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 GFP-Apoptin fusion gene (LV-GFP-AP) that can deliver Apoptin into haematopoietic cells efficiently. Apoptin selectively killed the human multiple myeloma cell lines MM1.R, MM1.S and the leukaemia cell lines K562, HL60, U937, KG1 and NB4. In contrast, normal CD34+ 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 cells and MM1.S cells. Among these kinases, PKCβ 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 PKCβ significantly reduced Apoptin phosphorylation. Furthermore, Apoptin mediated cell death proceeded via the up-regulation of PKCβ, activation of caspase-9/3, cleavage of the PKCδ catalytic domain and downregulation of the MERTK and AKT kinases. Collectively these results elucidate a novel pathway for Apoptin activation involving PKCβ and PKCδ. Further, they highlight the potential of Apoptin and its cellular regulators to purge bone marrow used in autologous transplantation for multiple myeloma.

Introduction

Stem cell rescue following high dose cytotoxic chemotherapy is considered curative and has gained extensive application worldwide as a therapeutic modality in several haematological malignancies (1-4). Allogeneic bone marrow transplant is the preferred choice for most type of leukemia’s, but due to the rarity of HLA compatible donor, 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 pharmacological and immunological approaches aiming at the
elimination of the leukemic cells have been developed but 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 haematological 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, fibroblast 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 threonine108 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 SH3 domain of phosphatidylinositol 3'-kinase (PI3-K) and protein kinase B (AKT) in MCF7 breast and PC3 prostate cancer cell lines. The activation of the PI3-K/AKT pathway by Apoptin results in the induction of the cyclin-dependent kinase CDK2, in turn leading to the phosphorylation of Apoptin (18, 19). In contrast, we have found no correlation between AKT activity in human cancer cell lines and their sensitivities to Apoptin killing (unpublished data).

In this study we have used a matched pair of multiple myeloma cell lines (MM1.R and MM1.S) that show resistance (R) or sensitivity (S) to dexamethasone; MM1.R being derived from MM1.S by continuous growth in dexamethasone. These cell lines showed a marked difference in sensitivity to Apoptin induced killing, MM1.R being clearly more sensitive than MM1.S. Microarray expression profiling of these cell lines showed that several kinases, including members of the PKC kinase family, are overexpressed in the Apoptin sensitive cell line MM1.R as compared to MM1.S cells. (see supplementary table 1)

PKC is a family of serine-threonine kinases, comprised of at least 13 known isoforms with a wide range of tissue distribution, subcellular localization and function. All PKC isoforms contain a
highly conserved C-terminal catalytic domain and N-terminal regulatory domain with an autoinhibitory pseudosubstrate sequence (20-22). It has been well established that the activation of the classic PKC isoforms (α, β1, β2 and γ) require Ca$^{2+}$ and a phospholipid, such as diacylglycerol (DAG) or phosphatidylserine (PS). These PKC isoforms also contain two cysteine-rich motifs within their N-terminal regulatory domain that facilitates phorbol ester (TPA) interaction and subsequent activation. The novel isoforms (δ, ε, η, μ, θ) are also activated by DAG and phorbol esters but lack a complete C2 domain, responsible for Ca$^{2+}$ interaction and are therefore Ca$^{2+}$ independent. The protein structures of atypical PKC isoforms (ζ, ι/λ), differs from the other members of the PKC family, missing both binding regions for Ca$^{2+}$ 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 non-stimulated 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 PKCδ can be activated by caspase 3 cleavage to release an active catalytic domain from the inhibitory N-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 PKCα, β, λ, ε, and ζ preferentially function to promote cell proliferation and survival, while PKCδ is a critical pro-apoptotic kinase in many cell types (26).

In order to evaluate Apoptin tumor specific toxicity in hematological malignancies, we developed a lentiviral vector encoding a GFP-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 PKCβ 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, USA). K562 (BCR/ABL cytogenetics), HL60 (TPA, retinoic acid differentiation), U937 (TPA differentiation) and KG1 (monosomy7, trisomy 8 cytogenetics) leukemic cells were obtained from the ATCC. NB4 (t15,17 translocation) was obtained from German Collection of Microorganisms and Cell Cultures. HCT116 (KRAS mutation analysis) was obtained from Prof Vogelstein (John Hopkins, Baltimore, USA). 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) by replacement of GFP by GFP-AP by blunt end ligation (see supplementary figure 1A). Lentiviral vectors were produced by co-transfection of 293T cells with a second generation packaging plasmid pCMVΔ8.91 and plasmid pMDG encoding VSV-G pseudotyped envelope (27). The titre of lentivirus was determined by quantification of viral core protein p24 by ELISA using a HIV-1 p24 capture assay kit (Perkin Elmer, UK). The values were normalized against a recombinant p24 protein standard. Virus numbers were then calculated based on the fact that a viral particle contains 2000 p24 molecules (28, 29). Comparable virus titres, based on p24 ELISA, were achieved for LV-GFP and LV-GFP-AP (supplementary table 2).

MTT proliferation

Leukemia cell lines were infected with LV-GFP or LV-GFP-AP in the presence of 4 μg/ml polybrene. Cells were seeded at 5x10^4, 10^4, and 10^3 per well in 96 well plates for MTT assays.
On day 2, 4, 6, 8, 10, 12, and 14 post-infection, 20 μl of 5mg/ml MTT in PBS was added to each well and incubated for 2-4 hours, 100 μl MTT solubilisation solution was added and incubated overnight. The OD was measured and the OD values 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 μg/ml of PI. The settings for FACS analysis were based on unstained parental, GFP transduced and PI stained control samples. The cells were analyzed on a BD FACS Canto II (Becton Dickinson, UK) and 10 000 events were acquired per sample. Fluorescence data was 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). CD34+ cells were isolated using anti-CD34+ microbeads, and an AutoMacs fractionation device (Miltenyi Biotec, UK) according to the manufacturer's instructions. CD34+ cells were cultured in Stemspan serum-free medium with cytokine cocktail (StemCell Technologies, Vancouver) for 48 hours. Mobilized CD34+ cells were infected with MOI of 100 of LV-GFP or LV-GFP-AP. Two days post-infection, infected CD34+ cells were analyzed for their progenitor function by an in vitro colony-forming assay (StemCell Technologies Catalog number #04434/04444).

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

Immunoprecipitation and Western blotting
For immunoprecipitation, cells were lysed in RIPA buffer on ice with freshly added protease inhibitor cocktail (Sigma). The cell debris was removed and 2 μg of anti-GFP (Abcam, UK) or
anti-PKCβ1 antibody (Santa Cruz) was added to the cell lysate for 30 minutes at room temperature under constant agitation. 20 μl of washed Bio-Adembeads PAG (Ademtech, France) was added to the cell lysate with antibody for 2-3 hours at 4°C and complexes were pulled-down using a magnet rack.

Proteins from different fractions of the cell were isolated using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas, UK, K0311) according to the manufacturer’s protocol.

For Western blotting, the proteins were separated on SDS-PAGE gels; electroblotted onto nitrocellulose membrane (GE Healthcare, UK) and blocked with 5% non-fat 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 detection system and exposed to ECL film (both Amersham Biosciences, UK). The blot was stripped with ReBlot plus strong antibody stripping buffer (Millipore, UK) and re-probed 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, H2N-SLITTTTPSRPRTA-CONH2 respectively (Eurogentec, Belgium). 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 and antibodies against phospho-AKT-Ser-473, phospho-PKCβ2-Thr-638, total AKT, PI3K/p85, caspase 3, caspase 9 and GFP for Western blotting were purchased from Cell Signalling.

**In vitro kinase assay**

2 μg of MBP-Apoptin fusion protein was added to 1x kinase buffer (Cell Signaling, UK) containing 200 μM ATP and 0.2 ng of PKCβ2 kinase (Cell Signaling, UK) or PKCβ1 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 PKCβ-specific inhibitor (Calbiochem, UK, cat. no. 539654) or AKT-specific inhibitor (Calbiochem, UK, cat. no. 124005). The reaction was stopped by adding 2x loading buffer and boiling for 5 minutes.
Construction of plasmids encoding PKCβ catalytic domains and transfection of 293T

The catalytic domains of PKCβ1 and PKCβ2 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 expression of the catalytic domain was verified by western blotting of transfected cells with PKCβ1 and PKCβ2 specific antibodies (Santa Cruz Biotechnology).

Immunohistochemistry and visualization by fluorescence Microscopy

5x10^4 HCT116-p53-/- or MM1.R cells were infected with lentiviral vector and at day 2 or 5 cells were fixed and permeabilised and incubated with antibody against PKCβ1 for 1 hour and texas red IgG secondary antibody for 1 hour. The cells were washed and covered with DAPI mounting medium (Vector Laboratories, UK) and visualized.

Statistical analysis

A Student 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 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 figure 2A, MM1.S cells have a comparable infectivity to the parental matched MM1.R cells, data not shown). CD34+ cells were less sensitive requiring a higher MOI of 100 to obtain 50-80% transduction efficiency. Furthermore, higher expression levels of the transgene were observed in the leukemic cell lines as compared to primary CD34+ cells or PBMCs (supplementary figure 2B 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 propidium iodide (PI) positive cells. As shown in Fig. 1A, GFP-Apoptin triggered apoptosis in transduced leukemia cells in a dose dependent manner, while no cell death was observed in untransduced or GFP-transduced parental cells (figure 1A). Furthermore, MTT cell survival assay showed over 90% cell death in a panel of leukemia cell lines infected with LV-GFP-AP while no significant killing was observed with LV-GFP infected and uninfected parental cells (figure 1B). Using a MOI of 100 we were able to infect over 80% of CD34+ cells with LV-GFP and over 50% with LV-GFP-AP respectively, but the transgene expression was 2-3 logs lower as compared with the leukemia cells (supplementary figure 2C). Nevertheless, after infection with LV-GFP-AP CD34+ cells remained viable and maintained the same differentiation capacity as control LV-GFP infected and uninfected CD34+ cells on methylcellulose colony-forming assay (figure 1C). This indicates that Apoptin expressing CD34+ 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+ and PBMC cells demonstrate 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 demonstrated that MM1.R cells were significantly more sensitive to Apoptin-induced cell death as assessed by MTT cell viability and FACS analysis (figure 2A and B). Importantly, increased sensitivity of MM1.R cells to Apoptin-induced cell death was associated with a higher level of Apoptin phosphorylation (figure 2C) detected by Western blot analysis using an Apoptin phospho-specific antibody which we have recently developed.

Expression profiling by Affymetrix microarray analysis showed the overexpression of several kinases in MM1.R cells as compared to MM1.S cells, including PKCβ (9 fold), c-mer proto-oncogene tyrosine kinase (MERTK, 5.9 fold) and diacylglycerol kinase (DGKH, 5.2 fold). The expression levels of PKCα (1.6 fold), PKCγ (1.5 fold) and PKCδ (1.4 fold) were slightly higher in MM1.R cells. In contrast, PKCe expression was higher (3.2 fold) in MM1.S cells. The detailed...
microarray data are provided in the supplementary materials. The highest expressing candidates PKCβ, MERTK and DGKH were further validated by quantitative RT-PCR using Sybrgreen Taqman master mix and Western blot analysis (figure 2D). These experiments confirmed the microarray analysis data.

PKCβ 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 and after 3 days total cell extracts were immunoprecipitated (IP) with GFP antibody (Abcam, UK). Western blot analysis of the IP complexes detected a clear co-immunoprecipitation of both PKCβ 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, PKCα and PKCδ but none of these proteins were found in the GFP-Apoptin immunocomplex (figure 3A). The reciprocal IP using anti-PKCβ1 (figure 3B) or PKCβ2 antibody confirmed the interaction of the PKCβ variants with GFP-Apoptin.

To further investigate whether Apoptin is phosphorylated by PKCβ, bacterial recombinant Apoptin fused to maltose binding protein (MBP-Apoptin) was used as substrate with either recombinant GST-PKCβ2 protein (Cell Signaling, UK) or with immunopurified PKCβ1. Western blot analysis confirmed enhanced phosphorylation of Apoptin by both PKCβ1 and PKCβ2 (Fig. 3C). Furthermore, a PKCβ-specific inhibitor resulted in diminished Apoptin phosphorylation whilst 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 demonstrated that the total amount of MBP-Apoptin supplemented was similar in different samples. To obtain evidence that the PKCβ isoforms also phosphorylated Apoptin in cells, we co-expressed Apoptin with the catalytic domains of PKCβ1 and β2 (pPKCβ1-CF or pPKCβ2-CF) or control pbabepuro in 293T cells. At 48 hrs after transfection total cell extracts were separated on SDS-PAGE and protein expression was analyzed by Western blot using anti-PKCβ1 or anti-PKCβ2 antibodies. The catalytic domain...
fragments (CF) of both PKCβ isoforms were detected by Western blotting with PKCβ variant specific antibodies (Fig. 3D). In agreement with the in vitro data, overexpression of PKCβ-CF resulted in a significant increase in Apoptin phosphorylation. In contrast, Apoptin was weakly phosphorylated by co-transfection with the empty vector control, representing the basal phosphorylation levels in 293T cells. Anti-GFP antibody was used on the same blot to demonstrate similar expression levels of GFP-Apoptin in different samples. Furthermore, cell death was increased when exogenous PKCβ1-CF or PKCβ2-CF were co-expressed with Apoptin (figure 3D).

**shRNA mediated knockdown or drug inhibition of PKCβ activity inhibits Apoptin phosphorylation**

To further show a causal link between PKCβ and Apoptin phosphorylation, MM.1R cells were infected with LV-GFP-AP and lentiviral vectors expressing shRNA against PKCβ. The successful knockdown of endogenous PKCβ1 and PKCβ2 protein levels by shRNA was demonstrated by Western blotting (Figure 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 co-expressing Apoptin together with PKCβ shRNA (supplementary figure 3A/B). This is not surprising as PKCβ isoforms are known to play an important role in cell proliferation and their inhibition is likely to suppress cellular growth. Moreover, we examined PKCβ-mediated Apoptin phosphorylation using PKCβ-specific or AKT-specific inhibitors (figure 4B). We found that 3 days after treatment with PKCβ inhibitor or AKT inhibitor, Apoptin phosphorylation was reduced. However, PKCβ inhibition decreased phosphorylation of Apoptin considerably more than inhibition of AKT. These results collectively suggest that PKCβ 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 that 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 decrease expression of MERTK and AKT as normalized to γ-tubulin (figure 5A). PKCβ1 and PKCβ2 protein levels remained unchanged but increased phosphorylated PKCβ1, PKCβ2 and AKT were detected in total cell extracts. Meanwhile, elevated levels of PKCβ1 and β2 in cytoplasmic membrane (CM) and nuclear fractions, representing kinase activities, were also detected in the Apoptin transduced cells (figure 5B). Interestingly, Apoptin expression also induced cleavage of caspase 9, caspase 3 and PKCδ 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. This data indicates that phosphorylation plays an important role in the subcellular localization of Apoptin (see figure 5B). These membranes are the sites where PKCβ is considered to be active (not in the cytosol) (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 PKCβ 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 (ER), Golgi, nucleus and nuclear membrane. To study the subcellular localization of PKCβ, 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 PKCβ, whereas the Apoptin transduced HCT116 cells had increased cytosolic levels and nuclear import of PKCβ (figure 5C). In the untransduced and GFP transduced MM1.R cells, PKCβ mostly concentrated near the nucleus in a pattern that resembled the endoplasmic reticulum. In the Apoptin transduced MM1.R cells, the activated PKCβ relocated to the nuclear membrane and also translocated into the nucleus, co-localizing with Apoptin. Apoptin expressing MM1.R cells clearly showed condensed apoptotic nuclei on day 5 post-infection (figure 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 anti-cancer 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 PKCβ. Deletion and point mutation studies have demonstrated the importance of the C-terminal domain (aa 80–121) of Apoptin for its nuclear accumulation and function (31-34). Apoptin was shown to be phosphorylated in this domain predominantly at threonine 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 PKCβ family as potential tumor specific Apoptin kinases. Our in vitro and in vivo kinase studies and the knockdown of PKCβ further confirmed that Apoptin was phosphorylated by PKCβ.

Ludwig et al., have recently shown that virus infection or the treatment with the viral protein haemagglutinin 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 PKCβ may be an important cellular component that interacts with viral proteins including Apoptin. Consequently PKCβ 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 PKCδ, 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 demonstrate an interaction between Apoptin and either the p85 subunit of PI3 kinase or AKT, but we could not entirely rule out the involvement of PI3K/AKT pathway in Apoptin phosphorylation either. Particularly, the 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 Maddika et al could be due to the different intrinsic cell features in the cell types used. Maddika et al. worked with MCF-7 cells which are known 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 et al., 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 PKCβ protein level was detected in MM1.R cells. It is generally accepted that the transient activation of PKCs supports cell survival while 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 which balances 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 PKCδ during Apoptin induced cell death in myeloma cell lines. Active caspase-8 or 9 can directly cleave and activate the effector caspases, such as caspase-3 and/or engage the intrinsic apoptotic pathway through the cleavage of the Bcl-2 homology 3 (BH3) 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 constitutes into the cytoplasm. PKCδ is a well characterized and ubiquitously expressed kinase with multiple functions (47). Full length PKCδ is located in the cytoplasm and has been shown to promote cell proliferation, whilst cleaved PKCδ is located in the nucleus and has proapoptotic functions. Exposure to numerous apoptotic stimuli results in the activation of PKCδ and its translocation to the nucleus. Our data indicates that Apoptin activates both PKCδ and caspase 3 and enhances their nuclear accumulation (data not shown). In the nucleus, PKCδ seems to be cleaved by activated caspase 3 to generate the constitutively activated proapoptotic cleaved form (48). Potential target substrates of PKCδ 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, p73β and STAT1 (transcription factors) (20, 23, 47). Taken together, our data suggest that cleavage of PKCδ 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 which have become resistant to dexamethasone. This study has led to the identification of tumor specific cellular targets such as PKCβ, whose modulation by shRNAs and small molecule drugs can induce strong anti-leukemia 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 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.

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**References**


Figure 1

A

% of PI+ cells


MOI 5  MOI 25  MOI 50

* U937  HL60  K562

B

% of control absorbance

LV-GFP

LV-GFP-AP

days post-infection

days post-infection

C

Number of colony

CFU-GM  CFU-G  CFU-M  CFU DC  Mixed Lineage  P-BFU-E  M-BFU-E  CFU-E

CD34+un  LV-GFP  LV-GFP-Ap

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Figure 4

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<th>0</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT inhibitor</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>80</td>
<td>μM</td>
</tr>
</tbody>
</table>

- p-GFP-Apoptin
- GFP-Apoptin
Figure 5

Panel A: Western blot analysis showing expression of various proteins in MM1.R, LV-GFP, and LV-GFP-AP cells at 24h, 48h, and 72h.

Panel B: Western blot analysis showing expression of p-GFP-Apoptin, GFP-Apoptin, GFP, PKCβ1, PKCβ2, and γ-tubulin in MM1.R, LV-GFP, and LV-GFP-AP cells.

Panel C: Immunofluorescence images of HCT116-p53-/- parental, LV-GFP-day 2, and LV-GFP-AP-day 2 cells showing PKCβ1 expression.

Panel D: Immunofluorescence images of MM1.R parental, LV-GFP-day 2, LV-GFP-AP-day 2, and LV-GFP-AP-day 5 cells showing PKCβ1 expression.
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