PCPH/ENTPD5 Expression Confers to Prostate Cancer Cells Resistance against Cisplatin-Induced Apoptosis through Protein Kinase Cα–Mediated Bcl-2 Stabilization

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Abstract
Prostate cancer (PCa) frequently develops antiapoptotic mechanisms and acquires resistance to anticancer drugs. Therefore, identifying PCa drug resistance determinants should facilitate designing more effective chemotherapeutic regimens. Recently, we described that the PCPH protein becomes highly expressed in human prostatic intraepithelial neoplasia and in PCa, and that the functional interaction between PCPH and protein kinase Cα (PKCα) increases the invasiveness of human PCa. Here, we report that the functional interaction between PCPH and a different PKC isoform, PKCα, confers resistance against cisplatin-induced apoptosis to PCa cells. This interaction elicits a mechanism ultimately resulting in the posttranslational stabilization and subsequent elevated expression of Bcl-2. Stable knockdown of either PCPH, mt-PCPH, or PKCα in PCa cells decreased Ser70-phosphorylated Bcl-2 and total Bcl-2 protein, thereby increasing their cisplatin sensitivity. Conversely, forced expression of the PCPH protein or, in particular, of the mt-PCPH oncoprotein increased the levels of phosphorylated PKCα concurrently with those of Ser70-phosphorylated and total Bcl-2 protein, thus promoting cisplatin resistance. Consistently, Bcl-2 knockdown sensitized PCa cells to cisplatin treatment and, more importantly, reversed the cisplatin resistance of PCa cells expressing the mt-PCPH oncoprotein. Moreover, reexpression of Bcl-2 in PCPH/mt-PCPH knockdown PCa cells reversed the cisplatin sensitization caused by PCPH or mt-PCPH down-regulation. These findings identify PCPH and mt-PCPH as important participants in the chemotherapy response of PCa cells, establish a role for PCPH/PKCα–Bcl-2 functional interactions in the drug response process, and imply that targeting PCPH expression before, or simultaneously with, chemotherapy may improve the treatment outcome for PCa patients. [Cancer Res 2009;69(1):102–10]

Introduction
Prostate cancer (PCa) is the most common non-skin cancer among men in developed countries and the second leading cause of cancer death (1). Although early PCa is generally treatable, most advanced cases eventually progress to a stage characterized by resistance to drugs such as cisplatin and other compounds normally effective against different cancer types (2) and by androgen independence, which jointly contribute to the lack of treatment response and the high mortality rates among patients with advanced PCa (3). Despite numerous studies on the subject, the molecular mechanisms underlying the acquisition of chemoresistance by advanced PCa are not clearly defined. Therefore, an improved understanding of the molecular pathways leading to chemoresistance will have direct clinical implications by increasing our capability to develop new treatment options that may overcome drug resistance in advanced PCa.

PCPH, a gene well conserved from yeast to humans that is expressed in a broad variety of normal mammalian tissues (4), was initially identified as an oncogene (mt-PCPH) activated in chemically initiated cells (5) by a single base-pair deletion within the coding region of the proto-oncogene that shifted the normal open reading frame and caused early translation termination, thus generating a mt-PCPH oncoprotein that is a truncated form of the normal polypeptide (6). The neoplastic transforming activity of the mt-PCPH oncoprotein is mediated by its ability to (a) provoke a Ras-independent, sustained activation of extracellular signal-regulated kinase (7), and/or (b) render cancer cells resistant to a variety of apoptosis-inducing stimuli (6, 7), including serum deprivation, hyperthermia, ionizing radiation, and chemotherapeutic drugs. This prosurvival activity represents a major functional difference between the normal PCPH protein and the mt-PCPH oncoprotein because expression of the normal protein consistently provided lower levels of protection against apoptotic agents, including chemotherapeutic drugs and radiation (6–8). The broad range of stress-inducing stimuli to which mt-PCPH responded and the variety of cell systems in which mt-PCPH exerted survival-promoting effects suggested that this protein may interact with diverse signaling pathways leading to apoptosis. Indeed, our laboratory showed that PCPH is identical to CD39L4 (later renamed ENTPD5), confirmed that both PCPH and mt-PCPH have ATP diphosphohydrolase (apyprase) activity (9), and established that the resistance to various stress stimuli elicited by mt-PCPH was mediated by its enhanced intrinsic ability to decrease phosphate donor availability for the kinases involved in the various stress-induced phosphorylation cascades with which it interacts (10).

Our laboratory reported frequent alterations of PCPH in a rodent model of mammary carcinogenesis (11) as well as in human tumor
cells and solid tumors (12–15), strongly suggesting its possible involvement in cancer development. Consistent with this notion, we recently reported (16) that, whereas the PCPH protein is either not expressed or expressed at nearly undetectable levels by normal human prostate epithelial cells, its expression increases in benign prostatic hyperplasia, becomes greatly elevated in prostatic intraepithelial neoplasia, and remains at high levels in prostate carcinoma (PCa). In addition, experiments with PCA cell lines showed that expression of the mt-PCPH oncoprotein enhanced their invasiveness. Further mechanistic analyses showed that mt-PCPH expression increased the levels of protein kinase Cα (PKCa), which in turn up-regulated the expression of collagen I (COL1A1 and COL1A2) genes, thereby contributing to the greater invasive ability of the PCA cells (16).

Impairment of the mechanisms of apoptotic response in cancer cells may be a critical factor in tumor development as well as a major barrier to effective treatment (17). The Bcl-2 protein plays a central role in determining whether or not cells will undergo apoptosis (18). The antiapoptotic functions of Bcl-2 are regulated by posttranslational modifications, including phosphorylation (19), which determine whether Bcl-2 remains active or it is targeted for degradation via the ubiquitin/proteasome system (20). Phosphorylation of specific amino acid residues of Bcl-2 has different functional consequences. For instance, phosphorylation of the serine residue at position 70 (Ser70) in the Bcl-2 primary sequence is required for its antiapoptotic function (21), whereas that of Ser87 seems to be responsible for its proteasome-dependent degradation (22). Several kinases, including PKCa, Raf-1, cyclic AMP–dependent protein kinase, c-jun NH2-terminal kinase, and AMP–dependent protein kinase, have been shown to phosphorylate Bcl-2 (23, 24).

In this report, we describe that PCPH and, particularly, mt-PCPH expression confer resistance against cisplatin-induced apoptosis to human PCA cells. PCPH and mt-PCPH knockdown increased cisplatin sensitivity, whereas PCPH and mt-PCPH overexpression promoted cisplatin resistance. Mechanistic studies showed that PCPH and mt-PCPH confer resistance to cisplatin-induced apoptosis by inducing the phosphorylation of PKCa, which in turn phosphorylates and stabilizes the antiapoptotic protein Bcl-2 by rendering it resistant to proteasome-mediated degradation.

Materials and Methods

Cell culture and reagents. LNCaP and PC-3 cells were cultured as described (16). Oligonucleotide primers were from Bio-Synthesis, Inc., or Invitrogen. Rottlerin, G6076, and the PKCa inhibitor were purchased from Calbiochem/EMD Biosciences, Inc. Plasmids for the expression of normal PCPH or the mt-PCPH oncoprotein were described previously (16). The CalBiochem/EMD Biosciences, Inc. Plasmids for the expression of normal PCPH or the mt-PCPH oncoprotein were described previously (16). Antibodies against PKCa, anti–PKCa, anti–phospho(Thr638)-PKCa, anti–phospho(Ser70)-Bcl-2, anti–Bcl-2, anti–cleaved caspase-3, or anti-GAPDH; washed with Tween 20 in PBS; incubated with peroxidase-conjugated secondary antibody; and the signal was then detected using a chemiluminescence-based system (Pierce). Western blot analyses were repeated at least thrice for each protein tested.

Reverse transcription-PCR. Total RNA (3 μg), extracted using the RNeasy Mini Kit (Qiagen), was used for CDNA synthesis with SuperScript III Reverse Transcriptase (Invitrogen). PCR primers for Bcl-2 and GAPDH were designed using Oligo 6.0 software (National Bioscience). Amplification of a 750-bp Bcl-2 fragment was carried out using the primers 5′-GTTGAG-GAGCTCTTCAGGGAC-3′ (forward) and 5′-AGGCACCCAGGTGTAG-CAGA-3′ (reverse). GAPDH was amplified as described (16). For each set of primers, the number of cycles was adjusted so that the reaction end points fell within the exponential phase of product amplification, thus providing a semiquantitative estimate of relative mRNA abundance. Reverse transcription-PCR (RT-PCR) determinations were carried out at least thrice for each relevant transcript.

Statistical analysis. For assays requiring statistical analysis, ANOVA or Student’s t-tests were used to assess the significance of differences between groups or individual variables, respectively. P < 0.05 was considered significant.

Results

Expression of PCPH confers resistance to cisplatin-induced apoptosis in PCA cells. To explore whether PCPH and/or mt-PCPH expression modified the chemosensitivity of human PCA cells, we used LNCaP cells in which PCPH and mt-PCPH, which are normally expressed at relatively high levels, had been simultaneously knocked down (16) by stable expression of a PCPH-specific shRNA (shPCPH), and PC-3 cells in which PCPH or mt-PCPH, which are not normally expressed, had been ectopically overexpressed (16). Cells were treated with various concentrations (up to 10 μg/mL) of cisplatin, chosen as a prototype anticancer drug to which PCa, especially advanced PCa, is generally considered to be resistant (2, 25), and the proportions of live and death cells were determined 24 hours later. LNCaP cells expressing shPCPH (LNCaP/shPCPH) were significantly more sensitive to cisplatin than control LNCaP cells transfected with a nonspecific, sequence scrambled (Sc) shRNA (LNCaP/Sc). Importantly, cells expressing shPCPH were more sensitive to treatment with 5 μg/mL cisplatin than were the control cells exposed to cisplatin at 10 μg/mL (Fig. 1A, top). Conversely, PC-3 cells expressing mt-PCPH (PC-3/mt-PCPH) were significantly more resistant to cisplatin treatment than control PC-3 cells (PC-3/V) transfected with empty vector DNA (Fig. 1B, top). Interestingly, control PC-3/V cells were more sensitive to 5 μg/mL cisplatin than were PC-3/mt-PCPH cells exposed to 10 μg/mL cisplatin. PC-3 cells expressing PCPH (PC-3/PCPH) were also more resistant to cisplatin, but the differences detected were not statistically significant relative to PC-3/V control cells (Fig. 1B, top). The apoptotic nature of the cisplatin-induced cell death was confirmed by the detection of activated, cleaved caspase-3 (Fig. 1A and B, bottom), the extent of which correlated tightly with the sensitivity to cisplatin of the various cell lines tested. Taken together, these findings strongly suggested that the presence of hexamethine bromide at the recommended multiplicity of infection; and infected cells were selected in media with 100 ng/mL puromycin.

Immunoblot analysis. Methods for the preparation of total cellular extracts in the presence of a protease inhibitor cocktail, SDS-PAGE electrophoresis of cellular proteins (50 μg), and transfer to nylon membranes were as previously described (16). Membranes were incubated with anti-PCPH, anti-PKCa, anti–phospho(Thr638)-PKCa, anti–PKCa, anti–phospho(Ser70)-Bcl-2, anti–Bcl-2, anti–cleaved caspase-3, or anti-GAPDH; washed with Tween 20 in PBS; incubated with peroxidase-conjugated secondary antibody; and the signal was then detected using a chemiluminescence-based system (Pierce). Western blot analyses were repeated at least thrice for each protein tested.
resistance of PCa cells to cisplatin-induced apoptosis could be modulated by the level of expression of PCPH and especially of mt-PCPH.

**Inhibition of PKCα sensitizes PCa cells to cisplatin-induced apoptosis.** We recently reported that PCPH regulates PKCα in PCa cells. Increased PCPH expression up-regulated PKCα, and shRNA-mediated PCPH knockdown down-regulated PKCα expression (16). Although PKCα activation is involved in, and sometimes required for, the initiation of apoptosis (26), certain studies showed that PKCα down-regulation could also result in chemosensitization (27).

To test whether PCPH conferred resistance to cisplatin-induced apoptosis by regulating PKCα or other PKC isoform, we pretreated PCa cells expressing various levels of PCPH or mt-PCPH with known pharmacologic inhibitors of different PKCs for 45 minutes before the addition of 10 μg/mL cisplatin to the cultures. Treatments included the PKCα-specific (28) Gö6976 (3 nmol/L), a PKCβ-specific (29) inhibitor (21 nmol/L), and the PKCδ-specific (30) rottlerin (3 μmol/L). Inhibition of PKCα with Gö6976 sensitized LNCaP/Sc control cells to cisplatin-induced apoptosis, significantly increasing the levels of death to >70%, compared with the 33% observed in LNCaP/Sc cells treated with cisplatin alone (Fig. 1C, left). Interestingly, the levels of death caused by the addition of Gö6976 plus cisplatin were similar to those observed in LNCaP/shPCPH cells treated with cisplatin alone, which were not changed by the simultaneous addition of Gö6976 and cisplatin (Fig. 1C, left). In the case of PC-3-derived cell lines, PKCα inhibition significantly increased the level of cisplatin-induced death from ~25% to nearly 60% in PC-3/mt-PCPH cells and from ~30% to nearly 50% in PC-3/PCPH cells, whereas no differences were observed in PC-3/V control cultures treated with cisplatin alone or in combination with Gö6976 (Fig. 1D, left). Cell death levels were not modified by pretreatment of LNCaP or PC-3 cells with Gö6976 alone (Fig. 1C and D, left). In all cases, the extent of caspase-3 activation, detected by cleaved caspase immunoblotting (Fig. 1C and D, right), correlated well with the changes in sensitivity to cisplatin caused by PKCα inhibition in the various cell lines tested. Chemical inhibition of either PKCα (Supplementary Fig. S1) or PKCβ (data not shown) before cisplatin treatment did not modify the cellular response to cisplatin, suggesting that PKCα and PKCβ are not involved in the mechanism of resistance to cisplatin-induced apoptosis promoted by PCPH or mt-PCPH expression. These data strongly suggested that PCPH and/or mt-PCPH expression conferred resistance to cisplatin-induced cell death by a mechanism that involves PKCα.

**Figure 1.** PCPH expression confers resistance to cisplatin-induced apoptosis in prostate cancer cell lines. Exponentially growing cultures of LNCaP cells expressing shPCPH or scrambled control shRNA (A) and PC-3 cells expressing empty vector, PCPH, or mt-PCPH (B) were treated with cisplatin at the indicated concentrations. V, empty vector; Cp, cisplatin. LNCaP cells expressing shPCPH or scrambled shRNA (C) and PC-3 cells expressing empty vector, PCPH, or mt-PCPH (D) were treated with 3 nmol/L Gö6976 (G6) 45 min before the treatment with 10 μg/mL cisplatin. After 24 h, viable and dead cell counts were determined using the trypan blue exclusion assay. Columns, mean; bars, SD. *, P < 0.05. Caspase-3 activation (A and B, bottom; C and D, right) was detected after the indicated treatments by Western blot with an antibody specific for active cleaved caspase-3. GAPDH was used as loading control in all cases. Experiments were replicated at least thrice.
PCPH expression maintains elevated cellular levels of phosphorylated PKCα. The functional activation of PKCα, similar to other PKC isotypes, is regulated by serine/threonine trans- and auto-phosphorylation reactions (31). To investigate whether PCPH and/or mt-PCPH expression affected PKCα activity, we performed immunoblot analyses of extracts from PCa cells expressing different levels of PCPH or mt-PCPH to evaluate the phosphorylation status of the threonine residue at position 638 (Thr638), which is an autophosphorylation site indicative of activation of PKCα (32, 33). Results showed that, relative to LNCaP/Sc control cells, PCPH knockdown in LNCaP/shPCPH cells did not affect the total levels of PKCα but dramatically decreased the levels of phosphorylated PKCα (Fig. 2A, left). In the case of PC-3–derived cultures, expression of either PCPH or mt-PCPH did not alter the total levels of PKCα protein relative to PC-3/V controls, but markedly increased the levels of phosphorylated PKCα (Fig. 2A, right). The direct correlation detected between the cellular levels of PCPH and/or mt-PCPH and those of phospho(Thr638)-PKCα strongly suggested that PCPH and mt-PCPH may be involved in regulating PKCα phosphorylation and activation in PCa cells.

Because the expression of Bcl-2 has been associated with cisplatin resistance in different tissues (34) and PKCα has been reported to mediate chemoresistance through Bcl-2 phosphorylation at Ser70 (21), we examined the levels of the antiapoptotic protein Bcl-2 and its phosphorylation status in PCa cells expressing various levels of PCPH or mt-PCPH. PCPH knockdown in LNCaP/shPCPH cells efficiently down-regulated Bcl-2 expression and reduced the levels of phospho(ser70)-Bcl-2 (Fig. 2A, left). Conversely, in PC-3–derived cultures, ectopic expression of mt-PCPH (PC-3/mt-PCPH cells) dramatically increased the levels of total and phosphorylated Bcl-2 relative to control PC-3/V cells, whereas PCPH expression produced more moderate expression increases (Fig. 2A, right). These results were consistent with those obtained with LNCaP-derived cultures and strongly suggested that PCPH and, especially, mt-PCPH expression regulate Bcl-2 expression levels and phosphorylation in PCa cell lines. The fact that no Bcl-2 expression differences were observed at the RNA level (data not shown) in any of the LNCaP- or PC-3–derived cell lines expressing various levels of PCPH or mt-PCPH strongly suggested that the regulatory action of PCPH and mt-PCPH on Bcl-2 expression was effected through mechanisms involving the posttranslational modification of Bcl-2.

Exposure to diverse apoptotic stimuli may increase or decrease the phosphorylation status of specific amino acid residues of Bcl-2, respectively leading to its stabilization or promoting its degradation via the proteasome system (22, 35). To further investigate whether the action of PCPH and mt-PCPH on the cisplatin response of PCa cells may be mediated through PKCα and/or Bcl-2, we investigated the effect of cisplatin exposure on PKCα and Bcl-2 expression and phosphorylation in cells expressing different levels of PCPH or mt-PCPH. Results showed that the levels of total PKCα protein were not significantly modified by cisplatin in any of the LNCaP- or PC-3–derived cell lines tested, relative to the cisplatin-un-treated controls (Fig. 2B and C), whereas cisplatin treatment decreased the phospho(Thr368)-PKCα levels in both LNCaP/Sc (Fig. 2B, left) and PC-3/V (Fig. 2C, left) control cell lines. Compared with the cisplatin effect on LNCaP/Sc cells (Fig. 2B, left), cisplatin treatment of LNCaP/shPCPH did not further decrease the already greatly reduced levels of phospho(Thr368)-PKCα provoked by PCPH knockdown (Fig. 2B, right). Most interestingly, compared with the cisplatin effect on PC-3/V cells (Fig. 2C, left), the observed down-regulatory effect of cisplatin on phospho(Thr368)-PKCα was substantially lessened in PC-3 cells expressing PCPH (Fig. 2C, middle) and completely blocked by mt-PCPH expression (Fig. 2C, right). These data were consistently paralleled by results on the effect of cisplatin on the levels of Bcl-2 expression and phosphorylation, which were markedly reduced in both LNCaP/Sc (Fig. 2B, left) and PC-3/V (Fig. 2C, left) control cell lines. Whereas...
PCPH knockdown in LNCaP/shPCPH cells did not modify the cisplatin response, the observed down-regulation effect of cisplatin on Bcl-2 and phospho-Bcl-2 was substantially diminished in PC-3 cells expressing PCPH (Fig. 2C, middle) and completely prevented by mt-PCPH expression (Fig. 2C, right). Taken together, these results showed that expression of PCPH or mt-PCPH results in the phosphorylation and subsequent posttranslational stabilization and protection of Bcl-2 against cisplatin-induced degradation, and strongly suggested the involvement of PKCα in this process.

PKCα knockdown sensitizes PCa cells to cisplatin-induced apoptosis by enhancing Bcl-2 down-regulation. We showed above that pharmacologic inhibition of PKCα sensitized PCa cells to cisplatin-induced apoptosis (Fig. 1C and D). However, because Gö6976 could also provoke a variety of nonspecific effects (36), and to define a role for PKCα in the PCPH/mt-PCPH-mediated cisplatin resistance of PCa cells, PKCα was knocked down by shRNA-mediated transfection into LNCaP and PC-3 cells (Fig. 3A), and PKCα knockdown cells were exposed to cisplatin (10 μg/mL) for 24 hours. Results (Fig. 3B) showed that, relative to the Sc-transfected controls, PKCα down-regulation caused statistically significant increases in cisplatin-induced cell death in both PCa cell lines (~35% increase in LNCaP cells and ~27% in PC-3 cells), which correlated well with the respective increases observed in caspase-3 activation (Fig. 3C). In addition, immunoblot analyses showed that specific PKCα down-regulation decreased the levels of both total and phospho(Ser70) Bcl-2 in both LNCaP and PC-3 cells (Fig. 3D), strongly suggesting that PKCα phosphorylates Bcl-2 in PCa cells, thus protecting the Bcl-2 protein from proteasome-mediated degradation. Results from similar experiments carried out with LNCaP and PC-3 cells in which PKCγ was knocked down by transfection with a specific shRNA construct (16) showed that neither the cellular sensitivity to cisplatin nor the Bcl-2 expression levels were modified by PKCγ knockdown (Supplementary Fig. S2), showing that PKCγ does not participate in the mechanism by which PCPH or mt-PCPH promotes resistance to cisplatin-induced apoptosis in PCa cells. These data, along with the fact that PKCα knockdown essentially recapitulated the effects on cisplatin sensitivity and Bcl-2 expression and phosphorylation provoked by PCPH knockdown, confirmed that PKCα acts downstream of PCPH and mt-PCPH in the process.

Ectopic reexpression of Bcl-2 prevents the sensitization of LNCaP cells to cisplatin-induced apoptosis promoted by PCPH/mt-PCPH knockdown. To determine whether Bcl-2 indeed mediated the resistance to cisplatin-induced apoptosis promoted by PCPH or mt-PCPH, we first used a shRNA-based approach to knock down Bcl-2. LNCaP cells were infected with several lentiviral preparations expressing different shRNAs designed to target Bcl-2 or with a nonspecific shRNA. The shBcl2-5 preparation effectively knocked down Bcl-2 expression, whereas shBcl2-1 did not work (Fig. 4A) and was used as a negative control in experiments in

![Figure 3](https://example.com/figure3.png)

**Figure 3.** PKCα knockdown decreased the levels of Bcl-2, sensitizing prostate cancer cells to cisplatin-induced apoptosis. LNCaP (left) and PC-3 (right) cells were stably transfected with shPKCα or scrambled shRNA. PKCα knockdown was ascertained by Western blot (A), and cells expressing decreased PKCα levels were treated with cisplatin, at 10 μg/mL, for 24 h. Then, viable and dead cell counts were determined by trypan blue exclusion assay (B). Columns, mean; bars, SD. *, P < 0.05. Caspase-3 activation was determined as described in previous figure legends (C). Levels of Bcl-2 and phospho(Ser70) Bcl-2 were determined by Western blot analysis with specific antibodies (D). GAPDH was used as loading control in all cases.
which the lentivirus-infected PCa cells were treated with cisplatin (at 5 or 10 μg/mL). Live and dead cell counts determined after 24 hours showed that Bcl-2 knockdown significantly increased the cellular sensitivity to cisplatin-induced apoptosis (Fig. 4B). Cells infected with the specific shBcl2-5 construct were significantly more sensitive (≈40% cell death) to 5 μg/mL cisplatin than Sc control cells (≈25% cell death) and cells infected with the inactive shBcl2-1 preparation (≈23% cell death). When treated with 10 μg/mL cisplatin, cell death levels in cultures infected with shBcl2-5 were >50%, compared with the 25% and 27% levels obtained with the Sc and shBcl2-1 preparations, respectively (Fig. 4B, top). In all cases, the levels of cell death observed correlated well with the extent of caspase-3 activation detected under the different experimental conditions (Fig. 4B, bottom). Because these results provided strong evidence in support of a role for Bcl-2 as a mediator of the effect of PCPH and mt-PCPH on the response of PCa cells to cisplatin, we transfected LNCaP/shPCPH cells, in which PCPH knockdown had reduced the Bcl-2 expression levels (Fig. 2, left), with a Bcl-2 expression vector to determine the effect of restoring Bcl-2 expression to its normal levels (Fig. 4C) on the cellular sensitivity to cisplatin. Treatment of PCPH knockdown LNCaP cells reexpressing Bcl-2 (LNCaP/shPCPH + Bcl-2) with cisplatin (10 μg/mL) showed that Bcl-2 reexpression reversed the cisplatin sensitivity of LNCaP/shPCPH cells (≈70% cell death after 24 hours) to levels of resistance (≈31% cell death) similar to those (≈30% cell death) of the LNCaP/Sc control cultures (Fig. 4D, top). In all cases, the observed levels of cell death correlated well with the extent of caspase-3 activation detected under the different experimental conditions (Fig. 4D, bottom). These results showed that PCPH knockdown sensitized PCa cells to cisplatin-induced apoptosis through Bcl-2 down-regulation.

**Bcl-2 knockdown sensitizes PC-3 cells to cisplatin-induced apoptosis.** PC-3/mt-PCPH cells were more resistant to cisplatin-induced apoptosis than PC-3/V or PC-3/PCPH cells (Fig. 1B). In addition, Bcl-2 was up-regulated to greater expression levels in PC-3/mt-PCPH cells than in PC-3/PCPH cells and PC-3/V control cultures (Fig. 2A). To investigate whether the levels of Bcl-2 were responsible for the different degrees of resistance of these cells to cisplatin-induced apoptosis, we knocked down Bcl-2 in the three PC-3–derived cell lines expressing various levels of PCPH or mt-PCPH (Fig. 5A) by infection with the shBcl2-5 lentiviral preparation described above. Treatment of the Bcl-2 knockdown cells and the corresponding Sc control cultures with cisplatin (10 μg/mL) consistently increased the cisplatin sensitivity of all cell lines tested (Fig. 5B). The increase in cisplatin sensitivity brought about by Bcl-2 knockdown in PC-3/V cells did not reach statistical significance, whereas the increase in cisplatin sensitivity promoted in PC-3/PCPH and PC-3/mt-PCPH cells were statistically significant (Fig. 5B). The observed levels of cell death correlated tightly with the extent of caspase-3 activation detected under the different experimental conditions (Fig. 5C). Taken together, these results suggested that the up-regulation of Bcl-2 produced by expression of PCPH and especially of mt-PCPH is responsible for the resistance to cisplatin-induced apoptosis in the PC-3–derived cells.

**Discussion**

The study described here represents the first report on the involvement of the PCPH protein and the mt-PCPH oncoprotein in determining the chemoresistance of human PCa. Using shRNA-mediated gene expression knockdown, ectopic protein expression, and reexpression studies, our results, which are consistent among PCa-derived cell lines manipulated to express different levels of PCPH or mt-PCPH, show that expression of PCPH and particularly of mt-PCPH in PCa cells antagonizes the cisplatin-induced apoptotic process by enhancing the activating phosphorylation of...
PKCα at Thr638 and increasing the total expression and phosphorylated levels of Bcl-2. This antiapoptotic effects ultimately rendered PCa cells resistant to apoptotic cell death, thus favoring tumor cell survival and malignant proliferation. Results reported here identify a new pathway (PCPH/mt-PCPH→PKCα→Bcl-2) that, taking into consideration that PCPH/mt-PCPH expression increases at the prostatic intraepithelial neoplasia stage and is maintained at high levels in malignant PCa, may likely contribute to the acquisition of chemoresistance by PCa cells during tumor progression. Indeed, the same pathway (PCPH/mt-PCPH→PKCα→Bcl-2) was also found to be functional in the cisplatin response of C4-2 cells, a more metastatic LNCaP-derived line that expresses both PCPH and mt-PCPH (Supplementary Fig. S3), and PCPH knockdown also sensitized CWR22Rv1 PCa cells, which only express PCPH, to cisplatin treatment (data not shown). We propose a mechanistic paradigm for the sequence of events that, in the presence of PCPH or especially mt-PCPH, culminates in the acquisition by PCa cells of resistance to cisplatin-induced apoptosis. The model (Fig. 6) takes into account two scenarios: (a) In the absence of PCPH or mt-PCPH, phosphorylation of Bcl-2 at Ser70 by PKCα protects Bcl-2 from proteasome-mediated degradation, thereby creating an antiapoptotic environment; this setting is subverted when exposure to cisplatin promotes PKCα inactivation and, consequently, decreases the levels of phosphoSer70 Bcl-2, thus favoring the proteasome-mediated degradation of nonphosphorylated Bcl-2 and ultimately resulting in the apoptotic death of the cells. (b) The expression of PCPH or, more efficiently, of mt-PCPH prevents the cisplatin-induced dephosphorylation of PKCα at Thr638, maintaining it in an activated state that phosphorylates Bcl-2 at Ser70 and prevents its degradation, eventually leading to increased phosphorylated Bcl-2 levels that protect PCa cells against apoptosis.

The significance of PKC activation in the cellular response to apoptosis-inducing stimuli, including cisplatin, has long been recognized (31, 37, 38). Although there are still controversies about how PKCs influence apoptosis, the general consensus is that the antiapoptotic or proapoptotic function of individual PKC isoforms is regulated through a spatiotemporally coordinated cascade of PKC activation (39) that is differentially triggered by diverse stimuli and is also dependent on the distinct isoform repertoires found in different cellular contexts. This complex interplay among PKC isoforms leads to the establishment of unique and redundant response pathways, which determine the outcome of the cellular exposure to different apoptosis-inducing agents (40, 41). Most of what is known on the role of individual PKC isoforms in the regulation of apoptosis in human PCa cells derives from mechanistic studies about the cell death–inducing activity of phorbol esters, a group of carcinogenesis promoters and well-characterized PKC activators (31, 42). Results from several laboratories showed that, in response to phorbol esters, PKCε preferentially mediates survival signaling, that PKCδ and PKCζ are proapoptotic kinases, and that PKCδ is essential for the apoptosis process, whereas PKCα and PKCε are not so stringently required and may have redundant functions under certain conditions (41, 43, 44). Unfortunately, there is little information available on the expression of different PKC isoforms in tumor specimens from PCa patients (16, 45), thus making it difficult to translate these observations to the physiology of PCa and their response to treatment.

The mechanism of cisplatin-induced apoptosis in PCa cells is not so well characterized, and there is little information on the involvement of individual PKC isoforms. Our findings show that the apoptotic process triggered by cisplatin in PCa cells is quite different from that elicited by phorbol esters. In contrast to the latter case, PKCδ is dispensable for cisplatin-induced apoptosis (Supplementary Figs. S1 and S2), and cisplatin causes the down-regulation, rather than the increase, of the levels of active, Thr638-phosphorylated PKCα (Fig. 2B and C). The fact that, similar to PCPH/mt-PCPH knockdown, PKCα knockdown also sensitizes LNCaP cells to cisplatin (Fig. 3) provides strong evidence in support of the model for PCPH/mt-PCPH→PKCα→Bcl-2.
The regulation of Thr638-phosphorylated PKC influences the response of PCa cells to cisplatin irrespective of their androgen responsiveness status. Our results also show a clear mechanism of promotion of resistance to cisplatin-induced apoptosis by PCPH and mt-PCPH. PKCα phosphorylates Bcl-2 at Ser70, preventing Bcl-2 from degradation. Treatment with cisplatin produces PKCα dephosphorylation or inactivation, which decreases the levels of phosphorylation of Bcl-2. The ubiquitin/proteasome pathway degrades dephosphorylated Bcl-2. Once the levels of Bcl-2 decrease, the proapoptotic protein BAX is released, activating the apoptotic pathway. The expression of mt-PCPH prevents cisplatin-induced PKCα dephosphorylation. Activated PKCα prevents Bcl-2 degradation. High levels of Bcl-2 protect prostate cancer cells against apoptosis induced by cisplatin.

The existence of a functional interaction between PCPH and PKCα. Moreover, the fact that PKCα knockdown also efficiently sensitized PC-3 cells, which are androgen independent, to cisplatin (Fig. 3) strongly suggests that such PCPH-PKCα interaction influences the response of PCa cells to cisplatin irrespective of their androgen responsiveness status. Our results also show a clear role for PCPH and especially for mt-PCPH in preventing the down-regulation of Thr638-phosphorylated PKCα (Fig. 2B and C) and, consequently, in providing resistance to cisplatin-induced apoptosis (Fig. 1). Whether PCPH and mt-PCPH expression prevent the dephosphorylation of preexisting Thr638-phosphorylated PKCα or somehow stimulate the Thr638-phosphorylation process remains to be elucidated. The involvement of PKCα, rather than PKCδ, in the response of PCa to cisplatin-induced apoptosis is likely a cell type–specific characteristic, as PKCδ has been reported to be the major determinant of the response to cisplatin in other tumor cells (46, 47). Our data agree with reports indicating that chemical- or ribozyme-mediated inhibition of PKCα sensitized PCa cells to cisplatin and other anticancer drugs (36, 48), and are also consistent with the fact that the expression of both PCPH/mt-PCPH (16) and PKCα (45) is elevated in PCa. Results from our experiments on the susceptibility to cisplatin-induced apoptosis of LNCaP and PC-3 cells in which Bcl-2 was knocked down, as well as those from studies on the effect of reexpressing Bcl-2 in PCPH knockdown LNCaP cells, conclusively identify Bcl-2 as a mediator of the prosurvival function of PCPH and mt-PCPH. These findings are also consistent with current knowledge on the central role of Bcl-2 in determining the life-or-death outcome after apoptotic stimulation of PCa and other tumor cells (49) and on the regulation by PKCα-mediated phosphorylation of the susceptibility of Bcl-2 to proteasome-dependent degradation (34, 49, 50).

A direct comparison between the relative antiapoptotic activities of PCPH and mt-PCPH was not possible in LNCaP cells because, although they express both PCPH and mt-PCPH, the single base pair difference between the normal and mutated mRNAs (6) did not allow the selective knockdown of one or the other. In this regard, ectopic expression of PCPH or mt-PCPH in PC-3 cells, in which they are not endogenously expressed, became more informative. Our results on the response to cisplatin of PCPH- and mt-PCPH–expressing PC-3 cells agree with previous reports on the prosurvival function of PCPH and mt-PCPH that assigned a greater antiapoptotic activity to the mt-PCPH oncoprotein than to the normal PCPH protein in the response to ionizing radiation (8), as well as with our previous results from the characterization of a PCPH/mt-PCPH-inducible system developed to study their involvement in mechanisms of chemoresponse of primary mouse embryo fibroblasts.3 Furthermore, although Western immunoblotting is not a quantitative technique, it was consistently apparent that the expression levels of PCPH and mt-PCPH achieved in PC-3 cells were quite similar (16), and this is consistent with previous estimates indicating that a substantially greater expression of PCPH was required to attain levels of prosurvival activity directly comparable to those induced by the expression of mt-PCPH (8, 10).

It would be extremely interesting to investigate whether PCPH and/or mt-PCPH may also play a role in the response of PCa to radiation, usually successful in the treatment of localized disease, or to chemotherapeutic agents currently under investigation, such as docetaxel, which yields modest benefits to patients with disseminated PCa. The fact that in the same cellular system (cultured mouse embryo fibroblasts) PCPH/mt-PCPH conferred resistance to radiation by interacting with the mammalian target of rapamycin pathway (8) while modulating a different pathway to promote resistance to chemotherapeutic drugs3 suggests that a similar involvement of PCPH/mt-PCPH with different signaling pathways in the response to different stress stimuli may also be the case in PCa cells. Experiments designed to test these possibilities are currently ongoing in our laboratory.

Finally, it is important to note that PCPH and mt-PCPH seemed to be able to functionally interact with two different PKC isoforms in PCa cells [through PKCδ, to modulate their invasiveness (16), and through PKCα, to regulate their chemoresponse], and that invasiveness and chemoresistance are properties typically acquired during tumor progression to advanced PCa. Therefore, on the basis of the increased levels of PCPH along the process of malignant

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3 O.M. Tirado et al, unpublished data.
PCa progression reported previously (16) and of the increased probability of accumulating mt-PCPH mutations during advanced PCa progression stages, it seems reasonable to propose that PCPH and mt-PCPH may be important contributors to the development of the malignant phenotype of PCa cells. In this context, targeted blockage of PCPH/mt-PCPH expression may be an effective strategy to sensitize PCa to therapeutic treatment and a useful approach to improve the treatment outcome for advanced PCa patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

References

PCPH/ENTPD5 Expression Confers to Prostate Cancer Cells Resistance against Cisplatin-Induced Apoptosis through Protein Kinase Cα–Mediated Bcl-2 Stabilization

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