Down-regulation of protein kinase Cη potentiates the cytotoxic effects of exogenous tumor necrosis factor–related apoptosis-inducing ligand in PC-3 prostate cancer cells

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a highly promising candidate for the treatment of cancer because it elicits cell death in the majority of tumor cells while sparing most normal cells. Some cancers, however, display resistance to TRAIL, suggesting that treatment with TRAIL alone may be insufficient for cancer therapy. In the present study, we explored whether the apoptotic responsiveness of PC-3 prostate cancer cells to TRAIL could be enhanced by targeting the novel protein kinase C (PKC) isoform η. Transfection of PC-3 cells with second-generation chimeric antisense oligonucleotides against PKCη caused a time- and dose-dependent knockdown of PKCη, as revealed by real-time RT-PCR and Western blot analyses. Knockdown of PKCη resulted in a marked amplification of TRAIL's cytotoxic activity. Cell killing could be substantially prevented by the pan-caspase inhibitor z-VAD-fmk. In addition, PKCη knockdown and administration of TRAIL significantly synergized in activation of caspase-3 and internucleosomal DNA fragmentation. Knockdown of PKCη augmented TRAIL-induced dissipation of the mitochondrial transmembrane potential and release of cytochrome *c* from mitochondria into the cytosol, indicating that PKCη acts upstream of mitochondria. We conclude that PKCη represents a considerable resistance factor with respect to TRAIL and a promising target to exploit the therapeutic potential of TRAIL. [Mol Cancer Ther 2004;3(7):773–81]

Introduction

Resistance to chemotherapy, including androgen deprivation therapy in case of prostate carcinomas, is a severe clinical problem, and considered as the result of curtailed apoptosis (1). Chemotherapy activates the apoptotic machinery only indirectly, more effective outcomes, thus, might be accomplished by direct induction of apoptosis. In this respect, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) or Apo-2 ligand, a member of the tumor necrosis factor α (TNF) family of cytokines, is a highly promising candidate: it provokes cell death in the majority of cancer cells while sparing most normal cells (2-4). Importantly, TRAIL acts independently of p53, making it a potentially effective means against chemoresistant tumors (5). Some cancers, however, fail to respond to TRAIL's cytotoxic effects (6), suggesting that treatment with TRAIL could be improved by targeting TRAIL resistance factors.

TRAIL triggers apoptosis by binding to the death-inducing receptors TRAIL-R1 (DR4) (7) or TRAIL-R2 (DR5) (8), resulting in receptor ligation. In turn, a death-inducing signaling complex (DISC) is formed by recruitment of the adaptor protein Fas-associated death domain and caspase-8 to the activated receptors (9). This leads to cleavage and activation of caspase-8 and the initiation of two different apoptotic pathways, depending on the cell type (10). In type I cells, activated caspase-8 directly processes and activates downstream executioner caspases, such as caspase-3, -6, and -7, in a mitochondrial-independent manner. In type II cells, the mitochondria integrate death signals mediated by proteins of the Bcl-2 family: activated caspase-8 cleaves Bid into a truncated form (tBid) that translocates to the mitochondria, promoting the release of cytochrome *c* into the cytosol (11). This results in the formation of the ''apoptosome'' consisting of cytochrome *c*, Apaf-1, and caspase-9, in turn leading to the activation of caspase-3 (12).

The apoptotic responsiveness to TRAIL can be affected at several levels, for example, through the expression of the so-called ''decoy'' receptors TRAIL-R3 (DcR1) (8) and TRAIL-R4 (DcR2) (13), or by overexpression of FLICE-inhibitory protein (14). Other mechanisms of resistance lie in the TRAIL-induced activation of NF-κB (15), which is

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responsible for the inducible expression of anti-apoptotic proteins (16), or, in type II cells, in overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-2 (17) and Bcl-xL (18). Resistance to TRAIL has been shown to be overcome by cotreatment with conventional chemotherapeutic drugs. For example, in androgen-independent prostate cancer cells, the cytotoxic efficacy of TRAIL could be augmented by paclitaxel (19), actinomycin D, or gemcitabine (20), as well as cisplatin, etoposide, or doxorubicin (21). On the other hand, the combination of TRAIL with chemotherapy bears the risk of eliciting apoptosis in otherwise TRAIL-resistant normal cells (22).

Protein kinase C (PKC), a family of at least 10 serine/threonine protein kinases can be classified into three groups, based on their structural and biochemical properties (23): phorbolester-responsive and Ca^{2+}-dependent ''conventional'' PKCs (α, β, γ), phorbolester-responsive but Ca^{2+}-independent ''novel'' PKCs (δ, ε, η, θ, μ), and phorbolester-unresponsive and Ca^{2+}-independent but still phosphatidylserine-activated ''atypical'' PKCs (ι, ζ). A number of studies have implicated several isoforms in regulation of apoptosis: for example, it has been shown that inactivation of PKCs sensitizes tumor cells to drug-induced apoptosis and, moreover, that overexpression of PKCs confers protection against apoptosis (24).

Recent studies point to a special role of the novel PKC isozyme η in drug resistance and regulation of apoptosis: PKCη expression levels were reported to correlate with drug resistance and drug resistance associated genes in specimens obtained from patients: (1) breast cancer (25), (2) ovarian cancer cells from ascites aspirates (26), and (3) AML blasts (27). With respect to tumors of the prostate, a significantly higher expression of PKCη has been found in prostate carcinoma than in benign prostatic hyperplasia (28). In addition, several studies showed that PKCη might be an effective inhibitor of the apoptotic machinery: (1) overexpression of PKCη in a breast cancer cell line attenuates tumor necrosis factor α–induced cell death by preventing activation of caspase-8 and -7 (29, 30); (2) in glioblastoma cell lines, PKCη seems to mediate the proliferative response to phorbolester (31) and to block UV- and γ-irradiation–induced apoptosis (32); (3) in human keratinocytes, UV-induced activation of caspase-3 is reduced by overexpression of PKCη (33); and (4) PKCη is reported to be a potent activator of Raf1 (34), a protein kinase implicated in the development of drug resistance (35).

These findings urged us to initiate a systematic approach based on chimeric second generation antisense oligonucleotides to analyze the effects of PKCη down-regulation (''knockdown'') on the sensitivity of cancer cells to anti-neoplastic agents. In a recent study, we showed that PKCη knockdown was capable of sensitizing A549 lung carcinoma cells to the cytostatics vincristine and paclitaxel (36). These studies were extended to the effects of TRAIL on PC-3 prostate cancer cells. Here, we show that treatment with antisense oligonucleotides targeted against PKCη potentiates TRAIL-induced apoptosis.

Materials and Methods

Cell Culture

PC-3 prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (medium and supplements were purchased from Biochrom, Berlin, Germany). Cells were cultivated at 37°C in a humidified 5% CO_2 incubator and routinely passaged when 90% to 95% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of *Mycoplasma* with *Mycoplasma* detection reagents from Roche (Mannheim, Germany).

Oligonucleotides

As recently described, a directed antisense strategy was applied to suppress PKCη expression in PC-3 cells (36). Briefly, a series of different oligonucleotide sequences complementary to the PKCη mRNA was screened. Oligonucleotides were 20 bases in length containing a phosphorothioate backbone and 2′-alkoxy modifications on the ribose residues at bases 1 to 5 and 16 to 20 at the 5′ and 3′ ends, respectively. Oligonucleotides were screened for their antisense activity to reduce PKCη expression using real-time PCR analysis. The sequence of the most effective PKCη antisense oligonucleotide (ETA-ASO) was 5′-AGGCCCGTACAGCATTTCCT-3′ (position 1,762 on PKCη mRNA). This sequence shows no cross-hybridization to the mRNAs of the other PKC isoforms. An independent inactive oligonucleotide (control oligonucleotide) was used as a control for monitoring effects of the nucleotide lipid complex in the cellular system. The sequence of control oligonucleotide was 5′-TAACCACTCAGCCTAGCGTC-3′.

Oligonucleotide Treatment

Oligonucleotides were delivered to cells as complexes with Argfectin-50 (Atugen, Berlin-Buch, Germany), which were allowed to form in HEPES-buffered (20 mmol/L) standard growth medium without fetal calf serum and antibiotics at 37°C for 20 minutes. PC-3 cells were plated either at 10,000 cells/well in 96-well plates or at 200,000 cells/well in 6-well plates in medium supplemented with 10% fetal calf serum (without antibiotics) and allowed to adhere for at least 24 hours. Immediately before treatment with oligonucleotides, the medium was replaced by fresh one and cells were incubated with 1.2 μg/mL Argfectin-50 and 50 nmol/L of oligonucleotides until harvested. Cells were incubated for additional 48 hours before application of TRAIL (Peprotech, Rocky Hill, NJ; distributed by Tebu, Offenbach, Germany), allowing for a maximum of reduction of Bcl-2 and Bcl-XL expression before addition of TRAIL.

Quantitative Real-time RT-PCR

RNA was prepared with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification of mRNA expression was carried out using the Abi Prism 7700 (Applied Biosystems, Langen, Germany) sequence-detection system (TaqMan). Oligonucleotide primers and fluorescently labeled TaqMan

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probes were designed using Primer Express 1.0 software (Applied Biosystems). Sequences were: forward primer: 5′-ATGCTGTACGGGCCTGCA-3′, reverse primer: 5′-CGTGACCACAGAGCATCTCATAGA-3′, probe: 5′-FAM-AACACGCCCATTGCCCACCAGTCTA-TAMRA-3′ (PCR product of 70 bp); primers and probes were from Metabion, Martinsried, Germany. The 18s rRNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. TaqMan 18s rRNA primers and a 5′-VIC-labeled probe were used according to the manufacturer's instructions (Applied Biosystems). Reverse transcription and PCR were done in a one-step RT-PCR following the manufacturer's protocol (Applied Biosystems). Reactions for all samples were done in triplicate in 96-well optical plates using 5 ng of RNA, 10 μl of 2× TaqMan master mix (Applied Biosystems), 100 nmol/L probe, 300 nmol/L of each primer, 1 μL of 20× 18s rRNA mix to a final volume of 20 μL. Thermocycler conditions comprised an initial holding stage at 48°C for 30 minutes followed by 95°C for 10 minutes. This was followed by a two-step TaqMan PCR program consisting of 95°C for 15 seconds and 60°C for 1 minute for 40 cycles.

Western Blot Analysis

Cell were lysed on ice for 15 minutes in 40 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with a protease inhibitor cocktail (Roche) followed by brief sonification. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL) according to the manufacturer's instructions. For immunoblotting, 60 μg of total cellular protein per lane were separated by standard SDS-PAGE on 10% gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). After blocking in PBS containing 5% dry milk and 0.05% Tween 20, PKCη was immunodetected using rabbit anti-PKCη polyclonal antibody (dilution 1:5000; Santa Cruz Biotechnology, Heidelberg, Germany). Peroxidase-conjugated goat anti-rabbit IgGs (dilution 1:25,000; Dianova, Hamburg, Germany) followed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) were used for detection. Densitometric analyses were done with Kodak 1D software.

Cytotoxicity Assay

Alamar Blue assay is a simple way of measuring cytotoxicity and cell proliferation; it measures metabolic activity through the chemical reduction of Alamar Blue by living cells. The assays were done in triplicate in 96-well flat-bottom microtiter plates. After a 24-h incubation with TRAIL, 1/10 volume of Alamar Blue (Biosource, Solingen, Germany) solution was added and cells were incubated at 37°C for an additional 3 hours. The absorbance of the wells was measured at 560/595 nm using a Wallac Victor (Perkin-Elmer, Rodgau-Jügesheim, Germany) fluorometer. Results are expressed as a percentage of relative cell numbers of control cells that were neither exposed to oligonucleotides nor TRAIL. The pan-caspase inhibitor z-VAD-fmk (Alexis, Grünberg, Germany) was administered 1 hour before treatment with TRAIL.

Cytofluorometric Analysis of DNA Content

To determine DNA content, cells were analyzed for propidium iodide incorporation. Cells were harvested 24 hours after treatment with TRAIL, washed twice with PBS, and fixed in 70% ethanol at −20°C for at least 30 minutes. After centrifugation, cells were resuspended in PBS containing 1% glucose, 50 μg/mL RNase A (Roche), and 50 μg/mL propidium iodide and incubated in the dark at room temperature for 30 minutes. Flow cytometry analysis was done on a Becton Dickinson (Heidelberg, Germany) FACSCalibur using CellQuest software. Data were gated to exclude debris and aggregates; 20,000 cells were analyzed in each sample. Apoptotic cells were detected as sub-G_1 peak. Results are expressed as percentage apoptotic cells. The caspase-3 inhibitor z-DEVD-fmk (Calbiochem, Schmalbach, Germany) was administered 1 hour before treatment with TRAIL.

Caspase-3 Activity

Caspase-3 activity was measured 3.5 hours after treatment with TRAIL using the synthetic fluorogenic substrate Ac-DEVD-AFC (Bachem, Heidelberg, Germany). Cells were lysed in 10 mmol/L Tris-HCl, 10 mmol/L $NaH_2PO_4/NaHPO_4$ (pH 7.5), 130 mmol/L NaCl, 1% Triton X-100, and 10 mmol/L $Na_4P_2O_7$ and then incubated with 20 mmol/L HEPES (pH 7.5), 10% glycerol, 2 mmol/L DTT, and 25 μg/mL Ac-DEVD-AFC at 37°C for 2 hours. The release of trifluoromethylcoumarin (AFC) was analyzed on a Wallac Victor fluorometer using an excitation/emission wavelength of 390/510 nm. Caspase-3 activities were calculated as a ratio of emission of treated cells to untreated cells.

Cytofluorometric Analysis of Mitochondrial Transmembrane Potential ($\Delta\psi_m$)

The accumulation of the cationic lipophilic fluorochrome dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in the mitochondrial matrix is directly proportional to $\Delta\psi_m$ (37). $\Delta\psi_m$ was determined 24 hours after treatment with TRAIL. Cells were incubated with 50 nmol/L 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes, Eugene, OR; distributed by Mobitec, Göttingen, Germany) at 37°C for 30 minutes. After washing, at least 10,000 cells were analyzed using a FACSCalibur and CellQuest software. Data were gated to exclude debris.

Cytofluorometric Determination of Cytochrome *c* Release from Mitochondria

This flow cytometry–based method allows the differential detection of cytosolic but not mitochondrial cytochrome *c* (38). Release of cytochrome *c* from mitochondria into the cytosol was analyzed by direct staining with FITC-conjugated anti-cytochrome *c* monoclonal antibody (Santa Cruz Biotechnology). Cells were harvested 24 hours after treatment with TRAIL, washed twice with PBS, and fixed in 1% paraformaldehyde in PBS at room temperature for at least 30 minutes. After washing with PBS, cells were permeated with 0.05% Triton X-100 in PBS containing 2% bovine serum albumin and 10 μL FITC-anti-cytochrome *c* antibody in the dark at 4°C for 20 minutes. Unbound antibody was washed with PBS and flow cytometry analysis was carried out on a FACSCalibur using CellQuest software.

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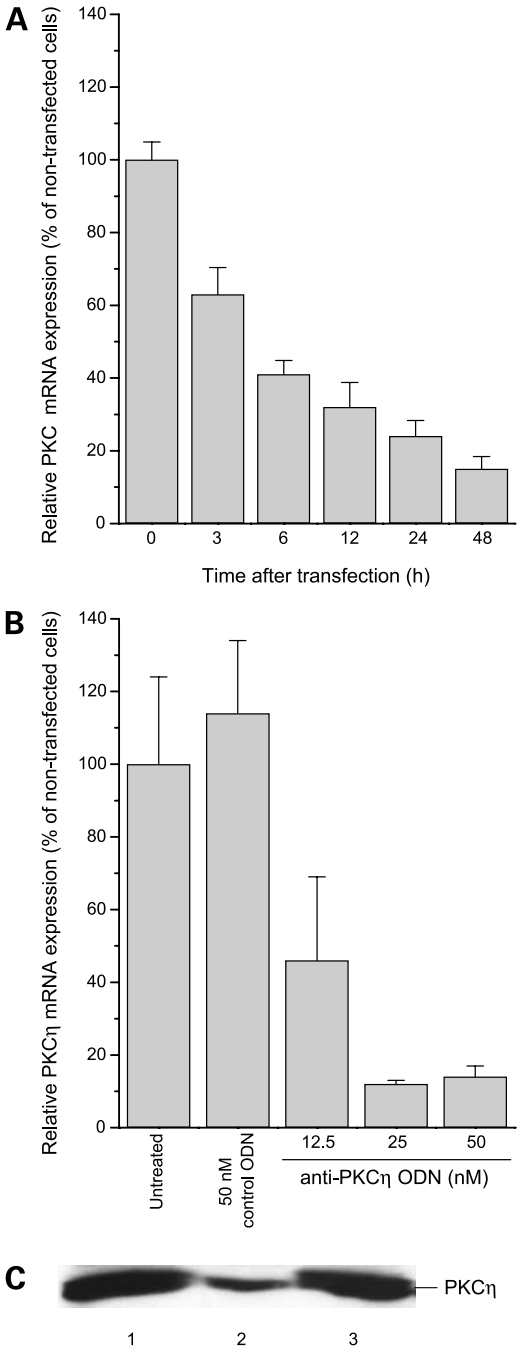

Figure 1. Antisense knockdown of PKCη by chimeric antisense oligonucleotides. **A** and **B,** PKCη mRNA expression assessed by quantitative real-time RT-PCR (TaqMan). **A,** kinetics of PKCη knockdown. PC-3 cells were transfected with ETA-ASO at a concentration of 50 nmol/L and incubated for the indicated times. *Columns*, mean values of two quantifications in duplicate, expressed as percentages of non-transfected cells; *bars*, SD. **B,** dose-response relationship of PKCη knockdown. PC-3 cells were incubated with the indicated concentrations of ETA-ASO for 48 hours. *Columns*, mean values of two quantifications, expressed as percentages of non-transfected cells; *bars*, SD. **C,** immunoblotting for PKCη. PC-3 cells were transfected with oligonucleotides (*ODNs*) at a concentration of 50 nmol/L and incubated for 48 hours. Total protein (60 μg) was separated by a 10% polyacrylamide gel. *Lane 1*, non-transfected cells; *lane 2*, ETA-ASO–treated cells; *lane 3*, control oligonucleotide–treated cells.

Statistical Analysis

Statistical significance of differences between experimental groups was determined using the paired two-tailed Student's *t* test.

Results

Time- and Dose-Dependent Inhibition of PKCη mRNA Expression by a Chimeric Antisense Oligonucleotide

We first analyzed the effect of the treatment with ETA-ASO on PKCη gene expression in PC-3 prostate carcinoma cells by real-time RT-PCR analyses. Figure 1A shows the kinetics of ETA-ASO treatment: PKCη mRNA knockdown was time dependent, with a 60% decrease achieved as early as 6 hours after transfection. Figure 1B depicts the dose dependence of the antisense oligonucleotide: starting at doses as low as 12.5 nmol/L, a marked inhibition of PKCη mRNA expression could be observed. At 25 to 50 nmol/L, treatment with ETA-ASO down-regulated the mRNA by at least 80%. PKCη mRNA expression was hardly affected by the control oligonucleotide at 50 nmol/L compared with non-manipulated cells. A prominent knockdown of PKCη was also accomplished on protein level, as revealed by Western immunoblotting (Fig. 1C). The densitometric quantification of the corresponding bands showed that PKCη was reduced to 28% after transfection with ETA-ASO. On immunoblots, PKCη is occasionally detected as a doublet (29, 31), the higher molecular weight band possibly representing the autophosphorylated form of the protein. The disappearance of this band after treatment with ETA-ASO might be attributable to a reduction of PKCη to a level too low to allow autophosphorylation. No difference in PKCη expression could be observed between non-transfected (lane 1) and control oligonucleotide–transfected (lane 3) cells.

Treatment with ETA-ASO Enhances Sensitivity to TRAIL

In initial experiments on the effects of PKCη knockdown on the sensitivity to TRAIL, cell viability of PC-3 cells was monitored using Alamar Blue assay. Forty-eight hours after transfection, cells were exposed to varying concentrations of TRAIL for 24 hours. As presented in Fig. 2A, non-transfected cells displayed only very weak responsiveness at high doses (100 to 300 ng/mL TRAIL). In contrast, in cells treated with ETA-ASO, TRAIL evoked a marked dose-dependent cytotoxic effect, with 50% cell killing at 300 ng/mL TRAIL. It should be noted that PKCη knockdown per se significantly reduced cell viability, which might be attributable to a protective effect of PKCη against spontaneous cell death in PC-3 cells. However, some reduction in viability was also observed in the control oligonucleotide–treated cells, likely explicable by non-specific cytotoxic effects of the nucleotide lipid complexes.

Amplification of TRAIL Responsiveness by Pretreatment with ETA-ASO Involves Apoptosis

Next, we investigated whether PKCη knockdown would sensitize PC-3 cells to TRAIL by facilitating the apoptotic responsiveness. Apoptosis was evaluated by using the

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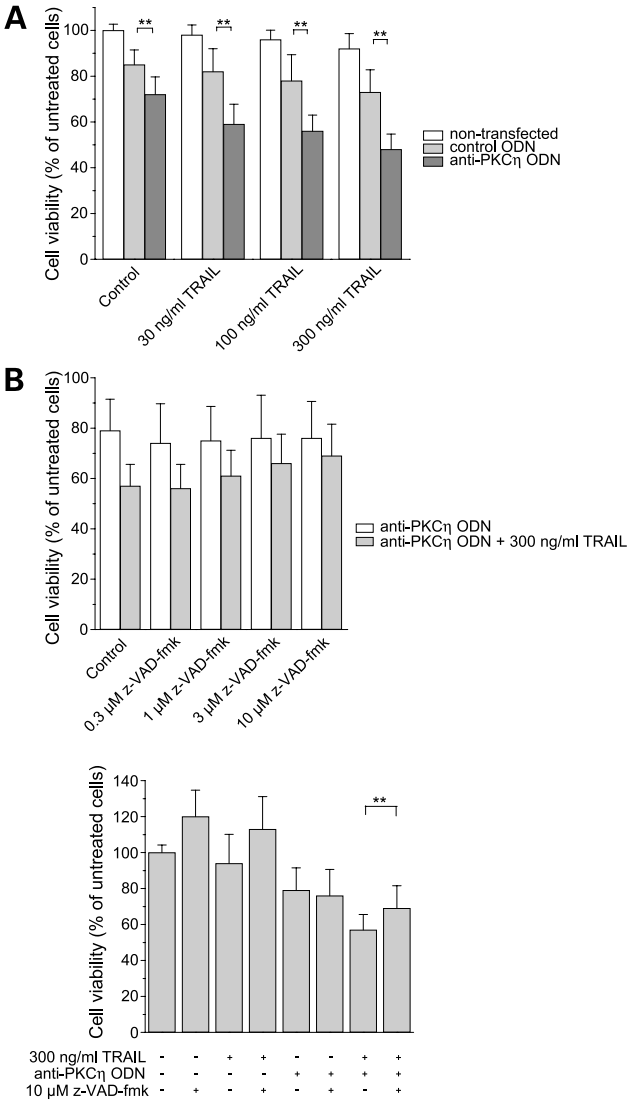

Figure 2. PKCη knockdown sensitizes PC-3 cells to TRAIL. Cells were transfected with 50 nmol/L of oligonucleotides. Forty-eight hours after transfection, cells were exposed to TRAIL for 24 hours. **A,** dose-response relationship of the cytotoxic effect of TRAIL. Cytotoxic effects were assessed by Alamar Blue assay; cell viabilities are expressed as the ratio of absorbance of treated to untreated cells. *Columns*, means of four experiments in triplicates; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$. **B,** effect of z-VAD-fmk on TRAIL-induced cell death as determined by Alamar Blue assay. z-VAD-fmk was applied 1 hour before TRAIL administration; cells were then incubated for 24 hours. *Columns*, means of two experiments in triplicates; *bars*, SD. **, $P < 0.01$.

broad-spectrum irreversible caspase inhibitor z-VAD-fmk and by cell cycle analysis. First, the effect of z-VAD-fmk on TRAIL-mediated cell death was monitored by Alamar Blue assay. As illustrated in Fig. 2B, the pan-caspase inhibitor prevented TRAIL-induced killing of PKCη knockdown cells in a concentration-dependent fashion. Second, cells were assessed for apoptosis by staining the nuclei of ethanol-fixed cells with propidium iodide and determining the DNA content by cytofluorometry. Typically, sub-G_1

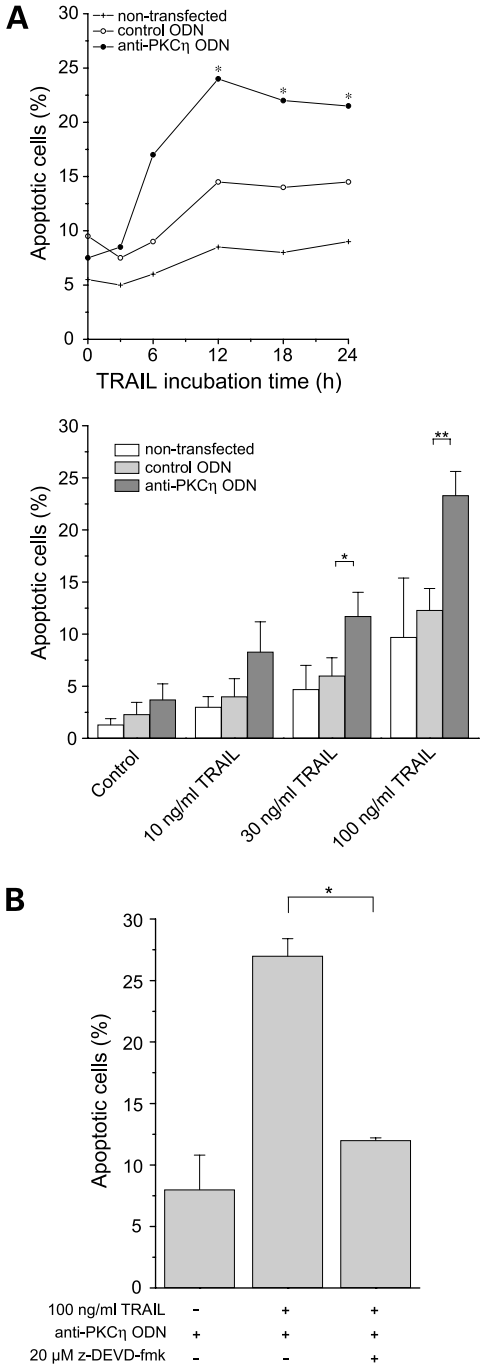

Figure 3. Potentiation effect of PKCη knockdown on TRAIL-mediated apoptosis. PC-3 cells were transfected with 50 nmol/L of oligonucleotides and treated with TRAIL 48 hours later. **A,** kinetics and dose-response relationship of TRAIL-induced apoptosis. For the kinetics, cells were treated with 100 ng/mL TRAIL; for the dose-response relationship, cells were exposed to TRAIL for 24 hours. DNA fragmentation was determined by cytofluorometric cell cycle analysis; apoptotic cells were detected as sub-G_1 peak. *Points*, means of two (kinetics) experiments. *Columns*, means of three (dose-response relationship) experiments. *, $P < 0.05$. **B,** effect of z-DEVD-fmk on TRAIL-induced apoptosis as assessed by cytofluorometric cell cycle analysis. z-DEVD-fmk was applied 1 hour before TRAIL treatment; cells were then incubated for 24 hours. *Columns*, means of two experiments; *bars*, SD. *, $P < 0.05$.

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cells are indicative of apoptosis. The results are demonstrated in Fig. 3A: it is clear that TRAIL-induced apoptosis is time- and dose-dependent both in non-transfected and transfected cells. When exposed to 100 ng/mL TRAIL for 24 h, not more than 12% of non-transfected or control oligonucleotide–transfected cells underwent apoptosis. However, 23% of cells treated with the combination of ETA-ASO plus the same dose of TRAIL became apoptotic. As was the case for the determination of cell viability, cells treated with ETA-ASO as a single agent revealed a weak increase in apoptosis, further pointing to PKCη as a relevant survival factor. Again, however, some non-specific toxicity was observed in the control oligonucleotide–transfected cells.

Characterization of the Sequelae of PKCη Knockdown on TRAIL-Induced Apoptosis

Caspase-3. To gain further insight into the mechanisms by which PKCη depletion sensitized PC-3 cells to TRAIL-induced apoptosis, we examined the involvement of caspases and mitochondria. The activation of caspase-3 is a hallmark of apoptotic cell death in many cell types (39). Thus, we first assessed whether the caspase-3 inhibitor z-DEVD-fmk could prevent TRAIL from inducing apoptosis. As shown in Fig. 3B, 20 μmol/L z-DEVD-fmk blocked TRAIL-mediated apoptosis in PKCη knockdown cells. The remaining effect of TRAIL might be explicable by residual caspase-3 activity or, alternatively, by caspase-6 or -7 activities. In a second step, we determined the kinetics of TRAIL-induced caspase-3 activity: a pronounced increase of caspase-3 activity was detectable 2 hours after administration of 100 ng/mL TRAIL and attained a maximum after 4 hours in non-transfected and control oligonucleotide–transfected cells (Fig. 4). Interestingly, in PKCη knockdown cells, caspase-3 was more expeditiously activated and reached its peak activity after 2 hours already. We next assessed the dose-response relationship: in non-transfected cells, TRAIL at 100 ng/mL led to a 6-fold raise in caspase-3 activity. However, in cells treated with ETA-ASO, 100 ng/mL TRAIL activated caspase-3 by more than 20-fold, whereas in control oligonucleotide–transfected cells, only a 12-fold increase in caspase-3 activity could be observed.

Mitochondrial Transmembrane Potential ($\Delta\psi_m$). TRAIL has been reported to harness the mitochondrial pathway of apoptosis that is triggered by an early permeabilization of mitochondrial membranes and the subsequent release of cytochrome *c* into the cytosol concomitant with the activation of caspase-9 (40, 41). Initially, we studied the effect of TRAIL on mitochondrial depolarization by determining $\Delta\psi_m$. In control cells, a faint decline in $\Delta\psi_m$ was detected after a 12-h incubation with 100 ng/mL TRAIL (Fig. 5A). In contrast, in PKCη knockdown cells, TRAIL provoked a $\Delta\psi_m$ decay as early as 3 hours after administration. At 24 hours after treatment with 300 ng/mL TRAIL, dissipation of $\Delta\psi_m$ occurred in less than 20% of non-transfected or control oligonucleotide–transfected cells but in nearly 60% of ETA-ASO-transfected cells. Again, down-regulation of PKCη by itself evoked a pro-apoptotic reaction, corroborating that PKCη serves as guardian to prevent cell death. The pan-caspase inhibitor z-VAD-fmk at 10 μmol/L abolished TRAIL-triggered loss of $\Delta\psi_m$ (Fig. 5B), demonstrating that the mitochondrial apoptotic function is both in non-transfected cells as well as in PKC knockdown cells in principal caspase dependent.

Cytochrome c Release. To evaluate whether the observed drop of $\Delta\psi_m$ was associated with the release of pro-apoptotic factors from mitochondria, we determined cytosolic cytochrome *c* by using FITC-conjugated

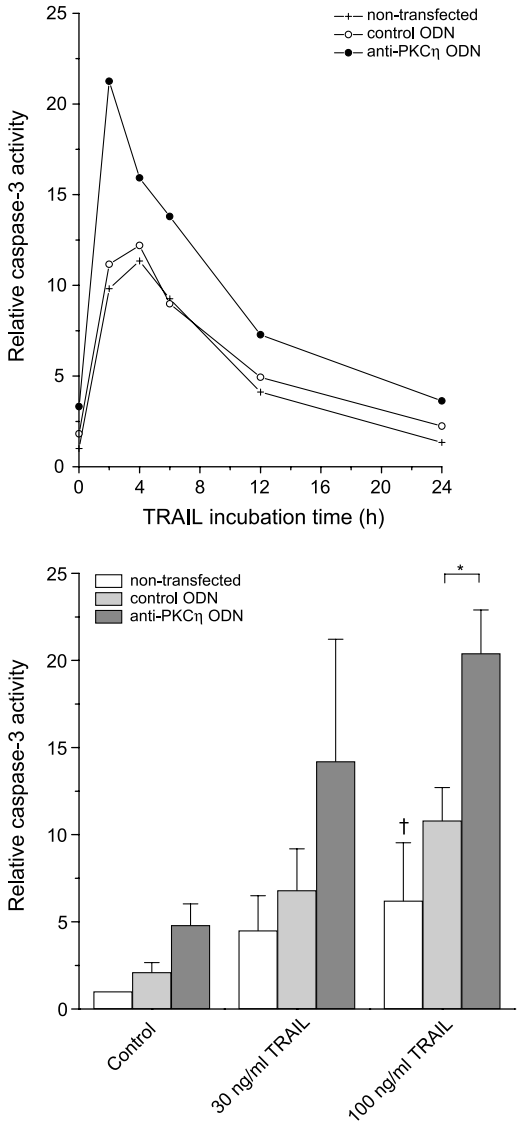

Figure 4. PKCη knockdown amplifies TRAIL-triggered caspase-3 activity. PC-3 cells were transfected with 50 nmol/L of oligonucleotides and treated with TRAIL 48 hours later. For the kinetics, cells were treated with 100 ng/mL TRAIL; for the dose-response relationship, cells were exposed to TRAIL for 3.5 hours. Relative caspase-3 activities are the ratio of treated cells to untreated cells. *Points*, means of two (kinetics) experiments. *Columns*, means of four (dose-response relationship) experiments; *bars*, SD. *, $P < 0.05$; non-transfected cells treated with 100 ng/mL TRAIL versus non-transfected cells: †, $P < 0.05$.

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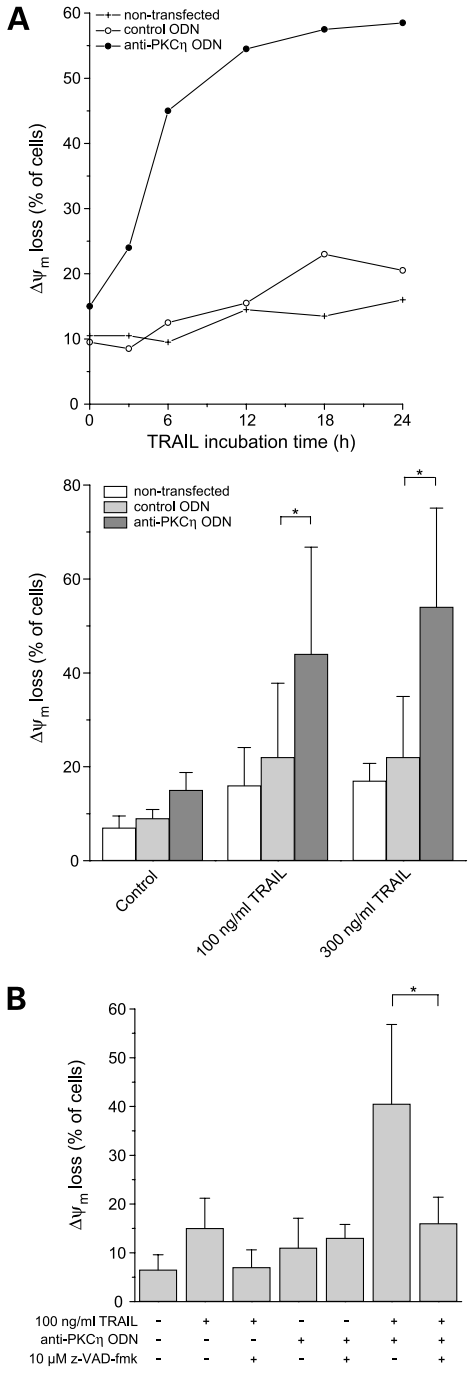

Figure 5. PKCη knockdown amplifies TRAIL-mediated $\Delta\psi_m$ dissipation. PC-3 cells were transfected with 50 nmol/L of oligonucleotides and treated with TRAIL 48 hours later. **A,** kinetics and dose-response relationship of TRAIL-induced $\Delta\psi_m$ loss. For the kinetics, cells were treated with 100 ng/mL TRAIL; for the dose-response relationship, cells were exposed to TRAIL for 24 hours. $\Delta\psi_m$ was assessed by cytofluorometric analysis of 3,3′-dihexyloxacarbocyanine iodide staining. *Points*, means of two (kinetics) experiments; *bars*, SD. *Columns*, means of four (dose-response relationship) experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$. **B,** effect of z-VAD-fmk on TRAIL-induced $\Delta\psi_m$ dissipation as assessed by cytofluorometric analysis of 3,3′-dihexyloxacarbocyanine iodide staining. z-VAD-fmk was applied 1 hour before TRAIL treatment; cells were then incubated for 24 hours. *Columns*, means of four experiments; *bars*, SD. *, $P < 0.05$.

anti-cytochrome *c* antibody in fixed cells. As depicted in Fig. 6A, treatment with 100 ng/mL TRAIL resulted in a time-dependent release of cytochrome *c* into the cytosol. In the control incubations, cytosolic cytochrome *c* levels increased from 5% to about 20% after 24 hours. In the PKCη knockdown cells, however, a 24-h treatment with TRAIL resulted in a cytosolic cytochrome *c* level of nearly 70%. The TRAIL-mediated cytochrome *c* release was largely blocked by 10 μmol/L z-VAD-fmk (Fig. 6B), further substantiating that the involvement of mitochondria in apoptosis is strictly caspase dependent. Of note, cells

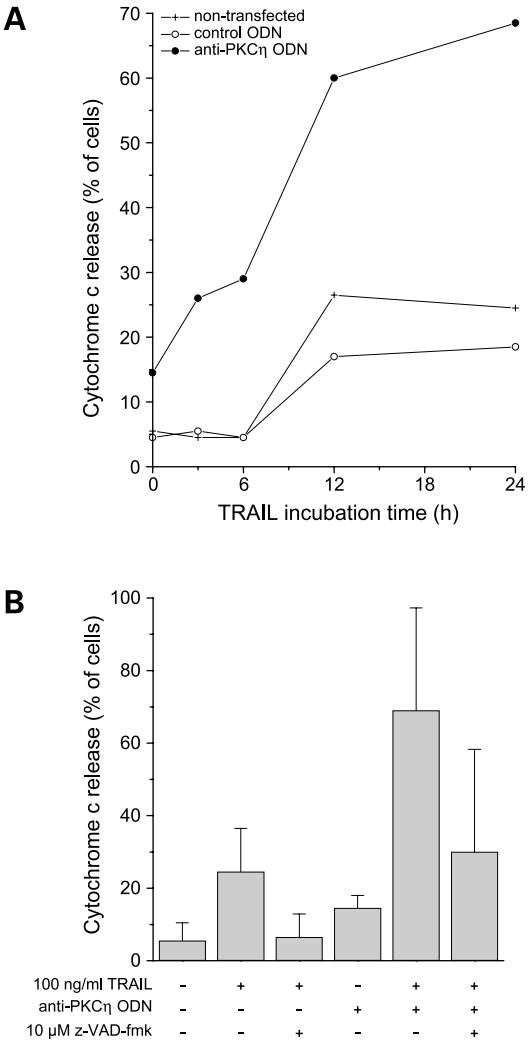

Figure 6. PKCη knockdown amplifies TRAIL-induced cytochrome *c* release. PC-3 cells were transfected with 50 nmol/L of oligonucleotides and treated with TRAIL 48 hours later. **A,** kinetics of cytochrome *c* release provoked by 100 ng/mL TRAIL. Cytosolic cytochrome *c* was detected by cytofluorometric analysis of FITC-anti-cytochrome *c* antibody. *Points*, means of two experiments. **B,** effect of z-VAD-fmk on TRAIL-induced cytochrome *c* release as determined by cytofluorometric analysis of cytosolic cytochrome *c*. z-VAD-fmk was applied 1 hour before TRAIL administration; cells were then incubated for 24 hours. *Columns*, means of two experiments; *bars*, SD.

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treated with ETA-ASO in the absence of TRAIL revealed an increase in cytochrome *c* release. Therefore, one can hypothesize that sole down-regulation of PKCη could facilitate the mitochondrial pathway of apoptosis.

Discussion

A common molecular strategy used by tumor cells to evade apoptosis is the up-regulation of anti-apoptotic proteins or the down-regulation of pro-apoptotic ones. Antisense oligonucleotides are useful tools for biological research (42) and could be used to validate candidate proteins. The ability to suppress the expression of individual genes with a high degree of specificity could distinguish the antisense technology from other modes of treatment. Nonetheless, there is a considerable debate on the specificity of antisense oligonucleotides, particularly, first-generation antisense oligonucleotides were shown to exert various non-specific effects (42). This has led to establish new chemical modifications by which specificity as well as efficacy could be markedly increased (42). In the present study, we used a chimeric second-generation antisense oligonucleotide, allowing efficient knockdown of PKCη gene expression at nanomolar concentrations, as revealed by real-time RT-PCR analysis and Western blotting. The strict time and dose dependence of the knockdown strongly suggest a specific effect of the antisense oligonucleotides.

We demonstrate that PKCη knockdown is accompanied by a substantial sensitization of PC-3 cells to TRAIL. This is evidenced by several different read-outs. First, down-regulation of PKCη resulted in a significant amplification of TRAIL's cytotoxic activity, as revealed by Alamar Blue assay (Fig. 2A). Second, PKCη knockdown amplified TRAIL-induced apoptosis, as shown by the assessment of sub-G_1 cells (Fig. 3A). Third, this sensitization crucially relied on activation of caspases, since it was significantly reversed by the broad-spectrum caspase inhibitor z-VAD-fmk (Fig. 2B) as well as the caspase-3–specific inhibitor z-DEVD-fmk (Fig. 3B). Fourth, down-regulation of PKCη led to a marked increase in TRAIL-triggered caspase-3 activity (Fig. 4). Fifth, PKCη knockdown affected mitochondrial activities, as shown for TRAIL-mediated $\Delta\psi_m$ dissipation (Fig. 5A) and cytochrome *c* release (Fig. 6B). z-VAD-fmk protected PC-3 cells from TRAIL-induced loss of $\Delta\psi_m$ (Fig. 5B) and cytochrome *c* release (Fig. 6B) both in non-transfected as well as in PKC knockdown cells. Thus, TRAIL-affected mitochondrial functions depended on caspase activity, indicating that caspases, for example, caspase-8, act upstream of mitochondria. As another implication of the observed effect of PKC knockdown on $\Delta\psi_m$ and cytochrome *c* release, it is suggested that PKCη acts upstream of mitochondria. This conclusion is in concordance with a previous study, which shows that PKCη regulates resistance to UV- and γ-induced apoptosis by preventing caspase-9 activation (32). However, the precise mechanism(s) by which PKC might modulate the apoptotic responsiveness to TRAIL still awaits elucidation.

In advanced prostate cancer, currently used cytotoxic chemotherapies are mostly limited to a palliative benefit (43, 44), and systemic administration of the death receptor ligands tumor necrosis factor α and FasL is hampered by their adverse effects (45). TRAIL still holds promise as a therapeutic agent due to its apparent lack of toxicity for normal cells (2-4). Several studies, however, demonstrate that many cancer cell lines are unresponsive to the cytotoxic effects of TRAIL (46-48). Unfortunately, the molecular determinants of TRAIL resistance are not fully understood (6). Among the suggested explanations are the expression of TRAIL decoy receptors and a high expression of FLICE-inhibitory protein. Other suggestions point to increased cytosolic concentrations of anti-apoptotic Bcl-2 family members or reduced concentrations of pro-apoptotic ones. Furthermore, it has been reported that PKC inhibitors sensitize cancer cells to TRAIL (49-51). Our study provides the first evidence that the PKC isozyme η is an important factor to control TRAIL sensitivity. Hence, PKCη defines a novel—and possibly more tractable—target in an adjuvant treatment to exploit the therapeutic potential of TRAIL.

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