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PCPH/ENTPD5 expression confers to prostate cancer cells resistance against cisplatin-induced apoptosis through PKCα-mediated Bcl-2 stabilization

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Abstract

Prostate cancer (PCa) frequently develops anti-apoptotic mechanisms and acquire 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 (PIN) and in PCa, and that the functional interaction between PCPH and protein kinase Cδ (PKCδ) 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 (Cp)-induced apoptosis to PCa cells. This interaction elicits a mechanism ultimately resulting in the post-translational 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 Cp 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 Cp resistance. Consistently, Bcl-2 knockdown sensitized PCa cells to Cp treatment and, more importantly, reversed the Cp resistance of PCa cells expressing the mt-PCPH oncoprotein. Moreover, re-expression of Bcl-2 in PCPH/mt-PCPH knocked-down PCa cells reversed the Cp 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 prior to, or simultaneously with, chemotherapy may improve the treatment outcome for PCa patients.

Keywords

prostate cancer; cisplatin resistance; protein kinase α; Bcl-2; PCPH/mt-PCPH expression

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Conflicts of Interest: None

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 (Cp) 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 chemo-resistance by advanced PCa are not clearly defined. Therefore, an improved understanding of the molecular pathways leading to chemo-resistance 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 (*i*) provoke a Ras-independent, sustained activation of ERK (7), and/or (*ii*) render cancer cells resistant to a variety of apoptosis-inducing stimuli (6,7), including serum deprivation, hyperthermia, ionizing radiation, and chemotherapeutic drugs. This pro-survival 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 demonstrated that PCPH is identical to CD39L4 (later renamed ENTPD5), confirmed that both PCPH and mt-PCPH have ATP diphosphohydrolase (apyrase) 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 while 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 (BPH), becomes greatly elevated in prostatic intraepithelial neoplasia (PIN), and remains at high levels in prostate carcinoma (PCa). In addition, experiments with PCa cell lines demonstrated that expression of the mt-PCPH oncoprotein enhanced their invasiveness. Further mechanistic analyses showed that mt-PCPH expression increased the levels of Protein Kinase Cδ (PKCδ), 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 anti-apoptotic functions of Bcl-2 are regulated by post-translational 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 anti-apoptotic function (21), whereas that of Ser87 appears to be responsible for its proteasome-dependent degradation (22). Several kinases, including PKCα, Raf-1, PKA, JNK and others, have been shown to phosphorylate Bcl-2 (23,24).

In this report we describe that PCPH and, particularly, mt-PCPH expression confer resistance against Cp-induced apoptosis to human PCa cells. PCPH and mt-PCPH knockdown increased Cp sensitivity, whereas PCPH and mt-PCPH over-expression promoted Cp resistance. Mechanistic studies demonstrated that PCPH and mt-PCPH confer resistance to Cp-induced apoptosis by inducing the phosphorylation of protein kinase Cα (PKCα), which in turn phosphorylates and stabilizes the anti-apoptotic 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. (Lewisville, TX) or Invitrogen (San Jose, CA). Rottlerin, Gö6976 and the PKCβ inhibitor were purchased from Calbiochem/EMD Biosciences, Inc. (San Diego, CA). Plasmids for the expression of normal PCPH or the mt-PCPH oncoprotein were described previously (16). The rabbit polyclonal anti-PCPH antiserum, produced by BioSysthesis, Inc., was previously described (11-15). Antibodies against PKCα and against PKCδ were from BD Biosciences-Pharmingen (San Diego, CA). Antibodies against phospho(Thr638)-PKCα, Bcl-2, cleaved caspase 3, and phosphor(Ser70)-Bcl-2 were purchased from Cell Signaling (Beverly, MA). GAPDH (Abcam Ltd., Cambridge, England) was used for loading normalization. All other general reagents were from Sigma-Aldrich (St. Louis, MO).

Selection of cells stably expressing shRNA constructs

Cell lines expressing shRNA constructs targeting PCPH and PKCδ were generated as described (16). Cells stably expressing shRNA against PKCα or the appropriate control plasmids (obtained from Origene, Rockville, MD) were generated using the same general procedure, but in this case LNCaP and PC-3 cells were transfected with shRNA using Lipofectamine (Invitrogen), and selected in media with 100 ng/ml puromycin. Validated Mission® shRNA lentiviral particles targeting Bcl-2 expression were purchased from Sigma-Aldrich. Cells were plated 24 h before infection, viral particles were added in the presence of hexadimethrine 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-PKCα, anti-phospho(Thr638)-PKCα, anti-PKCδ, anti-phospho(Ser70)-Bcl2, anti-Bcl-2, anti-cleaved caspase 3, or anti-GAPDH, washed with Tween 20 in phosphate-buffered saline (PBS), incubated with peroxidase-conjugated secondary antibody, and the signal was then detected using a chemiluminescence-based system (Pierce, Rockford, IL). Western blot analyses were repeated at least three times for each protein tested.

Reverse transcription-PCR

Total RNA (3 μg), extracted using the RNeasy™ Mini Kit (Qiagen, Valencia, CA), 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, Plymouth, MN). Amplification of a 750-bp *Bcl-2* fragment was carried out using the primers 5′-GTGGAGGAGCTCTTCAGGGAC-3′ (forward) and 5′-AGGCACCCAGGGTGATGCAAG-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 semi-quantitative estimate of relative mRNA abundance. RT-PCR determinations were carried out at least three times 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 regarded as significant.

Results

Expression of PCPH confers resistance to Cp-induced apoptosis in PCa cells

To explore whether PCPH and/or mt-PCPH expression modified the chemo-sensitivity of human PCa, we utilized 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 over-expressed (16). Cells were treated with various concentrations (up to 10 μg/ml) of Cp, chosen as a prototype anti-cancer 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 h later. LNCaP cells expressing shPCPH (LNCaP/shPCPH) were significantly more sensitive to Cp than control LNCaP cells transfected with a non-specific, sequence scrambled (Sc) shRNA (LNCaP/Sc). Importantly, cells expressing shPCPH were more sensitive to treatment with 5 μg/ml Cp than were the control cells exposed to Cp at 10 μg/ml (Fig. 1A, top). Conversely, PC-3 cells expressing mt-PCPH (PC-3/mt-PCPH) were significantly more resistant to Cp 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 Cp than were PC-3/mt-PCPH cells exposed to 10 μg/ml Cp. PC-3 cells expressing PCPH (PC-3/PCPH) were also more resistant to Cp, but the differences detected were not statistically significant relative to PC-3/V control cells (Fig. 1B, top). The apoptotic nature of the Cp-induced cell death was confirmed by the detection of activated, cleaved caspase 3 (Fig. 1A and 1B, bottom), the extent of which correlated tightly with the sensitivity to Cp of the various cell lines tested. Taken together, these findings strongly suggested that the resistance of PCa cells to Cp-induced apoptosis could be modulated by the level of expression of PCPH and, especially, of mt-PCPH.

Inhibition of PKCα sensitizes PCa cells to Cp-induced apoptosis

We recently reported that PCPH regulates PKCδ in PCa cells. Increased PCPH expression up-regulated PKCδ and shRNA-mediated PCPH knockdown down-regulated PKCδ expression (16). Although PKCδ activation is involved in, and sometimes required for, the initiation of apoptosis (26), certain studies showed that PKCδ down-regulation could also result in chemo-sensitization (27). To test whether PCPH conferred resistance to Cp-induced apoptosis by regulating PKCδ or other PKC isoform, we pretreated PCa cells expressing various levels of PCPH or mt-PCPH with known pharmacological inhibitors of different PKCs for 45 min before

the addition of 10 μg/ml Cp to the cultures. Treatments included the PKCα-specific (28) Gö6976 (3 nM), a PKCβ-specific (29) inhibitor (21 nM), and the PKCδ-specific (30) rottlerin (3 μM). Inhibition of PKCα with Gö6976 sensitized LNCaP/Sc control cells to Cp-induced apoptosis, significantly increasing the levels of death to over 70% compared to the 33% observed in LNCaP/Sc cells treated with Cp alone (Fig. 1C, left). Interestingly, the levels of death caused by the addition of Gö6976 plus Cp were similar to those observed in LNCaP/shPCPH cells treated with Cp alone, which were not changed by the simultaneous addition of Gö6976 and Cp (Fig. 1C, left). In the case of PC-3-derived cell lines, PKCα inhibition significantly increased the level of Cp-induced death from about 25% to nearly 60% in PC-3/mt-PCPH cells and from over 30% to nearly 50% in PC-3/PCPH cells, whereas no differences were observed in PC-3/V control cultures treated with Cp alone or in combination with Gö6976 (Fig. 1D, left). Cell death levels were not modified by pre-treatment of LNCaP or PC-3 cells with Gö6976 alone (Fig. 1C and 1D, left). In all cases, the extent of caspase 3 activation, detected by cleaved caspase immunoblotting (Fig. 1C and 1D, right), correlated well with the changes in sensitivity to Cp caused by PKCα inhibition in the various cell lines tested. Chemical inhibition of either PKCδ (Suppl. Fig. 1) or PKCβ (data not shown) prior to Cp treatment did not modify the cellular response to Cp, suggesting that PKCδ and PKCβ are not involved in the mechanism of resistance to Cp-induced apoptosis promoted by PCPH or mt-PCPH expression. These data strongly suggested that PCPH and/or mt-PCPH expression conferred resistance to Cp-induced cell death by a mechanism that involves PKCα.

PCPH expression maintains elevated cellular levels of phosphorylated PKCα

The functional activation of PKCα, similar to other PKC isoforms, 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(Thr368)-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 Cp resistance in different tissues (34) and PKCα has been reported to mediate chemo-resistance 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 phosporylated 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 regulates 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 post-translational 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 Cp response of PCa cells may be mediated through PKCα and/or Bcl-2, we investigated the effect of Cp 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 Cp in any of the LNCaP- or PC-3-derived cell lines tested, relative to the Cp-untreated controls (Fig. 2B and 2C), whereas Cp 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 to the Cp effect on LNCaP/Sc cells (Fig. 2B, left), Cp treatment of LNCaP/shPCPH did not decrease further the already greatly reduced levels of phospho(Thr368)-PKCα provoked by PCPH knockdown (Fig. 2B, right). Most interestingly, compared to the Cp effect on PC-3/V cells (Fig. 2C, left), the observed down-regulatory effect of Cp 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 Cp 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. While PCPH knockdown in LNCaP/shPCPH cells did not modify the Cp response, the observed down-regulation effect of Cp 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 demonstrated that expression of PCPH or mt-PCPH results in the phosphorylation and subsequent post-translational stabilization and protection of Bcl-2 against Cp-induced degradation, and strongly suggested the involvement of PKCα in this process.

PKCα knockdown sensitizes PCa cells to Cp-induced apoptosis by enhancing Bcl-2 down-regulation

We showed above that pharmacological inhibition of PKCα sensitized PCa cells to Cp-induced apoptosis (Figs. 1C and 1D). However, because Gö6976 could also provoke a variety of non-specific effects (36), and to define a role for PKCα in the PCPH/mt-PCPH-mediated Cp-resistance response of PCa cells, PKCα was knockdown by shRNA-mediated transfection into LNCaP and PC-3 cells (Fig. 3A), and PKCα-knockdown cells were exposed to Cp (10 μg/ml) for 24 h. Results (Fig. 3B) showed that, relative to the Sc-transfected controls, PKCα down-regulation caused statistically significant increases in Cp-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 PKCδ was knocked down by transfection with a specific shRNA construct (16) showed that neither the cellular sensitivity to Cp nor the Bcl-2 expression levels were modified by PKCδ knockdown (Suppl. Fig. 2), demonstrating that PKCδ does not participate in the mechanism by which PCPH or mt-PCPH promote resistance to Cp-induced apoptosis in PCa cells. These data, along with the fact that PKCα knockdown essentially recapitulated the effects on Cp-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 re-expression of Bcl-2 prevents the sensitization of LNCaP cells to Cp-induced apoptosis promoted by PCPH/mt-PCPH knockdown

To determine whether Bcl-2 indeed mediated the resistance to Cp-induced apoptosis promoted by PCPH or mt-PCPH, we first used a shRNA-based approach to knockdown Bcl-2. LNCaP cells were infected with several lentiviral preparations expressing different shRNAs designed to target Bcl-2 or with a non-specific shRNA. The shBcl2-5 preparation effectively knocked down Bcl-2 expression, while shBcl2-1 did not work (Fig. 4A) and was used as a negative control in experiments in which the lentivirus infected PCa cells were treated with Cp (at 5 or 10 μg/ml). Live/dead cell counts determined after 24 h showed that Bcl-2 knockdown significantly increased the cellular sensitivity to Cp-induced apoptosis (Fig. 4B). Cells infected with the specific shBcl2-5 construct were significantly more sensitive (~40% cell death) to 5 μg/ml Cp 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 Cp, cell death levels in cultures infected with shBcl2-5 were greater than 50% compared to the 25% and 27 % levels obtained with the Sc and the 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 Cp, 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 Cp. Treatment of PCPH-knockdown LNCaP cells re-expressing Bcl-2 (LNCaP/shPCPH+Bcl-2) with Cp (10μg/ml) demonstrated that Bcl-2 re-expression reversed the Cp-sensitivity of LNCaP/shPCPH cells (~70% cell death after 24 h) 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 demonstrated that PCPH knockdown sensitized PCa cells to Cp-induced apoptosis through Bcl-2 down-regulation.

Bcl-2 knockdown sensitizes PC-3 cells to Cp-induced apoptosis

PC-3/mt-PCPH cells were more resistant to Cp-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 Cp-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 Cp (10μg/ml) consistently increased the Cp sensitivity of all cell lines tested (Fig. 5B). The increase in Cp-sensitivity brought about by Bcl-2 knockdown in PC-3/V cells did not reach statistical significance, whereas the increases in Cp 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 PCPH and, especially, by mt-PCPH expression is responsible for the resistance to Cp-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 chemoresistant response of human PCa. Using sh-RNA-mediated gene expression knockdown, ectopic protein expression and re-

expression studies, our results, which are consistent among PCa-derived cell lines manipulated to express different levels of PCPH or mtPCPH, show that expression of PCPH and, particularly, of mt-PCPH in PCa cells antagonizes the Cp-induced apoptotic process by enhancing the activating phosphorylation of PKCα at Thr638 and increasing the total expression and phosphorylated levels of Bcl-2. This anti-apoptotic 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 PIN 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 [PCPH/mt-PCPH → PKCα → Bcl-2] pathway was also found to be functional in the Cp response of C4-2 cells, a more metastatic LNCaP-derived line that expresses both PCPH and mt-PCPH (Suppl. Fig. 3), and PCPH knockdown also sensitized CWR22Rv1 PCa cells, which only express PCPH, to Cp treatment (data not shown). We propose a mechanistic paradigm for the sequence of events that, in the presence of PCPH or, specially, of mt-PCPH, culminates in the acquisition by PCa cells of resistance to Cp-induced apoptosis. The model (Fig. 6) takes into account two scenarios: 1) 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 anti-apoptotic environment; this setting is subverted when exposure to Cp promotes PKCα inactivation and, consequently, decreases the levels of phosphoSer70 Bcl-2, thus favoring the proteasome-mediated degradation of non-phosphorylated Bcl-2 and ultimately resulting in the apoptotic death of the cells; and 2) the expression of PCPH or, more efficiently, of mt-PCPH prevents the Cp-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 Cp, has long been recognized (31,37,38). Although there are still controversies regarding how PKCs influence apoptosis, the general consensus is that the anti- or pro-apoptotic 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 demonstrated that, in response to phorbol esters, PKCε preferentially mediates survival signaling, that PKCα and PKCδ are pro-apoptotic 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 Cp-induced apoptosis in PCa cells is not so well characterized, and there is little information on the involvement of individual PKC isoforms. Our findings demonstrate that the apoptotic process triggered by Cp in PCa cells is quite different from that elicited by phorbol esters. In contrast to the latter case, PKCδ is dispensable for Cp-induced apoptosis (Suppl. Figs. 1 and 2), and Cp causes the down-regulation, rather than the increase, of the levels of active, Thr638-phosphorylated PKCα (Figs. 2B and 2C). The fact that, similar to PCPH/mt-PCPH knockdown, PKCα knockdown also sensitizes LNCaP cells to Cp (Fig. 3) provides

strong evidence in support of 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 Cp (Fig. 3) strongly suggests that such PCPH-PKCα interaction influences the response of PCa cells to Cp 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 (Figs. 2B and 2C) and, consequently, providing resistance to Cp-induced apoptosis (Fig. 1). Whether PCPH and mt-PCPH expression prevent the dephosphorylation of pre-existing 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 Cp-induced apoptosis is likely a cell type-specific characteristic, as PKCδ has been reported to be the major determinant of the response to Cp in other tumor cells (46,47). Our data agree with reports indicating that chemical or ribozyme-mediated inhibition of PKCα sensitized PCa cells to Cp 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 Cp-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 re-expressing Bcl-2 in PCPH knockdown LNCaP cells conclusively identify Bcl-2 as a mediator of the pro-survival 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 anti-apoptotic 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 Cp of PCPH- and mt-PCPH-expressing PC-3 cells agree with previous reports on the pro-survival function of PCPH and mt-PCPH that assigned a greater anti-apoptotic 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 chemo-response of primary mouse embryo fibroblasts (Tirado *et al*., unpublished data). 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 pro-survival 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 mTOR pathway (8) while modulating a different pathway to promote resistance to chemotherapeutic drugs (Tirado *et al*., unpublished data) 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 appeared to be able to functionally interact with two different PKC isoforms in PCa cells (through PKC-δ, to modulate their

invasiveness (16), and through PKC-α, to regulate their chemo-response), and that invasiveness and chemo-resistance 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BPH, benign prostatic hyperplasia; Cp, cisplatin; PBS, phosphate-buffered saline; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia; Sc, sequence scrambled control; PKC, protein kinase C.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics. CA Cancer J Clin 2007;57:43–6. [PubMed: 17237035]
2. Oh WK, Tay MH, Huang J. Is there a role for platinum chemotherapy in the treatment of patients with hormone-refractory prostate cancer? Cancer 2007;109:477–86. [PubMed: 17186531]
3. DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. Pathological and molecular aspects of prostate cancer. Lancet 2003;361:955–64. [PubMed: 12648986]
4. Velasco JA, Zimonjic DB, Popescu NC, et al. Tissue-specific expression, evolutionary conservation and localization of the *cph* proto-oncogene on Syrian hamster chromosome X. Oncogene 1996;12:2713–17. [PubMed: 8700531]
5. Velasco JA, Castro R, Avila MA, et al. *cph*, a novel oncogene which cooperates with H-ras in the transformation of NIH3T3 fibroblasts. Oncogene 1994;9:2065–69. [PubMed: 8208552]
6. Velasco JA, Avila MA, Notario V. The product of the cph oncogene is a truncated, nucleotide-binding protein that enhances cellular survival to stress. Oncogene 1999;18:689–701. [PubMed: 9989819]
7. Recio JA, Paez JG, Maskeri B, Loveland M, Velasco JA, Notario V. Both normal and transforming PCPH proteins have guanosine diphosphatase activity but only the oncoprotein cooperates with Ras in activating extracellular signal-regulated kinase ERK1. Cancer Res 2000;60:1720–28. [PubMed: 10749145]
8. Tirado OM, Mateo-Lozano S, Sanders S, Dettin LE, Notario V. The PCPH oncoprotein antagonizes the proapoptotic role of the mammalian target of rapamycin in the response of normal fibroblasts to ionizing radiation. Cancer Res 2003;63:6290–98. [PubMed: 14559816]
9. Paez JG, Recio JA, Rouzaut A, Notario V. Identity between the PCPH proto-oncogene and the CD39L4 (ENTPD5) ectonucleoside triphosphate diphosphohydrolase gene. Int J Oncol 2001;19:1249–54. [PubMed: 11713596]
10. Recio JA, Paez JG, Sanders S, Kawakami T, Notario V. Partial depletion of intracellular ATP mediates the stress-survival function of the PCPH oncoprotein. Cancer Res 2002;62:2690–94. [PubMed: 11980669]

11. Solanas M, Escrich E, Rouzaut A, Costa I, Martínez A, Notario V. Deregulated expression of the *PCPH* proto-oncogene in rat mammary tumors induced with 7,12-dimethylbenz[a] anthracene. Mol Carcinog 2002;33:219–27. [PubMed: 11933075]
12. Rouzaut A, Recio JA, Notario V. Expression of the protein product of the PCPH proto-oncogene in human tumor cell lines. Radiat Res 2001;155:181–7. [PubMed: 11121232]
13. Blanquez MJ, Regadera J, Mariño J, Newman RE, Notario V. Gradual deregulation and loss of PCPH expression in the progression of human laryngeal neoplasia. Mol Carcinog 2002;35:186–95. [PubMed: 12489110]
14. Blanquez MJ, Arenas MI, Conde I, Tirado OM, Paniagua R, Notario V. Deregulated expression of the PCPH proto-oncogene in human breast cancers. Int J Oncol 2004;25:821–30. [PubMed: 15375529]
15. Regadera J, Blanquez MJ, Gonzalez-Peramato P, et al. PCPH expression is an early event in the development of testicular germ cell tumors. Int J Oncol 2006;28:595–604. [PubMed: 16465363]
16. Villar J, Arenas MI, Maccarthy CM, Blanquez MJ, Tirado OM, Notario V. PCPH/ENTPD5 expression enhances the invasiveness of human prostate cancer cells by a protein kinase Cδ dependent mechanism. Cancer Res 2007;67:10859–68. [PubMed: 18006831]
17. McKenzie S, Kyprianou N. Apoptosis evasion: the role of survival pathways in prostate cancer progression and therapeutic resistance. J Cell Biochem 2006;97:18–32. [PubMed: 16216007]
18. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–56. [PubMed: 12209154]
19. Kutuk O, Letai A. Regulation of Bcl-2 family proteins by posttranslational modifications. Curr Mol Med 2008;8:102–18. [PubMed: 18336291]
20. Breitschopf K, Haendeler J, Malchow P, Zeiher AM, Dimmeler S. Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. Mol Cell Biol 2000;20:1886–96. [PubMed: 10669763]
21. Ruvolo PP, Deng X, May WS. Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia 2001;15:515–22. [PubMed: 11368354]
22. Basu A, Haldar S. Signal-induced site specific phosphorylation targets Bcl2 to the proteasome pathway. Int J Oncol 2002;21:597–601. [PubMed: 12168105]
23. Zhao M, Eaton JW, Brunk UT. Bcl-2 phosphorylation is required for inhibition of oxidative stress-induced lysosomal leak and ensuing apoptosis. FEBS Lett 2001;509:405–12. [PubMed: 11749964]
24. Jiffar T, Kurinna S, Suck G, et al. PKC alpha mediates chemoresistance in acute lymphoblastic leukemia through effects on Bcl2 phosphorylation. Leukemia 2004;18:505–12. [PubMed: 14737078]
25. Choy H, Park C, Yao M. Current status and future prospects for satraplatin, an oral platinum analogue. Clin Cancer Res 2008;14:1633–38. [PubMed: 18347164]
26. Yoshida K. PKCdelta signaling: mechanisms of DNA damage response and apoptosis. Cell Signal 2007;19:892–901. [PubMed: 17336499]
27. Okhrimenko H, Lu W, Xiang C, et al. Roles of tyrosine phosphorylation and cleavage of protein kinase Cdelta in its protective effect against tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis. J Biol Chem 2005;280:23643–52. [PubMed: 15774464]
28. Spitaler M, Wiesenhofer B, Biedermann V, et al. The involvement of protein kinase C isoenzymes alpha, epsilon and zeta in the sensitivity to antitumor treatment and apoptosis induction. Anticancer Res 1999;19:3969–76. [PubMed: 10628339]
29. Tanaka M, Sagawa S, Hoshi J, et al. Synthesis of anilino-monoindolylmaleimides as potent and selective PKCbeta inhibitors. Bioorg Med Chem Lett 2004;14:5171–4. [PubMed: 15380221]
30. Basu A, Woolard MD, Johnson CL. Involvement of protein kinase C-delta in DNA damage-induced apoptosis. Cell Death Differ 2001;8:899–908. [PubMed: 11526445]
31. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer 2007;7:281–94. [PubMed: 17384583]
32. Keranen LM, Dutil EM, Newton AC. Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. Curr Biol 1995;5:1394–1403. [PubMed: 8749392]
33. Kitatani K, Idkowiak-Baldys J, Hannun YA. Mechanism of inhibition of sequestration of protein kinase C alpha/betaII by ceramide. Roles of ceramide-activated protein phosphatases and

phosphorylation/dephosphorylation of protein kinase C alpha/betaII on threonine 638/641. J Biol Chem 2007;282:20647–56. [PubMed: 17504762]

34. Cho HJ, Kim JK, Kim KD, et al. Upregulation of Bcl-2 is associated with cisplatin-resistance via inhibition of Bax translocation in human bladder cancer cells. Cancer Lett 2006;237:56–66. [PubMed: 16009487]
35. Wang L, Chanvorachote P, Toledo D, et al. Peroxide is a key mediator of Bcl-2 downregulation and apoptosis induction by cisplatin in human lung cancer cells. Mol Pharmacol 2007;73:119–27. [PubMed: 17911532]
36. Aaltonen V, Koivunen J, Laato M, Peltonen J. PKC inhibitor Go6976 induces mitosis and enhances doxorubicin-paclitaxel cytotoxicity in urinary bladder carcinoma cells. Cancer Lett 2007;253:97–107. [PubMed: 17320279]
37. Dempsey EC, Newton AC, Mochly-Rosen D, et al. Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 2000;279:L429–38. [PubMed: 10956616]
38. Musashi M, Ota S, Shiroshita N. The role of protein kinase C isoforms in cell proliferation and apoptosis. Int J Hematol 2000;72(1):12–9. [PubMed: 10979203]
39. Collazos A, Diouf B, Guérineau NC, et al. A spatiotemporally coordinated cascade of protein kinase C activation controls isoform-selective translocation. Mol Cell Biol 2006;26:2247–61. [PubMed: 16508001]
40. Gavrielides MV, Frijhoff AF, Conti CJ, Kazanietz MG. Protein kinase C and prostate carcinogenesis: targeting the cell cycle and apoptotic mechanisms. Curr Drug Targets 2004;5:431–43. [PubMed: 15216909]
41. Yin L, Bennani-Baiti N, Powell CT. Phorbol ester-induced apoptosis of C4-2 cells requires both a unique and a redundant protein kinase C signaling pathway. J Biol Chem 2005;280:5533–41. [PubMed: 15611126]
42. Gonzalez-Guerrico AM, Meshki J, Xiao L, Benavides F, Conti CJ, Kazanietz MG. Molecular mechanisms of protein kinase C-induced apoptosis in prostate cancer cells. J Biochem Mol Biol 2005;38:639–45. [PubMed: 16336777]
43. Brodie C, Blumberg PM. Regulation of cell apoptosis by protein kinase c delta. Apoptosis 2003;8:19–27. [PubMed: 12510148]
44. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal 2007;19:1633–42. [PubMed: 17537614]
45. Cornford P, Evans J, Dodson A, et al. Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. Am J Pathol 1999;154:137–44. [PubMed: 9916928]
46. Mohanty S, Huang J, Basu A. Enhancement of cisplatin sensitivity of cisplatin-resistant human cervical carcinoma cells by bryostatin 1. Clin Cancer Res 2005;11:6730–7. [PubMed: 16166454]
47. Albihn A, Lovén J, Ohlsson J, Osorio LM, Henriksson M. c-Myc-dependent etoposide-induced apoptosis involves activation of Bax and caspases, and PKCdelta signaling. J Cell Biochem 2006;98:1597–614. [PubMed: 16572399]
48. Orlandi L, Binda M, Folini M, et al. Ribozyme-mediated inhibition of PKCalpha sensitizes androgen-independent human prostate cancer cells to cisplatin-induced apoptosis. Prostate 2003;54:133–43. [PubMed: 12497586]
49. Basu A, DuBois G, Haldar S. Posttranslational modifications of Bcl2 family members--a potential therapeutic target for human malignancy. Front Biosci 2006;11:1508–21. [PubMed: 16368533]
50. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007;26:1324–37. [PubMed: 17322918]

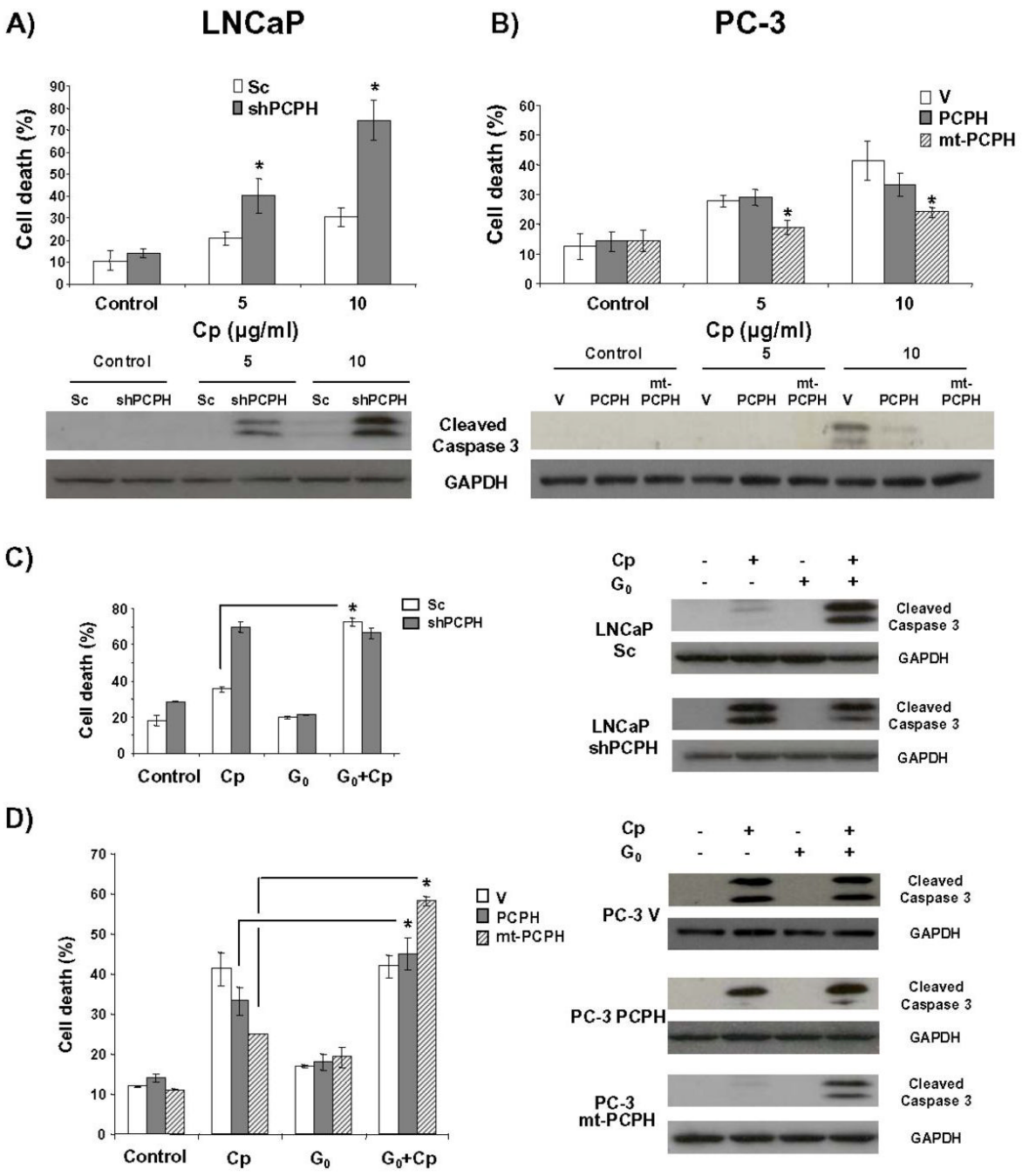

Figure 1. PCPH expression confers resistance to cisplatin-induced apoptosis in prostate cancer cell lines

Exponentially growing cultures of LNCaP cells (**A**) expressing shPCPH or scrambled (Sc) control shRNA and PC-3 cells (**B**) expressing empty vector (V), PCPH or mt-PCPH were treated with cisplatin (Cp), at the indicated concentrations. LNCaP cells (**C**) expressing shPCPH or scrambled (Sc) shRNA and PC-3 cells (**D**) expressing empty vector (V), PCPH or mt-PCPH were treated with 3 nM Gö6976 (α) 45 min before the treatment with 10 μg/ml cisplatin (Cp). 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 panels; C and D, right panels) was detected after the indicated treatments by western blot using

an antibody specific for active cleaved caspase 3. GAPDH was used as loading control in all cases. Experiments were replicated at least three times.

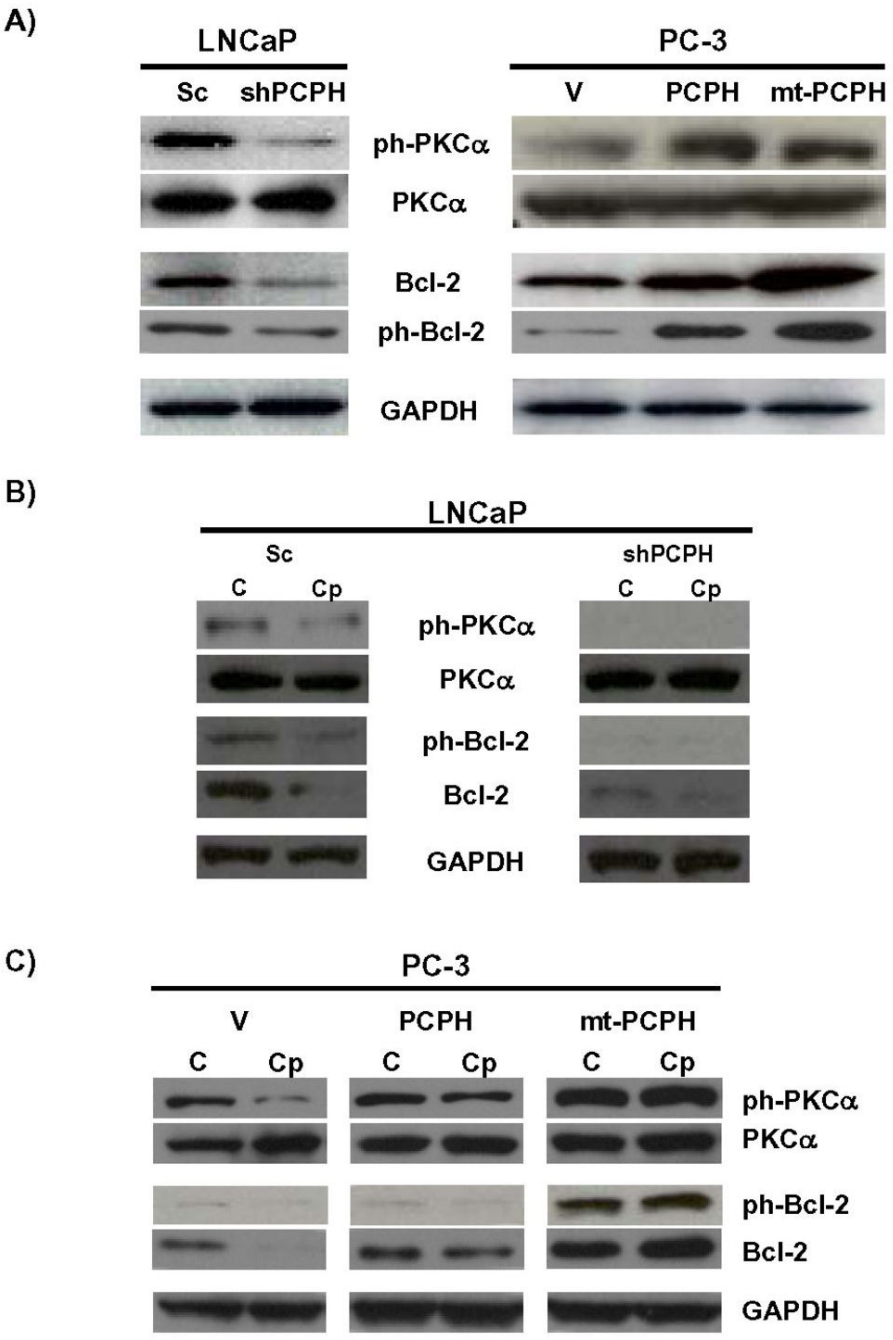

Figure 2. PCPH expression regulates PKCα phosphorylation and Blc-2 expression levels and phosphorylation in prostate cancer cell lines

(**A**) Western blot analysis of extracts from LNCaP cells (left panels) expressing shPCPH or scrambled (Sc) shRNA showing that PCPH knockdown decreases the phosphorylation of PKCα (top) and the expression levels and phosphorylation of Bcl-2 (bottom). Western blot analysis of extracts from PC-3 cells (right panels) expressing empty vector (V), PCPH or mt-PCPH showing that PCPH and mt-PCPH expression increase the levels of phosphorylated PKCα as well as the expression levels and phosphorylation of Bcl-2. (**B**) Immunoblot analysis showing that, relative to untreated control (C) cultures, treatment of LNCaP cells expressing shPCPH (right) or control (Sc) shRNAs with cisplatin (Cp), at 10 μg/ml, for 24 h, decreased

the levels of phosphorylated PKCα (ph-PKCα**)**, phosphorylated Bcl-2 (ph-Bcl-2) and total Bcl-2 protein, but did not change total PKCα protein levels. (**C**) Western blot analysis showing that expression of mt-PCPH in PC-3 cells prevents, and expression of PCPH minimizes, the down-regulation of phosphorylated PKCα (ph-PKCα**)**, phosphorylated Bcl-2 (ph-Bcl-2) and total Bcl-2 protein produced by cisplatin (Cp) treatment, but did not change total PKCα protein levels relative to untreated controls (C). GAPDH was used as loading control in all cases. All experiments were repeated at least three times.

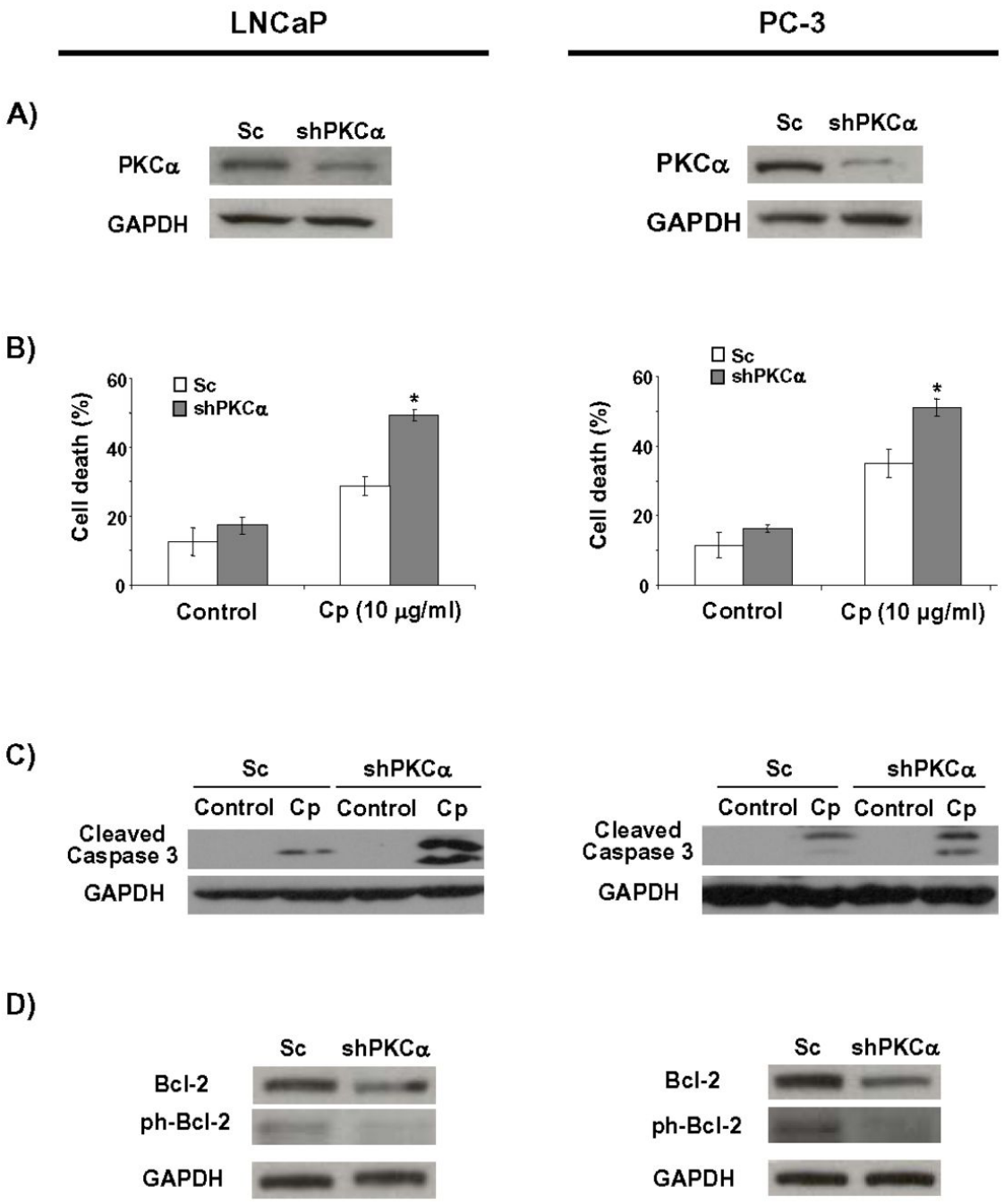

Figure 3. PKCα knockdown decreased the levels of Bcl-2, sensitizing prostate cancer cells to cisplatin-induced apoptosis

LNCaP (left panels) and PC-3 cells (right panels) were stably transfected with shPKCα or scrambled (Sc) shRNA. PKCα knockdown was ascertained by western (**A**), and cells expressing decreased PKCα levels were treated with cisplatin (Cp), at 10 μg/ml, for 24 h. Then, viable and dead cells 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 (ph-Bcl-2) were determined by western blot analysis with specific antibodies (**D**). GAPDH was used as loading control in all cases.

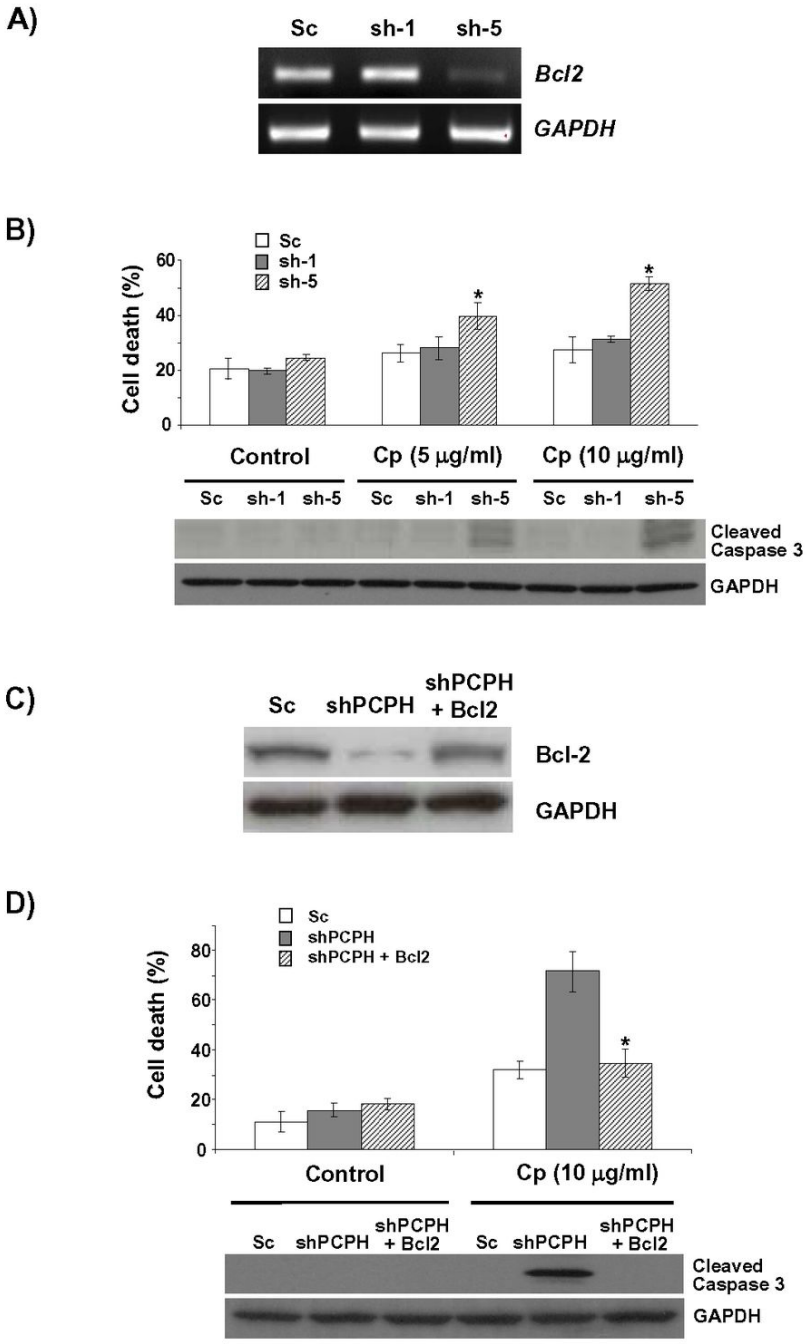

Figure 4. Bcl-2 expression confers resistance to cisplatin-induced apoptosis in LNCaP cells

LNCaP cells were infected with lentiviral particles generated with shRNA constructs shBcl2-1 (sh-1) or shBcl2-5 (sh-5), to target Bcl-2, or with a sequence scrambled (Sc) shRNA, as described under material and methods. Bcl-2 knockdown was ascertained by RT-PCR (**A**) using GAPDH expression levels as reference. Exponentially growing cultures of the cell lines shown were treated with cisplatin (Cp), at the indicated concentrations, for 24 h. Then, viable and dead cell counts were determined using the trypan blue exclusion assay (**B**, upper panel). Columns mean; bars SD. *, P <0.05. Caspase 3 activation (**B**, bottom panel) was determined as described in previous figure legends. The role of Bcl-2 in the process was studies by transfecting PCPH knockdown LNCaP cells with a Bcl-2 expression construct. Bcl-2 re-

expression was confirmed by western blot analysis of extracts from LNCaP cells expressing scrambled (Sc), shPCPH, or shPCPH + Bcl-2 (**C**), and the sensitivity of these cells to exposure to Cp (10μg/ml) for 24h was established by determining live and dead cell counts using the Trypan blue exclusion test (**D**, upper panel) and caspase 3 activation (**D**, bottom panel). GAPDH was used as loading control in all cases. All experiments were repeated at least three times.

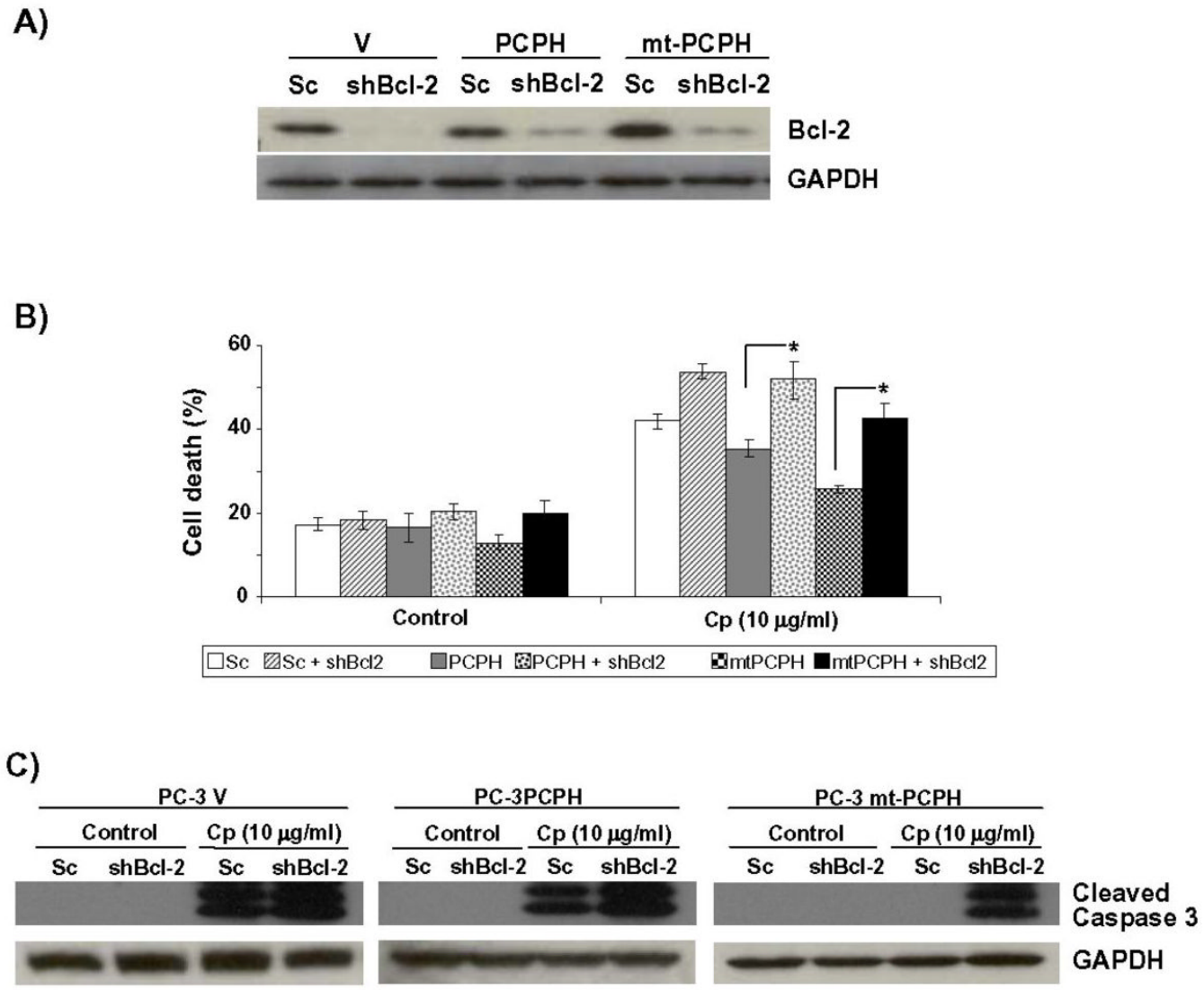

Figure 5. Bcl-2 down-regulation reverts the antiapoptotic effect of mt-PCPH overexpression

A, PC-3 cells expressing empty vector (V), PCPH, or mt-PCPH were infected with shBcl2 or scrambled (Sc) shRNA. Levels of Bcl-2 protein were measured by western blot (**A**). Cells were treated with 10µg/ml cisplatin (Cp) and the levels of death (**B**) were determined using Trypan blue exclusion assay. Columns, mean; bars SD. *, P <0.05. Experiments were carried out at least three times. Caspase 3 activation was measured by western blot (**C**), using levels of GAPDH as loading control.

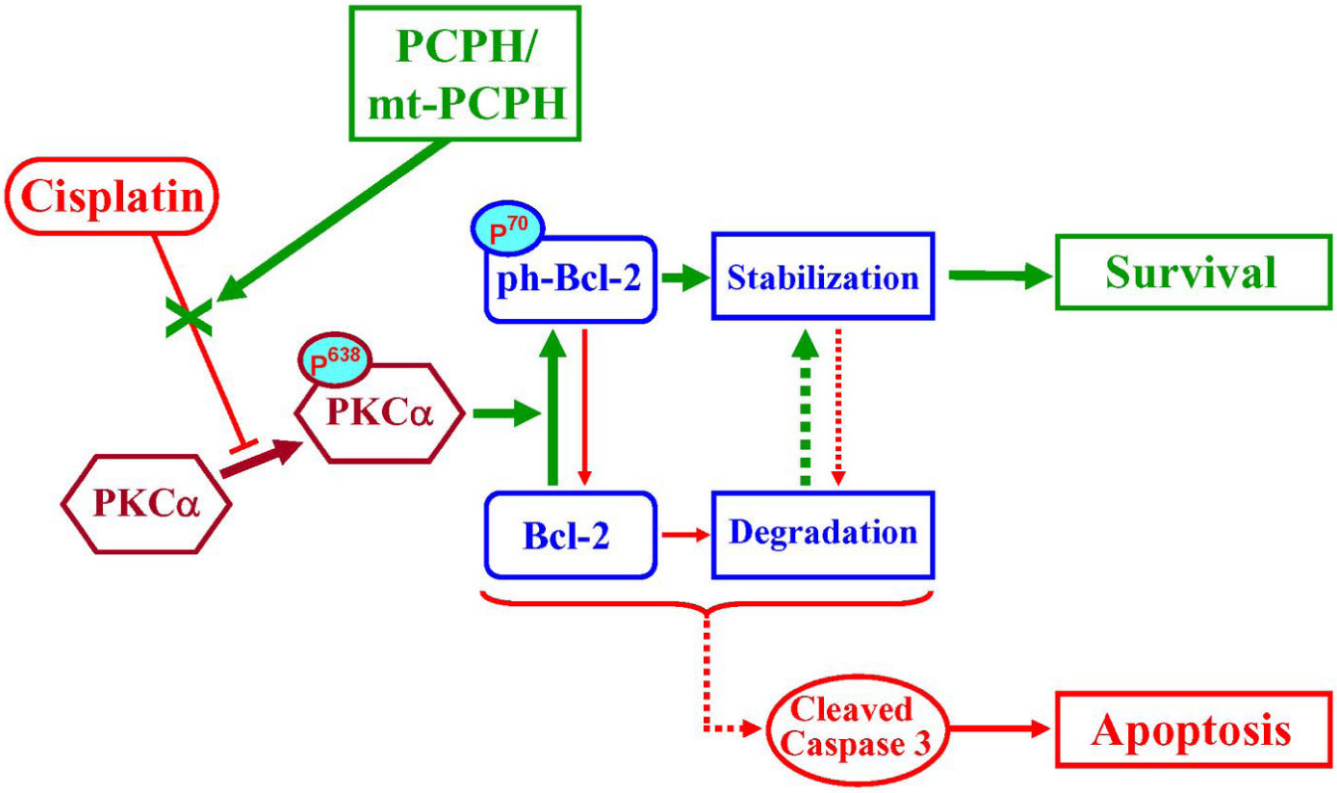

Figure 6. Model proposed for the mechanism of promotion of resistance to cisplatin-induced apoptosis by PCPH and mt-PCPH

PKCα phosphorylates Bcl-2 at serine 70, 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 pro-apoptotic 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.