

Suppression of casein kinase 2 sensitizes tumor cells to antitumor TRAIL therapy by regulating the phosphorylation and localization of p65 in prostate cancer

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Abstract. In the United States, prostate cancer (PCa) is the most commonly diagnosed cancer in males. For PCa at the late hormone-refractory stage, substantial improvement in treatment strategies is critically needed. TNF-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent, but both intrinsic and acquired resistance to TRAIL poses a huge problem in establishing clinically effective TRAIL therapies. In the present study, we examined the role played by casein kinase 2 (CK2) in the TRAIL-induced nuclear factor κ -light-chain-enhancer of activated B cell (NF- κ B) pathway in a PCa cell line. Downregulation of CK2 combined with a sub-dose of TRAIL suppressed p65 phosphorylation at serine 536. The combination treatment of TRAIL and the CK2 inhibitor decreased p65 nuclear translocation. Under the treatment of a sub-dose of TRAIL, downregulation of CK2, using both genetic and pharmacological approaches, decreased the transcriptional activity of NF- κ B and the expression of NF- κ B downstream anti-apoptosis genes. Therefore, we provided novel molecular mechanistic insight reporting that CK2 regulates the sensitivity of PCa cells to the antitumor effect of TRAIL. This is important for understanding how the TRAIL pathway is disrupted in PCa and may help to develop an effective combinatorial therapy for PCa.

Introduction

In the United States, prostate cancer is the most commonly diagnosed cancer in males (1). Treatment strategies for PCa are based on androgen deprivation, but in the late hormone-refractory stage, it is still necessary to develop improved treatment strategies. TNF-related apoptosis-inducing ligand (TRAIL), a new member of the tumor necrosis factor (TNF) family, was shown to possess the ability to induce apoptosis in a wide range of human cancer cell lines without significant cytotoxicity towards normal cells (2,3). Yet, previous studies show that both intrinsic and acquired resistance to TRAIL poses a huge problem in establishing clinically efficacious TRAIL therapies. Combinatorial therapy represents a promising strategy for treating cancers that are resistant to TRAIL (4-12).

It is increasingly evident that several anti-apoptotic pathways including those regulated by nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) are critical in modulating the effects of TRAIL (4-6). Constitutive NF- κ B activation has been implicated in resistance to TRAIL (7). As a member of the TNF superfamily, upon receptor ligation, TRAIL simultaneously activates intrinsic (or mitochondrial) and extrinsic cell death pathways (8), as well as survival signaling via transcription of NF- κ B (9,10). In the static state, NF- κ B stays in the cytoplasm forming a complex with inhibitor of κ B (I κ B). Binding of TRAIL (11) to the receptors activates the inhibitor of κ B-kinase (IKK). I κ B is phosphorylated by activated IKK and is then ubiquitinated and targeted for proteolysis. The degradation of I κ B allows NF- κ B to translocate to the nucleus where it binds to NF- κ B response elements, which activate transcription of target genes, such as Bcl-XL (12), carrying out antitumor function.

Casein kinase 2 (CK2), a most highly conserved and ubiquitously protein serine/threonine kinase, has been recognized as a key player in cell growth and proliferation as well as in the regulation of apoptotic activity in cells. Due to the general pro-survival effects of CK2 activity, its inhibitors are considered as potential anticancer drugs (13,14). Their application appears

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particularly promising in combination with other anticancer agents whose effective concentrations may be reduced in this manner. In the case of death ligand TRAIL, its synergism with CK2 inhibitors has been demonstrated (15,16). Downregulation of CK2 sensitizes prostate cancer cells to TRAIL-mediated apoptosis through effects on caspase-3, -8, and -9, and mitochondrial apoptotic proteins (15). Yet, the effect of CK2 on the TRAIL-induced NF- κ B pathway remains largely unclear.

In the present study, we investigated the effects of CK2 on TRAIL-mediated NF- κ B signaling. We found that under the stimulation of TRAIL, downregulation of CK2 with a specific inhibitor (TBB, 4,5,6,7-tetrabromobenzotriazole) or specific small interfering RNA (siRNA) decreased the phosphorylation of the subunit of NF- κ B, p65, and blocked the translocation of p65 into the nucleus from the cytosol. These effects inhibited the activation of p65, and then decreased the expression of downstream pro-apoptotic genes. Thus, downregulation of CK2 sensitizes prostate cancer cells to TRAIL-mediated apoptosis through the suppression of NF- κ B signaling.

Materials and methods

Cell line, cell culture and reagents. The prostate cancer cell line PC-3 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-3 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin, 100 U/ml penicillin, and 0.25 μ g/ml amphotericin B. Cells were incubated at 37°C with 5% CO₂. TBB was purchased from Calbiochem (San Diego, CA, USA) and TRAIL was from R&D Systems (Minneapolis, MN, USA).

Cell transfection. Transfections were performed by electroporation using an Electro Square Porator ECM 830 (BTX) (17) or by using Lipofectamine 2000 (Invitrogen). siRNA pool specific for CK2 α and non-specific (NS) control siRNAs were purchased from GE Health Dharmacon (Lafayette, CO, USA). Cells were harvested 48-72 h after transfection. Approximately 75-90% transfection efficiencies were routinely achieved.

Western blot analysis. Protein samples were prepared by lysing cells in modified RIPA buffer [1X PBS, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)]. Lysates (50-100 μ g) were separated on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed with the specific primary antibody and HRP-conjugated secondary antibody and then visualized by chemiluminescence. Antibodies against CK2 α (C-18) and ERK2 (D-2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against p65 (610868) was purchased from BD (Biosciences, San Jose, CA, USA). Antibody against β -tubulin was purchased from Sigma-Aldrich Co. LLC. Antibodies against histone 3 (9715) and phospho-NF- κ B p65 (Ser536, 3031) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Nuclear cytoplasmic fractionation. Cell fractionation was performed as described previously (18). Cells were washed with PBS twice, then lysed in cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT). Complete Mini

(Roche) protease inhibitor (PIC) was added fresh containing 0.1% NP-40 for 10 min on ice. The pellet was washed 3 times with buffer A after centrifugation at 6,500 rpm for 3 min. The supernatant (cytoplasmic fraction) was transferred to a new tube and centrifuged for 10 min at high speed to clear debris/membranes. The crude nuclear fraction pellet was washed with 1 ml of buffer A for 3 times. The pellet was resuspended in an equal volume of cold buffer B (20 mM HEPES pH 7.9, 0.42 mM NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT; PIC added fresh). Samples were rotated at 4°C for 30 min; centrifuged for 20 min at high speed, and the supernatant was transferred to a new tube (nuclei). Histone 3 and β -tubulin were used as nuclear and cytoplasmic markers, respectively.

Semi-quantitative and real-time RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with cDNA samples using the IQ SYBR-Green Supermix and ABI Prism 7900 platform (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The 2^{- $\Delta\Delta$ Ct} method was used to calculate the relative expression level by normalization at glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) levels. The following primer sequences were used: *BCL-XL*, 5'-gatctggtcccttcgacgtagttt-3' and 5'-ctaaggggtaagggttgccaat-3'; *TRAF2*, 5'-agaacattgtctgcgctcctgaacc-3' and 5'-tctccaagaccttctgctccaagt-3'; and *GAPDH*, 5'-accactctcca cctttgac-3' and 5'-tggtgctgtagccaattcgtt-3'.

Luciferase reporter assay. For luciferase reporter assays, the cells were harvested 24 h after transfection, and firefly and *Renilla* luciferase activities in cell lysates were measured using a Dual-Luciferase kit (Promega). *Renilla* luciferase activity of the cells was used as internal controls. Cell transfection was performed as described previously. Approximately 75-90% transfection efficiencies were routinely achieved.

Cell viability assay. Cell viability was monitored by absorbance using the MTS assay according to the manufacturer's instructions (Promega). Briefly, PC-3 cells were plated in 96-well plates at a density of 2,500 cells/well and treated with 60 μ M of TBB for 3 h, and then treated with or without 10 ng/ml of TRAIL. At 0, 24 and 48 h after the TBB treatment, 20 μ l of CellTiter 96R Aqueous One Solution reagent (Promega) was added to the cells. After incubation for 90 min at 37°C in a cell incubator, cell viability was measured in a microplate reader at 490 nm.

Statistical analysis. All values are expressed as means \pm SD. Comparison between two mean values was made by an independent-samples t-test. Statistical significance was set at P<0.05.

Results

Downregulation of CK2 combined with a sub-dose of TRAIL suppresses p65 phosphorylation. In a previous study (15), we clarified that CK2 suppressed TRAIL-induced apoptosis via its effects on the activation of caspases, DNA fragmentation,

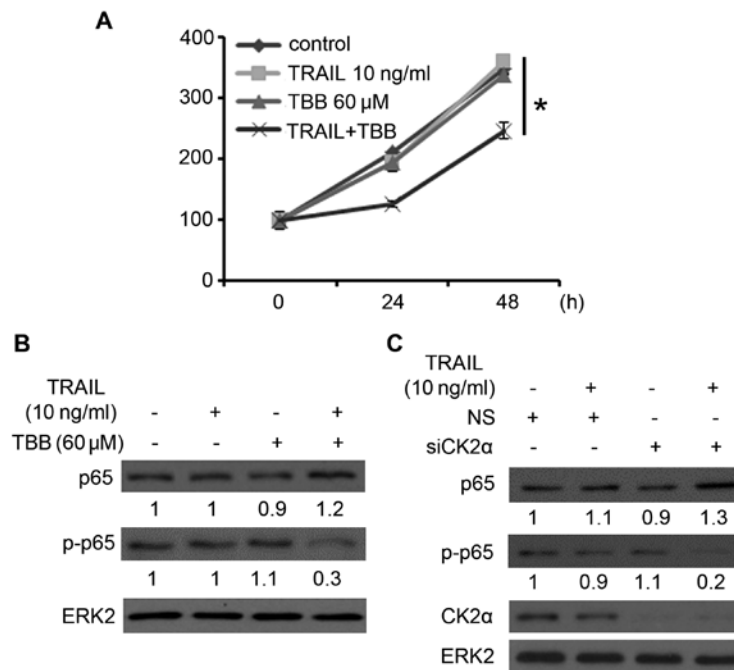


Figure 1. Downregulation of CK2 suppresses TRAIL-induced p65 phosphorylation. (A) Cell viability assay was carried out in the PC-3 cells as described in Materials and methods. Error bars, SD; * $P < 0.05$. (B) PC-3 cells were treated with 60 μ M of TBB for 3 h, and then maintained for 21 h with or without 10 ng/ml of TRAIL. Protein expression of p65, p-p65 (phosphorylation levels of p65 at serine 536), and ERK2 were analyzed by western blotting. The levels of p65 and p-p65 were quantified, normalized to ERK2 levels, and expressed relative to mock-treated cells. (C) PC-3 cells were transfected with NS control or CK2 α siRNA. After 24 h of transfection, the cells were treated with or without 10 ng/ml of TRAIL for 24 h. p65, p-p65, CK2 α and ERK2 protein expression levels were analyzed by western blotting. The levels of p65 and p-p65 were quantified, normalized to ERK2 levels, and expressed relative to mock-treated cells. TRAIL, TNF-related apoptosis-inducing ligand; NS, non-specific.

and also by blocking the mitochondrial apoptosis machinery. Here, we wanted to ascertain whether CK2 has an effect on the TRAIL-induced NF- κ B pathway. Since we wanted to ascertain the mechanism involved in the increased sensitivity of PCa cells to the antitumor effect of TRAIL, we used TRAIL at concentrations that did not induce significant apoptosis alone (10 ng/ml), in the PC-3 cells (15).

Firstly, we employed an MTS assay to assess the effects of a sub-dose of TRAIL and TBB on prostate cancer cell viability. As shown in Fig. 1A, cell viability was significantly reduced following the combination therapy of sub-dose TRAIL and TBB, but almost no change was noted following treatment of a sub-dose TRAIL or TBB alone. This confirmed that suppression of CK2 sensitized the PCa cells to the antitumor effect of TRAIL.

In order to study the effect of CK2 on the TRAIL-induced NF- κ B pathway, PC-3 cells were treated with 60 μ M of TBB for 3 h, and then maintained for 21 h with or without 10 ng/ml of TRAIL (15). After 24 h, the cells were harvested for western blot assay to assess the expression level of NF- κ B subunit p65 (p65) and the phosphorylation levels of p65 at serine 536 (p-p65), which play an important role in the activation of the NF- κ B pathway (19,20). As shown in Fig. 1B, the treatment of cells with TBB followed by TRAIL resulted in a slight increase in p65 expression but a marked decrease in the phosphorylation levels of p65. PC-3 cells were then treated with non-specific or CK2 α -specific siRNA. After 24 h of transfection, the cells were treated with or without 10 ng/ml of TRAIL. After another 24 h, the cells were harvested for western blot assay. After knockdown of CK2 α , the level of p-p65 (Ser536) was significantly downregulated following the treatment of

TRAIL (Fig. 1C). Thus, downregulation of CK2 α combined with a sub-dose of TRAIL suppressed the phosphorylation of p65 at serine 536 in the PCa cells.

The combination treatment of TRAIL and TBB regulates p65 nuclear translocation. p65 nuclear localization is the rate-limiting step in the activation of the NF- κ B pathway, thus we investigated the effect of CK2 on TRAIL-induced p65 nuclear localization. PC-3 cells were treated with 60 μ M of TBB for 3 h, and then maintained for 21 h with or without 10 ng/ml of TRAIL. The cytoplasmic and nuclear separation technology was employed to assess the expression of p65. As shown in Fig. 2, treatment of TBB followed by TRAIL resulted in an increase in p65 expression in the cytoplasm. Yet, the expression of p65 in the nucleus was decreased following the combination treatment of a sub-dose of TRAIL and TBB. These data indicate that the combination treatment decreased the nuclear translocation of p65.

Downregulation of CK2 combined with a sub-dose of TRAIL suppresses NF- κ B transcriptional activity. To further understand the changes associated with CK2 and the TRAIL-induced NF- κ B pathway, we investigated the transcriptional activity of NF- κ B regulated by CK2 following the treatment of a sub-dose of TRAIL. PC-3 cells were treated with non-specific or CK2 α -specific siRNA. After 24 h of transfection, the cells were treated with or without 10 ng/ml of TRAIL for another 24 h. The knockdown effect of CK2 α is shown in Fig. 3A. Treatment of 10 ng/ml of TRAIL or knockdown of CK2 α alone did not affect the NF- κ B luciferase activity. Yet, when CK2 α was knocked down and a sub-dose

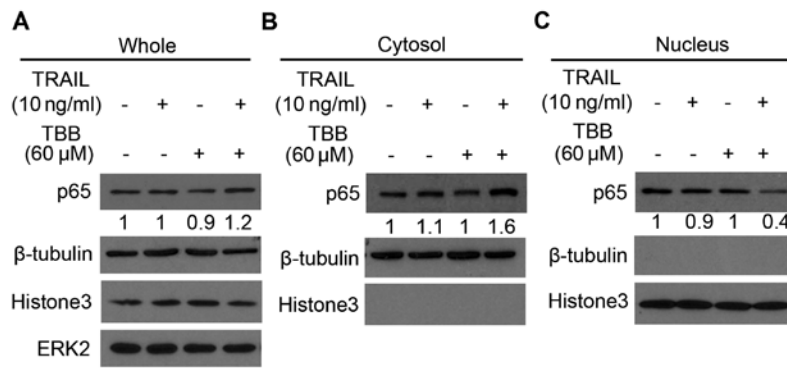


Figure 2. Combination treatment of TRAIL and TBB regulates p53 nuclear translocation. PC-3 cells were treated with 60 μM of TBB for 3 h and then maintained for 21 h with or without 10 ng/ml of TRAIL. Nuclear cytoplasmic fractionation was performed. In the lysates of whole cells (A), the cytoplasm (B) and the nucleus (C), p53, β-tubulin, histone 3 and ERK2 protein expression levels were analyzed by western blotting. The levels of p53 were quantified, normalized to loading control, and expressed relative to mock-treated cells. TRAIL, TNF-related apoptosis-inducing ligand.

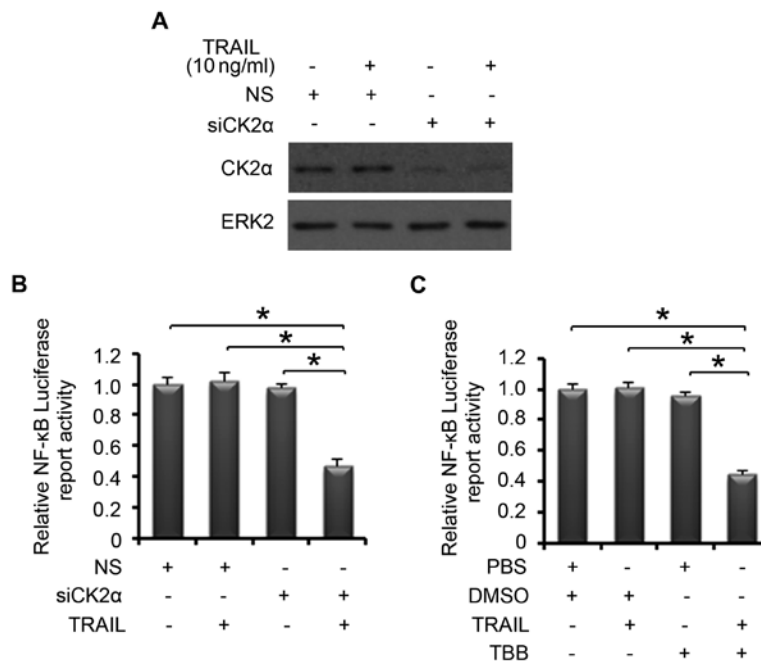


Figure 3. Effect of CK2 on the TRAIL-induced NF-κB transcriptional activity. (A) PC-3 cells were treated with NS or CK2α-specific siRNA. After 48 h of transfection, CK2α and ERK2 protein expression levels were analyzed by western blotting. (B) PC-3 cells were transfected with NF-κB firefly luciferase reporter, and *Renilla* luciferase as a control using Lipofectamine 2000, and then treated with or without 10 ng/ml of TRAIL for another 24 h. Firefly was normalized to *Renilla* luciferase activity for every measurement in all experiments. Columns, mean values among three replicates; error bars, SD; *P<0.05. (C) PC-3 cells were transfected with NF-κB firefly luciferase reporter, and *Renilla* luciferase as control using Lipofectamine 2000, treated with 60 μM of TBB for 3 h, and then maintained for 21 h with or without 10 ng/ml of TRAIL. Firefly was normalized to *Renilla* luciferase activity for every measurement in all experiments. Columns, mean values among three replicates; error bars, SD; *P<0.05. TRAIL, TNF-related apoptosis-inducing ligand; NS, non-specific; siRNA, small interfering RNA.

of TRAIL was added, the NF-κB luciferase activity decreased significantly (Fig. 3B). Consistent results were found in the combination of TRAIL and TBB treatment. The treatment of 10 ng/ml of TRAIL or 60 μM of TBB alone did not affect the NF-κB luciferase activity, while the NF-κB luciferase activity decreased significantly following treatment with TBB followed by TRAIL (Fig. 3C). Thus, following treatment of a sub-dose of TRAIL and downregulation of CK2α, using both genetic and pharmacological approaches, the transcriptional activity of NF-κB was decreased in the PCa cells.

Downregulation of CK2 combined with a sub-dose of TRAIL suppresses NF-κB downstream apoptosis-related gene

expression. Furthermore, we assessed the mRNA expression level of NF-κB downstream anti-apoptotic genes (21), *BCL-XL* and *TRAF2*. The PC-3 cells were treated with non-specific or CK2α-specific siRNA. After 24 h of transfection, the cells were treated with or without 10 ng/ml of TRAIL for another 24 h. When we knocked down CK2 and the sub-dose TRAIL was added, the expression of *BCL-XL* and *TRAF2* was decreased significantly (Fig. 4A and B). When the cells were treated with TBB followed by TRAIL, the expression of these genes was also significantly decreased (Fig. 4C and D). Thus, following treatment of a sub-dose of TRAIL, downregulation of CK2 decreased the expression of the NF-κB downstream anti-apoptotic genes in the PCa cells.

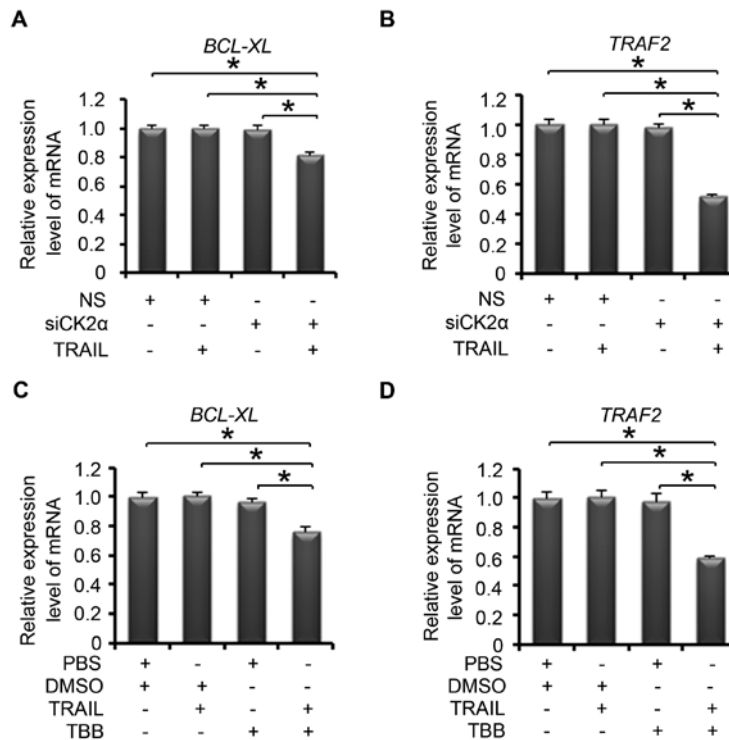


Figure 4. Effect of CK2 on expression of TRAIL-induced NF- κ B downstream apoptosis-related genes. (A and B) PC-3 cells were treated with NS or CK2 α -specific siRNA. After 24 h of transfection, the cells were treated with or without 10 ng/ml of TRAIL for another 24 h. Cells were harvested and RNAs were isolated and subjected to real-time PCR for *BCL-XL* (A) and *TRAF2* (B) expression. *GAPDH* cDNA was used as a control for the normalization. Columns, mean values among three replicates; error bars, SD; * $P < 0.05$. (C and D) PC-3 cells were treated with 60 μ M of TBB for 3 h, and then maintained for 21 h with or without 10 ng/ml of TRAIL. Cells were harvested and RNAs were isolated and subjected to real-time PCR for *BCL-XL* (C) and *TRAF2* (D) expression. *GAPDH* cDNA was used as a control for the normalization. Columns, mean values among three replicates; error bars, SD; * $P < 0.05$. TRAIL, TNF-related apoptosis-inducing ligand; NS, non-specific; siRNA, small interfering RNA.

Discussion

Since the mid-1990's, TRAIL has been used as a target of several anticancer therapies. To improve the effectiveness of TRAIL therapy, efforts should be focused on the understanding of how the TRAIL pathway is disrupted in individual cancers and which combination therapies can be utilized most effectively. Similar to previous studies (22,23), we found that the cell viability was significantly reduced following the combination therapy of a sub-dose of TRAIL and TBB. Suppression of CK2 sensitized the PCa cells to the antitumor effect of TRAIL.

CK2, a highly conserved and ubiquitous protein Ser/Thr kinase, plays important roles in tumorigenesis (24,25). Previous studies found that CK2 downregulation sensitized cells to TRAIL-induced cell apoptosis, but the mechanism was mostly related to the TRAIL-induced activation of intrinsic and extrinsic cell death pathways (15). NF- κ B is a dimeric transcription factor consisting of p50, p52, p65/relA, relB and c-rel subunits. Constitutive NF- κ B activation is a common event in cancer (26,27), and has been implicated in resistance to TRAIL (7). CK2 acts at multiple levels in NF- κ B activation, such that it targets not only I κ B, but also IKKi/IKKe and p65 (28-30). In the present study, we found that following treatment of a sub-dose of TRAIL in combination with downregulation of CK2 the transcriptional activity of NF- κ B was decreased and the expression of the NF- κ B downstream anti-apoptotic genes was also decreased in the PCa cells. These results revealed that the downregulation of CK2 sensitized the

tumor cells to the antitumor therapy of TRAIL at least partially due to the suppression of NF- κ B transcriptional activity and the downregulation of NF- κ B downstream anti-apoptotic genes. Reduction in the expression of anti-apoptotic genes allowed the tumor cells to be more sensitive to TRAIL-induced apoptosis, thereby alleviating TRAIL resistance to some extent.

CK2-regulated phosphorylation and localization of p65 are important for the sensitization of tumor cells to TRAIL. Phosphorylation of the p65 subunit at Ser-536 is an alternative to classical NF- κ B activation. It serves as an integrator for multiple signaling pathways during NF- κ B activation (31,32). In the present study, downregulation of CK2 combined with a sub-dose of TRAIL almost blocked the phosphorylation of p65 at Ser-536. This is an important mechanism of the inhibition of p65 activity.

An indispensable mechanism involved in the regulation of NF- κ B transcriptional activity and NF- κ B downstream anti-apoptotic gene expression is NF- κ B nucleus translocation. The import of NF- κ B from the cytoplasm to the nucleus is the key step in the activation of the NF- κ B pathway. Based on our data, the combination treatment of TRAIL and the CK2 inhibitor decreased p65 nuclear import. The reduction in p65 nuclear translocation caused a decrease in NF- κ B downstream anti-apoptotic gene expression resulting in a pro-apoptotic effect.

In summary, we provide molecular insight into the mechanisms by which CK2 regulates the sensitivity of PCa cells to the antitumor effect of TRAIL in a completely new perspective.

Downregulation of CK2 combined with a sub-dose of TRAIL suppressed the phosphorylation of p65 at Ser-536. The combination treatment of TRAIL and a CK2 inhibitor decreased p65 nuclear translocation. These effects finally led to the suppression of NF- κ B transcriptional activity and the downregulation of expression of NF- κ B downstream anti-apoptotic genes, thereby promoting TRAIL-induced apoptosis. Understanding the molecular mechanisms may lead to the identification of a novel therapeutic strategy to overcome the resistance of TRAIL in the treatment of PCA.

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