were washed and analyzed by flow cytometry.

#### Isolation of Nuclear Proteins

Nuclear extracts were prepared by the modified procedure of Dignam et al. (42). Following treatment with curcumin for 24 hours, LNCaP cells were washed three times with PBS and incubated on ice for 15 minutes in hypotonic buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.6% NP40]. Cells were vortexed gently for lysis, and nuclei were separated from the cytosol by centrifugation at  $12,000 \times g$  for 1 minute. Nuclei were resuspended in buffer C [20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride] and shaken for 30 minutes at 4°C. Nuclear extracts were obtained by centrifugation at  $12,000 \times g$ , and protein concentration was measured by Bradford assay (Bio-Rad, Richmond, CA). NF- $\kappa$ B in nuclear extracts was detected by Western blotting as described below.

#### NF- $\kappa$ B Binding Activity

NF- $\kappa$ B (p65) binding activity in nuclear extracts was measured using the ELISA-based TransAm NF- $\kappa$ B kit. In this assay, NF- $\kappa$ B binds to the immobilized oligonucleotide containing the NF- $\kappa$ B consensus sequence (5'-GGGA-CTTCC-3'), which is detected by sandwich ELISA. The detection limit for TransAm NF- $\kappa$ B kit is  $<0.4 \text{ ng}/\text{mL}$  purified p65.

#### Western Blot Analysis

Total cellular proteins were isolated by detergent lysis [1% Triton X-100 (v/v), 10 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 2 mmol/L sodium vanadate, 5  $\mu\text{g}/\text{mL}$  leupeptin, 1  $\mu\text{g}/\text{mL}$  aprotinin, 1  $\mu\text{g}/\text{mL}$  pepstatin, and 10  $\mu\text{g}/\text{mL}$  4-2-aminoethyl-benzenesulfonyl fluoride] as described by Keane et al. (43). Lysates were clarified by centrifugation at  $14,000 \times g$  for 10 minutes at 4°C, and protein concentrations were determined by Bradford assay. Samples (50  $\mu\text{g}$ ) were boiled in an equal volume of sample buffer [20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mmol/L Tris-HCl (pH 7.5), and 640 mmol/L 2-mercaptoethanol] and separated on 10% to 14% SDS-PAGE gels. Proteins resolved on the gels were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in 10 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl with 0.05% Tween 20 and probed with protein specific antibodies to caspase-3, poly(ADP-ribose) polymerase, NF- $\kappa$ B (p65), or phospho-I $\kappa$ B $\alpha$ . Immunocomplexes were visualized with enhanced chemiluminescence detection system from Amersham (Arlington Heights, IL).

#### Intracellular Localization of NF- $\kappa$ B

LNCaP cells cultured on cover slips for 24 hours were treated with curcumin (50  $\mu\text{mol}/\text{L}$ ) for 4 hours. After treatment, cells were washed twice and fixed with cold 4% paraformaldehyde for 1 hour. After washing in PBS, cells were blocked, permeabilized, and incubated overnight at 4°C with rabbit anti-ReiA (p65) antibody (1:500 dilution). After incubation, cells were treated with Texas red-conjugated anti-rabbit IgG in the dark at room temperature for 2 hours. Cells were washed, mounted with mounting medium containing 4',6-diamidino-2-phenylindole, and examined under fluorescent microscope.

#### DNA Transfections

The transcriptional activity of constitutive nuclear NF- $\kappa$ B was measured in NF- $\kappa$ B-dependent luciferase reporter gene expression assay. Briefly, LNCaP cells were plated in six-well plates for 24 hours and transiently transfected using the LipofectAMINE reagent (Invitrogen) with p3 $\kappa$ B-Luc (2  $\mu\text{g}$  DNA) expression plasmid containing three HIV  $\kappa$ B sites upstream of the thymidine kinase minimal reporter and the luciferase cDNA. Cells were incubated at 37°C for 24 hours and lysed to prepare cell extracts. The luciferase activity in cell extracts (50  $\mu\text{L}$ ) was measured in a luminometer using reagents and instructions provided with the luciferase assay system (Promega). In some cultures, LNCaP cells were also transfected with pCMV-I $\kappa$ B $\alpha$  vector (2  $\mu\text{g}$ ) expressing the dominant negative mutant I $\kappa$ B $\alpha$  (A32/36) from Clontech (Palo Alto, CA). The mutant I $\kappa$ B $\alpha$  contains serine-to-alanine mutations at residues 32 and 36, which confers resistant to phosphorylation and subsequent proteasome-mediated degradation but still binds to NF- $\kappa$ B.

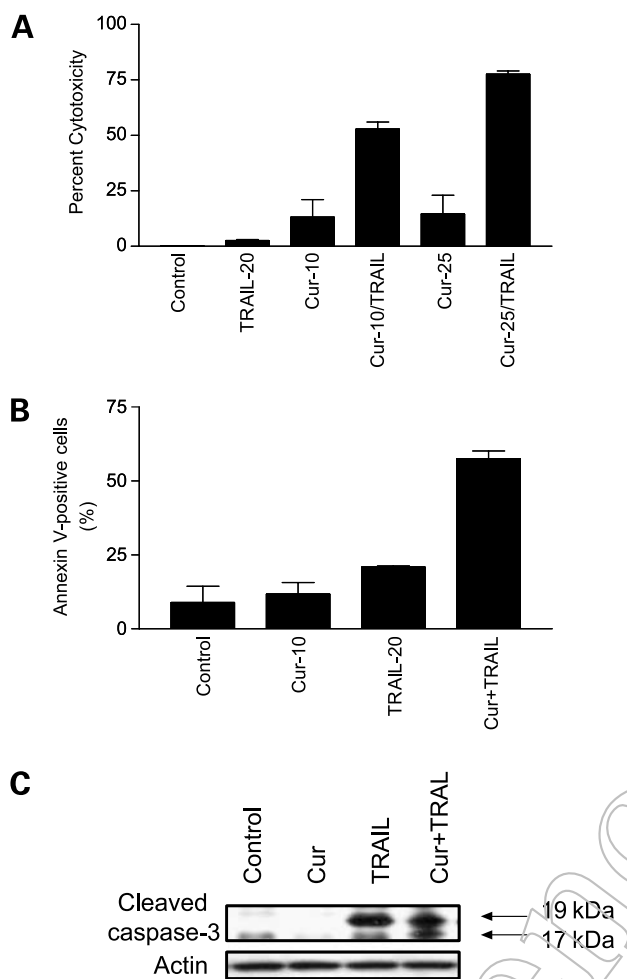
#### Statistical Analysis

Data are presented as means  $\pm$  SD. Interaction between TRAIL and curcumin was tested by two-way ANOVA. The degree of interaction was expressed as the percentage difference between the combined TRAIL and curcumin response and the sum of the responses to TRAIL and curcumin alone.

## Results

### Combined Curcumin and TRAIL Treatment Induces Cytotoxicity in Prostate Cancer Cells

The induction of cytotoxicity in LNCaP prostate cancer cells by curcumin (10 and 25  $\mu\text{mol}/\text{L}$ ), TRAIL (20 ng/mL), and combination of curcumin and TRAIL was calculated from the loss of cell viability measured by the MTS assay. As shown in Fig. 1A, treatment of LNCaP cells with curcumin (10 or 25  $\mu\text{mol}/\text{L}$ ) or TRAIL alone induced little cytotoxicity ( $\sim 10\%$ ). In contrast, simultaneous exposure of LNCaP cells to curcumin at both 10 and 25  $\mu\text{mol}/\text{L}$  and TRAIL resulted in significantly enhanced cytotoxicity (52% and 77%, respectively). On the other hand, the viability of PZ-HPV-7 human prostatic epithelial cells, which are transformed but are not tumorigenic, was not affected at these concentrations of curcumin or TRAIL when applied separately or in combination (data not shown). Overall, the



**Figure 1.** **A**, curcumin sensitizes prostate cancer cells to TRAIL-induced cytotoxicity. LNCaP cells ( $2 \times 10^4$ ) were treated with curcumin (10 or 25  $\mu\text{mol/L}$ ), TRAIL (20 ng/mL), or combination of curcumin and TRAIL for 48 hours. Cell viability was measured by the MTS assay using CellTiter 96 Aqueous One Solution Proliferation Assay System. **B**, TRAIL-induced binding of Annexin V-FITC to LNCaP cells. LNCaP cells were treated with curcumin (10  $\mu\text{mol/L}$ ), TRAIL (20 ng/mL), or curcumin-TRAIL combination for 48 hours. Cells were resuspended in 0.5 mL of binding buffer, reacted with 2  $\mu\text{L}$  Annexin V-FITC for 30 minutes at room temperature, and analyzed for Annexin V-FITC binding by flow cytometry. **C**, combined curcumin and TRAIL treatment activates caspase-3. Whole cell lysate proteins (50  $\mu\text{g}$  per lane) isolated from untreated LNCaP cells or those treated with TRAIL, or curcumin, or curcumin + TRAIL for 20 hours were fractionated on 10% to 14% SDS-PAGE gels. Proteins were transferred from the gel to nitrocellulose membrane and probed with antibodies to cleaved caspase-3 or actin using enhanced chemiluminescence. Similar results were obtained in three independent experiments.  $P < 0.044$ , 10  $\mu\text{mol/L}$  curcumin + 20 ng/mL TRAIL;  $P < 0.018$ , 25  $\mu\text{mol/L}$  curcumin + 20 ng/mL TRAIL.

amount of cytotoxicity induced with combined curcumin and TRAIL treatment was 320% and 489% higher for 10 and 25  $\mu\text{mol/L}$  curcumin-TRAIL combinations than cytotoxicity that would be induced if the combined effect was simply additive.

To determine whether apoptosis is the mode of cell death induced by combination of curcumin and TRAIL,

LNCaP cells treated with curcumin, TRAIL, or curcumin-TRAIL combination for 48 hours were examined by flow cytometry for binding of Annexin V-FITC to phosphatidylserine, which is externalized to the outer surface of the plasma membrane in cells undergoing apoptosis. As shown in Fig. 1B, there was little binding of Annexin V-FITC to untreated LNCaP cells or those treated with curcumin alone (9% and 12%, respectively). The binding of Annexin V-FITC was increased somewhat following treatment of cells with TRAIL (21%). However, the binding of Annexin V-FITC with combined treatment with curcumin and TRAIL (58%) was 76% higher than what would be expected if the effect was merely additive. The  $P$  value for the test for interaction was 0.003.

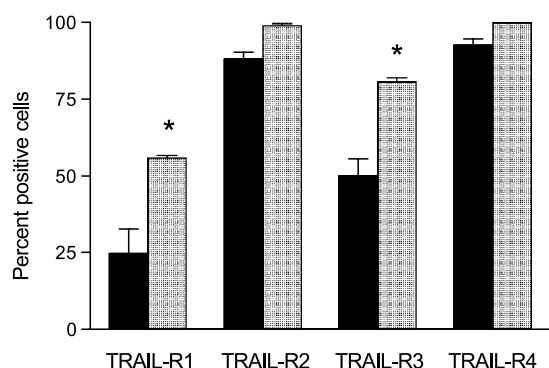
To further investigate induction of apoptosis, we performed immunoblot analysis of whole cell lysate to determine cleavage of procaspase-3. Untreated LNCaP cells and those treated with curcumin (10  $\mu\text{mol/L}$ ) for 20 hours showed little or no cleaved fragments (19 and 17 kDa) of caspase-3 (Fig. 1C, lanes 1 and 2). The cleavage of procaspase-3 was increased in cells treated with TRAIL alone (20 ng/mL; lane 3). However, by far the most processing of procaspase-3 was observed in cells treated with curcumin-TRAIL combination (lane 4). Thus, enhanced binding of Annexin V-FITC and activation of effector caspase-3 demonstrate induction of apoptosis in cells treated with curcumin-TRAIL combination.

#### Effect of Curcumin on Expression of TRAIL-R on LNCaP Cells

Because TRAIL-induced apoptosis in LNCaP cells was enhanced by curcumin, we considered the possibility that curcumin might enhance apoptosis by TRAIL by modulating the expression TRAIL-R. TRAIL transmits the proapoptotic signal by interacting with death receptor 4 (DR4 or TRAIL-R1) and death receptor 5 (DR5 or TRAIL-R2). Both TRAIL-R1 and TRAIL-R2 have cytoplasmic death domains that can transduce death signals and activate caspases and NF- $\kappa\text{B}$  (44). TRAIL also binds to decoy receptors Dcr1 (TRAIL-R3) and Dcr2 (TRAIL-R4), which have truncated death domains and are incapable of transducing death signals (45). We examined LNCaP cells for the expression of TRAIL-R and the effect curcumin has on their expression by flow cytometry. Figure 2 shows that LNCaP cells express all of the four TRAIL-R. The high-affinity death signaling TRAIL-R2 is more abundantly expressed than TRAIL-R1. In addition, both decoy receptors (TRAIL-R3 and TRAIL-R4) are also heavily expressed on LNCaP cells. Treatment with curcumin (10  $\mu\text{mol/L}$ ) for 48 hours up-regulated the expression of TRAIL-R1 without altering the expression of TRAIL-R2. Curcumin also increased the expression of TRAIL-R3 without affecting TRAIL-R4 expression. These results indicate that curcumin selectively increases the expression of TRAIL-R1 and TRAIL-R3 on LNCaP cells.

#### LNCaP Cells Express NF- $\kappa\text{B}$ and Curcumin Inhibits It

We next investigated the possibility that constitutively active NF- $\kappa\text{B}$  mediates the resistance of LNCaP cells to



**Figure 2.** Effect of curcumin on TRAIL-R. Monolayers of LNCaP cells were treated with curcumin (10  $\mu\text{mol/L}$ ) for 48 hours. Untreated (*black bars*) or treated (*gray bars*) cells ( $1 \times 10^6$ ) were blocked with Fc block (anti-CD16/CD32) and reacted with primary goat anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3, or anti-TRAIL-R4 (5  $\mu\text{g/mL}$  each) for 45 minutes on ice. Cells were washed and incubated with phycoerythrin-conjugated donkey anti-goat secondary antibody for 45 minutes on ice. Cells were analyzed for TRAIL-R expression on a FACScan flow cytometer. \*,  $P < 0.05$ , compared with control TRAIL-R1 and TRAIL-R2.

TRAIL, and potentiation of TRAIL killing of these cells might involve inhibition of NF- $\kappa\text{B}$  activation by curcumin. Immunoblot analysis of nuclear extracts prepared from untreated and treated cells revealed the expression of constitutive NF- $\kappa\text{B}$ , which was inhibited following treatment with curcumin for 20 hours in a dose-dependent manner (Fig. 3A). NF- $\kappa\text{B}$  was also suppressed in cells treated with curcumin-TRAIL combination. On the other hand, treatment with TRAIL alone had no effect on NF- $\kappa\text{B}$  expression. Similar results were obtained in experiments in which DNA binding activity of NF- $\kappa\text{B}$  to the  $\kappa\text{B}$  consensus sequence was measured. These measurements also showed activated NF- $\kappa\text{B}$  in LNCaP cells, which is inhibited by curcumin (Fig. 3B). These results were further corroborated by immunocytochemical examination of NF- $\kappa\text{B}$  in LNCaP cells. Control cells exhibited intense nuclear localization of NF- $\kappa\text{B}$  as detected by treatment with anti-RelA (p65) primary antibody followed by staining with Texas red-conjugated secondary antibody (Fig. 3C). Cells treated with curcumin (50  $\mu\text{mol/L}$ ) for 4 hours showed diffused cytoplasmic expression of RelA. Taken together, these results demonstrate that LNCaP cells express constitutively active NF- $\kappa\text{B}$ , which is inhibited by curcumin.

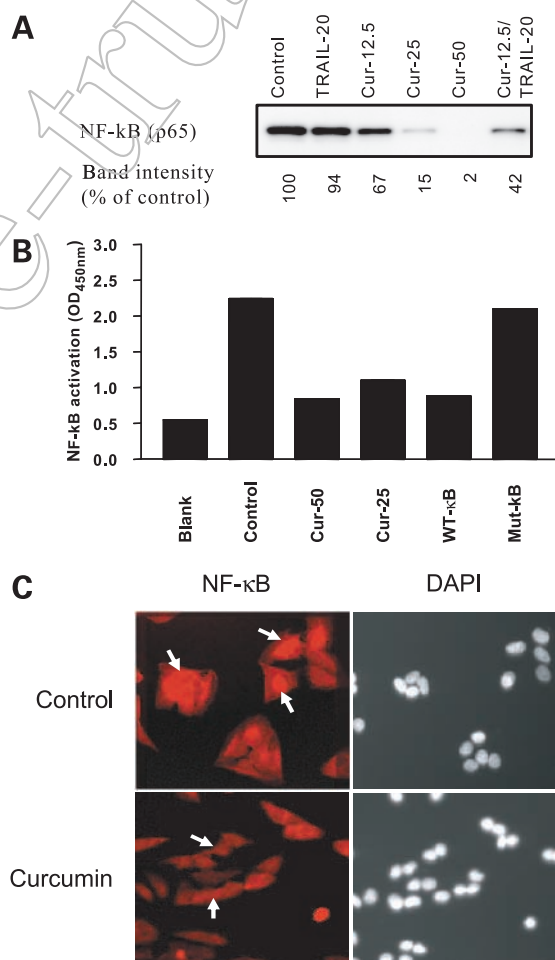
#### Constitutive NF- $\kappa\text{B}$ in LNCaP Cells Is Transcriptionally Active

To examine whether constitutive NF- $\kappa\text{B}$  is transcriptionally active, LNCaP cells were transiently transfected with NF- $\kappa\text{B}$  regulated p3 $\kappa\text{B}$ -Luc reporter construct to measure NF- $\kappa\text{B}$ -dependent reporter gene expression. As shown in Fig. 4, the relative luciferase activity of LNCaP cells transfected with p3 $\kappa\text{B}$ -Luc reporter vector was  $\sim 75$ -fold higher compared with cells transfected with the control plasmid without  $\kappa\text{B}$  sites. The luciferase activity was suppressed when transfected cells were treated with curcumin at 50  $\mu\text{mol/L}$ . Luciferase activity was also suppressed when cells were double transfected with

p3 $\kappa\text{B}$ -Luc and dominant negative pCMV-I $\kappa\text{B}\alpha\text{M}$  (mutant) vectors. The inhibition of NF- $\kappa\text{B}$  activity by curcumin or pCMV-I $\kappa\text{B}\alpha\text{M}$  was further confirmed by immunoblotting of nuclear NF- $\kappa\text{B}$  (*top*). These results indicate that constitutive NF- $\kappa\text{B}$  in LNCaP cells is transcriptionally active.

#### Pretreatment with Curcumin Sensitizes LNCaP Cells to TRAIL by Inhibiting NF- $\kappa\text{B}$

To determine whether the mechanism by which curcumin sensitizes LNCaP cells to TRAIL-induced apoptosis involves inhibition of NF- $\kappa\text{B}$ , we pretreated LNCaP cells with curcumin at 25  $\mu\text{mol/L}$  for 48 hours followed by extensive washing. Cells were treated at 25  $\mu\text{mol/L}$  curcumin because, at this concentration, curcumin almost completely blocks NF- $\kappa\text{B}$  expression (Fig. 3A). After treatment with curcumin, cells were incubated with TRAIL (20 ng/mL) for 48 hours to determine cytotoxicity. As

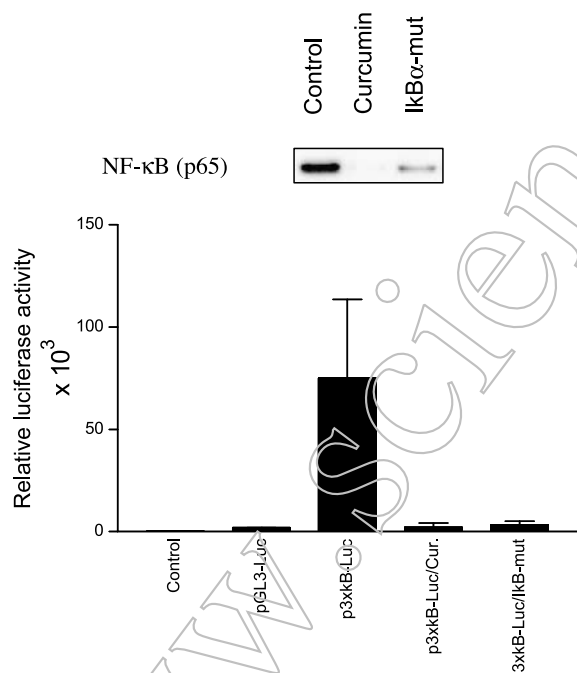


**Figure 3.** Constitutive NF- $\kappa\text{B}$  expression in LNCaP cells and its inhibition by curcumin. Nuclear extracts were prepared from untreated LNCaP cells (*Control*) or those treated with curcumin (12.5 to 50  $\mu\text{mol/L}$ ) for 20 hours and analyzed for NF- $\kappa\text{B}$  by Western blotting (**A**) or colorimetrically (**B**) as described in Materials and Methods. **C**, immunocytochemical detection of NF- $\kappa\text{B}$  in LNCaP cells. Cells were treated with curcumin (50  $\mu\text{mol/L}$ ) for 4 hours and reacted with anti-RelA (p65) antibody followed by Texas red-conjugated secondary antibody for immunocytochemical detection of NF- $\kappa\text{B}$ . Similar results were obtained in three identical experiments.

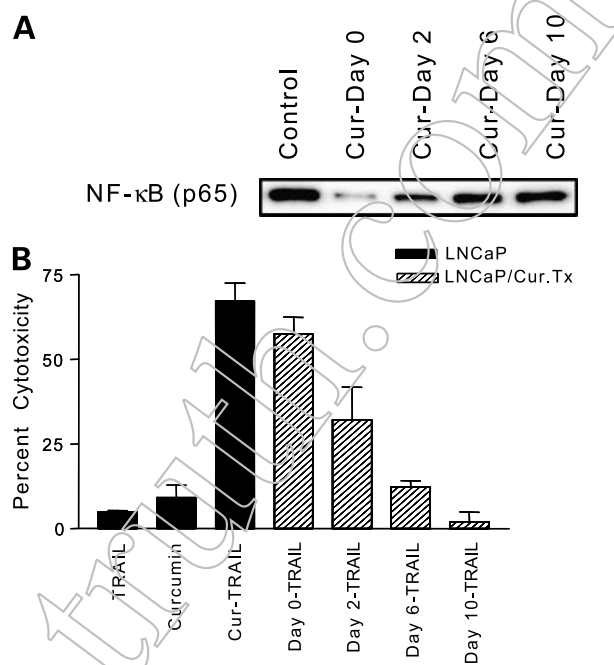
shown in Fig. 5A, pretreatment with curcumin for 48 hours resulted in the suppression of NF- $\kappa$ B (day 0). Following the removal of curcumin from cultures, NF- $\kappa$ B began to recover and reached the pretreatment levels between days 6 and 10. The induction of sensitivity to TRAIL in pretreated cells was tightly associated with the level of NF- $\kappa$ B (Fig. 5B). Induction of cytotoxicity in pretreated cells was most pronounced on day 0 (58%), a time at which NF- $\kappa$ B was maximally suppressed. The progressive decrease in percentage cytotoxicity from days 2 to 10 corresponded with the reappearance of nuclear NF- $\kappa$ B in treated cells. Thus, these data demonstrate a clear correlation between constitutive NF- $\kappa$ B and resistance/sensitivity to TRAIL.

#### Inhibition of NF- $\kappa$ B by Ectopic Expression of I $\kappa$ B $\alpha$ Restores Susceptibility of LNCaP Cells to TRAIL

Results shown in Fig. 5 demonstrated that the level of NF- $\kappa$ B determines the resistance or susceptibility of LNCaP cells to TRAIL. These data, however, do not provide direct evidence that NF- $\kappa$ B mediates resistance of LNCaP cells to TRAIL because, besides inhibiting NF- $\kappa$ B, curcumin may also induce additional biochemical changes in cells that could contribute to the sensitization of LNCaP cells to TRAIL. To test this more directly, LNCaP cells were



**Figure 4.** Constitutive NF- $\kappa$ B is transcriptionally active in LNCaP cells. LNCaP cells grown to 60% to 70% confluency were transfected with control vector (*pGL3-Luc*), p3 $\kappa$ B-Luc, or p3 $\kappa$ B-Luc and dominant negative I $\kappa$ B $\alpha$ M (*I $\kappa$ B $\alpha$ -mut*) expression vectors (2  $\mu$ g plasmid DNA) for 24 hours. Culture medium was replaced with fresh medium, and in some wells, cells transfected with p3 $\kappa$ B-Luc vector were treated with curcumin (50  $\mu$ M) for 20 hours. Cell lysates were prepared, and luciferase activity was determined in a luminometer using the luciferase assay system. Nuclear extracts prepared from p3 $\kappa$ B-Luc transfected LNCaP cells treated with curcumin or transfected with I $\kappa$ B $\alpha$  expression vector were analyzed for NF- $\kappa$ B by Western blotting (*top*). Similar results were obtained in two separate experiments.



**Figure 5.** Curcumin inhibits NF- $\kappa$ B and augments induction of cytotoxicity by TRAIL. LNCaP cells were treated with curcumin (25  $\mu$ M) for 20 hours. Cells were extensively washed in PBS to remove curcumin and tested for expression of nuclear NF- $\kappa$ B and induction of cytotoxicity by TRAIL. **A**, western blot analysis of nuclear NF- $\kappa$ B on days 0, 2, 6, and 10 after removal of curcumin from the cultures. **B**, induction of cytotoxicity by TRAIL (20 ng/mL) in cells pretreated with curcumin (*hatched bars*) and induction of cytotoxicity in untreated LNCaP cells by curcumin, TRAIL, or curcumin-TRAIL combination (*solid bars*). Identical results were obtained in three separate experiments.

transiently transfected with pCMV-I $\kappa$ B $\alpha$ M dominant negative vector for targeted molecular ablation of NF- $\kappa$ B and tested for suppression of NF- $\kappa$ B and susceptibility to TRAIL. The expression of NF- $\kappa$ B was dramatically reduced on day 2 after transfection and returned to the pretransfection level by day 12 (Fig. 6A). Day 2 cells with highly decreased level of NF- $\kappa$ B showed significantly enhanced susceptibility to TRAIL in the absence of curcumin (Fig. 6B). On the other hand, day 12 transfected cells expressed NF- $\kappa$ B at a level comparable with that for control cells and were resistant to TRAIL. However, these cells remained susceptible to the combined curcumin-TRAIL treatment. These results provide direct evidence that NF- $\kappa$ B mediates resistance of LNCaP cells to TRAIL.

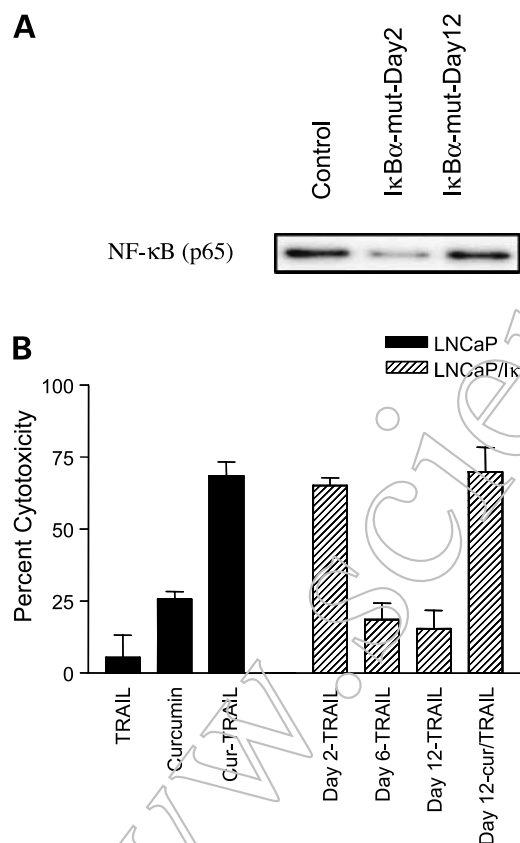
#### Curcumin Inhibits I $\kappa$ B $\alpha$ Phosphorylation

To determine whether inhibition of NF- $\kappa$ B by curcumin is due to inhibition of the degradation of I $\kappa$ B $\alpha$ , we measured the effect of curcumin on phosphorylation of I $\kappa$ B $\alpha$ , an essential event for its proteolytic degradation. As shown in Fig. 7A, treatment of LNCaP cells with TRAIL increased the phosphorylation of I $\kappa$ B $\alpha$  (*lane 2* versus *lane 1*). Phosphorylation of I $\kappa$ B $\alpha$  was partially to completely inhibited by curcumin at 12.5  $\mu$ M (*lane 2*) after 4- and

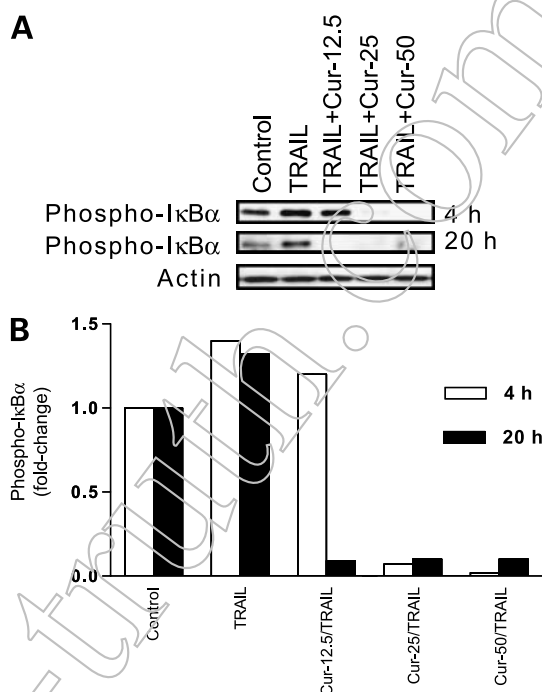
20-hour treatment, respectively. At higher concentrations (25 to 50  $\mu\text{mol/L}$ ), curcumin completely inhibited  $\text{I}\kappa\text{B}\alpha$  phosphorylation (lanes 4 and 5) after 4-hour treatment. These data imply that curcumin inhibits constitutive  $\text{NF-}\kappa\text{B}$  by suppressing phosphorylation and degradation of  $\text{I}\kappa\text{B}\alpha$ .

## Discussion

Recent studies have shown that plant-derived dietary compounds provide protection against the development of cancers and other diseases (46, 47). We and others have shown previously that food additive curcumin inhibits proliferation and induces apoptosis in several tumor cell lines (26-30). We have also demonstrated that curcumin sensitizes hormone-sensitive LNCaP human prostate cancer cells to TRAIL-induced apoptosis by promoting cleavage of procaspases and release of cytochrome *c* from mitochondria (32). Here, we report that suppression of the constitutively active  $\text{NF-}\kappa\text{B}$  in LNCaP cells is a major event by which curcumin chemosensitizes prostate cancer cells to



**Figure 6.** Expression of dominant negative  $\text{I}\kappa\text{B}\alpha$  inhibits  $\text{NF-}\kappa\text{B}$  and increases susceptibility to TRAIL. LNCaP cells were transfected with dominant negative pCMV- $\text{I}\kappa\text{B}\alpha$  vector expressing mutant  $\text{I}\kappa\text{B}\alpha$  (A32/36). Transfected cells were harvested to measure  $\text{NF-}\kappa\text{B}$  and sensitivity to TRAIL. **A**, western blot analysis of  $\text{NF-}\kappa\text{B}$  in control and transfected cells (days 2 and 12). **B**, cytotoxic response of days 2, 6, and 12 transfected cells to TRAIL (20 ng/mL) or day 12 cells to curcumin-TRAIL combination (cur/TRAIL; hatched bars) and cytotoxic response of control LNCaP cells to TRAIL, curcumin, or combined curcumin-TRAIL (solid bars). Similar results were obtained in three independent experiments.



**Figure 7.** Curcumin inhibits phosphorylation of  $\text{I}\kappa\text{B}\alpha$ . **A**, LNCaP cells were treated with TRAIL (20 ng/mL) or combined TRAIL-curcumin (12.5, 25, and 50  $\mu\text{mol/L}$ ) simultaneously for 4 or 20 hours. Cell lysates were prepared and analyzed by Western blotting to determine phosphorylated  $\text{I}\kappa\text{B}\alpha$  using antibodies against phosphorylated  $\text{I}\kappa\text{B}\alpha$ . **B**, histogram showing the relative levels of phospho- $\text{I}\kappa\text{B}\alpha$ . Identical results were obtained in two separate experiments.

TRAIL-induced cytotoxicity. The blockade of  $\text{NF-}\kappa\text{B}$  activation by curcumin involved repression of the phosphorylation of  $\text{I}\kappa\text{B}\alpha$ , an event that primes  $\text{I}\kappa\text{B}\alpha$  for proteasomal degradation.

Hormone-sensitive LNCaP cells are only slightly sensitive to TRAIL or curcumin alone. However, these two agents together induced a strong apoptotic response characterized by the binding of Annexin V and activation of effector caspase-3. Other investigators have shown DNA-damaging chemotherapeutics doxorubicin, etoposide, and CPT-11 to potentiate apoptosis by TRAIL in a variety of tumor cell lines (48-52). Because administration of these agents is limited by systemic toxicity, curcumin, which is a pharmacologically safe agent, provides an alternative approach to sensitize tumor cells to TRAIL without toxicity.

Curcumin may enhance the sensitivity of tumor cells to TRAIL by modulating the expression of TRAIL-R on tumor cells. Fluorescent-activated cell sorting analysis revealed that LNCaP cells express death signal-inducing TRAIL-R1 (DR4) and TRAIL-R2 (DR5) as well as decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2). Curcumin increased the expression of TRAIL-R1 but not of TRAIL-R2. It also increased the expression of TRAIL-R3 without altering TRAIL-R4 expression. It is unclear whether increase in TRAIL-R1 by curcumin accounts for increased

sensitivity to TRAIL because inhibitory TRAIL-R3 was also increased by curcumin. It should be noted, however, that TRAIL-R data presented are the percentage of cells expressing each receptor, not the total receptor abundance. Therefore, the possibility remains that curcumin sensitizes LNCaP cells to TRAIL by altering the balance of death and decoy TRAIL-R in favor of death-inducing TRAIL-R1 and TRAIL-R2. NF- $\kappa$ B not only plays a critical role in the transcription of genes involved in immune and inflammatory responses (33), cell proliferation/differentiation (53), or cell transformation (54) but also acts as a survival factor by protecting tumor cells by inhibiting apoptosis (34-37). Because NF- $\kappa$ B protects tumor cells from apoptosis, we considered the possibility that resistance of prostate cancer cells to TRAIL might also be due to increased NF- $\kappa$ B activity. LNCaP cells were found to express transcriptionally active NF- $\kappa$ B. Treatment with curcumin inhibited NF- $\kappa$ B in a dose-dependent fashion. Others have also shown curcumin to suppress constitutive and inducible NF- $\kappa$ B activity in several cell types (54-56). Because activated NF- $\kappa$ B induces proliferation in tumor cells and therefore curtails apoptosis, this might explain why LNCaP cells are resistant to TRAIL.

Our results also demonstrate that constitutively active NF- $\kappa$ B in LNCaP cells is not merely an epiphenomenon irrelevant to the resistance of these cells to TRAIL, because suppression of NF- $\kappa$ B through pretreatment with curcumin or transfection with superrepressor I $\kappa$ B $\alpha$  (A32/36) sensitized LNCaP cells to TRAIL. This result agrees with previous studies showing that constitutive NF- $\kappa$ B renders tumor cells resistant to TRAIL (54, 57, 58), and its inhibition with various agents leads to the sensitization of tumor cells to TRAIL-induced apoptosis (59-61).

The mechanism by which curcumin inhibits NF- $\kappa$ B is unclear. In resting cells, NF- $\kappa$ B remains sequestered in the cytoplasm in a functionally inactive form noncovalently bound to an inhibitory protein, I $\kappa$ B $\alpha$  (62). On stimulation of cells with mitogens, antigens, or cytokines, I $\kappa$ B $\alpha$  is phosphorylated and degraded allowing NF- $\kappa$ B to translocate to the nucleus where it binds to the  $\kappa$ B motifs in the promoter region of the responsive genes. Our result demonstrating the inhibition of phosphorylation of I $\kappa$ B $\alpha$  indicates that curcumin inhibits NF- $\kappa$ B signaling by preventing I $\kappa$ B $\alpha$  phosphorylation and its degradation. These results are consistent with previous reports in which curcumin was shown to inhibit phosphorylation of I $\kappa$ B $\alpha$  (54, 63). Whether curcumin inhibits I $\kappa$ B $\alpha$  phosphorylation in LNCaP cells by blocking IKK activity, the kinase that phosphorylates I $\kappa$ B $\alpha$  remains to be determined.

It is also not entirely clear how suppression of NF- $\kappa$ B by curcumin promotes apoptosis, but it may be related to the effect of curcumin on NF- $\kappa$ B target genes that regulate apoptosis. Curcumin down-regulates the expression of NF- $\kappa$ B-dependent antiapoptotic Bcl-2 and Bcl-xL in prostate cancer cell lines (29). The inhibition of NF- $\kappa$ B by curcumin may also attenuate members of the inhibitor of apoptosis proteins family, which are regulated by NF- $\kappa$ B (64, 65). Besides NF- $\kappa$ B, other molecular targets of cur-

cumin such as cell survival signal protein kinase B/Akt, c-Jun NH<sub>2</sub>-terminal kinase, and peroxisome proliferator-activated receptor  $\gamma$  (66-68) may also contribute to the chemosensitization of prostate cancer cells to TRAIL.

In conclusion, present studies demonstrate that constitutively active NF- $\kappa$ B mediates resistance of prostate cancer cells to TRAIL, and the mechanism by which curcumin sensitizes them to TRAIL involves suppression of NF- $\kappa$ B activation through inhibition of I $\kappa$ B $\alpha$  phosphorylation and its degradation. Although the finding that a relatively nontoxic agent such as curcumin can restore the responsiveness of LNCaP cells to TRAIL is promising, additional prostate cancer cell lines will have to be tested before the combined curcumin-TRAIL treatment regimen could be considered for clinical application. It is also unclear whether the effective (sensitizing) concentration of curcumin can be achieved *in vivo* because of the poor bioavailability of orally administered curcumin (69). This difficulty may be surmounted, however, by coformulation with piperine, which has been shown to inhibit hepatic and intestinal glucuronidation and increase the bioavailability of curcumin (70).

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