

## Crucial Roles for Protein Kinase C Isoforms in Tumor-Specific Killing by Apoptin

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### Abstract

The chicken anemia virus–derived protein apoptin induces apoptosis in a variety of human malignant and transformed cells but not in normal cells. However, the mechanisms through which apoptin achieves its selective killing effects are not well understood. We developed a lentiviral vector encoding a green fluorescent protein–apoptin fusion gene (LV-GFP-AP) that can efficiently deliver apoptin into hematopoietic cells. Apoptin selectively killed the human multiple myeloma cell lines MM1.R and MM1.S, and the leukemia cell lines K562, HL60, U937, KG1, and NB4. In contrast, normal CD34<sup>+</sup> cells were not killed and maintained their differentiation potential in multilineage colony formation assays. In addition, dexamethasone-resistant MM1.R cells were found to be more susceptible to apoptin-induced cell death than the parental matched MM1.S cells. Death susceptibility correlated with increased phosphorylation and activation of the apoptin protein in MM1.R cells. Expression array profiling identified differential kinase profiles between MM1.R and MM1.S cells. Among these kinases, protein kinase C $\beta$  (PKC $\beta$ ) was found by immunoprecipitation and *in vitro* kinase studies to be a candidate kinase responsible for apoptin phosphorylation. Indeed, shRNA knockdown or drug-mediated inhibition of PKC $\beta$  significantly reduced apoptin phosphorylation. Furthermore, apoptin-mediated cell death proceeded through the upregulation of PKC $\beta$ , activation of caspase-9/3, cleavage of the PKC $\delta$  catalytic domain, and downregulation of the MERTK and AKT kinases. Collectively, these results elucidate a novel pathway for apoptin activation involving PKC $\beta$  and PKC $\delta$ . Further, they highlight the potential of apoptin and its cellular regulators to purge bone marrow used in autologous transplantation for multiple myeloma. *Cancer Res*; 70(18); 7242–52. ©2010 AACR.

### Introduction

Stem cell rescue following high-dose cytotoxic chemotherapy is considered curative and has gained extensive application worldwide as a therapeutic modality in several hematologic malignancies (1–4). Allogeneic bone marrow transplant is the preferred choice for most types of leukemias, but due to the rarity of HLA-compatible donors, autologous bone marrow transplantation is still an alternative therapeutic option. However, disease relapse remains a primary cause of death, partially due to inefficient elimination of contaminated clonogenic tumor cells from the autografts. Multiple pharmacologic and immunologic approaches aiming at the elimination of

leukemic cells have been developed; however, to date, these purging methods have shown only limited efficacy, restricting their clinical applications (5, 6). In addition, several gene therapy–based trials have been conducted for the selective removal of contaminating epithelial cancer cells from autografts. However, these strategies have been shown to be inefficient for purging of leukemia cells mainly due to the lack of efficient gene delivery into hematologic malignant cells (7, 8).

Apoptin, a chicken anemia virus–derived protein, has been shown to possess tumor-specific cytotoxicity (9, 10). Its expression induces apoptosis in human tumor and transformed cells, but there is little or no cytotoxic effect in many normal human cell lines derived from different tissues, including peripheral blood mononuclear cells, fibroblasts, and epithelial cells (11–13). Several studies have shown that the tumor-specific killing of apoptin correlates with its phosphorylation and its subcellular localization (14, 15). In cancer cells, apoptin is localized in the nucleus and is phosphorylated on Thr-108 by an as yet unknown kinase (16, 17), whereas in normal cells apoptin is detected in the cytoplasm and is essentially unphosphorylated. Recent studies have reported that apoptin interacts with the p85 Src homology 3 (SH3) domain of phosphatidylinositol 3'-kinase (PI3K) and protein kinase B (AKT) in MCF7 breast and PC3 prostate cancer cell lines. The activation of the PI3K/AKT pathway by apoptin results in the induction of the cyclin-dependent kinase CDK2, in turn leading to the phosphorylation of apoptin (18, 19).

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In contrast, we have found no correlation between AKT activity in human cancer cell lines and their sensitivities to apoptin killing.<sup>3</sup>

In this study, we used a matched pair of multiple myeloma cell lines (MM1.R and MM1.S) that show resistance (R) or sensitivity (S) to dexamethasone, with MM1.R being derived from MM1.S through continuous growth in dexamethasone. These cell lines showed a marked difference in sensitivity to apoptin-induced killing, with MM1.R being clearly more sensitive than MM1.S. Microarray expression profiling of these cell lines showed that several kinases, including members of the protein kinase C (PKC) family, are overexpressed in the apoptin-sensitive cell line MM1.R compared with MM1.S cells (see Supplementary Table S1).

PKC is a family of serine-threonine kinases, composed of at least 13 known isoforms with a wide range of tissue distribution, subcellular localization, and function. All PKC isoforms contain a highly conserved COOH-terminal catalytic domain and NH<sub>3</sub>-terminal regulatory domain with an autoinhibitory pseudosubstrate sequence (20–22). It has been well established that the activation of the classic PKC isoforms ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2, and  $\gamma$ ) requires Ca<sup>2+</sup> and a phospholipid, such as diacylglycerol (DAG) or phosphatidylserine (PS). These PKC isoforms also contain two cysteine-rich motifs within their NH<sub>2</sub>-terminal regulatory domain that facilitate phorbol ester (TPA) interaction and subsequent activation. The novel isoforms ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\mu$ ,  $\theta$ ) are also activated by DAG and phorbol esters; however, they lack a complete C2 domain responsible for Ca<sup>2+</sup> interaction and are therefore Ca<sup>2+</sup> independent. The protein structures of atypical PKC isoforms ( $\zeta$ ,  $\iota$ / $\lambda$ ) differ from the other members of the PKC family, missing both binding regions for Ca<sup>2+</sup> and DAG or phorbol esters. Activation of atypical isoforms is dependent on other phospholipids, such as PS, inositol lipids, and phosphatidic acid.

Most PKC isoforms are present in the cytosol in nonstimulated cells. Upon stimulation, several PKC isoforms migrate to the plasma membrane where interaction with DAG induces full activation. A number of activated PKC isoforms can also translocate to the nucleus where they can be targeted by lipid coactivators or other activated protein kinases (23). Furthermore, some PKC isoforms such as PKC $\delta$  can be activated by caspase-3 cleavage to release an active catalytic domain from the inhibitory NH<sub>2</sub>-terminal regulatory domain.

Increased level of PKC or differential activation of PKC isoforms has been linked to a variety of cancers, including breast, lung, thyroid, and adenomatous pituitary cancers as well as leukemias (24, 25). There is emerging evidence that PKCs play key roles in the regulation of cell growth, apoptosis, and differentiation of hematopoietic cells. Studies involving small interfering RNA knockdown and genetic disruption of individual PKC isoforms in mice have shown that PKC $\alpha$ ,  $\beta$ ,  $\lambda$ ,  $\epsilon$ , and  $\zeta$  preferentially function to promote cell proliferation and survival, whereas PKC $\delta$  is a critical proapoptotic kinase in many cell types (26).

To evaluate apoptin tumor-specific toxicity in hematologic malignancies, we developed a lentiviral vector encoding a green fluorescent protein–apoptin fusion gene (LV-GFP-AP) that can be efficiently delivered into hematopoietic cells. This strategy enabled us to identify novel apoptin-interacting cellular targets. Here, we provide strong evidence for the role of PKC family kinases, particularly PKC $\beta$  variants, in apoptin phosphorylation and consequently its tumor-specific cytotoxicity.

## Materials and Methods

Additional details about the materials and methods can be found in the Supplementary Data.

### Cell lines

MM1.S (dexamethasone-sensitive) and MM1.R (dexamethasone-resistant) cell lines were obtained from Dr. Tai (Dana-Farber, Boston, MA). K562 (*BCR/ABL* cytogenetics), HL60 (TPA, retinoic acid differentiation), U937 (TPA differentiation), and KG1 (monosomy 7, trisomy 8 cytogenetics) leukemic cells were obtained from the American Type Culture Collection. NB4 (t15,17 translocation) was obtained from German Collection of Microorganisms and Cell Cultures. HCT116 (*KRAS* mutation analysis) was obtained from Prof. Vogelstein (Johns Hopkins, Baltimore, MD). All cell lines were obtained since 2006 and were tested for cell line-specific defects before batch freezing (see above for specific assays). Cells are regularly tested to ensure the absence of *Mycoplasma* contamination, and cell morphology is regularly checked to ensure the absence of cross-contamination of cell lines.

### Construction of lentiviral vector LV-GFP-AP, lentivirus production, and titration

LV-GFP-AP was constructed by cloning GFP-apoptin derived from pCMV-GFP-AP (13) into the LV-GFP lentiviral construct (27) through replacement of GFP by GFP-AP by blunt end ligation (see Supplementary Fig. S1A). Lentiviral vectors were produced by cotransfection of 293T cells with the second-generation packaging plasmid pCMV $\Delta$ 8.91 and plasmid pMDG encoding VSV-G–pseudotyped envelope (27). The titer of lentivirus was determined by quantification of viral core protein p24 through enzyme-linked immunosorbent assay (ELISA) using a HIV-1 p24 capture assay kit (Perkin-Elmer). The values were normalized against a recombinant p24 protein standard. Virus numbers were then calculated based on the fact that a viral particle contains 2,000 p24 molecules (28, 29). Comparable virus titers, based on p24 ELISA, were achieved for LV-GFP and LV-GFP-AP (Supplementary Table S2).

### MTT proliferation

Leukemia cell lines were infected with LV-GFP or LV-GFP-AP in the presence of 4  $\mu$ g/mL polybrene. Cells were seeded at  $5 \times 10^4$ ,  $10^4$ , and  $10^3$  per well in 96-well plates for MTT assays. On days 2, 4, 6, 8, 10, 12, and 14 postinfection, 20  $\mu$ L of 5 mg/mL MTT in PBS were added to each well and samples were incubated for 2 to 4 hours. MTT solubilization solution (100  $\mu$ L) was then added, and samples were incubated overnight. The optical density (OD) was measured, and the OD values

<sup>3</sup> Unpublished data.

were converted into percentages of the control absorbance. The average values were obtained from triplicates.

#### Flow cytometric analysis

Apoptosis was assessed by propidium iodide (PI) staining. Briefly, cells were pelleted and resuspended in PBS with 20  $\mu\text{g}/\text{mL}$  of PI. The settings for fluorescence-activated cell sorting (FACS) analysis were based on unstained parental, GFP-transduced, and PI-stained control samples. The cells were analyzed on a BD FACSCanto II (Becton Dickinson), and 10,000 events were acquired per sample. Fluorescence data were analyzed by FlowJo software.

#### Methylcellulose colony-forming assay

Normal human bone marrow cells were obtained from volunteers undergoing open heart surgery at King's College Hospital (Ethics Committee number 05-03-125).  $\text{CD34}^+$  cells were isolated using anti- $\text{CD34}^+$  microbeads, and an AutoMacs fractionation device (Miltenyi Biotec) according to the manufacturer's instructions.  $\text{CD34}^+$  cells were cultured in Stemspan serum-free medium with cytokine cocktail (StemCell Technologies) for 48 hours. Mobilized  $\text{CD34}^+$  cells were infected with LV-GFP or LV-GFP-AP at a multiplicity of infection (MOI) of 100. Two days postinfection, infected  $\text{CD34}^+$  cells were analyzed for their progenitor function by an *in vitro* colony-forming assay (StemCell Technologies).

#### Purification of maltose-binding protein–apoptin fusion protein

Apoptin cDNA was cloned into pMALc2 (NEB) in frame with the maltose-binding protein (MBP), and protein was purified from BL21 codon plus *E. coli* cells (Stratagene) using an Amylose Resin kit (NEB) according to the manufacturer's recommendations.

#### Immunoprecipitation and Western blotting

For immunoprecipitation, cells were lysed in radioimmuno-precipitation assay buffer on ice with freshly added protease inhibitor cocktail (Sigma). The cell debris was removed, and 2  $\mu\text{g}$  of anti-GFP (Abcam) or anti-PKC $\beta$ 1 antibody (Santa Cruz Biotechnology) were added to the cell lysate for 30 minutes at room temperature under constant agitation. Twenty microliters of washed Bio-Adebeads PAG (Ademtech) were added to the cell lysate with antibody for 2 to 3 hours at 4°C, and complexes were pulled down using a magnet rack.

Proteins from different cell fractions were isolated using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas) according to the manufacturer's protocol.

For Western blotting, the proteins were separated on SDS-PAGE gels, electroblotted onto nitrocellulose membrane (GE Healthcare), and blocked with 5% nonfat dried milk for 1 hour at room temperature. The membrane was incubated with a specific primary antibody and a horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescent (ECL) detection system and exposed to ECL film (both from Amersham Biosciences). The blot was stripped with ReBlot plus strong antibody stripping buffer (Millipore) and reprobed with a different antibody as required.

Antibodies against Thr-108-phosphorylated apoptin or total apoptin were raised in rabbit against peptides H2N-SLITTT(PO3H2)PSRPRTA-CONH2 and H2N-SLITTTPSRPRTA-CONH2, respectively (Eurogentec). All antibodies used in the study were purchased from Santa Cruz Biotechnology except antibodies against MERTK, DGKH, and GFP for immunoprecipitation, which were purchased from Abcam. Antibodies against phospho-AKT-Ser-473, phospho-PKC $\beta$ 2-Thr-638, total AKT, PI3K/p85, caspase-3, caspase-9, and GFP for Western blotting were purchased from Cell Signaling.

#### *In vitro* kinase assay

Two micrograms of MBP-apoptin fusion protein were added to 1 $\times$  kinase buffer (Cell Signaling) containing 200  $\mu\text{mol}/\text{L}$  ATP and 0.2 ng of PKC $\beta$ 2 kinase (Cell Signaling) or PKC $\beta$ 1 pulled down from MM1.R cell lysate. The reaction mixture was incubated for 30 minutes at room temperature. Reactions were carried out under different conditions, with kinase or without kinase, with PKC $\beta$ -specific inhibitor (Calbiochem) or AKT-specific inhibitor (Calbiochem). The reaction was stopped by adding 2 $\times$  loading buffer and boiling for 5 minutes.

#### Construction of plasmids encoding PKC $\beta$ catalytic domains and transfection of 293T

The catalytic domains of PKC $\beta$ 1 and PKC $\beta$ 2 were PCR amplified from cDNA derived from MM1.R cells and cloned into the retroviral vector pBabePuro. The forward primer introduces a start codon in a Kozak context. The sequences were verified, and the expression of the catalytic domain was verified by Western blotting of transfected cells with PKC $\beta$ 1- and PKC $\beta$ 2-specific antibodies (Santa Cruz Biotechnology).

#### Immunohistochemistry and visualization by fluorescence microscopy

HCT116-p53 $^{-/-}$  or MM1.R cells ( $5 \times 10^4$ ) were infected with a lentiviral vector. At day 2 or 5, cells were fixed, permeabilized, and incubated with antibody against PKC $\beta$ 1 for 1 hour and Texas red IgG secondary antibody for 1 hour. The cells were washed and covered with 4',6-diamidino-2-phenylindole mounting medium (Vector Laboratories) and visualized.

#### Statistical analysis

Student's *t* test was used to determine significance.

## Results

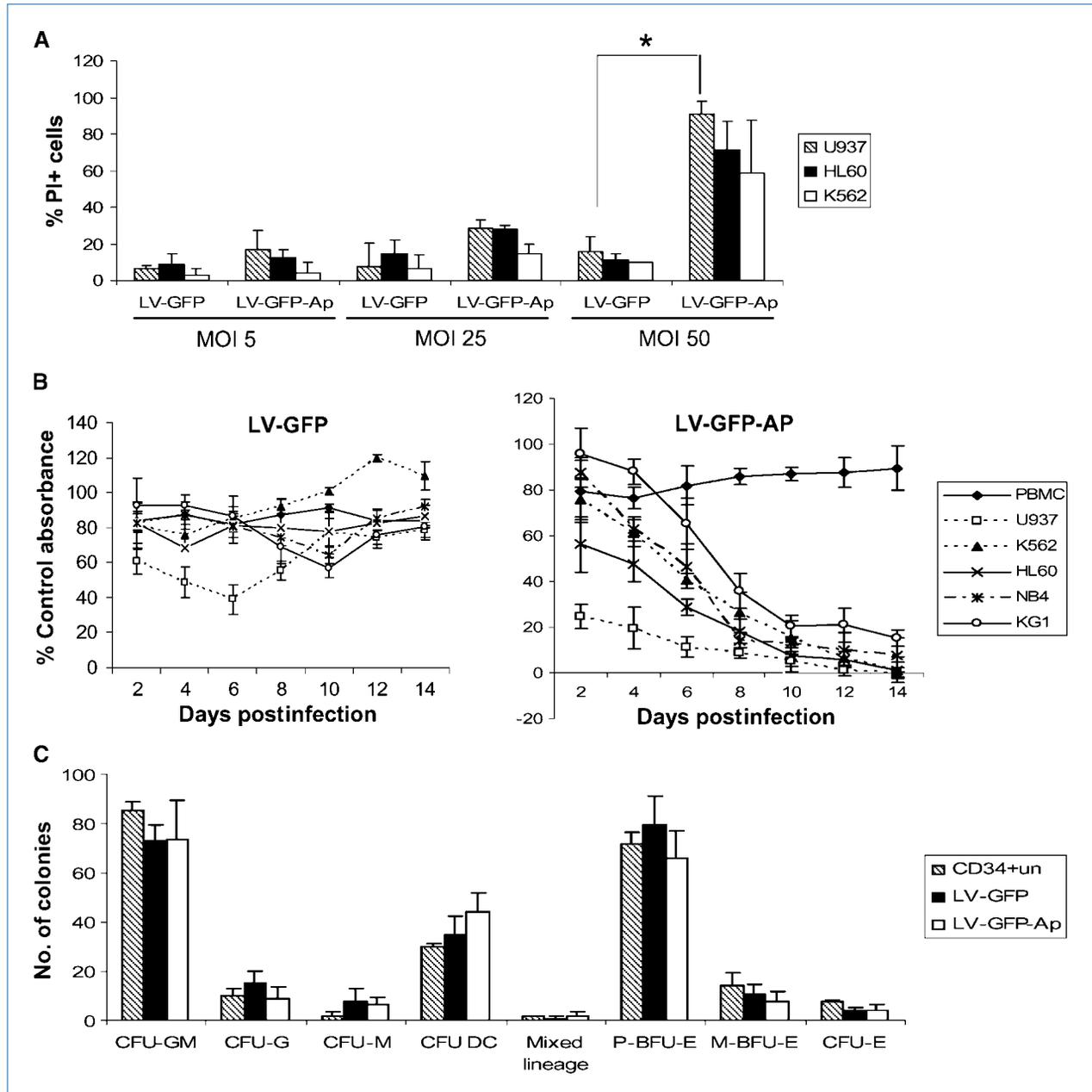
#### Apoptin induces tumor-specific killing of leukemia cells

A panel of leukemia cell lines as well as primary  $\text{CD34}^+$  and PBMC cells were infected with LV-GFP or LV-GFP-AP lentiviral vectors. In general, the leukemia cell lines were found to be more sensitive to lentiviral infection than normal cells. In particular, multiple myeloma MM1.R and MM1.S cells were highly sensitive to infection, requiring only a MOI of 2 to achieve 100% transduction (Supplementary Fig. S2A; MM1.S cells have a comparable infectivity to the parental matched MM1.R cells; data not shown).  $\text{CD34}^+$  cells were less sensitive, requiring a higher MOI of 100 to obtain 50% to 80% transduction efficiency. Furthermore, higher expression levels of

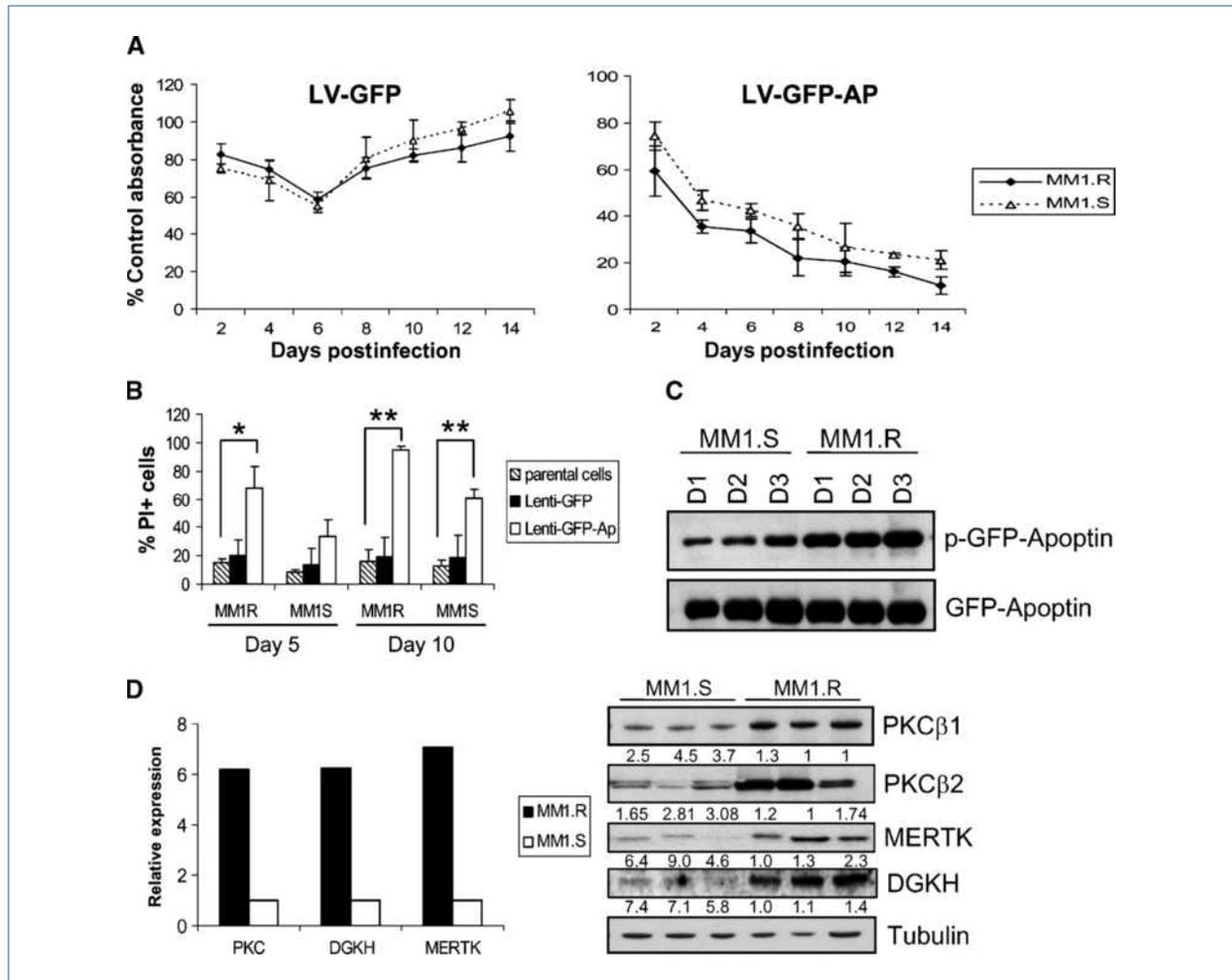
the transgene were observed in the leukemic cell lines compared with primary CD34<sup>+</sup> cells or PBMCs (Supplementary Fig. S2B and C).

To examine apoptin-mediated cell death,  $1 \times 10^5$  cells were infected with equal MOIs for LV-GFP and LV-GFP-AP virus. After 5 days, cell death was determined by FACS analysis of PI-positive cells. As shown in Fig. 1A, GFP-apoptin triggered

apoptosis in transduced leukemia cells in a dose-dependent manner, whereas no cell death was observed in untransduced or GFP-transduced parental cells (Fig. 1A). Furthermore, MTT cell survival assay showed more than 90% cell death in a panel of leukemia cell lines infected with LV-GFP-AP, whereas no significant killing was observed with LV-GFP-infected and uninfected parental cells (Fig. 1B). Using a MOI of 100, we were



**Figure 1.** Apoptin kills leukemia cell lines but does not kill normal bone marrow CD34<sup>+</sup> cells. A, cell death was measured by FACS analysis of PI-positive cells on day 5 postinfection. The mean values were calculated from three independent experiments. \*,  $P < 0.05$ . B, cell viability of MOI 100 infected cells was measured by MTT assay at the indicated time points. The values were converted to percentage of control-untransduced parental cells. The mean values and SD were obtained from three independent experiments each performed in triplicate. C, colony-forming assay of CD34<sup>+</sup> cells. Mobilized normal CD34<sup>+</sup> cells were infected with a MOI of 100 and 48 h later seeded in methylcellulose-based medium for the evaluation of colony formation. After 12 to 14 d, the different lineage colonies were counted and the mean values were obtained from two independent experiments each in duplicates.



**Figure 2.** Sensitivity to apoptin killing is correlated to PKC $\beta$  expression in myeloma cells. A, cell viability was measured by MTT assay of MOI 2 infected cells at the indicated time points. The values were determined by the percentage of the value measured from control-untransduced parental cells. The mean values and SD were obtained from three independent experiments performed in triplicate. B, cell death was measured by FACS analysis of PI-positive cells on day 5 or day 10 of MOI 2 infected cells, respectively. The mean values were derived from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . C, differential phosphorylation of apoptin protein was detected by Western blotting in MM1.R and MM1.S cells expressing GFP-apoptin. D1, D2, and D3 represent day 1, day 2, and day 3 postinfection. D, relative expression of PKC $\beta$ , DGKH, and MERTK in MM1.R and MM1.S cells measured by real-time quantitative RT-PCR (left) and their protein levels were measured by Western blotting (right). PKC $\beta$  protein level was detected by variant-specific antibodies. Protein samples were prepared from three independent cell populations. Numbers below each lane represent the quantified level of protein as compared to the control.

able to infect more than 80% of CD34<sup>+</sup> cells with LV-GFP and more than 50% with LV-GFP-AP, respectively, but the transgene expression was 2 to 3 logs lower compared with the leukemia cells (Supplementary Fig. S2C). Nevertheless, after infection with LV-GFP-AP, CD34<sup>+</sup> cells remained viable and maintained the same differentiation capacity as control LV-GFP-infected and uninfected CD34<sup>+</sup> cells on methylcellulose colony-forming assay (Fig. 1C). This indicates that apoptin-expressing CD34<sup>+</sup> cells function normally as hematopoietic progenitors and can differentiate into multiple lineages. The low infectivity, low expression, and lack of toxicity shown by the transduced CD34<sup>+</sup> and PBMC cells show an important potential for LV-GFP-AP as a purging agent in autologous bone marrow transplant for leukemia.

#### Identification of apoptin kinase(s) by microarray analysis

Two variants of multiple myeloma cells, which originated from the same patient, are characterized by resistance (MM1.R) or sensitivity (MM1.S) to dexamethasone. Using a MOI of 2, we showed that MM1.R cells were significantly more sensitive to apoptin-induced cell death as assessed by MTT cell viability and FACS analysis (Fig. 2A and B). Importantly, increased sensitivity of MM1.R cells to apoptin-induced cell death was associated with a higher level of apoptin phosphorylation (Fig. 2C) detected by Western blot analysis using an apoptin phospho-specific antibody that we have recently developed.

Expression profiling by Affymetrix microarray analysis showed the overexpression of several kinases in MM1.R cells

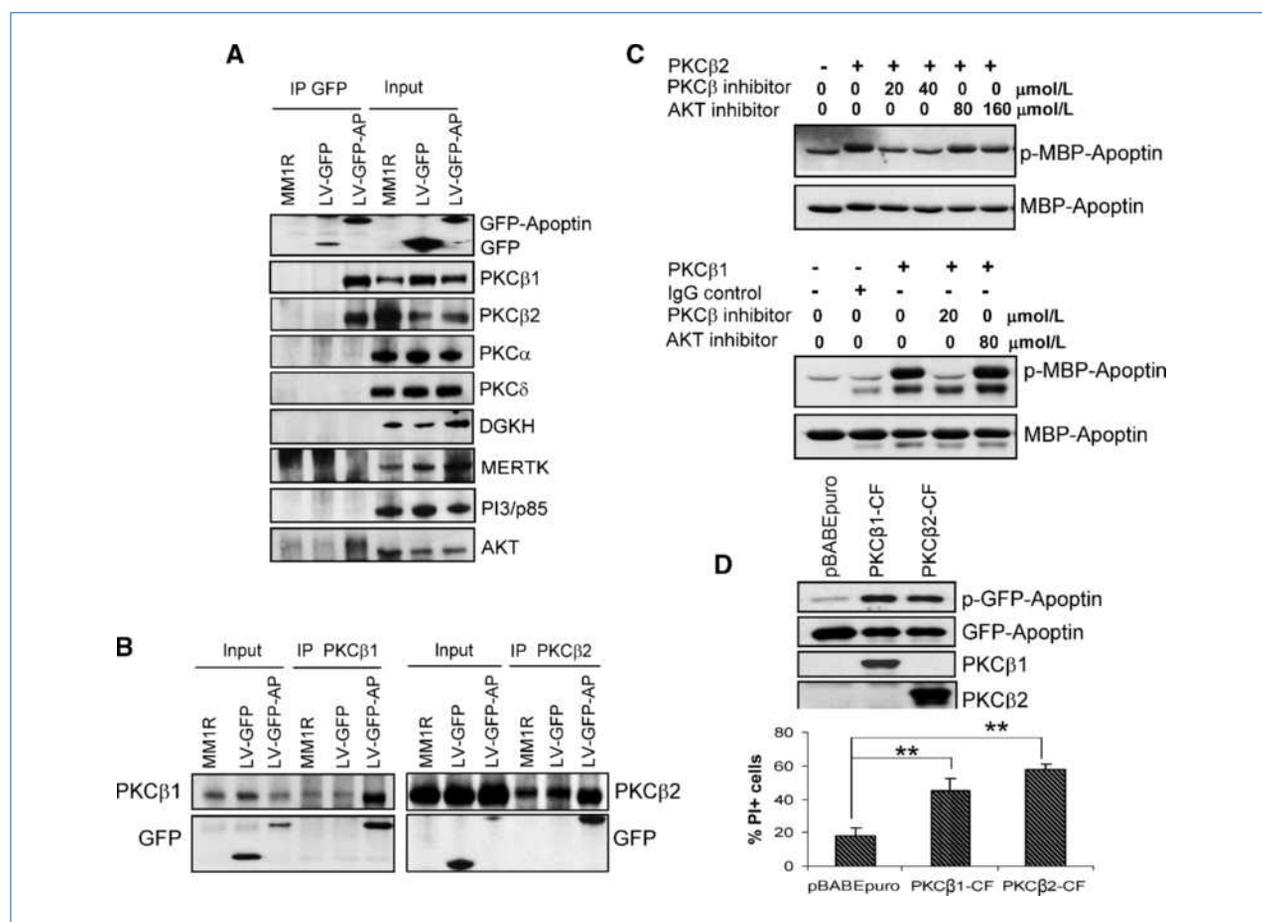
compared with MM1.S cells, including PKC $\beta$  (9 fold), *c-mer* proto-oncogene tyrosine kinase (MERTK, 5.9-fold), and diacylglycerol kinase (DGKH, 5.2-fold). The expression levels of PKC $\alpha$  (1.6-fold), PKC $\gamma$  (1.5-fold), and PKC $\delta$  (1.4-fold) were slightly higher in MM1.R cells. In contrast, PKC $\epsilon$  expression was higher (3.2-fold) in MM1.S cells. The detailed microarray data are provided in the supplementary materials. The highest expressing candidates PKC $\beta$ , MERTK, and DGKH were further validated by quantitative reverse transcriptase-PCR (qRT-PCR) using Sybr Green Taqman master mix and Western blot analysis (Fig. 2D). These experiments confirmed the microarray analysis data.

### PKC $\beta$ interacts and phosphorylates apoptin *in vitro* and *in vivo*

To further investigate the role of the identified kinases in the phosphorylation of apoptin, MM1.R cells were infected

with LV-GFP or LV-GFP-AP. After 3 days, total cell extracts were immunoprecipitated with GFP antibody (Abcam). Western blot analysis of the IP complexes detected a clear IP of both PKC $\beta$  variants with GFP-apoptin fusion protein but not with GFP alone. The precipitated complexes were also analyzed using antibodies against DGKH, MERTK, AKT, PI3K/p85, PKC $\alpha$ , and PKC $\delta$ , but none of these proteins were found in the GFP-apoptin immunocomplex (Fig. 3A). The reciprocal IP using anti-PKC $\beta$ 1 (Fig. 3B) or PKC $\beta$ 2 antibody confirmed the interaction of the PKC $\beta$  variants with GFP-apoptin.

To further investigate whether apoptin is phosphorylated by PKC $\beta$ , bacterial recombinant apoptin fused to MBP (MBP-apoptin) was used as substrate with either recombinant GST-PKC $\beta$ 2 protein (Cell Signaling) or with immunopurified PKC $\beta$ 1. Western blot analysis confirmed enhanced



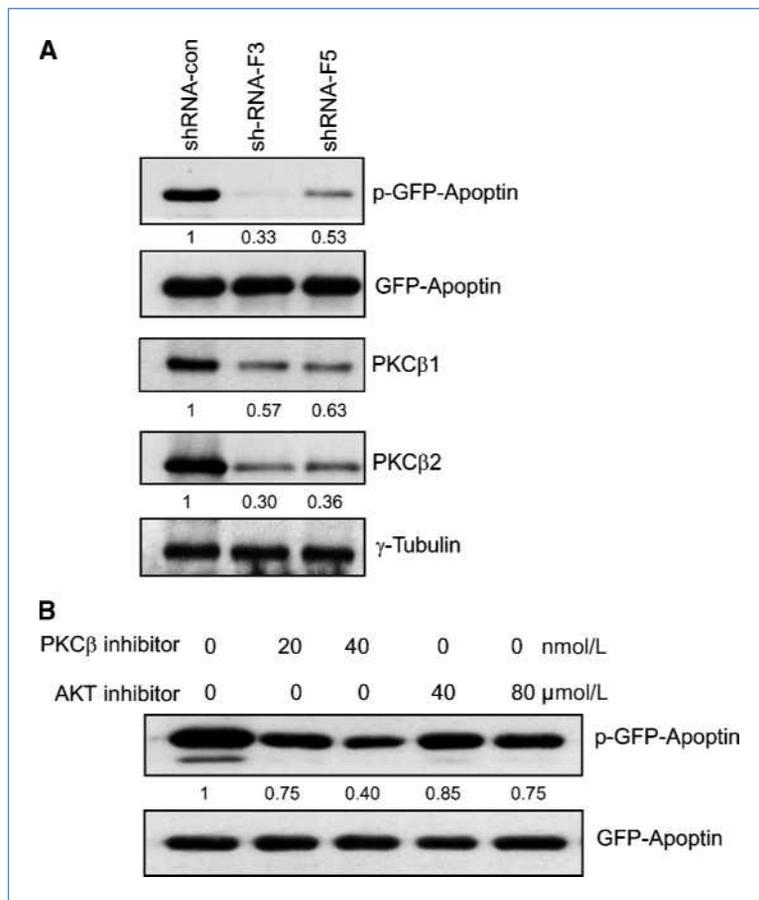
**Figure 3.** Both PKC $\beta$  variants interact with apoptin and phosphorylate apoptin *in vitro* and *in vivo*. MM1.R cells were lysed in RIPA buffer on day 3 postinfection. A, Immunoprecipitation (IP) with anti-GFP antibody. B, IP with anti-PKC $\beta$ 1 antibody (left) and anti-PKC $\beta$ 2 antibody (right). The immunocomplexes were separated on SDS-PAGE gels, and proteins were detected by the indicated antibodies. C, an *in vitro* kinase assay showed MBP-apoptin phosphorylation by recombinant PKC $\beta$ 2 (top) and PKC $\beta$ 1 purified from MM1.R cell lysates by IP with a PKC $\beta$ 1 antibody or an unspecific antibody (lane 2, bottom). The lower bands are heavy chains from the IP cross-reacting with the secondary antibody. The phosphorylation of MBP-apoptin is blocked by a PKC $\beta$  inhibitor, but not by an Akt inhibitor. D, increased phosphorylation of apoptin was detected in 293T cells 48 h after cotransfection of LV-GFP-AP with pPKC $\beta$ 1-CF and pPKC $\beta$ 2-CF. Expression of PKC $\beta$ 1 and PKC $\beta$ 2 catalytic fragments in 293T cells was detected by Western blot using PKC $\beta$ 1- and PKC $\beta$ 2-specific antibodies. The same membranes were reprobed with a phosphorylated apoptin-specific antibody and anti-GFP antibody. Increased cell death was detected by FACS analysis in 293T cells 5 d after cotransfection of LV-GFP-AP with pPKC $\beta$ 1-CF and pPKC $\beta$ 2-CF. The mean values and SD were obtained from triplicate experiments. \*\*, P < 0.01.

phosphorylation of apoptin by both PKC $\beta$ 1 and PKC $\beta$ 2 (Fig. 3C). Furthermore, a PKC $\beta$ -specific inhibitor resulted in diminished apoptin phosphorylation, whereas an AKT-specific inhibitor had no such effect. The activity of the AKT inhibitor was confirmed by Western blot analysis of AKT phosphorylation in the presence or absence of AKT inhibitor on total cell lysate from MM1.R cells (data not shown). The level of total apoptin detected by the apoptin antibody showed that the total amount of MBP-apoptin supplemented was similar in different samples. To obtain evidence that the PKC $\beta$  isoforms also phosphorylated apoptin in cells, we coexpressed apoptin with the catalytic domains of PKC $\beta$ 1 and PKC $\beta$ 2 (pPKC $\beta$ 1-CF or pPKC $\beta$ 2-CF) or control pBabePuro in 293T cells. At 48 hours after transfection, total cell extracts were separated on SDS-PAGE and protein expression was analyzed by Western blotting using anti-PKC $\beta$ 1 or anti-PKC $\beta$ 2 antibodies. The catalytic domain fragments (CF) of both PKC $\beta$  isoforms were detected by Western blotting with PKC $\beta$  variant-specific antibodies (Fig. 3D). In agreement with the *in vitro* data, overexpression of PKC $\beta$ -CF resulted in a significant increase in apoptin phosphorylation. In contrast, apoptin was weakly phosphorylated by cotransfection with the empty vector control, representing the basal phosphorylation levels in 293T cells. Anti-GFP antibody was used on the same blot

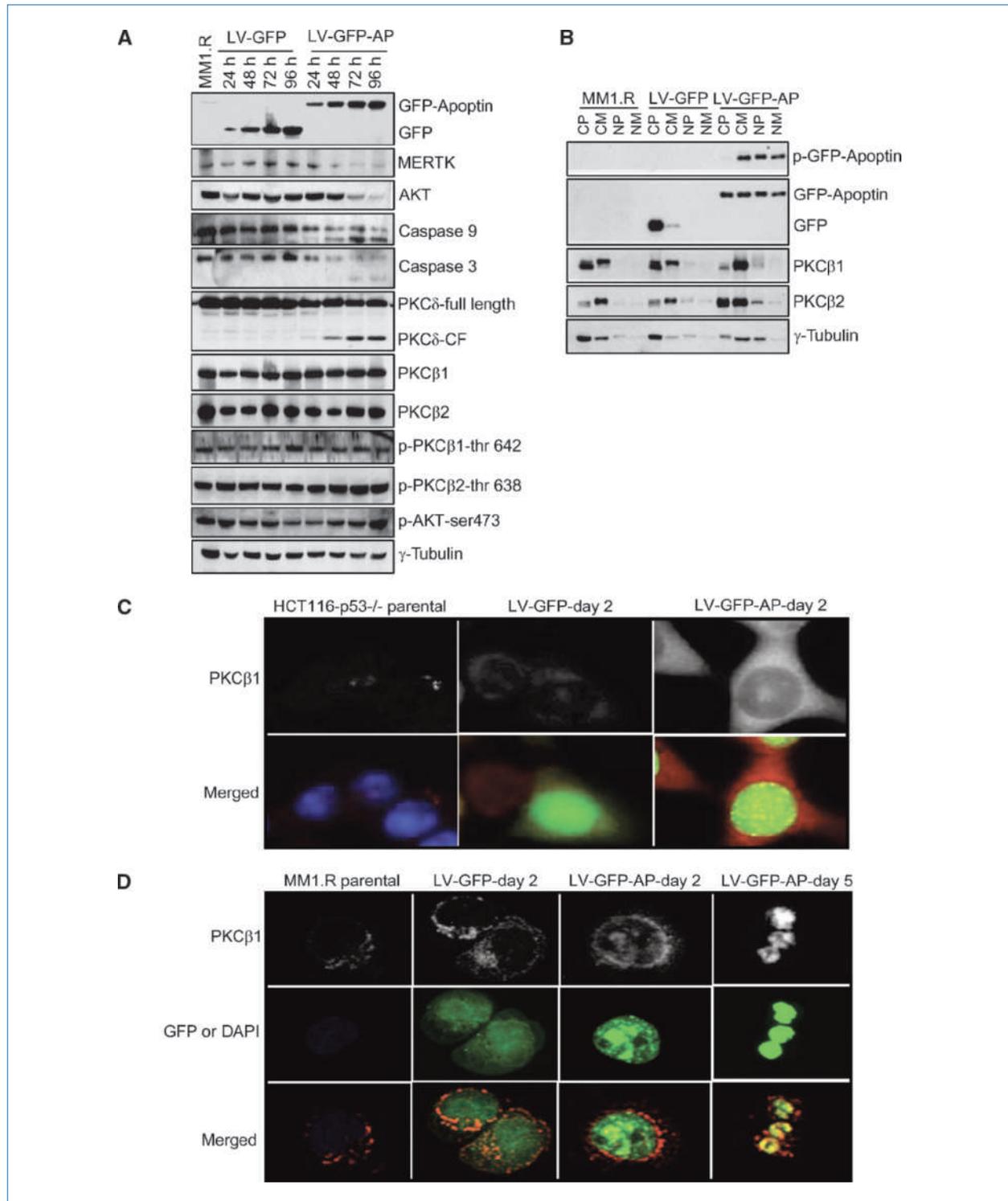
to show similar expression levels of GFP-apoptin in different samples. Furthermore, cell death was increased when exogenous PKC $\beta$ 1-CF or PKC $\beta$ 2-CF were coexpressed with apoptin (Fig. 3D).

#### shRNA-mediated knockdown or drug inhibition of PKC $\beta$ activity inhibits apoptin phosphorylation

To further show a causal link between PKC $\beta$  and apoptin phosphorylation, MM1.R cells were infected with LV-GFP-AP and lentiviral vectors expressing shRNA against PKC $\beta$ . The successful knockdown of endogenous PKC $\beta$ 1 and PKC $\beta$ 2 protein levels by shRNA was shown by Western blotting (Fig. 4A). A strong reduction in apoptin phosphorylation was observed at 3 days after transduction with shRNA. However, FACS analysis of PI-positive cells showed increased rather than decreased cell death in cells coexpressing apoptin together with PKC $\beta$  shRNA (Supplementary Fig. S3A and B). This is not surprising as PKC $\beta$  isoforms are known to play an important role in cell proliferation and their inhibition is likely to suppress cellular growth. Moreover, we examined PKC $\beta$ -mediated apoptin phosphorylation using PKC $\beta$ -specific or AKT-specific inhibitors (Fig. 4B). We found that apoptin phosphorylation was reduced 3 days after treatment with PKC $\beta$  inhibitor or AKT inhibitor. However, PKC $\beta$  inhibition



**Figure 4.** Apoptin phosphorylation is regulated by PKC $\beta$  expression and PKC $\beta$  activity. A, PKC $\beta$  knockdown resulted in reduced apoptin phosphorylation. MM1.R cells were coinfecting with lentiviral vector encoding GFP-apoptin and lentiviral construct encoding either shRNA-con (no homology to human genome), or shRNA-F3 and shRNA-F5 directed against PKC $\beta$ . The levels of phosphorylated apoptin and the knockdown of PKC $\beta$  were detected by Western blotting of cell lysates obtained 3 d after infection. The same blot was probed to detect phosphorylated apoptin, GFP-apoptin, PKC $\beta$ 1, PKC $\beta$ 2, and  $\gamma$ -tubulin. B, apoptin phosphorylation is reduced by inhibition of PKC $\beta$  activity. MM1.R cells were infected with LV-GFP-AP. PKC $\beta$  and AKT inhibitors were applied on day 1 postinfection with the indicated concentrations; on day 3, the cell lysates were detected by immunoblotting with apoptin phosphospecific and GFP antibodies. Numbers below each lane represent the quantified level of protein as compared to the control.



**Figure 5.** Apoptin differentially regulates cellular protein kinases. A, kinase protein levels and their activities detected by Western blotting from whole cell lysates of transduced MM1.R cells. B, protein levels detected by Western blotting from subcellular fractions of transduced MM1.R cells on day 3. CP, proteins isolated from the cytoplasmic fraction; CM, proteins from the cell membrane fraction; NP, proteins isolated from the nuclear fraction; NM, proteins bound to the nuclear membrane. C, apoptin upregulates PKC $\beta$  in HCT116-p53<sup>-/-</sup>. HCT116-p53<sup>-/-</sup> cells were fixed and labeled with anti-PKC $\beta$ 1 antibody on day 2 postinfection. D, PKC $\beta$  colocalized with apoptin in the nucleus of transduced MM1.R cells. The cells were fixed and stained with anti-PKC $\beta$ 1 antibody on the indicated days postinfection, and representative images were taken by fluorescence microscopy. Apoptin-expressing MM1.R cells clearly showed condensed apoptotic nuclei on day 5 postinfection.

decreased phosphorylation of apoptin considerably more than inhibition of AKT. These results collectively suggest that PKC $\beta$  is an important apoptin kinase, but other intricate pathways may be involved in apoptin phosphorylation and regulation of its activation.

### Apoptin differentially regulates cellular kinases and kinase activities

We further studied the effect of apoptin on the expression and activation of other cellular candidate kinases, which were either reported to play a role in the apoptin activity or were identified in our microarray expression profiling.

Infection of MM1.R cells with either control LV-GFP or LV-GFP-AP resulted in a time-dependent increase of GFP or GFP-AP expression. However, apoptin expression resulted in a decreased expression of MERTK and AKT as normalized to  $\gamma$ -tubulin (Fig. 5A). PKC $\beta$ 1 and PKC $\beta$ 2 protein levels remained unchanged, but increased phosphorylated PKC $\beta$ 1, PKC $\beta$ 2, and AKT were detected in total cell extracts. Meanwhile, elevated levels of PKC $\beta$ 1 and  $\beta$ 2 in cytoplasmic membrane and nuclear fractions, representing kinase activities, were also detected in the apoptin-transduced cells (Fig. 5B). Interestingly, apoptin expression also induced cleavage of caspase-9, caspase-3, and PKC $\delta$ , which correlated with apoptin-induced cell death.

Furthermore, we found that in tumor cells, the cytosolic fraction of the apoptin protein was not phosphorylated, whereas the plasma membrane-bound, nuclear, and nuclear membrane-associated apoptin protein was significantly phosphorylated. These data indicate that phosphorylation plays an important role in the subcellular localization of apoptin (see Fig. 5B). These membranes are the sites where PKC $\beta$  is considered to be active (not in the cytosol; ref. 30). Similar results were obtained with different cell lines infected with LV-GFP-AP (data not shown), suggesting that apoptin is phosphorylated by the activated PKC $\beta$  at cellular membranes.

It is widely accepted that the biological functions of PKC isoforms depend on their intracellular localization. PKC isoforms can be localized to multiple cellular compartments, including the plasma membrane, endosomes, endoplasmic reticulum, Golgi, nucleus, and nuclear membrane. To study the subcellular localization of PKC $\beta$ , MM1.R and p53-deficient HCT116 cells (sensitive to apoptin killing) were infected with LV-GFP (MOI 2) or LV-GFP-AP (MOI 8), respectively. The untransduced and GFP-transduced HCT116 cells showed a low level of cytosolic distribution of PKC $\beta$ , whereas the apoptin-transduced HCT116 cells had increased cytosolic levels and nuclear import of PKC $\beta$  (Fig. 5C). In the untransduced and GFP-transduced MM1.R cells, PKC $\beta$  mostly concentrated near the nucleus in a pattern that resembled the endoplasmic reticulum. In apoptin-transduced MM1.R cells, activated PKC $\beta$  relocated to the nuclear membrane and also translocated into the nucleus, colocalizing with apoptin. Apoptin-expressing MM1.R cells clearly showed condensed apoptotic nuclei on day 5 postinfection (Fig. 5D).

## Discussion

Recently, several viral and cellular proteins that specifically kill tumor cells have been identified. Such proteins as well as their cellular interacting and regulatory targets are important candidates for anticancer therapeutics. In the present study, we have identified a novel cellular pathway involved in the sensitization of cancer cells to apoptin.

Apoptin has been shown to be predominantly localized in the nucleus of cancer cells, whereas in normal cells its nuclear accumulation is severely impaired (13, 14). Several studies indicated that phosphorylation of apoptin is crucial for its nuclear localization and cytotoxic activity (9, 10). Here, we provide evidence that the phosphorylation and nuclear migration of apoptin in tumor cells is mediated by PKC $\beta$ . Deletion and point mutation studies have shown the importance of the COOH-terminal domain (amino acids 80–121) of apoptin for its nuclear accumulation and function (31–34). Apoptin was shown to be phosphorylated in this domain predominantly at Thr-108 in cancer cells (16, 17). Analysis of the apoptin amino acid sequence for potential phosphorylation sites using NetPhos software (<http://www.cbs.dtu.dk/services/NetPhos>) indicated seven putative phosphorylation sites corresponding to the PKC kinase consensus motifs (S/TXK/R or S/TXXK/R). These sites included Thr-108, which has been previously shown to be phosphorylated in tumor cells but not in normal cells (31, 32). Microarray expression profiling of multiple myeloma cell lines with different sensitivity to apoptin identified the PKC $\beta$  family as potential tumor-specific apoptin kinases. Our *in vitro* and *in vivo* kinase studies and the knockdown of PKC $\beta$  further confirmed that apoptin was phosphorylated by PKC $\beta$ .

Ludwig and colleagues have recently shown that virus infection or the treatment with the viral protein hemagglutinin resulted in a rapid activation of PKC isoforms (35). Furthermore, besides the well-established role of PKCs in activating the downstream RAF/MEK/ERK kinases, PKCs have been found to directly phosphorylate several viral proteins (36, 37). Our study provides further evidence that PKC $\beta$  may be an important cellular component that interacts with viral proteins including apoptin. Consequently, PKC $\beta$  phosphorylates apoptin and triggers its nuclear migration where it induces the activation of multiple signaling events, involving caspase-9, caspase-3 activation, and cleavages of PKC $\delta$ , shifting the equilibrium from survival signaling toward the activation of the cell death machinery.

Recently, apoptin has been shown to interact with the SH3 domain of p85 regulatory subunit of PI3K and its downstream effector AKT kinase (18, 19, 38). Interestingly, the interaction of apoptin with AKT seems to trigger the nuclear trafficking of AKT in parallel with apoptin, resulting in the AKT-mediated phosphorylation of downstream mitogenic cyclin-dependent kinase CDK2 and consequent phosphorylation of apoptin by CDK2 in the nucleus. However, in this study, we have been unable to show an interaction between apoptin and either the p85 subunit of PI3K or AKT, but we could not entirely rule out the involvement of PI3K/AKT pathway in apoptin phosphorylation either. Particularly, inhibition with an AKT inhibitor showed decreased apoptin

phosphorylation to some extent. In our study, apoptin expression resulted in AKT activation and rapid AKT protein degradation, which may prevent detection due to a sensitivity problem. The inconsistencies between our study and that of Maddika and colleagues could be due to the different intrinsic cell features in the cell types used. MCF7 cells, which they used in their experiments, have previously been shown by others to be caspase-3 deficient (39). It has been reported that AKT kinases are functionally inactivated by caspase-3 cleavage in response to a variety of apoptotic stimuli and growth factor withdrawal (40, 41). A nuclear translocation of AKT with apoptin in MCF7 cells reported by Maddika and colleagues could therefore be due to the lack of cleavage of AKT in the caspase-3-deficient MCF7 cells, resulting in a full-length, active AKT that could capture and accompany apoptin into the nucleus.

In contrast to AKT, which was destabilized by apoptin, a consistent, stable PKC $\beta$  protein level was detected in MMLR cells. It is generally accepted that the transient activation of PKCs supports cell survival, whereas a sustained activation of PKCs induces apoptosis (21). Activation of PKCs in certain leukemia cell lines (HL60, U937, and K562) by TPA has been shown to be associated with growth arrest and terminal differentiation (42–44). This effect is also often accompanied by apoptosis.

Controlled cellular proliferation involves multiple mechanisms that balance increased cell numbers with subsequent cell death. This complex process is orchestrated by many kinases and caspases (45). We observed increased cleavage of caspase-9, caspase-3, and PKC $\delta$  during apoptin-induced cell death in myeloma cell lines. Active caspase-8 or caspase-9 can directly cleave and activate effector caspases, such as caspase-3, and/or engage the intrinsic apoptotic pathway through the cleavage of the Bcl-2 homology 3 protein Bid (46). Cleaved Bid translocates to the mitochondria, where it triggers activation of the intrinsic apoptotic pathway by promoting activation of the Bcl-2 proteins Bax and Bak, which induces loss of mitochondrial membrane potential and release of proapoptotic mitochondrial components into the cytoplasm. PKC $\delta$  is a well-characterized and ubiquitously expressed kinase with multiple functions (47). Full-length PKC $\delta$  is located in the cytoplasm and has been shown to promote cell proliferation, whereas cleaved PKC $\delta$  is located in the nucleus and has proapoptotic functions. Exposure to numerous apoptotic stimuli results in the activation of PKC $\delta$  and its translocation to the nucleus. Our data indicate that apoptin activates both PKC $\delta$  and caspase-3 and enhances their nuclear accumulation (data not shown). In the nucleus, PKC $\delta$  seems to be cleaved by activated caspase-3 to generate the constitutively activated proapoptotic cleaved form (48). Potential target substrates of PKC $\delta$  are nuclear proteins that are involved in apoptotic

cell death such as lamin B (nuclear structural protein), DNA-dependent protein kinase (DNA-PK), Rad9 (cell cycle checkpoint protein), p53, p73 $\beta$ , and STAT1 (transcription factors; refs. 20, 23, 47). Taken together, our data suggest that cleavage of PKC $\delta$  and caspase-3 is also involved in the regulation of apoptin-mediated apoptosis, but downstream of apoptin. This is consistent with previous reports showing that caspase-3 is required for apoptin-induced apoptosis (12).

Cancer remains one of the leading causes of death, and many cancer patients relapse as they become resistant to conventional therapies. There is mounting evidence that cancer cells, including leukemia, have an intrinsic ability to prevent apoptosis (49). Here, we provide important evidence that the ectopic expression of apoptin can restore the failing apoptosis program in leukemia and overcome intrinsic or acquired resistance to cell death. Furthermore, apoptin was able to effectively eliminate multiple myeloma cells that had become resistant to dexamethasone. This study has led to the identification of tumor-specific cellular targets such as PKC $\beta$ , whose modulation by shRNAs and small-molecule drugs can induce strong antileukemia effects.

In conclusion, the present study provides novel mechanisms for apoptin regulation by protein phosphorylation involving the PKC pathway. This knowledge can be applied to understanding the role of these kinases in response to treatment with a variety of anticancer agents. Importantly, the evidence that our newly developed lentiviral vector expressing apoptin has no effect on CD34<sup>+</sup> progenitor colony formation while effectively killing multiple myeloma cells further supports apoptin as an important and ideal *ex vivo* purging agent in the autologous bone marrow transplantation for human multiple myeloma.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Crucial Roles for Protein Kinase C Isoforms in Tumor-Specific Killing by Apoptin

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