Apoptin Interacts with and Regulates the Activity of Protein Kinase C Beta in Cancer Cells

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Running Title: Apoptin activates and is regulated by PKCβ

Abbreviations: CAV = chicken anaemia virus; PKC = protein kinase C; HGyV = human Gyrovirus; Thr-108 = threonine-108; PLA = proximity ligation assay; CKAR = C kinase activity reporter; FRET = fluorescence resonance energy transfer; TCSPC = time-correlated single-photon counting; FLIM = fluorescence lifetime imaging microscopy; CFP/YFP/GFP/RFP = cyan / yellow / green / red fluorescent protein; Cdk2 = cyclin-dependent kinase 2; NF-κB = nuclear factor kappa B; ANOVA = analysis of variance

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Abstract

Apoptin, the VP3 protein from chicken anaemia virus (CAV), induces tumour cell-specific cell death and represents a potential future anti-cancer therapeutic. In tumour but not in normal cells, Apoptin is phosphorylated and translocates to the nucleus, enabling its cytotoxic activity. Recently, the β isozyme of protein kinase C (PKCβ) was shown to phosphorylate Apoptin in multiple myeloma cell lines. However, the exact mechanism and nature of interaction between PKCβ and Apoptin remain unclear. Here we investigated the physical and functional link between PKCβ and CAV-Apoptin as well as with the recently identified Apoptin homologue derived from human Gyrovirus (HGyV).

In contrast to HCT116 colorectal cancer cells the normal colon mucosa cell lines expressed low levels of PKCβI and showed reduced Apoptin activation, as evident by cytoplasmic localisation, decreased phosphorylation and lack of cytotoxic activity. Co-immunoprecipitation and proximity ligation assay (PLA) studies identified binding of both CAV- and HGyV-Apoptin to PKCβI in HCT116 cells. Using Apoptin deletion constructs the N-terminal domain of Apoptin was found to be required for interacting with PKCβI. FRET-based PKC activity reporter (CKAR) assays by fluorescence lifetime imaging microscopy (FLIM) showed that expression of Apoptin in cancer cells but not in normal cells triggers a significant increase in PKC activity.

Collectively, the results demonstrate a novel cancer specific interplay between Apoptin and PKCβI. Direct interaction between the two proteins leads to Apoptin-induced activation of PKC and consequently activated PKCβI mediates phosphorylation of Apoptin to promote its tumour-specific nuclear translocation and cytotoxic function.
Introduction

Apoptin was originally identified as the apoptosis-inducing VP3 protein from chicken anaemia virus (CAV), the first member of the Gyrovirus genus [1]. In recent years several other viruses resembling the overall organisation of CAV have been identified, including the first human Gyrovirus HGyV [2] which contains a VP3 protein with similar properties as CAV-Apoptin [3]. Importantly, Apoptin has the ability to selectively kill various human tumour or transformed cells with little cytotoxic effect in normal cells [4-7]. However, the precise cellular mechanisms of Apoptin-induced cell death and its mode of tumour selectivity remain unclear [8]. In general, Apoptin triggers caspase-dependent cell death via the intrinsic apoptotic pathway [9, 10], which occurs independently of p53 but seems to require the activity of pro-apoptotic TAp73 isoforms from the p53 family [11, 12].

CAV-Apoptin is a small protein of 14 kDa, rich in proline, serine, threonine and basic amino acids. It contains a bipartite nuclear localisation signal and a nuclear export signal, which facilitate shuttling of the protein between the nucleus and cytoplasm [13-15], as well as several potential phosphorylation sites, including threonine-108 (Thr-108). In tumour cells but not in normal cells Apoptin is phosphorylated on Thr-108 [16], which promotes its accumulation in the nucleus of tumour cells whereas in normal cells it predominantly localises to the cytoplasm. Both phosphorylation and nuclear localisation are important for its pro-apoptotic function in tumour cells [15]. The tumour-specific phosphorylation of Apoptin has generated interest in identifying cellular kinases with increased activity in tumour cells that might be responsible for Apoptin phosphorylation and therefore its tumour-specific activation.

A number of kinases including cyclin-dependent kinase 2 (Cdk2) have been
implicated in Apoptin phosphorylation [17]. Recently, the beta isoform of protein kinase C (PKC\(^\beta\)) was shown to interact with Apoptin and regulate its phosphorylation and cytotoxic activity in multiple myeloma cells [18].

PKC is a family of serine-threonine kinases, comprising at least 13 known isoforms that differ in their tissue distribution, subcellular localisation and function [19]. They play key roles in the regulation of essential biological processes including proliferation, apoptosis and differentiation. Consequently, elevated levels or differential activation of PKC isoforms have been linked to various types of cancer including colon cancer and leukaemias [20-22]. All isoforms share a similar structure, comprising a highly conserved catalytic domain, a regulatory domain and an autoinhibitory pseudosubstrate, and can be further subdivided into classical, novel or atypical isoforms depending on the composition of regulatory elements [23].

In unstimulated cells the majority of PKC isoforms are located in the cytosol and translocate to the plasma membrane upon stimulation for full activation by lipid co-activators. Although this event has served as the hallmark for PKC activation, it is unclear to what extent translocation to the plasma membrane actually corresponds to catalytic activity and substrate phosphorylation. Genetically encodable biosensors based on fluorescence resonance energy transfer (FRET) between fluorescent proteins provide a novel tool to study signalling processes in cells, such as activation of kinases [24] or Rho GTPases [24-26]. FRET refers to a non-radiative type of energy transfer between an excited donor fluorophore and an acceptor fluorophore in close proximity. This process depletes the donor population in the excited state, reducing the fluorescence intensity as well as the fluorescence lifetime of the donor and sensitising emission from the acceptor fluorophore. For the detection of PKC activity
Violin et al. developed CKAR (C kinase activity reporter) which comprises a PKC substrate sequence linked to CFP and YFP as well as the FHA2 phosphothreonine-binding domain [27]. Activation of PKC triggers a conformational change that reduces FRET between the two fluorophores, which can be measured using ratiometric imaging of CFP to YFP fluorescence intensity. In contrast to intensity-based methods for the detection of altered FRET, fluorescence lifetime imaging microscopy (FLIM) is independent of fluorophore concentrations and stoichiometry, requires only measurement of donor fluorescence and represents a state-of-the-art technology to study cellular signalling events [28, 29].

Here we show that the activity of Apoptin in human cancer and normal cells derived from colon mucosa correlates with respective expression levels of PKCβI. Both CAV- and HGyV-Apoptin were found to interact and co-localise with PKCβI in the nucleus of cancer cells. Using a modified CKAR reporter plasmid in a FLIM setup we detected Apoptin-induced activation of PKC in cancer cells but not in normal cells. Taken together, the data highlight an important role of PKCβI for the regulation of Apoptin in human cancer cells, which indicates a novel anti-proliferative function for PKCβI in response to apoptotic stimuli. In addition to elucidating the tumour-specific regulation of Apoptin the information obtained could be important for the design of future PKCβ-targeted strategies.
Results

PKCβI expression levels correlate with Apoptin activity

Apoptin phosphorylation and its apoptotic activity are regulated by the PKC isoforms βI and βII in multiple myeloma cell lines [18]. We therefore investigated the link between PKCβ and Apoptin in the human colorectal cancer cell line HCT116, which we found to be highly sensitive to Apoptin [11]. HCT116 cells demonstrated significantly higher levels of PKCβI expression than NCM460 cells derived from normal colon mucosa (Fig. 1a) while PKCβII levels did not show any marked difference (Fig. S1a).

Western blot analysis of HCT116 and NCM460 cells expressing FLAG-tagged Apoptin showed a significantly lower Apoptin phosphorylation in normal cells compared to cancer cells by approximately 40% (Fig. 1b). This decreased phosphorylation status of Apoptin was associated with a cytoplasmic expression pattern of Apoptin in NCM460 cells as shown by fluorescence and confocal microscopy, whereas in HCT116 cells Apoptin was exclusively present in the nucleus (Fig. 1c, d). In contrast to HCT116 cells, NCM460 cells remained completely resistant to Apoptin-induced apoptosis following infection with an adenoviral vector to express Apoptin (Fig. 1e). Transient knockdown of PKCβI in HCT116 cells to nearly undetectable levels was associated with a significant decrease in Apoptin phosphorylation by approximately 50% measured as the ratio of phosphorylated Apoptin (P-Apoptin) to total GFP-Apoptin (Fig. 1f).

The differential activity and expression of Apoptin was confirmed using another normal colon mucosa cell line, NCM356, with significantly lower expression levels of
PKCβI (Fig. S1a-d). Overall the results suggest a correlation between PKCβI expression levels and the tumour-specific activity of Apoptin, indicated by its phosphorylation, nuclear localisation and cytotoxicity.

**Apoptin interacts with PKCβI in the nucleus of colon cancer cells**

A number of cellular proteins have been identified as Apoptin binding partners and regulators of its function and tumour cell-specific cytotoxicity. To test whether CAV-Apoptin and its newly discovered homologue HGyV-Apoptin can directly bind to PKCβI in colon cancer cells, HCT116 cells were co-transfected with HA-tagged PKCβI and plasmids expressing FLAG-tagged Apoptin. Both CAV- and HGyV-FLAG-Apoptin were present in immunocomplexes pulled down from total cell extracts using anti-FLAG antibodies (Fig. 2a). As expected from their respective amino acid sequences, HGyV-Apoptin was detected at a slightly higher molecular weight than CAV-Apoptin [3]. Similarly, the reciprocal immunoprecipitation using anti-HA antibodies confirmed binding of Apoptin to PKCβI in HCT116 cells (data not shown).

The interaction between Apoptin and endogenous PKCβI was further analysed using the proximity ligation assay (PLA) which allows the direct detection and localisation of protein interactions *in situ* [30]. Staining of HCT116 cells transfected with FLAG-Apoptin using antibodies against Apoptin and FLAG served as a positive control for all PLA experiments (Fig. 2b). To detect binding of Apoptin to PKCβI, HCT116 cells were transfected with CAV- or HGyV-FLAG-Apoptin or the pcDNA3 control plasmid and stained with antibodies directed against FLAG and PKCβI, followed by PLA analysis. Red fluorescent PLA signals, indicating a site of protein
interaction, were detected in cells expressing both types of Apoptin but not cells transfected with the control vector (Fig. 2c). Corresponding to the expression pattern of Apoptin these were predominantly localised in the nucleus, indicating nuclear translocation of PKCβI in response to Apoptin expression. Quantification of positive PLA foci showed a significantly increased number of signals in Apoptin-expressing cells compared to control cells (Fig. 2c). The clear interaction between Apoptin and PKCβI observed by PLA is unlikely to be affected by cell death as no significant induction of apoptosis was observed at the same time point following transfection of Apoptin plasmids (data not shown).

We next investigated the importance of the N-terminal part of Apoptin for binding to PKCβ. This region contains a leucine-rich stretch and has been implicated in interaction of Apoptin with other cellular proteins. In contrast to cells expressing full-length GFP-Apoptin, HCT116 cells transfected with an Apoptin mutant lacking amino acids 1 to 73 showed no significant increase in the total number of PLA signals compared to the GFP control (Fig. 2d). This data suggests that the C-terminal half of Apoptin alone is not sufficient to mediate binding to PKCβI and that interacting regions are located within the N-terminus. Additional Apoptin mutants need to be tested in order to precisely map the region/s required for interacting with PKCβI.

**FRET-based measurements of PKC activity**

PKCβ-mediated Apoptin phosphorylation and Apoptin-induced translocation of PKCβ to the nucleus suggest a novel regulatory loop between Apoptin and PKCβ activities. To monitor PKC activity we employed a modified version of the PKC activity reporter CKAR [27] which consists of the two fluorophores GFP and RFP linked by a PKC substrate sequence and the FHA2 phosphothreonine-binding domain.
(Fig. 3a). As illustrated, PKC-induced phosphorylation of the substrate triggers a conformational change, resulting in a decrease of FRET from GFP to RFP and consequently an increase in the fluorescence intensity and lifetime of the donor fluorophore GFP. We detected alterations in FRET by measuring the two-photon excited fluorescence lifetime $\tau$ of GFP using a time-correlated single-photon counting system (TCSPC) established for FLIM [31]. Mapping of GFP lifetimes across single cells expressing CKAR allowed for the visualisation of PKC activity and calculation of average GFP lifetimes (Fig. 3b). Comparison of a number of cells transfected with the reporter plasmid to cells expressing only the donor fluorophore GFP showed a clear decrease of average GFP lifetimes from 2.14 to 1.86 ns, indicating FRET between GFP and RFP with a FRET efficiency of 13.3% (Fig. 3b).

The ability of CKAR used in this TCSPC/FLIM setup to measure alterations in PKC activity was tested in HCT116 cells transfected with CKAR followed by treatment with the PKC agonist TPA or Gö6976, an inhibitor of classical PKC isoforms. As shown on representative FLIM images TPA treatment, as expected, triggered a shift to higher GFP lifetimes, indicating activation of PKC, whereas GFP lifetimes were reduced following treatment with Gö6976 (Fig. 3c). This was reflected in a corresponding increase or decrease of average GFP lifetimes compared to untreated cells, suggesting that CKAR is suitable for the detection of changes in PKC activity following pharmacological modulation.

**Apoptin expression induces cancer specific PKC activation**

To investigate the effect of Apoptin on PKC activity, HCT116 cells were transfected with CKAR followed by infection with an adenoviral vector to express Apoptin (Ad-Apoptin). Apoptin expression was detected by Western blot analysis after
5 hours and further increased until 16 hours post-infection with Ad-Apoptin (Fig. 4a). Phosphorylated Apoptin was detectable after 8 and 16 hours, suggesting activity of the Apoptin kinase before or at that time point. As shown on representative FLIM images Apoptin expression was associated with a time-dependent increase in PKC activity as indicated by a shift to higher GFP lifetimes in Apoptin-treated cells (Fig. 4b). Measurement of average GFP lifetimes confirmed a significant increase in PKC activity at 8 and 16 hours post-infection similar to control treatment with TPA. A later drop of GFP lifetimes to basal levels might be due to apoptosis as a similar reduction of PKC activity was observed in cisplatin-treated cells (data not shown). These results suggest Apoptin-induced activation of PKC, correlating with the expression and phosphorylation levels of Apoptin at the respective stage in human cancer cells.

Apoptin phosphorylation and therefore activity of the Apoptin kinase represents an important aspect of the tumour cell-specific cytotoxic function of Apoptin. We therefore investigated whether Apoptin expression in normal colon mucosa cells with lower expression levels of PKCβI (Fig. 1a, Fig. S1a) would trigger activation of PKC as observed in cancer cells. In contrast to HCT116 cells, which showed a significant increase in PKC activity following infection with Ad-Apoptin, PKC activation remained unaltered in normal NCM356 cells at any time point (Fig. 4c). Similarly, compared to HCT116 cells Apoptin only triggered a minor increase in PKC activity in normal NCM460 cells (Fig. 4d), suggesting that this differential effect of Apoptin on the activation status of PKC is not cell type-specific. Apoptin-induced PKC activation therefore seems to occur predominantly in tumour cells but not in normal cells and might depend on the presence of PKCβI.
Discussion

Specific killing of tumour cells while minimising damage to normal tissues remains a major challenge in the development of effective anti-cancer agents. In recent years, several cellular or viral proteins that selectively induce cell death in cancer cells have been identified, including Apoptin which was originally discovered as the apoptosis-inducing protein from CAV [8]. Gaining further understanding of their regulation and cellular targets is essential for their development into future therapeutic agents as well as the identification of novel tumour-specific targets and processes.

In this study we investigated the physical and functional interaction between Apoptin and PKC. The PKC signalling pathway has predominantly been associated with survival, proliferation and inhibition of apoptosis and is therefore an important target for the development of anti-cancer agents.

Tumour cell-specific phosphorylation of Apoptin on Thr-108 by a cellular kinase represents an important step for its accumulation in the nucleus and cytotoxic activity [16] and PKCβ was identified as a potential Apoptin kinase, but so far only in multiple myeloma cells [18]. Several cancer types, including multiple myeloma, show increased expression or activity of PKCβ, suggesting that PKCβ might represent a tumour-specific target responsible for sensitising cells to Apoptin [20-22].

We therefore investigated the dynamics of the interplay between Apoptin and PKCβI in HCT116 cells, a colorectal cancer cell line expressing high levels of PKCβI and displaying high sensitivity to Apoptin. Co-immunoprecipitation and PLA studies convincingly showed direct binding and co-localisation of Apoptin and PKCβI. This interaction seemed to require the N-terminal region of Apoptin which has been shown
to be involved in both binding of Apoptin to other cellular proteins [32-34] as well as Apoptin multimerisation [35]. Importantly, interaction with Apoptin triggered nuclear translocation of PKCβI; to our knowledge this is a novel phenomenon in respect to PKCβI activity. The importance of this nuclear localisation on the activity of PKCβI is currently unclear, however it may cause changes to PKCβI accessibility for its substrates and/or its function [18]. Notably, Apoptin has been shown to trigger nuclear accumulation of the related kinase Akt, switching its pro-survival to pro-apoptotic activity in prostate cancer cells [36].

In contrast to HCT116 cells, two cell lines derived from normal colon mucosa were found to express almost undetectable levels of PKCβI. Increased expression levels of PKCβI in HCT116 cells correlated with enhanced activation of Apoptin, demonstrated by increased expression and phosphorylation levels of Apoptin, its translocation to the nucleus as well as induction of apoptosis in cancer cells while normal colon mucosa cells remained resistant to Apoptin expression. Knockdown of PKCβI in HCT116 cells reduced Apoptin phosphorylation, albeit not completely which might be due to residual Apoptin phosphorylation by other regulatory kinases such as PKCβII or Cdk2.

Interestingly, HGyV-Apoptin, a recently identified VP3 homologue derived from the first human Gyrovirus HGyV [2, 3], was also shown to interact with PKCβI. An online kinase prediction assay of the HGyV-Apoptin amino acid sequence (http://www.cbs.dtu.dk/services/NetPhos) revealed several potential phosphorylation sites matching the PKC consensus motif (S/TXK/R or S/TXXK/R) including Thr-111 which, like Thr-108 of CAV-Apoptin, is adjacent to a putative NES within the
respective protein. This suggests a similar mode of tumour-specific regulation of HGyV-Apoptin by PKCβI-mediated phosphorylation as identified for CAV-Apoptin.

Using CKAR, a FRET-based biosensor for PKC activation, we showed a transient increase in PKC activity following Apoptin expression in HCT116 cells similar to treatment with known PKC activators. The demonstrated increase in PKC activity correlated with the presence of phosphorylated Apoptin, suggesting a link between Apoptin expression, PKC activation and Apoptin phosphorylation. In contrast to HCT116 cancer cells, normal colon mucosa cell lines displayed significantly reduced and delayed activation of PKC following Apoptin expression. These data support an important role for PKC in the tumour cell-specific regulation of Apoptin.

Despite the biosensor’s lack of discrimination between various classical and novel PKC isoforms [37], the reduced PKC activation in normal colon mucosa cells lacking PKCβI indicates specific activation of PKCβI by Apoptin. However, a potential involvement of other PKC isoforms, including PKCβII or PKCδ, cannot be excluded and should be further investigated. Importantly, PKCδ is generally considered to be a pro-apoptotic PKC isoform that is cleaved and activated in response to many pro-apoptotic stimuli [38] including Apoptin expression and might represent a link between Apoptin-induced PKC activation and induction of caspase-dependent apoptosis [18].

It remains to be determined how the presence of Apoptin triggers activation of PKC. Using in vitro kinase assays we have previously shown PKC-mediated Apoptin phosphorylation after incubation with PKCβ alone without any additional PKC activators in the reaction [18], suggesting that the presence of Apoptin alone is sufficient to activate PKC.
Allosteric activation of PKCβII has been suggested to involve exposure of arginine-19 to proteolysis, which is shielded by a cluster of acidic residues under non-stimulated conditions [39]. Apoptin is rich in basic amino acids, which could potentially bind to these acidic residues on PKCβ to expose Arg-19 and thereby trigger PKC activation.

In conclusion, this study has established an important tumour-specific link between Apoptin and PKCβI in colon cancer cells. Remarkably, this interaction induces not only PKC-dependent phosphorylation of Apoptin but also nuclear translocation and activation of PKC. In addition to phosphorylating Apoptin, modulation of PKC activity by Apoptin could trigger other yet unknown cellular pathways shifting the balance from proliferation to apoptosis.
Materials and Methods

Cell Lines and Reagents

HCT116 cells were obtained from ATCC and maintained in McCoy’s 5A medium supplemented with 10% foetal bovine serum, 50 µg/ml streptomycin and 100 µg/ml penicillin (PAA Laboratories). NCM356 and NCM460 cells were purchased from INCELL Corporation. Cisplatin was obtained from the Guy’s Hospital Pharmacy. Adenoviral vectors for the expression of Apoptin (Ad-Apoptin) or GFP (Ad-GFP) were generated as described previously [40]. For viral infections, virus stocks were diluted in culture medium to achieve the desired MOI (multiplicity of infection) and added to the cells.

Transient Transfection

pCAV-FLAG-AP for the expression of CAV-FLAG-Apoptin was obtained from Dr. Jose Teodoro (Goodman Cancer Research Centre, Montreal, Canada), plasmids for full-length or truncated GFP-Apoptin (pEPI-GFP-Apoptin 1-121 or 74-121) were provided by Dr. Gualtiero Alvisi (Monash University, Clayton, Australia). The expression vector for HGyV-FLAG-Apoptin (pHGyV-FLAG-AP) was generated as described previously [3]. pHA-PKCβ1 was obtained from Professor Alexandra Newton (University of California, San Diego, CA). pGFP/RFP CKAR was modified from the original reporter construct pCFP/YFP CKAR [27] by Dr. Melanie Keppler (King’s College, London). Control vectors used were pcDNA3.1 and peGFP-C1 (Life Technologies).

HCT116 cells were transiently transfected using X-tremeGENE HP transfection reagent according to the manufacturer’s protocol (Roche Applied Science). In short, a
mix of plasmid DNA and transfection reagent was prepared in OptiMEM reduced-serum medium (Life Technologies) and added dropwise to the cells after incubation for 20 min at room temperature.

**Transient siRNA Knockdown**

siGENOME Human PRKCB siRNA SMARTpool targeting PKCβI (targeting sequences GAACCAAGGUCCCGGAAGA, GGAUGAAACUGACCGAUUU, CCAAGUCUAUGUCCAAGGA, AGAGUAAGGGCAUCAUUUA) and GAPDH control siRNA were obtained from Dharmacon (GE Healthcare). Cells were transfected with siRNAs using the X-tremeGENE siRNA transfection reagent as recommended by the manufacturer (Roche Applied Science). In short, siRNA and transfection reagent were prepared separately in OptiMEM reduced-serum medium (Life Technologies), mixed carefully and added dropwise to the cells after incubation for 20 min at room temperature.

**Lentivirus production**

Lentiviral expression constructs for GFP (Lenti-GFP) or CAV-GFP-Apoptin (Lenti-GFP-AP) were constructed as previously described [18]. Lenti-FLAG-AP for the expression of CAV-FLAG-Apoptin was generated by insertion of the FLAG-Apoptin gene from pCAV-FLAG-AP into the lentiviral backbone used for Lenti-GFP.

Lentiviruses were produced in HEK293T cells transfected with the second-generation packaging plasmid pCMVΔ8.91 and plasmid pMDG encoding VSV-G-pseudotyped envelope as well as the construct of interest by calcium phosphate precipitation. The virus-containing supernatant was harvested at 24 and 48
hours post-transfection, put through a 0.45 μm filter, concentrated by centrifugation for 6 hours at 13,000 x g and stored at -70°C. For infection lentiviral stocks were diluted in a minimal amount of medium supplemented with 5 µg/ml polybrene (Santa Cruz Biotechnology) and added to target cells.

**Flow cytometry**

Cells were cultured on 12-well plates at a density of 1 x 10^5 cells per well and infected the next day. Cells were collected after 40 hours after by trypsinisation and washing in PBS. Apoptosis was quantified by staining with Annexin V-APC (BD Biosciences) and propidium iodide (PI, Sigma-Aldrich). Data was acquired on a BD FACSCantoII flow cytometer (BD Biosciences) and analysed using FlowJo software (Tree Star, Inc.)

**Immunoprecipitation**

Cells were seeded on 6-well plates at a density of 2 x 10^5 cells per well and transfected the next day as indicated. At 2 days post-transfection cells were collected by scraping and washing with cold PBS and centrifuged for 15 min at 16,000 x g. The obtained cell pellet was resuspended in 250 μl to 500 μl radioimmunoprecipitation assay buffer (Cell Signalling), sonicated and incubated for 1 hour on ice. After centrifugation at 4°C and 16,000 x g for 10 min the supernatant was transferred to a new tube and incubated with 2 μg of anti-HA or anti-FLAG antibody for 30 min under agitation at 4°C. 20 μl of washed Bio-Adembeads PAG (Ademtech) was added and incubated with the sample for 3 hours at 4°C. Using a magnetic rack, beads were washed and isolated from the solution and proteins were removed from the beads by resuspending in Laemmli sample buffer and boiling for 5 min at 95°C.
Western blot analysis

Cells were cultured on 6-well plates at a density of $2 \times 10^5$ cells per well and treated the next day. Cells were collected by trypsinisation, washed in PBS and re-suspended in lysis buffer (2 mM MgCl$_2$, 25 mM HEPES, 2 mM EGTA, 0.1% Triton X-100) supplemented with protease inhibitors. Following 30 min incubation on ice, protein lysates were obtained by centrifugation for 15 min at 16,000 x g. 40 to 60 µg of protein was used for further SDS-PAGE and transfer of proteins to nitrocellulose membranes (Sigma-Aldrich). Membranes were incubated with specific primary and secondary antibodies as indicated and developed using an enhanced chemiluminescent system.

Antibodies used for immunoblotting were: β-actin, Tubulin, FLAG (Sigma-Aldrich), PKCβI, PKCβII (Santa Cruz Biotechnology), GFP and HA (Cell Signalling). Antibodies directed against total or Thr-108-phosphorylated Apoptin (P-Apoptin) were obtained as described previously [18]. Secondary HRP-coupled anti-rabbit and anti-mouse antibodies were obtained from GE Healthcare and Sigma-Aldrich, respectively. The relative expression or phosphorylation levels of proteins were determined using ImageJ software.

Immunofluorescence

Cells were grown on Falcon 8-chamber culture slides (BD Biosciences) at a density of $2 \times 10^4$ to $3 \times 10^4$ cells per well and transfected the next day. After 2 days cells were washed in PBS, fixed in 4% paraformaldehyde for 10 min and permeabilised with 0.2% Triton X-100 for 10 min. After permeabilisation cells were washed in PBS, blocked using 3% BSA in PBS-T for 30 min and incubated with a primary anti-FLAG
antibody for 90 min and a secondary FITC-conjugated anti-mouse antibody (Sigma-Aldrich) for 1 hour. Cells were washed between incubations in PBS and all steps were carried out at 37°C. Cells were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) to stain nuclei. Images were acquired on an Olympus BX61 automated fluorescence microscope or an A1R Si Confocal System and Eclipse Ti-E Inverted Microscope (Nikon) for confocal microscopy.

**Proximity Ligation Assay (PLA)**

Cells were seeded in Falcon 8-chamber culture slides (BD Biosciences) at a density of 3 x 10^4 cells per well and transfected the next day. After 2 days cells were washed in PBS, fixed in 4% paraformaldehyde for 10 min, washed again and permeabilised with 0.2% Triton X-100 for 10 min. After permeabilisation cells were blocked in blocking solution for 30 min and then incubated with the indicated primary antibodies for 2 hours at 37°C in a humidity chamber. All further steps were carried out according to the manufacturer’s instructions (Duolink In Situ kit, Olink) in a reaction volume of 80 μl per well with three washing steps in Buffer A between incubations. Briefly, cells were incubated with PLA probes for 1 hour followed by a ligation reaction for 30 min and incubation with an amplification mix for 100 min. After mounting in DAPI-containing mounting medium images were acquired on the Olympus BX61 automated fluorescence microscope using Cell^F software (Olympus) and analysed using Blobfinder software (Olink) to automatically detect and quantify positive PLA signals.

**Fluorescence Lifetime Imaging Microscopy (FLIM)**

Cells were seeded on sterile coverslips in 24-well plates at a low density and 24 hours later transfected with pGFP/RFP CKAR or peGFP-C1. The next day cells were
infected with Ad-Apoptin at an MOI of 10 to express Apoptin for 2 up to 24 hours before fixation in 4% paraformaldehyde for 10 min. Control cells were treated with 200 nM TPA (12-O-Tetradecanoylphorbol-13-Aacetate, Cell Signalling) for 30 min or 50 nM Gö6976 (Calbiochem) for 120 min. To reduce autofluorescence fixed cells were treated with 1 mg/ml sodium borohydride for 5 min and mounted on slides in 8 μl FluorSave mounting medium (Calbiochem).

Time-domain FLIM was performed using a home-built two-photon laser-scanning microscope based on the modified Nikon Eclipse Ti-E inverted microscope. A mode-locked Ti:Sapphire laser (Coherent, Chameleon Vision) with repetition rate of 80 MHz and pulse width of ~140 fs was tuned to 890 nm providing a photoexcitation source for GFP. GFP fluorescence (500/40 nm bandpass) was detected with a 40x/1.3NA objective lens and a hybrid photo multiplier tube (PMT, HPM-100) in conjunction with a time-correlated single-photon counting (TCSPC, SCP-150) card (both from Becker & Hickl). Images were acquired at 256 x 256 pixels with peak photon counting rates kept below 10⁷ photons per second to avoid pulse pile-up. The GFP fluorescence lifetime τ was calculated by fitting bi-exponential fluorescence decay models to the images using TRI2 software (Paul Barber, University of Oxford). Output files were analysed to produce a distribution of the GFP lifetime across the cells and calculation of average donor lifetimes in single cells.

**Statistical Analysis**

Statistical analysis of results obtained from at least three independent experiments was performed using GraphPad Prism 6 software and one- or two-way analysis of variance (ANOVA) or two-tailed Student’s t test as appropriate and indicated.
Conflict of Interest

The authors declare no conflict of interest.

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References


**Supplementary Material**

Fig. S1 Correlation of PKCβI Levels and Apoptin Activity in HCT116 and NCM356 Cells

**Legends to Figures**

**Fig. 1 Correlation of PKCβI Levels and Apoptin Activity in Colon Cancer and Normal Cells**

(a) PKCβI expression in HCT116 and NCM460 cells was investigated by Western blot analysis of total cell lysates, showing results from 2 separate cultures. Bars represent PKCβI expression normalised to β-Actin; error bars indicate SEM (n = 7). Statistical significance was determined by unpaired two-tailed Student’s t test (*** P < 0.001). (b) HCT116 and NCM460 cells were infected with Lenti-GFP (GFP) or Lenti-CAV-FLAG-AP (Apo) at an MOI of 5 for 2 days. Total cell lysates were analysed by immunoblotting with the indicated antibodies; blots were cut and combined at the indicated line. Bars represent ratios of phosphorylated Apoptin (P-Apoptin) to FLAG-Apoptin; error bars indicate SEM (n = 4). Statistical significance was determined by unpaired two-tailed Student’s t test (** P < 0.01). (c/d) HCT116 and NCM460 cells were transfected with CAV-FLAG-AP, fixed after 24 hours and stained with a primary mouse anti-FLAG and secondary FITC-conjugated anti-mouse antibody and DAPI for detection of nuclei. Representative widefield (c, original magnification 60x, bar = 10 μm) and confocal (d, original magnification 100x, bar = 20 μm) fluorescence images are shown. Bars represent the percentage of Apoptin-expressing cells with nuclear localisation of Apoptin; error bars indicate SEM (n = 3). Statistical significance was determined by unpaired two-tailed Student’s t test (*** P < 0.001). (e) HCT116 and NCM460 cells were infected...
with Ad-GFP or Ad-Apoptin at an MOI of 10 for 40 hours before cell death detection by Annexin V/PI staining and FACS analysis. Bars represent the mean percentage of Annexin V positive cells; error bars indicate SEM (n = 4). Statistical significance was determined by two-way ANOVA (*** P < 0.001). (f) HCT116 cells were transfected with PKCβI or GAPDH control siRNA and infected 24 hours later with Lenti-CAV-GFP-AP at an MOI of 5. Total cell lysates were collected after 2 days for Western blot analysis with the indicated antibodies. Bars represent ratios of phosphorylated Apoptin (P-Apoptin) to total GFP-Apoptin; error bars indicate SEM (n = 3). Statistical significance was determined by one-way ANOVA (* P < 0.05, ns P > 0.05).

Fig. 2 Interaction and Co-localisation of CAV-Apoptin and H GyV-Apoptin with PKCβI in the Nucleus of Cancer Cells

(a) HCT116 cells were co-transfected with CAV-FLAG-AP (CAV) or HGyV-FLAG-AP (HGyV) and HA-PKCβI. Immunocomplexes pulled down with anti-FLAG antibodies and total cell lysates prepared in RIPA buffer at 2 days post-transfection were subjected to Western blot analysis with the indicated antibodies. (b) HCT116 cells were transfected with CAV-FLAG-AP or pcDNA3 followed by PLA staining using anti-FLAG and anti-P-Apoptin antibodies and counterstaining of nuclei with DAPI. Representative PLA images are shown (original magnification 60x, bar = 10 µm). (c) HCT116 cells were transfected with CAV-FLAG-AP, HGyV-FLAG-AP or pcDNA3 and analysed by PLA at 2 days post-transfection using anti-FLAG and anti-PKCβI antibodies. Representative images indicating nuclei counterstained with DAPI and PLA signals are shown (original magnification 60x,
bar = 10 μm). Bars represent average number of PLA foci per cell calculated from at least 100 cells (excluding cells with 0 signals); error bars indicate SEM (n = 3). Statistical significance was determined by one-way ANOVA (* P < 0.05, ** P < 0.01). (d) HCT116 cells were transfected with peGFP-C1 or the indicated pEPI-GFP-Apoptin plasmids and analysed by PLA using anti-GFP and anti-PKCβI antibodies at 2 days post-transfection. Representative images indicating nuclei counterstained with DAPI, PLA signals or GFP expression are shown (original magnification 60x, bar = 10 μm). Bars represent average number of PLA foci per cell, localised to either the nucleus or the cytoplasm, calculated from at least 100 GFP-expressing cells; error bars indicate SEM (n = 3). Statistical significance was determined by one-way ANOVA (* P < 0.05, ns P > 0.05).

**Fig. 3 Measurement of PKC activity by FRET/FLIM using CKAR**

(a) Structure of the PKC activity reporter CKAR, comprising GFP and RFP linked by a PKC substrate sequence and the FHA2 phosphothreonine-binding domain of Rad53p. PKC-induced phosphorylation of the substrate sequence triggers a conformational change, resulting in reduced FRET measured as an increase of the fluorescence lifetime τ of the donor GFP (adapted from [27]). (b) HCT116 cells were transfected with pGFP/RFP CKAR or peGFP-C1 and fixed for FLIM analysis the following day. A representative FLIM image of a single cell, indicating the distribution of GFP lifetime (τ) is shown (bar = 10 μm). Each symbol represents the average τ of a single cell; lines indicate the mean value of τ for the entire sample. (c) HCT116 cells were transfected with pGFP/RFP CKAR and treated the following day with 200 nM TPA for 30 min or 50 nM Gö6976 for 120 min before fixing for FLIM
analysis. Representative FLIM images indicating $\tau$ are shown (bar = 10 $\mu$m). Bars represent changes in average $\tau$ compared to untreated cells, corresponding to PKC activity, from at least 15 cells per sample; error bars indicate SEM ($n = 5$). Statistical significance was determined by one-way ANOVA (* $P < 0.05$, *** $P < 0.001$).

**Fig. 4 Apoptin-induced Activation of PKC Isoforms in Colon Cancer Cells but not Normal Cells**

(a) HCT116 cells were infected with Ad-Apoptin at an MOI of 10 and cell lysates were collected at the indicated time points for Western blot analysis. Blots were cut and combined at the indicated line. (b) CKAR-expressing HCT116 cells were infected with Ad-Apoptin at an MOI of 10 before fixing for FLIM analysis at the indicated time points. Representative FLIM images indicating $\tau$ are shown (bar = 10 $\mu$m). Bars represent changes in average $\tau$ compared to untreated cells calculated from at least 15 cells per sample; error bars indicate SEM ($n = 3$ for 2h/5h, $n = 5$ for 8h/16h/24h). Statistical significance was determined by one-way ANOVA (* $P < 0.05$, *** $P < 0.001$). (c/d) HCT116 and NCM356 (c) or NCM460 (d) cells were transfected with pGFP/RFP CKAR and infected the following day with Ad-Apoptin at an MOI of 10 for 8 or 16 hours before fixing for FLIM analysis. Bars represent changes in average $\tau$ compared to untreated cells calculated from at least 10 cells per sample; error bars indicate SEM ($n = 3$). Statistical significance was determined by two-way ANOVA (** $P < 0.01$).
Fig. S1 Correlation of PKCβI Levels and Apoptin Activity in HCT116 and NCM356 Cells

(a) PKCβI and PKCβII expression levels in HCT116, NCM356 and NCM460 cells were determined by Western blot analysis. (b) HCT116 and NCM356 cells were infected with Lenti-GFP (GFP) or Lenti-CAV-FLAG-AP (Apo) at an MOI of 5. Total cell lysates were collected after 2 days for Western blot analysis with the indicated antibodies; blots were cut and combined at the indicated line. (c) HCT116 and NCM356 cells were transfected with CAV-FLAG-AP, fixed after 24 hours and stained with a primary mouse anti-FLAG and secondary FITC-conjugated anti-mouse antibody. Nuclei were detected by counterstaining with DAPI. Representative fluorescence images are shown (magnification 60x, bar = 10 µm). (d) HCT116 and NCM356 cells were infected with Ad-GFP or Ad-Apoptin at an MOI of 10 and collected at 40 hours post-infection for cell death detection by Annexin V/PI staining and FACS analysis. Bars represent the percentage of Annexin V positive cells; error bars indicate SEM (n = 4). Statistical significance was determined by two-way ANOVA (***, P < 0.001).
Fig. 1

(a) Western blots showing PKCβ1 and β-Actin expression in HCT116 and NCM460 cells. PKCβ1 expression is normalized to β-Actin.

(b) Western blots showing FLAG, P-Apoptin, GFP, and Tubulin expression in HCT116 and NCM460 cells under different conditions. P-Apoptin expression is normalized to FLAG-Apoptin.
Fig. 1 continued

c)

FLAG-Apoptin

DAPI

FLAG

Merge

HCT116

NCM460

d)

FLAG-Apoptin

HCT116

NCM460

f)

GFP-Apoptin

- GAPDH PKCβ siRNA

GFP

41 kDa

P-Apoptin

41 kDa

PKCβ

79 kDa

Tubulin

55 kDa

e)

% Nuclear Apoptin

% Annexin V+ Cells

HCT116

NCM460

Control Ad-GFP Ad-Apoptin

P-Apoptin / GFP-Apoptin

Control siRNA GAPDH PKCβ
Fig. 2

(a) Western blot analysis showing the expression of FLAG and HA proteins. Input and IP: FLAG columns compare the expression under different conditions.

(b) Immunofluorescence images showing DAPI staining, PLA, and merge for pcDNA3, CAV, and HGyV treatments.

(c) quantification of PLA signals per cell for different treatments.
Fig. 2 continued

d

![Immunofluorescence images with DAPI, PLA, Merge, and GFP channels showing eGFP-C1 and Apoptin 1-121, Apoptin 74-121 with bar graphs comparing the number of signals per cell in cytoplasm and nucleus.](image)

- **DAPI**: Nuclear staining
- **PLA**:lked to specific epitopes for quantification
- **Merge**: Overlay of DAPI and PLA
- **GFP**: Green fluorescence protein

![Bar graph showing comparison of signal counts between GFP, Apoptin 1-121, and Apoptin 74-121 in cytoplasm and nucleus.](image)

- **Cytoplasm**
- **Nucleus**

*ns* indicates no statistically significant difference; *p* < 0.05
**Fig. 3**

(a) Schematic representation of the interaction between FHA2-P and GFP, showing the FRET process with wavelengths 395 nm and 607 nm. The diagram also illustrates the action of PKC and the effects of TPA and Gö6976 on GFP and RFP expression.

(b) Fluorescence lifetime imaging (FLIM) of GFP and GFP/RFP constructs under untreated, TPA, and Gö6976 conditions. The graph shows the distribution of fluorescence lifetime (τ GFP, nanoseconds) with color coding.

(c) Comparison of average fluorescence lifetime (τ GFP, nanoseconds) across different treatments: Untreated, TPA, and Gö6976. The bar graph indicates a significant change in fluorescence lifetime under TPA treatment compared to Untreated and Gö6976.
Fig. 4

(a) Western blot analysis of Ad-Apoptin. Time points: 2h, 5h, 8h, 16h, 24h. Labeling for Apoptin, P-Apoptin, and β-Actin are shown with respective molecular weights of 14 kDa and 43 kDa.

(b) Fluorescence imaging over time with Ad-Apoptin. Color scale indicates τ GFP (ns) from 1.6 to 2.0.

(c) Bar graph showing average change in τ GFP (ns) for HCT116 and NCM356. Time points: Untreated, Apoptin 8h, Apoptin 16h. * indicates statistical significance.

(d) Bar graph showing average change in τ GFP (ns) for HCT116 and NCM460. Time points: Untreated, Apoptin 8h, Apoptin 16h. * indicates statistical significance.
Fig. S1

(a) Western blot analysis of PKCβI, PKCβII, and Tubulin in HCT116, NCM356, and NCM460 cell lines. Tubulin band at 55 kDa.

(b) Western blot analysis of FLAG, P-Apoptin, and GFP in HCT116 and NCM356 cell lines. Apoptin band at 19 kDa.

(c) Immunofluorescence images of FLAG-Apoptin in NCM356 cells. DAPI, FLAG, and Merge images are shown.

(d) Bar graph showing % Annexin V+ cells in HCT116 and NCM356 cell lines. There is a significant difference between control and Ad-Apoptin groups.